Replication of Equine Infectious Anemia Virus in Engineered Mouse NIH 3T3 Cells

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We employed the equine lentivirus equine infectious anemia virus (EIAV) to investigate the cellular restrictions for lentivirus replication in murine NIH 3T3 cells. The results of these studies demonstrate that NIH 3T3 cells expressing the EIAV receptor ELR1 and equine cyclin T1 supported productive replication of EIAV and produced infectious virions at levels similar to those found in a reference permissive equine cell line. The studies presented here demonstrate, for the first time, differential levels of restriction for EIAV and human immunodeficiency virus type 1 (HIV-1) replication in murine cells and suggest that these differences can be exploited to reveal critical virus-cell interactions required for HIV-1 assembly and budding of lentivirus particles.

To date, efforts to establish a murine model of lentivirus infection and disease have been hampered by the realization of a series of host cell restrictions to human immunodeficiency virus type 1 (HIV-1) replication that impede critical steps in HIV-1 infection, gene expression, and virus assembly and budding (1, 2, 4, 6, 9, 17, 18). In our previous studies of host cell receptors and assembly mechanisms of the equine lentivirus equine infectious anemia virus (EIAV), we made several observations suggesting that murine cells may be able to support EIAV replication with only minimal engineering. First, we observed that transiently transfected murine NIH 3T3 cells transiently expressing the EIAV receptor ELR1 were susceptible to EIAV infection at levels that were similar to those achieved in permissive equine cell lines, indicating no obvious host restrictions to early steps in virus entry (11, 19). Second, we discovered that murine NIH 3T3 cells transduced with EIAV Gag plasmids produced virus particles at levels similar to those of transfected equine cell lines, indicating a lack of host cell restrictions to late steps in virus replication leading to Gag assembly and virus budding (10). While murine NIH 3T3 cells were found to be permissive for EIAV entry and budding, previous studies have shown that expression of EIAV provirus requires the presence of equine cyclin T1 (cycT1) and that human and murine forms of cycT1 fail to effect efficient Tat-mediated transactivation of the EIAV proviral long terminal repeat (3, 16). Based on these individual observations with EIAV, we hypothesized that murine NIH 3T3 cells engineered to express the ELR1 receptor and equine cycT1 would be permissive for EIAV replication.

To test this hypothesis, the NIH 3T3 cell line (ATCC CRL-1658) was stably transduced with murine leukemia virus retroviruses expressing either ELR1 or equine cycT1, respectively. The ELR1-expressing retrovirus vector was designed to express the full-length ELR1 receptor protein as described previously (19). The equine cycT1 retrovirus vector was constructed from the functional 300-amino-acid fragment of equine cycT1 described by Bieniasz et al. (3). The equine cycT1 plasmid (provided by B. R. Cullen) was modified to contain a hemagglutinin (HA) tag. The expression construct contains a ribosome internal entry site and has the gene for enhanced green fluorescent protein inserted following the cycT1 gene to provide a screening marker. Vesicular stomatitis virus-pseudotyped retroviruses expressing ELR1 or cycT1 were made as described previously (19). The ELR1-expressing NIH 3T3 cell line, designated NIH3T3(ELR1), was generated by ELR1-expressing retrovirus infection, followed by G418 selection. The ELR1-transduced NIH 3T3 cells were then infected with the cycT1-expressing retrovirus, followed by cell sorting for enhanced green fluorescent protein-positive cells with a FACS Aria cell sorting system (BD Biosciences, San Jose, CA). ELR1 and cycT1 expression levels were analyzed by flow cytometry and compared to those in the permissive equine dermal (ED) cell line routinely used to produce EIAV. The results indicate that about 90% of the transduced cells were ELR1 positive with a mean fluorescent intensity of 680 (Fig. 1A), similar to the level of ELR1 expression detected on the surface of the reference ED cells (89% positive, mean fluorescent intensity of 1,100) (Fig. 1B). In addition, more than 50% of the transduced NIH 3T3 cells expressed equine cycT1 (Fig. 1C).

The expression of the equine cycT1 and ELR1 proteins of the expected molecular weights by the transduced cells was also confirmed by Western blotting of cell lysates (data not shown). The product NIH 3T3 cell line expressing ELR1 and equine cycT1 is designated NIH3T3(ELR1/cyc).

We have shown in previous studies that NIH 3T3 cells transiently transfected with ELR1 are permissive for EIAV entry, but not for productive virus replication (19). In the present study, the specificity of EIAV entry into stably transduced NIH3T3(ELR1) cells was assayed by evaluating the ability of a reference anti-ELR1 serum to block EIAV entry into NIH3T3(ELR1) cells. For these studies, EIAV infection was measured by assaying the level of EIAV cDNA produced in
infected cells at 24 h postinfection, as described previously (19). As summarized in Fig. 2A, EIAV entry into NIH3T3 (ELR1) cells was blocked in a dose-dependent manner by the ELR1-specific antiserum, with more than 90% inhibition observed at a 1:33 dilution of the antiserum and 50% inhibition at a serum dilution of 1:1,000. These results demonstrate receptor-specific EIAV entry into NIH3T3(ELR1) cells.

We next examined if EIAV infection of NIH3T3(ELR1) cells was successful in producing integrated EIAV provirus. For this experiment, NIH3T3(ELR1) cells and the permissive ED cell line were infected in parallel with EIAV at a multiplicity of infection (MOI) of 1.0. At 14 days postinfection, high-molecular-weight cellular DNA was extracted from the respective cells and analyzed for proviral DNA with our standard real-time PCR assay (19). The results of this assay (Fig. 2B) revealed about $10^{6.5}$ DNA copies per $10^5$ NIH3T3(ELR1) cells and about $10^{5.5}$ DNA copies per $10^5$ ED cells following infection, with no detectable proviral DNA observed in control NIH 3T3 cells lacking ELR1 expression or in ED cells not exposed to the virus. Thus, these data confirm that EIAV infection of NIH3T3(ELR1) cells supports the early steps of EIAV replication, from infection to provirus integration, as efficiently as that of a reference equine cell line routinely used for EIAV production.

While NIH3T3(ELR1) cells supported the early steps of EIAV infection, we were unable to detect the production of virus particles in the supernatant of infected cells (see Fig. 3), suggesting a block in virus replication following provirus integration, and consistent with the expected requirement for equine cycT1 for efficient expression of the EIAV provirus in murine cells.

FIG. 1. ELR1 and cycT1 expression in NIH3T3(ELR1/cyc) cells. NIH 3T3 cells transduced with ELR1 and cycT1 were incubated with an anti-ELR1 polyclonal antibody for surface staining of ELR1 (A) or an anti-HA monoclonal antibody for intracellular staining of HA-tagged equine cycT1 (C). Cells were then stained with anti-rabbit–allophycocyanin or anti-rat–Cy5, respectively. For comparison, ED cells were stained in parallel with an anti-ELR1 polyclonal antibody or with preimmune (pre) rabbit serum (B). Stained cells were analyzed by flow cytometry with a BD FACScalibur. Max, maximum.

FIG. 2. Specificity of EIAV entry into NIH3T3(ELR1) cells and provirus production. (A) Specificity of ELR1-mediated entry into engineered NIH3T3(ELR1) cells as demonstrated by blocking with ELR1-specific rabbit serum antibodies. NIH 3T3 cells expressing ELR1 were incubated with EIAV in the presence of medium containing the indicated dilutions of rabbit anti-ELR1 serum or control preimmune serum. Virus entry was detected at 24 h postinfection by real-time PCR to quantify viral DNA produced during late reverse transcription (19). The percentage of virus entry was calculated by comparison of the viral DNA copy number observed at each immune serum dilution to the levels observed without the control preimmune rabbit serum. (B) EIAV proviral DNA production in NIH3T3(ELR1) cells compared to permissive ED cells. Cells were incubated with EIAV at an MOI of 1.0, and high-molecular-weight proviral DNA was quantified by using a real-time PCR at 14 days after infection.
To address this restriction, we next tested the replication competence of EIAV in NIH3T3(ELR1/cyc) cells stably expressing both ELR1 and equine cycT1. For this assay, equal numbers of NIH3T3(ELR1/cyc) cells, NIH3T3(ELR1) cells, NIH 3T3 cells, and permissive ED cells were infected in parallel with EIAV at an MOI of 1.0 and reverse transcriptase (RT) activity in cell culture supernatants was assayed to quantify the levels of virus replication (12). The virus replication profiles presented in Fig. 3 clearly demonstrate that NIH3T3(ELR1/cyc) cells were able to support virus replication at levels similar to those of permissive ED cells, as measured by the production of supernatant RT activity. In contrast, EIAV did not replicate in normal NIH 3T3 cells or in NIH3T3(ELR1) cells, as evidenced by the lack of supernatant RT production from these nonpermissive cells. Thus, these data reveal for the first time the ability of a minimally engineered murine cell line to achieve productive infection by a lentivirus.

Finally, to complete the evaluation of EIAV replication in NIH3T3(ELR1/cyc) cells, we evaluated the infectivity of the virions produced in the engineered mouse cells compared to virions produced in the ED cells. For this evaluation, supernatant virus infectivity titers were determined by using a standard infectious-center assay (7) and the concentration of virus particles in cell supernatants was quantified by real-time PCR assays of EIAV genomic RNA (5). As summarized in Table 1, at 30 days after infection, the supernatant from infected NIH3T3(ELR1/cyc) cells contained about \( 0.9 \times 10^5 \) infectious units (IU)/ml virus and the supernatant from the ED cells contained \( 3.3 \times 10^5 \) IU/ml. Quantification of the supernatant viral genomic RNA produced by the two cell lines revealed similar levels of \( 5.2 \times 10^4 \) copies per ml produced from NIH3T3(ELR1/cyc) cells and \( 5.9 \times 10^4 \) copies per ml from ED cells. Taking the ratio of RNA copies per IU to normalize virion infectivity indicates that the virus produced by NIH3T3(ELR1/cyc) cells displayed \( 5.7 \times 10^4 \) RNA copies/IU, while the virus produced by ED cells was calculated to be \( 1.8 \times 10^4 \) RNA copies/IU. Thus, these results indicate that the quality of the virus produced in engineered NIH 3T3 cells was similar to that of the virus produced by a permissive equine cell line in terms of virion infectivity.

Taken together, these experiments represent the first successful engineering of a murine cell line to achieve productive replication of a natural unmodified lentivirus. Numerous efforts to engineer permissive murine cells for HIV-1 replication have revealed host restrictions at virtually every step of the virus replication cycle, indicating the lack of appropriate host factors to mediate critical steps in HIV-1 entry, gene expression, and assembly and budding (1, 1, 8, 9, 13, 15, 17). While it is not clear what differences between EIAV and HIV-1 are responsible for the different replication phenotypes in mouse cell lines, it is evident that EIAV is intrinsically able to utilize murine host cell machinery and to avoid restriction factors to a greater extent than HIV-1, especially as related to late stages of virus infection leading to assembly and budding. In this regard, the murine cells lacked only the EIAV receptor ELR1 and the necessary equine cycT1 protein to support EIAV replication. In contrast to HIV-1, EIAV is able to effectively use mouse cell factors for late replication stages of assembly and budding. Based on these differences between the two lentiviruses, it should now be possible to use chimeric constructs of HIV-1 and EIAV to map critical virus determinants of replication potential and to identify critical host cell factors that mediate lentivirus replication in murine cells.

EIAV has one of the simplest genomes characterized to date among lentiviruses, containing only three regulatory genes (\( rev, tat \), and \( S2 \)) in addition to the canonical \( gag, pol \), and \( env \) genes found in all retroviruses. We have previously proposed that EIAV may represent a link in genomic organization and replication properties between simple retroviruses and the more complex lentiviruses (14). The ability of EIAV to establish productive replication in minimally engineered murine cells is consistent with this proposed linkage between simple retroviruses like murine leukemia viruses and complex lentiviruses like HIV-1.

![FIG. 3. EIAV replication in NIH 3T3, NIH3T3(ELR1), NIH3T3 (ELR1/cyc), and ED cells. Equal numbers of the indicated cells (3 \( \times 10^5 \) well) were infected in parallel with EIAV at an MOI of 1.0, and virus replication kinetics were followed at the indicated times by measurement of supernatant RT activity as described previously (12). Cell cultures were maintained for the indicated times with medium replaced every 3 to 4 days after sample collection for RT assays. Duplicate supernatant samples were assayed at each time point, and the Student \( t \) test method was used to evaluate the statistical significance of differences between mean values. Background RT levels were determined by assay of supernatants from the respective uninfected cells.](http://jvi.asm.org/)

![TABLE 1. Infectivity of EIAV produced in NIH 3T3(ELR1/cyc) and ED cells.](http://jvi.asm.org/)

<table>
<thead>
<tr>
<th>Cell line(^a)</th>
<th>Infectivity (IU/ml)(^b)</th>
<th>No. of viral RNA copies/ml(^d)</th>
<th>RNA/IU ratio(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED</td>
<td>( 3.3 \times 10^3 )</td>
<td>( 5.9 \times 10^9 )</td>
<td>( 1.8 \times 10^6 )</td>
</tr>
<tr>
<td>NIH3T3(ELR1/cyc)</td>
<td>( 0.9 \times 10^3 )</td>
<td>( 5.2 \times 10^9 )</td>
<td>( 5.7 \times 10^6 )</td>
</tr>
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\(^a\) Equal numbers of the indicated cells (3 \( \times 10^5 \) well) were infected in parallel with EIAV at an MOI of 1.0. Cell culture supernatants were maintained with changes of medium every 3 to 4 days. Supernatants were harvested at 30 days postinfection and assayed for EIAV infectivity and genomic RNA content.

\(^b\) Virus infectivity was measured by infectious-center assay as previously described (7).

\(^c\) Viral genomic RNA content was assayed by real-time PCR as previously described (5).

\(^d\) Normalization of virion infectivity by calculating the ratio of viral genomic RNA to the infectious virus titer. Numbers of IU and viral cDNA copy numbers are averages of duplicate samples.
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REFERENCES


