Cell cycle phase dependent productivity of a recombinant Chinese hamster ovary cell line

Roshni L. Dutton · Jenö Scharer ·
Murray Moo-Young

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Abstract A Chinese Hamster Ovary cell line, CHO1-15₅₀₀, producing recombinant human tissue type plasminogen activator (tPA) via the dihydrofolate reductase (DHFR) amplification system, was studied in batch culture. In this system both DHFR and tPA are under the control of the strong constitutive viral SV40 early promoter. Employing the cumulative viable cell-hour approach, the specific productivity of tPA had maxima in the lag ($0.065 \text{ pg cell}^{-1} \text{ h}^{-1}$) and early decline ($0.040 \text{ pg cell}^{-1} \text{ h}^{-1}$) population growth phases. The viable population was assigned into four subpopulations (G1, S, G2/M phase, and Apoptotic cells) using flow cytometric analysis. As expected, intracellular DHFR was maximally expressed during the S cell cycle phase. The production of tPA, however, was found to be a direct linear function of the G1 phase, with a subpopulation specific productivity of $0.080 \text{ pg c-h}^{-1}$. Productivity maxima in the lag and early decline corroborate the flow cytometric data, indicative that this recombinant tPA production occurs primarily in the G1 cell cycle phase, not

the S phase. This suggests that endogenous regulatory mechanisms are important controlling influences on the production of recombinant tPA in this ovarian cell line. Productivity from recombinant cell lines cannot be inferred from either the plasmid construct or the host cell alone. Elucidation of the relationship between expression of recombinant protein and the cell cycle phases of the host cell is a major component of the characterization of the animal cell production system. This information facilitates rational process design, including operating mode, modelling and control, and medium formulation.

Keywords Cell cycle associated protein productivity · Chinese hamster ovary (CHO) · DHFR · Regulation of expression · Specific productivity · Tissue plasminogen activator (tPA)

Abbreviation

CH_{vol} volumetric cell-hours

R. L. Dutton (✉)
BioProcess Assist (BPA) Ltd, 8 Maple Leaf Lane,
Aberfoyle, ON, Canada N1H 6H9
e-mail: roshni.dutton@sympatico.ca

J. Scharer · M. Moo-Young
Department of Chemical Engineering, University
of Waterloo, Waterloo, ON, Canada

Introduction

In recombinant protein production by animal cell culture, one of the most important characteristics determining specific productivity is the relationship between protein expression and the phases of

the cell cycle. Expression patterns of various secreted recombinant proteins from Chinese hamster ovary (CHO) host cells have been evaluated from measurements including external protein, internal protein, and messenger RNA of the protein, or inferred from the plasmid construct (Chevalot et al. 1994; Croughan and Wang 1990; de Boer et al. 2004; Fann et al. 2000; Gu et al. 1993; Hayter et al. 1993). However, synthesis of a complete protein is regulated at many stages. Even after accurate translation of the relevant mRNA, many proteins produced in animal cells undergo a number of post-translational modifications and processing steps before the mature protein appears (Cartwright 1994). It seems that these processing steps, including secretory mechanisms where applicable, may be limiting in cells that produce high levels of heterologous mRNA (Freedman et al. 1995). In addition to being the rate-limiting step, secretion may itself be a cell cycle dependent process (Gu et al. 1993).

In the CHO1-15₅₀₀ cell line, transcription of *tPA* and of *dhfr* are both under the influence of the constitutive SV40 early promoter. Given constitutive transcription, production of DHFR and tPA can be expected to be independent of population growth phase. However, the expression patterns of a protein and of its mRNA can differ (James et al. 1996; Ohta et al. 1996). DHFR, a key enzyme in the synthesis of DNA, begins to be expressed during the late G1 cell cycle phase, with maximum expression observed during the S cell cycle phase. Endogenous DHFR hnRNA is present at approximately constant levels throughout the cell cycle, while the levels of DHFR mRNA increase from the G1 cell cycle phase throughout the S cell cycle phase with accompanying increases in the intracellular levels of the DHFR protein (Johnson 1984). This implies that native *dhfr* is transcribed constitutively, but that post-transcriptional modifications are regulated and tied to the cell cycle. The increase in the rate of DHFR synthesis during the S cell cycle phase is due to an increase in the content of mature DHFR mRNA and perhaps an increase in the efficiency of translation of the message, but not due to a change in the rate of transcription (Johnson 1984).

When the gene of a protein such as tPA is co-transfected with *dhfr* into a DHFR-negative host cell, and methotrexate selective pressure is applied, both genes are frequently co-inserted as stable constructs into various chromosomal regions (Kubbies and Stockinger 1990). This observation has led many workers to assume that with this type of plasmid construct, tPA should be co-expressed with DHFR during the S cell cycle phase (Banik et al. 1996; Gu et al. 1993; Mariani et al. 1981). Even assuming that transcription of *tPA* occurs coincidentally with transcription of *dhfr*, there is no reason to suppose post-transcriptional modifications of the hnRNA molecules are under the same control mechanisms, let alone subsequent translation of the mature mRNA molecules and protein trafficking of the intracellular DHFR and secreted tPA. There is no a priori reason to suppose that post-transcriptional through post-translational processing of the complete DHFR and tPA proteins would be co-regulated. In this study tPA and DHFR production from the CHO 1-15₅₀₀ cell line is examined in conjunction with the cell cycle.

Materials and methods

Recombinant tPA in CHO

The cell line used in all experiments was a CHO cell (CHO1-15₅₀₀) producing recombinant human tissue type plasminogen activator (tPA), via the dihydrofolate reductase (DHFR) vector and amplification system developed by Genentech Inc. (US patent 4,766,075, 1988). The strong constitutive viral SV40 early promoter directs transcription of both recombinant proteins. The plasmid was transfected into *dhfr*⁻ CHO-DUX B11 cells, and *dhfr*⁺ cells were selected by growth in glycine, hypoxanthine, and thymidine deficient medium. Amplification in 500 nmol L⁻¹ MTX resulted in a stable cell line producing amounts of tPA in excess of 1 µg/million cells/day (ATCC # CRL 9606).

Upon obtaining the CHO1-15₅₀₀ cell line from ATCC, the population was expanded serially for 10 days (approximately 14 population doublings) as an adherent monolayer in T-flasks. The

expanded cell population was then frozen in aliquots of 2.0×10^7 cells, in 1.25 mL of 7% dimethylsulfoxide (DMSO) (Sigma-Aldrich) in FBS (Flow Laboratories), and stored in the vapour phase of a liquid nitrogen dewar for use as the seed cultures for the experiments. Inocula for experiments were prepared from seed culture in T-flasks as described below. Inocula were expanded for between 5 and 10 population doublings.

Seed culture and inocula were grown in Ham's F12 basal medium (Sigma-Aldrich), supplemented with 50 IU L⁻¹ penicillin and 50 mg L⁻¹ streptomycin (MP Biomedicals), 4 g L⁻¹ HEPES buffer (Sigma-Aldrich), and 2 g L⁻¹ sodium bicarbonate (Sigma-Aldrich), and the glucose content enriched to 40 mmol L⁻¹ (Sigma-Aldrich). The basal medium was further supplemented at time of use with 4 mmol L⁻¹ glutamine (Sigma-Aldrich) and 10% v/v FBS (seed culture) or 5% v/v FBS (inocula) (Flow Laboratories).

HB-CHO serum free basal medium (Irvine Scientific) was supplemented with 50 IU L⁻¹ penicillin and 50 mg L⁻¹ streptomycin, 4 g L⁻¹ HEPES buffer, and 2 g L⁻¹ sodium bicarbonate, and the glucose content enriched to either 8 mmol L⁻¹ or 40 mmol L⁻¹. The basal medium was further supplemented at time of use with 4 mmol L⁻¹ glutamine and 10 mL L⁻¹ HB-CHO supplement.

Experimental design

Cells were propagated as dispersed suspensions in 1,500 mL (1,000 mL working volume) spinner flasks, agitated at 150 rpm, and housed in a 37 °C, 95% humidity incubator, supplied with 5% CO₂ in air. These normally attached cells converted to suspension culture rapidly in the absence of serum and under the shearing imparted by the agitation rate. Normal population growth curves are attained in the first batch culture period with this cell line, so that an adaptation period is not required. An adequate supply of oxygen was introduced to the system by headspace aeration (Glacken et al. 1983). An inoculum consisting of 0.25×10^6 cells mL⁻¹ was charged into 100% fresh pre-warmed and gassed medium. Apart from the

HEPES and bicarbonate-CO₂ buffering systems, no additional pH control was employed.

The CHO1-15₅₀₀ cell line was propagated in up to four replicates as a 1 L dispersed suspension batch culture in 1.5 L spinner flasks agitated at 150 rpm with serum free HB-CHO medium containing either 8 mmol L⁻¹ or 40 mmol L⁻¹ glucose. Growth and tPA productivity patterns were evaluated with respect to the whole population and to the subpopulations residing within each of the cell cycle phases. Flow cytometry was employed in assigning the cells to the sub-populations.

A second set of experiments was designed to examine productivity during the G1 cell cycle phase. Density-arrested populations were prepared by growing cell populations as adherent monolayers in 225-cm² T-flasks, using medium containing 5% FBS. Once confluence was achieved, the populations were maintained for two months by replacing the medium daily to ensure that nutrient limitation and biowaste toxicity were not factors affecting cell physiology.

The pattern of expression of intracellular DHFR with respect to the cell cycle was also investigated. Growth and relative DHFR productivity patterns from four replicate cultures were evaluated with respect to the subpopulations residing within each of the cell cycle phases. Flow cytometry was employed in assigning the cells to the sub-populations and in measuring the relative fluorescence associated with intracellular DHFR.

Cell density and viability measurements

Suspended cells were counted with a haemocytometer under an inverted microscope. Adherent cells were detached prior to counting with 25 mg mL⁻¹ of the serine protease porcine trypsin in phosphate buffered saline. Cell viability was determined using the Trypan Blue Exclusion Test (Phillips 1973). The cells were counted on five fields of a haemocytometer. The counts were carried out within one minute of dye addition to ensure that only the dead cells stained blue. Only dark staining cells were considered nonviable. It was noted that the suspended viable CHO cells were highly refractive when viewed under the light microscope.

Counts of refractive and opaque cells precisely matched counts of non-stained and darkly stained cells, respectively. The two methods of determination of viability were used in conjunction. Percent viability determinations were repeatable to $\pm 2\%$.

Measurement of extracellular tPA

Tissue plasminogen activator concentration was determined by a micro volume modification of the Chromozym t-PATM spectrophotometric assay (Boehringer Mannheim). Chromozymt-PATM is cleaved by tPA to yield the chromophore 4-nitraniline, which is detected at 405 nm. Absorbance had a linear correlation to pure tPA (Sigma-Aldrich) concentration between 0 and $4 \mu\text{g mL}^{-1}$ ($r^2 = 0.9991$). Triplicates of $10 \mu\text{L}$ samples, diluted as necessary in Tris buffer (100 mmol L^{-1} Tris, pH 8.5; Tween 80, 0.15% w/v) (Sigma-Aldrich), were placed in 96-well microplates, with $50 \mu\text{L}$ of 1 mmol L^{-1} Chromozym t-PATM in Tris buffer. Plates were incubated at 37°C for 1 h. The cleavage reaction was halted with 10% w/v citric acid (Sigma-Aldrich), and absorbance was measured immediately. Results for serum-free samples were accurate to $\pm 5\%$, while serum-containing samples were less accurate, at $\pm 12\%$. The activity of tPA was confirmed by the ability to dissolve a standard fibrin clot containing plasminogen, as monitored by our piezoelectric based method (Hayward et al. 1998).

Relative specific DNA and RNA content (cell cycle phase)

Cell cycle phase measurements were made with a three colour EPICS XL-MCL flow cytometer with System II Software (Coulter Corporation) using standard optical configuration. A gate was employed to exclude cellular debris and cell aggregates.

Greater than 99% of the samples fell within the $8\text{--}20 \mu\text{m}$ flow cytometric gate through mid-exponential phase in all cultures. Thereafter the gated percentage of samples fell to approximately 95%. Most excluded particles had a diameter greater than $20 \mu\text{m}$, and were most likely 2–5 cell aggregates,

which could be observed microscopically from the mid exponential phase until the late decline phase. Aggregates were excluded from consideration in interpreting these data, due to the complexity they add to the segregation of the population on the basis of DNA content. It was only in the late decline phase that the gated sample portions fell below 90%, and cellular debris indicative of cell lysis was seen under the microscope.

The DNA and RNA content per cell was determined with an Acridine Orange staining method (Darzynkiewicz and Hoffmann 1994). Stained samples were excited with the 488 nm line from a 5 W argon ion laser at 400 mW, within 2–10 min of staining. Fluorescence emission at 520 nm from DNA, and at 640 nm from RNA, was detected by passing the excited samples through the bandpass filters of a tri-channel flow cytometer. Mean fluorescence intensities were calculated from the 256 channel, integrated linear fluorescence histograms (System II Software, Coulter). The percentage of the population within each of the G1, S, G2/M, and A subpopulations was calculated from the proportion of the sample having relative DNA associated fluorescence (emission at 520 nm) of 1, >1 but <2 , 2, and <1 , respectively (Milner et al. 1996). The data were fitted to Gaussian distributions for the Apoptotic cells, G1 and G2/M phase cells. An extended polynomial fit was employed for the S phase cells (Multicycle DNA Analysis Software, Phoenix Flow Systems).

Intracellular DHFR content

DHFR content of individual cells was measured by an adaptation of the methods of Assaraf et al. (1989) and Kaufman et al. (1978). Aliquots of permeabilized sample were prepared as above. The DHFR was then immediately stained by adding MTX ligated to the fluorochrome fluorescein (F-MTX) (Molecular Probes Inc), and incubating on ice for 2 min. The cells were washed three times with basal medium to remove unbound F-MTX by centrifuging at $2,000g$, decanting the supernatant, and resuspending the cell pellet by gentle vortexing in the initial volume of basal medium. F-MTX analysis utilized excitation at

488 nm line from a 5 W argon ion laser at 400 mW, within 20–30 min of staining. Fluorescence emission was detected at 520 nm by passing the excited samples through the bandpass filters of a tri-channel flow cytometer. Mean fluorescence intensities were calculated from the 256 channel integrated linear fluorescence histograms. Relative DHFR content was assessed qualitatively from fluorescence peak heights and widths.

Data analysis

Cumulative volumetric cell-hours

The biological capacity for production can be quantitated as the cumulative volumetric cell-hours ($Xvdt$, CH_{vol}), as described previously (Dutton et al. 1998):

$$CH_{vol} = \sum_{j=1}^{f-1} \frac{(Xv_{j+1} - Xv_j)}{\ln(Xv_{j+1} / Xv_j)} (t_{j+1} - t_j) \quad (1)$$

The viable cell concentration, Xv , is calculated from the measured total cell concentration and the determined fractional viability at each sampling point, t .

The distribution of cellular DNA content provides a basis for classifying cells into G1, S, G2/M phase, or A fraction. The fractions of the population in each subcompartment, f_{G1} , f_S , $f_{G2/M}$, and f_A , are determined with the Multicycle DNA Analysis Software for each sampling point. The cumulative volumetric cell-hours spent in each phase by the population is then calculated by restricting the context of Eq. (1) as formulated in Eq. (2):

$$CH_{Sub\ vol} = \sum_{j=1}^{n-1} \frac{(Xv_{j+1}f_{Subj+1} - Xv_jf_{Subj})}{\ln \frac{(Xv_{j+1}f_{Subj+1})}{(Xv_jf_{Subj})}} (t_{j+1} - t_j) \quad (2)$$

where Sub = G1, S, G2/M, or A subpopulation.

Specific productivity

The specific protein production rate is evaluated as a function of the length of time spent in the culture by viable cells, as discussed elsewhere

(Dutton et al. 1998). Specific productivity (q_P , mass/cell-hour) is equal to the slope of the measured cumulative product titre (P , mass/volume) linearly regressed against the calculated cumulative volumetric cell-hours:

$$q_P = \frac{P}{CH_{vol}} \quad (3)$$

$$q_{P_{Sub}} = \frac{P}{CH_{Sub\ vol}} \quad (4)$$

Results and discussion

Batch population growth and tPA production

A representative batch culture of CHO1-15₅₀₀ cells is depicted in Fig. 1. Productivity of tPA is a function of cell-hours, rather than just the number of cells in the system. Thus, the first step in considering productivity kinetics is to convert cell concentrations into volumetric cell-hours, CH_{vol} . This integral is the population time available for metabolic activity, both primary and secondary, including the processing of endogenous and recombinant proteins (Dutton et al. 1998). As shown in Fig. 1, the cell-hours in a batch culture continue to increase rapidly through the early decline phase. It is noteworthy that with this cell line, approximately 60% of the total cell-hours accumulated during the decline phase. CH_{vol} is a direct quantification of the “load” on the medium, while the cumulative volumetric cell-hours attained (i.e. the maximum of CH_{vol}) is a quantification of the success both of population growth and maintenance and of medium utilization by the population. This culture attained a remarkable maximum CH_{vol} of 450×10^6 c-h mL⁻¹. By comparison, a typical hybridoma batch suspension culture attains a maximum CH_{vol} of approximately 150×10^6 c-h mL⁻¹ (Dutton et al. 1999).

As anticipated, productivity extended well beyond the exponential growth phase (Lloyd et al. 2000), with approximately 30% of the extracellular tPA accumulating during the early decline phase. The CHO1-15₅₀₀ cell line exhibits

Fig. 1 Chinese hamster ovary cells, CHO1-15₅₀₀, producing recombinant human tissue type plasminogen activator (tPA). Cells were grown as a 1 L dispersed suspension culture using a serum free medium. **(A)** Cell density and cumulative product titer. **(B)** Cumulative volumetric cell-hours (CHvol) and cumulative product titer

growth-phase-associated productivity as indicated by three distinct lines that are of constant slope within each growth phase, with the slope being a quantitative measure of the specific productivity. In two batch cultures of CHO1-15₅₀₀ cells (Fig. 2) that differed only by initial glucose concentration, these patterns of productivity within the batches were similar, with the highest rate of specific productivity occurring in the lag phase, followed by the early decline and exponential stages. Between batches, the specific productivity was similar in all stages of the cultures, with the exception of the early decline phase, in which the specific productivity decreased with the initial glucose concentration. Surprisingly, under conditions of glucose limitation, the specific productivity increased during the early decline phase to approximately twice that of the exponential phase (Fig. 2B). The specific productivity from these cultures was not consistent with S-phase specific protein production, the suggested pattern for this vector construct, in which maximum specific productivity

Fig. 2 Cumulative protein concentration as a function of the cumulative volumetric cell-hours. The slope of the curve is the specific productivity. The specific productivity of recombinant human tPA from the CHO1-15₅₀₀ cell line is growth-phase-associated. The specific productivity changes with culture conditions. **(A)** High initial glucose concentration of 40 mmol L⁻¹. **(B)** Low initial glucose concentration of 8 mmol L⁻¹

would be expected during the exponential growth phase (Banik et al. 1996; Gu et al. 1993; Kubbies and Stockinger 1990; Mariani et al. 1981).

As expected, productivity in both cultures became negative in the late decline phase, which can be attributed to the combination of declining secretion and enhanced degradation of extracellular tPA (Fig. 2). Excluding the late decline phase from consideration, the specific productivity was found to be inversely related to the specific growth rate (i.e. negatively growth-associated) in the CHO1-15₅₀₀ cell line. This finding is in agreement with several investigators who have correlated reduced specific growth rate to increased recombinant protein productivity for CHO cell lines utilizing the DHFR amplification system (Fox et al. 2005; Fussenegger et al. 1997; Hayduk and Lee 2005; Hendrick et al. 2001; Kaufmann et al. 1999; Yoon et al. 2003, 2005). Although not conclusive, this negative growth-association suggests

that the protein may be preferentially or exclusively produced during the G1 cell cycle phase.

The segregated population

Subpopulations of cells in G1, S, G2/M, and A were identified based on the distribution of relative DNA content per cell, as determined through flow cytometric analyses. The population dynamics are presented in Fig. 3. Commencing with an inoculum of 0.25×10^6 viable cells per millilitre, cells accumulated in the G1 phase during the first three days of the batch culture. This is consistent with G1 arrest associated with the lag phase, in response to the step change in environment associated with inoculation. Typically, a large proportion of a culture resides in the G1 cell cycle phase immediately after inoculation, as can be observed for this CHO cell line where approximately 77% of the population was found to be in G1 during the lag

population phase (Fig. 3). It can be seen from Fig. 3 that the population exited the lag phase in partial synchrony, with peaks in the subpopulations representing the S, G2/M, and G1 phases.

Following the lag phase, some of the cells entered the A (apoptotic) subcompartment, but the majority of the cells re-entered the cycling fraction of the population. This was seen as an increase of the S phase subpopulation between 100 and 200 h of batch culture (Fig. 3).

The re-entry of the G1 subpopulation into the cycling fraction following the lag phase appears to be consistent with the probabilistic model proposed by Smith and Martin (1973), in which random re-entry of G1 cells into the cycling fraction is coupled with a portion of the population not arrested in G1, thereby preventing complete synchrony of the population. Peaks in the S phase at approximately 125, 200, and 290 h preceded peaks in the G2/M phase (Fig. 3),

Fig. 3 Population growth dynamics of CHO1-15₅₀₀ cells in dispersed suspension batch culture using serum free medium, from an inoculum grown in serum containing medium. The population is divided into four subpopulations on the basis of relative DNA per cell. **(A)** Entire viable population. The population accumulates in G1 during the lag phase and then traverses the cell cycle in partial synchrony. The population accumulates in the apoptotic subcompartment during the late decline phase. **(B)** Cycling fraction of the viable population (A subpopulation excluded). The cycling fraction accumulates in G1 both during the lag phase and again during the late decline phase

supporting the interpretation of partially synchronized traverse of the viable population through the cell cycle.

After approximately three population doublings, the cells entered the decline phase (Fig. 1A). Under the conditions of this experiment, the viable cell concentration decreased smoothly through most of the decline phase, at a constant specific rate of 0.01 h^{-1} . By the late decline phase, most of the viable population was dying through apoptosis, and the rate of decline diminished (Figs. 1, 3).

At 300 h, well into the decline phase, cells once again accumulated in the G1 phase (Figs. 1, 3). This is consistent with the findings of Leelavatcharamas et al. (1999) who have further shown that supplementing with depleted nutrients during the population decline phase promotes the accumulated G1 cells to traverse the S and G2/M phases. Here, in the absence of limiting nutrient supplementation, at approximately 340 h there was a sharp decrease of cells in all of the cycling phases and a corresponding increase in the size of the A subpopulation. The fact that cells accumulated in the G1 phase just prior to the increase in the A subpopulation suggests that cells enter apoptosis from the G1 phase. This is substantiated by considering the relative size of the subpopulations of only the cycling fraction of the viable cells (Fig. 3B). It can be clearly seen that almost the entire cycling fraction resided in the G1 phase by the end of the batch culture.

In asynchronous growth, 50–80% of the viable population resides in the S cell cycle phase during the early exponential growth phase (Lakhotia et al. 1992), which corresponds to a 12- to 20-h cell cycle. The proportion in S phase decreases through the remainder of the exponential growth phase, as the proportion in G1 phase increases, presumably as cells arrest in G1 in response to diminishing nutrient pools (Lakhotia et al. 1992). In asynchronous growth, the proportion of the population in G2/M remains relatively constant at 8–10% until the late exponential growth phase, when it begins to increase. The proportion of the population in S phase during the exponential phase of a batch culture typically varies between 25% and 45% (Leelavatcharamas et al. 1999), suggesting that batch cultures are not typically

completely asynchronous. In this culture, the proportion of the viable population in S phase never rose above 60%; while the proportion in G1 remained high during early exponential growth phase, falling to approximately 20% only in mid and late exponential growth phase.

The partially synchrony of this culture was anticipated as a response to the step change in environmental conditions encountered at inoculation, primarily due to the removal of serum. However flow cytometric analyses of batch cultures that differed in terms of the initial concentrations of lactate, glucose, glutamine and serum, all followed a remarkably similar trend (data not shown). In all cases, it was seen that asynchrony was only achieved for about 24 h during the early exponential phase. The viable population was accumulated in the G1 phase during the first 3 days of culture, and resided primarily in the apoptotic state by the end of the batch culture.

Recombinant protein productivity as a function of cell cycle

Cells from a population residing within a given (or specific) cell cycle phase are in a more or less similar physiological state. Thus, it is reasonable to expect that if protein were produced only during a particular cell cycle phase, then the rate of production per cell-hour within that cell-cycle phase subpopulation would be approximately constant. When the tPA concentration was plotted as a function of the individual subpopulations (Fig. 4), it could be seen that the tPA production was directly proportional to the cell-hours that the viable population spends in the G1 phase, strongly suggesting that secretion occurs primarily during this cell cycle phase. Empirical correlations can certainly be developed to describe a relationship between accumulation of tPA and each of the other subpopulations (Fig. 4). However, for these non-linear relationships to hold, the tPA production rate must be variable in each of these cell cycle phases over the duration of the batch, which clearly is less parsimonious.

The specific productivity calculated as a function of the G1 cell cycle phase was $0.080\text{ pg cell-h}^{-1}$

Fig. 4 Production of the recombinant human tPA is a linear function of the G1 cell cycle phase cell-hours, with a calculated specific productivity of $0.080 \text{ pg c-h}^{-1}$. This pattern suggests that tPA is produced during the G1 cell cycle phase in the CHO1-15₅₀₀ cell line. (Top left) Recombinant protein as a function of the G1 phase

cell-hours. (Top right) Recombinant protein as a function of the S phase cell-hours. (Bottom left) Recombinant protein as a function of the G2/M phase cell-hours. (Bottom right) Recombinant protein as a function of the A subpopulation cell-hours

(Fig. 4, top left). The whole-population specific productivity during the lag phase was 75% of this value (Fig. 2), correlating well with the proportion of the population that was in the G1 cell cycle phase during the lag period (Fig. 3a), and further substantiating the interpretation that production of tPA is associated with this cell cycle phase.

These findings suggest that a key aspect of batch culture operation for optimum tPA production from this CHO cell line is the maximization of the proportion of the population in the G1 cell cycle phase, rather than the achievement and prolongation of a maximum specific growth rate during the exponential growth phase. An additional optimization strategy would be prevention of the onset of apoptosis after cessation of growth, at which time cells are accumulating in the G1 cell cycle phase. Identification of regulatory mechanisms for cell cycle progression and of associated environmental manipulation are emerging areas of research (Balcarcel and Stephanopoulos 2001; Bjorklund et al. 2006; Carvalhal et al. 2003; deZengotita et al. 2001; Ibarra et al. 2003; Sun et al. 2004; Sunstrom et al. 2000).

Protein production from a density-arrested population

A density-arrested population was prepared in order to further test the apparent G1 phase association of tPA production. From the analysis of the DNA profile from this culture (Fig. 5), less than 12% of the population was seen to be in S and G2/M phases, while less than 4% of the population could be assigned to the apoptotic compartment. Volumetric productivity was maintained at an approximately constant value, $0.381 \text{ mg L}^{-1} \text{ h}^{-1}$, throughout the test period, with a corresponding whole-population specific productivity of $0.067 \text{ pg c-h}^{-1}$, and G1 phase specific productivity of $0.081 \text{ pg c-h}^{-1}$. These specific productivities correlate well with the 84% of the population that resided within the G1 cell cycle phase (Fig. 5), and confirm that tPA production is associated with the G1 cell cycle phase for this CHO cell line, in agreement with Hendrick and co-workers (2001). The cellular and molecular mechanisms underlying the effect of growth arrest on the productivity of mammalian cells are poorly

Fig. 5 Density arrested population of CHO1-15₅₀₀ cells. Cells were maintained as a confluent monolayer on a 225-cm² T-flask, using medium containing 5% FBS. The majority of the population resides in the G1 cell cycle phase. The whole population specific productivity was calculated to be 0.067 pg c-h⁻¹. The G1 associated specific productivity, calculated to be 0.081 pg c-h⁻¹, correlates well with the proportion of the population in the G1 cell cycle phase (84%)

understood (Al-Fageeh et al. 2006). Here, however, the specific productivity correlated to the G1 cell cycle phase was found to be the same for the density arrested population as for the population in batch culture.

DHFR expression

To examine DHFR expression patterns in the CHO1-15₅₀₀ cell line, the specific DHFR levels in the different cell cycle phases were investigated (Fig. 6). The partial synchrony of the population is apparent in this experiment as well. About 73.5% of the cells of a population resided in the G1 phase during the lag phase, with about 16% in the S phase. The corresponding DHFR associated fluorescence profile showed two distinct populations, a group comprising approximately 60% of the population with a relative DHFR content of 1, and a group comprising approximately 20% of the population with a relative DHFR content of 10. Populations in early and late exponential phase both showed good correlations between the proportion of cells in G1 phase and a relative DHFR content of 1, and between the proportion of cells in S phase and a relative DHFR content of 10. In the late decline phase, with essentially all of the viable population residing within the A compartment (98.4%), there was no detectable

DHFR-associated fluorescence. Collectively, these observations suggest that there is about 10 times as much DHFR in S phase cells as in G1 phase cells, and that DHFR is not expressed during apoptosis. This finding is in agreement with that of Kubbies and Stockinger (1990).

Fig. 6 Association of the production of DHFR to the cell cycle phases. Relative DHFR associated fluorescence intensity is compared to the proportion of the population in each of the subpopulations. DHFR production correlates with the G1 and S cell cycle phases, at relative concentrations of 1 and 10, respectively. DHFR is not produced during apoptosis

The role of DHFR expression

DHFR, a key enzyme for thymidine synthesis, is essential for the replication of DNA. Endogenous DHFR begins to be expressed during the late G1 cell cycle phase, with maximum expression observed during the S cell cycle phase. In the CHO1-15₅₀₀ cell line, transcription of the *dhfr* gene is under the control of the strong constitutive viral SV40 early promoter. Transcription, therefore, should presumably be cell cycle phase independent.

The expression pattern of the recombinant DHFR seen in this study is consistent with that of endogenous DHFR. This is indicative of physiological expression-control mechanisms of the protein synthesis. It is known that the expression patterns of a mRNA and the appearance of the corresponding mature protein can differ (James et al. 1996; Ohta et al. 1996). It should not be surprising, therefore, that the synthesis of both recombinant and endogenous DHFR are cell-cycle-phase-associated, irrespective of the nature (i.e. constitutive or induced) of the transcription of the *dhfr* gene.

This observation, coupled with the physiological role of DHFR, has led investigators to assume that given the plasmid construct used in the CHO1-15₅₀₀ cell line (in which both recombinant proteins are co-transfected on the same plasmid and under the control of the same strong constitutive viral SV40 early promoter), tPA should be co-expressed with DHFR during the S cell cycle phase (Banik et al. 1996; de Boer et al. 2004; Gu et al. 1993; Mariani et al. 1981).

Often DHFR based rCHO cell expression systems (in which a second foreign protein gene has been co-transfected with *dhfr* into the CHO host cell), have been reported solely in terms of the complete proteins. It has become generally assumed that production of the second recombinant protein is coincident with the appearance of DHFR during S phase. Gu and co-workers (1993) found that the intracellular level (synthesis) of recombinant β -galactosidase in CHO cells increased exclusively during the S phase. In their system, *dhfr* was under the control of the SV40 promoter, while β -galactosidase was under the control of the constitutive hCMV promoter. These authors did not

calibrate synthesis of DHFR with the phases of the cell cycle. Banik and co-workers (1996) found that the intracellular level of recombinant β -galactosidase in CHO cells was directly proportional to the cell-specific growth rate. This suggests that although the synthesis of this recombinant protein is under the influence of the constitutive SV40 early promoter, it is nevertheless S-phase-associated. Similarly, the production of recombinant human interferon- γ by CHO cells was reported to be growth-associated (i.e. S-phase-dependent, Dutton et al. 1998), but the promoter was not identified by the authors, and it was unclear as to whether intracellular or extracellular human interferon- γ levels were measured (Hayter et al. 1993). This evidence of S-phase-associated synthesis of these proteins is obviously not related to the initiation of transcription by constitutive promoters.

When a second foreign protein gene is co-transfected with *dhfr*, but the two genes are under the control of different promoters, it should not be unexpected that the timing of the expression of the two genes, inclusive of transcription through post-translational modifications and secretion, may differ. The system used in the CHO1-15₅₀₀ cell line utilizes the constitutive SV40 early promoter for the expression of both proteins (tPA and DHFR), ensuring co-transcription. However, it is evident from this study (Figs. 5, 6) that the appearance of secreted tPA and the synthesis of DHFR may not occur in concert. Whether the genes are transcribed in concert or not, post-transcriptional modifications, translation, post-translational modifications, and protein trafficking, which collectively result in the appearance of the complete protein, can be subject to different controlling influences (Cartwright 1994).

In agreement with the understanding of DHFR gene transcription and protein synthesis patterns, it was found in this study that DHFR levels are associated with the G1, S, and G2/M fractions of the cell population, in a cell cycle phase-dependent manner. DHFR protein appearance is apparently not restricted to the S phase, but is present in the G1 phase as well, albeit at an order of magnitude lower level. In spite of a single promoter controlling the transcription of both recombinant proteins, the appearance of the complete tPA protein was found to be associated

with the G1 phase. It is likely that regulatory mechanisms beyond gene transcription influence tPA protein synthesis and secretion. The relationship between transcriptional and post-translational regulation of protein expression and cell cycle progression is beginning to be explored (Andersen et al. 2000; Jensen et al. 2006; Sherlock 2006).

The appearance of a secreted protein in the culture medium is the endpoint of spatio-temporal processing. Secreted proteins are directed into the secretory pathway within the cell, while intracellular proteins are not. In the CHO1-15₅₀₀ cell line, DHFR is an intracellular protein while tPA is a secreted protein. For this reason alone, it is not surprising that post-transcriptional processing may diverge for these two recombinant proteins.

The role of native plasminogen activator control mechanisms

Tissue plasminogen activator, a highly regulated serine protease involved in multiple biological processes, is highly conserved from species to species (Rouf et al. 1996). Expression patterns of tPA vary from tissue to tissue and organ to organ, as well as with physiological state (S. Gyorffy et al. in preparation; Inaba et al. 1998; Shen et al. 1997). Many different mammalian cell types produce tissue plasminogen activator, and the expression of endogenous plasminogen activator in cultured CHO cells has been reported (Warner 1999).

Production (expression) of tPA can be induced in response to various stimuli. For example, it has been observed that cardiac muscle cells, that do not normally produce tPA, will produce tPA in response to myocardial infarction and ischemia where it plays a role in tissue repair (S. Gyorffy et al. in preparation).

The activity of plasminogen activators has been reported to be increased in mitotic fibroblasts (Aggeler et al. 1982). These workers found that plasminogen activators were secreted from fibroblasts in culture during the G2 and M phases, with the activity being about twice that associated with the early cell cycle phases. This cell-cycle-specific secretion of native

plasminogen activator occurred both in serum-free medium and in medium containing 5% serum. The authors suggest that a physiological role for plasminogen activators on cultured mitotic fibroblasts may be to break cell-matrix attachments and alter surface proteins and proteoglycans, allowing cells to round up for division. As these adherent cell populations reached confluence, they became arrested in the G1 phase, and the secretion of plasminogen activators decreased by several fold.

Observations in the current study confirm the cell adhesion and G1 arrest patterns described by these authors: It was found in our study that adherent confluent cells of the CHO1-15₅₀₀ cell line arrested in G1 and became very firmly attached to the substratum, requiring extended exposure to trypsin to achieve detachment. In contrast, however, tPA production from the CHO1-15₅₀₀ cell line was observed to occur preferentially during the G1 cell cycle phase (Figs. 4, 5). This is indicative that control mechanisms for expression of the same protein differ in different cell types, where the protein may play different physiological roles. Moreover, the structure of this serine protease is highly conserved between species, often varying only in single amino acid substitutions (Rouf et al. 1996). Hence, cross-species native regulatory mechanisms can potentially impact the production of recombinant tPA.

The activity of native tissue plasminogen activator in mammalian ovary cells is relatively high. For example, the human ovary, which is relatively rich in tPA expression, yields 210 Units per gram of fresh tissue (Rouf et al. 1996). Recombinant porcine follicle stimulating hormone has been shown to induce gene expression of tPA in cultured rat granulosa cells in vitro (Inaba et al. 1998). This work not only demonstrated the inducibility of tPA expression, but also showed that tPA expression in one mammal responded to an inducing molecule from another mammal.

Shen and co-workers (1997) investigated hormonal regulation of tPA expression in the ovary of female Wistar rats. They showed that the temporal regulation of tPA biosynthesis following induction depends on the cell types and size

classes in the various ovarian compartments. Turnover of differentiated ovarian cells is low, with the cells residing primarily in the G1 or G0 state, so that expression and secretion of native ovarian tPA is likely associated with the G1 cell cycle phase. This pattern of productivity is consistent with that observed from the CHO cell line in our current study.

Transformed cells contain and secrete excess levels of plasminogen activators relative to normal cells (Scott et al. 1987). The maximal plasminogen activator activity in the culture supernatant (maximal secretion of enzyme) of a rat prostate adenocarcinoma cell line was found to correspond with the S/G2 cell cycle interface (Scott et al. 1987). In accordance with this finding, the levels of intracellular plasminogen activators have been shown to decrease during the active cell cycle, and to accumulate in cells arrested in G1 (Loskutoff and Paul 1978). This is in contrast to both the processing pattern of native ovarian tPA and the production pattern of the recombinant tPA in our current study, and is suggestive that expression of recombinant tPA in the transformed but non-tumorigenic CHO1-15₅₀₀ ovary cell line is influenced by native ovarian control mechanisms rather than the physiological state associated with metastasis.

The apparent G1 association of tPA production from CHO cells observed in this study is in agreement with the work of Altamirano et al. (2001) who observed tPA production from a growth-arrested CHO cell population. This is in contrast, however, with the findings of Fann et al. (2000), who in an experimental design limited to investigation of the exponential population growth phase, found a direct correlation of tPA production from CHO cells to the specific growth rate, implying S cell cycle phase dependence. It is also in contrast to the findings of Kubbies et al. (1990) and Scott et al. (1987) who found a direct correlation of tPA production with the S phase. Finally, Aggeler et al. (1982) observed peak tPA production during the G2/M phases. Of course, in all these cases, the confounding factor remains consistency of analysis and interpretation of the experimental data. As seen in Fig. 5, in our data there were seemingly short, more or less linear regions in the tPA response curves with respect to

any of the cell cycle phases that could be interpreted as phase dependence.

Concluding remarks

Production of recombinant tPA, which is measured as the accumulation of the secreted protein in the culture medium, was found to be a linear function of the viable population cell-hours in the G1 phase. This pattern strongly suggests that tPA production from CHO1-15₅₀₀ cells is G1 phase associated, which is in contrast to the S phase associated maximum intracellular DHFR content. Association of protein productivity with the G1 cell cycle phase contra-indicates process designs that maximize the exponential growth phase. On the contrary, processing conditions that promote extended G1 cell cycle phase or arrest of the cell population in G1 should serve to maximize productivity.

It appears that post-transcriptional through post-translational processing of the complete DHFR and tPA proteins are not co-regulated. Native regulatory systems for the individual proteins may play a significant role in their processing. Native ovarian tPA secretion is likely associated with G1 phase, as is the secretion of tPA from CHO1-15₅₀₀ cells. A systems analysis approach could be employed to further elucidate the productivity pattern of this recombinant protein, the impact of the host cell regulatory machinery and the influence of population history (e.g. condition of inoculum) and of the culture environment (e.g. nutrient concentrations).

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