A Block to Human Immunodeficiency Virus Type 1 Assembly in Murine Cells

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Human immunodeficiency virus type 1 (HIV-1) does not replicate in murine cells. We investigated the basis of this block by infecting a murine NIH 3T3 reporter cell line that stably expressed human CD4, CCR5, and cyclin T1 and contained a transactivatable HIV-1 long terminal repeat (LTR)-green fluorescent protein (GFP) cassette. Although the virus entered efficiently, formed provirus, and was expressed at a level close to that in a highly permissive human cell line, the murine cells did not support M-tropic HIV-1 replication. To determine why the virus failed to replicate, the efficiency of each postentry step in the virus replication cycle was analyzed using vesicular stomatitis virus G pseudotypes. The murine cells supported reverse transcription and integration at levels comparable to those in the human osteosarcoma-derived cell line GHOST.R5, and human cyclin T1 restored provirus expression, consistent with earlier findings of others. The infected murine cells contained nearly as much virion protein as did the human cells but released less than 1/500 the amount of p24\textsuperscript{\textregistered} into the culture medium. A small amount of p24\textsuperscript{\textregistered} was released and was in the form of fully infectious virus. Electron microscopy suggested that aberrantly assembled virion protein had accumulated in cytoplasmic vesicular structures. Virions assembling at the cell membrane were observed but were rare. The entry of M-tropic JR.FL-pseudotyped reporter virus was moderately reduced in the murine cells, suggesting a minor reduction in coreceptor function. A small reduction in the abundance of full-length viral mRNA transcripts was expectedly observed.

HIV-1 fails to replicate in murine cells for several reasons, the best studied of which is the block to virus entry. The HIV-1 envelope glycoprotein (Env) binds with high affinity to hu-CD4 but does not bind measurably to murine (mu-CD4) (29). In addition, HIV-1 Env fails to interact with mu-CCR5 (2). Unexpectedly, some T-tropic HIV-1 Env proteins are able to use mu-CXCR4 for infection (5, 48). Coexpression of transfected hu-CD4 and hu-CCR5 or hu-CXCR4 in murine cells in culture allows the entry of HIV-1 (4). Alternatively, the entry block can be circumvented by pseudotyping HIV-1 with amphotropic Moloney murine leukemia virus Env or vesicular stomatitis virus G (VSV-G), which allows entry through different receptors.

Several murine models have been developed for studies of AIDS pathogenesis. Mice transgenic for either the entire or partial human immunodeficiency virus type 1 (HIV-1) genome develop symptoms with similarities to AIDS. In one model, mice expressing HIV-1 Nef developed a wasting syndrome characterized by the loss of CD4\textsuperscript{\textregistered} cells (21, 45). In another model, SCID mice were reconstituted with human peripheral blood lymphocytes or fetal thymus and liver and then inoculated with HIV-1 (37). These have been useful for studies on mechanisms of CD4\textsuperscript{\textregistered} cell depletion and for evaluation of therapeutic strategies.

In addition to the entry block in murine cells, HIV-1 long terminal repeat (LTR)-directed transcription is inefficient in murine cells due to weak Tat activity in rodent cells (1). Using transfected reporter constructs, transactivation of LTR transcription by Tat was 10- to 25-fold less active in rodent cells. The deficiency in Tat function in rodent cells could be complemented in rodent/human somatic cell hybrids containing human chromosome 12 (1, 23, 38). The active gene was recently identified as cyclin T1 (51), a partner for the cyclin-dependent kinase CDK9 in the transcription elongation factor P-TEFb (19, 34, 55). Cyclin-T1/CDK9 binds the TAR transactivation region of nascent HIV-1 transcripts, promoting hyperphosphorylation of serine and threonine residues in the cytoplasmic tail domain of RNA polymerase II (reviewed in...
MATERIALS AND METHODS

**Derivation of murine and human reporter cell lines.** Murine reporter cells were derived by transfecting NIH 3T3 cells with pM-LTR-EGFP-1, which contains the HIV-1 LTR linked to the enhanced green fluorescent protein (EGFP) gene. Transfectants were selected in medium (Dulbecco's modified Eagle's medium plus 10% fetal bovine serum) containing 0.4 mg of G418 per ml. The resulting drug-resistant population was sorted by fluorescence-activated cell sorting (FACS) for those expressing low but detectable green fluorescence. The sorted cells were then infected with pBABE-CCR5 retroviral vector stock and selected 2 days later in medium containing 0.2 μg of puromycin per ml. Puromycin-resistant cells were picked by FACS for those that stained with the anti-CCR5 monoclonal antibody 2D7 (Phamingen). The cells were then infected with the retroviral vector pMX-CD4, based on the vector pMX (39), that contains the hu-CD4 gene. Sorted cells were then infected with pBABE-cyclin T1 retroviral vector stock and selected 2 days later with 0.4 mg of hygromycin per ml. Individual drug-resistant clones were expanded and analyzed for their response to transfected Tat expression vector. A clone was chosen and named MGT5.cyT for murine/GFP/T4/CCR5/cyclin T1.

**Analysis of genotype that results from the presence of a tyrosine in place of serine.** In addition to the natural stop codon, there is a single (A→C) point mutation that results in a tyrosine in place of serine at amino acid position 3860 (reference 17). Phosphorylation increases the processivity of the transcription complex, allowing the synthesis of the full-length HIV-1 primary RNA transcript. Mu-cyclin T1 is competent to bind Tat, but the resulting complex fails to bind TAR, a phenotype that results from the presence of a tyrosine in place of cysteine at residue 261 (7, 18, 27). Transfection of a human cyclin T1 (hu-cyclin T1) expression vector restored Tat function in rodent cells in an HIV-1 LTR reporter vector assay and in infected murine cells (7, 18, 27, 51).

**Murine NIH 3T3 cells expressing transected hu-CD4, hu-CCR5, and hu-cyclin T1 failed to support HIV-1 replication.** While the cyclin T1 allowed for more efficient expression of the HIV-1 provirus, infected cells produced only low levels of p24Gag, suggesting the presence of additional blocks to HIV-1 replication in the murine cells. Here we investigated the basis of this additional block in the murine cells by measuring the efficiency of each step of virus replication in NIH 3T3 reporter cells expressing these three human cofactors. Consistent with earlier findings (18), the murine cells were not permissive for HIV-1 replication; however, they efficiently supported several of the steps in the virus life cycle. Virion structural proteins were expressed at high level but, instead of assembling at the cell membrane, were trapped in cytoplasmic vesicular structures. As a result, Gag was poorly processed and virions were released only at low levels. Interestingly, the small amount of Gag protein that was released from the cells was in the form of infectious virus, suggesting that the block to replication in murine cells is not absolute.

**Particle-free infection.** Infectious virions were released only at low levels. Interestingly, the small amount of Gag protein that was released from the cells was in the form of infectious virus, suggesting that the block to replication in murine cells is not absolute.

**Western blotting.** Cells were infected with NL4-3 (SVS-G) and, 3 days later, harvested. Cell lysates were prepared. Virions were pelleted at 100,000 × g of culture supernatant at 35,000 rpm in an SW40Ti rotor for 1 hr and lysed in lysis buffer (100 mM NaCl, 10 mM EDTA, 20 mM Tris [pH 7.5], 1% Triton X-100, 1% sodium deoxycholate). Cell lysates were prepared by removing medium and washing the infected cells with phosphate-buffered saline, and lysing in 500 μl of lysis buffer. Protein in the lysates was quantitated by using Coomassie blue reagent (Bio-Rad) and stored at −80°C until use. Lysates containing 10 μg of protein were separated by electrophoresis on a polyacrylamide gel, transferred to a nitrocellulose filter, and probed with serum from an AIDS patient (1:500 dilution; gift of D. Richman, University of California San Diego) followed by horseradish peroxidase-conjugated rabbit anti-human antibody (BioSource International). Filters were developed using enhanced chemiluminescence reagents (Amersham) and quantitated using a PhosphorImager.

**Density gradient centrifugation of virions.** Supernatants (3 ml) from murine and human cells that had been infected 3 days earlier at high multiplicity of infection (MOI) with NL4-3 (SVS-G) were overlaid on 20 to 60% linear sucrose gradients. The gradients were centrifuged at 35,000 rpm in an SW40Ti rotor for 12 h and fractionated into 10, 0.9-ml fractions. The density of each fraction was determined by refractometer. The fractions were then diluted with 2 volumes of TNE (10 mM Tris [pH 7.5], 100 mM NaCl, 1 mM EDTA) and centrifuged for 1 h at 35,000 rpm in an SW55Ti rotor. Pelleted virions were resuspended in 100 μl of lysis buffer. p24Gag was quantitated in the fractions by ELISA.

**Electron microscopy.** MGT5 and MGT5.cyT cells were seeded on large glass coverslips in 10-cm dishes (106 per dish) and 1 day later were infected with NL4-3 (SVS-G) at a MOI of 0.5. The cells were fixed 72 h postinfection in situ for 20 min with cold 2.5% glutaraldehyde in 100 mM Pipes (pH 6.9). Fixed cells were transferred from the coverslip with a rubber policeman, collected by centrifugation, fixed three times in cold 100 mM Pipes, and postfixed in 1% osmium tetroxide in Pipes for 30 min at 4°C. After extensive washing in 100 mM Pipes, the cells were embedded in low-melting-temperature agarose (Sigma) and further treated with 1% tannic acid for 10 min. Finally, agar blocks were dehydrated in ethanol and embedded in ERL resin. Silver-gray sections were stained with lead citrate and uranyl acetate. The sections were examined in a Philips CM120 electron microscope at 60 kV.

**RESULTS**

Expression of hu-CD4, hu-CCR5, and hu-cyclin T1 in murine cells allows for HIV-1 infection but not replication. To analyze the blocks to HIV-1 replication in murine cells, we derived reporter cell lines MGT5 and MGT5.cyT, based on NIH 3T3 fibroblasts (Fig. 1A). MGT5 was established by first introducing an HIV-1 LTR-EGFP cassette into NIH 3T3 cells and then transducing hu-CCR5 and hu-CD4 with retroviral vectors. Individual cell clones were evaluated by flow cytometry, and a clone with low-level constitutive green fluorescence and uniform CCR5 and CD4 expression was chosen. hu-cyclin T1 was introduced into MGT5 by retroviral vector transduction, resulting in MGT5.cyT. These cells are the murine equivalents of GHOST.R5 (24), a HOS-derived cell line that expresses a replication-competent HIV-1 isolates JR.FL and MDM were expanded on phytohemagglutinin-activated human peripheral blood mononuclear cells and then amplified on HOS.CD4.CCR5 cells. MDM is a rapidly replicating CCR5-dependent patient isolate (provided by R. Connor, Aaron Diamond AIDS Research Center). OM10.1 cells are derivatives of the promyelocytic cell line HL-60, which harbors a latent HIV-1 provirus that can be induced by tumor necrosis factor alpha (TNF-α).

Quantitative-competitive PCR quantitation of proviral copy number. Genomic DNA was prepared from 106 murine and 106 human cells 3 days postinfection and then cleaved with SphI to decrease its viscosity. Aliquots of genomic DNA (1 μg) were mixed with decreasing quantities of competitor pNL-3 containing a 1.4-kb deletion in env in env (nt 6628 to 9026). The mixture was amplified by PCR using primers RC9 and RC-12 (11), which flank env. Amplified proviral and competitor plasmid were separated by electrophoresis through 1% agarose, visualized by ethidium bromide staining, and quantified by densitometry.

**Northern blotting.** Total cellular RNA was isolated from infected cells 3 days earlier with NL4-3 (SVS-G) using Triazol (Life Technologies). RNA (10 μg) was separated by electrophoresis on an agarose-formaldehyde gel and transferred to a nylon filter. The filter was hybridized to an [α-32P]dCTP-labeled HIV-1 LTR probe derived from the XhoI-BglII fragment (nt 8265 to 8419) of HIV-1gag. The filters were exposed to film and subsequently quantitated using a PhosphorImager (Molecular Dynamics). The filter was then stripped and reprobed with an antisense oligonucleotide to glyceraldehyde phosphosphate dehydrogenase mRNA (nt 1017 to 1037).

**Electron microscopy.** MGT5 and MGT5.cyT cells were seeded on large glass coverslips in 10-cm dishes (106 per dish) and 1 day later were infected with NL4-3 (SVS-G) at a MOI of 0.5. The cells were fixed 72 h postinfection in situ for 20 min with cold 2.5% glutaraldehyde in 100 mM Pipes (pH 6.9). Fixed cells were transferred from the coverslip with a rubber policeman, collected by centrifugation, fixed three times in cold 100 mM Pipes, and postfixed in 1% osmium tetroxide in Pipes for 30 min at 4°C. After extensive washing in 100 mM Pipes, the cells were embedded in low-melting-temperature agarose (Sigma) and further treated with 1% tannic acid for 10 min. Finally, agar blocks were dehydrated in ethanol and embedded in ERL resin. Silver-gray sections were stained with lead citrate and uranyl acetate. The sections were examined in a Philips CM120 electron microscope at 60 kV.

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presses CD4 and CCR5 and contains LTR-EGFP. The murine cell lines expressed cell surface CD4 at levels approximating those of GHOST.R5 (Fig. 1B). hu-CCR5 was expressed at a slightly higher level in MGT5.cyT than in MGT5, possibly due to a transcriptional effect of cyclin T1. Expression of cyclin T1 could not be directly quantitated in these cells because of the presence of the endogenous murine cyclin T1, but it was verified by Western analysis in NIH 3T3 cells expressing HA-tagged cyclin T1 in cells prepared in parallel (data not shown).

Although fibroblasts are not natural targets of HIV-1 in vivo, NIH 3T3 cells support HIV-1 reverse transcription and integration (28, 40) and, when transfected with hu-CD4 and CCR5 or CXCR4, support entry (3). In contrast, lymphoid cells from hu-CD4/CXCR4 transgenic mice were poorly infectable by reporter virus (data not shown). Moreover, human fibroblastic cell lines, such as HOS.CD4.CCR5 or HeLa.CD4, are highly permissive for HIV-1 replication, indicating the ability of HIV-1 to replicate efficiently in nonlymphoid cells once the entry block is removed.

To determine whether hu-cyclin T1 would restore high-level expression of integrated HIV-1 provirus in murine cells, MGT5 and MGT5.cyT were infected with NL4-3 (VSV-G). This virus has a higher infectivity than wild-type HIV-1 and was therefore used to achieve high levels of infection in the murine cells in the absence of virus replication. In addition, because NL4-3 requires CXCR4 and cannot use CCR5, the NL4-3(VSV-G) pseudotype was restricted to a single round of replication in the GHOST.R5 cells. This allowed a comparison between the human cell line and the murine cell line, which supports only a single round of virus replication (as shown below). At 2 days postinfection, the majority of MGT5.cyT and GHOST.R5 cells were brightly fluorescent (Fig. 2A). Fluorescent cells were not observed in the infected MGT5 culture.

Quantitation of the number of fluorescent cells and their intensity by flow cytometry showed that the GHOST.R5 and MGT5.cyT cells were infected to a similar extent over a range of input virus levels (Fig. 2B). MGT5 had considerably fewer fluorescent cells, although they contained equivalent numbers of HIV-1 proviruses, as shown below. The fluorescence intensity of the MGT5.cyT was considerably higher than that of MGT5 but was still two- to threefold lower than that of GHOST.R5. Thus, cyclin T1 largely, but not completely, restored the deficiency in the ability of the murine cell to activate the HIV-1 LTR.

Expression of the three human cofactors, however, was not sufficient to cause the murine cells to become permissive for HIV-1-replication. Infection of the three cell lines with the M-tropic HIV-1 isolates MJM and JR.FL did not result in production of detectable amounts of supernatant p24 gag over a 2-week period (Fig. 2C). Thus, HIV-1 replication was blocked in the murine cells at a postentry, posttranscription stage of virus replication.

Efficient HIV DNA synthesis and integration in the murine cells. To evaluate the ability of the murine cells to support HIV-1 DNA synthesis and integration, viral DNA in the infected murine and human cells was measured by quantitative-competitive PCR. Latently infected OM10.1 cells that harbor a single HIV-1 provirus were used for standardization. GHOST.R5 cells contained a proviral load similar to that of OM10.1. The MGT5 and MGT5.cyT provirus loads were about twofold lower (Fig. 3). It was not clear whether the slightly increased amount of virus DNA in the GHOST.R5 cells was due to a small increase in DNA synthesis or to experimental variability. Although the GHOST.R5 cells contained more viral DNA than the murine cells, they did not show a correspondingly greater proportion of fluorescent cells. This could indicate that the murine cells expressing hu-cyclin T1 are more efficient at activating the integrated LTR-EGFP than are the

FIG. 1. Derivation of MGT5 and MGT5.cyT reporter cells. (A) NIH 3T3 cells were transfected with pLTR.EGFP, which contains the HIV-1 LTR linked to the EGFP gene. hu-CD4, hu-CCR5, and hu-cyclin T1 were introduced using pBABE retroviral vectors (36). MGT5.cyT was transduced with BABE.CyT.hygro; MGT5 contains control vector BABE.hygro. (B) FACS analysis of CCR5 and CD4 on MGT5, MGT5.cyT, GHOST.R5, and NIH 3T3 cells.
human cells. GHOST.R5 contains an HIV-2 LTR-EGFP, which may be less responsive to HIV-1 Tat than is the HIV-1 LTR-EGFP in MGT5 cyT. In this analysis, the DNA measured is likely to represent integrated provirus, since unintegrated forms tend to be rapidly lost (41), although some contribution of unintegrated circles cannot be excluded. Taken together, these data suggest that the murine cells support reverse transcription and integration with an efficiency similar to that of highly permissive human cells and that cyclin T1 affected proviral expression but not any other early steps in virus replication.

Murine cells synthesize and splice HIV-1 RNA. In HIV-1-infected cells, the quantity of HIV-1 mRNA is controlled by the LTR and by Tat while the characteristic pattern of fully spliced, partially spliced, and unspliced viral mRNA transcripts is regulated by Rev (reviewed in reference 42). While the intensity of EGFP fluorescence in the infected MGT5 cyT cells suggested that HIV-1 RNA had been synthesized, this was not indicative of Rev activity, since the LTR-EGFP was activated by Tat, which is synthesized from a Rev-independent, fully spliced transcript. To evaluate the production of HIV-1 mRNA transcripts in murine cells, HIV-1 transcripts in the NL4-3(VSV-G)-infected cells were visualized by Northern blotting. At 3 days postinfection, the murine MGT5 cyT cells were found to contain the characteristic unspliced, singly spliced, and multiply spliced mRNA transcripts (Fig. 4), although at a slightly altered ratio. The proportion of the unspliced transcript was somewhat reduced compared to that in the human cells (unspliced/multiply spliced ratio of 11.4 in GHOST.R5 and 5.0 in MGT5 cyT), and the relative amount of unspliced transcript was about threefold lower in the murine cells. Real-time PCR quantitation of these transcripts confirmed these results (data not shown). Thus, Rev function was intact in the murine cells. The reduction in unspliced transcript could have been due to a small reduction in Rev activity in the murine cells or to more rapid degradation of the long transcript. Transfecting an expression vector for the Rev cofactor Crm-1/exportin-1 (42) did not alter the ratio of transcripts in the murine cells (data not shown). In addition, while the human cells produced threefold more HIV-1 mRNA transcripts than did the murine cells, they contained two- to fourfold more proviruses. Thus, on a per provirus basis, the murine cells produced mRNA transcripts at a level comparable to that of the human cells.

Murine cells synthesize and process HIV-1 proteins but fail to release virions efficiently. Production of viral proteins by the NL4-3(VSV-G)-infected murine and human cell lines was evaluated by Western analysis of cell lysates and supernatants. This analysis showed that the infected GHOST.R5 and MGT5 cyT cells contained readily detectable amounts of HIV-1 structural proteins (Fig. 5). Lysates of GHOST.R5 and MGT5 cyT contained Gag polyproteins pr55 gag and pr160 gag/pol, the Gag-processing intermediate pr41, and fully processed Gag proteins p24 CA and p17 MA. The presence of pr160 gag/pol at an abundance similar to that of GHOST.R5 suggested that the translational frameshifting event required for synthesis of the Gag-Pol polyprotein occurred in the murine cells at a frequency comparable to that in human cells, consistent with an earlier report by Moosmayer et al. (35). Importantly, while the pr55 gag polyprotein was present at a similar abundance in the murine and human cells, fully processed CA was significantly reduced in abundance in the murine cells. Quantitation of the band intensities showed that pr55 gag was actually present at slightly higher level in the MGT5 cyT than in the GHOST.R5 cells but that CA was reduced 7.7-fold in the murine cells (Table 1). In GHOST.R5, CA was...
about 46% as abundant as pr55*Gag*, while in MGT5.cyT, CA was about 5% as abundant.

While virion polyprotein precursor proteins were present at similar abundance in the murine and human cells, the amount of virion protein that could be pelleted from culture supernatants of infected MGT5.cyT cells was dramatically reduced (Fig. 5). This could not be explained by the disintegration of the murine released virions into nonpelletable subunits, because p24*Gag* measurement of supernatants without centrifugation showed that they contained less than 1/500 as much p24*Gag* (see Fig. 7B). Thus, the murine cells failed to secrete virions efficiently.

It was possible that the block to virus assembly and secretion in the murine cells was due to failure of the cells to produce a sufficient quantity of virion structural proteins. This could have been the case if virus assembly required a threshold concentration for aggregation and condensation of the virion. To test whether this was the case, we analyzed virion production in the latently infected human cell line OM10.1, in which virus expression can be controlled by TNF-α (9). OM10.1 cells were exposed to decreasing concentrations of the inducer TNF-α.

Viral proteins were then visualized by Western analysis and quantitated by p24*Gag* ELISA. This showed that by decreasing the concentrations of TNF-α, it was possible to titrate viral protein production down to low levels (Fig. 6). As the amount of viral protein in the cell lysates decreased, the ratio of unprocessed to processed Gag remained similar. This suggested that virions were able to assemble even with a very low abundance of precursor proteins, an abundance considerably lower than that of the infected MGT5.cyT cells (Fig. 6A, top panel). Moreover, the human cells efficiently released small quantities of virions in response to low doses of TNF-α (Fig. 6A, bottom panel). This was evident by Western analysis of the virions and by the consistency of p24*Gag* levels in the supernatant and lysates in response to decreasing levels of TNF-α stimulation (Fig. 6B). Taken together, these data suggest that HIV-1 assembly is efficient in human cells even in the presence of only low levels of virion protein. Thus, the murine cells appear to have a specific defect in their ability to assemble virions.

**Murine cells produce small numbers of infectious virions.** Although the murine cells released only small amounts of p24*Gag*, it remained possible that this was in the form of infec-
tious virions. To test whether the p24<sup>core</sup> had been released from the cells in the form of virions, supernatants from NL4-3(VSV-G)-infected MGT5.cyT cells were subjected to sucrose density gradient analysis (Fig. 7A). Supernatant, without pelleting, was layered on a sucrose density gradient and centrifuged to equilibrium. Analysis of gradient fractions showed that p24<sup>core</sup> banded at 1.14 g/ml, a density consistent with that of virions and similar to the density of virions produced by the human cell line 293T. Similar results were obtained with GHOST.R5 cells, but these were less efficient at producing virions than were the 293T cells (data not shown). A high-density peak was present in the 293T-derived gradient, possibly corresponding to aberrantly formed virions secreted by these cells.

Infectivity of the mouse cell-produced virus was determined by end-point dilution analysis. This showed that the MGT5.cyT cells produced a low but significant titer of infectious virus, a finding that was reproducible over three repetitions of the experiment (Fig. 7B). This finding was not due to carryover of input virus, since the MGT5-derived supernatant never showed any measurable titer in the limiting-dilution assay. The 50% tissue culture infective dose (TCID<sub>50</sub>) for the mouse cell-derived virus was about 35-fold lower than that derived from the human cell line, but when normalized for p24<sup>core</sup> it had an infectivity only marginally reduced from that of the human cell line-derived virus. Thus, there is no absolute block to HIV-1 replication in the murine cell line; the virus that buds from the mouse cells is as infectious as that from human cells.

**Aberrant structures accumulate in vesicles.** Electron microscopic analysis of the infected MGT5.cyT cells showed rare, budding structures at the plasma membrane that were of normal size and morphology (Fig. 8C). Such structures were not observed in infected MGT5 cells or uninfected controls. A few extracellular immature (Fig. 8D) and mature (Fig. 8E) particles were detected near the plasma membrane of infected MGT5.cyT cells but not in uninfected or MGT5 cells. The virions were of typical size and appearance, and the characteristic cone-shaped internal core structure of mature HIV-1 virions was apparent in a small number of instances (Fig. 8E). No budding structures at intracellular membranes and no cytoplasmic or vesicle-associated capsids or virus-like particles were observed.

While the cellular architecture was well preserved, large numbers of electron-dense structures were present in the cytoplasm of the infected MGT5.cyT cells but not in uninfected control cells. These structures often appeared as multivesicular bodies or multilaminate myelin figures characteristic of lamellar lysosomes (Fig. 8A). Lamellar lysosomes and vesicular primary lysosomes were sometimes joined, segregating portions of the cytoplasm containing the dense material (Fig. 8A). In addition, infected but not uninfected MGT5.cyT cells frequently exhibited contrast-rich membranes associated with the lysosomal

![FIG. 4. Murine cells produce unspliced and spliced HIV-1 transcripts.](http://jvi.asm.org/)

![FIG. 5. HIV-1-infected murine cells synthesize viral proteins but fail to efficiently release virions.](http://jvi.asm.org/)

**FIG. 3.** Murine cells support efficient HIV-1 provirus formation. Cells were infected with NL4-3(VSV-G) at an MOI of 1. Genomic DNA was prepared 2 days later, and proviral copy numbers were determined by quantitative-competitive PCR. Competitor DNA deleted between the NheI and HpaI sites flanking env was added in the amounts indicated above each lane. DNA from OM10.1 cells containing a single provirus per cell was used for calibration. The proviral load per cell calculated from band intensities normalized to OM10.1 was as follows: GHOST.R5 = 1, MGT5 = 0.5, and MGT5.cyT = 0.25.
complexes (Fig. 8B). These structures correspond to thick electron-dense layers, which were often close to but clearly separated from cisternae of the endoplasmic reticulum (Fig. 8B). Similar structures were also detected in infected MGT5 cells but at a much lower frequency (data not shown). Assuming that the electron-dense material was viral Gag precursor protein, these observations suggest that virion proteins had not been targeted correctly to the cell membrane but had been inappropriately targeted to vesicles.

CCR5 and CD4 on the murine cells mediate entry with reduced efficiency. In the experiments described above, VSV-G pseudotypes that bypass CD4/coreceptor-mediated entry were used to achieve efficient infection. To evaluate the efficiency of CD4/coreceptor-mediated entry in the murine cells, MGT5.cyT and GHOST.R5 cells were infected with a mixture of two luciferase reporter viruses: CCR5-specific JR.FL pseudotyped firefly luciferase reporter virus and VSV-G pseudotyped Renilla firefly reporter virus (in which the firefly reporter gene was replaced by Renilla luciferase). This procedure allowed the measurement of both viruses in dually infected cultures, permitting normalization for any postentry differences between the cell types. In GHOST.R5 cells, the JR.FL pseudotype induced luciferase activity close to (about 75%) that of the VSV-G pseudotype (Fig. 9A). In MGT5.cyT cells, JR.FL entry was less efficient than that of VSV-G (about 20%). This difference was not due to postentry differences between the two cell lines, since the VSV-G pseudotype was used to normalize for such differences. Nor was the difference due to limiting amounts of CD4 or CCR5, since these were expressed in ample amounts in the murine cells, as shown above (Fig. 1B). Thus, hu-CD4/hu-CCR5 on the murine cells mediated HIV-1 entry but at significantly reduced efficiency with JR.FL Env.

The reduced amount of hu-CD4/hu-CCR5-mediated entry in the murine cells was not due to an inability of the murine cells to support Env-mediated fusion. This was shown in a syncytium formation assay in which 293T cells transiently expressing a transfected Env expression vector were cocultured with the murine human cell lines (Fig. 9B). MGT5.cyT cells formed large multinucleated syncytia, consistent with earlier findings (3), and these were more pronounced than in the GHOST.R5 cells. Syncytia were not formed in control cocultures (Fig. 9B). These findings suggested that reduced entry into the murine cells was not due to inefficiency of the fusion reaction in the MGT5.cyT cells but perhaps was due to some other, less well understood activity of hu-CD4/hu-CCR5 during entry.

### DISCUSSION

Murine NIH 3T3 fibroblasts stably expressing hu-CD4, hu-CCR5, and hu-cyclin T1 fail to support HIV-1 replication primarily because of a block to virus assembly. Interestingly, the cells supported the other steps of the HIV-1 replication cycle. Reverse transcription/integration, proviral transcription, protein synthesis, and frameshifting were nearly as efficient as in human cells, while production of unspliced HIV-1 mRNA transcripts and CD4/CCR5-mediated virus entry were somewhat reduced. In contrast, the block to virus assembly was dramatic. Upon infection with NL4-3(VSV-G) pseudotypes, the murine cells produced large amounts of Gag and Gag-Pol, but this accumulated in the cells in vesicular structures and was largely not released. The Gag proteins were inefficiently pro-

| Cell line | pr55 | pr41 | CA | MA |
|-----------|------|------|----|----|
| GHOST.R5  | 156,892 | 91,022 | 73,755 | 54,725 |
| MGT5.cyT | 214,315 | 45,180 | 9,561 | 10,298 |
| MGT5     | 4,852 | ND | ND | 110 |

**a** Relative intensity of bands detected by PhosphorImager analysis.

**b** ND, not determined.

**c** Ratio of band intensities in GHOST.R5 cells to those in MGT5.CyT cells.
cessed by the viral protease, probably as a result of their failure to assemble, consistent with earlier findings in transfected rodent cells (35). Importantly, the infected murine cells produced small amounts of virions, and these were as infectious on a per-particle basis as those from human cells, consistent with findings by Garber et al. (18), who used transfected NIH 3T3 cells.

Provirus expression, which is nearly undetectable in NIH 3T3 cells, was largely restored by expressing hu-cyclin T1, consistent with earlier findings (18). The small reduction in LTR-EGFP activation in the infected MGT5.cYT cells could have been caused by competition from the endogenous mu-cyclin T1, which is thought to have dominant negative activity on the human protein (6, 27), or to inefficient LTR function resulting from incompatibilities between murine transcription factors and LTR binding-site sequences. We could not distinguish between these two possibilities. Decreased Tat activity does not appear to account for the failure of the virus to replicate in MGT5.cYT cells, since upon infection, the cells produced ample amounts of virion structural proteins.

An earlier report (49) suggested that there is a block to Rev function in murine cells; however, this did not appear to be the case in our study. Judging by the ratio of spliced to unspliced HIV-1 mRNA transcripts and indirectly by the accumulation of viral structural proteins which are translated from Rev-dependent mRNA transcripts, Rev function was largely intact in the murine cells. A threefold reduction in the relative abundance of the unspliced transcript in the murine cells was noted, perhaps reflecting a small reduction in Rev activity; however, this could also be caused by some instability of the unspliced

![Graph](http://jvi.asm.org/Downloaded from http://jvi.asm.org/)

**FIG. 7.** Murine cells produce infectious virus. (A) Murine and human cells were infected with NL4-3(VSV-G). At 3 days postinfection, the density of virions in the supernatant was measured by sucrose density gradient centrifugation. (B) p24 gag was quantitated in cell lysates and from pelleted virions. (C) The infectious titer (TCID 50 ) of supernatant virus was measured by end-point dilution on CEM.LTR.GFP cells. The ratio of TCID 50 to supernatant p24 gag is 154 and 236 for the MGT5.cYT- and GHOST.R5-produced viruses, respectively.
transcript, which, because of its failure to be encapsidated by assembling virions, may be more rapidly degraded by cellular RNase. Transfecting the murine cells with Crm-1/exportin-1 expression vector, the human Rev cofactor (42), did not alter viral protein production or mRNA transcript ratios (data not shown).

While some steps of the virus replication cycle were somewhat less efficient in the murine cell line (mRNA synthesis and virus entry), the block to assembly was drastic. Although the murine cells contained amounts of Gag polyprotein at levels comparable to that in the human cells, the levels of p24\textsuperscript{gag} released were more than 500-fold reduced. The reason for the failure to assemble virions is unknown. Processing of the Gag polyprotein by the viral protease was inefficient; however, this was more likely to have been a consequence of the failure of the polyprotein molecules to assemble than its cause, since processing is not required for budding (16). In addition, it was not likely to have been due to a failure to incorporate cyclophilin, since incorporation of this cellular protein is not required for assembly or budding (53). Nor was it due to an inappropriate association of Env with Gag at the cell membrane, since cells infected with Env\textsuperscript{−} NL4-3(VSV-G) also failed to assemble virions (data not shown).

The inability of the murine cells to support HIV-1 assembly is presumably caused either by the absence of a required human-specific host factor or by the presence of an inhibitory murine factor. HIV-1 assemblies at the cell membrane by a process similar to that of the type C retroviruses (16). The Gag polyproteins are transported to the cell membrane in an energy-dependent process (31) that may be mediated by vesicles (22, 25) and that is directed by specific domains of MA. MA contains an N-terminal basic patch and an N-terminal myristate that are required for membrane attachment (16, 46, 54). Deleting a portion of MA results in targeting of viral assembly to intracellular membranes (14). Adding a src membrane-targeting sequence to an intracisternal A-type particle Gag redirected it to assemble at the cell membrane, release, and process (52). In the infected murine cells, inappropriate targeting

FIG. 8. Ultrathin-section electron microscopic analysis of infected MG5T.cyT cells. (A) Overview of an infected MG5T.cyT cell. (B) Detail of an infected cell, revealing a complex vesicular cytoarchitecture. Note the thickened contrast-rich membranes situated in close association with cisternae of the endoplasmic reticulum (arrowheads). Adjacent is a dense body (D), also surrounded by a thickened membrane. (C to E) Budding structures and immature and mature HIV-1 particles, respectively.
of the Gag polyprotein could have caused the accumulation of virion proteins in vesicular structures that we observed by electron microscopy. Targeting to the cell membrane could involve cellular cofactors, such as those that mediate protein folding or intracellular transport, any of which could be altered or lacking in the murine cells. Using an in vitro assembly system, Lingappa et al. (31) detected at least two host cofactors, one detergent sensitive and the other insensitive, that were required for the assembly of HIV-1 capsids. Whether these activities are present in murine cells is not known. In addition, the chaperonin T-complex polypeptide has been implicated as playing a role in hepatitis B virus assembly (32). A similar factor could play a role in HIV-1 assembly.

Alternatively, murine cells might contain an inhibitor of virus assembly. The expression of variant HIV-1 Gag molecules in human cells interferes with the production of infectious particles (50). By analogy, endogenous murine retroviral Gag proteins or fortuitous interaction of the Gag polyprotein with murine cellular proteins could interfere with assembly. Because the virus has evolved to replicate in human cells, it would not have been selected to avoid interference by murine cellular proteins. Our data do not allow us to distinguish between the two types of models; however, supporting the former hypothesis, Trono and Baltimore found that fusing a human cell line to HIV-1-infected NIH 3T3 cells boosted the release of p24<sub>agg</sub> (49).

The finding that the murine cells produced virions with an infectivity similar to that of virions from human cells indicates
that there is no absolute block to HIV-1 replication in murine cells. The failure to support replication probably results from the state of viral activation in a CD4

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