doi:10.1016/j.ymthe.2006.05.022

G2 Cell Cycle Arrest and Cyclophilin A in Lentiviral Gene Transfer

Shangming Zhang,[1] Guiandre Joseph,[2] Karen Pollok,[3] Lionel Berthoux,[4] Lakshmi Sastry,[1] Jeremy Luban,[4] and Kenneth Cornetta[1,2,5,*]

[1]*Department of Medical and Molecular Genetics,* [3]*Herman B. Wells Center for Pediatrics Research,* [5]*Department of Medicine, and* [2]*Department of Microbiology and Immunology, Indiana University School of Medicine, Indianapolis, IN 46202, USA*
[4]*Department of Microbiology and Medicine, College of Physicians and Surgeons, Columbia University, 701 W. 168th Street, New York, NY 10032, USA*

**To whom correspondence and reprint requests should be addressed at the Department of Medical and Molecular Genetics, Indiana University School of Medicine, 975 West Walnut Street, Indianapolis, IN 46202, USA. Fax: +1 317 274 2262. E-mail: kcornette@iupui.edu.*

Available online 9 August 2006

Lentiviral vectors derived from the human immunodeficiency virus-1 (HIV-1) have a higher propensity to transduce nondividing cells compared to vectors based on oncoretroviruses. We report here that genistein, a previously known protein tyrosine kinase (PTK) inhibitor and G2 cell cycle arrest inducer, significantly enhanced lentiviral transduction in a dose-dependent manner. Increased transduction, as measured by vector expression, was seen in a variety of human cell lines, murine primary lymphocytes, and primary human CD34$^+$ peripheral blood progenitor cells as well. Increased vector expression was also associated with an increase in vector DNA copy number, as assessed by quantitative PCR. Genistein-mediated G2 cell cycle arrest, rather than PTK inhibition, appears to be the major factor responsible for increased gene transfer. Genistein also increases cyclophilin A (CypA) protein, a cellular protein important for efficient HIV-1 infection. While we show that CypA$^{-/-}$ Jurkat cells transduce poorly with lentiviral vectors, genistein does increase gene transfer in CypA-deficient cells. CypA and G2 cell cycle arrest appear to be two independent factors important for efficient lentiviral gene transfer. The role of genistein and other G2-arresting agents may be useful for improving the efficiency of lentiviral gene therapy.

Key Words: human immunodeficiency virus-1, lentiviral vector, gene therapy, genistein, cyclophilin A, cyclosporin A, vesicular stomatitis virus glycoprotein

INTRODUCTION

Lentiviral vectors derived from the human immunodeficiency virus-1 (HIV-1) are promising tools for gene therapy. Lentiviral vectors pseudotyped with envelopes such as the vesicular stomatitis virus glycoprotein (VSV-G) and Ross River virus (RRV) glycoprotein can be used to transfer genes into nonproliferating cells, including hematopoietic stem cells, neurons, and muscle cells [1–6]. Gene transfer of lentiviral and other integrating vectors requires both viral and cellular proteins. For example, cyclophilin A (CypA), an 18-kDa cytoplasmic protein, was originally identified as the cytosolic cyclosporin A (CSA)-binding protein and subsequently shown to be a peptidylprolyl *cis–trans*-isomerase (PPIA) [7,8]. It has been well documented that CypA is specifically incorporated into HIV-1 virions by interacting with the Gag polyprotein but not into virions of other retroviruses [9–16]. Importantly, both host and virion-associated CypA is required for the HIV-1 infectivity [17,18]. The reverse transcription of HIV-1 was strikingly reduced in CypA-deficient Jurkat cells or CSA-treated target cells [17]; virion-associated CypA is believed to mediate HIV-1 attachment to target cells [19,20].

To understand better the host factors modulating lentiviral vector transfer, we screened a variety of inhibitors such as the protein tyrosine kinase (PTK) inhibitors genistein and tyrphostin 1 and 23, p38 mitogen-activated protein kinase (MAPK) inhibitor SB203580, extracellular signal-regulated protein kinase (ERK) inhibitor PD98059, phosphatidylinositol 3 (PI3)-kinase inhibitor LY294002, and G-protein-coupled receptor inhibitor pertussis toxin (PTX) at commonly used concentrations, to determine their potential effects on lentiviral gene transduction. Genistein and tyrphostin 1

1525-0016/$30.00

and 23 have been reported to promote adeno-associated virus type 2 (AAV2) transduction by altering the phosphorylation status of FKBP52 protein [21–24]; p38 MAPK plays a critical role in HIV-1 replication [25–27]; ERK is involved in the regulation of an early step in HIV-1 infection [28–30]; inhibition of the PI3-kinase signaling pathway suppresses HIV-1 infection post-viral entry and post-reverse transcription [31]; and HIV-1 production can be inhibited by PTX [32,33]. Of the inhibitors tested, only genistein significantly enhanced lentiviral gene expression in multiple human cell lines, murine primary lymphocytes, and human primary CD34$^+$ progenitor cells, but reduced retroviral transduction. Genistein also increased total vector DNA, suggesting that increased gene expression occurs predominantly from integrated vector DNA. The action of genistein on gene transduction was envelope independent. It appears that genistein-mediated G2 cell cycle arrest, as opposed to PTK inhibition, is the major contributor to genistein's action on lentiviral transfer.

RESULTS

Genistein Enhances Lentiviral Vector Transduction in Cell Lines and Primary Cells in a Dose-Dependent Manner

In an attempt to identify the host factors important for lentiviral vector transfer, we screened several classes of inhibitors, to determine their effects on lentiviral gene transduction. We found that only genistein increased the percentage of enhanced green fluorescent protein (eGFP)-positive 293 cells (Fig. 1). We saw increased gene transfer by genistein in a variety of cell lines, including 293, K562, and HEL cells (Figs. 2A–2C) and primary mouse lymphocytes (Fig. 2D). Importantly, exposure of G-CSF-mobilized human CD34$^+$ peripheral blood pro-

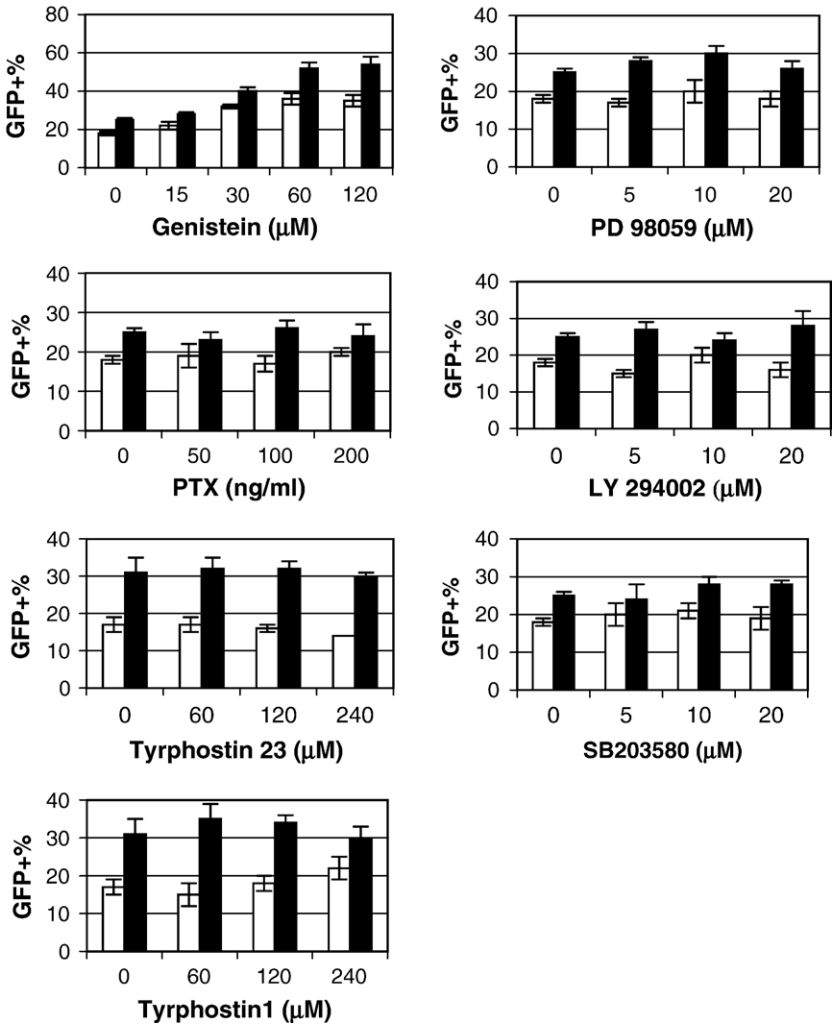

FIG. 1. Genistein increases transgene eGFP expression in 293 cells. 1×10^5 293 cells per well were seeded into six-well plates and incubated overnight. Cells were treated with increasing concentrations of genistein, PD98059, LY294002, SB203580, AG490, PTX, or DMSO as a control for 4 h, followed by transduction with VSV-G (filled column) or RRV (open column) lentiviral supernatant at m.o.i. of 0.2 for an additional 4 h in the presence of 8 μg/ml Polybrene. 4 h later viral supernatants were completely removed and cells were incubated for another 72 h before eGFP expression was analyzed by a FACScan.

doi:10.1016/j.ymthe.2006.05.022

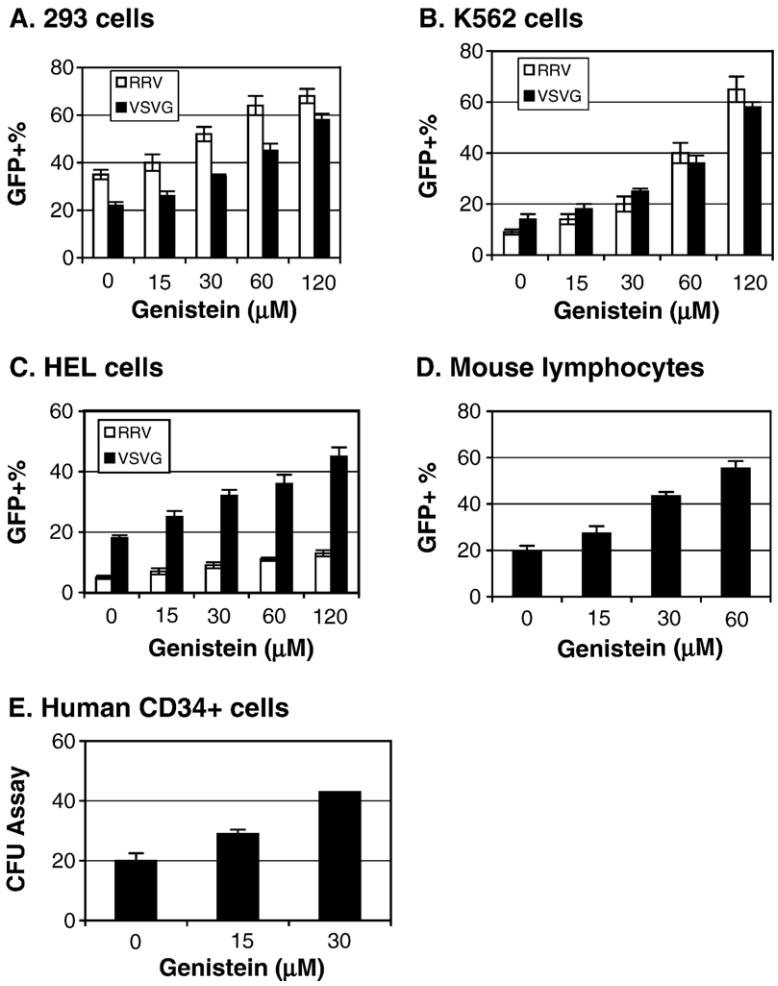

FIG. 2. Genistein enhances lentiviral transduction in cell lines and primary cells in a dose-dependent manner. (A, B, C) 293, K562, and HEL cells were treated with varying concentrations of genistein or DMSO for 4 h, followed by transduction with VSV-G or RRV lentiviral supernatant at m.o.i. of 0.2–0.5 as described above. eGFP expression was analyzed 72 h after transduction by FACScan. (D) Mouse lymphocytes were treated and transduced with VSV-G lentiviral supernatants at m.o.i. of 15 as described under Materials and Methods. eGFP expression was analyzed by a FACScan 72 h after transduction. (E) Frozen human peripheral CD34$^+$ cells were cultured and treated as described under Materials and Methods. A colony-forming unit assay was used to determine the colony formation and eGFP expression.

genitor cells to 30 μM genistein increased the number of eGFP-positive hematopoietic progenitor colonies by twofold (Fig. 2E). Genistein at this concentration did not significantly affect the number of total colonies or their differentiation into burst-forming unit–erythroid and colony-forming unit–granulocyte–macrophage colonies (data not shown). We had previously determined that cytotoxicity, as measured by the MTT assay, occurred in primary cells exposed to concentrations $\geq$60 μM, and immortalized cell lines exposed to concentrations >60 μM (data not shown). This is consistent with a previous report, in which 50 μM genistein induced a slight increase in apoptosis [34].

Genistein-Treated Jurkat Cells Maintain Increased Total Vector DNA and Transgene Expression 4 weeks Posttransduction

While genistein has been associated with an increase in HIV-1 replication [34], the precise mechanism involved remains to be defined. As shown in Fig. 3A, Jurkat cells pretreated with genistein for 4 h, followed by a 4-h vector exposure in medium containing genistein, demonstrated an increase in eGFP expression. To determine whether the increased vector DNA copy was related to integrated forms, or merely an increase in the number of unintegrated vector genomes, we monitored transduced Jurkat cells for vector expression (GFP) and vector DNA over a 4-week period. As seen in Fig. 3B, at 24 h posttransduction, there was approximately a twofold increase in the number of total vector DNA copies in the presence of 30 μM genistein. This trend continued throughout the 4-week period. While genistein also increased the number of two-LTR circles at the early time points, circles represented only a small portion of total vector DNA (Fig. 3C). At the 4-week time point, two-LTR circles were still marginally detectable but their levels were similar in the genistein-treated and untreated cultures. In contrast, the genistein-treated cells maintained a higher level of total vector DNA, consistent with a true increase in integrated forms.

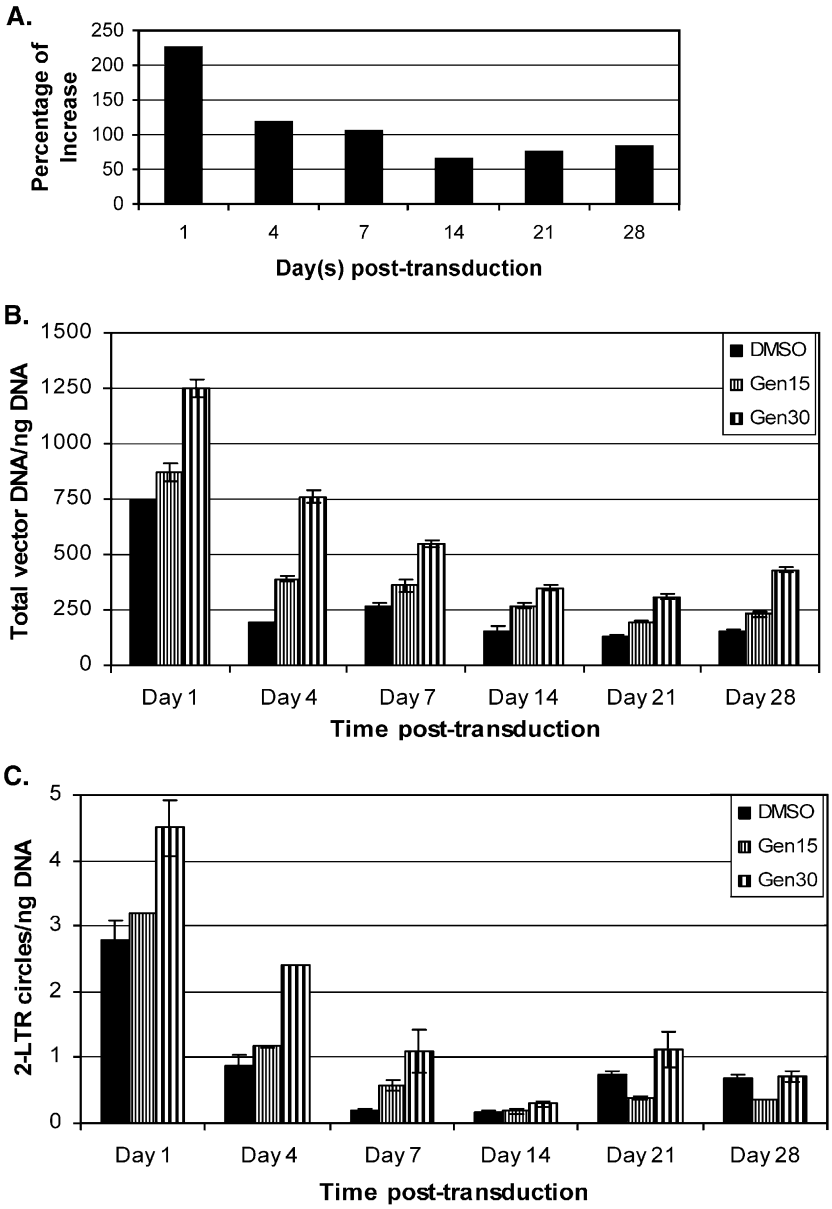

FIG. 3. Genistein increases total vector copy number. Jurkat cells were treated as indicated for 4 h followed by exposure to VSV-G-pseudotyped lentiviral supernatants at m.o.i. of 2.5 for an additional 4 h. Culture supernatants were then removed and refed with fresh medium. Cells were analyzed at various time points by FACS analysis and DNA was prepared for evaluating vector copy number. (A) Percentage of increase in GFP-positive cells (30 μM genistein treated compared to untreated) demonstrated the persistence of vector-expressing cells. (B) Real-time PCR was performed to determine copy number of lentiviral vector as described under Materials and Methods. (C) Determination of copy number of two-LTR circular vector DNA by quantitative PCR assay.

Genistein Decreases Retroviral Gene Transduction

To determine if genistein could facilitate expression of retroviral vectors derived from murine leukemia viruses, we pretreated 293 cells with varying concentrations of genistein for 4 h followed by a 4-h exposure to the amphotropically pseudotyped retroviral vector MFG-eGFP [35] at m.o.i. of 5 and 50. We analyzed the eGFP expression by FACScan 72 h posttransduction. In sharp contrast to the findings with lentiviral vectors, genistein treatment was associated with a dose-dependent decrease in gene transfer (Fig. 4A). To ensure this observation was vector and not envelope related, we used an eGFP-expressing lentiviral vector pseudotyped with the ecotropic envelope to transduce NIH/3T3 cells and compared it to the ecotropically packaged MFG-eGFP retroviral vector. As shown in Fig. 4B, genistein mediated opposite effects in lentiviral and retroviral vectors. In addition, the gene transduction is increased in VSV-G-, RRV-, and ecotropically packaged lentiviral vectors, suggesting this effect is receptor independent.

G2 Arrest Partially Contributes to the Action of Genistein on Lentiviral Transduction

Given that retroviral vectors transduce nondividing cells poorly compared to lentiviral vectors, we considered whether inhibition of retroviral transduction by genistein

doi:10.1016/j.ymthe.2006.05.022

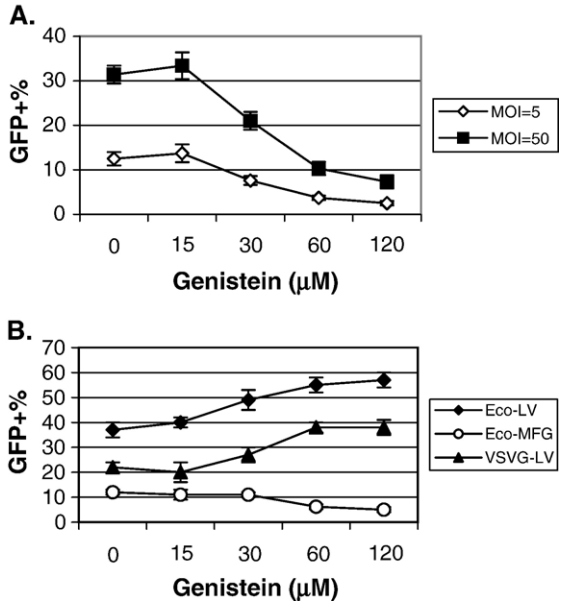

FIG. 4. Increased lentiviral transduction by genistein is vector, not envelope, dependent. (A) 293 cells were treated and transduced with MFG-eGFP retroviral supernatants at m.o.i. of 5 and 50. eGFP expression was analyzed by a FACScan 72 h later after transduction. (B) NIH/3T3 cells were transduced with ecotropic or VSV-G lentiviral or ecotropic-pseudotyped MLV supernatants (m.o.i. 0.2) for an additional 4 h following 4 h genistein pretreatment. eGFP expression was determined 72 h posttransduction.

was related to its ability to cause G2 cell cycle arrest. To test this, we analyzed cell cycle status and vector expression in transduced 293 cells treated with and without genistein. As shown in Fig. 5A, genistein is a powerful inducer of G2 arrest in 293 cells and the level of G2 arrest correlates with the level of gene transfer. Pentoxyfylline, which is known to reverse genistein G2 arrest [34,36], completely reversed G2 arrest and markedly reduced vector transduction (Fig. 5B). We saw a similar result also in Jurkat cells (data not shown). This finding is consistent with data generated from chronically HIV-1-infected promyelocytic cell lines in which pentoxyfylline also reversed the effect of genistein on HIV-1 replication [34]. Fig. 5C presents additional evidence for the role of G2 arrest in lentiviral transduction, as nocodazole-induced G2 arrest also increased lentiviral transfer. In contrast to the increased lentiviral gene transfer seen with G2 cell cycle arrest, G0 arrest induced by serum deprivation decreased lentiviral transduction (Fig. 5D).

Genistein Increases CypA Levels

CypA is known to interact specifically with the capsid protein of HIV-1, and CypA-deficient cells show a marked decrease in HIV-1 replication [9–16]. CypA does not interact with murine retroviral proteins and other lentiviruses such as HIV-2 and simian immunodeficiency

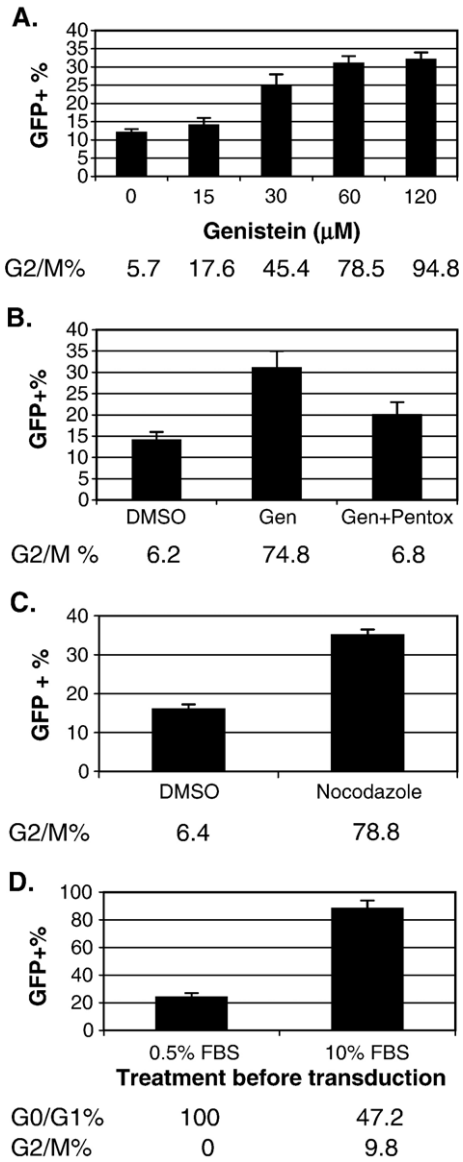

FIG. 5. G2 cell cycle arrest mediates increased lentiviral transduction by genistein. (A) Increased gene transfer by genistein correlated well with genistein-mediated G2 arrest. 293 cells were treated with varying concentrations of genistein followed by VSV-G lentiviral supernatant transduction and cell cycle analysis. (B) Pentoxyfylline (Pentox) reversed genistein-mediated G2 arrest and partially blocked increased lentiviral gene transduction by genistein simultaneously. 293 cells were treated with 60 μM genistein in the presence or absence of 1 mM Pentox. eGFP expression and cell cycle analysis were performed. (C) 293 cells were treated with 0.5 μM nocodazole, followed by transduction with VSV-G-pseudotyped lentiviral supernatants at m.o.i. of 0.5 and cell cycle analysis. (D) Lentiviral gene transfer was significantly diminished in 293 cells arrested at G0. 293 cells were cultured with DMEM containing 0.5% FCS for 72 h to induce G0 arrest. At the end of 3 days, cells were transduced with VSV-G-pseudotyped lentiviral supernatants at m.o.i. of 0.5 and cell cycle analysis was also done.

virus [9–16]. Since we determined that genistein increased lentiviral but not retroviral gene transfer, we explored whether upregulation of CypA may play a role in the genistein effect. We measured CypA protein levels by Western blot in cells treated with 60 μM genistein for 0.5–16 h. CypA protein was significantly increased at 8 h and continued to be elevated 16–24 h after genistein treatment in both 293 cells (Fig. 6A) and K562 cells (data not shown). It is likely that increased CypA protein level following genistein treatment is due to a decreased degradation of CypA, given the time course and our observation that proteasome inhibitors also increase CypA level (data not shown).

To determine if the effect of genistein on CypA protein levels was dose dependent, we treated 293 cells with increasing concentrations of genistein and transduced them simultaneously. As demonstrated in Fig. 6B, genistein markedly increased CypA expression in a dose-dependent manner, and the percentage of eGFP-positive cells increased from 19% in untreated cells to 56% in 60 μM genistein-treated cells. We obtained similar results from K562 cells (data not shown). Interestingly, nocodazole increased gene transfer and G2 arrest but did not increase CypA levels (Fig. 6C).

Cell Cycle Status and CypA Appear to Be Independent Factors Important for Lentiviral Transduction

HIV-1 replication in CypA-deficient cells is markedly decreased, although CypA is not an absolute requirement for replication [17]. As little is known about the level of CypA required for efficient lentiviral gene transfer, we examined lentiviral transduction in CypA$^{-/-}$ Jurkat cells in which the gene encoding CypA (*PPIA*) was deleted by homologous recombination [17]. As shown in Fig. 6D, lentiviral transduction was greatly diminished but not completely eliminated in CypA$^{-/-}$ Jurkat cells. Reexpres-

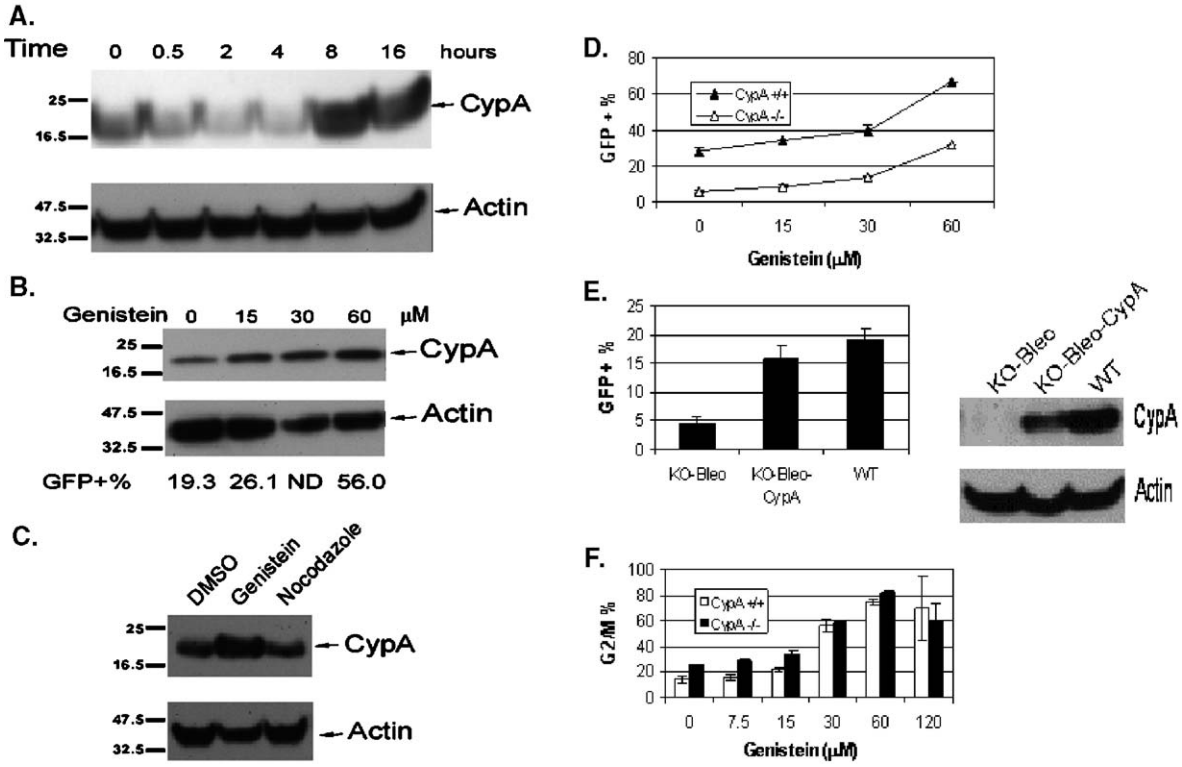

FIG. 6. CypA is not involved in the improved lentiviral transduction by genistein but is important for efficient lentiviral transduction. (A) Time course of CypA expression following genistein treatment. 293 cells were treated with 60 μM genistein for the indicated times and Western blot was performed for CypA expression. The membrane was stripped and reprobed with anti-actin as a loading control. (B) eGFP expression correlates with CypA expression levels. 293 cells were treated with increasing concentrations of genistein for 16 h. Whole cell lysates were subjected to Western blot for CypA expression. The pretreated cells were also transduced with VSV-G lentiviral supernatants at m.o.i. of 0.5. eGFP expression was analyzed. (C) 293 cells were treated with genistein or nocodazole and whole cell lysates were subjected to Western blot for CypA expression. (D) Lentiviral transduction is compromised in CypA$^{-/-}$ cells. 1×10^5 CypA$^{+/+}$ or CypA$^{-/-}$ Jurkat cells per well were seeded into six-well plates. Cells were treated, transduced with VSV-G-pseudotyped lentiviral supernatants at m.o.i. of 10, and analyzed as described above. (E) Reexpression of CypA and transduction in CypA$^{-/-}$ Jurkat cells. CypA$^{-/-}$ Jurkat cells were transiently transfected with a CypA expression plasmid using the Nucleofector Kit (Amaxa Biosystem), followed by VSV-G-pseudotyped lentiviral supernatants at m.o.i. of 10, and eGFP expression was analyzed as described above. CypA expression was determined by Western blot. The membrane was stripped and reprobed with anti-actin as a loading control. (F) CypA$^{+/+}$ or CypA$^{-/-}$ Jurkat cells were treated with increasing concentrations of genistein for 16 h followed by cell cycle analysis.

doi:10.1016/j.ymthe.2006.05.022

sion of CypA in CypA$^{-/-}$ Jurkat cells using electroporation of a CypA expression plasmid increased lentiviral transduction to levels similar to those seen in CypA$^{+/+}$ cells (Fig. 6E). Interestingly, genistein increased gene transfer in both CypA$^{+/+}$ and CypA$^{-/-}$ cells in a dose-dependent manner (Fig. 6D) and the increase correlates with the extent of G2 cell cycle arrest (Fig. 6F). CSA did abrogate the increase in gene transfer and G2 cell cycle arrest (data not shown). Unfortunately, CSA alone significantly decreased the number of CypA$^{+/+}$ and CypA$^{-/-}$ cells in G2 phase and, therefore, was not helpful in differentiating the role of cell cycle and CypA (data not shown). Nevertheless, the finding of increased gene transfer in G2-arrested CypA$^{-/-}$ cells, along with the ability of nocodazole to increase gene transfer without altering CypA (Fig. 6C), indicates that the increase in transduction associated with G2 arrest is not mediated by an increase in CypA.

DISCUSSION

To understand better the postreceptor processing of lentiviral vectors, we evaluated several classes of inhibitors of signal transduction for their effects on gene transfer. Only genistein significantly altered gene transfer, presumably by inducing G2 arrest through down-regulation of CDC25 and topoisomerase II [37,38]. This conclusion is supported by a number of findings. First, the PTK inhibitors tyrphostins failed to induce G2 arrest and did not increase gene transfer. Second, genistein is known to increase HIV-1 replication linked to its ability to cause G2 arrest [34]. G2 arrest appears important in HIV-1 replication as the accessory protein Vpr is known to induce G2 arrest [39–42], the HIV-1 LTR is most active in G2 [39,43], and the HIV-1 leader internal ribosome entry site is active during the G2/M phase of the cell cycle [44]. Our observation indicates that the important role of G2 in HIV-1 replication extends to third-generation lentiviral vectors. As the lentiviral vectors used contain defective LTRs and do not contain Vpr, additional mechanisms may play a role in regulation of HIV-1 expression during G2.

Genistein along with other protein tyrosine kinase inhibitors had been reported to promote AAV2 transduction by altering phosphorylation status of FKBP52 protein [21–24]. However, inhibition of tyrosine kinases does not appear to be the predominant mechanism by which genistein increased lentiviral transduction, as other known PTK inhibitors such as tyrphostin 1 and 23 failed to increase gene transfer. Of course, while it is possible that genistein treatment could upregulate receptors for pseudotyped lentiviral vectors, our observation that the effect of genistein is similar in three distinctly different vector pseudotypes suggests that the mechanism of increased gene transfer is not receptor mediated.

While cell cycle has long been known to play a role in the integration of retroviral and lentiviral vectors, cell cycle arrest per se does not explain the mechanism by which gene transfer rates are increased. We considered whether upregulation of CypA could be a mediator of the increased gene transfer noted during G2 cell cycle arrest. We show that CypA-deficient cells show much lower gene transfer than CypA-expressing cells, confirming the importance of this protein in lentiviral gene transfer. We did find an increase in CypA after genistein treatment that correlated with G2 arrest. On further study, we found that genistein also mediated an increase in gene transfer in CypA-deficient cells. Furthermore, nocodazole inhibition of G2 arrest increased gene transfer but did not increase CypA levels. Together these finding indicate that genistein significantly increases lentiviral vector integration and transgene expression, predominately through its ability to induce G2 cell cycle arrest. CypA deficiency markedly decreases gene transfer, but it still remains to be determined if altered expression in cells expressing CypA at endogenous levels can be manipulated to improve gene transfer.

These findings have relevance to HIV-1 biology and lentiviral gene therapy. The use of lentiviral vectors combined with agents that alter the cell cycle provides a model for postinfection processing without interference from propagating HIV-1 and can serve as a tool to determine the molecular mechanisms involved in lentiviral vector gene transfer. The use of agents that induce cell cycle arrest may also provide a means of improving gene transfer in research and clinical applications.

MATERIALS AND METHODS

Cell lines and other reagents. The human embryonic kidney epithelial cell line 293 and the K562 (human chronic myelogenous leukemia), Swiss mouse NIH/3T3, and HEL92.1.7 (human erythroleukemia) cell lines were obtained from the American Tissue Culture Collection. All cell lines were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum (FCS; Hyclone, Logan, UT, USA), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. CypA$^{+/+}$ and CypA$^{-/-}$ Jurkat cells have been previously described. They were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Genistein, nocodazole, dexamethasone, tyrphostin-1, tyrphostin-23, pentoxyfylline, cyclosporin A, SB203580, PD98059, LY294002, and PTX were purchased from Calbiochem (La Jolla, CA, USA). Compounds were dissolved in DMSO except for pentoxyfylline and PTX, which were dissolved in DMEM, and CSA, which was dissolved in ethanol. The final concentration of DMSO or ethanol was 0.1% (v/v). Rabbit anti-cyclophilin A antibodies were purchased from the Upstate Biolabs (Lake Placid, NY, USA).

Vector production. Lentiviral vectors were produced by transient transfection of 293T cells as described previously [45,46]. Vector-containing supernatants were harvested 48 h after transfection, filtered through a 0.45-μm syringe filter, and then stored at −80°C. Infectious titers were determined using 293 cells as previously described. A stable E86 cell line transfected with MFG-eGFP retrovirus vector expressing the eGFP was

provided by Karen Pollok [47]. The MFG-eGFP retroviral vector was also amphotropically pseudotyped using the GP+envAM12 cell line [35] and cell-free supernatants were obtained by growing cells to confluence, refeeding with fresh medium, collecting supernatant 18 h after refeeding, filtering through a 0.45-μm filter, and storing at −70°C until use.

Preparation and transduction of human CD34⁺ cells. Frozen human peripheral CD34⁺ cells were thawed, resuspended in complete IMDM containing 100 ng/ml human stem cell factor (SCF), and incubated overnight followed by a stimulation for 24 h with granulocyte colony-stimulating factor, SCF, and MGDF (100 ng/ml each). Cells were pretreated with genistein for 4 h and then transduced with ultrafiltered and concentrated VSV-G-pseudotyped lentiviral supernatants (m.o.i. 100) for 18 h after a removal of genistein. Finally cells were plated in triplicate in methylcellulose-based medium H4434 (Stem Cell Technology) for colony formation analysis. Colonies were assessed for morphology and eGFP expression after 14 days incubation as previously described [47].

Mouse lymphocyte preparation, treatment, and transduction. Total spleen and lymph node cells from CH3 mice purchased from Harlan Bioproducts (Indianapolis, IN, USA) were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and activated for 72 h with 2 μg/ml plate-bound anti-CD3 (clone 145-2C11) as previously described [48]. Nonadherent cells were washed twice with complete medium and pretreated with genistein for 4 h followed by transduction with VSV-G lentiviral vector supernatants at m.o.i. of 15 overnight in the presence of 8 μg/ml Polybrene. Culture supernatants were removed the next day and cells were refed with fresh complete medium. eGFP expression was analyzed 72 h later by fluorescence-activated cell sorting (FACScan; Becton–Dickinson, San Jose, CA, USA). Similarly, 293, NIH/3T3, HEL, or K562 cells were treated, transduced, and analyzed.

Vector DNA detection in transduced Jurkat cells by real-time PCR. Total vector DNA and two-LTR circular vector DNA were detected using a modified real-time PCR assay initially described by Butler *et al*. [49]. Briefly, the modifications adapted to this assay include the following: Total cellular DNA was prepared from transduced Jurkat cells treated with either DMSO or 15 or 30 μM genistein for 4 h, using a Puregene DNA Purification Kit (Gentra Systems, Inc., Minneapolis, MN, USA), from which a 500-ng sample was used for each reaction. To determine total vector DNA copy number, a standard curve was generated using the copy numbers from serial dilutions of plasmid DNA corresponding to the vector plasmid used for vector production as a reference. To determine the copy number of two-LTR circular vector DNA, a standard curve was generated using the copy numbers from serial dilutions of a two-LTR circle reference plasmid that was generated upon the introduction of a two-LTR fragment into a cloning vector, pCR4-TOPO (Invitrogen, Carlsbad, CA, USA). Primer and probe concentrations and the PCR cycling conditions used were described previously [49]. Each sample was run in duplicate and data analysis was performed using Sequence Detection Systems 1.9.1 software (Applied Biosystems, Foster City, CA, USA).

Western blotting. 293 cells at about 80% confluence or K562 cells at 5×10^5 cells/ml were treated with genistein as indicated. Whole cell lysates were made using 1× cell lysis buffer (New England Biolabs) containing a protease inhibitor cocktail (Roche, Indianapolis, IN, USA). Equal amounts (about 30 μg) of cell lysates were resolved on 4–12% SDS–PAGE, followed by Western blot using rabbit anti-CypA polyclonal antibodies. The membranes were stripped and reprobed with anti-actin as a loading control.

Cell cycle analysis. Cells were treated with compound as indicated for 8 h. Cell culture supernatants were completely removed and the cells were incubated overnight before propidium iodide (Sigma, St. Louis, MO, USA) staining [34]. Briefly, 2×10^5 cells were washed once with PBS. Cells were incubated with 200 μl of 10 mg/ml RNase A (Roche) and 200 μl of propidium iodide (50 μg/ml) on ice for 30 min and analyzed by FACScan. Cell cycle status was analyzed using ModFitLZ v2.0 software.

ACKNOWLEDGMENTS

We thank Dr. Xiao-Fan Wang (Department of Pharmacology and Cancer Biology, Duke University Medical Center) for the CypA expression plasmid. The Indiana University Vector Production Facility is a NIH-designated National Gene Vector Laboratory (U42RR11148) and this work was supported, in part, by a Core Centers of Excellence in Molecular Hematology grant (PHS P50DK49218) and a core laboratory supporting grant (PHSP01HL53586). K.C. is supported in part by the Indiana Genomic Initiative created through a grant from the Lilly Endowment, Inc. We are also grateful to Yi Xu, Teresa Johnson, and Jing Yao for their technical assistance.

RECEIVED FOR PUBLICATION SEPTEMBER 23, 2005; REVISED MAY 22, 2006; ACCEPTED MAY 31, 2006

REFERENCES

1. Amado, R. G., and Chen, I. S. (1999). Lentiviral vectors—The promise of gene therapy within reach? *Science* **285:** 674–676.
2. Blomer, U., Naldini, L., and Kafri, T. (1997). Highly efficient and sustained gene transfer in adult neurons with a lentiviral vector. *J. Virol.* **71:** 6641–6649.
3. Naldini, L., *et al.* (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. *Science* **272:** 263–267.
4. Podsakoff, G. M. (2001). Lentiviral vectors approach the clinic but fall back: National Institutes of Health Recombinant DNA Advisory Committee review of a first clinical protocol for use of a lentiviral vector. *Mol. Ther.* **4:** 282–283.
5. Lever, A. M. (2000). Lentiviral vectors: progress and potential. *Curr. Opin. Mol. Ther.* **2:** 488–496.
6. Watson, D. J., Kobinger, G. P., Passini, M. A., Wilson, J. M., and Wolfe, J. H. (2002). Targeted transduction patterns in the mouse brain by lentivirus vectors pseudotyped with VSV, Ebola, Mokola, LCMV, or MuLV envelope proteins. *Mol. Ther.* **5:** 528–537.
7. Handschumacher, R. E., Harding, M. W., Rice, J., Drugge, R. J., and Speicher, D. W. (1984). Cyclophilin: a specific cytosolic binding protein for cyclosporin A. *Science* **226:** 544–547.
8. Gething, M. (1992). Protein folding in the cells. *Nature* **355:** 33–45.
9. Franke, E. K., Yuan, H. E., and Luban, J. (1994). Specific incorporation of cyclophilin A into HIV-1 virions. *Nature* **372:** 359–362.
10. Luban, J. (1996). Absconding with the chaperone: essential cyclophilin–Gag interaction in HIV-1 virions. *Cell* **87:** 1157–1159.
11. Luban, J., Bossolt, K. L., Franke, E. K., Kalpana, G. V., and Goff, S. P. (1993). Human immunodeficiency virus type 1 Gag protein binds to cyclophilins A and B. *Cell* **73:** 1067–1078.
12. Thali, M., *et al.* (1994). Functional association of cyclophilin A with HIV-1 virions. *Nature* **372:** 363–365.
13. Braaten, D., Franke, E. K., and Luban, J. (1996). Cyclophilin A is required for the replication of group M human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus SIV(CPZ)GAB but not group O HIV-1 or other primate immunodeficiency viruses. *J. Virol.* **70:** 4220–4227.
14. Saphire, A. C., Bobardt, M. D., and Gallay, P. A. (2000). Human immunodeficiency virus type 1 hijacks host cyclophilin A for its attachment to target cells. *Immunol. Res.* **21:** 211–217.
15. Yin, L., Braaten, D., and Luban, J. (1998). Human immunodeficiency virus type 1 replication is modulated by host cyclophilin A expression levels. *J. Virol.* **72:** 6430–6436.
16. Franke, E. K., and Luban, J. (1996). Inhibition of HIV-1 replication by cyclosporine A or related compounds correlates with the ability to disrupt the Gag–cyclophilin A interaction. *Virology* **222:** 279–282.
17. Braaten, D., and Luban, J. (2001). Cyclophilin A regulates HIV-1 infectivity, as demonstrated by gene targeting in human T cells. *EMBO J.* **20:** 1300–1309.
18. Saphire, A. C., Bobardt, M. D., and Gallay, P. A. (2002). Cyclophilin A plays distinct roles in human immunodeficiency virus type 1 entry and postentry events, as revealed by spinoculation. *J. Virol.* **76:** 4671–4677.
19. Anonymous (1999). Host cyclophilin A mediates HIV-1 attachment to target cells via heparans. *EMBO J.* **18:** 6771–6785.
20. Pushkarsky, T., *et al.* (2001). CD147 facilitates HIV-1 infection by interacting with virus-associated cyclophilin A. *Proc. Natl. Acad. Sci. USA* **98:** 6360–6365.
21. Qing, K., Wang, X. S., Kube, D. M., Ponnazhagan, S., Bajpai, A., and Srivastava, A. (1997). Role of tyrosine phosphorylation of a cellular protein in adeno-associated virus 2-mediated transgene expression. *Proc. Natl. Acad. Sci. USA* **94:** 10879–10884.
22. Qing, K., *et al.* (1998). Adeno-associated virus type 2-mediated gene transfer: correlation of tyrosine phosphorylation of the cellular single-stranded D sequence-binding protein with transgene expression in human cells in vitro and murine tissues in vivo. *J. Virol.* **72:** 1593–1599.
23. Qing, K., Hansen, J., Weigel-Kelley, K. A., Tan, M., Zhou, S., and Srivastava, A. (2001). Adeno-associated virus type 2-mediated gene transfer: role of cellular FKBP52 protein in transgene expression. *J. Virol.* **75:** 8968–8976.

doi:10.1016/j.ymthe.2006.05.022

24. Mah, C., *et al.* (1998). Adeno-associated virus type 2-mediated gene transfer: role of epidermal growth factor receptor protein tyrosine kinase in transgene expression. *J. Virol.* **72:** 9835 – 9843.
25. Cohen, P. S., *et al.* (1997). The critical role of p38 MAP kinase in T cell HIV-1 replication. *Mol. Med.* **3:** 339 – 346.
26. Kumar, S., Orsini, M. J., Lee, J. C., McDonnell, P. C., Debouck, C., and Young, P. R. (1996). Activation of the HIV-1 long terminal repeat by cytokines and environmental stress requires an active CSBP/p38 MAP kinase. *J. Biol. Chem.* **271:** 30864 – 30869.
27. Shapiro, L., Heidenreich, K. A., Meintzer, M. K., and Dinarello, C. A. (1998). Role of p38 mitogen-activated protein kinase in HIV type 1 production in vitro. *Proc. Natl. Acad. Sci. USA* **95:** 7422 – 7426.
28. Jacque, J. M., *et al.* (1998). Modulation of HIV-1 infectivity by MAPK, a virion-associated kinase. *EMBO J.* **17:** 2607 – 2618.
29. Yang, X., Chen, Y., and Gabuzda, D. (1999). ERK MAP kinase links cytokine signals to activation of latent HIV-1 infection by stimulating a cooperative interaction of AP-1 and NF-kappaB. *J. Biol. Chem.* **274:** 27981 – 27988.
30. Yang, X., and Gabuzda, D. (1999). Regulation of human immunodeficiency virus type 1 infectivity by the ERK mitogen-activated protein kinase signaling pathway. *J. Virol.* **73:** 3460 – 3466.
31. Francois, F., and Klotman, M. E. (2003). Phosphatidylinositol 3-kinase regulates human immunodeficiency virus type 1 replication following viral entry in primary $CD4^+$ T lymphocytes and macrophages. *J. Virol.* **77:** 2539 – 2549.
32. Alkhatib, G., Locati, M., Kennedy, P. E., Murphy, P. M., and Berger, E. A. (1997). HIV-1 coreceptor activity of CCR5 and its inhibition by chemokines: independence from G protein signaling and importance of coreceptor downmodulation. *Virology* **234:** 340 – 348.
33. Lin, Y. L., Mettling, C., Portales, P., Reynes, J., Clot, J., and Corbeau, P. (2002). Cell surface CCR5 density determines the postentry efficiency of R5 HIV-1 infection. *Proc. Natl. Acad. Sci. USA* **99:** 15590 – 15595.
34. Gozlan, J., Lathey, J. L., and Spector, S. A. (1998). Human immunodeficiency virus type 1 induction mediated by genistein is linked to cell cycle arrest in G2. *J. Virol.* **72:** 8174 – 8180.
35. Markowitz, D., Goff, S., and Bank, A. (1988). Construction and use of a safe and efficient amphotropic packaging cell line. *Virology* **167:** 400 – 406.
36. Poon, B., Jowett, J. B., Stewart, S. A., Armstrong, R. W., Rishton, G. M., and Chen, I. S. (1997). Human immunodeficiency virus type 1 vpr gene induces phenotypic effects similar to those of the DNA alkylating agent, nitrogen mustard. *J. Virol.* **71:** 3961 – 3971.
37. Li, Y., and Sarkar, F. H. (2002). Gene expression profiles of genistein-treated PC3 prostate cancer cells. *J. Nutr.* **132:** 3623 – 3631.
38. Suzuki, K., *et al.* (2002). Genistein, a soy isoflavone, induces glutathione peroxidase in the human prostate cancer cell lines LNCaP and PC-3. *Int. J. Cancer* **99:** 846 – 852.
39. Gummuluru, S., and Emerman, M. (1999). Cell cycle- and Vpr-mediated regulation of human immunodeficiency virus type 1 expression in primary and transformed T-cell lines. *J. Virol.* **73:** 5422 – 5430.
40. He, J., Choe, S., Walker, R., Di Marzio, P., Morgan, D. O., and Landau, N. R. (1995). Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. *J. Virol.* **69:** 6705 – 6711.
41. Zander, K., *et al.* (2003). Cyclophilin A interacts with HIV-1 Vpr and is required for its functional expression. *J. Biol. Chem.* **278:** 43202 – 43213.
42. Zhou, Y., and Ratner, L. (2000). Phosphorylation of human immunodeficiency virus type 1 Vpr regulates cell cycle arrest. *J. Virol.* **74:** 6520 – 6527.
43. Goh, W. C., *et al.* (1998). HIV-1 Vpr increases viral expression by manipulation of the cell cycle: a mechanism for selection of Vpr in vivo. *Nat. Med.* **4:** 65 – 71.
44. Brasey, A., *et al.* (2003). The leader of human immunodeficiency virus type 1 genomic RNA harbors an internal ribosome entry segment that is active during the G2/M phase of the cell cycle. *J. Virol.* **77:** 3939 – 3949.
45. Kahl, C. A., Marsh, J., Fyffe, J., Sanders, D. A., and Cornetta, K. (2004). Human immunodeficiency virus type 1-derived lentivirus vectors pseudotyped with envelope glycoproteins derived from Ross River virus and Semliki Forest virus. *J. Virol.* **78:** 1421 – 1430.
46. Dull, T., *et al.* (1998). A third-generation lentivirus vector with a conditional packaging system. *J. Virol.* **72:** 8463 – 8471.
47. Pollok, K. E., *et al.* (2001). Differential transduction efficiency of SCID-repopulating cells derived from umbilical cord blood and granulocyte colony-stimulating factor-mobilized peripheral blood. *Hum. Gene Ther.* **12:** 2095 – 2108.
48. Zhang, S., and Kaplan, M. H. (2000). The p38 mitogen-activated protein kinase is required for IL-12-induced IFN-gamma expression. *J. Immunol.* **165:** 1374 – 1380.
49. Butler, S. L., Hansen, M. S., and Bushman, F. D. (2001). A quantitative assay for HIV DNA integration in vivo. *Nat. Med.* **7:** 631 – 634.