Effect of temperature on recombinant protein expression in Semliki Forest virus infected mammalian cell lines growing in serum-free suspension cultures

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Abstract

The firefly luciferase gene was introduced into the Semliki Forest virus (SFV) vector and high titer recombinant SFV particles generated. The broad host range of SFV allowed efficient infection and high level expression of four mammalian cell lines growing in serum-free suspension cultures. The incubation temperature had dramatic effects on the level and duration of recombinant protein expression. For example, the luciferase activity was significantly higher in the rodent BHK and CHO cell lines incubated at 33 °C compared to 37 °C when harvested 19 h post-infection. At 33 °C the specific expression levels increased 10–20 fold during prolongation of the post-infection time up to 50 h. In contrast, a significant decrease in luciferase activity was observed from 26 h post-infection for cell cultures incubated at 37 °C. Only a slight temperature effect on luciferase expression was seen in the human cell line HEK293 and no effect was observed for the subclone293(EBNA).

Introduction

Recombinant protein expression is an essential part of modern molecular biology. Studies on gene function and regulation have been significantly facilitated by application of appropriate expression techniques to mammalian cells. Many different vectors and transfection methods have been developed to create both transient (Fong et al., 1992) and stable expression (Nakajima et al., 1992) systems. For large scale expression of recombinant proteins in mammalian cells it has been essential to establish the methodology for efficient and cost-effective production (Blasey et al., 1996). In these cases, much attention has been focused on cell culture growth conditions and viability. Cell death or apoptosis has become an increasingly important factor in these bioprocesses. To prevent cell death, or at least prolong the viability of the culture, two approaches have been taken. Stable expression of anti-apoptotic genes have shown a significant ef-

fect on cell viability. For example, the introduction of the Bcl-2 gene into RIN cells resulted in strong decrease of cell death after infection with Semliki Forest virus (SFV) (Lundstrom et al., 1997). Another approach has been to enhance the cell survival by addition of anti-apoptotic chemicals to the cell culture. It has recently been reported that the addition of Z-VADfmk (N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) to Sindbis virus infected AT3-*neo* cells prolonged the CAT (chloramphenicol acetyltransferase) expression for at least 24 h and maintained cell viability for 48 h longer (Mastrangelo and Betenbaugh, 1998).

The incubation temperature of cell cultures might also influence cell behaviour. When adherent BHK cells were grown at a lower temperature (30 °C) slower growth rate and glucose consumption rate were observed, resulting in reduced lactate production (Weidemann et al., 1994). Maximum cell density and productivity were not affected. Similar results were obtained for suspension cultures of CHO cells, where

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the lower temperatures promoted the resistance of the cells to shear stress (Kretzmer et al., 1997). There was therefore an indication that lowering of the temperature might be a feasible parameter for process optimization.

Here we have investigated the effect of cell culture temperature on the expression levels of a reporter gene, namely firefly luciferase. We have applied the highly efficient SFV vectors to generate high titer recombinant SFV particles expressing the luciferase gene. Due to the broad host range of SFV several mammalian cell lines growing in serum-free suspension cultures could be efficiently infected. The expression levels of luciferase were monitored in the different host cells at time points from 19 to 65 h post-infection after incubation at the normal growth temperature of 37 °C and the lowered temperature of 33 °C, respectively.

Materials and methods

Cell culture

The rodent cell lines BHK-21 C13-2P (ECACC No 84111301) and CHO-dhfr⁻ (ATCC CRL9096) were adapted to serum-free growth in DHI medium (Schlaeger, 1996) and the human HEK293 cells (ATCC CRL1573) and the subclone 293(EBNA) (Invitrogen, Leek, The Netherlands) were adapted to serum-free growth in HL medium (Schumpp and Schlaeger, 1990). Both fortified powder media, calcium-free DHI and calcium-free HL, were developed in-house and purchased from Life Technologies (Basel, Switzerland). The cells were routinely cultured in spinner flasks (Bellco, Inotech AG, Dottikon, Switzerland) with 95–105 rpm using about 70% of the recommended working volume. If 33 °C incubation were used for SFV infection BHK and CHO cells were adapted for two passages at lower temperature. HEK293 and 293(EBNA) cells which do not grow well at 33 °C were used from a 37 °C preculture for such an experiment.

Expression of luciferase from SFV vectors

The coding region of the firefly luciferase gene was cloned into the *Sma*I site of the pSFV1 vector resulting in pSFV1-Lux. *In vitro* transcribed RNA from pSFV1-Lux and pSFV-Helper2 (Berglund et al., 1993) were coelectroporated into BHK cells and virus-containing medium collected from the cells 24 h later as earlier

described (Lundstrom et al., 1994). The recombinant SFV particles were activated with α -chymotrypsin (500 μ g mL⁻¹) (Boehringer Mannheim) for 15 min at 25 °C and the reaction terminated with Aprotinin (250 μ g mL⁻¹) (Sigma) prior to infection of mammalian cell lines. The expression of recombinant luciferase was verified by metabolic labeling with [³⁵S] methionine followed by SDS-PAGE and autoradiography.

Luciferase assay

The enzymatic activity of luciferase was measured at the different time points post-infection as follows. 2 × 1 ml samples were centrifuged for 5 min at 2000 rpm at 4 °C and the supernatants carefully removed. The cell pellets were resuspended mechanically and 1 mL ice cold lysis buffer (Boehringer Mannheim) added. The cell lysates were mixed, incubated on ice for 13 min and centrifuged at 12 000 rpm for 10 min at 4 °C to remove cell debris. The supernatants were stored frozen at -20 °C until analyzed. After thawing, the samples were diluted in lysis buffer and assayed in the Lumat LB9501 (Berthold AG, Regensdorf, Switzerland) as earlier described (Legendre et al., 1996). The protein concentration of the cell extracts was determined using the BCA protein assay reagent (Pierce, Rockford, IL, USA).

Results and discussions

The introduction of the coding region of the luciferase gene into the pSFV1 vector made it possible to *in vivo* package recombinant SFV-Lux particles with an approximate titer of 5×10^8 infective units mL⁻¹. The titer was indirectly estimated by determination of the titer of SFV-LacZ virus *in vivo* packaged in parallel with SFV-Lux. The luciferase expression was verified by infection of BHK cells growing in monolayer cultures on 6 well plates. The SFV-infected cells were subjected to metabolic labeling 18 h post-infection, which resulted in high-level expression of luciferase as identified by the presence of a strongly labeled 66 kD band in SDS-PAGE (not detectable in uninfected control cells [data not shown]). The morphological changes in the cells were also dramatic and made it possible to microscopically distinguish infected cells from uninfected ones.

BHK, CHO, HEK293 and 293(EBNA) cells grown in serum-free suspension cultures achieving cell densities of $3.5\text{--}5.0 \times 10^6$ cells mL⁻¹ were subjected to

Figure 1. SFV-infection of BHK cells grown in suspension cultures at 37 °C. A) Cells adjusted to various concentrations in 15 mL volume, infected with 0.1 mL SFV-Lux and the luciferase activity determined at 19 h post-infection. B) 15 mL spinner cultures with 7.5×10^5 cells mL⁻¹ infected with 0.1 mL SFV-Lux virus were assayed at different post-infectious time points. C) 7.5×10^5 cells mL⁻¹ infected with different SFV-Lux concentrations and assayed at 19 h post-infection.

infection studies with recombinant SFV-Lux virus. The BHK cells were chosen to study the effect of the cell density and virus concentration on expression levels. This was because the BHK cells were used for *in vivo* packaging of SFV-Lux particles and were known to be efficiently infected by SFV in suspension cultures from previous studies (Blasey et al., 1996). A relatively broad cell density, from $6.0\text{--}9.0 \times 10^5$ cells mL⁻¹, resulted in high luciferase expression (Figure 1A). For all further studies, cell densities of $7.0\text{--}7.5 \times 10^5$ cells mL⁻¹ were used. Next the optimal post-infection time for luciferase expression was determined. Our results indicated that the best harvest time would be 19 to 23 h post-infection (Figure 1B). The effect of virus concentration was studied by infection of 15 mL spinner cultures with different virus concentrations (0.025–0.3 mL). These amounts correspond to estimated multiplicity of infection (MOI) values from 1 to 15. The maximal luciferase activity was obtained with a MOI of 10 with no further increase in expression levels with higher MOI values (Figure 1C). For practical reasons all further experiments were performed with 0.1 mL virus/15 mL culture (MOI 5).

Using the conditions described above we compared the expression levels of luciferase in different host cells by infection of two rodent cell lines, Baby Hamster Kidney (BHK) and Chinese Hamster Ovary (CHO), with SFV-Lux virus. Previous studies had shown that CHO cells were infected with SFV as ef-

ficiently as BHK cells (Lundstrom et al., 1994). We have also demonstrated the high efficiency of HEK293 and 293(EBNA) cells by SFV-lacZ infection and by X-gal staining (Lundstrom, unpublished results). The infected spinner cultures were incubated at 33 and 37 °C, respectively. The 33 °C incubation temperature was chosen because it was shown recently that elevated production was observed with a recombinant CHO cell line at lower temperature (Kretzmer et al., 1997). The expression levels of luciferase were much higher in both BHK and CHO cells cultured at 33 °C than at 37 °C (Figure 2A and B). Additionally, while the luciferase activity rapidly decreased from 26 h post-infection onwards at 37 °C there was a significant increase in expression levels at 33 °C that continued until 65 h post-infection. As shown below no difference in viable cell numbers was observed during post-infection at 33 °C (Table 1). The increase in luciferase activity at the different time points for cells cultured at 33 °C in comparison to 37 °C is shown in Figure 2C. At 50 h post-infection more than 10-fold increase in luciferase levels was measured in CHO cells and almost 20-fold in BHK cells. The influence of other temperatures on the luciferase expression was not analyzed.

Likewise, we studied the temperature effect on the human HEK293 cell line and its subclone 293(EBNA). Surprisingly, we could not detect a significant difference in the expression levels of luciferase at the two temperatures studied (Figure 3). Even though the ex-

Figure 2. Kinetics of SFV-Lux infected BHK and CHO cells. 20 mL spinner cultures (7.0×10^5 cells mL^{-1} , from a 37 °C culture) infected with 0.135 mL of SFV-Lux virus were incubated at 33 and 37 °C, respectively for 65 h. 2×1 mL aliquots were taken at 19, 26, 41, 50 and 65 h post-infection and assayed for luciferase activity (expressed as relative light units/mg protein of the soluble fraction of the cell lysate). A) BHK cells; B) CHO cells. C) The relative increase of luciferase activity at different time points for SFV-Lux infected cells incubated at 33 °C compared to 19 h at 37 °C.

Figure 3. Kinetics of SFV-Lux infected HEK293 and 293(EBNA) cells. 20 mL spinner cultures (7.0×10^5 cells mL^{-1}) infected with 0.135 mL of SFV-Lux virus were incubated at 33 and 37 °C, respectively for 65 h. 2×1 mL aliquots were taken at 19, 26, 41, 50 and 65 h post-infection and assayed for luciferase activity (expressed as relative light units/mg protein of the soluble fraction of the cell lysate). A) HEK 293 cells; B) EBNA cells. C) The relative increase of luciferase activity at different time points for SFV-Lux infected cells incubated at 33 °C compared to 19 h at 37 °C.

Table 1. Viable cell density during SFV post-infection. The viable cell counts of the time kinetics of SFV infection were measured microscopically. Non-infected cells in brackets. Values are in 10^5 cells mL^{-1} .

Cell line		BHK	CHO	HEK293	293(EBNA)
0 h	33 °C	_____	7	_____	
	37 °C	_____	7	_____	
19 h	33 °C	14 (12.6)	8 (14.4)	8 (7.6)	7 (7.0)
	37 °C	22 (19.0)	10 (16.7)	8 (8.2)	8 (9.2)
41 h	33 °C	16 (40)	10 (42.0)	6 (10.0)	8 (10.0)
	37 °C	22 (40)	23 (52.0)	8 (17.8)	9 (17.0)
65 h	33 °C	11 (40)	9 (48.0)	4 (10.3)	4 (9.5)
	37 °C	21 (38)	28 (44.0)	7 (20.0)	7 (21.0)

pression levels were already relatively high at 19 h post-infection (almost 5-fold higher than for BHK cells) a 2-fold increase in luciferase activity levels was detected in HEK293 cells at 41 h post-infection at 33 °C. There was almost no difference in the expression levels seen at 33 °C for the 293(EBNA) cells. Thus, no improvement in luciferase expression levels could be achieved with human cell lines at the lower temperature (Figure 3C).

To analyze the differences in cell growth and viability properties for cultures incubated at 33 and 37 °C, we determined the number of viable cells in the SFV-Lux infected cultures and in non-infected samples. The viable cell numbers of infected BHK and CHO cells were lower at 33 °C but no significant difference was observed between 19 and 65 h (Table 1). Incubation at 37 °C showed some differences between BHK and CHO cells. Whereas the BHK cell numbers remained constant during 19 and 65 h post-infection, the cell density of CHO continued to increase. We could therefore conclude that the observed temperature effect on the expression level in both rodent cell lines was independent of the reduced cell growth rate at 33 °C compared to 37 °C. Additionally, the experiments also demonstrated that the infection of the cells in the spinner cultures was highly efficient since the cells divided poorly during the entire post-infection period. In contrast uninfected BHK and CHO cells grew within 40 h to cell densities of $4\text{--}5 \times 10^6$ cells mL^{-1} .

Infected HEK293 and 293(EBNA) cells did not show differences in viable cell numbers during two days incubation at 33 and 37 °C. Further growth of both cell lines was immediately inhibited after SFV infection.

In this study we have been able to show a dramatic increase in luciferase expression levels by simply lowering the growth temperature of SFV-Lux infected cell cultures of rodent BHK and CHO cell lines from 37 to 33 °C. This procedure resulted in up to 20-fold higher expression levels and, moreover, the expression phase could be prolonged to about 50 h. However, only a minor effect was seen for the human cell line HEK293 and no effect was detected for the subclone 293(EBNA). Both HEK cell lines grow poorly at low temperatures, in contrast to the rodent cells which grow normally. We therefore postulate that our finding could be important for further development of recombinant protein expression techniques to increase yields, especially for poorly expressed proteins. Obviously, it will be crucial to follow up studies on expression of some more complex mammalian recombinant proteins under these same conditions to evaluate if similar improvements in quantity can be achieved without loss in the quality of the product. Furthermore, it could be interesting to measure other (lower) temperatures for the expression of recombinant proteins in rodent cells using the SFV vector system.

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