表 1　HIV 感染症の病態と HLA 遺伝子型の相関

相関する HLA	HIV 感染症に与える影響	文献
HLA-A, B, C homozygosity	エイズへの進行促進	2, 3
*HLA-B**35	エイズへの進行促進	4, 5
*HLA-B**57, *HLA-B**27	病態進行の遅延	6
*HLA-A**01-*B0**8-*DRB1**03	エイズへの進行促進	7, 8

態遅延と相関する効果によって，見かけ上 *HLA-B* アリルの重要性がクローズアップされたのかもしれない。また，HIV がヒトからヒトへ伝播することを考えると，*HLA-A**02 のように遺伝子頻度の高い *HLA* アリルに対してはすでに HIV は適応しているため，免疫応答をうけにくいのかもしれない[10,11]。いずれにしても，HIV 感染者の体内で HIV 感染制御に最も有効性の高い CTL 応答の実体の解明が待たれる。

2. HIV ゲノムの変異による CTL 応答からの逃避

HIV はさまざまな変異を獲得して抗 HIV 阻害剤に耐性を示すことが知られている。CTL による免疫応答に対しても，CTL エピトープあるいはその周辺領域に変異を獲得して，CTL から逃避することが明らかになってきた[12]。数百人の HIV 感染者を用いた大規模な HIV の遺伝子配列を感染者の HLA 遺伝子型に沿って調べたところ，各 HLA 分子に共通した変異が認められた。こうした変異は，その HLA 分子が提示する CTL エピトープの内部の配列であったり，ごく近傍に位置していた。機能的な解析から，実際に CTL による傷害活性やサイトカイン産生活性を示さなくなることが明らかとなった。こうした変異のうち，CTL エピトープ内部の変異は HLA 分子との結合や TCR との相互作用に影響して，CTL による免疫応答から逃れると考えられる。また最近，CTL エピトープの近傍の変異によって，ペプチド断片を生成する蛋白質のプロセッシングパターンを変えてしまうことにより，エピトープペプチドが生成されなくなるという新しい逃避メカニズムが報告された[13,14]。こうしたことを考え合わせると，HIV ゲノムの変異による CTL 免疫応答からの逃避は，当初考えられていたよりもかなり広範囲にわたって起きていることが強く示唆される。

それではこうした CTL エスケープ変異は，HIV 感染症の病態進行にどのようにかかわるのだろうか？　HLA-B*27 拘束性の Gag エピトープは，ドミナントなエピトープで非常に強い CTL 応答を誘導することが知られている。HIV 感染後数年にわたって血中ウイルス量が低く維持されていた患者では，このエピトープに特異的な CTL 応答が認められた[15]。そうした患者の一部では，このエピトープ領域に一つの変異を認めたが，それとほぼ同時期に急激なウイルス量の増加が観察された。この変異は，HLA-B*27 分子との結合を失わせる変異であったため，変異獲得後は CTL による免疫応答がほぼ完全に失われていた[15]。こうしたことから，HIV が変異を獲得して CTL による免疫応答から逃れる結果，HIV 感染症の病態が進行することは明らかである。しかしながら，CTL エスケープ変異と病態進行の相関を示す症例の報告はいまだ限られており，具体的にどんな，あるいはどの CTL エスケープ変異が病態進行と関連するのか，エスケープ変異に対して獲得免疫系はどのように対応するのかなど明らかになっていない問題は多い。

HIV ゲノムの変異にかかわる大きな因子の一つとして，HIV の複製効率への影響がある。もし CTL エピトープの領域が HIV の複製にとって必須であった場合，CTL エスケープ変異は HIV の複製効率を低下させ，結果として HIV の増殖抑制に働くかもしれない（図 1）。こうした事例が SIV 感染モデルを用いた研究から示された[16]。さらに，ウイルス複製能を低下させる CTL エスケープ変異を持ったウイルスが MHC の異なる他の個体に伝播するケースでは，変異はもとのアミノ酸に戻りウイルス複製能が回復することが SIV 感染モデルおよび HIV 感染症患者で明らか

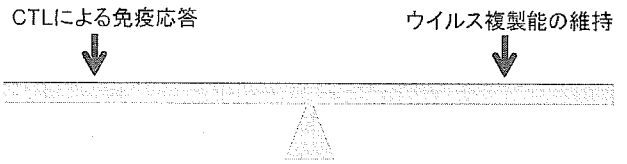

図 1　CTL エスケープ変異にかかわる要因
HIV に対する CTL の免疫応答の選択圧とウイルス複製能を維持する選択圧のバランスが，CTL エスケープ変異の出現に影響する。他にもさまざまな要因が考えられるが，それらがどの程度 HIV によるエスケープ変異獲得に関与するか現在さかんに研究されている。

にされた[17,18]。一方，HIV あるいは SIV が MHC を共有する他の個体に伝播したケースでは，エスケープ変異は保存されることから[17,18]，CTL 免疫応答による選択圧とウイルス複製能のバランスが，CTL エスケープ変異の出現を決める因子であると示唆された(図1)。しかしながら，$CD4^+$ T 細胞による細胞性免疫，抗体や自然免疫系あるいは他の生体内抗ウイルス因子など，CTL 以外の要因による抗ウイルス効果も，CTL エスケープ変異の出現に影響すると考えられる。実際，強い CTL 応答を示すにもかかわらず，エピトープおよびその周辺に変異が認められない例も多く(上野，未発表データ)，宿主が持つ HIV に対する選択圧については防御免疫系以外の要因も含め，今後の詳細な解析が待たれる。HIV による CTL エスケープ変異の出現要因や，変異によるエスケープ後の抗ウイルス免疫応答の解明という課題は，ワクチン開発に重要な情報を提供するだけでなく，持続的に感染する病原体に対するヒト免疫応答の性質を明らかにする上で，非常に興味深いモデルである。

3. CTL による抗ウイルス活性の効率

MHC テトラマーやサイトカイン産生細胞の染色法を用いて HIV 特異的な $CD8^+$ T 細胞の頻度を測定すると，慢性 HIV 感染者では HIV 特異的な CTL は十分に存在するが，抗ウイルス機能を測定するとその機能は減弱している。その原因として，$CD8^+$ T 細胞が機能的な CTL として十分に成熟できないというモデルが提唱されたが[19]，未だこの全容は解明されていない。我々は，HIV 特異的 $CD8^+$ T 細胞中には，エピトープ特異性は示すが HIV 感染細胞に作用できないサブセットがいるのではないかと考え，これまでに樹立した HIV 特異的 CTL クローンの抗ウイルス活性を再検討した。その結果，HLA-B*35 拘束性で逆転写酵素由来のペプチド（IPLTEEAEL）に特異的な CTL 55 は，MHC テトラマーに結合し，合成ペプチドをパルスした標的細胞を殺傷するにもかかわらず，HIV 感染細胞に対して全く機能しないことを見出した[20]。さらに興味深いことに，この患者の末梢リンパ球を数年にわたり経時的に解析したところ，CTL 55 タイプの CTL の頻度が増しており，逆に同一エピトープに特異的だが抗ウイルス活性に優れた CTL サブセットの頻度が減少していた。このことは，個々の CTL の機能が経時的に減少するのではなく，抗ウイルス活性機能が劣った別の CTL サブセットが増えてしまうために，個体全体の CTL による抗ウイルス機能が減弱化するという新しいモデルを示唆している（上野ら，投稿準備中)。

また，オックスフォード大学のグループは *HLA-B**08* を持つ長期未発症者（long-term nonprogressor ; LTNP）の Nef エピトープ（FLKEKGGL）に応答する CTL を解析した。その結果，$V\beta$ 13.2 という TCR を持つ CTL は，他の TCR を持つ CTL に比べて増殖能に優れ，エピトープ変異にも応答できる機能を有していた[21]。

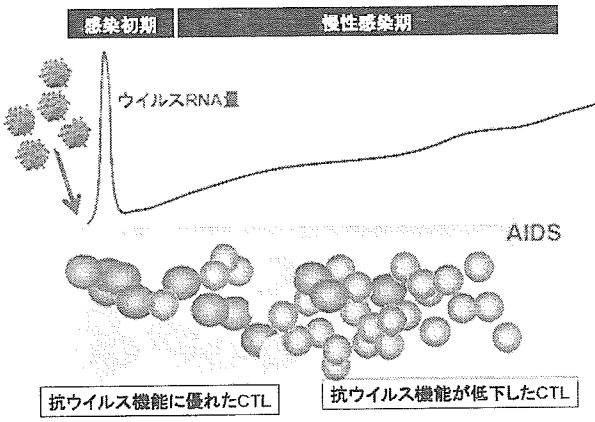

図 2 CTL による抗ウイルス免疫応答の経時変化
CTL による免疫応答は感染初期には効率的に HIV の複製を制御するが，HIV の排除には至らない。慢性感染成立後の HIV に対する CTL 応答を解析すると，HIV 特異的 CD8 T 細胞数は十分認められるにもかかわらず，CTL の抗ウイルス機能は低下している。抗ウイルス機能に優れた初期の CTL が時間とともにその機能を喪失するのか，あるいは機能が低下した CTL が後から誘導されるのだろうか。慢性感染ウイルスに対するヒト CTL 応答には未解明の問題が多く残されている。

こうした結果は，抗原特異性が同一であっても，T 細胞レセプター（TCR）が異なる CTL サブセット間では，抗ウイルス活性に差があることを示している。したがって，HIV 感染者の抗ウイルス免疫応答の活性を評価するには，抗原特異的な T 細胞の数よりもむしろ CTL の機能を包括的に解析する方法が必要である。一方，TCR 依存的に抗ウイルス活性に優れた CTL サブセットが一部の感染者で実在することを考えると，抗ウイルス活性に優れた TCR をコードする遺伝子を他の感染者の CD8 T 細胞に導入することにより，効率的な抗ウイルス機能を持った CTL を大量に作り出すことが可能である[22]。こうした情報を基に，抗ウイルス活性に優れた CTL の実体の解明や，こうした CTL を効率的に誘導するためのワクチン開発研究が期待される。

4. CTL による抗ウイルス活性のエピトープ依存性

CTL は HIV 由来の複数のエピトープペプチドに応答する。一つの HLA アリルあたり平均しておおよそ 10 種類程度のペプチドが CTL エピトープとして認識されている。

表 2　HLA-B51 拘束性エピトープに特異的な CTL の抗ウイルス活性

蛋白質	エピトープ	ウイルス増殖阻害	細胞傷害活性	サイトカイン産生
IN	LPPVVAKEI	＋＋＋	＋＋＋	＋＋
RT	TAKTIPSI	＋＋＋	＋＋＋	＋＋＋
Gag	NANPDCKTI	＋＋	＋	＋＋
Rev	VPLQLPPLERL	＋	−	＋

エピトープ特異性は，CTL の抗ウイルス活性に影響するのだろうか。個々のエピトープは，それが由来する蛋白質の発現量，プロテアソームなど蛋白質分解酵素による消化効率，HLA 分子との結合活性などが異なるため，最終的に感染細胞表面で提示される量はエピトープごとに異なっている。実際 Yang らは，CTL の抗ウイルス活性は，その CTL が抗原ペプチドを認識する感度よりも，どの抗原を認識するかという抗原特異性の影響が大きいことを報告した[23]。

また我々は，HLA-B*51 拘束性の四つの異なるエピトープについて，CTL の抗ウイルス活性を比較した（表 2）。これまでは CTL 活性を測定する標的細胞には B 細胞由来の細胞株が頻繁に用いられていたが，この実験では HIV-1 を感染させる標的細胞にヒト末梢血から分離した $CD4^+$ T 細胞を用いた。また Nef 蛋白質は HLA クラス I 分子の細胞表面での発現量を低下させ，CTL による傷害活性から逃避することが知られている。Nef 陽性の HIV-1 株（NL432）を用いることにより，CTL にとってより厳しい条件下で抗ウイルス機能を評価する実験系を確立した[24]。その結果，逆転写酵素やインテグレース由来エピトープに特異的な CTL では顕著な抗ウイルス活性を認めたが，Rev 特異的な CTL は抗ウイルス活性をほとんど示さなかった（表 2）。*HLA-B*51* はエイズ病態進行の遅延と相関していることを考えると，HLA-B*51 拘束性の CTL が Nef による HLA 分子の発現低下にもかかわらず効率的な抗ウイルス活性を示したことは，こうした CTL が病態進行を遅延させる要因となっているのかもしれない。

おわりに

これまでウイルス感染症に対する免疫応答の研究は，主に急性感染ウイルスをモデルを中心として進められてきた。HIV 感染症のように，長期にわたって持続的に抗原が存在する感染症に対して，ヒト獲得免疫系がどのように応答するのか，未解明な問題が多く残されている。ウイルス特異的 CTL の抗ウイルス機能が一様ではなく，ウイルス複製を制御できない CTL サブセットが長期に渡って存在するなど，急性感染症とは異なり，慢性 HIV 感染症に際立った感染免疫学的現象が見出されてきている。今後は，クロスプレゼンテーションなど抗原提示系や自然免疫系との相互作用を通じて，HIV の抗原変異がヒト免疫応答に与える長期的な影響や抗ウイルス機能に優れた CTL の実体とその成立メカニズムなどが統合的に解析されることにより，獲得免疫系による HIV の制御機構と破綻メカニズムの解明，合わせてそれを修復する新たな方法論の開発が期待される。

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Susceptibility of Mink (*Mustera vision*)-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 (*Mustera 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

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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 (*Mustera 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 Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) for 30 min at room temperature. After centrifugation, cells were washed with Ca^{2+}- and Mg^{2+}-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 (Sigma) 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 ng 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 derivative that stably expresses human CCR5, GHOST-hi5 (27), was obtained through 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 puromycin (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 pBMGCCR5 (33) and selected in medium with 700 μg of hygromycin per ml. Clones with surface expression of either human CD4 (Mv.1.Lu-CD4) or both human CD4 and CCR5 (Mv.1.Lu-CD4-CCR5) 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 R7/3/SF162, R7/3/SF162P3, and R7/3/SF33 were constructed as described previously (21). A vesicular stomatitis virus G (VSV-G)-expressing plasmid (pVSV-G) was purchased from Clontech. For expression of HIV-1 SF162, SF162P3, SF33, and IIIB gp160, each *env* gene was subcloned into the β-actin-based expression vector pCAGGS (28). Single-round replication-competent luciferase reporter virus stocks were produced in 293T cells transfected with an equal amount of pNL4-3 Luc E^-R^- and pVSV-G or various HIV-1 envelope expression vectors by using Lipofectamine 2000. Culture supernatants were harvested at 48 h posttransfection, 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 R7/3/SF162P3 proviral plasmid and pVSV-G. 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-ng equivalents of luciferase reporter viruses and cultured for 48 h. Infected cells were lysed with 100 μl of cell lysing 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 B9506; 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 R7/3/SF162, R7/3/SF162P3, and R7/3/SF33 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 (5×10^5) were plated onto 60-mm-diameter plates. Transient transfections were done with 2 μg of a luciferase reporter plasmid, 1 μg of pAct-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 posttransfection. Lysates were prepared from a portion of the transfected cells by using cell lysing buffer (Luc PGC-50) and assayed for luciferase activities. Another portion was used to prepare cell lysates for β-galactosidase (β-Gal) 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 SuperScriptII (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′-ATGGAGGGAGAGAGGAAGAAC-3′–5′-ATGAGAAAGGAGAT TCTGGGC-3′ and 5′-CAATGTGAAGTCACAATATGC-3′–5′-TTTACTTAG GAAGGGGTGGAAG-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 R7/3/162 proviral plasmid by using the primer sets 5′-GCTTGCCTTGAGTGCTTCAAG-3′–5′-CCCATC TCTCTCCTTCTAGCCTCC-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 *Hin*dIII, 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-^{32}P]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 R7/3/162P3(VSV-G)-infected cell lines with the TRIzol reagent, was hybridized to the antisense RNA probe overnight and digested with an RNase A-RNase T_1 mixture (RNase cocktail; Ambion). Protected fragments that corresponded to

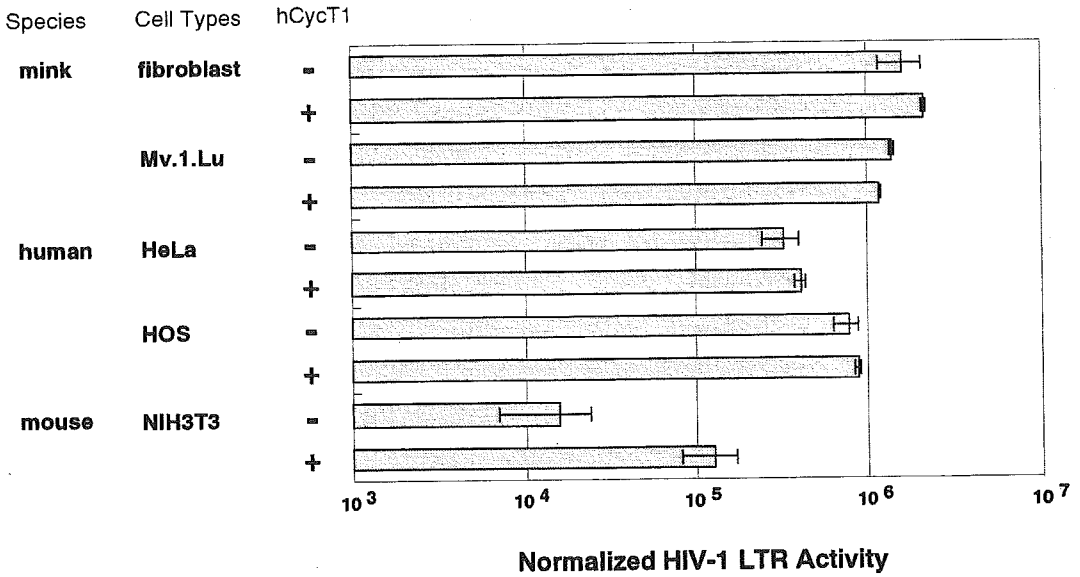

FIG. 1. The HIV-1 LTR exhibits significant activity in mink cells. Mink fibroblasts and Mv.1.Lu, human HeLa, HOS, and mouse NIH 3T3 cells were transfected with 2 μg of pNL4-3 Luc E^-R^-, 1 μg of pAct-β-gal, and 2 μg of pBC12/CMV/hCycT1 (expression plasmid for human CycT1) or 2 μg of cytomegalovirus immediate-early promoter-based vector pcDNA3.1 (empty vector). Luciferase and β-Gal activities in cell lysates were 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.

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 GHOST-hi5 cells. Cells (7×10^5) plated onto 60-mm-diameter plates were transfected by using Lipofectamine 2000 with 3 μg of the pRcCMV-CD8-SF2Nef expression construct (2). As a control, a chimeric CD8 fusion protein with Nef in the antisense orientation (pRcCMV-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 FACSCalibur.

$TCID_{50}$ determination. The infectious titer (50% tissue culture infective dose [$TCID_{50}$]) of HIV-1 in cell culture supernatants was determined by end point limiting dilution on PHA–IL-2-activated human PBMC from HIV-1-seronegative donors 5 days after inoculation.

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. vison*) 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 (4, 11) but is conserved in mink 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

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                             <---------------------------------------------------------------------------
human CycT1    1 MEGERKNNNKRWYFTREQLENSPSRRFGVDPDKELSYRQQAANLLQDMGQRLNVSQLTINTAIVYMHRFYMIQSFTQFPGNSVAPAALFLAAKVEEQPKK 100
murine CycT1   1 ------------------------------S-----------------------------------------------HRY-M----------------- 100
mink CycT1     1 ----------------------------L-------N-----------------------------------------HR-------------------- 100
                 ----------------------------------------------------------------------------------------------------
human CycT1  101 LEHVIKVAHTCLHPQESLPDTRSEAYLQQVQDLVILESIILQTLGFELTIDHPHTHVVKCTQLVRASKDLAQTSYFMATNSLHLTTFSLQYTPPVVACVC 200
murine CycT1  101 ---------------------------------------------------------------------------------------------------- 200
mink CycT1   101 ---------A---------------------------------------T-------------------------------------------------- 200
                 -- Cyclin box ---------------------------->          *
human CycT1  201 IHLACKWSNWEIPVSTDGKHWWEYVDATVTLELLDELTHEFLQILEKTPNRLKRIWNWRACEAAKKTKADDRGTDEKTSEQTILNMISQSSSDTTIAGLM 300
murine CycT1 201 -------------------------------------------------S-----R----YQ--M---P----A--N------------T---------- 300
mink CycT1   201 -------------------------------------------------------R-----Q--R-----------N----------------------- 300

human CycT1  301 SMSTSTTSAVPSLPVSEESSSNLTSVEMLPGKRWLSSQPSFKLEPTQGHRTSENLALTGVDHSLPQDGSNAFISQKQNSKSVPSAKVSLKEYRAKHAEEL 400
murine CycT1 301 ----AS--------S------S----D--Q-E-------P----AA-------S---I------Q----S--G----A---------------------- 400
mink CycT1   301 -----SA-------AT-------SN----Q-E-------P----HA-----------M------Q----------------------------------- 400

human CycT1  401 AAQKRQLENMEANVKSQYAYAAQNLLSHHDSHSSVILKMPIEGSENPERPFLEKADKTALKMRIPVAGGDKAASSKPEEIKMRIKVHAAADKHNSVEDSV 500
murine CycT1 401 --------------------------- --------------S---------D----S-----L---S----V--------------S-G-----I---- 499
mink CycT1   401 ----------------------------------------M----------------A---------S-------------------S------------ 500

human CycT1  501 TKSREHKEKHKTHPSNHHHHHNHHSHKHSHSQLPVGTGNKRPGDPKHSSQTSNLAHKTYSLSSSFSSSSSTRKRGPSEETGGAVFDHPAKIAKSTKSSSL 600
murine CycT1 500 ---------QR---------------R---L---A-PVS---S---------T----------TL-----------P----A---------------- - 598
mink CycT1   501 ----------------------------------A-----------------TF----------------------P----------------------I 600

human CycT1  601 NFSFPSLPTMGQMPGHSSDTSGLSFSQPSCKTRVPHSKLDKGPTGANGHNTTQTIDYQDTVNMLHSLLSAQGVQPTQPTAFEFVRPYSDYLNPRSGGISS 700
murine CycT1 599 --P--P----T-L----------P------------M------P------A--S----------------------AP-----HS-GE-M---A-A---- 698
mink CycT1   601 -----P----A-L----------P------------M-------------A---------------------------P--D--HS-GE-----A--M--- 700

human CycT1  701 RSGNTDKPRPPPLPSEPPPPLPPLPK 726
murine CycT1 699 ---T---------------------- 724
mink CycT1   701 ---------L---------------- 726
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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.*

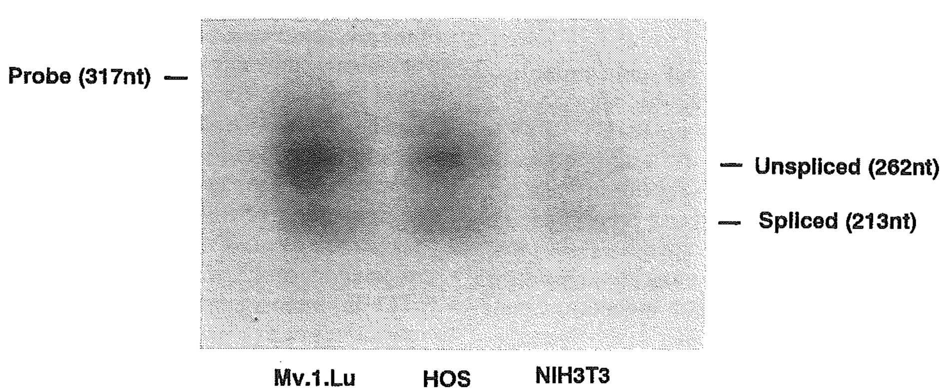

FIG. 3. RNase protection analysis of HIV-1 transcripts in infected mink, human, and mouse cells. The indicated HIV-1 receptor-negative cells were infected with VSV-G-pseudotyped R7/3/162P3. Forty-eight hours later, total RNA was extracted from infected cells and analyzed by RNase protection assay. The 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.

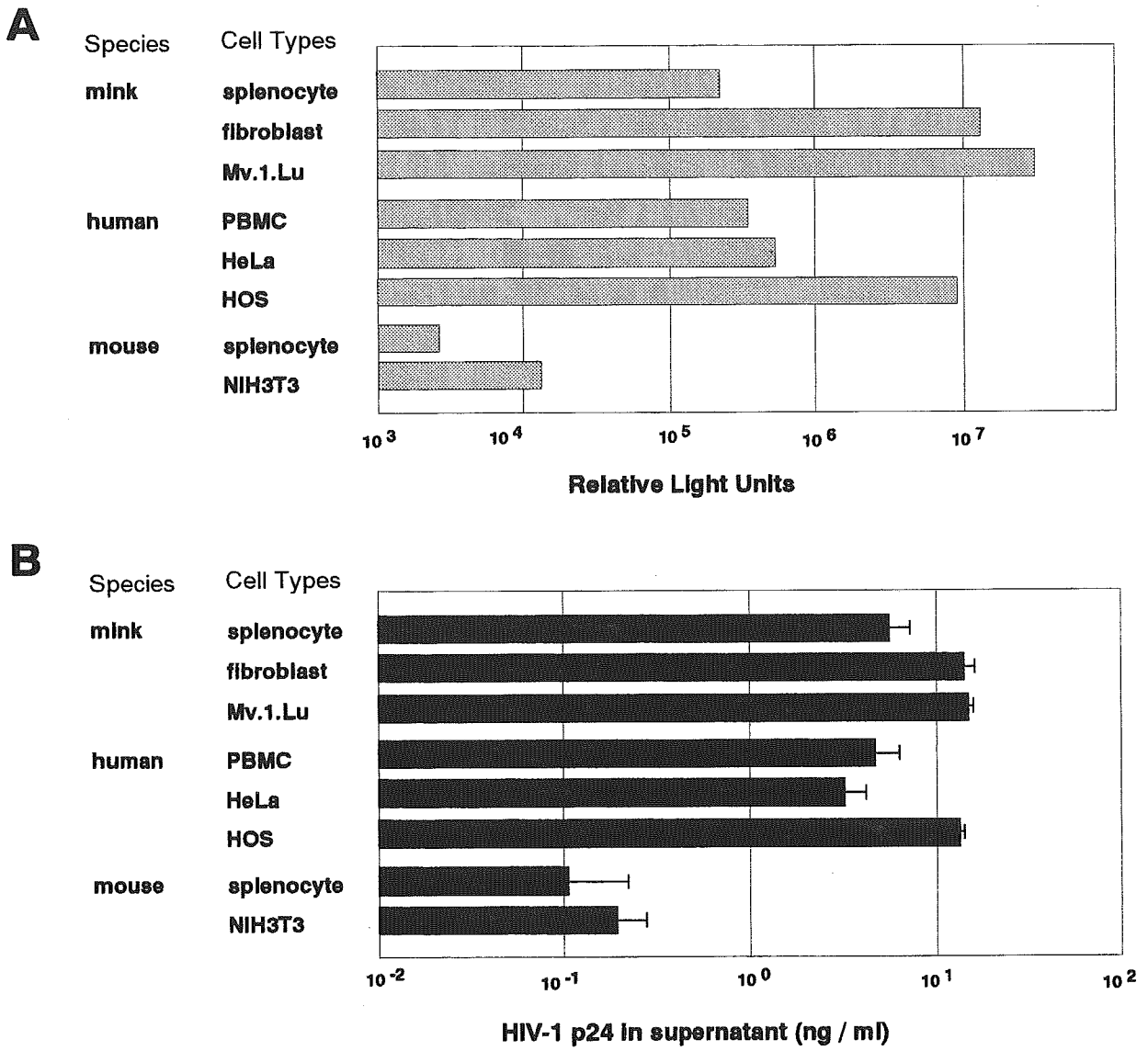

FIG. 4. Primary mink cells as well as the mink Mv.1.Lu cell line support all postentry steps in the HIV-1 replication cycle. (A) ConA–IL-2-activated mink splenocytes, mink fibroblasts, and the Mv.1.Lu cell line were inoculated with 15 ng of the p24 equivalent NL4-3 Luc E^-R^- reporter viruses pseudotyped 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).

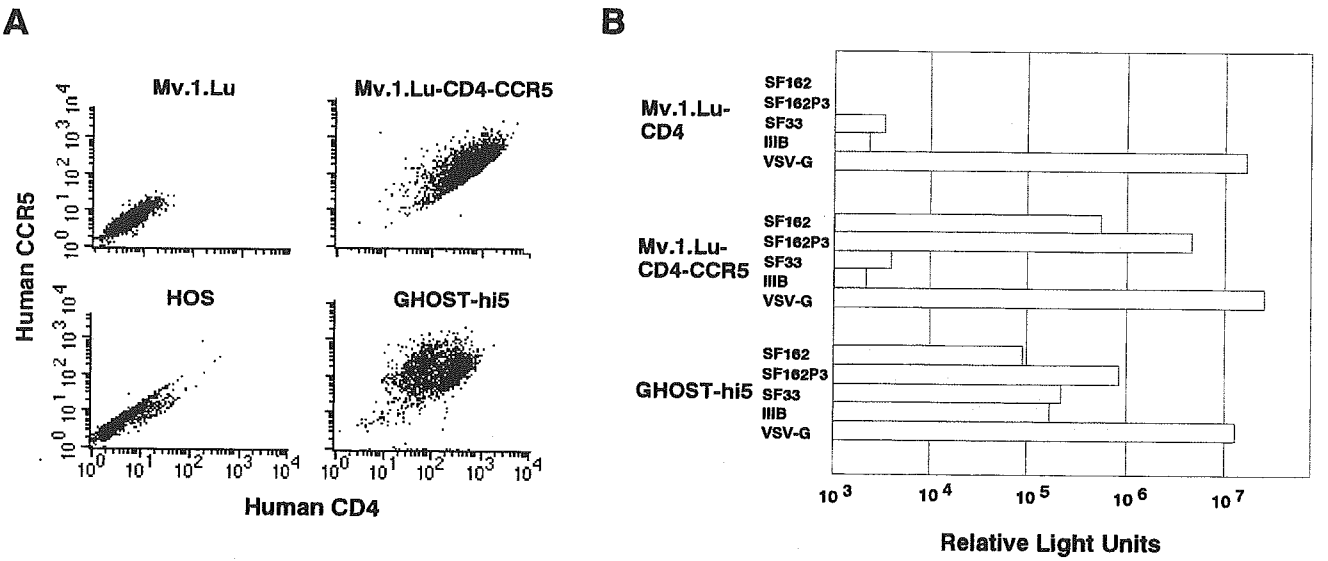

FIG. 5. The mink cell line Mv.1.Lu can be rendered permissive to HIV-1 R5 virus infection by coexpression of human CD4 and CCR5. (A) Flow cytometry analysis of human and mink cell lines for determination of expression of human CD4 and CCR5. The parental cells were used as negative controls. (B) Single-round 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

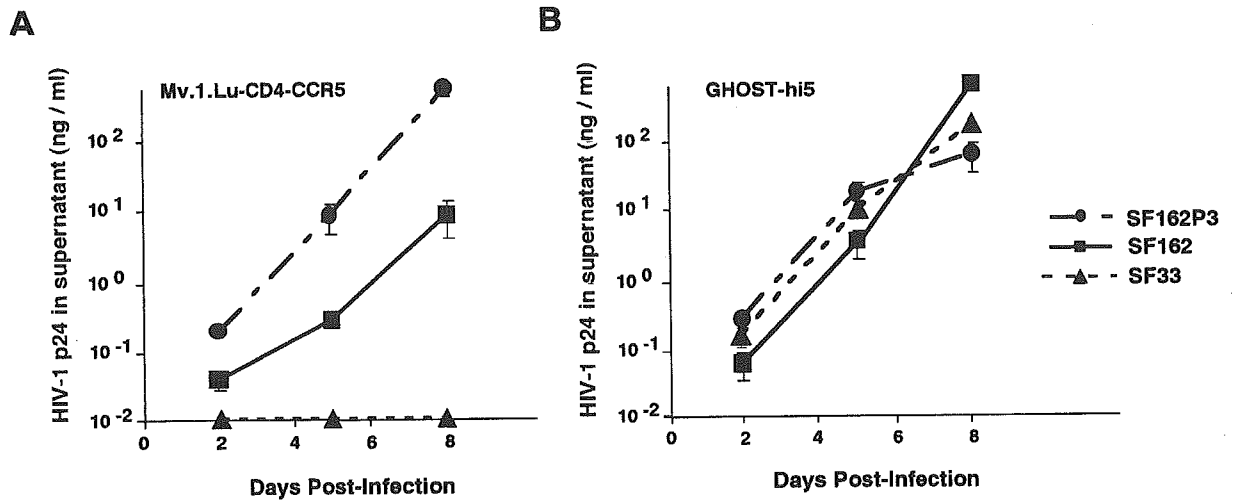

FIG. 6. Mink Mv.1.Lu-CD4-CCR5 (A) and human GHOST-hi5 (B) cells (2×10^5) were infected at the same input dose (15 ng of p24 equivalent) with R5 R7/3/162 and R7/4/162P3. Virus replication was monitored by the amounts of p24 CA produced in the culture supernatants on days 2, 5, and 8. Results shown are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

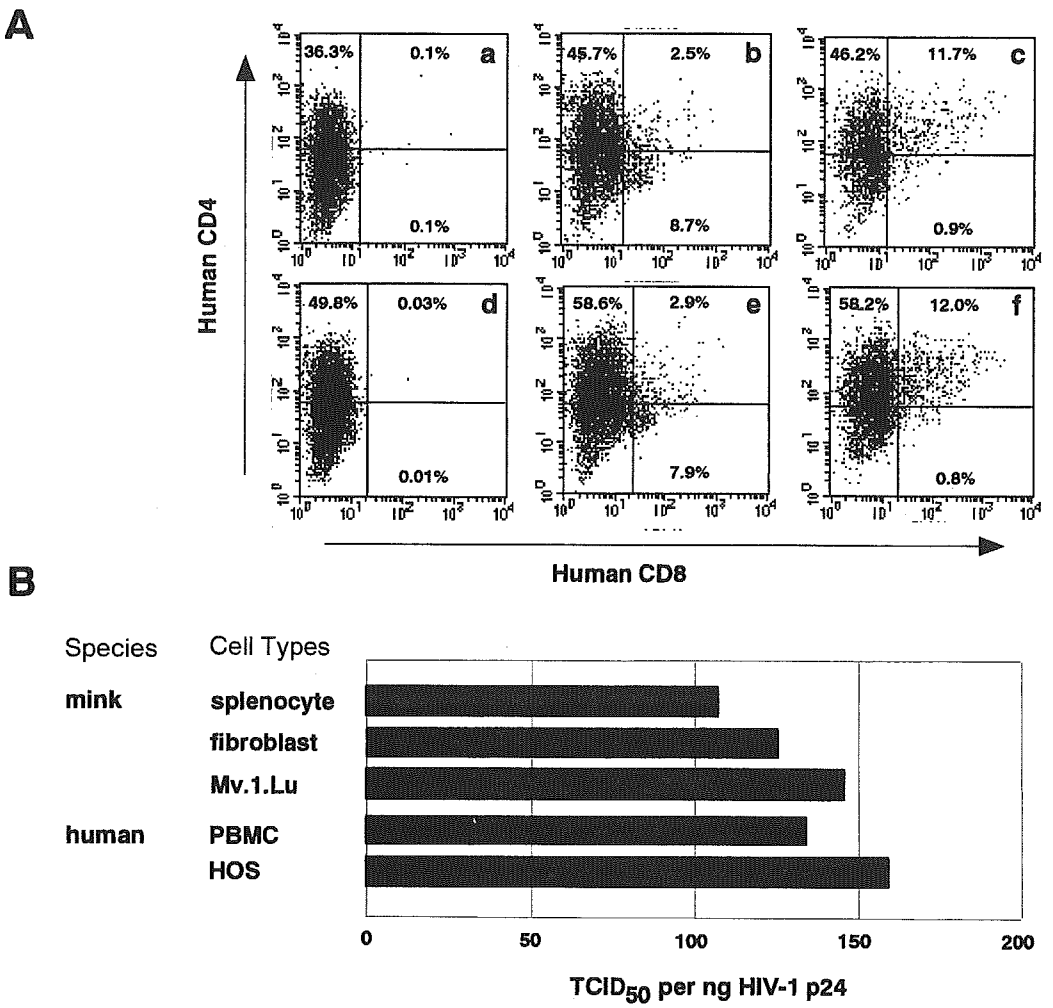

FIG. 7. (A) Mink cells support Nef function in CD4 down-regulation. Wild-type chimeric CD8/Nef or CD8/antisense Nef expression plasmid was transfected into Mv.1.Lu-CD4-CCR5 (a, b, and c) and GHOST-hi5 (d, e, and f) cells, and cell surface CD4 expression on CD8-positive transfected cells (i.e., Nef-expressing 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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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.

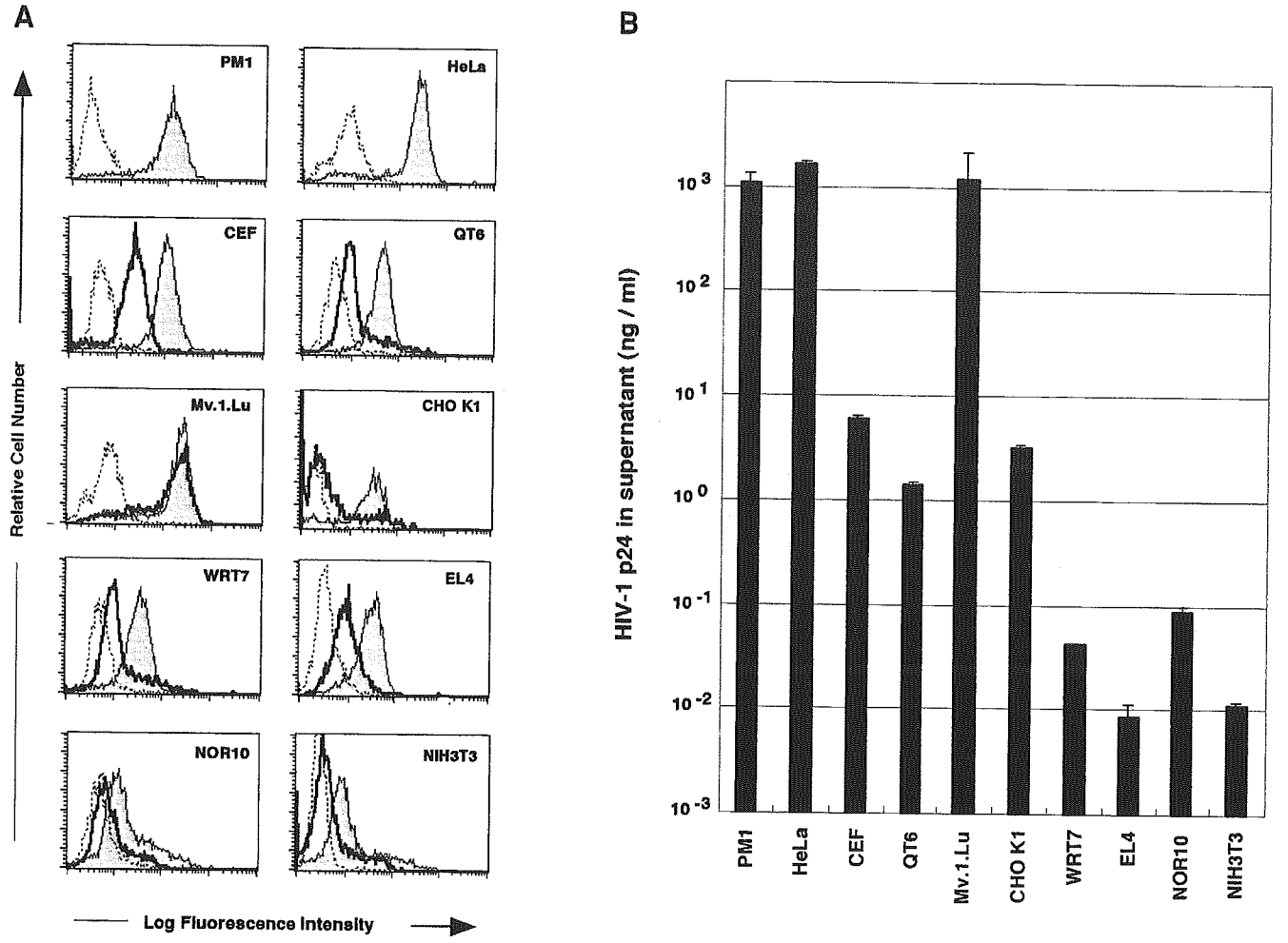

FIG. 1. Human cyclin T1 restores the intracellular HIV-1 Gag expression but not virion assembly in the murine cells. (A) Human or nonhuman cell lines with (in gray shaded area) or without (solid tracing) transduced hu-cyclin T1 were infected with HIV-puro (VSV-G). After selection in the presence of puromycin, 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

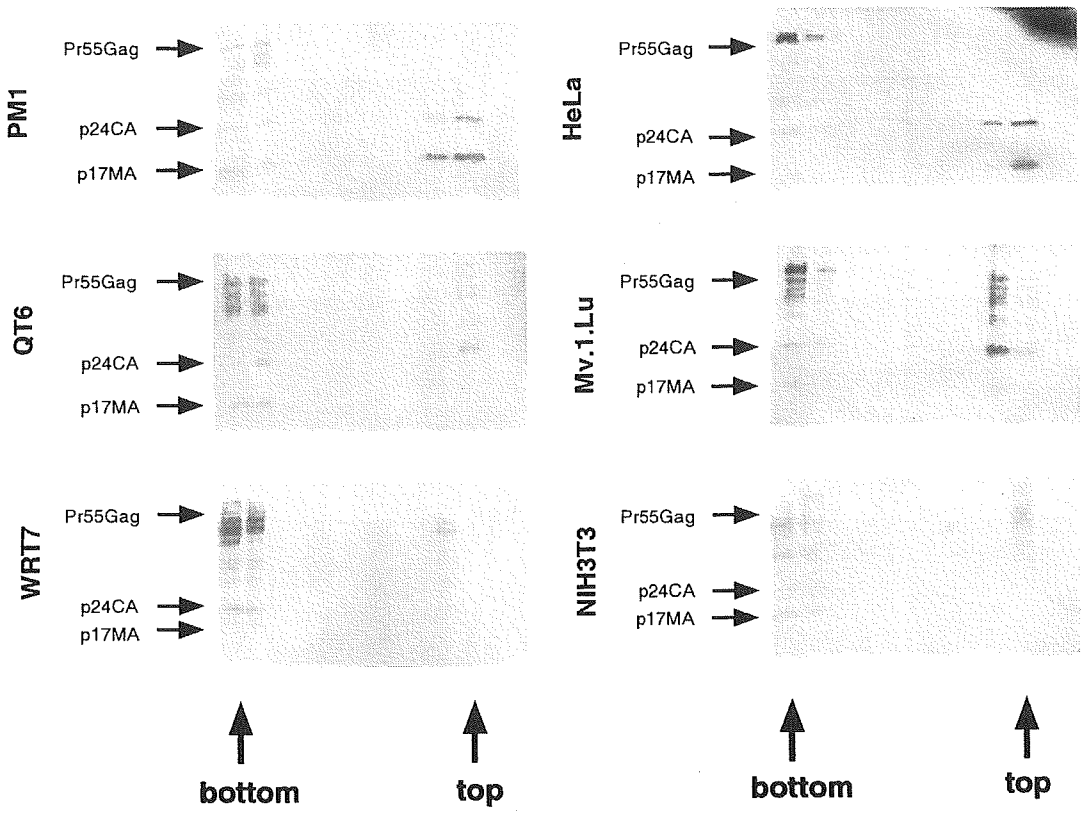

FIG. 2. Membrane floatation centrifugation with p17MA, p24CA, and Gag precursors. Postnuclear supernatants were prepared and subjected to membrane floatation centrifugation. Aliquots of each fraction were subjected to denaturating by 15% SDS–PAGE followed by electroblotting onto a PVDF membrane and developed 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

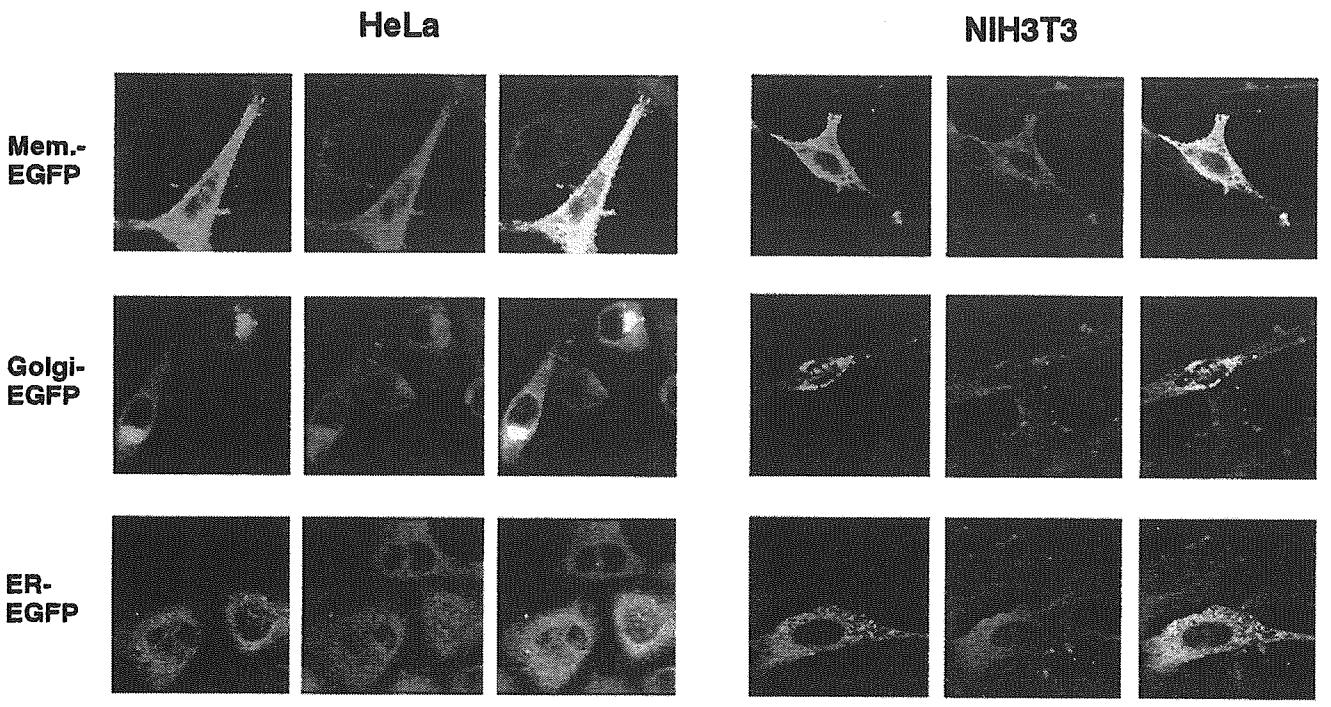

FIG. 3. Confocal microscopy analysis of intracellular Gag polyprotein. HeLa and NIH3T3 cells expressing HIV-1 *gag* and *pol* genes were transiently transfected with pEYFP vectors (CLONTECH) encoding yellow–green fluorescent variants of the enhanced green fluorescent proteins (EYFPs) targeted to the 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.

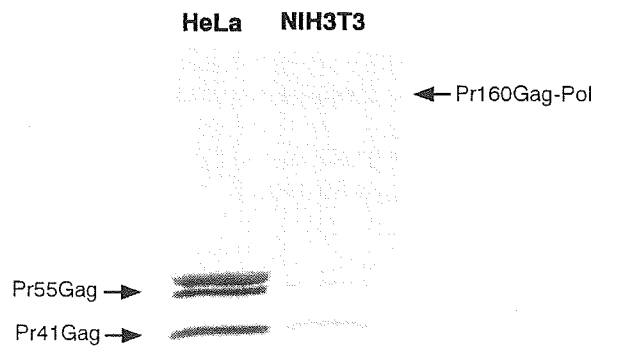

FIG. 4. Gag protein myristoylation. Cells were metabolically labeled with [9,10(n)-^{3}H]myristic acid. Cells were lysed, immunoprecipitated with an anti-p24 CA MAb, and separated by 10% SDS–PAGE subsequent to the visualization using autography.

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

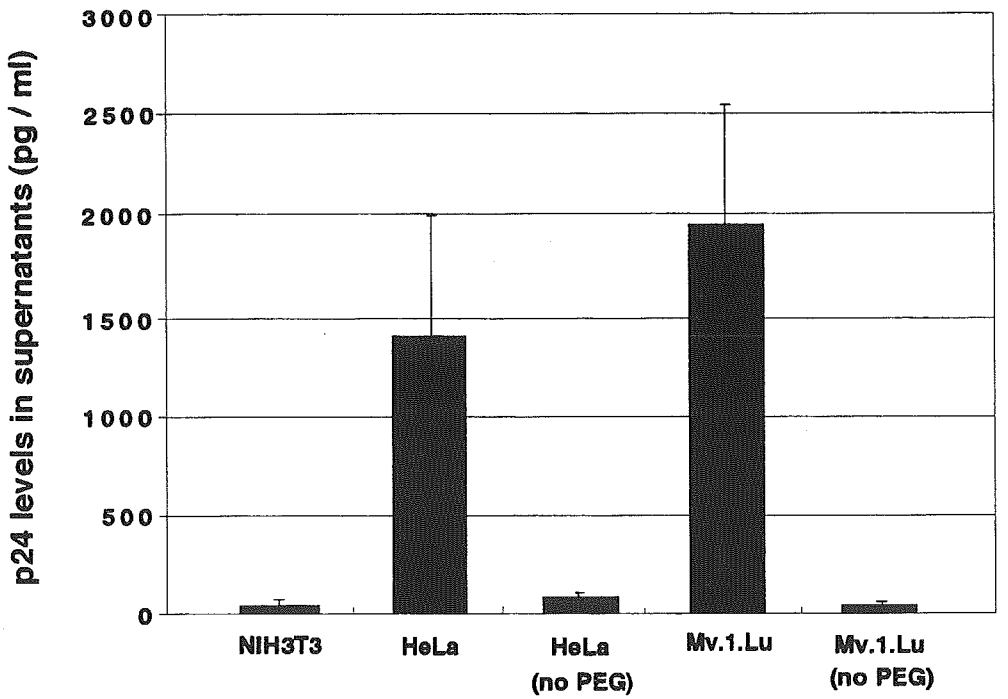

FIG. 5. Heterokaryons between murine cells stably expressing hu-cyclin T1 and HIV-1 *gag, pol* genes with human or mink cells. Murine 3T3-hCycT1 carrying HIV-1 *gag–pol* genes was cultured along with an equal number of human HeLa or mink Mv.1.Lu cells. Cocultivated cells were fused with PEG, and subsequent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