Kinetic studies of recombinant rabies virus glycoprotein (RVGP) cDNA transcription and mRNA translation in *Drosophila melanogaster* S2 cell populations

R. M. Astray · S. A. C. Jorge · M. A. N. Lemos ·
A. Y. Yokomizo · V. L. L. Boldorini ·
A. L. P. Puglia · O. G. Ribeiro · C. A. Pereira

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Abstract Recombinant rabies virus glycoprotein (RVGP) was expressed in cell membranes of stably transfected *Drosophila* S2 cells using constitutive and inducible promoters. Although with quantitative differences of RVGP expression in both systems, the cDNA transcription, as evaluated by relative RVGP mRNA levels measured by qRT-PCR, sustained the amount of RVGP producing cells and the RVGP volumetric (Π_{RVGP}) productivity. At the transition to the stationary cell growth phase, once the cell culture slowed down its rate of multiplication, an accumulation of RVGP mRNA and RVGP was clearly observed in both cell populations. Nevertheless, cell cultures performed under sub-optimal temperatures indicated that an envisaged increase in the RVGP production is

not only dependent on cell growth rate, but essentially on optimal cell metabolic state.

Keywords S2 cells · Rabies virus glycoprotein · qRT-PCR · mRNA · Cell culture

Abbreviations

RVGP	Rabies virus glycoprotein
qRT-PCR	Quantitative reverse transcriptase-polymerase chain reaction
X	Cell concentration (cell/mL)
P _c	Rabies virus glycoprotein cell content ($\mu\text{g}/10^7$ cells)
μ	Specific cell growth rate (h^{-1})
Π_{RVGP}	Volumetric productivity ($\text{ng mL}^{-1} \text{h}^{-1}$)

R. M. Astray (✉) · S. A. C. Jorge · M. A. N. Lemos ·
A. Y. Yokomizo · V. L. L. Boldorini ·
A. L. P. Puglia · C. A. Pereira (✉)
Laboratório de Imunologia Viral, Instituto Butantan,
Av. Vital Brasil 1500, São Paulo 05503-900, Brazil
e-mail: renatoastray@butantan.gov.br

C. A. Pereira
e-mail: grugel@butantan.gov.br

S. A. C. Jorge
e-mail: sacjorge@butantan.gov.br

M. A. N. Lemos
e-mail: manlemos@butantan.gov.br

A. Y. Yokomizo
e-mail: adriana.biotec@cristalia.com.br

V. L. L. Boldorini
e-mail: boldorini@butantan.gov.br

A. L. P. Puglia
e-mail: analiapp@butantan.gov.br

Present Address:

A. Y. Yokomizo
Cristália Produtos Químicos e Farmacêuticos Ltda,
Itapira, Brazil

O. G. Ribeiro
Laboratório de Imunogenética, Instituto Butantan,
Av. Vital Brasil 1500, São Paulo 05503-900, Brazil
e-mail: oribeiro@butantan.gov.br

Introduction

Rabies is one of the most lethal infectious diseases, being responsible for 55,000 deaths/year worldwide, despite prophylactic treatment saving at least 330,000 deaths every year (World Health Organization 2007). The major rabies virus antigen able to induce immune response is the transmembrane glycoprotein rabies virus glycoprotein (RVGP) (Perrin et al. 1985). In order to confer immunity against rabies, the RVGP has to be oligomerized and present the antigenic site III (Gaudin et al. 1992), which is developed after complex post-translational modifications (Gaudin 1997; Dietzschold 1977), a characteristic of its expression in eukaryotic organisms (Butler 2004; Jorge et al. 1996). The RVGP is present in virus envelope which surrounds a negative strand RNA tightly encased by the nucleocapsid protein. After infection, a viral polymerase transcribes the viral RNAs directly from RNA genome, generating capped, methylated and polyadenylated monocistronic mRNAs. Translation takes place at polyribosomes except for the transmembrane glycoprotein (RVGP) which is translated and glycosylated on endoplasmic reticulum membrane-associated polyribosomes (Wagner 1991).

The *Drosophila melanogaster* Schneider 2 (S2) cells have been used as an efficient eukaryotic expression system (McCarroll and King 1997; Moraes et al. 2012). The two most utilized promoters are the constitutive actin promoter and the inducible metallothionein promoter which is activated by the addition of heavy metal in the culture medium (Chung and Keller 1990a, b). Several complex glycoproteins were already expressed in the S2 cell system, using these promoters (Mallender et al. 2001; Zhang et al. 2007; Scotter et al. 2006; Brillet et al. 2006; Kim et al. 2005; Johansson et al. 2007; Jennings et al. 2006; Li et al. 2005; Lim et al. 2004; Lee et al. 2007). Aiming the expression of high levels of RVGP under the control of these promoters in the S2 cell system, for vaccination as well as structure/function evaluation, many studies were already carried out on cell growth and heterologous recombinant protein expression kinetics (Yokomizo et al. 2007; Galesi et al. 2008; Swiech et al. 2008a; Batista et al. 2009; Ventini et al. 2010; Lemos et al. 2009), as well as on metabolism and synthesis of secondary products (Swiech et al. 2008b, c) and culture medium formulation and supplementation (Galesi et al. 2007; Batista et al. 2008, 2011;

Mendonça et al. 2008, 2009). For constitutive RVGP expression using the actin promoter, instead of a gradual and sustained increase of RVGP, we have observed a sharp RVGP increase, at the beginning of the stationary cell growth phase, which could not be associated with the culture system or the cell culture media, pH, oxygen concentration or substrate change (Galesi et al. 2008; Ventini et al. 2010; Batista et al. 2011). In the present study, in view of better understanding the RVGP expression profile and exploring more in detail the kinetics of heterologous RVGP cDNA transcription we measured the RVGP mRNA and RVGP in different S2 cell cultures. The peak of RVGP mRNA and RVGP synthesis observed at the transition to the stationary cell growth phase indicated that an optimization of RVGP production could be combined with decreased cell growth rates providing suitable environmental and metabolic cell culture conditions are supplied.

Materials and methods

Recombinant cell populations and cell culture

Drosophila melanogaster S2 cells (DES[®], Invitrogen-Life Technologies, Carlsbad, CA, USA) were transfected with the RVGP cDNA under the control of a constitutive (actin—Ac) or an inducible (metallothionein—Mt) promoter (Yokomizo et al. 2007; Lemos et al. 2009). Originated S2 cell populations were named S2AcRVGP-2k and S2MtRVGP-Hy, respectively. Cell cultures were performed in 25 cm² T-flasks and adapted to growth in suspension for 48 or 72 h with an inoculum of 5×10^5 cells/mL in 20 mL of serum-free medium SF900II[®] (Invitrogen) in 100 mL shake flasks (Schott, Elmsford, NY, USA), at 100 rpm and 28 °C. Unless indicated, cell cultures were then performed at 28, 25 or 22 °C using cell populations cultured at the given temperature for 10 serial passages in order to allow them a metabolic adaptation. All cell cultures were performed in triplicates. The RVGP expression in S2MtRVGP-Hy was induced with the addition of 500 µM of CuSO₄ at the indicated time. Cell viability was determined by trypan blue exclusion method (Doyle and Griffiths 1998). Flow cytometry samples (10⁶ cells) were immediately treated as described in a following section. For ELISA, the samples (10⁶ cells) were centrifuged (1,000×g/3 min),

the supernatant discarded, the pellet washed once with PBS with sucrose 320 mM, centrifuged again ($3,000\times g/1$ min), and frozen immediately at -20°C . For qRT-PCR, cell samples (3×10^6 cells) were centrifuged ($1,000\times g/3$ min), the supernatant discarded, the pellet washed once with PBS, centrifuged again ($3,000\times g/1$ min), resuspended with 1 mL of Trizol[®] (Invitrogen) and frozen at -80°C .

Analysis of RVGP mRNA synthesis

RNA extraction

The total RNA was extracted from cell samples using the guanidine Isothiocyanate–phenol–chloroform method (Chomczynski and Sacchi 1987) and quantified by Quant-it[®] RNA assay kit (Invitrogen) that allows individual quantification of total RNA in DNA and RNA mixed samples. For removal of residual DNA, 3 μg of total RNA were treated with 3 U of RNase-free DNase I (Promega, Madison, WI, USA) at 37°C for 30 min, in the presence of RNase inhibitor (RNase-Out[®], Invitrogen). After the incubation time, the DNase was inactivated with the enzymatic inhibitor EGTA at 65°C for 10 min.

Primers for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

The following primers were designed and used for reverse transcription and amplification of RVGP mRNA:

RVGP_{r3} 5'-AGCCGCAAGTCTCACTCCC-3' (RT), RVGP₉ 5'-CTGACTACCACTGGCTTCG-3' (forward), RVGP_{r1} 5'-TGTAATCGTGGTTAGTG-GAGC-3' (reverse).

After alignment verification (Clustal X, 1.83[®]), the primers sequences for actin-42 (*D. melanogaster*—Ac mRNA) amplification were obtained (Southon et al. 2004):

Ac42f 5'-GCTTCGCTGTCTACTTTCCA-3' (forward)
Ac42r 5'-CAGCCCCGACTACTGCTTAGA-3' (reverse)

By using these sets of primers, fragments of 177 and 105 bp were generated after RVGP mRNA and Ac mRNA amplification, respectively.

qRT-PCR

For reverse transcription, 600 ng of DNase-treated RNAs were incubated at 37°C for 50 min with

reverse specific primers, buffer, DTT and 200 U of M-MLV (Invitrogen). The qRT-PCR was performed according to published guidelines (Nolan et al. 2006). Briefly, initially the PCR products were detected (177 and 105 bp for, respectively, RVGP and actin) by 1 % agarose gel. In preliminary assays we determined the primer concentration (200 and 300 nM for, respectively, forward and reverse primers), the cDNA concentration (7.2 ng/mL) evaluating the absence of dimers, lowest Ct, highest end point fluorescence, and absence of signal in the non template controls. Data were normalized for each experiment individually against RVGP mRNA level at time 0 h of cell culture kinetics. Relative quantification (R) of RVGP mRNA was determined by using a mathematical model (Pfaffl 2001)

$$R = \frac{(E_{RVGP})^{(Ct_{normalizer} - Ct_{sample})}}{(E_{actin})^{(Ct_{normalizer} - Ct_{sample})}}$$

where amplification efficiencies (E) of target and housekeeping genes, the cycle threshold of normalizer and sample for both genes are considered. The amplification efficiencies were determined by performing the amplification of five serial tenfold dilutions of concentrated cDNA. The efficiencies were obtained from the regression line of Ct to log of dilutions, being 2.13 for RVGP and 2.23 for actin. The qRT-PCR was established on the CHROMO4[™] Thermal Cycler-200 (Bio-rad, Hercules, CA, USA). The reactions were performed in triplicate with the Platinum[®] Sybr[®] green qPCR supermix UDG kit (Invitrogen) with 90 ng of cDNA in a final volume of 12.5 μL in optical microtubes (MicroAmp[®], Applied Biosystems-Life Technologies). Amplifications were performed using the following temperature profile: 50°C for 2 min, 95°C for 2 min and (95°C for 15 s, 55°C for 30 s, 72°C for 30 s) $\times$ 34. Melting curves were measured from 60 to 90°C . Nine replicates of one cell sample, from RNA extraction to qPCR, showed adequate interassay reproducibility (standard deviation of 0.21 and 0.32 Ct for, respectively, actin and RVGP).

RVGP quantification

ELISA

The RVGP concentration in cell lysates was determined by ELISA (Perrin et al. 1996). Microplates

were sensitized (3 h at 37 °C) with monoclonal D1 anti-RVGP antibodies, specific to the RVGP trimeric form. Samples of S2 cells were lysed with detergent as already described (Astray et al. 2008) and incubated in microplates. Bound RVGP was recognized by antibodies labeled with peroxidase. Absorbance was measured at 492 nm in an absorbance microplate reader (Multiskan™ EX, MJ-Labsystems, Milford, MA, USA). The RVGP cell contents (P_c), expressed in $\mu\text{g}/10^7$ cells were calculated by comparison of the OD measured for samples with the OD measured for RVGP standard-curve.

Flow cytometry

Samples of S2 cells expressing RVGP on the membrane were incubated (4 °C/30 min) with monoclonal D1 anti-RVGP antibody coupled with fluorescein (Pasteur Institute, Paris, France) diluted 1:400 in PBS with 2 % of fetal bovine serum (FBS). After antibody labeling, samples were fixed in PBS with p-formaldehyde (4 %) before data acquisition in FACSCalibur™ (Becton-Dickinson, Franklin Lakes, NJ, USA) flow cytometer. Percentage of RVGP producer cells was always assessed by flow cytometry.

Cell concentration (X), RVGP cell content (P_c), specific cell growth rate (μ), and RVGP volumetric productivity (Π_{RVGP})

The cell concentration (X) was expressed as the natural logarithm of X ($\ln X$). Values of RVGP cell content (P_c) were determined by ELISA in samples with known cell concentration (X). The specific cell growth rates (μ) were calculated at the indicated periods of cell culture as linear regression of the natural logarithm of viable cell concentration ($\ln X$) versus culture time. The RVGP volumetric productivity (Π_{RVGP}) was calculated by dividing the RVGP volumetric concentration by the culture time. The nomenclature and calculations are in accordance with published guidelines (Augusto et al. 2010). For RVGP and RVGP mRNA statistical data analysis we used the Student T test.

Results

As shown in Fig. 1, the constitutive heterologous gene expression in S2AcRVGP-2k cell cultures presented a

peak of the relative amount of RVGP mRNA at 84 h (R_{MAX} : 7.1). It occurred at the same time of highest values of RVGP producer cells in culture (55 %), RVGP cell content (P_c : $0.31 \mu\text{g}/10^7$ cells) and RVGP volumetric productivity (Π_{RVGP} : $6.2 \text{ ng mL}^{-1} \text{ h}^{-1}$). These values were observed at the transition from the exponential to the stationary cell growth phase. The μ of 0.042 h^{-1} at 24–72 h dropped to 0.026 h^{-1} at 72–80 h. At this point the $\Pi_{RVGP MAX}$ was at least twofold higher than Π_{RVGP} values attained during exponential cell growth phase and the amount of RVGP producer cells increased from 28 % at 72 h to 55 % at 84 h. The data pointed out to an accumulation of RVGP mRNA and RVGP when the cells slow down its multiplication rate.

In order to extend the previous observations and to compare the constitutive (actin promoter) RVGP expression system with an inducible one, we analysed the RVGP mRNA and RVGP expression using the inducible metallothionein promoter provided by S2MtRVGP-Hy cells. Cell cultures were induced at the cell inoculum, so mirroring the constitutive expression. As shown in Fig. 2 the promoter induction led to significantly better RVGP expression. Higher initial RVGP mRNA level and Π_{RVGP} were clearly observed already at 24 h. During exponential cell growth phase the RVGP mRNA, Π_{RVGP} and % of RVGP producer cells remained at high levels (respectively, R: 3.6, $16 \text{ ng mL}^{-1} \text{ h}^{-1}$ and 68 % at 48 h). The number of RVGP producer cells remained essentially constant during exponential cell growth phase leading to progressively increasing values of Π_{RVGP} , both being sustained by regular RVGP mRNA synthesis.

In contrast to the constitutive S2AcRVGP-2k cell system, specific RVGP (P_c) values were already very high soon after induction ($1.14 \mu\text{g}/10^7$ cells at 24 h) and showed progressive decreasing levels ($0.59 \mu\text{g}/10^7$ cells at 60 h) in function of exponential cell growth. A sharp increase was then observed in all RVGP values (RVGP mRNA, Π_{RVGP} , % of RVGP producer cells and P_c) when the cell multiplication slowed down. The μ of 0.052 h^{-1} at 0–60 h dropped to 0.006 h^{-1} at 60–72 h. At 72 h a high cell density (2×10^7 cells/mL) was attained with a high number of RVGP producer cells (82 %) and maximal volumetric ($\Pi_{RVGP MAX}$: $34.2 \text{ ng mL}^{-1} \text{ h}^{-1}$) and specific ($P_{c MAX}$: $1.25 \mu\text{g}/10^7$ cells) RVGP values.

The data obtained with S2AcRVGP-2k cells and mainly those with S2MtRVGP-Hy cells clearly

Fig. 1 Kinetics of S2AcRVGP-2k cell growth and RVGP mRNA (a) and RVGP expression (b). Suspension cell cultures were performed at 28 °C. $\ln X$ natural logarithm of cell concentration (filled circle), RVGP mRNA relative RVGP mRNA (fold difference) compared to time 0 h (grey columns), μ calculated by the regression line of $\ln X$ dots corresponding to

cell growth phase, Π_{RVGP} RVGP volumetric productivity (filled triangle), % of RVGP producer cells anti-RVGP-FITC labeled cells measured by flow cytometry (white columns), P_c RVGP cell content measured by ELISA (filled square). Data shown are the average of 3 different experiments. * Statistically different from the preceding values ($P < 0.05$)

demonstrated a cellular RVGP accumulation during the transition of exponential to a stationary cell growth phase.

Increasing values of the % of RVGP producer cells in S2AcRVGP-2K cell cultures, correlated with the specific RVGP expression (P_c) values. The increasing levels of P_c allowed the detection of RVGP producing cells by the flow cytometry assay (Fig. 1b). Conversely, for S2MtRVGP-Hy cells, the % of RVGP producer cells were already very high after induction (68 % at 24 h) and the P_c values decreased in function of time and exponential cell growth (Fig. 2b). Although different kinetic patterns of specific RVGP synthesis (P_c) were observed for constitutive (S2AcRVGP-2k cells)

and inducible (S2MtRVGP-Hy cells) cell systems, altogether the data clearly pointed out to an accumulation of RVGP mRNA and RVGP when the cells slowed down its multiplication rate. Experiments performed with different initial cell concentrations, leading to variations in the kinetic pattern, confirmed these observations (data not shown).

In view of studying the influence of overall cell metabolic state towards an optimization of RVGP production, we performed experiments aiming to slow down the cell growth rate by cultivating them under suboptimal temperature conditions. Cells adapted or not to grow at 22 or 25 °C were tested for cell growth, RVGP

Fig. 2 Kinetics of S2MtRVGP-Hy cell growth and RVGP mRNA (a) and RVGP expression (b) upon $CuSO_4$ induction at culture onset. Suspension cell cultures were performed at 28 °C. $\ln X$ natural logarithm of cellular concentration (filled circle), RVGP mRNA relative RVGP mRNA (fold difference) compared to time 0 h (grey columns), μ calculated by the regression line

corresponding to growth phase, Π_{RVGP} RVGP volumetric productivity (filled triangle), % of RVGP producer cells anti-RVGP-FITC labeled cells measured by flow cytometry (white columns), P_c RVGP cell content measured by ELISA (filled square). Data shown are the average of 3 different experiments. * Statistically different from the preceding values ($P < 0.05$)

mRNA and RVGP synthesis as compared to the usual 28 °C. Data shown in Table 1 indicated that lower temperatures of culture affected more the gene transcription, RVGP mRNA translation and RVGP synthesis than the cell growth rate. At suboptimal temperature conditions of cell culture, lower values for μ_{MAX} , P_{cMAX} , $\Pi_{RVGP MAX}$ and RVGP mRNA were always observed.

Discussion

In previous studies we have demonstrated the practicality of *D. melanogaster* S2 cells to produce a

recombinant RVGP at high levels upon cDNA transfection using plasmid vectors with constitutive (actin) and inducible (metallothionein) promoters (Moraes et al. 2012). The RVGP could then be detected in the cell membranes by quantitative approaches such as ELISA and flow cytometry. By using these assays, specific and volumetric RVGP synthesis as well as the number of RVGP producing cells could be evaluated.

Nevertheless, in studies aiming the improvement of recombinant protein expression, often the amount of mRNA coding for the protein of interest is not considered. In order to study more in detail the kinetics of RVGP synthesis by recombinant S2 cell populations

Table 1 Cell growth, RVGP mRNA and RVGP synthesis in S2AcRVGP-2k cell cultures at optimal and suboptimal temperatures

Temperature (°C)	X_{MAX} ($\times 10^7$ cells/mL) ^a	μ_x (h^{-1}) ^b	$P_{c,MAX}$ ($\mu g/10^7$ cells) ^c	Π_{RVGP} ($ng\ mL^{-1}\ h^{-1}$) ^d	RVGP mRNA ^e
28	1.1	0.038	1.63	14.8	1
28/25*	0.9	0.024	1.21	9.0	0.23
25	1.2	0.035	0.81	7.8	0.27
22	0.6	0.026	0.83	5.0	0.22

Cell cultures were performed after adaptation to the indicated temperature

* The cell culture had the temperature shifted from 28 to 25 °C at the beginning of exponential cell growth phase

^a Maximal cell concentration

^b Exponential cell growth rate

^c Maximal RVGP specific productivity

^d Maximal RVGP volumetric productivity

^e Average value for exponential cell growth phase

and establish parameters for future improvements in bioprocess control and optimization, we carried out the present study. S2 cell cultures showing a constitutive (S2AcRVGP-2k) or inducible (S2MtRVGP-Hy) expression were performed. Together with RVGP synthesis we evaluated the kinetics of RVGP mRNA by using qRT-PCR. Our data show that the kinetics of heterologous gene transcription/metabolisation, as measured by the specific mRNA evaluation by qRT-PCR, can be a valuable and helpful approach in optimizing bioprocesses.

The classical pattern of a cell culture in a batch mode of operation is represented by an adaptation followed by an exponential cell growth phase. Then for reasons due essentially to limitation of nutrient and/or accumulation of metabolites the cells enter into stationary cell growth phase preceding cell death.

Our kinetic studies of S2AcRVGP-2k cell growth, RVGP mRNA and RVGP expression (Fig. 1) showed during the exponential cell growth phase a progressive increase of P_c and Π_{RVGP} while the RVGP mRNA and the % of producer cells remained at constant levels. The highest values were observed when the cell cultures were at the transition from exponential to the stationary cell growth phase, which was confirmed and more clearly evidenced with the inducible S2MtRVGP-Hy cell cultures. They showed also better expression levels (Fig. 2) and in general confirmed our previous observations of RVGP productivity (Lemos et al. 2009). For this cell population, at the exponential cell growth phase, we observed higher initial levels of RVGP producer cells and Π_{RVGP} , accompanied by characteristic levels of RVGP mRNA. The higher μ

observed among these cultures in this phase can be associated with decreasing P_c levels.

The volumetric RVGP productivity (Π_{RVGP}) is directly related to biomass increase (cells/mL) and to specific RVGP production (P_c). Nevertheless, when the cell cultures enter the stationary phase the biomass increase is very low and only increasing values of P_c contribute to increase Π_{RVGP} . At this point a sharp increase in RVGP cell content (P_c) and consequently in volumetric productivity (Π_{RVGP}) was shown to be directly linked to high and sustained RVGP mRNA synthesis and translation. The kinetics of quantitative RVGP mRNA detection demonstrated sustained levels during the whole culture time. After 72–84 h when limiting conditions of cell culture took place and cell cultures started to loose suitable environmental conditions for cell growth and for gene transcription/translation, the RVGP mRNA levels showed decreasing values. At this point we observed also a decreased synthesis of RVGP.

In contrast to the RVGP, the RVGP mRNA detected does not represent only synthesis, but also consumption due to metabolization for the translation process. Our data showed that, for both constitutive and inducible systems, once the cells slowed down its rate of multiplication, an accumulation of RVGP mRNA and RVGP was observed in cells. Together with the data of decreasing P_c values during the exponential S2MtRVGP-Hy cell growth phase, it leads to the evidence that high cell growth rate limits the RVGP mRNA and protein accumulation in cells. At the transition from the exponential to the stationary cell culture phase cell growth slowed down and RVGP

cDNA transcription and RVGP mRNA translation remained active at least for a certain period of time and both RVGP mRNA and RVGP (Pc and Π_{RVGP}) accumulation were observed. It allowed us to hypothesize that an increased RVGP accumulation in cells (volumetric production) would be attained in cell cultures showing lower cell growth rate, provided that suitable environmental and metabolic cell culture conditions for heterologous protein synthesis were supplied.

Considering optimizations for the synthesis of heterologous recombinant protein by genetically modified cell populations, the easiest approach of favouring the cell multiplication in view of obtaining high levels of recombinant cell product may not be always the indicated one. A rational approach would be to somehow drive the cell metabolism redirecting cell resources used for cell proliferation to the recombinant protein expression (Palomares et al. 2004; Dinnis and James 2005).

Although bioprocess approaches using suboptimal cell culture temperatures for improving recombinant protein or virus production are well known (Butler 2004), our data with the S2 cell system (Table 1) suggest that overall measures in view of favouring gene transcription and translation by inhibiting cell multiplication through temperature shifting, may fail to lead to higher recombinant protein production. In contrast to other systems (Butler 2004), in our recombinant S2 cell system the temperature shifting affected mainly gene expression than cell growth. In fact some protein expression pathways were already shown to be affected by temperature shifting, such as gene regulation (Nie et al. 2006), or posttranscriptional regulatory mechanisms (Washburn et al. 2003). Specific approaches interfering with cell metabolism should be envisaged for tentative successful results. In this sense, it may be of particular interest the induction of a cell cycle arrest, as it could interfere with cell growth without affecting translation rates (Palomares et al. 2004; Yoon et al. 2006; Fujii-Taira et al. 2009).

In conclusion, molecular analytical tools for bioprocess development are becoming available. We have used the qRT-PCR for this purpose and our study showed that the S2 cells expressing an heterologous gene present high amounts of accumulated mRNA at the transition to stationary cell growth phase, when the recombinant protein level is high. These findings can be helpful for establishing better strategies for cell

cultures in view of the production of recombinant protein. This work brings relevant information for researches aiming to better understand the mechanisms underlying the expression pathway (promoter-RNA-protein) in protein synthesis systems.

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