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Human Immunodeficiency Virus Type 1 *vpr* Gene Induces Phenotypic Effects Similar to Those of the DNA Alkylating Agent, Nitrogen Mustard

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The product of the human immunodeficiency virus type 1 (HIV-1) *vpr* gene induces cell cycle arrest in the G_2 phase of the cell cycle and is characterized by an accumulation of the hyperphosphorylated form of cdc2 kinase. This phenotype is similar to the effect of DNA-damaging agents, which can also cause cells to arrest at G_2. We previously reported that Vpr mimicked some of the effects of a DNA alkylating agent known as nitrogen mustard (HN_2). Here we extend these earlier observations by further comparing the activation state of cdc2 kinase, the kinetics of G_2 arrest, and the ability to reverse the arrest with chemical compounds known as methylxanthines. Infection of cells synchronized in the G_1 phase of the cell cycle with a pseudotyped HIV-1 resulted in arrest at G_2 within 12 h postinfection, before the first mitosis. Similar to that induced by HN_2, Vpr-induced arrest led to a decrease in cdc2 kinase activity. Vpr-mediated G_2 arrest was alleviated by methylxanthines at concentrations similar to those needed to reverse the G_2 arrest induced by HN_2, and cells proceeded apparently normally through at least one complete cell cycle. These results are consistent with the hypothesis that Vpr induces G_2 arrest through pathways that are similar to those utilized by DNA-damaging agents.

The human immunodeficiency virus type 1 (HIV-1), in addition to its structural genes, *gag*, *pol*, and *env*, also encodes several accessory genes, some of which are vital for viral replication and pathogenesis. Vpr is a 15-kDa basic protein whose role in HIV-1 pathogenesis is unclear. It is virion associated through interaction with Gag p6, and has been proposed to participate in transport of the virus core into nondividing cells, thus allowing infection of cells such as macrophages (2, 7, 15, 17, 19, 22, 33, 47). It has also been proposed that Vpr may assist in virus assembly through its association with Gag (19). Vpr has also been reported to have a weak *trans*-activation activity and may function to *trans*-activate the long terminal repeat during early stages of transcription (5). The related simian immunodeficiency virus contains two *vpr*-related genes (*vpr* and *vpx*), which are thought to have arisen by duplication of a single ancestral gene (39, 45). One group has reported that mutations in both of these genes were necessary for a decreased replication rate and a nonpathogenic virus when inoculated into rhesus macaques (16). However, other studies reported that mutations in *vpr* alone led to slower progression to AIDS (20, 23).

An additional function of Vpr appears to be perturbation of the cell cycle. Vpr has been shown to be necessary and sufficient to cause accumulation of human T cells in the G_2 phase of the cell cycle (18, 21, 36, 37). The ability of Vpr to arrest cells in G_2 has also been described for the fission yeast, *Schizosaccharomyces pombe*, suggesting interactions with highly conserved components of the cell cycle (48). Indeed, one effect of Vpr-induced G_2 arrest is an inactivation of the cdc2 kinase, although Vpr does not appear to directly inhibit cdc2 kinase (3, 18, 21, 36). cdc2 kinase is a major checkpoint control protein in the G_2-to-M transition and is regulated by (i) phosphorylation by Wee1 and CAK and (ii) dephosphosphorylation by cdc25, proteins which, in turn, are themselves regulated by phosphorylation events (29, 30, 40). Maintenance of the cdc2 kinase in its inactive state would prevent entry into mitosis. Agents that damage DNA, such as alkylating chemicals and ionizing radiation, can also lead to the arrest of cells in G_2 by preventing the activation of cdc2 kinase (25, 27, 31). A G_2 arrest induced by DNA damage is thought to allow cells to complete DNA repair before entry into mitosis.

We hypothesized that there could be similarities in the mechanism of the G_2 block induced by Vpr and drugs such as nitrogen mustard (HN_2). We therefore further compared the phenotypes of arrest of these two agents and the effects of compounds capable of alleviating the G_2 arrest induced by HN_2 on cells arrested by Vpr. HIV-1 infection of G_1-synchronized cells revealed that Vpr exerts its effects within 12 h postinfection, resulting in arrest at the first G_2 phase. We found that the G_2 arrests induced by HN_2 and Vpr had similar phenotypes, including the inactivation of cdc2 kinase via hyperphosphorylation. Chemical agents known as methylxanthines, which reverse the G_2 arrest induced by HN_2, were also effective at similar concentrations in alleviating the G_2 arrest of Vpr-expressing cells, allowing them to progress apparently normally through the cell cycle.

MATERIALS AND METHODS

Virus and infection. Viral stocks of HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/vesicular stomatitis virus G protein (VSV-G) and HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G were generated by electroporation of COS cells. Briefly, 10^7 cells were resuspended in electroporation medium (RPMI with 20% fetal calf serum [FCS]) and electro-

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porated at 960 μF and 230 V with 10 μg each of plasmid NLthyΔBgl or NLthyΔBglVprX, along with VSV-G. NLthyΔBgl and NLthyΔBglVprX were derived from HIV-$1_{NL4\text{-}3\text{-}luc}$env(−) (34) by cloning the *thy* 1.2 gene into the *Xho*I and *Mlu*I restriction sites, replacing the luciferase gene. Supernatants were collected at 48 and 72 h posttransfection, centrifuged at 3,000 rpm for 5 min, filtered through a 0.45-μm-pore-size filter, and ultracentrifuged in a Beckman L3-50 centrifuge with a SW28 rotor at 25,000 rpm for 90 min. Viral pellets were resuspended in 0.1× Hanks balanced salt solution overnight at 4°C and stored in the presence of 10% FCS at −70°C. HeLa cells were infected with the viral stock in the presence of 10 μg of Polybrene (Sigma, St. Louis, Mo.) per ml at 37°C for 4 h, washed with phosphate-buffered saline (PBS), and resuspended in Dulbecco's modified Eagle medium with 10% calf serum (Gemini, Calabasas, Calif.). The titers of both viruses were determined by infecting 2×10^5 HeLa cells with various amounts of virus (5 to 20 μl) and assaying at day 2 postinfection for the percentage of Thy 1.2-positive cells.

Cells. HeLa cells were synchronized with thymidine as described previously (6). Cells were treated with 2 mM thymidine for 19 to 21 h, washed with PBS, and grown in Dulbecco's modified Eagle medium with 10% calf serum for 9 h. A second thymidine block was performed by adding 2 mM thymidine for an additional 17 to 19 h. After the second thymidine block, cells were washed with PBS and infected with the retroviral stocks, as described above, or treated with nitrogen mustard (5 μM) (Sigma) for 30 min at 25°C. Pentoxifylline and caffeine (1 mM) (Sigma) were added after HN_2 treatment or infection with either HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G or HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G.

Transfection of HeLa cells. Plasmids BSVprthy and BSVprXthy, that contained an untranslated intron of the cytomegalovirus immediate-early promoter, have been described previously (21). HeLa cells were electroporated with plasmid DNA (20 μg) at 230 V and 960 μF. Culture medium was replaced at 24 h posttransfection, and at 48 h posttransfection, cells were harvested and stained for Thy 1.2 and DNA content.

Flow cytometry. The method of double staining for surface marker Thy 1.2 and DNA content was adapted from the method of Schmid et al. (38). Cells were stained with anti-Thy 1.2 fluorescein isothiocyanate-conjugated monoclonal antibody (Caltag, Burlingame, Calif.) diluted 1/200 in fluorescence-activated cell sorter (FACS) buffer (PBS with 2% FCS and 0.01% sodium azide) and incubated on ice for 20 min. Cells were washed with FACS buffer and resuspended in hypotonic propidium iodide (PI) buffer (PI [100 μg/ml], sodium citrate [1 mg/ml], 0.3% Triton X-100, and RNase A [2 μg/ml]). All stained cells were acquired on a FACScan 2 apparatus (Becton-Dickinson) and analyzed with the Lysis 2 software package.

Cell lysis and immunoprecipitation. HeLa cells were lysed on ice in lysis buffer (Hanks buffered salt solution with 1% Nonidet P-40, 50 mM β-glycerophosphate, 10 mM NaF, 1% aprotinin, 1 mM sodium orthovanadate and leupeptin [1 μg/ml]). Immunoprecipitations were performed with glutathione *S*-transferase (GST)–$p13^{suc}$ bound to agarose beads (catalog no. 14-122; Upstate Biotechnology, Lake Placid, N.Y.), a monoclonal antibody specific for cyclin B (Santa Cruz Biotechnology) or anti-cdc2 monoclonal antibody (Santa Cruz Biotechnology). Histone H1 kinase assays were performed as previously described (31). Briefly, lysates from 10^6 cells were incubated with GST-$p13^{suc}$-agarose beads, or anti-cdc2 or anti-cyclin B followed by protein A/G plus agarose (Santa Cruz Biotechnology), at 25°C for 1 h. Complexes were washed three times with lysis buffer, washed twice with kinase buffer (50 mM Tris, 10 mM $MgCl_2$), and incubated at 30°C for 30 min in kinase buffer containing 1 mM dithiothreitol, 10 μCi of [γ-^{32}P]ATP, and 1 μg of histone H1 (Boehringer-Mannheim Biochemicals). The reaction mixtures were analyzed by autoradiography after separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Western blot analysis was performed with anti-cdc2 antibody and developed with the enhanced chemiluminescence assay (Amersham).

Immunofluorescence staining. Following infection, HeLa cells were grown on chamber slides (Fisher). Cells were fixed with 1% paraformaldehyde for 15 min. After fixation, cells were permeabilized with 0.1% Triton X-100 (Sigma) for 10 min, and the DNA was stained with Hoechst 33342 (1 μg/ml) for 5 min and washed with FACS buffer. All procedures were performed at 25°C.

Thymidine incorporation. Cultures were incubated with [^{3}H]thymidine (50 μCi/ml) at 37°C for 4 h. Cells were harvested and lysed in 0.5% Triton X-100. Lysates were added to glass fiber filters, washed three times with distilled H_2O, washed twice with ethyl alcohol, and counted in a scintillation counter.

RESULTS

The G_2 arrest induced by HIV-1 Vpr and nitrogen mustard results in inactivation of cdc2 kinase. The HIV-1 *vpr* gene product has been reported to be responsible for the G_2 arrest observed in HIV-1-infected cells (3, 18, 21, 36, 37). We further investigated the phenotype of the cell cycle arrest by generating high-titer infectious HIV-1. The resulting infection and subsequent G_2 arrest of a high percentage of the cells in the culture facilitated the biochemical analysis of infected cells. An Env-defective HIV-1 was pseudotyped with the VSV-G envelope, allowing concentration to high infectious titers. We confirmed that the pseudotyped HIV-1 was capable of inducing G_2 arrest by infecting HeLa cells and demonstrating cell cycle arrest. Infection with HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G resulted in 98% of the cell population arrested in G_2 (Fig. 1A). In contrast, infection with Vpr mutant virus [HIV-$1_{NL4\text{-}3\text{-}thy}$vprX env(−)/VSV-G] that contains a frameshift mutation at the carboxyl terminus of *vpr*, resulting in an unstable Vpr protein (33, 35), showed only a minor effect on the cell cycle profile. This effect of the Vpr mutant virus was seen only at the highest multiplicities of infection and was not consistently observed.

cdc2 kinase is a key regulatory protein of the G_2-to-M transition. During G_2, cdc2 kinase is inactive and becomes active as the cells enter mitosis. The active and inactive states differ by phosphorylation states and can be distinguished by SDS-PAGE, with the inactive hyperphosphorylated form of cdc2 kinase migrating as the slower species (11, 42). We have previously shown that cells arrested by transfection with a Vpr expression vector had an increase in the hyperphosphorylated form of cdc2 kinase (21). Here we show that infection by HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G is similarly characterized by the presence of hyperphosphorylated cdc2 kinase (Fig. 1B).

We further extended these studies by comparing the in vitro kinase activity of cdc2 kinase from HIV-1-infected cells to that of HN_2-treated cells. The hyperphosphorylated cdc2 kinase present in HN_2-treated cells had a decreased ability to phosphorylate H1 histone in vitro (31). Synchronized cells were used to allow greater discrimination of cell cycle stages and to facilitate biochemical and cellular analysis. HeLa cells were initially synchronized in G_1 by a double thymidine block to obtain cells that, upon release, progress through the cell cycle as a synchronous population. Under these conditions, uninfected HeLa cells entered S, G_2, and M at 2, 10, and 12 h postrelease, respectively (data not shown). Some cells were treated with HN_2 to obtain G_2-arrested cells for comparison in our assays. In addition, nocodazole, which prevents microtubule polymerization and arrests cells in the M phase, was added to all the samples at the time of release to allow better discrimination between cells that were arrested before M and those that were capable of entering M. Cells were infected with HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G or HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G and harvested 12 h postrelease.

As expected, microscopic analysis revealed that the majority of cells in the mock-infected and Vpr mutant virus [HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G]-infected cultures had entered M (data not shown). In contrast, cultures infected with HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G or treated with HN_2 had less than 10% of the cell population in mitosis. It is noteworthy that following infection of synchronized cells in G_1, more than 90% of HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G-infected cells were incapable of reaching mitosis. Thus, Vpr exerts its effects on the cell cycle within 12 h postinfection, prior to the onset of the first mitosis. HIV-1 Vpr also resulted in a more prolonged arrest of cells in G_2 compared to HN_2. HN_2 treatment results in an extended delay of several hours in G_2, during which cells repair the DNA damage. In contrast to the entry into mitosis at 12 h post-thymidine release by uninfected synchronized HeLa cells, cells treated with HN_2 did not start to enter mitosis until 20 h postrelease. Cells infected with HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G were blocked in G_2 for an even longer time, with less than 10% of cells in mitosis at 24 h postrelease. This may be due to the stability and constitutive expression of Vpr compared to the brief exposure of cells to HN_2.

cdc2 kinase was immunoprecipitated by using $p13^{suc}$-agarose beads. $p13^{suc}$ is a protein of undetermined function that is found in a complex with cdc2 kinase in eukaryotic cells and

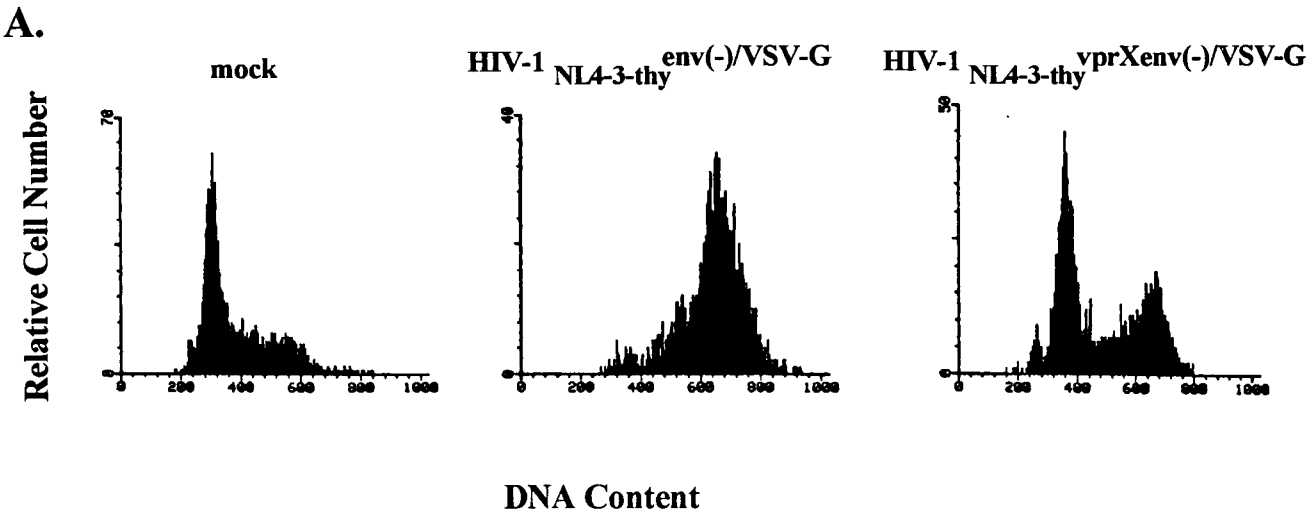

FIG. 1. Infection of HeLa cells with HIV-1 causes cell cycle arrest. Cells (10^6) were infected with virus to obtain infection of 90 to 100% of the cells, as described in Materials and Methods. At day 1 postinfection, cells were analyzed for cell cycle profile (A) and the phosphorylation state of cdc2 kinase (B). (A) DNA content of nuclei from mock-, HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G-, or HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G-infected HeLa cells. At day 1 postinfection, the cells were stained with PI, and 5×10^3 cells were analyzed by flow cytometry, as described in Materials and Methods. The histogram indicates relative cell numbers (*y* axis) as a function of relative DNA content (*x* axis). (B) Western blot analysis of cdc2 kinase. Total cell lysates (10 μg) were separated by SDS-PAGE (15% polyacrylamide) and analyzed for the phosphorylation state of cdc2 kinase by Western blot analysis, as described in Materials and Methods. The upper and lower arrows indicate the hyperphosphorylated and nonhyperphosphorylated forms of cdc2 kinase, respectively.

which can specifically immunoprecipitate cdc2 kinase. Western blot analysis revealed approximately equal amounts of total cdc2 kinase immunoprecipitated from cells infected with HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G or HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G (Fig. 2A). As reported above, cells arrested in G_2 by treatment with HN_2 or by infection with HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G showed an accumulation of the slower-migrating, hyperphosphorylated form of cdc2 kinase. In contrast, the major species of cdc2 kinase present in Vpr mutant virus [HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G]-infected and mock-infected cells was a faster-migrating, nonhyperphosphorylated form of cdc2 kinase. The in vitro kinase assay revealed a reduction in the ability of cdc2 kinase from both HN_2-treated and HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G-infected cells to phosphorylate H1 histone relative to HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G- or mock-infected cells (Fig. 2B). The same result was obtained when the immunoprecipitation and in vitro kinase assays were repeated with a monoclonal antibody specific for cdc2 kinase (Fig. 2C).

Treatment with pentoxifylline restores the cdc2 kinase activity and cell cycle progression in cells arrested in G_2 by Vpr or nitrogen mustard. Based on the similarity of cdc2 kinase inactivation in Vpr- and HN_2-arrested cells, we next investigated whether agents which can alleviate the G_2 arrest induced by DNA-damaging agents could also alleviate the arrest induced by Vpr. We evaluated the ability of pentoxifylline, a methylxanthine previously shown to alleviate HN_2-mediated G_2 arrest (31, 32), to alleviate the arrest induced by HIV-1. Mock- and HIV-1 Vpr mutant virus-infected cells were also treated with pentoxifylline to determine the effect of pentoxifylline alone on cell cycle progression.

Cells were harvested for immunoprecipitation at 12 h postrelease, when mock-infected cells were still undergoing mitosis. Immunoprecipitation of cdc2 kinase was performed with an antibody raised against cyclin B, a protein that also forms a complex with cdc2 kinase. Cells arrested in G_2 by HIV-$1_{NL4\text{-}3\text{-}thy}$ env(−)/VSV-G or by treatment with HN_2 again showed an increase in hyperphosphorylated cdc2 kinase, as well as decreased kinase activity relative to the mock- and HIV-$1_{NL4\text{-}3\text{-}thy}$vprX env(−)/VSV-G-infected cells (Fig. 3A). Pentoxifylline had no effect on the migration during SDS-PAGE or on the kinase activity of cdc2 kinase from mock- or Vpr mutant virus [HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G]-infected cells (Fig. 3B). Addition of pentoxifylline to either HN_2-treated cells or cells infected with HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G resulted in a decrease in the hyperphosphorylated cdc2 kinase species, with a concomitant increase in cdc2 kinase activity. We conclude that pentoxifylline, a chemical agent which restores cdc2 kinase activity in cells treated with DNA-damaging agents, has a similar effect on cells arrested by Vpr.

Analysis of the cell cycle profile was performed at 18 h postrelease, a time when uninfected cells have entered G_1 (Fig. 4). In agreement with the reactivation of cdc2 kinase, treatment of HN_2-treated cells with pentoxifylline reduced the percentage of cells in G_2 from 65 to 9%, with a concomitant increase of cells in G_1 from 35 to 91%, relative to cells exposed to HN_2 alone. Pentoxifylline treatment of HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G-infected cultures also resulted in a decrease in the G_2 population from 88 to 54%. The ability of pentoxifylline to alleviate the entire population of cells arrested by HN_2 as compared to the alleviation of a proportion of the cells ar-

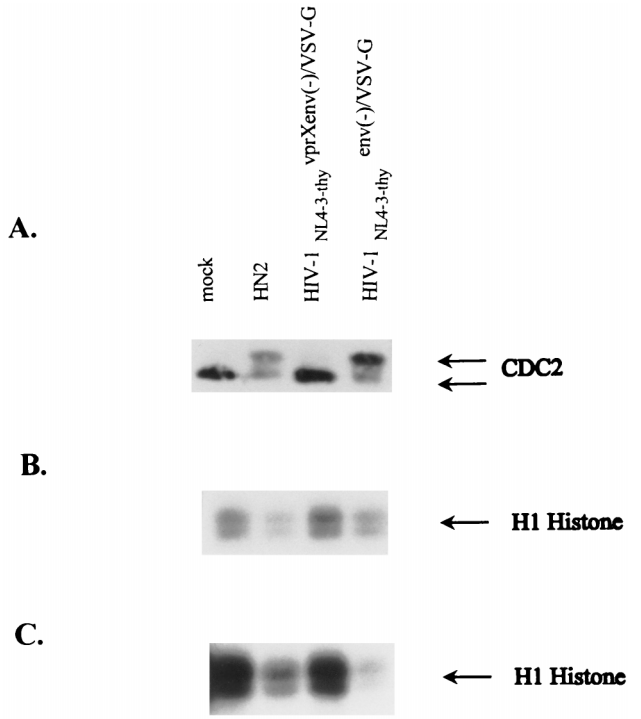

FIG. 2. HeLa cells arrested in G_2 by HIV-1 Vpr accumulate inactive cdc2 kinase. HeLa cells (2×10^6) were synchronized in G_1 by a double thymidine block, as described in Materials and Methods. Upon release, cells were mock infected, exposed to HN_2 (5 μM) for 30 min, or infected with HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G or HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G to achieve infection of 90 to 100% of the cells. Cells were lysed at 12 h post-thymidine release, and lysates from 10^6 cells were used for immunoprecipitation by using GST-$p13^{suc}$-agarose beads (A and B). Lysates from the remaining 10^6 cells were used for immunoprecipitation by anti-cdc2 (C). (A) Western blot analysis of cdc2 kinase. Lysates from 10^6 cells were immunoprecipitated with 25 μl of GST-$p13^{suc}$-agarose beads, as described in Materials and Methods. Half of the bound complexes were used for Western blot analysis, and the other half were used in the histone H1 kinase assay (B). For Western blot analysis, bound complexes were separated by SDS-PAGE (15% polyacrylamide) and an anti-cdc2 monoclonal antibody was used to detect the phosphorylation state of cdc2 kinase, as described in Materials and Methods. The upper and lower arrows indicate hyperphosphorylated and nonhyperphosphorylated cdc2 kinase, respectively. (B) Histone H1 kinase assay using complexes bound to GST-$p13^{suc}$-agarose beads. Half of the bound complexes were used in a histone H1 kinase assay, as described in Materials and Methods, and separated by SDS-PAGE (12% polyacrylamide). The arrow indicates phosphorylated histone H1. (C) Histone H1 kinase assay using complexes immunoprecipitated with anti-cdc2. Lysates from 10^6 cells were incubated with anti-cdc2 antibody, followed by protein A/G plus agarose, as described in Materials and Methods. Half of the bound complexes were used in a histone H1 kinase assay and separated by SDS-PAGE (12% polyacrylamide). The arrow indicates phosphorylated histone H1.

rested by HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G is likely due to the brief exposure of cells to HN_2, while Vpr would be constitutively expressed. There was no effect of pentoxifylline on the cell cycle profile of either mock- or Vpr mutant virus (HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G)-infected cultures.

We assessed the effects of varying the times at which pentoxifylline was added. In the experiments described above, pentoxifylline was added immediately after infection and maintained throughout the experiment. Pretreatment of cells for 24 h with pentoxifylline, with subsequent removal of the drug before infection with HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G, did not prevent the G_2 arrest induced by Vpr (data not shown). However, adding pentoxifylline 24 h postinfection did result in alleviation. Therefore, pentoxifylline must be present during the G_2 arrest to have any effect.

Alleviation of HIV-1 Vpr-mediated G_2 arrest by pentoxifylline allows cells to progress through mitosis. We examined the ability of cells in which the arrest induced by Vpr had been alleviated by pentoxifylline to progress normally through the cell cycle. Using a fluorescent DNA intercalating dye, Hoechst 33342, we examined the progression from G_2 into M through microscopic analysis of individual cells. At 12 h postrelease from the thymidine block, as measured by the presence of cells with visibly condensed chromosomes, more than 50% of the uninfected cells with or without pentoxifylline were undergoing mitosis (Fig. 5A and B). All phases of mitosis were evident. Similarly, more than 50% of the HeLa cells infected by Vpr mutant virus [HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G] were at a visible stage of mitosis, showing no alteration in the timing of mitotic entry upon addition of pentoxifylline (Fig. 5E and F). Cells that had been treated with HN_2 showed few mitotic cells visible at this time point. As expected, pentoxifylline treatment of HN_2-treated cells resulted in an increase in the proportion of mitotic cells (Fig. 5C and D). Cultures infected with HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G contained few mitotic cells. Similar to cells treated with HN_2 and pentoxifylline, the addition of pentoxifylline to HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G-infected cells also resulted in an increase in mitotic cells, with all stages of mitosis visible (Fig. 5G and H). HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G-infected cells treated with pentoxifylline appeared to exit M at a rate similar to that of mock-infected cultures, with few mitotic cells visible 14 h postrelease (data not shown). Therefore,

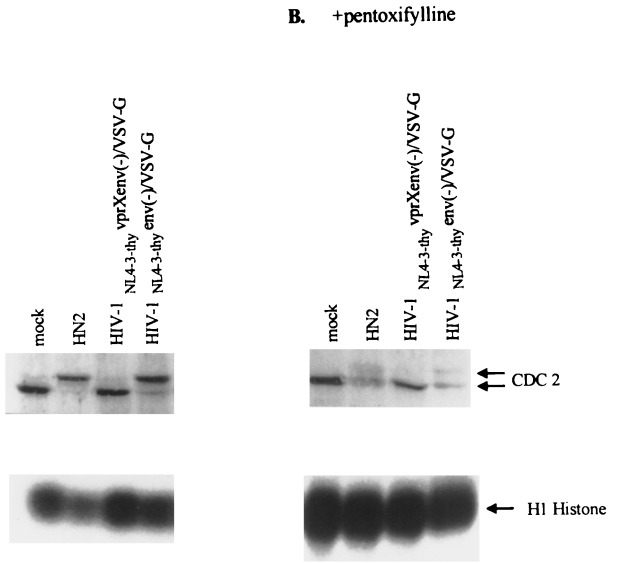

FIG. 3. Treatment of HeLa cells arrested in G_2 by Vpr with pentoxifylline restores cdc2 kinase activity. HeLa cells (2×10^6) were synchronized in G_1 by a double thymidine block, as described in Materials and Methods. Upon release, cells were mock infected, exposed to HN_2 (5 μM) for 30 min, or infected with HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G or HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G to achieve infection of 90 to 100% of the cells. Pentoxifylline (1 mM) was added immediately after HN_2 treatment or after infection (B). Cells were harvested 12 h postrelease, and lysates from 10^6 cells were immunoprecipitated with anti-cyclin B antibody, as described in Materials and Methods. Following incubation with protein A-Sepharose, half of the bound complexes were subjected to Western analysis (upper panels) or histone H1 kinase assay (lower panels), as described in Materials and Methods. Shown are results of analyses of cdc2 kinase from non-pentoxifylline-treated cells (A) and cells treated with pentoxifylline (B). The upper panels are Western blots probed with anti-cdc2 antibody, and the upper and lower arrows to the right of panel B indicate the hyperphosphorylated and nonhyperphosphorylated forms of cdc2 kinase. The lower panels show results of the histone H1 kinase assays performed on immunoprecipitated complexes from non-pentoxifylline-treated cells (A) and cells treated with pentoxifylline (B).

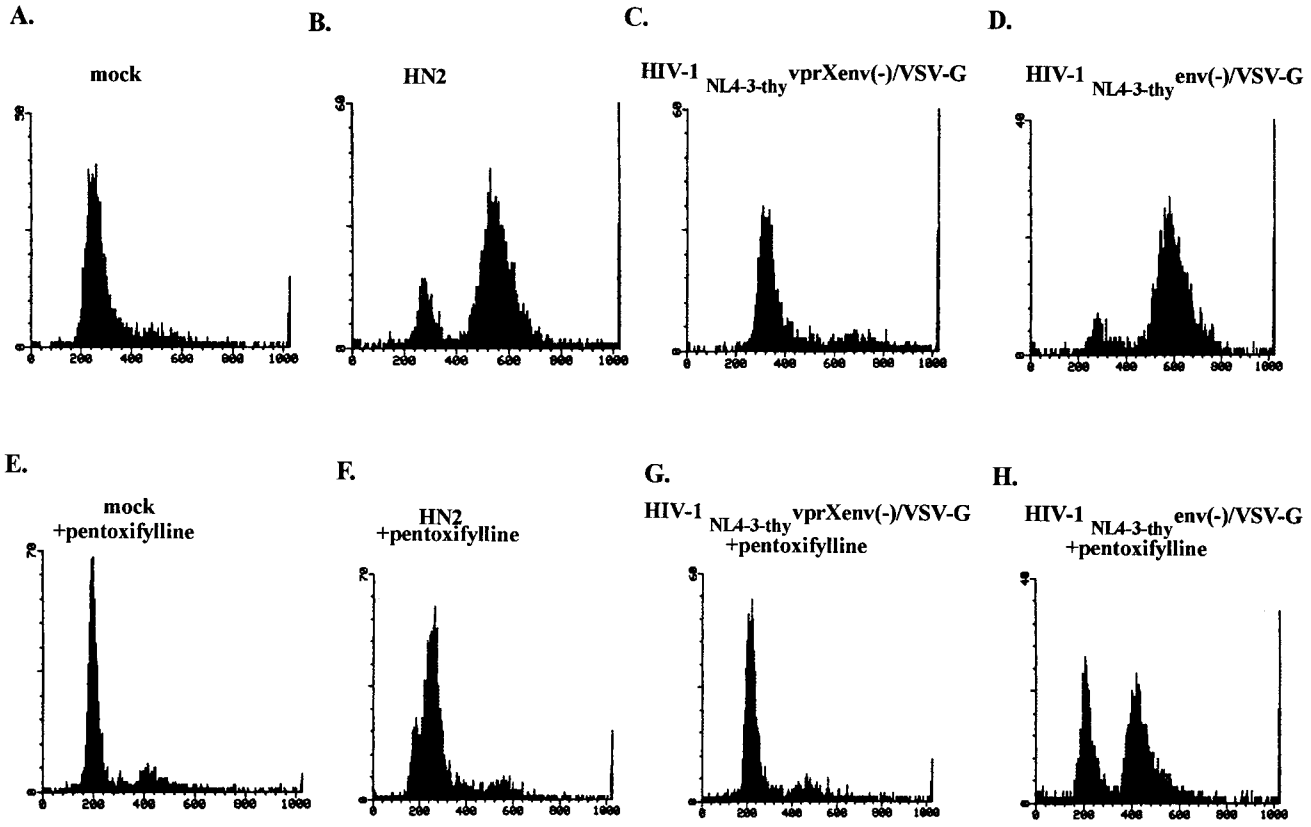

FIG. 4. Treatment with pentoxifylline alleviates the G_2 arrest induced by HIV-1 Vpr. HeLa cells (10^6) were synchronized in G_1 by a double thymidine block, as described in Materials and Methods. Upon release, cells were mock infected, exposed to HN_2 (5 μM) for 30 min, or infected with HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G or HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G to achieve infection of 90 to 100% of the cells. Pentoxifylline (1 mM) was added after HN_2 treatment or after infection (E to H). At 18 h postrelease, cells were stained with PI, and 5×10^3 cells were analyzed by flow cytometry, as described in Materials and Methods. The histograms indicate relative cell number (*y* axis) as a function of relative DNA content (*x* axis). (A and E) Mock infected; (B and F) HN_2 treated; (C and G) HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G infected; (D and H) HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G infected.

pentoxifylline treatment of HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G-infected cells allowed the cells to enter, progress through and exit M in a manner similar to uninfected cells.

Pentoxifylline allows Vpr-arrested cells to progress through S phase. As described above, by microscopic (Fig. 5) and flow cytometric analyses (Fig. 4), we have shown that cells arrested by Vpr and subsequently treated with pentoxifylline were released from G_2 and were capable of entering mitosis and G_1. We next investigated whether these cells could continue through the cell cycle and enter S phase. The ability of the cells to undergo DNA synthesis was evaluated by [^{3}H]thymidine uptake. Flow cytometric analysis showed that synchronized uninfected cells reached mitosis and reentered G_1 and S at 12, 14, and 20 to 22 h postrelease, respectively (data not shown). Uninfected cells with or without pentoxifylline and Vpr mutant virus [HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G]-infected cells with or without pentoxifylline had similar cell cycle progressions, entering S at approximately 20 h postrelease, and continued to incorporate [^{3}H]thymidine for several hours (Fig. 6A and C). HN_2 treatment resulted in a transient arrest of about 8 h, after which cells started to enter and progress through S phase. Treatment of HN_2-arrested cells with pentoxifylline alleviated this transient arrest, resulting in uptake of [^{3}H]thymidine at 20 to 22 h postrelease (Fig. 6B). HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G-infected cells did not incorporate [^{3}H]thymidine at any time during the course of the experiment. However, cells infected with the same virus but subsequently treated with pentoxifylline started to enter S at approximately 22 h postrelease, as indicated by the increase in [^{3}H]thymidine uptake, which continued for several hours (Fig. 6D). This entry into S correlated with the increase of cells in G_1, as measured by flow cytometric analysis at 18 h postrelease, from 5% in untreated HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G-infected cells to 30% in HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G-infected cells treated with pentoxifylline (data not shown). Thus, pentoxifylline treatment of Vpr-arrested HeLa cells not only alleviated the G_2 arrest but also allowed cells to continue through a second round of DNA synthesis.

Pentoxifylline and other methylxanthines can alleviate the G_2 arrest induced by Vpr alone. The experiments described above showed that pentoxifylline can alleviate the G_2 arrest induced by Vpr when expressed in the context of an HIV infection. We next tested whether a G_2 arrest induced by Vpr expressed in the absence of other viral components could also be alleviated by pentoxifylline treatment. HeLa cells were transfected with a vector that expresses both the cell surface *thy* 1.2 gene and either the *vpr* or mutant *vpr* (*vprX*) gene. This allowed us to specifically study the effect of pentoxifylline on cells expressing Vpr by analyzing the cell cycle profile of the Thy 1.2-positive cells. We also tested whether another methylxanthine, caffeine, could alleviate the effects of Vpr. Caffeine, like pentoxifylline, has also been reported to reverse the G_2 delay of DNA-damaged cells (13, 24, 28).

As shown previously, the Thy 1.2-positive subpopulation from cells transfected with BSVprthy showed an increase in the

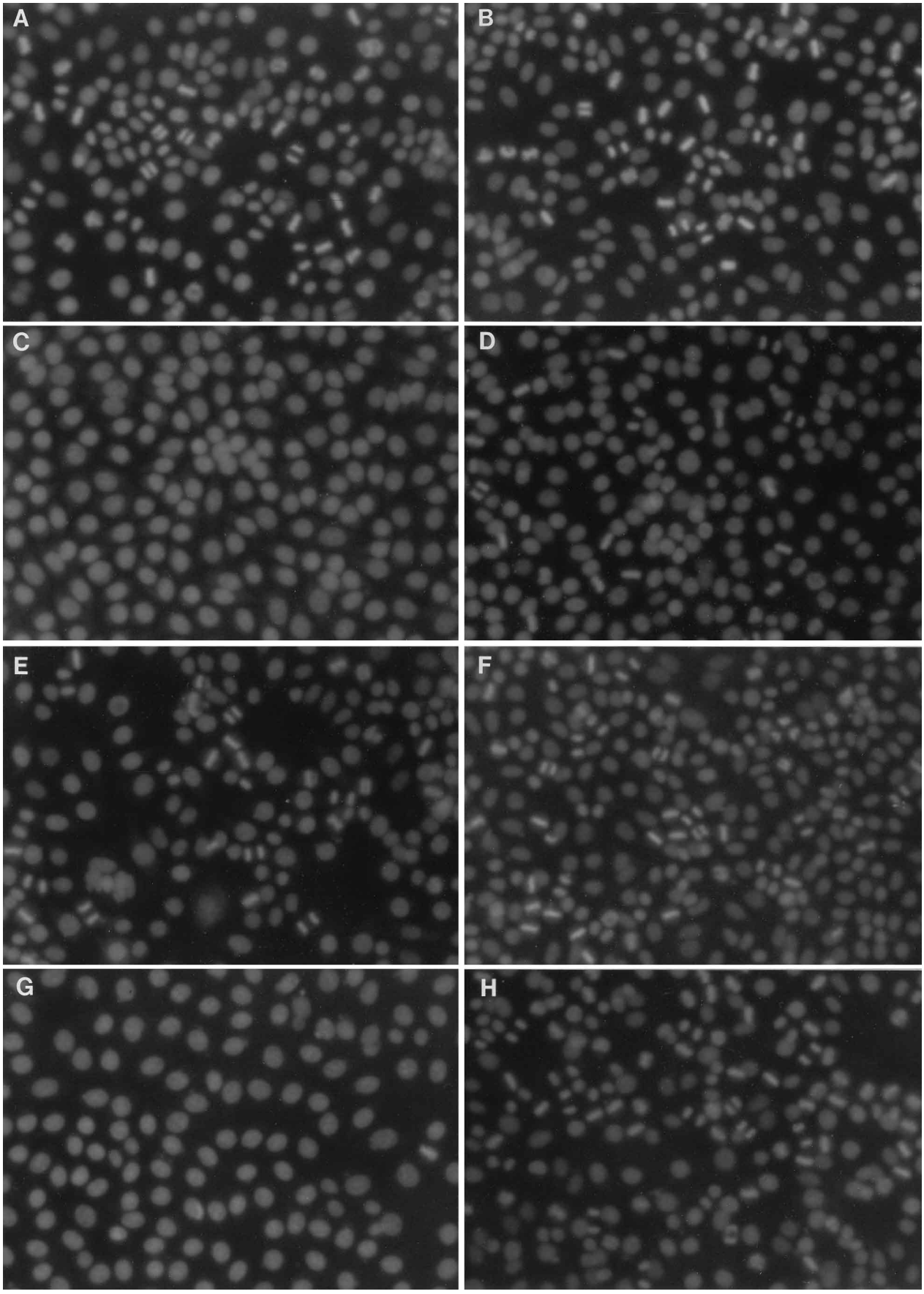
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FIG. 5. Treatment with pentoxifylline allows cells arrested by HIV-1 Vpr to enter mitosis. HeLa cells (10^5) were plated on four-chamber slides and synchronized by a double thymidine block. Upon release, cells were mock infected, exposed to HN_2 (5 μM) for 30 min, or infected with HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G or HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G to achieve infection of 90 to 100% of the cells, as described in Materials and Methods. Pentoxifylline (1 mM) was added to one set of slides immediately after HN_2 treatment or infection (B, D, F, and H). Microscopic analysis after staining with Hoechst 33342, as described in Materials and Methods, was performed at 12 h postrelease. (A and B) mock-infected cells; (C and D) HN_2-treated cells; (E and F) HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G-infected cells; (G and H) HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G-infected cells.

G_2 population relative to cells transfected with a vector expressing mutant Vpr (BSVprXthy) (Fig. 7A). Compared with untreated BSVprthy cells, treatment with pentoxifylline or caffeine decreased the percentage of Thy 1.2-positive cells in the G_2 phase of the cell cycle from 85 to 56 and 48% and increased the percentage in G_1 from 12 to 37 and 45%, respectively. There was no effect of either methylxanthine treatment on the cell cycle profile of Thy 1.2-positive cells from mutant Vpr (BSVprXthy)-transfected cultures. In agreement with the flow cytometric analysis, Western analysis revealed a reduction in the hyperphosphorylated form of cdc2 kinase in BSVprthy-transfected cells treated with pentoxifylline or caffeine compared with untreated BSVprthy cells (Fig. 7B). There was no effect on cdc2 kinase in cells transfected with mutant Vpr (BSVprXthy) and treated with pentoxifylline or caffeine.

The concentration of pentoxifylline required to reverse the G_2 arrest induced by HN_2 in vitro has been reported to be in the range 0.5 to 2 mM (13, 14, 44). We found that the same concentrations of pentoxifylline were also effective in reversing the arrest induced by Vpr (Fig. 8). Concentrations of pentoxifylline below 0.25 mM did not result in significant changes in the percentage of cells arrested in G_2 by HN_2 treatment or by BSVprthy.

We considered the possibility that the decrease in cells arrested in G_2 could be due to an effect of the methylxanthines on the expression of Vpr. Since both the *vpr* and *thy* 1.2 genes are under the control of the cytomegalovirus immediate-early promoter in the BSVprthy vector, we assessed the effect of methylxanthines on expression by comparing the percentages and fluorescence intensities of Thy 1.2-positive cells from untreated and treated cultures. There was no difference in either the percentage or intensity of Thy 1.2 staining in BSVprthy, BSVprthy with pentoxifylline, or BSVprthy with caffeine cultures (data not shown). We also analyzed the effects of methylxanthines on the expression level and cellular localization of Vpr by detecting a Vpr protein that had been fused at the amino terminus to the influenza hemagglutinin nonapeptide (35). The levels of expressed Vpr protein were analyzed by Western blot analysis, using an antibody against the nonapeptide, and the levels of Vpr were not reduced in the presence of pentoxifylline (data not shown). When cells were examined by immunofluorescence, pentoxifylline treatment did not affect the previously reported nuclear localization of Vpr (data not shown). Therefore, it was unlikely that the methylxanthines had alleviated the G_2 arrest by affecting Vpr expression or localization.

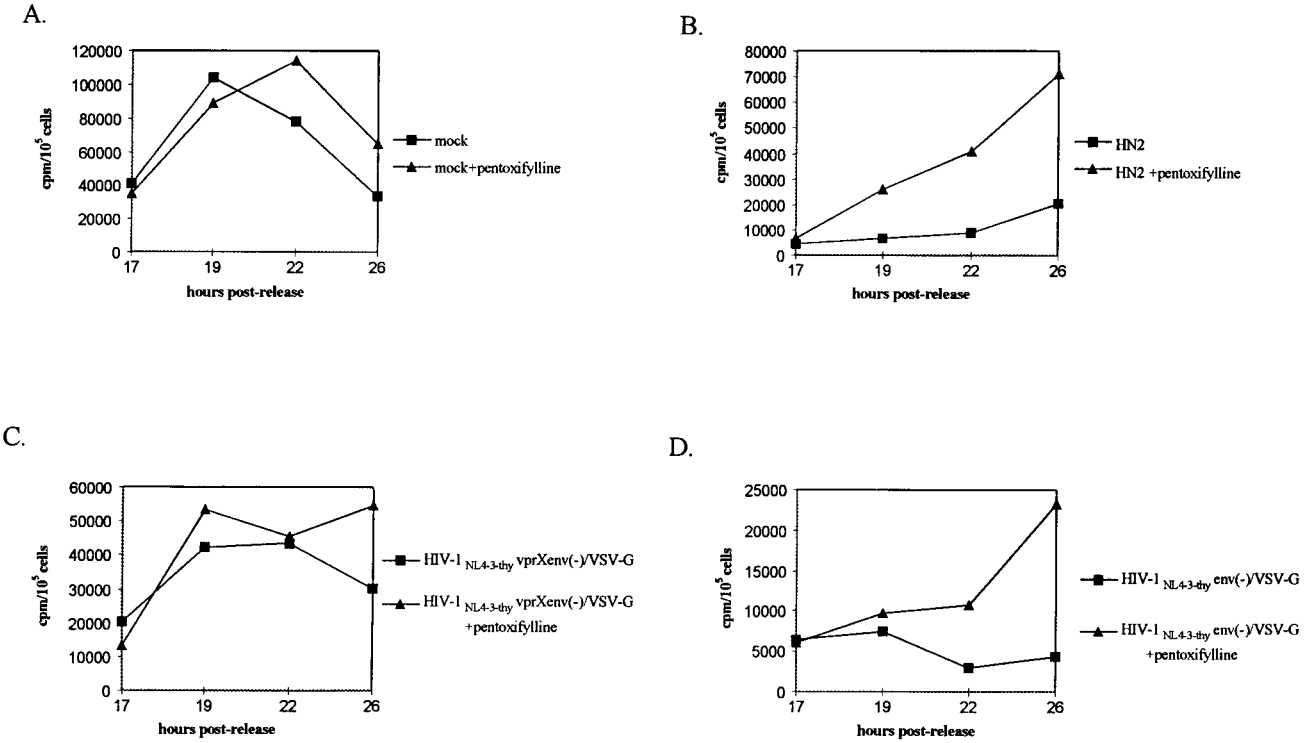

FIG. 6. Pentoxifylline treatment allows Vpr-arrested cells to progress through S phase. Synchronized HeLa cells (10^5) were mock infected, HN_2 treated, or infected with HIV-1, as described in Materials and Methods. Pentoxifylline (1 mM) was added to one set of samples immediately after HN_2 treatment or after infection. [^{3}H]thymidine (50 μCi/ml) was added to cultures for 4 h at the indicated times postrelease. As described in Materials and Methods, lysates were harvested, collected on glass fiber filters, and counted on a scintillation counter. The data are representative of three independent experiments. (A) Mock infected; (B) HN_2 treated; (C) HIV-$1_{NL4\text{-}3\text{-}thy}$vprXenv(−)/VSV-G infected; (D) HIV-$1_{NL4\text{-}3\text{-}thy}$env(−)/VSV-G infected.

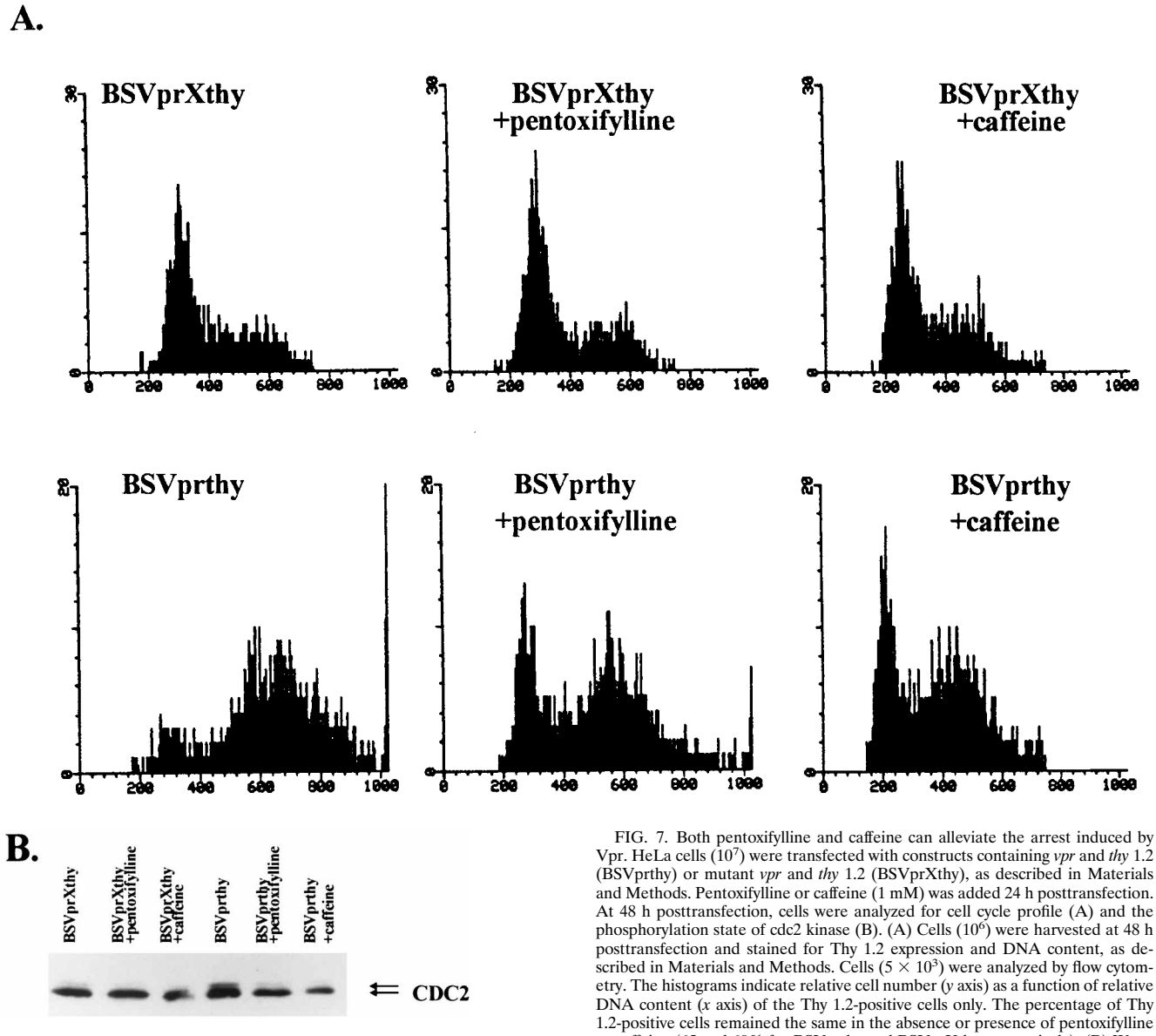

FIG. 7. Both pentoxifylline and caffeine can alleviate the arrest induced by Vpr. HeLa cells (10^7) were transfected with constructs containing *vpr* and *thy* 1.2 (BSVprthy) or mutant *vpr* and *thy* 1.2 (BSVprXthy), as described in Materials and Methods. Pentoxifylline or caffeine (1 mM) was added 24 h posttransfection. At 48 h posttransfection, cells were analyzed for cell cycle profile (A) and the phosphorylation state of cdc2 kinase (B). (A) Cells (10^6) were harvested at 48 h posttransfection and stained for Thy 1.2 expression and DNA content, as described in Materials and Methods. Cells (5×10^3) were analyzed by flow cytometry. The histograms indicate relative cell number (*y* axis) as a function of relative DNA content (*x* axis) of the Thy 1.2-positive cells only. The percentage of Thy 1.2-positive cells remained the same in the absence or presence of pentoxifylline or caffeine (65 and 60% for BSVprthy and BSVprXthy, respectively). (B) Western blot analysis of cdc2 kinase. At 48 h posttransfection, cells were lysed and 10 μg of total cell lysates/sample was separated by SDS-PAGE (15% polyacrylamide). Western blot analysis for the phosphorylation state of cdc2 kinase was performed as described in Materials and Methods. The upper and lower arrows indicate the hyperphosphorylated and nonhyperphosphorylated forms of cdc2 kinase, respectively.

Several other methylxanthine derivatives, such as theophylline and theobromine (8), were also effective in alleviating the Vpr-induced G_2 arrest (Table 1). Of the compounds tested, pentifylline appeared to be the most effective at the lowest concentration (0.1 mM) tested. Therefore, other members of the methylxanthine family can function in alleviating the G_2 arrest induced by Vpr.

DISCUSSION

Pentoxifylline alleviates the G_2 arrest induced by Vpr. We and others have previously shown that infection with HIV-1 encoding Vpr results in cells becoming arrested in the G_2 phase of the cell cycle (3, 21, 36, 37). The cell cycle arrest induced by Vpr is characterized by the accumulation of the inactive hyperphosphorylated form of cdc2 kinase. The inactivation of cdc2 kinase is similar to what is observed in cells arrested in G_2 by treatment with DNA-damaging agents such as HN_2 (31, 32). Based on the decrease in the G_2 population and the reduction in the hyperphosphorylated form of cdc2 kinase in Vpr-arrested cells treated with pentoxifylline or caffeine, we conclude that a class of chemical agents known as methylxanthines, previously shown to be effective in reversing the G_2 arrest induced by HN_2, can also alleviate the arrest induced by Vpr. The effect of pentoxifylline on cells arrested by Vpr, namely, the reactivation of the cdc2 kinase and the ability of treated cells to enter mitosis, reenter G_1, and initiate DNA synthesis, parallels the effect of pentoxifylline treatment on HN_2-arrested cells. In addition to pentoxifylline, other members of the methylxanthine family were also demonstrated to be effective in reversing the Vpr-induced cell cycle arrest.

Others have reported that addition of pentoxifylline had no effect on the cell cycle arrest caused by Vpr (3). This discrep-

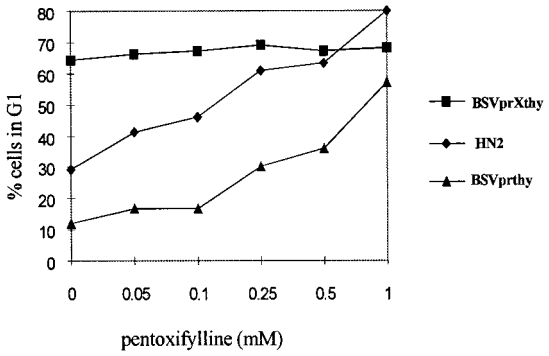

FIG. 8. Alleviation of HN_2- or Vpr-mediated arrest occurs at similar pentoxifylline concentrations. HeLa cells (10^7) were transfected with BSVprthy or mutant Vpr (BSVprXthy), as described in Materials and Methods, and seeded at a density of 1.5×10^5 cells/well in 12-well plates. Untransfected cells were also seeded in 12-well plates at the same cell density. At 24 h posttransfection, the untransfected cells were treated with HN_2 (5 μM) for 30 min, as described in Materials and Methods. Various concentrations of pentoxifylline were subsequently added to cells either treated with HN_2 or transfected with BSVprthy or BSVprXthy, as indicated. At 40 h posttransfection or 18 h post-HN_2 treatment, 10^5 cells were stained for Thy 1.2 expression and DNA content and 5×10^3 cells were analyzed by flow cytometry, as described in Materials and Methods. The percentages of Thy 1.2-positive cells in G_1 for BSVprthy- or BSVprXthy-transfected cells were determined for each concentration of pentoxifylline. Similar results were obtained in two independent experiments.

ancy may be explained by the different experimental protocols. Although a similar pseudotyped HIV-1 virus was used to express Vpr, selection for infected cells occurred for several days prior to the addition of pentoxifylline in the previous report. In contrast, we investigated the effects of pentoxifylline early in the infection process, within 12 h postinfection, without selective pressure. Consistent with the data of Bartz et al. (3), we also observed that cells that had been arrested by Vpr for >3 days were refractory to reversal by pentoxifylline (data not shown). This observation may be explained by our recent finding that the cells begin to undergo apoptosis following several days of arrest by Vpr (43).

Vpr appears to specifically affect the G_2 checkpoint. Specific points, termed checkpoints, exist within the cell cycle which ensure the completion of one phase before initiation of the subsequent phase (29, 40). One of these checkpoints exists at the transition from G_2 to M and is controlled by the cdc2 kinase. Activation of cdc2 kinase via dephosphorylation at Thr 14 and Tyr 15, with concomitant phosphorylation of Thr 161 as well as complex formation with cyclin B, is required for entry into mitosis. Cells will arrest at the G_2 checkpoint in response to DNA damage or failure to complete DNA synthesis. Arrest at the G_2 checkpoint is due to inactivation of cdc2 kinase, either by aberrant expression of cdc2 kinase and/or cyclin B or alteration of the phosphorylation state of cdc2 kinase.

The phenotypes of HN_2- and Vpr-induced cell cycle arrest have a number of similarities. Exposure of cells to either HN_2 or Vpr resulted in arrest in the G_2 phase of the cell cycle. Cells arrested in G_2 by HN_2 or Vpr accumulated the inactive, hyperphosphorylated form of cdc2 kinase that correlated with a suppression of cdc2/cyclin B kinase activity. Neither HN_2 nor Vpr appeared to adversely affect the levels of cdc2 kinase or cyclin B (18, 31). Finally, we have shown here that drugs such as pentoxifylline and caffeine, which could reverse the G_2 arrest induced by HN_2, were also capable of alleviating the G_2 arrest induced by Vpr. These observations are consistent with our hypothesis that HIV-1 Vpr appears to induce G_2 arrest by a mechanism similar to that utilized by DNA-damaging agents, which potentially interferes with signal transduction pathways that ultimately converge on cdc2 kinase.

Vpr-arrested cells alleviated by methylxanthine treatment progress in an apparently normal manner through the cell cycle. However, removal of pentoxifylline after cells had progressed through S resulted in arrest of cells at the next G_2 phase (data not shown). This further suggests that Vpr may be specifically targeting components involved in the regulation of the G_2 phase, resulting in arrest, without permanently adversely affecting other cell cycle phases.

It is unclear at present how Vpr interacts with pathways that may be utilized by cells in response to DNA-damaging agents. Recently, an interaction between Vpr and uracil DNA glycosylase (UNG), a highly conserved enzyme responsible for the removal of uracil present in DNA due to the misincorporation of uracil or deamination of cytosine, was described (4). It is noteworthy that recent reports have indicated that overexpression of a species of UNG can result in transient arrest of human osteosarcoma (Saos) cells in G_1 (41). In addition, several leukemic cell lines which have high UNG activity also have an increased proportion of cells in G_1 (46). To date, we are not aware of any reports implicating involvement of UNG at a G_2 checkpoint; however, given the observations mentioned above, it is possible that an interaction between Vpr and UNG could contribute to the observed G_2 arrest.

A role for methylxanthines in treating HIV-1 disease. Pentoxifylline, as well as caffeine, theophylline, and theobromine, are members of the methylxanthine family which have been used in a number of clinical settings (8, 12). These compounds have been shown to inhibit the production of cytokines such as tumor necrosis factor alpha (TNF-α). They are inhibitors of phosphodiesterase activity, thus resulting in accumulation of cyclic AMP. Methylxanthines are also antagonists of adenosine receptors via competitive inhibition of binding sites (26). Pentoxifylline has been used routinely in the treatment for intermittent claudication (peripheral vascular disease) (1, 12). Treatment with pentoxifylline reduces blood viscosity by affecting erythrocyte membrane fluidity, inhibiting platelet aggregation, and reducing fibrinogen levels. Methylxanthines have been used to increase sensitivity to tumor cell chemother-

TABLE 1. Other members of the methylxanthine family can alleviate the G_2 arrest induced by Vpr[a]

Methylxanthine	G_1/G_2 ratio[b] at methylxanthine concn (mM):		
	0	0.1	0.5
None	0.09		
Pentoxifylline		0.10	0.51
Caffeine		0.10	0.40
Theophylline		0.10	0.35
Theobromine		0.09	0.30
Pentifylline		0.40	0.43

[a] HeLa cells (10^7) were transfected with BSVprthy and seeded at a density of 1.5×10^5 cells/well in 12-well plates. At 24 h posttransfection, the indicated methylxanthine, at either 0.5 or 0.1 mM, was added. Pentoxifylline (Sigma), caffeine (Aldrich), theophylline (Sigma), and theobromine (Sigma) were solubilized to 100 mM in H_2O. Pentifylline (Sigma) was solubilized to 100 mM in 50% acetone. Acetone alone at the concentration used in the dilution had no effect on the G_2 arrest induced by BSVprthy. At 48 h posttransfection, 10^5 cells were stained for Thy 1.2 expression and DNA content and 5×10^3 cells were analyzed by flow cytometry. The percentage of Thy 1.2-positive cells remained the same (41%) in the absence or presence of the methylxanthines.

[b] The ability of each methylxanthine to alleviate the Vpr-mediated arrest is expressed as the ratio of Thy 1.2-positive cells in G_1 versus Thy 1.2-positive cells in G_2. The methylxanthine was considered effective if it produced a minimum of a twofold increase in the G_1/G_2 ratio over that of untreated BSVprthy-transfected cells. Similar results were obtained in two independent experiments.

apy, and treatment with methylxanthines in conjunction with alkylating agents has been shown to abrogate the G_2 delay. A resulting enhancement of tumor cell cytotoxicity was shown both in vitro and in vivo (14, 24, 44) and is likely due to the replication and persistence in daughter cells of chromosomes damaged by the alkylating agents.

The effect of pentoxifylline on reducing TNF-α production has led to clinical trials in AIDS patients (9, 10). AIDS patients have increased levels of TNF-α, which may contribute to the wasting syndrome. Despite the decrease in TNF-α production by in vivo administration of pentoxifylline, there was no effect on disease progression or viral load. However, the concentration of pentoxifylline achievable in patients (5 to 10 μM) would have been insufficient to have an effect on Vpr, given the much higher levels required in vitro (0.5 to 1 mM) to alleviate the cell cycle arrest. The effects of pentoxifylline and caffeine as lead compounds may assist in the identification of other more potent derivatives which are effective at concentrations achievable in vivo. Studies with simian immunodeficiency virus mutants suggest that Vpr plays a critical role in viral pathogenesis (20, 23). Therefore, drugs which block some of the effects mediated by Vpr, such as cell cycle arrest, might be expected to have therapeutic value. However, we cannot predict based upon the current data whether a reversal of the Vpr-mediated effect on the cell cycle would have a beneficial role in either immune dysfunction or viral load.

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