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The role of the cell cycle in determining gene expression and productivity in CHO cells

D.R. Lloyd, V. Leelavatcharamas, A.N. Emery & M. Al-Rubeai
Centre for Bioprocess Engineering, School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK

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Abstract

Understanding the relationships between cell cycle and protein expression is critical to the optimisation of media and environmental conditions for successful commercial operation of animal cell culture processes. Using flow cytometry for the analysis of the early phases of synchronised batch cultures, the dependency of product expression on cell cycle related events has been evaluated in a recombinant CHO cell line. Although the production of recombinant protein is initially found to be cell cycle related, the maximum specific protein productivity is only achieved at a later stage of the exponential phase which also sees a maximum in the intracellular protein concentration. Subsequent work suggests that it is the batch phase/medium composition of cultures which is the major determinant of maximum specific productivity in this cell line. Furthermore the effect of the positive association between S phase and specific productivity is subordinate to the effect of batch phase/medium composition on the specific productivity of batch cultures.

Abbreviations: AMLP – adenovirus major late promoter; βgal (*lacZ*) – β-galactosidase, product of the *Escherichia coli lacZ* gene; CHO – Chinese hamster ovary; CMV – cytomegalovirus; DHFR – dihydrofolate reductase; DNA – deoxyribonucleic acid; ELISA – enzyme linked immunosorbent assay; FCS – foetal calf serum; FITC – fluorescein isothiocyanate; FS – forward scattered light; hSEAP – human secreted alkaline phosphatase; IU – international units; MAb – monoclonal antibody; mRNA – messenger ribonucleic acid; MTX – methotrexate; PA – plasminogen activator; PBS – phosphate buffered saline; PI – propidium iodide; PSAP – prostate specific acid phosphatase; RNase – ribonuclease; SS – 90° side scattered light; SV40 – simian virus 40; TMPS – thymidine monophosphate synthetase; tPA – tissue-like plasminogen activator; uPA – urokinase-like plasminogen activator; μ – specific growth rate; χ_v – cell density; t – time; a, b, c and d constants; P – production (of interferon-γ).

Introduction

The overall growth or death rate of any cell culture is determined by the equilibrium between the cell death rate and the rate of cell division. In turn, the rate of cell division is determined by cell cycle duration and the number of dividing (mitotic) cells in the population. Although the duration of the cell cycle or its individual phases may vary between different cell lines, cell cycle events always occur in sequence and checkpoints ensure that, if preceding events are not completed correctly, the cell is arrested and will eventually die if the damage is not repaired. Defining the relationship between cell cycle phase and protein expression is crucial to understanding the dynamics of product elaboration and such knowledge must, in turn, be incorporated in the specification of the optimal culturing protocol for successful commercial operation.

Literature on the relationship between cell cycle and protein expression is inconsistent (Table 1). Product expression (or at least the maximum rate of expression) has not only been shown to be related to different cell cycle phases by different workers but has been shown to be aphasic by others. However,

Table 1. Variation in phase expression of different products with cell line and promoter

Cell line	Promoter	Product	Phase of maximal expression	Reference
Mouse leukaemia	endogenous	DHFR	aphasic	Matherly et al., 1989
CHO	endogenous	DHFR	aphasic	Feder et al., 1989
Human osteosarcoma	endogenous	DHFR	aphasic	Feder et al., 1989
Mouse emrbyo fibroblast	endogenous	DHFR	aphasic	Fedral et al., 1989
Human prostate epithelium	endogenous	PSAP	all except early G_1	MHertz et al., 1991
Hybridoma	endogenous	MAb	G_1/G_0 (mainly)	Kromenaker and Srienc 1991
CHO	AMLP	DHFR	G_1 (mainly)	Kubbies and Stockinger 1990
Hybridoma	endogenous	MAb	G_1/S	Al-Rubeai and Emery 1990
Mouse hybridoma	endogenous?	MAb	G_1 (arrested)	Terada et al., 1996
Mouse plasmacytoma	endogenous	immuniglobulin	late G_1/earlyS	Garatum-Tjeldstø et al., 1976
Human lymphoid cell	endogenous	immunoglobulin	late G_1/S	Buell and Fahey 1969
CHO	SV40	h SEAP	G_1 (arrested)	Fussenegger et al., 1997
CHO	endogenous	DHFR	G_1/S[1]	Darzynkiewocz et al., 1996
Mouse leukaemia	endogenous	TMPS	S phase	Matherly et al., 1989
CHO	CMV	β gal (lacZ)	S phase	Gu et al., 1993
CHO	SV40	tPA	S (mainly)	Kunnies and Stockinger 1990
Mouse L cell	MMTV	β gal (lacZ)	S (predominantly)	Gu et al., 1996
CHO	endogenous	DHFR	S phase	Mariani et al., 1981
Rat adenocarcinoma	endogenous	tPA	shortly after S	Scott et al., 1987
Rat adenocarcinoma	endogenous	uPA	shortly after S	Scott et al., 1987
CHO	endogenous	PA	G_2/M	Aggeler et al., 1982

[1] gene expression activates at G1/S transition.

these observations do not necessarily contradict each other and may be a true reflection of the systems used, since a variety of cells, products and constructs were studied. Cell cycle related productivity could vary with cell line, the nature of the recombinant gene expressed or the promoter/enhancer used to generate product expression. Nevertheless, some results do directly conflict. Mariani et al. (1981), using cultures synchronised by mitotic shake off and ^{35}S-methionine protein labelling, showed that DHFR expression under the control of an endogenous promoter in CHO cells was S phase related. In contrast Feder et al. (1989), using phase enriched cell populations generated by centrifugal elutriation with ^{35}S-methionine protein labelling, DHFR activity assay and measuring DHFR mRNA, showed that the same expression was aphasic. Directly conflicting results such as these may still represent valid differences because, although the two groups used the same cell line, there were differences in their experimental techniques and there may have been some clonal variation in the 8 years between the papers. However, as such subtle differences between cells seem unlikely to be responsible for such major differences in their behaviour, an alternative explanation should be sought. Prime candidates must include the history or condition of the inoculum, population heterogeneity during batch phases and the influence of changing nutrient conditions on cellular composition.

The aim of this work is to determine the relative effects of cell cycle phase and culture chemical environment on recombinant gene expression (intracellular product accumulation and specific productivity) in recombinant CHO cells.

Materials and methods

Cell Culture

CHO 320 cells producing human interferon-γ were kindly provided by Glaxo-Wellcome Research and Development, Beckenham, UK. This recombinant cell line contained an insert for human interferon-γ under the control of the SV40 promoter/enhancer which was co-amplified with the DHFR gene by the presence of MTX. Cells were grown in stirred batch suspension cultures in RPMI 1640 (Gibco, Paisley, UK) with 5%

FCS (Gibco, Paisley, UK) and mM MTX (Sigma, Poole, UK) at 37 °C.

Cell density measurements

Cell density and viability of culture samples were measured by haemacytometer using trypan blue exclusion.

Cell cycle analysis

1×10^6 cells were harvested, washed with PBS, fixed with cold 70% ethanol and stored at –20 °C until analysed. Cells were washed in PBS, then resuspended in RNase solution (250 μg ml^{-1} RNase (Sigma, Poole, UK) in PBS) and incubated at 37 °C for 30 minutes. PI (Sigma, Poole, UK) was added to a final concentration of 50 μg ml^{-1} and the preparation was incubated at room temperature for a further 10 minutes. The relative cellular DNA content of stained cells was measured using an EPICS Elite flow cytometer (Coulter Electronics, Luton, UK) equipped with an argon laser emitting 15 mW at 488 nm. FS was collected using a neutral density filter and the standard cross beam mask; 90° SS was collected using a 488 nm band pass filter; propidium iodide fluorescence was collected using a 488 nm long pass, follwed by a 635 nm band pass filter. FS signal integral, SS signal integral, PI fluorescence signal integral and PI fluorescence signal peak were recorded. Single cells were selected for analysis by using the distribution of Pl fluorescence signal integral against Pl fluorescence signal peak to discriminate doublets and debris (Al-Rubeai, 1998). The relative size of Pl fluorescence signal integral (DNA content) in single cells was plotted as a frequency histogram. The proportions of cells in phases G_0/G_1, S and G_2/M were determined from the latter by cell cycle analysis using Multicycle software (Phoenix Flow Systems, San Diego, USA).

Product analysis

Secreted product concentration was measured by ELISA. Where results are quoted in IU, measurements were made using a triple sandwich ELISA. An ELISA plate was coated with polyclonal anti-interferon-γ capture antibody (R1PA, kindly provided by Dr P Hayter, then at The University of Kent, UK) and incubated overnight at 4 °C. After washing and blocking, standard (kindly provided by Glaxo-Wellcome R and D, Beckenham, UK) or diluted samples were added in duplicate to the appropriate wells followed by incubation at room temperature. After washing, monoclonal anti-interferon-γ detection antibody (20B8, Dr P Hayter, as above) was added, followed by further incubation at room temperature. After washing, the plate was probed with anti mouse immunoglobulin peroxidase conjugate (Sigma, Poole, UK) followed by further incubation at room temperature. Then, after final washing, freshly prepared substrate solution (o-phenylene diamine (Sigma, Poole, UK) and H_2O_2 (BDH, Poole, UK)) was added, followed by incubation in the dark at room temperature until the lowest concentration of standard showed an appreciable colour change. The reaction was stopped by acidification and absorbance was read at 492 nm using an ELISA plate reader (SLT Spectra, Tecan, Reading, UK). Absorbance was converted to interferon-γ concentration (IU ml^{-1}) by interpolation from the standard curve.

Where results are quoted in mass terms the measurements were made using a DuoSeT kit (Genzyme, West Malling, UK), following the manufacturer's protocol supplied with the kit. Briefly, an ELISA plate was coated with anti-interferon-γ capture antibody and incubated overnight at 4 °C. After washing and blocking, standard or diluted sample was added in duplicate to the appropriate wells followed by incubation at room temperature. After washing, peroxidase conjugated anti-interferon-γ detection antibody was added, followed by further incubation at room temperature. After final washing 3,3',5,5' tetramethylbenzidine liquid substrate system (Sigma, Poole, UK) was added, followed by incubation in the dark at room temperature. The reaction was stopped by acidification and absorbance was read at 492 nm using an ELISA plate reader as before. Absorbance was converted to interferon-γ concentration (ng ml^{-1}) by interpolation from the standard curve.

Intracellular interferon-γ was measured by flow cytometry after direct immunofluorescence staining. Briefly, 3×10^5 cells were harvested and washed with block buffer (0.5% BSA (Sigma, Poole, UK) in PBS) and resuspended in block buffer. An equal volume of fixative (1% formaldehyde in PBS, prepared freshly from ρ-formaldehyde (Agar Scientific, Stanstead, UK)) was added followed by incubation at 4 °C. Fixed cells were washed in block buffer and resuspended in 190 μl block buffer. 10μl permeablising solution (2% saponin (Sigma, Poole, UK) in PBS) and then 1 μl FITC conjugated anti-interferon-γ (Pharmingen, San Diego, USA) were added followed by dark incubation at 4 °C. Immunostained cells were

washed with PBS then analysed by flow cytometry using an EPICS Elite flow cytometer with FS and SS collected as described before; FITC fluorescence was collected using a 488 nm long pass, followed by a 525 nm band pass filter. FS signal integral, SS signal integral, FITC fluorescence signal integral, FITC fluorescence signal integral log and FITC fluorescence signal integral:FS signal integral ratio were recorded. Single cells were selected for analysis by using the distribution of FS against SS to discriminate doublets and debris. The relative size of FITC fluorescence signal integral log was plotted as a frequency histogram and its mean defined the amount of interferon-γ per cell. Using FS as an indicator of cell size allowed correction of the amount of interferon-γ per cell to concentration per cell. Thus the relative size of FITC fluorescence signal integral:FS ratio was plotted as a frequency histogram and its mean defined the interferon-γ concentration per cell.

Curve fitting and non-linear regression analysis

Curve fitting and non-linear regression analysis were performed using SigmaPlot scientific graphing software (Jandel, Erkrath, Germany).

Batch culture experiments

Cultures were (partially) synchronised either by late exponential nutrient deprivation of the inoculum (Leelavatcharamas, 1996), or by release from thymidine block. Briefly; cells taken from an exponential culture were transferred to fresh medium containing 10% FCS, 1 μM MTX and 3 mM thymidine (Sigma, Poole, UK) and incubated for 18–24 h followed by washing once in fresh thymidine-free medium before use. Inocula were transferred to fresh medium in an appropriately sized stirred flask and incubated at 37 °C. At each time point, the smallest appropriate volume samples were taken off-line for cell cycle, cell density and product analysis.

The effect of medium condition on cell growth and productivity

A population containing a relatively high percentage (50–60% at t = 0) of S phase CHO 320 cells was taken from a culture which had previously been inoculated with cells from a very late exponential phase and then incubated in fresh medium for 17 h. A population with a relatively low percentage (20–30% at t = 0) of S phase cells was taken from a mid-exponential phase culture. In both cases cells were harvested by centrifugation and replicate samples were resuspended either in fresh or spent medium (cell free supernatant from the end of a batch culture) and incubated for 2 and 3 h. Cell density was measured by haemacytometer (as described above), interferon-γ concentration was measured by ELISA (as described above) and cell cycle was measured flow cytometrically (as described above). All three parameters were measured at 0, 2 and 3 hours after the start of the experiment.

Results and discussion

Productivity and the cell cycle in a CHO 320 culture synchronised by nutrient deprivation

Changes in the intracellular interferon concentration and the specific secreted productivity were apparently closely associated with the observed percentage of S phase cells. Both reached their highest values in the early exponential phase of the growth curve at about the same times as, if not slightly before the maximum specific growth rate (seen in Figure 1) and declined after the mid-exponential phase. Thus the intracellular interferon-γ concentration and specific productivity were apparently related not only to the cell cycle (proportion of S phase cells present) but also to the culture growth rate and batch phases. However, at this time, no causative relationship between any of the observations could be established. Furthermore, it was possible that the frequency of observations in the lag and early exponential phases of the culture (every 6 hours) did not allow all the changes which were taking place at that time to be seen.

A more detailed investigation of the lag and early exponential phases in a CHO 320 batch culture synchronised by nutrient deprivation

Cell density and interferon-γ production by CHO 320 cells during the first 2 days of a batch culture started with a late exponential inoculum are shown in Figure 2. As in most animal cell culture work, culture growth is defined by the increase in the number of viable cells present. Use of this commonly accepted definition explains the absence of growth during the first 20 hours of the culture i.e. the lag phase.

At the start of the culture the majority of cells were in (early?) G_1 (data not shown), thus the number of cells present remained constant while the cells traversed the cell cycle until, about 20 hours later, the

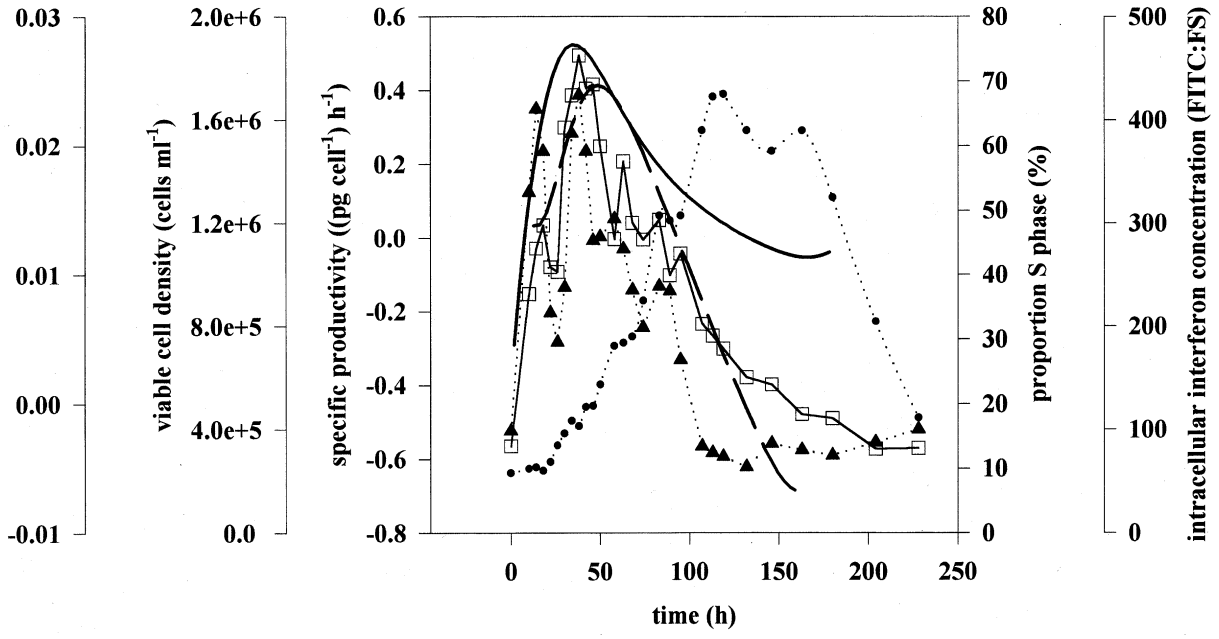

Figure 1. CHO 320 growth, cell cycle phase, specific production rate and intracellular interferon-γ concentration; — specific production rate, --- specific growth rate; ● cell density; ▲ S phase cells; □ intracellular interferon-γ concentration.

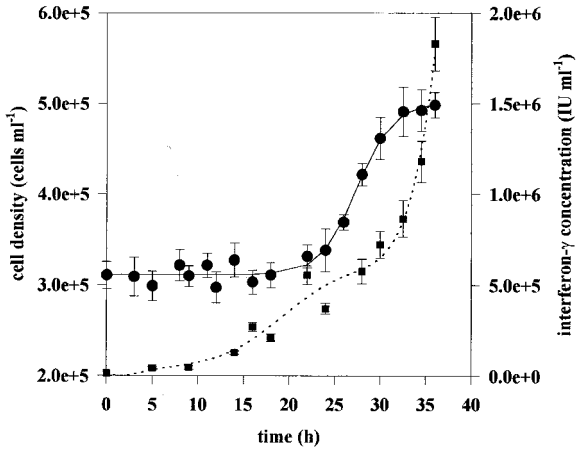

Figure 2. Growth and production in the early part of a CHO 320 batch culture synchronised using a late exponential inoculum; ● cell density; ■ interferon-γ concentration; bars = standard error.

first cells exited mitosis as 2 daughter cells, which was manifest by the number of cells present starting to increase. Then the last cells of the inoculation cohort exiting mitosis was manifest by the apparent stalling of the increase in cell density at about 35 hours. Thus one can infer that the cell cycle transit time of these cells under the given conditions varies from about 20 to about 35 hours. With such a wide variation in cell cycle transit time, usually due to variation in the duration of G_1, (Zetterberg, 1996) it is not surprising that cultures rapidly loose synchrony. In contrast the interferon-γ concentration increased approximately exponentially from the start of the culture, with the exception of a 'blip' at about 20–25 hours.

Previous work (Al-Rubeai et al., 1997) has shown that the error in manual cell desnity measurements is of the order of 10%; greater than the real differences which might be expected between consecutive points. To obviate the effect of these errors on subsequent derived parameters, the cell density curve was smoothed by determining the best fit regression line between the observed points. The specific growth rate (μ) at any point x_v, t) on the curve was then obtained using the slope of the line at that point (dx_v/dt), simply from:

$$\mu \approx \left(\frac{1}{x_v}\right)\left(\frac{dx_v}{dt}\right) \tag{1}$$

The curve fit for the x_v, t data in Figure 2 was based on a logistic function given by:

$$x_v = \frac{a}{(1 + e^{b(t-c)})} + d \tag{2}$$

Using the SigmaPlot curve fitting program, the constants a, b, c and d were obtained:

$$x_v = \frac{1.89}{(1 + e^{-0.52(t-27.41)})} + 3.12 \tag{3}$$

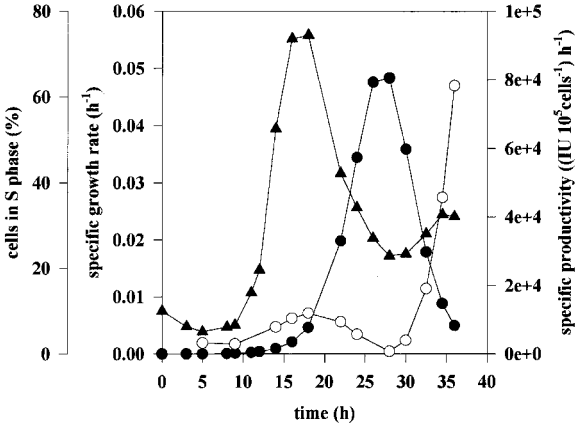

Figure 3. Relative changes in S phase cells, specific growth and production rates in the early part of a CHO 320 batch culture synchronised using a late exponential inoculum; ● specific growth rate; ▲ S phase cells; ○ specific production rate, error bars are not appropriate.

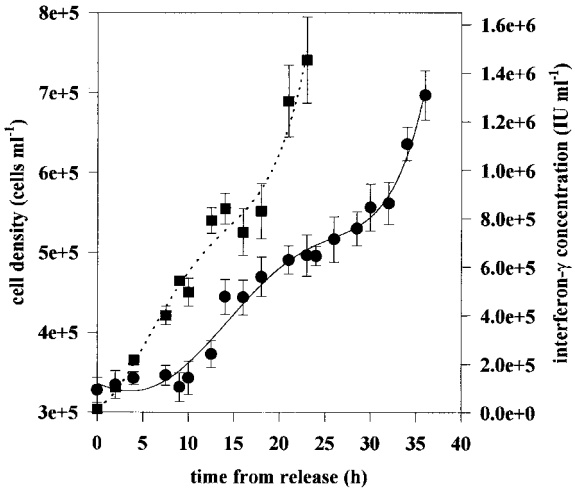

Figure 4. Growth and production in the early part of a CHO 320 batch culture synchronised by release from thymidine block; ● cell density; ■ interferon-γ concentration, bars = standard error.

and this is shown as the solid line in Figure 2. The gradient of the curve is given as:

$$\frac{dx_v}{dt} = -a \cdot b \cdot \exp(b(t-c)) \cdot (1+\exp(b(t-c))^{-2} \quad (4)$$

The results for the values of μ calculated using equation (1) are shown in Figure 3.

Using a similar approach, the interferon-γ production data was also smoothed using the polynomial:

$$P = 14600 - 19500t + 9400t^2 - 1400t^3 +$$

$$98.6t^4 - 3.1t^5 + 0.04t^6 \quad (5)$$

and, using the same strategy as for the specific growth rate calculation, a specific productivity could be calculated as *dP/dt* divided by x_v and this is also shown in Figure 3.

The results in Figure 3 clearly demonstrated how the specific interferon-γ productivity rate varies during the first 36 h cultivation of CHO 320 batch cultures. As in earlier work (Leelavatcharamas et al., 1996) the proportion of cells in S phase peaked approximately $10\frac{1}{2}$ h before the specific growth rate. The specific interferon-γ productivity also showed a small peak, coincident with the S phase peak, before the specific growth rate peak. However, the specific productivity then increased sharply at the end of the culture period, when there was a second, less distinct, S phase peak; thus the cell cycle could be presumed to be exerting a much smaller effect on productivity than at the time of the first major S phase peak.

These results suggest that although a higher rate of interferon-γ production may occur during S phase, in agreement with other work using CHO cells (Gu et al., 1993, 1996; Kubbies and Stockinger, 1990; Mariani et al., 1981; Matherly et al., 1989 all showed increased product expression in S phase), there must be other factor(s) which have a much greater influence on the cellular and volumetric productivity of a culture. It is also noteworthy that this apparent cell cycle relationship is different from that found with hybridomas whose specific productivity increased with an increase in the proportion of G_1 phase cells, not S phase cells (Al-Rubeai and Emery, 1990).

An investigation of the lag and early exponential phases in a CHO 320 batch culture synchronised by thymidine block

The cell density and secreted interferon-γ produced by CHO 320 cells after release from thymidine block are shown in Figure 4. Both growth and production curves were smoothed following similar principles to those described above. After release from the thymidine block, cell density did not increase for approximately 10 h after which it increased sharply, levelled out and then increased again at 30 h of incubation, i.e. synchronous division was maintained (at least in part) over two cycles. The interferon-γ concentration started to increase immediately after release from the thymidine block but there were also two sharp increases in interferon-γ concentration which are clearly

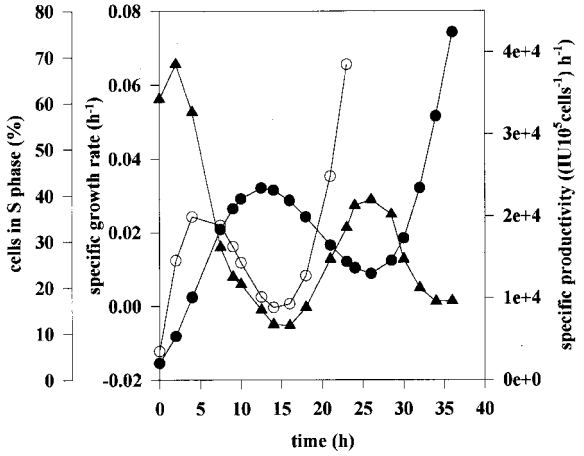

Figure 5. Relative changes in S phase cells, specific growth and production rates in the early part of a CHO 320 batch culture synchronised by release from thymidine block; ● specific growth rate; ▲ S phase cells; ○ specific production rate, error bars are not appropriate.

not coincident with accelerations in growth. Specific growth rate and specific productivity were obtained by using the same strategy as described earlier and are shown in Figure 5.

A second synchronisation method was used to determine whether the effects seen using nutrient deprivation were real phenomena or simply an artefact of that method. Although it was expected that RNA and protein synthesis could continue during thymidine block and that specific interferon-γ productivity would recover rapidly afterwards, it was nonetheless, as in the culture synchronised by late exponential nutrient deprivation, depressed in the first cycle. Lambert and Studzinski (1969) suggested that abnormal cellular composition caused by excess thymidine was found to revert to control levels by the time of the first mitosis. Thus it was possible that interferon-γ production was still depressed after cells were released from the block. However, after the first mitosis, specific interferon-γ productivity recovered; it peaked before the specific growth rate and started to increase again during the decline of specific growth rate. We can see (at least up to 25h) that specific interferon-γ productivity increased with the increase of percentage of S phase. These results confirmed the observation from the previous experiment that the secretion of interferon-γ was maximal during S phase; perhaps because the CV40 promoter used to drive interferon-γ gene expression is S phase specific (as suggested by Banik et al., 1996).

However in both cases; nutrient deprivation, where cells 'arrest' in (early) G_1 and thymidine block, where cell accumulate at the G_1-S boundary; there is a peculiar 'first cycle' phenomenon. The specific productivity peaks at about the same time as the first major S phase peak; clearly demonstrating the relationship between S phase and product elaboration/secretion but while there is a similar increase in specific productivity at the time of the second S phase peak, the amount of specific productivity at that time is substantially greater; almost an order of magnitude greater in the case of nutrient deprivation (Figure 3); despite the S phase peak being smaller (as the culture begins to loose synchrony). Thus the relationship between increased productivity and maximal S phase remains, but superimposed on that relationship must be some other(s) to explain the difference in magnitude between the first and second specific productivity increases. If specific productivity was simply related to S phase, the first specific productivity peak should be greater then the second, not the inverse which was seen experimentally using 2 different synchronisation methods.

It is demonstrable (Al-Rubeai et al., 1991, 1992; Leelavatcharamas et al., 1996) that an increase in cell number is preceded by an increase in the fraction of S phase cells. The apparent association of interferon-γ production with the S phase, consequently, may lead to interferon-γ production being interpreted as growth associated (Hayter et al., 1991, 1993). The claim that interferon-γ production could be uncoupled from cell proliferation in glutamine-deficient medium, made by Hayter et al. (1991), may have arisen because their cells were arrested throughout the cell cycle because of glutamine starvation. Thus there was no cell division but interferon-γ could be produced by arrested cells. Arrested cells are likely to elaborate more product than cycling cells in any phase (Edwards and Al-Rubeai, 1998; Fussenegger et al., 1997; Terada et al., 1996). However, these studies address a different question to that which this work addresses. Although our cultures had been arrested/synchronised, they were cycling, dividing, growing (increase in cell number) 'normal' cultures when they were examined. In contrast, these other works described the situation in cells which were arrested when they were studied. Normally cycling and growing cells will, of course, utilise a large proportion of available substrates for biomass synthesis and only a small proportion will be directed to recombinant product synthesis (or 'housekeeping'). However, in the case of arrested cells,

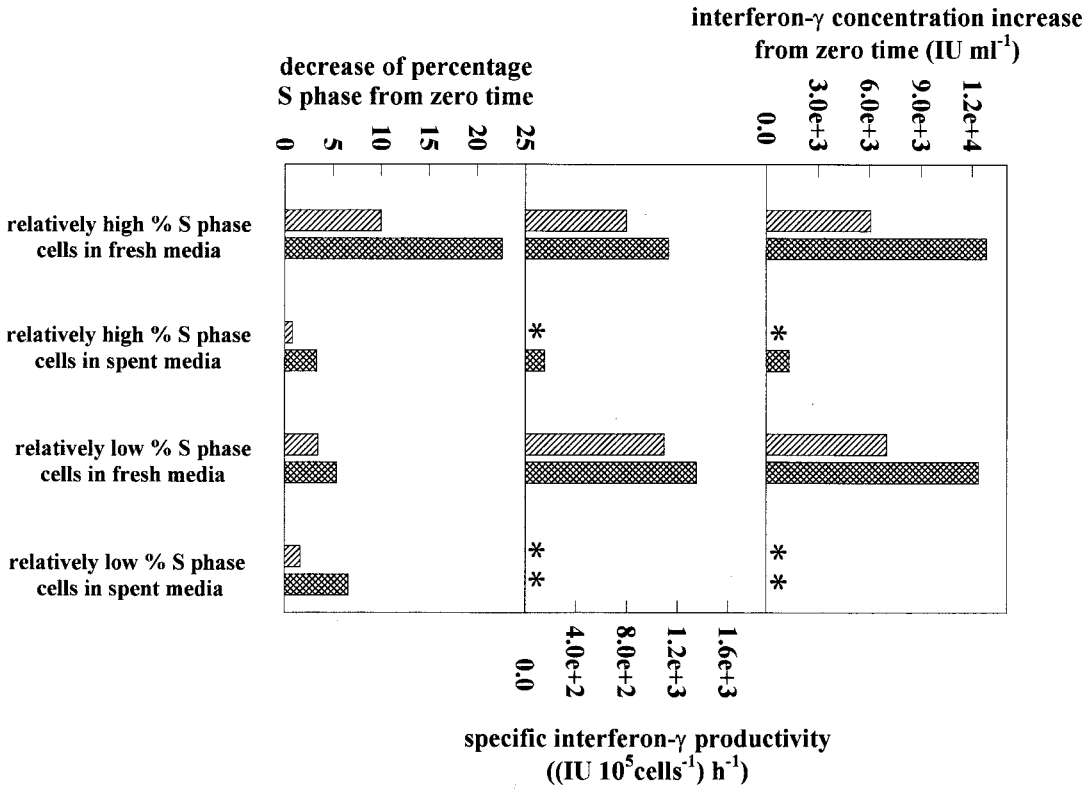

Figure 6. The relative effects of medium condition and proportion of S phase cells present on interferon-γ concentration and specific production rate in CHO 320; ▨ = 2h; ▩ = 3h; ∗ = below limits of detection.

all the resources that would have been directed towards biomass synthesis become available for product synthesis; hence substantially higher specific productivities are possible with arrested cells.

The effect of nutrient conditions on cell growth and productivity

The effect of nutrient condition on interferon-γ production by cell populations containing different proportions of cells in each phase of the cycle is shown in Figure 6. Cell densities were constant throughout the duration of the experiment. It was obvious that interferon-γ was only produced in significant quantity when cells were grown in fresh medium, irrespective of the cell cycle composition of the population. Thus it can be claimed that medium composition has a more dominant influence on specific productivity than the cell cycle phase composition of the culture in question. These results support the indication (Figure 1) that the changes in specific productivity seen during batch culture are due to passage through the batch growth phases and are associated with medium condition (nutrient availability, waste accumulation etc.) and not the proportion of S phase cells in the population. In other words, the batch phase/culture environment is the major determinant of specific productivity and although there is a positive relationship between S phase and specific productivity, this relationship is subordinate to the effect of medium composition.

Conclusion

Gene expression (intracellular product concentration and specific productivity) was throught to be cell cycle related because it varied with cell cycle and batch phase. However, the evidence in this work shows that the batch phase/medium condition of a culture is the major determinant of specific productivity in that culture. Superimposed on the major effect of batch phase/medium condition, the proportion of S phase cells in a population has a positive association with both intracellular product accumulation and the specific productivity of that population but this association is subordinate to the effect of batch phase/medium

condition and only has a minor influence on the specific productivity of a batch culture.

Further work using centrifugal elutriation to produce 'purified' populations in different cell cycle phases, without any confounding batch phase or culture history variables, could be attempted in order to further test cell cycle and productivity relationships. Such techniques could also be used to investigate the effect of different promoters on cell cycle/size related specific productivity and to determine whether these relationships are valid for other recombinant cells.

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Address for correspondence: Dr. M. Al-Rubeai, Centre for Bioprocess Engineering, School of Chemical Engineering, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.
Phone: +121-414-3888; Fax: +121-414-5324;
E-mail: m.al-rubeai@bham.ac.uk