The cell cycle phase affects the potential of cells to replicate *Autographa californica* multiple nucleopolyhedrovirus

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Summary. – We investigated the effect of growth phase of suspension culture of insect Sf9 cells on cell cycle phase distribution, cell viability and *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) production. The cell culture showed a maximum cell viability and potential to replicate the virus at the peak of G_1 phase cells in the culture, while the minimum cell viability coincided with the peak of G_2/M phase cells. These results indicate that the G_1 phase plays a substantial role in the ability of cells to replicate the baculovirus and may help to develop a baculovirus infection dynamics model and control the expression of foreign genes.

Keywords: cell cycle; baculovirus; viability; flow cytometry; infection

Introduction

The baculovirus expression vector system (BEVS) has become one of the most widely used systems for production of recombinant proteins, such as biopesticides and vaccines (Bonning and Hammock, 1992; Kost *et al.,* 2005; Cox, 2008). A thorough understanding of the *in-vitro* kinetics of virus infection helps to design and scale up processes that involve recombinant or wild-type baculovirus infections. A number of mathematical models have been published to describe the infection of insect cells with baculoviruses. Licari and Bailey (1992) developed a model to optimize the time of infection and MOI for a simulated culture; Dee and Shuler (1997) depicted a detailed model that represents the process of infection pictorially; Enden *et al.* (2005) modeled the infection of insect cells in suspension culture at low MOI. These models are very useful in understanding the dynamics of baculovirus-infection process. In addition, many researchers

found that the productivity of the expression system is greatly affected by time of infection (TOI) (Kioukia *et al.,* 1995; Lecina *et al.,* 2006; Power *et al.,* 2010). In fact, the principle of TOI is determined by the distribution of cell cycle and the nutrient condition of the cell culture. If the nutrients are sufficient in the culture at the time of infection, the cell cycle is more significant in affecting the cell status (Elena *et al.,* 1997; Braunagel *et al.,* 1998; Probst *et al.*, 2009; Baumann, 2010). However, none of these models takes cell cycle into account. A number of studies about the effect of the cell cycle on the recombinant protein expression or wild-type baculovirus infection have been reported. In mammalian cells, gene transfer and recombinant protein production were affected by host cell cycles (Springett *et al.,* 1989; Miller *et al.,* 1990; Gu *et al.,* 1993; Fussenegger *et al.,* 1997). Gu *et al.* (1993) and Fussenegger *et al.* (1997) concluded that higher recombinant productivity occurred after infection in either the S phase or in the G_1 phase. In the BEVS, TN-368 cells synchronized in the S phase were more susceptible to AcMNPV infection than cells exposed in the G_2/M phase (Lynn and Hink, 1978). The green fluorescent protein expression corresponded to the profile of the G_1 cell cycle in the BEVS and the infection yield at G_1 or S phase-infection was 1.5–1.8-fold higher than that at G_2/M phase-infection (Saito *et al.,* 2002). An increased viral genome replication

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and recombinant protein production were observed when the infection occurred at later stages of the cell cycle (Haas *et al.,* 2005), which is different from Saito′s results (2002). Therefore, it is clear that the cell cycle distribution of host cells has an important role in the baculovirus infection progression and the amount of progeny virus (non-occluded virus, NOV; occluded virus, OV) or recombinant protein produced. It is, however, not clear how the host cell cycle participates in the virus replication or recombinant gene expression in the BEVS. If the effects of cell cycle on virus production or recombinant gene expression were elucidated, it would be valuable in developing a baculovirus infection dynamics model and in controlling the expression of useful foreign genes to increase the productivity of the recombinant protein and virus.

In this work, we investigated the viability of Sf9 cells, distribution of cell cycle phases and potential to propagate AcMNPV in different growth phases of suspension cell culture. The obtained results lead us to deduce that the G_1 phase plays an essential role in the ability of Sf9 cells to replicate the baculovirus.

Materials and Methods

Cells and viruses. The insect cell line used in this study was Sf9 (CCTCC-GDC0008), a sub-clone of the primary cell line IPBL-Sf-21. Cells were routinely maintained in 25-cm^2 culture flasks (Corning) at 27°C in Grace′s medium (Gibco) supplemented with 10% (*v/v*) FBS (Gibco, AU). For suspension culture, 0.05% (*w/v*) Pluronic F-68 (Sigma) was added. All cell counting was done with a hemocytometer using trypan blue (0.5% *w/v*) exclusion to distinguish dead cells. wt AcMNPV (CCTCC GDV114) was obtained from Professor Qi Yipeng, Wuhan University, China. Sf9 cells were infected with Ac-MNPV at a MOI of 200 NOVs/cell. It must be noted that the unit of MOI is a ratio of viral particles to cell, which is counted by FCM. The traditional unit of MOI is PFU/cell or TCID$_{50}$/cell, both of which are determined by statistics. Obviously, MOI determined by FCM could be many times bigger than by statistics.

Cell viability assay. MTT (Sigma) stock solution was prepared by dissolving 5 mg/ml in culture medium and filtering through a 0.22 μm filter. Cells from $5×10^3$ to $2.5×10^4$ per well were seeded in flat-bottomed 96-well plates in 100 μl of fresh medium. Medium without cells was used as control. 30 μl MTT was added to each well of the plates, which were then incubated under agitation for 4 hr at 27°C. After incubation, the plates were centrifuged (700×g, 10 min) and the supernatants were carefully discarded with an injector. The formazan crystals were solubilized with 100 μl DMSO. Plates were shaken gently for approximately 10 min before A$_{570}$ was measured.

Virus titer assay (flow cytometry of total virus particles). An improved FCM was used to quantitate the baculovirus titer according to Shen *et al.* (2002) and Brussaard *et al.* (2004). Before FCM analysis, virus samples were fixed with 0.1% (*w/v*) paraformaldehyde for 30 min at 4°C, and then stained with a specific nucleic acid dye, SYBR Green I (Molecular Probes), at the dilution $1×10^{-4}$ for 10 min at 80°C. Yellow-green fluorescence microspheres (1 μm in diameter, Molecular Probes) were added to the baculovirus as an internal reference. Samples were analyzed using a FACSCalibur flow cytometer (Becton Dickinson, BD), the threshold was set on green fluorescence to eliminate the background interference. All the parameters were collected in logarithmic model and a total of at least 10,000 events were recorded for each sample. To avoid coincidence of viral particles (i.e., two or more particles being simultaneously within the sensing zone), the samples were diluted so that the total event rate was below 500 events/sec.

Cell cycle phase analysis. $1–2×10^6$ cells were collected in 5 ml Falcon tube, and then washed twice with PBS (pH 7.0). Cells were fixed and permeabilized by adding 1 ml ice-cold 70% ethanol slowly while gently vortexing, the fixed cells were stored at -20°C until the commencement of the cell cycle analysis. Prior to analysis, fixed cells were washed once in PBS and resuspended in 100 μl of RNase A (GenScript) for 30 min at 37°C, 400 μl of propidium iodide (GenScript) was then added, incubated for another 30 min at 4°C and protected from light. At least $1×10^4$ cells were counted in each sample and cell cycle phase distribution was analyzed by ModFit LT 3.2 software (Verity Software House).

Results

Cell cycle progression in suspension culture

First, the cell cycle progression of Sf9 cells in suspension culture was investigated. Cells in late exponential phase were diluted with fresh medium at the same initial density of $6×10^5$ cells/ml in shake flasks. Cell count and cell cycle analysis were taken every 12 hrs, and the results are shown in Fig. 1 and Table 1.

The distribution of exponentially growing cells in the three cell cycle phases is approximately identical at 48 hr post inoculation (Fig. 1 and Table 1). Similar result was reported by Braunagel *et al.* (1998). More evidently, the proportion of cells in G_1 and S phase reached their maximum of 66.7%, and, in particular, the number of cells in G_1 is the highest at this time point. The ratio of cells in G_1 to (G_1+S) also reached its maximum of 44.2% at this time point compared (Table 1), coinciding with a minimum distribution of cells in the G_2/M phase. In addition, it is worth noticing that the percentage of cells in the G_2/M phase started to increase rapidly at 96 hrs, accompanied by the decrease in the number of cells in G_1 phase and S phase. After approximately 108 hrs, cells started to enter into the stationary phase. After 132 hrs, cells entered into a death phase and the percentage of cells in G_2/M phase reached 75.8%. It is suggested that G_2/M phase is the "resting phase" of insect cells similar to the mammalian G_1/G_0 resting phase (Fertig *et al.,* 1990). As it is well known, cell activity is higher in exponential phase but lower in stationary phase. It
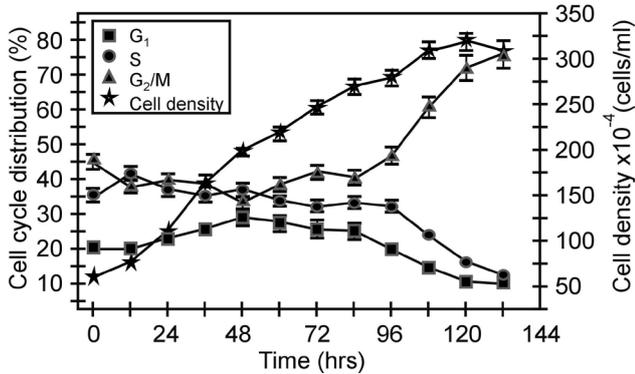
Fig. 1

Distribution of cell cycle phases in various phases of suspension cell culture growth

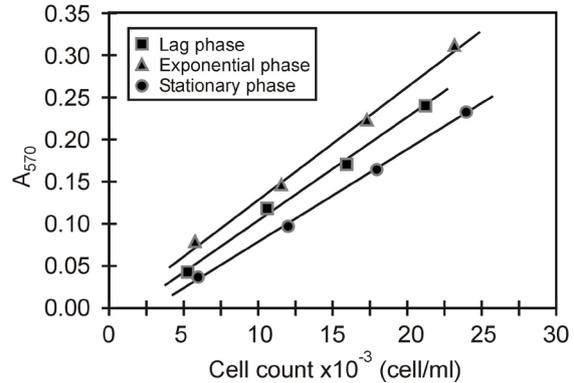Means of three replicates with their range are shown.

Fig. 2

The cell viability in various phases of suspension culture growth

Means of six replicates with their range are shown.

can been seen from Table 1 that maximum proportions of (G_1+S) phase and the ratio of G_1 to (G_1+S) phase were reached in the exponential phase, therefore, we can assume that Sf9 cells in G_1 and S phase have a better cellular activity than in G_2/M phase and that G_1 phase plays a much important role in cellular viability. On the other hand, the decline in the cellular viability and accumulation of cells in the G_2/M phase that occurs upon reaching the stationary phase of cultures are related to the depletion of carbon source. However, the cellular viability in the earlier growth phase with the replacement of medium is still lower than in the exponential phase, where there is a higher proportion of cells in G_1 and S phases (Fig. 2). Therefore, it can be concluded that the cellular viability is closely related to the cell cycle and that the G_1 phase plays a much important role in the cellular viability.

Effect of growth phase of suspension culture on cell viability and virus production

To confirm the assumption made above, we firstly investigated the effect of the growth phase on cell activity. Sf9 cells were seeded into three groups of shake flasks at

Table 1. Distribution of cell cycle phases in various phases of growth of suspension cell culture

Time/hr	G_1	S	G_2/M	(G_1+S)	$G_1/(G_1+S)$
0	20.1	35.0	44.9	55.1	36.5
12	20.2	41.9	37.9	62.1	32.5
24	23.1	36.9	40.0	60.0	38.5
48	29.5	37.2	33.3	66.7	44.2
72	25.5	32.2	42.0	57.7	44.2
96	20.0	32.3	46.8	52.3	38.2
120	10.7	16.7	72.0	27.4	39.1

the same initial cell density (6×10^5 cells/ml). At 12 hrs (lag phase), 48 hrs (exponential phase), 108 hrs (stationary phase) after inoculation, cells were sampled individually for cell viability assay (Fig. 2). In addition, baculovirus was added at the same time (MOI = 200), and the production of progeny virus (NOV and OV) was determined using FCM (Fig. 3 and Fig. 4).

The cell viability assay is a widespread colorimetric method for measuring cell proliferation and cytotoxicity. It is based on the capacity of succinic dehydrogenase of viable cells to transform the MTT tetrazolium salt into MTT formazan (Mosmann *et al.*, 1983). As shown in Fig. 2, a good linear relationship between absorbance (A) and cell number per well was observed when cell density was below 3×10^4 cells/well. In addition, it can be easily discerned that cells from the exponential phase reached higher absorbance values than lag phase and stationary phase with the same cell number. This result indicated that cells in the exponential phase have higher viability than in other growth phases.

Further, the effect of growth phase on the baculovirus production was examined (Fig. 3 and Fig. 4). The results in Fig. 3 show that the cells infected in the exponential phase yielded the highest overall titer of 3.69×10^9 NOVs/ml at 72 hrs post infection. For lag and stationary infection, maximum virus titers were 2.39×10^9 and 2.02×10^9 NOVs/ml, respectively. These results suggest that when cells were infected in the exponential phase, the NOV titer is 1.54- and 1.83-fold higher than that obtained after the infection in lag and stationary phases, respectively. For OVs production (Fig. 4), OVs density after the infection in the exponential phase is 1.4- and 5.3-fold higher than that after the infection in lag and stationary phase, respectively.

Given the results of MTT analysis and the FCM analysis of the cell ability to replicate the baculovirus, it can be con-
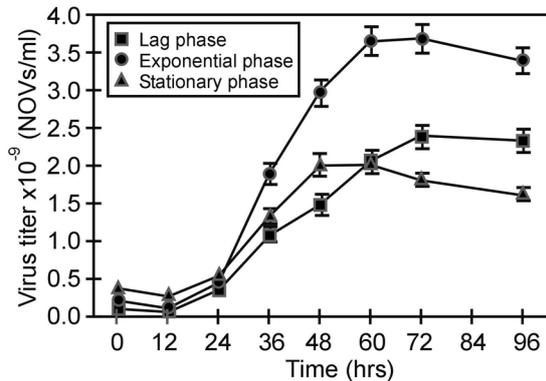
Fig. 3

Production of NOVs by suspension cell culture in various growth phases

Means of three replicates with their range are shown.

Fig. 4

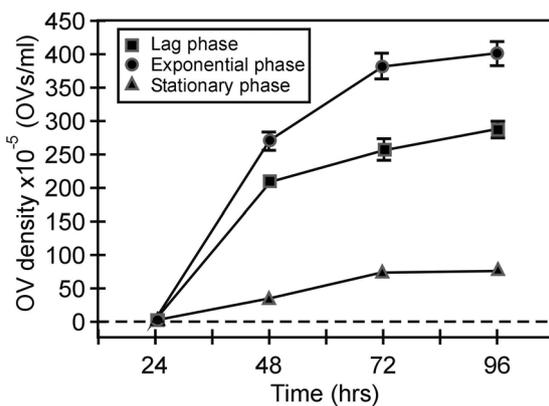**Production of OVs by suspension cell culture in various growth phases**

Means of three replicates with their range are shown.

cluded that Sf9 cells have higher viability and stronger ability to replicate the baculovirus when infected in the exponential phase than in other growth phases. On the other hand, cells in the G_1 phase have a better cellular activity than in S and G_2/M phase. Therefore, it can be deduced that G_1 phase plays a substantial role not only in cell viability, but also in baculovirus infection.

Discussion

Many models for insect cell-baculovirus expression vector system have shown that TOI is one of the most important factors in baculovirus infection. In fact, the virtual differ-

ence in TOI is the difference in cell cycle distribution. In this work, we investigated the cell viability of Sf9 insect cells and their ability to replicate baculovirus in different cell cycle phases using MTT and FCM. The results indicate that cells in the exponential phase, when the amount of cells in the G_1 or (G_1+S) phase reached their maximum, have the highest viability and infection at this stage results in highest virus production. That means that cells in G_1 phase are more active than in S or G_2/M phase and have better ability to replicate the baculovirus.

As it is well known, after viral nucleocapsids uncoat into the nucleoplasm of the host cell, the viral genomic DNA is replicated by means of the host cell transcription machinery (Carstens *et al.,* 1980; Kelly and Wang, 1981). During the G_1 phase, cell components such as enzymes, which recognize a group of early viral genes to initiate their transcription (mRNA production) or translation (protein synthesis), are abundant and active in order to start DNA and RNA synthesis. These may be the reasons that the infection in the G_1 phase results in more effective virus production. Saito *et al.* (2002) made a similar conclusion that the infection in the G_1 phase of the cell cycle is more effective for recombinant expression and infection yield than in the G_2/M phase. However, Lynn and Hink (1978) showed that the efficiency of infection in the S phase of TN-368 cells was higher than that in the G_2 phase. They speculated that cell membrane might change during the cell cycle progression. This result seems to be different from our present results and Saito's conclusion. This might be because the method of cell cycle distribution assay was limited in 1978 or because of the differences of the cell lines. Since Saito et al. and we used the advanced FCM to determine cell cycle distribution, we believe that the conclusion drawn by Saito and us is more convincible. The lowest virus production was observed after infection in the stationary phase, although cells in this phase were infected at a higher cell density than in the other two phases. This may result from the cells (72% in G_2/M phase) in this phase having a reduced capacity for viral DNA synthesis and protein synthesis. In addition, because OV is produced in the very late stages of viral infection, more nutrients are consumed in the culture at this stage, the output of OV is far below the output after the infection in other phases.

Although it may not be realistic to synchronize the cell cycle in the recombinant protein and/or baculovirus insecticide production using the BEVS, it is very necessary to understand whether the cell cycle affects viral replication or recombinant gene expression. Through the quantitative relationship of recombinant protein expression and/or virus replication with the profile of the cell cycle in the BEVS (Saito *et al.,* 2002), a mathematical model could be established. This model would predict and simulate the practical process of recombinant protein and/or baculovirus insecticide production according to the measured cell cycle phase distribution

at the time of infection. The present work has proved that the phase of the cell cycle plays a substantial role in the cellular activity and effectivity of baculovirus infection, which may provide be helpful in developing the baculovirus infection dynamics model and controlling the expression of useful foreign genes using the BEVS.

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