Increased production of a secreted glycoprotein in engineered CHO cells through amplification of a transcription factor

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Abstract

Expression levels of reporter protein driven by Mouse Mammary Tumor Virus Promoter system were improved by expressing its specific transcription factor (glucocorticoid receptor) from a different expression vector. The vector that expresses glucocorticoid receptor (GR) also contained dihydrofolate reductase (*dhfr*) gene as a selection marker. In the present study we amplified the glucocorticoid receptor gene (*gr*) along with the *dhfr* gene by adapting the cell lines to increasing concentrations of methotrexate, an antifolate analog. Stepwise increases in the volumetric titers of a secreted reporter glycoprotein, Secreted Alkaline Phosphatase (SEAP), were observed in recombinant Chinese hamster ovary (CHO) cells growing in increased concentrations of methotrexate. Western and RT-PCR analysis showed that this increase in volumetric titers is associated with higher levels of GR expressed in CHO cells growing in increased concentration of methotrexate. A stably transfected cell line growing in 10^{-6} M methotrexate was grown in suspension culture and induced with 10^{-7} M dexamethasone. The SEAP volumetric titers reached a peak of $\sim 23 \mu\text{g ml}^{-1}$ on the 5th day after induction. Inducing these cells with increasing concentrations of dexamethasone resulted in increased specific productivity. These high volumetric productivities were further increased in fed-batch bioreactors.

Introduction

Production of foreign glycoproteins by mammalian cell cultures is superior to their production in microbial or in insect cell lines in terms of posttranslational modifications (Zang et al., 1995). The Chinese hamster ovary (CHO) cell line is the most widely used industrial host cell line for expressing heterologous glycoproteins. This is due to its relative ease of cultivation at large scale and due to its high stability of chromosomally integrated heterologous genes (Reff, 1993). One of the key issues in overexpressing foreign proteins is whether to use a constitutive promoter in the expression vector that allows the production during exponential phase or to use an inducible expression vector that allows the growth phase to be separated from production phase by adding the inducer after the

cell density reaches higher levels. Production in an exponential growth system may lead to lower titers due to a lower number of cells during the early or mid-exponential growth phase (Pendse et al., 1992), while induction of cells for production after achieving medium or high cell densities may lead to higher titers, limited primarily by cell death due to nutrient limitation (Mazur et al., 1999). Therefore, an efficient method of increasing the product titers involves optimizing time and level of induction, and maintaining the viability of induced cells as long as possible through nutrient supplementation.

Multiple expression vectors using Mouse Mammary Tumor Virus-Long Terminal Repeat (MMTV-LTR) as promoters have been reported as a highly inducible expression system (Ko et al., 1989; Gu et al., 1996) in mouse L cells. MMTV-LTR is activated by

glucocorticoid hormones through the interaction of the activated hormone-glucocorticoid receptor (GR) complex with the glucocorticoid response element (GRE) on the MMTV-LTR (Boronat et al., 1997). When MMTV promoter is stably introduced into host cells, the long terminal repeat is organized into an array of at least six nucleosomes (Richard-Foy and Hager, 1987). This chromatin structure inactivates MMTV promoter by preventing the binding of transcriptional factors such as nuclear factor 1 and octamer proteins, to the MMTV promoter (Cordingley et al., 1987). The binding of the hormone receptor complex on the GRE disrupts the nucleosome structure. Concomitantly, the other transcription factors bind to the MMTV promoter and activate the process of transcription (Lee and Archer, 1994). This association of the steroid hormone and recruitment of the transcriptional factors is reported to be dynamic and not static (McNally et al., 2000). The bound hormone-receptor complex is disengaged from its binding site through an ATP dependent chromatin remodeling process (Hager et al., 2000), causing the inactivation of transcription of downstream genes. In order to continuously maintain the active state of expression, intracellular GR level should be more than that of ATP and chromatin remodeling fraction. However, the levels of GR in normal CHO cells are reported to be insufficient for significant activation of MMTV promoter (Hirst et al., 1990). Our recent studies also showed that MMTV promoter driven reporter protein (Secreted Alkaline Phosphatase, SEAP) production titers were very low when the expression vector is stably transfected in to CHO-DG44 cells. However, co-transfection of the same host cells with a second plasmid expressing the transcription factor GR, which was again driven by another MMTV promoter led to production of SEAP of about $5 \mu\text{g ml}^{-1}$, which is 8 fold higher titer than from the constitutive SV40 promoter in another stably transfected CHO cell line (James et al., 2000).

In the present study, we have investigated the possibility of further increasing the expression levels of the secreted reporter glycoprotein by increasing the GR levels in the cells. As the expression vector for GR also contained *dhfr* as a selection marker, increasing the GR expression levels could easily be accomplished by utilizing the gene amplification protocols for dihydrofolate reductase, with increasing concentration of the anti-folate, methotrexate.

Materials and methods

Amplification of glucocorticoid receptor gene

The construction of plasmids pMMap (neo) and pM-Mgr (*dhfr*) and their transfection details are described earlier (James et al., 2000). The CHO cells stably transfected with the above plasmids are referred to as MMapG to indicate the two MMTV promoters ('MM') driving the expression of the two different proteins SEAP ('ap') and GR on an amplifiable vector ('G'). To amplify pMMgr (*dhfr*), MMapG cells were plated in α -MEM(-) (containing L-glutamine but no ribonucleosides or deoxyribonucleosides) with 400 mg l^{-1} Geneticin or G418 (Life Technologies, Rockville, MD), 10% dialyzed fetal calf serum (α -G) with increasing concentration of methotrexate (MTX) from 10^{-8} to 10^{-6} M in a stepwise manner. MMapG cells stably growing in 10^{-8} M MTX (hereafter referred to as MMapG-8) were seeded into 10^{-7} M MTX and cells growing well in 10^{-7} M MTX (hereafter referred to as MMapG-7) were seeded in 10^{-6} M MTX concentration. The cells that survived in 10^{-6} M MTX are referred to as MMapG-6 in later discussions. Pools of resistant cells growing at each concentration of MTX were selected after 5 days and maintained as adherent cultures for two weeks in the same medium. The cells were adapted to serum free IS-CHO (Irvine Scientific, Santa Clara, CA) medium containing G418 and MTX and were maintained in T75 flasks before seeding into spinner batch culture experiments.

Induction studies in tissue culture flasks

Cells growing in varying concentration of MTX were split into two T75 flasks. After two days one flask was induced with 10^{-7} M dexamethasone (a synthetic glucocorticoid hormone analog). Cells were further incubated for two more days before measuring cell density and SEAP concentration.

Batch suspension cultures

The pools of MMapG-6 cells were cloned by limiting dilution in 96 well plates and a single clonal population with high SEAP productivity was isolated. This clonal population was adapted to serum free IS-CHO medium containing G418 and MTX and was used in both the batch suspension cultures and the bioreactor scale-up studies. Each batch culture was started at a density of 2×10^5 cells ml^{-1} in 100 ml of fresh IS-CHO serum-free medium containing G418 and MTX

in spinner flasks as described earlier (James et al., 2000). Dexamethasone was added at predetermined levels to MMapG6 cells when the cell density reached approximately 7×10^5 cells ml^{-1} . Cells were sampled daily by recovering 1.2 ml of the culture medium, 0.2 ml of which was used for determining cell concentration using the Trypan blue dye exclusion procedure. The remaining 1.0 ml was frozen for SEAP enzymatic activity measurements.

Secreted alkaline phosphatase assay

SEAP enzymatic activity assay was initially performed using the Great EscApe SEAP Fluorescence Detection Kit (Clontech labs, Palo Alto, CA) as recommended by the supplier. Assay was carried out in 96-well plates, in kinetic mode with sampling every 2 min for 18 min (10 measurements) with 15 sec shaking time between each cycle, in an HTS 7000 BioAssay Reader (Perkin-Elmer, Norwalk, CT). The temperature of the 96 well plate was maintained at 37 °C. The level of SEAP in each sample was calculated from the mean slope (in RFU/min) using a standard curve obtained with the positive control provided in the kit. The positive control included in the kit was Placental Alkaline Phosphatase (PLAP), which is an untruncated version of SEAP. After performing these assays, it was brought to our attention that our reporter protein SEAP has a significantly different activity than the positive control, PLAP. In addition, we also determined that the positive control included with the kit contained significantly less PLAP than listed. As a consequence, previously determined values for SEAP concentration were recalculated by comparing the PLAP positive controls with a SEAP positive control that was purified in our laboratory (Lipscomb, 2002) using a SEAP assay modified from Cullen and Malim (1992).

Briefly, SEAP was purified by affinity chromatography with an anti-PLAP antibody bound to agarose (Sigma, St. Louis, MO), followed by ion exchange FPLC using a Protein-Pak DEAE-5PW column (Waters, Milford, MA) and a Pharmacia FPLC. The level of SEAP was determined by adding 100 μl 2 $\times$ SEAP buffer (2 M Diethanolamine, 1 mM MgCl_2 and 20 mM L-Homoarginine) to 100 μl sample in a 96 well plate. The plate was heated to 37 °C for ~10 min and 20 μl of 120 mM *p*-Nitrophenol phosphate was added to each well. The light absorbance was measured at 405 nm every 5 min over the next 30 min while continuing to incubate the plate at 37 °C using the

HTS 7000 BioAssay Reader. The level of SEAP in each sample was calculated from the mean slope (in AU/min) using a standard curve obtained with the purified SEAP control. Values previously determined using the Great EscApe SEAP Fluorescence Detection Kit and the included PLAP standard were recalculated by comparing the mean slope of the purified SEAP control to that of the PLAP control. Specific protein production rate was calculated as the protein accumulation rate between successive culture samples divided by the average viable cell number concentration.

Estimation of GR protein by western blotting

GR protein levels in the induced and the uninduced cells were estimated by western blot analysis. The hybridoma cell line ATCC CRL-2173 was obtained from American Type Culture Collection (ATCC) (Rockville, MD) to produce an anti-GR antibody. These cells were grown in Serum Free Hybridoma medium (Life Technologies, Rockville, MD). The supernatant was treated with equal volumes of super saturated ammonium sulphate solution. The resulting precipitate was separated by centrifugation and antibody was purified using protein G column. Purified antibody was checked for homogeneity using 10% SDS-PAGE. 1:100 dilution of this antibody was used for the blotting studies.

A sample of 10^7 cells from both induced and uninduced spinner flasks were centrifuged and pelleted at 1000 rpm. The pelleted cells were washed with cold phosphate buffered saline (PBS). PBS was removed by centrifugation. The cell pellet was solubilized in 1 ml of RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton-X 100, 1% Sodium deoxycholate, 1% SDS and 100 μl of protease inhibitor cocktail (Sigma, St. Louis, MO)). The mixture was kept on ice for 2 h and the insoluble debris was removed by centrifugation at $14\,000 \times g$ for 30 min. Supernatant was transferred into a fresh tube. The protein content of the supernatant was measured by Bio-Rad Protein Assay kit (Bio-Rad, Richmond, CA) using Bovine Serum Albumin as a standard protein. To 75 μl of the supernatant 75 μl of 2X gel-loading buffer (100 mM Tris, 25% glycerol, 2% SDS, 0.01% bromophenol blue pH 6.8, 10% β -mercapto-ethanol) was added and loaded on to a 10% SDS polyacrylamide gel. Proteins separated on SDS-PAGE were transferred on to a PVDF membrane at 24 volts for 2 h using Genie electrophoretic blotter (Idea Scientific Company, Minneapolis, MN). The membrane was washed with phosphate buf-

ferred saline with tween (PBST) and then put in 20 ml of 5% non fat dry milk solution (blocking solution). After 2 h the membrane was washed thrice with PBST and was incubated with anti-GR antibody (1:100 dilution) overnight. Membranes were washed again with PBST to remove any unbound primary antibody and then further incubated with secondary antibody (anti-mouse IgG conjugated to horse radish peroxidase developed in sheep) for 6 h and the color was developed using Opti-CN kit (Biorad, Richmond, CA). The intensity of the bands was measured using Gel doc (Bio-Rad, Richmond, CA).

Quantification of GR mRNA by RT-PCR

Samples containing 10^7 viable cells from both induced and uninduced culture were collected every day. Total RNA was isolated using RNeasy minikit (Qiagen). The protocol for isolation of total RNA from animal cells was strictly followed as per manufacturers recommendations. RNA was quantified by measuring the absorbance at 260 nm. For the synthesis of 1st strand cDNA, 10 μ g of total RNA was taken in a 200 μ l microfuge tube to which 1 μ l of oligo dT primer (12) was added. Volume was made to 12 μ l by adding sterile nuclease free water. The contents were mixed and heated to 70 °C for 10 min and quickly chilled on ice. To the cooled sample 4 μ l of 5X first strand buffer, 2 μ l of 0.1 M DTT and 1 μ l of 10 mM dNTP mix were added. The contents were mixed and incubated at 42°C. After 2 min, 1 μ l (200 units) of Superscript^{TM} Reverse-transcriptase (Life Technologies, Rockville, MD, Cat. No. 18064-014) was added and further incubated at 42 °C for 50 min. Heating at 70 °C for 15 min inactivated the reaction. The samples were chilled on ice and to remove RNA complementary to cDNA, 1 μ l (2 units) of E.coli RNase H was added to the tube and further incubated at 37 °C for 20 min.

For PCR amplification, a 10 μ l aliquot of the cDNA containing soup was transferred into two separate 200 μ l microfuge tubes. To each tube, 10 μ l of 10X PCR buffer (200 mM Tris HCl (pH 8.4), 500 mM KCl), 3 μ l of 50 mM MgCl₂, 2 μ l of 10 mM dNTP mix, 1 μ l each of 5' and 3' primers (10 μ M), 1 μ l of Taq DNA polymerase (5 U ml⁻¹) were added and the final volume was adjusted to 100 μ l by adding nuclease free sterile water. The primers for rat *gr* were generated using vector NTI suite (Informax, Inc). The 5' primer contained TGGACTCCAAAGAAT and 3' primer contained CTAGTATCGCCTTTGCCCAT.

These primers generate a band of 1.9 kb corresponding to the entire sequence of *gr*. The PCR amplification steps were performed for 50 cycles with an annealing temperature of 55 °C. 20 μ l of the PCR product was electrophoresed on a 1% agarose gel and intensity of each band was calculated using Gel Doc (Bio-Rad, Richmond, CA) and expressed as relative intensity.

Bioreactor scale-up studies

IS-CHO medium (1200 ml) containing 400 mg l⁻¹ of Geneticin and 10⁻⁶ M methotrexate was transferred to a previously sterilized 2 l New Brunswick Celligen bioreactor vessel using a peristaltic pump and seeded with 2.5 × 10⁵ cells ml⁻¹ from spinner flasks. Dissolved oxygen and pH were controlled at 30% and 7.0, respectively, by pumping carbon dioxide, oxygen, nitrogen and air. Dexamethasone was added at 10⁻⁷ M concentration when the cell count reached 8 × 10⁵ cells ml⁻¹. Cells were sampled daily by collecting 15 ml of the culture medium, 0.2 ml of which was used for determining cell concentration using the Trypan blue dye exclusion procedure, and the rest was frozen for SEAP activity measurements. Osmolality was measured using the Advanced Micro osmometer (Advanced instruments Inc, Norwood, MA).

Results

Increasing concentrations of methotrexate

Pools of resistant MMapG cells growing at increasing concentrations of MTX were maintained in serum containing α -G medium for 2 weeks and then adapted to serum free ISCHO medium at similar MTX concentrations in tissue culture flasks (T-75). The cells growing in ISCHO medium without any methotrexate were labeled as MMapG, and the other cell lines were labeled MMapG-8, MMapG-7 and MMapG-6, where in the number X indicates the concentration (10^{-X} M) of MTX to which they were adapted. Cells were maintained by subsequent seeding in to fresh IS-CHO medium. Cells actively growing in varying concentrations of MTX were split into two T75 flasks for induction studies. The results in Figure 1a show that specific production rate (pg cell⁻¹ day⁻¹) after two days of induction with 10⁻⁷ M dexamethasone from MMapG-8 cells in tissue culture flasks was 3 times higher than that from MMapG, and the MMapG-7 and MMapG-6 cells show much higher activity.

Figure 1. Expression of secreted alkaline phosphatase and glucocorticoid receptor by MMapG cells growing in ISCHO medium with varying concentration of methotrexate in tissue culture flasks (T75). (a) Specific protein production per cell. (b) Quantification of GR by western blot analysis. Lanes 1 MMapG cells uninduced, Lane 2 MMapG cells induced with 10^{-7} M dexamethasone, Lane 3 MMapG-8 cells uninduced, Lane 4 MMapG-8 cells induced with 10^{-7} M dexamethasone, Lane 5 MMapG-7 cells uninduced, Lane 6 MMapG-7 cells induced with 10^{-7} M dexamethasone, Lane 7 molecular weight markers, Lane 8 MMapG-6 cells uninduced and lane 9 MMapG-6 cells induced with 10^{-7} M dexamethasone. (c) Quantification of GR mRNA by RT-PCR and agarose gel. Lane 1 MMapG cells uninduced, Lane 2 MMapG cells induced with 10^{-7} M dexamethasone, Lane 3 MMapG-8 cells uninduced, Lane 4 MMapG-8 cells induced with 10^{-7} M dexamethasone, Lane 5 1 Kb DNA ladder, Lane 6 MMapG-7 cells uninduced, Lane 7 MMapG-7 cells induced with 10^{-7} M dexamethasone, Lane 8 MMapG-6 cells uninduced, Lane 9 MMapG-6 cells induced with 10^{-7} M dexamethasone.

Since the plasmid pMMgr contains another expression cassette with a SV40 promoter driven *dhfr* gene, any amplification of *dhfr* by increased concentration of MTX should also amplify *gr* gene. Moreover, as all the cells growing in different concentrations of MTX are derived from the same clone, the increase in SEAP production is due to amplified production of GR in these cell lines.

Equal volumes (75 μ l) from uninduced and induced cells were loaded onto SDS-PAGE and transferred to PVDF membrane, by electro blotting, for Western blot analysis of GR. This analysis revealed that the uninduced cells have no or very little GR. It could not be detected by our technique for all the cell lines growing in different concentrations of MTX (Figure 1b: Lanes 2, 5, 7 and 9). For the induced cell lines growing in increased concentration of MTX two different bands at molecular weights approximately $\sim$ 90 kDa and $\sim$ 40 kDa were seen (Figure 1b: Lanes 1, 4, 6, and 8). It is reported in the literature that the molecular weight of Gr is $\sim$ 92 kDa (Hirst et al., 1990). The second band at $\sim$ 40 kDa may be a truncated form of this GR that needs to be investigated. Hirst et al. (1990) also reported two bands at 92 and 45 kDa in their western blot analysis for wild type mouse glucocorticoid receptor. The intensities of both low and high molecular weight bands were taken together to estimate the relative intensity of GR with increased concentration of MTX. Compared to the intensities of GR bands from MMapG cells, the intensities of GR bands in MMapG-8, MapG-7 and MMapG-6 cells were 1.4, 1.6 and 1.7 times higher, respectively.

Equal amount of total RNA (10 μ g) from induced and uninduced cells was subjected to RT-PCR under identical conditions. In all the cell lines grown in increasing concentration of MTX the uninduced cells did not show any band at 1.9 kb corresponding to rat GR (Figure 1c: Lanes 1, 3, 6 and 8). However, a clear 1.9 kb band is seen in all the induced cells, with increasing intensities from cells growing in increased concentrations of MTX (Figure 1c: Lanes 2, 4, 7 and 9). The relative intensities are 1.1, 1.4 and 1.5 times higher in MMapG-8, MMapG-7 and MMapG-6 cells compared to MMapG cells. Thus the increase in SEAP productivity with increase in MTX concentration could be correlated to increased *gr* mRNA and GR protein levels in these cells.

The total intracellular protein in induced and uninduced cells was measured using the Bio-Rad total protein assay and shown in Table 1 and summarized here. These measurements show a step-wise increase

in the total intracellular protein in both uninduced and induced cells grown in increasing concentration of MTX. For the uninduced cells, these intracellular protein increases at higher MTX concentration are consistent with the amplified production of dihydrofolate reductase protein at increasing concentrations of MTX. At each MTX level, the induced cells show a significant increase in total intracellular protein, which is consistent with the induced overproduction of the intracellular transcription factor, GR proteins from MMTV promoter, upon addition of 10^{-7} M dexamethasone.

The total RNA isolated from the cells growing in increasing concentrations of MTX are given in Table 1 and summarized here. The trends in the total RNA inside each cell follow the trends in the total intracellular protein very closely. For uninduced cells grown at increasing concentration of MTX, the total RNA content per cell shows a stepwise increase, suggesting higher transcription from amplified *dhfr* gene content. At each MTX level, the induced cells contain a slightly higher amount of total RNA, which is consistent with the increased transcription of both *gr* and *seap* genes, when induced with 10^{-7} M dexamethasone.

Production of SEAP in MMapG-6 cells in suspension culture

Since MMapG-6 cells showed higher productivity compared to all other cell lines, we conducted a more detailed kinetic study on the productivity of secreted reporter glycoprotein, SEAP. The MMapG-6 cells were cloned and a single clonal population producing a high level of SEAP was isolated and analyzed in suspension culture. It is seen from the cell concentration data (Figure 2a) that MMapG-6 cells slow down in their growth upon induction with 10^{-7} M dexamethasone on day 3. The slowing down of growth was also seen in our previous studies (James et al., 2000) and follows our structured kinetic model prediction that the growth rate is reduced due to the production of heterologous proteins in the recombinant CHO cells (Kompala, 1999).

The volumetric concentration of SEAP in the induced cells increased to a peak of about 23 μ g ml $^{-1}$ on the 9th day (6 days after induction) and dropped slightly to 19 μ g ml $^{-1}$ on 10th day (7 days after induction) (Figure 2b). This SEAP concentration was significantly greater than that obtained in SV40 or unamplified MMapG cultures (James et al., 2000). There

Figure 2. Production of secreted alkaline phosphatase by MMapG-6 cells in spinner flasks upon induction by 10^{-7} M dexamethasone on day 3. (a) Cell concentration. (b) Volumetric SEAP protein concentration (symbols represent the average of six measurements indicated by error bars). (c) Total intracellular protein.

Table 1. Total protein and total RNA in MMapG cells growing in increasing concentrations of methotrexate

	Methotrexate concentration	Total protein		Total RNA	
		Uninduced pg cell ⁻¹	Induced ^a pg cell ⁻¹	Uninduced pg cell ⁻¹	Induced ^a pg cell ⁻¹
MMapG	0	236	288	6.2	6.9
MMapG-8	10 ⁻⁸ M	242	299	6.6	7.4
MMapG-7	10 ⁻⁷ M	251	305	6.8	7.4
MMapG-6	10 ⁻⁶ M	265	330	7.1	7.8

^a Cells were induced with 10⁻⁷ M dexamethasone.

was insignificant or no SEAP production in uninduced cells during the entire culture period (Figure 2b).

Equal volume (75 μ l) of cell lysate from both induced and uninduced cells at different time intervals was subjected to SDS-PAGE for western blot analysis. The uninduced cells of MMapG-6 cells did not show any GR protein in their cell lysate (Figure 3a: Lanes 1, 3, 5, 7, 10, 12, 14). In the induced cells, GR protein bands appeared within 1 day of induction (4th day of the culture) and the intensity remained the same until the 9th day and dropped slightly by the 10th day (Figure 3a: Lanes 2, 3, 6, 8, 11, 13 and 15). These data suggest that the intracellular GR protein content per cell increases within 1 day after induction and maintains a steady state until the 9th day and during this period, SEAP productivity increased with time (Figure 2b).

Total intracellular protein in cell samples taken at different time intervals (Figure 2c) revealed that upon induction with 10⁻⁷ M dexamethasone the intracellular total protein in induced cells increased to about 240 pg cell⁻¹ within one day, and this is about 30 pg cell⁻¹ more than that in the uninduced cells. This difference is maintained throughout the cycle. Since MMTV promoter, upon induction with dexamethasone, also drives expression of *gr* gene, the increase in total intracellular protein in inducible cells is due to the induced overexpression of GR. This large increase (~12%) in the total intracellular protein upon induction has also been observed in our earlier work with the expression of intracellular β -galactosidase in mouse L cells (Gu et al., 1996).

Similar to our earlier RT-PCR results with different cells growing in increasing concentration of MTX, the uninduced cells of MMapG-6 cells did not show any band corresponding to *gr* cDNA, with respect to time (Figure 3b: Lanes 1, 3, 5, 7, 9, 12 and 14). In the induced cells with respect to time, there was a slight

increase in the intensity of the band for *gr* cDNA from the 4th to the 5th day (one day after induction) and the intensity remained almost the same until the 9th day and then reduced by the 10th day (Figure 3b: Lanes 2, 4, 6, 8, 10, 13, 15).

The data of western blot analysis (Figure 3a) and RT-PCR data (Figure 3b) clearly indicate that the increase in SEAP productivity in MMapG-6 cells is due to increased expression of the specific transcription factor, glucocorticoid receptor, upon induction.

Effect of dexamethasone on SEAP production by MMapG-6 cells

GR can actively induce transcription from MMTV-LTR only when it is bound to glucocorticoid hormone. Thus even if GR is available in plenty, the concentration of glucocorticoid hormone in the cells might be a limiting factor, if not provided in sufficient quantities, for the efficient transcription of this expression system. To address the possible effects of hormone limitation, we induced the MMapG-6 cells with varying concentrations of dexamethasone. These experiments showed a strong dose dependent response in cell concentration, volumetric SEAP titers and specific production with increase in dexamethasone concentration (Figure 4). The volumetric production (Figure 4b) increased from the peak titer of 10 μ g ml⁻¹ with the induction by 10⁻⁸ M dexamethasone, to about 19 and 23 μ g ml⁻¹, in cases of induction with 10⁻⁷ M and 10⁻⁶ M dexamethasone concentrations, respectively. These results indicate that there is a potential to increase our titers further in this expression system, if we can avoid the detrimental effect of dexamethasone on cell growth. The product concentration reached a peak value of about 23 μ g ml⁻¹ within 2 days after induction with 10⁻⁶ M dexamethasone, followed by an extracellular degradation, most likely by

Figure 3. Profile of glucocorticoid receptor in MmapG-6 cells grown in spinner flasks. (a) Quantification of GR protein in induced and uninduced cells by western blotting. Lanes 1, 3, 5, 7, 9, 12, 14 are for uninduced cells from day 4, 5, 6, 7, 8, 9 and 10 days, respectively. Lanes 2, 4, 6, 8, 10, 13 and 15 are for induced cells from 4, 5, 6, 7, 8, 9 and 10 days. Lane 11 is the molecular weight marker. (b) Quantification of *gr* mRNA by RT-PCR and agarose gel electrophoresis. Lanes 1, 3, 5, 7, 9, 12, 14 are for uninduced cells from day 4, 5, 6, 7, 8, 9 and 10 days, respectively. Lanes 2, 4, 6, 8, 10, 13 and 15 are for induced cells from 4, 5, 6, 7, 8, 9 and 10 days. Lane 11 is the molecular weight marker.

proteases released from dead cells. At 10^{-7} M dexamethasone concentration, the product titers increased up to 3 days after induction and reached a maximum of $19 \mu\text{g ml}^{-1}$. Cells treated with 10^{-6} M dexamethasone showed highest per cell specific production rate that reached a peak of $16 \text{ pg cell}^{-1} \text{ day}^{-1}$ after 1 day of induction (Figure 4c).

Bioreactor cultures

MMapG-6 cells were grown in 1.5 l bioreactors, which were maintained at constant pH and dissolved oxygen of 7.0 and 30%, respectively. The cells were induced with 10^{-7} M dexamethasone on 5th day after the cell concentration reached $7.1 \times 10^5 \text{ cells ml}^{-1}$. Glucose, glutamine and MEM vitamin solution were also added at day 5 (along with dexamethasone) and at day 8 (Figure 5a). We also observed that supplying additional nutrients at the time of induction and after 4 days after induction did not improve the cell viability. The cell concentration gradually decreased to 5×10^5

cells ml^{-1} with viability falling below 50% after 12 days. The cell death may not be due to factors such as pH, DO and nutrient limitation, but may likely be due to the accumulation of metabolic by-products, like ammonia and lactate.

The production of SEAP (Figure 5b) showed a steady increase from the day of induction to about $25 \mu\text{g ml}^{-1}$ on the 10th day (5 days after induction). The specific production rate (Figure 5c) also increased rapidly within the two days after induction and maintained a high specific productivity of $\sim 9.5 \text{ pg cell}^{-1} \text{ day}^{-1}$ for next four days. In our earlier studies (James et al., 2000), we obtained a peak rate of $0.3 \text{ pg cell}^{-1} \text{ day}^{-1}$ using the constitutive SV40 promoter and $2.3 \text{ pg cell}^{-1} \text{ day}^{-1}$ for SEAP in un-amplified MMapG cells. Thus, in MMapG-6 cells there is almost a 32-fold increase over SV40 and a 4-fold increase over MMapG cells.

Figure 4. Effect of dexamethasone on production of secreted alkaline phosphatase by MMapG-6 cells in spinner flasks. (a) Cell concentration. (b) Volumetric SEAP protein production (symbols represent the average of six measurements indicated by error bars). (c) Specific SEAP protein production rate.

Figure 5. Secreted alkaline phosphatase production by MMapG-6 cells in bioreactors. (a) Cell concentration. (b) Volumetric SEAP protein production (symbols represent the average of six measurements indicated by error bars). (c) Specific SEAP protein production rate (+ Indicates addition of 0.8 g of glucose and 52.8 mg of glutamine along with 10 ml of filter sterilized 100 X MEM vitamin solution).

Discussion

Previous reports (Yamamoto, 1985 and Vanderbilt et al., 1987) have identified that heterologous protein expression from the glucocorticoid-inducible MMTV promoter is limited by the unavailability of its specific transcription factor, glucocorticoid receptor, in mammalian cells. Ko et al. (1989) and Gu et al. (1996) have shown that inducible expression of an intracellular reporter protein (β -galactosidase) from MMTV promoter can be significantly improved by the co-transfection of the *gr* gene in mouse L cells. We have recently demonstrated (James et al., 2000) that secreted reporter glycoprotein SEAP can be overexpressed (up to $5 \mu\text{g ml}^{-1}$) from the inducible MMTV expression system in the commonly used industrial host, CHO cell lines, through coordinated expression of the glucocorticoid receptor from a second MMTV expression vector.

In the present study, we have taken advantage of the *dhfr* selection marker, that we have constructed into the second expression vector and co-amplified the *gr* gene copy number through the *dhfr* gene amplification with methotrexate. After several weeks of culturing the stably transfected cells at increasing methotrexate levels, the GR mRNA (based on the intensities of cDNA after PCR) and protein levels were shown (Figure 1) to be significantly higher on the 2nd day after dexamethasone induction, in cells grown in higher concentrations of methotrexate. These experiments demonstrate the feasibility of increasing heterologous protein expression levels by amplifying *gr* gene copy number.

Focussing in further studies on a single gene-amplified cell clone (MMapG-6), we have characterized the molecular intermediates underlying the very high expression levels (Figures 2 and 3) of the secreted reporter glycoprotein upon induction with dexamethasone.

We have also demonstrated from our co-transfection experiments that in our induced cell lines, the specific production rate of SEAP increases with increased concentrations of dexamethasone (Figure 4). This indicates that there is further potential in increasing the production titers in our expression system if one can overcome the detrimental effects of dexamethasone on cell growth.

Culturing this *gr* gene-amplified cell clone MMapG-6 in a microprocessor-controlled bioreactor provides a more accurate control of pH, dissolved oxygen and nutrient concentration and there by achieves

a slightly higher volumetric titer (Figure 5) compared to the SEAP titer in the spinner flask cultures (Figures 2 and 4). Addition of a fresh medium 3 or 4 days after induction in fed-batch culture may extend the bioreactor culture longevity over that in spinner flask cultures. However, the accumulation of proteases from the dead cells in fed-batch cultures may play a strong role in the degradation of the secreted reporter protein over the extended culture operation. This extracellular degradation of secreted protein may be reduced by selective removal of dying cells, before they release their proteases into culture medium, through selective perfusion devices such as the inclined settlers (Batt et al., 1990; Searles et al., 1994).

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