Rapid expression of recombinant proteins in modified CHO cells using the baculovirus system

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Abstract

Baculovirus containing the mammalian CMV promoter, in place of the insect polyhedron promoter (BacMam), has been used to transiently transfect COS, CHO and CHO-E1a (CHO cells expressing the E1a transcriptional activator). Using this system for the expression of a cellular adhesion factor (SAF-3) Fc fusion protein in CHO-E1a, we found that levels of expression were highest with a MOI of 100, 20mM sodium butyrate, at 34 °C. Production increased further if the cells were resuspended in fresh medium, about 3×10^6 cells ml⁻¹, prior to addition of the virus. These conditions were used to express 3 secreted proteins, SAF-3-Fc, CD40-hexa his and Asp 2-Fc, and, at 2 to 6 days post infection, protein levels ranged from 4 ug ml⁻¹ to 25 ug ml⁻¹. Based on these results, the BacMam system represents a viable technique for producing protein at ug ml⁻¹ levels in a relatively short period of time.

Introduction

There are a variety of ways of introducing foreign DNA into the nucleus of mammalian or insect cells. These methods include calcium phosphate coprecipitation, DEAE-Dextran, electroporation (Kriegler 1990), lipofectamine (Harms et al. 1997) and viral transduction (Hofmann et al. 1995; Boyce 1996; Condreay et al. 1999). Obtaining stable transfectants using any of these methods can be slow, elaborate and time consuming. Transient production of recombinant proteins is often desirable for the initial screening studies of new recombinant molecules in a discovery environment. However, none of the fore-mentioned transfection methods offer a simple, rapid and straightforward manner to produce milligram quantities of protein with the possible exception of viral transduction or viral infection as in the case of Semliki Forest virus (Lundstrom et al., 2001) or traditional Baculovirus systems. The Semliki Forest virus system offers the advantages of high infection efficiencies and good protein production levels but carries with it the

disadvantage of possible infectivity to humans. Baculoviral infection of insect cells, though rapid, has some disadvantages due to 'insect-like' post-translational modifications. Recently, several groups have reported using baculovirus *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) as a carrier of foreign DNA into mammalian and insect cells in a non-lytic fashion (Carbonell et al. 1985; Shoji et al. 1992; Boyce et al. 1996; Yap et al. 1997; Condreay et al. 1999; Duisit et al. 1999; Kost and Condreay 1999; Lee 2000; Pieroni et al. 2001; Tani et al. 2001; van Loo et al. 2001). These publications demonstrated the expression of β -galactosidase, luciferase or the green fluorescent protein (GFP) using a mammalian promoter in a variety of mammalian cells, and noted the varied susceptibility of mammalian cells to recombinant baculovirus (BacMam). Upon BacMam infection of mammalian cells, chromosomal integration of baculoviral DNA does occur (Merrihew et al. 2001), however, no viral amplification or cell lysis was detected (Tjia et al. 1983; Shoji et al. 1997).

In this study we describe the use of baculovirus AcMNPV as a vector to transduce mammalian cells with genes to express secreted or transmembrane fusion proteins for the purpose of transient expression. In addition, we utilized a CHO line overexpressing the E1a gene product to enhance expression levels of the BacMam system. In this manner, we were able to express at mg L^{-1} concentrations three different secreted fusion proteins in mammalian cells.

Materials and methods

Cell lines

Three cell lines were evaluated in this study for transduction and expression with BacMam baculovirus: the dhfr deleted Chinese hamster ovary (CHO) cell line (DG-44; Urlaub 1983), a CHO line expressing the adenovirus E1a gene, which transactivates the CMV promoter, (CHOE1a; Trill – manuscript in preparation), and COS-7 (Gluzman 1981). Both CHO cell lines were adapted for growth in suspension in a proprietary serum-free media (SB-SFM) and were expanded in 500-ml Corning disposable Erlenmeyer flasks. COS-7 cells were grown as an adherent monolayer in D-MEM + 10% FBS and were expanded in 850 cm^2 roller bottles (Corning). Baculovirus used for this study was propagated in *Spodoptera frugiperda* cells (SF9) in IPL-41 media containing 5% FBS.

Generation of BacMam vector and recombinant inserts

The BacMam vector was constructed from the shuttle vector pFastBac1. The *polh* promoter in pFastBac1 was deleted by digestion with the unique blunt restriction enzymes SnaB I and Stu I, followed by self-closure. Complete expression cassettes, including a *cmv* promoter, the gene of interest, DHFR gene and selectable marker were subcloned on a NotI fragment

Not I Not I
 | cmv promoter gene-of-interest dhfr NeoR |

and inserted into the unique Not I site which remains from the pFastbac1 multiple cloning site.

Bacmids were generated in DH10Bac cells, and BacMam virus stocks were prepared according to the Invitrogen/ Life Technologies Bac-to-Bac protocol.

Genes-of-interest

SAF-3-Fc fusion. The cDNA encoding amino acids 1-347 of Sialoadhesin family member-3 (SAF-3) (GenBank accession number E30064) was subcloned upstream of the cDNA encoding amino acids 99 to 330 of human IgG1.

Asp2-Fc fusion. The cDNA encoding amino acids 1-460 of Asp-2 (GenBank accession number AF204943) was subcloned upstream of the cDNA encoding amino acids 99 to 330 of human IgG1. The Asp2 construct contained the following mutations N223Q and N354Q.

His tagged CD40 ligand. The cDNA encoding amino acids 114 to 261 of CD40 ligand (Prosite accession number P29965) was subcloned downstream of the TPA signal sequence, a gp120 epitope and 6-his tag.

Expression of SAF-3-Fc in CHO and COS-7 cells infected with recombinant baculovirus

CHO and CHOE1a cells were propagated in Corning Erlenmeyer disposable 500-ml flasks in SB-SFM medium to a density of $3\text{--}4 \times 10^6$ cells per ml and a viability of at least 95%.

For each transduction, sufficient cell suspension to give a final cell pellet of 1.5×10^8 was transferred to conical centrifuge tubes and centrifuged at 1000 RPM for 5 minutes. The cell pellets were suspended in 10 ml of fresh medium, and transferred to 125-ml Corning disposable Erlenmeyer flasks. The BacMam viruses were added to these flasks at the indicated MOI and incubated at either at 34 °C or 37 °C in a 5% CO_2 atmosphere. Two hours post transduction each flask was brought up to a final volume of 50 ml with fresh medium containing sodium butyrate at a final concentration of 20 mM. The flasks were incubated with shaking at 150 RPM at the indicated temperatures.

COS-7 cells were propagated in 850 cm^2 roller bottles in D-MEM + 10% FBS and removed by trypsinization. Trypsin was neutralised with FBS-containing medium, and the cell suspension was centrifuged at 1000 rpm. The resulting cell pellet was suspended in fresh medium to give a final density to seed Cytodex 3 microcarriers (Sigma Scientific Co.) at 2.0×10^4 cells per cm^2 . In this manner 0.5 gram of Cytodex-3 microcarriers placed in a 125 ml spinner flask were seeded and incubated at 37 °C in an atmosphere of 5% CO_2 and stirred at 100 RPM. Supernatant from these microcarriers was removed via a stainless steel screen in one of the side spouts in

Table 1. Expression of SAF-3-Fc^a in various cell lines with and without 20 mM sodium butyrate (BA) at 37°C

Day	COS-7	COS-7 + 20mM BA	CHO-E1A	CHO-E1A + 20mM BA
1	0.035	0.072	0.031	1.39
2	0.046	0.573	0.053	3.80
3	0.065	0.876	0.078	4.75

^a SAF-3-Fc titers in mg/L

the flask and 10 ml of fresh medium was added back. These cells were transduced with BacMam virus to give the desired MOI, incubated at either 34 °C or 37 °C and stirred at 100 RPM. At the end of 2 hours of incubation, cultures were brought to a final volume of 50 ml with fresh medium containing sodium butyrate at a final concentration of 20 mM and the flasks were returned to the incubator. All cultures were sampled as indicated. The titers of the BacMam viruses were determined as previously described (Mannix 2000) using Sf9 cells.

Expression of Asp2Fc and CD40-his in CHO E1a cells infected with recombinant baculovirus

Conditions for the expression of Asp2Fc and CD40-his were identical to Table 3, Protocol A.

Product assay

Expression levels of the SAF-3-Fc and ASP2-Fc fusions were quantitated using an HPLC based Protein A binding assay. Aliquots of culture media were filtered through a 0.45 micron filter and injected onto a 2.1 mm × 30 mm Protein A ImmunoDetection Sensor Cartridge (Perseptive Biosystems, Framingham MA) equilibrated in 20 mM Na₂HPO₄ / 150 mM NaCl pH 7.2. The bound material was eluted using 20 mM H₃PO₄ / 150 mM NaCl pH 2.0 and quantitated based on absorbance at 210 nm relative to a human pooled IgG calibration standard. CD40-hexahis was assayed via Western blot analysis using an anti-hexahis antibody.

Results

A BacMam virus expressing SAF-3-Fc was used to transfect COS and CHO E1a cells at 37 °C in the presence and absence of sodium butyrate (Table 1). In 3 days post infection very low but essentially equal levels of SAF-3-Fc were found in the supernatants

Table 2. Expression of SAF-3-Fc^a by CHO and CHO-E1A at 34 °C with 20 mM sodium butyrate

Days	CHO-E1A	CHO
1	4.50	2.4
2	15.50	3.8
3	23.10	4.3
6	29.60	4.8

^a SAF-3-Fc titers in mg L⁻¹

Table 3. Impact of time of addition of 20 mM sodium butyrate on expression of SAF-3-Fc^a

Day	Protocol A	Protocol B	Protocol C	Protocol D
1	4.25	2.20	4.35	4.25
2	13.55	6.70	13.25	13.55
3	19.75	10.30	17.80	19.30
6	24.90	15.10	20.25	22.50

^a SAF-3-Fc titers in mg L⁻¹ with a moi of 100.

A: Cells were spun and suspended in 10 ml of fresh medium, and virus was added. At 2 hours post infection, 40 ml of fresh medium containing sodium butyrate (20 mM) were added.

B: Cells propagated to a cell density of 3.0×10^6 ml (3 days) virus added followed by the addition of sodium butyrate.

C: Cells spun, cell pellet suspended in 50 ml of fresh media virus added and sodium butyrate added immediately.

D: Cells spun, cell pellet suspended in 50 ml of fresh media virus added. Sodium butyrate added 2 hours post infection.

of both COS and CHO E1a cultures. The addition of sodium butyrate increased expression levels in both COS-7 and CHO E1a about 13 and 60-fold respectively.

Our past experience with protein secretion in CHO suggested that expression levels of Fc containing proteins in the culture medium could be increased when the cells are incubated 34 °C (Ramos, unpublished observations). Both CHO and CHO E1a cells were transduced with BacMam and incubated at 34 °C (Table 2).

At day 1 the effect of E1a is clearly evident with about a 2-fold higher level of SAF-3-Fc in CHO E1a compared to CHO. By day 3 the level of SAF-3-Fc doubled in CHO but expression appeared to plateau with the level of SAF-3-Fc at day 6 only 11% higher. With CHO E1a, however, there was a 5 to 6.5-fold increase in the expression levels at days 3 and 6 respectively. At 34 °C, the expression levels at day 3 were about 5-fold higher than those at 37 °C. Since the CMV promoter-SAF-3-Fc DNA fragment was cloned into a single unique NotI site in Baculoviral DNA, two orientations of the insert were possible. In order

to exclude orientation effects, BacMam containing the SAF-3-Fc gene in both orientations were used to transfect CHOEl_a. Sodium butyrate was added and the cells were incubated at 34 °C post infection. While the kinetics of expression were about the same, there was a 20% increase in expression (21.2 mg L⁻¹ vs 16.7 mg L⁻¹) when transcription of the insert was in the same direction as transcription of the viral DNA. These results suggest orientation may have some effect on transcription.

In all of the experiments up to this point we had collected 1.5×10^8 cells by centrifugation, suspended the pellet in 10 ml of fresh medium, and added the virus. After a 2-hour incubation at 34 °C, 40 ml of fresh medium containing sodium butyrate (Protocol A) were added to the culture. While this is a relatively straightforward process, the additional manipulations of the cells make the process more cumbersome so we examined some alternative approaches (Table 3).

In Protocol B the virus and sodium butyrate were directly added to the culture after the cells reached a density of 3×10^6 . This approach would do away with centrifugation and suspension in fresh medium and represents the simplest approach. In Protocols C and D, the cells were harvested by centrifugation and suspended in 50ml of fresh medium and sodium butyrate was added immediately (C) or after 2 hours (D). It is clear from the data in Table 3 that the worst condition was adding the virus and sodium butyrate directly to the cells in spent medium as the expression level decreased 40%. The other process changes had only a modest effect on expression suggesting that fresh medium was the most critical factor for improving the level of protein expression.

In order to examine the robustness of this protocol, we examined the expression of 2 other proteins, Asp2-Fc and CD40-hexahis, using Protocol A. The levels of Asp2-Fc reached 4 mg L⁻¹ at day 4 while the level of CD40 reached a level of about 10 mg L⁻¹ at day 2.

Discussion

Our data demonstrate that the BacMam expression system can quite successfully be used to produce recombinant proteins in mammalian cell lines. CHOEl_a, which expresses the viral transcriptional activator El_a, was a better host than either CHO or COS-7 cells indicating the importance of transcriptional activation in this system. Additional factors that improved expression levels were sodium butyrate and the

temperature of incubation. Levels of expressed protein were significantly improved (13 to 60-fold) by including 20 mM sodium butyrate, an inhibitor of histone deacetylase (Kruh, 1982), in the medium. The maximum effect was observed if the sodium butyrate was added 2 hours after the virus. The effect of lower temperature (34 °C) on the expression level of SAF-3Fc was significant improving expression another 5-fold.

One advantage of this process is that all of the expressed recombinant proteins were obtained in only three to four days after transduction of the mammalian cells. Thus, one can use this process as a transient expression system for early evaluation of proteins of interest while stable cell lines are being cloned, amplified and evaluated.

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