Packaging Cell Lines for Simian Foamy Virus Type 1 Vectors

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Foamy viruses are nonpathogenic retroviruses that offer several unique opportunities for gene transfer in various cell types from different species. We have previously demonstrated the utility of simian foamy virus type 1 (SFV-1) as a vector system by transient expression assay (M. Wu et al., J. Virol. 72:3451–3454, 1998). In this report, we describe the first stable packaging cell lines for foamy virus vectors based on SFV-1. We developed two packaging cell lines in which the helper DNA is placed under the control of either a constitutive cytomegalovirus (CMV) immediate-early gene or inducible tetracycline promoter for expression. Although the constitutive packaging expressing cell line had a higher copy number of packaging DNA, the inducible packaging cell line produced four times more vector particles. This result suggested that the structural gene products in the constitutively expressing packaging cell line were expressed at a level that is not toxic to the cells, and thus vector production was reduced. The SFV-1 vector in the presence of vesicular stomatitis virus envelope protein G (VSV-G) produced an insignificant level of transduction, indicating that foamy viruses could not be pseudotyped with VSV-G to generate high-titer vectors. The availability of stable packaging cell lines represents a step toward the use of an SFV-1 vector delivery system that will allow scaled-up production of vector stocks for gene therapy.

Foamy viruses have several characteristics that make them ideal for developing a viral vector system for gene transfer. These viruses are found in several mammalian species (12), and no disease has been associated with foamy virus infection (12,31). Furthermore, foamy viruses can be propagated in a variety of cell types from different species both in vivo and in cell culture systems (12,20). Recently, several groups have demonstrated the utility of foamy virus vector (21,25,27). Like human immunodeficiency virus (HIV) DNA, foamy virus DNA can enter the nucleus of G1/S-phase-arrested cells (26). A comparative gene transfer study with murine leukemia virus (MuLV)- and human foamy virus (HFV)-based vectors indicated that HFV replicates better than MuLV in stationary-phase culture (25). This observation suggests efficient gene transduction with foamy virus vectors in nonreplicating cells. Furthermore, the efficiency of transduction in primate hematopoietic cells by foamy virus vectors compared favorably with results obtained for MuLV vectors (11). Conversely, others have reported that HFV productive infection is cell cycle dependent (3). The reasons for these contradictory results are not clear. Recently, we identified a cis-acting element located in the pol gene that is critical for simian foamy virus type 1 (SFV-1) vector transduction (32). A cis-acting element in the corresponding region of the HFV pol gene is also required for vector construction (5,10). The necessity of sequences in the pol gene for vectors has not previously been found for retroviruses.

Foamy virus infections result in marked cytopathology in a variety of cultured cell lines (20,22). The cytopathic effect of foamy virus infection is characterized by formation of intracellular vacuoles in multinucleated giant cells and in some cases balloon formation. The mechanism by which foamy viruses kill cells remains unresolved, although Mergia et al. have reported that SFV-1 induces apoptosis of infected cells (19). Retroviral envelope proteins can induce syncytium formation, creating multinucleated giant cells (16,28). The cytopathic effect of foamy virus infection also includes cell fusion, potentially mediated by the interaction of the env gene product and cell receptor. Establishing a packaging cell line containing the env gene can, therefore, be an inherent obstacle for foamy virus vector development. Transient vector production is easily attainable with an SFV-1 vector (32). However, this limits large-scale vector production. In this report, we describe the development of the first packaging cell lines for an SFV-1 vector in which the structural genes are under the control of a constitutive or inducible promoter for expression. These stable packaging cell lines will be advantageous to scale up SFV-1 vector stocks for gene therapy.

Plasmid constructs. All recombinant DNA manipulations were performed by standard techniques (17). Plasmids were derived from an infectious proviral DNA clone of pSFV-1 (21). The constructions of pV7-9 and pV7-5 have been described previously (32). pCV7-9 was derived by replacing the U3 domain (except the last 36 bases) of the SFV-1 long terminal repeat (LTR) with the cytomegalovirus (CMV) immediate-early gene promoter (Fig. 1). To generate plasmid pCGP, synthetic DNA containing the 5’ end of SFV-1 splice donor sequence (positions 1336 to 1357) was cloned into the pCI vector (Promega, Madison, Wis.) downstream the CMV promoter. The coding sequence of the gag-pol region of SFV-1 was cloned at the SmaI cloning site. For selection, the hygromycin resistance gene under the control of the TK (thymidine kinase) promoter and poly(A) signal was removed from the pCEP vector (Invitrogen, San Diego, Calif.) and subcloned into the SaI site of plasmid pCPG, generating the SFV-1 envelope expression plasmid pCPG. The following methods were used to prepare the SFV-1 envelope expression plasmid pSVE by digestion with restriction enzymes EcoRI and ScaI and cloned into pCLMFG-LacZ and pCLMFG-GalsK, which were obtained from Jing-Kuan Yee (City of Hope, Duarte, Calif.) and Inder M. Verma.

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The promoter (CMV prm). The used in this study. In vector pCV7-9, the U3 domain is replaced with the CMV splice donor and acceptor sites in SFV-1 splice donor (SD) was cloned downstream from the CMV promoter. The 40 poly(A) signal (SV40 poly A). A synthetic DNA containing the 5' genes were placed under the control of the CMV promoter and the simian virus internal CMV promoter. In the packaging construct pCGPETH, the structural genes were placed under the control of the inducible tetracycline promoter (Tet prm). pCGP and pSE are pTGPETH is similar to pCGPETH except that the SFV-1 genes are under the control of the CMV promoter as depicted in Fig. 1 (pCGPETH).

We have Transient expressions to generate SFV-1 vectors. To generate a replication-defective helper packaging plasmid, the structural and the transactivator genes of SFV-1, restriction enzymes SphI and SalI were introduced in pUHD 10-3 downstream from the tetracycline operator (tetO) sequences in the multiple cloning sites. A fragment containing the SFV-1 5' splice donor site, the structural genes, and the transactivator gene was obtained from plasmid pCGPET by digestion with restriction enzymes SphI and SalI and cloned into the pUHD 10-3 vector downstream from the CMV promoter. The splice donor and acceptor sites in gag-pol-env-tas were retained. For selection, an expression cassette containing the TK promoter driving expression of the hygromycin resistance gene (Hyg) was included in the packaging construct. Plasmid pTGPETH is similar to pCGPETH except that the SFV-1 genes are under the control of the inducible tetracycline promoter (Tet prm). pCGP and pSE are packaging constructs containing SFV-1 gag-pol and env, respectively.

FIG. 1. Schematic representation of SFV-1 vector and packaging constructs used in this study. In vector pCV7-9, the U3 domain is replaced with the CMV promoter (CMV prm). The β-galactosidase reporter gene is expressed from the internal CMV promoter. In the packaging construct pCGPETH, the structural genes were placed under the control of the CMV promoter and the simian virus 40 poly(A) signal (SV40 poly A). A synthetic DNA containing the 5' end of the SFV-1 splice donor (SD) was cloned downstream from the CMV promoter. The splice donor and acceptor sites in gag-pol-env-tas were retained. For selection, an expression cassette containing the TK promoter driving expression of the hygromycin resistance gene (Hyg) was included in the packaging construct. Plasmid pTGPETH is similar to pCGPETH except that the SFV-1 genes are under the control of the inducible tetracycline promoter (Tet prm).

| TABLE 1. Titers of pCV7-9 vector packaged in cell lines exhibiting constitutive and inducible expression |
| --- | --- | --- | --- |
| Cell line | Titer (vector particles/ml) | Titer (vector particles/ml) |
| | | +Dox | +Doxa |
| 293-3 | (3.5 ± 1.3) × 103 | <0.1 | (1.3 ± 0.3) × 103 |
| 293-9 | (2 ± 1.7) × 104 | <0.1 | (6.2 ± 0.1) × 103 |
| 293-10 | (2.3 ± 0.4) × 103 | <0.1 | (2 ± 1.1) × 102 |
| 293-11 | (3.8 ± 0.3) × 102 | <0.1 | (4 ± 1.1) × 101 |

a Mean ± standard deviation for duplicate determinations.

b To induce the tetracycline promoter, cells were exposed to 15 ng of Dox per ml unless indicated otherwise.

c Titer obtained at a higher Dof concentration (>25 ng/ml).
transfected cells had a vector titer of 2.0 and fractionated into the 293-3 cell line. Supernatant harvested from the plasmid constructs of pCGPETH used to establish the 293-3 cell line; 1 and 4, DNA samples digested with restriction enzymes EcoRI and Asp718; 2 and 5, DNA samples digested with restriction enzyme EcoRI; 3 and 6, DNA samples digested with restriction enzymes Asp718 and SalI. Sizes of the restriction enzyme digest products are shown at the left and right.

Asp Eco

DNA samples digested with restriction enzyme Asp718 and SalI resulted in detection of two bands of 3.2 and 12.4 kb, whereas in the DNA from the 293-3 cell line the fragments were shifted to greater molecular sizes and appeared as a long smeared band, suggesting that the helper DNA was integrated at multiple sites of the genome. To demonstrate that the packaging cell line expresses all of the SFV-1 structural proteins, a smeared band, suggesting that the helper DNA was integrated at multiple sites of the genome. To demonstrate that the packaging cell line expresses all of the SFV-1 structural proteins, a vector with deletions in the gag, pol, and env genes was transfected into the 293-3 cell line. Supernatant harvested from the transfected cells had a vector titer of $2.0 \times 10^2$, indicating that the packaging cell line expresses the SFV-1 structural proteins. The packaging cell line was maintained in cell culture for over 4 months, and we observed no cytopathic effect due to continuous expression of the SFV-1 structural genes. Consistent with our result, HIV packaging cell lines constitutively expressing structural genes were established (4, 30). The success with the HIV packaging cell lines was attributed to creating the cell line by introduction of a selectable marker that was placed in plasmids separate from the ones expressing the structural genes (30). This presumably allowed the selection of cells producing tolerable levels of structural proteins, since it enables coexpression. Our construct, however, contained both the selectable marker and structural genes in one plasmid, where the 293-3 cell line allowed a tolerable level of viral protein expression.

**Inducible packaging cell line.** The constitutive expression of the structural protein of SFV-1 in the packaging cell line may contribute to cell death and may allow survival only of cells that express the structural genes at a tolerable level. This subsequently may reduce vector production. To remedy this potential problem, we replaced the CMV promoter with the inducible tetracycline promoter to limit the expression of the SFV-1 structural genes to the time of vector production. The tetracycline system involves the use of a trans-acting factor (rtTA) formed by fusion of the activation domain of HSV protein VP16 to the *Escherichia coli* tetracycline repressor protein (8). A promoter containing the *tetO* sequence can be stimulated by the rtTA transactivator (7, 9). To establish a tetracycline-inducible SFV-1 packaging cell line, the plasmid containing SFV-1 structural genes under control of the *tetO* promoter (pTGPETH) was cotransfected with rtTA (pUHG 17-1) into 293 cells. Hyg' cells were selected and screened for the presence of SFV-1 structural genes and rtTA by PCR. Positive cells were tested for SFV-1 vector production in the presence of the tetracycline analogue doxycycline (Dox). The level of SFV-1 vector production in the established cell line ranged from $4 \times 10^2$ to $1.3 \times 10^3$ particles/ml at a Dox concentration of 15 ng/ml (Table 1). No transducible vector was detected from these cell lines in the absence of Dox. The effect of vector production by a range of concentrations of Dox was tested with clone 293-24, which produced the highest level of vector. At a Dox concentration of 25 to 100 ng/ml, a higher titer of SFV-1 vector ($1.1 \times 10^3$ vector particles/ml) was obtained. This titer is fourfold higher than that produced by the constitutively expressing packaging cell line 293-3. The inducible cell line can also package the genome of the SFV-1 vector with deletions in *gag*, *pol*, and *env* sequences (p75-5-4.3k), generating vector particles with a titer of $9.0 \times 10^3$, indicating that the cell line expresses the SFV-1 structural proteins. Interestingly, a quantitative PCR analysis of DNA from 293-24 and 293-3 cells revealed that the copy number of packaging DNA in the constitutively expressing cell line was higher than that of DNA isolated from the inducible cell line (Fig. 3). To quantitatively measure packaging DNA in the two cell lines, a known concentration of serially diluted competitor DNA was used in a PCR as described previously (18). For DNA from the inducible cell line, the competitive DNA decreased with equivalency points of the competitive and target products at approximately $6.4 \times 10^{-3}$ ng, whereas the equivalency points for the DNA from the constitutively expressing cell line were at 0.8 ng. The packaging DNA copy number in the inducible DNA is at least 25 times less than that of the 293-3 cell line. This inverse relation of copy number of the packaging DNA and vector transduction in the two packaging cell lines indicated a better level of helper viral protein expression in the inducible packaging cell line than in the 293-3 cell line. The tetracycline-inducible promoter, therefore, provides an advantage over the constitutively expressed promoter for creating packaging cell lines.

**Pseudotyping SFV-1 vector system with VSV-G.** The vector titers obtained with the packaging cell lines that we created were comparable to the titers of HIV vectors reported for HIV packaging cell lines. Recent advances in vector construction have allowed the development of a system which involves packaging of retroviral vectors into envelopes containing vesicular stomatitis virus envelope glycoprotein G (VSV-G). With this system, higher transduction levels were achieved with the same retroviral vector.
with an MuLV or HIV backbone when pseudotyped with VSV-G (1, 34). Furthermore, retroviral vector particles containing VSV-G envelopes are less fragile than retroviral envelopes, allowing high pseudotyped vector particle concentrations to be attained by centrifugation without loss of infectivity (34). To enhance the titer of the SFV-1 vector, we attempted to pseudotype the SFV-1 vector backbone with VSV-G. We transfected pCGP, pCV7-9, and pHCMV-G (VSV-G-expressing plasmid) into 293 cells to determine if high-titer SFV-1 vector can be obtained. The pCGP and pCV7-9 constructs did not contain the SFV-1 envelope (Fig. 1). Therefore, retroviral vectors generated from pCGP and pCV7-9 showed no infectious particles. Similarly, supernatant harvested from a MuLV vector (pCLMFG-LacZ) transfected into the established 293GP cell line (kindly provided by Sybille L. Sauter, Chiron, Inc., San Diego, Calif.) containing the MuLV gag-pol genes contained no infectious virus particles. A very high (>5 × 10⁶ particles/ml) virus particle titer was obtained from 293GP cells transfected with MuLV vector and pHCMV-G. In contrast, when the SFV-1 vector (pCV7-9 and pCGP) was pseudotyped with VSV-G, a titer of 39 particles/ml was observed when the SFV-1 envelope (pSE [Fig. 1]) instead of VSV-G was supplied in trans. These results suggested that foamy viruses could not be pseudotyped with VSV-G to generate a high-titer vector. Consistent with our data, it has been reported that foamy viruses cannot release particles without co-expression of their envelope protein (2, 6). This observation may support the notion that foamy viruses are unique among retroviruses with respect to particle assembly.

Foamy viruses have several inherent features that make them ideal vectors for gene transfer; in particular, these viruses do not cause diseases and have a broad host range with respect to species and cell types. The development of the first stable packaging cell lines for foamy virus vector represents a step toward the use of an SFV-1 vector delivery system and will be advantageous for scaled-up production of vector stocks for gene therapy. These packaging cell lines allow vector production comparable to that of the HIV vectors without the application of any titer enhancement methods. Preliminary transient expression assays show that higher-titer SFV-1 vector production can be achieved in 293T cells (5 × 10⁶ vector particles/ml) than in 293 cells (2.4 × 10⁶ vector particles/ml) (33), which suggests that a stable packaging cell line producing higher-titer vector can be created with the 293T cell line. Furthermore, our transient expression assay showed efficient vector production with packaging DNAs that lack the 5′ and the gag-pol and envelope genes under separate transcriptional units. Therefore, a packaging cell line that yields high-titer vector particles but would not generate replication-competent virus can be established by using 293T cells.

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