Gene Ther Mol Biol Vol 4, 23-31. December 1999.

Semliki Forest virus vectors for *in vitro* and *in vivo* applications

Review Article

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Abbreviations: **SFV**, Semliki Forest virus; **CTL,** cytotoxic T-cell; **GFP**, green fluorescence protein; **GPCRs**, G-protein coupled receptors; **hNK1R**, human neurokinin-1 receptor

Key words: Semliki Forest virus, gene therapy, vaccine, LacZ, G-protein coupled receptors

Received: 16 December 1999; accepted: 20 December 1999

Summary

Rapid virus generation, broad host range, efficient RNA replication in the cytoplasm, and high expression levels are features that have made the use of Semliki Forest virus (SFV) vectors attractive. High-level expression of G-protein coupled receptors has allowed specific binding and functional studies in a variety of mammalian cell lines. Furthermore, high infection efficiency (75-90%) has greatly facilitated gene expression and localization studies in primary neurons in culture. The establishment of SFV infections of large-scale suspension cultures has resulted in the production of hundreds of milligrams of recombinant receptors now available for structural studies. SFV vectors have been shown to preferentially infect neurons in hippocampal slice cultures, which will facilitate studies on gene expression, transport, and protein localization in neuronal tissue. Injection of replication-deficient SFV vectors into rat brain resulted in local, high-level transient expression *in vivo*. Recent vector improvements have included the generation of SFV vectors with low-to-moderate transgene expression resulting in more physiological expression levels that are similar to those seen in native tissue. Novel SFV vectors, which have recently been developed, permit prolonged survival of infected host cells.

I. Introduction

Recombinant protein expression is an essential part of molecular biology research and drug discovery nowadays. As the sequencing of the human genome is approaching its completion, the requirement for fast and high-level expression of novel gene sequences is rapidly increasing. Several viral and non-viral vector systems have been developed for this purpose.

Semliki Forest virus (SFV), a member of the alphavirus family, is an enveloped virus with a single-stranded RNA genome. To generate an expression system the SFV genome was split on two plasmids as cDNA molecules (Liljeström and Garoff, 1991). The cloning vector contains the four nonstructural SFV genes (nsP1-4), which code for the replicase complex, responsible for RNA replication. The subgenomic SFV 26S promoter is located at the 3' end of the nsP4 gene and drives the expression of the foreign gene of interest inserted immediately downstream of nsP4. The helper vector contains the SFV structural genes encoding the capsid and membrane proteins, required for packaging of infectious particles. RNA molecules are transcribed *in vitro* from both the cloning and helper vector and transfected into BHK cells either by electroporation or lipofection for *in vivo* packaging of recombinant SFV particles (**Fig. 1**). High-titer virus stocks (10^8-10^9 infectious particles/ml) harvested 24 h post-transfection are ready for infection of host cells without any further purification or concentration. The second-generation helper vector pSFV-Helper2 contains three point mutations in p62 (precursor for the E2 and E3 membrane proteins), which will result in production of conditionally infectious SFV particles (Berglund et al., 1993). These virus stocks require

treatment with α-chymotrypsin to restore the infectivity. The broad host range of SFV allows infection of a wide variety of mammalian cell lines and primary cell cultures (Lundstrom, 1999). Due to the presence of the nonstructural SFV genes in the recombinant SFV RNA introduced into the host cell, extensive RNA replication will occur directly in the cytoplasm. Generation of large quantities of RNA results in high-level expression of foreign genes. However, due to the absence of the SFV structural genes, no further virus particles are generated, which leads to only transient expression from these suicidal vectors.

SFV vectors have been applied for high-level expression of many topologically different proteins. Successful expression of nuclear, cytoplasmic, membrane, and secreted proteins have been reported (Liljeström and Garoff, 1991, Lundstrom 1997). The advantages of using SFV vectors are the rapid production of high-titer virus and the broad host range that allows efficient infection of a variety of mammalian and insect cell lines, primary cell cultures (Lundstrom 1999) as well as neurons *in vivo* (Lundstrom et al., 1999).

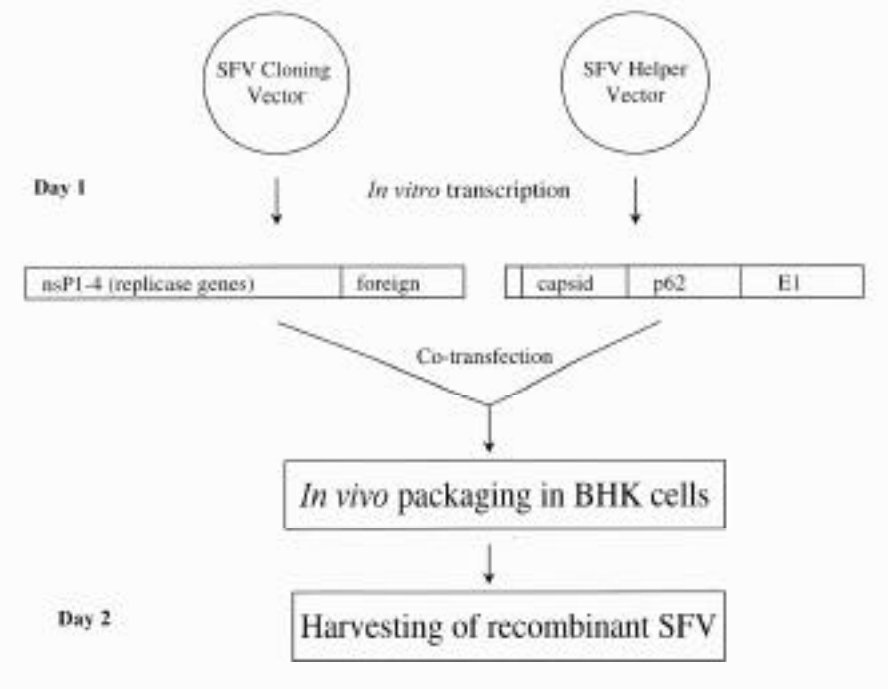

Fig. 1A. Schematic presentation of recombinant SFV particle production. The gene of interest is cloned into the SFV cloning vector and subjected together with SFV-Helper vector to *in vitro* transcription with SP6 RNA polymerase. RNA molecules are co-transfected into BHK cells for *in vivo* packaging of recombinant SFV particles.

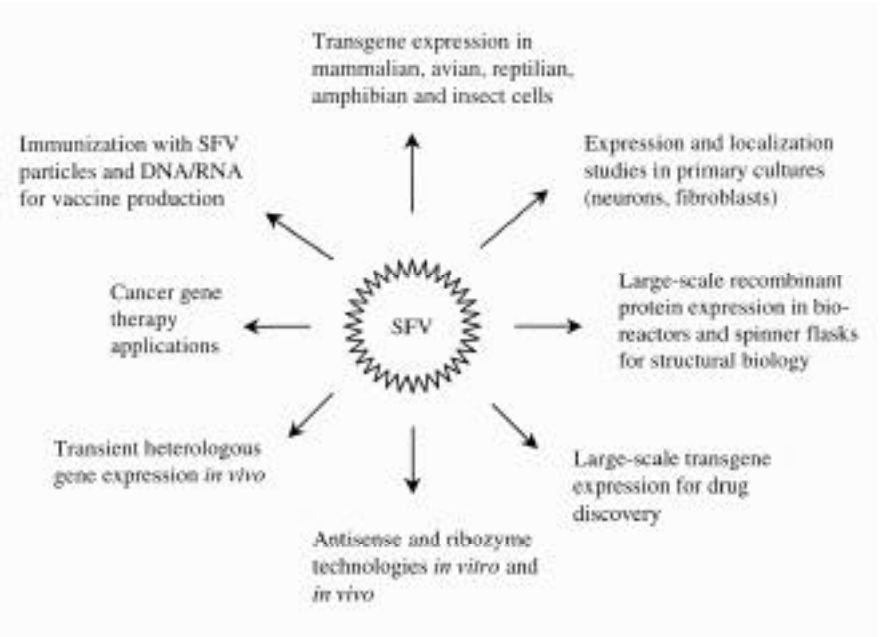

Fig. 1B. Possible applications of SFV vectors.

In this review, we describe the versatility of the SFV expression system. We have chosen to focus on SFV vectors but comparable studies have been performed with similar vectors for other types of alphaviruses, namely Sindbis virus and Venezuelan Equine Encephalitis virus. SFV vectors can be efficiently used for receptors previously known to be difficult to express at high levels. The expression has also been expanded to large-scale production, e.g., to facilitate receptor purification for structural studies. Furthermore, the replication-deficient nature of the vectors has allowed *in vivo* studies in rat brain. Initial studies indicated that there is a great potential for the use of SFV vectors for cancer gene therapy. SFV has also been proven a good candidate as a vector to be used in vaccine production. Finally, we will discuss recent improvements of SFV vectors.

II. Recombinant gene expression

Although many types of recombinant proteins have been expressed from SFV vectors, we will focus here on G-protein coupled receptors (GPCRs). Several GPCRs have most efficiently been expressed from SFV vectors (Lundstrom 1999). Metabolic labeling experiments demonstrated high expression levels of the human neurokinin-1 receptor (hNK1R) (Lundstrom et al., 1994). However, SFV-infection caused inhibition of the endogenous gene expression and also triggered the induction of apoptosis, resulting in host cell death within 48 to 72 hours. This was also reflected in the expression pattern measured by specific radioligand binding for different receptors. The maximal expression time and duration of measurable binding activity varied from one receptor to another. For instance, the highest levels of expression for hNK1-R were obtained at 16 hours post-infection, whereas the hamster α_{1b}-adrenergic receptor reached maximal activity as late as 40 hours post-infection (Scheer et al., 1999). Saturation assays on isolated membranes revealed extremely high binding activity with B_{max} values up to 80 pmol receptor / mg protein. Binding experiments carried out on whole cells indicated receptor densities of >6 x 10^6 receptors / cell. GPCRs could be efficiently expressed in several different mammalian host cells (BHK, CHO, COS-7, HEK293 cells, etc.). Additionally, high infection rates (75-90%) were obtained in primary rat hippocampal neurons. Two rat odorant receptors, I7 and OR5, as well as the *C. elegans* odr-10 receptor, were expressed in these neurons and were found to be localized at the plasma membrane by immunofluorescence microscopy (Monastyrskaia et al., 1999). This observation is particularly interesting, since the expression of these olfactory receptors from the same SFV vectors in BHK cells did not target the receptors to the plasma membrane.

Functional coupling of GPCRs to G proteins could be demonstrated in CHO, BHK and COS-7 cells by measuring agonist-induced intracellular Ca^{2+}-release (Fura-2, FLIPR), inositol phosphate accumulation, cAMP stimulation and GTPγS-binding. Enhanced functional responses could be obtained for the α_{1b}-adrenergic receptor expressed from an SFV vector in COS-7 cells after co-infection with SFV expressing the α_{1b}-adrenergic receptor and the Gαq subunit (Scheer et al., 1999). Interestingly, quadruple infections with SFV vectors for the α_{1b}-adrenergic receptor, Gαq as well as Gβ2 and Gγ2 subunits resulted in further increased response to epinephrine stimulation. These results indicate that the decrease in endogenous G-protein levels (due to the inhibition of host cell protein expression) in SFV-infected cells can be compensated by multiple infections.

The adaptation of mammalian cell lines (BHK, CHO, HEK293, rat C6 glioma cells) to suspension culture has facilitated large-scale production of recombinant receptors for both drug screening purposes as well as for structural studies. Recently, the hexa-histidine tagged mouse serotonin 5-HT_3 receptor was expressed in bioreactors (11.5 liter) to yield large quantities of highly purified receptor (Lundstrom et al., 1997; Hovius et al., 1998). Preliminary data from cryo-EM and gel filtration chromatography confirmed postulations that the 5-HT_3 receptor is a homo-pentameric channel. The discovery that the SFV capsid sequence contains a translation enhancement signal has made it possible to further increase transgene expression levels (Sjöberg et al., 1994). Sjöberg and co-workers showed that fusion of the full-length capsid gene to a transferrin receptor resulted in efficient release of the recombinant tranferrin receptor from the fusion by capsid-protein mediated autolytic cleavage. The transferrin receptor yield was approximately 10-fold higher when expressed as a fusion protein to the SFV capsid. We have fused the human neurokinin-1 receptor (hNK1R) to the full-length capsid sequences and achieved 5- to 10-fold higher expression levels. Additionally, the capsid protein is efficiently cleaved from the hNK1R (**Fig. 2**). This has allowed large-scale production of recombinant hNK1R that has been subjected to solubilization and purification activities with the aim to obtain 2D- and 3D-crystals for high-resolution structure determination studies.

III. In vivo studies

To study the feasibility of SFV vectors for *in vivo* applications, replication-deficient SFV-LacZ virus was injected into the striatum and amygdala of adult male Wistar rats. Animals were injected with 10^5 infectious particles and subjected to behavioral studies for 28 days. No differences in body weight, body temperature, feeding behavior, spontaneous exploration, sensorimotor function and muscular capacity were observed between virus-injected rats and control animals (Lundstrom et al., 1999). Histological examinations revealed high local β-galactosidase expression levels (**Fig**. **3**). Maximal expression was observed 1-2 days post-injection, and the reporter gene expression decreased with time. Some weak X-gal staining was still visible at 28 days post-injection. However, this was most likely due to the high stability of the recombinant β-galactosidase enzyme. *In situ*

hybridization studies confirmed the transient nature of SFV-mediated gene expression where no LacZ mRNA could be detected any more 4 days post-injection.

The SFV infection pattern in rat brain was remarkably neuron-specific. Similar results were found in rat hippocampal slices at 1-5 days after infection with SFV vectors encoding β-galactosidase, green fluorescence protein (GFP), and GFP fused to the ionotropic glutamate receptor 1 subunit (**Fig**. **4**). Upon injection of recombinant SFV into the pyramidal cell layer, the majority of transgene-positive cells were neurons (>90% for GFP), with pyramidal cells, interneurons and granule cells expressing high levels of β-galactosidase and GFP (Ehrengruber et al., 1999).

IV. Gene therapy applications

SFV vectors have been used for indirect gene therapy applications to produce infectious recombinant Moloney murine leukemia virus particles in BHK cells (Li and Garoff, 1996). The *gag-pol* and *env* genes as well as a recombinant retrovirus genome (LTR-ψ^+-*neo*-LTR) were introduced into individual SFV vectors and were co-transfected into BHK cells to generate extracellular virus-like particles that possessed reverse-transcriptase activity. Recently, it has been shown that intron-containing sequences could also be efficiently packaged by the SFV expression system (Li and Garoff, 1998). The rapid virus production and relatively high titers obtained (4 x 10^6 colony forming units/ml) make this an attractive alternative for retrovirus production.

The high expression levels achieved from SFV vectors combined with their ability to shut down host cell protein synthesis and to induce apoptosis have increased the interest in cancer gene therapy applications. SFV-LacZ virus infection of prostate tumor cell lines and of biopsies from patients revealed a strong apoptotic effect measured by flow cytometry (Hardy et al., manuscript in preparation). Furthermore, it has been demonstrated that pre-immunization with self-replicating SFV-LacZ RNA could protect mice from tumor challenge (Ying et al., 1999). Therapeutic immunization with SFV-LacZ RNA also prolonged survival of BALB/c mice with established tumors. SFV vectors were tested in vivo in a murine B16 tumor model (Asselin-Paturel et al., 1999). SFV vectors expressing the p35 and p40 subunits of interleukin-12 from two subgenomic promoters (Zhang et al., 1999) were injected into tumors. This led to significant tumor regression and inhibition of tumor blood vessel formation. No sign of toxicity was observed in SFV-IL12-treated mice. The anti-tumor effect could be enhanced by repeated injections and, most encouragingly, no antiviral response to SFV was detected.

The broad host range of alphaviruses has been of some concern for using these vectors for gene therapy applications. Attempts to target Sinbis virus vectors have recently been carried out. IgG-binding domains of protein A were introduced into the E2 membrane protein, which reduced the infection rate of BHK cells by a factor of 10^5 (Ohno et al., 1997). Targeted infection could be achieved for human cell lines treated with monoclonal antibodies reacting with cell-surface antigens. Similar approaches have been taken for SFV vectors, where protein A domains have been introduced into various regions of E1 and E2 membrane proteins. However, the second generation SFV vectors possess the additional safety feature of conditional infectivity compared to Sindbis virus (Berglund et al., 1993). This should further enhance the selective advantage of targeting IgG-bound cells vs. non-bound cells by a factor of 10^5. The development of chimeric SFV vectors is now in progress.

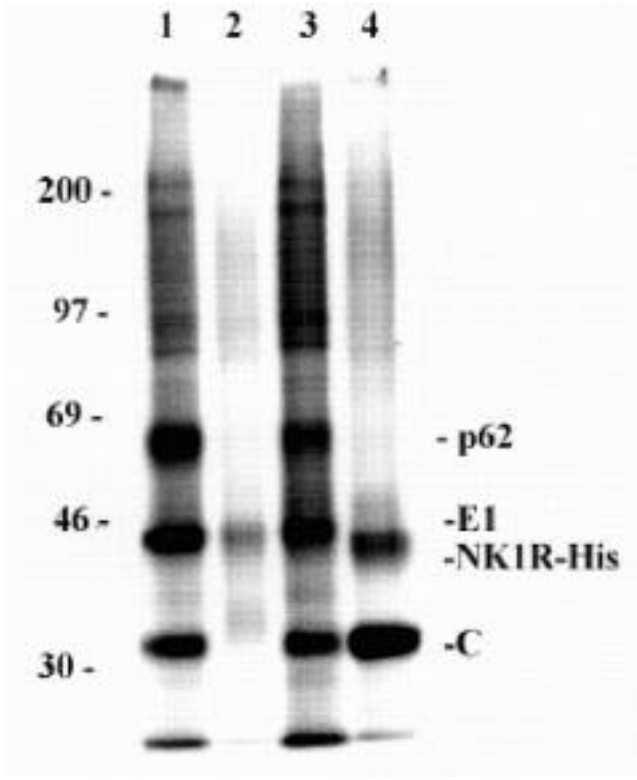

Fig. 2. Metabolic labeling of BHK cells electroporated and infected with SFV vectors. BHK cells co-electroporated with pSFV1-NK1R (**lane 1**) and pSFVCAP-NK1R-His (**lane 3**) and RNA from pSFV-Helper2. BHK cells infected with SFV-NK1R (**lane 2**) and SFVCAP-NK1R-His (**lane 4**) virus. Pulse-labeling with [^{35}S]-methionine was carried out after 4 h (electroporations) and 16 h (infections). C, capsid; E1, SFV E1 membrane protein; p62, precursor for SFV E2 and E3 membrane proteins; NK1R, neurokinin-1 receptor; NK1R-His, neurokinin-1 receptor with hexa-histidine tag; CAP-NK1R, capsid-neurokinin-1 receptor fusion.

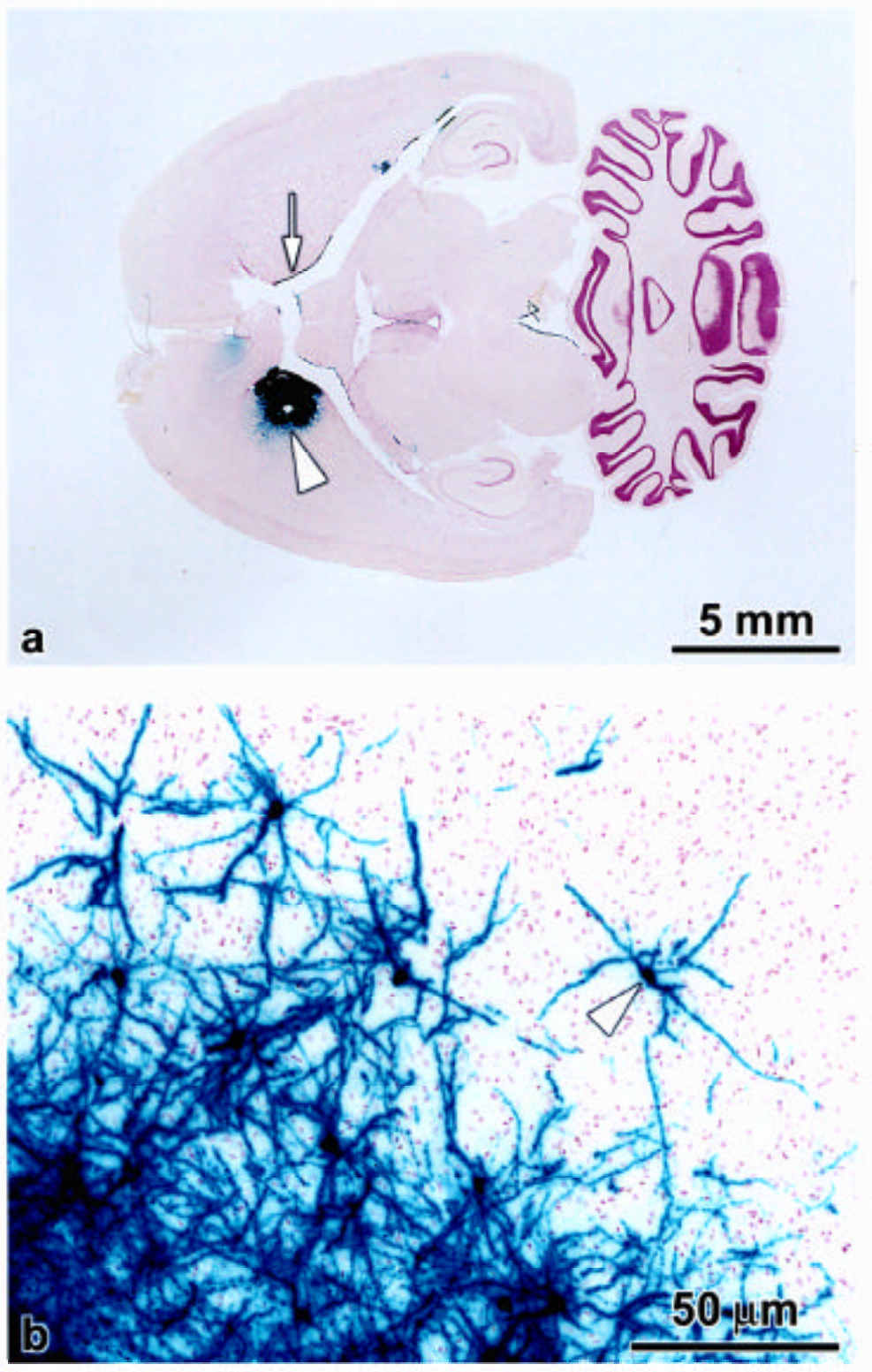

Fig. 3. Expression of recombinant β-galactosidase in rat brain. SFV-LacZ virus (10^5 particles per injection) was injected into adult male Wistar rat brain (striatum and amygdala) and X-gal staining performed at 1 day post-injection. (**A**) Whole brain section. Arrow indicates injection site, arrowhead spread of virus into the ventricles. The counter-staining seen in cerebellum is nonspecific. (**B**) Higher magnification, showing expression of β-galactosidase in neuronal processes.

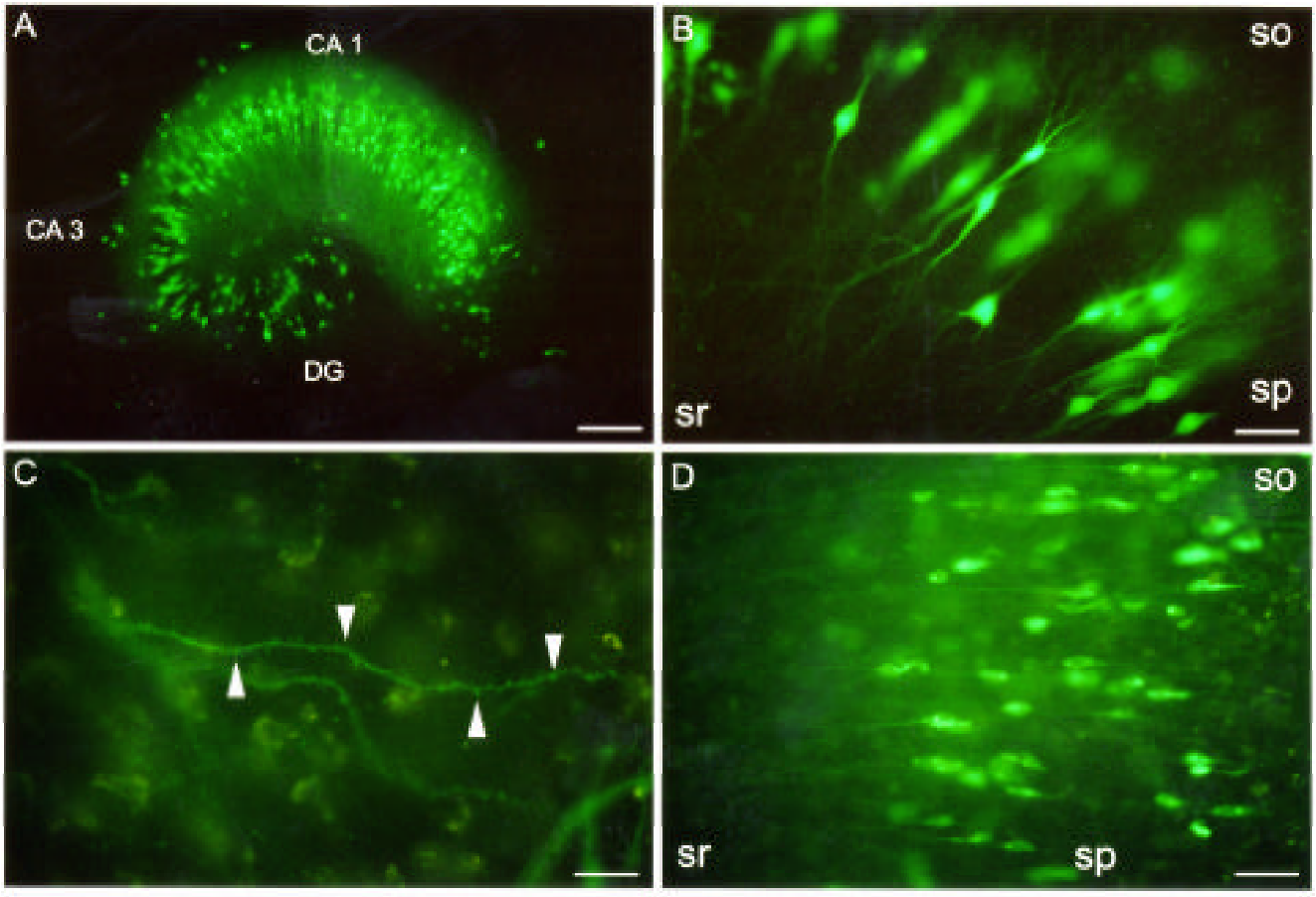

Fig. 4. Recombinant SFV-mediated gene transfer into pyramidal cells of cultured hippocampal slices from postnatal rats. (A, B, and **C)** Expression of the GFP reporter gene. Fluorescence illuminations of living slices at 11-16 days in culture and 2 (A and C) and 5 days (B) after injection of 10^4-10^5 infectious SFV-GFP particles into the pyramidal cell layer. **(B)** GFP-positive CA1 pyramidal cells. **(C)** Apical dendrites from an infected CA1 pyramidal cell. Note the GFP-positive spines, which are typical of pyramidal cells (arrowheads). **(D)** SFV-mediated expression of an N-terminal GFP fusion of the ionotropic glutamate receptor 1 subunit. Fluorescence illumination from the CA1 region of a living slice at 29 days in culture and 4 days post-infection. The cDNA for the GFP fusion construct was provided by Drs. Rolf Sprengel and Volker Mack (Max-Planck-Institute for Medical Research, Heidelberg, Germany). Abbreviations: so, stratum oriens; sp, stratum pyramidale; sr, stratum radiatum. Bars: 300 μm (A), 75 μm (B and D), and 25 μm (C).

V. Vaccine production

SFV vectors have been shown to be potential candidates in vaccine production. Intravenous injections of recombinant replication-deficient SFV particles expressing the influenza NP gene led to humoral responses with high antibody titers in BALB/c mice (Zhou et al., 1994). As few as 100 infectious SFV-NP particles induced a strong cytotoxic T-cell (CTL) response and, after one booster injection, a CTL-memory lasting for more than 40 days was generated (Zhou et al., 1995). Pigtail macaques immunized with recombinant SFV particles expressing simian immunodeficiency virus (SIV) gp160 were protected against lethal disease (Mossman et al., 1996). Recently SFV particles expressing P815 tumor antigen generated a strong CTL response and demonstrated protection against P815 tumor challenge (Colmenero et al., 1999).

In addition to application of recombinant SFV particles, direct injection of nucleic acids have been used for SFV vectors. Injection of self-replicating RNA containing the influenza NP gene into the quadriceps muscle of mice resulted in high humoral responses (Zhou et al., 1994). The advantage of using naked RNA for immunization is the high safety due to the rapid degradation of the injected RNA and the lack of integration into the host genome. Recently, naked RNA vectors expressing the HIV-1 gp41 glycoprotein were injected intramuscularly into mice for the generation of monoclonal antibodies (Giraud et al., 1999). Layered plasmid DNA vectors that utilize an RNA polymerase II expression cassette to drive the transcription of a self-amplifying RNA (replicon) vector have been engineered

for SFV (Berglund et al., 1998). These SFV DNA vectors allowed the direct use of plasmid DNA and resulted in higher levels of humoral and cellular immune responses than for conventional DNA plasmids, immunizing animals against normally lethal challenges with influenza virus. In another study, SFV RNA and DNA vectors and recombinant SFV particles were applied to generate monoclonal antibodies against prion proteins (Krasemann et al., 1999).

VI. Vector development

Despite the various applications and the high efficiency of SFV vectors, there are still areas where improvements are possible. The extreme transgene expression levels obtained from SFV vectors are not comparable to physiological levels anymore. More moderate expression levels would therefore match better the endogenous gene expression. One approach to achieve this is by site-directed mutagenesis of the subgenomic 26S promoter. It has been shown that a 3-nucleotide insertion in the Sindbis virus subgenomic promoter region resulted in a 100-fold reduction of the promoter activity (Raju and Huang, 1991). Very recently, a series of amino acid substitutions and insertions into the SFV 26S subgenomic promoter resulted in significantly lower reporter gene expression (Lundstrom et al., manuscript in preparation). Depending on the type of mutation in the 26S subgenomic promoter, expression levels of 1%, 3%, and 30% of the wild-type promoter activity were obtained. Despite these reduced expression levels, these mutant viruses still substantially inhibited host cell protein synthesis and, therefore, had cytotoxic effects.

Novel SFV vectors with lower cytopathogenicity would be beneficial. Non-cytopathogenic Sindbis vectors have been described (Agapov et al., 1998). This change in phenotype was due to a single amino acid substitution in the nsP2 gene (Pro726Ser). However, titer yields of this virus were rather low and the non-cytopathogenic phenotype was mainly restricted to BHK and Vero cells. Recently, an SFV strain with reduced virulence and only minor inhibition of host cell DNA synthesis was described. Analysis of the nuclear localization signal in the nsP2 gene (Pro^{647}-Arg^{648}-Arg^{649}-Arg^{650}-Val^{651}) revealed a point mutation ($Arg^{649}\rightarrow Asp$) in this SFV strain which results in the retention of the nsP2 protein to the cytoplasm and thus prevents interference with host cell gene transcription (Rikkonen, 1996). Site-directed mutagenesis of nsP2 at Arg^{650} (to Asp) and Ser^{259} (to Pro) resulted in an SFV mutant with significantly higher transgene expression. Interestingly, in contrast to the conventional SFV vector, the host protein synthesis was not as dramatically reduced (**Fig**. **5**). The reduced cytopathogenicity was observed in various mammalian cell lines (BHK, CHO, and HEK293 cells) and primary rat hippocampal neurons and the survival of host cells was substantially prolonged (Lundstrom et al., manuscript in preparation). Such novel non-cytopathogenic vectors will be extremely useful, not only for expression studies, but also for antisense and ribozyme applications.

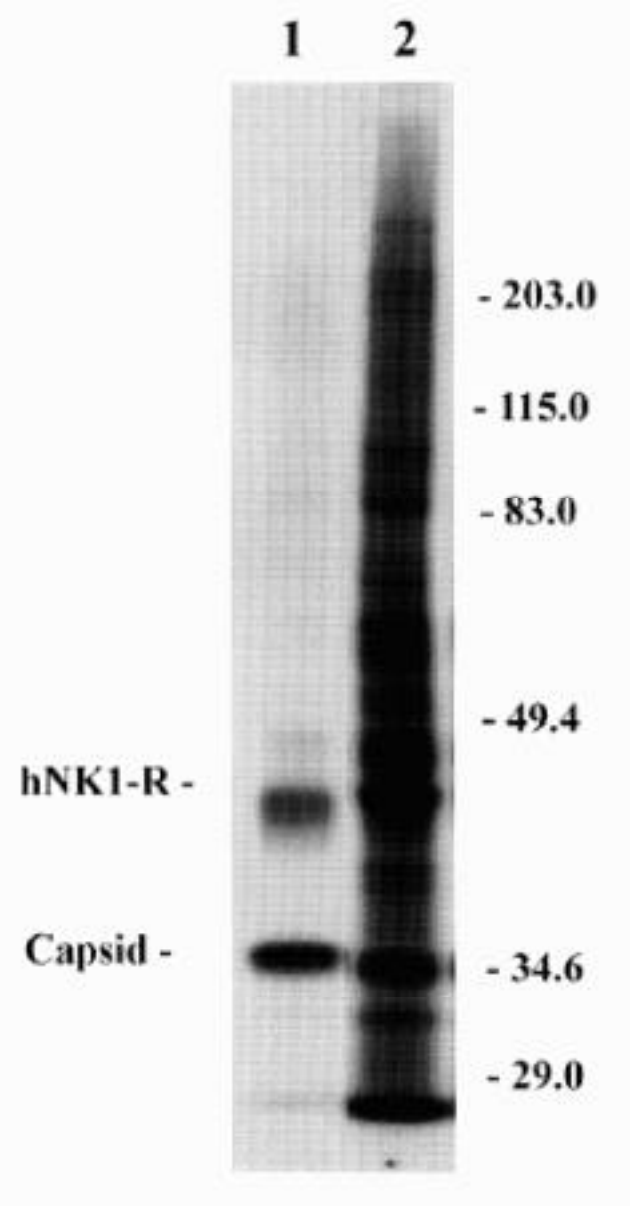

Fig. 5. Metabolic labeling of BHK cells infected with recombinant SFV vectors. Pulse-labeling of BHK cells infected with SFVCAP-NK1R-His (lane 1) and $SFV^{259\text{-}650}$CAP-NK1R-His (lane 2) with [^{35}S]-methionine 16 h post-infection. hNK1R, human neurokinin-1 receptor; $SFV^{259\text{-}650}$, SFV vector with $Ser^{259}\rightarrow$Pro and $Arg^{650}\rightarrow$Asp mutations in nsP2.

VII. Conclusions and future prospects

SFV vectors can be used for a variety of applications in modern molecular biology and drug discovery. Rapid high-titer virus production, broad host range, direct cytoplasmic RNA replication and extremely high yields of expressed recombinant protein, have made SFV vectors attractive alternatives to other expression vectors.

Additional improvements in vector design will further increase the application range of SFV. In the meanwhile, SFV vectors have been used in animal models for initial gene therapy experiments. The application of SFV vectors to man is soon to come.

Acknowledgements

The authors thank Danielle Hermann, Raquel Herrador, Daniel Rotmann and Marie-Therese Zenner for their skillful assistance in the SFV vector construction and virus production, and Lotty Rietschin and Lucette Heeb for the preparation of rat hippocampal slice cultures. We are grateful to Martine Maco and Martine Kapps for their assistance in the neurosurgical injections and to Jürg Messer for his help in imaging of brain sections. This work was supported by the Swiss National Science Foundation (grant no, 31-57'125.99 to M.U.E.).

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