Recombinant protein production using the Semliki Forest Virus expression system

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Abstract

We report here the successful scale up of transient recombinant protein expression to litre scale using Semliki Forest Virus System. The expression of bacterial β -galactosidase was initially compared in BHK and CHO cells and the conditions for optimal infection of BHK cells were identified. 10% FCS in a medium at pH 6.9 and infection in small volumes were found to be optimal. A high MOI results in an increased recombinant protein yield. Stirring does not affect the infection process. Finally we applied these optimal conditions to the production of a microsomal enzyme, human cyclooxygenase-2 in suspension spinners. Five independent productions at the 1 litre scale yielded reproducible substantial amounts of recombinant protein (16 mg microsomal protein 10^9 cells⁻¹) with an average specific activity of 3942 ± 765 pg PGE₂ μ g⁻¹ microsomal protein 5 min⁻¹.

Introduction

The Semliki Forest Virus expression system (Liljeström and Garoff, 1991a) presents an alternative for expressing heterologous genes in mammalian cells in culture. Co-transfection of *in vitro* transcribed recombinant and helper RNA into HBK cells leads to *in vivo* packaging of only recombinant RNA (which specifically carries the packaging signal) and production of infectious, but non replicative, recombinant virus particles at high titer (approximately 10^9 virus particles ml⁻¹). The broad host range makes the SFV expression system particularly attractive for functional studies in different cell lines. Another interesting feature is the high expression level obtained. The use of the extremely efficient SFV 26S promoter has resulted in recombinant bacterial β -galactosidase levels of up to 25% of total cell protein (Liljeström and Garoff, 1991). The fast and simple generation of virus has allowed production of a recombinant protein at small scale much faster than using a stable recombinant cell line approach. Finally, recombinant proteins can be produced with authentic post-translational modifica-

tions, based on the specific processing capacities of the host cell. Several types of recombinant proteins have already been produced at a small scale with the SFV system. HIV envelope proteins gp120 and gp160 were produced in BHK, HeLa and MOLT4 cell lines (Paul *et al.*, 1993). The plasminogen activator inhibitor type 2 (Mikus *et al.*, 1993) has been expressed and characterised. Complex seven transmembrane proteins such as Neurokinin-1, Neurokinin-2 and Dopamine D3 receptors (Lundström *et al.*, 1994; 1995) have been expressed in BHK, CHO, COS and HOS cells. Olkkonen *et al.* (1993) performed functional studies on heterologous proteins expressed in rat primary neurons and Lundström (unpublished results) examined the cellular localisation of catechol-o-methyltransferase. With recent advances in the sequencing of the human genome and the accumulation of DNA sequences without a known function, it is clear that the SFV technology will also give simple and rapid access to the gene product and contribute to the understanding of its biological function.

The ability of the virus to infect almost any eukaryotic cell raises a biological safety issue, in terms of

potential environmental release and of operator safety. These requirements have been met by the production of only conditionally infectious recombinant virus particles due to three mutations in the pSFV-Helper2 plasmid (Berglund *et al.*, 1993).

In this study, our objective was to optimize the infection procedure to be able to quickly produce research grade material at one litre scale. We have examined the influence of various process parameters such as the nature of the cell line, cell culture technology, environmental conditions and infection parameters on the expression efficiency. Finally, we also report on scale up experiments in a stirred system for the production of recombinant human cyclooxygenase-2 (hCOX-2). Cyclooxygenase or prostaglandin G/H synthase (PGHS) catalyses the rate limiting step in the conversion of arachidonic acid to prostaglandins and thromboxane. The date, two isoforms of cyclooxygenase have been cloned, COX-1 and COX-2. Whereas COX-1 is expressed constitutively in most tissues and may account for the housekeeping role of cyclooxygenase, COX-2 is rapidly induced in response to a wide range of biological and inflammatory mediators (Xie *et al.*, 1992, Lee *et al.*, Hla *et al.*, Pritchard *et al.*, 1994). These two separate functions for the COX isoforms have allowed the hypothesis that compounds which selectively inhibit COX-2 will provide the next generation of nonsteroidal anti inflammatory drugs (NSAIDS) (Xie *et al.*, 1992). Such compounds should have a reduced side effect profile by allowing COX-1 to perform its beneficial housekeeping role. Thus, we have developed a process for the production of sufficient quantities of COX-2 to support high throughput compound screens.

Materials and methods

Medium was a mixture of Ham's F12 and Iscove's 1:1 (Seromed, FRG), 4 mM glutamine and 10% FCS (Seromed, FRG).

Cell maintenance. BHK cells were split 3 times a week at a ratio of 1/20–1/40. After detachment with trypsin (0.5 g l^{-1}) / (EDTA (0.2 g l^{-1}), cell clumps were disrupted by repeated pipetting. CHO cells were split 3 times a week at a ratio of 1/4 to 1/10. Cultures were expanded in 225 cm^2 T-flasks.

Culture system for production: Cox-2 production was carried out in 21-Bellico spinners, which were run at 60 rpm.

Glucose and lactate were assayed with an enzymatic analyser (YSI 2000, Yellow Springs Instruments, USA).

Plasmids. The plasmids pSFV3-LacZ, pSFVHelper-1 and pSFVHelper-2 have been described elsewhere (Liljeström and Garoff, 1991; Berglund *et al.*, 1993). Plasmid pSFV1-hCOX-2 was constructed by PCR amplification of the coding region for the human cyclooxygenase-2 gene (received as pCDNA1-hCOX-2 from Dr. Tim Hla, American Red Cross, Holland Laboratory, Rockville, USA) as follows. A 5' end primer with a *BAM* site and an optimal Kozak-sequence (GCACC) and homologous sequences starting with hCOX-2 initiation ATG and a 3' end primer with a *BAM*H1 site were designed. The resulting PCR fragment, amplified with PFU polymerase (Stratagene) for 35 cycles (94°C 1 min, 60°C 2 min and 72°C 3 min), was cloned into the *Bam*H1 site of the pSFV1 vector and the accuracy of the construct verified by DNA sequencing.

Generation of recombinant SFV. *In vitro* RNA transcripts were made from pSFV3-LacZ, pSFV1-hCox-2 and pSFV Helper plasmids (Lundström *et al.*, 1994) and the RNA products were co-electroporated into BHK cells according to Liljeström and Garoff (1991b). The *in vivo* packaging of virus particles was completed 24 hours after electroporation, virus stocks collected and stored at -80°C after eliminating cell debris by centrifugation.

Virus titration. A method for the exact titration of the nonreplicating SFV has to be developed. Therefore the titer of the LacZ virus stock was estimated as follows. Defined quantities of BHK cells were infected with serial dilutions of virus. About 20 hours post infection, the cells were stained with X-gal and the percentage of blue β -Gal expressing cells determined. Taking into account the dilution factor, the volume of the virus suspension added, the number of cells per plate and the fraction of blue cells: the virus concentration could be estimated. For recombinant SFV viruses like the SFV-hCOX-2, no direct titer estimations could be carried out. However, since both SFV-LacZ and SFV-hCox-2 were generated in parallel and showed similar levels of expression of the viral structural proteins in pulse-labeled electroporated cells, we assumed the titers to be in the same range.

Virus activation. Virus stocks generated with pSFV-Helper2 (Berglund *et al.*, 1993) are totally non-infectious unless the p62 precursor is cleaved into E2 and E3 membrane proteins. Full infectivity is achieved by treatment with $200 \mu\text{g ml}^{-1}$ (final concentration)

Figure 1. BHK cells were infected for 1 hour in T25 flasks with different amounts of the recombinant SFV-LacZ virus (MOI 40, 120, 200, 400). β -Gal assay by FACS was performed 20 hours after infection.

of α -chymotrypsin (Sigma, USA) for 15 min at room temperature followed by inactivation of the protease activity with Aprotinin (Sigma, USA) at $400 \mu\text{g ml}^{-1}$ final concentration.

Infection experiments. Cells were inoculated into T25-flasks with 5 ml medium. Virus solution was added to yield the MOI indicated in the graphs. For some experiments, the virus containing solution was completely removed after a defined virus-cell contact time, as indicated in the text. In this case, cultures were washed once with culture medium and 5 ml of fresh medium were added immediately after.

Following this protocol, we could separate the phase of protein expression after infection (20 hours cultivation time p.i.) from the infection itself (virus/cell attachment and cell penetration), thus allowing to study the influence of different parameters on the infection process.

For optimising pH of infection, we adjusted the medium pH in the T25 flasks with HEPES and acetic acid respectively. After several hours in the CO_2 incu-

bator, when the culture pH was stabilised, the pH was again measured and cells were infected.

Expression labeling of recombinant proteins. Metabolic labeling was performed 5 hours post electroporation and 20 hours post infection respectively. Protein expression was verified by running lysates of pulse labeled cells on a 10% SDS gel (Novex) followed by autoradiography.

β -Gal assay by FACS. Relative β -Gal concentration in cells was analysed by FACS (Becton Dickinson, Belgium) using the 'FluoReporter lacZ' (Molecular probes, Inc., USA) according to the manufacturer's instructions. The assay is based on the enzymatic release of fluoresceine by β -Gal from fluoresceine-di- β -D-galactoside. The mean fluorescence (MFI) as FACS readout reflects the amount of active β -Gal in the cells.

COX-2 microsome preparation. $4 \cdot 10^8$ cells from a suspension spinner culture were pelleted by centrifugation at $300 \times g$, washed once in ice-cold PBS and resuspended in 10 ml of buffer (0.1 M TRIS-HCl pH 7.4

Figure 2. In T25 flasks, CHO cells were infected for 5 hours with different amounts of the recombinant SFV-LacZ virus (MOI 4, 40, 400). β -Gal assay by FACS was performed 20 hours after infection.

containing protein inhibitors: 200 μ M PMSF, 100 μ M APMSF, 2 mM Benzamidine, 100 μ M Pepstatin, 10 μ M E64, 10 μ M TLCK, 100 μ M Antipain, 100 μ M Leupeptin; all from Sigma, USA). Cells were broken by sonication on ice and debris and nuclei were eliminated by a short centrifugation (10 min at $10,000 \times G$). The microsomes were collected by a 45 min. spin at $200,000 \times g$. The microsome pellet was resuspended in 1 ml of TRIS buffer, aliquoted in 100 μ l fractions and frozen at -80°C .

COX-2 analysis, PGE2 EIA assay. COX-2 was analysed with a prostaglandin E2 enzyme immuno-assay using a 96 well micro-titer plate. The reaction volume was 50 μ l: 5 μ l (20 μ g) microsomal protein, 5 μ l (10 μ M) Hematin, 5 μ l (10 mM) phenol, 25 μ l buffer (200 mM TRIS, 2 mM EDTA, pH8) and 5 μ l water. The reaction was initiated with the addition of 5 μ l 300 μ M arachidonic acid. After 5 minutes at room temperature, the reaction was terminated by adding 25 μ l of 1M acetic acid and the mixture neutralised with 25 μ l 1N NaOH. The rest of the assay was performed according to the manufacturer's instructions (Amersham UK Ltd).

Results and discussion

In these studies, we used well characterised and robust cell lines, such as CHO and BHK cells. They are used to express a large variety of different recombinant proteins and their large scale culture is well established.

In order to define conditions for the scale up of the SFV expression system, we decided to investigate a series of parameters, which we hypothesised would have an influence on infection and expression of recombinant protein.

Influence of MOI on expressed β -Gal level. BHK cells were infected with the SFV-LacZ virus at a multiplicity of infection (number of infectious virus particles per cell, MOI) of 40, 120, 200 or 400 β -Gal expression was determined 20 hours post infection (Figure 1). A similar experiment was run with CHO cells but in this case, cells were infected for 5 hours at MOIs of 4, 40 or 400 (Figure 2). For both cell lines, we reproducibly found that the MOI had a strong influence on the maximum protein concentration.

Figure 3. Comparison of BHK and CHO cells, which were infected in parallel with the SFV-LacZ virus at MOI 40. Analysis was done 20 hours post infection.

The reason for the modulation of recombinant protein expression by the MOI has not been precisely identified. Interestingly, X-Gal staining of cells infected with the SFV-LacZ virus revealed 100% infectivity at MOI 4. This suggests therefore that due to the occurrence of multiple infections, when higher MOIs were used, more copies of the RNA molecules enter the cells and consequently lead to a higher recombinant protein expression.

Comparison of beta-Gal expression in CHO and BHK cells. In order to compare the susceptibility of the two cell lines to SFV infection, CHO and BHK cells were infected in parallel (Figure 3). In T-flasks CHO cells produced a much lower amount of recombinant protein compared to BHK cells. A further drop in β -Gal concentration was observed when CHO cells were infected in suspension cultures (2 independent experiments). BHK cells appeared to express β -Gal equally well after infection in T-flasks or in spinners (data not shown). Therefore, the optimisation experiments were continued with BHK cells.

Influence of pH on infection. Given the objective of scaling up the SFV production process in suspension systems we investigated whether the pH had to be controlled to allow optimal infection and, if so, in what range.

BHK cells were infected for one hour at pHs between 6.5–7.8, medium was exchanged thereafter with fresh medium and cells were analysed 20 hours post-infection. In two independent experiments we identified an optimum at pH 6.9 (Figure 4) for infection. This indicates that an efficient infection required a tight and accurate pH control. Thus in further experiments in spinner systems the pH was adjusted with a 1 M HEPES solution to pH 6.9 prior to infection.

Influence of virus concentration on infection. In T-flasks, identical quantities of BHK cells were infected with the same MOI (virus particle per cell) but with a variable volume of medium resulting in different virus concentrations (virus particle per unit volume). After a one hour contact time, the virus-containing medium was replaced by fresh medium. 20 hours post infection, we found the highest β -Gal concentration in the cells which had been infected in the smallest volume (Figure

Figure 4. BHK cells in T25 flasks (duplicates) were infected for 1 hour with SFV-LacZ at different pH. After infection, virus was taken off and exchanged for culture medium. In two independent experiments the pH 6.9–7.0 was identified as optimal for infection.

5). Thus, the effect of *virus concentration* on infection is another factor which strongly influenced the expression level. A high level of expression was achieved if the first step of infection, the virus/cell contact and attachment, was performed at high densities of both cells and viruses, i.e. in small volumes. However, in order to support cell metabolism for the required time of protein expression fresh medium must be added to provide more nutrients. To match these opposing needs we established the following production protocol for spinner cultures. BHK cells were infected in fresh medium at 10%–20% of the final culture volume. The medium in the spinner was adjusted to pH 6.9 and virus was added to the culture. After 1.5 hours the culture was expanded to its final volume by addition of fresh medium.

Culture scale up. To express recombinant protein with the SFV at the litre scale, the scale-up of BHK culture is another important issue. We had grown cells in flasks and transferred them into spinner systems for infection.

Performing both the infection and protein expression phases in a spinner, provided the advantage that the infection can be completed in one step and cells could be rapidly harvested by centrifugation, thereby avoiding any detachment procedures, which might have a negative effect on the product quality.

In another approach, we tested the CellCube system and obtained very satisfactory cell growth (Blasey *et al.*, 1995) yielding on average $3.3 \cdot 10^9$ cells for the smallest CellCube unit of 2 m². This method was subsequently used for culture volumes of several litres (data not shown). For infection, cells were detached, pelleted and, after resuspension, transferred into a spinner, as described above.

Summary

We have identified a set of optimal conditions for large scale recombinant protein production with the SFV system:

Figure 5. BHK cells in T25 flasks (duplicates) were infected with SFV-LacZ at the same MOI but different medium volumes and therefore different virus concentrations. β -Gal assay by FACS was performed 20 hours post infection.

- BHK as host cell line allows good expression after infection.
- The expression level is positively correlated to MOI (indicated infection at high MOI).
- pH is controlled at 6.9.
- Infection is optimal at high virus concentration (low volume), as shown in flask experiments.

In other experiments (data not shown here) we further observed that FCS has an influence on infection (for BHK 10% FCS appears to be optimal, two independent experiments), that shear – up to 120 RPM in 100 ml Bellco spinners – does not influence the infection process and that SFV infection results in an increase in metabolic activity (two independent experiments).

Application of optimized conditions to hCOX-2 production

Having optimized the conditions for SFV infection we applied this protocol to generate material for high throughput compound screens. As an example, we present results on the production of the human

Figure 6. SDS gel of BHK cell lysate after pulse labelling. Cells were infected with the SFV-hCOX-2 virus. A strong band at 72 kD represents the highly expressed hCOX-2.

cyclooxygenase-2 (hCOX-2). These data were collected from experiments performed at the 1 litre scale with cells generated in T-flasks.

In an effort to express the human COX-2 with the SFV, the gene was subcloned into pSFV1, as described in Materials and Methods, and subsequently, a virus stock was generated. For hCOX-2 production, we applied the optimized conditions listed above. Each single batch was run with BHK cells generated in 20 T225 flasks ($= 10^9$ cells). Cells were detached, pelleted and resuspended in 200 ml medium with 10% FCS, pH 6.9. Infection was started by addition of 40 ml virus stock solution per 10^9 cells (MOI 40, estimated as described in Materials and Methods). Two hours after infection, medium was added to a final 1000 ml culture volume. The culture was harvested 17 hours post infection by centrifugation and microsomes were prepared. To verify expression, a sample of infected cells was removed from the spinner culture and analysed by pulse labeling. The major product was a polypeptide corresponding to a molecular weight of 72 kD, which is the expected size for fully glycosylated hCOX-2 (Figure 6).

The analysis of microsomal preparations from 5 different spinner productions demonstrated high level expression of hCOX-2. The mean activity was 3942 ± 765 pg PGE_2 μg^{-1} microsomal protein 5 min^{-1} . Each spinner yielded approximately 16 mg microsomal protein.

These data demonstrate that production of recombinant protein using the SFV according to our protocol reproducibly yields high levels of expression.

During this work, we became aware of several important question which essentially need to be addressed in the near future. Developing a method for titration of the nonreplicative virus is one of the most urgent needs. Presently we are also screening other cell lines for optimal expression of different protein models (cytosolic, secreted and membrane bound protein). We also observed some variation in expression between different infections, which will have to be addressed.

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