Improved Retroviral Packaging Lines Derived from Spleen Necrosis Virus

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Using highly efficient gene expression vectors, we constructed new retroviral packaging lines derived from spleen necrosis virus. Core proteins are expressed from the murine leukemia virus promoter and enhancer followed by the tripartite leader sequence of an adenovirus. Using different plasmids for envelope expression, we found that the efficiency of vector transduction is dependent on the level of gag-pol expression. The level of envelope expression did not have a measurable impact on vector virus titers. The new helper cell lines do not contain any sequences homologous to vector genomes. They transduce standard retrovirus vectors with titers up to 10^6 colony forming units per milliliter of supernatant tissue culture medium. No replication-competent virus was observed. © 1995 Academic Press, Inc.

INTRODUCTION

Retroviral packaging lines and retroviral vectors are widely used to investigate various aspects of retrovirus replication, to study cell differentiation, to make transgenic animals, and recently, to transduce therapeutic genes into humans (for reviews, see Temin, 1986; Kohn et al., 1989; Gilboa, 1990; Tournoyer and Caskey, 1993; Morgan and Anderson, 1993). In the past decade, several retroviral packaging lines have been developed (reviewed by Miller, 1990). Most of them have been derived from murine leukemia virus (MLV), avian leukosis virus (ALV), and reticuloendotheliosis viruses (REV), e.g., reticuloendotheliosis virus strain A, REV-A, and spleen necrosis virus, SNV (reviewed by Dornburg, 1995).

The first REV-A-based packaging line (termed C3A2 cells) was constructed more than a decade ago by Watanabe and Temin (1983) and was one of the first packaging lines made. C3A2 cells were derived from D17 cells, a dog osteosarcoma cell line, by transfection of two plasmid constructs containing defective proviruses of REV-A. The provirus in one plasmid contained a deletion of the encapsidation sequence (E) including the splice donor site and, therefore, produced gag-pol proteins only. The other plasmid had a deletion of E plus most of gag-pol to express envelope. C3A2 cells are high producer lines giving virus titers of 10^6 to 10^7 CFU/ml supernatant medium with standard retroviral vectors. However, the use of C3A2 cells is very limited, because these cells occasionally produce replication-competent virus (Hu et al., 1987). The emergence of replication-competent virus results from recombination of helper cell sequences with vector sequences (a repair of the deleted encapsidation sequence).

To reduce the probability of homologous recombination, several modifications have been made. For example, Highkin and colleagues (1991) replaced the downstream LTR of these defective proviruses with the polyadenylation signal sequence of simian virus 40 (SV40). In another approach, Meyers et al. (1991) constructed a vector in which both gag-pol and env are expressed from the SV40 immediate early promoter in a single plasmid construct. This expression vector contains SNV sequences upstream of the first ATG codon of gag-pol including the splice donor site. Thus, env is expressed from a spliced mRNA similar to wild-type SNV. Poly(A) addition is mediated by the polyadenylation sequence of SV40.

To completely avoid problems of homologous recombination, Dougherty et al. (1988) constructed a SNV-based retroviral helper cell line in which gag-pol and envelope are expressed from heterologous promoters. In this packaging cell line (termed DSN), gag-pol is expressed from the cytomegalovirus immediate early promoter. Polyadenylation is mediated by the polyadenylation signal of simian virus 40. Envelope is expressed from the Rous sarcoma virus LTR promoter. DSN cells were reported to be free of replication-competent virus.

In all such modified packaging lines, vector virus titers were much lower than those obtained from C3A2 cells, for unknown reasons. In this paper, we describe two new, highly efficient SNV-derived packaging lines (termed DSH298 and DSH134G). The gag-pol and env expression plasmids were made using new universal gene expression vectors constructed in our laboratory (Shey et al., 1993). In contrast to MLV, in which the encapsidation sequence extends into the gag-pol coding region (Bender et al., 1987), this appears not to be
the case for SNV (Watanabe and Temin, 1982; Embretson and Temin, 1987). Thus, the SNV sequences present in these gene expression vectors have no overlap with retroviral vector sequences, avoiding risks of homologous recombination of the vector with helper cell sequences.

MATERIALS AND METHODS

Nomenclature

All retroviral sequences used in this study were derived from SNV, an avian reticuloendotheliosis virus (Purchace and Witter, 1975). Plasmid constructs are prefixed with the letter "p" to distinguish them from virus (pJD214HY vs JD214HY). The hygromycin B phosphotransferase gene (Gritz and Davies, 1983) is referred to as hygro. Hygro' refers to the phenotype of hygromycin resistance. Similarly, the neomycin (G418) resistance gene (Jorgensen et al., 1979) is called neo, while the phenotype is G418'. Selection of cells that express the Escherichia coli gene coding for xanthine–guanine phosphoribosyltransferase from plasmid pSV2gpt is referred to as gpt selection (Mulligan and Berg, 1981).

Construction of the gag–pol and envelope expression plasmids

Plasmids pSB5 and pRD114 (Fig. 2A) are universal eucaryotic gene expression vectors and have been described recently (Sheay et al., 1993). Briefly, they contain the MLV promoter and enhancer, followed by a multiple cloning site (derived from pBluescript) and the polyadenylation sequence of simian virus 40. pRD114 has the adenovirus tripartite leader (AVII) for further enhancement of gene expression. pRD136 has been made in two cloning steps using standard cloning procedures (Sambrook et al., 1989). First, the carboxy-terminal part of the SNV gag–pol coding region (a HindIII to Hpal fragment) has been cloned into pRD114. The Hpal site downstream of the pol coding region has been created by site-directed mutagenesis for cloning reasons. The resulting plasmid has been named pRD135. Next, the amino-terminal part of gag–pol (an Eagi to HindIII fragment, isolated from pBR1; Dougherty et al., 1999) has been inserted into Eagi plus HindIII-digested pRD135 to give plasmid pRD136 (Fig. 2C). pRD134 and pML29 have been made by inserting the SNV envelope gene (a SacI to Avtrl fragment, isolated from plasmid pBR102; Dougherty et al., 1989) into pRD114 or pSB5, respectively (Fig. 2C). More details of the cloning strategies are available upon request.

Retroviral vectors

The retroviral vector pJD214HY (Fig. 3, top) contains all cis-acting sequences required for normal retroviral replication (Dougherty and Temin, 1987). pLM31 has been derived from pJD214HY and contains the internal ribosomal entry site of a picornavirus followed by a multiple cloning site (mcs). In plM41, the SNV envelope gene has been inserted into this mcs (Fig. 3). pPO11 is a self-inactivating vector and has been described recently (Olson et al., 1994). Briefly, the right side U3 has been replaced with an XhoI linker. The U3 region of the left LTR has been replaced with the immediate early promoter of cytomegalovirus. The simian virus 40 polyadenylation signal immediately following the U5 of the right LTR has been shown to increase vector titers (Dougherty and Temin, 1987).

Cells

D17 were obtained from the ATCC. D17-C3A2 and D17-DSN are REV-A- or SNV-derived helper cells, respectively, and have been described earlier (Watanabe and Temin, 1983; Dougherty et al., 1989). All cell lines have been adapted to grow in 6% calf serum. Selection for resistance to hygromycin was performed at 80 µg/ml for D17, 60 µg/ml for C3A2, and 100 µg/ml for DSN cells.

Transfections and infection

The dimethyl sulfoxide–Polybrene method (Kawai and Nishizawa, 1984) was used for all transfections. Infections were performed as described (Dornburg and Temin, 1988). Virus titers are expressed as colony forming units per milliliter (CFU/ml) of tissue culture medium supernatant.

RNA isolation and Northern blots

mRNA was isolated using the mRNA isolation kit purchased from Invitrogen and following the protocol of the supplier. Northern blots were performed as described (Sambrook et al., 1989).

FACS

Plasma membrane localization of the envelope proteins was determined by a fluorescence-activated cell sorter (FACS). The primary antibody, a polyclonal antibody R850, was a gift from Dr. Orozlan of the National Cancer Institute. The secondary antibody used in this method was a goat anti-rabbit IgG conjugated with fluorescein purchased from Pierce. For FACS analysis, 10⁶ envelope-expressing cells were used. The cells were incubated with 100 µl of anti-REV-A envelope antibody (diluted 1:50 in PBS) on ice for 0.5 hr. Cells were washed twice with 4 ml of PBS plus 0.5% sodium azide and resuspended in 100 ml of anti-IgG (diluted 1:200 in PBS). The secondary antibody was incubated on ice for 0.5 hr. Cells were washed once as before, resuspended in 0.4 ml of PBS plus 0.5% sodium azide, and analyzed by flow cytometry. The green fluorescence was measured at 520 nm.

Reverse transcriptase assays

Reverse transcriptase (RT) assays were performed as described (Goff et al., 1981).
RESULTS

In order to obtain a highly efficient retroviral packaging cell line, first we determined the natural ratio of gag–pol (genomic) to envelope mRNAs in D17 cells infected with replication-competent SNV and REV-A virus. We hypothesized that mimicking this ratio in a packaging line would result in high-vector-virus-producing cells. The ratio of gag–pol to env mRNA was determined by Northern blot analysis using total mRNA isolated from D17 cells infected with SNV or REV-A. We found that in both SNV- and REV-A-infected cells, the ratio of gag–pol (genomic) and env mRNAs was approximately 1 to 1 (Fig. 1). Surprisingly, we also found two more RNA species (4.5 and 7 kb, respectively) that make up less than 5% of the total viral RNA. At this time, we do not know whether such mRNAs code for any viral proteins or whether they are dead-end products of aberrant splicing events using cryptic splice donor or acceptor sites. The high-molecular-weight band above 15 kb is most probably dimeric RNA.

In a second approach toward highly efficient packaging cells, we constructed a series of universal eucaryotic gene expression vectors. We found that insertion of the adenovirus tripartite leader sequence downstream of various promoters enhanced gene expression up to 18-fold. These vectors have been described in detail recently (Sheay et al., 1993). Two of these plasmids (pSB5 and pRD114, Fig. 2) were used to construct gag–pol and env expression vectors (Fig. 2).

Plasmid DNAs that contain overlapping regions of homology can undergo homologous recombination at rather high frequencies when cotransfected into mammalian cells (Folger et al., 1982; Ayares et al., 1986; Lin et al., 1987). Although the region of homology between the gag–pol and the env expression plasmids is rather short (183 bp in our constructs), the viral protein expression plasmids were transfected in two consecutive steps to avoid any possibility of homologous recombination during transfection. Two different approaches were made.

In one approach, the plasmid pRD134, which was expected to express high levels of envelope, was first transfected along with pSV2neo. After G418 selection, single colonies were isolated and several cell lines were established. Expression of envelope was monitored by Northern and by FACS analysis (data not shown). The cell line with the highest envelope expression was used for transfection of plasmid pRD136 to express gag–pol proteins. In the latter transfection, plasmid pSV2gpt was cotransfected, followed by gpt selection. Twenty gpt-resistant cell colonies were isolated to establish individual cell lines. The production of retroviral particles was measured in RT assays with supernatant medium harvested from confluent cultures. The biological activity was tested in a transient transfection assay using the retroviral vector pCXL, which transduces the bacterial β-galactosidase (lacZ) gene (Mikawa et al., 1992). One cell line that transferred the vector significantly more frequently than the rest of the cell clones was identified. This cell line was termed DSH134G (D17 cell-derived, SNV helper cell line, containing pRD134, clone G) and was used for further analysis.

In the other approach, the gag–pol expression plasmid was transfected first. Following the protocol described above, 1 of 20 cell lines was identified that produced high levels of RT. This cell line (termed DSG913; D17 cell derived SNV gag–pol producer, clone 13) was used for further analysis. Biological activity was tested with a bicistronic vector, transducing the hygro as well as the SNV envelope gene (pIM41, Fig. 3). In this vector, the SNV envelope gene is expressed from an internal ribosomal entry site of a picorna virus. Plasmid pIM41 was transfected into DSG913 cells, followed by hygro selection. Virus was harvested from confluent cultures and virus titers were determined by infecting fresh D17 cells in series dilutions. Hygro was transferred with titers of about 10⁶ CFU/ml supernatant tissue culture medium (Table 1). Next, plasmid pIM29 was transfected into DSG913 cells in cotransfection with pSV2neo. After G418 selection, 20 cell colonies were isolated and cell lines were established from these clones. Using a transient transfection assay with the retroviral vector pCXL as described above, two cell lines were identified that produced significantly higher levels of infectious virus than the rest of the cell clones. One of these cell lines (termed DSH928, D17 cell derived, SNV helper cell line with plasmid pIM29, clone B) was further analyzed.

Virus production by the new helper cell lines was determined using the retroviral vectors pJD214-HY and pPO111.R1. It was compared to that of two other REV-derived retroviral packaging lines (C3A2 and DSN). pJD214-HY is a standard retroviral vector which ex-
presses the hygro gene from the LTR promoter. pPO111.R1 is a self-inactivating retroviral vector constructed in our laboratory recently (Olson et al., 1994). In this vector, almost all U3 sequences of the right LTR are deleted. The U3 region of the left LTR has been replaced with the immediate early promoter of cytomegalovirus. As a result of the mode of retroviral replication, the deletion of the U3 region of the right LTR is copied to both LTRs after one round of retroviral replication. Thus, the passenger gene (hygro) has to be expressed from an internal promoter.

The retroviral vectors pJD214HY and pPO111.R1 were transfected into DSH29B, DSH134G, C3A2, and DSN cells, followed by hygromycin selection. Virus was harvested from confluent cultures and virus titers were determined. We found that the new packaging cell lines DSH29B and DSH134G transferred hygro at equal efficiencies (Table 1). The efficiency of gene transfer was almost as high as that of C3A2 cells. It was about 50-fold higher than that of DSN cells (Table 1).

We were surprised to find that the DSH29B, DSH134G, and DSgp13 cells containing pLM41 produced infectious virus at approximately the same efficiency, because the level of envelope expression was expected to differ by more than 1 order of magnitude among those packaging lines. This hypothesis is based on our earlier findings using similar plasmids that contained the bacterial chloramphenicol acetyltransferase (cat) gene (Shea et al., 1993). To determine the factors that influence particle formation, the level of cell surface envelope expression and RT activity of particles in tissue culture supernatants were measured by FACS analysis and RT assays, respectively.

We found that DSgp13, DSH29B, and DSH134G cells produced about the same amount of RT activity (Fig. 4B). RT activity was much higher than that of DSN cells (Fig. 4B). Quantitative analysis by scanning blots of four different assays (data not shown) revealed that the RT activity of the DSH cells was about 50-fold higher than that of DSN cells. Expression of env, however, differed markedly among the different cell lines. As expected, DSH134G cells had the highest level of envelope expression, followed by DSH29B. DSgp13 cells transfected with the bicistronic retroviral envelope expression vector ex-
pressed env at significantly lower levels (Fig. 4A). Env
expression of DSN cells, which is driven by the Rous
sarcoma virus (RSV) LTR promoter (Dougherty et al.,
1988) was hardly above background values in this assay
(Fig. 4A). These different levels of env expression coin-
cide very well with promoter strength measured in plas-
mids containing the SNV, MLV, or RSV promoter with or
without the AVI sequence expressing the bacterial cat
gene (Sheay et al., 1993). Further, these data indicate
that envelope density on the viral surface does not have
a major impact on the efficiency of infection.

Earlier, it was reported that the DSN helper cells,
which express gag–pol and env genes from different
plasmids under the control of heterologous promoters,
are free of replication-competent virus (Dougherty et al.,
1989). To test whether this also applies to the DSH cells,
the following infectivity studies were performed. Helper
cells were kept growing in tissue culture for more than
2 months. This time period would allow even miniscule
amounts of helper virus to replicate and spread through
the culture. After that time period, vector pJD214HY was
transfected into such cells, followed by hygro selection.
After another 6 weeks, virus was harvested and fresh
D17 cells were infected with undiluted tissue culture me-
dium. Infected cells were selected for hygromycin resis-
tance. After 3 weeks undiluted supernatant medium har-
vested from confluent D17 cells was used to infect fresh
D17 cells. Infected cells were selected for hygromycin
resistance. Replication-competent virus can further pas-
sage the pJD214HY hygro vector to fresh D17 cells with
very high efficiency (titers about 10^6). However, no hygro-
mycin-resistant cell clones have been observed. Further,
RT test did not reveal measurable enzyme activity in su-
pernats of infected D17 cells. In addition, PCR analy-
sis with primers specific for gag–pol and env did not

![Diagram of retroviral vectors](image)

**FIG. 3.** Retroviral vectors. pJD214HY is a standard retroviral vector, having complete LTRs. pPO111-R1 is a self-inactivating retroviral vector. U3
has been deleted from the right LTR. The U3 region of the left LTR has been replaced with the promoter and enhancer of the cytomegalovirus
immediate early promoter (CMVIE). R, U5, and all other sequences are derived from SNV. E extends up to the EcoRI site 5 bp before the start codon
of gag. Plasmid pIM31 has been derived from pJD214HY. It contains the internal ribosomal entry site of a picornavirus (Koo et al., 1992) followed
by a multiple cloning site. In pIM42 and pIM41, the neo or the SNV envelope gene were inserted into the multiple cloning site of pIM31.

**TABLE 1**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Vector</th>
<th>Titer (CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3A2</td>
<td>pJD214HY</td>
<td>7 × 10^6</td>
</tr>
<tr>
<td>DSN</td>
<td>pJD214HY</td>
<td>1 × 10^4</td>
</tr>
<tr>
<td>DSGp13</td>
<td>pJD214HY</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td>pIM41</td>
<td>1 × 10^6</td>
</tr>
<tr>
<td>DSH26B</td>
<td>pJD214HY</td>
<td>5 × 10^6</td>
</tr>
<tr>
<td></td>
<td>pPO111-R1</td>
<td>5 × 10^6</td>
</tr>
<tr>
<td>DSH34G</td>
<td>pJD214HY</td>
<td>5 × 10^6</td>
</tr>
<tr>
<td></td>
<td>pPO111-R1</td>
<td>3 × 10^6</td>
</tr>
</tbody>
</table>

*Note. Retroviral vector titers obtained from RevA- or SNV-derived retroviral packaging lines. The helper cell lines were transfected with the
vector indicated (Fig. 3). After antibiotic selection, virus was harvested from confluent cultures and fresh D17 cells were infected. Virus
titers are expressed as colony forming units (CFU)/ml of tissue culture supernatant medium.*
reveal recombinant SNV sequences in infected cells (data not shown). These data indicated that both DSH29B and DSH134G cells did not produce replication-competent virus.

**DISCUSSION**

The main goal of the research in our laboratory is the development of retroviral vectors for cell type-specific gene transfer. Using anti-hapten single-chain antibodies fused to the envelope, we and others have shown that retroviral vector particles that display scAs on the viral surface are competent for infection (Russell et al., 1993; Chu et al., 1994; Chu and Dornburg, 1995). Working with chimeric envelopes of SNV and MLV as well as an scA-env directed against a cell surface protein, we found that particles displaying such chimeras were only infectious on human cells if wild-type envelope was coexpressed in the packaging lines. Vector particles containing the SNV wild-type envelope alone do not infect human tissues (Chu et al., 1994). Thus, SNV-derived retroviral vectors appear to be good candidates for the development of gene delivery systems specific for human cells. Therefore, attempts were made to develop an SNV-derived, safe, and highly efficient packaging line.

In the first step towards this goal, we measured the ratio of gag-pol to env messages. We found that in D17 cells infected with replication-competent SNV or REV-A viruses, about 50% of the genomic RNA was spliced (Fig. 1). Surprisingly, we also found two more viral RNA species in both SNV- and REV-A-infected cells. However, the SNV genome does not reveal open reading frames of significant lengths other than gag-pol and env (data not shown). Thus, it remains unclear whether one or both RNAs code for viral proteins. However, it is interesting to note that wild-type SNV-infected cells are highly resistant
against superinfection, whereas cells expressing high levels of envelope alone exhibit only a moderate level of superinfection interference (Federspiel et al., 1989; Delwart and Panganiban, 1989). Thus, it may be speculated that one or both hypothetical proteins derived from such minor RNA species play a role in the establishment of superinfection interference (similar to nef in HIV) (Schwartz et al., 1993).

The REV-A-derived helper cell line C3A2 and other MLV-based helper cells have been reported to sporadically produce replication-competent helper virus. Such viruses arose by recombination of vector sequences with the helper cell genome (Hu et al., 1987). Further, plasmid DNAs that contain overlapping regions of homology can undergo homologous recombination at rather high frequencies when cotransfected into mammalian cells. Although the SNV sequences in pLM29 as well as pRD134 share only 183 bp of homology with SNV sequences in pRD136, the construction of the packaging line was performed in two consecutive transfection steps. This protocol was found to reduce the risk of formation of replication-competent virus in other retroviral vector systems (Markowitz et al., 1988; Danos and Mulligan, 1988).

After 2 months in culture, we did not detect any replication-competent virus by infectivity studies or PCR analysis. However, it was found that several helper cells, which were originally characterized as helper-free, produced replication-competent virus after long periods of cultivation (Otto et al., 1994). Thus, DSH cells need to be monitored for much longer periods to establish a helper-free status. However, we consider the possibility of formation of replication-competent virus extremely low, since there is no overlap between vector and helper cell sequences.

Two retroviral packaging lines (DSH29B and DSH-134Q) have been made by taking alternative routes of transfection. In each case, high levels of SNV protein expression has been monitored. We found that in both DSH29B and DSH134Q cells gag-pol was expressed at about the same level (Fig. 4). Although these cell lines express env with significantly different efficiencies, they give rise to about equal vector virus titers. Vector virus was transduced with even slightly higher efficiency from cell lines that express env at significantly lower levels. However, this small increase of the vector virus titer may be a feature of the vector pLM41. This hypothesis is supported by the finding that vector pLM42, which is identical to pLM41 except that it contains the G418 resistance gene in place of the envelope gene, transduced the hypo gene five times less efficiently than pLM41 (Table 1). DSN cells express envelope at levels not detectable by FACS analysis. In summary, these data indicate that the efficiency of env expression is not crucial for the efficiency of infectivity.

Gag-pol expression in DSN cells is about 50-fold less than that of DSH cells. This finding is surprising, because we found that the CMV promoter, which is used in DSN cells to express gag-pol, is very strong in D17 cells (Sheay et al., 1993). However, the gag-pol expression vector in DSN cells contains an additional ATG codon about 100 bp upstream of the actual gag-pol ATG codon. The upstream ATG codon was part of the multiple cloning site used to insert the gag-pol sequence. Thus, many translation products may not fold into functional viral proteins. Vector virus titers from DSN cells are about 50-fold lower than those obtained from DSH cells. These data clearly indicate that the level of gag-pol expression determines the efficiency of vector virus production. Release of RT activity from DSgpo13 cells was as high as that from DSH cells expressing envelope. These data further show that envelope is not required to form budding virus particles. However, envelope-negative particles were not infectious.

In summary, we have constructed new, highly efficient packaging lines derived from spleen necrosis virus. We show that virus titers up to 10^6 can be obtained with such helper cell lines. The new helper cells contain SNV protein coding sequences with no overlap to retroviral vector genomes. Thus, there is no risk of homologous recombination which could reprogram a replication-competent helper virus.

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