

RESEARCH

A NEW GENERATION OF ANIMAL CELL EXPRESSION VECTORS BASED ON THE SEMLIKI FOREST VIRUS REPLICON

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We have developed a novel DNA expression system, based on the Semliki Forest virus (SFV) replicon, which combines a wide choice of animal cell hosts, high efficiency and ease of use. DNA of interest is cloned into SFV plasmid vectors that serve as templates for *in vitro* synthesis of recombinant RNA. The RNA is transfected with virtually 100% efficiency into animal tissue culture cells by means of electroporation. Within the cell, the recombinant RNA drives its own replication and capping and leads to massive production of the heterologous protein while competing out the host protein synthesis. The expression system also includes an *in vivo* packaging procedure whereby recombinant RNA is packaged into infectious virus particles using cotransfection with packaging-deficient helper RNA molecules. The resulting high titer recombinant virus stock can be used to infect a wide range of animal cells with subsequent high expression of the heterologous gene product, but without expression of any structural proteins of the helper. The infected cells produce protein for up to 75 hours post infection after which the heterologous product can constitute as much as 25% of the total cell protein. The general utility of the system is demonstrated

through the expression of human transferin receptor, mouse dihydrofolate reductase, chick lysozyme and *Escherichia coli* β -galactosidase.

A central technique in basic and applied molecular biology is the expression of cloned genes from carrier vectors, for which purpose numerous expression systems are available. While the prokaryotic and yeast expression systems are highly efficient and easy to use, expression systems for use in higher eukaryotic cells are still hampered by difficulties such as insufficient levels of protein production, restrictions in host range, and/or complexity of the system. Of the existing expression systems for higher eukaryotic cells the baculovirus vector is perhaps the most efficient in terms of protein production¹. However, it can only be used in insect cells, which, for example, are known to glycosylate proteins differently than animal cells. The system also relies on the construction of recombinant virus stocks through homologous recombination, a laborious technique especially when large series of genetic variants have to be analyzed. The most efficient mammalian cell expression system is based on vaccinia virus. However, this also uses recombinant viruses that express the heterologous gene upon infection. Alternatively, expression from recombinant plasmids can be obtained using helper virus constructs. In this case the plasmid must be transfected into the cells, where huge amounts of helper proteins are made^{2,3}.

In this communication we describe an expression system which overcomes these obstacles. It is highly efficient, easy to use and has a very broad host range. The system functions in an unorthodox fashion, consisting of a self-replicating and self-transcribing RNA molecule that only relies on the host cell translational machinery for function. The RNA can be used both for direct transfection of

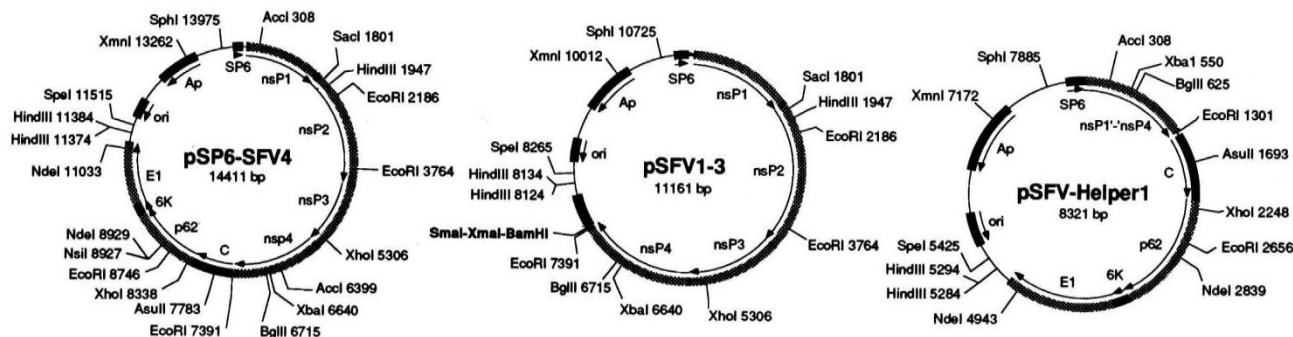


FIGURE 1 SFV expression vector system. To construct the SFV expression plasmids, the coding region of the pSP6-SFV4 structural genes was deleted between coordinates *Asu*II (7783) and *Nde*I (11033). The vectors retain the promoter region of the 26S subgenomic RNA and the last 49 amino acids of the E1 protein as well as the complete noncoding 3' end of the SFV genome. The

three expression vectors pSFV1-3 have practically the same size and differ only by a few base pairs in the area of the polylinker site (see Fig. 2). The pSFV-Helper1 represents pSP6-SFV4 with a deletion between the restriction sites *KpnI* (3597) and *KpnI* (5841) within the nonstructural region of the plasmid.

cells as well as for production of recombinant virus that only expresses the heterologous portion of a recombinant. The system is based on the Semliki Forest virus (SFV) replicon, which has been used extensively as a model in both virology and cell biology⁴⁻⁷. The use of another alphavirus (Sindbis) as an expression vector has earlier been reported⁸, but due to technical difficulties has not found wider use.

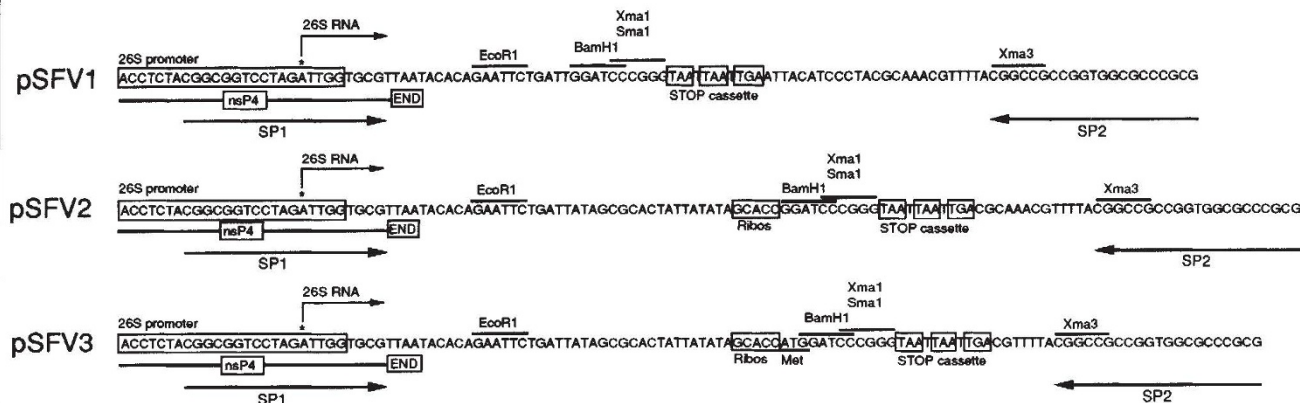
SFV has a single-stranded RNA genome of positive polarity, and replication of this capped and polyadenylated RNA starts upon the initial translation of the 5' two-thirds of the genomic RNA, producing a polyprotein which by autoproteolytic events is post-translationally cleaved into four nonstructural proteins (nsP1-nsP4). These proteins are responsible for the replication of the plus strand genome into full-length minus strands which later in infection are copied into new plus strand genomes⁶. The minus strands also serve as templates for the synthesis of a shorter subgenomic RNA species colinear with the last one-third of the plus-strand genome. All

structural proteins of the virus are encoded by this sub-genomic RNA. The capsid protein binds to newly made full-length positive strand genomes to form cytoplasmic nucleocapsids. The other three structural proteins are membrane proteins that are translocated through the endoplasmic reticulum membrane and transported to the cell surface where they meet the nucleocapsid during virus budding⁹⁻¹³.

The life cycle of SFV has several features that makes the virus an attractive candidate for development of a DNA expression system: SFV RNA genome is infectious due to its positive polarity, i.e. it functions directly as an mRNA. The infecting RNA molecule encodes its own RNA replicase, which in turn drives an efficient RNA replication and transcription. As a consequence, practically all ribosomes of the infected cell will be enrolled in the synthesis of the virus encoded proteins thus overtaking host protein synthesis. SFV replication occurs in the cell cytoplasm, where the virus replicase transcribes and caps the RNA for production of the viral proteins. This obviates many

FIGURE 2 Polylinker region of SFV vector plasmids pSFV1-3. The position of the promoter for the subgenomic 26S RNA is boxed, and the first nucleotide to be transcribed is indicated by an asterisk. In pSFV1 the *Bam*HI-*Sma*I-*Xma*I polylinker cassette is situated 31 bases downstream of the transcription initiation site. For pSFV2, the cloning site is positioned immediately after the ribosome binding site of the SFV capsid gene. The initiation motive of the capsid gene is identical to the consensus sequence³⁷.

For pSFV3, the polylinker is positioned three nucleotides further downstream, i.e. immediately following the initial AUG codon of the SFV capsid gene. The three translation stop codons in all three reading frames following the polylinker are boxed. Sequencing primers (SP) needed for checking both ends of an insert have been designed to hybridize either to the promoter region (SP1), or to the region following the stop codon cassette (SP2).



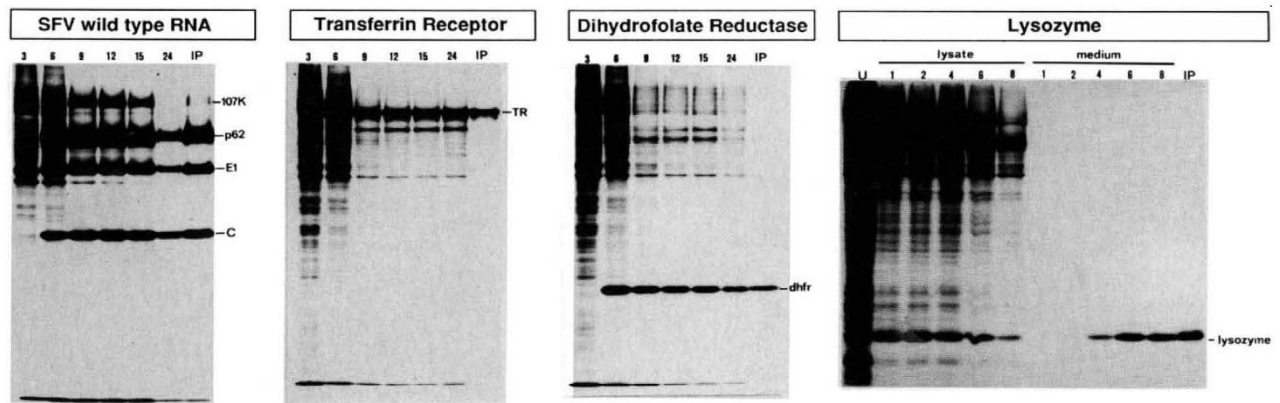


FIGURE 3 Expression of heterologous proteins in BHK cells upon RNA transfection by electroporation. Cells were transfected with RNAs of wild type SFV, pSFV1-dhfr, and pSFV1-TR, pulse-labeled at 3, 6, 9, 12, 15, and 24 hours p.e. Equal amounts of lysate were run on a 12% gel. Note that the faster migrating band in the TR experiment represents an unglycosylated form of the TR protein, and is not recognized by the monoclonal antibody.

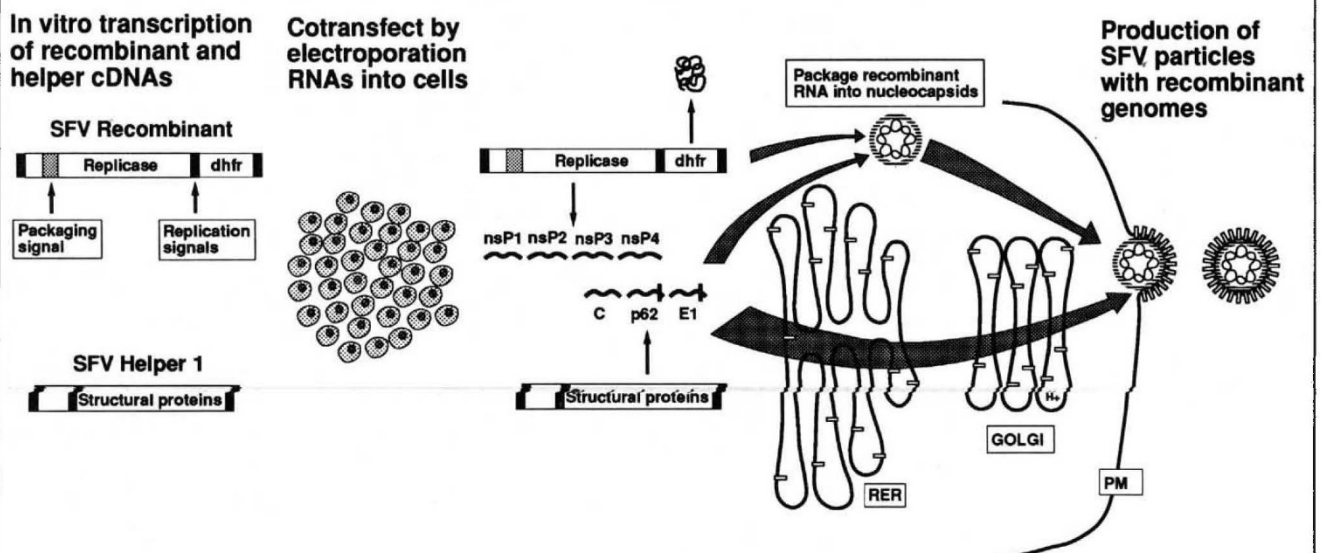
The 9 hour sample was also used for immunoprecipitation (IP) of the SFV, the dhfr and the transferrin receptor proteins. Cells transfected with pSFV1-lysozyme were pulse-labeled at 9 hours p.e. and then chased for the times (hours) indicated. Equal portions of lysate or medium was loaded on a 13.5% gel. IP represents immunoprecipitation from the 1 hour chase lysate sample. The U-lane is a lysate of labeled but untransfected cells.

problems that are encountered in the conventional "nuclear" DNA expression systems, such as mRNA splicing, limitations in transcription factors, problems with capping efficiency and mRNA transport. The cytopathogenic effects appear rather late during infection, and thus there is an extensive time window from about 4 hours after infection up to at least 48 hours after infection during which a high expression level is combined with negligible morphological change. SFV has a very broad host range as a consequence of the natural life cycle, which includes transmission through arthropod vectors to wild rodents and birds. Under laboratory conditions, SFV infects cultured mammalian (e.g. human, monkey, hamster, mouse, porcine), avian (e.g. chicken), reptilian (e.g. viper), amphibian (e.g. frog) and insect (e.g. mosquito, *Drosophila*) cells¹⁴⁻¹⁸.

RESULTS

The SFV expression plasmids. As a basis for the construction of the SFV expression vectors, we used a cDNA clone, pSP6-SFV4, spanning the complete genome of SFV (Fig. 1). This plasmid can be used for *in vitro* production (with SP6 RNA polymerase) of full-length SFV transcripts that will initiate virus replication upon introduction into animal cells¹⁹. To allow for heterologous inserts, the subgenomic (26S) coding region for the structural proteins of the virus was deleted from pSP6-SFV4, and a *Bam*HI-*Xma*I-*Sma*I polylinker sequence, followed by a cassette of translational stop codons in all three reading frames, was inserted downstream from the 26S promoter site^{20,21} to create the final vectors, pSFV1, pSFV2 and pSFV3 (Figs. 1 and 2). To maintain self-replication, the vectors also retain the complete nonstruc-

FIGURE 4 Schematic presentation of *in vivo* packaging of pSFV1dhfr RNA into infectious particles using *trans* complementation.



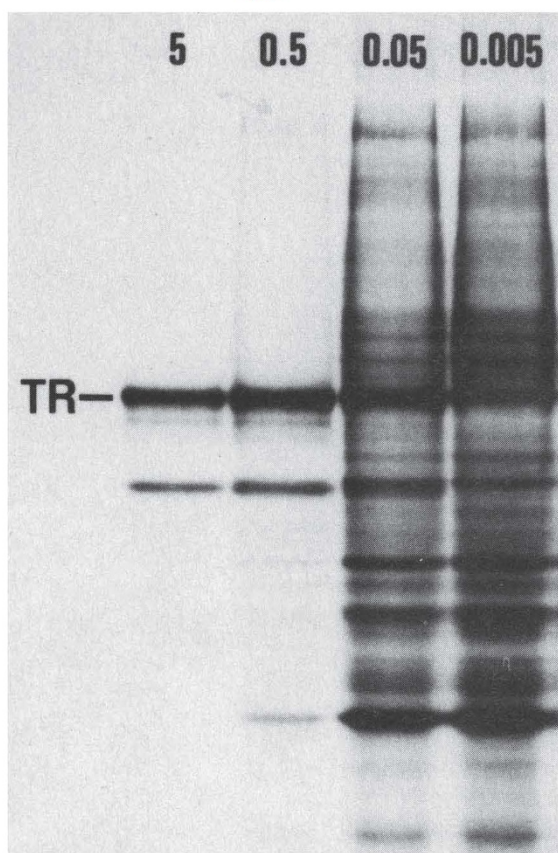


FIGURE 5 Expression of human transferrin receptor in BHK cells after infection by *in vivo*-packaged particles carrying pSFV1-TR recombinant RNA. Virus (200 μ l) diluted in MEM (including 0.5% BSA and 2 mM glutamine) was overlaid on cells to give m.o.i. values ranging from 5 to 0.005. After 1 hour at 37°C, complete BHK medium was added and growth continued for 9 hours, at which time a 10 minute pulse and 10 minute chase was performed, and the cells dissolved in NP-40 lysis buffer. Of the 300 μ l lysate (corresponding to about 30,000 cells) 10 μ l was run on the 10% gel, processed for autoradiography, dried and exposed for 2 hours at -70°C.

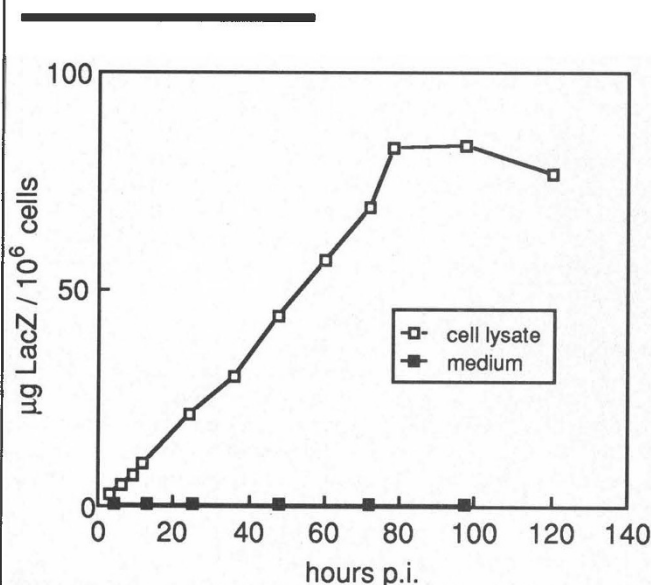


FIGURE 6 Expression of LacZ from a pSFV3-lacZ recombinant. Recombinant RNA was packaged *in vivo* using Helper-1 as described in the text. The resulting virus stock was used to infect cell monolayers at m.o.i. 5, after which production of LacZ was monitored both from the total cell lysates and growth medium.

tural region of the SFV genome encoding the viral replicase complex nsP1-4, and the short 5' (nt 1-247) and 3' (nt 11,423-11,441) regions required in *cis* for RNA replication²²⁻²⁶.

Expression of heterologous proteins from pSFV1. To test heterologous gene expression, the genes encoding mouse dihydrofolate reductase (dhfr, a 21 kD cytoplasmic protein), human transferrin receptor (TR, a 90 kD membrane protein), and chicken lysozyme (a 14 kD secreted protein) were inserted into the pSFV1 vector. *In vitro*-made RNA of the dhfr and TR constructs was electroporated into BHK cells, with RNA of wild type SFV as a control (Experimental Protocol). At different times post electroporation (p.e.) cells were pulse-labeled for 10 minutes followed by a 10 minute chase, whereafter the cells were lysed and directly analyzed by SDS PAGE and autoradiography (Fig. 3). At 3 hours p.e. hardly any exogenous proteins were made (SFV mRNA synthesis does not peak until about 6 hours after infection⁶), and almost all labeled proteins were of host origin, although a substantial decrease of host protein synthesis had already occurred (compare Fig. 3, lane U). In contrast, by 6 hours p.e., the exogenous proteins were synthesized with high efficiency, and severe inhibition of host protein synthesis was evident. This was even more striking at 9 hours p.e. This result confirms our earlier findings¹⁹ that the transfection frequency in practice was 100%. Efficient production of the heterologous proteins was (in this experiment) followed up to 24 hours p.e. Since chicken lysozyme is a secretory protein, its expression was analyzed both from cell lysates and from the growth medium. Cells were pulse-labeled for 10 minutes at 9 hours p.e. and then chased up to 8 hours, during which time almost all labeled lysozyme molecules were transported to the medium (Fig. 3).

***In vivo* packaging of recombinant RNA into infectious SFV particles.** To develop an *in vivo* packaging system for the introduction of recombinant RNA into cells by normal infection, we constructed a helper vector, pSFV-Helper1 (Fig. 1). The vector retains the 5' and 3' signals needed for RNA replication as well as the complete structural region including its promoter, but lacks a region at the end of nsP1 which is required to package SFV RNA into nucleocapsids^{25,27}. The principle of the helper-catalyzed packaging is shown in Figure 4. When helper RNA is cotransfected with a pSFV recombinant derivative, the recombinant genome provides the enzyme for RNA replication and transcription of both incoming RNAs, whereas the helper supports the production of the structural proteins via its subgenomic region. Since the helper lacks the region for RNA packaging, RNA derived from this vector will not be packaged and hence, transfections with recombinant and helper only produce virus particles that carry recombinant derived RNA. The viruses produced provide a one-round virus stock and after infection these particles will only express the heterologous protein.

To test the packaging system, *in vitro*-made RNA of pSFV1-TR was mixed with helper RNA at different ratios and these mixtures cotransfected into BHK cells. Cells were grown for up to 72 hours after which the culture medium was collected and the virus particles sedimented by ultracentrifugation. The number of infectious units (i.u.) was determined by indirect immunofluorescence staining for TR in BHK cells that had been infected with serial dilutions of the purified recombinant virus stock. A helper to recombinant ratio of 1:1 gave the highest titre, and maximum virus yields were obtained by growing the cells for 36 hours p.e. On the average, 5×10^6 cells (the amount used per one electroporation) produced 5×10^9 i.u. TR expression from the recombinant virus was tested

by infecting BHK cells at different multiplicities of infection (m.o.i.), and infection with about 1 infective unit per cell caused TR production with virtually complete inhibition of host protein synthesis, indicating that all cells had been infected (Fig. 5). Moreover, there was no expression of helper-encoded proteins (even at m.o.i. 100, data not shown), indicating that helper RNA is not packaged.

Protein production by the SFV vector. To determine the amount of protein expressed by the system, the gene encoding the *Escherichia coli* β -galactosidase protein, LacZ, was cloned into pSFV3. RNA from the recombinant construct was packaged *in vivo* into SFV particles and the virus stock used to infect BHK cells. An initial test showed that about one particle per cell is enough to start a complete infection, since there were essentially no differences in expression levels using m.o.i. ranging from 1 to 100. Thus, the self-replicating mechanism of the virion quickly saturates the cytoplasmic translation system with virus specific mRNA. Time-course experiments showed that production of LacZ continued up to about 80 hours, after which synthesis ceased, probably due to limitation of host components. However, the cells remained sealed throughout the experiment, since no LacZ leaked into the growth medium (Fig. 6). The amount of LacZ produced was assayed using purified LacZ as standard, and 5×10^6 cells produced 400 μ g of protein, corresponding to about 25% of total cell protein. Figure 7 shows an SDS-PAGE analysis of corresponding lysates staining the gel with Coomassie brilliant blue. The LacZ is by far the most prominent protein in the cell lysate.

DISCUSSION

An essential prerequisite for the successful design of an SFV expression system was the earlier development of a new RNA transfection procedure based on electroporation, whereby virtually all cells receive the vector RNA¹⁹. This allows full expression of the recombinant genomes, and is also the key to the production of a high titre stock of recombinant virus using only one round of replication. Although expression analysis can be made using electroporated cells, for most purposes it is probably more practical to use the recombinant stock, which consists of about 5×10^9 infectious units per electroporation. This is sufficient to perform about 10^5 labeling experiments involving 10^4 cells, each allowing clear product identification using SDS-PAGE and autoradiography with an overnight exposure, without prior antibody-mediated protein concentration from the cell lysates. The LacZ expression experiment showed that a single electroporation can be used for the production of about 3×10^{17} molecules, and as one packaging stock can be used to infect 5×10^9 cells it can be used to produce a total of 400 mg active β -galactosidase.

Xiong and coworkers recently described the expression of the chloramphenicol acetyltransferase (CAT) protein using a Sindbis virus vector⁸. Although the overall principle of their vector is similar to that of SFV, the total protein production obtained after initial transfection of cells remained quite low, probably due to inefficient (0.1%) transfection. They also described the packaging of Sindbis recombinant genomes into virus particles using wild-type virus as helper. However, 6–7 passages of the mixed virus stock were necessary to obtain a preparation of virus, which contained a titre of the recombinant virus high enough to be useful for protein expression. The necessity for a high number of passages is also most likely due to the low transfection efficiency. Moreover, since the Sindbis helper consists of an infectious virus derivative, coinfections will lead to high expression of virus proteins,

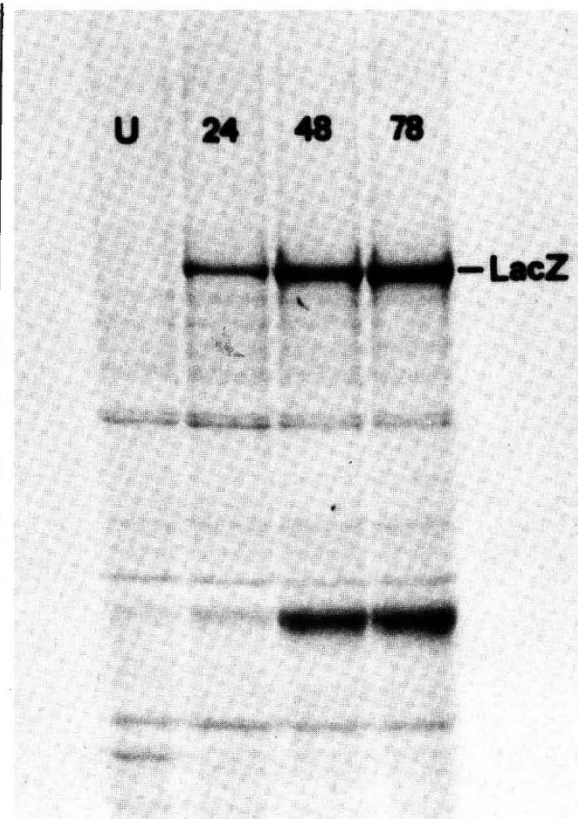


FIGURE 7 Accumulation of LacZ in cells infected with pSFV3-lacZ recombinant virus (see Fig. 6). Lysates were prepared at 24, 48, and 78 hours p.i. Lane U, 48 hour lysate of uninfected cells. Of total cell lysate protein 25 μ g was loaded in each lane. After electrophoresis the gel was stained with Coomassie brilliant blue. At the 48 and 78 hour timepoints a strong band besides LacZ can be seen. The identity of this band, which has a molecular weight of about 65 kD, is unclear. Pulse-labeling showed that this protein is synthesized only very late after infection. It does not react with antibodies against SFV nsP proteins, and might represent a heat-shock protein.

which might hamper analysis of the heterologous gene products.

An important question relating to the usefulness and safety of the SFV expression system is whether any wild type SFV particles could arise after cotransfection with recombinant and helper RNAs. Recently, recombination was reported to occur between defective Sindbis virus transcripts, and was assumed to be due to template switching during RNA replication²⁸. To date, however, we have not detected any wild type SFV in our recombinant stocks, as assayed by either immunofluorescence or pulse-labeling experiments (note the absence of SFV structural proteins in Fig. 5).

A major advantage of the SFV system is that a high titer recombinant virus stock can be produced by a single (overnight) transfection experiment. There is no need for tedious selection/screening, plaque purification or amplification steps for the production of a recombinant virus stock. This feature of the SFV expression system is especially important in experiments where the phenotypes of a large number of mutants have to be characterized. Furthermore, the recombinant virus can be used to transduce the recombinant genome in a "natural" and non-leaky way into a large variety of cells including insect and most higher eukaryotic cell types. This property should be especially useful when cell-type specific post-translational

modifications are required for the activity of the expressed protein.

EXPERIMENTAL PROTOCOL

General. Cell culture, DNA techniques, *in vitro* mutagenesis, immunoprecipitations, immunofluorescence and SDS-polyacrylamide gel electrophoresis were done as described previously^{9,19}. Polyclonal rabbit anti-mouse dhfr was a kind gift from E. Hurt (European Molecular Biology Laboratory, Heidelberg, FRG). SFV nonstructural proteins nsP1-4 were immunoprecipitated as described earlier²⁹. Anti-E1, anti-E2³⁰, and anti-C³¹ monoclonal antibodies and rabbit anti-lysozyme³², and anti-nsP1-4²⁹ have been described. The human transferrin receptor was precipitated with the monoclonal antibody OKT-9 in ascites fluid. This preparation was provided by Thomas Ebel at our laboratory using a corresponding hybridoma cell line obtained from ATCC (American Type Culture Collection).

In vitro transcription and transfection. These have been described in detail previously¹⁹. Briefly, RNA was synthesized at 37°C for 1 hour in 10–50 µl reactions containing 1–5 µg of *Spe*I-linearized plasmid DNA, 40 mM Hepes-KOH (pH 7.4), 6 mM MgCl₂, 2 mM spermidine-HCl, 5 mM dithiothreitol (DTT), 100 µg/ml of nuclease free BSA, 1 mM each of ATP, CTP and UTP, 500 µM of GTP, 1 mM of m⁷G(5')ppp(5')G, 1 unit/µl of RNasin and 100–500 units/ml of SP6 RNA polymerase. For transfection by electroporation, RNA was added either directly from the *in vitro* transcription reaction or diluted with transcription buffer containing 5 mM DTT and 1 unit/µl of RNasin. Cells were trypsinized, washed once with complete BHK-cell medium and once with PBS (without MgCl₂ and CaCl₂) and finally resuspended in PBS to give 10⁷ cells/ml. Cells (0.5 ml) were transferred to a 0.2 cm cuvette (BioRad, Richmond, CA), 5–50 µl (5–10 µg) of RNA solution added and electroporation done at room temperature by two consecutive pulses at 1.5 kV/25 µF using a BioRad Gene Pulser apparatus with its pulse controller unit set at maximum resistance. The cells were diluted at least 1:20 in complete BHK-cell medium and transferred onto tissue culture plates.

Construction of recombinant pSFV1. The dhfr gene was isolated from pGEM2-dhfr³³ as a *Bam*HI-*Hind*III fragment, blunted with Klenow fragment and inserted into *Sma*I-cut pSFV1. The transferrin receptor gene was first transferred from pGEM1-TR³³ as an *Xba*I-*Eco*RI fragment into pGEM7ZF(+) and subsequently from there as a *Bam*HI fragment into pSFV1. A *Bam*HI fragment from pGEM2 carrying the lysozyme gene³⁴ was transferred into pSFV1. The *Escherichia coli lacZ* gene was isolated as a *Bam*HI fragment from the plasmid pMC1871³⁵.

Assay of β-galactosidase. Cells were washed once with PBS and then lysed in NP-40 buffer (1% NP-40, 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2 mM EDTA) for 10 min on ice. To assay for LacZ³⁶, a small amount of lysate was diluted in Z-buffer (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM MgSO₄, 40 mM 2-mercaptoethanol), ONPG (*o*-nitrophenyl-β-D-galactopyranoside, 4 mg/ml in Z-buffer) was added and the mixture incubated at 37°C before measurement of the resulting yellow color at 420 nm. Purified β-galactosidase protein was used as standard.

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References

- Bishop, D. H. L. 1990. Gene expression using insect cells and viruses, p. 62–67. *In: Current Opinion in Biotechnology*. M. Rosenberg and B. Moss (Eds.). Current Opinion Ltd., London.
- Moss, B. 1990. Regulation of Vaccinia virus transcription. *Ann. Rev. Biochem.* **59**:661–688.
- Moss, B., Elroy-Stein, O., Mizukami, T., Alexander, W. A. and Fuerst, T. R. 1990. New mammalian expression vectors. *Nature* **348**:91–92.
- Garoff, H., Kondor-Koch, C. and Riedel, H. 1982. Structure and assembly of alphaviruses. *Curr. Top. Microbiol. Immunol.* **99**:1–50.
- Simons, K. and Fuller, S. 1987. The budding of enveloped viruses: A paradigm for membrane sorting?, p. 139–150. *In: Biological Organization: Macromolecular Interactions at High Resolution*. R. K. Burnett and R. H. Vogel (Eds.). Academic Press, New York.
- Strauss, E. G. and Strauss, J. H. 1986. Structure and replication of the alphavirus genome, p. 35–90. *In: The Togaviridae and Flaviviridae*. S. S. Schlesinger and M. J. Schlesinger (Eds.). Plenum Press, New York.

- Schlesinger, S. S. and Schlesinger, M. J. (Eds.). 1986. *The Togaviridae and Flaviviridae*, p. 453. Plenum Press, New York.
- Xiong, C., Levis, R., Shen, P., Schlesinger, S., Rice, C. M. and Huang, H. V. 1989. Sindbis virus: An efficient, broad host range vector for gene expression in animal cells. *Science* **243**:1188–1191.
- Wahlberg, J. M., Boere, W. A. and Garoff, H. 1989. The heterodimeric association between the membrane proteins of Semliki Forest virus changes its sensitivity to mildly acidic pH during virus maturation. *J. Virol.* **63**:4991–4997.
- Garoff, H., Huylebroeck, D., Robinson, A., Tillman, U. and Liljestrom, P. 1990. The signal sequence of the p62 protein of Semliki Forest virus is involved in initiation but not in completing chain translocation. *J. Cell Biol.* **111**:867–876.
- Metsikkö, K. and Garoff, H. 1990. Oligomers of the cytoplasmic domain of the p62/E2 membrane protein of Semliki Forest virus bind to the nucleocapsid *in vitro*. *J. Virol.* **64**:4678–4683.
- Liljestrom, P. and Garoff, H. 1991. Internally located cleavable signal sequences direct the formation of Semliki Forest virus membrane proteins from a polyprotein precursor. *J. Virol.* **65**:147–154.
- Lusa, S., Garoff, H. and Liljestrom, P. 1991. Fate of the 6K membrane protein of Semliki Forest virus during virus assembly. *Virology* **185**:000–000.
- Griffin, D. E. 1986. Alphavirus pathogenesis and immunity, p. 209–250. *In: The Togaviridae and Flaviviridae*. S. S. Schlesinger and M. J. Schlesinger (Eds.). Plenum Press, New York.
- Clark, H. F., Cohen, M. M. and Lunger, P. D. 1973. Comparative characterization of a C-type virus-producing cell line (VSW) and a virus-free cell line (VH2) from *Vipera russelli*. *J. Natl. Cancer Inst.* **51**:645–657.
- Leake, C. J., Varma, M. G. R. and Pudney, M. 1977. Cytopathic effect and plaque formation by Arboviruses in a continuous cell line (XTC-2) from the toad *Xenopus laevis*. *J. Gen. Virol.* **35**:335–339.
- Stollar, V. 1980. Defective interfering alphaviruses, p. 427–457. *In: The Togaviruses*. R. W. Schlesinger (Ed.). Academic Press, New York.
- L'Héritier, P. 1977. About the infection of *Drosophila* female germ line cells by Sigma viruses. *Ann. Microbiol. (Inst. Pasteur)* **128A**:119–131.
- Liljestrom, P., Lusa, S., Huylebroeck, D. and Garoff, H. 1991. *In vitro* mutagenesis of a full-length cDNA clone of Semliki Forest virus: the 6,000-molecular-weight membrane protein modulates virus release. *J. Virol.* **65**:4107–4113.
- Levis, R., Schlesinger, S. and Huang, H. V. 1990. Promoter for Sindbis virus RNA-dependent subgenomic RNA transcription. *J. Virol.* **64**:1726–1733.
- Grakoui, A., Levis, R., Raju, R., Huang, H. V. and Rice, C. M. 1989. A cis-acting mutation in the Sindbis virus junction region which affects subgenomic RNA synthesis. *J. Virol.* **63**:5216–5227.
- Tsiang, M., Weiss, B. G. and Schlesinger, S. 1988. Effects of 5'-terminal modifications on the biological activity of defective interfering RNAs of Sindbis virus. *J. Virol.* **62**:47–53.
- Niesters, H. G. M. and Strauss, J. H. 1990. Mutagenesis of the conserved 51-nucleotide region of Sindbis virus. *J. Virol.* **64**:1639–1647.
- Niesters, H. G. and Strauss, J. H. 1990. Defined mutations in the 5' nontranslated sequence of Sindbis virus RNA. *J. Virol.* **64**:4162–4168.
- Levis, R., Weiss, B. G., Tsiang, M., Huang, H. and Schlesinger, S. 1986. Deletion mapping of Sindbis virus DI RNAs derived from cDNAs defines the sequences essential for replication and packaging. *Cell* **44**:137–145.
- Kuhn, R. J., Hong, Z. and Strauss, J. H. 1990. Mutagenesis of the 3' nontranslated region of Sindbis virus RNA. *J. Virol.* **64**:1465–1476.
- Weiss, B., Nitschko, H., Ghattas, I., Wright, R. and Schlesinger, S. 1989. Evidence for specificity in the encapsidation of Sindbis virus RNAs. *J. Virol.* **63**:5310–5318.
- Weiss, B. G. and Schlesinger, S. 1991. Recombination between Sindbis virus RNAs. *J. Virol.* **65**:4017–4025.
- Peränen, J., Takkinen, K., Kalkkinen, N. and Kääriäinen, L. 1988. Semliki Forest virus-specific non-structural protein nsP3 is a phosphoprotein. *J. Gen. Virol.* **69**:2165–2178.
- Boere, W. A. M., Harmsen, T., Vinje, J., Benaissa-Trouw, B. J., Kraaijeveld, C. A. and Snippe, H. 1984. Identification of distinct antigenic determinants on Semliki Forest virus by using monoclonal antibodies with different antiviral activities. *J. Virol.* **52**:575–582.
- Greiser-Wilke, I., Moennig, V., Kaaden, O.-R. and Figueiredo, L. T. M. 1989. Most alphaviruses share a conserved epitopic region on their nucleocapsid protein. *J. Gen. Virol.* **70**:743–748.
- Kondor, K. C., Bravo, R., Fuller, S. D., Cutler, D. and Garoff, H. 1985. Exocytic pathways exist to both the apical and the basolateral cell surface of the polarized epithelial cell MDCK. *Cell* **43**:297–306.
- Zerial, M., Melançon, P., Schneider, C. and Garoff, H. 1986. The transmembrane segment of the human transferrin receptor functions as a signal peptide. *EMBO J.* **5**:1543–1550.
- Krieg, P., Strachman, R., Wallis, E., Tabe, L. and Colman, A. 1984. Efficient expression of cloned complementary DNAs for secretory proteins after injection into *Xenopus* oocytes. *J. Mol. Biol.* **180**:615–643.
- Casadaban, M. J., Martinez-Arias, A., Shapira, S. K. and Chou, J. 1983. β-galactosidase gene fusions for analyzing gene expression in *Escherichia coli* and yeast. *Meth. Enzymol.* **65**:293–308.
- Silhavy, T. J., Berman, M. L. and Enquist, L. W. 1984. Experiments with Gene Fusions, p. 303. Cold Spring Harbor Laboratory Press, New York.
- Kozak, M. 1989. The scanning model for translation: an update. *J. Cell Biol.* **108**:229–241.