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Cell Cycle Arrest in G_2/M Promotes Early Steps of Infection by Human Immunodeficiency Virus

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We have identified four small molecules that boost transduction of cells by human immunodeficiency virus (HIV) and investigated their mechanism of action. These molecules include etoposide and camptothecin, which induce DNA damage by inhibiting religation of cleaved topoisomerase-DNA complexes, taxol, which interferes with the function of microtubules, and aphidicolin, which inhibits DNA polymerases. All four compounds arrest the cell cycle at G_2/M, though in addition high concentrations of aphidicolin arrest in G_1. We find that early events of HIV replication, including synthesis of late reverse transcription products, two-long terminal repeat circles, and integrated proviruses, were increased after treatment of cells with concentrations of each compound that arrested in G_2/M. Stimulation was seen for both transformed cell lines (293T and HeLa cells) and primary cells (IMR90 lung fibroblasts). Arrest in G_1 with high concentrations of aphidicolin boosted transduction, though not much as with lower concentrations that arrested in G_2/M. Arrest of IMR90 cells in G_1 by serum starvation and contact inhibition reduced transduction. Previously, the proteasome inhibitor MG132 was reported to increase HIV infection—here we investigated the effects of combinations of the cell cycle inhibitors with MG132 and obtained data suggesting that MG132 may also boost transduction by causing G_2/M cell cycle arrest. These data document that cell cycle arrest in G_2/M boosts the early steps of HIV infection and suggests methods for increasing transduction with HIV-based vectors.

Human immunodeficiency virus (HIV) replication is modulated by the state of the host cell cycle. The HIV-1 viral protein Vpr, for example, arrests cell cycle progression at G_2/M, which allows more efficient transcription from the viral long terminal repeat (6). Vpr has been proposed to regulate cell cycle progression by inhibiting dephosphorylation to activate Cdc2-cyclin B (5, 6, 9, 17, 23) and by activating expression of the cyclin-dependent kinase inhibitor p21/Waf1/Cip1 (3). Arrest of cells by chemical agents such as genistein has also been reported to increase HIV promoter activity and viral production from infected cells (7). Here we report that arrest of cells in G_2/M can also increase the efficiency of earlier steps of the viral life cycle prior to integration.

A variety of antitumor agents such as etoposide (ETP) and camptothecin (CPT) arrest the cell cycle in G_2/M by activating a DNA damage-responsive checkpoint. ETP inhibits topoisomerase II by trapping the enzyme in a complex with cleaved DNA and thereby induces DNA double-strand breaks (13). CPT is an inhibitor of DNA topoisomerase I that blocks DNA religation of covalent topoisomerase I-DNA complexes, trapping DNA nicks (13). DNA synthesis through stalled topoisomerase I covalent complexes potentially generates DNA double-strand breaks.

We also studied two additional compounds that arrest the cell cycle. The antitumor agent taxol/paclitaxel (TAX) blocks the cell cycle in G_2/M by modulating microtubule polymerization. Aphidicolin (APC) inhibits DNA polymerases delta and epsilon. In the presence of high levels of APC, cell cycle progression is blocked in G_1.

Here we report that all four of these agents, when applied to cells at optimized concentrations, can boost early steps of HIV transduction. After initiation of HIV-1 infection, the HIV RNA genome is reverse transcribed to yield a double-stranded linear DNA copy of the viral genome. The viral cDNA translocates to the host cell nucleus, where linear viral DNA becomes integrated into the host chromosome. Some viral DNAs can follow dead-end pathways, in which the viral DNA becomes circularized by homologous recombination to yield one-long terminal repeat (1-LTR) circles or ligated end to end by the cellular nonhomologous DNA end-joining (NHEJ) pathway (10, 14) to form 2-LTR circles. We find that G_2/M cell cycle arrest promotes accumulation of late reverse transcription (RT) products, 2-LTR circles, and integrated proviruses. A lesser boost in transduction could be seen after arrest of cells in G_1 by treatment with high concentrations of APC, but lower concentrations, which arrested in G_2/M, showed a more robust boost. The increased transduction due to G_2/M arrest could be detected in several transformed cell lines and a primary cell type. Further studies allow aspects of the stimulatory mechanism to be proposed.

MATERIALS AND METHODS

Cells, viruses, and HIV-based vectors. 293T, IMR90, and HelaP4 cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin-glutamine. HIV R9 and NL4-3 viruses were produced by transfection of 293T cells with pR9 and pNL4-3. The self-inactivating HIV-based vector SM2-GFP inducing single-cycle infection was produced from the inducible cell line SKSM2 as described previously (8). HIV-based vector HIV-LTR-Tat-IRES-GFP (pEV731) was originally described by

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Jordan et al. (11). Multiplicity of infection (MOI) refers to the titer determined on 293T cells.

Viral infections and DNA isolation. HIV virus or HIV-based vector containing supernatants were added to cells in minimal volume with DEAE-dextran (20 μg/ml) and incubated at 37°C. After 3 h, cells were washed with phosphate-buffered saline (PBS) and fresh medium was added. Cells were collected at 12, 24, 48, and 72 h postinfection and washed once with PBS, and DNA was harvested using the DNeasy tissue kit (QIAGEN, Valencia, CA).

Treatment to induce G_2/M cell cycle arrest. ETP, CPT, TAX, and APC were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) and dissolved in dimethyl sulfoxide. MG132 was purchased from BioMol (Plymouth Meeting, PA). Cells in a density of 2×10^5/ml were treated with the indicated concentrations of each compound. Cells were cultured in drug-containing medium for the entire time period of the experiments.

Cell cycle analysis by fluorescence-activated cells sorting (FACS). Cells were collected and fixed by resuspending them in 0.5 ml of 70% ethanol for 30 min and then centrifuged at 2,000 rpm ($800 \times g$) for 10 min and washed in ice-cold PBS. The cell pellets were resuspended in 0.5 ml PBS containing 50 μg/ml propidium iodide (Sigma-Aldrich Chemical Co., St. Louis, MO) and 100 μg/ml RNase (Invitrogen, Carlsbad, CA), incubated at 37°C for 30 min, and then analyzed using a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

Quantitative real-time PCR (TaqMan). DNA samples were quantitated by optical density at 260 nm and brought to a concentration of 25 ng/μl before use in quantitative PCR. The primers used to detect late RT products, 2-LTR circles, and the number of proviral DNA were as follows: LRT-F (5′-TGTGTGCCCG TCTGTTGTGT-3′); LRT-R (5′-GAGTCCTGCGTCGAGAGAGC-3′); LRT-P [5′-(FAM)-CAGTGGCGCCCGAACAGGGA-(TAMRA)-3′]; 2-LTR-F (5′-AA CTAGGGAACCCACTGCTTAAG-3′); 2-LTR-R (5′-CCACAGATCAAGGA TATCTTGTC-3′); 2-LTR-P [5′-(FAM)-ACACTACTTGAAGCACTCAAGGC AAGCTTT-(TAMRA)-3′]; Alu forward, LRT-F (above); Alu-R (5′-TGCTGG GATTACAGGCGTGAG-3′); Alu probe, LRT-P (above). Reaction mixtures contained 1× TaqMan universal master mix (PE-Applied Biosystems, Branchburg, NJ), 300 nM forward primer, 300 nM reverse primer, 100 nM probe primer, and 250 ng of template DNA. After initial incubations at 50°C for 2 min and 95°C for 10 min, 40 cycles of amplification were carried out for 15 s at 95°C followed by 1 min at 60°C. Data were analyzed using the ABI Prism 7700 sequence detection system (PE-Applied Biosystems, Foster City, CA). Results were analyzed with ABI Prism SDS software. The highest values are normalized to 100% to facilitate comparisons among multiple cell types. To avoid PCR artifacts due to carryover of DNA after transfection to generate HIV vector stocks, viral stocks were prepared from an inducible cell line (8). Early time points after infection (analyzed in some experiments) showed low values as expected (data not shown). All experiments shown in the figures as a single panel were carried out side by side with the same viral stocks.

Transcriptional profiling. Total RNA was isolated from cells using an RNeasy Midi kit (QIAGEN, Valencia, CA) and concentrated to 0.5 μg/μl in Tris-EDTA buffer. Labeled RNA (cRNA) was prepared as described in the Affymetrix protocol and was hybridized to Affymetrix GeneChip HG-U133A. Data were processed using Affymetrix GeneChip MicroArray Suite software version 5.1. Significantly affected genes were identified using the Significance Analysis of Microarray (SAM) package (http://www-stat.Stanford.EDU/~tibs/SAM/). Statistical analysis of gene ontology employed the Fischer exact test as implemented by EASE (http://david.niaid.nih.gov/david/ease.htm) without correction for multiple comparisons.

RESULTS

Arresting 293T cells in G_2/M boosts transduction with HIV-based vectors. ETP, CPT, TAX, and APC were tested for their effects on transduction of 293T cells by an HIV-based vector (SM2-GFP), which expresses green fluorescent protein (GFP) under the control of a cytomegalovirus (CMV) promoter. Cells were simultaneously treated with increasing concentrations of each inhibitor. Two days later the percentage of GFP-positive cells was quantified by FACS analysis and the cell cycle status of the cultures was analyzed (Fig. 1). A peak in transduction was observed when cells were treated with 2 μM ETP, 0.4 μM CPT, 2 μM APC (Fig. 1A), or 25 nM TAX (Fig. 1B). Transduction was not increased as much in the presence of higher concentrations of the drugs. These higher concentrations resulted in increased toxicity to cells, which probably accounted for the diminished transduction.

Increased HIV-1 transduction correlated closely with cell cycle arrest in G_2/M. About 80% of cells treated with 2 μM ETP or 0.4 μM CPT were arrested in G_2/M as shown by FACS analysis (Fig. 1C). With APC, 58% of cells were arrested in G_2/M at 2 μM, the optimum concentration for increasing transduction. At 10 μM APC, arrest at G_1 predominated (63% of cells), but there was only a slight increase in transduction under these conditions (Fig. 1A).

TAX, which arrests cells in G_2/M by disregulating microtubule polymerization, also boosted transduction (Fig. 1B), with the maximum transduction at 25 nM correlating with the most complete block in G_2/M. Thus, the stimulatory effect of cell cycle arrest is not restricted to agents that cause accumulation of DNA ends (either by disrupting DNA replication or trapping topoisomerase intermediates).

Arresting IMR90 cells in G_2/M boosts transduction with HIV-based vectors. To assess the effects of ETP, CPT, TAX, and APC in a primary cell type, infection was studied in IMR90 cells, which are primary lung fibroblasts (Fig. 2). In this case as well, all four compounds boosted transduction. Optimal concentrations of compounds for boosting transduction were the same as for 293T cells. The cell cycle profiles for the IMR90 cells treated with the four compounds were somewhat more heterogeneous than for 293T cells, though the directions were similar. Treatment of cells with 2 μM ETP or CPT resulted in predominant arrest in G_2/M (47% and 49%), though more cells were arrested in other cell cycle stages than for 293T. APC arrested predominantly in G_2/M at 0.4 μM (32%) and at G_1 at 2 μM (59%). TAX at 25 nM showed a greatly increased population of cells in G_2/M (39%), though G_1-arrested cells were the most frequent (42%). Thus, as for 293T cells, the concentrations of small molecules that maximized the fraction of cells in G_2/M were the most effective in increasing transduction with the HIV-based vector.

Assays with another HIV-based vector. The data in Fig. 1 and 2 document that arrest in G_2/M boosts transduction by the HIV vector SM2-GFP in two cell types (293T and IMR90). To test the generality of these findings, infection was compared with another HIV-based vector, HIV-LTR-Tat-IRES-GFP. This vector as well showed a boost in transduction in the presence of 2 μM ETP (Fig. 3A).

Assays with replication-competent HIV. To test whether stimulation could also be seen with replication-competent HIV, HelaP4 indicator cells were treated with 2 μM ETP and infected with HIV-1 stocks generated using two different HIV DNA clones (R9 and NL4-3). The number of infected centers recovered was increased 3.9- and 3.3-fold in the treated cells compared to nontreated cells (Fig. 3B and C). These results indicate that cycle arrest induced by ETP enhances transduction of replication-competent HIV as well as HIV-based vectors.

Effects of G_2/M arrest on transcription of integrated HIV-1 proviruses does not fully explain the boost in transduction. Cell cycle arrest in G_2/M induced by Vpr has been reported to maximize viral production because this stage of the cell cycle is optimal for LTR transcription (6). To assess the part of the viral life cycle affected in our experiments, we tested the effects of ETP on transcription of marker genes in the HIV-based

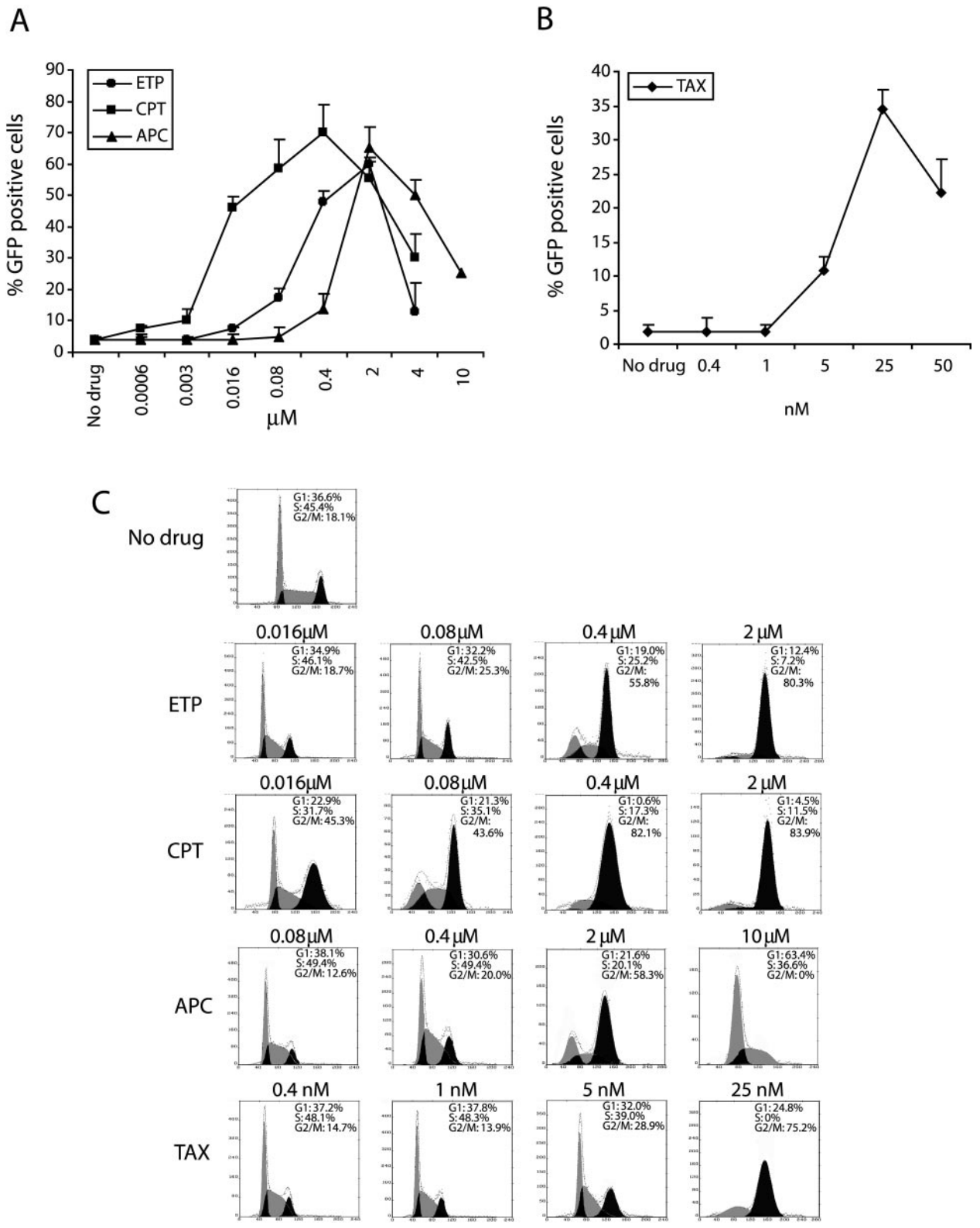

FIG. 1. Increased HIV transduction in arrested 293T cells. 293T cells were infected with HIV-based vector SM2-GFP (MOI, 0.05) and simultaneously treated with various concentrations of ETP, CPT, or APC (A) or TAX (B). Two days later the percentage of GFP expression was measured by FACS analysis. (C) Cell cycle analysis of 293T cells treated with increasing concentrations of ETP, CPT, TAX, and APC and infected with HIV-based vector SM2-GFP (measured after 48 h of treatment). The *y* axis denotes cell count and the *x* axis represents DNA content. The percentages of cells in the G_1, G_2/M, and S phases of the cell cycle were calculated by using Multicycle software.

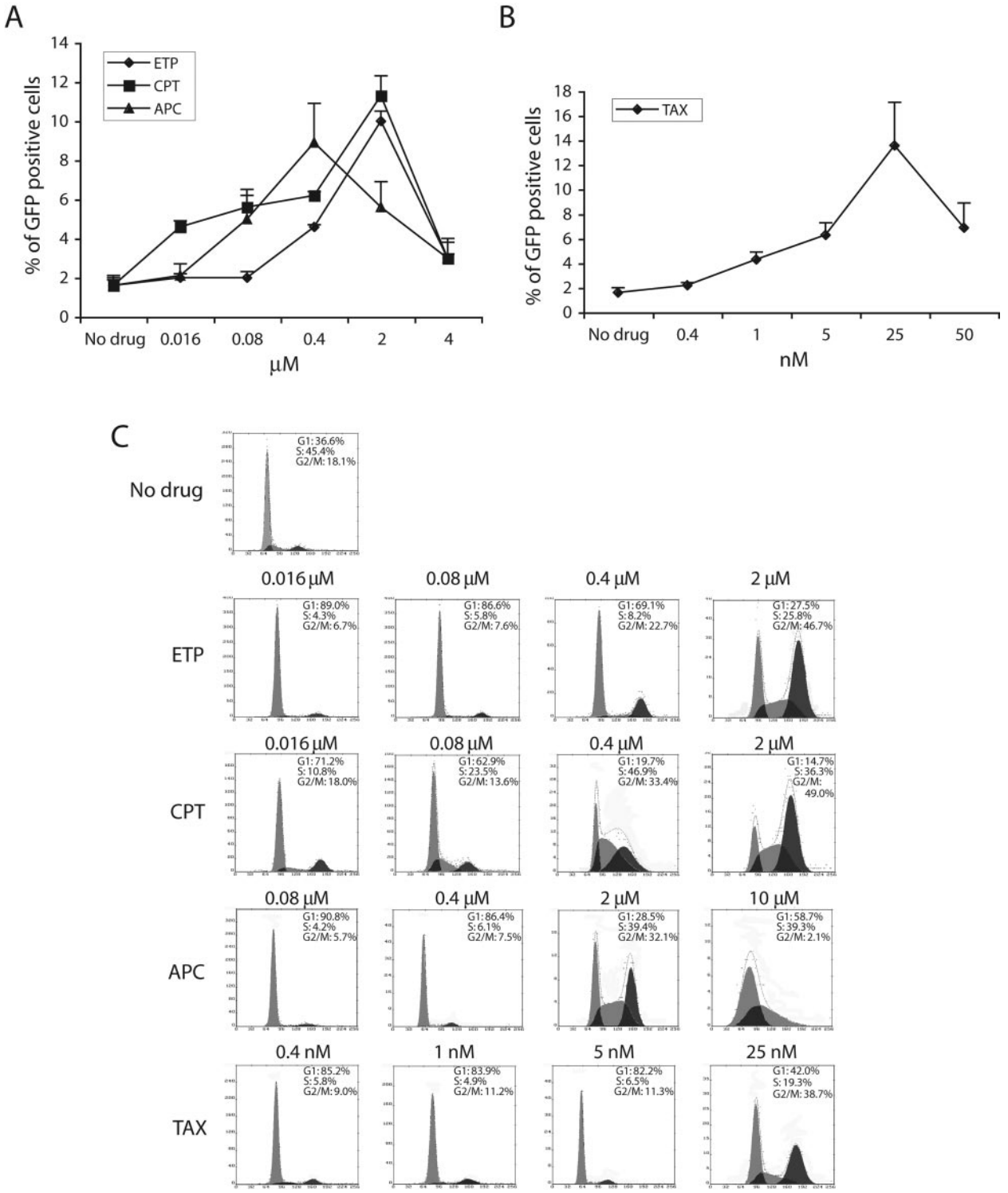

FIG. 2. Increased HIV transduction in arrested IMR90 cells. IMR90 cells were infected with HIV-based vector SM2-GFP (MOI, 0.05) and simultaneously treated with various concentrations of ETP, CPT, or APC (A) or TAX (B). Two days later the percentage of GFP expression was measured by FACS analysis. (C) Cell cycle analysis of IMR90 cells treated with increasing concentrations of ETP, CPT, TAX, and APC and infected with HIV-based vector SM2-GFP (measured after 48 h of treatment). The *y* axis denotes cell count and the *x* axis represents DNA content. The percentages of cells in the G_1, G_2/M, and S phases of the cell cycle were calculated by using Multicycle software.

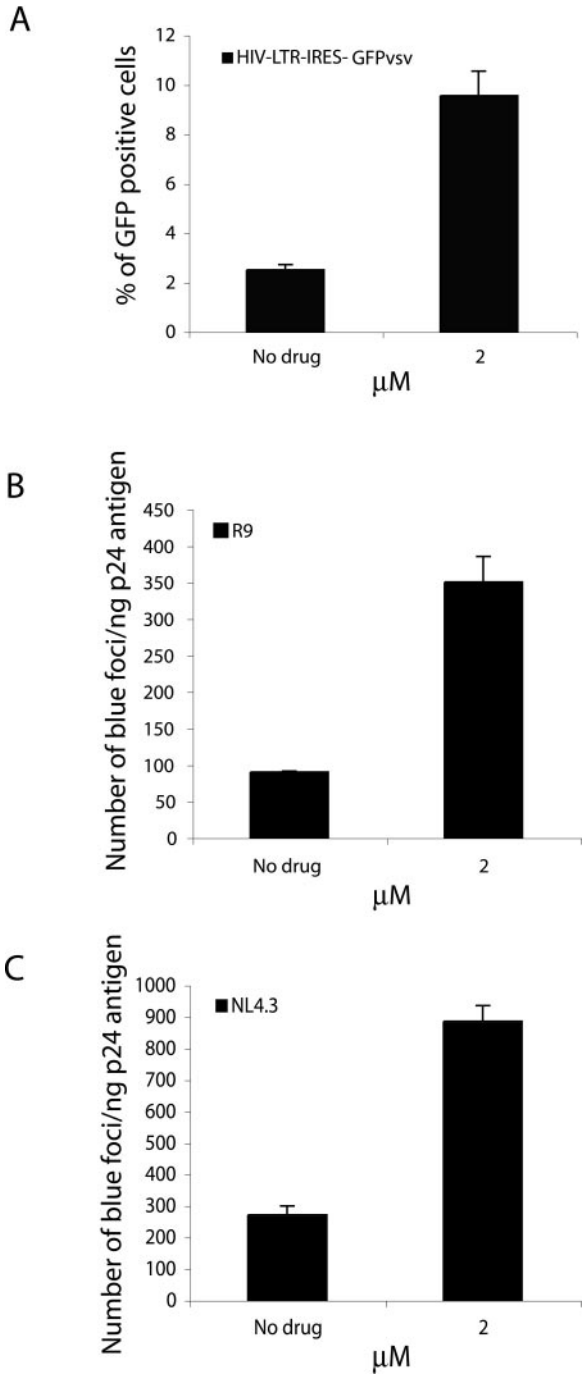

FIG. 3. Treatment with ETP boosts transduction with wild-type HIV, with another HIV-based vector, and in another cell type (HeLaP4 cells). (A) 293T cells were infected with HIV-based vector HIV-LTR-Tat-IRES-GFP at an MOI of 0.03 and treated with 2 μM ETP. Forty-eight hours later, the percentage of GFP expression was measured by FACS analysis. (B) HIV R9 or (C) HIV NL4-3 were used to infect HelaP4 indicator cells in the presence or absence of 2 μM ETP. After 48 h, cells were stained for β-galactosidase activity and the number of blue foci was counted.

vector studied here. In the SM2-GFP vector the transcription of GFP is under the control of the CMV promoter-enhancer.

We infected 293T cells with SM2-GFP at four different MOIs and then cultured the cells for 3 days, sufficient time for integration to be mostly completed (1). We then treated infected cells with various concentrations of ETP. Forty-eight hours later, the number of GFP-positive cells was determined by FACS analysis. Treatment with 2 μM ETP resulted in 1.2-, 3-, 1.7-, and 3.5-fold increases in transcription of integrated proviruses when cells were infected with MOIs 0.5, 0.1, 0.05, and 0.02, respectively (Fig. 4). Thus, the CMV promoter driving GFP in the HIV vector used was also up-regulated slightly by G_2/M arrest, as seen with the HIV LTR (6). However, it seems unlikely that the increase in transcription explains the increase in titer seen after treatment in Fig. 1 to 3 because (i) the increase of titer was often in the five- to sixfold range, greater than the observed increase in transcription, and (ii) direct analysis of viral DNA accumulation at short times after infection showed an increase (described below).

Reversibility of the cell cycle block. The toxicity of these treatments was assessed by exposing 293T cells or IMR90 cells to 2 μM ETP, 1 μM CPT, or 25 nM TAX for 12 h and then asking what fraction of cells could return to growth after wash out of the drug (data not shown). For IMR90 cells, exposure to each of the treatments resulted in cell cycle arrest, but after wash out of the drug, the cells appeared morphologically normal and resumed doubling at the normal rate. Exposure of 293T cells to TAX followed by wash out allowed most of the cells to return to growth. CPT treatment was more toxic, but a substantial fraction of cells recovered after wash out and prolonged incubation. The 293T cells did not recover from ETP treatment. Thus, the G_2/M block that results in increased transduction is reversible in IMR90 (primary) cells and reversible under some circumstances in 293T (transformed) cells, an issue of importance for possible uses of this method for increasing titer with HIV-based vectors.

Increased accumulation of reverse transcription products, 2-LTR circles, and integrated proviruses in arrested 293T cells. To assess the effects of G_2/M arrest on early steps of HIV replication, 293T cells were treated with ETP, CPT, or TAX

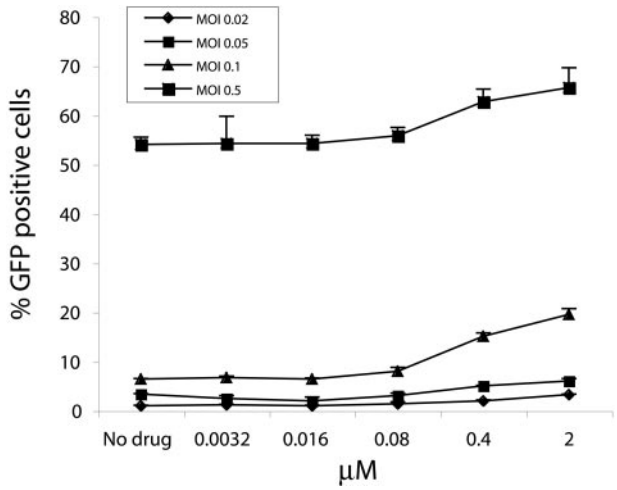

FIG. 4. Effects of G_2/M arrest on transcription from integrated HIV proviruses. 293T cells were infected with SM2-GFP at the four different multiplicities indicated. Three days later, infected cells were treated with the indicated concentrations of ETP. Forty-eight hours later, the percentage of GFP-positive cells was determined by FACS analysis.

and then infected with the HIV-based vector SM2-GFP. Virus was removed 3 h after application to cells and cellular DNA samples were harvested at 12, 24, 48, and 72 h. The copy number of late RT products, 2-LTR circles, and integrated provirus was then measured by quantitative fluorescence-monitored real-time PCR (Fig. 5). G_2/M cell cycle arrest was verified by FACS analysis after 12, 24, 48, and 72 h as in previous experiments (data not shown).

In untreated cells, late reverse transcription products reached a maximum in abundance after 12 h (Fig. 5A; also data not shown). By 72 h post infection, the viral DNA copy number declined around 10-fold. Treatment of infected cells for 12 h with ETP, CPT, and TAX resulted in 5-, 4-, and 3.3-fold increase in the peak production of late RT products, respectively. The increased copy number was sustained throughout the time course except in the case of TAX, where the numbers declined to near wild-type levels. The observed decline correlated with increased toxicity at longer times (data not shown). These data indicate that G_2/M arrest causes a substantial increase in total HIV DNA synthesis, and the effect was prominent in as little as 12 h.

The formation of 2-LTR circles was next measured by quantitative PCR (19), which monitors the ligation of the viral DNA ends together by the host cell NHEJ pathway (10, 14). The number of 2-LTR circles peaked 24 h postinfection in untreated cells. G_2/M cell cycle arrest by ETP, CPT, and TAX increased the number of 2-LTR circles about threefold (Fig. 5B).

The number of integrated proviruses formed was next measured using quantitative Alu PCR (1, 2). The number of proviruses increased up to 72 h after infection. G_2/M cell cycle arrest increased the number of proviruses formed by about fivefold (Fig. 5C).

In summary, the accumulation of HIV DNA in the untreated 293T cells generally paralleled the profiles seen previously (1, 2, 15), while arrest of the cell cycle in G_2/M resulted in increased accumulation of all the DNA forms examined. The quantitative increase in abundance in viral DNA paralleled the observed increase in viral titer.

Increased accumulation of reverse transcription products, 2-LTR circles, and integrated proviruses in arrested IMR90 cells. To test the generality of these findings, we compared infection of human primary lung fibroblasts (IMR90) in dividing and arrested states. Cells were infected with SM2-GFP, samples were harvested after 12, 24, 48, and 72 h, and DNA forms were quantitated. Figure 6A through C show that treatment with ETP boosted transduction in this primary cell type, as was seen in 293T cells. After the 72-h time course, the number of proviruses recovered was increased about threefold over dividing cells. Cell cycle analysis confirmed that the cells were arrested primarily in G_2/M (Fig. 2; also data not shown).

We next investigated whether arresting IMR90 cells in G_1 also boosted transduction. To achieve G_1 arrest, IMR90 cells were grown to confluence and then starved for serum by switching to 0.5% fetal bovine serum in the culture medium. Cells were maintained under these conditions for 7 days prior to infection, and G_1 arrest was confirmed by FACS (data not shown). Comparison of relative transduction by using the GFP marker showed that G_1 arrest by this method resulted in a 4.5-fold reduction in transduction (Fig. 6D). Analysis of viral DNA metabolism confirmed that there was a decrease in total reverse transcription products, 2-LTR circles, and integrated proviruses (data not shown). Thus, arrest of IMR90 cells in G_2/M was most effective in increasing the accumulation of viral DNA.

Comparison of the effects of proteasome inhibitor MG132 to the effects of ETP, CPT, and TAX. Previous studies revealed that treatment of cells with proteasome inhibitors could boost accumulation of early HIV infection intermediates and increase HIV titers (2, 18). To explore the relationship between the action of proteasome inhibitors and CPT, ETP, and TAX, we compared treatment with both types of inhibitors simultaneously. We reasoned that if the two classes of compounds were working in independent pathways, then we might observe synergistic increases in HIV transduction, while if they were working by similar pathways we might not see any further increase with both treatments versus only one.

293T cells were treated with 5 μM MG132 plus 2 μM ETP, 1 μM CPT, or 25 nM TAX, or with each independently. Cells were infected with the HIV-based vector SM2-GFP, and 2 days later HIV transduction was quantified by FACS measurement. MG132 treatment resulted in 4.6-fold increase in HIV transduction when compared to untreated cells, paralleling previous studies (Fig. 7) (2, 18). Treatment with ETP, CPT, and TAX boosted transduction, as seen in previous experiments (5-, 4.3-, and 3.3-fold, respectively). No further boost of HIV transduction was seen when ETP-, CPT-, and TAX-treated cells were exposed simultaneously to 5 μM MG132 (Fig. 7). In the case of CPT plus MG132, a reduction in transduction was seen, which we infer to be due to the cumulative toxicity of the two treatments. These data are consistent with the idea that all four small molecules are acting via the same mechanism.

DISCUSSION

We report that arresting the cell cycle in G_2/M by small molecule treatments boosted early steps of HIV replication. Analysis of the part of the viral life cycle affected revealed increases in formation of late reverse transcription products, 2-LTR circles, and integrated proviruses. Thus, cell cycle arrest at G_2/M can boost both early and late steps of infection, the latter at the level of transcription, as previously reported (5, 6).

G_2/M arrest and increased transduction by HIV. The boost in early events was seen with a type I topoisomerase inhibitor (CPT), a type II topoisomerase inhibitor (ETP), a modulator of tubulin polymerization (TAX), and an inhibitor of DNA polymerase (APC), indicating that increased transduction was not specifically dependent on DNA damage or microtubule disruption. The boost was seen in cell lines (293T and HeLa) and primary cells (IMR90).

The extent of the increase was most prominent for arrest in G_2/M, though a modest boost by arrest in G_1 could be seen in the presence of high levels of APC. IMR90 cells arrested in G_1 by serum starvation and contact inhibition, in contrast, showed less transduction than in dividing cells. Evidently the transduction rate following arrest in G_1 is sensitive to the arrest protocol and not just the stage in the cell cycle. The boost in transduction was optimal at intermediate concentrations of the small molecules used, likely reflecting a balance between fa-

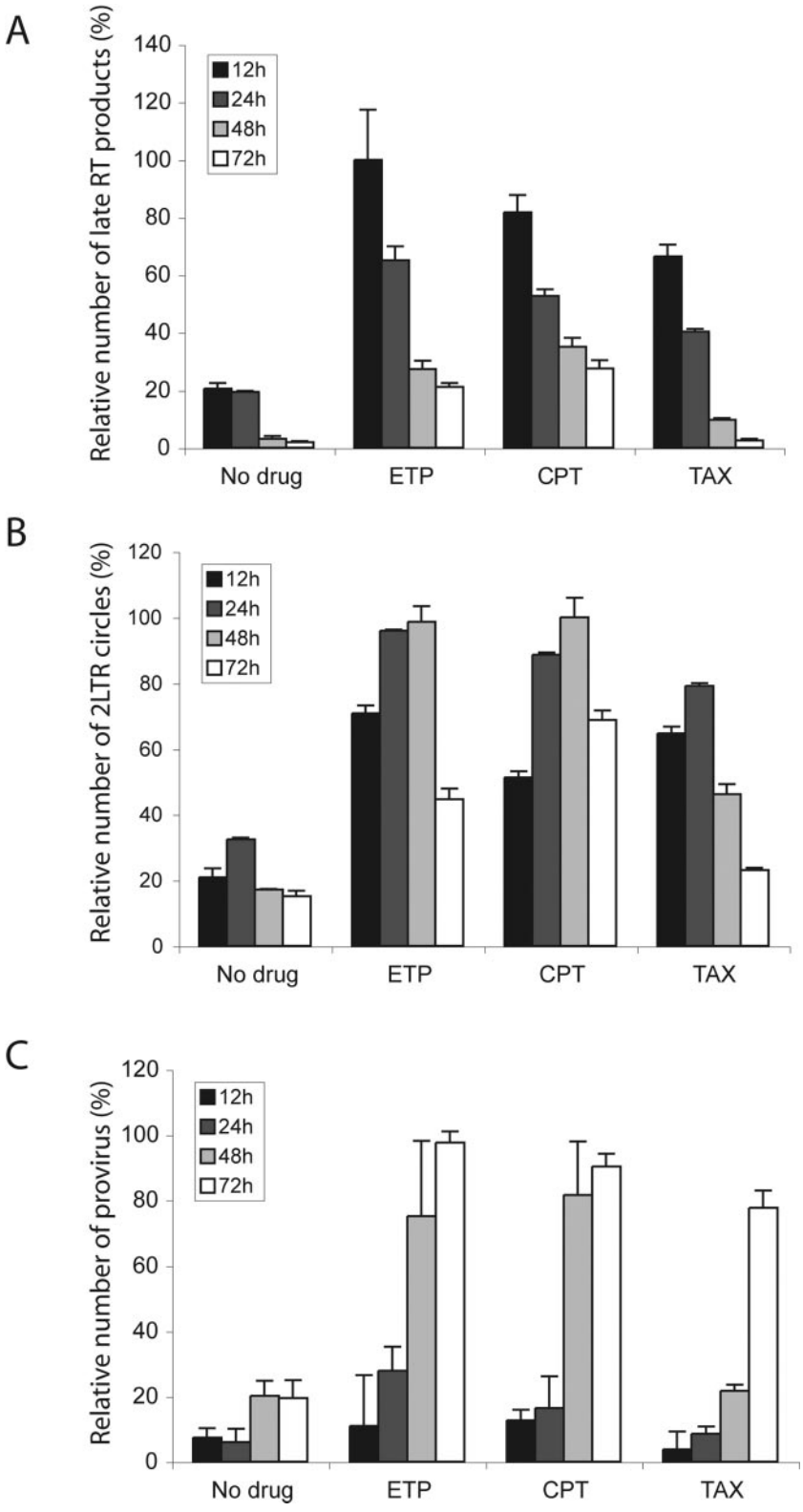

FIG. 5. G_2/M cell cycle arrest boosts accumulation of total reverse transcription products, 2-LTR circles, and integrated proviruses in 293T cells. 293T cells were not treated (no drug) or treated with ETP (2 μM), CPT (1 μM), and TAX (25 nM). Twenty-four hours later, cells were infected with the HIV-based vector SM2-GFP and incubated at 37°C for 3 h. Virus was removed after 3 h, and cell samples were collected 12, 24, 48, and 72 h postinfection. The numbers of (A) late RT products, (B) 2-LTR circles, and (C) HIV provirus were measured by fluorescence-monitored real-time PCR (TaqMan).

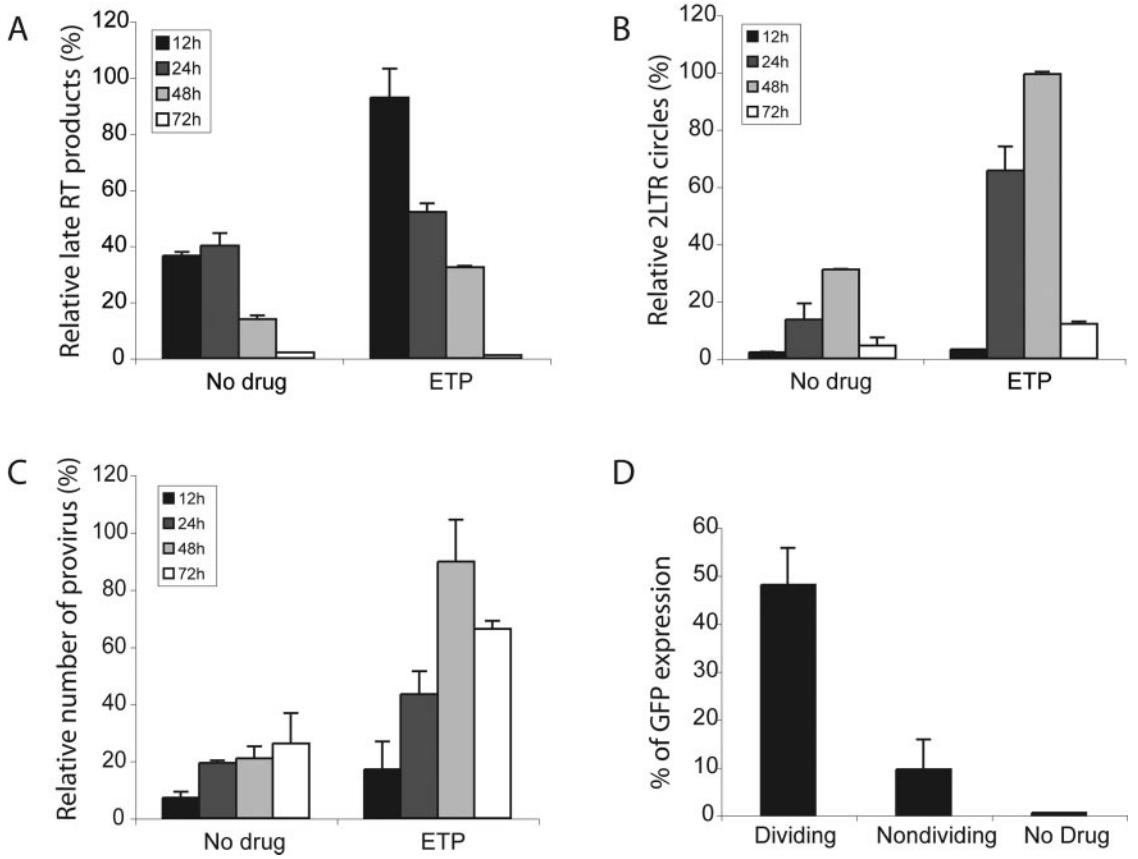

FIG. 6. G_2/M but not G_1 arrest boosts accumulation of total reverse transcription products, 2-LTR circles, and integrated proviruses in IMR90 cells. IMR90 cells were not treated (no drug) or were treated with ETP (2 μM). Twenty-four hours later, cells were infected with the HIV-based vector SM2-GFP and incubated at 37°C for 3 h. Virus was removed after 3 h, and cell samples were collected 12, 24, 48, and 72 h postinfection. The numbers of late RT products (A), 2-LTR circles (B), and HIV proviruses (C) were quantified by fluorescence-monitored real-time PCR. (D) Dividing IMR90 cells and G_1-arrested IMR90 cells were infected with HIV at an MOI of 10 (as determined by titration of the viral stock on 293T cells), and 2 days later the extent of infection was determined by FACS analysis.

vorable effects of G_2/M arrest and disfavorable toxicity at higher concentrations.

We were also able to detect another effect of G_2/M arrest, the previously reported increase in gene expression from the integrated HIV provirus. However, an increase in synthesis of the viral DNA was seen as early as 12 h after infection, so an increase in viral transcription does not fully account for the increase in transduction seen here.

We favor the view that the G_2/M arrest results in a change in cellular physiology that is more conducive to HIV infection, but an alternative would be that a simple increase in cell surface area due to arrest promoted entry. The increase in cell volume due to arrest is likely to be about twofold for a cell stalled just prior to division. For a sphere, an increase in twofold of volume results in roughly a 1.56-fold increase in surface area. In our experiment, the magnitudes of the increases in transduction we measured were around three- to fivefold. Thus, the rate of transduction would need to be extremely sensitive to cellular surface area to explain the observations, and we know of no evidence for this. At present it seems more logical to assume that some change in cellular physiology accounts for the increase in transduction.

The boost in HIV transduction following treatment with the

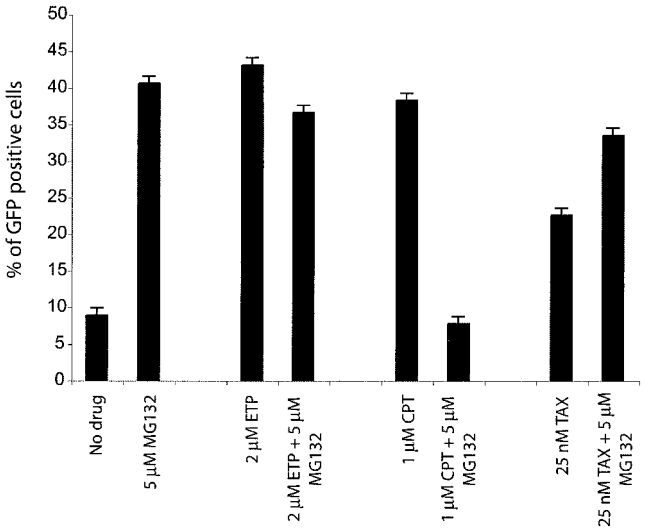

FIG. 7. Comparison of the effects of proteasome inhibitor MG132 to the effects of ETP, CPT, and TAX. 293T cells were treated with 5 μM MG132 plus 2 μM ETP, 1 μM CPT, or 25 nM TAX, or with each drug independently. Cells were infected with the HIV-based vector SM2-GFP, and 2 days later HIV transduction expressed as percentage of GFP expression was quantified by FACS measurement.

proteasome inhibitor MG132 has been interpreted as due to decreased proteolysis of HIV replication intermediates (2, 18). However, treatment with proteasome inhibitors also arrests the cell cycle in G_2/M by disrupting the regulated proteolysis of cell cycle control proteins (12). Thus, an additional possible explanation for the boost in transduction by MG132 is that arrest in G_2/M itself boosts transduction independent of proteasome inhibition.

Possible mechanisms for increased production of early HIV DNAs in G_2/M-arrested cells. A variety of mechanisms, none mutually exclusive, could potentially explain the observed boost in transduction. One relatively trivial model would posit that simply arresting the cell cycle increased transduction by concentrating viral replication complexes in nondividing cells. According to this idea, cell division would dilute early viral intermediates prior to integration, so that after division there would be fewer viral replication complexes per cell to carry out integration. Arguing against this idea are the fact that (i) RT intermediates are increased in abundance in as little as 12 h, before much dilution due to cell division could take place, and (ii) infected center assays show the boosting effect (the dilution effect would be expected to result in fewer cells per infected center but not fewer total infected centers). Thus, we infer that a change in cellular physiology is responsible for the observed increase in HIV transduction.

We find that the extent of the boost in transduction did not depend strongly on whether the HIV envelope or vesicular stomatitis virus G protein was used to deliver HIV replication complexes to cells. Thus, we favor the idea that the stimulation of transduction by cell cycle arrest was not due to increased entry but rather to action at later steps, such as stimulating uncoating, increasing reverse transcription, or decreasing degradation of viral replication intermediates.

Possible models for the increase in transduction assessed in light of transcriptional profiling data. Several models for the mechanism of the transduction boost could be addressed by analyzing transcriptional profiling data. We treated 293T cells with 2 μM CPT and prepared two RNA samples from independent treated and untreated cultures (four samples total). RNAs were then analyzed using HG-U133A microarrays, which query the activity of about 20,000 genes. Genes affected by camptothecin treatment were first identified using the SAM package (22). The data were analyzed using a stringent criteria for significance, accepting a false discovery rate of only 1% and demanding that genes change their expression level twofold or more. This yielded 103 genes that increased in activity and 72 that decreased. Such an analysis assumes that the factors responsible for boosting transduction are regulated at the transcriptional level, which of course is only one possibility. However, this type of information can help constrain mechanistic hypotheses.

Genes for protein turnover are of particular interest, since decreasing the rate of proteolysis of HIV replication intermediates might result in increased transduction. Recently, it has been shown that the rhesus monkey TRIM5-α protein is a dominant inhibitor of HIV replication (21). At least 40 genes for TRIM family members exist in humans and so are candidate factors mediating HIV degradation. However, of the 20 different TRIM genes queried by the HG-U133A microarray, none were significantly repressed by CPT treatment. In addition, there was no significant enrichment for genes down-regulated by CPT in the "protein catabolism" category, which includes genes involved in protein degradation more broadly. Thus, this analysis did not strengthen the idea that transcriptional repression of genes involved in protein degradation boosted HIV transduction.

Another known antiviral protein acting early during infection is APOBEC3G (20). However, the gene for APOBEC3G was not repressed by CPT treatment, nor were other APOBEC family members. Thus, transcriptional down-regulation of APOBECs does not seem to account for the boost in transduction either.

DNA repair pathways can act both positively and negatively on the early steps of retroelement replication, so it was of interest to examine the response of genes encoding DNA repair proteins. Genes in the categories "damaged DNA binding" and "nucleotide excision repair" were significantly enriched among the activated genes. Possibly activation of these pathways helps stabilize the unintegrated HIV DNA.

Another possible model postulates that cellular factors for trafficking of preintegration complexes within cells might be increased in abundance or activity, thereby increasing transduction. In possible support of this idea, genes for intermediate filaments were significantly induced by CPT ($P = 0.0058$), raising the possibility that HIV traffics along intermediate filaments in CPT-treated cells.

In summary, no clear-cut explanation for the increase in viral DNA production following CPT treatment emerged from this analysis. However, the transcriptional profiling data does help to generate and rule out some possibilities.

Possible connection to function of HIV Vpr. HIV Vpr arrests the cell cycle in G_2/M and thereby boosts HIV infection. Previous studies have emphasized the role of Vpr-mediated G_2/M arrest in boosting transcription from the HIV LTR (6). Data presented here raise the possibility that Vpr may also boost transduction via G_2/M arrest by increasing synthesis of viral DNA. Such a mechanism would require that Vpr protein is either imported into target cells in HIV particles in sufficient quantities to achieve cell cycle arrest or expressed from unintegrated DNA. It is uncertain whether the needed levels of Vpr accumulate (but see references 4 and 16). Further studies are required to determine whether such a mechanism operates during normal infection.

More broadly, data presented here indicate that cell cycle arrest at G_2/M can boost accumulation of HIV DNA and invite further investigation of possible biological roles. Further studies of the mechanism by which G_2/M arrest stimulates infection may provide insight into interactions between HIV and the host cell, and the technique for boosting transduction described here may be useful in increasing transduction by HIV-based vectors.

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