Large scale transient 5-HT₃ receptor production with the Semliki Forest Virus Expression System

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Abstract

The expression of recombinant proteins with the Semliki Forest Virus (SFV) system has been scaled up to bioreactor scale. As a model protein for this study the human 5-HT₃ receptor was chosen. The gene for the receptor was subcloned into the SFV expression plasmid pSFV1. Virus production by *in vivo* packaging and production of the recombinant protein was scaled up, the latter to a reactor volume of 11.5 l. A VibromixTM agitation system was chosen to overcome aggregation problems of BHK cells in suspension. In the process, cells were first grown to a density of 10⁶ cells/ml, the medium was then exchanged with fresh medium and the culture was infected with the recombinant virus at an estimated multiplicity of infection of 30. 24 h post infection we measured an expression level of 3 million functional 5-HT₃ receptors per cell. For harvesting, the cells were pelleted by centrifugation. The receptor protein was purified in a single step (Hovius et al., 1998) by exploiting the hexa-His tag at minimal protein loss (51% yield). Experiments to optimise expression resulted in yields up to 8 million receptors per cell, when the pH of a suspension culture was controlled at pH 7.3. Rapid virus generation and protein production, high protein yields as well as successful large scale application have made the SFV expression system attractive to produce large quantities of recombinant protein in a very short time. After optimisation of the expression conditions (in particular by setting the pH at 7.3), yields were increased twofold.

Introduction

Significant progress has recently been made in the development and scale up of transient mammalian gene expression systems in exploiting both, viral as well as DNA vector mediated gene expression strategies (Bernard and Blasey, 1999; Wurm and Bernard, 1999).

For DNA vector mediated expression, COS cells were scaled up to litre volume (Blasey et al., 1996). However, this approach suffers limitations such as large DNA requirement and the demand for a large scale transfection procedure. The latter may be overcome now by using continuous electroporation systems (Parham et al., 1999), by applying polyethyleneimine (Schlaeger et al., 1999) or calcium phos-

phate transfection protocols at reactor scale (Jordan et al., 1998). An example for the large scale use of this method is the production of the fusion protein CD40Fc, which was reported to yield 10 l of serum free supernatant and a total of 30 mg purified protein (Blasey et al., 1996). As an alternative, the HEK293EBNA system, which allows episomal replication of the EBNA carrying plasmid, can be operated with transfections at small scale before scale up. Expanding the transfected cell population under selective pressure is an easy route to reach larger volumes of culture. With this system we have successfully produced a soluble interleukine receptor at several milligrams per litre (Blasey et al., unpublished data).

Amongst the viral systems, Adenovirus, Vaccinia virus and the Semliki Forest virus are the ones most reported on for gene expression on large scale. Adenovirus was scaled up in a reactor to the 3 l working volume, yielding 90 mg/l protein tyrosine phosphatase (Garnier et al., 1994). The largest scale described for a mammalian viral system however is known for Vaccinia (Barrett, 1989). In the biopharmaceutical industry it is being used for the production of an HIV vaccine in a 40 l perfusion bioreactor.

We report here on the scale-up of one of the most recent viral expression systems which is based on one of the alphaviruses, the Semliki Forest Virus (SFV) (Liljeström and Garoff, 1991a). The SFV offers distinct advantages over other mammalian systems with respect to expression levels and speed. A limitation comes from the viral production protocol. Because the SFV expression system is not based on a replication competent virus it requires co-transfection of *in vitro* transcribed recombinant and helper RNA into BHK cells to produce infectious, replication deficient recombinant virus particles at high titer (approximately 10^9 virus particles/ml). Very recently a stable alphavirus packaging cell line has been described yielding a viral titre of 10^7 particles per ml (Polo et al., 1999). The SFV can then virtually infect most mammalian, insect and amphibian cells. Thus by choosing the host, we can select cell specific post-translational modification patterns. In theory, only recombinant RNA molecules should be packed into SFV particles. However, generation of replication-proficient virus (RPV) has been observed at low frequency (Berglund et al., 1993). Biosafety limitations due to these RPVs have been overcome by mutating the viral spike proteins in the pSFV-Helper2 plasmid (Berglund et al., 1993). This results in the generation of only conditionally infectious recombinant virus particles. Recently, in another approach, the capsid and the mutated viral spike proteins were expressed from two separate helper plasmids (Smerdou and Liljeström, 1999).

Alternatively, SFV based expression vectors have been designed to be used as DNA vectors (Berglund et al., 1998). After transfection, a strong viral promoter such as CMV will drive the transcription of the SFV replicase gene. The replicase is responsible for the efficient RNA replication that generates hundred thousand copies of mRNA molecules leading to high level recombinant protein expression. Although these DNA vectors have been useful for vaccination, their application for recombinant protein expression in particular on large scale is limited by the same factors as

mentioned above for the DNA vector mediated expression by COS cells. The same is true for transfection of cells with only the *in vitro* generated recombinant RNA, generated from the pSFV vectors.

Since the SFV expression system was established, a large number of various recombinant proteins have been expressed at small scale (Table 1) by infecting various different cell lines (Lundström 1999). We have earlier applied the SFV technology to the expression of human cyclooxygenase 2 (hCOX2) in a suspension spinner system, up to a volume of 1 l (Blasey et al., 1997). At this scale we could rapidly satisfy the demand for large amounts of active cytosolic recombinant protein preparations requested to run high-throughput compound screens. Starting from the plasmid, the protein preparation was available in one week, thus demonstrating the potential for rapid gene expression.

For the work presented here, our objective was to demonstrate the feasibility of the production of mouse serotonin 5-HT₃ receptor. This 5-HT₃ receptor is a ligand-gated cation channel and belongs to the group of the acetylcholine receptors with a postulated pentameric structure. The receptor itself has been of great therapeutic interest because it is a target in chemotherapy induced emesis. Production was done in a suspension culture of BHK cells, using a mechanically mixed bioreactor at a volume of 11.5 l. The protein was purified and is now used as starting material for structural studies and for the development of biosensors.

Materials and methods

Cell culture medium for all experiments described was a mixture of Ham's F12 and Iscove's 1:1 (Seromed, FRG), 4 mM glutamine and 10% FCS.

Cell maintenance. Baby hamster kidney cells (BHK) were split 3 times a week at a ratio of 1/20–1/40. After detachment with trypsin (0.5 g/l)/EDTA (0.2 g/l), cell clumps were disrupted by repeated pipetting.

Cell lines used in receptor expression study. Adherent cell lines: CHO (Chinese hamster ovary cells), NIE115 (mouse neuroblastoma), COS M6 (transformed green monkey kidney cells); HeLa (human adenocarcinoma). Suspension cell lines: Raji (human lymphoma), NSO (mouse myeloma), TK6 (human lymphoblast), RPMI 8226 (human plasmacytoma), Hyb179 (mouse hybridoma).

Table 1. Proteins expressed from SFV vectors

Enzymes	
β -gal (up to 25% of cell protein)	P Liljeström (1991)
Mouse dehydrofolate reductase	P Liljeström (1991)
Chicken lysozyme	P Liljeström (1991)
Terminal deoxynucleotide transferase	V Ciccarone (1993)
NEDD-2	B Allet (1996)
Human cyclooxygenase-2	HD Blasey (1997)
Rat and human catechol O-methyltransferase	I Ulmanen (1997)
Thyroid peroxidase	H Bikker (1997)
Receptors	
Human transferrin receptor	P Liljeström (1991)
Polymeric immunoglobulin receptor	E Ikonen (1993)
Mouse 5-HT ₃ receptor	P Werner (1994)
Human neurokinin receptor-1	K Lundström (1994)
Dopamine receptor 3	K Lundström (1995)
Human neurokinin receptor-2	K Lundström (1995)
Adenosin A3 receptor	M Patel (1997)
Human prostaglandin receptor EP-4	FH Marschall (1997)
Human 5-HT ₁ alpha receptor	J Stables (1997)
Human P _{2x1} , rat P _{2x2} , rat P _{2x3} , rat P _{2x4}	K Lundström (1997)
GABAA alpha1 and beta2 subunits	GH Gorri (1997)
Metabotropic glutamate receptor 8	Malherbe P (1999)
Opioid	K Lundström (1999)
Galanin	K Lundström (1999)
Histamin	K Lundström (1999)
Odorant receptors: rat I7 and OR5 and C. elegans odr-10	K. Monastyrskaya (1999)
G α : Gq, Gi, Go, Gs, G16	K Lundström (1999)
α 1b-adrenergic receptor, G α q, G γ 2, G β 2	A. Scheer (1999)
Viral proteins	
HIV gp120, gp160	NL Paul (1993)
Hamster measles virus protein HNT-PI F and Ph26H	KB Hummel (1994)
Moloney murine leukemia virus gag and env	M Suomalainen (1994)
Influenza virus nucleoprotein	X Zhou (1995)
Human papilloma type 16 capsid protein	P Heino (1995)
Vesicular stomatitis virus glycoprotein G	MM Rolls (1996)
GB virus-C E2 envelope protein	TJ Pilot-Matias (1996)
GP4 structural glycoprotein of Lelystad virus	JJ Meulenbergh (1997)
HN and F of human parainfluenza virus type-1	T Bousse (1997)
Respiratory-syncytial virus glycoprotein-G	J Meanger (1997)
Murine leukemia virus envelopes	I Lebedeva (1997)
Capsid protein of hepatitis E virus (HEV)	J Torresis (1997)
Infectious-bronchitis virus (IBV) gray strain nucleocapsid protein	SH Seo (1997)
Rotavirus genes: vp2 and vp6 of group A rotavirus (SA-11), vp6 of porcine group C rotavirus (Cowden/AmC-1)	M Nilson (1998)
Human Cytomegalovirus	A McCue Lee (1998)
Haemagglutinin protein of measles virus	F Bouche (1998)
Gonadotropin-releasing hormone receptor	K Marheineke (1998)
Herpes simplex virus type 1 DNase.	E Kehm (1998)

Table 1. (continued)

Other proteins	
Rab 12, 22, 24	VM Olkkonen (1993)
Plasminogen activator inhibitor type 2	P Mikus (1993)
Rab 8, VIP-21caveolin	VM Olkkonen (1993)
Yeast syntaxin Sso2p	J Jantti (1994)
IL-4	HD Blasey (pers. communication)
Rabbit pIgR	M de Hoop (1995)
Human APP	B De Strooper (1995)
Presenilin	DG Cook (1996)
Mitochondrial precursor protein	A Huckrieder (1996)
Amyloid beta peptide, ApoE isoforms	S Aleshkow (1997)
Human IL-12p40 and p35	J Zhang (1997)
Nucleotide exchange factor Vav	R Arudchandran (1999)

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

Plasmids. The plasmids pSFV3-LacZ, pSFVHelper-2 have been described elsewhere (Liljeström and Garoff, 1991; Berglund et al., 1993). The pSFV-5-HT₃ plasmid has been described earlier (Werner et al., 1994). To facilitate purification, an in-frame hexahistidine tag was introduced at the C-terminus of the 5-HT₃ receptor by PCR technique. The amplified 5-HT₃ receptor gene was subcloned into the BamHI site of the pSFV1 vector.

Generation of recombinant SFV. In vitro RNA transcripts were prepared from pSFV-5-HT₃ and pSFV-Helper2 plasmids (Lundström et al., 1994) and co-electroporated into BHK cells according to Liljeström and Garoff (1991b). The *in vivo* packaging of virus particles was completed 24 h after electroporation, virus stocks were collected and stored at -80 °C after removing cell debris by centrifugation (10 min, 240 g).

Virus titration. For recombinant SFV viruses like the SFV-5-HT₃, no direct titer estimations can be carried out because the non-replicating virus does not allow plaque formation. However, since both SFV-LacZ and SFV-5-HT₃ were generated in parallel and showed similar levels of expression of the viral structural proteins in pulse-labelled electroporated cells, we assumed the titers to be in the same range. The SFV-LacZ was titrated by infecting BHK cells with different numbers of virus after serial dilution. Staining of the LacZ expressing cells was performed one day after infection with X-gal. The number of blue (infected) cells were determined and based on the dilution the titers estimated.

Figure 1. VIBROMIX™: the mechanical stirring device, which allows to grow adherent BHK cells as a single cell suspension culture.

Virus activation. Virus stocks generated with pSFV-Helper2 (Berglund et al., 1993) are non-infectious unless the p62 precursor is cleaved into E2 and E3 membrane proteins. Full infectivity is achieved by treatment with 200 µg/ml (final concentration) of α-chymotrypsin (Sigma, USA) for 15 min at room temperature followed by inactivation of the protease activity with aprotinin (Sigma, USA) at 400 µg/ml final concentration.

Large scale production was carried out in a mechanical mixed reactor from MBR (Switzerland) with a working volume of 11.5 l. Oxygen was supplied via silicone tubing (length 28 m, Ø 1.2 mm, thickness 0.2 mm). Our objective for the scale up of BHK cell culture was to obtain a single cell suspension culture i.e. with no aggregation of cells (cell aggregates prevent optimal virus infection therefore potentially

Figure 2. Adherent and non-adherent cell lines were grown and after a medium change infected with the recombinant SFV to express the 5-HT₃ receptor (a single experiment). The cells were collected 24 h after infection and a binding assay performed. As shown, BHK showed best 5-HT₃ receptor expression.

reduce recombinant protein yield). Therefore, mixing was carried out with a VIBROMIX™ device (Gerber+Pfenninger, Switzerland): 2 stainless steel plates of 5.5 cm diameter, fixed on a vibrating shaft (vibration: 50 Hz, amplitude 1.5 mm). The plates (Figure 1) carry 20 conical holes each. The medium change was carried out by external crossflow filtration with a hollow fiber microfilter (CellFlow, MICROGON, USA, 0.39 m², 0.22 µm pore size). *Reactor harvest*: the culture was transferred into 250 ml centrifuge tubes (Corning, USA) and spun for 5 min at 420 g in a RC3B centrifuge (Sorvall, USA). The pellets were washed in PBS and frozen at -80 °C.

Optimisation of expression pH: a glass spinner from Bellco (USA) was equipped with a pH probe (Ingold, Switzerland) which was connected to a pH controller (Bioengineering) controlling two peristaltic pumps. The pH was adjusted by addition of 5% (w/v) NaOH and 10% (v/w) acetic acid. BHK cells from T225 flasks cultures were transferred with 270 ml medium into the spinner (at 3·10⁵ cells/ml) and infected with the recombinant SFV at an estimated MOI of 30.

Purification of the 5-HT₃ receptor: A suspension of cell membranes was incubated in the detergent C₁₂E₉ (nonylphenylethylene glycol monododecyl ether; in 10 mM HEPES, 1 mM EDTA, pH 7.4) for 1 h. Non-

solubilised material was removed by centrifugation at 100000 g. The receptor was purified in one step by chromatography on NTA-agarose (Qiagen, Hilden, Germany) immobilised metal-ions as described in detail elsewhere (Hovius et al., 1998).

Protein analysis: the protein was analysed by SDS-PAGE. Samples were run on a 10% gel (Laemmli, 1970) in parallel with a molecular weight marker from BioRad (Switzerland). The protein was visualized by silverstain. Deglycosylation was performed with peptide N-glycosidase F from Boehringer Mannheim (Germany), as described by the manufacturer.

5-HT₃ receptor assay: The K_d and total binding were determined by binding of [3H]-GR65630 in absence or presence of the 5-HT₃ receptor antagonist quipazine. Assays were done in duplicates.

Results

In a first expression attempt we were interested in identifying the cell line best suited for receptor production. Although it is known that SFV infects most mammalian cells (Liljeström and Garoff, 1991a), we could show that there were significant differences in the level protein expression in different cell lines. The

Figure 3. Large scale recombinant gene expression with SFV: (1) subcloning gene of interest into expression plasmid, (2) RNA generation from recombinant and helper plasmid, (3) co-transfection of RNAs into BHK cells to yield in-vivo packaging of recombinant virus, (4) scale up of BHK cell culture, (5) medium renewal, (6) virus activation, (7) Infection of the BHK culture in the bioreactor, (8) one day production, (9) harvest, (10) protein purification.

5-HT₃ receptor production was highest in BHK cells compared to 9 other cell lines (Figure 2). We therefore chose BHK cells as host cell line for the production. BHK cells have been widely used to express a large variety of different recombinant proteins. At this stage we had not included the BHK21C13-2P (ATCC) a BHK which was adapted to suspension. In first experiments however it showed comparable expression to our adherent BHK cell line when expressing the soluble placental alkaline phosphatase SPAP (data not shown).

The scale up of the complete production process is shown in Figure 3. and described in the following paragraphs.

The use of the VIBROMIX™ agitation device allowed to grow the adherent BHK cells as a single cell suspension culture. The oscillation of the plates induces mixing of the culture and shear forces which provide conditions for non aggregated, single cell growth of BHK. We had not been able to achieve this with other approaches like media modifications: basal media with low calcium and magnesium levels,

addition of heparin or Darwan® – a detergent (Keay, 1975).

The BHK cell culture was grown in the 11.5 l reactor to a density of 10^6 cells/ml. Since the medium nutrients have been widely used up at this stage and because the cells after infection have a significantly increased metabolic rate (Blasey et al., 1997) it is obvious that providing fresh nutrients for the production phase is essential. We therefore exchanged about 90% of the medium with fresh medium. The culture was then infected by addition of 330 ml activated virus, containing an estimated 10^9 virus per ml to yield a MOI of 30. The pH at infection was pH 7.3, but not controlled. The cells were harvested 20 h post-infection by centrifugation.

Two reactor runs for the production of 5-HT₃ receptor with SFV infected BHK cells yielded very high expression: $3.2 \cdot 10^6$ and $3.0 \cdot 10^6$ receptors per cell. This is significantly higher compared to what we had generally obtained from stable, amplified recombinant cell lines which typically would express a few hundred thousand receptors per cell.

Optimisation of expression pH: In spinner cultures we compared 5-HT₃ receptor expression at pH 6.9 (which is optimal for infection as reported earlier (Blasey et al., 1997)) and 7.3 (identified to be optimal for SPAP expression by BHK after SFV-SPAP infection, not shown). The pH was adjusted before infection and kept constant throughout the experiment. Results (average from two independent experiments each) showed a difference by a factor of two: the yield was $4.0 \cdot 10^6$ 5-HT₃ receptors per cell for pH 6.9 and $8.3 \cdot 10^6$ 5-HT₃ receptors per cell for pH 7.3.

Purification and analysis of the 5-HT₃ receptor: the receptor protein yield was determined to be 15 mg per 11.5 l batch. The protein preparation showed high purity by SDS-PAGE as one band at 65 kD was observed corresponding to the glycosylated receptor (Figure 4). After deglycosylation with peptide N-glycosidase F the molecular weight of the receptor band was decreased to 49 kD. The specific ligand binding activity was found to be 5050 ± 50 pmol [³H]-GR65630 binding sites per mg protein.

The molecular size of the receptor complex was determined by gel filtration chromatography to be approximately 280 kD, which is in good agreement with the calculated molecular mass of 270 kD for a pentameric structure. The secondary structure of the purified receptor protein was probed by circular dichroism and appeared to be dominated by α helical elements (48%) and poor in non-regular structure (9%) (Hovius et al., 1998).

Pharmacological studies displacing [³H]-GR65630 in the presence of serotonin, quipazine, ondansetron and granisetron demonstrated that the receptor protein generated by SFV had an identical pharmacological profile to the wildtype 5-HT₃ (Hovius et al., 1998).

Discussion

In conclusion, the 5-HT₃ receptor protein production with the SFV system allows very high level expression of proteins, up to 8 million receptors per cell. Subcloning of a gene into the SFV expression system, generating virus and running a production at litre scale (spinner, reactor) only takes 2–4 weeks. If in addition we consider the wide range of cells which can be used for expression, providing the cell specific post translational modifications requested, it is obvious that the benefits of the SFV expression system are setting new standards. SFV in its present state can consequently be expected to speed up protein studies and in particular

Figure 4. SDS PAGE of purified 5-HT₃ receptor. Left Lane: molecular weight marker. Right lane: purified 5-HT₃ receptor.

drive drug target identification and compound screens as well as target validation in the exploratory units of pharmaceutical companies.

A bottleneck to be addressed for the SFV system is the large scale generation of virus, which is presently done by *in vivo* packaging after transfection of recombinant and helper RNA. However, packaging cell lines for easy virus production were developed for alphavirus (Polo et al., 1999), which will be most useful for large scale virus production.

Recent literature reports additional strategies for further improvement. A modification of the expression plasmid carrying the first 102 bases of the viral capsid gene function as translational enhancer allow to increase heterologous protein synthesis at the level of wildtype polypeptide (Sjoberg et al., 1994). Also, the optimisation of production parameters may yield further increase of expression levels, as was shown recently for the influence of the temperature (Schlaeger and Lundström, 1998).

We here have presented our results on the first reactor runs with the SFV expression system. BHK cells were grown in suspension using a specially designed reactor system at the scale of 11.5 l. Medium exchange before infection allowed the culture to express more than 3 million 5-HT₃ receptors per cell before harvesting at 24 h post infection. Although expression

was very high, studies on pH optimisation revealed the potential for further increase of the receptor yield to 8 million receptors per cell when the pH was controlled at 7.3.

In summary we conclude that the SFV expression system results in very high level expression of recombinant proteins in flask as well as in reactor cultures while offering the advantage of very rapid production of large amounts of recombinant proteins.

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