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Retrovirus Packaging Cells Based on 10A1 Murine Leukemia Virus for Production of Vectors That Use Multiple Receptors for Cell Entry

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10A1 murine leukemia virus can enter cells by using either of two different cell surface phosphate transport proteins, the gibbon ape leukemia virus receptor Glvr-1 (Pit-1) or the amphotropic retrovirus receptor Ram-1 (Pit-2). Glvr-1 and Ram-1 are widely expressed in different tissues, but the relative amounts of each are highly variable. We have developed retrovirus packaging cell lines based on 10A1 virus to take advantage of this dual receptor utilization to improve gene transfer rates in somatic cells of animals and humans, in which the relative levels of the two receptors are not always known. Optimization of the Env expression vector allowed the generation of packaging lines that produce helper-free vector titers up to 10^7/ml. By interference analysis, we found that a 10A1 pseudotype retroviral vector can utilize Ram-1 for efficient entry into mouse, rat, and human cells and can utilize Glvr-1 for entry into mouse and human cells but not for entry into rat cells. The 10A1 pseudotype vector efficiently enters mouse cells by using Glvr-1, while entry into human cells is much less efficient. Thus, the 10A1 pseudotype packaging cells may be advantageous compared with the standard amphotropic packaging cells because vectors produced by the cells can use an additional receptor for cell entry. These packaging cells will also be useful to further explore the complicated pattern of receptor usage conferred by the 10A1 viral surface protein.

Retrovirus packaging cells provide useful tools for a variety of gene transfer applications. Recent improvements include the design of packaging cells to produce vectors having a vesicular stomatitis virus G-protein coat for expanded host range (5, 17), vectors that are resistant to human serum (8), and vectors that target new cell surface proteins (15, 36). However, there is still room for improvement, because not all cell types can be efficiently transduced by using existing retroviral vectors. For example, treatment of genetic and acquired disease in humans would be greatly facilitated by the ability to transfer genes into hematopoietic stem cells but transduction of these cells in large animals and humans remains inefficient.

Amphotropic retrovirus packaging cells were developed more than 10 years ago (7, 20, 22, 37) and are still commonly used because of the wide range of cell types from different species, including humans, that these vectors can transduce. More recently, we developed packaging cells based on gibbon ape leukemia virus (GALV) (21) that produce vectors that use a different receptor for cell entry and are capable of transducing myeloid, lymphoid, and airway epithelial cells at higher rates than amphotropic vectors do (1, 2, 4, 39). The GALV and amphotropic retrovirus receptors (25, 28, 38) are related phosphate transport proteins that exhibit extensive but different patterns of tissue-specific expression (16). The GALV receptor Glvr-1 (Pit-1) is most highly expressed in bone marrow, whereas the amphotropic receptor Ram-1 (Pit-2) is most highly expressed in the heart. Recently, we showed that 10A1 murine leukemia virus (MLV) can use either mouse or human Glvr-1 or rat or human Ram-1 for cell entry (26). Given the differential expression of Glvr-1 and Ram-1 on cells from different tissues, we hypothesized that packaging cells having a 10A1 pseudotype might be advantageous, especially for gene transfer in humans.

Here we describe the construction of 10A1 pseudotype packaging cells that produce helper-free vectors at high titer. These vectors can use Glvr-1 or Ram-1 for entry into mouse cells. While vectors with a 10A1 pseudotype efficiently utilize Ram-1 for entry into humans cells, they inefficiently utilize Glvr-1 for entry into the three human cell types studied and can use only Ram-1, but not Glvr-1, for entry into one rat cell line studied. Thus, the receptor utilization pattern for 10A1 MLV is more complex than previously suspected.

MATERIALS AND METHODS

Nomenclature. Cells that contain a virus or vector are indicated by the cell name followed by a slash and the name of the virus, e.g., NIH 3T3/10A1 or PA317/LXSN. A retroviral vector in its viral form is indicated by the vector name followed, in parentheses, by the name of the helper virus or packaging cells used to pseudotype the vector, e.g., LAPSN(10A1) or LAPSN(PA317). Throughout this paper, the pseudotype of a retroviral vector refers to the viral envelope protein present on the vector virions that determines the cell surface receptor utilization pattern of the vector. For example, vectors produced by PA317 cells will be referred to as having an amphotropic pseudotype because of the presence of amphotropic Env proteins in the virions, even though the Gag and part of the Pol proteins in these virions are derived from Moloney murine leukemia virus (MoMLV) (20). Transduction is defined as transfer and expression of a gene mediated by a viral vector.

Cell culture. The following cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum: NIH 3T3 thymidine kinase-deficient (TK^-) cells (20), 208F rat embryo fibroblasts (31), 293 adenovirus-transformed human kidney cells (12), PA317 amphotropic retrovirus-packaging cells (20), and PE501 ecotropic retrovirus-packaging cells (24). Human foreskin fibroblasts (HFF) were grown in Dulbecco's modified Eagle's medium with 15% fetal bovine serum. PG-4 Moloney murine sarcoma virus-infected G355 cat cells (a gift from Donald Blair [9, 13]) were grown in McCoy's medium with 15% fetal bovine serum.

Replication-competent retroviruses. The pB6 plasmid contains a circularly permuted clone of the 10A1 retrovirus (29) and was a gift from Alan Rein, NCI-Frederick Cancer Research and Development Center, Frederick, Md. The pB6 plasmid was cut with *Sal*I to release the 10A1 segment, religated at low DNA

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concentration to recircularize the 10A1 virus, and introduced into NIH 3T3 cells by calcium phosphate-mediated transfection. The cells were passaged for 2 weeks to allow complete virus spread and were then used in interference assays and to make 10A1 virus for infection of other cells. Plasmid pAM (22) contains a hybrid amphotropic retrovirus constructed by replacing the *env* gene of MoMLV with that of 4070A amphotropic virus (6). NIH 3T3 cells were transfected with pAM, passaged for 2 weeks to allow complete virus spread, and used in interference assays and to produce amphotropic helper virus for infection of other cells. The helper virus produced from pAM is referred to as AM-MLV. Mv 1 Lu mink cells (ATCC CCL 64) producing GALV SEATO strain were a gift from Maribeth V. Eiden, National Institutes of Health, Bethesda, Md., and were used to produce GALV for infection of other cells.

Retroviral vector assay. For the LAPSN vector assay, target cells were seeded at 10^5 cells per 6-cm dish or at 5×10^4 cells per 35-mm (diameter) well of a six-well plate on day 1, the cells were fed with fresh medium containing 4 μg of Polybrene (Sigma) per ml and the LAPSN vector on day 2, and the cells were fixed with glutaraldehyde and stained for alkaline phosphatase expression as described previously (10) on day 4. Assay for the *neo* expression vector LNL6 (3) was identical except that instead of staining for alkaline phosphatase, the medium was changed to medium containing 0.75 mg of G418 (active) per ml on day 3 and G418-resistant colonies were stained with Coomassie brilliant blue G (Sigma; 1 g/liter in 40% methanol–10% acetic acid) and counted on day 8.

Helper virus assays. To perform the PG-4 S^+L^- helper virus assay, PG-4 cells were seeded at 10^5 cells per 6-cm dish on day 1, the cells were fed with fresh medium plus 4 μg of Polybrene per ml and test virus on day 2, and foci were counted on day 7. To perform the marker rescue assay for helper virus, NIH 3T3 cells were plated at 10^5 cells per 6-cm dish on day 1, fed with fresh medium plus 4 μg of Polybrene per ml and 0.5 ml of test virus containing the LAPSN vector on day 2, and trypsinized and split 1:10 every 2 to 3 days for 2 weeks while being kept at high density to facilitate potential virus spread. Confluent dishes of cells were fed on day 15, and medium was harvested from the cells and tested for LAPSN vector presence on day 16. The passaged cells were also stained for alkaline phosphatase to ensure that they had been transduced with the LAPSN vector and therefore were capable of producing the vector if helper virus were present. The cells were 20 to 100% alkaline phosphatase positive in these assays.

Sequence analysis. DNA fragments to be sequenced were cloned into Bluescript II (Stratagene, La Jolla, Calif.), and double-stranded plasmid DNA was prepared. Sequencing was performed on both strands of the insert by the dideoxy sequencing method with M13 dye primers and Sequenase DNA polymerase to cycle the reaction (PRISM kit; Applied Biosystems, Foster City, Calif.) and an Applied Biosystems 373A DNA sequencer and sequence analysis software. Sequences were compiled with a text editor and checked for accuracy against the primary fluorescent-dye profile data.

Nucleotide sequence accession numbers. The GenBank accession numbers for the sequences of the 4070A and the 10A1 murine leukemia viruses that were determined here are U51729 and U51730, respectively.

RESULTS

10A1 Env expression plasmids. To make retrovirus packaging cells for production of vectors with a 10A1 pseudotype, we chose to introduce a 10A1 Env expression plasmid into well-characterized LGPS clone 91-22 cells (21) that express MoMLV Gag-Pol proteins and that were derived from NIH 3T3 cells. 10A1 Env and hybrid Env expression plasmids (Fig. 1) were tested by introducing the plasmids into LGPS cells that contained a retroviral vector and by measuring the titer of vector produced. Note that the carboxy ends of all of the Env constructs (3′ of the *Cla*I site [Fig. 1]) consisted of MoMLV *env* sequences, but this does not affect the interpretation of the results, because the amino acid sequence in this region is identical for the MoMLV, 10A1, and amphotropic 4070A viruses.

We initially constructed a 10A1 Env expression plasmid designed to minimize or eliminate overlap of sequences in the construct with those present in retroviral vectors or in the Gag-Pol expression vector. The Env expression plasmid pL10A1E (Fig. 1) has no homologous overlap at the 3′ end with our standard retroviral vectors (24) and minimal overlap at the 5′ end with the 3′ end of the MoMLV *pol* gene present in the Gag-Pol expression plasmid. Surprisingly, this construct had poor activity in the transient-expression assay for Env function compared with a similar plasmid encoding an amphotropic Env protein, pLAE2 (Fig. 1). Analysis of the pL10A1E plasmid with multiple restriction enzymes showed the expected band patterns, indicating that the plasmid had the correct structure, but we have not sequenced the entire Env coding region to check for subtle defects. Two independent plasmid clones were tested, with similar results.

Ott and Rein (30) have shown that a hybrid Env consisting of the amino terminus of the 10A1 Env protein fused to the carboxy terminus of the 4070A amphotropic Env at a common *Eco*RI site confers a 10A1 pseudotype. A hybrid Env expression plasmid with this structure, pL10AME, performed at least as well as the amphotropic Env construct in the transient vector rescue assay (Fig. 1), and a preliminary experiment suggested that the vector made with the pL10AME plasmid had the receptor utilization properties of 10A1 virus (data not shown). These results indicated that there was some defect in the 3′ end of the 10A1 *env* gene in the pL10A1E plasmid. We were able to make high-titer virus by using the original 10A1 helper virus plasmid pB6 (29), so that either the defect is not severe and revertants with wild-type virus replication properties are generated during helper virus replication in culture or the defect occurred during propagation of the pB6 plasmid or during construction of the pL10A1E plasmid.

An amphotropic Env expression plasmid that incorporated the region that normally lies upstream of the *env* gene in the retrovirus produced a higher vector titer in the transient vector rescue assay (pLAE1 [Fig. 1]). Two similar constructs in which the critical regions in the amino terminus of the amphotropic Env were replaced with 10A1 sequences (pBX and pSX) also gave a higher titer, although not as high as the amphotropic construct pLAE1 (Fig. 1). Vectors made with the pBX construct, in which only the *Bsr*GI-to-*Xho*I region of the amphotropic *env* gene was replaced with 10A1 sequences, or vectors made with the pSX construct had 10A1 interference properties (Fig. 1).

In one final construct (pSX2), we included the splice donor present after the retroviral long terminal repeat (LTR) that is normally used with a splice acceptor just upstream of *env* in MLVs to generate the spliced *env* mRNA. This construct produced the highest vector titer of all constructs tested in the transient vector rescue assay. In summary, while we could not make high titer vectors by using the wild-type 10A1 *env* sequences, we were able to make such vectors by using hybrid 10A1-amphotropic constructs. The highest vector titers were obtained by including the retroviral splice donor and acceptor sequences that are normally used to make the spliced retroviral *env* mRNA.

Generation of 10A1-pseudotype retrovirus-packaging cell lines. To generate stable retrovirus-packaging cells that expressed the hybrid 10A1 Env protein, we used LGPS clone 91-22 cells (21). LGPS cells were made by cotransfection of an MoMLV Gag-Pol expression vector with the herpes simplex virus TK gene into NIH 3T3 TK^- cells followed by selection of the cells in medium containing hypoxanthine, aminopterin, and thymidine (HAT medium). The Env expression construct that yielded the highest vector titer in the transient vector production assay (pSX2 [Fig. 1]) was introduced into LGPS cells by cotransfection (24) with either a mutant methotrexate-resistant dihydrofolate reductase gene (*dhfr**) contained in plasmid pFR400 (35) or a hygromycin phosphotransferase (*hpt*) gene contained in plasmid pSV2Δ13-hyg (a gift from Paul Berg, Stanford University). The ratio of selectable marker plasmid to Env expression plasmid was 1:20 or 1:100. Cell colonies containing the genes were selected in 100 nM methotrexate with dialyzed fetal bovine serum or 0.4 mg of hygromycin per ml, respectively, and were isolated by using cloning rings. Cell lines were generated with either marker so that a 10A1 packaging cell line would be available for use with any dominant marker.

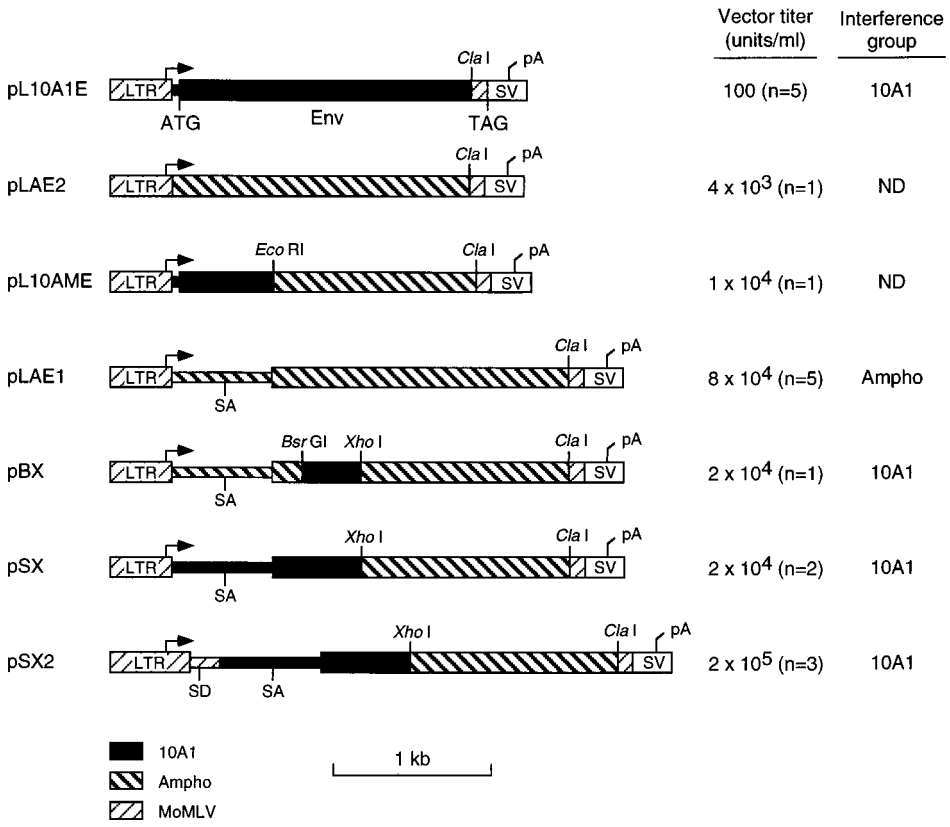

FIG. 1. Env expression constructs and their characterization. Each Env expression plasmid contains an MoMLV LTR promoter (the transcription start site is indicated by an arrow) truncated at the 5′ end to a *Sau*3AI site just upstream of the transcriptional enhancers in the LTR, the Env coding region, the early polyadenylation signal (pA) from simian virus 40 (SV) inserted just after the Env stop codon, and plasmid sequences (not shown) derived from the bacterial poison sequence-minus plasmid pML-1 (18). LTR-*env* junctions were *Sma*I-*Bsr*BI for pL10A1E and pL10AME; *Sma*I-*Pfl*MI (blunted) for pLAE2; *Sma*I-*Sph*I (blunted) for pLAE1, pBX, and pSX; and *Bsr*BI-*Sph*I (blunted) for pSX2. To test for functional activity, the chimeric envelope expression constructs were transfected into LGPS/LNL6 or LGPS/LAPSN cells by using the calcium phosphate technique, the culture medium was changed the next day, and the medium was harvested 2 days after transfection. Vector titers in the harvested medium were determined by using NIH 3T3 cells as targets for transduction and are mean values with the number of replicate assays indicated. Independent titer determinations varied no more than threefold from the mean. The vector titer was <1 U/ml when no DNA or an irrelevant plasmid was used for transfection. Similar results were obtained with the LNL6 and LAPSN vectors in these assays. Interference grouping was determined by measuring the titers of the vectors on NIH 3T3, NIH 3T3/AM-MLV, and NIH 3T3/10A1 cells. Vectors indicated as being in the 10A1 interference group gave equivalent titers (within twofold) on NIH 3T3 and NIH 3T3/AM-MLV cells, while the vector in the amphotropic interference group had a titer on NIH 3T3 cells that was $>10^4$ higher than that measured on NIH 3T3/AM-MLV cells. Vector titers measured on NIH 3T3/10A1 cells were <2 U/ml for all vectors tested for interference grouping. ND, not done.

Clone designations all begin with PT (packaging cells having a tenA1 pseudotype) followed by a number from 1 to 74 for clones cotransfected with *dhfr** or from 100 to 125 for clones cotransfected with *hpt*.

Two methods were used to screen packaging cell clones for their ability to produce a retroviral vector. The first method involved introduction of the LAPSN vector into the cells by calcium phosphate-mediated transfection followed by assay of virus production 2 days after transfection. Later assays were simplified by elimination of the transfection step and introduction of the LAPSN vector by exposure of the cells to LAPSN vector made by PE501 cells (multiplicity of infection ≈1). This can be done because transduction by ecotropic pseudotype vectors produced from PE501 cells is unaffected by expression of the 10A1 Env or MoMLV Gag-Pol proteins. The vector titer was measured 2 days after LAPSN transduction. The titers of LAPSN vector produced by either technique were similar.

We analyzed 56 *dhfr** and 17 *hpt* cotransfected cell clones for vector production ability. The titers of vector produced from packaging cell clones made by using either marker or by using either ratio of selectable marker plasmid to pSX2 Env plasmid (1:20 or 1:100) were similar. LAPSN titers up to 10^6/ml were made from some packaging cell clones in this transient vector production assay.

Analysis of stable vector-producing packaging cell clones. Seven of the *dhfr**-cotransfected packaging cell clones that produced the highest vector titers in the transient vector production assay were chosen for further analysis. The cells were transduced with LAPSN(PE501) vector and selected in G418 for 5 days to ensure the presence of the LAPSN vector in every cell. LAPSN vector titers produced from these packaging cell clones ranged from 2×10^6 to 1×10^7 focus-forming units (FFU)/ml.

LAPSN vector preparations from three of these cell lines (PT28/LAPSN, PT42/LAPSN, and PT67/LAPSN) were analyzed for receptor utilization in NIH 3T3 cells. LAPSN vector from all three clones could utilize a receptor other than the amphotropic receptor for cell entry, and the interference properties of LAPSN produced by these packaging cells closely matched those of LAPSN produced by using 10A1 helper virus to pseudotype the vector (Table 1).

LAPSN vector preparations from these three packaging cell

TABLE 1. The LAPSN vector produced by 10A1-pseudotype packaging cells can use a receptor other than the amphotropic receptor for entry into NIH 3T3 mouse cells

LAPSN pseudotype	Vector titer (FFU/ml) on target cells[a]:		
	NIH 3T3	NIH 3T3 + AM-MLV	NIH 3T3 + 10A1
PT28	2×10^6	1×10^6	40
PT42	2×10^6	8×10^5	40
PT67	3×10^6	2×10^6	90
10A1	3×10^7	2×10^7	500
PA317	1×10^7	30	20
PE501	2×10^6	1×10^6	1×10^6

[a] Values are means of duplicate assays in a single experiment; the results varied by no more than 21% from the mean (except for the value of 20, for which the numbers of colonies per plate were small and varied by 47% from the mean). The experiment was performed once.

lines were also analyzed for the presence of replication-competent retrovirus by using the PG-4 S^+L^- assay and a marker rescue assay. Use of the PG-4 S^+L^- assay was complicated by significant PG-4 cell fusion following exposure of the cells to more than 50 μl of medium from the packaging cells, making it difficult to distinguish helper virus-induced foci from areas of cell death due to cell fusion. However, 50- and 10-μl samples of the LAPSN vector preparations induced no foci in the PG-4 S^+L^- assay, indicating a helper virus titer of <20 FFU/ml, while 10A1 virus produced by NIH 3T3 cells gave a titer of 4×10^6 FFU/ml in the same assay. We also tested the LAPSN vector preparations for the presence of helper virus in a marker rescue assay. In this case, cell fusion induced by large amounts of the LAPSN vector did not complicate the assay. Duplicate 0.5-ml samples of vector from PT28/LAPSN, PT42/LAPSN, and PT67/LAPSN cells scored negative for helper virus in this assay, indicating the absence of helper virus (<1 U/ml) in the LAPSN preparations from the three clones tested.

Sequence analysis of 10A1 and 4070A Pol/Env regions. We sequenced the 10A1 and 4070A viruses from a common *Sph*I site in the 3′ end of the *pol* gene to the *Eco*RI site in the *env* gene (10A1) or the *Pfl*MI site just upstream of the Env start codon (4070A) to confirm the amino acid sequence in 10A1 responsible for its utilization of multiple receptors and to define the previously unsequenced region of the *pol* genes of 10A1 and 4070A present in the Env expression plasmids. These sequences are compared with each other and that of MoMLV in Fig. 2. The sequence of the 10A1 *env* region was different from the published sequence (29) at seven bases (circled in Fig. 2), none of which resulted in changes from the previously predicted amino acid sequence of the gp70 protein, although three changes at bases 719 to 721 (Fig. 2) do change a predicted amino acid in the Env leader peptide. There are only six amino acid differences between 10A1 and 4070A viruses in the *Bsr*GI-to-*Xho*I region of Env (boxed codons in Fig. 2); because the pBX Env expression construct had a 10A1 receptor utilization pattern (Fig. 1), these changes must be responsible for the difference in receptor utilization between 10A1 and 4070A viruses, in agreement with the results of Ott and Rein (30).

The 10A1 and 4070A virus sequences are similar upstream of *env*, with scattered base differences and a 3-bp insertion in 10A1 at bases 615 to 617. Relatively long regions of identity between 10A1 and MoMLV upstream of *env* raise the possibility of recombination in this region to join the *gag-pol* and *env* sequences present in the 10A1 pseudotype packaging cells, which might facilitate helper virus production, but additional recombination events would still be required to regenerate the viral LTRs (Fig. 3) (see Discussion).

GALV receptor utilization by a 10A1 pseudotype vector is variable in cells from different species. One goal of these studies was to produce retroviral vectors capable of using either the Ram-1 or Glvr-1 receptors for entry into human and other cell types. However, in initial studies with vectors produced in the Env expression plasmid transfection studies, we found that 10A1 pseudotype vectors might not effectively use Glvr-1 in some cells, in particular in 208F rat cells (data not shown). To explore this issue, we performed interference studies with 208F cells (Table 2). We used the wild-type 10A1 virus for these studies instead of the 10A1 packaging cells to rule out any effect of MoMLV and 4070A virus sequences present in the 10A1 packaging cells. LAPSN(10A1) vector efficiently transduced 208F cells and 208F cells previously infected with GALV but only poorly transduced 208F cells previously infected with AM-MLV amphotropic virus. An identical pattern was observed for amphotropic LAPSN(PA317) vector. In contrast, GALV pseudotype LAPSN(PG13) vector efficiently transduced 208F cells and 208F cells previously infected with AM-MLV amphotropic virus but poorly transduced 208F cells previously infected with GALV. These results show that a 10A1 pseudotype vector cannot utilize the GALV receptor for entry into 208F rat cells.

We next used interference analysis to examine the ability of 10A1 pseudotype LAPSN vector to use the GALV receptor in primary human fibroblasts (HFF) (Table 3). The LAPSN(PA317) vector efficiently transduced HFF and HFF infected with GALV, confirming that GALV binds to a different receptor from that used by amphotropic virus for cell entry. Similarly, LAPSN(PG13) efficiently transduced HFF and HFF infected with amphotropic virus, confirming that amphotropic virus binds to a different receptor from that used by GALV for cell entry. LAPSN(PA317) poorly transduced HFF infected with amphotropic virus, and LAPSN(PG13) poorly transduced HFF infected with GALV, as expected because of receptor blockage. These results confirm that GALV and amphotropic virus use different receptors for entry into human cells and thus show no interference. LAPSN(10A1) vector transduced HFF cells previously infected with amphotropic virus at a significantly higher rate than the amphotropic pseudotype LAPSN(PA317) vector did but at lower efficiency than the LAPSN(10A1) vector transduced HFF or HFF cells previously infected with GALV. Also, GALV pseudotype LAPSN(PG13) vector transduction of HFF cells was significantly inhibited by previous infection with 10A1 virus. These results indicate that 10A1 pseudotype vectors can use the GALV receptor Glvr-1 for entry into human cells but use Glvr-1 less efficiently than they use the amphotropic receptor Ram-1.

Since the properties of 10A1 pseudotype vectors may differ for cells from different individuals or tissue sources, we performed interference analyses on additional human cell lines from different individuals and tissues, including 293 embryonic kidney cells (Table 4) and IB3 airway epithelial cells (data not shown). In both cases, the results were similar to those of experiments with HFF and indicate that 10A1 pseudotype vectors can use both Ram-1 and Glvr-1 for cell entry but use the GALV receptor less efficiently.

In a final experiment, we performed interference analysis with the same pseudotyped LAPSN vectors on NIH 3T3 cells (Table 5). Note that GALV does not infect murine cells and therefore the interference properties of GALV could not be tested. 10A1 virus blocked transduction by amphotropic pseudotype LAPSN(PA317) vector, showing that 10A1 binds to and presumably can enter cells by using the amphotropic

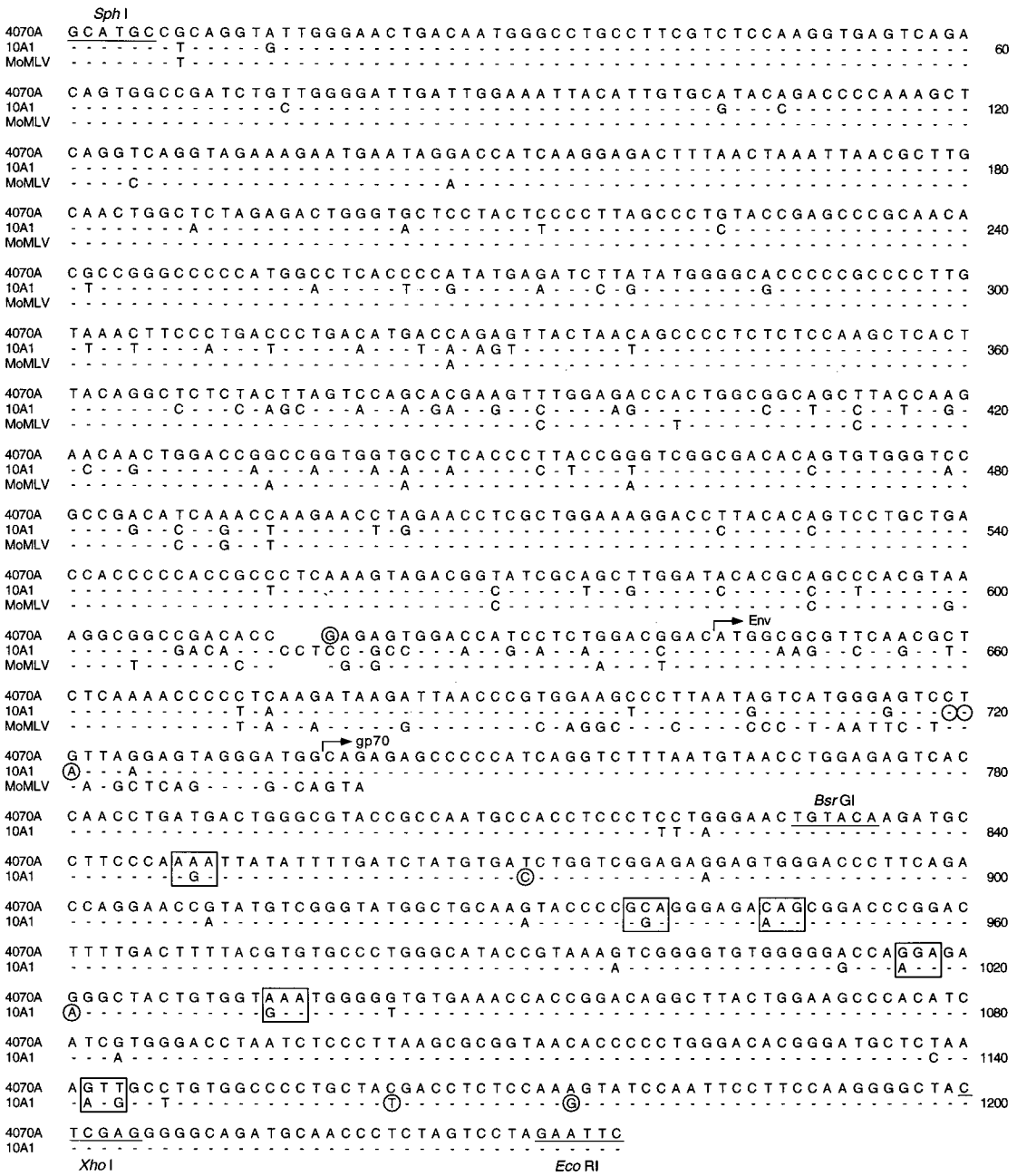

FIG. 2. Sequence analysis of 10A1 and 4070A *pol-env* regions. 10A1 virus was sequenced from an *Sph*I site in the *pol* gene to the *Eco*RI site in the *env* gene. 4070A amphotropic virus was sequenced from an *Sph*I site in the *pol* gene to the *Pfl*MI site 12 bases upstream of the *env* start codon. The rest of the 4070A (29) and the MoMLV (34) sequences depicted were obtained from GenBank and checked against the original publications. The entire sequence of 4070A is shown. For the other sequences, dashes indicate bases that are identical to those in 4070A while letters indicate base differences. Blank spaces in the 4070A and MoMLV sequences indicate the position of a 3-base insertion in 10A1 virus. MoMLV bases after base 737 are not shown because the following sequence was very divergent from those of 4070A and 10A1 and could not be meaningfully aligned. Circled bases indicate sequence differences between our data and previous sequence analysis of 4070A and 10A1 (29). Boxes indicate codons within gp70 that encode different amino acids in 10A1 and 4070A viruses and that determine the different receptor utilization patterns of the two viruses. The Env start codon and the beginning of the mature gp70 Env protein produced following cleavage of the leader sequence are indicated by arrows.

receptor. The LAPSN(10A1) vector had the same titer on NIH 3T3 cells or NIH 3T3 cells previously infected with AM-MLV, showing that a 10A1 pseudotype vector can enter cells by using a receptor other than the amphotropic receptor. Cells infected with AM-MLV or 10A1 virus were fully susceptible to transduction by the ecotropic virus LAPSN(PE501), which uses a different receptor for entry from that used by the amphotropic or 10A1 viruses, showing that inhibition of LAPSN(PA317) and LAPSN(10A1) transduction in these cells was due solely to receptor blockage and not to some general toxicity of the replication-competent viruses. These results and previous results showing that the mouse homolog of the human GALV receptor can function as a receptor for GALV-pseudotype vectors in CHO cells (26) indicate that 10A1 pseudotype vectors can efficiently transduce NIH 3T3 murine cells by using Glvr-1 as a receptor for cell entry. Because 10A1 virus can

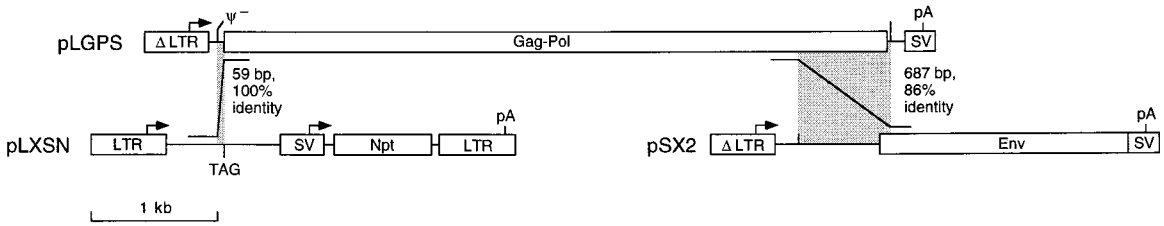

FIG. 3. Areas of overlap between retroviral vector sequences and the Gag-Pol and Env expression plasmids in the 10A1 packaging cells that could facilitate the generation of replication-competent retrovirus by homologous recombination. Possible recombination events with the LXSN vector (24) are shown as a typical example. A third event to add the 3′ LTR to the end of the *env* sequences (not shown) would be required to generate helper virus, but there is no homology between *env* and vector sequences in this region. Protein-coding regions are shown as boxes, Npt indicates neomycin phosphotransferase, arrows indicate promoters, pA indicates a polyadenylation signal, ΔLTR indicates an LTR with a 5′ truncation to the *Sau*3AI site upstream of the enhancers in the LTR, SV indicates simian virus 40 sequences, ψ^- indicates a deletion of the retroviral packaging signal (21), and TAG indicates the conversion of the Gag ATG start codon to a stop codon in LXSN (24).

block transduction by an amphotropic vector, 10A1 can bind Ram-1 and presumably can also use Ram-1 as a receptor for entry into murine cells.

DISCUSSION

We have generated 10A1 pseudotype packaging cell lines that produce vectors at high titer (up to 10^7 FFU/ml) from uncloned populations of vector-transduced packaging cells. Presumably, clones that produce even higher titer can be isolated from these populations, as has been shown for other packaging cells. The assay that we have used to measure the vector titer probably underestimates the titer somewhat, because the target cells were seeded at relatively low density prior to vector exposure, and we have shown that the apparent titer of a retroviral vector is almost directly dependent on the initial cell plating density (19). In addition, these assays were performed without trypsinization and subcultivation of the cells, as is often done in assays for transfer of the *neo* gene. Thus, vector titers produced by the 10A1 pseudotype packaging cells are at least as good as the best previously described packaging cells (23). Similar high-titer packaging cell lines were obtained by cotransfection of the Env expression plasmid with either the *dhfr** or *hpt* gene, the best clones being PT67 and PT105, respectively. Thus, any dominant marker gene can be used in at least one of these two cell lines.

A total of three recombination events would be required in the 10A1 pseudotype packaging cells to generate helper virus from retroviral vector sequences and the independently transfected *gag-pol* and *env* genes. Because we included 10A1 viral sequences upstream of the *env* gene to increase vector titer and there is significant homology between this region and the 3′ end of the MoMLV *gag-pol* sequences, there is the potential for homologous recombination between these sequences (Fig. 3). There is also homologous overlap between the extended packaging signal present in the LN series of retroviral vectors (24) and the 5′ end of the *gag* gene (Fig. 3), but there is no homologous overlap between the 3′ vector sequences and 3′ *env* sequences present in the 10A1 pseudotype packaging cells. We have verified the absence of helper virus (<1 FFU/ml) in several different vector-producing 10A1 pseudotype packaging cell lines, but more sensitive assays performed on multiple large batches of vectors are required to satisfactorily address this issue. There is a very low incidence of helper virus production in packaging cell lines in which only two recombination events are necessary to generate helper virus (e.g., PA317 cells), and we predict an even lower rate of helper virus production for the 10A1-based packaging cells described here.

We previously showed that a retroviral vector pseudotyped with wild-type 10A1 virus can use human and rat Ram-1 and human and mouse Glvr-1 for efficient entry into CHO cells (26). While 10A1 pseudotype vectors are normally able to transduce CHO cells, we found that conditioned medium from CHO cells blocked the hamster receptors for 10A1 virus but did not interfere with the transduction of CHO cells expressing the receptors from the other species tested. In the presence of the CHO conditioned medium, the titer of the 10A1 pseudotype LAPSN vector was between 3×10^5 and 1×10^6 FFU/ml on CHO cells transfected with each of the four receptors and was <500 FFU/ml assayed on untransfected cells. These results contrast with results presented here that show inefficient transduction by the 10A1 pseudotype LAPSN vector in human cells in which Ram-1 is blocked by amphotropic helper virus but in which Glvr-1 is available for virus entry (Tables 3 and 4). These apparently conflicting results might be explained by high-level expression of human Glvr-1 in the transfected CHO cells and low Glvr-1 expression in the human cells studied or by differences in posttranslational modification

TABLE 2. The LAPSN vector with a 10A1 virus pseudotype can use the amphotropic receptor, but not the GALV receptor, for entry into rat 208F cells

LAPSN pseudotype	Vector titer (FFU/ml) on target cells[a]:		
	208F	208F + AM-MLV	208F + GALV
10A1	1×10^7	500	1×10^7
PA317	2×10^6	200	2×10^6
PG13	2×10^5	2×10^5	100

[a] Values are means of duplicate assays in a single experiment; the results varied by no more than 14% from the mean. The experiment was repeated twice with similar results.

TABLE 3. The LAPSN vector with a 10A1 virus pseudotype can use the amphotropic receptor, and can inefficiently use the GALV receptor, for entry into HFF

LAPSN pseudotype	Vector titer (FFU/ml) on target cells[a]:			
	HFF	HFF + AM-MLV	HFF + GALV	HFF + 10A1
PA317	2×10^6	2	2×10^6	2
PG13	3×10^5	2×10^5	20	5×10^4
10A1	6×10^6	5×10^3	6×10^6	8

[a] Values are means of duplicate assays in a single experiment; the results varied by no more than 10% from the mean (except for values of <10, for which the numbers of colonies per plate were small and varied by up to 100% from the mean). The experiment was repeated once with similar results.

TABLE 4. The LAPSN vector with a 10A1 virus pseudotype can use the amphotropic receptor, and can inefficiently use the GALV receptor, for entry into human 293 cells

LAPSN pseudotype	Vector titer (FFU/ml) on target cells[a]:			
	293	293 + AM-MLV	293 + GALV	293 + 10A1
PA317	8×10^4	40	8×10^4	5
PG13	2×10^5	2×10^5	20	1×10^4
10A1	3×10^5	8×10^3	3×10^5	2×10^2

[a] Values are means of duplicate assays in a single experiment; the results varied by no more than 20% from the mean (except for the value of <10, for which the numbers of colonies per plate were small and varied by 33% from the mean). The experiment was repeated once with similar results.

of human Glvr-1 in CHO and human cells, both of which might lead to more efficient transduction in the CHO cells compared with the human cells. However, it is still clear that a GALV pseudotype vector can efficiently transduce the human cells tested regardless of the level of expression or posttranslational modification of Glvr-1 in these cells, showing that the use of Glvr-1 by 10A1 virus is restricted in comparison with its use by GALV. The possibility that 10A1 pseudotype vector efficiently uses Glvr-1 in some human cell types remains to be determined.

MoMLV ecotropic and 4070A amphotropic retroviruses have quite different *env* sequences, reflecting the utilization of unrelated cell surface receptors by these viruses and the isolation of these viruses from different mice more than 10 years apart (14, 27, 32). The 10A1 and 4070A *env* sequences are much more similar, reflecting the similarity in receptor usage and the derivation of 10A1 by passage in mice of the 1504A amphotropic retrovirus (33), which is closely related to 4070A (6). Given this background, we were surprised to find near complete identity of 4070A and MoMLV in the region at the 3′ end of the *pol* gene, with a 178-bp region of complete identity (bases 147 to 325) (Fig. 2). Analysis of the construction history of the plasmids used for sequencing of 10A1 and 4070A strongly indicates that we did not inadvertently substitute MoMLV sequences for 4070A in our analysis. In addition, the independently sequenced 10A1 virus *pol* region is very similar to that of MoMLV, and since 10A1 was derived from an amphotropic virus related to 4070A, these data support the conclusion that the 4070A is similar to MoMLV in this region. These results are puzzling, given the rapid mutation rate of retroviruses during replication and the very different histories of isolation of MoMLV and 4070A. In addition, amphotropic viruses are thought to be exogenous viruses in mice (11), and one would expect that even if the amino acid sequence of the viral proteins were conserved, the nucleic acid sequence would diverge owing to the degeneracy of the amino acid code. Perhaps this region of *pol* in the MoMLV and 4070A viruses derives from a conserved endogenous retrovirus sequence in mice that is frequently incorporated into replicating retroviruses, perhaps by template switching during reverse transcription.

TABLE 5. The LAPSN vector with a 10A1 pseudotype can use a receptor other than the amphotropic receptor for entry into NIH 3T3 murine cells

LAPSN pseudotype	Vector titer (FFU/ml) on target cells[a]:			
	NIH 3T3	NIH 3T3 + AM-MLV	NIH 3T3 + 10A1	NIH 3T3 + MoMLV
PA317	5×10^6	40	3	4×10^6
10A1	7×10^6	6×10^6	2×10^2	6×10^6
PE501	3×10^6	2×10^6	2×10^6	40

[a] Values are means of duplicate assays in a single experiment; the results varied by no more than 14% from the mean (except for the value of <10, for which the numbers of colonies per plate were small and varied by 33% from the mean). The experiment was repeated once with similar results.

In summary, we have been able to generate retrovirus-packaging cell lines based on 10A1 MLV that have the advantages of the best amphotropic packaging cells but also produce vectors capable of using an alternative receptor (Glvr-1) for cell entry. Utilization of Glvr-1 in murine cells is efficient and may be useful for gene transfer studies in mice since no other retrovirus is known to use the murine homolog of Glvr-1 for entry, including GALV itself. Unfortunately, usage of Glvr-1 as a receptor for 10A1 pseudotype vector entry is inefficient in the human cell types studied here. The efficient use of human Glvr-1 for 10A1 vector entry into CHO cells suggests the possibility that Glvr-1 is efficiently used in some human cell types, as a result of either higher levels of Glvr-1 expression or differences in posttranslational modifications. Thus, the 10A1 pseudotype packaging cells may be useful for human gene transfer experiments and should help clarify the complicated receptor usage pattern of 10A1 virus.

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