



Enhanced productivity of G1 phase Chinese hamster ovary cells using the GADD153 promoter

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Abstract

Productivity of three different promoters at various cell cycle stages and under two distinct growth conditions was examined in Chinese hamster ovary cells. Under the Growth Arrest and DNA Damage inducible GADD153 promoter, productivity of the short half-live variant of the enhanced green fluorescent protein (d2EGFP) and the secreted alkaline phosphatase (SEAP) was highest at the G1 phase of the cell cycle and at serum starvation, while under the cytomegalovirus (CMV) or the simian virus SV40 promoter, productivity was highest at S-phase and in complete medium. These results indicate the utility of the GADD153 promoter for production purposes under protein-free conditions.

Introduction

The production of recombinant proteins by mammalian cells typically takes place in protein-free medium, with a large proportion of cells in the G1 phase of the cell cycle. Because the cells do not need to devote cellular resources to biomass production, these can be employed for recombinant protein synthesis.

The relationship between cell cycle and protein expression is still unclear. Maximum culture productivity has been reported during exponential growth (Hayter *et al.* 1991) or when cultures have reached their maximum cell density (Tonouchi *et al.* 1992). Results reported by Lee *et al.* (1998), Banik *et al.* (1996) and Gu *et al.* (1994) show that cell cycle phase expression characteristics of the promoters is of greater importance to the expression of the transgenes under their control than any possible cell cycle effects or other culture parameters. The promoters typically used for production purposes, the cytomegalovirus (CMV) and the simian virus SV40 promoter, have been shown to be S-phase specific (Banik *et al.* 1996, Brightwell *et al.* 1997).

We developed an expression system for biopharmaceuticals in G1 phase Chinese hamster ovary (CHO) cells based on the Growth-Arrest and DNA

Damage inducible GADD153 promoter, which is specifically active under serum-free conditions (Fornace *et al.* 1989), and has, to our knowledge, not been used for production purposes before. In this work, the ability of the GADD153 promoter to drive expression of recombinant genes in CHO cells under different conditions was compared to that of the CMV and SV40 promoters. The reporter gene d2EGFP, a degradable variant of the enhanced green fluorescent protein, was chosen because of ease of its detection by flow cytometry and fluorescence plate reader, and its short half-live of 2 h allows a short on-off response in promoter activity (Bi *et al.* 2002). Productivity studies imitating production conditions in protein-free medium were conducted using the model bio-pharmaceutical secreted alkaline phosphatase (SEAP) (Yang *et al.* 1997). We found that at G1 phase and serum starvation, expression was highest under the GADD153 promoter compared to the CMV and SV40 promoters.

Materials and methods

Cell lines

Genetically engineered Chinese hamster ovary cells (CHO-K1, ATCC CCL61) expressed either d2EGFP or SEAP under the control of the GADD153, the CMV

or the SV40 promoter. Highly expressing clones were isolated and maintained in media containing 400 μg gentamicin ml^{-1} .

Cell culture

Medium used was either protein-free medium, a 1:1 (v/v) mix of Dulbecco's Modified Eagle's Medium (CSL, Australia) and Coon's F12 (Gibco BRL), or complete medium, which was protein-free medium supplemented with 10% (v/v) foetal calf serum (CSL, Australia). CHO cells were generally cultured in T-flasks in complete medium at 37 °C in a humidified atmosphere of CO₂/air (5:95 v/v). Cells were anchorage dependent, and detached by trypsinization when needed.

Synchronisation

Cultures were submitted to serum starvation for 32 h before addition of fresh complete medium containing 10 μg aphidicolin ml^{-1} . After 15 h, cells were released from aphidicolin block by returning the cells to drug-free complete medium. Samples were taken at this point and every 4 h afterward, and cell cycle status and d2EGFP fluorescence were determined.

Cell cycle analysis

Cells were detached, centrifuged, resuspended in PBS, fixed using ice-cold 70% (v/v) ethanol and stored at 4 °C. Before measurement the cells were washed in PBS, then resuspended in the staining solution containing 20 μg propidium iodide ml^{-1} , 2 mg DNase free RNase A ml^{-1} and 0.1% (v/v) TritonX-100 in PBS, and incubated for 30 min at room temperature. Flow cytometric analysis was performed using a Becton Dickinson flow cytometer, excitation 488 nm, emission 630 nm. DNA distributions were modelled using ModFit software (Verity Software House, Inc, Topsham, ME).

AlamarBlue assay

AlamarBlue (AccuMed International, Westlake, OH)/complete medium (10:90, v/v) was added to cells growing in microplates. To determine the background reading, the fluorescence was measured immediately on an F-max fluorescence plate reader (Molecular Devices) at 544 nm excitation and 590 nm emission. The plates were incubated for 4 h at 37 °C under a humidified atmosphere of CO₂/air (5:95 v/v) to allow

the colour to develop, and the fluorescence reading was repeated.

d2EGFP fluorescence

For micro plate cultures, fluorescence was determined using an F-max plate reader (Molecular Devices) with a 488/507 nm filter set, after replacing phenol-red containing media with sterile PBS. For intact cells, a Becton Dickinson flow cytometer using a 488 nm Argon laser was used.

SEAP assay

SEAP concentrations of the cellular supernatant were determined using the Great EscAPe SEAP Assay Kit (Clontech, Palo Alto, Ca) by fluorescent assay as per manufacturer's instruction.

Results and discussion

Gene expression from GADD153, CMV and SV40 promoters is cell cycle related

Cultures expressing d2EGFP under the control of the CMV, the SV40 and the GADD153 promoters were synchronised as described. EGFP fluorescence and cell cycle status was correlated at various time points after release from aphidicolin block, and compared to those of untreated asynchronous control cultures. Representative results for three clones of each cell line are shown in Figure 1.

Results for the cell line CHO-CMV-d2EGFP are shown in Figure 1A. Increased fluorescence of EGFP relative to controls was found in samples that had higher S-phase proportions than the control, indicating that these two factors were related. In contrast, samples with high G1 proportions showed decreased fluorescence relative to controls. For example, the highest fluorescence of 1.05 relative to controls was observed at T4, when 75% S-phase compared to 50% of S-phase in untreated controls; the lowest fluorescence of 0.47 relative to controls was recorded at T12, when no S-phase cells were detected.

Figure 1B shows results for cell line CHO-SV-d2EGFP and again indicate that relative EGFP expression of this cell line was dependent on the proportion of cells in S-phase. Expression increased when S-phase proportion increased relative to untreated control (26% in S-phase). Highest fluorescence of 2.2 was recorded at T4, when 99% of cells were in S-phase,

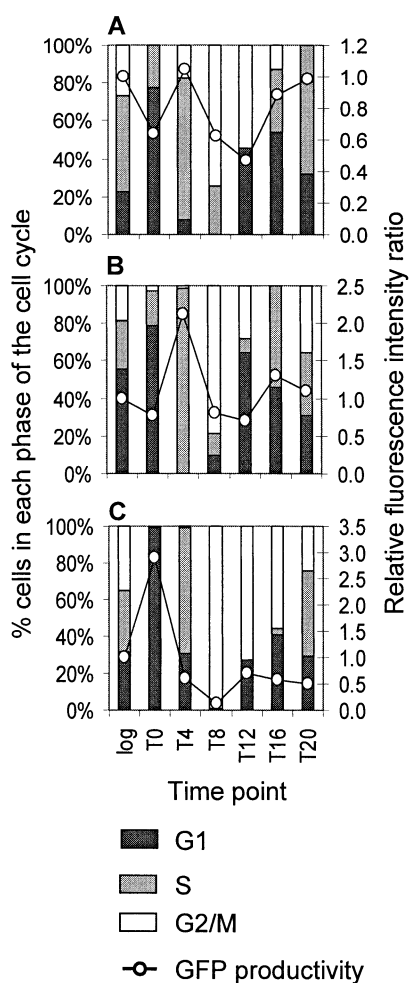


Fig. 1. Cell cycle distributions and EGFP fluorescence of clones after release from aphidicolin block, and untreated controls (log). Representative results of a total of 3 clones per cell line are shown. d2EGFP expression was under the control of the CMV (A), the SV40 (B) or the GADD153 (C) promoters. To allow the comparison of the increase or decrease of d2EGFP expression between samples, the relative fluorescence intensity ratio for each sample was calculated by using a value of 1 for the untreated control (log) of each clone. Average fluorescence per cell of controls are 1.8×10^{-5} fluorescence units under CMV, and 2×10^{-5} fluorescence units under SV40 and under GADD153 control, respectively. Under CMV (A) and SV40 control (B), fluorescence was highest at S phase, and under GADD153 control (C) fluorescence was highest at G1.

while lowest fluorescence was recorded at T12, when 64% were in G1, and only 8% of cells in S-phase.

Results for the cell line CHO-GADD153-d2EGFP are shown in Figure 1C. An increase of 2.9-fold above controls was observed at T0, when 100% of cells were in G1-phase, compared to 32% of controls; lowest fluorescence was observed at T8, when only 1% of cells were in G1-phase. These results indicate that

fluorescence of EGFP was highest in G1 phase of these cells. Virtually no increase in reporter activity was displayed by synchronised cultures that had the same proportion of G1 cells as the untreated (log) control (data not shown). This suggested that the increase in promoter activity was due to G1 growth arrest of the cells treated, rather than aphidicolin treatment *per se*.

For clones in which d2EGFP was controlled by the CMV and SV40 promoters, the cultures were more fluorescent when the proportion of cells in S-phase was high. These results confirmed higher activity of these promoters at S-phase reported by Banik *et al.* (1996) and Brightwell *et al.* (1997), and therefore supported the reliability of the assay and the results achieved for the GADD153 promoter. These indicated that the GADD153 promoter was active primarily in the G1-phase of the cell cycle.

Promoter activity in protein-free medium versus complete medium

Because protein production takes place in protein-free medium, we were interested to determine what proportion of cells enters G1 phase under these conditions, and whether the high G1 phase productivity of the GADD153 promoter after synchronisation translates into high productivity in protein-free medium. The cell cycle status of cells subjected to serum starvation for 24 h and 48 h was analysed and compared to cells growing in complete medium. Results shown in Figure 2 indicate that after 48 h in protein-free medium, a high proportion of cells (79% versus 51% in complete medium) were in G1 phase.

The ability of the GADD153, the CMV and the SV40 promoters to express d2EGFP under protein-free conditions was compared. Preliminary work showed that d2EGFP expression could be quantified by fluorescence plate reader in the cell lines under investigation. This method used for the detection of d2EGFP in 10 clones of the cell lines CHO-GADD-d2EGFP and CHO-CMV-d2EGFP and of 6 clones of the cell line CHO-SV-d2EGFP, after cells were maintained in complete medium, or in protein-free medium for 48 h. Fluorescence was normalised to a measure of cell number in each well of the test plate as estimated by the alamarBlue assay.

Results for each clone are shown in Figure 3. These graphs show that for CHO-GADD-d2EGFP, the average fluorescence per cell was higher in protein-free medium than in complete medium for all 10 clones tested. In CHO-CMV-d2EGFP and CHO-SV d2EGFP,

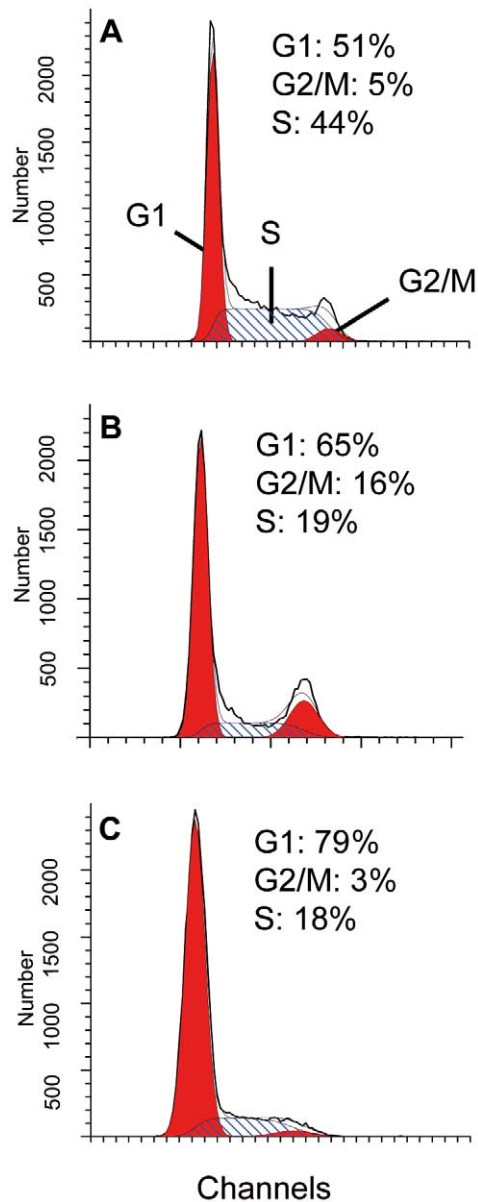


Fig. 2. Cell cycle distribution of CHO-K1 cells in protein-free medium. Representative results of 3 experiments. Cells were grown in complete medium for 24 h; monolayers were subsequently washed 3× with warm protein-free medium and medium replaced with fresh protein-free medium. Cells were harvested prior to medium changeover (A), 24 h post changeover (B) and 48 h post changeover (C), stained with propidium iodide, analysed for their DNA content by flow cytometry and the resulting cell cycle histograms were analysed using ModFit software (Verity Group). After 48 h in protein-free medium, G1 proportion increased mainly at the expense of S phase cells.

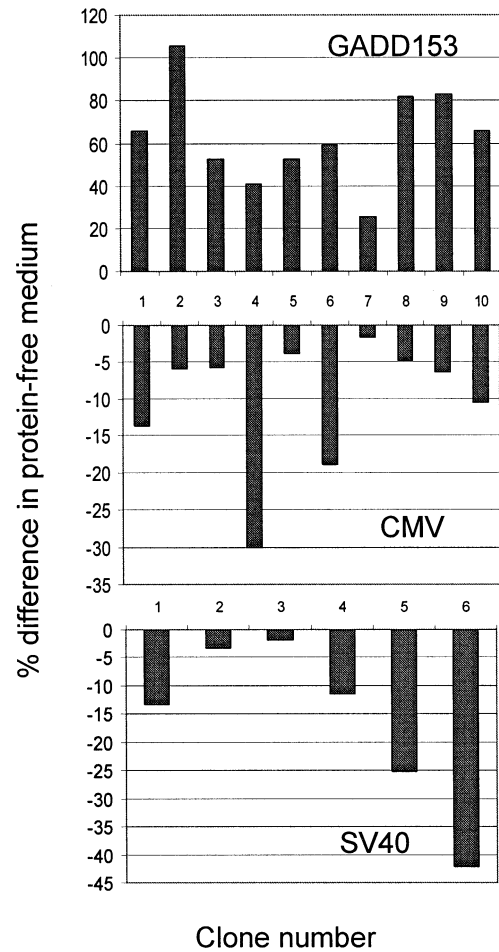


Fig. 3. Percent difference in d2EGFP fluorescence of clones in protein-free medium versus complete medium. Cells were seeded into a 96-well plate in triplicate. Fluorescence was read by plate reader after 24 h in complete medium and after 48 h in protein-free medium and normalised to cell numbers in each well as determined by alamarBlue assay. Average fluorescence per cell in complete medium was 5.7×10^{-5} fluorescence units under GADD153, 6.7×10^{-5} fluorescence units under CMV and 8.9×10^{-5} fluorescence units under SV40 control. To show the effect of culturing the cells in protein-free medium, results were expressed as % difference in protein-free medium.

the fluorescence was lower in protein-free medium than in complete medium for all clones tested. These results indicate that under protein-free conditions, when a high proportion of the culture is in G1 phase, the GADD153 promoter was more active than in cultures in complete medium undergoing rapid growth; the CMV and the SV40 promoters were more active in cells actively growing in complete medium.

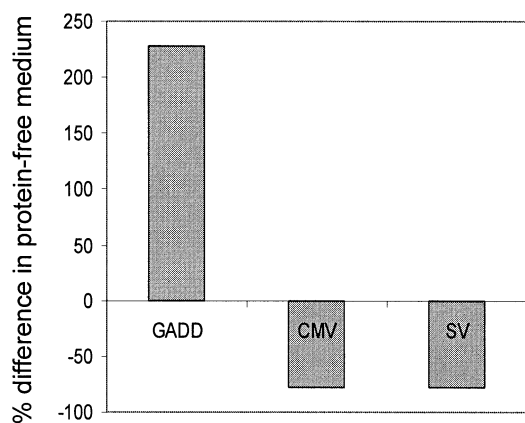


Fig. 4. Percent difference in SEAP productivity of cells in protein-free medium versus complete medium. Under the GADD153 promoter, productivity was 228% higher in protein-free medium than in complete medium, while under CMV and SV40 control, productivity was 78% lower protein-free medium than in complete medium. In complete medium, the average expression per cell over 24 h was 6.1×10^{-9} mg ml⁻¹ under GADD153, 4.8×10^{-9} mg ml⁻¹ under CMV and 3.8×10^{-9} mg ml⁻¹ under SV40 control.

SEAP productivity of three different cell lines in protein-free medium

To assess protein synthesis and secretion in protein-free medium, the intracellular reporter d2EGFP was replaced by the model bio-pharmaceutical secreted alkaline phosphatase (SEAP) (Yang *et al.* 1997). Clones expressing the reporter under the control of the three promoters were cultured in either complete medium or in protein-free medium. SEAP concentrations in the cellular supernatant were determined and expressed as % difference in protein-free medium versus complete medium. Results plotted in Figure 4 show that under the GADD153 promoter, the SEAP productivity per cell in protein-free medium was 228% higher than in complete medium, while under CMV and SV40 control, SEAP productivity decreased by 78% under the same conditions.

These results using a secreted reporter confirmed those achieved by detecting d2EGFP by plate reader. Recombinant protein expression under the GADD153 promoter was higher in protein-free medium, while expression under the CMV and the SV40 promoters were higher in complete medium.

Several investigators (Gu *et al.* 1994, Suzuki & Ollis 1990) have suggested that foreign protein production can be amplified by using bioreactor strategies

appropriate for the particular pattern in which the specific production varies with the growth rate. In the case of inverse-growth related productivity, the cultures may be grown at reduced growth rates in high-density fed-batch or perfusion cultures. If the G1-specific productivity under the GADD153 promoter shown in this paper is reproducible on a large scale in future experiments, this strategy will have an important effect on maximizing glycoprotein productivity of CHO cells.

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