Enzootic nasal tumor virus (ENTV) and jaagsiekte sheep retrovirus (JSRV) are closely related retroviruses that cause epithelial cancers of the respiratory tract in sheep and goats (6). Several lines of evidence indicate that Hyal2 is the primary entry receptor for JSRV. First, retroviral vectors bearing the JSRV Env can transduce human but not hamster cells, and phenotypic mapping of the human receptor in human/hamster radiation hybrid cells revealed a single locus responsible for susceptibility (17). Later analysis showed that only a single gene in this locus, the Hyal2 gene, conferred susceptibility to transduction in otherwise resistant cells, including hamster and mouse cells (18). Furthermore, expression of any of the other closely related paralogs of Hyal2 that are present in the human genome did not confer susceptibility to infection (18), again indicating that Hyal2 is the only gene in the human genome that can act as a receptor. Second, a hybrid protein consisting of the receptor-binding domain (SU, or surface domain) of JSRV Env linked to a human immunoglobulin G (IgG) constant domain (JSU-IgG) binds to cells that are susceptible to JSRV vector transduction but not to cells that are resistant (7). Expression of human Hyal2 protein in otherwise resistant cells results in strong binding of the JSU-IgG domain to the modified cells (7), indicating that Hyal2 is the primary determinant of JSRV binding to cells. Lastly, tight binding of a purified soluble form of human Hyal2 to purified JSU-IgG has been detected by surface plasmon resonance analysis, with a $K_d$ in the picomolar range (21), again indicating that Hyal2 is the main binding partner for the JSRV Env protein.

While Hyal2 also appears to be the primary receptor for ENTV (1, 3), there is additional complexity in these results. Retroviral vectors bearing the ENTV Env show a host range limited to cell lines from sheep and some cell lines from humans, while JSRV vectors can efficiently transduce sheep cells, most cell lines from humans, and monkey, dog, cow, and rabbit cells (3). Furthermore, while expression of either the human or sheep Hyal2 proteins in rodent cells renders them quite susceptible to JSRV vector transduction, ENTV vectors show poor transduction rates in these cells (3 and unpublished results).

A limitation of the host range analysis for ENTV vectors is the low titer of these vectors even on susceptible sheep cells. Here we have generated high-titer ENTV-based packaging cell lines and have reinvestigated these anomalies. We confirm and extend the results showing a limited host range for ENTV vectors, and we find that expression of human Hyal2 in several otherwise nonsusceptible rat cell lines is not sufficient to confer full ENTV vector susceptibility. We made a hybrid protein consisting of the receptor-binding (SU) domain of ENTV Env linked to a human IgG constant domain (ESU-IgG) and show that the ENTV Env SU domain can still bind to the human Hyal2 protein expressed on these rat cells at levels similar to those of other highly infectible cells. These results indicate the involvement of other factors, perhaps a coreceptor, in cell entry mediated by the ENTV Env protein.

**MATERIALS AND METHODS**

**Cell culture.** Cell lines used here included 208F (16) and Rat2 (20) rat embryo fibroblasts, normal rat kidney (NRK) cells (5), XC rat cells (19), 9L rat glioma...
cells (2), SSF-123 primary sheep skin fibroblasts (gift from William Osborne, University of Washington, Seattle), NIH 3T3 thymidine kinase-deficient mouse embryo fibroblasts (22), HT-1080 human fibrosarcoma cells (we used an approxi- 
mately diploid subclone of HT-1080 cells from ATCC CCL-121), D17 dog 
osteoosarcoma cells (ATCC CRL-6248), Vero African green monkey kidney 
epithelial cells (ATCC CCL-81), 293T human embryonic kidney cells (4), and 
P4.JSRV pseudotype (17) and PET67 10A1 murine leukemia virus (MLV) 
pseudotype (8) retrovirus packaging cells. Cells were grown in Dulbecco's mod-
dified Eagle medium (DMEM) with high glucose (4.5 g/liter) and 10% fetal bovine 
serum at 37°C in a 10% CO2-air atmosphere at 100% relative humidity.

Plasmid expression vectors. Plasmids used to express JSRV Env (pCSI-Jenv) 
and ENTV Env (pCSI-Env) were made by cloning the respective Env-coding 
regions into the pCSI expression vector, which includes a cytomegalovirus im-
mediate-early promoter, and splicing and polyadenylation signals from simian 
virus 40 (SV40) to drive cDNA expression. The ENTV Env and hybrid ENTV/
JSRV Env proteins were also expressed by cloning the coding regions into the 
pSx2 expression vector in place of the 10A1 MLV Env cDNA originally present in 
the vector (8), as described previously (5). The pSx2 vector employs a pro-
motor, enhancer, and splicing signals from Moloney MLV (MoMLV), and 
the early polyadenylation signal from simian virus 40 to drive transcription.

Retroviral vectors and virus production. The LAPSv vector (11) expresses 
human placental alkaline phosphatase (AP) from the retroviral long terminal 
repeat (LTR) and neomycin phosphotransferase (Neo) from an internal SV40 
early promoter. Retroviral vectors that express human Hya1 and Hya2 
(LHyalsN and LHyalsIN, respectively) were made as described previously (18) 
by inserting the Hya1 or Hya2 cDNAs into the LXSN retroviral vector (10).

For some experiments, retroviral vectors were made by transient CaPO4-
mediated transfection of ENTV, JSRV, or 10A1 MLV packaging 
cells; harvest of virus; transduction of ENTV, JSRV, or 10A1 MLV expressing 
the surface (SU) domain of ENTV Env (373 amino acid 
residues beginning with the Env start codon) to the human IgG constant region.
A similar plasmid (pCSI-ESU-IgG) that expresses the SU domain 
linked to the same human IgG constant region (ESU-IgG) was 
previously (7). A similar pattern of discordant results was ob-
tained in studies using the same target cells was nearly 
efficiency of transduction using the same target cells was nearly 
eightfold lower than the JSRV vector titer on human 293 cells (3), while in the other study, the 
ENTV vector titer was 100-fold lower than the JSRV 
titer vector on human 293 cells (3), while in the other study the 

efficiency of transduction using the same target cells was nearly 
eightfold lower than the JSRV vector titer on human 293 cells (3), while in the other study the 

Flow cytometry. Flow cytometry assays with ESU-IgG and JSU-IgG proteins 
were performed as described previously (7). Briefly, cells were harvested from 
culture dishes by incubation with 10 ml of phenotype-buffered saline (PBS) plus 1.488 g of EDTA per liter. Cells were then washed twice with 
PBS plus 2% fetal bovine serum albumin (wash buffer) and were counted with a 
hemacytometer. A total of 10^6 cells were transferred to a microcentrifuge tube and 
were pelleted by centrifugation at 1,000 × g for 5 min at 4°C. Cells were then 
resuspended in 100 μl of SU-IgG diluted in wash buffer and incubated on ice for 
2 h. In this and the following incubation step, the cells were resuspended every 
15 min. Following SU-IgG binding, cells were washed three times with 100 μl of 
wash buffer, were resuspended in 100 μl of appropriately diluted secondary 
antibody (Dako), and were incubated on ice for 1 h. Cells were washed twice, 
resuspended in 300 to 500 μl of wash buffer, and analyzed by using a Calibur 
fluorescence-activated cell sorter (FACS) (Becton Dickinson). Analysis of cell 
populations was performed using CellQuest software.

RESULTS

Comparison of ENTV Env expression plasmids for high-
titer vector production. Previous studies are inconsistent with 
regard to the ability of the ENTV Env to pseudotype MoMLV-
based retroviral vectors (1, 3). In both previous studies, virions 
were made using MoMLV-based vectors, MoMLV Gag-Pol 
proteins, and either the JSRV or JSRV Env protein. In one study, the ENTV vector titer was 100-fold lower than the JSRV 
vector titer on human 293 cells (3), while in the other study the 
efficiency of transduction using the same target cells was nearly 
the same (1). A similar pattern of discordant results was ob-
erved in primary sheep cells, although in one study the cells 
were sheep skin fibroblasts (3) while in the other study the cells 
were derived from the choroid plexus (1).

Different JSRV and ENTV Env clones and expression plas-
mids were used in these studies, and to determine whether 
these differences could explain the discordant results, we mea-
sured the titers of vectors made using the original plasmids 
(Table 1). The titers of the vectors made with the JSRV Env-
expressing plasmids were similar and matched that made with 
a vesicular stomatitis virus G protein (VSV-G) expression plas-
mid used as a positive control. The JSRV Env sequences in 
these plasmids are given in GenBank accession numbers 
Y18301 (pCSI-Jenv) and AF105220 (pCMV3JS21ΔGP). Titers of vectors made with the ENTV Env-expressing plasmids were both 
over 100-fold lower than those of the JSRV and VSV-G
vector stocks. The ENTV env sequence in the pCSI-env plasmid is given in GenBank accession number AF401741, and the sequence in pCMV3ENTV env genes, except that the titers of the vectors made by ENTV Env was not altered by replacement of its TM domain with that of JSRV.

**Construction of a high-titer ENTV packaging cell line.** To further study the host range conferred by the ENTV SU, we made an ENTV host range packaging cell line by expressing the EEJ Env protein (Fig. 1) in cells that express MoMLV Gag-Pol proteins. Although the Env used to make this packaging cell line is a hybrid between ENTV and JSRV, for simplicity we will call it an ENTV packaging line because, based on the results shown in Fig. 1 and discussed above, the primary determinant of the ENTV host range is in SU. NIH 3T3 cells expressing MoMLV Gag-Pol proteins (LGPS clone 91–22 cells [9]) were cotransfected with the hygromycin phosphotransferase-expressing plasmid pSV2hygro and the EEJ Env-expressing plasmid pSX2.EEJ at 1:20 or 1:100 ratios, the cells were selected in hygromycin, and resistant clones were isolated using cloning rings. To screen these clonal lines for packaging function, we transduced the cells with the LAPSN vector made using the PT67 packaging cell line, selected the cells in G418 for 5 days, harvested virus in G418-free medium exposed to confluent cells for 16 h, and assayed the medium for vector titer by using SSF cells as targets for transduction. Two of the LAPSNS-transduced clones made no LAPSN vector, 16 clones made vector with titers from $1 \times 10^3$ to $3 \times 10^4$ AP$^+$ focus-forming units/ml. We used the latter clone to make ENTV pseudotype LAPSNS vector for the following studies and named the packaging cell line PN229 (for “packaging cell line from ENTV, version 2, clone 29”).

**An ENTV vector produced by PN229 cells has a restricted host range compared to that of a JSRV vector.** LAPSN vectors made with the ENTV and JSRV packaging cell lines PN229 and PJ4, respectively, were added to cell lines from different species, and the apparent titers were determined (Table 2). Both vectors transduced sheep SSF cells at approximately equal rates, but they were basically unable to transduce 208F rat or NIH 3T3 mouse cells. Human HT-1080 cells were transduced at a 100-fold lower rate by the ENTV vector than by the JSRV vector (Table 2), similar to results shown in Fig. 1 that were obtained by using vectors made by transient transfection of the env genes, except that the titers of the vectors made by the packaging lines were higher than those made by transient transfection. In both Vero monkey cells and D17 dog cells, transduction by the ENTV vector could not be detected, while the titer of the JSRV vector was relatively high on both cell lines. Thus, the ENTV vector produced by PN229 cells clearly has a restricted host range compared to that of the JSRV vector.

**Multiple cell lines that are resistant to ENTV vector transduction are rendered susceptible following expression of human Hya2L in the cells.** Human HT-1080 cells were transduced at a 100-fold lower rate by the ENTV vector than by the JSRV.
TABLE 2. Cells from multiple species that are resistant to ENTV vector transduction become susceptible after expression of human Hyal2

<table>
<thead>
<tr>
<th>Target cell type</th>
<th>Expressed protein</th>
<th>Apparent titer of LAPSN vector (AP+ foci/ml) produced by packaging cells:</th>
<th>Ratio of JSRV to ENTV vector titers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PN229 (ENTV)</td>
<td>P34 (JSRV)</td>
</tr>
<tr>
<td>SSF</td>
<td>None</td>
<td>$4 \times 10^9$</td>
<td>$4 \times 10^9$</td>
</tr>
<tr>
<td>HT-1080</td>
<td>None</td>
<td>$2 \times 10^5$</td>
<td>$2 \times 10^5$</td>
</tr>
<tr>
<td>Hyal2</td>
<td>$1 \times 10^5$</td>
<td>$4 \times 10^5$</td>
<td>$4 \times 10^5$</td>
</tr>
<tr>
<td>D17</td>
<td>None</td>
<td>$&lt;5$</td>
<td>$7 \times 10^3$</td>
</tr>
<tr>
<td>Hyal2</td>
<td>$1 \times 10^5$</td>
<td>$3 \times 10^5$</td>
<td>$0.3$</td>
</tr>
<tr>
<td>Vero</td>
<td>None</td>
<td>$&lt;5$</td>
<td>$2 \times 10^4$</td>
</tr>
<tr>
<td>Hyal2</td>
<td>$1 \times 10^5$</td>
<td>$9 \times 10^4$</td>
<td>0.9</td>
</tr>
<tr>
<td>NIH 3T3</td>
<td>None</td>
<td>10</td>
<td>$&lt;5$</td>
</tr>
<tr>
<td>Hyal2</td>
<td>$6 \times 10^4$</td>
<td>$5 \times 10^4$</td>
<td>0.8</td>
</tr>
<tr>
<td>208F</td>
<td>None</td>
<td>$&lt;1$</td>
<td>$&lt;1$</td>
</tr>
<tr>
<td>oHyal2</td>
<td>400</td>
<td>$6 \times 10^3$</td>
<td>30</td>
</tr>
<tr>
<td>rHyal2</td>
<td>$&lt;1$</td>
<td>$9 \times 10^3$</td>
<td>$&gt;9,000$</td>
</tr>
</tbody>
</table>

* Cells expressing human or rat Hyal2 were generated by transduction with vectors encoding these proteins and Neo (LHyal2SN and LHyal2SN, respectively) followed by selection in G418. LAPSN vector titers were determined by infection of cells seeded the day before at $10^4$ cells per well (diameter, 3.5 cm) of 6-well plates. AP+ foci were counted 3 days after vector exposure. Results are means of two to five experiments each, and experimental values varied by no more than four-fold from the mean.

*2 Cells expressing ovine Hyal2 were generated by transfection with a plasmid that expresses ovine Hyal2 and Neo (pCR3.1Hyal2) followed by selection in G418.

hyaluronidase, and the apparent titers of both vectors on HT-1080 cells were significantly decreased from those observed for SSF sheep cells (Table 2). Transduction of the HT-1080 cells with the human Hyal2-expressing vector LHyal2SN rendered the cells as infectible by the JSRV vector as are SSF cells and nearly as infectible by the ENTV vector as are SSF cells (Table 2). Thus, human Hyal2 can mediate efficient entry of ENTV and JSRV vectors, but to do so it must be expressed at a higher level than is normally found on HT-1080 cells. Presumably, the large difference between ENTV and JSRV vector titers in unmodified HT-1080 cells reflects a lower affinity of ENTV Env for human Hyal2, but high-level human Hyal2 expression can compensate for the weaker interaction to promote efficient ENTV vector entry.

D17 dog fibroblasts and Vero monkey kidney cells were completely resistant to transduction by the ENTV pseudotype LAPSN vector, but they were rendered fully susceptible to vector transduction following expression of human Hyal2 in the cells (Table 2). Both of these cell lines showed moderate susceptibility to the JSRV pseudotype LAPSN vector, and their susceptibility was increased by expression of human Hyal2 in these cells (Table 2). These results indicate that the dog and monkey cells express an endogenous Hyal2 protein at a level that can mediate entry of the JSRV vector but that does not mediate ENTV vector entry. Expression of human Hyal2 is all that is required to render these cells fully susceptible to ENTV vector transduction, indicating that Hyal2 is the primary determinant of ENTV vector entry in these cells.

NIH 3T3 mouse fibroblasts were resistant to transduction by either JSRV or ENTV vectors, but they were relatively efficiently transduced by both vectors following expression of human Hyal2 in these cells. In summary, expression of human Hyal2 is all that is required to make the HT-1080, D17, Vero, and NIH 3T3 cells fully susceptible to ENTV vector transduction, indicating that Hyal2 is the primary determinant of ENTV vector entry in all of these cell lines.

208F rat fibroblasts are resistant to ENTV vector transduction, even after expression of human Hyal2 in the cells. 208F rat fibroblasts were completely resistant to ENTV and JSRV vector entry (Table 2). Similar to results discussed above for NIH 3T3 mouse cells, the 208F cells were relatively efficiently transduced by the JSRV vector following expression of human Hyal2 in the cells (Table 2). In contrast, 208F cells expressing human Hyal2 were only partially susceptible to ENTV vector transduction, the rate of transduction being 2,500-fold lower than that observed in D17 dog cells expressing human Hyal2 and over 100-fold lower than that of Vero monkey or NIH 3T3 mouse cells expressing human Hyal2 (Table 2). Furthermore, 208F cells expressing ovine Hyal2, the natural receptor of ENTV, were also poorly transduced by the ENTV vector (Table 2). Possible explanations for these results are that human and ovine Hyal2 made in the rat cells are made at a low level or are modified such that these proteins do not efficiently bind ENTV Env, that ENTV requires a coreceptor and the coreceptor ortholog in rat cells functions poorly, or that the cells make an inhibitor of ENTV vector transduction.

ENTV Env SU binds efficiently to 208F rat cells expressing human Hyal2. To address the possibility that 208F cells transduced with the human Hyal2 expression vector might not be able to bind ENTV Env either due to low Hyal2 expression, alternative posttranslational Hyal2 modification, or the presence of factors that can block binding, we made a hybrid protein consisting of the SU receptor-binding domain of the ENTV Env linked to a human IgG constant region (ESU-IgG) and used it to measure ENTV Env SU binding to various cell lines. We first showed that the ESU-IgG protein bound at high levels to NIH 3T3 cells expressing human Hyal2 (NIH 3T3/ Hyal2 cells) but bound poorly to the parental NIH 3T3 cells (Fig. 2, top panel). Binding of an analogous JSRV Env SU-IgG protein (JSU-IgG) to the NIH 3T3 cells expressing human Hyal2 was similar to that of ESU-IgG (Fig. 2, top panel). We obtained essentially the same results for ESU-IgG and JSU-IgG binding to 208F cells and 208F cells expressing human Hyal2, except that there is a small population of 208F/Hyal2 cells that did not bind either SU-IgG protein, which likely represents revertants in the population that no longer express human Hyal2 (Fig. 2, bottom panel). These results indicate that the more than 100-fold lower titer of the ENTV vector on 208F/Hyal2 cells compared to that for NIH 3T3/Hyal2 cells is not due to decreased expression of human Hyal2 on the 208F/Hyal2 rat cells or to an inability of the ENTV Env protein to bind the human Hyal2 protein expressed on the 208F cells. In addition, ESU-IgG binding to the parental 208F cells is at least 10-fold lower than that to 208F/Hyal2 cells, showing that rat Hyal2 expressed on 208F cells cannot effectively compete with Env binding to human Hyal2 and arguing against the possibility that the rat Hyal2 might competitively block virus entry mediated by human Hyal2.

We next measured binding of ESU-IgG protein to additional cells described in Table 2 that expressed human Hyal2 (Fig. 3). Note that here we used a lower concentration of ESU-IgG, so the binding curves are shifted ~10-fold lower in this experi-
ment. As shown above, ESU-IgG bound equally well to NIH 3T3 and 208F cells expressing human Hyal2 are similar. Dashed lines represent Hyal2-expressing cells without SU-IgG or antibody addition; dotted lines, Hyal2-expressing cells incubated with secondary antibody only; solid heavy lines, parental or Hyal2-expressing cells incubated with ESU-IgG and antibody; and solid thin lines, Hyal2-expressing cells incubated with JSU-IgG and antibody. All profiles were generated in the same experiment with the same reagents. Cells were incubated with ESU-IgG and JSU-IgG at a concentration of 10 μg/ml (0.12 μM).

To better address whether the affinity of ENTV Env for human Hyal2 might be different for 208F/Hyal2 cells than for other cell types, we measured human Hyal2-specific binding over a range of ESU-IgG concentrations for 208F/Hyal2 and NIH 3T3/Hyal2 cells (Fig. 4). Specific binding was determined by subtracting ESU-IgG binding to the parental cell lines from that of the human Hyal2-expressing cell lines. ESU-IgG showed saturable and specific binding to 208F/Hyal2 and NIH 3T3/Hyal2 cells, with somewhat more binding to the 208F/Hyal2 cells (Fig. 4A). Scatchard analysis of these data showed similar binding affinities, with $K_d$ values of 170 and 180 nM for 208F and NIH 3T3 cells, respectively (Fig. 4B). These data show that differences in maximum Env binding or binding affinity cannot explain the poor transduction of 208F/Hyal2 cells compared to that of NIH 3T3/Hyal2 cells.

Multiple rat cell lines expressing human Hyal2 are resistant to ENTV vector transduction. To explore whether resistance to ENTV vector transduction in 208F rat cells expressing human Hyal2 is a unique property of these cells or is representative of cells from rats, we measured ENTV and JSRV vector transduction of several additional rat cell lines after transduction with a retroviral vector expressing human Hyal2 or with a vector expressing human Hyal1 as a control (Table 3). We tested cell lines from several rat strains and from different rat tissues, including normal rat kidney (NRK) cells from Osborne-Mendel rats (5), Rat2 embryo fibroblasts from Fischer rats (20), XC rat cells derived from a tumor induced by Rous sarcoma virus in Wistar rats (19), and 9L glioma cells from Fischer rats (2). All of the cell lines transduced with the control vector expressing human Hyal1 were completely resistant to ENTV or JSRV vector transduction, as are 208F cells. After transduction with a retroviral vector encoding human Hyal2, the cells became quite susceptible to JSRV vector transduction but were only poorly transduced by the ENTV vector, as we
found for the 208F cells. Thus, rat cell lines derived from various tissues and from different strains of rats all had phenotypes similar to that of 208F rat cells.

To determine whether human Hyal2 was expressed on the rat cell lines transduced with the Hyal2 vector and was capable of binding ENTV Env, we measured ESU-IgG binding to the cell lines (Fig. 5). All of the cell lines, except the 9L/Hyal2 cells, exhibited high human-Hyal2-specific ESU-IgG binding. We have not further explored the reason for the low but detectable level of ESU-IgG binding to the 208F/Hyal2 cells but are even less susceptible to ENTV vector transduction (20 AP⁺ foci/ml; Table 3) than are the 208F cells (400 AP⁺ foci/ml; Table 2). Together these results show that multiple cell lines from different strains and tissues of rats are resistant to ENTV vector transduction, even after expression of human Hyal2 in these cells.

208F cells do not secrete an inhibitor of infection. CHO cells secrete a factor that inhibits infection of the cells by amphotropic MLV (12, 13), and we considered the possibility that the 208F cells might secrete a similar inhibitory factor. To test this, we mixed 208F/Hyal2 cells with D17/Hyal2 cells at various ratios (while keeping the total number of cells constant), grew the cells together overnight, and measured ENTV vector transduction of the cultures to see if factors secreted by the 208F/Hyal2 cells might block transduction of the D17/Hyal2 cells (Table 4). An equal mixture of 208F/Hyal2 and D17/Hyal2 cells was transduced at about half the rate of D17/Hyal2 cells alone, and, given that there were half as many D17/Hyal2 cells in the mixed culture, this result shows that there is no inhibition of D17/Hyal2 cell transduction in the presence of 208F/Hyal2 cells. 208F/Hyal2 cells alone were transduced at a rate 1,000-fold lower than that for the D17/Hyal2 cells, showing that we can ignore the contribution of 208F/Hyal2 cell transduction to the transduction rates measured in the mixed cell cultures. These results indicate that the 208F cells do not secrete an inhibitor of transduction but do not rule out the possibility of a cell-autonomous inhibitor in the 208F cells.

<table>
<thead>
<tr>
<th>Target cell type</th>
<th>Expressed protein</th>
<th>Apparent titer of LAPSN vector (AP⁺ foci/ml) produced by packaging cells</th>
<th>Ratio of JSRV to ENTV titers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat2</td>
<td>Hyal1</td>
<td>&lt;1</td>
<td>2,5</td>
</tr>
<tr>
<td></td>
<td>Hyal2</td>
<td>20</td>
<td>2 × 10³</td>
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<tr>
<td></td>
<td></td>
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<td>1,000</td>
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<tr>
<td>NRK</td>
<td>Hyal1</td>
<td>&lt;1</td>
<td>20</td>
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<td></td>
<td>Hyal2</td>
<td>20</td>
<td>8 × 10³</td>
</tr>
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<td>XC</td>
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<td>&lt;1</td>
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</tr>
<tr>
<td></td>
<td>Hyal2</td>
<td>7</td>
<td>1 × 10³</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>140</td>
</tr>
</tbody>
</table>

* Cells expressing human Hyal1 or human Hyal2 were generated by transduction with vectors encoding these proteins and Neo (LHyal1SN and LHyal2SN, respectively) followed by selection in G418. LAPSN vector titers were determined by infection of cells seeded the day before at 10⁴ cells per well (diameter, 3.5 cm) of 6-well plates. AP⁺ foci were counted 3 days after vector exposure. Results are means of at least two experiments. Values from different experiments varied by no more than three-fold.

Table 4. ENTV vector transduction of D17 dog cells is unaffected by coculture with 208F rat cells*

<table>
<thead>
<tr>
<th>No. of cells (× 10⁴) seeded</th>
<th>Apparent titer (AP⁺ foci/ml) of ENTV vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>D17/Hyal2</td>
<td>1.1 × 10⁵</td>
</tr>
<tr>
<td>208F/Hyal2</td>
<td>4.5 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>9.0 × 10⁴</td>
</tr>
<tr>
<td></td>
<td>1.8 × 10⁵</td>
</tr>
</tbody>
</table>

* D17 and 208F cells that express human Hyal2 were seeded at the indicated numbers into wells of 6-well plates. The next day the cells were exposed to LAPSN vector made from PN229 ENTV pseudotype packaging cells, and 2 days after vector exposure the cells were stained for AP. Results are means of two wells for experiment 1 and of 4 wells for experiment 2.
bated with ESU-IgG at a concentration of 10 μg/ml (0.12 μM). All profiles were generated in the same experiment with the same reagents. Cells were incubated with ESU-IgG and secondary antibody. All profiles were generated in the same experiment with the same reagents. Cells were incubated with ESU-IgG and secondary antibody. Dotted lines, Hyal2-expressing cells incubated with secondary antibody; solid thin lines, cells transduced with a vector encoding human Hyal1 and incubated with ESU-IgG and secondary antibody; solid heavy lines, cells transduced with a vector encoding human Hyal2 and incubated with ESU-IgG and secondary antibody. All profiles were generated in the same experiment with the same reagents. Cells were incubated with ESU-IgG at a concentration of 10 μg/ml (0.12 μM).

FIG. 5. The ENTV SU domain binds specifically and at high levels to most rat cells transduced with a vector encoding human Hyal2. The cell type analyzed is given at the top right of each panel. Dashed lines represent Hyal2-expressing cells without SU-IgG or antibody addition; dotted lines, Hyal2-expressing cells incubated with secondary antibody only; solid heavy lines, cells transduced with a vector encoding human Hyal2 and incubated with ESU-IgG and secondary antibody; solid thin lines, cells transduced with a vector encoding human Hyal1 and incubated with ESU-IgG and secondary antibody. All profiles were generated in the same experiment with the same reagents. Cells were incubated with ESU-IgG at a concentration of 10 μg/ml (0.12 μM).

DISCUSSION

To investigate the receptor requirements and host range of ENTV, we previously made retroviral vectors with MoMLV core components and the ENTV Env (3). These vectors had low titers, making host range studies difficult. However, the previous work also indicated that a hybrid Env consisting of the JSRV SU linked to the ENTV TM could more effectively pseudotype MoMLV-based vectors while maintaining the host range of vectors bearing the wild-type ENTV Env. Here we have generated a packaging cell line that expresses the hybrid ENTV/JSRV Env and produces high-titer vectors, and we have confirmed that these vectors have the host range conferred by the wild-type ENTV Env.

The poor performance of the wild-type ENTV Env with MoMLV-based vectors might be due to defects in the TM region of the particular clone of ENTV used, to incompatibilities of the ENTV Env with MoMLV components in the virions, or simply to poor Env protein production in the mouse cells used to make the vectors. Dirks et al. sequenced the ENTV Env clone used here (GenBank accession no. AF401741) and found no amino acid differences between the encoded TM domain of Env sequence and that of a previously derived sequence (GenBank accession no. Y16627) (3). Three additional clones of the ENTV Env TM region isolated from three different sheep exhibiting enzootic nasal adenocarcinoma have been recently isolated (14); the amino acid sequence of one of these clones matches our sequence exactly, one shows a single conservative amino acid difference, and one shows four different, mostly conservative, amino acid differences. These results indicate that the TM region of the ENTV Env clone used here is representative of other wild-type isolates and is thus not defective. In particular, the large difference in amino acid sequence in the membrane-spanning and cytoplasmic domains of the ENTV and JSRV Env proteins appears to be characteristic of these viruses and not due to a major artifact in these regions of the ENTV Env clone that we have used.

Several possibilities might explain the poor susceptibility of human Hyal2-expressing rat cells to ENTV vector transduction: (i) human Hyal2 does not mediate efficient entry because it is modified or is expressed at a low level in rat cells compared to that in other cells, (ii) there is a dominant-acting inhibitor(s) of infection in rat cells, or (iii) cofactors required for ENTV vector entry are poorly expressed or are less functional in rat cells than in other cells. We have shown that human Hyal2 is expressed well on the rat cell lines (with the exception of rat 9L cells) based on their ability to bind relatively large amounts of the ENTV Env SU domain (ESU-IgG) compared to those of highly infectible cell lines from other species. In addition, the affinity of ESU-IgG for human Hyal2 expressed on 208F or NIH 3T3 cells was similar, further supporting the conclusion that human Hyal2 expressed on 208F rat cells is fully functional and is expressed at high levels.

We have not found evidence for production of a secreted inhibitor of infection by 208F cells in experiments using mixed cultures of 208F/Hyal2 and D17/Hyal2 cells. Our results do not rule out the presence of a cell-autonomous inhibitor of ENTV vector transduction in the 208F/Hyal2 cells. However, our findings that human Hyal2 expressed by these cells binds ESU-IgG at levels and with an affinity similar to that of cells that are efficiently transduced and that human-Hyal2-dependent JSRV vector transduction is not blocked by the putative factor indicate that any potential inhibitors do not interact with human Hyal2.

We propose that ENTV and JSRV cell entry requires Hyal2 and an as-yet unidentified coreceptor, and the variable transduction of different cell types by the viruses is dependent on variable interaction of the viruses with both factors. For example, it is clear that JSRV Env can mediate infection by using a wider range of Hyal2 orthologs from different species than can the ENTV Env. Our results suggest the involvement of a coreceptor in 208F cell entry mediated by ENTV Env, and given the similarity of the extracellular domains of ENTV and JSRV Env proteins, it is reasonable to assume that JSRV would have a similar requirement. There is even a suggestion of a coreceptor requirement for JSRV Env-mediated 208F cell entry, because the titer of the JSRV vector on 208F/Hyal2 cells is...
down about 10-fold from that observed for SSF sheep cells and for several other cell lines expressing human Hyal2 (Table 2).

Many retroviruses require a multiple-membrane-spanning protein as a receptor for cell entry (15), and one can hypothesize that virus binding to such proteins allows close juxtaposition of the viral and cellular membranes to facilitate fusion. Binding to a protein with a single transmembrane segment or to a glycosylphosphatidylinositol (GPI)-anchored protein might leave the virus too far away from the cell membrane to allow fusion. Although there are several examples of retroviruses that utilize single-pass or GPI-linked membrane proteins as primary entry receptors (15), the involvement of coreceptors in these cases has not been ruled out.

The best analogy for the proposed use of a coreceptor by ENTV and JSRV is provided by human immunodeficiency virus (HIV) and other lentiviruses (15 and references therein). While the single-membrane-spanning protein CD4 is the primary binding receptor for HIV, the virus also requires a seven-transmembrane G-coupled protein for cell entry. This requirement was discovered based on the finding that expression of human CD4 alone in nonsusceptible mouse cells did not promote virus entry, indicating another factor was required. Like CD4, Hyal2 is not a multiple-membrane-spanning protein but is linked to the cell membrane by a GPI anchor. As we have shown here, expression of human Hyal2 in 208F cells is not sufficient to promote efficient transduction by an ENT vector. For HIV, cell lines existed that expressed CD4 but not the coreceptors, making it relatively straightforward to screen for and clone the required factors. In the case of the ENTV, a screen for the coreceptor would require detecting an increase in transduction rate mediated by expression of an additional protein, making such a screen technically more difficult but achievable. Future work will be aimed at determining whether rat cell resistance to ENTV infection is indeed due to the lack of an efficient coreceptor by analysis of somatic cell hybrids between rat and human or dog cells and, if so, is due to identification of the functional coreceptor gene.

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