Foamy Virus Vectors

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Human foamy virus (HFV) is a retrovirus of the spumavirus family. We have constructed vectors based on HFV that encode neomycin phosphotransferase and alkaline phosphatase. These vectors are able to transduce a wide variety of vertebrate cells by integration of the vector genome. Unlike vectors based on murine leukemia virus, HFV vectors are not inactivated by human serum, and they transduce stationary-phase cultures more efficiently than murine leukemia virus vectors. These properties, as well as their large packaging capacity, make HFV vectors promising gene transfer vehicles.

The three major subfamilies of retroviruses are oncoviruses, such as murine leukemia virus (MLV); lentiviruses, such as human immunodeficiency virus (HIV); and foamy viruses or spumaviruses (51). Foamy viruses have been isolated from many vertebrate species, including primates, and are not known to cause disease (14), even in accidentally infected research personnel (47). Previous reports of human diseases associated with foamy virus infection have not been confirmed by an extensive analysis of the prevalence of foamy viruses in human populations (47). Human foamy virus (HFV) was originally derived from a human nasopharyngeal carcinoma cell line (1); however, subsequent studies indicate that it may be a chimpanzee virus variant (3, 47).

Foamy viruses have the largest genome size of all the retroviruses (9). The HFV genomic RNA is 11,021 nucleotides in length and, in addition to the *gag*, *pol*, and *env* genes found in all retroviruses, contains three open reading frames (*bel1* to *bel3*) downstream from the *env* gene that are unique to spumaviruses (9, 10, 25) (see Fig. 1). Multiple splicing patterns predict that as many as six gene products are encoded by the *bel* reading frames (32), one of which (Bel1) is a transactivator of transcription from the viral long terminal repeat (LTR) promoter (17, 40, 49). The HFV genome also contains a second promoter in the *env* gene that directs transcription of the *bel1* gene early in infection (20, 21).

HFV has been cloned as an infectious provirus (22, 39), and a recent report describes the construction of replicating foamy virus vectors with deletions in the *bel2-3* region (45). We have constructed replication-incompetent transducing vectors by replacing a portion of the *env* gene and *bel1* promoter with reporter genes. These vectors can be packaged as viral particles by cotransfection with helper plasmids. In this work, we have studied the transducing properties of HFV vectors and compared them with vectors based on MLV. Our results suggest that HFV vectors offer several potential advantages over currently available viral vectors and that they may prove useful for a wide variety of gene transfer applications.

MATERIALS AND METHODS

Cell culture. The cultured cells used included normal human foreskin fibroblasts (34), human 293 cells (11), COS-7 cells (simian virus 40 [SV40]-transformed African green monkey kidney cells; ATCC CRL 1651), Vero African green monkey kidney cells (ATCC CCL 81), MDBK bovine kidney cells (ATCC CCL 22), sheep choroid plexus cells (ATCC CRL 1700), Cf2Th canine thymus cells (ATCC CRL 1430), CCC-81 cat cells transformed with Moloney murine sarcoma virus (8), AH 927 cat cells (from J. Overbaugh; originally from W. Nelson-Rees), NRK normal rat kidney cells (6), 208F rat fibroblasts (37), NIH 3T3 TK⁻ mouse fibroblasts (50), PA317 mouse amphotropic MLV vector packaging cells (28), CHO-K1 Chinese hamster ovary cells (16), BHK21 baby hamster kidney cells (24), FAB hamster HFV β-galactosidase indicator cells (53), and primary White Leghorn chicken embryo fibroblasts (from P. Neiman). Unless otherwise indicated, all cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) at 37°C in 10% CO_2. FBS was heat inactivated for 30 min at 56°C except as indicated in the legend to Fig. 5. Nonessential amino acids were added to CHO-K1 cultures.

Stationary-phase 208F and human fibroblast cultures were prepared by maintaining confluent cultures for 2 to 3 weeks in DMEM containing 5% FBS and 10^{-6} M dexamethasone as described before (30, 43). Dividing cultures were prepared by treating them with trypsin and plating the cells at a density of either 2.5×10^5 cells per 35-mm well in six-well plates or 4×10^5 cells per 60-mm dish the day before infection. The percentage of cells passing through S phase every 24 h was determined by autoradiography (43).

Vector construction and production. Relevant restriction sites are shown in Fig. 1. pFdelGP is a deletion derivative of pHSRV13 (22) lacking the internal *Nco*I fragment. pFGPXhoI (not shown) is a deletion derivative of pHSRV13 made by digestion with *Bsp*EI and *Bpu* 1102, end-filling with the Klenow fragment of DNA polymerase I, and insertion of an *Xho*I linker (5′-CCTCGAGG-3′). pFGPSN was made by inserting an *Nhe*I-*Xho*I fragment of pLXSN (29) containing the SV40 early promoter and neomycin phosphotransferase gene into the *Xho*I site of pFGPXhoI. pFGPMAP was made by inserting a *Sal*I-*Bsp*MI fragment of pALAPSN (43) containing the MLV LTR promoter and the human placental alkaline phosphatase gene into the *Xho*I site of pFGPXhoI.

Vector stocks were made by calcium phosphate cotransfection of BHK21 cells with 10 μg each of vector plasmid and helper plasmid per 6-cm dish, further culture for 3 to 11 days, with medium changes every 2 to 3 days, collection of culture medium, and filtration through cellulose acetate filters (0.45-μm pore size). Stocks were stored in aliquots at −76°C. Unless otherwise indicated, stocks were prepared by transfecting with pFGPMAP and pHSRV13 (FGPMAP) or pFGPSN and pFdelGP (FGPSN). Stocks of the MLV vector LAPSN (31) were prepared in PA317 cells as described before (29).

Transduction assays. Unless otherwise indicated, all cells were plated at 2×10^5 cells per 35-mm well the day before infection, and infections were performed by adding dilutions of vector stocks in the presence of Polybrene (4 μg/ml; Sigma). Polybrene did not increase the transduction efficiency of HFV vectors (data not shown) but was included to improve transduction of control cultures by MLV vectors. Transduction of G418 resistance was performed by infecting on day 1, treating with trypsin, plating the cells at different dilutions in DMEM with 10% FBS on day 2, and adding active G418 to 0.50 mg/ml (human fibroblasts) or 0.70 mg/ml (FAB cells and 208F cells) on day 3 (human fibroblasts and 208F cells) or day 2 (FAB cells). Surviving colonies were counted after staining with Coomassie brilliant blue G on days 7 to 10, when all cells in control cultures were dead. Transduction of alkaline phosphatase was measured by histochemical staining 2 days after infection and counting individual stained foci of cells (7). Helper virus expressing the *bel1* gene was assayed in parallel with vector assays by staining FAB cells for β-galactosidase 2 days after infection (53).

RESULTS

Production of foamy virus vectors. We replaced a portion of the *env* gene of the pHSRV13 HFV provirus (22) with either

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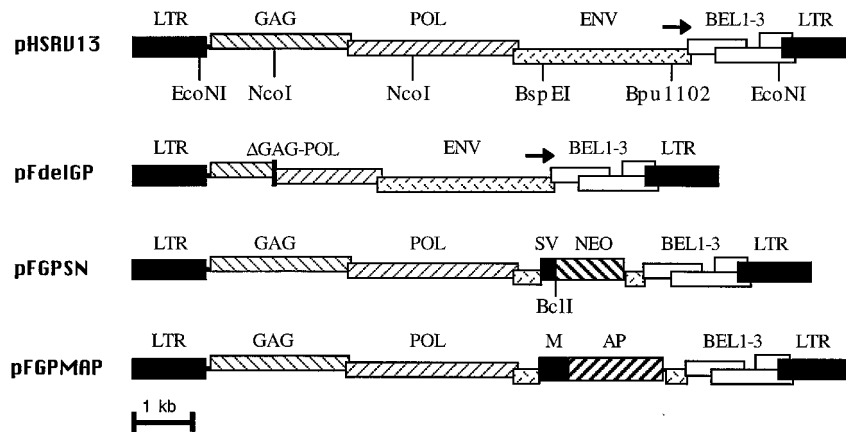
FIG. 1. Foamy virus constructs. The structures of HFV constructs used to generate vector stocks are shown. The positions of the viral *gag*, *pol*, *env*, and *bel* genes are indicated, as are the HFV LTRs and the internal viral promoter (arrow). Also shown are the pFdelGP *gag-pol* deletion and the SV40 early promoter (SV), neomycin phosphotransferase gene (NEO), MLV LTR promoter (M), and alkaline phosphatase gene (AP) present in the pFGPSN and pFGPMAP vectors. The locations of relevant restriction sites are shown below the constructs.

the neomycin phosphotransferase gene under the control of the SV40 early promoter or the human placental alkaline phosphatase gene under the control of the MLV LTR promoter to make the vector constructs pFGPSN and pFGPMAP, respectively (Fig. 1). This *env* deletion prevents the synthesis of a functional envelope protein and also disrupts the internal *bel1* promoter required for high-level expression of the Bel1 transactivator protein. Vector stocks were generated by cotransfection of BHK21 cells with a vector plasmid construct and either the wild-type pHSRV13 plasmid or the pFdelGP helper construct, containing a deletion in the *gag* and *pol* genes (Fig. 1).

Functional vector particles were measured by infecting FAB cells with filtered culture medium collected at different times after transfection. FAB cells contain an integrated copy of a nuclear β-galactosidase gene under the control of the HFV LTR promoter (53), so infection by viruses expressing the *bel1* gene leads to transactivation of the HFV LTR, the production of β-galactosidase, and blue histochemical nuclear staining. As the pFGPSN and pFGPMAP vector constructs do not produce high levels of Bel1, infection by vector particles does not lead to detectable β-galactosidase expression, and transduction can be measured by G418 resistance (FGPSN) or purple histochemical staining for alkaline phosphatase (FGPMAP). Although both titering methods measure gene expression from an internal promoter, there could be differences in titering efficiency as it relates to virion particle numbers.

After transfecting BHK21 cells with pFGPSN and pHSRV13, vector titers were approximately 10-fold lower than helper virus titers, while transfection with pFGPSN and pFdelGP produced nearly equivalent vector and helper titers after 7 days (Fig. 2A and B). The higher helper titers obtained with pHSRV13 were not unexpected, as this construct produces replication-competent virus, while pFdelGP can only produce helper particles in the presence of *gag* and *pol* genes encoded by the vector construct. When BHK21 cells were transfected with pFGPSN and pHSRV13 and then cultured in the presence of G418 to ensure that all cells infected with helper virus also contain vector sequences, vector titers were as high as helper titers (Fig. 2C). With the same transfection procedures, the pFGPMAP construct produced similar titers, ranging from 1×10^3 to 5×10^4 FAB cell transducing units at 5 to 7 days after transfection (data not shown). We have also produced vector stocks by transfecting NIH 3T3 TK⁻ cells, 293 cells, and COS-7 cells (data not shown).

Transduction of different cell types by foamy virus vectors. We screened a series of cell lines from different species to determine the range of cells that can be transduced by HFV vectors. Fourteen cell lines from species ranging from humans to chickens were all transduced by the FGPMAP vector (Table 1). The small differences in transduction efficiencies did not correlate with evolutionary divergence among species, cell histological type, or state of cellular transformation.

Integration of vector genomes. The structure of vector genomes present in transduced cells was determined by Southern analysis. Independent G418-resistant clones were isolated from rat 208F cells infected with FGPSN and expanded to more than 10^7 cells before high-molecular-weight DNA was isolated. Figure 3 shows the results of *Eco*NI and *Bcl*I restriction digests from six representative G418-resistant clones probed with *neo* sequences. *Eco*NI cleaves inside both terminal repeats of pFGPSN, producing an 8.9-kb fragment containing the FGPSN coding regions, while *Bcl*I cleaves 5′ to the *neo* gene, producing fragments with 3′ *Bcl*I sites in adjoining chromosomal DNA (see Fig. 1). Three of these cell lines (clones 1 to 3) contained the *Eco*NI fragment expected from intact proviruses bounded by LTRs. Two transductants (clones 4 and 6) contained smaller proviruses, which further analysis showed contained the *gag-pol* deletion present in the pFdelGP helper construct (data not shown). We have analyzed a total of 10 transductants: 6 contained intact proviruses, 3 contained *gag-pol* deletions from pFdelGP, and 1 (clone 5) contained a provirus with a larger than expected *Eco*NI fragment, which we have not characterized further (Fig. 3 and data not shown). Digestion with *Bcl*I produced various sizes of *neo*-hybridizing restriction fragments, consistent with random integration. No integrated helper virus was detected in any of the clones analyzed when the blots were probed with viral sequences (data not shown).

Effect of cell proliferation on transduction. We compared the transduction rates of HFV vectors on stationary-phase and dividing cultures of human fibroblasts and rat 208F cells. Infections were performed at low multiplicities to avoid coinfection with helper virus. Stationary-phase cultures were prepared by maintaining confluent cell monolayers in 5% FBS and 10^{-6} M dexamethasone under conditions that resulted in approximately 4% (human fibroblasts) or <1% (208F cells) of the cells passing through S phase each day (30, 43). Dividing cultures were subconfluent cells grown in 10% FBS, with 60% (human
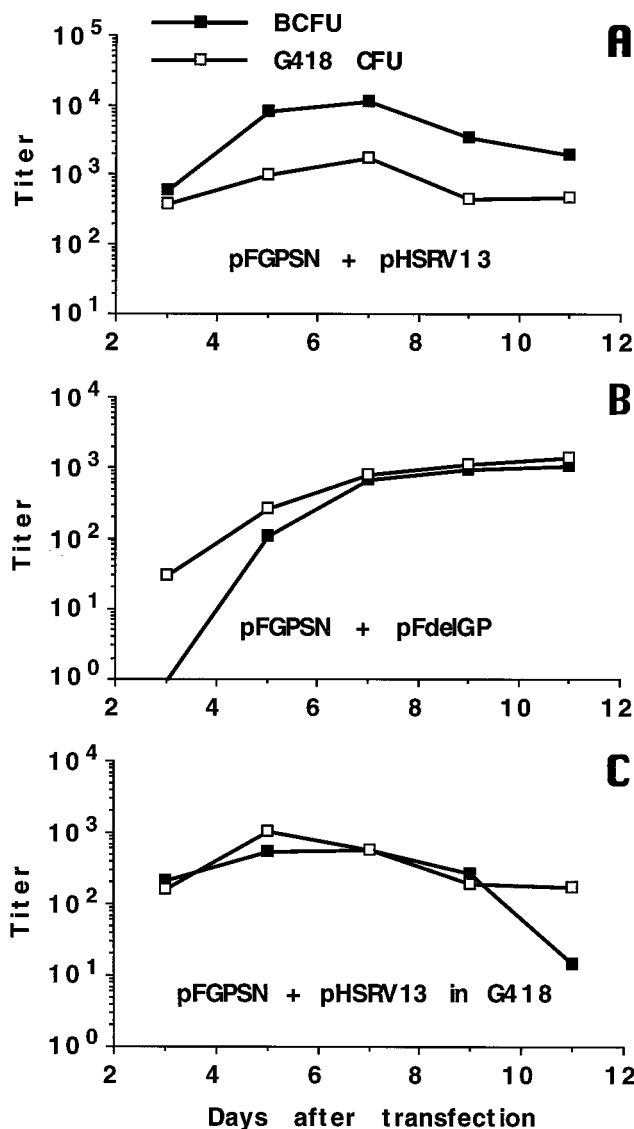
FIG. 2. Foamy virus vector production. BHK21 cells were cotransfected with pFGPSN and pHSRV13 (A and C) or pFdelGP (B) and then cultured for an additional 11 days. The medium was changed the day after transfection (day 1) and then collected and replaced every 2 days afterwards. Collected medium was filtered and used to infect FAB cells. Vector titers were measured by the production of G418-resistant colonies (G418 CFU), and helper titers were measured as blue-cell-forming units (BCFU) after staining for β-galactosidase. In panel B, the 3-day BCFU titer was <1/ml. In panel C, G418 (700 μg of active drug per ml) was added to the culture on day 2. Titers are per milliliter.

TABLE 1. Transduction of different cell types by the HFV vector FGPMAP[a]

Species	Cell type	AP (FFU/ml)	Relative transduction efficiency[b]
Human	Normal fibroblasts	17,000	1.00
Monkey	COS-7	2,300	0.13
	Vero	4,300	0.25
Steer	MDBK	2,400	0.14
Sheep	Choroid plexus	2,900	0.17
Dog	Cf2Th	1,600	0.09
Cat	CCC-81	16,000	0.93
	AH 927	2,500	0.15
Rat	NRK	9,200	0.54
	208F	3,900	0.23
Mouse	NIH 3T3 TK⁻	5,100	0.30
Hamster	CHO-K1	6,400	0.37
	FAB	8,400	0.49
Chicken	Embryo fibroblasts	2,700	0.16

[a] Each cell type was infected with FGPMAP and stained for alkaline phosphatase (AP) expression 2 days later, and the number of AP focus-forming units (FFU) per milliliter (FFU/ml) of vector stock was determined. All results are the averages of two independent measurements that varied by less than 36% from the mean. No AP foci were observed in uninfected control cultures.
[b] Relative transduction efficiency is the AP FFU/ml on each cell type divided by the AP FFU/ml on normal human fibroblasts.

erating at the time of infection and higher than those obtained with MLV vectors. In a G418 resistance assay, which requires that cells be treated with trypsin after the infection and allowed to divide during selection, the S/D ratio was 1.0 for HFV vectors and 0.14 for MLV vectors. The higher S/D ratios obtained with the G418 resistance assay suggest that efficient vector gene expression occurs after these cells are stimulated to divide.

Human serum does not inactivate HFV vectors. Human serum is known to inactivate several animal retroviruses, including MLV (52), while human retroviruses such as human T-cell leukemia virus and HIV are not inactivated by human serum, which may contribute to their pathogenicity (2, 15). We measured transduction by HFV and MLV vectors after incubating them with three different human serum samples. As shown in Fig. 5, there was no decrease in the transduction efficiency of FGPMAP after serum exposure under conditions that inactivated MLV vectors (42, 48). Thus, HFV is similar to
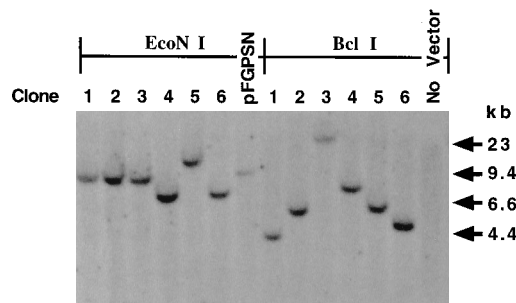
fibroblasts) or >90% (208F cells) of the cells passing through S phase each day (43) (data not shown). If transduction occurs only in proliferating cells, stationary-phase cultures should be transduced at approximately 7% or <1% the rate of dividing cultures for human fibroblasts and 208F cells, respectively.

Figure 4 shows the relative transduction efficiencies of HFV and MLV vectors on stationary-phase and dividing cultures, plotted as the stationary-to-dividing efficiency ratio (S/D ratio). In an alkaline phosphatase assay which can be performed without stimulating cultures to divide, the S/D ratio for HFV vectors was 0.18 on human fibroblast cultures and 0.07 on 208F cells. While these results show that HFV vectors preferentially transduce proliferating cells, the ratios are higher than those expected if transduction occurred only in cells actively prolif-

FIG. 3. Integration of foamy virus vector genomes. Southern analysis was performed with a *neo* gene probe and 10 μg of high-molecular-weight DNA isolated from six independent G418-resistant clones of 208F cells transduced by FGPSN (lanes 1 to 6) or untransduced 208F cells (No Vector lane). The pFGPSN lane contains 10 pg of pFGPSN plasmid DNA mixed with untransduced 208F cell DNA. Samples were digested with *Eco*NI or *Bcl*I as indicated. The positions of size standards from the same gel are shown.
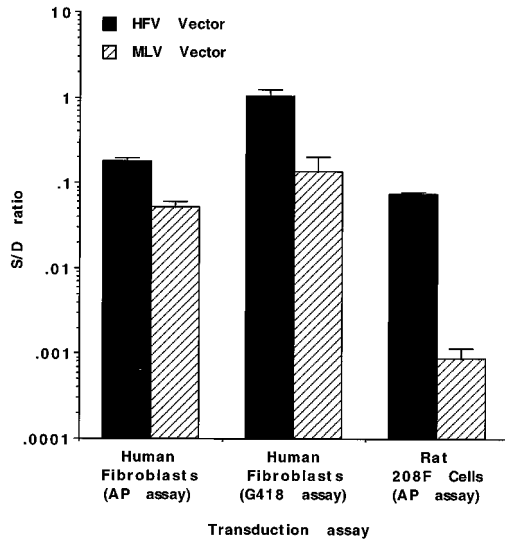
FIG. 4. Transduction in stationary-phase and dividing cultures. Stationary-phase and dividing cultures of human fibroblasts and rat 208F cells were transduced with the HFV vector FGPMAP (solid columns, alkaline phosphatase [AP] assay), the HFV vector FGPSN (solid columns, G418 assay), and the MLV vector LAPSN produced in PA317 cells (hatched columns) and then stained for alkaline phosphatase expression (AP assay) or selected for G418 resistance (G418 assay). The S/D ratio is the vector titer measured in stationary-phase cultures divided by that in dividing cultures. Mean values are plotted with standard errors. FGPMAP infections contained less than one transducing particle and less than 10 helper particles per 1,000 cells. FGPSN infections contained less than one transducing particle and less than 0.1 helper particle per 5,000 cells.

known human retroviruses in its resistance to human complement.

DISCUSSION

Vector production. The transient cotransfection procedure that we have used generated vector stocks with titers as high as 5×10^4 per ml. Wild-type HFV can be grown to titers of up to 10^9/ml under optimal conditions (53), so higher vector titers may be possible with improved packaging systems. Vector stocks free of helper virus were generated at early times after transfection by using the pFdelGP deleted helper construct (see Fig. 2B). However, a single recombination event between vector and helper constructs could have generated replication-competent helper virus at later time points. Further modifications of the helper construct should prevent the formation of replication-competent helper virus, as was the case for MLV packaging lines (27).

Host range. Every vertebrate cell type that we tested was transduced by HFV vectors, suggesting that the receptor used for viral entry is a common cell surface molecule. Previous studies concluded that some cell lines, including COS-7 cells, were resistant to HFV infection, based on the absence of observed cytopathic effects (3, 20, 22) and decreased virus production (23). However, COS-7 cells were transduced by HFV vectors in our experiments. Thus, it is possible that virus entry, reverse transcription, and gene expression can occur without cytopathic effects (syncytium formation) or the production of functional viral particles in some cell types.

Provirus integration. Cultures infected with foamy viruses usually contain high copy numbers of episomal, linear cDNA molecules that may be generated by repeated infections (18, 26, 38). As these cultures are producing viral particles, the
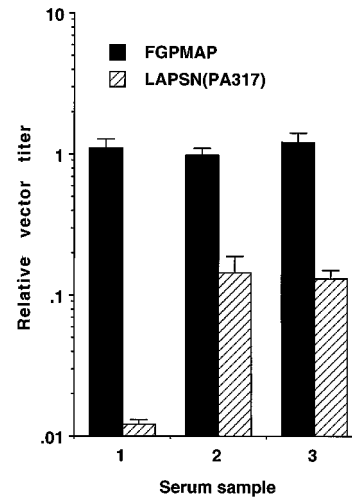

FIG. 5. HFV vectors are not inactivated by human serum. Stocks of FGPMAP and LAPSN(PA317) were incubated with an equal volume of serum from three different individuals for 30 min at 37°C and then used to transduce rat 208F cells as described before (42). Results are shown as the titer of vector stocks incubated with human serum divided by the titer of stocks incubated under identical conditions with heat-inactivated serum (30 min at 56°C). Mean values are plotted with standard errors. Heat-inactivated serum samples did not decrease vector titers compared with the titers after incubation with DMEM containing 10% FBS (data not shown).

episomal viral DNA molecules could be transcriptionally active. Although foamy viruses contain reverse transcriptase and integrase genes that should allow the formation of an integrated provirus, the limited life span of actively infected cells and excess of episomal molecules make the detection of integrated proviruses problematic. Integrated simian foamy virus 3 genomes were detected in infected Vero cells grown for more than 50 passages in the presence of 3′-azido-3′-deoxythymidine to prevent cytopathic effects (46). However, the efficiency of integration is not known, and the entire foamy virus life cycle could occur without chromosomal integration in most infected cells.

All of the G418-resistant transductants that we analyzed contained integrated vector proviruses, most of which appeared to be randomly integrated, intact vector genomes bounded by LTRs. Thus, foamy virus vectors integrate in a typical retroviral pattern. The deleted proviruses that we detected were presumably created by recombination between vector and helper sequences, which could have happened during the transfection procedure or during reverse transcription. Transductants contained single-copy proviruses and no helper genomes, so vector integration occurred in the absence of helper virus.

Effects of cell proliferation on transduction. Oncoviruses such as MLV require cell proliferation for efficient infection (13), while lentiviruses such as visna virus and HIV do not (12, 19). In the case of MLV, mitosis is required for nuclear entry of viral particles (41), and vectors based on MLV require cell division for efficient transduction (30). Although syncytium formation and virus production are decreased when confluent cultures are infected with foamy viruses (23, 35, 36), it is not known if viral DNA synthesis and gene expression require cell proliferation. Our findings suggest that HFV vectors preferentially transduce dividing cultures, but the transduction efficiency on stationary-phase cultures is higher than for MLV vectors and higher than expected if transduction occurs only in cells proliferating at the time of infection. The HFV Gag

protein contains a functional nuclear localization signal (44), so it is possible that nuclear entry of HFV particles is independent of mitosis, as is the case for HIV (4, 5), and the decreased transduction of stationary-phase cells could be due to a block in the HFV life cycle after nuclear entry. The high transduction rate in stationary-phase cultures obtained with the G418 resistance assay indicates that HFV vectors efficiently enter non-proliferating cells and that functional vector genomes survive at least until these cultures are stimulated to divide the day after infection.

Potential in gene transfer applications. Vectors based on MLV and adeno-associated virus are the only integrating vector systems currently available, and both are limited by genome size, host range, and cell proliferation requirements (27, 33, 43). The potential advantages of foamy virus vectors include a larger genome size, wide host range, and improved transduction of nondividing cells. HFV vectors are not inactivated by human serum and do not require the addition of polycations such as Polybrene for efficient transduction, so they may be especially well suited for in vivo gene delivery. Because Bel1 is required for high-level transcription from the viral LTR, integrated Bel1-deficient vectors should not activate downstream cellular genes by promoter insertion. These properties, as well as the lack of pathogenicity associated with foamy virus infections, suggest that foamy virus vectors may be safe and effective in gene therapy protocols.

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