Pseudotype Formation of Murine Leukemia Virus with the G Protein of Vesicular Stomatitis Virus

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Mixed infection of a cell by vesicular stomatitis virus (VSV) and retroviruses results in the production of progeny virions bearing the genome of one virus encapsidated by the envelope proteins of the other. The mechanism for the phenomenon of pseudotype formation is not clear, although specific recognition of a viral envelope protein by the nucleocapsid of an unrelated virus is presumably involved. In this study, we used Moloney murine leukemia virus (MoMLV)-based retroviral vectors encoding the gene for neomycin phosphotransferase to investigate the interaction between the VSV G protein and the retroviral nucleocapsid during the formation of MoMLV(VSV) pseudotypes. Our results show that VSV G protein can be incorporated into the virions of retrovirus in the absence of other VSV-encoded proteins or of retroviral envelope protein. Infection of hamster cells by MoMLV(VSV) pseudotypes gave rise to neomycin phosphotransferase-resistant colonies, and addition of anti-VSV serum to the virus preparations completely abolished the infectivity of MoMLV(VSV) pseudotypes. It should be possible to use existing mutants of VSV G protein in the system described here to identify the signals that are important for the formation of MoMLV(VSV) pseudotypes.

The assembly of enveloped animal viruses is characterized by selective inclusion of the viral genome and accessory virion proteins into budding viral particles (21). Although the mechanisms for selective encapsidation are not well characterized, it has been postulated that the recognition of viral envelope proteins within the plasma membrane by the viral nucleocapsids represents one probable control point for packaging specificity. Using internal image anti-idiotype antibodies, Vaux et al. have shown that the nucleocapsid of Semliki Forest virus contains a specific receptor for the cytoplasmic tail of the virion E2 spike glycoprotein (23), and they suggested that a specific receptor-ligand-like interaction between the two is likely to be critical in the organization of the budding of Semliki Forest virus and related viruses from infected cells. In apparent contrast to this high degree of specificity is the well-known phenomenon of pseudotype formation, in which mixed infection of a cell by vesicular stomatitis virus (VSV), a member of the rhabdovirus family, and retroviruses results in the production of progeny virions bearing the genome of one virus encapsidated by the envelope proteins of the other (7, 12, 28). Such phenotypically mixed viruses form plaques on appropriate indicator cells and can be neutralized by sera raised against the specific envelope protein.

The mechanism for the inclusion of the envelope protein of one virus into the virions of an unrelated virus is uncertain. Sequence comparison of VSV G protein and retrovirus envelope proteins reveals no significant sequence similarity among these proteins (19, 25). It is also difficult to determine whether G protein alone in the absence of other VSV-encoded proteins can participate in pseudotype formation. Pseudotypes do not form between VSV and alphaviruses such as Semliki Forest virus (29) even though pseudotypes may form between two alphaviruses (2) or between alphaviruses and related flaviviruses such as lactic dehydrogenase virus (9). In some cases, phenotypic mixing is unilateral, as in the case of VSV with fowl plague virus (FPV) or VSV with Sindbis virus. The pseudotype virus particle VSV(FPV) containing the VSV genome encapsidated by the envelope protein of FPV or VSV(Sindbis) (29) has been demonstrated, but reverse pseudotypes containing the FPV or Sindbis virus genome with the VSV G protein have not been detected. Thus, in some cases, specific recognition of a viral envelope protein by the nucleocapsid of an unrelated virus seems to be involved in pseudotype formation. An understanding of how two distinctive viruses interact in the production of pseudotypes should shed light on the mechanisms of assembly of viral components during the budding process.

In this study, we used Moloney murine leukemia virus (MoMLV)-based retroviral vectors encoding the gene for neomycin phosphotransferase (Neo) to investigate the interaction between the VSV G protein and the retroviral nucleocapsid during the formation of MoMLV(VSV) pseudotypes. Our results indicate that VSV G protein alone is sufficient to interact with the nucleocapsid of MoMLV in the formation of MoMLV(VSV) pseudotypes and that the VSV G protein can be incorporated into the virions of retrovirus efficiently.

MATERIALS AND METHODS

Plasmid construction. The MoMLV-based retroviral vector pLRNL (Fig. 1) contains the Neo resistance (Neo^r) gene under control of the promoter of Rous sarcoma virus (RSV) (10). A single *Bam*HI is immediately upstream of the RSV promoter. Into this *Bam*HI site, a 1.7-kb *Bam*HI fragment containing the entire coding region of the VSV G gene (18) was inserted, giving rise to construct pLGRNL (Fig. 1). Alternatively, a 2.7-kb *Xba*I fragment containing the envelope (*env*) gene of amphotropic retrovirus 4070A (3) was inserted into the same *Bam*HI site in pLRNL to form construct pLARNL (Fig. 1). Plasmid pSVGP (Fig. 1) was constructed by inserting a 5.8-kb *Hind*III-*Sca*I fragment containing the simian virus 40 (SV40) early promoter and the *gag* and *pol* regions from pSAM into the mammalian expres-
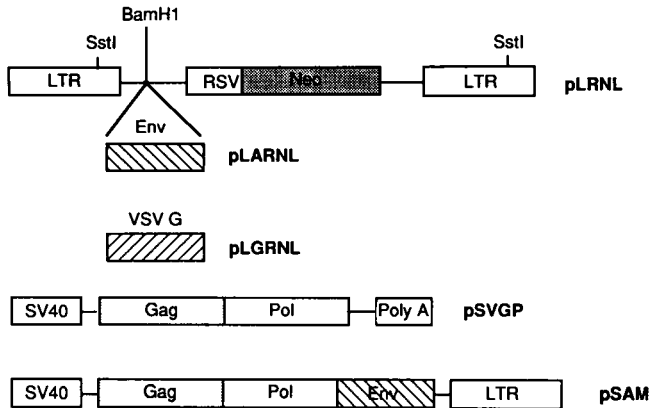
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FIG. 1. Retroviral vectors and packaging constructs. In pLRNL, the Neo[r] gene expressed from the RSV promoter was inserted between the LTRs of MoMLV. The env gene of 4070A virus was inserted at the single BamHI site of pLRNL to form pLARNL. pLGRNL is similar to pLARNL except that the env gene was replaced by the VSV G gene. The packaging construct pSVGP contains the gag and pol genes derived from MoMLV under control of the SV40 enhancer and the early promoter. Poly A, The polyadenylation signal derived from SV40. Construct pSAM, containing the gag, pol, and env genes of MoMLV, has been described previously (14). The figure is not drawn to scale.

sion vector pcD (15). Plasmid pSAM (Fig. 1), containing the gag region of MoMLV, a hybrid pol region between MoMLV and amphotropic virus 4070A, and the env region of 4070A, has been described elsewhere (14).

Cell culture and generation of virus. Cells were grown in Dulbecco modified Eagle medium with high glucose supplemented with 10% fetal calf serum. Hypoxanthine phosphoribosyltransferase-deficient 208F cells were derived from Fischer rat cells by selection in 6-thioguanine (17). Thymidine kinase-deficient BHK cells were derived from BHK-21 cells by selection with 5-bromodeoxyuridine (11). LNSV cells were derived from SV40-transformed hypoxanthine phosphoribosyltransferase-deficient Lesch-Nyhan cells (8), and HeLa cells were obtained from the American Type Culture Collection. To generate virus, 10 μg of the vector DNA and 10 μg of pSAM or pSVGP were cotransfected into cells by using the calcium phosphate precipitation procedure (6). Culture medium was collected 36 h after transfection and filtered through a 0.45-μm-pore-size filter. To determine virus titers, cells were infected overnight with filtered supernatants in the presence of Polybrene (4 mg/ml). Infected cells were selected in medium containing G418 (400 mg/ml), and colonies were scored about 14 days after infection.

Antibody neutralization and complement-mediated virolysis. Rabbit anti-VSV serum was purchased from Lee Biomolecular Research Laboratories, Inc. Virus supernatants in a final volume of 200 μl of Dulbecco modified Eagle medium–10% fetal calf serum containing various amounts of specific antisera, complement, or equivalent volumes of normal serum were heated at 37°C for 45 min. The mixture was then added to culture media in the presence of Polybrene (4 μg/ml). Infected cells were selected in G418-containing medium as described above.

DNA analysis. Genomic DNA was prepared as described previously (13). DNA samples were digested with appropriate restriction enzymes, electrophoresed on 0.8% agarose gels, and transferred to nylon membranes. A ^{32}P-labeled DNA probe containing the complete Neo gene prepared by

TABLE 1. Transient virus production from BHK cells following cotransfection by pSAM with either pLRNL or pLGRNL

Virus	Cell infected	Titer (CFU/ml)
LRNL	208F	480
	BHK	<10
LGRNL	208F	380
	BHK	260

priming with random deoxyhexanucleotides (Amersham) was used to hybridize with the filters. Filters were washed with 0.1× SSC–0.5% sodium dodecyl sulfate at 53°C several times and subjected to autoradiography.

RESULTS

Infection of hamster cells with the amphotropic N2 virus. We have studied the interaction between the VSV G protein and the nucleocapsid of MoMLV by introducing the Neo gene as a selectable marker with MoMLV(VSV) pseudotypes into cells incapable of supporting wild-type retrovirus infection. Since it is known that hamster cells are not susceptible to infection with MoMLV-based retroviruses because of the absence of retrovirus-specific cell surface receptors (5), we assayed the MoMLV(VSV) pseudotypes on BHK cells. In initial experiments, we confirmed that this cell line is indeed refractory to the infection of retrovirus. Amphotropic N2 virus containing the Neo gene inserted between the long terminal repeats (LTRs) of MoMLV was prepared from the producer cell line PA317/N2 and titered on both BHK cells and rat 208F fibroblasts, a cell line that is susceptible to retrovirus infection. We observed a 10[5]-fold decrease in Neo[r] CFU in BHK cells compared with that in rat 208F cells (data not shown); thus, the BHK cell line that we used failed to support infection by N2 virus containing the amphotropic retroviral envelope protein.

Generation of MoMLV(VSV) pseudotypes. To produce MoMLV(VSV) pseudotypes, we used several MoMLV-based retroviral vectors (Fig. 1). The retroviral vector pLRNL contains the Neo gene inserted between the LTRs of MoMLV, and expression of the Neo gene is controlled by the promoter of RSV (10). Construct pLARNL contains the gene encoding the amphotropic MoMLV envelope protein inserted immediately upstream of the RSV-Neo cassette in pLRNL. Construct pLGRNL is similar to pLARNL except that the MoMLV env gene was replaced with the gene encoding the VSV G protein (19). To generate infectious virus, either pLGRNL or pLRNL was cotransfected into BHK cells with the helper vector pSAM, which expresses all MoMLV-encoded genes from the SV40 early promoter (14). Supernatants from transfected BHK cells were collected at 48 h posttransfection and used to infect susceptible 208F cells and resistant BHK cells.

The ability of each of these constructs to produce virus capable of conferring Neo resistance is shown in Table 1. As expected, virus derived from pLRNL was able to infect 208F cells efficiently but failed to infect BHK cells. In contrast, virus derived from pLGRNL could infect not only 208F cells but also, to a lesser extent, BHK cells, as indicated by the appearance of Neo[r] colonies. Transfection of pLGRNL alone into BHK cells failed to generate Neo[r] virus (data not shown). Since the virus derived from pLGRNL encoded VSV G protein, we postulate that G protein produced in pLGRNL-transfected BHK cells was incorporated into at least some of the retroviral virions, producing MoMLV-

TABLE 2. Transient virus production from BHK cells following cotransfection by pSVGP with pLRNL, pLARNL, or pLGRNL

Expt no.	Virus	Cell infected	Titer (CFU/ml)
1	LRNL	208F	<10
		BHK	<10
	LARNL	208F	280
		BHK	<10
	LGRNL	208F	440
		BHK	680
2	LRNL	208F	<10
		BHK	<10
	LARNL	208F	260
		BHK	<10
	LGRNL	208F	480
		BHK	720

TABLE 3. Neutralization of MoMLV(VSV) pseudotypes with anti-VSV antibody

Virus[a]	Normal rabbit serum	Anti-VSV serum			Titer (CFU/ml) in:	
		1:500	1:100	1:20	208F	BHK
LARNL	−	−	−	−	240	<10
	+	−	−	−	230	<10
	−	−	+	−	230	<10
	−	−	−	+	220	<10
LGRNL	−	−	−	−	620	880
	+	−	−	−	600	880
	−	+	−	−	110	150
	−	−	+	−	<10	<10
	−	−	−	+	<10	<10

[a] Supernatants were harvested 48 h after cotransfection of pSVGP with pLARNL or pLGRNL into BHK cells.

(VSV) pseudotypes capable of infecting hamster cells. The results in Table 1 also indicate that G protein alone, without the participation of other VSV-encoded proteins, is sufficient for the formation of MoMLV(VSV) pseudotypes.

To determine whether the presence of MoMLV envelope protein is necessary for the formation of MoMLV(VSV) pseudotypes, we cotransfected each retroviral construct in Fig. 1 with pSVGP into BHK cells. Construct pSVGP contains the *gag* and *pol* genes of MoMLV expressed from the SV40 early promoter, but the MoMLV *env* gene is absent. The virus expressing the Neo[r] marker was titered separately on 208F and BHK cells (Table 2). As expected, no infectious virus was detected in cells cotransfected with pLRNL and pSVGP, since the MoMLV *env* gene was absent in both plasmids. Virus derived from pLARNL containing the MoMLV *env* gene gave a Neo[r] titer of 200 to 300 CFU/ml in 208F cells, but this preparation failed to infect BHK cells. Virus derived from pLGRNL containing the VSV G gene could infect both 208F and BHK cells with similar efficiencies. Since the LGRNL virus was generated in the complete absence of MoMLV *env* protein, we conclude that VSV G protein can be assembled into the murine retroviral particles without the participation of any retrovirus-encoded envelope protein.

The specificity of infection with the MoMLV(VSV) pseudotype can be ascertained by treatment with neutralizing antiserum specific to VSV. Virus generated transiently by cotransfection of pLARNL or pLGRNL with pSVGP was incubated at 37°C for 45 min either with normal rabbit serum or with various dilutions of anti-VSV serum. The virus was then assayed for the formation of Neo[r] colonies on 208F and BHK cells. Anti-VSV serum at a dilution of 1:100 markedly reduced the infectivity of LGRNL virus on both cell types, whereas the titer of LARNL on 208F cells was not affected by exposure to VSV antiserum (Table 3). This observation further supports the hypothesis that VSV G protein is assembled into the virions of LGRNL virus.

Incorporation of VSV G protein into retroviral particles seemed to be efficient, since virus transiently generated by cotransfecting pLGRNL or pLARNL with pSVGP gave similar Neo[r] titers on 208F cells (Table 2). To determine whether the process of incorporating G protein into retroviral virions is equally efficient in the presence of retroviral envelope protein, we cotransfected pLGRNL with pSAM into BHK cells and analyzed the proportions of pure pseudotypes and phenotypically mixed particles by anti-VSV antibody-directed, complement-mediated virolysis. Without any treatment, transiently generated LGRNL virus

gave Neo[r] titers of 380 CFU/ml on 208F cells and 260 CFU/ml on BHK cells (Table 4). Addition of normal rabbit serum to the same virus preparation had no effect on virus titer. In contrast, the titer dropped to 270 CFU/ml on 208F cells and to the background level on BHK cells when the virus was pretreated with a 1:20 dilution of the anti-VSV antibody. This result indicates a titer of LGRNL virus containing only G protein in this preparation of approximately 110 CFU/ml on 208F cells. To determine the fraction of virions containing only the retroviral envelope protein and the fraction of virions containing both the retroviral envelope protein and the VSV G protein, we treated the virus preparation with a combination of rabbit complement and the anti-VSV antibody. The potentiating effect of complement-mediated virolysis has been shown previously to eliminate VSV(MoMLV) pseudotypes efficiently (27). Addition of rabbit complement alone to the virus preparation slightly reduced the Neo[r] titers to 320 CFU/ml on 208F cells and 230 CFU/ml on BHK cells (Table 4), probably because of the nonspecific interaction of fetal calf serum in the media with rabbit complement. Virus pretreated with a 1:20 dilution of the anti-VSV antibody together with rabbit complement gave a Neo[r] titer of 90 CFU/ml on 208F cells, suggesting that this population of Neo[r] virus contains only the retroviral envelope protein in the virion. This interpretation is consistent with the results shown in Table 4, in which similar

TABLE 4. Antibody complement-mediated virolysis of MoMLV(VSV) pseudotypes

Virus[a]	Normal rabbit serum	Anti-VSV serum	Rabbit complement[b]	Titer (CFU/ml) in:	
				208F	BHK
LARNL	−	−	−	480	<10
	+	−	−	460	<10
	−	−	+	410	<10
	−	+	+	400	<10
LGRNL	−	−	−	380	260
	+	−	−	380	250
	−	−	+	320	230
	−	+	−	270	<10
	−	+	+	90	<10

[a] Supernatants were harvested 48 h after cotransfection of pSAM with pLARNL or pLGRNL into BHK cells.

[b] Supernatants were treated with either 10 µl of normal rabbit serum or 10 µl of anti-VSV serum at a 1:20 dilution with or without 10 µl of rabbit complement. All reactions were done in a final volume of 200 µl, heated at 37°C for 45 min.
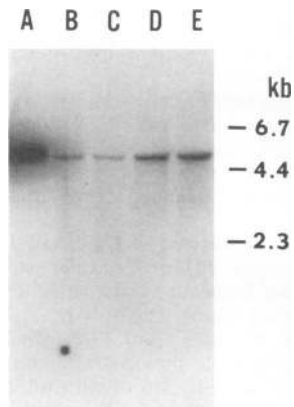
FIG. 2. Southern analysis of LGRNL-infected cells. Genomic DNA was isolated from two different Neo^r 208F clones (lanes B and C) and two different Neo^r BHK clones (lanes D and E) that were infected with LGRNL virus. DNA (10 μg) was cut with *Sst*I, run on a 1% agarose gel, transferred to a nylon-type filter, and probed with a Neo gene-specific probe. Lane A contained 5 ng of pLGRNL plasmid DNA digested with *Sst*I to serve as a marker.

TABLE 5. Relative susceptibilities of different cell lines to MoMLV(VSV) pseudotypes

Infected cell	Titer (CFU/ml)[a] of:	
	LARNL	LGRNL
208F (rat fibroblast)	250	450
BHK (hamster kidney)	<10	680
LNSV (human fibroblast)	16	180
HeLa (human carcinoma)	12	30

[a] Supernatants were harvested 48 h after cotransfection of pSVGP with pLARNL or pLGRNL into BHK cells. An equal volume of each supernatant was then applied to different cell types, and Neo^r titers were determined 2 weeks after G418 selection. The experiment was repeated three times, and the results of one experiment are presented.

treatments of LARNL virus containing only retroviral envelope protein in the virions had little effect on its Neo^r titer when measured on 208F cells. Thus, it is likely that VSV G protein can be efficiently incorporated into pseudotype virus even in the presence of retroviral envelope protein.

Analysis of LGRNL proviral DNA in infected cells. Retroviral DNA integrates into host chromosomes in a fashion that maintains the linear organization of the viral genome (22). To establish that each LGRNL-infected clone contained proviral sequences in the expected arrangement, chromosomal DNA from several Neo^r clones was digested with the restriction enzyme *Sst*I. *Sst*I cleaves only once in each LTR of LGRNL and is therefore expected to yield a 5-kb fragment (see Fig. 1). When the chromosomal DNA from two BHK clones and two 208F clones was cleaved with *Sst*I and hybridized to a probe containing the Neo gene, a fragment of about 5 kb was detected (Fig. 2, lanes B to E), and the size of this fragment was identical to that of the *Sst*I-cleaved pLGRNL plasmid DNA (Fig. 2, lane A). Thus, like wild-type retrovirus DNAs, the LGRNL proviral DNA appears to have an uninterrupted and unrearranged organization in the infected cells.

Infection of human cells with LGRNL virus. The retroviral *env* gene that we used in this study is derived from the *env* regions of amphotropic virus 4070A (3). The envelope glycoprotein encoded by this gene can bind to cell surface receptors present on cells from a wide range of mammalian species, including human cells. However, several human lines were more refractory to amphotropic viral infection than were murine cells. The Neo^r titer of LARNL in human cells such as HeLa cells or LNSV fibroblasts was about 20-fold lower than that in rat 208F cells (Table 5). While LGRNL virus infected HeLa cells with an efficiency similar to that of LARNL virus, it infected LNSV fibroblasts much more efficiently than did LARNL virus (Table 5). The major block to infection of LNSV cells with retrovirus may therefore be due to inefficient interaction of cell surface receptors with retroviral envelope protein. Since VSV is known to infect HeLa cells efficiently, the restriction in infecting HeLa cells by MoMLV(VSV) pseudotypes is probably due to postpenetration events.

DISCUSSION

Through the use of retroviral vectors expressing the Neo^r selectable marker, we have shown that VSV G protein can be incorporated into the virions of retrovirus in the absence of other VSV-encoded proteins or of retrovirus envelope protein. The process is efficient and results in the production of infectious virus with titers comparable to that of *env*-containing virus. Infection of hamster cells by MoMLV-(VSV) pseudotypes gave rise to Neo^r colonies, and addition of anti-VSV serum to the virus preparations completely abolished the infectivity of MoMLV(VSV) pseudotypes. The presence of VSV G protein in the virions is therefore correlated with the broadened host range of the retroviral vectors.

The final assembly of VSV from its components takes place at the plasma membrane of infected cells. Viral nucleocapsid (N) protein is preassembled with viral RNA to form the nucleocapsids in the cytosol of the infected cell. The viral matrix (M) protein and the nucleocapsids then associate with the plasma membrane and are budded off from the cell, picking up the G-protein-containing lipid bilayer in the process (21). Weiss and Bennett (24) have shown that VSV(RSV) pseudotypes are formed in RSV-infected cells superinfected with the G-protein mutant *ts*045 of VSV, while RSV(VSV) pseudotypes were produced in the same cells infected with the M-protein mutant *ts*G31, suggesting that VSV M protein is not required for the assembly of G protein into retrovirus virions. Our studies are consistent with their conclusion. In addition, since G protein is the sole VSV-encoded protein used in our study, it is clear that no VSV gene products other than G protein are required for the formation MoMLV(VSV) pseudotypes.

In this study, we obtained similar Neo^r titers when transiently generated LGRNL and LARNL viruses were assayed on 208F cells. Since expression of both the retrovirus *env* gene and the VSV G gene is regulated by the same MoMLV LTR, the amounts of transiently produced proteins in the transfected BHK cells are likely to be similar, although we cannot exclude the possibility that the stabilities of the two proteins are different. This observation suggests that the interaction between the nucleocapsids of retrovirus and the VSV G protein is as efficient as with authentic retroviral envelope protein. Alternatively, similar virus titers may indicate more efficient infection of 208F cells by MoM-LV(VSV) pseudotypes present at lower titers. The receptors for murine retroviruses have been shown to be cell surface proteins (1), whereas there is some evidence that a membrane phospholipid may be the receptor for VSV (20), possibly accounting for the wide host range of VSV. Our

observed Neo^r titers of MoMLV(VSV) pseudotypes therefore may simply reflect increased cellular susceptibility to infection with MoMLV(VSV) pseudotypes than with *env*-containing MoMLV.

Although the nucleocapsid of Semliki Forest virus contains a specific receptor for the cytoplasmic tail of the viral E2 spike glycoprotein, the mechanism of alphavirus budding is not directly applicable to other enveloped viruses. Retroviruses such as RSV are able to produce virions by using mutant forms of the envelope protein that lack a cytoplasmic tail (16), suggesting that if an interaction between the envelope protein of retrovirus and its nucleocapsid is required to mediate the incorporation of the glycoprotein into the envelope of budding viral particles, it can occur entirely within the lipid bilayer, presumably within the hydrophobic anchor domain of the envelope protein. This result contrasts somewhat with those of Whitt et al. (26), who reported that VSV G proteins with only one or three of the membrane-proximal amino acids of its cytoplasmic domain or G protein with foreign cytoplasmic domains of 3, 10, or 128 amino acids were not detected in VSV virus particles. This difference may reflect fundamental differences in the budding processes of retroviruses and other enveloped viruses such as VSV. Use of many existing mutants of VSV G protein in the system described here should make it possible to identify the signals that are important for the formation of MoMLV-(VSV) pseudotypes. This, in turn, should shed light on the mechanisms used to aggregate specific viral components during the budding process.

Currently, retroviral vectors provide the most efficient method for introducing new genetic information into mammalian cells. Since retroviruses containing amphotropic envelope protein in their virions can infect human cells, they represent a potentially useful tool for genetic approaches to the treatment of genetic disorders in humans (4). However, as shown in Table 5, human cells are relatively resistant to amphotropic retrovirus infection. The presence of relatively few amphotropic retrovirus receptors, inefficient reverse transcription in the cytoplasm, or accelerated degradation of unintegrated viral DNA in human cells may account for the inefficient infection of human cells by the retroviral vectors. The results described here indicate that MoMLV(VSV) pseudotypes can overcome at least some of the potential blocks for infection by broadening the host range of the retroviral vectors, thus facilitating more efficient transfer of foreign genes into human cells. The development of stable packaging cell lines constitutively expressing VSV G protein would facilitate even further the generation of retroviral vectors with wider host range and with increased efficiency of infection in human cells.

ACKNOWLEDGMENTS

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