JOURNAL OF VIROLOGY, Jan. 2005, p. 87–94 Vol. 79, No. 1
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.1.87–94.2005

Improved Enzootic Nasal Tumor Virus Pseudotype Packaging Cell Lines Reveal Virus Entry Requirements in Addition to the Primary Receptor Hyal2

Neal S. Van Hoeven[1,2] and A. Dusty Miller[1]*

Division of Human Biology[1] and Molecular and Cellular Biology Program,[2] Fred Hutchinson Cancer Research Center, Seattle, Washington

Received 10 June 2004/Accepted 26 August 2004

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. Both viruses use the glycosylphosphatidylinositol (GPI)-anchored cell surface protein hyaluronidase 2 (Hyal2) as a receptor for cell entry, and entry is mediated by the envelope (Env) proteins encoded by these viruses. Retroviral vectors bearing JSRV Env can transduce cells from a wide range of species, with the exception of rodent cells. Because of the low titer of vectors bearing ENTV Env, it has been difficult to determine the tropism of ENTV vectors, which appeared to transduce cells from sheep and humans only. Here we have developed high-titer ENTV packaging cells and confirm that ENTV has a restricted host range compared to that of JSRV. Most cells that are not transduced by JSRV or ENTV vectors can be made susceptible following expression of human Hyal2 on the cells. However, five rat cell lines from different rat strains and different tissues that were engineered to express human Hyal2 were still only poorly infected by ENTV vectors, even though the ENTV Env protein could bind well to human Hyal2 expressed on four of these cell lines. These results indicate the possibility of a coreceptor requirement for these viruses.

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.

* Corresponding author. Mailing address: Fred Hutchinson Cancer Research Center, 1100 Fairview Ave. N., Room C2-105, Seattle, WA 98109-1024. Phone: (206) 667-2890. Fax: (206) 667-6523. E-mail: dmiller@fhcrc.org.

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 approximately diploid subclone of HT-1080 cells from ATCC CCL-121), D17 dog osteosarcoma cells (ATCC CRL-6248), Vero African green monkey kidney epithelial cells (ATCC CCL-81), 293T human embryonic kidney cells (4), and PJ4 JSRV pseudotype (17) and PT67 10A1 murine leukemia virus (MLV) pseudotype (8) retrovirus packaging cells. Cells were grown in Dulbecco's modified Eagle medium (DMEM) with high glucose (4.5 g/liter) and 10% fetal bovine serum at 37°C in a 10% CO_2-air atmosphere at 100% relative humidity.

Plasmid expression vectors. Plasmids used to express JSRV Env (pCSI-Jenv) and ENTV Env (pCSI-Eenv) were made by cloning the respective Env-coding regions into the pCSI expression vector, which includes a cytomegalovirus immediate-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 (3). The pSX2 vector employs a promoter, enhancers, 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 LAPSN 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 Hyal1 and Hyal2 (LHyal1SN and LHyal2SN, respectively) were made as described previously (18) by inserting the Hyal1 or Hyal2 cDNAs into the LXSN retroviral vector (10).

For some experiments, retroviral vectors were made by transient $CaPO_4$-mediated transfection of Env expression plasmids into NIH 3T3 cells that express MoMLV Gag-Pol proteins (LGPS clone 91–22 cells [9]) and that contain the LAPSN vector. The cells were fed the day after transfection, and vector-containing medium was collected the day after feeding. Vector preparations were filtered through 0.45-μm-pore-size filters and were stored at −70°C.

Stable vector-producing cell lines were generated as described previously (10) by transient transfection of the vector plasmids into PE501 ecotropic packaging cells; harvest of virus; transduction of ENTV, JSRV, or 10A1 MLV packaging cells; and isolation and screening of G418-resistant clones for high-titer virus production. To harvest vectors, culture medium was incubated with vector-producing packaging cells for 12 to 16 h and the medium was harvested and filtered through 0.45-μm-pore-size filters and stored at −70°C.

Vector infections were done in the presence of 4 μg of Polybrene (Sigma) per ml, and transduction was quantitated 2 to 3 days after vector exposure by fixing cells with 3.7% formaldehyde, staining the cell monolayers for AP expression, and counting foci of AP-positive cells.

Production of IgG-tagged SU domains of ENTV and JSRV Env proteins. Construction of a plasmid (pCSI-JSU-IgG) encoding the surface (SU) domain of JSRV Env linked to a human IgG constant region (JSU-IgG) has been described previously (7). A similar plasmid (pCSI-ESU-IgG) that expresses the SU domain of ENTV Env linked to the same human IgG constant region (ESU-IgG) was generated by fusing the N-terminal SU portion of ENTV Env (373 amino acid residues beginning with the Env start codon) to the human IgG constant region. Following plasmid construction, the Env open reading frame was sequenced and found to be identical to the published ENTV *env* sequence (GenBank accession no. AF401741).

Hybrid SU-IgG proteins were generated by transient transfection of 293T cells. Briefly, cells were seeded at 2.5×10^6 cells per 10-cm-diameter dish and were transfected with 20 μg of pCSI-ESU-IgG or pCSI-JSU-IgG plasmid per dish 18 h later. Approximately 12 h after transfection, culture medium was replaced with 10 ml of DMEM supplemented with 10% Ultra-Low-IgG fetal bovine serum (Gibco). Cells were then incubated at 37°C, 10% CO_2 for 48 h. Following incubation, medium containing SU-IgG was collected, supplemented with 0.2% sodium azide, and filtered through a 0.45-μm-pore-size filter to remove cellular debris.

ESU-IgG and JSU-IgG proteins were purified by fast protein liquid chromatography using 1 ml of protein A-Sepharose (Amersham). A total of 300 ml of protein-containing culture medium was loaded onto the column at a flow rate of 1 ml/min. Protein was eluted in buffer containing 100 mM citrate and 0.2% sodium azide at pH 3.8. The elution buffer was then exchanged with a buffer containing 20 mM sodium phosphate, pH 7.4, using a 15-ml Centricon ultrafiltration device with a cutoff of 10 kDa (Millipore). The purity and immunoreactivity of the proteins were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting. The specificity of the reagents was verified in flow cytometry assays using NIH 3T3 and NIH 3T3/Hyal2 cells.

TABLE 1. The titer of vector produced by transient transfection of ENTV Env expression plasmids is much lower than that of JSRV Env plasmids[a]

Transfected *env* plasmid	Titer of LAPSN vector (AP^+ foci/ml) measured on SSF cells
None	<2
pCSI-Jenv	3×10^3
pCMV3JS21ΔGP	4×10^3
pCSI-Eenv	10
pCMV3ENTVΔGP	<2
pMDG (VSV-G)	5×10^3

[a] Vectors were produced by transient transfection of the indicated plasmids into NIH 3T3 cells that make MoMLV Gag-Pol proteins and that contain the LAPSN vector, as described in Materials and Methods. Plasmids pCMV3JS21ΔGP and pCMV3ENTVΔGP have been previously described (1). Titers of viral stocks were determined by infection of sheep skin fibroblast (SSF) cells seeded the day before at 10^5 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 experiments. Experimental values varied by up to four-fold from the mean.

Flow cytometry. Flow cytometry assays with ESU-IgG and JSU-IgG proteins were performed as previously described (7). Briefly, cells were harvested from confluent 10-cm-diameter dishes by incubation with 10 ml of phosphate-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 $1{,}000 \times g$ 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 ENTV 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 observed 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 plasmids were used in these studies, and to determine whether these differences could explain the discordant results, we measured 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 plasmid 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

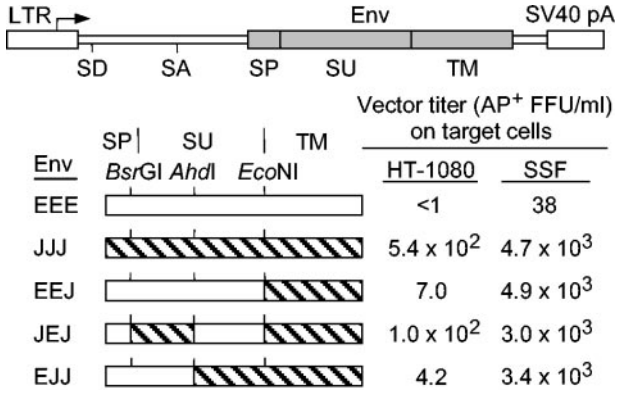

FIG. 1. Transduction of human and sheep cells by vectors bearing chimeric ENTV/JSRV Env proteins. At the top is the expression cassette used to express the Env proteins. The arrow indicates the transcription start site, and the abbreviations are the following: LTR, retroviral long terminal repeat promoter; SD, splice donor; SA, splice acceptor; SP, endoplasmic reticulum signal peptide; SU, Env surface subunit; TM, Env transmembrane subunit; and SV40 pA, simian virus 40 polyadenylation signal. Hybrid Env proteins (open boxes represent ENTV Env, hatched boxes represent JSRV Env) were made using the indicated restriction enzyme sites, and the relationship of these sites and the SP/SU and SU/TM cleavage sites are shown. LAPSN vectors bearing the indicated Env proteins were made by transient transfection as described in Materials and Methods. SSF and HT-1080 cells, seeded at 10^5 per well (diameter, 3.5 cm) of 6-well plates, were exposed to the vectors 1 day later and were stained to detect AP^+ foci 2 days after vector exposure. Values are averages of two experiments with duplicate determinations in each experiment. Data are from reference 3.

vector stocks. The ENTV env sequence in the pCSI-Eenv plasmid is given in GenBank accession number AF401741, and the sequence in pCMV3ENTVΔGP has not been reported. These results indicate that the ENTV Env sequences used in both prior reports are inherently over 100-fold less active than JSRV Env in pseudotyping MoMLV-based retroviral vectors. We hypothesize that the discrepancy in results might be explained by the difference in vector production systems used. In the one study reporting similar ENTV and JSRV vector titers, the vectors were produced by transient transfection of 293 cells (1), and the high protein synthetic capacity of this system may have compensated for inefficiencies in ENTV Env function. Here and in the other previous study (3), vectors were produced by transient transfection of NIH 3T3 cells.

Vectors pseudotyped with hybrid ENTV/JSRV Env proteins exhibit dramatically increased titers while still maintaining the ENTV host range. ENTV and JSRV Env proteins are quite similar over most of their length but differ considerably in the membrane-spanning and cytoplasmic regions of their transmembrane (TM) domains (1, 3). A possible explanation for the poor ability of the ENTV Env to pseudotype MoMLV-based retroviral vectors is that the cytoplasmic domain of ENTV cannot interact properly with the MoMLV virion core, leading to poor incorporation of ENTV Env into virions. Consistent with this hypothesis, a previous study showed that replacement of the TM domain of the ENTV Env with that of JSRV resulted in a hybrid Env (construct EEJ; Fig. 1) that could rescue vectors with an efficiency similar to that of JSRV Env (construct JJJ; Fig. 1) when the vectors were assayed on sheep cells (3). Vectors made with the hybrid Env transduced human HT-1080 cells much less well than they did sheep SSF cells, similar to what is observed with vectors bearing the parental ENTV Env (construct EEE; Fig. 1). Additional hybrids showed that the host range specificity differences between JSRV and ENTV Env proteins mapped to the N-terminal half of the Env surface (SU) domain (constructs JEJ and EJJ; Fig. 1), consistent with results obtained with other retroviruses. Together these results indicate that the host range conferred 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 LAPSN-transduced clones made no LAPSN vector, 16 clones made vector with titers from 1×10^3 to 3×10^4, and 1 clone produced the LAPSN vector with a titer of 3×10^5 AP^+ focus-forming units/ml. We used the latter clone to make ENTV pseudotype LAPSN 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 Hyal2 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[a]

Target cell type	Expressed protein	Apparent titer of LAPSN vector (AP^+ foci/ml) produced by packaging cells:		Ratio of JSRV to ENTV vector titers
		PN229 (ENTV)	PJ4 (JSRV)	
SSF	None	4×10^5	4×10^5	1
HT-1080	None	200	2×10^4	100
	Hyal2	1×10^5	4×10^5	4
D17	None	<5	7×10^3	>1,400
	Hyal2	1×10^6	3×10^5	0.3
Vero	None	<5	2×10^4	>4,000
	Hyal2	1×10^5	9×10^4	0.9
NIH 3T3	None	10	<5	<0.5
	Hyal2	6×10^4	5×10^4	0.8
208F	None	<1	<1	
	Hyal2	400	3×10^4	75
	oHyal2[b]	200	6×10^3	30
	rHyal2	<1	9×10^3	>9,000

[a] Cells expressing human or rat Hyal2 were generated by transduction with vectors encoding these proteins and Neo (LHyal2SN and LrHyal2SN, respectively) followed by selection in G418. LAPSN vector titers were determined by infection of cells seeded the day before at 10^5 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.

[b] Cells expressing ovine Hyal2 were generated by transfection with a plasmid that expresses ovine Hyal2 and Neo (pCR3.1oHyal2) followed by selection in G418.

vector, 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-

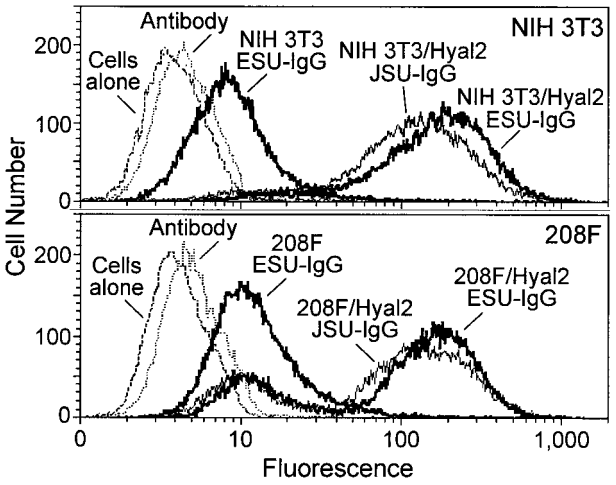

FIG. 2. Binding levels of ENTV and JSRV Env SU domains to NIH 3T3 and 208F cells expressing human Hyal2 are similar. Dashed lines represent Hyal2-expressing cells without SU-IgG or antibody addition; dotted lines, Hyal2-expressing cells incubated with secondary antibody only; solid heavy lines, parental 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).

ment. As shown above, ESU-IgG bound equally well to NIH 3T3/Hyal2 mouse and 208F/Hyal2 rat cells. Binding of ESU-IgG to Vero cells expressing human Hyal2 (Vero/Hyal2 cells) was significantly lower than binding to 208F/Hyal2 cells, yet Vero/Hyal2 cells are 250-fold more infectible than are 208F/Hyal2 cells. The D17 cells expressing human Hyal2 are a mixture of two discrete populations of cells that bind small or large amounts of ESU-IgG, likely due to the accumulation of revertants that express little or no human Hyal2. The high-binding population binds more ESU-IgG than any of the other cell lines, perhaps explaining the very high ENTV vector transduction rates observed for these cells (Table 2). In conclusion, the level of ENTV Env binding to human Hyal2 expressed on 208F cells is at least as high as that for highly infectible NIH 3T3 and Vero cells. Thus, the poor transduction of 208F/Hyal2 cells cannot be explained by insufficient human Hyal2 expression or poor binding of the ENTV Env to the human Hyal2 expressed on 208F/Hyal2 cells.

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.

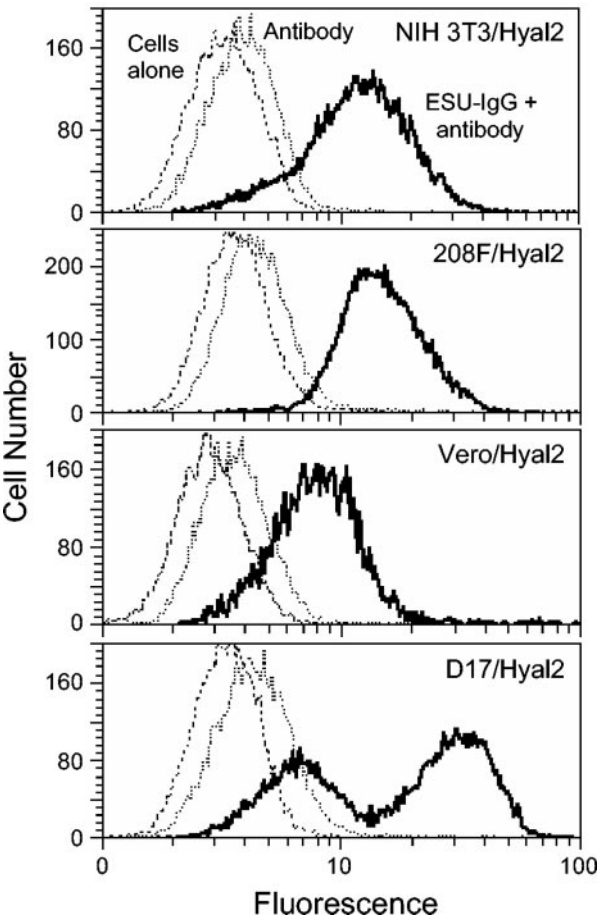

FIG. 3. Binding of the ENTV Env SU domain to human Hyal2 expressed on cells from different species. The human Hyal2-expressing cell type analyzed is given at the top right of each panel. Dashed lines represent cells without ESU-IgG or antibody addition; solid thin lines, cells incubated with secondary antibody only; solid heavy lines, cells 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 ~0.5 μg/ml.

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

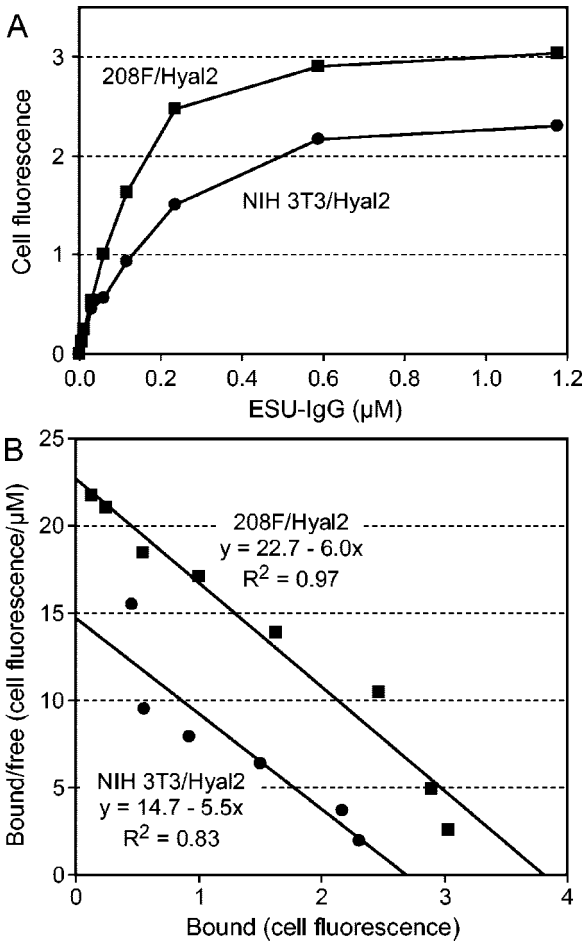

FIG. 4. Specific binding of ESU-IgG to human Hyal2 expressed on 208F and NIH 3T3 cells. (A) Parental or human Hyal2-expressing 208F or NIH 3T3 cells were incubated with various concentrations of ESU-IgG, were incubated with secondary antibody, and were analyzed by FACS. At each ESU-IgG concentration, the geometric mean fluorescence of parental cells (208F or NIH 3T3) was subtracted from that of human Hyal2-expressing cells (208F/Hyal2 or NIH 3T3/Hyal2) to obtain the human Hyal2-specific binding values, in arbitrary units, and these values are plotted against the ESU-IgG concentration. All results were generated using the same reagents and the same FACS settings and were repeated once, with similar results. (B) Scatchard analysis of the data from panel A is shown. Best-fit lines, their equations, and the Pearson correlation coefficients are indicated. K_d values (micromolars) are equal to -1/slope.

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 human-Hyal2-specific ESU-IgG binding to the 9L/Hyal2 cells. Impressively, the NRK/Hyal2 cells bind more ESU-IgG than do 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

TABLE 3. Multiple rat cell lines are resistant to ENTV vector transduction even after expression of human Hyal2 in the cells[a]

Target cell type	Expressed protein	Apparent titer of LAPSN vector (AP^+ foci/ml) produced by packaging cells:		Ratio of JSRV to ENTV titers
		PN229 (ENTV)	PJ4 (JSRV)	
Rat2	Hyal1	<1	<5	
	Hyal2	20	2×10^4	1,000
NRK	Hyal1	<1	<5	
	Hyal2	20	8×10^3	400
XC	Hyal1	<1	<5	
	Hyal2	14	3×10^3	200
9L	Hyal1	<1	<5	
	Hyal2	7	1×10^3	140

[a] 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 10^5 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.

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 4. ENTV vector transduction of D17 dog cells is unaffected by coculture with 208F rat cells[a]

No. of cells ($\times 10^4$) seeded		Apparent titer (AP^+ foci/ml) of ENTV vector	
D17/Hyal2	208F/Hyal2	Expt 1	Expt 2
5.0	0.0	1.1×10^5	1.8×10^5
2.5	2.5	4.5×10^4	9.0×10^4
0.0	5.0	200	180

[a] 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.

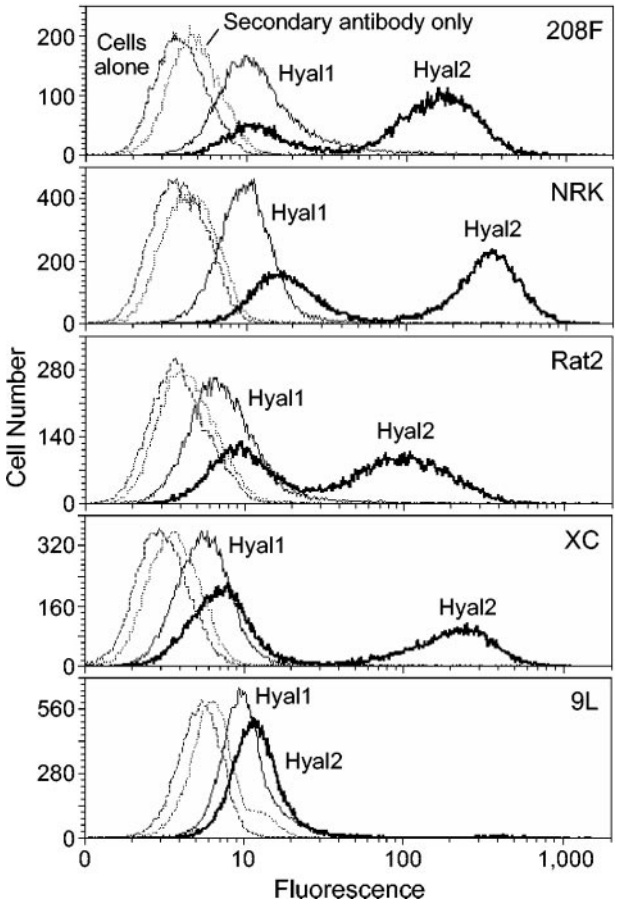

FIG. 5. The ENTV SU domain binds specifically and at high levels to most rat cells transduced with a vector encoding human Hyal2. The cell type analyzed is given at the top right of each panel. Dashed lines represent Hyal2-expressing cells without SU-IgG or antibody addition; dotted lines, Hyal2-expressing 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 ENTV 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.

ACKNOWLEDGMENTS

We thank Clarissa Dirks for sharing unpublished data and for helpful discussions.

This work was supported by NIH grants DK47754 and HL54881 (A.D.M). N.S.V.H. was supported in part by NIH training grant T32-CA09437.

REFERENCES

1. **Alberti, A., C. Murgia, S.-L. Liu, M. Mura, C. Cousens, M. Sharp, A. D. Miller, and M. Palmarini.** 2002. Envelope-induced cell transformation by ovine betaretroviruses. J. Virol. **76:**5387–5394.
2. **Barker, M., T. Hoshino, O. Gurcay, C. B. Wilson, S. L. Nielsen, R. Downie, and J. Eliason.** 1973. Development of an animal brain tumor model and its response to therapy with 1,3-bis(2-chloroethyl)-1-nitrosourea. Cancer Res. **33:**976–986.
3. **Dirks, C., F.-M. Duh, S. K. Rai, M. I. Lerman, and A. D. Miller.** 2002. Mechanism of cell entry and transformation by enzootic nasal tumor virus. J. Virol. **76:**2141–2149.
4. **DuBridge, R. B., P. Tang, H. C. Hsia, P. M. Leong, J. H. Miller, and M. P. Calos.** 1987. Analysis of mutation in human cells by using an Epstein-Barr virus shuttle system. Mol. Cell. Biol. **7:**379–387.
5. **Duc-Nguyen, H., E. N. Rosenblum, and R. F. Zeigel.** 1966. Persistent infection of a rat kidney cell line with Rauscher murine leukemia virus. J. Bacteriol. **92:**1133–1140.
6. **Fan, H.** 2003. Jaagsiekte sheep retrovirus and lung cancer. Curr. Top. Microbiol. Immunol. **275:**1–248.
7. **Liu, S.-L., F.-M. Duh, M. I. Lerman, and A. D. Miller.** 2003. Role of virus receptor Hyal2 in oncogenic transformation of rodent fibroblasts by sheep betaretrovirus env proteins. J. Virol. **77:**2850–2858.
8. **Miller, A. D., and F. Chen.** 1996. Retrovirus packaging cells based on 10A1 murine leukemia virus for production of vectors that use multiple receptors for cell entry. J. Virol. **70:**5564–5571.
9. **Miller, A. D., J. V. Garcia, N. von Suhr, C. M. Lynch, C. Wilson, and M. V. Eiden.** 1991. Construction and properties of retrovirus packaging cells based on gibbon ape leukemia virus. J. Virol. **65:**2220–2224.
10. **Miller, A. D., and G. J. Rosman.** 1989. Improved retroviral vectors for gene transfer and expression. BioTechniques **7:**980–990.
11. **Miller, D. G., R. H. Edwards, and A. D. Miller.** 1994. Cloning of the cellular receptor for amphotropic murine retroviruses reveals homology to that for gibbon ape leukemia virus. Proc. Natl. Acad. Sci. USA **91:**78–82.
12. **Miller, D. G., and A. D. Miller.** 1993. Inhibitors of retrovirus infection are secreted by several hamster cell lines and are also present in hamster sera. J. Virol. **67:**5346–5352.
13. **Miller, D. G., and A. D. Miller.** 1992. Tunicamycin treatment of CHO cells abrogates multiple blocks to retrovirus infection, one of which is due to a secreted inhibitor. J. Virol. **66:**78–84.
14. **Ortin, A., C. Cousens, E. Minguijon, Z. Pascual, M. P. Villarreal, J. M. Sharp, and M. De las Heras.** 2003. Characterization of enzootic nasal tumour virus of goats: complete sequence and tissue distribution. J. Gen. Virol. **84:**2245–2252.
15. **Overbaugh, J., A. D. Miller, and M. V. Eiden.** 2001. Receptors and entry cofactors for retroviruses include single and multiple transmembrane-spanning proteins as well as newly described glycosylphosphatidylinositol-anchored and secreted proteins. Microbiol. Mol. Biol. Rev. **65:**371–389.
16. **Quade, K.** 1979. Transformation of mammalian cells by avian myelocytomatosis virus and avian erythroblastosis virus. Virology **98:**461–465.
17. **Rai, S. K., J. C. DeMartini, and A. D. Miller.** 2000. Retrovirus vectors bearing jaagsiekte sheep retrovirus Env transduce human cells by using a new receptor localized to chromosome 3p21.3. J. Virol. **74:**4698–4704.
18. **Rai, S. K., F.-M. Duh, V. Vigdorovich, A. Danilkovitch-Miagkova, M. I. Lerman, and A. D. Miller.** 2001. Candidate tumor suppressor HYAL2 is a glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor for jaagsiekte sheep retrovirus, the envelope protein of which mediates oncogenic transformation. Proc. Natl. Acad. Sci. USA **98:**4443–4448.
19. **Svoboda, J., P. Chyle, D. Simkovic, and I. Hilgert.** 1963. Demonstration of the absence of infectious Rous virus in rat tumour XC, whose structurally intact cells produce Rous sarcoma when transferred to chicks. Folia Biol. (Prague) **9:**77–81.
20. **Topp, W. C.** 1981. Normal rat cell lines deficient in nuclear thymidine kinase. Virology **113:**408–411.
21. **Vigdorovich, V., R. K. Strong, and A. D. Miller.** 2005. Expression and characterization of a soluble, active form of the jaagsiekte sheep retrovirus receptor, Hyal2. J. Virol. **79:**79–86.
22. **Wei, C. M., M. Gibson, P. G. Spear, and E. M. Scolnick.** 1981. Construction and isolation of a transmissible retrovirus containing the src gene of Harvey murine sarcoma virus and the thymidine kinase gene of herpes simplex virus type 1. J. Virol. **39:**935–944.