



Regulated autocrine growth of CHO cells

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Received 27 July 1999; accepted 21 February 2000

Key words: autocrine growth, CHO, hGH production, IGF-I, *lac*, metallothionein, transferrin

Abstract

The goal of this work was to engineer a CHO cell line capable of autocrine growth in a fully defined protein-free medium. This was accomplished by stable integration of the genes encoding insulin-like growth factor I (IGF-I) and transferrin into the genome of a CHO-K1 cell line. The *lac* operator/repressor system was used to regulate the expression of the IGF-I gene with the *lac* operator sequence being placed upstream of the coding sequence for IGF-I. The expression of the *lac* repressor protein was driven by a modified metallothionein promoter allowing repressor expression to be regulated by the culture medium. The cell line called *Super CHO^r* (*r* for regulated) was able to grow in protein-free medium in an autocrine fashion with a doubling time of 20–24 hr, either attached to microcarriers or as aggregate suspension cultures. Upon addition of metal to the culture medium, the repressor protein was produced and bound to the operator sequences shutting down the expression of IGF-I and arresting the growth of the cells. Expression of the human growth hormone (hGH) gene and production of hGH was induced by the presence of metal ions. It was possible to release the cells from growth arrest in the presence of metal by the addition of isopropyl β -D-thiogalactopyranoside (IPTG), which prevented binding of the repressor to its operator sequences. The ability to grow CHO cells in fully defined protein-free medium and to be able to regulate their growth rate offers a number of advantages for the use of these cells as hosts for the production of recombinant DNA derived proteins.

Introduction

Mammalian cell cultures are most commonly used for the production of proteins which require post-translational modification for full biological activity. Such modifications do not occur in bacterial cells. The demand for such proteins is increasing rapidly. In particular, the Chinese Hamster Ovary (CHO) cell line has proven to be an effective and reliable mammalian cell host for the production of post-translationally modified proteins.

CHO cells are usually grown in media containing either fetal calf serum (FCS) which contains key proteins required for cell growth, or more defined media with lower total protein concentrations than FCS media. There are several problems associated with the use

of such media. From a regulatory viewpoint there are concerns regarding the use of animal derived materials and the inherent possibility of introducing adventitious agents to the culture. There are also concerns regarding the cost, batch to batch variability, and product purification difficulties associated with the use of such media. Although there are serum- or protein-free media described for the culture of CHO cells (Zang et al., 1995) they are not really satisfactory, because they were developed for one special cell clone. CHO cells can be adapted to protein-free medium. However, this process can take a long time. Alternatively, CHO cells can be genetically engineered to overexpress certain proteins to render the cell protein-free competent (Pak et al., 1996).

Early attempts to develop autocrine CHO cells in-

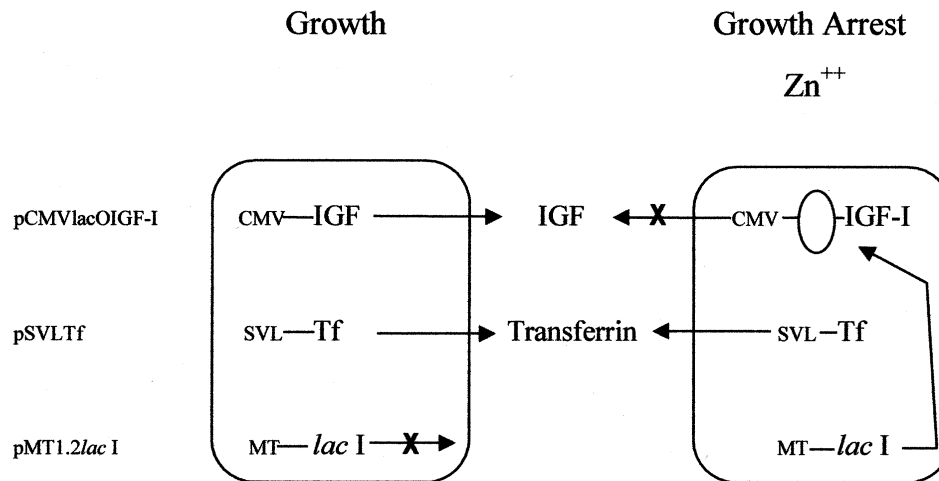


Figure 1. Strategy used to obtain regulated growth of CHO-K1 cell line. Mammalian expression vectors pCMV/lacOIGF-I, pSVLTf and pMT1.2lac I were transfected into CHO-K1 as described in Materials and Methods. Under growth conditions, in the absence of metal, pCMVlac-I expresses IGF-I when the *lac* repressor is not bound to its operator sequences which is located between the CMV promoter and the IGF-I coding sequence. Constitutive expression of transferrin, an iron transport protein, is under the control of the SV40 late promoter. The metal inducible metallothionein promoter controls the expression of the *lac* repressor, and in the absence of inducer, repressor is not produced. Growth arrest is accomplished by the addition of metal to the culture medium. In the presence of metal, repressor is expressed and binds to its operator sequences preventing further expression of the growth factor. Under growth conditions, cells secrete IGF-I and transferrin, while in growth arrest, cells secrete only transferrin.

involved cloning the gene coding for insulin into the cells (Hunt et al., 1996). Such cells failed to process insulin to the native form, and even after overcoming processing problems by introducing a furin recognition site between the C peptide/A chain junction, insulin levels never accumulated sufficiently to allow autocrine growth. Previously we have shown that it is possible to use IGF-I rather than insulin as the mitogenic agent (Hunt et al., 1997). Cells expressing IGF-I and transferrin were found to grow autonomously in completely protein-free medium at the same growth rate and to the same final cell density as the parent cell line (CHO-K1) grown in insulin, transferrin and selenium supplemented medium (Pak et al., 1996).

In this paper, the development of a CHO cell line capable of regulated autocrine growth is reported for the first time. A bacterial *lac* operator/repressor system modified for eukaryotic application was used for regulating TGF-I expression. This system has been shown to be a potent and specific gene regulatory mechanism effective in mammalian cells (Hu et al., 1990). By incorporating a *lac* repressor binding site within the growth factor gene and introducing the gene together with an optimal metal-inducible *lac*I gene into the same CHO cells, regulated expression of the growth factor gene was achieved by addition of metal to the growth medium. A modified inducible metallothionein

promoter (McNeill et al., 1989) was chosen for its low basal level expression and used to regulate the expression of the *lac*I gene.

Such CHO cells capable of regulated, autocrine growth have considerable potential as production hosts for recombinant DNA derived biopharmaceuticals. Not only are they capable of growing well in low cost fully defined protein-free medium with the inherent regulatory advantages, but they also offer the possibility of easily controlling the growth rate of the cells as required. The cells are capable of growing at maximum growth rates during the early batch phase of culture, then it is possible to decrease their growth rate during fed-batch or perfusion culture when recombinant protein expression is occurring at a maximal rate.

Materials and methods

Mammalian expression vectors. pSVLTf (Pak et al., 1996) contains human transferrin cDNA cloned into the expression vector pSVL (Pharmacia). PM1.2LacI (Hunt, 1995) contains the bacterial *lac*I coding sequence modified for mammalian expression and nuclear localisation of the repressor protein (Hannan et al., 1993) and the metallothionein promoter modified

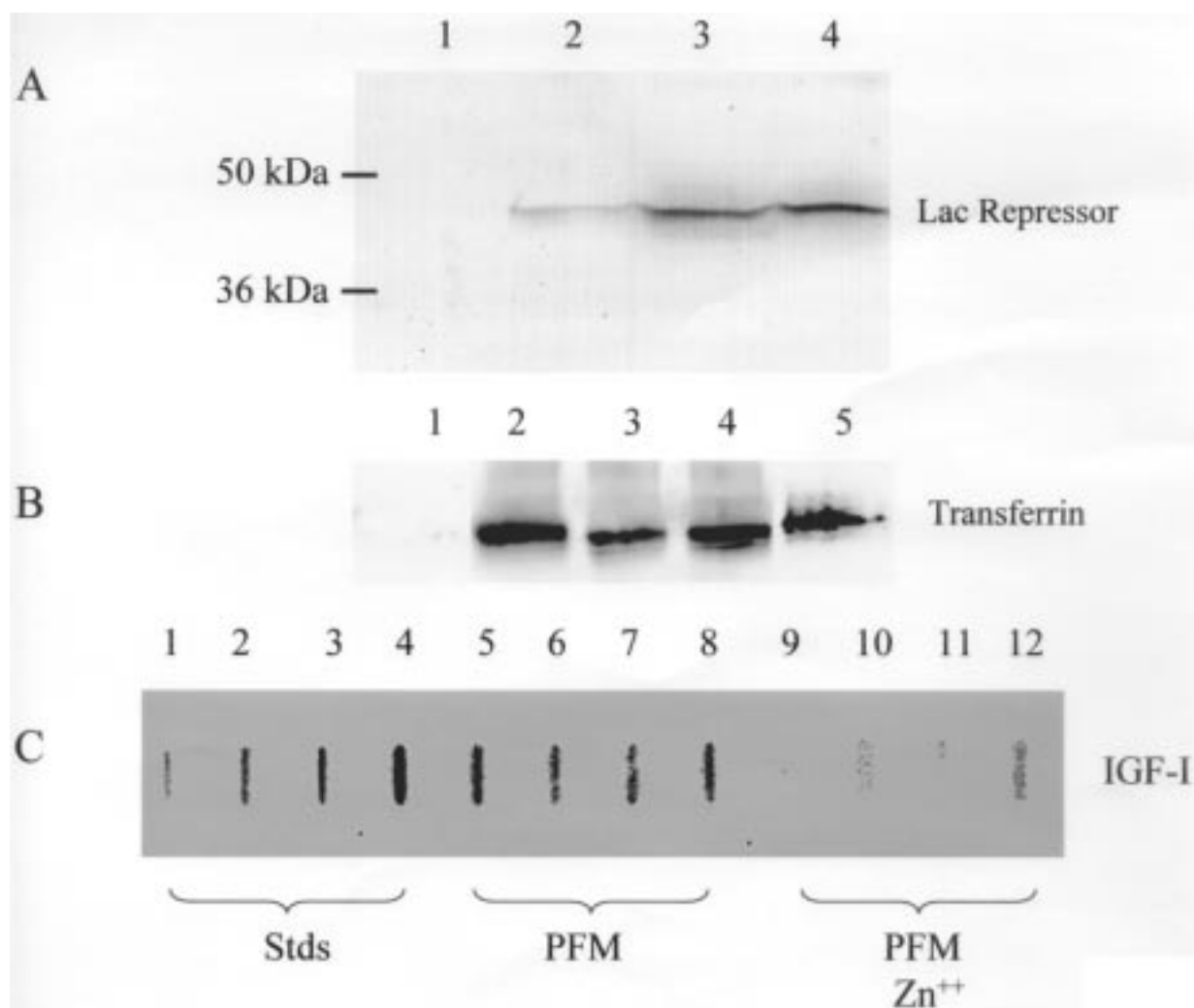


Figure 2. Detection of *lac* repressor, transferrin and IGF-I in regulated *Super-CHO*^F cell line, ISS-2CS-10. A) Western blot of cellular lysate for the detection of *lac* repressor in CHO cells (lane 1) and *Super-CHO*^F cells in protein-free medium (PFM) in the absence of 50 μ M ZnSO₄ (lane 2); and 24 and 48 h after addition of 50 μ M ZnSO₄ (lanes 3 and 4 respectively). B) Western blot of conditioned media for the detection of transferrin in CHO cells (lane 1) and *Super-CHO*^F cells PFM in the absence of 50 μ M ZnSO₄ (lane 2); and 24 and 48 h after addition of 50 μ M ZnSO₄ (lanes 3 and 4 respectively). Transferrin standard, 50 ng (Sigma), lane 5. C) Slot blot of conditioned media for the detection of IGF-I in *Super-CHO*^F cells in PFM in the absence of 50 μ M ZnSO₄ from days 1 to 4 (lanes 5 to 8) and after addition of 50 μ M ZnSO₄ days 1 to 4 (lanes 9 to 12). IGF-I protein standards (Boehringer Mannheim) 2.5-, 5-, 1-, 20 ng (lanes 1 to 4).

for high level inducible expression (McNeal et al., 1989). Plasmid pCMVlacOIGF-I (Hunt, 1995) has the IGF-I coding sequence driven by the human cytomegalovirus promoter and modified *lacO* sequence inserted in optimal sites within the CMVIGF-I gene to regulate IGF-I expression by the *lac* repressor (Hannan et al., 1993). Plasmid pCMVlacOIGF-I encodes the hygromycin B resistance gene. The coding sequence for hGH was cloned into pNK (Bailey et al., 1999) containing the antibiotic resistance gene neomycin. Expression of hGH is controlled by the

modified metallothionein M2.6 promoter (McNeill et al., 1989).

Transfections and cell line isolation. The Chinese hamster ovary cell line, CHO-K1 (ATCC CCL 61), was transfected with plasmids encoding transferrin, *lac* repressor and IGF-I. Cells were transfected with linearized plasmids using a molar ratio of 1:1:02 (pSVLTf:pM1.2lac:pCMVlacOIGF), using a calcium phosphate co-precipitation method. Transfected cells were selected in the presence of 1 mg ml⁻¹ hygromy-

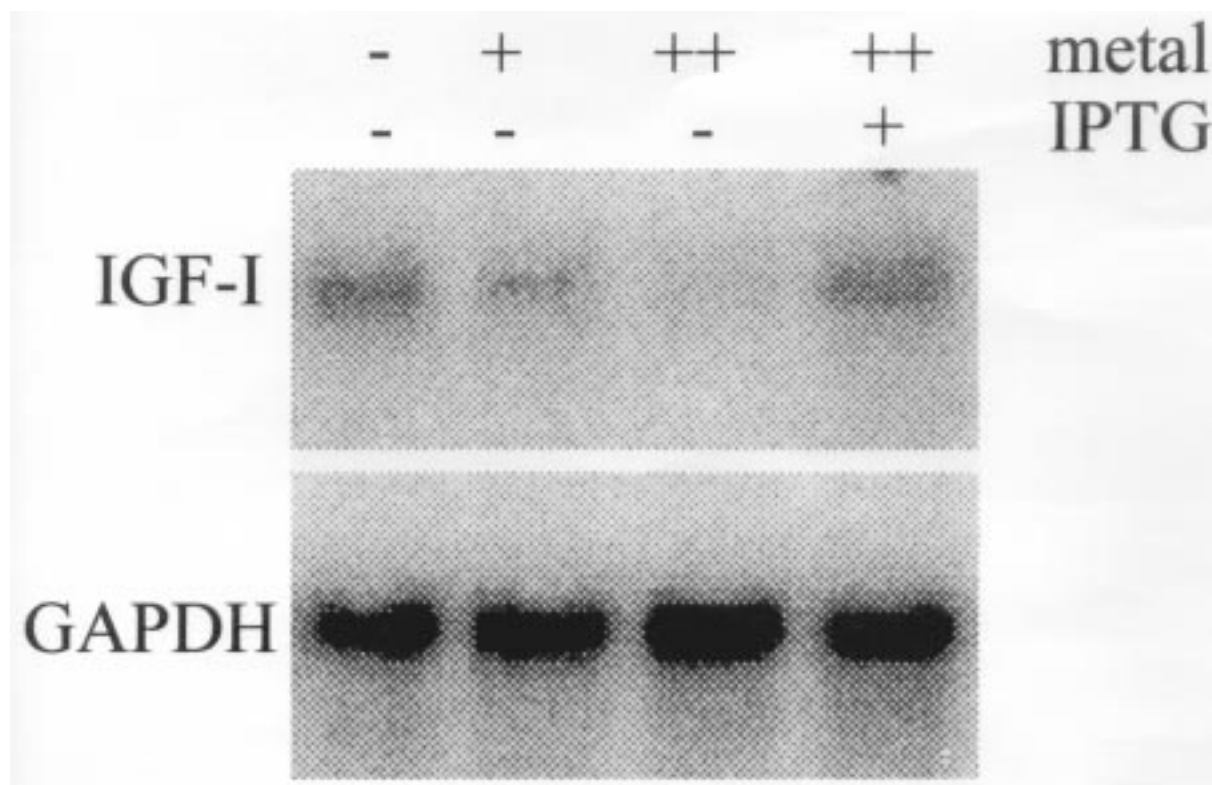


Figure 3. Northern blot of IGF-I mRNA expression in CHO cells capable of regulated growth. Cells were cultured in medium with and without metal (++ = 50 μM ZnSO_4 and 1 μM CdCl_2 , + = 25 μM ZnSO_4 and 0.5 μM CdCl_2) and +/- IPTG (20 mM). GAPDH mRNA was probed to control for cellular RNA levels.

cin B (Boehringer Mannheim). Hygromycin-resistant cells were cloned using limiting dilution plating. After selecting for autocrine growth in protein-free medium (PFM: DMEM:Coon's F12, CSL, Australia), a clone, designated ISS-3CS-10, was similarly transfected with linearized pNKhGH. Transfected cells were selected in the presence of G418, followed by limiting dilution plating. After screening for autocrine growth and hGH production, the clone designated IH10-54 was isolated and used for further study.

Cell culture. Cells were grown in suspension in 250 ml spinner flasks (Techne, UK) containing 100 ml DMEM:Coon's F12 (1:1) medium (CSL, Australia) with the addition of 2×10^{-8} M sodium selenite (Sigma) and 0.2% pluriol (BASF). Initial cell concentrations were $1.6\text{--}2.0 \times 10^5$ cells ml^{-1} . For growth regulation studies, 50 μM ZnSO_4 and/or 50 μM isopropyl-1thio- β -D-galactoside (IPTG) was added when the cell density reached 8×10^5 cells ml^{-1} . Samples were taken every day and viable cell counts were performed using trypan blue exclusion method. To determine cell

number, an aliquot of cells was removed from spinner flasks and cells were counted in a haemocytometer. A trypan blue dye exclusion (Doyle A and Newell DG, 1996) was used to distinguish viable from non viable cells. Regulated growth of *Super-CHO*^r clone ISS-3CS-10 is accomplished by the addition/removal of 50 μM ZnSO_4 . Once a cell concentration of $7\text{--}8 \times 10^5$ cells ml^{-1} was reached, the medium was replaced with fresh protein-free medium containing 50 μM ZnSO_4 . After 72 h the medium was again replaced with fresh protein-free medium without ZnSO_4 .

Preparation and analysis of RNA. Cells were grown as indicated in the text and RNA was extracted and isolated as described previously (Hunt et al., 1997). A CHO derived cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control in Northern blot analysis by first stripping the membrane of bound IGF-I probe and repeating hybridization procedure using GAPDH probe.

Protein detection and determination methods. Transferrin was detected by western blotting as previously described (Pak et al., 1996). Transferrin from conditioned media was visualized using a sheep anti-human transferrin (Serotec) and an alkaline phosphatase-conjugated donkey anti-sheep IgG (Serotec) respectively. For the detection of *lac* repressor cells were solubilized in RIPA buffer (150 mM NaCl, 1.0% NP-40, 1% deoxycholate, 0.1% SDS, 50 mM Tris pH 8.0) and incubated on ice for one hour, and then centrifuged at 14,000 rpm for 15 min. Proteins in the clear supernatant were separated on a 4–20% Tris glycine polyacrylamide gel and repressor protein was detected using a rabbit anti-*lac* repressor polyclonal sera (Stratagene) followed by an alkaline phosphatase-conjugated anti-rabbit IgG. IGF-I was detected in the conditioned medium using an enhanced chemiluminescent (ECL) dot blot method (Sunstrom et al., 1998). Protein concentration of hGH in the conditioned medium was determined using reverse phase high performance liquid chromatography (HPLC) using a Hamilton PRP-∞ column (Hamilton, cat no 79470).

Results and discussion

Cell line engineering

To obtain a cell line capable of regulated autocrine growth, CHO cells were transfected with the plasmids: pSVLtf, pCMV*lac*OIGF and pM1.2*lac*I (Figure 1). A modified *lac* repressor/operator system was chosen to regulate IGF-I expression. Regulation of gene expression was achieved by engineering the IGF-I gene to contain *lac* operator (*lac*O) DNA sequences and controlled by the *lac* repressor DNA-binding protein. An optimal *lac* operator (*lac*O) DNA sequence was chosen for maximal regulation of gene expression (Sadler et al., 1983; Simons et al., 1984). The *lac* repressor gene expression is in turn regulated by the metal inducible promoter to allow for the switching on and off of growth factor production by addition of heavy metal to the medium. The metallothionein promoter, M1.2 (McNeall et al., 1989) having low basal activity and high induction ratio was chosen for the regulated expression of the *lac*I gene. The *lac*I gene was modified for nuclear targeting of the repressor protein (Hannan et al., 1993). Regulated IGF-I mRNA expression by metal was demonstrated in a CHO cell line stably transfected with the pCMV*lac*OIGF construct which was described (Hunt, 1995). While the

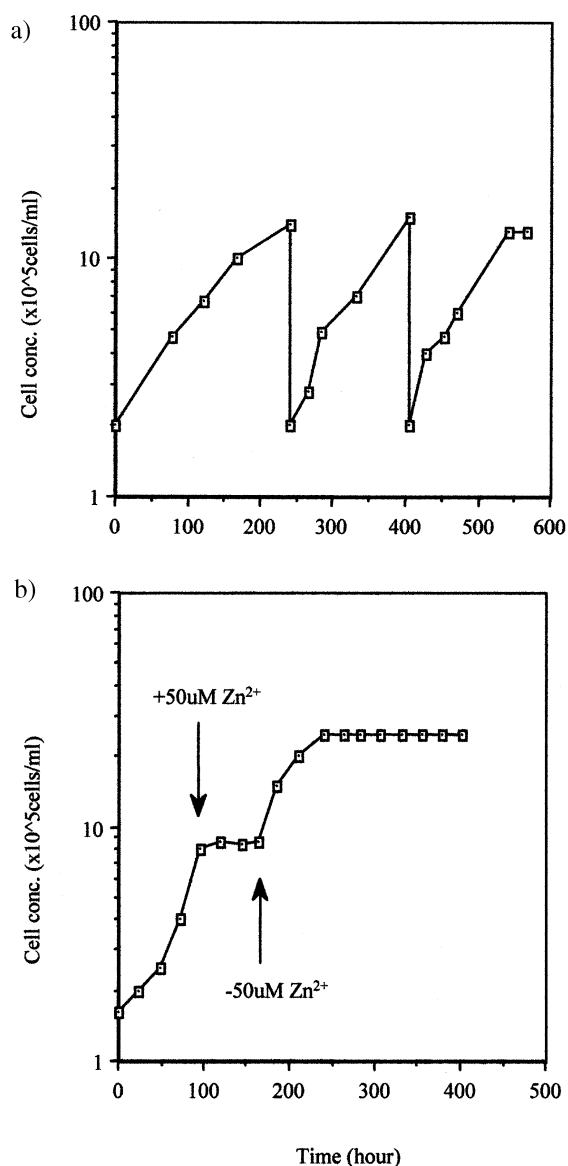


Figure 4. Autocrine growth of the regulated *Super-CHO*^F cell line. A *Super-CHO*^F clone ISS-3CS-10 was grown as small aggregates in protein-free medium without metal. When a concentration of 1.5×10^6 cells ml⁻¹ was reached, the culture was split 1:7 and fresh medium was added. Growth was observed for greater than 600 h. B. Regulated growth of *Super-CHO*^F clone ISS-3CS1-10 is accomplished by the addition/removal of 50 μM ZnSO₄. Once a cell concentration of $7-8 \times 10^5$ cells ml⁻¹ was reached, the medium was replaced with fresh protein-free medium containing 50 μM ZnSO₄. After 72 h the medium was again replaced with fresh protein-free medium without ZnSO₄.

expression of *lac* repressor and IGF-I is regulated by the addition of metal, expression of transferrin gene is constitutive driven by the SV40 late promoter. It was thought that the expression of transferrin, an iron transporting protein, would be beneficial to the cells both in growth and growth arrest phases.

Several clones were examined for the production of transferrin, IGF-I and *lac* repressor in protein-free medium (PFM), and cell lines that expressed all three proteins were expanded. Conditioned medium was analyzed for secreted IGF-I and transferrin by dot blot and Western blot respectively and cell lysate was analyzed by Western blot for *lac* repressor content. Results are shown for the cell line ISS-3CS-10 (Figure 2). Samples were taken from actively growing cells before and after the addition of metal. In Figure 2B, the levels of transferrin remains constant both in the absence, lane 2, and in the presence of metal, lanes 3 and 4. In Figure 2C, IGF-I is present in only trace amounts after three days in metal-containing medium. However, a slight increase in IGF-I was detected in the conditioned medium on the fourth day. This is perhaps due to depletion of metal in the medium leading to insufficient suppression of IGF-I expression. However, this may indicate that ZnSO₄ concentration should be maintained to sustain IGF-I gene repression. This suggests that the production of IGF-I in this cell line can be repressed in the presence of metal. Cellular concentrations of the *lac* repressor protein increased upon the addition of metal to the medium (compare lanes 2, 3 and 4). The addition of 50 μM ZnSO₄ was necessary and sufficient to induce *lacI* mRNA synthesis from the metallothionein M1.2 promoter (data not shown). Although synthesis of the *lac* repressor occurs in these cells at low levels in the absence of metal, it does not appear sufficient to repress growth factor synthesis.

Regulated expression of IGF-I mRNA is shown in Figure 3. RNA was analyzed by Northern hybridization and IGF-I mRNA levels were normalized against GAPDH mRNA levels. Repression levels of 80% and 90% were observed in the presence of 25 μM ZnSO₄ and 0.5 μM CdCl₂ (+), and 50 μM ZnSO₄ and 1 μM CdCl₂ (++) respectively. The addition of both ZnSO₄ and CdCl₂ results in a strong induction of the metallothionein promoter (Bailey et al., 1999). Addition of the strong anti-repressor, isopropyl β-D-thiogalactopyranoside (IPTG) led to a de-repression of IGF-I expression indicating the specificity of the repression.

Autocrine and regulated growth

The cell line, ISS-3CS-10, is able to grow in an autocrine fashion in suspension (Figure 4a) with a doubling time of 20–24 h in protein-free medium. Growth and doubling time in the absence of metal are comparable to that of an engineered CHO-K1 cell line that secreted IGF-I and transferrin constitutively (Pak et al., 1996). This demonstrates that the basal level of repressor is not sufficient to repress growth factor synthesis. To show that the growth of these cells can be regulated by the addition of metal, 50 μM ZnSO₄ was added to the medium of an actively growing culture (Figure 4b). This resulted in the complete cessation of cell division. Cell numbers remained constant for 72 h until the medium was replaced with PFM without ZnSO₄. Upon removal of metal, cell growth was restored. This indicates that cell growth can be tightly regulated by the addition/removal of ZnSO₄. In the absence of ZnSO₄, IGF-I accumulates in the medium since basal levels of *lac* repressor protein do not inhibit transcription of the gene and cells continue to divide and increase in number. Growth factor accumulates in the medium and its synthesis is not inhibited by *lac* repressor protein. When 50 μM ZnSO₄ is added to the culture medium, this is sufficient to induce expression of *lacI* gene and repressor levels accumulate in the cell to sufficient levels to bind to its operator upstream of the IGF-I gene and repress growth factor expression. In the absence of growth factor, cells are arrested and remain in this state while ZnSO₄ is present in the medium. Cells resume growth when ZnSO₄ is removed from the medium and *lac* repressor levels are reduced to a minimum.

To demonstrate that arrested cell proliferation was not due to the toxic effect of 50 μM ZnSO₄, but in fact due to the repression of IGF-I, IPTG was added to the medium at the same time as ZnSO₄ addition. It is known that IPTG prevents repressor binding to its operon which is located upstream of the IGF-I coding sequence; thus, in this system results in the de-repression of IGF-I expression. Figure 5 shows that the addition of both 50 μM ZnSO₄ and 50 μM IPTG had no effect on cell growth, and that cells continued to proliferate in the presence of metal if IPTG is also present. This demonstrates the combined use of the *lac* operator and repressor and the metal inducible promoter in the regulated growth of CHO cells. Therefore it is possible to regulate IGF-I expression and hence the growth rate of the cells by the level of zinc in the growth medium. This offers the possibility of shifting

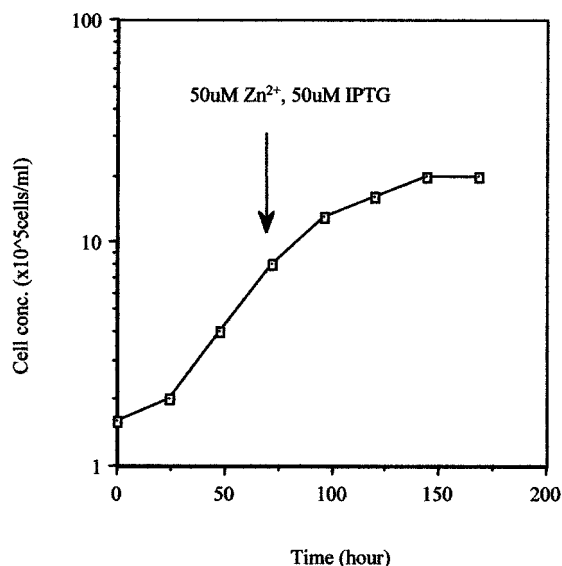


Figure 5. Cell growth in the presence of ZnSO_4 and IPTG. Cells were grown in suspension in protein-free medium. When cell density reached 8×10^5 cells ml^{-1} , the medium was replenished and supplemented with $50 \mu\text{M}$ ZnSO_4 and $50 \mu\text{M}$ IPTG.

the cell line from a phase of active growth to one of low or no growth as required in a production process.

Recombinant protein production is regulated

To demonstrate the usefulness of such cell lines for the production of recombinant proteins, a mammalian expression vector was used to express human growth hormone (hGH) under the control of the metal inducible metallothionein promoter (Figure 6). The expression vector also contains the entire metallothionein gene to confer protection to the cells in high concentrations of metal. The cell line used in this experiment was a stable line obtained by G418 selection but for which there had been no selection or amplification to obtain higher expressing clones. Addition of ZnSO_4 to the medium resulted in cell growth arrest due to repression of IGF-I production and induction of hGH expression. While the cells were in the arrested stage, induction of hGH synthesis was observed and continued for an extended period in fully defined protein-free medium. Cell specific productivity was measured in this cell pool at cell arrest phase and was observed to be $0.9 \mu\text{g}/10^6$ cells per day.

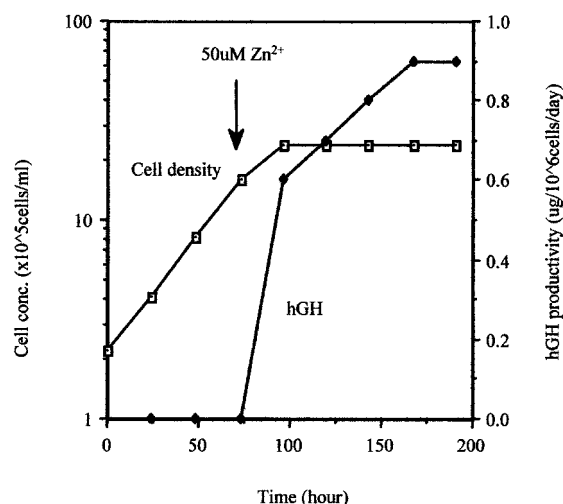


Figure 6. Regulated growth and recombinant protein production. The growth and productivity of the *Super-CHO*^T cell line, IH10-54 (a clonal isolate of ISS-3CS-10 producing hGH) can be regulated with $50 \mu\text{M}$ ZnSO_4 . Aliquots of the supernatant were taken at various times before and after addition of ZnSO_4 to measure hGH production.

Conclusion

The development of autocrine CHO cells allows the cells to be grown in fully defined, protein-free medium. Such medium has a number of cost and regulatory advantages. In previous work we had shown that by cloning the gene coding for IGF-I into the cells, in addition to the transferrin gene, it was possible to get constitutive growth of the CHO cells at high growth rates. In such cells, growth is maximal at all times. In this paper we have demonstrated that it is possible to use the *lac* repressor system to regulate the expression of IGF-I synthesis and hence the growth rate of the CHO cells. Such cells are then ideally suited to the production of recombinant DNA derived biopharmaceuticals. To generate the cell numbers required in the production process, the cells can grow at high rates in low cost fully defined medium. When the required cell concentration is reached, the growth rate can be slowed or stopped by the addition of zinc to the medium. In such a state the cells are ideally suited to fed-batch or perfusion operation, where the cells are provided with the nutrients required to synthesise the recombinant protein over extended periods of time.

Acknowledgements

Sybille Hunt was supported by a grant from the Industrial Research and Development Board, Department of Industry, Science and Technology, Australia. Sugiyono was supported by AUSAID and Charles Bailey by the Australian Post-graduate Research Award Scheme.

References

- Bailey CJ, Baig M, Gray PP and Sunstrom N-A (1999) A rapid selection/amplification procedure for high-level expression of recombinant protein in a metal-amplifiable mammalian expression system. *Biotechnol Techniques* 13: 615–619.
- Doyle A and Newell DG (1996) Cell quantification. In: Doyle A, Griffiths JB and Newell DG (eds), *Cell and Tissue Culture: Laboratory Procedures*. John Wiley and Sons.
- Hannan GN, Lehnert SA, MacAvoy ES, Jennings PA and Molloy PL (1993) An engineered PGK promoter and lac operator-repressor system for the regulation of gene expression in mammalian cell. *Gene* 130: 133–239.
- Hu MC and Davidson N (1990) A combination of derepression of the lac operator-repressor system with positive induction by glucocorticoid and metal ions provides a high-level-inducible gene expression system based on the human metallothionein IIA promoter. *Mol Cell Biol* 10: 6141–6151.
- Hunt, SMN (1995) The regulated expression of growth factor genes in Chinese hamster ovary cells. Doctoral Thesis. University of New South Wales.
- Hunt SMN, Pal SCO, Bridges MW, Gray PP and Sleigh MJ (1997) Chinese hamster ovary cells produce sufficient recombinant insulin-like growth factor I to support growth in serum-free medium. *Cytotechnology* 24: 55–64.
- Hunt SMN, Tait AS, Gray PP and Sleigh MJ (1996) Processing of mutated human proinsulin to mature insulin in the non-endocrine cell line, CHO. *Cytotechnology* 21: 279–288.
- McNeill J, Sanchez A, Gray PP, Chesterman CN and Sleigh JM (1989) Hyperinducible gene expression from a metallothionein promoter containing additional metalresponsive elements. *Gene* 81–88.
- Pak SCO, Hunt SMN, Bridges MW, Sleigh MJ and Gray PP (1996) Super-CHO – A cell line capable of autocrine growth under fully defined protein-free conditions. *Cytotechnology* 22: 139–146.
- Sadler JR, Sasmor H and Betz JL (1983) A perfectly symmetric lac operator binds the lac repressor very tightly. *Proc Natl Acad Sci USA* 80: 6785–6789.
- Simons A, Tils D, Wilcke-Bergmann B and Muller-Hill B (1984) Possible ideal lac operator, Eschericia coli lac operator-like sequences from eukaryotic genomes lack the central G-C pair. *Proc Natl Acad Sci USA* 81: 1624–1628.
- Sunstrom NA, Baig M, Cheng L, Payet D, Sugiyono and Gray PP (1998) Recombinant insulin-like growth factor-I (IGF-I) production in *Super-CHO* results in the expression of IGF-I receptor and IGF binding protein 3. *Cytotechnology* 28: 91–99.
- Zang M, Trautmann H, Gandor C, Messi F, Asselbergs F, Leist C, Fiechter A and Reiser J (1995) Production of recombinant proteins in chinese hamster ovary cells using a protein-free cell medium. *Bio/Technology* 13: 389–392.