Production of infectious recombinant Moloney murine leukemia virus particles in BHK cells using Semliki Forest virus-derived RNA expression vectors

(Semliki Forest virus expression system/retrovirus assembly/gene therapy)

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ABSTRACT   We describe a heterologous, Semliki Forest virus (SFV)-driven packaging system for the production of infectious recombinant Moloney murine leukemia virus particles. The gag–pol and env genes, as well as a recombinant retrovirus genome (LTR–ψ^+–neo^R–LTR), were inserted into individual SFV1 expression plasmids. Replication-competent RNAs were transcribed in vitro and introduced into the cytoplasm of BHK-21 cells using electroporation. The expressed Moloney murine leukemia virus structural proteins produced extracellular virus-like particles. In these particles the gag precursor was processed into mature products, indicating that the particles contained an active protease. The protease of the gag–pol fusion protein was also shown to be active in a trans-complementation assay using a large excess of Pr65gag. Moreover, the particles possessed reverse transcriptase (RT) activity as measured in an in vitro assay. Cotransfection of BHK-21 cells by all three SFV1 constructs resulted in the production of transduction-competent particles at 4×10^6 colony-forming units (cfu)/ml during a 5-hr incubation period. Altogether, 2.9×10^7 transduction-competent particles were obtained from about 4×10^6 transfected cells. Thus, this system represents the first RNA-based packaging system for the production of infectious retroviral particles. The facts that no helper virus could be detected in the virus stocks and that particles carrying the amphotropic envelope could be produced with similar efficiency as those that carry the ecotropic envelope make the system very interesting for gene therapy.

Retroviruses are positive, single-stranded RNA viruses that replicate via a chromosomally integrated DNA intermediate (provirus) (1, 2). After entering into a new cell, viral RNA is transcribed into double-stranded DNA using a virus-specific RT (3). The viral genome is transported into the nucleus, where it is integrated into the host DNA with the aid of a second virus-specific enzyme, the integrase (4). Expression of viral genes is initiated by transcription of provirus into RNA. The latter is transported from the nucleus into the cytoplasm, where it is either used for packaging into progeny particles, or as mRNA for translation of viral gag, gag–pol, and env precursor proteins. gag and gag–pol precursors are translated from full-length RNA, whereas the env precursor is made from a subgenomic RNA, which is derived from the longer form by RNA splicing. Translation of full-length RNA yields mostly gag precursor proteins. However, gag–pol fusion products are also synthesized, either because of suppression of the gag stop codon (5) or because of ribosomal frameshifting (6–8). The pol gene encodes a third enzyme, an aspartyl protease (PR), which is responsible for almost all of the maturation cleavages of the viral precursor proteins (9).

Because of their characteristic life-cycle, retroviruses are widely used as vectors for stable expression of heterologous genes in mammalian cells. These systems have also been adapted for the purpose of gene therapy in humans (10, 11). One of the most important innovations was the successful construction of helper cell lines that synthesize all retroviral proteins, but which do not produce infectious particles because they lack a transduction-competent genome (12). Transfection of these cells by recombinant proviral DNA that encodes the RNA-encapsidation signal results in production of particles carrying the recombinant genome. These particles are infectious, but of the so-called suicide type; that is, they cannot spread because they do not express structural proteins of the virus. This feature is essential especially if the particles are to be used in human gene therapy.

The best characterized packaging systems today are those derived from Moloney murine leukemia virus (MoMuLV) (13). Although preliminary experiments have shown that it is feasible to use recombinant particles produced by these systems for gene therapy, further improvement is required. Ideally, the production system should be fast and easy to use and yield a high titer stock of transduction competent particles, which is devoid of any replication proficient helper viruses. In addition, its flexibility is important; for instance, it should be possible to choose freely among surface protein genes that code for envelope proteins with different targeting specificity. These considerations have aroused interest to produce recombinant retroviruses by transient expression systems rather than by the standard packaging cell lines (14, 15). We and others have recently developed expression systems based on the alphaviruses (Semliki Forest and Sindbis virus; refs. 16 and 17). These systems monopolize on the ability of the alphavirus RNA genome to self-replicate very efficiently in the cell cytoplasm. The major advantages of alphavirus systems, as compared with other expression systems, are their ease of use, and the high levels of RNA and protein produced in transfected cells. Therefore, we were interested in determining if the Semliki Forest virus (SFV) system could be used to reconstitute assembly of infectious MoMuLV particles.

MATERIALS AND METHODS

Cell Culture. BHK-21 cells (American Type Culture Collection) were cultured in BHK-21 medium (GIBCO) containing 5% fetal calf serum, 10% tryptose phosphate broth, 20 mM Hepes, and 2 mM glutamine. NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum, 4500 mg/liter glucose, and 2 mM glutamine.

Abbreviations: SFV, Semliki Forest virus; MoMuLV, Moloney murine leukemia virus; RT, reverse transcriptase; PR, protease; cfu, colony-forming unit.
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Plasmid Constructs. The essential features of the SFV-based constructs used in this work are shown in Fig. 1.

pSFV1/Pr80env and pSFV-C/Pr65gag. pSFV1/Pr80env and pSFV-C/Pr65gag have been described (18). pSFV1/Pr80env contains a DNA copy of the MoMuLV Pr80env gene under the control of the SFV subgenomic promoter. In pSFV-C/Pr65gag the coding sequence of MoMuLV Pr65gag is fused to the capsid (C) gene of SFV. RNA transcribed from the latter plasmid can be used for high level expression of Pr65gag, due to a translational enhancer present in the SFV C gene (19). In SFV-C/Pr65gag RNA-transfected cells, a C-gag fusion protein is initially produced. SFV C posesses autoproteolytic activity and therefore cotranslationally cleaves itself off from the nascent chain giving C and free Pr65gag. The latter is further processed by myristoylation.

pSFV1/gag-pol and pSFV-C/gag-pol. pSFV1/gag-pol was made by inserting the MoMuLV gag-pol cDNA from pNCA (20) into the *Bam*HI site of pSFV1 via subcloning steps in pGEM-7Zf(+) (Promega) and M13mp18 (GIBCO/BRL). The two *Spe*I sites in the gag-pol cDNA were removed by site-directed mutagenesis, using the oligonucleotide 5'-GGGG-GGTTGTTTGACGAGTGCCTCTACTGCATGGGGGGCC-AGAATGACGAGTGGCTGTCCCATGGT-3' (21). pSFV-C/gag-pol was made by ligating the *Not*I–*Bsm*I fragment (14,410 bp) of pSFV1/gag-pol and the *Not*I–*Bsm*I fragment (2,723 bp) of pSFV-C/Pr65gag.

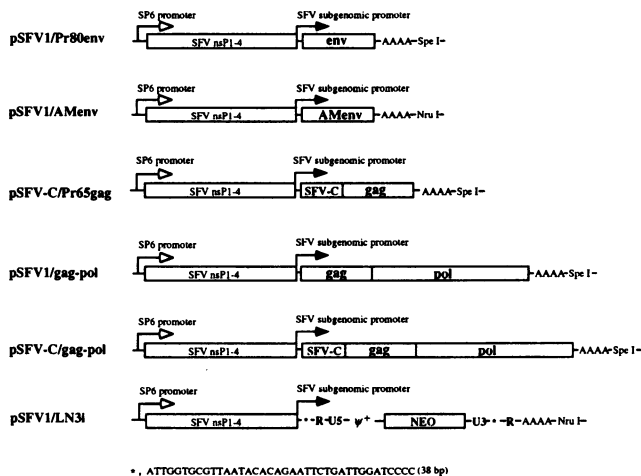*, ATTGGTGCGTTAATACACAGAATTCTGATTGGATCCCC (38 bp)

FIG. 1.   SFV vector constructs. Only the SFV recombinant region of each construct is shown. This region extends from the SP6 promoter (open arrow) to either the *Spe*I or *Nru*I site. All constructs contain, in the 5' to 3' direction, (*i*) the 5' replication signals of SFV RNA, (*ii*) genes encoding the SFV replication complex [nonstructural proteins (nsp) 1–4], (*iii*) the internal subgenomic promoter of SFV (solid arrow), (*iv*) the 3' replication signals of the SFV–RNA, and (*v*) the poly(A) tract of the SFV genome. pSFV1/Pr80env and pSFV1/gag-pol contain the regions encoding MoMuLV env protein and gag-pol protein, respectively. pSFV1/AMenv contains the region encoding the amphotropic envelope protein. In pSFV-C/Pr65gag and pSFV-C/gag-pol the regions encoding the MoMuLV proteins are fused to that of SFV capsid (C) gene. pSFV1/LN3i contains the 5' R-U5, the encapsidation signal (ψ⁺), the *neo*ᴿ gene, and the 3' U3-R sequences from the retrovirus vector pLN, and 38 SFV-specific bases (denoted with *) both before the R region at the 5' end and between the 3' U3 and R region. The SFV recombinant region is transcribed into RNA using SP6 RNA polymerase following linearization of the DNA with *Spe*I or *Nru*I depending on the construct. The SFV polymerase complex both replicates the RNA and transcribes the 3' portion from the internal subgenomic promoter into a subgenomic mRNA from which the env precursor protein (SFV1/Pr80env and SFV1/AMenv), the SFV-C-gag fusion protein (SFV-C/Pr65gag), the gag-pol precursor protein (SFV1/gag-pol), or the SFV-C-gag-pol fusion protein (SFV-C/gag-pol) is translated. Note that coding regions indicated are not to scale.

pSFV1/LN3i. pSFV1/LN3i was made by inserting a recombinant MoMuLV gene (R-U5-ψ⁺-neoᴿ-U3-R) from pLN (22) into the *Sma*I site of pSFV1-*Nru*I plasmid vector. pSFV1-*Nru*I is a derivative of pSFV1, made by deleting a 527-bp *Stu*I–*Hin*dIII fragment (7603–8130) from pSFV1 and changing the unique *Spe*I site to an *Nru*I site. This plasmid was a kind gift from Peter Liljeström (Karolinska Institute). The recombinant retroviral genome in pSFV1/LN3i is flanked at the 5' end by 38 SFV-specific bases (part of which encodes the internal SFV promoter). According to the model for retroviral DNA synthesis by RT, a strong-stop DNA is synthesized near the 5' end of the retrovirus RNA genome (23). This strong-stop DNA then jumps to the 3' end, and the exposed R sequence hybridizes with the complementary R sequence at the 3' end of the retrovirus RNA genome for synthesizing the minus-strand DNA. The 38 SFV specific bases inserted in front of the 5' end R region would interrupt the minus-strand DNA synthesis. To facilitate the conversion of the RNA into double-stranded DNA, we also inserted the same 38-base long SFV sequence between the 3' U3 and R regions. This was done by fusion-PCR (24) using Vent DNA polymerase (New England Biolabs) and a subcloning step in pUC18.

pSFV1/AMenv. Plasmid pSFV1/AMenv contains the coding sequence of the murine amphotropic virus (4070A) envelope protein. The amphotropic envelope gene fragment from pPAM3 (25) was first inserted into pUC18 by subcloning steps to make pUC18/AMenv. The plasmid pSFV1/AMenv was made by inserting the *Sma*I-*Hpa*I fragment (1976 bp) from pUC18/AMenv into the *Sma*I site of pSFV1-*Nru*I.

RNA Transcription and Transfection. Run-off transcripts were produced *in vitro* from *Spe*I-linearized pSFV-C/Pr65gag, pSFV1/gag-pol, pSFV-C/gag-pol, pSFV1/Pr80env, *Nru*I-linearized pSFV1/LN3i, pSFV1/AMenv using SP6 RNA polymerase (26). RNA (20 μl of each) was transfected into 8 × 10⁶ BHK-21 cells by electroporation. Fifty to 75% of the cells survived the transfection step.

Metabolic Labeling of Transfected Cells. Transfected cells were added to 9 ml of complete BHK-21 medium, plated onto three 33-mm culture dishes, and incubated at 37°C. At 8 hr after electroporation, transfected cells were washed twice with phosphate-buffered saline (PBS) and starved by incubation at 37°C for 30 min in 2 ml of methionine-free minimum essential medium (MEM; GIBCO), supplemented with 20 mM Hepes. Media were then replaced with 0.5 ml of methionine-free MEM containing 100 μCi (1 Ci = .376Bq) of [³⁵S]methionine per ml. After a 30-min pulse, cells were washed twice with MEM containing 20 mM Hepes and 150 μg/ml of unlabeled methionine (chase medium). Incubation was then continued in chase medium for different times. At the end of each chase period, culture media were collected, cell monolayers were washed once with PBS and then solubilized in 0.3 ml of lysis buffer [1% sodium dodecyl sulfate (SDS)/10 mM iodoacet-amide]. Media samples and cell lysates were clarified by centrifugation using an Eppendorf centrifuge, at 6000 rpm, for 6 min.

Protein Analysis. Cell lysates (0.3 ml) were diluted to 3 ml with NET buffer (150 mM NaCl/1 mM EDTA/50 mM Tris·HCl, pH 7.5/0.1% Nonidet P-40/0.25% gelatine/0.02% sodium azide). To immunoprecipitate MoMuLV-specific proteins, 5 μl of polyclonal pig anti-MoMuLV antiserum (HC 185, Quality Biotech, Camden, NJ) and 40 μl of protein A-Sepharose (Pharmacia) slurry [1:1 (vol/vol) in 10 mM Tris·HCl] were added to 1 ml of diluted cell lysate, and samples were incubated overnight at 4°C. Immunoprecipitates were washed as described (27) and analyzed by SDS/12% PAGE under reducing conditions. Extracellular particles in media samples were pelleted through a 20% sucrose cushion at 17,000 rpm, for 2 hr, at 10°C, using a Beckman JA18.1 rotor. Pellets were analyzed by SDS/PAGE as described above. Gels were dried and exposed to Fuji film. Dried gels were also exposed

to a Bas-III Image Plate (Fuji Photo Film), and the amount of radioactivity in bands was quantitated using the Fuji Bio-Image analyzer system BAS2000 (Fuji Photo Film).

RT Assay. This was done essentially as described in ref. 28. Briefly, a 10-μl aliquot of each medium sample was added to a 50-μl aliquot of a reaction cocktail, giving a final reaction mixture containing 50 mM Tris·HCl (pH 8.3), 20 mM DTT, 0.6 mM $MnCl_2$, 60 mM NaCl, 0.05% Nonidet P-40, 1 μg of poly(rA)p(dT)$_{12-18}$ (Pharmacia), and 10 μCi of [α-^{32}P]dTTP (Amersham). After incubation at 37°C for 1 hr, 10 μl of each sample was spotted onto sheets of dry Hybond-N$^+$ nucleic acid transfer membrane (Amersham). Membranes were washed at room temperature with gentle agitation, 3 × 20 min in 500 ml of 0.3 M NaCl/0.03 M sodium citrate and twice briefly in 500 ml of 95% ethanol. After air-drying, membranes were exposed to a BAS-III Image Plate and radioactivity was quantitated as above.

RNA Analysis. Transfected BHK-21 cells were plated onto 33-mm culture dishes and incubated for 2 hr at 37°C. Media were removed and replaced with 1-ml aliquots of medium containing 1 μg/ml actinomycin D (Sigma/Aldrich). After incubation for 2 hr at 37°C, media were replaced with 1-ml aliquots of medium containing 1 μg/ml actinomycin D and 75 KBq [^{14}C]uridine (2.1 GBq/mmol, DuPont). After incubation for 6 hr at 37°C, cellular RNA was isolated using TRIzol Reagent (GIBCO) as described by the manufacturer. RNA was dissolved in RNase-free H_2O and subjected to electrophoresis through 0.7% agarose gels containing formaldehyde (29). Gels were dried, and radiolabeled RNA was visualized by autoradiography.

Production of Infectious Recombinant Retrovirus Particles. BHK-21 cells were transfected by electroporation with SFV1/LN3i, SFV1/Pr80env (or SFV1/AMenv), and SFV1/gag–pol (or SFV-C/gag–pol) RNAs. The transfected BHK-21 cells were diluted into 9 ml of complete BHK medium, and 6 ml of the cell suspension (containing 3–4 × 10^6 living cells) was plated onto a 60-mm culture dish (Nunclon, Naperville, IL). The cells were incubated at 37°C, and the media were harvested at 5-hr intervals from the same dish and replaced with 2-ml aliquots of fresh complete BHK medium. The media were passed through a 0.45-μm filter and stored at −130°C. Neo^R transduction-competent retrovirus particles were titrated on NIH 3T3 cells. Therefore, NIH 3T3 cells were seeded at 5 × 10^5 cells per dish (60 mm) on day one. On day two, 1-ml aliquots of 10-fold serial dilutions of media samples were added to cell monolayers in the presence of 4 μg/ml Polybrene (Sigma). After incubation for 2 hr at 37°C, 1-ml aliquots of medium containing 4 μg/ml Polybrene was added to each dish, and incubation was continued at 37°C. On day three, 24 hr after incubation, the cells were split 1:100 into selection medium containing 1 mg/ml G418 (Geneticin, GIBCO). On day nine, the selection medium was replaced with fresh one. On day 15, G418-resistant colonies were stained with methylene blue (0.5% in 50% methanol) and counted. Virus titers are given as colony-forming units (cfu)/ml. They were calculated by multiplying the number of colonies with the dilution times and divided by 2 to account for cell doubling.

Multiplication-Proficient Particle Assay. 3T3Zip*neo*SV-(X)p cells (30), an NIH 3T3-derived cell line that harbors recombinant provirus consisting of the MoMuLV long terminal repeats (LTRs), a packaging signal, and the *neo*^{R} gene were used in this assay: Transfection of these cells by the genes encoding the MuLV gag–pol and env proteins results in the production of infectious particles containing the *neo*^{R}-recombinant genome. 3T3Zip*neo*SV(X)p cells were infected with the supernatant medium containing 2.6 × 10^6 infectious recombinant retrovirus particles in the presence of 4 μg/ml Polybrene. The infected cells were passaged for 8 days. Then the medium was replaced with fresh medium and the cells were incubated at 37°C. After a 24-hr incubation, the medium was

collected, passed through a 0.45-μm filter and analyzed for the presence of *neo*^{R}-transduction-competent particles by titration on NIH 3T3 cells as described above. Wild-type amphotropic retroviruses (4070A) used as a positive control were obtained from a producing cell line, a kind gift from Dinko Valario as in ref. 30.

RESULTS

RNA Transcribed from pSFV1/gag–pol Directs the Synthesis of Functional MoMuLV PR. To determine if the SFV-expression system could be used to produce retrovirus-like particles in which the viral gag and gag–pol precursors are in their mature cleaved form, the full-length coding region of gag–pol was cloned into the pSFV1 vector under the control of the SFV-subgenomic promoter. The resulting plasmid (pSFV1/gag–pol) was used as a template for *in vitro* transcription of the corresponding recombinant SFV–RNA. BHK-21 cells were transfected with SFV1/gag–pol RNA and metabolically labeled for 30 min with [^{35}S]methionine 8 hr after transfection. SDS/PAGE was used to analyze cell-associated and extracellular MoMuLV proteins at times from 15 min to 2 hr of chase. Fig. 2*A* (lanes 1–5) show MoMuLV proteins that were immunoprecipitated from cell lysates: Pr65gag and one of its cleavage products, the viral capsid protein p30, are clearly visible. Fig. 2*A* (lanes 6–10) show the proteins of the released gag–pol particles: These particles contained the Pr65gag cleavage products, p30 and pp12. Two additional gag products, p15 (matrix protein) and p10 (nucleocapsid protein), are not visible because they lack methionines. Therefore SFV1/gag–pol RNA expressed functional viral PR, because the particles contained fully processed structural proteins.

PR Expressed from SFV1/gag–pol RNA Cleaves Pr65gag Expressed from a Separate Replicon. To further check that PR functioned correctly, we tested its ability to cleave Pr65gag expressed from a separate replicon. SFV1/gag–pol RNA and SFV-C/Pr65gag RNA, the latter from which proteins are expressed at about a 10-fold higher level, were cotransfected into BHK-21 cells. Cells were pulse-labeled and chased, and cell-associated and extracellular MoMuLV proteins were analyzed by SDS/PAGE. Fig. 2*B* shows expression of uncleaved Pr65gag in cells transfected with SFV-C/Pr65gag RNA alone: As expected, about 10-fold more of Pr65gag was obtained in comparison with the SFV1/gag–pol RNA transfected cells (Fig. 2*A*). Fig. 2*B* (lanes 1–5) shows uncleaved intracellular Pr65gag, the amount of which decreased with time as gag particles were released from cells (Fig. 2*B*, lanes 6–10). Fig. 2*C* shows SDS/PAGE analysis of cell-associated and extracellular virus-specific proteins from cells cotransfected with SFV-C/Pr65gag and SFV1/gag–pol RNAs at the ratio of 1:1. Most of the Pr65gag expressed from SFV-C/Pr65gag RNA was processed to p30 and pp12. Efficiency of cleavage was increased when SFV-C/Pr65gag and SFV1/gag–pol RNAs were transfected at a ratio of 1:2 (data not shown). We conclude that PR expressed from SFV1/gag–pol RNA functioned efficiently.

RNA Transcribed from pSFV1/gag–pol Directs the Synthesis of Functional MoMuLV RT. RT activity released from SFV1/gag–pol RNA-transfected BHK-21 cells at different times after transfection was analyzed by a direct assay as described in *Materials and Methods*, and results are shown in Fig. 3. RT activity in media samples increased over 5-fold between 4 and 10 hr after transfection. This suggests that sub-viral particles containing RT activity were continuously released from cells.

A Recombinant Retrovirus Genome, Which Has Been Produced in the Cytoplasm, Is Incorporated into Infectious Retrovirus Particles. The retroviral recombinant genome containing the *neo*^{R} gene from pLN was subcloned into pSFV1 downstream of the SFV subgenomic promoter. The final construct, pSFV1/LN3i is shown schematically in Fig. 1. RNA
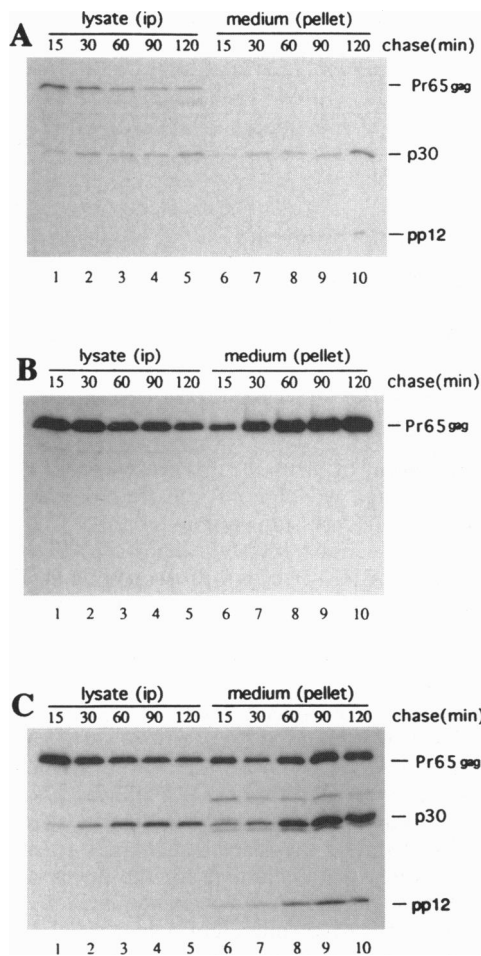
FIG. 2. Functional PR is produced from the recombinant SFV1/gag–pol RNA. SFV1/gag–pol RNA and SFV-C/Pr65gag RNA were transfected into BHK-21 cells, either separately or in combination (RNA ratio was 1:1). Transfected cells were pulse-labeled with [^{35}S]methionine for 30 min and chased as indicated. Viral proteins in cell lysates were immunoprecipitated with polyclonal serum against MoMuLV. Released particles were recovered from media by pelletation through a 20% sucrose cushion. Immunoprecipitates and pelleted particles were then analyzed by SDS/PAGE. Equivalent portions of immunoprecipitates and pelleted particles were loaded on gel. (*A*) SFV1/gag–pol transfected cells. (*B*) SFV-C/Pr65gag transfected cells. (*C*) SFV-C/Pr65gag and SFV1/gag–pol cotransfected cells.

was made and transfected into cells. The synthesis of genomic and subgenomic SFV RNAs was followed by labeling with
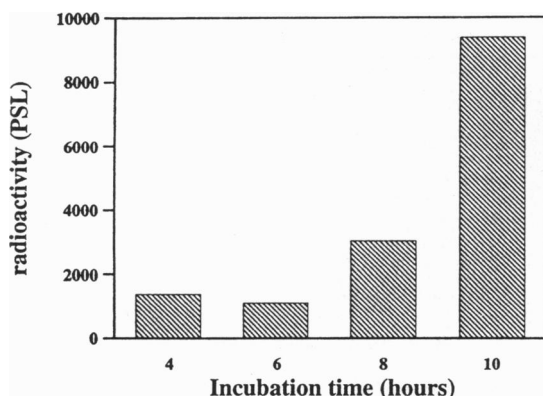

FIG. 3. RT activity in the medium of BHK-21 cells transfected with SFV1/gag–pol RNA. RT activity is expressed as PSL, a unit of radiation dose used in Fuji Bio-Image analyzer system BAS2000.

[^{14}C]uridine in the presence of actinomycin D, as described in *Materials and Methods*. As shown in Fig. 4 (lane 1), high levels of both SFV1/LN3i genomic and subgenomic RNAs were transcribed.

Next we investigated whether the recombinant retrovirus RNA genome that was transcribed in the cytoplasm would be encapsidated to form infectious recombinant particles. For this purpose, BHK-21 cells were cotransfected with the SFV1/gag–pol, SFV1/Pr80env, and SFV1/LN3i RNAs. The RNA analyzes are shown in Fig. 4 (lane 4). This shows that the genomic and subgenomic RNAs of SFV1/gag–pol, SFV1/Pr80env, and SFV1/LN3i were all produced in the cotransfected cells. The particle production was followed in a time course experiment. Cotransfected cells were incubated for a total of 25 hr. At 5-hr intervals, the medium was collected and replaced by fresh one. The presence of infectious recombinant particles in the media was tested by the ability to transduce the *neo*^{R} gene into NIH 3T3 cells. This was assayed by the selection of G418-resistant colonies. The results are shown in Table 1. During the first 5-hr incubation, 3.7×10^4 infectious particles were produced per milliliter; this increased to 6.5×10^5–1.1×10^6 transduction-competent particles per milliliter during the subsequent intervals.

To increase the production of infectious particles, we made the pSFV-C/gag–pol construct. This encodes the translation enhancing RNA sequence of the SFV capsid gene in front of the gag–pol gene (Fig. 1). The expression of gag–pol products in cells transfected with SFV-C/gag–pol RNA is shown in Fig. 5. This is much higher than that of the corresponding products in SFV1/gag–pol RNA transfected cells. Quantitation of radioactivity in bands shows that the difference is about 5-fold. When the SFV-C/gag–pol RNA was used in a cotransfection/time course experiment we found that the production of infectious particles was considerably increased (Table 1). The titer in most 5-hr media samples was about 4×10^6 cfu/ml. Control experiments showed that no transduction-competent particles were released into media of cells transfected with SFV1/LN3i and SFV-C/gag–pol, SFV1/LN3i and SFV1/Pr80env or SFV1/LN3i and SFV1/AMenv RNAs, respectively. We conclude that a retrovirus genome, which has been produced in the cell cytoplasm using the SFV expression system, can be encapsidated by coexpressed packaging proteins into a high titer stock of transduction-competent recombinant retrovirus.

Multiplication-Proficient Particles Were Not Detected. The possible presence of multiplication-proficient particles in culture media containing 2.6×10^6 infectious recombinant particles was tested by addition of media samples to
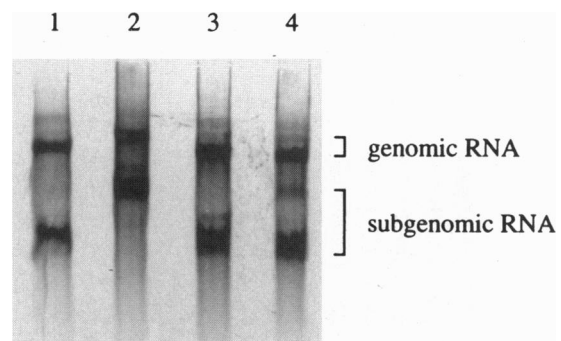

FIG. 4. RNA analysis. BHK-21 cells were transfected with SFV1/LN3i RNA (lane 1), SFV1/gag–pol RNA (lane 2), SFV1/Pr80env RNA (lane 3), or all three RNAs (lane 4). Transfected cells were labeled with [^{14}C]uridine for 6 hr in the presence of actinomycin D. Cellular RNAs were isolated and separated on 0.7% agarose gels containing formaldehyde. Radiolabeled bands were visualized by autoradiography. The positions of the replicated genomic and transcribed subgenomic RNAs are indicated.

Table 1. Release of infectious recombinant retrovirus particles from transfected BHK-21 cells

Exp.	RNA*	G418^R cfu/ml				
		0–5 hr	5–10 hr	10–15 hr	15–20 hr	20–25 hr
1	SFV1/LN3i + SFV-C/gag–pol	—	0	—	—	—
2	SFV1/LN3i + SFV1/Pr80env	—	0	—	—	—
3	SFV1/LN3i + SFV1/AMenv	—	0	—.	—	—
4	SFV1/LN3i + SFV1/gag–pol + SFV1/Pr80env	3.7×10^4†	8.0×10^5	1.1×10^6	8.5×10^5	6.5×10^5
5	SFV1/LN3i + SFV-C/gag–pol + SFV1/Pr80env	7.3×10^4	4.0×10^6	4.0×10^6	2.1×10^6	4.0×10^6
6	SFV1/LN3i + SFV-C/gag–pol + SFV1/AMenv	1.0×10^5	2.2×10^6	2.3×10^6	2.0×10^6	3.4×10^6

In each experiment about 4×10^6 transfected BHK-21 cells were plated into a 60-mm culture dish and incubated at 37°C. The medium was collected and replaced at 5-hr intervals. Media samples were passed through a 0.45-μm filter and stored at -130°C before being used for titration. —, not analyzed.
*RNA used for transfection of BHK-21 cells.
†NIH 3T3 cells were incubated with diluted medium of transfected BHK-21 cells and then subjected to G418 selection. The numbers refer to resistant colonies formed after 12 days incubation.

3T3Zip*neo*SV(X)p cells. Media from uninfected 3T3Zip*neo*SV(X)p cells and cells infected with wild-type amphotropic retrovirus (4070A) were used as negative and positive controls, respectively. The infected 3T3Zip*neo*SV(X)p cells were passaged for 8 days. When the cells were about 50% confluent, culture media were replaced with fresh media and cells were incubated for another 24 hr. The replacement media were then assayed for the presence of particles capable of transducing the *neo*^{R} gene into NIH 3T3 cells (marker rescue). No colonies were obtained for media from 3T3Zip*neo*SV(X)p cells infected with either the recombinant particles produced by the SFV expression system or the negative-control media. In contrast, about 4000 colonies were obtained using the positive-control media containing wild-type retrovirus.

Production of Particles with Amphotropic Envelope. The infectious recombinant retrovirus particles described above contain the ecotropic envelope glycoprotein. This can only target the virion to rodent cells. To broaden the host range of target cells for the particles and to make the system suitable also for human cells, for instance in the context of gene therapy, we set up experiments for the production of MoMuLV particles, which were pseudotyped with the amphotropic envelope glycoprotein. First we constructed an SFV1 based expression plasmid for the amphotropic envelope protein (pSFV1/AMenv in Fig. 1), which could be used for the *in vitro* transcription of corresponding RNA. This was used in a cotransfection experiment with SFV1/LN3i and SFV-C/gag–pol RNAs for particle production. As can be seen in Table 1 a high titer stock was also obtained when this envelope protein was used.

DISCUSSION

In a wild-type retrovirus infection, as well as in all packaging systems used today for production of recombinant retrovi-

ruses, transcription of virus-specific RNAs occur in the nucleus. In this study, we demonstrate that the cytoplasmic, RNA-based SFV expression system can be used for the production of infectious MoMuLV particles at high titer. Cotransfection of BHK-21 cells with recombinant SFV RNA genomes encoding the gag–pol, env, and a recombinant LTR-ψ^+-*neo*^{R}-LTR retroviral genome, resulted in production of about 4×10^6 transduction-competent recombinant particles per milliliter of culture media. Altogether, 2.9×10^7 recombinant particles were produced in 4×10^6 cells during a 20-hr incubation. We conclude that transcription of provirus in the nucleus can be bypassed during retrovirus production. This supports the idea that all retrovirus assembly events, including genome encapsidation, can take place in the cytoplasm of infected cells. It should therefore be generally feasible to use RNA based expression technology for the development of a new recombinant-retrovirus packaging system.

Potentially, replication competent helper virus can be generated in our system through recombination (31). However, this would require three separate recombination events: one between the 5' end of the recombinant *neo*^{R}-containing genome and the 5' end of the gag–pol genome, a second between the 3' end of gag–pol and the 5' end of the env genome, and a third between the 3' end of env and the 3' end of the recombinant *neo*^{R}-encoding genome. Since, in addition, homologous regions between different constructs are minimal, we think it is very unlikely that such recombination events would occur. Indeed, we could not detect any multiplication-proficient retrovirus in our recombinant stock. It should also be noted that our system cannot produce any multiplication-competent SFV-like particles because the genes encoding SFV structural proteins are not included in this system. Thus, the SFV-based recombinant retrovirus production system appears to be both efficient and helper free.
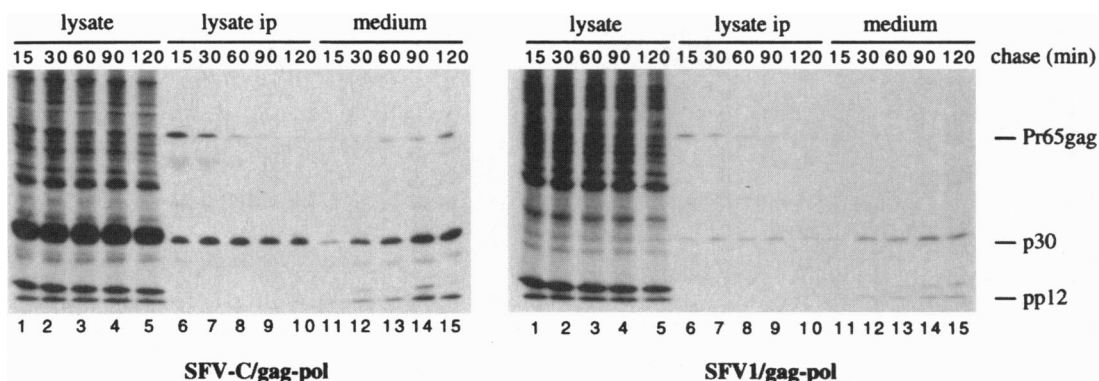

FIG. 5. The gag precursor production is more efficient in cells transfected with SFV-C/gag–pol RNA than in cells transfected with SFV1/gag–pol RNA. BHK-21 cells were transfected with SFV-C/gag–pol RNA or SFV1/gag–pol RNA by electroporation. The cell-associated and extracellular MoMuLV proteins were analyzed as described in the legend of Fig. 2.

The fact that we were able to produce recombinant particles with the amphotropic envelope at high titers makes the SFV system very interesting for the purpose of human gene therapy. The major advantage with this system is that it is convenient to use: once the required recombinant plasmids have been constructed, a recombinant retrovirus stock can be prepared within 1 day. For comparison, it takes a few months to establish a good producer cell line for a certain recombinant retrovirus. The present system is also very versatile. For instance, the targeting of the recombinant particles can easily be changed by using another envelope protein (or membrane protein) specifying RNA construct in the cotransfection. Finally, this system appears very useful for the production of recombinant retroviruses which carry a gene for a toxic product. Such recombinant particles can only be made by transient production systems which avoid the culturing of the producer cells for long time periods. In this system the packaging time is limited to about 1 day.

Landau and Littman (14) have reported another transient expression system in which simian virus 40-derived DNA vectors were used to drive the production of MoMuLV particles in COS-7 cells. During a 72-hr incubation period they obtained titers of 2.3×10^4 transduction-competent particles per milliliter from cells cotransfected with three expression plasmids, which encoded the gag–pol precursor, the env precursor, and a recombinant genome respectively. This should be compared with the titer of 2×10^6 cfu/ml that we obtained in BHK 21 cells after cotransfection with the corresponding SFV RNA constructs. When using a two plasmid system, i.e., a gag–pol env and a recombinant genome construction, Landau and Littman obtained a titer of 1.3×10^5 and 0.9×10^5 cfu/ml for ecotropic and amphotropic virus, respectively. More recently, Pear and coworkers (15) have described another transient DNA transfection system for production of recombinant retrovirus particles. They used the highly transfectable Ad5 transformed embryonic kidney cell line 293 for the generation of a packaging cell line BOSC 23 containing separate expression units for the MoMuLV gag–pol and env precursor proteins. When transfected transiently with a *neo*^{R} gene containing recombinant retrovirus DNA, Pear and coworkers obtained helper free virus stocks containing 3.9×10^6 transduction-competent particles per milliliter. This is similar to the virus titer we obtained. However, our system is more useful when manipulations of the retrovirus packaging genes are desired.

The SFV based recombinant retrovirus production system reported in this work represents a novel approach. We foresee several modifications of the system that are likely to increase its production efficiency. Altogether, we think that it represents a useful system for the production of transduction-competent retrovirus particles and that it has the potential to become important for the purpose of gene therapy in future.

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