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

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Received 29 July 1991/Accepted 28 April 1992

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 titrated by drug selection of infected target cells. We have used this method to produce relatively high 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, or 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 pMVMV (6) (not shown). Each contains MLV long terminal repeats, the Neo' 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' gene).

To produce virus, COS-7 cells (1.2 × 10⁶) 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 × 10⁵ 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 pMVMV, 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 × 10⁶/ml and ranged from 3 × 10⁵ to 14 × 10⁶/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
 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 (10⁵ 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 Neo²-selectable marker was produced by cotransfecting a 3T3 cell stock, 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 Neo² 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 × 10⁵ 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,
virus produced by transfection with SV-Ψ−E-MLV, DOL, and SV-AMLV-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 105/ml) is due at least in part to the relatively low cloning efficiency of QT6 cells and to the low level of avian leukemia virus receptor expression on these cells (1).

As is the case for pseudotypes produced by coinfected 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-AMLV-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 [35S]methionine-labeled virions. Densiometry of the autoradiogram of this gel showed a ratio of 3.5 to env of 3.5 for MLV particles and 13.1 for MLV(RSV) (Fig. 2, lanes 2 and 3). Since RSV gp85env contains one methionine residue, compared with two in gp70env, 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 gp85env 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 oncoprotein, whose product is toxic to cells (10).

We thank Lung Chang for the RSV env cDNA, Mark Levi for construction of the Env− MLV provirus, Michael Glotzer for densitometry, Heidi Stuhlmann for the 3T3 cell line used for marker rescue, and Paul Bates, John Young, Michael Loichie, and Harold Varmus for helpful discussion.

This work was supported by a grant from the Cancer Research Institute/Carolyn Spitzer Memorial Fund to N.R.L. and by grants from NIH and the Howard Hughes Medical Institute to D.R.L.

REFERENCES

1. Bates, P. Personal communication.

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**TABLE 2. Production of MLV pseudotyped by RSV envelope glycoprotein, using Env− and Env+ packaging vectors**

<table>
<thead>
<tr>
<th>env vector</th>
<th>Packaging vector</th>
<th>Titer (10⁶ colonies/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>SV-Ψ−E-MLV</td>
<td>QT6 0.0 HOS 0.0</td>
</tr>
<tr>
<td>SV-A-MLV-env</td>
<td>SV-Ψ−E-MLV</td>
<td>QT6 0.0 HOS 110.1</td>
</tr>
<tr>
<td>SV-RSV-env</td>
<td>SV-Ψ−E-MLV</td>
<td>QT6 6.1 HOS 0.0</td>
</tr>
<tr>
<td>None</td>
<td>SV-Ψ−env−MLV</td>
<td>QT6 0.0 HOS 0.0</td>
</tr>
<tr>
<td>SV-A-MLV-env</td>
<td>SV-Ψ−env−MLV</td>
<td>QT6 0.0 HOS 4.2</td>
</tr>
<tr>
<td>SV-RSV-env</td>
<td>SV-Ψ−env−MLV</td>
<td>QT6 4.2 HOS 0.0</td>
</tr>
</tbody>
</table>

* 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.