Safety aspects related to recombinant protein expression from Semliki Forest virus vectors

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Received 10 January 2001; accepted 24 April 2001

Key words: mutant vectors, recombinant proteins, safety aspects, Semliki Forest virus

Abstract

Semliki Forest virus vectors (SFV) have been developed for efficient transgene expression to result in high receptor yields (50–200 pmol receptor/mg protein) in a variety of mammalian host cells. Transfer of the SFV technology to mammalian cells growing in suspension cultures has made it feasible to produce hundreds of milligrams of receptor proteins in a short time. Large-scale production, however, raises the questions of the safety of handling virally infected cells for down-stream processing. Analysis of cell culture medium and SFV-infected cells revealed that some infectious particles were still present. Replacement of virus-containing medium at 2 h post-infection efficiently removed the majority of infectious replication-deficient SFV particles. Washes with PBS further reduced the number of infectious particles significantly both in the medium and associated with cells to levels that allowed safe handling of SFV-infected cells outside the cell culture facility for biochemical, pharmacological, or electrophysiological assays or down-stream processes in connection to receptor purification. Furthermore, engineering of novel temperature-sensitive mutant SFV vectors resulted in temperature-controlled transgene expression, which completely eliminates the risk of contaminating laboratory personnel.

Introduction

Viral vectors have played an important role in recombinant gene expression because of their high capacity of gene delivery and strong promoter activity. On the other hand, viral vectors capable of infecting mammalian cells, and particularly human cells, have always posed a threat concerning safety issues, especially when applied for large-scale recombinant protein production, where large volumes of infected cells are handled. It is therefore of utmost importance that the highest possible safety standards are met when viral vectors are employed.

The Semliki Forest virus (SFV) expression system is based on an expression vector containing the viral nonstructural genes and the foreign gene(s) of interest and a helper vector supplying the structural genes *in trans*. The replication-deficient recombinant SFV particles generated from co-transfections of RNA from the expression and helper vectors are capable of

one round of infection of host cells. After infection the viral recombinant RNA will be amplified directly in the cytoplasm due to the presence of the replicase complex (encoded by the nonstructural genes), which will lead to extreme overexpression of the foreign gene of interest. SFV vectors have earlier demonstrated high-level expression of topologically different (nuclear, cytoplasmic, membrane and secreted) proteins (Liljeström and Garoff, 1991; Lundstrom, 1999). The broad host range of SFV has allowed expression studies in a variety of cell lines (mammalian, insect, amphibian, reptilian cells) and in primary cell cultures (fibroblasts, hepatocytes, neurons). Particularly, G protein-coupled receptors (GPCRs), which due to their seven transmembrane domain topology have been difficult to express from many vectors, were efficiently expressed from SFV vectors (Lundstrom et al., 1994). It was also possible to transfer the SFV technology to mammalian cells adapted to growth in suspension cultures (Blasey et al., 1997). This has sub-

Figure 1. Infection kinetics of SFV-GFP on adherent BHK cells. BHK cells on 6-well plates were infected with SFV-GFP at a multiplicity of infection (MOI) of 10 for 2, 5, 15, 30, 60 and 120 min. Virus-containing medium was carefully removed and the cells washed with PBS. GFP expression was microscopically visualized at 24 h post-infection.

Table 1. Relative fluorescence (RFU) measurements and microscopic examination of adherent BHK cells re-infected with SFV-GFP after various treatments of cells after preliminary infections

Virus removal	PBS-wash		RFU	PBS-wash		Sample	RFU	%
	1×	3×		1×	3×			
–	–	–	20046	–	–	Medium	3757	50
–	–	–		–	–	Cells	2186	5–10
+	–	–	25635	–	–	Medium	680	1–2
+	–	–		–	–	Cells	2819	<10
+	+	–	25606	–	–	Medium	407	<0.1
+	+	–		–	–	Cells	1997	5
+	–	+	24949	–	–	Medium	429	<0.1
+	–	+		–	–	Cells	1872	<5
–	–	–	19936	+	–	Cells	3116	5
–	–	–	19717	–	+	Cells	2337	5
+	–	–	27079	+	–	Cells	3063	5
+	–	–	27045	–	+	Cells	2505	5
+	+	–	27446	+	–	Cells	2710	5
+	+	–	26957	–	+	Cells	2697	5
+	–	+	27270	+	–	Cells	3582	5
+	–	+	24157	–	+	Cells	1706	<5
+	–	–	38160	+	–	Cells ^a	718	<0.1
+	–	–	38798	–	+	Cells ^a	742	<0.1
+	+	–	39331	+	–	Cells ^a	783	<0.1
+	+	–	37948	–	+	Cells ^a	729	<0.1
+	–	+	38736	+	–	Cells ^a	908	<0.1

^a Cells filter sterilized (0.2 μ m).

stantially facilitated the production of large quantities of recombinant receptors. Using this technology, it has been possible to produce large amounts of the mouse serotonin 5-HT₃ receptor (Lundstrom et al., 1997) and of the human neurokinin-1 receptor (Lundstrom et al., 1999).

Because it has become feasible to infect mammalian cells with SFV vectors in large volumes in spinner flasks and bioreactors the safety aspects of using these vectors need to be addressed in detail. The safety concern is for the personnel involved in virus preparation and handling of large-scale cultures as well as for those individuals outside the cell culture facility involved in protein expression assays (enzymatic assays, pharmacological studies, electrophysiological recordings) and down-stream processing events. It is important to point out that the SFV expression system is based on an attenuated virus strain with a significantly reduced virulence in mice compared to wild-type SFV. The original SFV expression system generated a minor fraction of replication- proficient viruses (RPVs) through homologous recombination

events (Liljeström and Garoff, 1991). The amplification of RPVs could be eliminated by the introduction of three point mutations in the p62 precursor (for E2 and E3 membrane proteins) gene in the second generation SFV helper vector, which resulted in generation of conditionally infectious particles (Berglund et al., 1993). As a consequence of this modification, no RPVs could be detected even after infection with extreme multiplicity of infection (MOI) values, which further increased the already high safety standards of using SFV vectors.

In this study, we have examined the kinetics of recombinant SFV infections in BHK-21 cells. The presence of infectious SFV particles in the culture medium and associated with cells was also investigated after test infections with SFV-GFP. Clearly, untreated cell culture medium and cell preparates contained infectious particles after overnight incubation. However, exchange of medium at 2 h post-infection substantially decreased the number. Moreover, washes with PBS dramatically reduced the amount of infectious SFV particles associated with medium or cells. An-

SFV-GFP infection of BHK-21 cells

MOI 30, Infection time 2 h, overnight incubation at 37°C

Figure 2. Presence of infectious SFV particles in the medium after various treatments. Medium from adherent BHK cells infected with SFV-GFP was analyzed after no treatment, removal of virus-containing medium or a single wash with PBS for the presence of GFP-positive cells after infection of freshly plated cells by measurement of relative fluorescence (RFU).

Table 2. Relative fluorescence (RFU) measurements of suspension culture of CHO cells infected with SFV-GFP after re-infection of adherent BHK cells

Sample	PBS × 1	PBS × 3	Filter- sterilization	RFU
Medium	–	–	–	1187
Cells	–	–	–	21501
Cells	–	–	+	2882
Cells	+	–	–	12808
Cells	+	–	+	3138
Cells	–	+	–	10982
Cells	–	+	+	2726

other addition to the safe handling of SFV vectors has been the construction of temperature-sensitive SFV mutants. These vectors showed high GFP expression at 31 °C, but a complete inhibition of transgene expression was seen at the non-permissive temperature (37 °C).

Materials and methods

Cell cultures

BHK-21 and CHO-K1 cells were cultured in a 1:1 mixture of Dulbecco's modified F12 medium (Gibco BRL) and Iscove's modified Dulbecco's medium (Gibco BRL) supplemented with glutamine and 10% FCS. CHO cells adapted to growth in serum-free medium (DHI) in suspension cultures (Schlaeger and Lundstrom, 1998) were incubated at 37 and 31 °C.

SFV constructs

The SFV constructs pSFV2gen-GFP and pSFV-PD-GFP for expression of Green Fluorescence Protein (GFP) have been described elsewhere (Lundstrom et al., 1999). The construct pSFV-PDTE-GFP containing two temperature-sensitive mutations (one in nsP2 and another in nsP4) was earlier described (Lundstrom et al., 2000).

SFV-GFP infection of BHK-21 cells

MOI 30, Infection time 2 h, overnight incubation at 37°C

Figure 3. Presence of cell-associated infectious SFV particles. Cell samples were used as infectious material on freshly plated BHK cells to analyze the presence of cell-associated SFV-GFP particles by measurement of relative fluorescence (RFU) after removal of virus-containing medium, PBS washes and filter-sterilization.

Generation of recombinant SFV particles

Preparation of replication-deficient SFV particles was carried out using the pSFV-Helper2 vector (Berglund et al., 1993) as previously described (Lundstrom et al., 1994). Briefly, *in vitro* transcribed RNA from the pSFV2gen-GFP, pSFV-PD-GFP and pSFV-PDTE-GFP were co-electroporated with pSFV-Helper2 RNA into BHK-21 cells and mature SFV particles harvested 24 h later. Prior to infection, the generated virus stocks were filter-sterilized and activated with α -chymotrypsin (Roche Molecular Biochemicals) at room temperature for 15 min. The reaction was terminated by addition of trypsin inhibitor, aprotinin (Sigma). Virus stocks were stored at -20°C for short time (2–3 weeks) and were kept at -80°C for long-term storage (several years).

SFV-mediated GFP expression

Approximate estimations of titers of SFV stocks were obtained by infection of known numbers of BHK cells with serial dilutions of SFV-GFP virus followed by

counting of GFP-positive cells. This procedure resulted in quantitation of the minimal number of infectious particles per ml. We have earlier demonstrated that the number of GFP-positive cells correlate directly with the amount of applied SFV particles in organotypic hippocampal slices (Ehrensgruber et al., 2001). The multiplicity of infection (MOI) values also showed a good correlation to recombinant protein expression levels (Blasey et al., 1997).

Adherent BHK-21 and CHO-K1 cells were infected with different MOIs of wild-type and mutant SFV-GFP vectors. Likewise, infections of CHO cells in spinner flasks (25 ml) were carried out as earlier described (Schlaeger and Lundstrom, 1998). GFP fluorescence was visualized by microscopic examination using a Zeiss Axiovert 100 microscope and photographed using a Leica DM 1RB microscope and the Leica DC200 software. Relative fluorescence was measured from cells on 6 well plates in a Fluorocan Ascent fluorometer (LabSystems).

SFV-GFP infection of BHK-21 cells

Infection of CHO cells in 25 ml spinner culture,
re-infection of BHK-21 cells on 6-well plates
RFU measured after overnight incubation at 37°C

Figure 4. Presence of infectious SFV particles in the medium and associated to cells in spinner flask cultures. GFP-positive cells were measured for relative fluorescence (RFU) on freshly plated adherent BHK cells with samples from medium and cells (untreated, PBS-washed, filter-sterilized) from a 25 ml spinner culture of CHO cells.

Results and discussion

SFV vectors have previously showed high expression levels of GPCRs (Lundstrom et al., 1994, 1999) and ligand-gated ion channels (Lundstrom et al., 1997). Large-scale fermentor (11.5 l) cultures of BHK cells expressing the mouse serotonin 5-HT₃ receptor yielded 1–2 mg receptor protein per liter culture. Efficient purification through the C-terminally engineered hexa-histidine tag allowed structural characterization studies on the receptor (Hovius et al., 1998). Size exclusion affinity chromatography suggested that the size of the whole 5-HT₃ receptor channel was approximately 280 kD, which is in good agreement with the postulated pentameric structure of the channel. The purified subunit showed in SDS-PAGE a size of 65 kD, the expected size of a glycosylated receptor. Furthermore, preliminary cryo-EM studies also indicated that the channel structure is pentameric.

In attempts to further increase the expression levels from SFV vectors, a fusion of the SFV capsid gene to

the human neurokinin-1 (hNK1R) receptor was engineered. It has earlier been demonstrated that the capsid sequence contains a translation enhancer, which can increase the transgene expression 5–10 fold (Sjöberg et al., 1994). Additionally, fusion of the full-length capsid sequence to a recombinant gene led to efficient cleavage of the capsid protein, due to the presence of an autocatalytic cleavage property in the capsid. The fusion construct SFVCAP-hNK1RHisFLAG resulted in 5–10 fold higher expression levels compared to expression of the hNK1R alone from the SFV vector. Yields of 5–10 mg receptor protein per liter CHO cell cultures were obtained. Preliminary immuno-EM studies and cell fractionation suggested that up to 80% of the recombinant hNK1Rs were located in intracellular structures and not transported to the plasma membrane (Lundstrom, unpublished results). However, the receptors showed high specific functional binding activity. Extensive purification and 2D and 3D crystallization attempts are in progress.

PDTE day 3 p.i. MOI 10 @ 31°C

PDTE day 3 p.i. MOI 10 @ 37°C

Figure 5. Expression of GFP from the temperature-sensitive SFV-PDTE vector. BHK cells were infected with SFV-PDTE-GFP at an MOI of 10. Cells were incubated at 31 (A) and 37 °C (B), respectively, and GFP-positive cells were visualized by fluorescence microscopy.

We have here studied the kinetics of the infection process of replication-deficient SFV particles. Adherent BHK cells were infected with SFV-PD-GFP virus at an MOI of 10 for different lengths of time by applying virus directly to the cell culture medium. The virus-containing medium was removed after 2, 5, 15, 30, 60 and 120 min. Cells were carefully washed with PBS, fresh medium added and the GFP expression was visualized after 24 h by fluorescence microscopy (Figure 1). A significant proportion of the BHK cells were GFP-positive already after a 2 min contact with the host cells. Obviously increased incubation time resulted in elevated infection rates and it was evident that within 2 h, 100% infectivity was achieved. This observation indicated that 2 h is sufficient to obtain efficient infection, where after the cells can be washed of excess virus to exclude the presence of any infectious particles in the medium and in connection with the gene product to be harvested. We could also demonstrate that increased MOI values resulted in elevation of RFUs (relative fluorescence units) most likely due to multiple infections of host cells in a similar way as earlier described for SFV-mediated β -galactosidase expression in CHO cells (Blasey et al., 1997).

For characterization of recombinant proteins expressed from SFV vectors by pharmacological assays (radioligand binding, functional coupling to G proteins), electrophysiological recordings, as well as for down-stream processing of large-scale cultures, it is necessary to remove the cell cultures from the safety containment level 2 (SL2) laboratory environment required for work with SFV vectors in US and Europe (in Germany the use of pSFV-helper2 based SFV vectors have recently been relaxed to SL1). We have here studied the presence of infectious SFV particles in the culture medium and associated with SFV-infected cells. The effects of virus removal and one or multiple washes with PBS were analyzed at different time points during an expression study (Table 1). BHK cells were infected with SFV-GFP virus (MOI 10) for 2 h, where after no treatment was performed, virus-containing medium was removed, or additional PBS washes were carried out. Cells were allowed to express GFP over night and samples from medium and cells collected prior to or after further washes with PBS. To investigate the presence of infectious SFV particles, freshly plated BHK cells were infected with samples collected after the above described treatments and analyzed for GFP expression by fluorescence microscopy and relative fluorescence measurement. Obviously, the untreated culture medium still

contained reasonably high levels of infectious SFV particles after over night incubation, because SFV is relatively resistant to degradation (Figure 2). However, removal of the virus-containing medium and replacement with fresh medium significantly reduced the number of infectious particles. Furthermore, already a single wash with PBS eliminated the majority of infectious particles. Additional PBS washes further reduced the number. To determine whether infectious SFV particles were associated with cells, SFV-GFP infected BHK cells were subjected to different treatments (Table 1), detached and used as infectious material on freshly plated BHK cells. A similar pattern as for the cell culture medium was observed. Untreated cells still contained infectious SFV particles, whereas PBS washes, either 2 h post-infection or/and before harvesting, efficiently reduced the infection rate (Figure 3). Filter-sterilization substantially reduced the amount of detected infectious particles. Because SFV particles can efficiently penetrate 0.22 μ m filters, and these are used as a standard sterilization procedure of virus stock preparation (see the Section Materials and methods), the most likely explanation for the reduced infection rate of filter-sterilized material is that the GFP-positive cells from the first round of infection could re-attach and therefore resulted in false-positive signals in the freshly plated cells for the non-filtered samples. Alternatively, virus could be separated from cells by centrifugation, but using that approach the risk of contamination by non-pelleted cell debris is higher.

In a similar study as described for adherent cells, we studied the content of infectious SFV particles in the medium from a spinner flask culture of CHO cells after infection with SFV-GFP. Cells were also assayed for the presence of infectious SFV particles after treatment with 1 and 3 washes with PBS, respectively. The medium contained infectious SFV particles, but surprisingly, the number was very low (Figure 4). Infection of fresh cultures of BHK cells with the cell-associated material from the spinner culture demonstrated a high frequency of GFP-positive cells. However, as described for the adherent cells, these are mainly the cells that had been infected in the first round of infection and could reattach to the plate in the second round. This could be demonstrated by filter-sterilization of the material, which allows the penetration of virus particles, but not cells (Table 2). PBS washes of cells further reduced the infection rate.

It has recently been demonstrated that single point mutations in the nonstructural SFV (Lundstrom et al., 2000) or Sindbis virus (Boorsma et al., 2000) genes

can dramatically change these alphavirus vectors to temperature-sensitive. For instance, point mutations in the nsP2 (M780T) and nsP4 (G324E) genes of SFV resulted in temperature-dependent transgene expression. The combination of these mutations with two other mutations in nsP2 (S259P, R650D) led to a quadruple mutant SFV vector SFV-PDTE that showed a significantly decreased cytotoxicity to host cells as well as a strictly temperature-sensitive phenotype. Not a single GFP-positive cell could be detected at 37 °C even after careful microscopic examination (Figure 5). The expression levels of GFP were approximately 30-fold higher from this mutant compared to the wild-type SFV vector. Because no GFP expression was observed at the non-permissive temperature (37 °C), this mutant vector could further increase the already high safety standards of using the SFV system.

In conclusion, the SFV expression system has been used for rapid high-level expression of recombinant receptors at the 10-liter scale. Our study indicated that high safety standards required for viral vectors could be achieved. Generation of conditionally infectious particles, based on the second-generation helper vector, guarantees safe production of large volumes of SFV vectors under highly controlled conditions. Moreover, we could demonstrate that safe handling of medium and cells from SFV-infected cultures can be obtained. If desired, mutant vectors that only express transgene proteins at reduced growth temperature (31 °C) can be applied.

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