Development of an Avian Leukosis-Sarcoma Virus Subgroup A Pseudotyped Lentiviral Vector

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We are using avian leukosis-sarcoma virus (ALSV) vectors to generate mouse tumor models in transgenic mice expressing TVA, the receptor for subgroup A ALSV. Like other classical retroviruses, ALSV requires cell division to establish a provirus after infection of host cells. In contrast, lentiviral vectors are capable of integrating their viral DNA into the genomes of nondividing cells. With the intention of initiating tumorigensis in resting, TVA-positive cells, we have developed a system for the preparation of a replication-deficient, HIV-1-based lentiviral vector, pseudotyped with the envelope protein of ALSV subgroup A (EnvA). The HIV(ALSV-A) vector retains the requirement for TVA on the surface of target cells and can be produced at titers of 5 × 10^5 infectious units (IU)/ml. By inserting the central polypurine tract (cPPT) from the HIV-1 pol gene and removing the cytoplasmic tail of EnvA, the pseudotype can be produced at titers approaching 10^9 IU/ml and can be concentrated by ultracentrifugation to titers of 10^7 IU/ml. HIV(ALSV-A) also infects embryonic fibroblasts derived from transgenic mice in which TVA expression is driven by the β-actin promoter. In addition, this lentivirus pseudotype efficiently infects these fibroblasts after cell cycle arrest, when they are resistant to infection by ALSV vectors. This system may be useful for introducing genes into somatic cells in adult mouse transgenic animals and allows evaluation of the effects of altered gene expression in differentiated cell types in vivo.

Avian leukosis-sarcoma viruses (ALSV) have the ability to infect avian cells efficiently and replicate to high titer. Mammalian cells, however, are resistant to infection by these avian retroviruses and produce undetectable levels of infectious virus when rare infections occur (32). In 1993, Bates et al. cloned the gene that encodes the receptor for subgroup A ALSV (ALSV-A), termed TVA, and demonstrated that exogenous production of TVA on the surface of mammalian cells was both necessary and sufficient for the efficient infection of mammalian cells by ALSV-A (1, 34). Since susceptibility to infection is conferred by TVA, tissue- and cell type-specific infection in vivo can be achieved by expressing TVA from cell type- or tissue-specific promoters in transgenic animals. Federspiel et al. first demonstrated the utility of this system through specific infection of myocytes in which expression of TVA was directed by the α-actin promoter (6). Subsequent studies have demonstrated that this phenomenon is not restricted to a single cell type (8, 13, 21).

We are utilizing the TVA system to generate mouse tumor models for several types of human malignancy (8). In the mouse models generated to date, the target organs are readily accessible at birth, allowing the delivery of replication-competent ALSV vectors at a time when the target cells are still actively proliferating. However, in other organ systems, such as the pancreas, the target cells are not accessible at birth and proliferate very slowly in the adult animal (5). Like other classical retroviruses, ALSV vectors require cells to be actively dividing for the establishment of a provirus to occur (16, 19, 28). Therefore, for infection of nondividing cells in vivo, a retroviral vector that can generate a provirus in the absence of cell division is required.

Lentiviruses can integrate viral DNA into the genomes of nondividing cells (16, 19, 28). Naldini et al. have previously described the generation of a replication-deficient human immunodeficiency virus type 1 (HIV-1)-based vector pseudotyped with the vesicular stomatitis virus (VSV) envelope glycoprotein (VSVG) (22, 23). This vector can be generated at titers of 10^6 infectious units (IU)/ml and can infect many species and cell types. In addition, this vector was also shown to be more effective than a VSVG pseudotyped murine leukemia virus (MLV) vector at infecting several cell lineages in adult animals in vivo (15, 22, 23). Subsequently, other HIV-based pseudotypes have been described, as well as vectors based on other lentiviruses (25, 26, 27, 30).

To expand the utility of TVA technology, we sought to develop a replication-deficient, HIV-1-based lentiviral vector, pseudotyped with the envelope glycoprotein for ALSV subgroup A, named HIV(ALSV-A). We show here that this lentiviral vector can be produced at titers greater than 5 × 10^4 IU/ml and that it is stable during ultracentrifugation and can thus be concentrated to titers of 10^7 IU/ml. This vector retains the specificity of ALSV-based vectors, infecting only those mammalian cells engineered to express TVA. Further, this vector infects primary cells from multiple mouse tissues in culture and infects cell cycle-arrested mouse embryonic fibroblasts (MEFs) that are resistant to infection by ALSV vectors. The development of this pseudotyped lentivirus vector will
allow the expansion of TVA-directed gene delivery to include nondividing and terminally differentiated cells.

MATERIALS AND METHODS

Plasmids. A self-inactivating lentiviral vector plasmid pCS-CG expressing green fluorescent protein (GFP) from an internal cytomegalovirus (CMV) promoter was used as a transfer vector (20). The packaging plasmids pCMVΔR 8.2 (encoding all accessory proteins), and pCMVΔR 8.91 (deleted for all accessory proteins) were used to express the HIV-1 gag, pol, rev, and tat gene products (22, 37). The ALSV-A envelope protein (EnvA) was expressed from plasmid pCB6WTA, and the VSVG envelope glycoprotein was expressed from plasmid pMD.G. To generate the plasmid pcs-CG-cPPT, a 118-bp fragment of the central polypurine tract was amplified from plasmid pCMVΔR 8.91 utilizing the primers cPPT 5'-GCGGCCGCCCCTCTTTCACAAAATCCCTTATCTTATCTTAATTTTGGT-GA-3' and cPPT 5'-GCGGAGATCCGTTACCTTCCAAGTCAG-3', digested with BamHI and BglII, and inserted into pc8-CG at the BsmHI site upstream of the internal CMV promoter. The plasmid pCB6WTAΔ513 was generated by amplification of EnvA from the 5'-untranslated region to codon 513 with primers EnvA 5'-GCGGCCGCTCGAGGCAGAGGAGACCACAT ACCC-3' and EnvA 3'-GCGGCCGCGTCGTCGAGATCAGATTGGCA AAGGGCAAG-3', digestion of the PCR product with Apol and BsmHI, and insertion into pc8-CG digested with the same restriction enzymes. pCB6WTA/VCT was generated by amplifying the same region of EnvA and fusing it in frame to the last 90 coding base pairs of the VSVP envelope glycoprotein gene, amplified with primers VSVG cyto (5'-GCCGCTGTCGAGGAGACCACAT ACCC-3') and VSVG 3'-GCGGGGATCCGTTACCTTCCAAGTCAGATTGGCA AAGGGCAAG-3', digestion of the PCR product with Apol and BsmHI, and insertion into pc8-CG sites of pCB6. All PCRs were performed using Pfu Turbo DNA polymerase (Stratagene) in 10% dimethyl sulfoxide under the following amplification conditions: 94°C for 5 min, followed by 30 cycles at 94°C for 45 s, 50°C for 1 min, and 72°C for 2 min.

Vector production. Replication-deficient lentiviral vectors were generated by transfection of three plasmids into 293T cells using calcium phosphate as previously described (12, 29). The packaging plasmids pMD.G (10, 22) and pCMVΔR 8.2, which encodes the HIV structural and accessory proteins; and either pCB6WTA, encoding the ALSV-A envelope glycoprotein, or pMD.G, which encodes the VSVG envelope glycoprotein—were cotransfected into 293T cells, and the viral supernatant was harvested 60 to 65 h after transfection. The collected supernatants were placed on 293 cells engineered to express TVA (293-TVA cells) and parental 293 cells. More than 95% of 293-TVA cells were infected by the EnvA pseudotyped viral particles [HIV(EnvA)], whereas the parental 293 cells were not infected (Fig. 1). However, both cell lines were equally susceptible to infection by VSVG pseudotyped HIV particles [HIV(VSVG)] (Fig. 1B). These data demonstrate that the ALSV-A envelope can pseudotype HIV-1-based vectors and that these pseudotyped vectors require the presence of TVA on the surface of target cells for infection. 293-TVA cells infected with the HIV(EnvA) vector maintained expression of the GFP cassette for greater than 10 months (data not shown).

Having demonstrated that EnvA can pseudotype an HIV vector, we next sought to determine whether HIV accessory proteins, such as vpr, vpu, and nef, affect the infectious titer of the pseudotype. Vector particles were generated with plasmid pCMVΔR 8.2, which encodes all of the accessory proteins, or pCMVΔR 8.91, in which all accessory protein genes are deleted. Similar infectious titers were obtained with both constructs, suggesting that the HIV accessory proteins do not strongly influence the ability of the generated vector to infect 293-TVA cells (Table 1).

The HIV-1 central polypurine tract and altered ALSV-A envelopes increase the vector titer. The work of Naldini et al., and subsequently of several other groups, demonstrated that the HIV(VSVG) vector can be generated at titers of ca. 10⁷ to 10⁸ particles per ml, whereas the titer of the HIV(EnvA) vector was consistently less than 10⁴ IU/ml (15, 22, 36). Since the only difference between the two pseudotypes is the envelope glycoprotein and since the cytoplasmic tail of the transmembrane segment of the envelope is the only region that contacts the HIV core, we hypothesized that the cytoplasmic tail of the ALSV-A envelope might be inhibiting particle formation. We therefore generated a plasmid encoding EnvA truncated at residue 513, just beyond the membrane spanning region (pCB6 WTAΔ513), and a plasmid encoding a chimera in which the cytoplasmic tail of EnvA was replaced with that of VSVG (pCB6WTA VCT). These modified ALSV-A envelope proteins are shown in schematic form in Fig. 2A. Substitution of full-length EnvA with either of these proteins resulted in a fivefold increase in infectious titer to 1.7 × 10⁶ IU/ml as determined by serial dilution (Fig. 2C).

Recent work has demonstrated the presence of a short purine-rich sequence (cPPT) within the HIV-1 pol gene that acts to generate a “plus-strand” flap in the double-stranded viral
The mean. are from two experiments done in duplicate. The error is standard deviation from the mean.

pCMV-H9004 R8.2 All 4.6
pCMV-H9004 R8.91 None 4.3

The HIV accessory proteins are encoded by vif, vpr, vpu, and nef. The data are from two experiments done in duplicate. The error is standard deviation from the mean.

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When both the cPPT sequence and the altered envelopes were included in the vector particles, a synergistic effect was observed, with the titers increased by ca. 20-fold to 6.5 × 10^4 IU/ml (Fig. 2C). In subsequent experiments, we have achieved virus preparations with titers of 10^5 IU/ml that can be concentrated by ultracentrifugation to titers of 10^7 IU/ml after a 100-fold reduction in volume. In our experiments we routinely recover 75 to 100% of the infectious particles.

When we determined the physical titers of the ALSV-A pseudotypes by using an HIV-1 p24 ELISA, we measured 5.62 ± 0.92 ng of p24/ml for the vector generated with wild-type EnvA and minus cPPT, where 1 ng of p24 is equivalent to ca. 1,000 to 5,000 viral particles (Fig. 2C) (36). This corresponds to a physical titer >15 times higher than the infectious titer determined by serial dilution on 293-TVA cells, suggesting that the majority of p24 was present in noninfectious viral particles. In contrast, we find that the titers determined by serial dilution and p24 ELISA for the HIV(VSVG) vector were similar (data not shown), implying a high specific infectious activity. When we measured the levels of p24 in vector particles with the cPPT sequence and modified envelope proteins, we observed that these changes did not increase the physical titer, although the infectious titers of these vectors were 5- to 20-fold higher (Fig. 2C). Therefore, the insertion of the cPPT sequence into the expression cassette and the removal of the cytoplasmic tail of EnvA act to increase the specific infectivity of the HIV(ALSV-A) vector (Fig. 2C).

**Host range of HIV(ALSV-A).** Our main purpose in developing the HIV(ALSV-A) pseudotype was to generate a vector for in vivo gene delivery to stationary cells in TVA transgenic mice. We therefore tested the ability of HIV(ALSV-A) to infect primary mouse cells in culture, as well as cells from two additional nonprimate species, the rat and the chicken, to judge the host range of the pseudotype. The HIV(ALSV-A) vector was 5 to 10 times less efficient at infecting rodent cells than human 293-TVA cells and was a further 10-fold less efficient at infecting chicken cells (Table 2). In comparison, the ALSV-A vector, RCAS-GFP, infects MEFs ca. 30% as efficiently as 293-TVA cells (Table 2). In contrast to the HIV-(ALSV-A) vector, the HIV-GFP(VSVG) vector was able to infect human, rat, and chicken cells with equal efficiency, although it was only half as efficient at infecting MEFs (Table 2). It is possible that the reduced infection of MEFs observed with all vectors reflects a difference between primary cells and immortalized cell lines.

**Infection of nondividing cells by HIV(ALSV-A).** The advantage of lentiviral vectors over classical retroviral vectors is their ability to infect nondividing cells (16). We therefore examined the ability of HIV(ALSV-A) to infect cells arrested during the cell cycle. MEFs from transgenic mice carrying the β-actin TVA transgene were arrested by either gamma irradiation or colcemid treatment. Cells were infected 30 h after the induction of arrest, and cell cycle arrest was verified through propidium iodide staining and flow cytometric analysis. The HIV-(ALSV-A) pseudotype infected the arrested cells as efficiently as it did exponentially growing cells, whereas RCAS-GFP was...
only 10 to 16% as efficient at infecting arrested cells as exponentially growing cultures (Table 3). Next, we determined the ability of HIV(ALSV-A) to infect cells that had exited the cell cycle into G0. We allowed MEFs to exit the cell cycle into G0 by growing them to confluence and maintaining the cultures in 0.1% serum for 3 days after confluence. Cells were then infected with either RCAS-GFP or HIV-GFP(ALSV-A) vectors. Only HIV(ALSV-A) was able to infect the G0 cells (data not shown). Thus, like other HIV-based vectors, the ALSV pseudotype retains the ability to infect cells arrested during the cell cycle, as well as those which have exited the cell cycle into G0.

**DISCUSSION**

ALSV cannot efficiently infect mammalian cells unless these cells are engineered to express the ALSV receptor on their surface (32). The TVA system provides a mechanism to deliver genes in a tissue-specific manner in vivo (6). The gene delivery vectors currently used with this system are all ALSV-based vectors and, as such, have an absolute requirement for cell division for the establishment of infection (16, 19, 28). Indeed, in their initial test of the TVA system in vivo, utilizing /H9251\-actin TVA transgenic mice, Federspiel et al. found that the ability of the ALSV vector to infect cells in the myocyte lineage dramat-

**TABLE 2. Relative infection efficiency and host range of RCAS-GFP, HIV-GFP(ALSV-A), and HIV-GFP(VSVG) vectors**

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell line</th>
<th>Relative infection efficiencya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>RCAS-GFP</td>
</tr>
<tr>
<td>Human</td>
<td>293-TVA</td>
<td>1.00</td>
</tr>
<tr>
<td>Mouse</td>
<td>β-Actin TVA MEF</td>
<td>0.31</td>
</tr>
<tr>
<td>Rat</td>
<td>Rat1a TVA</td>
<td>0.76</td>
</tr>
<tr>
<td>Chicken</td>
<td>DF-1</td>
<td>ND</td>
</tr>
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</table>

a For each vector, the infection efficiency for 293-TVA cells is set to 1. All other values are displayed relative to this infection efficiency. The data are from a single representative experiment done in triplicate. ND, not determined.
We have described here the production of a replication-deficient, ALSV-A pseudotyped HIV-1 vector, HIV(ALSV-A). This virus, like the HIV(VSVG) pseudotype, is not produced in the target cells and therefore does not spread to infect neighboring cells. We have demonstrated that infection by HIV(ALSV-A) requires the presence of TVA on the surface of mammalian cells. Thus, using TVA transgenic mice, HIV(ALSV-A) infection can be cell type or tissue restricted in vivo. HIV(ALSV-A) can be generated at infectious titers approaching 10^9 IU/ml and is stable during ultracentrifugation and can therefore be concentrated to titers of 10^7 IU/ml. This titer should be adequate for many in vivo and in vitro applications, particularly in circumstances in which ALSV vectors are ineffective because cells are in a resting state. Our data suggest, at least for 293 cells, that the absence of the HIV accessory proteins does not affect the ability of the pseudotype to infect these cells. However, an extensive study of multiple cell types was not conducted, and it is possible that there are cell types in which specific HIV-1 accessory proteins may affect infection efficiency. Indeed, there are conflicting reports in the literature concerning the requirement of the accessory factors in certain cell types (2, 15, 24).

The ability of EnvA to pseudotype the HIV vector is poor relative to the VSVG envelope glycoprotein, as demonstrated by its relatively low physical and infectious titers. While several heterologous envelopes, such as those of MLV, can pseudotype HIV-based vectors, others, such as that of gibbon ape leukemia virus (GALV), have failed to effectively pseudotype HIV-1-based vectors (27, 30; N. Chinnasamy and R. A. Morgan, unpublished data). We were able to increase the infectious titer of the ALSV pseudotype fivefold by using altered ALSV-A envelope proteins without increasing vector particle formation, suggesting that there are conformational constraints present in wild-type EnvA pseudotyped vectors that prevent proper interaction with receptors. Consistent with this idea, Stitz et al. recently reported that while wild-type GALV envelope cannot form an infectious pseudotype, chimeric GALV or MLV envelopes are capable of forming an infectious HIV-based pseudotype (30).

The HIV(ALSV-A) vector demonstrated the ability to infect cells of human and rodent origin, as did the ALSV-A vector RCAS-GFP. Although the titers for RCAS-GFP and HIV-GFP(ALSV-A) were determined by using different methods, an approximate comparison of the efficiencies of infection can be made. We found that, while both vectors infect 293-TVA cells with similar efficiency, RCAS-GFP is three times more efficient than HIV-GFP(ALSV-A) at infecting dividing MEFs. However, we found that this difference is not apparent for all cell types. Astrocytes from transgenic mice in which TVA expression is driven by the nestin promoter are infected at similar efficiency by both vectors. The HIV(ALSV-A) vector infects chicken cells poorly relative to its ability to infect cells of human and rodent origin. In contrast, we find that an HIV (VSVG) pseudotype is able to infect the chicken cell line DF-1 as efficiently as it does cells of human and rodent origin. One possible reason for the discrepancy between the two pseudotypes may lie in the different entry mechanisms of VSV and ALSV. ALSV utilizes glycoprotein receptors on the membrane surface, whereas VSV has been shown to fuse directly with membrane phospholipids (1, 17, 31, 33, 34). However, we have not formally investigated whether there are differences in other steps during the establishment of infection. Another possible explanation for the difference may be that endogenous TVA expression on DF-1 cells is very low in comparison to that generated in the 293 cell line and MEFs by the potent CMV and ß-actin promoters.

We have further demonstrated in this work that HIV(ALSV-A) can readily infect primary murine cells that are arrested in the cell cycle or have exited the cell cycle. MEFs treated with either colcemid or gamma irradiation to induce cell cycle arrest were far less susceptible to infection by ALSV vectors but were equally susceptible to infection by HIV (ALSV-A) relative to logarithmically growing cells. Thus, the HIV(ALSV-A) pseudotype has the characteristic ability of other lentiviral vectors to infect nondividing cells.

Currently, the TVA system and ALSV vectors are used predominantly to generate mouse tumor models. Recently, however, Doetsch et al. and Murphy and Leavitt have used this system to monitor precursors of the glial and megakaryocyte cell lineages, respectively (4, 21). Previous studies have demonstrated effective gene delivery to liver, brain, and lung epithelia and to cells of the hematopoietic lineage by HIV (VSVG), illustrating that lentivirus vectors can deliver genes efficiently to nondoning dividing cells in vivo (3, 11, 14, 15, 18, 22, 23). These studies indicate that the HIV(ALSV-A) pseudotype should also provide an effective means of gene delivery to stationary cells in vivo. Thus, the development of the HIV (ALSV-A) pseudotype provides another valuable tool for groups interested in manipulating nondoning and terminally differentiated cells in vivo as a means to dissect molecular signaling and developmental pathways in these cells.

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REFERENCES


2. Chinnasamy, D., N. Chinnasamy, M. J. Enriquez, M. Otsu, R. A. Morgan,