Engineering Chinese hamster ovary (CHO) cells to achieve an inverse growth – associated production of a foreign protein, β -galactosidase

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Abstract

Protein synthesis in mammalian cells can be observed in two strikingly different patterns: 1) production of monoclonal antibodies in hybridoma cultures is typically *inverse* growth associated and 2) production of most therapeutic glycoproteins in recombinant mammalian cell cultures is found to be growth associated. Production of monoclonal antibodies has been easily maximized by culturing hybridoma cells at very low growth rates in high cell density fed-batch or perfusion bioreactors. Applying the same bioreactor techniques to recombinant mammalian cell cultures results in drastically reduced production rates due to their growth associated production kinetics. Optimization of such growth associated production requires high cell growth conditions, such as in repeated batch cultures or chemostat cultures with attendant excess biomass synthesis. Our recent research has demonstrated that this growth associated production in recombinant Chinese hamster ovary (CHO) cells is related to the S (DNA synthesis)-phase specific production due to the SV40 early promoter commonly used for driving the foreign gene expression. Using the stably transfected CHO cell lines synthesizing an intracellular reporter protein under the control of SV40 early promoter, we have recently demonstrated in batch and continuous cultures that the product synthesis is growth associated. We have now replaced this S-phase specific promoter in new expression vectors with the adenovirus major late promoter which was found to be active primarily in the G1-phase and is expected to yield the desirable *inverse* growth associated production behavior. Our results in repeated batch cultures show that the protein synthesis kinetics in this resulting CHO cell line is indeed *inverse* growth associated. Results from continuous and high cell density perfusion culture experiments also indicate a strong *inverse* growth associated protein synthesis. The bioreactor optimization with this desirable *inverse* growth associated production behavior would be much simpler than bioreactor operation for cells with growth associated production.

Introduction

Two strikingly different patterns of the specific glycoprotein production rate versus mammalian cell growth rates are commonly found in the literature. The first pattern, observed primarily in monoclonal antibody production from murine hybridoma cells, shows an *inverse* relationship between the specific (per cell) an-

tibody production rate and the cell growth rate. This essentially means that the specific production rate increases several fold as the specific growth rate decreases in continuous chemostat or perfusion cultures (Miller et al., 1988; Batt et al., 1990; Suzuki and Ollis, 1990). Repeated observations of higher specific monoclonal antibody production rate in many growth limiting culture conditions, such as increased osmolality (Ozturk and Palsson, 1991a), high ammonia concentrations, low and high pH (Miller et al., 1988; Ozturk and Palsson, 1991b) suggest the hypothesis that reduced cell growth rate under these different sub-optimal conditions may be the common mechanistic

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factor causing higher antibody production rates. The second pattern of glycoprotein production rate versus the cell growth rate is observed increasingly in recombinant mammalian cell cultures. In an early example of this pattern, the specific antibody production rate in transfected myeloma cell lines is found to be strongly proportional to growth rate (Robinson and Memmert, 1991). Further examples of this directly proportional relationship between specific production rate and cell growth rate have been observed during the production of recombinant glycoproteins in CHO cells at elevated pCO₂ levels (Aunins and Henzler, 1993; Kimura and Miller, 1996) and at varying serum concentrations (Leelavatharamas et al., 1994).

These two different patterns of protein synthesis have been reconciled by hypothesizing different cell cycle phase specific expression in a mathematical model for an intracellular reporter protein (Gu et al., 1994). The *inverse* relationship between the specific monoclonal antibody production rate and the cell growth rate has been generated by a previous cell cycle model (Suzuki and Ollis, 1989), which assumes that the cells secrete the antibody only during the G1 phase of the mammalian cell cycle. Experimental confirmations of the G1 phase expression of antibody by hybridoma cells have now been obtained by several researchers (Suzuki and Ollis, 1990; Ramirez and Mutharasan, 1990; Kromenaker and Srienc, 1991). Prediction of the *proportional* relationship between the intracellular reporter protein content and the cell growth rate is based on the assumption that the reporter protein is synthesized only during the S (DNA synthesis) phase of the cell cycle (Gu et al., 1994).

Several experimental studies suggest that commonly used promoters and enhancer elements driving the expression of foreign proteins are active maximally in the S phase. Mariani et al. (1981) found S phase specific synthesis of dihydrofolate reductase (DHFR), presumably due to its native promoter, in CHO cells. Kubbies and Stockinger (1990) found that the expression of tissue plasminogen activator (tPA) controlled by the SV40 early promoter in CHO cells was again S phase specific. The expression of β -galactosidase under the control of the cytomegalovirus (CMV) promoter in CHO cells was also shown to be S phase specific (Gu et al., 1993). Kubbies and Stockinger (1990) also found that the expression of DHFR controlled by the adenovirus major late (AML) promoter in CHO cells occurred primarily during G1 phase.

These literature reports suggest that the cell cycle phase in which the foreign genes are expressed is not controlled by the structural gene or by the site of integration on the chromosome, but by the promoter and enhancer elements used in the vector for their expression. For example, changing the promoter and enhancer elements driving the expression of the same structural gene *dhfr* (from its native promoter to the AML promoter) appears to alter the cell cycle phase in which DHFR is expressed from S to G1 phase. Further, the two distinct cell cycle phase expression results (Kubbies and Stockinger, 1990) from the two expression cassettes (SV40 early promoter driving the tPA expression and AML promoter driving the expression of DHFR) on a single plasmid stably transfected and presumably integrated together on the chromosome indicate that cell cycle phase expression characteristics of the two different promoters override any possible cell cycle effects prevalent at the random site of integration on the chromosome. Based on such observations we have hypothesized that the upstream promoter and enhancer elements determine the cell cycle phase in which the downstream structural genes are expressed. The expression of β -galactosidase driven by the S-phase specific SV40 promoter in stably transformed CHO 202 cells was found to be strongly growth associated (Banik et al., 1996), as predicted by Gu et al. (1994). In this paper, we determine the intracellular levels of the same reporter protein, β -galactosidase, expressed from the G1 phase specific AML promoter in stably transfected CHO 202 cells at different growth rates in batch and continuous suspension cultures to investigate whether the predicted *inverse* growth associated production pattern is obtained.

Materials and methods

Cell line construction and maintenance

Escherichia coli XL1-Blue (F' [*proA*⁺ *proB*⁺ *lacI*^q *lacZDM15* Tn10 (*Tet*^R)]/*supE44* *hsdR17* *recA1* *gyrA46* *thi* *relA1* *lac*⁻) (Stratagene, CA) was used for plasmid maintenance and construction. Plasmid (*Amp*^r) containing cells were selected using Luria broth (LB) medium containing 100 mg L⁻¹ ampicillin sodium salt (Sigma), as described in Sambrook et al. (1989).

Restriction endonucleases, alkaline phosphatase, and T4 DNA ligase were obtained from New England Biolabs, MA and Gibco, NY. Plasmid miniprep

Figure 1. Plasmid maps of (a) pAD β , (b) pMAMneo, and (c) pA β GneoR showing relative size, restriction sites, and location of inserted DNA.

and DNA electrophoresis were conducted as described in Sambrook et al., (1989). DNA fragments were purified from agarose gels using the GENECLAN kit (Bio 101, CA). Large-scale plasmid DNA preparation from *E. coli* was carried out using the Qiagen Plasmid Maxi kit (Qiagen, CA). *E. coli* transformations were performed using the calcium chloride method as described earlier by Sambrook et al. (1989).

Plasmid pAD β (Clontech, CA; Figure 1a) carries the bacterial *lacZ* gene under the control of the adenovirus major late (AML) promoter. Transcription of the *lacZ* gene is terminated by employing the polyadenylation (poly A) sequence from the simian virus SV40. The unique SalI site downstream to the

SV40 poly A sequence in the plasmid pAD β was used as a cloning site for the neomycin resistance (*neo*^r) selection marker. Plasmid pMAMneo (Clontech, CA; Figure 1b) contains the *neo*^r gene driven by the SV40 early promoter, and its transcription is also terminated by the SV40 poly A sequence. The *neo*^r gene confers resistance to the antibiotic G418, a neomycin analog, and provides a selection marker in screening plasmid-transfected mammalian cells. This *neo*^r expression cassette (2.9 Kb) was isolated by a BamHI restriction enzyme digestion; both ends of this BamHI fragment was subsequently adapted to SalI. After ligation of this fragment into the unique SalI site of the plasmid pAD β , an insertion with transcription in the reverse

Figure 2. a) Batch culture kinetics of viable and total cell concentration. CHO cells were stably transfected with the plasmid pA β GneoR and grown in suspension culture in spinner flasks. b) The volumetric β -galactosidase concentrations and the intracellular B-gal content versus batch culture time. While the volumetric concentration appears to follow the increase in cell concentration shown on the semilog graph in Figure 2, the intracellular content clearly shows a large increase in the pre-exponential growth phase and gradual decline during mid and post exponential growth phases.

direction as the *lacZ* gene were desired to prevent the potential structural instability due to the homologous recombination between two copies of the SV40 poly A sequence. The construct, including direction of this insertion was confirmed by a double digestion with BglII and XhoI; the final plasmid was designated pA β GneoR (Figure 1c).

Host cell line CHO-202 was stably transfected with plasmid pA β GneoR constructed as described above (containing the *lacZ* gene under the influence of the AML promoter and the gene for neomycin resistance driven by the SV40 early promoter) using LipofectinTM as recommended by the manufacturer (Life Technologies NY.). The transfected cells were cultured and cloned under a selection pressure of G418 (400 $\mu\text{g mL}^{-1}$), and a high producing cell line (2GAR-24) was used in all the experiments described below.

Medium and supplements

The stably transfected clones were routinely maintained in α -MEM (Life Technologies, NY) medium supplemented with 10% fetal calf serum (FCS, Life Technologies). The selected clone was adapted for growth in serum free IS-CHO medium (Irvine Scientific, CA), containing 8 g L⁻¹ glucose and 8 mM glutamine. Antibiotics, penicillin (100 units mL⁻¹) and streptomycin (10⁻⁴g mL⁻¹), were added to the medium. The medium was further supplemented with the neomycin analog G418 (400 $\mu\text{g mL}^{-1}$) for selection pressure.

Cell culture and analytical methods

The recombinant 2GAR-24 cells were adapted to suspension culture in spinner flasks (Techne, Cambridge, U.K.) agitated on a magnetic stirrer at a speed of 45 rpm placed inside a controlled environmental chamber at 37 °C with 5% CO₂. All the data shown in this paper were obtained from cells growing in suspension. Batch culture experiments were carried out in 350 mL spinner flasks with a working volume of 150 mL. Cells were seeded at a density of 1–2 $\times 10^5$ cells mL⁻¹.

Continuous culture experiments were carried out in a 1.5 L Celligen bioreactor (New Brunswick, NJ) with a working volume of 1.1 L. The dissolved oxygen was controlled at 40% air saturation and the pH was maintained at 7.2 $\pm$ 0.1 by sparging a mixture of air, nitrogen, carbon dioxide and oxygen into the culture medium.

Samples from the spinner flasks and the bioreactor were collected at fixed time intervals, and the cell numbers (both viable and total) were determined by counting on a haemocytometer slide after staining with 0.4% trypan blue solution. The activity of intracellular β -galactosidase was measured using the standard o-nitrophenyl β -D-galactoside (ONPG) assay described in more detail by Banik et al. (1996). This kinetic assay is highly reproducible and accurate with a standard error of about 5%.

Results

Batch culture

Stably transfected CHO cells (2GAR 24) were grown in spinner flasks for eight sequential batch cultures, to confirm reproducibility of the results. Data from a single representative batch culture are shown in Figures 2a and b. The cells were seeded at an initial density of 1.8 $\times 10^5$ viable cells mL⁻¹. The cells show an initial lag period and a slow growth rate during the first 24 h. Cells grew exponentially at their maximum specific growth rate till about 48 h, after which the cell growth slowed and cells reach their maximum density at 96 h. The volumetric β -galactosidase concentration (ng mL⁻¹) was found to increase with increase in cell number and reached a maximum value at the end of the exponential phase (Figure 2b). The intracellular β -galactosidase content expressed as ng 10⁻⁶ cells increased rapidly during the initial lag/slow-growth period of 24 h after which a gradual decrease in the intracellular content was observed (Figure 2b).

Continuous culture studies

The continuous culture experiments with the 2GAR 24 cells were carried out in a 1.5 L Celligen bioreactor with a working volume of 1.1 L. The cells were seeded at an inoculum density of 1.8 $\times 10^5$ cells mL⁻¹ and allowed to grow in batch culture initially. Continuous feeding and removal of medium were started during mid-exponential phase. The dilution rates were varied stepwise from 0.3 to 0.65 day⁻¹ after a steady state was well established at each setting. At the dilution rate of 0.65 day⁻¹ steady state concentration of cells could not be reached and wash-out conditions were observed, consistent with $\mu_{\text{max}} \approx 0.76$ day⁻¹ from Figure 2a.

The viable and total cell number concentrations during the continuous culture are shown in Figure 3a.

Figure 3. a) Viable and total cell concentrations of stably transfected CHO cells in continuous bioreactor culture. The dilution rates are changed stepwise after a steady state is well established. Volumetric β -galactosidase concentration; b) and intracellular β -galactosidase content; c) in continuous suspension cultures at different dilution rates.

From these results it can be seen that the steady state concentration of cells was higher at the higher dilution rates. The profiles of the volumetric β -galactosidase concentrations and specific intracellular β -galactosidase content are shown in Figures 3b and c respectively. Although the volumetric β -galactosidase concentration increased along with cell number with an increase in dilution rate, the specific intracellular β -galactosidase content showed the opposite trend and was higher at the lower dilution rates. This trend is clearly seen in Figure 4 where the steady state values of the intracellular β -galactosidase content are plotted as a function of the specific cell growth rate μ . The only exception to this trend occurred at the lowest di-

lution rate of 0.3 day⁻¹, where the cell viabilities were very low.

The specific cell growth rate μ is typically equal to the dilution rate when the cell viability is close to 100%, as in continuous cultures of bacterial cells. In chemostat cultures of mammalian cells, the cell viability is typically in the range of 90 to 95%. Hence the specific cell growth rate is calculated using the equation ($\mu = DX_T/X_V$) obtained from steady state cell balances. During the transient washout state observed at the highest dilution rate shown in Figure 3, the intracellular β -galactosidase content reaches a steady state after the initial transients during the first 200 hr, since the cells maintain their highest specific growth rate of

0.76 day^{-1} , as calculated from the transient balance and also confirmed by the batch culture data shown in Figure 2.

Discussion

Several investigators (Gu et al., 1994; Suzuki and Ollis, 1989) have suggested that foreign protein production can be maximized by utilizing different bioreactor operating strategies appropriate for the particular pattern in which the foreign production varies with the cell growth rate. For example, the *inverse*-growth associated relationship between the production rates and growth rate, may be exploited by growing cells at reduced growth rates in high cell density fed-batch or perfusion cultures to maximize the production of foreign proteins. A directly proportional relationship between the two will require maintaining the cells at high cell growth rates in repeated batch cultures or cause severely reduced production during the low growth fed-batch and perfusion cultures. For hybridoma cultures, which primarily exhibit the inverse relationship between the specific antibody production rate and cell growth rate (Miller et al., 1988; Suzuki and Ollis, 1990), the volumetric monoclonal antibody production rate can be increased several fold by growing cells at low specific growth rates in fed-batch (Bibila and Robinson, 1995) and continuous perfusion cultures (Batt et al., 1990).

In contrast, glycoprotein production in recombinant mammalian cells is typically found to be strongly growth associated with respect to their specific productivity (Aunins and Henzler, 1993; Leelavaatcharamas et al., 1994; Robinson and Memmert, 1991). This directly proportional pattern of specific production rate with growth rate requires constant high cell growth rates such as in repeated batch cultures for the maximization of production rate. However, as the CHO cells are merely the catalysts or mini-factories for the synthesis of glycoproteins it is undesirable to keep on producing these cells, when the real objective is to maximize the synthesis of glycoproteins. High cell density fed-batch or perfusion cultures, which represent the optimized production systems for hybridoma cells, however, cause severely reduced glycoprotein synthesis in cells possessing a directly proportional (relationship) between the production rate and growth rate.

The intracellular content of foreign protein has been predicted to either increase (for S-phase synthe-

Figure 4. The *inverse*-growth-associated pattern of intracellular β -galactosidase content versus the specific growth rate, obtained in chemostat cultures of CHO cells expressing β -galactosidase from the G1 phase specific AML promoter.

sis) or decrease (for G1-phase synthesis) with growth rate, depending on the cell cycle specific promoters that control the expression of foreign genes. Recent studies in our laboratory (Banik et al., 1996) have shown that the expression of an intracellular reporter protein (β -galactosidase) under the control of the S-phase specific SV40 promoter was growth associated in recombinant CHO cells, i.e., the steady state intracellular β -galactosidase content was found to increase almost linearly with the increasing specific cell growth rate in chemostat cultures. The same host cell line (CHO 202) stably transfected with a similar expression plasmid, but containing the G1-phase specific AML promoter for driving the expression of the same reporter protein, reverses the variation of intracellular β -galactosidase content with cell growth rate. The chemostat results summarized in Figure 4 show that the intracellular content of the reporter protein increases as the dilution rate and thereby the specific growth rate is decreased. Thus by simply switching to the G1 phase specific AML promoter, we have demonstrated that the intracellular foreign protein production can be switched from the undesirable growth associated pattern to the more desirable *inverse*-growth associated pattern.

The batch results shown in Figures 2a and b are consistent with these *inverse*-growth associated chemostat results in Figure 4 during the early pre-exponential slow growth phase, but not during the late-exponential slow growth period. Further in chemostat culture at the lowest dilution rate studied, the intracellular β -galactosidase content and the cell viability were significantly lower than the trend shown in Figure 4. It is suspected that the accumulated metabolic

by-products and dead cells in these conditions may have some bearing on these results. We are now re-investigating them through the selective removal of these components in a continuous perfusion bioreactor with an attached inclined settler (Searles et al., 1993; Batt et al., 1990). Nevertheless, the *inverse*-growth associated pattern of intracellular β -galactosidase reported here has been found to be reproducible for these stably transfected CHO cells in subsequent chemostat cultures at these medium to high cell growth rates. If this *inverse*-growth associated production pattern with the G1 phase specific AML promoter is reproduced with secreted reporter proteins in future experiments, such results will have significant impact on the maximizing of glycoprotein production rates from the slow growing CHO cells in high cell density fed-batch or perfusion bioreactors.

Conclusions

Switching from the S-phase specific SV40 promoter to the G1 phase specific AML promoter for driving the expression of the intracellular reporter protein β -galactosidase in a stably transfected CHO cell line, modifies intracellular β -galactosidase production from the growth associated to the more desirable *inverse*-growth associated pattern. These findings confirm our earlier predictions (Gu et al., 1994) concerning the effects of cell cycle phase specific expression characteristics. If these findings are reproduced with secreted reporter proteins in future chemostat cultures of recombinant CHO cells, they will have a large impact on maximizing glycoprotein synthesis in production scale bioreactors.

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