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Pseudotransduction of Hepatocytes by Using Concentrated Pseudotyped Vesicular Stomatitis Virus G Glycoprotein (VSV-G)–Moloney Murine Leukemia Virus-Derived Retrovirus Vectors: Comparison of VSV-G and Amphotropic Vectors for Hepatic Gene Transfer

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Recombinant retrovirus vectors are widely used for gene transfer studies. The recent development of a pseudotyped Moloney murine leukemia virus vector that contains the G envelope protein from the vesicular stomatitis virus allows for efficient concentration of vector and offers hope for potential use of these vectors for gene expression in vivo. A standard amphotropic vector expressing a serum marker protein, human alpha 1-antitrypsin, was infused into regenerating mouse liver and was 10-fold more efficient at achieving stable gene expression than was an equivalent pseudotyped vector. Discrepant results were obtained with cultured hepatocytes infected with an *Escherichia coli* β-galactosidase-producing pseudotype and amphotropic vector. High rates of β-galactosidase-positive cells were detected with the vesicular stomatitis virus G glycoprotein vector under culture conditions known to be relatively nonpermissive for retrovirus-mediated gene transfer. Subsequent studies demonstrated that β-galactosidase protein was concentrated and copurified during pseudotype vector preparation, resulting in high rates of protein transfer rather than stable gene transfer, a process referred to as pseudotransduction. The cotransfer of protein with concentrated pseudotyped retroviruses indicates that caution must be used when interpreting gene transduction efficiencies in gene therapy experiments.

Moloney retrovirus-based gene transfer vectors remain the only vehicle for which high-efficiency stable gene transfer in a wide variety of cell types has been demonstrated. The major disadvantages of this vector system are the inability to transduce nondividing cells; the relatively low concentration of virus, in the range of 10^6 to 10^7 CFU/ml; and the virus's instability, presumably the result of the envelope protein. The latter two limitations have recently been overcome through the use of a pseudotype envelope G protein from the vesicular stomatitis virus (VSV). By producing a pseudotyped vector, Burns et al. (1) were able to demonstrate that this vector could be concentrated to high titers of more than 10^9 CFU/ml, with almost 100% recovery, by ultracentrifugation. Additionally, the vector was demonstrated to transduce cells derived from a broad host range, most likely because the virus uses a universal lipid component of the membrane rather than a specific receptor for its entry. Because of the toxicity of the VSV G glycoprotein (VSV-G), the production of stable retroviral packaging cell lines has been difficult, and only recently have methods other than transient transfection methods been used to produce pseudotyped murine virus-based recombinant retrovirus vectors (20).

For the purposes of gene therapy, Yee et al. (22) demonstrated that a pseudotyped retrovirus vector expressing β-galactosidase (β-Gal) could transduce about 20-fold more primary mouse hepatocytes in culture than a standard amphotropic vector could, presumably because it was possible to use a greater multiplicity of infection (MOI) with concentrated virus stock. Further studies with the same pseudotyped vector showed occasional high rates of hepatic gene transfer into neonatal mouse liver in vivo (12). Importantly, for reasons that are not known, gene transfer was noted to occur without the need to stimulate hepatocyte replication. This result was extremely encouraging, because while use of amphotropic vectors in mice (5) and dogs (6) has led to indefinite gene expression, a partial (two-thirds) surgical hepatectomy was required and the number of transduced cells was only about 1%.

Our laboratory has recently developed a new method of inducing hepatic regeneration without a surgical hepatectomy by first transducing hepatocytes in vivo with a recombinant adenovirus that expresses a normal (9) or genetically modified (8) urokinase protein. The intracellular production of urokinase is believed to be hepatotoxic, resulting in the stimulation of hepatocyte proliferation. During the proliferative phase of liver regeneration, it was possible to transduce hepatocytes with standard amphotropic retrovirus vectors with a five- to ninefold improved efficiency over that of a standard partial hepatectomy model. Because this nonsurgical method of inducing liver regeneration allowed for multiple infusions of retrovirus over a period of about 7 days, high-titer pseudotype-based vectors were constructed in an attempt to permanently increase the number of genetically modified hepatocytes in vivo.

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TABLE 1. Percentage of β-Gal-positive hepatocytes after retroviral-mediated gene transfer in vivo[a]

Virus (amt [CFU])	% β-Gal-positive cells (SD) determined by staining of:	
	Frozen liver sections	Hepatocytes in culture
Amphotropic LBGpgk (3×10^6)	1.8 (0.1)	2.1 (0.3)
VSV-G LBGpgk (3×10^7)	4.4 (0.3)	4.6 (0.2)

[a] The percentages of β-Gal-positive cells were determined by staining frozen sections of liver (histochemical staining) or by staining hepatocytes in culture (hepatocyte population staining) from the same animal. The values represent the mean values and standard deviations from four animals per group.

MATERIALS AND METHODS

Cell lines. 208F rat embryo fibroblast and amphotropic retrovirus packaging cells were cultured in high-glucose Dulbecco's modified Eagle's medium (Gibco BRL), 10% Hyclone bovine calf serum (Hyclone), and penicillin-streptomycin (Pen/Strep; Gibco BRL) as previously described (5). The 293GP cell line (1) has been described previously and was grown in the same medium except that fetal calf serum (Gibco BRL) replaced the Hyclone calf serum.

Adenovirus vectors. The construction and preparation of Ad.PGKmuPA recombinant adenovirus have been previously described (8).

Amphotropic retrovirus vectors. The construction of amphotropic LBGpgk and LNAlb-hAAT retroviral vectors from mouse-derived NIH 3T3 fibroblasts has been previously described (5). The LBGpgk vector contains the sequence encoding the β-galactosidase–neomycin phosphotransferase fusion protein under the transcriptional control of the viral long terminal repeat. The LNAlb-hAAT vector expresses human alpha 1-antitrypsin (hAAT) from a portion (spontaneous deletion mutant) of the original albumin enhancer-promoter that was originally cloned into it (4a). Both vectors have a titer on 208F cells of about 2×10^6 to 4×10^6 CFU/ml. Retrovirus was collected by routine procedures (5). Freshly collected and filtered virus was used in all gene transfer experiments. For in vivo and in vitro gene transfer, Polybrene was added to 12 and 8 μg/ml, respectively.

Construction of VSV-G retrovirus vectors. VSV-G pseudotyped viruses were prepared from a human 293-derived cell line (1). The LBGpgk and LNAlb-hAAT amphotropic vectors were transduced into 293GP cells. One day later, 400 μg of G418 per ml was added to the culture media. About 20 G418-resistant clones were analyzed for each vector. The LBGpgk clones all stained positive for β-galactosidase, and the 20 clones from LNAlb-hAAT infection that expressed the highest levels of hAAT were transfected with pHCMV-G (22) (provided by Jane Burns), which contains the VSV-G gene under the transcriptional control of the immediate-early cytomegalovirus promoter. Viral supernatants collected 48 and 72 h later were filtered, Polybrene was added to 8 μg/ml, and titers were determined on 208F cells (5). Titers of pseudotype LBGpgk were determined by the number of G418-resistant colonies and also the number of β-galactosidase-positive colonies (prior to selection). Both methods led to titers within twofold of each other. Titers of the LNAlb-hAAT vector preparations were determined with G418-resistant colonies on 208F cells. Clones that gave the highest titers of virus were used in subsequent experiments.

Production of pseudotyped virus stocks. The 293GP-LBGpgk and 293GP-LNAlb-hAAT lines were grown in 10-cm-diameter dishes and transiently transfected with 25 to 30 μg of total DNA of pHCMV-G or pCMVβgal (14) (control) or a 1:1 DNA-plasmid mixture by calcium phosphate coprecipitation (21). The medium was replaced with fresh medium 8 to 10 h after transfection, and the pseudotype virus in supernatants was collected daily between 24 and 96 h after transfection. The pooled supernatants were filtered through a 0.45-μm-pore-size filter and/or centrifuged at $500 \times g$ for 5 min and then stored at −80°C.

The thawed viral supernatant was pelleted by ultracentrifugation in a Beckman model SW-L3-50 centrifuge in an SW28 rotor at 19,500 rpm and 4°C for 90 min. The pellet was resuspended for 1 to 3 h at 4°C in about 1/100 to 1/200 the original volume of supernatants in serum-free Dulbecco's modified Eagle's medium. The concentrated viruses were stored at −80°C. Thawed virus preparations were only used once. Viral titers were determined before and after concentration, and recovery was between 85 and 97%. Titers of frozen aliquots of concentrated vector were always determined before use. For infusion in animals or infection in culture, Polybrene was added to virus preparations to 12 and 8 μg/ml, respectively.

Isolation and infection of hepatocytes. Hepatocytes from 5- to 6-week-old C57BL/6 mice (Jackson Laboratories) were isolated by collagenase perfusion as previously described (15). Different densities of viable hepatocytes were plated in six-well PRIMARIA (Corning) dishes. Four hours after plating, the medium was changed to Williams E (Gibco BRL) containing 10% fetal calf serum (Gibco BRL), 1 mM glutamine, and Pen/Strep. The primary hepatocytes were infected for 2 h with 1 ml of retroviruses at 12, 24, and 48 h after plating. An equal volume of fresh medium was then added to the cells, and the complete medium was changed 24 h after infection. Supernatants were analyzed for hAAT by enzyme-linked immunosorbent assay (5), or cells were fixed and stained with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for β-Gal determination 24 h later.

Determination of β-Gal (LacZ) activities. Cytochemical stains for enzyme activity were performed by rinsing the cells twice with cold 1× phosphate-buffered saline (PBS) and then fixing them for 5 min with 0.5% glutaraldehyde in cold PBS. After being washed twice with PBS, the cells were stained with X-Gal (Gibco BRL) solution (11). Histochemical staining was performed on OCT-frozen sections as previously described (6). To quantitate enzyme activity, an *o*-nitrophenyl-β-D-galactopyranoside (ONPG) assay was used (11). *Escherichia coli* β-galactosidase (grade VI; Sigma Chemical) was used as a standard to establish a linear range of enzymatic activity. The assays were performed on the virus preparation directly or after disruption of retrovirus by freeze-thawing three times with or without the addition of 0.1% Triton X-100.

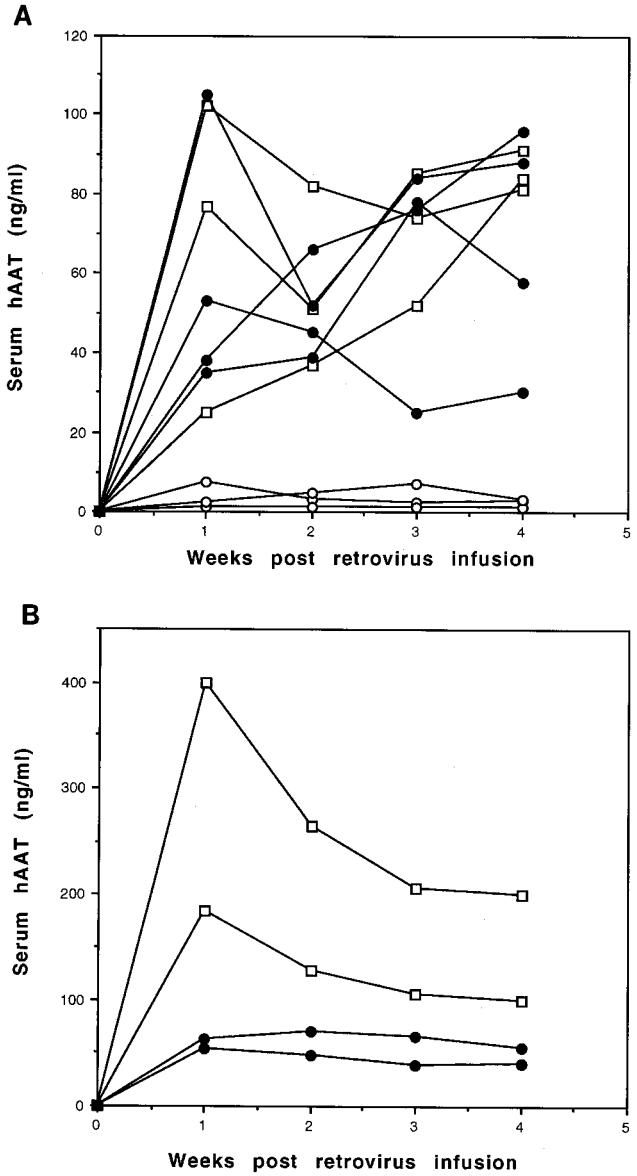

FIG. 1. Amphotropic and pseudotype hepatic retrovirus-mediated gene transfer in mice. A permanent indwelling portal-vein cannula was placed into C57BL/6 female mice. The animals were infused with 5×10^9 PFU of Ad.PGK-muPA as a stimulus for liver regeneration on day 0. At 4 (A) or 4 and 6 (B) days later, amphotropic or pseudotype LNAlb-hAAT retrovirus was infused via the portal-vein catheter. Periodic serum samples were analyzed in duplicate for hAAT. Open circles, VSV-G (3×10^6 CFU); solid circles, VSV-G (3×10^7 CFU); open squares, amphotropic (3×10^6 CFU). Each line represents results for an individual animal.

TABLE 2. LBGpgk retroviral transduction of primary mouse hepatocytes in culture[a]

Time (h)	Virus	% β-Gal-positive cells (mean ± SEM) at cell density (cells/well) and MOI					
		1×10^5			5×10^5		
		1	3	10	1	3	5 or 10
12	Amp[b]	0.6 ± 0.1	1.1 ± 0.09	1.5 ± 0.1	0.005 ± 0.0001	0.034 ± 0.002	0.06 ± 0.008
	VSV-G	0.3 ± 0.1	0.56 ± 0.1	14.6 ± 4.1	0.04 ± 0.002	16.8 ± 3.9	52 ± 7.2
24	Amp	4.2 ± 0.3	5.3 ± 0.3	6.2 ± 0.4	0.3 ± 0.1	0.4 ± 0.1	0.4 ± 0.11
	VSV-G	4.6 ± 0.2	6.2 ± 0.3	19.2 ± 4.0	0.54 ± 0.2	20.0 ± 4.1	44.0 ± 5.11
48	Amp	53.4 ± 3.1	61.7 ± 4.6	65.9 ± 3.1	4.8 ± 0.7	6.2 ± 0.8	6.83 ± 0.50
	VSV-G	56.6 ± 10.7	58.3 ± 4.1	60.2 ± 4.6	38.4 ± 6.1	42.7 ± 5.0	47.0 ± 2.80

[a] Hepatocytes from C57BL/6 mice were cultured at different densities in six-well dishes. Hepatocytes were transduced with either amphotropic or VSV-G LBGpgk virus at different MOIs and times after isolation. At the high density, it was not possible to maintain equal volumes and use equivalent MOIs. Thus, for amphotropic and VSV-G vectors, the MOIs were 5 and 10, respectively. The medium was changed 24 h after infection, and cells were stained with X-Gal 48 h after infection. For plates containing at least 1% blue cells, at least 2,000 cells per well were counted. For dishes containing less than 1% blue cells, all the blue cells were counted and divided by the total number of cells present in each well. The data represent the means ± standard errors of the mean from three independent experiments.

[b] Amp, amphotropic LBGpgk.

Viral infusions in mice. All surgical procedures, including administration of anesthesia, were performed in accordance with the University of Washington animal care committee guidelines. A permanent indwelling catheter was inserted in the portal vein as described previously (18). Ad.PGKmuPA vector (5×10^9 PFU) was used to induce liver regeneration (8, 9) prior to retrovirus administration. Recombinant retrovirus was infused via the portal-vein catheter in a final volume of about 1 ml over a 40-min period.

RESULTS

Amphotropic and pseudotyped retrovirus-mediated gene transfer in vivo. Mice were infused with 5×10^9 PFU of Ad.PGKmuPA adenovirus in order to induce liver regeneration (8, 9). Four days later the animals were infused via the portal vein with either 3×10^7 CFU of VSV-G pseudotype or 3×10^6 CFU of amphotropic LBGpgk virus. Three weeks later, the livers were removed and a small section from each was removed and frozen. The remaining liver was dissociated into hepatocytes and placed into culture. Both frozen sections and a population of hepatocytes were stained with X-Gal. The proportions of β-Gal-positive cells were determined, and the results are displayed in Table 1. The administration of a single infusion of 3×10^6 CFU of amphotropic virus transduced about 2% of cells, in accordance with previously reported studies (9). Concentrated pseudotyped virus infused at a 10-fold-greater titer (3×10^7 CFU) resulted in about twofold higher rates of gene transfer into about 4.5% of hepatocytes.

In an attempt to confirm the results of the studies mentioned above, a second set of studies, similar to those described above with the LNAlb-hAAT pseudotype VSV-G and amphotropic retroviral vectors that express the serum protein marker hAAT, was carried out (Fig. 1). A single infusion of 3×10^6 CFU of pseudotype or amphotropic vector was given 4 days after Ad.PGKmuPA induced liver regeneration. The hAAT concentrations in serum were 20- to 30-fold higher when amphotropic virus was used compared with those when pseudotyped virus was used (Fig. 1A). A 10-fold-greater titer of pseudotyped vector was required to increase serum hAAT concentrations into a range similar to that achieved with the amphotropic vector. The differences in hAAT expression were consistent with the number of transduced hepatocytes obtained with the pseudotyped and amphotropic LBGpgk vector, as summarized in Table 1.

In a more limited study, animals were given two retrovirus infusions on days 4 and 6 after adenovirus administration (Fig. 1B). The animals receiving two infusions of 3×10^6 CFU of amphotropic virus had slightly higher levels of hAAT than the mice that received two injections of 3×10^7 CFU of VSV-G pseudotyped vector. The studies with the LNAlb-hAAT vectors suggest that the amphotropic vector is about 1 order of magnitude more efficient at achieving stable gene expression when transduced into mouse liver in vivo.

Retrovirus-mediated gene transfer into primary mouse hepatocytes in culture. The pseudotyped and amphotropic vectors were produced in cell lines from different species. As a result, there are a number of host factors that may influence the number of infectious viral particles that reach hepatocytes after infusion in the portal vein (2, 16, 17). In order to determine whether or not the differences in gene transfer may have resulted from in vivo parameters, primary mouse hepatocytes were used for retrovirus-mediated gene transfer in vitro. Hepatocytes cultured under the appropriate conditions and in the appropriate media undergo brief periods of cellular division and are susceptible to retrovirus-mediated gene transfer. The two most critical factors are the time in culture and the cell density (3, 4, 7, 10, 19). The optimal time for amphotropic or ecotropic retrovirus-mediated gene transfer in mice is about 48 h postisolation (4, 10).

Different amounts of LBGpgk and LNAlb-hAAT pseudotyped or amphotropic vectors were added at various times after isolation to cells cultured at a high (nonpermissive) or low (permissive) density (Tables 2 and 3). Differences were obtained when LBGpgk (Table 2) and LNAlb-hAAT (Table 3) pseudotype vectors were used. Consistent with previous studies, amphotropic retrovirus-mediated gene transfer of either LNAlb-hAAT or LBGpgk was most efficient 48 h after isolation in cells cultured at low density. At 48 h after plating, there was a 10-fold greater percentage of β-Gal-positive cells in hepatocytes cultured at lower density than at high density (about 50 versus 5%) after infection with amphotropic LBGpgk virus (Table 2). Furthermore, increasing the MOI made only a slight difference in transduction efficiency.

Although the percentage of hepatocytes staining blue was 10-fold greater in the low-density culture, the absolute number of transduced cells in low-density culture was only about twofold greater than that in high-density culture (e.g., 1×10^5 cells $\times$ 0.53 = 5.3×10^4 transduced cells versus 5×10^5 cells $\times$ 0.048 = 2.4×10^4 transduced cells). If transduction with amphotropic LNAlb-hAAT is similar to that of LBGpgk, then as shown in Table 3, similar or slightly reduced amounts of hAAT

TABLE 3. LNAlb-hAAT retroviral transduction of primary mouse hepatocytes in culture[a]

Time (h)	Virus	Amt of hAAT (ng/dish/24 h [mean ± SEM]) at cell density (cells/well) and MOI					
		1×10^5			5×10^5		
		1	3	10	1	3	5 or 10
12	Amp[b]	<2	<2	5.8 ± 2.7	4.6 ± 2.0	9.5 ± 3.0	20.5 ± 3.0
	VSV-G	<2	<2	<2	<2	<2	<2
24	Amp	<2	6.2 ± 0.8	14.2 ± 0.60	5.1 ± 2.0	13.5 ± 2.0	24 ± 2.0
	VSV-G	<2	<2	<2	<2	<2	<2
48	Amp	14.7 ± 4.2	16.2 ± 3.9	17.0 ± 2.6	11.5 ± 2.0	24.5 ± 1.0	39.5 ± 3.0
	VSV-G	<2[c]	<2[c]	<2[c]	<2	<2	<2

[a] Hepatocytes from C57BL/6 mice were cultured at different densities in six-well dishes. Hepatocytes were transduced with either amphotropic or VSV-G LNAlb-hAAT virus at different MOIs and times after isolation. To maintain equal volumes during infection, cells cultured at the high density were transduced with amphotropic and VSV-G vector at MOIs of 5 and 10, respectively. The medium was changed 24 h after infection and collected for hAAT determination 24 h later. The values represent nanograms of hAAT per dish per 24 h (means ± standard errors of the means) from three independent experiments. The sensitivity of the assay was 1 ng/ml. The cultures contained 2 ml of medium.

[b] Amp, amphotropic LNAlb-hAAT.

[c] hAAT level was detectable above background but lower than the linear portion of the curve.

would be produced in high- versus low-density-cultured hepatocytes.

In contrast to the results obtained with the amphotropic vectors, the 48-h cultures resulted in similarly high rates (38 to 60%) of β-Gal-positive cells after VSV-G LBGpgk retrovirus-mediated gene transfer in both high- and low-density cultures. In fact, high percentages of β-Gal-positive cells (14 to 52%) were found under all culture conditions with a high MOI of VSV-G LBGpgk virus (Table 2). Unlike the concordant results with the LBGpgk and LNAlb-hAAT amphotropic vectors, the level of LNAlb-hAAT VSV-G-mediated gene expression was discordant with that of the LBGpgk VSV-G virus infection. Based on the absolute number of β-Gal-positive cells, the prediction was that hAAT production should be fourfold greater if LNAlb-hAAT VSV-G transduction in high-density-cultured cells were equivalent to that in LBGpgk VSV-G gene transfer. Low amounts of hAAT were detected with the LNAlb-hAAT pseudotype vectors when low-density cultures were infected at 48 h, but no hAAT was detectable in high-density cultures transduced with the vector.

Of important note, hepatocytes transduced with pseudotyped virus at MOIs of 3 or more showed increasing degrees of cytotoxicity as evidenced by granularity and some cell death. This by itself was not responsible for X-Gal staining, because hepatocytes transduced with pseudotyped LNAlb-hAAT showed similar rates of cell toxicity but did not stain with X-Gal. The hepatocytes that recovered after several days in culture were indistinguishable from nontransduced hepatocytes. Taken together, these results suggest that a significant proportion of β-Gal staining may not have resulted from retrovirus-mediated gene transfer.

β-Gal enzymatic activity is concentrated during VSV-G viral concentration. The relatively high rate of β-Gal-positive hepatocytes and the low level of hAAT expression after VSV-G retrovirus-mediated gene transfer were in apparent conflict. Because the β-Gal-positive cells were present under culture conditions that were relatively nonpermissive for retrovirus-mediated gene transfer, other sources of β-galactosidase enzymatic activity were investigated.

Amphotropic and pseudotype viral preparations were analyzed for the presence of β-Gal enzyme activity. Amphotropic virus and unconcentrated VSV-G vector had relatively low levels of enzyme activity (Table 4). However, concentration of the VSV-G vector led to a five- to sevenfold relative increase in enzyme activity per infectious particle. The level of β-Gal enzyme activity was slightly increased when the virus particles were disrupted by Triton X-100 and freeze-thaw treatment, suggesting that the enzyme is associated with the virus or copackaged in a lipid copurifying particle.

Pseudotransduction of VSV-G retrovirus vectors. The studies of LBGpgk VSV-G infection in hepatocytes suggest that a certain proportion of the β-Gal-positive cells were not the result of gene transfer but may have resulted from protein transfer. As proof that the transfer of antigen was responsible for the early high rate of β-Gal-positive cells, the 293GP-LNAlb-hAAT cell line was cotransfected with a nonretrovirus plasmid that expresses β-Gal (pCMVβgal) and pHCMV-G. Purified pseudotyped virus was used to infect 10^5 208F cells in a 24-well dish. Twenty-four hours later, the cells were stained with X-Gal. Cells infected with virus at MOIs of 1 and 10 showed <5 and about 45% blue staining, respectively. Control cells infected with concentrated LNAlb-hAAT alone had no blue staining.

In order to determine the relative proportion of stable transduction events with the β-Gal vectors, the LBGpgk pseudotype and amphotropic vectors were used to infect rapidly dividing 208F rat embryo fibroblasts. Two days later, parallel cultures were stained with X-Gal to establish a baseline for β-Gal-positive cells. Cultures were then split every several days, and the proportions of β-Gal-positive cells were determined (Fig. 2). Over a period of 3 weeks, the amphotropic transduced cultures maintained a relatively high and stable proportion of β-Gal-positive cells (about 30 to 45%). In direct contrast, the

TABLE 4. β-Gal enzyme activity in amphotropic and pseudotyped retroviral vectors[a]

Virus	U of β-Gal activity (mean [SD]) determined in expt			
	Non-concentrated vector	Concentrated vector	Freeze-thaw	Freeze-thaw with Triton X-100
Amphotropic	3.8 (0.4)	NA[b]	3.6 (0.3)	3.7 (0.4)
VSV-G	4.8 (0.4)	15.5 (1.1)	16.7 (1.8)	24.2 (2.1)

[a] The values listed are units of β-Gal activity per 10^6 CFU (means and standard deviations) from at least three separate experiments. For the freeze-thaw experiments, concentrated VSV-G virus was used.

[b] NA, not applicable.

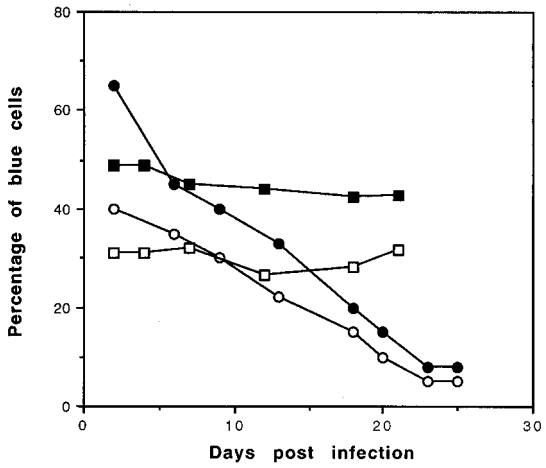

FIG. 2. VSV-G and amphotropic LBGpgk infection of 208F cells. 208F cells (10^5) were plated on six-well dishes the night before use. The cells were infected in duplicate with VSV-G or amphotropic LBGpgk virus. Two days later, one plate of cells from each experimental condition was stained with X-Gal and the percentages of blue cells were determined. The remaining cultures were split 1:5 every 2 to 4 days, and 1 to 2 days later, representative plates were stained with X-Gal. Open circles, VSV-G, MOI = 1; solid circles, VSV-G, MOI = 5; open squares, amphotropic LBGpgk virus, MOI = 1; solid squares, amphotropic LBGpgk virus, MOI = 5.

percentage of β-Gal-positive cells in VSV-G-infected cells decreased by almost 10-fold over a period of 3 weeks. The number of stable transduced cells was about 5 to 10%, 5- to 10-fold lower than that obtained with the amphotropic vector and consistent with the results obtained from the in vivo gene transfer studies. Importantly, these experiments demonstrate that pseudotransduction or antigen transfer was the major source of the high rates of β-Gal-positive cells after infection with purified pseudotyped LBGpgk virus.

DISCUSSION

The use of retrovirus for in vivo gene transfer has been hampered by the relatively poor rates of gene transfer (5, 6), which are in part due to the low titer of vector. At this time, it is not clear when vector titer will become rate limiting for hepatic gene transfer. However, the production of a pseudotyped retrovirus vector that can be concentrated to titers of more than 10^9 CFU/ml with recoveries of more than 90%, in theory, brings the possibility of therapeutic in vivo gene transfer a step closer to reality. This, in concert with the report of more than 20% transduction of neonatal mouse liver in the absence of stimulating hepatocyte regeneration (12), prompted us in these studies to investigate the use of VSV-G pseudotyped retrovirus vectors for hepatic gene transfer. The studies here directly compare the efficiencies of hepatic gene transfer with recombinant amphotropic and pseudotyped vectors that express the intracellular reporter β-galactosidase or the serum marker hAAT. The results obtained with these vectors suggest that gene transfer was about 10-fold less efficient, on an infectious particle basis, with the pseudotyped vector. There are a myriad of possible host factors that may influence hepatic gene transfer with different types of retrovirus vectors, most notably theoretical differences in complement inactivation (16) or preexisting humoral immunity (2). Although it is impossible to eliminate all host factor influences, the ~1-log-lower rate of what appears to be true gene transfer with the pseudotyped vector compared with that with the amphotropic vector in cultured hepatocytes and 208F cells makes host factors a less likely explanation for differences in in vivo gene transfer.

The demonstration that concentration of the pseudotyped virus coconcentrates β-Gal and can deliver the protein to cells in concert with the falloff of stable β-Gal staining in rapidly dividing cells proves that β-Gal protein was taken up by cells transduced with this vector. We observed in multiple cell types that infection of pseudotyped virus at MOIs of >3 led to transient cytotoxic effects, possibly because the VSV-G envelope protein is toxic to cells (1). At this time, it is not clear whether the toxicity is related to the viral particle itself or copurifying contaminants such as envelope proteins. Curiously, high rates of β-Gal transfer occurred in hepatocytes and 208F cells under nonpermissive retrovirus transduction conditions at the higher MOIs. The hypothesis is that a combination of a critical amount of β-Gal protein and cell toxicity is needed to pseudotransduce the cell with the β-Gal antigen.

The study of Yee et al. (22) showed that VSV-G retrovirus-mediated gene transfer into primary mouse hepatocytes was as high as 60% with a pseudotyped β-Gal vector at an MOI of 10, yet gene transfer after infection with an equivalent amphotropic vector (MOI = 1) was only 3%. Multiple studies carried out under permissive hepatocyte culture conditions, including this one, have demonstrated transduction efficiencies of 25 to 60% (3, 7, 10, 13, 19) with standard amphotropic or ecotropic vectors. Our study questions the ability of this vector to transduce hepatocytes at high efficiencies in vivo. Clearly, the previous study with neonatal mouse liver (12) is different from the studies performed here and there may be some inherent difference between adult and neonatal hepatocytes that affects their susceptibilities to retrovirus-mediated uptake. It is of interest that in neonatal liver studies, only low concentrations of a secretable marker were detected in the sera of recipients after VSV-G-retrovirus-mediated gene transfer (12).

This study clearly reveals that complicating factors may obscure true transduction efficiencies obtained with concentrated pseudotyped retrovirus vectors. Because it has not been possible to concentrate amphotropic vectors in a manner similar to that used for VSV-G vectors (1, 22), it is not clear whether pseudotransduction is unique to this type of retrovirus vector. Nevertheless, caution and rigorous confirmatory studies must be used to document true gene transduction as new vectors and novel gene delivery systems are developed.

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