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Packaging System for Rapid Production of Murine Leukemia Virus Vectors with Variable Tropism

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A method for rapidly producing helper-free murine leukemia virus (MLV) without using packaging cell lines is described. Viruses bearing ecotropic or amphotropic MLV or Rous sarcoma virus envelope glycoprotein and containing various retroviral vector genomes have been prepared with titers 30 to 40-fold higher than those produced by transient transfection of standard packaging cells. This system can be used to alter the cellular tropism of MLV by incorporating other envelope glycoproteins and to prepare retroviral vector stocks without establishing stable producer cell lines. This method will be particularly useful for preparing viruses that encode toxic proteins and for the rapid analysis of panels of mutant envelope glycoproteins.

Helper-free stocks of murine leukemia virus (MLV) containing a retroviral vector genome are typically made by transfecting a packaging cell line that produces the viral proteins with a retroviral vector plasmid encoding a genome to be packaged. Because viral titers resulting from transient transfection are usually low, packaging cells that have stably integrated the retroviral vector are usually required.

We have modified this approach for making MLV vectors to a simpler and more rapid method in which virus is produced in COS-7 cells transiently transfected with simian virus 40 origin (SV40 *ori*)-containing expression plasmids. Cotransfection with packaging vectors encoding MLV structural proteins and a retroviral vector encoding a packageable MLV transcript results in production of replication-defective virions that can be titered by drug selection of infected target cells. We have used this method to produce relatively high transient titers of MLV bearing the amphotropic, ecotropic, or Rous sarcoma virus (RSV) envelope glycoprotein.

Virus production in COS-7 cells. Vectors for expressing retroviral components in COS-7 cells are shown in Fig. 1. SV-Ψ⁻-E-MLV, SV-Ψ⁻-A-MLV, and SV-Ψ⁻-env⁻ are derived from Moloney MLV and contain the SV40 *ori*, a packaging site deletion, and, respectively, ecotropic, amphotropic, and deleted *env* genes. The *env* expression vectors SV-E-MLV-env, SV-A-MLV-env, and SV-RSV-env encode, respectively, the ecotropic, amphotropic, and RSV (subgroup A) envelope glycoproteins. In addition, three retroviral vectors were used to provide a source of packageable MLV transcript: DOL (7), HSG (4), and pMV7 (6) (not shown). Each contains MLV long terminal repeats, the Neo^r gene, and the packaging sequence, Ψ. HSG, in addition, contains *gag* region sequences to increase packaging efficiency (4); only DOL contains the SV40 *ori* (located at the 5′ boundary of the Neo^r gene).

To produce virus, COS-7 cells (1.2×10^6) were seeded in 10-cm-diameter dishes and transfected the next day with 10 μg of each plasmid (20 or 30 μg, total) by the calcium phosphate-chloroquine method (13). Supernatants were harvested 72 h after transfection, filtered through a 0.45-μm-pore-size filter, and immediately used to infect target cells. Virus-containing supernatants (0.5, 0.1, and 0.01 ml) were incubated in a total volume of 0.5 ml with 2.0×10^5 cells in a six-well culture dish in medium containing 4.0 μg of Polybrene per ml. After 2 to 4 h, 0.5 ml of fresh medium was added. Virus was removed after 12 h, and 2.0 ml of medium was added to each well. Two days after infection, 1/20 of the cells were transferred to selection medium (for HOS and 3T3, medium contained 400 μg of G418 per ml; for QT6 cells, medium contained 300 μg of G418 per ml). Cells were fed every 3 days, and after 10 to 12 days, colonies were stained. Colonies in the well containing the greatest number of discrete colonies were counted, normalized to 1.0 ml of virus, and multiplied by 5 to account for the 1:20 split and the approximately fourfold increase in cell number between the times of infection and selection.

Cotransfection with SV-Ψ⁻-E-MLV and DOL yielded high-titer virus that was dependent on the presence of the packaging vector and the SV40 *ori* of the retroviral vector (cotransfection with HSG or pMV7, neither of which has the SV40 *ori*, yielded an approximately 100- or 25-fold-lower viral titer, respectively; Table 1). Cotransfection of COS-7 cells with SV-Ψ⁻-A-MLV and DOL yielded virus of slightly lower titer than that produced with SV-Ψ⁻-E-MLV. This result is probably not due to different levels of envelope glycoprotein synthesis, since the two molecules showed similar intensities on immunoblots (8). In five additional experiments, COS-produced virus titers averaged 0.9×10^5/ml and ranged from 3×10^4 to 14×10^4/ml.

Ψ-2 packaging cells transfected with DOL produced virus with transient virus titers about 40-fold lower than those of COS-7 cells transfected with SV-Ψ⁻-E-MLV and DOL (Table 1). In a comparative experiment, virus titers were somewhat lower, but the ratio of COS- to Ψ-2-produced virus remained high (200:1; data not shown). This difference is not due solely to the level of virus produced, since there was only about twofold more reverse transcriptase activity recovered in COS cell supernatants than in Ψ-2 cell supernatants. This infectivity difference is probably due, in large part, to limiting amounts of packageable retroviral transcript in transiently transfected Ψ-2 cells. It may also be due, in part, to the presence in Ψ-2 cells of endogenous MLV proviruses whose transcripts could compete with those of the retroviral vector for encapsidation. Virus produced by

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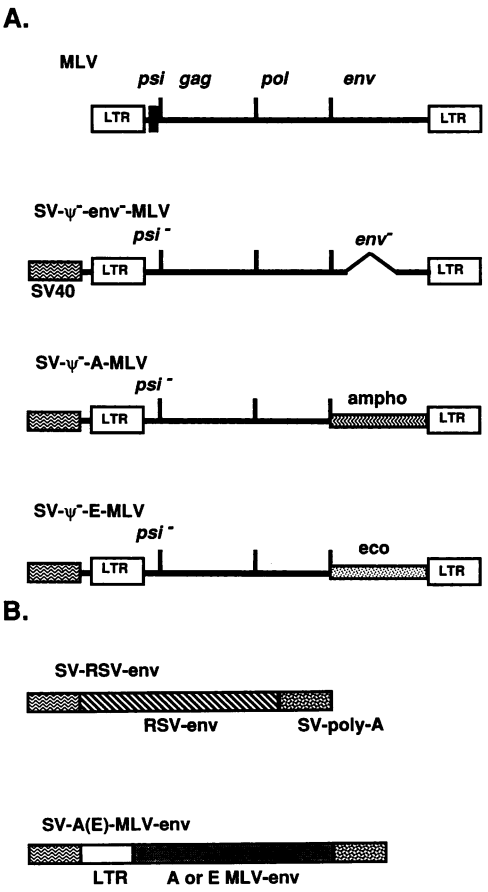

FIG. 1. Expression vectors for producing MLV in COS-7 cells. The MLV proviral structure is shown at the top of panel A. Structures of the vectors for expression of RSV envelope glycoprotein, SV-RSV-env, and of amphotropic (ampho) and ecotropic (eco) MLV envelope glycoprotein, SV-A-MLV-env and SV-E-MLV-env, are shown in panel B. SV-Ψ⁻-E-MLV consists of Ψ⁻-Moloney MLV (9) and the SV40 *ori* and enhancer from the vector pSV7d (12). SV-Ψ⁻-A-MLV is similar to SV-Ψ⁻-E-MLV except that it contains the *env* gene of the 4070a amphotropic strain of MLV at positions 5401 to 7846 (5). SV-Ψ⁻-env⁻-MLV is similar to the two vectors named above but carries a deletion of the *env* gene from positions 6076 to 7195. SV-E-MLV-env and SV-A-MLV-env contain the Moloney MLV ecotropic and 4070a amphotropic *env* genes and have been described previously (8). SV-RSV-env contains the *Sac*I fragment (positions 225 to 6865) of an *env* cDNA derived from the RSV Prague A strain provirus pJD100 (1a). LTR, long terminal repeat.

murine packaging cells has been shown to include a large proportion of particles containing an endogenous MCF-like RNA transcript (11) that is incorporated into particles at about the same level and transferred by infection at about the same frequency as is the transferred retroviral vector genome. Because COS-7 cells are of simian origin, they lack endogenous MLV sequences. Although the efficiency of packaging of simian endogenous virus transcripts into murine virions is unknown, it has been shown that reticuloendotheliosis virus particles package heterologous MLV transcripts inefficiently in the presence of the homologous transcript (2).

Absence of detectable wild-type MLV formed by recombination of input plasmids. The plasmids described here do not themselves encode replication-competent virus. However, recombination between input plasmids during transfection or during reverse transcription of copackaged retroviral and packaging vector RNA could generate a replication-competent genome. To test for the presence of low levels of such virus, ecotropic virus (10^5 PFU/ml) was prepared by using SV-Ψ⁻E-MLV and DOL. This virus (0.5 ml) was applied to 3T3 cells, which were then passaged for 16 days to allow for spread of wild-type virus that might be present. Supernatant from these cells showed background reverse transcriptase levels (<0.1 U/ml, similar to the value for uninfected control cells), while cells that had been infected on the same day with a small amount (approximately 100 infectious units) of replication-competent MLV showed readily detectable virus (38.4 U/ml).

To test more sensitively for replication-competent virus, a marker rescue assay was used. A cloned 3T3 cell line harboring an MLV provirus with a Neo^r-selectable marker was infected with 1 ml of virus-containing supernatants. After 4 days, supernatants from these cells were used to infect 3T3 cells, and drug-resistant colonies were counted 9 days later. Again, no replication-competent virus was detected in COS cell-derived supernatants, while control replication-competent MLV (approximately 100 tissue culture infectious units) resulted in 2,400 Neo^r colonies per ml. Therefore, this stock, although probably contaminated with a small amount of recombinant virus, contained less than one wild-type virion per 0.5×10^5 CFU.

For experiments in which it is desirable to even further reduce the likelihood of recombination, virus can be prepared by cotransfecting cells with the Env⁻ packaging vector, SV-Ψ⁻-env⁻-MLV, and one of the envelope expression vectors (Table 1, SV-E-MLV-env, SV-Ψ⁻-env⁻-MLV, and DOL). Although the titer of virus produced by this means is slightly reduced, an additional recombination step is required to form a replication-competent genome.

Pseudotype production. To determine whether this system could be used to produce pseudotyped MLV, COS-7 cells were transfected with SV-Ψ⁻-E-MLV and DOL alone or together with SV-A-MLV-env or SV-RSV-env. Virus produced by transfecting with SV-Ψ⁻-E-MLV and DOL alone contained only the ecotropic MLV envelope glycoprotein and did not infect QT6 or HOS cells (Table 2). In contrast,

TABLE 1. Comparison of titers of MLV produced in transient transfections of COS-7 cells and Ψ-2 packaging cells

Helper plasmid	Selectable genome	Producer cells	Titer (10^3/ml)[a]	RT[b] (U/ml)
SV-Ψ⁻-E-MLV	—[c]	COS-7	0.0	27.0
	DOL	COS-7	134.0	27.0
	HSG	COS-7	1.4	12.0
	pMV7	COS-7	4.7	NT[d]
SV-Ψ⁻-A-MLV	DOL	COS-7	88.0	NT
SV-Ψ⁻-env⁻-MLV + SV-E-MLV-env	DOL	COS-7	23.2	NT
None	—	Ψ-2	0.0	13.2
	DOL	Ψ-2	3.3	18.2
	HSG	Ψ-2	8.0	17.9

[a] G418-resistant 3T3 cell colonies.
[b] Reverse transcriptase activity (RT) was assayed as previously described (3). Purified MLV reverse transcriptase (Pharmacia, Piscataway, N.J.) was used as a standard and resulted in approximately 200,000 cpm for 4.0 U of input enzyme, with a background of approximately 500 cpm.
[c] —, none used.
[d] NT, not tested.

TABLE 2. Production of MLV pseudotyped by RSV envelope glycoprotein, using Env$^-$ and Env$^+$ packaging vectors[a]

env vector	Packaging vector	Titer (10^3 colonies/ml)	
		QT6	HOS
None	SV-Ψ$^-$-E-MLV	0.0	0.0
SV-A-MLV-env	SV-Ψ$^-$-E-MLV	0.0	111.0
SV-RSV-env	SV-Ψ$^-$-E-MLV	6.1	0.0
None	SV-Ψ$^-$-env$^-$-MLV	0.0	0.0
SV-A-MLV-env	SV-Ψ$^-$-env$^-$-MLV	0.0	40.0
SV-RSV-env	SV-Ψ$^-$-env$^-$-MLV	4.2	0.0

[a] COS-7 cells were transfected with or without 10 μg of envelope vector and with 10 μg each of packaging vector and DOL, encoding the selectable retroviral vector RNA.

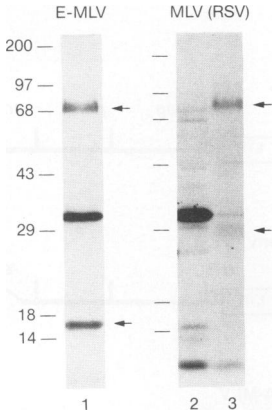

FIG. 2. Comparison of envelope glycoprotein levels on E-MLV and MLV(RSV) virions. COS-7 cells were transfected and 2 days later incubated with [^{35}S]methionine (100 μCi/ml in methionine-free Dulbecco modified Eagle medium with dialyzed 10% fetal calf serum) for 8 h. Supernatants were harvested and clarified by low-speed centrifugation at 400 × *g*, and virions were then pelleted by centrifugation for 1 h in an SW27 rotor at 24,000 rpm. Pelleted virions were dissolved in 100 μl of RIPA buffer (1.0% Nonidet P-40, 1.0% deoxycholate, 1.0% SDS, 10 mM Tris [pH 8.0], 1.0 mM EDTA, 0.15 M NaCl). Solubilized virions from the supernatants of cells transfected with SV-Ψ$^-$-E-MLV (lane 1) or SV-Ψ$^-$-env$^-$-MLV and SV-RSV-env (lanes 2 and 3) were immunoprecipitated with anti-MLV (lanes 1 and 2) or anti-RSV (lane 3). Immunoprecipitates were separated by SDS-polyacrylamide gel electrophoresis and exposed to X-ray film. Arrows on the left indicate MLV envelope glycoproteins gp70 and p15E; arrows on the right indicate RSV envelope glycoproteins gp85 and gp37. The major band in lanes 1 and 2 represents MLV p30^{gag}. Sizes are given in kilodaltons.

virus produced by transfection with SV-Ψ$^-$-E-MLV, DOL, and SV-A-MLV-env infected HOS but not QT6 cells, and that produced by transfection with SV-Ψ$^-$-E-MLV, DOL, and pSV-RSV-env infected QT6 but not HOS cells. This result suggested that the transfected COS-7 cells had released MLV pseudotypes containing the RSV envelope glycoprotein. The relatively low titer of the MLV(RSV) pseudotype virus (about 10^4/ml) is due at least in part to the relatively low cloning efficiency of QT6 cells and to the low level of avian leukosis virus receptor expression on these cells (1).

As is the case for pseudotypes produced by coinfecting cells with MLV and RSV (14), the envelope of the viruses studied here probably contains both RSV and MLV envelope glycoproteins. To test whether MLV pseudotypes with a pure RSV envelope glycoprotein could be produced, virus was prepared by using SV-Ψ$^-$-env$^-$-MLV and DOL and either SV-RSV-env or SV-A-MLV-env. Viral titers and cell tropism of viruses containing the RSV or A-MLV envelope glycoprotein were similar when formed either in the presence or in the absence of packaging vector-encoded ecotropic MLV envelope glycoprotein (Table 2). Pseudotype virus, therefore, forms with similar efficiency in the presence or absence of the E-MLV envelope glycoprotein; MLV envelope glycoprotein neither competes with the RSV envelope glycoprotein for binding sites on the MLV core particle nor is required for incorporation of the RSV envelope glycoprotein into the envelope. This result is analogous to what we observed previously in formation of human immunodeficiency virus (human T-cell leukemia virus) pseudotypes (8).

Efficiency of envelope glycoprotein incorporation. To determine the efficiency of RSV envelope glycoprotein incorporation relative to that of the MLV glycoprotein, the protein composition of viruses containing either the RSV or MLV envelope glycoprotein was examined by immunoprecipitation and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis of [^{35}S]methionine-labeled virions. Densitometry of the autoradiogram of this gel showed a ratio of *gag* to *env* of 3.5 for MLV particles and 13.1 for MLV(RSV) (Fig. 2, lanes 2 and 3). Since RSV gp85^{env} contains one methionine residue, compared with two in gp70^{env}, MLV(RSV) contains about half as much envelope glycoprotein as does MLV bearing the E-MLV envelope glycoprotein. These results suggest that the RSV envelope glycoprotein was incorporated efficiently into MLV core particles. Moreover, the incorporation of gp85^{env} is unlikely to be due to random inclusion of membrane proteins, since in control experiments, the membrane glycoproteins CD4 and CD8 failed to be incorporated into MLV particles (data not shown).

The method described here should prove advantageous for several applications. It saves time by obviating the need for stable retroviral vector-producing cell lines, since the titers of transient virus are adequate for most applications; it permits production of MLV pseudotypes containing an envelope glycoprotein of interest; and it reduces the problem of low titers associated with retroviral vectors that encode a toxic protein. Recently, this approach was successfully used to produce high-titer retroviruses containing the *abl* oncogene, whose product is toxic to cells (10).

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