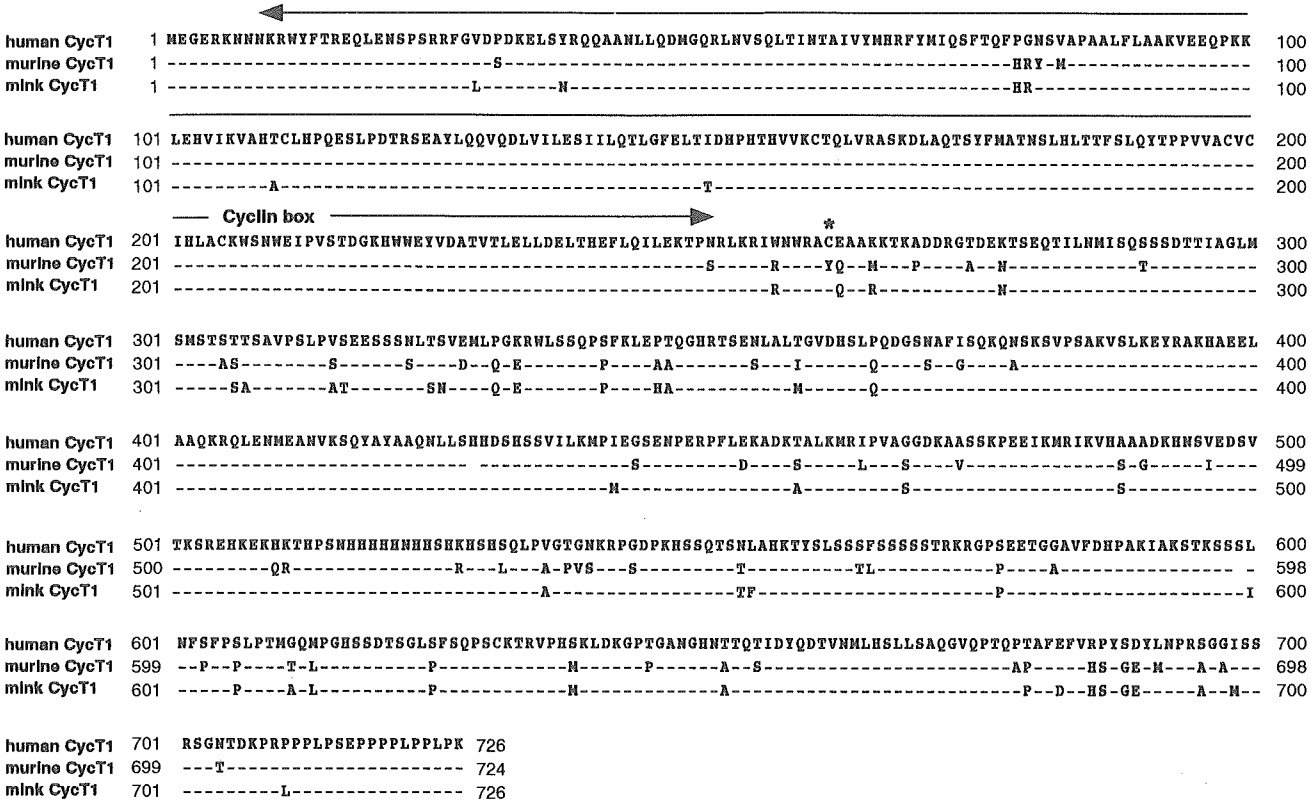

FIG. 2. Comparison of the predicted amino acid sequences of human (GenBank accession number AF048730), mouse (GenBank accession number AF095640), and mink CycT1s. The numbers are amino acid residue positions. Dashes indicate identical amino acids, and the conserved cyclin box is indicated by arrows. The cysteine residue at position 261, which can confer the ability of human CycT1 to mediate Tat function, is indicated by asterisk.

infected mink cells. Many authors have described a reduced level of unspliced genomic RNA in rodent cells (5, 22, 25, 31). The levels of unspliced and spliced HIV-1 mRNA in infected mink cells were therefore analyzed in RNase protection assays with a probe spanning the 5′ major splice donor (5). For infection, replication-competent, VSV-G-pseudotyped R7/3/162P3 viruses were used, with human HOS and murine NIH 3T3 cells expressing human CycT1 (18) serving as positive and negative controls, respectively. While the levels of spliced HIV-1 mRNA were similar in all of the cells analyzed, the amount of unspliced, full-length HIV-1 transcripts was low in murine NIH 3T3 cells expressing human CycT1 but was comparable in mink Mv.1.Lu and human HOS cells (Fig. 3).

Primary mink cells as well as the mink cell line Mv.1.Lu support substantial early and late HIV-1 gene expression. To investigate the potential for HIV-1 to replicate in cells from minks, ConA–IL-2-stimulated ex vivo cultures of primary mink splenocytes, fibroblasts, and the Mv.1.Lu cell line from *M.*

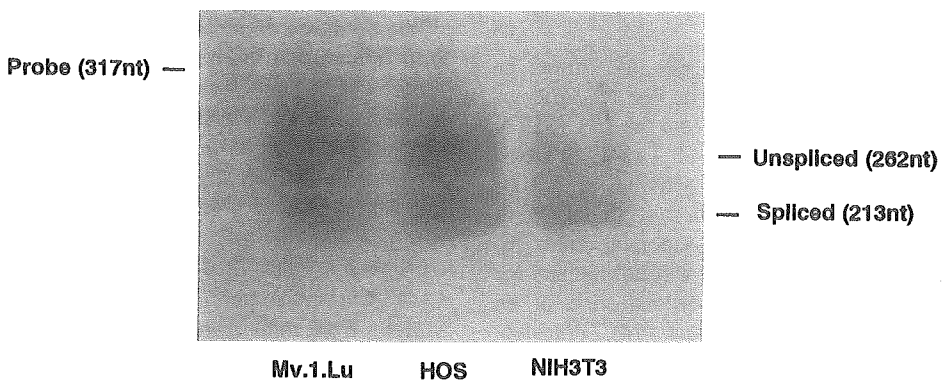

FIG. 3. RNase protection analysis of HIV-1 transcripts in infected mink, human, and mouse cells. The indicated HIV-1 receptor-negative cells were infected with VSV-G-pseudotyped R7/3/162P3. Forty-eight hours later, total RNA was extracted from infected cells and analyzed by RNase protection assay. The predicted migration of the 317-nucleotide (nt) undigested probe which spans the major 5′ splice donor site, resulting in two protected fragments of 262 and 213 nucleotides that correspond to unspliced or spliced HIV-1 RNA, respectively, are indicated.

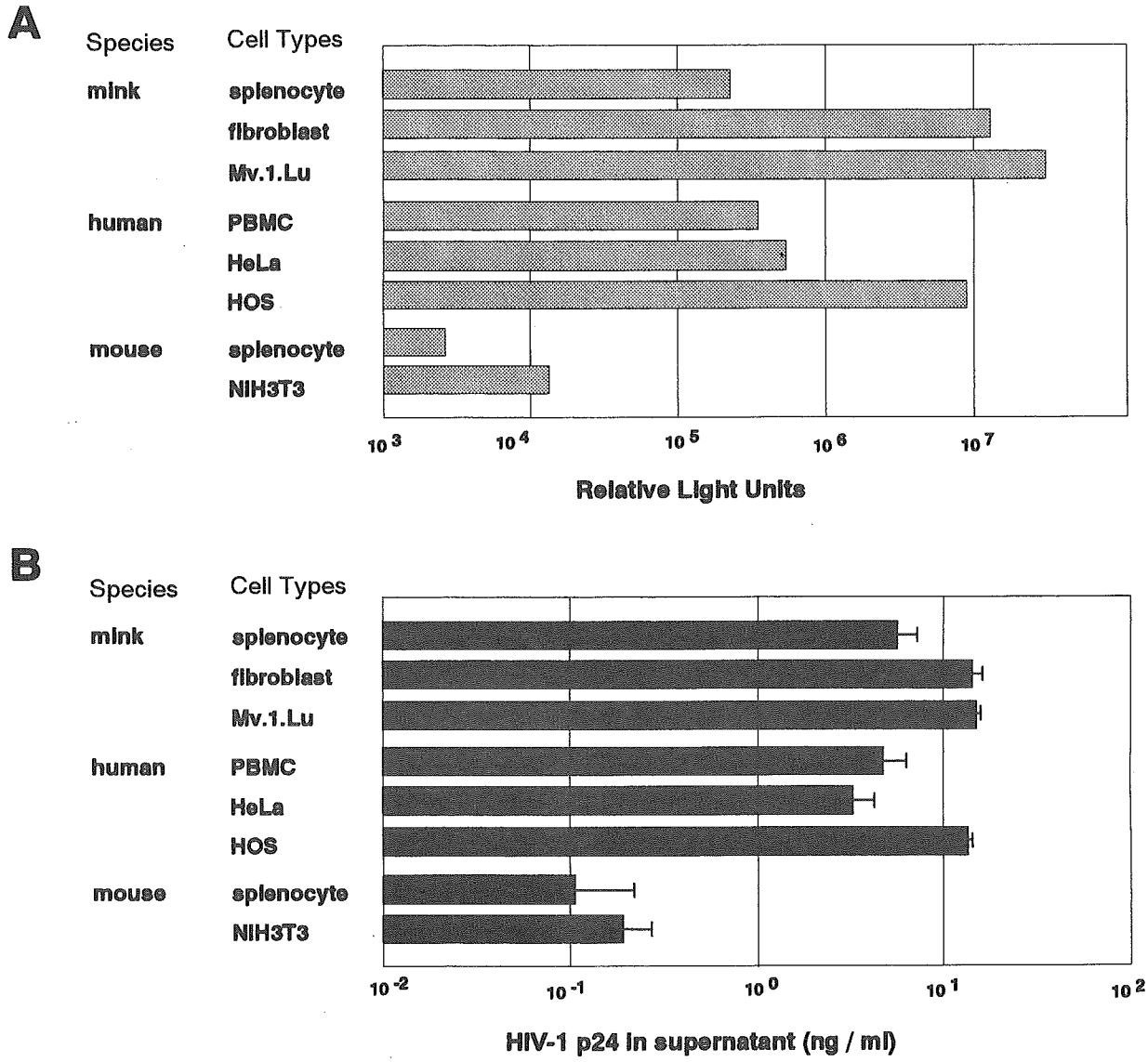

FIG. 4. Primary mink cells as well as the mink Mv.1.Lu cell line support all postentry steps in the HIV-1 replication cycle. (A) ConA–IL-2-activated mink splenocytes, mink fibroblasts, and the Mv.1.Lu cell line were inoculated with 15 ng of the p24 equivalent NL4-3 Luc E^-R^- reporter viruses pseudotyped with VSV-G envelope glycoprotein, and luciferase activity in the infected cells were measured at 3 days postinfection (in relative light units) as a marker of early HIV-1 gene expression. The data are representative of those from three independent experiments. (B) The cells were also infected with VSV-G-pseudotyped R7/3/162P3. The p24 antigen content in culture supernatants was determined at 3 days postinfection as a marker for expression and egress of a late, fully processed HIV-1 gene product. PHA-P–IL-2-activated human PBMC, HeLa cells, and HOS cells and ConA–IL-2-activated primary mouse splenocytes and NIH 3T3 cells served as control. Bars represent mean values from triplicate sample, with error bars representing standard deviations.

vison were infected. For these studies, VSV-G-pseudotyped replication-competent as well as single-round luciferase reporter viruses [R7/3/162P3(VSV-G) and NL4-3LucE^-R^-(VSV-G), respectively] were used. Infection with the single-round NL4-3LucE^-R^-(VSV-G) reporter virus allows quantitative measurement of early viral gene expression in the form of Tat-driven luciferase activity, while p24 CA antigen production in culture supernatants of cells infected with R7/3/162P3(VSV-G) gauges the relative expression and egress of a Rev-dependent, fully processed structural HIV-1 gene product. Parallel infections were made in PHA–IL-2-activated human PBMC, human HeLa, HOS cells, ConA–IL-2-activated mouse splenocytes, and NIH 3T3 cells. Infections in PHA–IL-2-activated human PBMC, which express both CD4 and CCR5, were carried out in the presence of the R5 entry inhibitor TAK-779 (10 μM) (1) to limit replication to a single round.

We found that primary mink lymphoid and fibroblast cells from three independent donors, as well as the Mv.1.Lu cell line, exhibited robust signals for both early (luciferase activity) (Fig. 4A) and late (p24 production) (Fig. 4B) HIV-1 gene expression following challenge with HIV-1(VSV-G) pseudotypes, while mouse splenocytes and NIH 3T3 cells yielded approximately 100- to 1,000-fold-lower signals (Fig. 4).

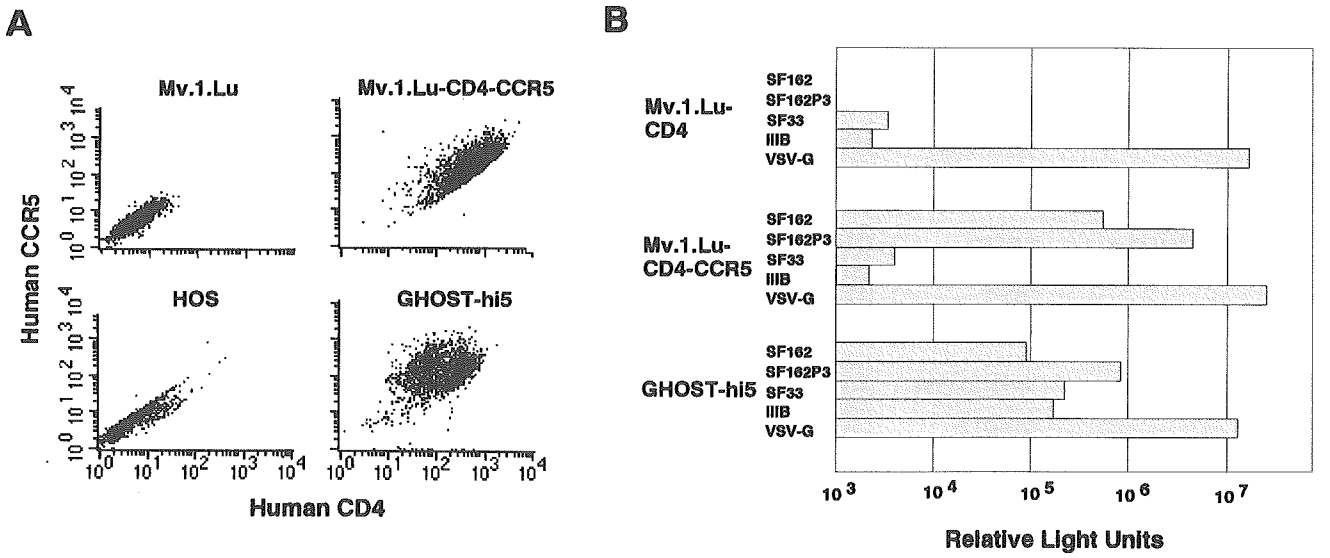

FIG. 5. The mink cell line Mv.1.Lu can be rendered permissive to HIV-1 R5 virus infection by coexpression of human CD4 and CCR5. (A) Flow cytometry analysis of human and mink cell lines for determination of expression of human CD4 and CCR5. The parental cells were used as negative controls. (B) Single-round infections with pseudotypes carrying different envelopes. Cells were infected with NL4-3 luciferase reporter viruses pseudotyped with HIV-1 R5 (SF162 and SF162P3), HIV-1 X4 (SF33 and IIIB), and VSV-G envelopes. The luciferase activity in the cell lysates was quantified as relative light units at 3 days postinoculation. Background luciferase activities, as determined by inoculation with virions containing no envelope glycoproteins, were subtracted from the values presented. The data are representative of those from three independent experiments.

Coexpression of human CD4 and CCR5 in mink Mv.1.Lu cells renders them permissive for HIV-1 replication. To determine if coexpression of human CD4 and the chemokine receptor CCR5 would allow for spread of HIV-1 infection in mink cells, we generated stable transfectants of the mink cell line Mv.1.Lu expressing human CD4 alone (Mv.1.Lu-CD4) or human CD4 and CCR5 (Mv.1.Lu-CD4-CCR5). Cell surface expression of human receptors was quantified by using flow cytometry, and abundant quantities of human CD4 and CCR5 on mink Mv.1.Lu-CD4 and Mv.1.Lu-CD4-CCR5 cells became evident (Fig. 5A). The human osteosarcoma-derived cell line GHOST-hi5, which had also been engineered to express both human CD4 and CCR5 (27), served as the control.

We first did infection studies with single-round NL4-3 luciferase reporter virus pseudotyped with different autologous or heterologous envelopes. The HIV-1 R5 envelope SF162 and SF162P3 pseudotypes efficiently infected Mv.1.Lu-CD4-CCR5 cells as well as GHOST-hi5 cells but failed to produce any signal in Mv.1.Lu-CD4 cells (Fig. 5B). Confirming the specificity, the HIV-1 X4 pseudotypes SF33 and IIIB did not infect Mv.1.Lu-CD4 cells or Mv.1.Lu-CD4-CCR5 cells but readily infected the human GHOST-hi5 cells expressing endogenous human CXCR4. Consistent with earlier findings, VSV-G pseudotypes comparably infected Mv.1.Lu-CD4, Mv.1.Lu-CD4-CCR5, and GHOST-hi5.

These findings indicate that mink Mv.1.Lu cells expressing human CD4 and coreceptor support HIV entry, which is comparable to findings with human GHOST-hi5 cells. Next, Mv.1.Lu-CD4-CCR5 cells were challenged with CCR5-sepcific replication-competent R7/3/SF162 and R7/3/SF163P3 viruses (Fig. 6), with GHOST-hi5 cells serving as controls. Cells were washed extensively with PBS following infection, and the p24 CA concentration in supernatants was monitored over the course of 8 days. Mv.1.Lu-CD4-CCR5 cells supported substantial levels of HIV-1 replication; the kinetics of p24 CA production indicated that the infection was not transient and spread through the culture. Compared to that in GHOST-hi5 cells, replication of R7/3/SF162 in MV.1.Lu-CD4-CCR5 cells appeared to be attenuated; the p24 CA concentration for Mv.1.Lu-CD4-CCR5 cells at 5 days postinfection was approximately 30-fold lower than that seen for human GHOST-hi5 cells. In contrast, levels of replication of R7/3/SF162P3 in MV.1.Lu-CD4-CCR5 and GHOST-hi5 cells were comparable; the massive multinuclear giant cell formation accompanied by cell death on day 5 postinfection of GHOST-hi5 cells with this virus limited virus spread in this culture. Thus, MV.1.Lu cells expressing human CD4 and CCR5 are permissive for productive and spread of HIV-1 R5 virus infection. In contrast, the HIV-1 X4 virus R7/3/SF33 did not productively infect Mv.1.Lu-CD4-CCR5 cells but did readily spread in the human GHOST-hi5 cells expressing endogenous human CXCR4.

Nef down-regulates CD4 in mink cells. HIV-1 Nef has been reported to facilitate viral infectivity and replication by down-regulation of cell surface CD4 (12, 20, 29). To determine whether mink cells support this Nef function, transient transfections in Mv.1.Lu-CD4-CCR5 and GHOST-hi5 cells were done with constructs expressing fusion proteins of human CD8 α chain and full-length HIV-1SF2 Nef (2). Chimeric proteins with HIV-1SF2 Nef in the antisense orientation were used as controls. CD4 displayed on the surface of transfected CD8-positive cells (i.e., Nef-expressing cells) was analyzed by flow cytometry at 40 h posttransfection. As expected, CD8/antisense Nef failed to down-modulate CD4 molecules on Mv.1.Lu-CD4-CCR5 cells as well as on GHOST-hi5 cells (Fig. 7A). In contrast, CD8/Nef was able to induce down-modulation of CD4 on both Mv.1.Lu-CD4-CCR5 and GHOST-hi5

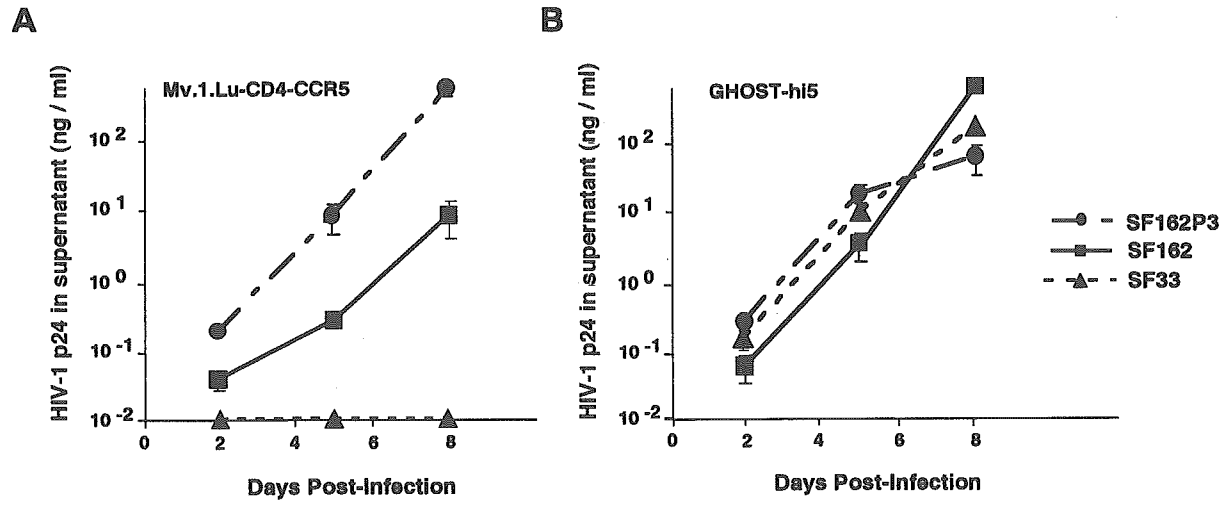

FIG. 6. Mink Mv.1.Lu-CD4-CCR5 (A) and human GHOST-hi5 (B) cells (2×10^5) were infected at the same input dose (15 ng of p24 equivalent) with R5 R7/3/162 and R7/4/162P3. Virus replication was monitored by the amounts of p24 CA produced in the culture supernatants on days 2, 5, and 8. Results shown are the means ± standard deviations from duplicate infections.

cells. CD4 expression was decreased by approximately 65.8% in Mv.1.Lu-CD4-CCR5 cells transfected with a CD8/Nef expression plasmid (mean fluorescence intensity, 60.89) compared to that in cells transfected with a CD8/antisense Nef (mean fluorescence intensity, 178.28). Similar observations were made in human GHOST-hi5 cells (Fig. 7A, panels d to f). These findings revealed that mink cells bear cellular host factors required to support a function of Nef that has been mechanistically linked to enhanced infectivity and replicative capacity of HIV-1 (20, 29).

HIV-1 produced by mink cells is as infectious as that produced by human cells. Finally, we addressed whether infected mink and human cell cultures produced comparable amounts of infectious virions. ConA–IL-2-activated ex vivo cultures of mink splenocytes, fibroblasts from *M. vison*, and the Mv.1.Lu cell line were infected with the R7/3/162P3(VSV-G) virus. Culture supernatants were harvested 3 days postinfection and then analyzed for both the p24 CA content and infectious titer ($TCID_{50}$ per milliliter) by using PHA–IL-2-activated human PBMC. No significant differences in relative titers of infectious HIV-1 released, defined as the ratio of the $TCID_{50}$ per milliliter to nanograms of p24, were observed among all cell types examined (Fig. 7B). Taken together, the abundant amounts of p24 in the culture supernatants of infected mink-derived cells were found to be as infectious as virus particles produced in human cells.

DISCUSSION

In the present study, we further characterized the potential of mink-derived cells to support HIV-1 replication. We find that the regulatory proteins Tat and Rev are fully functional in primary cells as well as cell lines of mink origin (Fig. 1 and 3). Infection of these cells with VSV-G-pseudotyped HIV-1 viruses demonstrates that once the entry block is removed, cells from *M. vision* pose no functional intracellular block to HIV-1 replication (Fig. 4). Indeed, mink cell lines engineered to express the CD4 and CCR5 receptors are permissive to HIV-1 R5 virus infection and replication (Fig. 5 and 6), with infectious virus particle production at titers that are comparable to those seen in human cells (Fig. 7B). These findings confirm and extend our previous observations made with mink cell lines (18) to primary mink fibroblasts and splenocytes and suggest that mink genetically engineered to express CD4 and the appropriate coreceptor could serve as a useful model for HIV infection.

A comparison of the deduced amino acid sequences of human and mink CycT1s revealed 93.5% homology (Fig. 2). The cysteine residue at amino acid 261 that is critical for interaction of human CycT1 with Tat is conserved in mink CycT1 and is likely to explain the functional integrity in mediating Tat *trans*-activation (Fig. 1). Rev also appears to be fully functional in mink cells. RNase protection assays showed comparable amounts of unspliced full-length HIV transcripts in mink and human cells (Fig. 3), consistent with our previous finding of an abundant level of p55 Gag, a protein that derives from the Rev-dependent p160 Gag-Pol precursor in the Mv.1.Lu cell line transduced with HIV-1 provirus (18). Nevertheless, it has been reported that while primary rat macrophages and microglia, as well as certain cell lines, synthesize Gag proteins and secrete significant concentrations of p24 CA, primary rat T lymphocytes do not do so (15, 16). Thus, the question of whether a tissue- or cell type-specific, rather than a species-specific, defect in Rev function also exists for *M. vision* requires further investigation.

Coexpression of human CycT1 in addition to CD4 and the appropriate coreceptor was found to be insufficient to render murine cells highly permissive for HIV replication (5, 11, 24, 25). In this regard, our finding that viral entry and replication in mink cell line Mv.1.Lu stably expressing only human CD4 and CCR5, and not human CycT1 in vitro, approach the level seen in human cells is significant. Compared to reference human GHOST-hi5 cells, Mv.1.Lu-CD4-CCR5 cells efficiently supported R5 HIV-1 entry (Fig. 5B). Extracellular p24 CA production in Mv.1.Lu-CD4-CCR5 cells infected with an R5

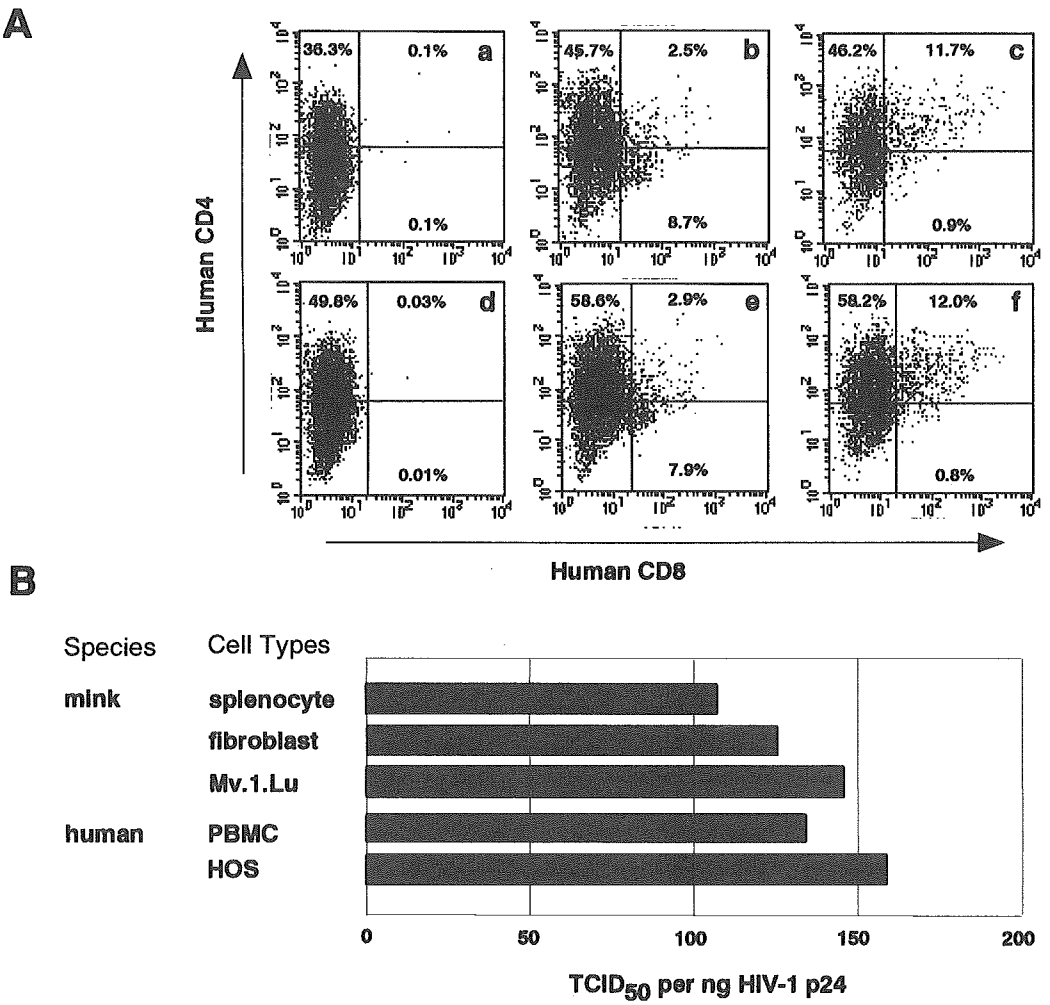

FIG. 7. (A) Mink cells support Nef function in CD4 down-regulation. Wild-type chimeric CD8/Nef or CD8/antisense Nef expression plasmid was transfected into Mv.1.Lu-CD4-CCR5 (a, b, and c) and GHOST-hi5 (d, e, and f) cells, and cell surface CD4 expression on CD8-positive transfected cells (i.e., Nef-expressing cells) was determined at 40 h posttransfection by flow cytometry as described in Materials and Methods. (a and d) Mock; (b and e) CD8/Nef; (c and f) CD8/antisense Nef. Establishment of the gate was based on the staining profiles of the nontransfected parental Mv.1.Lu and HOS cells, respectively. The percentage of events in each population is indicated. Representative results of experiments performed at least three times are shown. (B) Production of infectious mature virions by cells from mink. The relative infectivities of HIV-1 released by mink and human cells were determined as the ratio of $TCID_{50}$ measurements and p24 CA concentrations.

HIV-1 virus reached 600 ng/ml (Fig. 6A), much higher than those reported for rodent and rabbit cells (8, 16, 32). Furthermore, virus particles produced by infected mink cells are as infectious as those obtained from human cells (Fig. 7B), demonstrating a lack of substantial deficiencies in viral assembly, maturation, and egress in mink-derived cells. This is, to our knowledge, the first report of such highly permissive cells derived from a small-animal species.

Because of their well-characterized immune system and genetics and their short gestation time, rodents are the animals of choice for efforts directed at establishing a small-animal model for HIV and AIDS. However, due to the presence of multiple blocks to HIV infection and replication in cells of this species, it is far from clear that a robust rodent model for AIDS can be developed. Rabbits have a long history of serving as useful experimental models, especially for studies of humoral immune responses, but HIV replication efficiency and reproducibility are poor in rabbits (8, 9, 32, 35). Thus, there is a need to continue the search for novel animal model systems for the study of HIV disease.

Given the gaps in our understanding of the mink immune system and the ease of genetic manipulation in this species, the prospects of using *M. vision* as a small-animal model for HIV infection might be limited at present. Nevertheless, our findings of robust HIV replication in mink cells should raise hope in the search of other, more amendable small-animal species as model system for HIV infection, since our data demonstrate that intracellular blocks to HIV infection and replication in a small-animal species are not absolute.

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REFERENCES

1. **Baba, M., O. Nishimura, N. Kanzaki, M. Okamoto, H. Sawada, Y. Iizawa, M. Shiraishi, Y. Aramaki, K. Okonogi, Y. Ogawa, K. Meguro, and M. Fujino.** 1999. A small-molecule, nonpeptide CCR5 antagonist with highly potent and selective anti-HIV-1 activity. Proc. Natl. Acad. Sci. USA **96:**5698–5703.
2. **Baur, A. S., E. T. Sawai, P. Dazin, W. J. Fantl, C. Cheng-Mayer, and B. M. Peterlin.** 1994. HIV-1 Nef leads to inhibition or activation of T cells depending on its intracellular localization. Immunity **1:**373–384.
3. **Berger, E. A., P. M. Murphy, and J. M. Farber.** 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. **17:**657–700.
4. **Bieniasz, P. D., T. A. Grdina, H. P. Bogerd, and B. R. Cullen.** 1998. Recruitment of a protein complex containing Tat and cyclin T1 to TAR governs the species specificity of HIV-1 Tat. EMBO J. **17:**7056–7065.
5. **Bieniasz, P. D., and B. R. Cullen.** 2000. Multiple blocks to human immunodeficiency virus type 1 replication in rodent cells. J. Virol. **74:**9868–9877.
6. **Browning, J., J. W. Horner, M. Pettoello-Mantovani, C. Raker, S. Yurasov, R. A. DePinho, and H. Goldstein.** 1997. Mice transgenic for human CD4 and CCR5 are susceptible to HIV infection. Proc. Natl. Acad. Sci. USA **94:** 14637–14641.
7. **Connor, R., B. Chen, S. Choe, and N. R. Landau.** 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology **206:**935–944.
8. **Dunn, C. S., M. Mehtali, L. M. Houdebine, J.-P. Gut, A. Kirn, and A.-M. Aubertin.** 1995. Human immunodeficiency virus type 1 infection of human CD4-transgenic rabbits. J. Gen. Virol. **76:**1327–1336.
9. **Filice, G., P. M. Cereda, and O. E. Varnier.** 1988. Infection of rabbits with human immunodeficiency virus. Nature **335:**366–369.
10. **Fujinaga, K., R. Taube, J. Wimmer, T. P. Cuject, and B. M. Peterlin.** 1999. Interactions between human cyclin T1, Tat, and the transactivation response element (TAR) are disrupted by a cysteine to tyrosine substitution found in mouse cyclin T1. Proc. Natl. Acad. Sci. USA **96:**1285–1290.
11. **Garber, M. E., P. Wei, V. N. KewalRamani, T. P. Mayall, C. H. Herrmann, A. P. Rice, D. R. Littman, and K. A. Jones.** 1998. The interaction between HIV-1 Tat and human cyclin T1 requires zinc and a critical cysteine residue that is not conserved in the murine CycT1 protein. Genes Dev. **12:**3512–3527.
12. **Glushakova, S., J. Munch, S. Carl, T. C. Greenough, J. L. Sullivan, L. Margolis, and F. Kirchhoff.** 2001. CD4 down-modulation by human immunodeficiency virus type 1 Nef correlates with the efficiency of viral replication and with $CD4^+$ T-cell depletion in human lymphoid tissue ex vivo. J. Virol. **75:**10113–10117.
13. **Hart, C. E., C. Y. Ou, J. C. Galphin, J. Moore, L. T. Bacheler, J. J. Wasmuth, S. R. Petteway, Jr., and G. Schochetman.** 1998. Human chromosome 12 is required for elevated HIV-1 expression in human-hamster hybrid cells. Science **246:**488–491.
14. **Herbomel, P., B. Bourachot, and M. Yaniv.** 1984. Two distinct enhancers with different cell specificities coexist in the regulatory region of polyoma. Cell **39:**653–662.
15. **Keppler, O. T., F. J. Welte, T. A. Ngo, P. S. Chin, K. S. Patton, C.-L. Tsou, N. W. Abbey, M. E. Sharkey, R. M. Grant, Y. You, J. D. Scarborough, W. Ellmeier, D. R. Littman, M. Stevenson, I. F. Charo, B. G. Herndier, R. F. Speck, and M. A. Goldsmith.** 2002. Progress toward a human CD4/CCR5 transgenic rat model for de novo infection by human immunodeficiency virus type 1. J. Exp. Med. **195:**719–736.
16. **Keppler, O. T., W. Yonemoto, F. J. Welte, K. S. Patton, D. Iacovides, R. E. Atchison, T. Ngo, D. L. Hirschberg, R. F. Speck, and M. A. Goldsmith.** 2001. Susceptibility of rat-derived cells to replication by human immunodeficiency virus type 1. J. Virol. **75:**8063–8073.
17. **Kinter, A., J. Arthos, C. Cicala, and A. S. Fauci.** 2000. Chemokine, cytokines and HIV: a complex network of interactions that influence HIV pathogenesis. Immunol. Rev. **177:**88–98.
18. **Koito, A., H. Shigekane, and S. Matsushita.** 2003. Ability of small animal cells to support the post-integration phase of HIV-1 replication. Virology **305:**181–191.
19. **Kwak, Y. T., D. Ivanov, J. Guo, E. Nee, and R. B. Gaynor.** 1999. Role of the human and murine cyclin T proteins in regulating HIV-1 tat-activation. J. Mol. Biol. **288:**57–69.
20. **Lama, J., A. Mangasarian, and D. Trono.** 1999. Cell-surface expression of CD4 reduces HIV-1 infectivity by blocking Env incorporation in a Nef- and Vpu-inhibitable manner. Curr. Biol. **17:**622–631.
21. **Lue, J., M. Hsu, D. Yang, P. Marx, Z. Chen, and C. Cheng-Mayer.** 2002. Addition of a single gp120 glycan confers increased binding to dendritic cell-specific ICAM-3-grabbing nonintegrin and neutralization escape to human immunodeficiency virus type 1. J. Virol. **76:**10299–10306.
22. **Malim, M. H., D. F. McCarn, L. S. Tiley, and B. R. Cullen.** 1991. Mutational definition of the human immunodeficiency virus type 1 Rev activation domain. J. Virol. **65:**4248–4254.
23. **Mancebo, H. S., G. Lee, J. Flygare, J. Tomassini, P. Luu, Y. Zhu, J. Peng, C. Blau, D. Hazuda, D. Price, and O. Flores.** 1997. P-TEFb kinase is required for HIV Tat transcriptional activation *in vivo* and *in vitro*. Genes Dev. **11:**2633–2644.
24. **Mariani, R., B. A. Rasala, G. Rutter, K. Wiegers, S. M. Brandt, H. G. Krausslich, and N. R. Landau.** 2001. Mouse-human heterokaryons support efficient human immunodeficiency virus type 1 assembly. J. Virol. **75:**3141–3151.
25. **Mariani, R., G. Rutter, M. E. Harris, T. J. Hope, H. G. Krausslich, and N. R. Landau.** 2000. A block to human immunodeficiency virus type 1 assembly in murine cells. J. Virol. **74:**3859–3870.
26. **Moore, J. P., A. Trkola, and T. Dragic.** 1997. Co-receptors for HIV-1 entry. Curr. Opin. Immunol. **9:**551–562.
27. **Morner, A., A. Bjorndal, J. Albert, V. N. KewalRamani, D. R. Littman, R. Inoue, R. Thorstensson, E. M. Fenyo, and E. Bjorling.** 1999. Primary human immunodeficiency virus type 2 (HIV-2) isolates, like HIV-1 isolates, frequently use CCR5 but show promiscuity in coreceptor usage. J. Virol. **73:** 2343–2349.
28. **Niwa, H., K. Yamamura, and J. Miyazaki.** 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene **108:**193–199.
29. **Ross, T. M., A. E. Oran, and B. R. Cullen.** 1999. Inhibition of HIV-1 progeny virion release by cell-surface CD4 is relieved by expression of the viral Nef protein. Curr. Biol. **17:**613–621.
30. **Sawada, S., K. Gowrishankar, R. Kitamura, M. Suzuki, G. Suzuki, S. Tahara, and A. Koito.** 1998. Disturbed $CD4^+$ T cell homeostasis and in vitro susceptibility in transgenic mice expressing T cell line-tropic HIV-1 receptors. J. Exp. Med. **187:**1439–1449.
31. **Shukla, R. R., S. M. Marques, P. L. Kimmel, and A. Kumar.** 1996. Human chromosome 6- and 11-encoded factors support human immunodeficiency virus type 1 Rev function in A9 cells. J. Virol. **70:**9064–9068.
32. **Speck, R. F., M. L. Penn, J. Wimmer, U. Esser, B. F. Hague, T. J. Kindt, R. E. Atchison, and M. A. Goldsmith.** 1998. Rabbit cells expressing human CD4 and human CCR5 are highly permissive for human immunodeficiency virus type 1 infection. J. Virol. **72:**5728–5734.
33. **Tahara-Hanaoka, S., Y. Ushijima, H. Tarui, M. Wada, T. Hara, S. Imanishi, T. Yamaguchi, T. Hattori, H. Nakauchi, and A. Koito.** 2000. Differential level in co-down-modulation of CD4 and CXCR4 primed by HIV-1 gp120 in response to phorbol ester, PMA, among HIV-1 isolates. Microbiol. Immunol. **44:**489–498.
34. **Trono, D., and D. Baltimore.** 1990. A human cell factor is essential for HIV-1 Rev action. EMBO J. **9:**4155–4160.
35. **Tseng, C. K., M. A. Hughes, P. L. Hsu, S. Mahoney, M. Duvic, and S. Sell.** 1991. Syphilis superinfection activates expression of human immunodeficiency virus 1 in latently infected rabbits. Am. J. Pathol. **138:**1149–1164.
36. **Wei, P., M. E. Garber, S. M. Fang, W. H. Fischer, and K. A. Jones.** 1998. A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. Cell **92:**451–462.
37. **Zhu, Y., T. Pe'ery, J. Peng, Y. Ramanathan, N. Marshall, T. Marshall, B. Amendt, M. B. Mathews, and D. H. Price.** 1997. Transcription elongation factor P-TEFb is required for HIV-1 tat transactivation *in vitro*. Genes Dev. **11:**2622–2632.

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Ability of Small Animal Cells to Support the Postintegration Phase of Human Immunodeficiency Virus Type-1 Replication

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We examine the potential for a broad range of small animal cells, including rodent, mink, and avian cells, from multiple tissues to support postintegration steps of HIV-1 replication. These cells were engineered so as to support a stable expression of human cyclin T1 and were further transduced with HIV-1 *gag* and *pol* genes. Viral gene expression was activated by the presence of human cyclin T1, but, with the exception of mink cells, was not at the level seen in human cells. Furthermore, there were considerable defects in p24 CA release, in particular in the case of rodent cells. Fractionation of Gag proteins by sucrose floatation revealed that the Gag in human cells trafficked to membrane fractions and was processed to p24 CA and p17 MA efficiently. Confocal imaging demonstrated that Gag was localized in a punctate pattern at the plasma membrane as well as intracellular membrane *trans*-Golgi cisternae in these cells. In contrast, the majority of Gag in rodent cells was largely present in cytosolic complexes and remained unprocessed. Labeling with [9,10(n)-^{3}H]myristic acid showed a similar degree of N-myristoylated $Pr55^{gag}$ in rodent and human cells, indicating that while N-myristoylation of Gag was essential for membrane binding, it was not sufficient to confer membrane targeting specificity. Remarkably, despite the reduced level of intracellular Gag processing, mink Mv.1.Lu cells did not appear to differ significantly from human cells in support of virion assembly and release. Analysis of reciprocal heterokaryons suggested that the cellular factor(s) required for efficient assembly and release of infectious virions is lacking in murine cells but appears to be functionally present in mink as well as human cells. Our findings confirm and extend previous reports of multiple blocks to HIV replication in nonhuman cells that are most profound in murine cells. They also raise the possibility that other small animals, such as mink, could serve as novel model systems for studying HIV-1 infection and disease.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) is a lymphocytopathic virus that causes an immunodeficiency syndrome characterized by severe deficiency of $CD4^+$ T cell functions and significant $CD4^+$ T cell decline in infected individuals. Studies *in vivo* for understanding the mechanism of HIV-1 pathogenesis and for large-scale evaluation of the efficacy of potential anti-HIV-1 therapies or vaccines have been hampered since humans and certain nonhuman primates, such as chimpanzees, appear to be the sole hosts for HIV-1. With well-characterized immune systems and experimental utilities, including low cost and ease of genetic manipulations, transgenic mice and rats whose $CD4^+$ T lymphocytes could support HIV replication held promise as an ideal *in vivo* small animal model. However, it became clear that there are multiple blocks to HIV replication in these rodent cells. Mouse primary lymphocytes engineered to express receptors for HIV-1 infection, human CD4 (hu-CD4), and human CCR5 (hu-CCR5) or human CXCR4 (hu-CXCR4) were found to be susceptible to HIV-1 infection, with preintegration forms of provirus detected following infection (Browning *et al.*, 1997; Sawada *et al.*, 1998). This indicates that HIV-1 virions can bind to and fuse with mouse lymphoid cells expressing hu-CD4 and coreceptor, with subsequent reverse transcription of the genomic viral DNA. However, these mouse lymphoid cells exhibited few or no signs of productive infection (Browning *et al.*, 1997; Sawada *et al.*, 1998), which means that the replicative ability of HIV-1 could still be negatively controlled by the intracellular milieu of murine cells. Recently, transgenic rats that express hu-CD4 and hu-CCR5 on lymphocytes, macrophages, and microglia were shown to support HIV-1 replication at levels higher than those of comparable transgenic mouse models, raising the possibility that these animal species provide a useful small animal model of HIV-1 infection (Keppler *et al.*, 2002).

Intracellular blocks of HIV-1 replication in mouse cells had been well documented; transfection of HIV proviral DNA into mouse cells or introduction into transgenic mice was followed by gene expression, albeit much less efficient than that in permissive human cells (Levy *et al.*, 1986; Leonard *et al.*, 1988). This intracellular restriction could be partially overcomed by the introduction of human cyclin T1 (Wei *et al.*, 1998), species-specific cellular host factors that are required for the function of HIV-1

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regulatory protein Tat. However, HIV-1 does not establish productive infection in murine cells genetically engineered with these human cofactors (Garber *et al.,* 1998; Bieniasz and Cullen, 2000; Mariani *et al.,* 2000). Recently, additional blocks to the late phase of viral replication, including Gag polyprotein processing, virion assembly, and release, that result in the failure of the virus to spread have been reported (Bieniasz and Cullen, 2000; Mariani *et al.,* 2000, 2001).

In this study, we further examined this late phase intracellular regulation of HIV-1 replication and determined if the assembly defect is specific for rodent species. A broad range of cell types from different species stably expressing HIV-1 *gag/pol* as well as functional cyclin T1 genes were generated and examined for their abilities to support the postintegration phase of HIV-1 replication. Biochemical membrane fractionation studies and confocal microscopy using organelle-specific green fluorescence protein (GFP) vectors were used to track processing and localization of Gag proteins within the various cell types. Heterokaryons between assembly-resistant and permissive cells were tested for their potential to assemble and release virus in order to better understand the basis for the block in productive HIV replication. We found that among cells of small animal cells, the defect in Gag assembly/release is most prominent in the rodent species, although there are intraspecies variations. This defect in the infectious HIV-1 particle production of rodent cells is due to the lack of one or more species-specific cellular cofactors that facilitate specific targeting of Gag polyprotein to the plasma membrane prior to viral assembly and budding. Significantly, the finding that mink cells support HIV replication to a level that is comparable to that seen in human cells suggests that further evaluation of this small animal as a model for studies of HIV infection is warranted.

RESULTS

Differential synthesis of HIV-1 Gag/Pol in stable small animal cell lines expressing hu-cyclin T1

To further examine the basis for the block to HIV assembly and release in rodent cells, and to extend our knowledge to cells from other small animals, avian, mink, and rodent cell lines stably expressing the hu-cyclin T1 and the HIV-1 *gag, pol* genes were established. For this purpose, small animal cells, with or without the hu-cyclin T1 gene, were infected with the replication-incompetent, VSV-G pseudotyped HIV-puro virus and selected for puromycin resistance. The genome of HIV-puro contains full-length HIV-1_{NL43} proviral DNA with a frameshift in the *env* gene and an SV40 promoter regulating a puromycin-resistant gene. Intracellular HIV-1 Gag expression in the various cell lines was examined using anti-p24 CA MAb VAK4 followed by indirect immunofluorescent staining and flow cytometry. VAK4 recognized the carboxyl-terminal portion of Gag p24 CA and reacted with precursor $Pr55^{gag}$ and the intermediate precursor $Pr40^{gag}$ as well as fully processed p24 CA (Koito *et al.,* 1988). Thus, Gag protein expression including unprocessed precursors can be detected and quantitated based on fluorescence intensities.

Consistent with previous reports of the restoration of transactivation activity in hamster CHO and mouse NIH3T3 cells by hu-cyclin T1 expression (Bieniasz *et al.,* 1998; Garber *et al.,* 1998; Kwak *et al.,* 1999), an increase in Gag fluorescence, indicative of enhancement of proviral expression, was seen in all the rodent cells we examined (Fig. 1A). Gag expression was more pronounced in avian and mink cells but was robust in mink cells. The level of Gag expression in Mv.1.Lu cells was comparable to that in human PM1 and HeLa cells and was independent of hu-cyclin T1 expression. These findings indicate that mink cells, unlike those from rodent and avian sources, carry cellular host factors required to support the functions of HIV-1 Tat.

Defects in virus-like particle (VLP) release in rodent cell lines stably expressing HIV-1 Gag/Pol and hu-cyclin T1

To assess the efficiency of virus assembly and release in various cell lines, virus-like particle (VLP) production in culture supernatant was determined using a p24-specific ELISA assay. We found that human PM1, HeLa, and 293T cells carrying HIV-puro released considerable amounts of VLP into the culture supernatant, yielding p24 CA concentrations of approximately 1000 ng/ml (Fig. 1B). These cell lines stably secrete p24 CA, albeit with a slight reduction in expression levels after several months (data not shown). In contrast, HIV-1 Gag proteins were mainly cell-associated in rodent cells, even in the presence of hu-cyclin T1. Only small amounts of VLP, 4- to 5-log fold lower than the levels of p24 seen for human cells, were detected in culture supernatants of the mouse T cell line EL4, the muscle cell line NOR10, and fibroblast NIH3T3 cells (Figs. 1A and 1B). A similar pattern was observed in the murine T cell line BW5147, macrophage cell line RAW264 carrying HIV-1 *gag, pol,* and hu-cyclin T1 (data not shown). Intraspecies variations were evident. The murine muscle cell line NOR10 and rat myelomonocytic WRT7 cells showed approximately 5- to 10-fold higher levels of p24 release than other murine cells and the hamster cell line CHO showed significantly higher levels of p24 secretion, such being consistent with a report that this cell line could support at least some levels of HIV-1 replication (Bienasz and Cullen, 2000).

Nonmammalian avian cells, such as a chicken embrionic fibroblasts CEF and quail QT6 cells, produced considerably higher levels of VLP than the murine cells, albeit over 100-fold below those of the human cell lines.

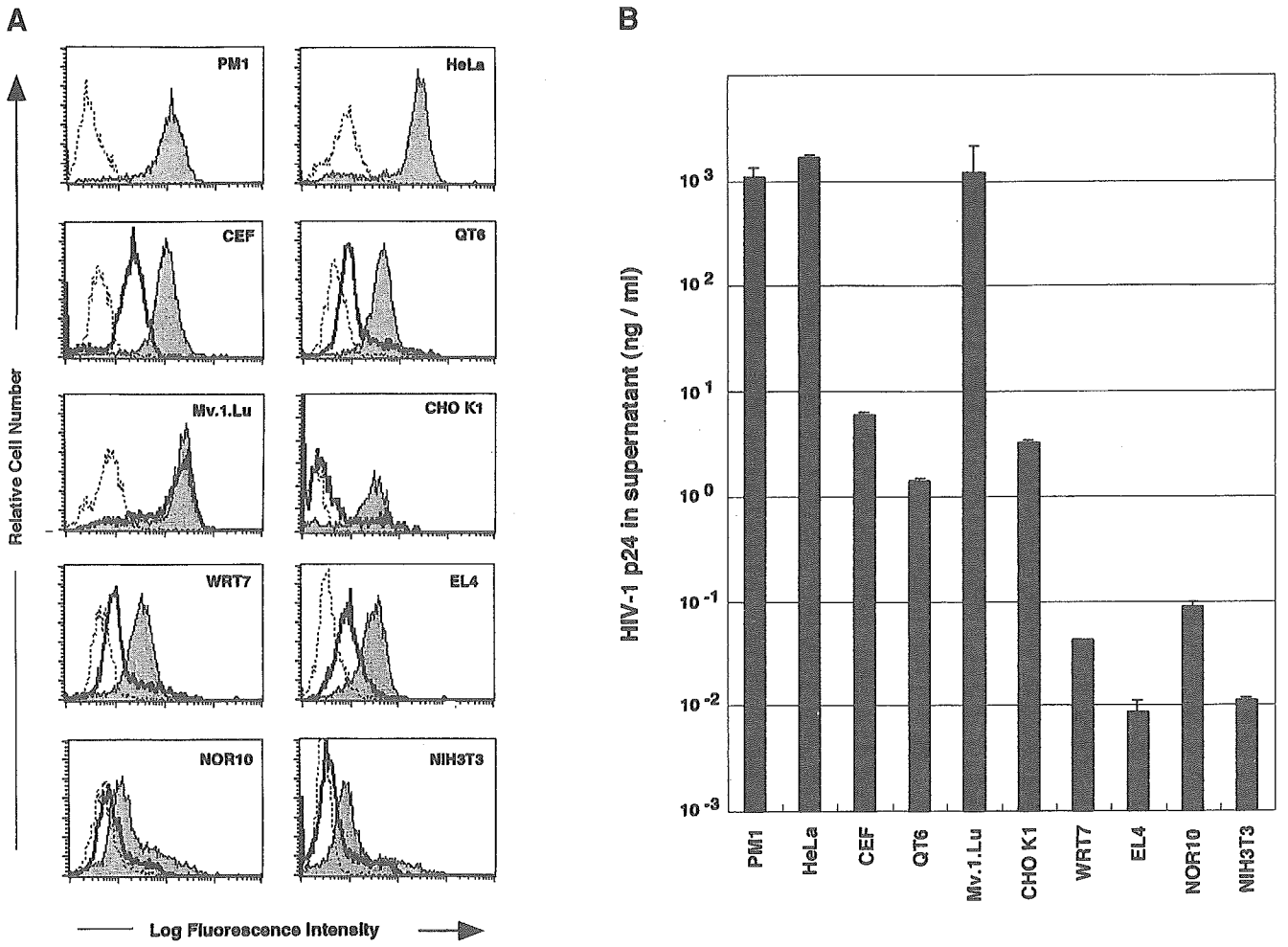

FIG. 1. Human cyclin T1 restores the intracellular HIV-1 Gag expression but not virion assembly in the murine cells. (A) Human or nonhuman cell lines with (in gray shaded area) or without (solid tracing) transduced hu-cyclin T1 were infected with HIV-puro (VSV-G). After selection in the presence of puromycin, intracellular expression of HIV-1 Gag proteins (p24 CA, Pr41Gag, and Pr55Gag) were detected by MAb against p24 CA subsequent to fixation and permeabilization. The dotted line indicates the staining of uninfected parental cells. (B) p24 levels in the culture supernatants. Culture supernatants of human or nonhuman cell lines with transduced hu-cyclin T1 expressing HIV-1 *gag* and *pol* genes were harvested and analyzed for p24 antigen content.

In the mink Mv.1.Lu cell line, the level of VLP release was comparable to that found in human cells. Thus, mink cells appear to support virus assembly and egress to a degree comparable to that of human cells.

Membrane targeting and proteolytic processing of HIV-1 pr55^{gag} are defective in rodent cells

To further examine the basis for the block in late phase HIV-1 replication in murine cells, we first looked at the intracellular localization of Gag polyprotein as well as the expression and processing of Gag using membrane floatation assays (Spearman *et al.*, 1997). We found that Gag in human PM1 and HeLa cells tended to float up through the sucrose cushion to the 10 to 65% sucrose interface, and this membrane-bound Gag was efficiently processed into p24 CA and p17 MA (Fig. 2). Differences in the targeting to membrane fractions as well as the level of intracellular processing of the Gag precursor were evident between human and murine cells. Gag was found predominantly in the bottom part of the gradient in an unprocessed form in rodent WRT7 and NIH3T3 cells; hence there was inefficient processing and targeting to the cell membrane. Profound defects in the degree to which p55^{gag} was processed were also noted in the murine lymphoid EL4 cell line (data not shown). An intermediate phenotype in membrane targeting and proteolytic processing of Pr55^{gag} were observed in avian QT6 cells. In mink Mv.1.Lu cells, Gag trafficked to the membrane fractions as efficiently as seen in human cell lines (Fig. 2), yet the majority of polyprotein remained unprocessed. Thus, the level of intracellular p55^{gag} processing does not correlate with membrane targeting efficiency. Since the level of p24 CA release in the mink cell line was comparable to that seen in human PM1 and

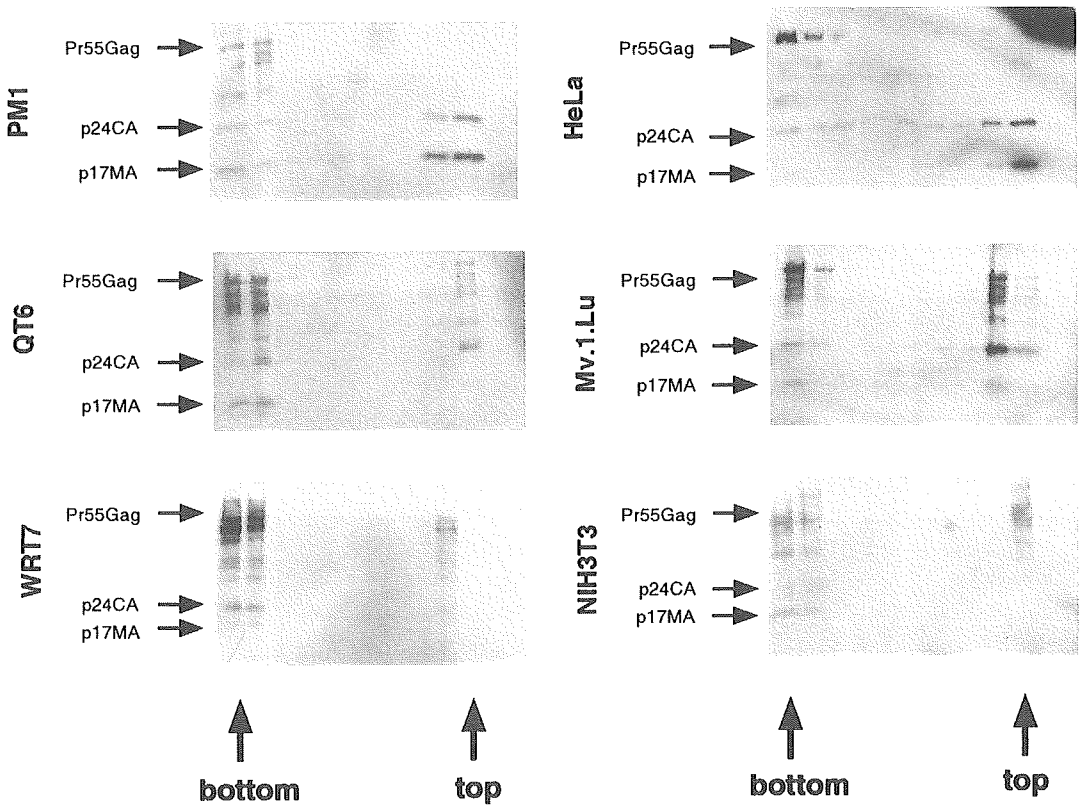

FIG. 2. Membrane floatation centrifugation with p17MA, p24CA, and Gag precursors. Postnuclear supernatants were prepared and subjected to membrane floatation centrifugation. Aliquots of each fraction were subjected to denaturating by 15% SDS–PAGE followed by electroblotting onto a PVDF membrane and developed with a mixture of anti-p17 MA and anti-p24 CA MAbs. Proteins were visualized by using the enhanced chemiluminescence method, subsequent to development with peroxidase-conjugated protein G.

HeLa cells (Fig. 1B), the efficacy of viral release from the cell does not correlate with the extent of intracellular processing of $p55^{gag}$.

Gag polyproteins localize primarily in large cytosolic complexes in murine cells

To determine intracellular localization of Gag polyproteins in the various cell types, plasmids encoding *Aequorea victoria* green fluorescent protein (GFP), variant EYFP fused to plasma membrane Golgi or ER-specific sequences, were used in transfection experiments. Laser scanning confocal microscopy, coupled with immunochemistry, was used to visualize the presence of Gag in each of the organelles. As expected, significant amounts of intracellular Gag polyproteins were expressed in human HeLa cells, some of which colocalized in a punctate pattern with the N-terminal neuromodulin-tagged EYFP at the plasma membrane (Fig. 3). This finding is consistent with previous observations made using cytological as well as biochemical methods showing that Gag polyprotein predominantly localizes in discrete regions or subdomains of the plasma membrane of human cells (Gottlinger *et al.*, 1989; Bryant and Ratner, 1990; Hermida-Matsumoto and Resh, 2000; Lindwasser and Resh, 2001). Gag polyprotein expressed in human cells also colocalized with EYFP-Golgi in the *trans* cisternae of the Golgi regions (Fig. 3), which means that HIV-1 Gag also targeted some intracellular membranes in human cells and not exclusively the plasma membrane. A similar overlay with EYFP-Golgi was observed in the case of human 293T cells (data not shown).

In contrast, analysis of Gag in murine NIH 3T3 cells revealed diffuse cytoplasmic staining with no clear localization to the plasma membrane (Fig. 3B), findings consistent with the failure of Gag to target appropriately in mouse cells (Mariani *et al.*, 2000, 2001; Bieniasz and Cullen, 2000). No costaining of Gag with ER was observed in human HeLa or murine NIH 3T3 cells.

HIV-1 Gag polyprotein in human and murine cells is similarly myristoylated

Mutation in the N-terminus of MA that blocks membrane targeting had been reported to result in viruses that failed to assemble or process efficiently in human cells (Freed *et al.*, 1994), a phenotype which is similar to that of wild-type HIV-1 Gag in murine cells (Bieniasz and Cullen, 2000; Marianii *et al.*, 2000, 2001; and this study). Covalent attachment of myristate, a 14-carbon saturated

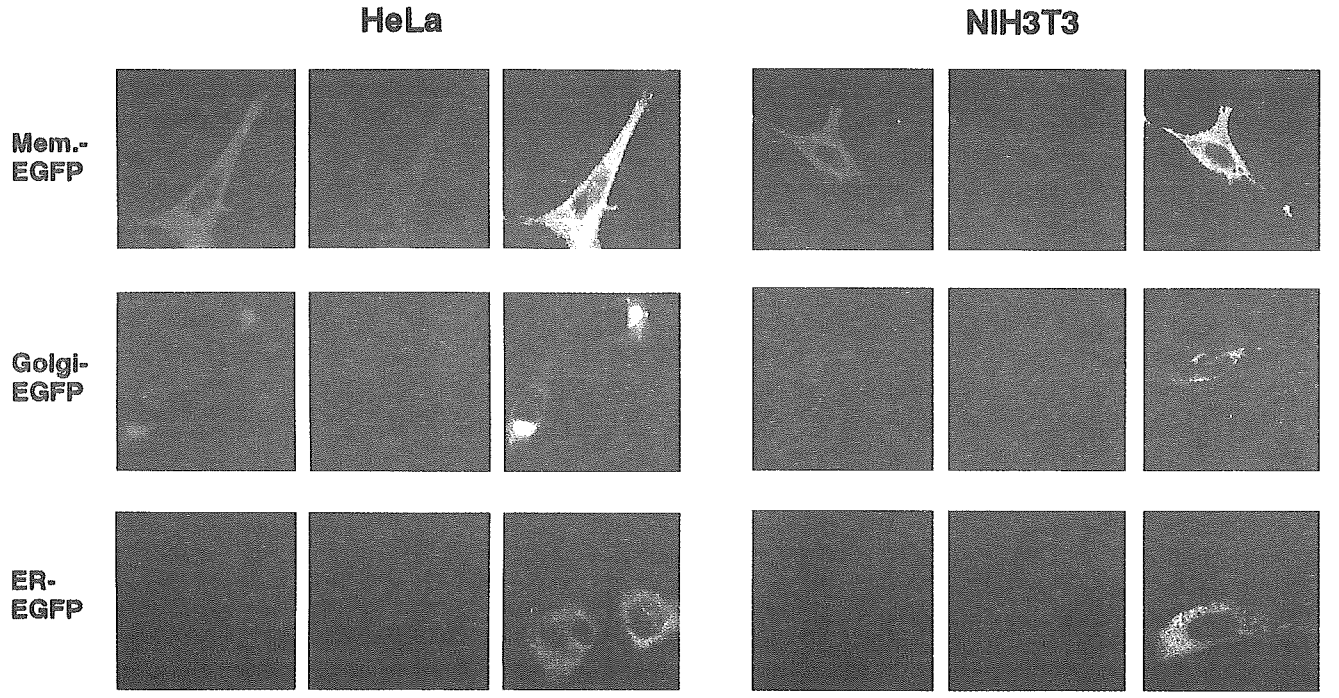

FIG. 3. Confocal microscopy analysis of intracellular Gag polyprotein. HeLa and NIH3T3 cells expressing HIV-1 *gag* and *pol* genes were transiently transfected with pEYFP vectors (CLONTECH) encoding yellow–green fluorescent variants of the enhanced green fluorescent proteins (EYFPs) targeted to the plasma membrane (pEYFP-Mem), *trans* medial Golgi (pEYFP-Golgi), or ER (pEYFP-ER). After fixation and permeabilization, cells were stained with MAbs against p24 CA and rhodamine-labeled antimouse IgG and analyzed by a confocal laser microscope. Colocalization of Gag and organelle-specific EFYP is indicated in yellow in the merged images (Merge).

fatty acid catalyzed by *N*-myristoyl transferase (NMT) to the Gly residue at position 2 of MA, had been shown to be essential for virus particle assembly and release (Bryan and Ratner, 1990; Gottlinger *et al.*, 1989). The N-terminal myristoylation of HIV-1 $Pr55^{gag}$ in murine cells has not been directly analyzed. Thus, it remains possible that species- or cell type-dependent differences in N-terminal myristoylation and/or multimerization form the basis for the apparent differences in Gag targeting/processing and virion morphogenesis in different cell types. To determine whether the $Pr55^{gag}$ translated in human and rodent cells is in the myristoylated form, cells expressing HIV-1 *gag, pol* genes were metabolically labeled with [9,10(n)-^{3}H]myristic acid. When HeLa cells expressing HIV-1 *gag–pol* genes were used, radiolabeled proteins of 55 and 41 kDa molecular size, corresponding to Gag precursor $Pr55^{gag}$ and intermediate precursor $Pr41^{gag}$, respectively, were clearly detected (Fig. 4). Radiolabeled Pr55 and Pr41 were also unequivocally identified in murine NIH3T3 cells. An additional band of 160 kDa was detected, but only in NIH3T3 cells. This band could represent the gag–pol precursor ($Pr160^{gag\text{-}pol}$) that is synthesized as a result of ribosomal frameshifting, a process shown to occur efficiently in rodent cells (Moosmayer *et al.*, 1991; Bieniasz and Cullen, 2000). In accordance with the expression levels of intracellular Gag polyproteins in human and murine cells, intensities of radiolabeled Pr55 and Pr41 bands in NIH3T3 cells were weaker than those found in HeLa cells. Taken together, evidence suggest that the lack of or inefficient membrane targeting activity of HIV-1 Gag observed in murine cells is not due to insufficient myristoylation. While myristoylation of HIV-1 Gag is essential for membrane binding, it is apparently not sufficient to determine membrane targeting specificity.

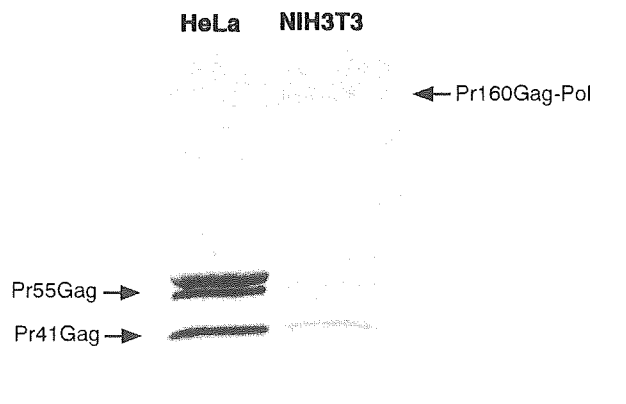

FIG. 4. Gag protein myristoylation. Cells were metabolically labeled with [9,10(n)-^{3}H]myristic acid. Cells were lysed, immunoprecipitated with an anti-p24 CA MAb, and separated by 10% SDS–PAGE subsequent to the visualization using autography.

TABLE 1

Titers of HIV-1-puro Packaged in Human, Mink, and Murine Cells Pseudotyped with Different Envelopes

Producing cells	Envelope	No. of Puro^r colonies on HOShCD4hCCR5[a]	
		0.5 ng[b]	5 ng
HeLa	HIV-1 SF162 Env	12 ± 3[c]	63 ± 15
	VSV-G	33 ± 13	306 ± 74
Mv.1.Lu	HIV-1 SF162 Env	8 ± 4	42 ± 13
	VSV-G	26 ± 14	280 ± 62
NIH3T3	HIV-1 SF162 Env	0	ND
	VSV-G	2 ± 1	6 ± 2

[a] HOS cells expressing both CD4 and CCR5.
[b] Input virus.
[c] Results are means ± standard deviations for duplicate determinations.

Production of infectious virions from murine cells is enhanced by heterokaryons formation between human or mink cells

Although rodent cells did release small amounts of p24 CA, it remained unclear whether this could be assembled in the form of infectious virions. To determine the relative infectivity of viruses produced from murine cells, pseudotyped HIV-puro viruses were prepared by transient transfection of cells with HIV-1 Env and VSV-G expression plasmids and assessed for their potential to infect HOShCD4hCCR5, a human osteosarcoma cell line that expresses hu-CD4 and hu-CCR5. Puromycin-resistant colonies were quantitated 10 days after infection. Highly infectious single-round virus was recovered from human HeLa cells carrying HIV-1 *gag, pol* genes when the virus was pseudotyped with envelopes from R5 HIV-1 or heterologous VSV (Table 1). As a control, no puromycin-resistant colonies were observed when 5 ng of VLP recovered from HeLa cells without Env was exposed to HOShCD4hCCR5 cells (data not shown). Comparable titers of infectious virus were recovered when the mink Mv.1.Lu cell was used as the producer cell. In contrast, a 20- to 50-fold reduction in infectivity of HOShCD4hCCR5 cells was observed when the single-round infectious virus was produced in murine NIH 3T3-hCycT1 cells.

Observations using reciprocal heterokaryons suggested that the reduced potential of rodent cells to produce infectious HIV-1 particles is attributed to the lack or functional disorder of a cellular cofactor(s) essential for virus assembly (Bieniasz and Cullen, 2000; Mariani *et al.*, 2001). To examine this further, murine NIH 3T3-hCycT1 cells carrying HIV-1 *gag, pol* genes were fused by PEG treatment with human HeLa as well as mink Mv.1.Lu cells. As can be seen in Fig. 5, fusion of murine NIH 3T3-hCycT1 cell expressing HIV-1 *gag, pol* genes with human HeLa or mink Mv.1.Lu cells, but not with murine NIH 3T3 cells, resulted in a significant increase (approximately 20- to 30-fold) in the level of p24 CA release into culture supernatants. This further demonstrates that the

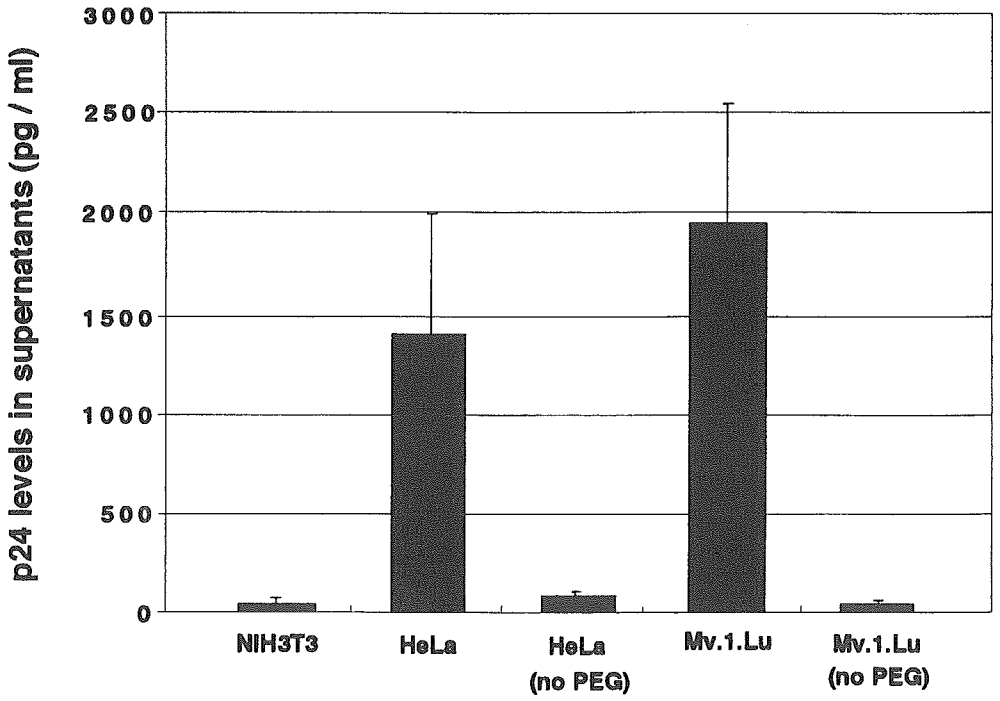

FIG. 5. Heterokaryons between murine cells stably expressing hu-cyclin T1 and HIV-1 *gag, pol* genes with human or mink cells. Murine 3T3-hCycT1 carrying HIV-1 *gag–pol* genes was cultured along with an equal number of human HeLa or mink Mv.1.Lu cells. Cocultivated cells were fused with PEG, and subsequent p24 CA production was analyzed after 24 h together with the non-PEG-treated control.

TABLE 2

Titer of HIV-1-puro Packaged in Murine 3T3 Cells Pseudotyped with VSV-G after Heterokaryon Formation between Human or Mink Cells

	No. of Puro^r colonies on HOShCD4hCCR5[a]	
Fusion partner	0.5 ng[b]	5 ng
HeLa	16 ± 3[c]	43 ± 15
Mv.1.Lu	20 ± 6	52 ± 12
NIH 3T3	2 ± 1	7 ± 2

[a] HOS cells expressing both CD4 and CCR5.

[b] Input virus.

[c] Results are means ± standard deviations for duplicate determinations.

assembly-resistant phenotype seen in murine NIH 3T3 cells can be complemented by phenotypic mixing with human as well as mink cells.

To verify that heterokaryons from murine and human or mink cells were also able to support the production of infectious virus, murine NIH 3T3-hCycT1 expressing HIV-1 *gag–pol* genes were transiently transfected with VSV-G vector and subsequently fused by PEG treatment with HeLa or Mv.1.Lu cells. The potential for pseudotyped viruses generated to infect hCD4hCCR5HOS cells was then determined. A greater than sevenfold increase in infectivity was observed for pseudotyped viruses produced from NIH 3T3 murine cells after heterokaryon formation between human or mink cells (Table 2). These findings clearly show that virions produced among murine cells can be rendered more infectious by phenotypic mixing with human as well as mink cells. This complementation in infectivity was a constant phenomenon in a large series of experiments. We conclude that murine NIH 3T3 cells lack some factor(s) necessary for the HIV-1 Gag assembly. These cellular cofactors appear to play crucial roles in mediating HIV-1 infectivity and are functional in both mink and human cells.

DISCUSSION

We obtained evidence for the heterogeneity in the potential of cells from small animal to support the late phase of HIV-1 replication. Among cells from nonhuman vertebrates, avian chicken embryonic fibroblasts (CEF), quail QT6, and mink (*Mustera vison*) lung Mv.1.Lu cells were found to support Gag assembly and release more efficiently than seen in rodent cells. A significant intraspecies variation was observed among rodent cells. Consistent with previous reports (Mariani *et al.*, 2000; Bieniasz and Cullen, 2001), the defects appear to be most profound in murine cells and can be partially overcome by fusion with permissive human cells (Tables 1 and 2), findings which suggest the requirement of a species-specific cellular factor(s) critical for HIV-1 assembly (Bieniasz and Cullen, 2001; Mariani *et al.*, 2001). However, this assembly cofactor does not appear to be human cell specific since HIV-1 Gag assembles efficiently in avian and mink cells (Fig. 1B), and fusion with mink cells also overcomes the assembly-defective phenotype seen in murine cells (Fig. 5). Indeed, efficient HIV-1 particle assembly and release has been reported to occur even in insect cells (Gheysen *et al.*, 1989).

The basis for the defect in infectious virus production in murine cells appears to be multiple. Gag gene expression was reduced in rodent cells, even in the presence of hu-cyclin T1 expression. Since Rev has been shown to be functional in rodent cells (Malim *et al.*, 1991), the lower expression of Gag expression could be due to instability of the unspliced transcripts that serve as mRNAs for the translation of Gag and Gag–Pol polyproteins. Alternatively, there is inefficient transcription even in the presence of heterologous hu-cyclin T1.

In addition to reduced Gag gene expression, defects in Gag trafficking were also observed in murine cells. The myristoylation and basic motif in the N-terminal region of $Pr55^{gag}$ are predicted to contribute to membrane interactions via hydrophobic and electrostatic interactions, respectively. Gag defective in myristoylation was shown to form large cytosolic complexes (Hermida-Matsumoto and Resh, 2000). In addition, acquisition of a myristoylated and/or palmitoylated sequence from murine leukemia virus matrix or human Src family kinase Fyn protein was reported to increase the assembly of HIV-1 Gag in murine and human cells (Chen *et al.*, 2001; Lindwasser and Resh, 2001; Reed *et al.*, 2002). Data from our radiolabeling studies, however, show that $Pr55^{gag}$ synthesized in human and murine cells is myristylated to a comparable degree, indicating that the murine form of NMT is functional with regard to transfer of myristate from myristoyl-CoA to $Pr55^{gag}$. Nevertheless, this myristoylated $Pr55^{gag}$ in murine cells does not appear to traffic appropriately. Membrane floatation analyses indicate that HIV-1 Gag is not efficiently targeted to cell membranes in rodent species. Confocal microscopy reveals that whereas Gag is membrane associated in human cells, a diffused cytoplasmic presence is detected in murine cells.

Localization of Gag polyprotein predominantly in a cholesterol-rich region of the plasma membrane, known as lipid rafts, has recently been reported, and a model for assembly of both the HIV-1 Gag and Env in this membrane microdomain is emerging (Nguyen and Hildreth, 2000; Rousso *et al.*, 2000; Lindwasser and Resh, 2001; Ono and Freed, 2001). Since removal of Gag multimerization domains has shown to increase, rather than decrease, recovery of Gag proteins from lipid rafts (Lindwasser and Resh, 2001), the assembly defect is unlikely to be due to the inability of the myristoylated HIV-1 Gag polyproteins to multimerize in murine cells. Thus, it is

tempting to speculate that the basis for the defect in assembly and release of infectious HIV virions of rodent cells is inefficient targeting of Gag polyproteins to the plasma membrane. The function of a cellular cofactor(s) that facilitates HIV assembly therefore could be to target viral proteins specifically to the plasma membrane.

Gag polyprotein was also found to localize in the *trans* cisternae of the Golgi regions (Fig. 3). This result appears to be at odds with data showing that only Gag with mutations in the N-terminal basic residue cluster localizes to a region partly overlapping the *trans* medial region of the Golgi apparatus (Lindwasser *et al.,* 2001). The reason for this discrepancy is not clear but may be related to the use of different probes to monitor this organelle. When anticaveolin-1 MAb and rhodamine-labeled secondary antibody were used, overlay with EYFP-Mem, but not with EYFP-Golgi, was observed (data not shown). Caveolins are present in caveolae, flask-shaped invaginations of the plasma membrane where there is a subset of lipid rafts (Schnitzer *et al.,* 1995). These results may mean that HIV-1 Gag in human cells localized partly at or near the Golgi and TGN-proximal region as well as in plasma membrane.

The mink Mv.1.Lu cell line appears to support the postintegration steps of HIV-1 replication with few, if any, deficiencies compared to findings in human cell lines. The block of HIV-1 infection in mink cells has been shown to occur prior to proviral integration. Transfection of full-length HIV-1 DNA into mink cells results in productive infection (Levy *et al.,* 1986), and HIV-1 replication in mink cells has also been demonstrated using phenotypic mixing with amphotropic murine leukemia virus (Canivet *et al.,* 1990) or polyethylene glycol treatment following HIV-1 adsorption (Clapham *et al.,* 1991). Our data complement and extend these earlier studies of HIV-1 infection in this cell species. Species-specific blocks in the function HIV-1 Tat and Rev function are absent in mink cells. In fact, gene expression was comparable to that found in human cells in the absence of exogenous hu-cyclin T1 expression. HIV-1 Gag polyproteins synthesized traffick to the plasma membrane as efficiently as in human cells. Furthermore, HIV-1 replication in Mv.1.Lu cells expressing huCD4 and huCCR5 *in vitro* approaches the level seen in human hCD4hCCR5HOS cells (Koito *et al.,* unpublished data); thus the mink serves as a useful small animal model for HIV-1 infection and disease.

In conclusion, there is an assembly block in rodent cells, especially in murine cells. Among various vertebral species, cell lines derived from avian and mink support assemble and release of HIV-1 virions. These results imply that a key assembly cofactor(s) is functional not only in human cells but also in many other vertebrae, but is absent in some rodent species. Additional studies are required to characterize this block(s) through identification of a species-specific cellular factor(s) that interacts with HIV-1 Gag during viral assembly for the development of murine models. Identification of such a human factor(s) may lead to elucidation of new targets for therapeutic intervention in HIV-1 infection and disease.

MATERIALS AND METHODS

Plasmids and reagents

The hu-cyclin T1 gene was amplified from pRC12/CMV/huCycT1 (Bieniasz *et al.,* 1998; provided by B. R. Cullen, Duke University, Durham, NC) and inserted into pBluescript (SKII$^+$) and the retroviral vector pLXSN (Miller and Rosman, 1989, purchased from Clonetech, Palo Alto, CA) to yield pSKII/hCycT1 and pLXSN/hCycT1, respectively. In this recombinant retroviral vector, the viral genomic mRNA is produced by LTR derived from murine Moloney leukemia virus (MoMuLV) and the bacterial gene encoding resistance to G418 is initiated at an internal SV40 promoter. Vesicular stomatatis virus G (VSV-G)-expressing plasmids (pVSV-G) were obtained from Clonetech. SV40-based HIV-1 Env expressing vector pSM-SF162-*env* has been described elsewhere (Koito *et al.,* 1994). pHIV-puro, which encodes full-length HIV-1_{NL43} proviral DNA with a frameshift in the *env* gene, and the SV40 promoter regulating puromycin-resistant gene, was provided by R. Sutton (Stanford University Medical Center, Stanford, CA). Plasmid pEYFP vectors encode yellow-green fluorescent variants with four amino acid substitutions of the *A. victoria* GFP that is optimized for brighter fluorescence and higher expression in mammalian cells (Ormo *et al.,* 1996). pEYFP vectors fused with the N-terminal plasma membrane targetting signal of neuromodulin (pEYFP-Mem), the N-terminal 81 amino acids of the precursor to the human β-1,4-galactosyltransferase (pEYFP-Golgi) or endoplasmic reticulum (ER) targeting sequence of calreticulin, and the ER retention sequence, KDEL (pEYFP-ER) were from Clontech. All plasmids were purified from transformed *Escherichia coli* strain DH5α using the CONCERT High Purity Plasmid Maxiprep System (GIBCO BRL, Grand Island, NY).

Anti-p24CA (clone VAK4; IgG_{2b}) monoclonal antibody (MAb) was as described elsewhere (Koito *et al.,* 1988). Anti-p17MA (LG20-13-15) MAb was obtained from the Chemo-Sero-Therapeutic Research Institute (Kikuchi, Kumamoto, Japan). Anticaveolin-1 polyclonal antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). Antimouse fluorescein isothiocyanate (FITC), antimouse rhodamine, and antirabbit rhodamine secondary antibodies were purchased from DAKO (Glostrup, Denmark).

Cells

The human T lymphoid cell line PM1, murine T lymphoma cell line EL4, BW5147, macrophage line RAW264, and WKA rat-derived myelomonocytic precursor cell line WRT7 (provided by M. Hosokawa; Hokkaido University,

Sapporo, Japan) were propagated in RPMI 1640 medium (GIBCO BRL). Human HeLa, 293T, the MoMuLV retroviral packaging cell line GP293 (CLONTECH), quail QT6, mink (*M. vision*) lung Mv.1.Lu (ATCC CCL-64), mouse muscle cell NOR10 (ATCC CCL-198), and fibroblast cell NIH3T3 were maintained in Dulbecco's modified Eagle's medium (GIBCO BRL). Chinese hamster ovary (CHO) K1 (ATCC CCL-61) cells were cultured in F-12 medium (Life Technologies, Rockville, MD). Chicken embryo fibroblasts (CEFs) were prepared and cultured as described (Shigekane *et al.*, 1999; provided by M. Sakaguchi; Kikuchi Research Center, Chemo-Sero-Therapeutic Research Institute). Human HOS cells stably expressing both the hu-CD4 molecule containing a cytoplasmic tail deletion and hu-CCR5 have been reported (Tahara-Hanaoka *et al.*, 2000). All media were supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (GIBCO BRL) and cells were cultured at 37°C and 5% CO_2 in a humidified chamber.

Nonhuman animal cell lines expressing hu-cyclin T1 were generated by transducing cells with hu-cyclin T1 retroviral expression vector pLXRN/hCycT pseudotyped with VSV-G followed by 4 weeks of selection in the presence of 0.5 mg/ml of G418 (Geneticin; GIBCO BRL). This hu-cyclin-T1-expressing retrovirus was generated by cotransfecting GP293 cells with pLXSN/hCycT and pVSV-G. Replication-incompetent HIV-puro pseudotypes were generated by cotransfecting 293T cells with pHIV-puro and pVSV-G. Transfections were done using Lipofectamine 2000 (Life Technologies, Gaithersburg, MD) according to the manufacturer's instructions. Culture supernatants were harvested at 2 days posttransfection and stored at −70°C.

FACS analysis

Staining for intracytoplasmic HIV-1 p24 was done using the Fix and Perm reagents (Caltag Laboratories, Burlingame, CA), as described (Atchison *et al.*, 1996), using a MAb to p24 CA (VAK4) and FITC-conjugated goat antimouse IgG. The cells were then washed with Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline PBS(−) and resuspended in staining medium (PBS(−), 0.05% NaN_3, and 3% FCS) containing propidium iodide (PI) (1 μg/ml). Amounts of intracellular p24 CA were then measured using a FACSCalibur (Beckton–Dickinson, San Jose, CA) by analyzing fluorescence using the Cell Quest software (Beckton–Dickinson).

Cell fractionation and sucrose floatation assays

The flotation assay was done as described (Spearman *et al.*, 1997) but with minor modifications. Cells (2×10^7) were harvested, rinsed twice with ice-cold PBS(−), resuspended in 300 μl of ice-cold hypotonic TE (10 mM Tris–HCl, pH 7.5, and 1 mM EDTA) buffer containing 10% (wt/vol) sucrose and a protease inhibitor cocktail (Roche; Mannheim, Germany) and then allowed to swell on ice for 15 min. These cells, then disrupted on ice by sonication (15 s, twice); nuclei and cell debris were pelleted by centrifugation (1000 *g* for 10 min at 4°C) and the resulting postnuclear extracts (250 μl) were then mixed with 1.25 ml of 85.5% (wt/vol) sucrose in TE buffer, adjusted to 80% (wt/vol) sucrose, placed at the bottom of a SW41 centrifuge tube, and overlaid with 65% (wt/vol) and 10% (wt/vol) sucrose in TE buffer. Standard volumes for this step gradient included 1.5 ml of 80% and 6 ml of 65% sucrose and the remaining portion of the centrifugation tube was filled with 10% sucrose in TE buffer. For centrifugation, we used a Beckman SW41 TI rotor at 30,000 rpm for 18 h at 4°C. Ten 1.0-ml fractions were collected from the top of the gradient. Aliquots of each fraction were separated by 15% polyacrylamide gel electrophophoresis containing sodium dodecyl sulfate (SDS–PAGE; Bio-Rad, Hercules, CA), transferred to polyvinylidine difluoride (PVDF) filters (Bio-Rad), and probed with a mixture of antibodies to p24 CA VAK4 and p17 MA LG20-13-15 followed by horseradish peroxidase-coupled protein G (Bio-Rad). Filters were developed using enhanced chemiluminescence reagents (ECL; Amersham, Buckinghamshire, England).

Immunofluorescence microscopy and confocal analysis

Cells transiently transfected with pEYFP-Mem, pEYFP-Golgi, or pEYFP-ER using Lipofectamine 2000 were reseeded into 35-mm poly-L-lysine-coated dish coverslips (Matsunami Glass Co., Osaka, Japan) and grown for 24 h at 37°C in DMEM containing 10% FCS, then fixed with 3.7% formaldehyde in PBS(−) for 15 min, permeabilized with 0.05% saponin and 0.2% bovine serum albumin (BSA) in PBS for 10 min at room temperature, and subsequently washed twice in PBS. For antibody staining, coverslips were incubated in a humid chamber at 37°C for 1 h with a MAb to p24 CA VAK4 for 1 h and with a rhodamine-conjugated rabbit antimouse IgG for 30 min at the appropriate dilution in 1% BSA and 0.02% Na azide in PBS(−). After three washings with PBS(−), these cells were mounted with FLUORESCENT MOUNTING MEDIUM (DAKO). For confocal microscopy, images were acquired with a Plan-Neofluar 100× oil immersion objective (Zeiss, Tokyo, Japan) and a Zeiss LSM 510 inverted laser scanning microscope. Beams (488 nm for FITC, 543 nm for He–Ne) from an argon and He–Ne laser were used. For two-color analysis, green and red emissions were simultaneously recorded through appropriate filters (505- to 530-nm bandpass filter for FITC and 585-nm long-pass filter for rhodamine) and stored in separate (red and green) image channels. Image quality was enhanced during data acquisition using the LSM line average feature. Postacquisition digital image enhancement was done using the LSM software.

N-Myristoylation

[9,10(n)-^{3}H]Myristic acid (catalog No. TRK907; Amersham), which was supplied in ethanol, was first vacuum dried and dissolved in dimethyl sulfoxide. Cells were metabolically labeled overnight with [9,10(n)-^{3}H]myristic acid (0.5 mCi/ml) in DMEM medium supplemented with 10% FCS. The cells were washed twice with PBS(−) and lysed in the radioimmunoprecipitation (RIPA; 0.15 M NaCl, 0.05 M Tris–HCl, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate) buffer containing a protease inhibitor cocktail (Roche). Proteins were immunoprecipitated with anti-p24 CA MAb VAK4 followed by immobilization on protein G–Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden). The immunoprecipitate was washed three times with RIPA buffer and solubilized in Laemmli's sample buffer. The radiolabeled proteins were size-fractionated by 10% SDS–PAGE, followed by autoradiography.

Pseudotyped virus production and transduction

HeLa, mink Mv.1.Lu, or murine NIH 3T3 carrying hucyclin T1 (3T3-hCycT1) cells expressing HIV-1 *gag, pol* genes (5×10^6 per 10-cm dish) were transfected using Lipofectamine 2000 with 10 μg of the pSM-SF162-env vector together with 5 μg of rev-expressing vector. Alternatively, replication-incompetent viruses were generated by transfection of 10 μg of VSV-G vector. The pseudotyped virus-containing culture supernatants of transfected cells were harvested at 48 h posttransfection. Supernatants were cleared of cellular debris by centrifugation at 500 *g* for 5 min and stored at −70°C, subsequent to a filtration through a 0.45-μm pore size filter and analyzed for HIV-1 p24 content by an enzyme-linked immunosorbent assay kit (ZeptoMetrix Corporation, Buffalo, NY). Virus pseudotyped by VSV-G was concentrated (141,000 *g* for 4 h at 4°C) and resuspended in a small amount of DMEM medium. A total of 10^6 target human osteosarcoma cells expressing both CD4 and CCR5 (HOShCD4hCCR5; Tahara-Hanaoka *et al.,* 2000) were plated on a 35-mm-diameter six-well culture plate and incubated with transfected cell media and 6 μg/ml of polybrene (Sigma Chemical Co., St. Louis, MO) for 3 h at 37°C, at which time 4 ml of complete medium was added and the incubation was continued. The medium was replaced 2 days later with medium containing 5 μg/ml of active puromycin for 10 days. The selection medium was changed every 2 to 3 days, and visible colonies arose typically in 6 to 9 days. These colonies were then stained with crystal violet.

Heterokaryon formation

Murine 3T3-hCycT1 cells (5×10^5) stably expressing HIV-1 *gag–pol* gene products were cocultured with an equal number of human HeLa, mink Mv.1.Lu, or murine NIH 3T3 cells in a culture plate (35-mm-diameter). After 12 h, medium was aspirated and the cells were washed once with serum-free medium. Then the cells were fused by adding 1.0 ml of a prewarmed 50% (wt/vol) polyethylene glycol [PEG (HYBRI-MAX; Sigma)] solution in DMEM for 2 min at 37°C. PEG was extensively washed, and heterokaryons were cultured for another 24 h. Culture supernatants was collected and the p24 CA content was monitored.

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REFERENCES

Atchison, R. E., Gosling, J., Monteclaro, F. S., Franci, C., Digilio, L., Charo, I. F., and Goldsmith, M. A. (1996). Multiple extracellular elements of CCR5 and HIV-1 entry: Dissociation from response to chemokines. *Science* **274,** 1924–1926.

Bieniasz, P. D., Grdina, T. A., Bogerd, H. P., and Cullen, B. R. (1998). Recruitment of a protein complex containing Tat and cyclin T1 to TAR governs the species specificity of HIV-1 Tat. *EMBO J.* **17,** 7056–7065.

Bieniasz, P. D., and Cullen, B. R. (2000). Multiple blocks to human immunodeficiency virus type 1 replication in rodent cells. *J. Virol.* **74,** 9868–9877.

Browning, J., Horner, J. W., Pettoello-Mantovani, M., Raker, C., Yurasov, S., DePinho, R. A., and Goldstein, H. (1997). Mice transgenic for human CD4 and CCR5 are susceptible to HIV infection. *Proc. Natl. Acad. Sci. UCA* **94,** 14637–14641.

Bryant, M., and Ratner, L. (1990). Myristoylation-dependent replication and assembly of human immunodeficiency virus 1. *Proc. Natl. Acad. Sci. USA* **87,** 523–527.

Canivet, M., Hoffman, A. D., Hardy, D., Sernatinger, J., and Levy, J. A. (1990). Replication of HIV-1 in a wide variety of animal cells following phenotypic mixing with murine retroviruses. *Virology* **178,** 543–551.

Chen, B. K., Rousso, I., Shim, S., and Kim, P. S. (2001). Efficient assembly of an HIV-1/MLV Gag-chimeric virus in murine cells. *Proc. Natl. Acad. Sci. USA* **98,** 15239–15244.

Clapham, P. R., Blanc, D., and Weiss, R. A. (1991). Specific cell surface requirements for the infection of CD4-positive cells by human immunodeficiency virus types 1 and 2 and by simian immunodeficiency virus. *Virology* **181,** 703–715.

Freed, E. O. (1998). HIV-1 gag proteins: Diverse functions in the virus life cycle. *Virology* **251,** 1–15.

Freed, E. O., Orenstein, J. M., Buckler-White, A. J., and Martin, M. A. (1994). Single amino acid changes in the human immunodeficiency virus type 1 matrix protein block virus particle production. *J. Virol.* **68,** 5311–5320.

Garber, M., Wei, P., KewalRamani, V., Mayall, T., Herrmann, C., Rice, A., Littman, D., and Jones, K. (1998). The interaction between HIV-1 Tat and human cyclin T1 requires zinc and a critical cysteine residue that is not conserved in the murine CycT1 protein. *Genes Dev.* **12,** 3512–3527.

Gheysen, D., Jacobs, E., de Foresta, F., Thiriart, C., Francotte, M., Thines, D., and De Wilde, D. (1989). Assembly and release of HIV-1 precursor Pr55gag virus-like particles from recombinant baculovirus-infected insect cells. *Cell* **59,** 103–112.

Gottlinger, H. G., Sodrosky, J. G., and Haseltine, W. A. (1989). Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus. *Proc. Natl. Acad. Sci. USA* **86,** 5781–5785.

Hermida-Matsumoto, L., and Resh, M. D. (2000). Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. *J. Virol.* **74,** 8670–8679.

Keppler, O. T., Welte, F. J., Ngo, T. A., Chin, P. S., Patton, K. S., Tsou, C.-L., Abbey, N. W., Sharkey, M. E., Grant, R. M., You, Y., Scarborough, J. D., Ellmeier, W., Littman, D. R., Stevenson, M., Charo, I. F., Herndier, B. G., Speck, R. F., and Goldsmith, M. A. (2002). Progress toward a human CD4/CCR5 transgenic rat model for de novo infection by human immunodeficiency virus type 1. *J. Exp. Med.* **195,** 719–736.

Koito, A., Harrowe, G., Levy, J. A., and Cheng-Mayer, C. (1994). Functional role of the V1/V2 region of human immunodeficiency virus type 1 envelope glycoprotein gp120 in infection of primary macrophages and sCD4 neutralization. *J. Virol.* **68,** 2253–2259.

Koito, A., Hattori, T., Matsushita, S., Maeda, Y., Nozaki, C., Sagawa, K., and Takatsuki, K. (1988). Conserved immunogenic region of a major core protein (p24) of human and simian immunodeficiency viruses. *AIDS Res. Hum. Retroviruses* **4,** 409–417.

Kwak, Y. T., Ivanov, D., Guo, J., Nee, E., and Gaynor, R. B. (1999). Role of the human and murine cyclin T proteins in regulating HIV-1 tat-activation. *J. Mol. Biol.* **288,** 57–69.

Leonard, J. M., Abramczuk, J. W., Pezen, D. S., Rutledge, R., Belcher, J. H., Hakim, F., Shearer, G., Lamperth, L., Travis, W., Fredrickson, T., Notkins, A. L., and Martin, M. A. (1988). Development of disease and virus recovery in transgenic mice containing HIV proviral DNA. *Science* **242,** 1665–1670.

Levy, J. A., Cheng-Mayer, C., Dina, D., and Luciw, P. A. (1986). AIDS retrovirus (ARV-2) clone replicates in transfected human and animal fibroblasts. *Science* **232,** 998–1001.

Lindwasser, O. W., and Resh, M. D. (2001). Multimerization of human immunodeficiency virus type 1 Gag promotes its localization to barges, raft-like membrane microdomains. *J. Virol.* **75,** 7913–7924.

Llopis, J., McCaffery, J. M., Miyawaki, A., Farquhar, M. G., and Tsien, R. Y. (1998). Measurement of cytosolic, mitochondrial, and Golgi pH in single living cells with green fluorescent proteins. *Proc. Natl. Acad. Sci. USA* **95,** 6803–6808.

Malim, M. H., McCarn, D. F., Tiley, L. S., and Cullen, B. R. (1991). Mutational definition of the human immunodeficiency virus type 1 Rev activation domain. *J. Virol.* **65,** 4248–4254.

Mariani, R., Rutter, G., Harris, M. E., Hope, T. J., Krausslich, H.-G., and Landau, N. R. (2000). A block to human immunodeficiency virus type 1 assembly in murine cells. *J. Virol.* **74,** 3859–3870.

Mariani, R., Rasala, B. A., Rutter, G., Wiegers, K., Brandt, S. M., Krausslich, H.-G., and Landau, N. R. (2001). Mouse-human heterokaryons support efficient human immunodeficiency virus type 1 assembly. *J. Virol.* **75,** 3141–3151.

Miller, A. D., and Rosman, G. J. (1989). Improved retroviral vectors for gene transfer and expression. *BioTechniques* **7,** 980–990.

Moosmayer, D., Reil, H., Ausmeier, M., Scharf, J.-G., Hauser, H., Jentsch, K. D., and Hunsmann, G. (1991). Expression and frameshifting but extremely inefficient proteolytic processing of the HIV-1 gag and pol gene products in stably transfected rodent cell lines. *Virology* **183,** 215–224.

Nguyen, D. H., and Hildreth, J. E. (2000). Evidence for budding of human immunodeficiency virus type 1 selectively from glycolipid-enriched membrane lipid rafts. *J. Virol.* **74,** 3264–3272.

Ono, A., and Freed, E. O. (2001). Plasma membrane rafts play a critical role in HIV-1 assembly and release. *Proc. Natl. Acad. Sci. USA* **98,** 13925–13930.

Ormo, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Remington, S. J. (1996). Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science* **273,** 1392–1395.

Reed, M., Mariani, R., Sheppard, L., Pekrun, K., Landau, N. R., and Soong, N.-W. (2002). Chimeric human immunodeficiency virus type 1 containing murine leukemia virus matrix assembles in murine cells. *J. Virol.* **76,** 436–443.

Rousso, I., Mixon, M. B., Chen, B. K., and Kim, P. S. (2000). Palmitoylation of the HIV-1 envelope glycoprotein is critical for viral infectivity. *Proc. Natl. Acad. Sci. USA* **97,** 13523–13525.

Sawada, S., Gowrishankar, K., Kitamura, R., Suzuki, M., Suzuki, G., Tahara, S., and Koito, A. (1998). Disturbed $CD4^+$ T cell homeostasis and *in vitro* susceptibility in transgenic mice expressing T cell line-tropic HIV-1 receptors. *J. Exp. Med.* **187,** 1439–1449.

Schnitzer, J. E., McIntosh, D. P., Dvorak, A. M., Liu, J., and Oh, P. (1995). Separation of caveolae from associated microdomains of GPI-anchored proteins. *Science* **269,** 1435–1439.

Shigekane, H., Kawaguchi, Y., Shirakata, M., Sakaguchi, M., and Hirai, K. (1999). The bi-directional transcriptional promoters for the latency-relating transcripts of the pp38/pp24 mRNAs and the 1.8 kb-mRNA in the long inverted repeats of Marek's disease virus serotype 1 DNA are regulated by common promoter-specific enhancers. *Arch. Virol.* **144,** 1893–1907.

Spearman, P., Horton, R., Ratner, L., and Kuli-Zade, I. (1997). Membrane binding of human immunodeficiency virus type 1 matrix protein *in vivo* supports a conformational myristyl switch mechanism. *J. Virol.* **71,** 6582–6592.

Tahara-Hanaoka, S., Ushijima, Y., Tarui, H., Wada, M., Hara, T., Imanishi, S., Yamaguchi, T., Hattori, T., Nakauchi, H., and Koito, A. (2000). Differential level in co-down-modulation of CD4 and CXCR4 primed by HIV-1 gp120 in response to phorbol ester, PMA, among HIV-1 isolates. *Microbiol. Immunol.* **44,** 489–498.

Wei, P., Garber, M. E., Fang, S. M., Fischer, W. H., and Jones, K. (1998). A novel CDK9-associated C-type cyclin interacts directly with HIV-1 Tat and mediates its high-affinity, loop-specific binding to TAR RNA. *Cell* **92,** 451–462.

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The impact of highly active antiretroviral therapy by the oral route on the CD8 subset in monkeys infected chronically with $SHIV_{89.6P}$

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Abstract

The objective of this study was to assess the impact of highly active antiretroviral therapy (HAART) by an oral route on the peripheral blood CD8 subset in the monkeys infected persistently with a pathogenic strain, $SHIV_{89.6P}$. Two rhesus macaques were inoculated intravenously with $SHIV_{89.6P}$, then treated with the combination of AZT, 3TC and Lopinavir/Ritonavir (LPV/RTV) as recommended in humans by the oral route with confectionery continued for 28 days. In one of two chronically infected macaques, MM260, the viral load was maintained in the range of 10^4–10^5 copies/ml before HAART. The plasma viral load and proviral DNA decreased dramatically during the treatment, and cessation of this therapy the viral load rebounded to the pre-treatment level but the proviral DNA rebound was delayed. The other monkey, MM242, had low viral loads (1.2×10^3– $< 5 \times 10^2$ copies/ml) both before and after HAART. $CD4^+$ and $CD8^+$ T cell counts and proviral DNA level were not significantly changed after the treatment. The percentages of $CD8^+CD45RA^-Ki67^+$ cells increased during (MM260) or after (MM242) HAART and the subset was maintained at a high percentage until 18 weeks post HAART in MM242. These findings suggest that this primate model might serve an important role in testing the virological and immunological efficacy of novel therapeutic strategies combined with HAART.

Keywords: $SHIV_{89.6P}$; Antiretroviral therapy; $Ki67^+$; Memory $CD8^+$ T cells; Proviral DNA; Animal model

1. Introduction

Pre-clinical approaches in non-human primate models of AIDS enable pertinent evaluations to be carried out and the possibility to determine precisely the conditions of efficacy can be determined (Le Grand et al., 1994). Macaques infected with pathogenic strains of the simian immunodeficiency virus (SIV) or related chimeras expressing the envelope of HIV-1 (simian/human immunodeficiency virus, SHIV) are currently relevant models of human HIV infection and AIDS (Haigwood, 1999; Nath et al., 2000; Nathanson et al., 1999; Tang et al., 2002). SIV and SHIV have biological properties similar to those of HIV, and infection of macaques with pathogenic isolates induces an immunodeficiency syndrome strikingly mimicking human AIDS (Reimann et al., 1996a,b). Animal models are also useful for understanding the complexity of the pathogenic mechanisms of HIV infection during antiviral treatment (Endo et al., 2000; Enose et al., 2002; Igarashi et al., 2001).

SHIVs contain HIV-1-derived segments encoding viral envelope glycoproteins and regulatory proteins such as Tat, Rev and Vpu in the SIV background (Li et al., 1992). SHIV containing the envelope glycoproteins of a primary HIV-1 isolate, 89.6, replicated in rhesus monkeys but did not deplete $CD4^+$ T lymphocytes or induce disease in these animals. Serial transfer

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of blood from $SHIV_{89.6}$-infected monkeys to naive monkeys generated a virus, termed $SHIV_{89.6P}$, that exhibited only a modest increase in replication in infected monkeys compared with $SHIV_{89.6}$. However, $SHIV_{89.6P}$ caused rapid loss of $CD4^+$ T lymphocytes and, subsequently, AIDS-like illness in inoculated monkeys (Reimann et al., 1996a,b). An animal system using $SHIV_{89.6P}$ acutely infected macaques with highly active antiretroviral therapy (HAART) was the same regimen used for humans (AZT+3TC+IDV) by the oral route (Le Grand et al., 2002, 2000; Thiebot et al., 2001) and a nasogastric catheter was placed for administering the drugs (Thiebot et al., 2001).

The objective of this study was to set up a novel AIDS model in monkeys and to evaluate the efficacy of the combination of AZT, 3TC and Lopinavir/Ritonavir (LPV/RTV), when administered by the oral route to the monkeys infected chronically with the pathogenic $SHIV_{89.6P}$.

2. Materials and methods

2.1. Cells and viruses

M8166 cells (Clapham et al., 1987) were grown in an RPMI-1640-based culture medium supplemented with 15% fetal calf serum (FCS: HyClone Laboratories, Logan, UT), 50 U/ml of penicillin and 50 mg/ml of streptomycin. $SHIV_{89.6P}$ (Reimann et al., 1996a) and HIV-1_{LAI} (Clavel et al., 1986) were used for the drug susceptibility assay.

2.2. Antiviral agents

Zidovudine (AZT) was purchased from Sigma (St. Louis, MO). Lamivudine (3TC) was a kind gift from R.F. Schinazi (Atlanta, GA). RTV was kindly provided by Abbott Laboratories (Abbott Park, Ill.). LPV was synthesized using published methods (Carrillo et al., 1998). Kaletra™ liquid (LPV/RTV) and Combivir® tablets (AZT/3TC) were purchased from Abbott Laboratories and GlaxoSmithKline, respectively.

2.3. Drug susceptibility assay

The sensitivities of $SHIV_{89.6P}$ against various drugs were determined as described previously with minor modifications (Maeda et al., 2001; Yoshimura et al., 1999). Briefly, M8166 cells (5×10^3 per ml) were exposed to 100 $TCID_{50}$ of $SHIV_{89.6P}$ and HIV-1_{LAI}, in the presence of various concentrations of drugs in 96-well microculture plates and incubated at 37 °C for 7 days. After 100 μl of the medium was removed from each well, 10 μl of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (7.5 mg/ml) in phosphate-buffered saline (PBS) was added to each well in the plate, followed by incubation at 37 °C for 2 h. After incubation, to dissolve the formazan crystals, 100 μl of acidified isopropanol containing 4% (v/v) Triton X-100 was added to each well and the optical density was measured in a microplate reader. All assays were performed in duplicate (S.D.: < 25%).

2.4. Monkeys

Two rhesus macaques (*Macaca mulatta*), MM260 and MM242, were infected intravenously with 1000 and 10 $TCID_{50}$ (50% tissue culture infectious dose) of a pathogenic $SHIV_{89.6P}$, respectively. $SHIV_{89.6P}$ was provided by K.A. Reimann and N.L. Letvin (Beth Israel Hospital, Boston, MA). However, because the stock $SHIV_{89.6P}$, which was not the original virus used in this study might have slight loss of pathogenicity in the course of stock virus preparation, the CD4 values of the monkeys inoculated with $SHIV_{89.6P}$ sometimes showed a some reversal (20–50% of the mean value before inoculation) after the drastic drop up to 10% at 2–3 weeks post inoculation. The two animals were treated with a combination of AZT (5 mg/kg), 3TC (2.5 mg/kg) and LPV/RTV (12/3 mg/kg) administered by the oral route with confectionery twice a day after an intravenous inoculation of $SHIV_{89.6P}$. Briefly, Combivir tablets (AZT/3TC) were ground and mixed with Kaletra™ liquid (LPV/RTV) then the drug-mixture was poured into the sweet confectionery that the monkeys are fond of. We monitored the compliance of drug delivery by observing the monkeys until they had finished eating the sweets. Treatment was initiated 38 (MM260) or 84 (MM242) weeks after the inoculation of $SHIV_{89.6P}$, and was continued for 28 days. Two uninfected, untreated animals (MM132 and MM244) were used as controls (Fig. 3A and C). Blood samples were collected periodically from the infected and healthy macaques for analysis of $CD4^+$ and $CD8^+$ cell counts, $Ki67^+$ percentage, viral loads, and proviral DNA.

2.5. Measuring of proviral DNA level

Proviral DNA levels in peripheral blood mononuclear cells (PBMC) from $SHIV_{89.6P}$-infected-monkey were measured using a novel hypersensitive nested PCR in the LTR region. The first PCR was carried out with SHIV-O-S (5′-AGGCATCATACCAGATTGGCA-3′) and SHIV-O-A (5′-ATTGAAGAGGGCTTTAAGCAA-3′) primers in the U3/R region. The first PCR reaction mixture consisted of 0.5 μg of the proviral DNA solution, 50 mM KCl, 10 mM Tris–HCl (pH 8.0), 2 mM $MgCl_2$, 0.01% gelatin, 0.2 mM dNTPs, 1.5 U of EX Taq DNA polymerase (Takara Shuzo Co., LTD.), and 15 pmol of each of the first PCR primers in a total volume of 30 μl. The PCR conditions used were an

initial 3 min at 94 °C, followed by 24 cycles of 30 s at 94 °C, 30 s at 65 °C, and 1 min at 70 °C with a final 10 min extension at 72 °C. The first PCR product was subsequently diluted 1000-fold, and subjected to a real-time PCR assay for measuring U3 DNA using SHIV-I-S (5′-AGACATTTGGCTGGCTATGGA-3′), SHIV-I-A (5′-AAGTTTGAGCTGGATGCATTA-3′) and SYBR Green PCR Master Mix (Perkin–Elmer-Applied Biosystems). For amplification and detection of PCR products we preheated the samples at 50 °C for 2 min and at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 30 s, using an ABI PRISM 7700 sequence detection system (Perkin–Elmer-Applied Biosystems). The SIVmac239 LTR sequence was cloned into pCR2.1 TOPO vector (Invitrogen), and served as a standard curve. The level of SHIV DNA was expressed as copies per micrograms of cellular DNA (copies/μg DNA). Under these conditions, the detection limit was 300 copies/μg DNA.

2.6. Detection of plasma viral RNA

Plasma viral loads were determined using Taqman RT-PCR kits (Perkin–Elmer, New Jersey, USA) and ABI Prism 7700 (Ui et al., 1999). Absolute copy numbers of viral RNAs were determined using standard plasma samples, the copy numbers, of which were determined by Chiron Corporation. Under these conditions, the detection limit was 500 copies/ml.

2.7. FACS analysis

Whole blood samples of the monkeys were stained with fluorecently labeled mouse monoclonal antibodies as follows; phycoerythrin (PE)-conjugated anti-human CD4 (NU-$T_{H/N}$, Nichirei, Japan), PerCP-conjugated anti-human CD8 monoclonal antibody (Leu-2a, BD PharMingen, CA). After hemolysis of the blood using a lysing solution (BD PharMingen), the, respectively, labeled lymphocytes were analyzed using FACSCalibur (BD PharMingen).

PBMCs were also isolated from the macaques using centrifugation on Ficoll–Hypaque density gradient centrifugation. The PBMCs were incubated with fluorochrome-labeled specific monoclonal antibodies against surface antigens, allophycocyanin (APC)-conjugated anti-human CD8 (RPA-T8, BD PharMingen) and PE-conjugated anti-human CD45RA (5H9, BD PharMingen), and also incubated with 7-amino-actinomycin D (7-AAD) (BD PharMingen) to exclude dead cells. After fixation and permeabilization, the cells were incubated with Ki67-FITC (B56, BD PharMingen). The stained cells were analyzed by four-color flow cytometry using a FACSCalibur (Kimura et al., 2002).

2.8. Statistical analysis

The $CD4^+$ and $CD8^+$ T cell counts between pre-HAART and baseline or post-HAART were determined statistically using Student's t-test.

3. Results

3.1. In vitro drug sensitivity of $SHIV_{89.6P}$ and HIV-1_{LAI}

We first tested two nucleoside reverse transcriptase inhibitors (NRTIs), AZT and 3TC, and two protease inhibitors (PIs), RTV and LPV, against $SHIV_{89.6P}$ and HIV-1_{LAI} in M8166 cells (Table 1). Antiviral activity of the two NRTIs was comparable against these two viruses. On the other hand, as shown in Table 1, $SHIV_{89.6P}$ had a high level of resistance to RTV (15–18-fold increase in IC_{50}) and a moderate level of resistance to LPV (5–6-fold) compared with the case against HIV-1_{LAI}. This finding is similar to a pattern of sensitivity of HIV-2 strains to PIs as reported (Yoshimura et al., 1999).

3.2. $SHIV_{89.6P}$-infected macaques were treated with HAART by the oral route

The replication of $SHIV_{89.6P}$ was inhibited by AZT, 3TC or LPV within the IC_{50} range between 0.007 and 0.47 μM in vitro (Table 1). We selected these three drugs for treatment to monkeys infected with $SHIV_{89.6P}$ because this combination is currently recommended for treatment of patients with HIV-1 infection. Two rhesus macaques were inoculated intravenously with 1000 and 10 $TCID_{50}$ of a cell-free stock of $SHIV_{89.6P}$ for MM260 and MM242, respectively. The monkeys, MM260 and MM242, became infected and were treated with HAART initiated at 38 and 64 weeks and continued until at 42 and 68 weeks post-inoculation, respectively. The animals were treated, as recommended in the case of humans, by the combination of AZT (5 mg/kg bid), 3TC (2.5 mg/kg bid) and LPV/RTV (12/3 mg/kg bid) after the intravenous inoculation of $SHIV_{89.6P}$. This treatment was administered by the oral route together with confectionery and was continued for 28 days. The animals (MM260 and MM242) were monitored for $CD4^+$ and $CD8^+$ T cell counts, viral loads in plasma, proviral DNA and $CD8^+$ T cell turnover.

MM260, the monkey with the high viral load showed a significant decline in $CD4^+$ but not $CD8^+$ T cell counts before HAART compared with the pre-infection level (598 ± 80 vs. 49 ± 16 per μl in mean $CD4^+$ count ± S.D., $P < 0.001$ determined by the Student's t-test, 288 ± 46 vs. 221 ± 81 per μl in mean $CD8^+$ count ± S.D., $P = 0.25$). $CD4^+$ and $CD8^+$ T cell counts