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Genetics

A general method for the generation of high-titer, pantropic retroviral vectors: Highly efficient infection of primary hepatocytes

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ABSTRACT **Retroviral vectors have been central components in many studies leading to human gene therapy. However, the generally low titers and inefficient infectivity of retroviral vectors in human cells have limited their use. We previously reported that the G protein of vesicular stomatitis virus can serve as the exclusive envelope protein component for one specific retroviral vector, LGRNL, that expresses vesicular stomatitis virus G. We now report a more useful general transient transfection scheme for producing very high-titer vesicular stomatitis virus G-enveloped pseudotypes from any Moloney murine leukemia-based retroviral vector without having to rely on the expression of the cytotoxic G protein from the retroviral vector itself. We also demonstrate very high efficiency of infection with a pseudotyped *lacZ* vector in primary mouse hepatocytes. We suggest that pseudotyped retroviral vectors carrying reporter genes will permit genetic studies in many previously inaccessible vertebrate and invertebrate systems. Furthermore, because these vectors represent retroviral vectors of sufficiently high titer to allow efficient direct retroviral-mediated *in vivo* gene transfer, we also suggest that pseudotyped vectors carrying potentially therapeutic genes will become useful to test the potential for *in vivo* gene therapy.**

The development of efficient gene-transfer techniques has led to important progress toward human gene therapy. Currently, the majority of approved gene therapy trials in humans rely on vectors derived from Moloney murine leukemia virus (Mo-MLV) (1, 2). These retroviral vectors infect replicating mammalian cells efficiently and stably transduce genes into the genome of target cells. Most importantly, the availability of murine retroviral packaging cell lines allows generation of replication-defective retroviral vectors that can deliver genes into target cells but cannot spread further (3, 4). However, the titers of retroviral vectors produced from these packaging cells are relatively low, and some human cells cannot be infected efficiently (5, 6). Attempts to concentrate retroviral vectors by physical methods such as filtration or ultracentrifugation have generally resulted in massive loss of infectious virus, presumably due to instability of the retroviral envelope protein, which is essential for the interaction of virions with the cell-surface receptor and for their entry into the cell.

It is known that mixed infection of a cell with retrovirus and vesicular stomatitis virus (VSV) (6, 7), a member of the rhabdovirus family, produces progeny virions bearing the genome of one virus encapsidated by the envelope protein of the other (8). Such phenotypically mixed viruses have been termed "pseudotypes." We have recently developed a method for pseudotyping Mo-MLV-derived retroviral vectors with the G glycoprotein of VSV. We have shown that the VSV-G pseudotyped vector can be concentrated to high titers by ultracentrifugation. This vector can also infect many cell types, such as hamster and fish cells, ordinarily resistant to infection with conventional retroviral vectors containing the amphotropic envelope protein (7).

Because stable expression of the VSV-G protein is toxic to most mammalian cells, it has not been possible to produce stable packaging cell lines for efficient production of pseudotyped retroviral vectors. The previously reported pseudotyped retroviral vector, LGRNL, contains the VSV-G gene controlled by the 5′ long terminal repeat (LTR) of Mo-MLV and the neomycin phosphotransferase (Neo) gene controlled by the promoter derived from Rous sarcoma virus (RSV) (6, 7). Infectious LGRNL virus was generated by transfection of pLGRNL DNA into human kidney 293GP cells constitutively expressing the Mo-MLV gag and pol proteins (7). Virus generated from transfected 293GP cells can spread to nontransfected 293GP cells in the same culture, thereby generating high-titer LGRNL virus. However, because production of pseudotyped virus with this method required the presence of the VSV-G gene in the viral genome and because stable expression of high-level VSV-G protein is toxic to most mammalian cells, studies of gene transfer into mammalian cells using LGRNL are quite limited.

On the basis of our previous observation that 293GP cells can be transfected with a high efficiency, we have developed a scheme for producing VSV-G pseudotypes of any retroviral vector by transient expression of the VSV-G gene in 293GP cells containing a provirus of interest. In these studies, we demonstrate that high-titer viruses can be produced by this method, that they can be concentrated to high titers by ultracentrifugation, and that they can infect mammalian cells with high efficiency. This class of vectors promises to allow many new kinds of direct *in vivo* gene delivery for therapeutic purposes. Furthermore, because of the very broad host range of the pseudotyped vectors, it should become possible to deliver useful marker genes to a variety of vertebrate and invertebrate organisms for more efficient genetic analysis than has been previously possible.

MATERIALS AND METHODS

Plasmid Construction. Construction of pLZRNL has been described (9). Plasmid pLPONL contains a 732-bp *Hin*dIII–*Eco*RV fragment derived from the 5′ nontranslated region of poliovirus type 2 (Lansing) (10) followed by the Neo gene. To construct pLSPONL, the 1.3-kb *Xho* I–*Bam*HI fragment from the hepatitis B virus genome spanning the coding region of the surface antigen (S antigen) gene (11) was inserted into the unique *Xho* I site immediately upstream of the poliovirus

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Abbreviations: Mo-MLV, Moloney murine leukemia virus; VSV, vesicular stomatitis virus; HCMV, human cytomegalovirus; S antigen, hepatitis B virus surface antigen; moi, multiplicity of infection; cfu, colony-forming units; SV40, simian virus 40; LTR, long terminal repeat; RSV, Rous sarcoma virus.

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sequence in pLPONL. Plasmid pHCMV-Bam contains the human cytomegalovirus (HCMV) immediate early promoter followed by the splicing and polyadenylylation signals derived from the rabbit β-globin gene. To construct pHCMV-G, the *Bam*HI fragment containing the VSV-G gene was isolated from pLGRNL (6) and inserted into the unique *Bam*HI site downstream from the 3′ splice signal in pHCMV-Bam.

Cell Lines and Virus Production. The 293GP, PA317, and rat 208F cells have been described (7, 12, 13). Cell line HT1080/LSHL (used for helper virus detection) was isolated after infection of human fibrosarcoma HT1080 cells with the retroviral vector LSHL containing the hygromycin-resistant gene controlled by the simian virus 40 (SV40) enhancer and the early promoter. The infected cells were selected in hygromycin (400 μg/ml)-containing medium, and a hygromycin-resistant clone was randomly picked and named HT1080/LSHL. The human epitheloid carcinoma line HeLa was obtained from American Type Culture Collection. The SV40-transformed human tracheal epithelial line 9HTEO⁻ was supplied by D. C. Gruenert (University of California, San Francisco). The primary human fibroblasts Basinger and TO-119 were obtained from the University of California at San Diego Medical Center. Preparation of primary mouse hepatocytes has been described (14). All cell lines in this study were maintained in high-glucose Dulbecco's modified essential medium (DMEM)/10% fetal calf serum. Primary mouse hepatocytes were grown in DMEM/high glucose/5% fetal calf serum, epidermal growth factor at 50 μg/ml/insulin at 50 μg/ml.

To generate the 293GP clone containing the provirus of interest, the plasmid containing the retroviral construct was transfected into PA317 cells with calcium phosphate coprecipitation (15), and the amphotropic retroviral vector generated 48 hr after transfection was collected, filtered through a 0.45-μm filter, and used to infect 293GP cells in the presence of Polybrene (8 μg/ml). Neoresistant 293GP clones were picked after selection in G418 (400 μg/ml)-containing medium, and expression of the inserted gene was determined (see below). The clone that produced the highest amount of the reporter gene was then expanded and used for subsequent production of the pseudotyped virus.

To generate the pseudotyped virus, the 293GP clone containing the provirus of interest was transfected with 20 μg of pHCMV-G using calcium phosphate coprecipitation. The culture medium was replaced with fresh medium 8 hr after transfection, and the pseudotyped virus was collected between 24 and 96 hr after transfection. The titer of the virus was determined on 208F cells as described (6).

Concentration of Pseudotyped Viruses and Detection of Helper Virus Contamination. Filtered culture supernatants were subjected to ultracentrifugation as described (7). To detect helper virus in the viral stocks, the HT1080/LSHL cells were infected with LSPONL(G) or LZRNL(G) at a multiplicity of infection (moi) of 5:1 colony-forming units (cfu) per cell in the presence of Polybrene (8 μg/ml). The infected cultures were maintained in medium/Polybrene for 1 week after superinfection, and the presence of rescued LSHL virus was determined by infection of 208F cells with supernatants harvested from the LSPONL- or LZRNL-infected HT1080/LSHL cells and selection in hygromycin (400 μg/ml)-containing medium.

Detection of VSV-G Protein and S Antigen. Expression of VSV-G protein on the surface of 293GP cells was determined by cell cytometry analysis using a monoclonal antibody to VSV-G protein (I1, IgG2a, provided by John Holland, La Jolla, CA) as described (7). The S antigen secreted into the culture medium was determined by a commercially available ELISA kit (AUSZYME monoclonal; Abbott) according to the manufacturer's instructions.

Detection of β-Galactosidase (LacZ) Activity. To prepare protein extracts for the LacZ activity assay, cells in a 100-mm tissue culture plate were scraped off, pelleted, and resuspended in 100 μl of 250 mM Tris·HCl, pH 7.8. The cells were subjected to four freeze/thaw cycles, and cell debris was removed by low-speed centrifugation. To determine LacZ activity, 5 μl of the cell extract was mixed with 500 μl of β-galactosidase buffer containing 50 mM Tris·HCl (pH 7.5), 100 mM NaCl, 10 mM $MgCl_2$ and *o*-nitrophenyl β-D-galactopyranoside at 0.75 mg/ml. The mixture was incubated at 37°C until yellow color appeared, and the reaction was terminated by addition of 500 μl of 10 mM EDTA solution. The optical density of the reaction was determined at a wavelength of 420 nm and normalized to the protein concentration of the cell extract. To detect the cells expressing the LacZ activity, cells were fixed for 15 min with 1.25% glutaraldehyde and stained for 4 hr with a solution containing 5 mM ferriferrocyanide, 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) at 400 μg/ml and 1 mM $MgCl_2$.

RESULTS

Transient Production of Pseudotyped Retroviral Vectors from 293GP Cells. To test the feasibility of generating high-titer pseudotyped retroviral vectors by transient expression of VSV-G in 293GP cells containing a provirus, we first determined the transient transfection efficiency of 293GP cells with plasmid pHCMV-G containing the VSV-G gene expressed from the immediate early gene promoter of HCMV. Expression of VSV-G was determined by flow cytometric analysis of cells stained with a monoclonal antibody specific for the G protein. Fig. 1 shows that ≈90% of the cells expressed VSV-G protein on the cell surface 40 hr after pHCMV-G transfection. This extremely high efficiency of transfection suggested that high-titer pseudotyped retroviral vectors might be produced transiently by pHCMV-G transfection of a 293GP clone previously infected with a retroviral vector.

To test this hypothesis, we introduced two murine leukemia virus-based retroviral vectors containing two different reporter genes (Fig. 2) into 293GP cells by infection. Plasmid pLZRNL contains the *E. coli* β-galactosidase (*lacZ*) gene controlled by the 5′ Mo-MLV LTR followed by the Neo gene controlled by the RSV promoter (9). Plasmid pLSPONL contains the gene encoding the major S antigen controlled by the 5′ Mo-MLV LTR. In addition, a DNA element corresponding to the internal

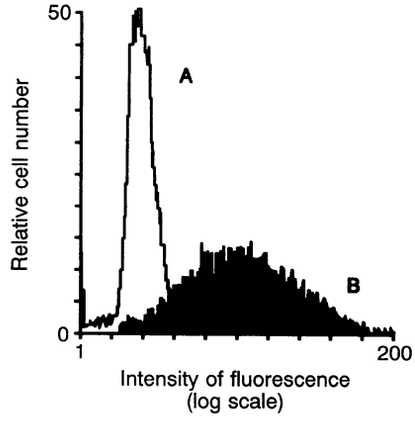

FIG. 1. Flow cytometric analysis of cell surface expression of VSV-G protein in 293GP cells. The mock-transfected (*A*) or pHCMV-G-transfected (*B*) 293GP cells were fixed and stained first with the monoclonal antibody I1 specific for VSV-G protein and then with fluorescein isothiocyanate-conjugated goat F(Ab′) fragment to mouse immunoglobulins (Cappel).

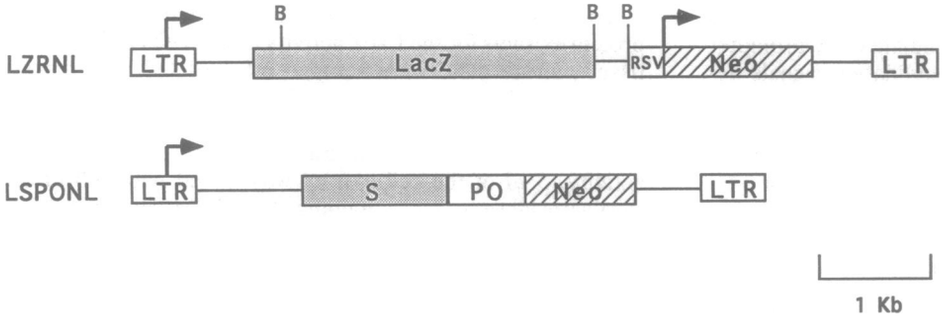

FIG. 2. Organization of retroviral vectors. Arrows indicate approximate locations of promoters and direction of transcription. LTR, Mo-MLV LTR; LacZ, the *Escherichia coli* β-galactosidase gene; RSV, LTR from RSV; Neo, neomycin phosphotransferase gene; S, S antigen gene; PO, internal ribosome entry site of poliovirus; B, *Bam*HI.

ribosome entry site of poliovirus (10) followed by the Neo gene was inserted immediately downstream from the S antigen gene. Previous work has shown that internal initiation of translation occurs efficiently in retroviral vectors carrying the poliovirus internal ribosome entry site sequence (16). To generate 293GP clones harboring the proviruses, we transfected plasmid pLZRNL or pLSPONL into amphotropic packaging line PA317 (12). Culture supernatant containing infectious LZRNL or LSPONL virus was collected 48 hr after transfection and used to infect 293GP cells. Colonies were picked after selection in G418-containing medium for 2 weeks, and the level of LacZ enzyme activity in LZRNL-infected cells or of S antigen secreted into the culture medium from LSPONL-infected cells was determined as described. The 293GP clone generating the highest LacZ activity (293GP/LZRNL) or S antigen (293GP/LSPONL) was selected and used for subsequent production of virus.

To generate pseudotyped virus, we transfected 20 μg of pHCMV-G into the 293GP/LZRNL or the 293GP/LSPONL clone using calcium phosphate coprecipitation (15). Culture supernatants were collected at various times after transfection, and the virus titer was determined on rat 208F fibroblasts. As shown in Table 1, titers $>10^5$ cfu/ml were obtained from a 100-mm tissue culture dish containing 6 ml of culture medium. Although syncytia formation in pHCMV-G-transfected 293GP cells became visible at 48 hr after transfection, the cells continued to produce infectious virus at a titer $>10^5$ cfu/ml for at least another 48 hr (Table 1). Generally, maximal production of pseudotyped viruses from pHCMV-G-transfected 293GP cells occurred between 48 and 72 hr after transfection. Transfection of the 293GP clones with a control plasmid pHCMV-Bam, containing no insert downstream from the HCMV promoter, produced no infectious virus (data not shown).

Concentration of Pseudotyped Retroviral Vectors by Ultracentrifugation. To determine whether the pseudotyped viruses can be concentrated without significant loss in titer, we sedimented the pseudotyped LZRNL and LSPONL vectors as described (7). Table 2 shows that 35% of the input LZRNL virus was recovered after one cycle of ultracentrifugation,

Table 1. Titers of retroviral vectors generated after transient transfection of pHCMV-G into 293GP cells

Virus	Time after transfection, hr	Virus titer,* cfu/ml
LZRNL	24	2.8×10^5
	48	1.0×10^6
	72	7.5×10^5
	96	2.1×10^5
LSPONL	24	1.0×10^5
	48	5.0×10^5
	72	2.0×10^6

*The retroviral vector was harvested from a 100-mm tissue culture dish containing 6 ml of culture medium. Virus titer was determined by infection of rat 208F fibroblasts with the culture medium and selection of G418-resistant colonies.

and the virus titer increased ≈300-fold. The titer of the LSPONL virus increased >100-fold after ultracentrifugation with virtually no loss of infectious virus. A number of other retroviral vectors have been similarly concentrated with comparable results (data not shown). Concentrated virus stocks were also assayed for the presence of replication-competent helper virus, and none was detected (data not shown). Thus, the method of transient pHCMV-G transfection into provirus-containing 293GP cells is an effective method for producing high-titer pseudotyped retroviral vectors. Consistent with our previous practice, the nature of the envelope protein is indicated in parentheses at the end of the vector name, with G denoting the VSV-G pseudotype and A denoting the amphotropic Moloney retroviral envelope.

Infectivity of the Pseudotyped Retroviral Vectors in Primary Rodent Hepatocytes. To compare the infectivity of amphotropic and pseudotyped viruses in mammalian cells, we infected several human-derived cell lines, including HeLa, 9HTEO$^-$ derived from human tracheal epithelial cells, and primary human fibroblasts derived from different tissues, at identical mois with the same batches of vector LSPONL(G) containing the VSV-G protein or of LSPONL(A) containing the amphotropic envelope protein and selected them in G418-containing medium. We have found that the pseudotyped virus is indistinguishable from the amphotropic virus in its ability to infect established human cell lines and primary human fibroblast cultures (Table 3).

Autologous transplantation of primary hepatocytes after retroviral-mediated gene transfer *in vitro* has been used as an approach to gene therapy for liver disease (17). However, this *ex vivo* approach is hindered by the fact that only 10–30% of the primary hepatocytes in culture can be infected by conventional retroviral vectors (5, 18, 19). To test whether a pseudotyped vector could mediate stable gene transfer into hepatocytes more efficiently than a vector containing the amphotropic envelope protein, we infected primary mouse hepatocytes with LSPONL(G) or LSPONL(A) and determined the amount of S antigen secreted into the culture medium. Fig. 3 shows that for LSPONL(A) virus, an increase in moi from 0.1:1 or 1:1 cfu per cell resulted in a 3-fold increase of S antigen secreted into the culture medium. A further increase of moi to 2:1 cfu per cell produced no significant increase in secreted S antigen. For LSPONL(G) virus, in contrast, an increase in moi proportionally increased S antigen secretion. At an moi of 5:1 cfu per cell, the LSPONL(G)-infected cells secreted 20-fold more S antigen than cells infected with the same virus at an moi of 0.1 cfu per cell. Fig. 3 also demonstrates that the pseudotyped virus can infect hepatocytes more efficiently than the conventional virus at the same moi as determined in rat 208F cells. At an moi of 2:1 cfu per cell, LSPONL(G)-infected cells secreted 3-fold more S antigen than LSPONL(A)-infected cells.

To examine the mechanisms leading to increased S antigen secretion by the pseudotyped vector, we infected primary mouse hepatocytes with either LZRNL(A) or LZRNL(G) and stained the cells histochemically with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) for LacZ activity 48 hr after

Table 2. Concentration of VSV-G pseudotyped retroviral vectors by ultracentrifugation

Virus	Virus titer,* cfu/ml		Fold conc	Total virus, cfu		Efficiency of recovery, %
	Preconc	Postconc		Preconc	Postconc	
LZRNL	6.7×10^5	2.0×10^8	298	3.4×10^8	1.2×10^8	35
LSPONL	8.0×10^5	1.0×10^8	125	2.1×10^7	2.0×10^7	95

Preconc, preconcentration, virus titer before ultracentrifugation; Postconc, virus titer after ultracentrifugation; conc, concentrated.
*Virus titers were determined on 208F cells as described for Table 1.

infection (Fig. 4). Even at the same moi of 1 cfu per cell, approximately three times as many hepatocytes were infected by LZRNL(G) virus as by LZRNL(A) virus (compare Fig. 4 *a* and *b*). At an moi of 1 cfu per cell normalized by titration in rat 208F cells, ≈3% of cells infected with LZRNL(A) expressed LacZ compared with ≈10% with LZRNL(G). When the moi of the pseudotyped vector was further increased to 5:1 and 10:1 cfu per cell, the percent LacZ-positive cells increased to ≈40% and 60%, respectively (Fig. 4 *c* and *d*). We were unable to achieve similar mois of the amphotropic *lacZ* retroviral vector LZRNL(A) because of its markedly lower titer. On the basis of these results, we conclude that primary mouse hepatocytes can be infected more efficiently with the pseudotyped virus than with the amphotropic virus. However, because the pseudotyped and the amphotropic vectors were produced by very different packaging cells, we cannot exclude the possibility that factors other than the presence of the VSV-G protein contribute to the high efficiency of infection with the pseudotyped vectors.

DISCUSSION

Our method permits the production of high-titer and broad-host-range (pantropic) forms of any Moloney-based retroviral vector for efficient delivery of therapeutic genes into mammalian cells. The use of our previously reported pseudotyped retroviral vector LGRNL is severely limited by the fact that it required expression of the VSV-G protein from the retroviral vector genome itself and by the cytotoxicity of the VSV-G protein. The scheme for generating pseudotyped retroviral vectors reported in the present study overcomes this problem and allows production of a VSV-G pseudotype of any retroviral vector.

The most important factors contributing to the success of the current scheme are the extremely high efficiency of transient transfection and the very high levels of expression of the gag and pol genes in the 293GP cells. Surprisingly, even with the ordinarily inefficient calcium phosphate coprecipitation method, the VSV-G gene can be transferred into up to 90% of 293GP cells. Insertion of the VSV-G gene under the control of the very strong HCMV promoter (20) is another factor contributing to production of high-titer pseudotyped vectors. Comparison of virus produced by transfection with pHCMV-G with that produced by transfection with pSV-G, in which the VSV-G gene is controlled by the SV40 enhancer and early promoter, showed that the titer of virus generated with pHCMV-G was ≈10,000-fold higher than that with pSV-G (data not shown). The inefficiency of the usually strong SV40 promoter-enhancer is probably due to expression in 293GP cells of the adenovirus E1A proteins, known to repress the SV40 enhancer function (21). Thus, the HCMV promoter is the preferred promoter for expression of the VSV-G gene in 293GP cells. Together, these two factors contribute to the production of pseudotyped viruses with a titer ranging from 10^5 to 10^6 cfu/ml. The great advantage of the method reported here, however, lies not only in the efficiency of initial virus production but also in the ability to concentrate the generated virus by simple ultracentrifugation to titers of 10^8–10^9 cfu/ml or greater.

Since our initial report of pseudotyping in 293GP cells, two reports have described the use of 293 cells for the production of relatively high titers of Moloney-based retroviral vectors (22, 23). The titers of retroviral vectors produced in these systems were certainly higher than those ordinarily produced by the traditional PA317 helper cell method, but these vectors described in these latter studies contained Moloney envelope proteins and would therefore not be stable to the concentration methods described by our laboratory group. Furthermore, their host range would be that of unmodified Moloney-based vectors, and improvements in infectability of some target cells would therefore be a consequence entirely of increased titer.

Due, at least partly, to the ability to produce pseudotyped retroviral vectors to very high titers, we have demonstrated that VSV-G pseudotyped vectors can infect primary mouse hepatocytes with great efficiency. This result is especially important because liver represents a potential target organ for clinical application of gene-transfer technology. Approaches to gene therapy of liver disease based on transplantation of autologous hepatocytes corrected *ex vivo* with retrovirus-

Table 3. Infectivity of the pseudotyped and the amphotropic viruses in human cells

Cell line	Neo^r titer, cfu/ml	
	LSPONL(G)	LSPONL(A)
208F (rat fibroblast)	1.2×10^6	8.2×10^5
HeLa (human kidney carcinoma)	4.6×10^5	1.0×10^5
9HTEO$^-$ (human tracheal epithelial cell)	2.2×10^6	5.0×10^5
Basinger (primary human fibroblast)	5.0×10^4	1.2×10^5
TO-119 (primary human fibroblast)	1.0×10^4	1.5×10^4

Neo^r, neomycin-resistant.

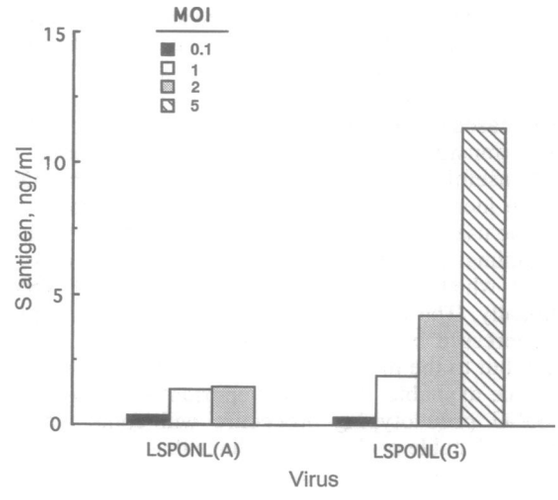

FIG. 3. Comparison of S antigen secretion from LSPONL(A)- and LSPONL(G)-infected primary mouse hepatocytes in culture. Primary mouse hepatocytes grown in a 60-mm culture dish were infected with either LSPONL(A) or LSPONL(G) at the indicated moi 2 days after seeding. Two days after infection, culture medium was collected and assayed for S antigen.

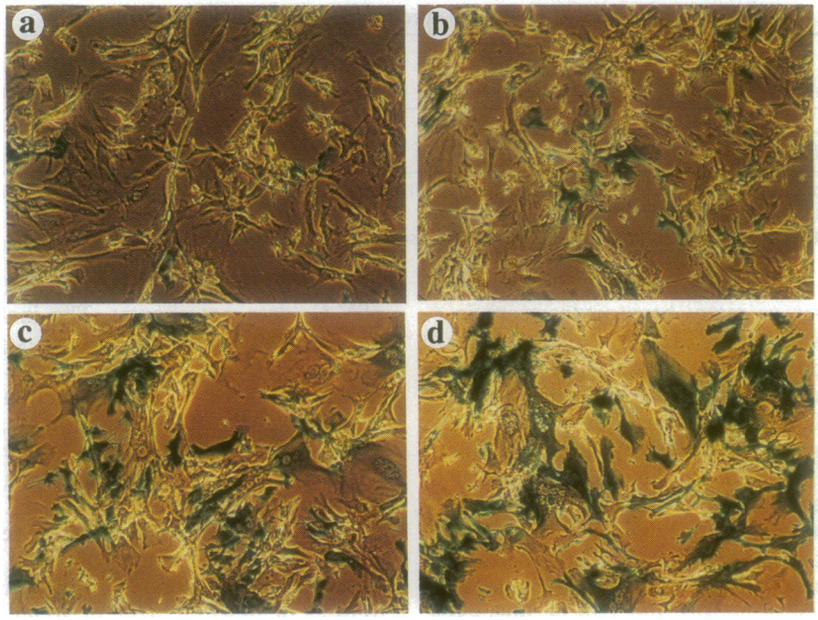

FIG. 4. Detection of β-galactosidase activity *in situ* in LZRNL(A)- and LZRNL(G)-infected primary mouse hepatocytes. (*a*) Hepatocytes infected with LZRNL(A) at an moi of 1:1. (*b–d*) Hepatocytes infected with LZRNL(G) at an moi of 1:1, 1:5, and 1:10, respectively. The β-galactosidase activity was detected *in situ* by 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining 48 hr after infection.

mediated gene transfer have been tested in animal models and applied to several human patients with familial hypercholesterolemia (17, 24, 25). More recently, Kay *et al.* (26) have shown that direct infusion of a retroviral vector containing the canine factor IX cDNA into the portal vein of factor IX-deficient hemophilic dogs resulted in persistent expression of low levels of canine factor IX and reductions of whole blood clotting and partial thromboplastin times. The presence of a high-titer pseudotyped virus that efficiently infects hepatocytes should facilitate direct gene transfer into liver *in vivo*, thereby circumventing the costs and time associated with the *ex vivo* approach to gene therapy for liver disease.

VSV-G pseudotyped vectors have been shown to infect not only mammalian cells but also cell lines derived from fish, *Xenopus laevis*, and insects (7). Because techniques for efficient gene transfer in many of these nonmammalian systems are not currently available, the retroviral vectors generated by the system described here should be useful for the study of gene function and for the establishment of transgenic organisms in lower vertebrate species.

As useful as the present method is for pseudotyping Moloney-based retroviral vectors, it suffers from the need for transient transfection, a step difficult to incorporate into clinically useful procedures for human gene therapy. Obviously, the ideal method for pseudotype production would involve the use of a stable packaging cell line that expresses no or minimal noncytotoxic amounts of VSV-G constitutively but which can express very high levels of VSV-G conditionally. The development of such cell lines requires tightly repressed but highly inducible conditional gene expression, but to the present time, such packaging lines have not become available. The next important improvement to the VSV-G pseudotyping procedure will come with the generation of stable packaging cell lines that are not susceptible to the cytotoxic effects of VSV-G protein.

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