

# Human Immunodeficiency Virus Type 1 Replication Is Modulated by Host Cyclophilin A Expression Levels

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**Human immunodeficiency virus type 1 (HIV-1) Gag and the cellular protein cyclophilin A form an essential complex in the virion core: virions produced by proviruses encoding Gag mutants with decreased cyclophilin A affinity exhibit attenuated infectivity, as do virions produced in the presence of the competitive inhibitor cyclosporine. The A224E Gag mutant has no effect on cyclophilin A affinity but renders HIV-1 replication cyclosporine resistant in Jurkat T cells. In contrast, A224E mutant virus is dead in H9 T cells, although replication is rescued by cyclosporine or by expression in *cis* of a Gag mutant that decreases cyclophilin A-affinity. The observation that disruption of the Gag-cyclophilin A interaction rescues A224E mutant replication in H9 cells prompted experiments which revealed that, relative to Jurkat cells, H9 cells express greater quantities of cyclophilin A. The resulting larger quantity of cyclophilin A shown to be packaged into virions produced by H9 cells is presumably disruptive to the A224E mutant virion core. Further evidence that increased cyclophilin A expression in H9 cells is of functional relevance was provided by the finding that Gag mutants with decreased cyclophilin A affinity are dead in Jurkat cells but capable of replication in H9 cells. Similarly, cyclosporine concentrations which inhibit wild-type HIV-1 replication in Jurkat cells stimulate HIV-1 replication in H9 cells. These results suggest that HIV-1 virion infectivity imposes narrow constraints upon cyclophilin A stoichiometry in virions and that infectivity is finely tuned by host cyclophilin A expression levels.**

One property of human immunodeficiency virus type 1 (HIV-1) that makes it unique among retroviruses is that the cytoplasmic host protein cyclophilin A is required for its replication (27). The capsid domain of the HIV-1 Gag polyprotein contains a proline-rich loop which forms a stable complex with cyclophilin A (7, 14, 17). The amino acid sequence of the proline-rich loop is shown in Fig. 1. Three residues located at the apex of the proline-rich loop, A220, G221, and P222, make intimate contact with the hydrophobic pocket of cyclophilin A (5, 9, 17, 45) and are required for binding to cyclophilin A in vitro, for packaging of cyclophilin A into virions, and for the production of infectious virions (6, 7, 14, 43). Mutant phenotypes relevant to this paper are summarized in Table 1.

The normal cellular function of cyclophilin A in vivo is not known. In vitro studies have demonstrated that cyclophilin A catalyzes the isomerization of peptidyl-prolyl bonds and that it also exhibits classic chaperone activity, increasing the yield of properly folded protein substrate (11, 15, 36). Structural studies of an uncomplexed HIV-1 Gag fragment and of Gag fragments in complex with cyclophilin A indicate that the peptidyl-prolyl bond connecting Gag residues G221 and P222 does not undergo isomerization as a result of binding to cyclophilin A (17, 18, 45). These data, together with the observation that HIV-1 replication requires the formation of a stable complex between Gag and cyclophilin A, suggest that cyclophilin A functions as a Gag chaperone rather than as a Gag isomerase (27).

Cyclophilin A was first discovered because of its high affinity for cyclosporine, a drug commonly used to suppress allograft rejection (19). Like Gag, cyclosporine also binds to the hydrophobic pocket of cyclophilin A (23, 29, 38). As a competitive inhibitor of the HIV-1 Gag-cyclophilin A interaction (28), cy-

closporine disrupts cyclophilin A incorporation into virions and, by doing so, attenuates virion infectivity (3, 13, 14, 22, 32, 37, 40).

Two Gag mutations, A224E and G226D, alter the sensitivity of HIV-1 replication to cyclosporine (1). The location of these mutations indicates not only that Gag binds to cyclophilin A but also that a gag-encoded function requires cyclophilin A. Also, since these mutations have no effect on Gag's affinity for cyclophilin A (4), they demonstrate that Gag's primary sequence requirements for binding to cyclophilin A can be distinguished from Gag's primary sequence requirements for dependence upon cyclophilin A (Table 1).

Interestingly, the phenotype of viruses bearing either the A224E mutation or the G226D mutation depends on the host cell in which viral replication is assayed. Mutant virus stocks bearing these mutations were originally selected by serial passage of HIV-1 in CD4<sup>+</sup> HeLa cells cultured in the continuous presence of a cyclosporine analog (1). In these cells the mutant viruses are cyclosporine dependent: they will not replicate in the absence of the drug. In Jurkat T cells, in contrast, these mutations confer cyclosporine resistance but not drug dependence (4): the mutant viruses are capable of replicating in the presence or in the absence of the drug.

After noting that A224E virus replication is drug dependent in H9 cells, we demonstrated that H9 cells express higher levels of cyclophilin A than do Jurkat cells. We compared the replication of a panel of Gag mutants in Jurkat cells and in H9 cells (Table 1), and the observed differences between the two cell lines suggest that HIV-1 replication is exquisitely sensitive to cyclophilin A expression levels.

## MATERIALS AND METHODS

**Plasmid DNAs and viruses.** Proviral DNAs were propagated in *Escherichia coli* JM109 clone 3226 (Life Technologies, Inc., Gaithersburg, Md.) at 30°C by standard methods (33). Supercoiled plasmids for use in transfection experiments were purified by using Plasmid Maxi kit (Qiagen, Chatsworth, Calif.). pNL4-3 is a plasmid containing a complete infectious clone of HIV-1 (2). The

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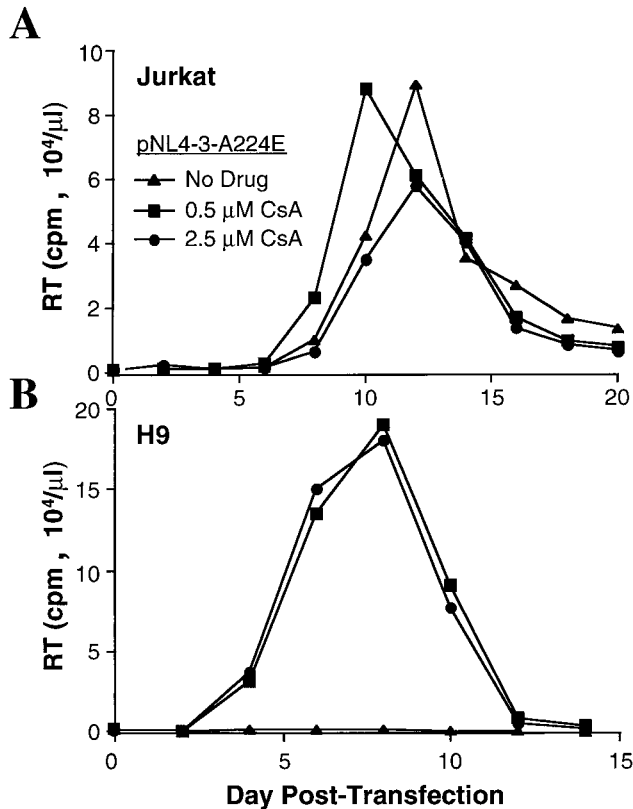


FIG. 2. HIV-1 A224E mutant virus is cyclosporine resistant in Jurkat cells and cyclosporine dependent in H9 cells. Infection of the Jurkat T-cell line (A) or of the H9 T-cell line (B) was initiated by transfection of proviral DNA pNL4-3 encoding the A224E amino acid mutation. Cyclosporine (CsA) was maintained in the cell culture medium at the indicated concentrations. The accumulation of extracellular virions in the culture supernatant resulting from viral spread through the culture is indicated by the reverse transcriptase (RT) activity in the supernatant at the indicated times posttransfection.

type replication kinetics shown in Fig. 3B). The A224E virus replication kinetics and magnitude of reverse transcriptase activity in H9 cells were identical at the two cyclosporine concentrations, indicating that there was no inhibition of replication at the higher dose relative to the lower one.

**Expression of the P222A mutation in *cis* rescues A224E virus replication in H9 cells.** We suspected that the rescue of A224E replication in H9 cells by cyclosporine was related to the drug's ability to function as a competitive inhibitor of the Gag-cyclophilin A interaction. If this is correct, expression in *cis* of a mutation that lowers Gag affinity for cyclophilin A would also rescue A224E virus replication in H9 cells. The A224E mutation was combined in *cis* with the mutation P222A. The latter mutation disrupts cyclophilin A affinity and virion packaging roughly fourfold (6), as does the double mutation P222A/A224E (4).

The P222A/A224E virus was able to replicate in Jurkat cells, although with delayed kinetics with respect to those of the wild type (Fig. 3A). In H9 cells, the P222A/A224E double mutant virus replicated at least as well as the wild-type virus (Fig. 3B). Thus, reduction of cyclophilin A incorporation by pharmacologic means with cyclosporine, or by genetic means with the P222A mutation expressed in *cis*, restores infectivity to the A224E mutant virus in H9 cells.

**Cyclophilin A expression levels in Jurkat and H9 cells.** The A224E mutation has no effect on cyclophilin A affinity or on the efficiency of cyclophilin A packaging into virions (4). The

fact that the A224E virus replicates well in Jurkat cells but does not replicate detectably in H9 cells unless rescued by factors which inhibit cyclophilin A packaging into virions suggested that the two cell lines might differ with respect to cyclophilin A expression levels. If H9 cells expressed higher levels of cyclophilin A than Jurkat cells and the packaging of relatively large quantities of cyclophilin A into virions was not tolerated by the A224E mutant virion core, cyclosporine or the P222A mutation would restore A224E infectivity in H9 cells by decreasing the levels of cyclophilin A in the virions.

To determine if H9 cells express higher levels of cyclophilin A than do Jurkat cells, total soluble protein from the two cell lines was probed in immunoblots with a rabbit anti-cyclophilin A antibody. Samples from Jurkat cells (Fig. 4A, lanes 1 and 3) and H9 cells (Fig. 4A, lanes 2 and 4) were normalized by cell number. Samples from Jurkat cells and H9 cells were also normalized for total protein content (Fig. 4A, lanes 5 to 9). Whether samples were normalized for cell number or for total cellular protein, it was evident that H9 cells express higher levels of cyclophilin A than do Jurkat cells. Similar results were obtained when cyclophilin A expression was examined by using cells grown at the lower density of  $2 \times 10^5$ /ml (data not shown). To compare relative protein levels, the H9 sample was serially diluted (Fig. 4A, lanes 6 to 9), and these semiquantitative immunoblots indicated that H9 cells express roughly threefold more cyclophilin A than do Jurkat cells.

**Virions produced in H9 cells contain more cyclophilin A than do virions produced in Jurkat cells.** Since H9 cells ex-

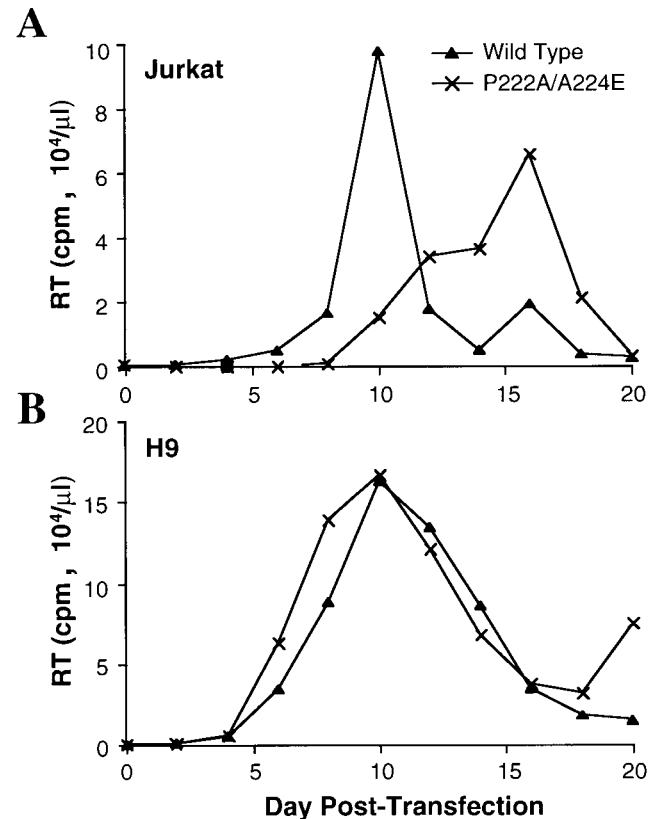


FIG. 3. P222A, a Gag mutation that confers reduced cyclophilin A affinity to the Gag polyprotein, rescues the replication of HIV-1 mutant A224E in H9 cells when the two mutations are expressed in *cis*. Jurkat T cells (A) or H9 T cells (B) were transfected with the wild-type proviral DNA pNL4-3 or with pNL4-3 encoding the P222A/A224E double mutation. RT, reverse transcriptase.

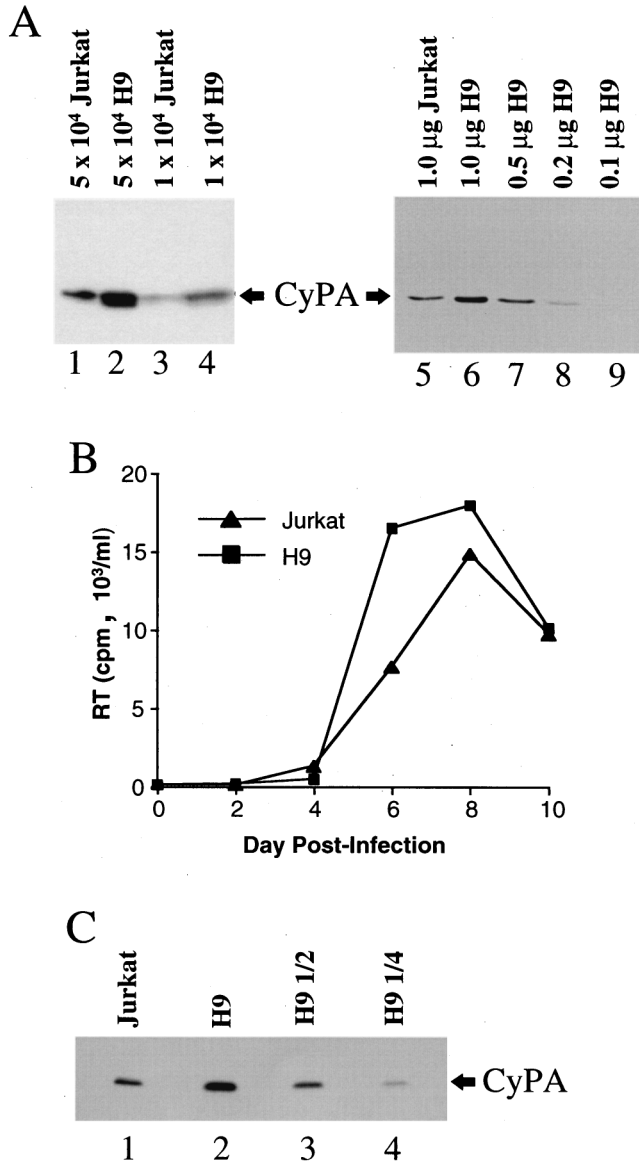


FIG. 4. H9 cells express more cyclophilin A (CyPA) than do Jurkat cells. (A) Jurkat cells (lanes 1, 3, and 5) and H9 cells (lanes 2, 4, and 6 to 9) were grown to a density of 10<sup>6</sup>/ml, lysed in radioimmunoprecipitation assay buffer, and processed for Western blotting with a primary anti-cyclophilin A antibody. Lanes 1 to 4, samples normalized by cell number. The quantity of soluble cell lysate loaded per lane, as measured by number of cell equivalents, is indicated above the lanes. Lanes 5 to 9, samples normalized by quantity of total protein in the soluble cell lysate. The numbers above the lanes indicate the amount of total protein loaded per well. (B) Replication of wild-type HIV-1 in Jurkat or H9 cells after initiation of infection with a common stock of virus produced by transfection of 293T cells with pNL4-3. RT, reverse transcriptase. (C) Virions were purified from the supernatants of the cultures used for panel B on day 8 postinfection. Virion-associated protein was probed in a Western blot with a primary anti-cyclophilin A antibody. Lanes 1 and 2, samples from Jurkat and H9 cell cultures, respectively, normalized for reverse transcriptase activity and capsid protein. Lanes 3 and 4, serial twofold dilutions of the H9 sample, as indicated.

press higher levels of cyclophilin A than do Jurkat cells, the cyclophilin A contents of virions produced by these two cell lines were compared. To obtain sufficient quantities of virions for biochemical analysis, infection was initiated in Jurkat and H9 cells by using a single virus stock produced by transient transfection of 293T cells with pNL4-3. Following infection, virion accumulations in the supernatants of the two cell lines

were roughly comparable, although the reverse transcriptase activity peaked slightly earlier and achieved higher magnitudes in the H9 cell cultures (Fig. 4B).

Virions were purified from supernatants taken at day 8 from the two cultures (Fig. 4B) by centrifugation through sucrose gradients as described in Materials and Methods. After normalization for capsid protein content and for reverse transcriptase activity, samples were probed in Western blots with anti-cyclophilin A antibodies. Virions produced in H9 cells were repeatedly found to contain greater quantities of cyclophilin A than virions produced in Jurkat cells. Results from a representative experiment are shown in Fig. 4C. Serial dilution of the sample produced in H9 cells indicated that these virions contained between two- and threefold more cyclophilin A than the virions produced by Jurkat cells (Fig. 4C).

**HIV-1 mutants with decreased cyclophilin A affinity replicate in H9 cells.** If the higher level of cyclophilin A expression in H9 cells than in Jurkat cells is of functional relevance to HIV-1 replication, mutations which decrease Gag polyprotein affinity for cyclophilin A would have more drastic effects on viral replication in Jurkat cells than in H9 cells. Therefore, the effect on viral replication of two mutations which decrease cyclophilin A affinity, G221A and P222A (6), was examined. The presence of either mutation was sufficient to inhibit the accumulation of extracellular virions in Jurkat T-cell cultures to levels below the limit of detection by assays for reverse transcriptase activity; no significant accumulation of virions was seen with either mutant G221A or P222A for up to 3 weeks of culture (Fig. 5A). Under identical conditions, the

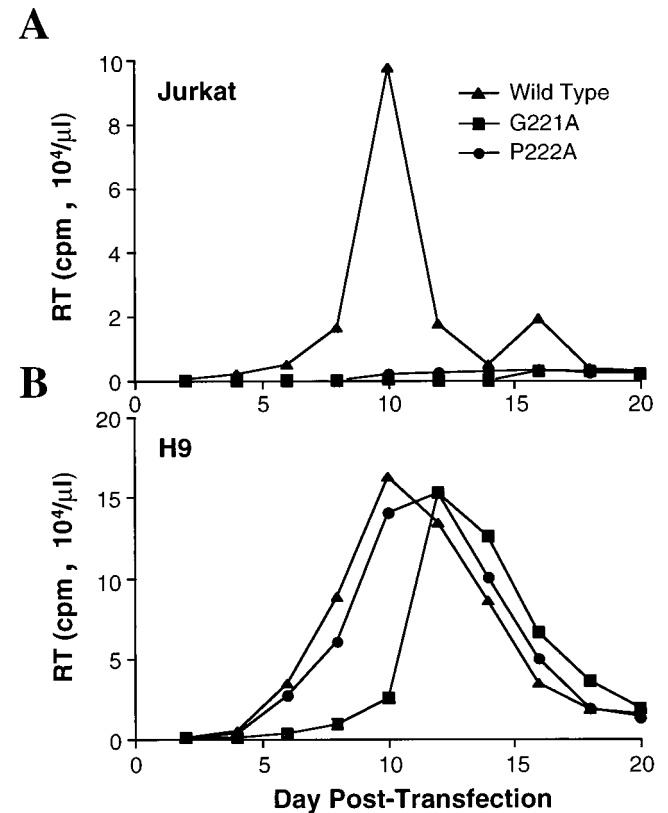


FIG. 5. HIV-1 proviruses expressing Gag mutants with attenuated cyclophilin A affinity cannot promote a spreading infection in Jurkat cells (A) but do replicate in H9 cells (B). Infection was initiated by direct transfection of wild-type or mutant proviral DNA pNL4-3 into the T-cell lines. RT, reverse transcriptase.

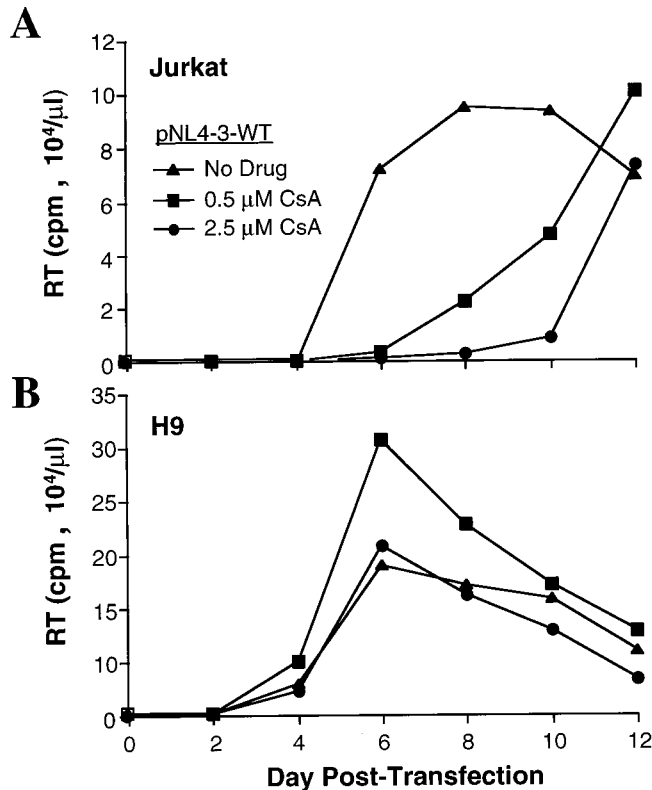


FIG. 6. HIV-1 replication in H9 cells is resistant to inhibition by cyclosporine. Infection of Jurkat T cells (A) or of H9 T cells (B) was initiated by transfection of wild-type (WT) proviral DNA pNL4-3. Cyclosporine (CsA) was maintained in the cell culture medium for the duration of the experiment at the indicated concentrations. RT, reverse transcriptase.

wild-type virus peaked at 10 days posttransfection (Fig. 5A).

In contrast to what was observed following transfection of the Jurkat cells, the G221A and P222A mutants were both capable of replicating quite well in H9 cells. Both mutant viruses peaked by 12 days posttransfection, in comparison with the replication peak at 10 days in these cells for the wild-type virus (Fig. 5B). Although the mutant viruses were able to replicate in H9 cells, there was a slight inhibition of replication, the magnitude of which correlated with the previously reported effect of these mutations on cyclophilin A binding and packaging: G221A has a larger effect than P222A on cyclophilin A affinity (6), and it produced a more significant reduction in replication in H9 cells.

**Replication of wild-type HIV-1 is cyclosporine resistant in H9 cells.** Additional evidence that higher-level cyclophilin A expression in H9 cells is of functional relevance was provided by comparing the effects of cyclosporine on replication of the wild-type virus in the two cell lines. Since cyclosporine competes with Gag for binding to cyclophilin A, a given concentration of the drug would be expected to disrupt HIV-1 replication more effectively in Jurkat cells than in H9 cells. Infection was initiated by transfection with wild-type pNL4-3, and cyclosporine was maintained continuously in the culture medium at 0, 0.5, or 2.5  $\mu$ M. In Jurkat cells, viral replication was inhibited by cyclosporine in a titratable fashion (Fig. 6A). When the identical experiment was conducted with H9 cells, no inhibition of viral replication was observed (Fig. 6B). In fact, in the presence of 0.5  $\mu$ M cyclosporine, the peak of reverse transcriptase activity was 75% higher than that produced without the drug. Even with 2.5  $\mu$ M cyclosporine, viral replication kinetics

and magnitude were as robust as those produced without the drug.

**Gag mutants with altered cyclophilin A affinity or cyclophilin A dependence do not rescue the replication of Vif-defective HIV-1 in H9 cells.** With respect to HIV-1 replication, cyclophilin A shares several features with the HIV-1 Vif protein (8, 12, 16, 21, 26, 34, 39). Both proteins are incorporated into virions, and both are required for reverse transcription after virions fuse membranes with susceptible target cells. Interestingly, as with the A224E mutant, H9 cells are nonpermissive for the replication of Vif-defective HIV-1. Vif is not required for HIV-1 replication in other cell lines, such as CEM-SS or, to a lesser extent, Jurkat cells.

To test the possibility that cyclophilin A and Vif perform related functions in HIV-1 replication, Vif-defective proviruses bearing various *gag* mutations that affect interactions with cyclophilin A were constructed. As expected, the Vif-defective virus was unable to replicate in H9 cells (Fig. 7). This virus replicated weakly in Jurkat cells but as well as the wild type in CEM-SS cells (data not shown). Since the P222A mutant was able to rescue A224E replication in H9 cells, the effect of the P222A, A224E, or P222A/A224E mutant on replication of the Vif-defective virus in H9 cells was tested. None of the Gag mutants were found to rescue Vif-defective viral replication in H9 cells (Fig. 7).

## DISCUSSION

By examining the effect of pharmacologic and genetic factors that alter the interaction between HIV-1 Gag and cyclophilin A in two T-cell lines that differ in cyclophilin A expression levels, we have obtained evidence that HIV-1 replication is modulated by levels of cyclophilin A expression in host cells. The A224E mutant replicates well in Jurkat cells but is dead in H9 cells (Fig. 2). The latter cell line expresses relatively high levels of cyclophilin A (Fig. 4), and by disrupting the interaction between Gag and cyclophilin A with the competitive inhibitor cyclosporine (Fig. 2) or by expressing the A224E mutation in *cis* with another Gag mutation that reduces cyclophilin A affinity (Fig. 3), the replication of the A224E virus in H9 cells was rescued. Conversely, Gag mutants that decrease cyclophilin A affinity and completely block viral replication in Jurkat cells (Fig. 5A) are, in effect, rescued by the higher con-

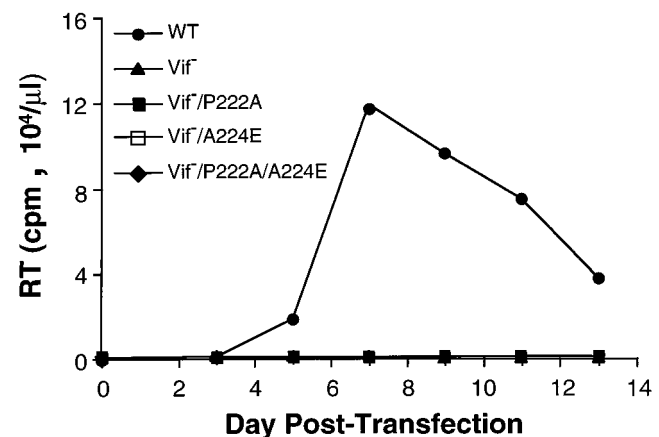


FIG. 7. HIV-1 Gag mutants expressed in *cis* do not rescue the replication of Vif-defective HIV-1 in H9 cells. Infection of H9 T cells was initiated by transfection of wild-type (WT) or mutant proviral DNA pNL4-3. RT, reverse transcriptase. The Vif<sup>-</sup> virus is pNL4-3 with a deletion in the *vif* open reading frame (21).

centrations of cyclophilin A expressed in H9 cells (Fig. 5B). Similarly, cyclosporine concentrations which effectively block HIV-1 replication in Jurkat cells have no effect on HIV-1 replication in the presence of the higher quantities of cyclophilin A expressed by H9 cells (Fig. 6B).

We believe that the differences in virus replication in Jurkat cells and H9 cells are explained by the higher levels of cyclophilin A expressed in H9 cells (Fig. 4A) and the resulting greater quantity of cyclophilin A packaged into the virions that these cells produce (Fig. 4C). Cyclophilin A is required for an early event in the infection cycle, after membrane fusion but before initiation of reverse transcription (6). Based on genetic and structural data, current models propose that cyclophilin A interferes with contacts between capsid monomers in the virion core (17, 27). At a ratio of 1:10 with respect to capsid (14, 37), cyclophilin A weakens the core structure to an extent required for virion uncoating. The linear decrease in infectivity observed with linear decreases in cyclophilin A packaging (6) is consistent with this model and suggests that the capsid/cyclophilin A ratio must be finely tuned for infectivity. When there is too much cyclophilin A, the core is unstable; when there is too little cyclophilin A, the core is too stiff for uncoating. Subtle weakening of capsid-capsid interactions by the A224E mutation would destabilize the core and permit virion uncoating to proceed in Jurkat cells in the absence of cyclophilin A. Since cyclophilin A affinity is not attenuated by the A224E mutation (4), a slight increase in cyclophilin A expression, as observed with H9 cells, would weaken capsid-capsid interactions further, rendering the virion core unstable. Consistent with this model is the observation that A224E virus replication in H9 cells is rescued by factors that decrease cyclophilin A packaging.

It remains a possibility that cellular properties other than cyclophilin A expression levels are responsible for the differences in viral phenotypes in Jurkat and H9 cells. It seems unlikely that differences in cyclophilin A primary structure could be responsible for the observations reported here, since there is absolute cyclophilin A amino acid conservation among four different primate species (42). Alternatively, Jurkat and H9 cells might differ with respect to the expression levels of an unknown factor that regulates the Gag-cyclophilin A interaction.

Although cyclophilin A accumulates to reasonably high levels in all cell lines and tissue types examined (25), threefold increases in cyclophilin A expression following heat shock in yeast (35) and following concanavalin A stimulation of mouse splenocytes (25) have been reported. The experiments presented here suggest that a threefold change in cyclophilin A expression could have profound effects on HIV-1 replication. Several steps of the HIV-1 life cycle, for example, reverse transcription (44) and proviral transcription (10, 24, 30), are regulated by the activation state of the cell. Perhaps increases in cyclophilin A expression are yet another cellular activation response that HIV-1 exploits to maximize viral production.

Disruption of HIV-1 *vif* leads to a cell-type-dependent phenotype similar to that of cyclophilin A-deficient virions (8, 12, 16, 21, 26, 34, 39). Although H9 cells are nonpermissive for replication of *Vif*-defective HIV-1 and for the A224E mutant, factors which rescue A224E replication do not rescue that of *Vif*-defective virus. These results suggest that the role of *Vif* in the early events of HIV-1 replication is distinct from the role of cyclophilin A.

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