Susceptibility of Mink (Mustela vison)-Derived Cells to Replication by Human Immunodeficiency Virus Type 1

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In vivo studies for understanding viral transmission and replication, host immune responses, and pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection would greatly benefit from the establishment of a small-animal model. In this study, we explored the potential of American mink (Mustela vison) as a susceptible host. We found that primary cells and cell lines derived from this species efficiently supported trans-activation of the HIV-1 long terminal repeat by Tat. Accordingly, the cysteine residue at position 261, which has been shown to be important for interaction of the human cyclin T1 with the HIV-1 regulatory protein Tat, is conserved in the mink homologue. No species-specific defect in Rev function could be detected in mink cells. In addition, primary splenocytes, fibroblasts, and the Mv.1.Lu cell line from American mink supported early as well as late HIV-1 gene expression following infection with vesicular stomatitis G protein-pseudotyped HIV-1 viruses, at levels comparable to those seen with permissive human cells. Furthermore, the mink Mv.1.Lu cell line stably expressing human CD4 and CCR5 receptors supported a spreading HIV-1 infection with few, if any, deficiencies compared to findings in human cell lines. This indicates the potential of HIV-1 to replicate in these cells once the blockade at the stage of virus entry has been removed. These results clearly show that cells from American mink generally pose no functional intracellular block to HIV-1 replication, and collectively they raise the possibility that this animal species could be engineered to support HIV-1 infection, providing a useful small-animal model for evaluating de novo infection by HIV-1.

Human immunodeficiency virus type 1 (HIV-1) replicates efficiently only in humans and certain nonhuman primates such as chimpanzees. Cellular entry has been considered to be a major restriction of HIV-1 replication in cells from nonhuman species. The identification of roles played by chemokine receptors as entry coreceptors with human CD4 (reviewed in references 3, 17, and 26) was seen to offer possibilities for overcoming species-specific restrictions to HIV-1 replication in rodent cells, leading to the development of a transgenic rodent model. However, primary lymphocytes from mice transgenic for human CD4 and either the human CCR5 or human CXCR4 coreceptor, while largely able to overcome the entry block, exhibited little or no sign of productive infection (6, 30). Recently, primary cells, especially macrophages and microglia from rats transgenic for human CD4 and CCR5 were found to support HIV-1 replication at levels higher than those described for comparable transgenic mouse models, but in vivo replication of HIV-1 in this host appeared to be limited (15).

Multiple intracellular steps at which the HIV-1 replication cycle is blocked have been noted, especially in cells from rodent species. The inability of the HIV-1-encoded trans-activator Tat to activate viral RNA transcription from the long terminal repeat (LTR) is one major restriction at the postintegration phase in rodent cells (13). This intracellular restriction could be partially overcome by the introduction of human cyclin T1 (CycT1) (4, 10, 11, 19, 36), indicating that human CycT1 is essential for Tat-mediated transcription. CycT1 is a component of the positive-transcription-elongation factor-β transcription factor complex (23, 37), which associates with the cyclin-dependent kinase CDK9. Human CycT1, in association with CDK9, interacts with HIV-1 Tat to form a heterodimer with high affinity for the trans-activation response element stem-loop at the 5’ ends of all nascent viral transcripts. This complex promotes hyperphosphorylation of the carboxy-terminal repeat domain of RNA polymerase II, causing increased transcriptional processivity (11). Human and murine forms of CycT1 are 90% identical at the amino acid level; a single amino acid change from cysteine to tyrosine at position 261 of murine CycT1 prevents it from interacting with Tat (4, 11, 19). Although expression of human CycT1 in mouse NIH 3T3 cells coexpressing human CD4 and an appropriate coreceptor allows HIV-1 to proceed through entry, reverse transcription, integration, and proviral gene expression, it is not sufficient to reconstitute the full replication cycle (5, 11, 25).

Reduced levels of unspliced genomic RNA synthesis as well as structural gene expression of HIV-1 have also been noted in rodent cells. However, the issue of whether HIV-1 Rev, which is known to associate with the cellular export factor CRM1, in cells from rodent species is functional or nonfunctional has been controversial (22, 34). More recent studies suggest a relative, rather than an absolute, limitation in the function of this regulatory protein in rodent cells (5, 25). Lastly, there are blocks to the late, posttranslational phase of viral replication, including Gag polyprotein processing, virion assembly, and release, that result in failure of the virus to spread. Although these blocks could be partially circumvented by human-mouse heterokaryon fusions, the underlying mechanism has yet to be understood.

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clarified (5, 18, 24, 25). This assembly defect of HIV-1 in murine cells further complicates the development of a permissive small-animal model of HIV-1 disease.

Nevertheless, the potential usefulness of a small-animal model of HIV-1 infection and disease warrants further effort directed at an assessment of the quantitative as well as qualitative limitations and blocks in the viral replication cycle in animals that may serve as hosts. Among the small-animal species studied, we previously observed efficient proviral gene expression and virion assembly and release in certain cell lines from American mink (Mustela vision) stably transduced with HIV-1 proviruses (18). The indication that mink-derived cells are permissive for postintegration steps in the HIV-1 replication cycle prompted us to extend these provocative findings with established cell lines to primary cultures, with the intent of further exploring mink as a potential small laboratory animal model for HIV infection.

MATERIALS AND METHODS

Primary cells and cell lines. Primary fibroblast and splenocytes were prepared from kidney and spleen, respectively, which had been removed aseptically from euthanized M. vision animals at 6 months of age. Primary fibroblasts from M. vision kidney were prepared by filtering tissue pieces through a nylon mesh screen (Falcon cell strainer; 70-μl pore size; Becton Dickinson) after treatment with 0.25% trypsin in Ca2+ - and Mg2+-free phosphate-buffered saline (PBS) for 30 min at room temperature. After centrifugation, cells were washed with Ca2+- and Mg2+-free PBS and cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Sigma) supplemented with 10% fetal calf serum (FCS) and antibiotics (stabilized penicillin-streptomycin solution; Sigma). Single-cell suspensions of splenocytes were prepared by pushing tissue pieces through a nylon mesh screen and purified on Lympholyte-M CL5030 (Cedarlane Laboratories). Activation of mink splenocytes was achieved by an initial overnight stimulation with 1 μg of concanavalin A (ConA) (Wako, Osaka, Japan) per ml and subsequent culturing in RPMI 1640 (Sigma) containing 15% FCS, human recombinant interleukin 2 (IL-2) (40 IU/ml) (Genzyme), 5 × 10−5 M 2-mercaptoethanol (GIBCO-BRL), nonessential amino acids (GIBCO-BRL), 1 mM sodium pyruvate (GIBCO-BRL), minimum essential medium vitamin solution (GIBCO-BRL), and antibiotics. Human peripheral blood mononuclear cells (PBMC) from healthy donors were prepared by using Ficoll-Paque (Ficoll-Paque PLUS; Amersham Pharmacia Biotech AB, Uppsala, Sweden) density centrifugation and then cultured in RPMI1640 containing 10% FCS and 40 IU of human recombinant IL-2 per ml after activation with 3 μg of phytohemagglutinin (PHA-P) (P) (per ml) for 2 days. Primary mouse splenocytes were prepared from spleens removed aseptically from euthanized female C57BL/6J mice by passage through a nylon mesh screen and purified on Lympholyte-MCL5030. Cells were cultured in RPMI 1640 containing 10% FCS and 4 g of recombinant murine IL-2 (R&D Systems, Inc.) per ml, followed by activation with 1 μg of ConA per ml. Mink Mv.1.Lu (NBL-7) cells (a mink-fibroblast-like cell line derived from the lung of a normal M. vision embryo or fetus with no detectable reverse transcriptase activity) was obtained from the RIKEN Cell Bank (Tsukuba, Japan) and maintained in DMEM supplemented with 10% FCS and antibiotics. 293T, HeLa, HOS, and NIH 3T3 cells were maintained in DMEM with 10% FCS and antibiotics. NIH 3T3 cells stably expressing human CycT1 (18) were maintained in DMEM supplemented with 10% FCS, antibiotics, and 200 μg of G418 (Geneticin; GIBCO-BRL) per ml. The CD4+ human osteosarcoma GHOST cell line that carries stably expressed human CCR5, GHOST-h5 (27), was obtained by the AIDS Research and Reference Reagent Program and was maintained in DMEM supplemented with 10% FCS, 200 μg of G418 per ml, 100 μg of hygromycin (Sigma) per ml, 1 μg of puroycin (Sigma) per ml, and antibiotics.

To generate mink cells expressing human CD4, parental mink Mv.1.Lu cells were transfected with pMOSCD4 (33) by using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol and selected in culture medium containing 1 mg of G418 per ml. To introduce human CCR5, Mv.1.Lu cells expressing human CD4 (Mv.1.Lu-CD4 cells) were transfected with pBMGCC5 (33) and selected in medium with 700 μg of hygromycin per ml. Clones with surface expression of >80% of CD4 (Mv.1.Lu-CD4+ cells) were selected. Mv.1.Lu-CD4+CCR5 cells were selected with phycoerythrin-conjugated CD4 monoclonal antibody (eBioscience) and fluorescein isothiocyanate-conjugated anti-CCR5 monoclonal antibody 2D7 (PharMingen) by flow cytometry (FACSCalibur; Becton Dickinson) after limiting dilution.

HIV-1 molecular clones, envelope expression vectors, and generation of pseudotyped and HIV-1 viral stocks. The pNL4-3 Luc E’R’ reporter plasmid (7) was obtained through the AIDS Research and Reference Reagent Program. The infectious HIV-1 molecular clones R7/3SF162, R7/3SF162P3, and R7/3SF33 were constructed as described previously (21). A vesicular stomatitis virus (VSV-G)-expressing plasmid (pSVS-VG) was purchased from Clontech. For expression of HIV-1 SF162, SF162P3, SF33, and IIB gp160, each env gene was subcloned into the β-actin-based expression vector pCAGGS (28). Single-round replication-competent virus stocks were produced in NIH 3T3 cells transfected with an equal amount of pNL4-3 Luc E’R’ and pSVS-VG or various HIV-1 envelope expression vectors by using Lipofectamine 2000. Culture supernatants were harvested at 48 h after transfection, passed through 0.45-μm pore-size filters, and frozen in aliquots at −80°C. The p24 contents of the viruses were determined with enzyme-linked immunosorbent assay kits (Cellular Products Inc.), as were those of standards provided by the manufacturer. To generate the replication-competent VSV-G-pseudotyped virus, 293T cells were cotransfected with an equal amount of the R7/3SF162P3 proviral plasmid and pSVS-VG. VSV-G is incorporated into the HIV-1 virions during production and mediates entry into cells from a broad range of vertebrate animals. HIV-1 virus stocks were produced in 293T cells transfected with respective proviral DNA clones. Culture supernatants were harvested, quantitated for p24 content, and frozen as described above.

Viral entry assay. Target cells were infected for 3 h with 15-μg equivalents of luciferase reporter viruses and cultured for 48 h. Infected cells were lysed with 100 μl of cell lysis buffer (Luc PGC-50, PicaGene; Wako), and 20 μl of each lysate was assayed for photon emission after the addition of 100 μl of luciferase assay substrate (Wako) with a luminometer (Lumat B906; Bertold). The protein concentration of each sample was determined with the Bio-Rad protein assay.

Infectivity assay. Target cells were exposed to HIV-1 SF162P3 (VSV-G) replication-competent pseudotypes or HIV-1 R7/3SF162, R7/3SF162P3, and R7/3SF33 proviruses for 3 h at 37°C. After being washed three times with PBS, cells were treated with trypsin (0.025%)–EDTA (0.27 mM) (Sigma) for 3 min at 37°C and then washed three times with complete medium. The p24 antigen in the medium was assayed immediately after the cell washing (day 0), and that in the culture supernatants was assayed periodically. Background levels, taken to be those on day 0, were subtracted from the amount of p24.

Transient transfection and luciferase assay. To determine the transcriptional activity of the HIV-1 LTR, cells (5 × 106) were plated onto 60-mm-diameter plates. Transient transfections were done with 2 μg of a luciferase reporter plasmid, 1 μg of Pαct-β-gal plasmid, and 2 μg of PBC12/CMV/hCycT1 (human CycT1 under the control of the cytomegalovirus promoter) (4) or pcDNA3.1, using Lipofectamine 2000. Cells were harvested at 48 h after transfection. Lysates were prepared from a portion of the transfected cells by using cell lysis buffer (Luc PGC-50) and assayed for luciferase activity. Another portion was used to prepare β-galactosidase (β-gal) (P) (Sigma)–gallate measurements to ensure comparable efficiency of transfection. β-Gal activity was measured by standard colorimetric methods with β-Gal detection kits (Invitrogen).

cDNA sequencing of mink CycT1. Total RNA was prepared from mink Mv.1.Lu cell by using the TRizol reagent (Invitrogen), and first-strand cDNA was generated with SuperScriptIII (Invitrogen) according to the manufacturer’s instructions, using oligo(dT) as a primer. The 5′ and 3′ halves of cDNA encoding the entire open reading frame of mink CycT1 were amplified by using primer sets (5′-ATGGAGGAGGAGGAAGAAC-3′–5′-ATGAGAAGAGAGATCAGGCGC-3′ and 5′-CAATGTTAGAATGACATAGTCCC-5′–3′TTTACTTAGAAGATGTTGAGAA-3′) designed based on the sequence of human CycT1 (GenBank accession number AF048730). Taq polymerase-amplified PCR products were cloned into a vector by using pCR2.1-TOPO TA cloning (Invitrogen), and a TA clone of each half was obtained and sequenced.

RNase protection assays. A 262-bp fragment (nucleotide 78 to 340 relative to the site of transcription) was PCR amplified from the R7/3SF162 proviral plasmid by using the primer sets 5′-GCTTGGCCTAGGTGCTCTACAAAGAC-3′–5′-CCCATCTCCTCCTCTAGGTCCTCCG-3′ and inserted into pcR2.1 TOPO TA vector containing the T7 promoter to provide a template for the synthesis of an antisense RNA probe. This plasmid was linearized with HindIII, and the antisense RNA probe spanning the HIV-1 major 5′ splice donor was generated by in vitro transcription with T7 polymerase in the presence of [α-32P]dCTP (Amersham) by using RNA transcription kits (Stratagene), heated to 85°C, and used as a probe in hybridization. Ten micrograms of total RNA, extracted from HIV-1-infected 293T cells, was hybridized with the TRGE luciferase reporter plasmid cDNA expressing T7 promoter to determine the assay procedures.

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Spliced and unspliced HIV-1 RNA were visualized by autoradiography after separation on a 5.0% denaturing acrylamide gel.

**CD4 down-regulation assay.** Transfection to assess CD4 down-regulation by Nef was performed with Mv.1.Lu-CD4-CCR5 and GHOS-T-hbs cells. Cells (7 × 10⁶) plated onto 60-mm-diameter plates were transfected by using Lipofectamine 2000 with 3 μg of the pReCMV-CD8-SF2Nef expression construct (2). As a control, a chimeric CD8 fusion protein with Nef in the antisense orientation (pReCMV-CD8-antisense SF2Nef) was used. At approximately 40 h posttransfection, cells were harvested and stained with a mixture of phycoerythrin-conjugated anti-CD4 (eBioscience) and fluorescein isothiocyanate-conjugated anti-CD8 monoclonal antibodies (PharMingen). Surface CD8 expression was used as a marker for Nef-expressing cells. CD4 down-regulation was determined by comparing the percentage of CD4 expression on CD8-positive cells transfected with CD8/Nef expression plasmid to that on cells transfected with CD8/antisense Nef by using a FACScan-Calibur.

**TCID₅₀ determination.** The infectious titer (50% tissue culture infective dose [TCID₅₀]) of HIV-1 in cell culture supernatants was determined 48 h after transfection, and the firefly luciferase activity was normalized to the β-Gal activity measured by standard colorimetric methods. Values are arithmetic means ± standard deviations from duplicate transfections. Results are representative of those from three independent experiments.

### RESULTS

**HIV-1 LTR activity is robust in mink cells and cannot be enhanced by human CycT1.** To characterize the efficiency of trans-activation and transcript elongation from the HIV-1 LTR in mink cells, the proviral plasmid pNL4-3 Luc E−R− was introduced by transfection, and luciferase activities in cellular lysates were quantified 48 h later. Both adherent primary mink fibroblasts and the Mv.1.Lu cell line from American mink (M. vision) were used, and HIV-1 LTR activity was normalized for variability in transfection efficiency by cotransfection of an LTR-independent β-Gal reporter construct (14). Transfections of human HeLa and HOS cells were included as positive controls, while mouse NIH 3T3 cells served as a negative control.

HIV-1 LTR activity that was 2 log units higher than that found in mouse NIH 3T3 cells and comparable to that of the human HeLa and HOS cells was observed in primary mink fibroblasts and Mv.1.Lu cells (Fig. 1). The high-level transcriptional activity observed in mink fibroblasts and Mv.1.Lu cells could not be further enhanced by cotransfection of an expression plasmid encoding human CycT1. In contrast, the luciferase signal in NIH 3T3 cells was significantly augmented in the presence of human CycT1. A single amino acid change at residue 261 from cysteine to tyrosine in murine CycT1 has been shown to be the major determinant in restriction of Tat-mediated HIV-1 LTR trans-activation in NIH 3T3 cells (4, 10, 11, 19, 36). The ability to trans-activate the HIV-1 LTR in mink cells suggests that the mink homologue of CycT1 is capable of forming functional complexes with HIV-1 Tat. To confirm this at the genetic level, the gene encoding CycT1 was isolated from cDNA of mink Mv.1.Lu cells, and the sequence was compared to those of human and murine CycT1s (GenBank accession numbers AF048730 and AF095640, respectively). Alignment of the predicted amino acid sequences showed that the CycT1s from human and mink have 726 amino acid residues, while mouse CycT1 has 724 amino acids (Fig. 2). There are 47 amino acid substitutions between human and mink CycT1, many of which are also found in mouse CycT1. Importantly, however, the cysteine residue at position 261 that is critical for productive interaction of human CycT1-Tat to the trans-activation response element is absent in mouse CycT1. The identity of residue 261 in mink CycT1 was further confirmed by preparing another set of RNA samples from mink primary fibroblasts and amplifying the region around residue 261 (data not shown). Collectively, these results suggest that the ability to support robust HIV-1 LTR activity is a property unique to M. vision among small-animal species.

Efficient spliced and unspliced HIV-1 mRNA syntheses in...
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′/H11032 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. 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.

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.
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. 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. Parallel infections were made in PHA-IL-2-activated human PBMC, HeLa, 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.

**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-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.
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-specific 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 CD8α 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.
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 (TCID50 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 TCID50 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. vison 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. vison 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
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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