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Effects of temperature shift on cell cycle, apoptosis and nucleotide pools in CHO cell batch cultues

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Abstract

Temperature reduction in CHO cell batch culture may be beneficial in the production of recombinant protein and in maintenance of viability. The effects on cell cycle, apoptosis and nucleotide pools were studied in cultures initiated at 37 °C and temperature shifted to 30 °C after 48 hours. In control cultures maintained at 37 °C, viable cells continued to proliferate until the termination of the culture, however, temperature reduction caused a rapid decrease in the percent of cells in S phase and accumulation of cells in G_1. This was accompanied by a concurrent reduction in U ratio (UTO/UDP-GNAc), previously shown to be a sensitive indicator of growth rate. Culture viability was extended following temperature shift, as a result of delayed onset of apoptosis, however, once initiated, the rate and manner of cell death was similar to that observed at 37 °C. All nucleotide pools were similarly degraded at the time of apoptotic cell death. Temperature reduction to 30 °C did not decrease the energy charge of the cells, however, the overall rate of metabolism was reduced. The latter may be sufficient to extend culture viability via a reduction in toxic metabolites and/or limitation of nutrient deprivation. However, the possibility remains that the benefits of temperature reduction in terms of both viability and productivity are more directly associated with cultures spending extended time in G_1.

Introduction

Mammalian cells grown in batch cultures for the production of recombinant proteins die via apoptosis (Fraňek *et al*., 1992; Singh *et al*., 1994a; Singh *et al*., 1994b; Moore *et al*., 1995). This can result in the premature termination of production cultures and consequential suboptimal product yields and/or product quality. We have previously shown that CHO cells grown under the conditions stated, died in an extremely reproducible manner, exiting the cell cycle predominantly from G_0/G_1; and those that remained viable continued to proliferate for the duration of the culture (Moore *et al*., 1995).

Reduction in operating temperature is a widely used manipulation of production cultures, and increased viability with variable effects on productivity have been reported under such conditions (Weidemann *et al*., 1994; Reuveny *et al*., 1993; Bloemkolk *et al*., 1992; Sureshkumar and Mutharasan, 1991; Giard *et al*., 1982). The effects of temperature reduction on batch culture cellular dynamics are not well understood, so an analysis of cell cycle characteristics and apoptosis was carried out in this study. In addition the intracellular nucleotide pools were investigated to determine potential association with the cell cycle distribution and the onset of apoptosis.

Apoptosis was measured concurrently with cell cycle analysis by flow cytometry using the terminal transferase assay (Gorczyca *et al*., 1992, 1993). Nucleotides were measured by HPLC after extraction using perchloric acid (Ryll and Wagner 1991).

Methods

Cells

Experiments were performed using Chinese hamster ovary (CHO) cells derived from a dihydrofolate minus (dhfr-) DUKX CHO host (Chasin and Urlaub, 1980). Cells were genetically engineered to secrete recombinant proteins using a dhfr/methotrexate selection method similar to that used by Kaufman and Sharpe, (1982). Observations were made in cultures grown in 2 liter bioreactors. Cells were grown as described previously (Moore *et al*., 1995). Reactors were equipped with calibrated dissolved oxygen, pH and temperature probes. Dissolved oxygen was controlled on-line through sparging with air and/or oxygen, and pH was controlled through additions of CO_2 or Na_2CO_3. Vessels were equipped with temperature jackets, and all cultures were grown at 37 °C for the first 48 hours, and then either maintained at this temperature for the duration of the culture, or reduced to 33 °C or 30 °C. Cultures were routinely terminated on day 13, however, some were allowed to progress to day 18 (see later). Cell viability was assessed by trypan blue exclusion and by measurement of total and supernatant lactate dehydrogenase (LDH) (Goergen *et al*., 1993).

Preparation of cells for analysis of cell cycle and apoptosis by flow cytometry

Cells were harvested by centrifugation each day for flow cytometry and fixed in 0.5% iso-osmotic formaldehyde (using EM grade formaldehyde, Polysciences, Warrington, PA) for 15–30 min on ice. After washing in PBS (pH 7.4), the cells were stored for up to 10 days in PBS plus 0.1% sodium azide and 0.1% BSA (pH 7.4) at 4 °C. Labeling was carried out according to the method originally described by Gorczyca *et al*., 1993, and as described previously (Moore *et al*., 1995), with minor modifications. Briefly, approximately 2×10^6 cells per sample were pelleted and resuspended in 50μl of a reaction solution containing 0.2M potassium cacodylate, 25mM Tris–HCl, 0.25mg/ml BSA, 2.5 mM $CoVl_2$, 25 units terminal transferase (Boehringer Mannheim, Indianapolis, IN), and 500pM FITC–ddUTP (Boehringer Mannheim). Samples were incubated at 37 °C for 30 min, washed twice in PBS and then resuspended in 1ml PBS plus 0.1% DNase-free RNase (Boehringer Mannheim) and 10μg/ml propidium iodide (Boehringer Mannheim). Cells were incubated in this solution for 1 hr at 37 °C and then submitted for analysis by flow cytometry. Each sample had a corresponding control processed as indicated above with the omission of terminal transferase enzyme. 20,000 cells were analyzed from each sample. Red (DNA) and green (dUTP) fluorescence of individual cells was measured with an Elite ESP Flow Cytometer (Coulter Corporation, Miami, FL), equipped with a 40mW argon laser. A 550nm long-pass dichroic filter was utilized to separate the green and red fluorescence signals. Green fluorescence intensity was quantified using a 525nm bandpass filter; red fluorescence using a 630nm long-pass filter. Cell cycle phase fraction analysis was performed in conjunction with the Multicycle software package (Phoenix Flow Systems, San Diego, CA) which utilizes the cell cycle fitting algorithm of Dean and Jett (1974).

DNA laddering

Cells were collected daily, pelleted by centrifugation at 900 × g, snap frozen and stored at –80 °C. Extraction of DNA was carried out according to the method by Tilly and Hsueh, (1993), and as described previously (Moore *et al*., 1995). 10μg DNA was incubated with 1μl 0.1mM TO–TO–1 (Molecular Probes, Eugene, OR) at room temperature for 1 hour, separated by electophoresis, visualized with a UV transilluminator (Fisher Scientific, Pittsburgh, PA) and photographed.

Nuceleotide extraction and quantification

Intracellular nucleotides and nucleotide surgars, specifically ATP, UTP, GTP, CTP, USP–GNAc, NAD, ADP and AMP were extracted and quantified as described earlier (Ryll and Wagner 1991). Briefly, $2 – 6 \times 10^6$ cells were cooled and pelleted at 200 × g for 3 minutes. Cell pellets were extracted using 0.5 M perchloric acid. Supernatants were pooled after repeating the extraction step, and neutralized using 2.5 M KOH in 1.5 M K_2HPO_4. The clear filtered supernatant was used for quantification using an ion–pair reversed phase HPLC method running on an HP1090 system (Hewlett Packard, Mountain View, CA). Elution gradient and flow rates were changed to accommodate higher back pressure with the HP system. Separation was carried out using the following gradient: 0–2.5 min 0% B at 1.3 ml/min, 2.5–16 min 0–40% B at 1.3 ml/min, 16–18 min 40–55% B at 1.3 ml/min, 18–19.5 min 55–65% B at 1.0 ml/min, 19.5–20 min 65–100% B at 1.0 ml/min, 20–60 min 100% B at 1.0 ml/min, 26–26.5 min 100–0% B at 1.0 ml/min, 26.5–27.5 min 0% B

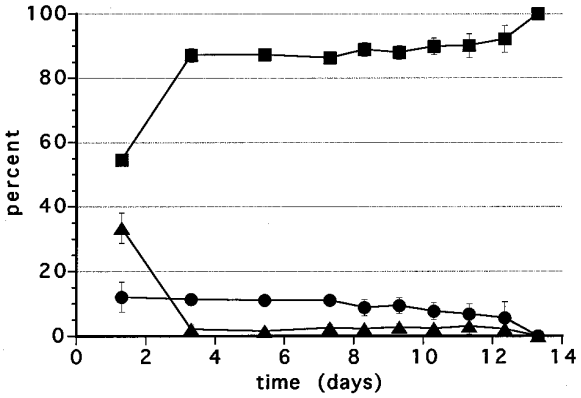

Figure 1. Proportion of viable cells in each phase of the cell cycle; G_1/G_0 (■), S phase (▲), and G_2/M (●), on days 1 to 14, in cultures initiated at 37°C and temperature shifted to 30°C at 48 hours. Analyzed by flow cytometry and expressed as a proportion of the total non-apoptotic cell population. n = 3–5, mean ± s.d.

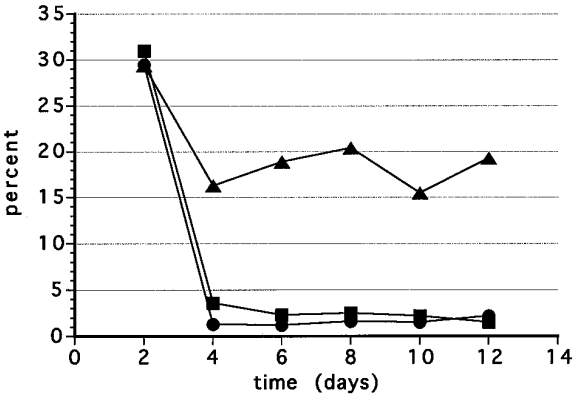

Figure 2. Proportion of viable cells in S phase from days 2 through 12 in cultures maintained at 37°C (▲), and in cultures initiated at 37°C and temperature shifted to 33°C (■), or to 30°C (●) at 48 hours. Analyzed by flow cytometry and expressed as a proportion of the total non-apoptotic cell population. (One data set from 2 independent experiments).

at 1.0 ml/min, 27.5–36 min 0% B at 1.3 ml/min. Quantification was done by integrating peak areas using calibration curves for all nucleotides in the appropriate range.

Results

Two different sets of experiments were performed. Experiment set one was performed comparing the effect of shifting the temperature to 33 °C and 30 °C; control cultures were maintained at 37 °C. Cultures in experiment set two were shifted to 30 °C and maintained for up to 18 days to follow the course of apoptosis and nucleotide pools at low temperature.

Cell cycle phase occupancies are shown in Figure 1. Curve fitting was carried out on DNA histograms up to Day 14, after which the extent of cell death was too great to yield reliable statistics. 31 hours after the initiation of the culture, cells were rapidly dividing, with 33.4±4.7% in S phase. Following a temperature shift to 30 °C, the cell population becomes almost stationary, dropping to 2.2±1.3% S phase on day 3.3, (mean ± s.d., n=3).

The temperature shift to 30 °C also resulted in the majority (87%) of cells entering G_0/G_1 and accumulating in this phase up to 100% on day 14. Conversely, maintenance of cells at 37 °C without a temperature shift resulted in 20% of cells remaining in S phase through Day 12 (Figure 2). These percentages represent cycling cells, however, as can be seen in Figure 3, non-cycling (non-viable) cells accumulated in a sub G_1

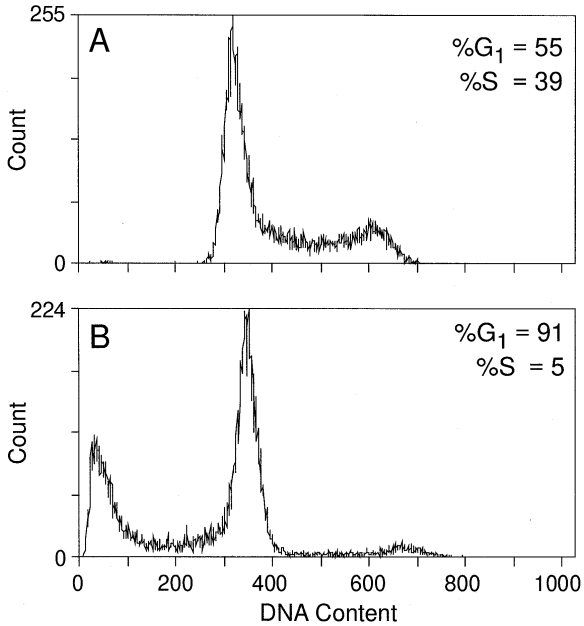

Figure 3. DNA histograms from CHO cells grown in batch culture analyzed on day 2 (A), prior to temperature shift, and on day 13 (B) following temperature reduction to 30°C at 48 hours. Note a high percent of cells in S phase on day 2, and a large hypodiploid population (leftmost peak) on day 13.

population. This population became significant at day 6 at 37 °C and day 10 at 30 °C (see Figure 5).

The sub G_1 or hypodiploid cells have exited the cell cycle by apoptosis (Telford *et al.*, 1991). The accumulation of cells in a hypodiploid population was coincident with the appearance of a clear laddering pattern

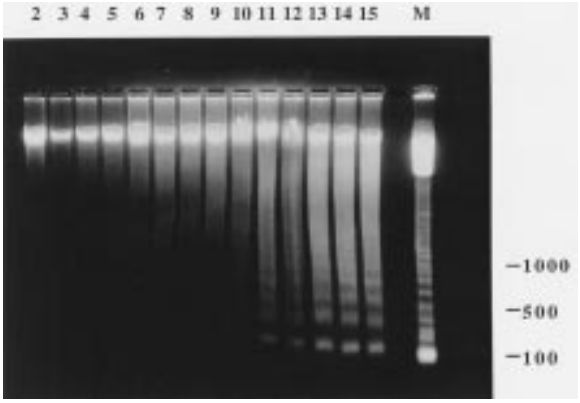

Figure 4. Time course of internucleosomal cleavage of CHO cell DNA: Electrophoretic pattern of 10μg DNA extracted on days 2 through 15, following a temperature shift to 30°C at 48 hours. M is a molecular weight marker. DNA was visualized using TO-TO-1 dye and UV transillumination.

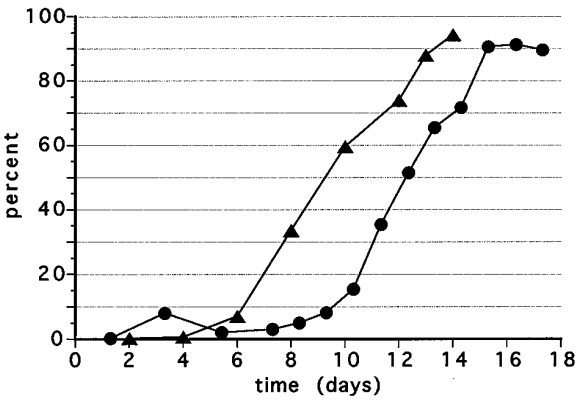

Figure 5. Proportion of apoptotic cells as a percent of the total cell population from days 1 through 17, analyzed by flow cytometry. Cultures maintained at 37°C (▲), and cultures temperature shifted to 30°C at 48 hours (●). (One data set from 2 independent experiments).

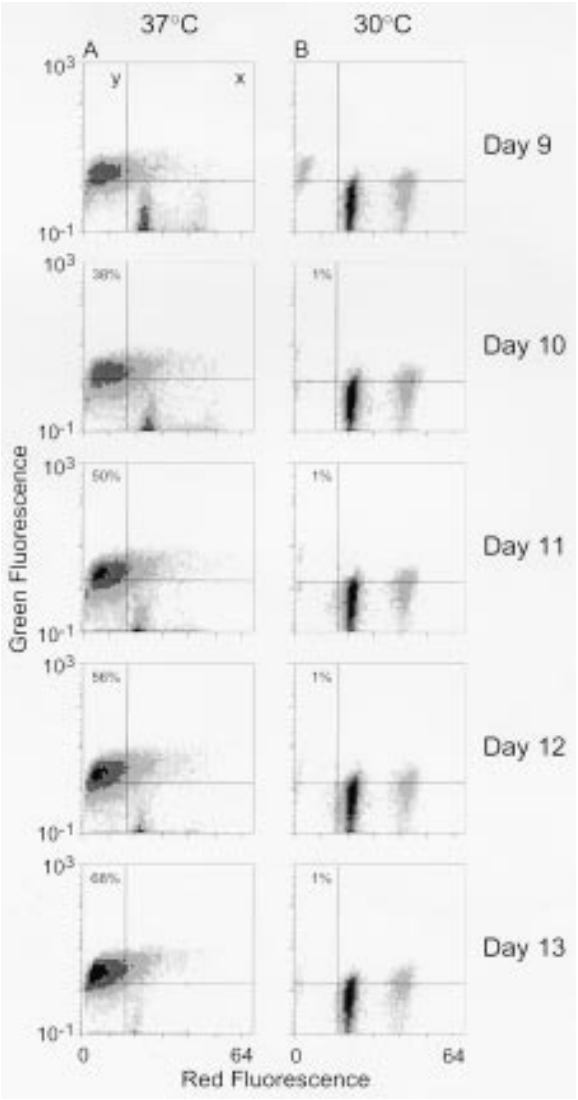

Figure 6. Two dimensional flow cytometric labeling of apoptotic cells and cell cycle. X axis (red fluorescence) is proportional to DNA content; Y axis (green fluorescence) represents end-labeling of apoptotic DNA fragments. Shown in panel A: CHO cells maintained at 37°C, analyses from days 9–13. Cells undergoing apoptotic endonuclease activity become labeled and move into population "x". Progression through the process of apoptosis results in the formation of a highly labeled, hypodiploid population "y". Shown in panel B: CHO cells which were temperature shifted to 30°C at 48 hours, analyses from days 9–13.

of extracted DNA from the same cultures, as shown previously at 37 °C (Moore *et al*., 1995), and currently at 30 °C, Figure 4. The percent of apoptotic cells as a fraction of the total cell population is shown in Figure 5. Reduction in temperature to 30 °C afforded approximately 4 days delay in the onset of significant apoptosis.

Figure 6 shows the flow cytometric pattern of apoptotic labeling together with cell cycle on days 9 to 13 at 37 °C and at 30 °C. In agreement with previous observations (Moore *et al*., 1995), cells were able to exit from any point in the cell cycle, but did so predominantly from G_0/G_1. As cells became apoptotic, the associated DNA strand breaks were labeled with fluoresceinated nucleotide, and are shown in population 'x'. As the cells progressed through the apoptotic process, DNA was lost from the cells and chromatin condensed resulting in the highly labeled hypodiploid population 'y'. This is most obvious in the cells maintained at 37 °C, since the cells grown at 30 °C showed

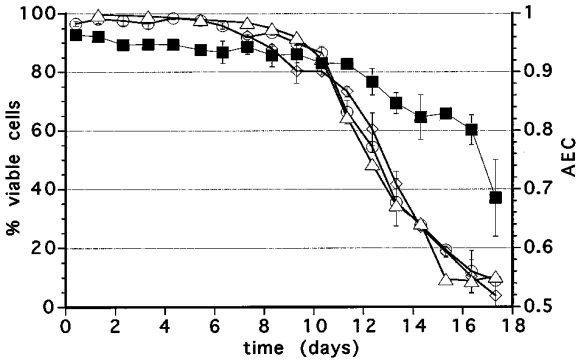

Figure 7. Adenylate energy charge (AEC) (■) on each day of an 18 day culture, which was temperature shifted from 37° C to 30° C at 48 hours. Also shown are the viabilities by trypan blue (○) and LDH (◊), and the percent non-apoptotic cells measured by flow cytometry (△). Mean ± s.d., n = 4.

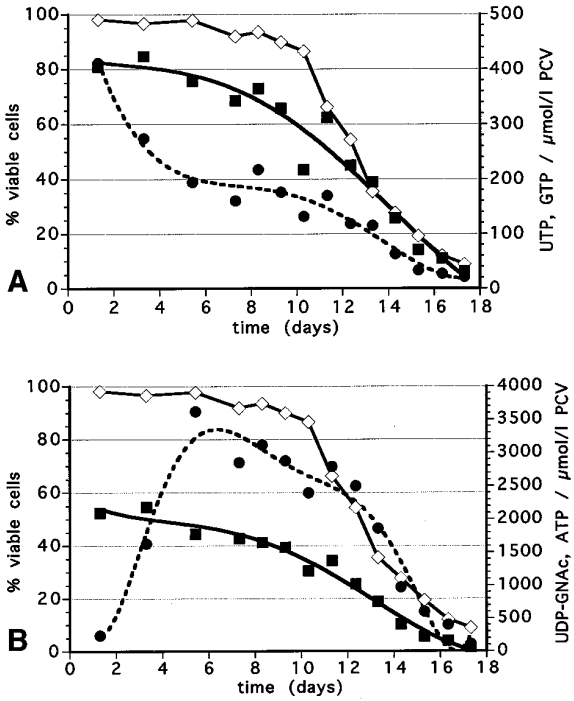

Figure 8. a. Quantification of UPT (●) and GTP (■) concentrations over the course of an 18 day culture initiated at 37° C and temperature shifted to 30° C after 48 hours. Also shown is the percent non-apoptotic cells as analyzed by flow cytometry (◊). b. Quantification of UDP-GNAc (●) and ATP (■) concentrations over the course of an 18 day culture initiated at 37° C and temperature shifted to 30° C after 48 hours. Also shown is the percent non-apoptotic cells as analyzed by flow cytometry (◊). Nucleotide concentrations are given in µmol/liter packed cell volume. Mean of duplicate determinations from 1 of 2 independent experiments.

minimal apoptosis during this time period. Note also the lack of cells in S phase in the lower temperature scatter plots. The percentage of the highly labeled hypodiploid population 'y' correlates well with the percentage of non-viable cells as measured by trypan blue uptake and LDH (see Figure 7). Reduction in temperature caused the cell population to be virtually arrested, i.e. from days 4 through 11 less than 2% of cells were in S phase. Proliferation was therefore minimal, (a proportion of the cells in the G_2/M peak are undoubtedly tetraploid G_1 cells, since CHO cells exhibit characteristic multinucleation). Cell death was also minimal during this time, the average percentage apoptosis per day was 2.3±0.4% (mean ± s.d., n=20). The rate of breakdown of apoptotic bodies in these cultures is unknown, however, it is known that they persist for some time *in vitro* in the absence of phagocytiizing cells. The change in total cell number (viable plus non-viable) during this period of 8 days was approximately 30%.

Nucleotide pools were analyzed in experiment set two. Data given are mean values from two parallel cultures and two independent extractions per culture and at each time point. The standard deviation between the two extractions for each culture was below 10% until day 10 and up to 20% after day 10.

The adenylate energy charge (AEC) (see Ryll and Wagner, 1992, for formulae), was not affected by a temperature shift to 30 °C (Figure 7). The AEC remained above 0.9 until the culture viability dropped below 80% indicating that the cells were able to maintain highly charged nucleotide pools at the lower temperature. Specific amounts of ATP, GTP, UTP and UDP–GNAc are given in Figures 8a and 8b. The ATP and GTP amounts were not immediately influenced by the temperature shift. In contrast the UTP amount showed a sharp decrease after the temperature shift suggesting that cells in S phase contained much greater amounts of UTP than cells in G_1. A decrease of UTP as a result of culture conditions is often associated with increased intracellular concentrations of UDP activated hexosamines UDP–GlcNAc and UDP–GalNAc (UDP–GNAc). This has been previously shown for hybridoma and BHK cells in batch and perfusion cultures (Ryll and Wagner, 1992). The specific UDP–GNAc amount increased sharply following the temperature shift suggesting that G_1 cells contain large amounts of these activated amino sugars. All triphosphates and nucleotide sugars pools were broken down when the viability of the cultures rapidly declined after day 10. The U ratio (UTP/UDP–GNAc),

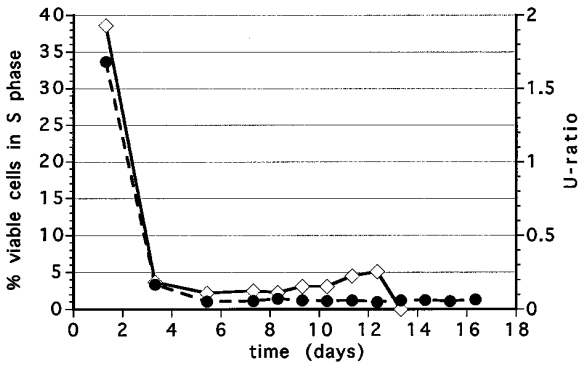

Figure 9. U-ratio (UTP/UDP-GNAc) (●) and percent of cells in S phase (◊) over the course of an 18 day culture initiated at 37°C and temperature shifted to 30°C after 48 hours. Mean of duplicate determinations from 1 to 2 independent experiments.

previously shown to be a very sensitive parameter indicating changes in growth rate (Ryll and Wagner, 1992), correlated very well with the percentage of cells in S phase, Figure 9.

Discussion

Temperature manipulation is a widely used practice in the optimization of batch culture performance (Giard *et al.*, 1992; Jenkins and Hovey, 1993; Weidemann *et al.*, 1994). However, such temperature shifts are not universally beneficial causing depressed productivity in some cases (Reuveny *et al.*, 1986; Bloemkolk *et al.*, 1992). The mechanisms behind these effects are not well understood, and it is thought that temperature reduction *per se* may not directly cause increased productivity, but rather such increases are secondary to effects on cell cycle and viability (Ramírez and Mutharasan, 1990). Recently, it has been acknowledged that cell death in batch culture is mediated via apoptosis (Fraňek *et al.*, 1992; Singh *et al.*, 1994a; Singh *et al.*, 1994b; Moore *et al.*, 1995). However, effects of temperature reduction on rates of apoptosis and correlation with cell cycle events have not been previously studied.

A reduction in temperature on Day 2 of batch culture resulted in a rapid and effective reduction in the percent of cells in S phase and accumulation of the cells in G_0/G_1. This is in agreement with effects previously observed in hybridoma cultures following temperature reduction (Bloemkolk *et al.*, 1992). As observed previously, (Moore *et al.*, 1995) viable cells in CHO cultures maintained at 37 °C continued to proliferate until the termination of the culture.

Temperature reduction additionally resulted in a very significant suppression of apoptotic cell death. This was not an inhibition of apoptosis, but rather a delay. Cultures extended to 18 days in length ultimately exhibited similar kinetics of cell death at 30 °C, but approximately 4 days later than cultures maintained at 37 °C.

Studies investigating cellular growth and death dynamics (carried out at 37 °C), appear to conform to the 'grow or die' theory (de la Broise *et al.*, 1991), which proposes that cells remain viable while they are able to cycle. It was unknown whether steps taken to encourage CHO cells to arrest in G_0/G_1 in which optimum productivity has previously been observed (Suzuki and Ollis, 1989; Coco–Martin *et al.*, 1992) would be at the cost of additionally encouraging cells to exit the cell cycle by apoptosis. The current study using temperature reduction has enabled us to oberve CHO cells in a state close to growth arrest. Rather than promoting cell death, these conditions suppressed cell death. Whether the ability of the cells to reside in G_0/G_1 is directly associated with the reduction in cell death is unknown.

Oxygen uptake rate measurements previously carried out on parallel cultures using the same CHO cell line described here showed a reduction of the specific oxygen uptake rate of approximately 50% (20–25 amol/s/cell) following temperature reduction to 30 °C (data not shown). Thus the cell specific ATP production rate for this CHO cell line is reduced from 200 to about 100 amol/s following the temperature shift. This calculation was done assuming a P/O (moles ATP produced per mole oxygen consumed) ratio of 2.2 (Müller *et al.*, 1986). Together with high, stable energy charge, and high, stable ATP concentrations this suggests that the overall metabolism was reduced at 30 °C. This may be the primary reason for the delayed onset of apoptosis induced by temperature reduction, however the possibility that more specific cellular protective events are also involved remains.

The involvement of nucleotide parameters in the regulation of specific productivity at different temperatures has been discussed for hybridoma cultures (Barnabe and Butler, 1994). In the current study, the correlation of nucleotide pools with the cell cycle distribution and the onset of apoptosis was investigated. No specific correlation could be found between nucleotide pools and increase in death rate after day 10. All observed nucleotide and nucleotide sugar pools (including NAD and CTP, data not shown) broke down reflecting the ultimate degradation of cells following apoptosis.

However, a specific reaction to the temperature shift was observed in the pools of UTP and UDP–GNAc. The specific amount of UTP decreased to 50% following the temperature shift, with a 10 fold increase in the specific amount of UDP–GNAc in the same time frame. The reduction of the percentage of cells in S phase can account for the reduction of the UTP amount. Assuming quantities of UTP proportional to the phases of the cell cycle, the amount of UTP in S and G_2 phase would be approximately 10 times higher than in G_1 (3 versus 0.2 fmol/cell). However, the huge increase observed in the concentration of UDP–GNAc cannot be explained in the same way, and it must be concluded that additional factors played a role in promoting the accumulation after day 2. The accumulation of ammonium in cell culture media has previously been described to be such a factor (Ryll *et al*., 1994). The ammonium concentration in the described CHO cultures reached levels of 6–8 mmol/l on day 3 (data not shown), and it is therefore possible that ammonia facilitated the intracellular accumulation of glucosamine and finally UDP–GNAc, leading to the huge expansion of the total pyrimidine pool (Zaharevitz *et al*., 1988; Ryll *et al*., 1994).

In conclusion, we have shown that reduction of temperature in CHO cell batch cultures caused a rapid decline in cell proliferation, with accumulation of cells in G_1. This was accompanied by a concurrent reduction in U ratio (UTP/UDP–GNAc). Cell viability was preserved in the temperature shifted cultures owing to a delay in the onset of apoptosis. However, once significant apoptosis began, the rate of cell death followed similar kinetics to cultures maintained at 37 °C, and all nucleotide pools were similarly degraded. Temperature reduction to 30 °C did not decrease the energy charge of the cells, however, the overall rate of metabolism was reduced. Whether all, or any of these effects of temperature shift can be explained by conservation of intracellular resources in the absence of growth, catabolite repression at reduced temperature or more specific molecular mechanisms remains to be defined.

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