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 correlated 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 G0/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+4.3k 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 in 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 3′ end of the splice donor. To construct pCGPET, the sequence containing the env and las genes from positions 6496 to 11208 was excised from pSFV-1 by digestion with restriction enzymes EcoRI and SacI and cloned into EcoRI and Smal sites of pCGP, generating the SFV-1 structural and transactivator gene expression cassette. 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 SalI site of plasmid pCGPET to obtain pCGPETH. The SFV-1 envelope expression plasmid pSE was constructed by placing the env gene from positions 6989 to 10207 into an expression vector containing the simian virus 40 early gene promoter. Plasmids pHCMV-G and pCLMFG-LacZ were obtained from Jing-Kuan Yee (City of Hope, Duarte, Calif.) and Inger M. Verma.
driven gene expression (24). Therefore, when an SFV-1 vector with the two LTRs is transfected into a packaging cell line, the tas gene product activates gene expression by the viral LTR. To improve SFV-1 vector production, we have replaced the U3 domain of the LTR with the CMV promoter and constructed a vector containing the lacZ gene (pCV7-9 [Fig. 1]). The efficiencies of lacZ gene transduction by the vectors pV7-9 and pCV7-9 were compared by using the pCGPETH helper plasmid in 293 cells. The pV7-9 vector produced a titer of $1.3 \times 10^3$ infectious particles/ml, as observed previously (32). Like the infectious clone pSVF-1, pCGPETH provided functions comparable to those of a helper plasmid. The SFV-1 vector where the U3 domain of the LTR was replaced with the CMV promoter showed a 10-fold increase ($2 \times 10^4$ particles/ml) in lacZ gene transduction. We observed a similar level of lacZ gene transduction when the vector titer was determined by infecting simian fibroblast (Cos-7) and feline fibroblast (CRFK) cell lines. As with other retroviral vector systems (14, 23, 29), transient higher-titer vector production can be achieved in the SFV-1 system by using the CMV promoter and a 293 cell line.

**Packaging cell lines that constitutively express the SFV-1 structural proteins.** Constitutive expression of the cytoplasmic retrovirus envelope protein is toxic to cells (16, 28). The protease of HIV has also been implicated in toxic effects of infected cells (13, 15). These observations indicate the difficulties of obtaining a stable packaging cell line with retroviruses that induce cytopathology. To develop a stable packaging cell line for SFV-1 vectors, we used plasmid pCGPETH, containing the SFV-1 structural genes and the tas gene. The plasmid was linearized with restriction enzyme SalI and transfected into 293 cells by a liposome-mediated method using Lipofectamine reagent (Life Technologies, Inc., Gaithersburg, Md.). Hygromycin-positive (Hyg') colonies were isolated and screened for the presence of the structural and tas genes by PCR (data not shown). We identified 13 Hyg' cells that were positive by PCR analysis. PCR-positive cells were further screened for the ability to package and transduce SFV-1 vectors containing the tas gene. Of the 13 PCR-positive cells, 4 were able to transduce SFV-1 vectors as determined by β-galactosidase assays (Table 1). The titers varied from $2.2 \times 10^3$ to $2.4 \times 10^3$ vector particles/ml. Cell line 293-3, which produced the highest number of SFV-1 vector particles, was further characterized. The integration pattern of the helper DNA in 293-3 packaging cells was assessed by Southern blot analysis of DNA. Digestion of DNA with EcoRI or EcoRI/Asp718 revealed the expected 4.7- and 5.2-kb or 1.5-, 3.7-, and 4.7-kb bands, respectively (Fig. 2). Southern analysis of plasmid pCGPETH revealed DNA fragments of the same sizes. Treatment of pCGPETH with Asp718

![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). pCGP and pSE are packaging constructs containing SFV-1 gag-pol and env, respectively.](http://jvi.asm.org/)
transfected cells had a vector titer of 2.0 
fected into the 293-3 cell line. Supernatant harvested from the 
DNA samples digested with restriction enzymes 
plasmid constructs of pCGPET 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 Asp718 and SalI. Sizes of the restriction en-
zeyme digest products are shown at the left and right.

Asp
Sal

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 
smear, suggesting that the helper DNA was integrated 
at multiple sites of the genome. To demonstrate that the pack-
caging cell line expresses all of the SFV-1 structural proteins, a 
vector with deletions in the gag, pol, and env genes was trans-
sected 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 continu-
ous 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 

FIG. 2. (A) Restriction enzyme map of pCGPET and probe used for Southern analysis. (B) Southern blot analysis of DNA isolated from packaging cell line 293-3. Lanes: 1 to 3, DNA samples obtained from 293-3 cells; 4 to 6, packaging 

pol 
gag 
env

zyme digest products are shown at the left and right.

FIG. 3. Quantitation by competitive PCR of packaging DNA from cell lines exhibiting constitutive and inducible expression. Serial fivefold dilutions of compet-
itor molecules ranging from 100 (lane 1) to 1.2 \times 10^{-3} (lane 8) ng were added to the reaction mixture containing 0.5 µg of DNA isolated from either the inducible (A) or constitutively expressing (B) cell line. A set of primers for the 
PCR was selected from the tetO region. The primers amplify both the competitor 
DNA molecules (401 bp) and the target molecules (697 bp).

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 10^4 to 1.3 \times 10^5 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 induc-
ible cell line can also package the genome of the SFV-1 vector 
with deletions in gag, pol, and env sequences (pV7-5+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 equiva-

cence points of the competitive and target products at approx-
imately 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 pack-
aging cell line than in the 293-3 cell line. The tetracycline-induc-
bile promoter, therefore, provides an advantage over the consti-
tutively 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 pack-
caging cell lines. Recent advances in vector construction have al-
lowed the development of a system which involves packaging of 
retroviral vectors into envelopes containing vesicular stomatitis 

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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 pCGG, pC7V-9, and pHMCMV-G (VSV-G-expressing plasmid) into 293 cells to determine if high-titer SFV-1 vector can be obtained. The pCGG and pC7V-9 constructs did not contain the SFV-1 envelope (Fig. 1). Therefore, retroviral vectors generated from pCGG and pC7V-9 showed no infectious particles. Similarly, supernatant harvested from an 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 × 10⁸ particles/ml) virus particle titer was obtained from 293GP cells transfected with MuLV vector and pHMCMV-G. In contrast, when the SFV-1 vector (pC7V-9 and pCGG) was pseudotyped with VSV-G, a titer of 39 particles/ml was obtained. This value was significantly lower than the titer of 3 × 10⁷ particles/ml 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 × 10⁷ vector particles/ml) than in 293 cells (2.4 × 10⁸ 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 las gene 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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REFERENCES


