INFLUENCE OF GENETIC FACTORS ON THE PRODUCTIVITY OF RECOMBINANT *CHINESE HAMSTER OVARY* (CHO) CELLS

Thesis by

Girish J. Pendse

In Partial Fulfillment of the Requirements

for the Degree of

Doctor of Philosophy

California Institute of Technology

Pasadena, California

1992

(Submitted May 8, 1992)

ACKNOWLEDGMENTS

I would like to express my gratitude and appreciation to Jay Bailey for his valuable advice, constant encouragement and financial support during my stay at Caltech.

I would also like to take this opportunity to thank Subhash Karkare and all the people I was fortunate to meet with at Amgen, for providing assistance and guidance during various stages of my thesis project.

It has been a pleasure to be a member of the Bailey group and meet new people with different ethnic and cultural backgrounds from different parts of the globe.

Most of all, I am very grateful to my wife, Nandagauri, for her tireless patience, understanding and encouragement which kept me going, especially in the last few months at Caltech.

Finally, I would like to express a deep sense of indebtedness to my parents and my brother Aditya, for their constant love, encouragement and support. It is due to the endless efforts and sacrifices of my parents, that I have been able to achieve my goals and aspirations.

ABSTRACT

In this work, influence of some genetic factors on the productivity of recombinant Chinese hamster ovary (CHO) cells has been investigated using a number of experimental techniques. The first system that we have studied is a CHO cell line producing Hepatitis B surface antigen (HbsAg). We have determined the effect of cloned gene dosage on cell growth and HbsAg synthesis and secretion. Gene copy numbers in each clone determined using Southern hybridizations were positively correlated with intracellular dihydrofolate reductase (DHFR) content using a flow cytometric assay. The mRNA levels quantitated using Northern hybridization followed by autoradiography and densitometry also gave the same trends. Flow cytometry experiments also show that the amplified clones exhibit a great deal of heterogeneity in single-cell DHFR content as compared to parental cells which were quite homogeneous with respect to intracellular DHFR content.

Batch culture experiments carried out in T flasks show that specific growth rates decrease with gene copy number. Secreted HbsAg titers and specific HbsAg secretion rates were found to increase with gene copy number. Specific glucose and glutamine consumption rates as well as specific lactate and NH_3 production rates did not vary significantly between the clones although the 1 μM clone which has the highest relative gene copy number among the clones we studied, exhibited metabolic rates that were slightly higher than those for the other clones.

Using pulse-chase experiments we found that efficiency of HbsAg secretion (fraction of total initial HbsAg (0 h chase) that is secreted into the extracellular medium during a 23.5 h chase) decreases and overall efficiency of HbsAg expression (relative amount of HbsAg secreted into the medium, [AU.mm/10^6 cells]/relative gene copy number) decreases whereas intracellular HbsAg degradation increases with cloned gene dosage. Comparisons between the relative gene copy number, relative mRNA level, relative amount of total initial HbsAg (0 h chase) and HbsAg secreted into the medium during the longest chase time of 23.5 h for various clones indicate that neither transcription nor translation is limiting at high cloned gene dosage. Our results show that some step after translation limits the overall productivity of these cells at high cloned gene dosage.

^{31}P NMR spectroscopy was carried out using a novel continuous flow packed bed reactor configuration to study the intracellular metabolism of recombinant CHO cells. NMR measurements of CHO cells growing in macroporous collagen microspheres indicate that intracellular phosphorylated metabolite levels including NTP is higher in cells producing HbsAg. These transformed cells producing HbsAg, which exhibit lower specific growth rate in the packed bed, may be utilizing these higher NTP amounts in the HbsAg secretory pathway instead of the cellular biosynthetic pathway.

The second system studied here is a CHO cell line producing secreted tissue plasminogen activator (tPA). ATP limitations may influence productivity of secreted cloned proteins in CHO cells and expression of *Vitreoscilla* hemoglobin

(VHb) has been suggested to increase ATP production efficiency in *Escherichia coli*. Based on these hypotheses, we have cloned the *Vitreoscilla* hemoglobin gene in CHO cells producing tPA (CHO-tPA) and obtained inducible intracellular expression of VHb in these transfected cells (VHb-CHO). We confirmed the presence of the VHb gene in the genomic DNA of these cells by Southern blots while Western blots were used to demonstrate the expression of VHb protein upon induction with dexamethasone. Batch culture experiments were carried out in order to determine the effect of VHb expression on growth and tPA secretion. We have shown that VHb-CHO cells have a lower specific growth rate compared to CHO-tPA cells but exhibit a significantly higher specific tPA production rate throughout the batch.

We have also investigated the effect of some growth factors on cell growth and monoclonal antibody productivity in batch hybridoma cultures. Growth factor addition did not affect specific growth rate of the cells significantly, but significant variation in monoclonal antibody production occurred depending on the growth factor present in the medium. These results indicate that growth factors affect some step in the monoclonal antibody synthesis and secretion pathway without significantly influencing the cell specific growth rate.

TABLE OF CONTENTS

CHAPTER 1

Introduction

1.1 Mammalian cell culture

In the past several years, dramatic advances in the fields of recombinant DNA technology coupled with bioprocess engineering have enabled the large scale production of a large variety of products such as viral vaccines, hormones, immunoregulators, growth factors, blood clotting factors and other proteins required for therapeutic, diagnostic and research purposes. A large number of biopharmaceuticals such as human growth hormone, tissue plasminogen activator (tPA), erythropoeitin (EPO), Factor VIII, interferons, granulocyte-macrophage colony stimulating factor (GM-CSF) and a large variety of monoclonal antibodies are being currently produced on a large scale using bacterial, yeast, insect cell and mammalian cell expression systems.[6,12,14,13,16,17,18,20,23] A number of expression systems for the production of heterologous proteins have been reviewed by Yarranton et al.[26]

Mammalian cell expression systems have become the hosts of choice for the production of a number of heterologous proteins because of their ability to perform various complex post-translational modifications such as protein folding, disulfide bond formation and glycosylation among others and to secrete active forms of the protein into the extracellular medium. Hence, some recombinant proteins produced using mammalian cells are more efficacious than the same ones produced using bacterial, yeast or insect cells. Various issues concerning high level production of proteins in mammalian cells have been reviewed recently by Kaufman.[8]

The ever-increasing demand for a large number of products using mammalian cell culture has stimulated much interest in the design and scale-up of bioreactors. Mammalian cells are generally more difficult and expensive to grow than bacteria or yeast. Since mammalian cells lack a cell wall which surrounds bacteria and yeasts, they are much more fragile and hence shear sensitive especially in large scale bioreactors. One problem that results because of this is the maintenance of adequate supply of oxygen especially in large scale cultures. They also exhibit complex nutritional requirements compared to bacteria or yeast. Moreover, nutrient depletion and toxic end product accumulation may limit the bioreactor productivity by reducing both the maximum achievable cell concentration and the specific productivity (g/cell/day). Many recent studies have focused on understanding and developing strategies to minimize if not overcome these limitations which become much more critical in large scale operations.[4,7,19,21]

Bioreactor optimization is one aspect of process development which includes initial development of high secreting clones, adaptation of cells to a serum-free medium, laboratory scale culture and optimization, development of scale-up strategies, and development of efficient downstream processing strategies. Traditional biochemical engineering has concentrated on various issues dealing with the bioreactor optimization, scale-up and downstream processing aspects of process development. We have taken a slightly different approach in that we have focused mainly on studying some biochemical processes occurring at the cellular level in order to get a fundamental understanding of

the influence of some genetic factors on recombinant CHO cell physiology and productivity.

1.2 Motivation for this work

A wide range of mammalian cell expression systems are available for both transient as well as long-term production of recombinant proteins. Gene amplification is a widely used method of achieving high level expression of recombinant proteins in mammalian cells by increasing the vector copy number. Several amplifiable markers are available including dihydrofolate reductase (DHFR)[10,25], adenosine deaminase (ADA)[8], ornithine decarboxylase (ODC)[3] and glutamine synthetase (GS)[1].

Introduction of a DNA vector into mammalian cells and its subsequent integration into the host cell chromosome for purposes of cloned gene expression perturbs native cellular functions at various levels. These disturbed functions include DNA replication and transcription, RNA and protein processing, and cellular energy and intermediary metabolism among others. Many of these perturbations can and do occur simultaneously. Many of these host-vector interactions have been studied in detail, mostly in *E. coli*[22,24] and *S. cerevisiae*[2] due to the relative complexity of mammalian cell metabolism. The effect of host-vector interactions on the metabolic characteristics of the host-vector system can have a tremendous impact on the yields of cloned gene products. Genetically engineered overexpressing CHO cells are quite different physiological entitities compared to wild-type CHO cells and also the parental unamplified

cells. Our aim was to study some of the physiological characteristics of different clones as a function of cloned gene dosage.

A number of recent studies have considered factors affecting secretion of heterologous proteins in CHO cells. Dorner and coworkers have shown that many such proteins are transiently associated with BiP (also called GRP78) which is a resident endoplasmic reticulum (ER) protein.[5] Dissociation from GRP78 may be a primary ATP-dependent step of the energy metabolism of recombinant CHO cells. Previous studies in our laboratory have shown that expression of the *Vitreoscilla* hemoglobin gene in *E. coli* resulted in higher final cell density and higher specific growth rate.[15] One hypothesis proposed to explain these observations is that expression of *Vitreoscilla* hemoglobin (VHb) results in increased ATP production because of increased proton translocation efficiency across the cell membrane. Current and ongoing research results from our laboratory are consistent with this hypothesis. Keeping in mind the potential benefits of availability of elevated intracellular ATP levels on cloned product secretion, our goal was to obtain inducible expression of VHb in recombinant CHO cells producing tPA and to investigate the effect of VHb expression on cell growth and productivity of these cells.

1.3 Scope of the thesis

A recombinant Chinese hamster ovary (CHO) cell line producing *adw* type Hepatitis B surface Antigen (HbsAg) was used in these experiments. Various clones containing increasing number of cloned gene copies were isolated by

the standard technique of sequential selection in increasing concentration of methotrexate (MTX) in the culture medium.[11] All clones were generously provided by Amgen. Our goals were two-fold: One was to investigate the effect of high level cloned gene expression on cell growth, and the second was to determine the effect of cloned gene dosage on some key processes involved in the synthesis and secretion of HbsAg such as transcription, translation and post-translational secretion into the extracellular medium. The aim of these experiments was to identify possible bottleneck(s) in the overall process of HbsAg synthesis and secretion that may arise as a consequence of overexpression resulting from gene amplification.

We have employed a number of experimental techniques to acheive our desired objectives. These include Southern and Northern hybridization for the determination of gene copy number, and mRNA level respectively, Pulse-chase/immunoprecipitation for measurement of intracellular HbsAg synthesis, degradation and secretion, flow cytometry for determination of intracellular DHFR content and finally, NMR spectroscopy to study the intracellular metabolism. Batch culture experiments provided information on the kinetics of growth and product formation in all clones.

We have also cloned the VHb gene in recombinant CHO cells producing tPA. Southern blot and Western blots were used in the initial experiments to establish the presence of VHb gene, and VHb protein respectively. Once this was demonstrated, we have investigated the effect of VHb expression on cell growth and tPA production in batch culture experiments.

Early in this project, we have also investigated the effect of growth factors on cell growth and monoclonal antibody productivity in batch hybridoma cultures. Cells adapted to a completely defined serum-free medium were used in batch culture experiments to study the effect of specific growth factors. These results are presented in Appendix A.

1.4 REFERENCES

1. **Bebbington, C.R., Hentschel,C.C.G., 1985**. The expression of recombinant DNA products in mammalian cells. *Trends Biotech.*, **3**: 1-5.

2. **Da Silva, N.A., 1988**. Host Plasmid Interactions and Regulation of Cloned Gene Expression in Recombinant Cells, Ph.D. Thesis, California Institute of Technology, Pasadena, CA.

3. **Chiang, T.R., McConlogue, L., 1988**. Amplification and expression of heterologous ornithine decarboxylase in chinese hamster cells. *Mol. Cell Biol.*, **8**: 764-769.

4. **Croughan, M.S., Hamel, J-F, Wang, D.I.C., 1987**. Hydrodynamic effects on animal cells grown in microcarrier culture. *Biotech. Bioeng.*, **29**: 130-141.

5. **Dorner A.J., Wasley, L.C., Kaufman, R.J. 1990**. Protein dissociation from GRP78 and secretion are blocked by depletion of cellular ATP levels. *Proc. Natl. Acad. Sci.*, **87**: 7429-7432.

6. **Fieschko, J., Lin, F.K., Browne, J., Strickland, T., Egrie, J., Andren, R., 1989**. Production and characterization of recombinant erythropoietin, Abstract, Japanese Association of Animal Cell Technology, annual meeting, Tsukuba, Japan, November 20-22.

7. **Glacken, M.W., 1988**. Catabolic control of mammalian cell culture. *Bio/Technology*, **6**: 1041-1050.

8. **Kaufman R.J., 1987**. High level production of proteins in mammalian cells. *Genetic Engineering: Principles and Methods*, Plenum Press, NY, **Vol. 9**, 155-198.

9. **Kaufman, R.J., Murta, P., Ingolia, D.E., Yeung, C-Y, Kellems, R. E., 1986**. Selection and amplification of heterologous gene encoding adenosine deaminase in mammalian cells. *Proc. Natl. Acad. Sci USA*, **83**: 3136-3140.

10. **Kaufman, R.J., Sharp, P.A., 1982**. Amplification and Expression of Sequences Cotransfected with a Modular Dihydrofolate Reductase Complementary DNA gene. *J. Mol. Biol.*, **159**: 601-621.

11. **Kaufman, R.J., Schimke, R.T. 1981**. Amplification and Loss of Dihydrofolate Reductase Genes in a Chinese Hamster Ovary Cell Line. Mol. Cell. Biol. **1**: 1069-1076.

12. **Kaufman, R.J., Wasley, L.C., Dorner, A.J., 1988**. Synthesis, processing and secretion of recombinant human Factor VIII expressed in mammalian cells. *J. Biol. Chem.*, **263**: 6352-6362.

13. **Kaufman, R.J., Wasley, L.C., Spliliotes, A.J., Gossels, S.D., Latt, S.A., Larsen, G.R., Kay, R.M., 1985**. Coamplification and Coex-

pression of Human Tissue-Type Plasminogen Activator and Murine Dihydrofolate Reductase Sequences in Chinese Hamster Ovary Cells. *Mol. Cell. Biol.*, **5**: 1750-1759.

14. **Kingsman, S.M., Kingsman, A.J., Mellor, J., 1987**. The production of mammalian proteins in *Saccharomyces cerevisiae. Trends in Biotechnology*, **5**: 53-57.

15. **Khosla, C., Bailey, J.E. 1988**. Heterologous expression of a bacterial hemoglobin improves growth properties of recombinant *Escherichia coli. Nature*, **331**: 633-635.

16. **Lin, F.K., Suggs, S., Lin, C.H., Browne, J.K., Smalling, R., Egrie, J.C., Chen,K.K., Fox, G.M., Martin, F., Satbinsky, Z., Badrawi, S.M., Lai, P.H., Goldwasser, E., 1985**. Cloning and expression of the human erythropoietin gene. *Proc. Natl. Acad. Sci. USA*, **82**: 7580-7584.

17. **Lubiniecki, A., Arathoon, R., Polastri, G., Thomas, J., Wiebe, M., Garnick, R., Jones, A., van Reis, R., Builder, S., 1989**. Selected strategies for manufacture and control of recombinant tissue plasminogen activator prepared from cell culture, in *Animal cell biology and technology for bioprocesses*, **Spier, R., Ed.**, Buttersworth, Kent, U.K., 442-451.

18. **Luckow, V.A., Summers, M.D., 1988**. Trends in the development of baculovirus expression vectors. *Bio/Technology*, **6**: 47-55.

19. **McQueen, A., Bailey, J.E., 1990**. Effect of ammonium ion and extracellular pH on hybridoma metabolism and antibody formation. *Bio/Technology*, **35**: 1067-1077.

20. **Nilsson, K., Birnbaum, S., Mosbach, K., 1988**. Microcarrier culture of recombinant Chinese hamster ovary cells for production of human interferon and human tissue type plasminogen activator

21. **Papoutsakis, E.T., 1991**. Fluid-mechanical damage of animal cells in bioreactors. *Trends in Biotechnology*, **9**: 427-437.

22. **Peretti, S.W. 1987**. Theoretical modeling and experimental investigation of host-plasmid interactions in recombinant *Escherichia coli*, Ph.D. Thesis, California Institute of Technology, Pasadena, CA.

23. **Schein, C.H., 1989**. Production of soluble recombinant proteins in bacteria. *Bio/Technology*, **7**: 1141-1149.

24. **Seo, J.H., Bailey, J.E., 1985**. Effect of recombinant plasmid content on growth properties and cloned gene product formation in *E. Coli*. *Biotech. Bioeng.*, **27**: 1668-1674.

25. **Subramani, S., Mulligan, R., Berg, P., 1981**. Expression of the Mouse Dihydrofolate Reductase Complimentary Deoxyribonucleic Acid in Simian Virus 40 Vectors. *Mol. Cell. Biol.*, **1**: 854-864.

26. **Yarranton, G.T., 1990**. Mammalian recombinant proteins: vectors and expression systems. *Curr. Opin. Biotechnology*, **1**: 133-140.

CHAPTER 2

Effect of cloned gene dosage on kinetics of cell growth and HbsAg production in batch cultures

2.1 SUMMARY

We have used a Chinese hamster ovary (CHO) cell line producing recombinant Hepatitis B surface antigen (HbsAg) to study the influence of cloned gene dosage on the kinetics of cell growth and HbsAg production in batch cultures.

Clones adapted to increasing MTX levels exhibit higher intracellular DHFR activity as determined using flow cytometry. These elevated intracellular DHFR levels were shown to be a consequence of *dhfr* gene amplification. Southern hybridization showed that the process of *dhfr* gene amplification resulted in coamplification of HbsAg genes in clones adapted to increasing MTX concentrations. Thus, both *dhfr* and HbsAg gene copy number increases with MTX level in the medium. The flow cytometry experiments also show that the amplified clones exhibit a great deal of heterogeneity in DHFR content as compared to parental cells.

Batch culture experiments with different clones show that specific growth rate decreases with cloned gene dosage. Secreted HbsAg titers and specific HbsAg secretion rates were found to increase with cloned gene dosage. Specific glucose and glutamine consumption rates as well as specific lactate and NH_3 production rates did not vary significantly between the clones although the 1 μM clone exhibited metabolic rates that were slightly higher than those for the other clones.

2.2 INTRODUCTION

Many proteins required for therapeutic and diagnostic use are being produced using mammalian cell culture. Gene amplification is a commonly used technique of achieving high level expression of recombinant proteins in mammalian cells. Among the various available amplifiable markers, dihydrofolate reductase (*dhfr*)[7,15] is perhaps the most widely studied and used in academia as well as in commercial applications including the production of important recombinant proteins such as tissue plasminogen activator (t-PA) and erythropoietin (EPO).[8,9]

Previous studies with *E. coli*[13] and *S. cerevisiae* (Baker's Yeast).[2] have shown the detrimental effects of high foreign plasmid copy number on specific growth rate and overall efficiency of foreign plasmid expression. The aim of this work was to investigate the influence of cloned gene dosage on host cell growth and cloned gene product synthesis and secretion. A recombinant Chinese hamster ovary (CHO) cell line producing *adw* type Hepatitis B surface Antigen (HbsAg) was used in these experiments.

Various clones containing increasing number of cloned gene copies were isolated by the standard technique of sequential selection in increasing concentration of methotrexate in the culture medium.[6] Dihydrofolate reductase (DHFR) is a housekeeping enzyme involved in the *de novo* synthesis purines, thymidylate and glycine (Figure 1). Methotrexate (MTX) is a folic analog which binds tightly to the catalytic pocket of DHFR thereby inactivating the

enzyme and killing the cells. One way the cells acquire resistance to increasing MTX concentrations is as a result of elevated intracellular levels of DHFR resulting from *dhfr* gene amplification.[6] Although the mechanisms of *dhfr* gene amplification are not completely understood, it has been found that regions of the chromosome, flanking the *dhfr* gene also undergo amplification, thereby amplifying the product gene of interest.[7,12]

The first issue we addressed was to determine whether increased levels of MTX in the extracellular medium resulted in elevated levels of intracellular DHFR levels. We then investigated whether this was a consequence of *dhfr* gene amplification and furthermore, had the process of *dhfr* gene amplification resulted in coamplification of the product gene of interest, namely, HbsAg. We also carried out experiments with all the clones to determine the effect of HbsAg gene coamplification on cell growth and HbsAg productivity in a batch culture. Flow cytometry was used to determine the intracellular DHFR content as a function of MTX levels in the medium. These experiments also provided information about the single-cell distribution of DHFR in the cell population. The *dhfr* and HbsAg gene copy numbers for the various clones have been measured using Southern hybridization. Batch culture experiments were carried in static *T* 75 flasks over a period of 6-7 days. The cloned gene product (HbsAg) titers were measured using a commercially available ELISA kit. Extracellular concentrations of metabolites such as glucose, lactate, glutamine and NH_3 were measured using commercially available kits.

2.3 MATERIALS AND METHODS

Cell culture

Various clones of Chinese Hamster Ovary cells (CHO) producing recombinant Hepatitis B Surface Antigen (HbsAg) were donated generously by Amgen Inc. (Amgen Center, Thousand Oaks, CA 91320-1789). These clones were the following: 1. dhfr$^-$ cells that lack the ability to constitutively express DHFR, 2. parental cells obtained by co-transfecting dhfr$^-$ cells with separate linearized vectors containing the *dhfr* and HbsAg genes (J. Browne, personal communication) and 3. amplified clones obtained by selecting subclones exposed to increasing levels of methotrexate such as 100 nM, 1 μM and 10 μM. The sub-cloning and selection of cells exposed to various MTX levels was done over a period of at least one month for each MTX level. Each higher amplified clone was obtained as a sub-clone of the lower amplified clone. At each stage, stock cells were frozen down for future use (J. Browne, personal communication). All our experiments were carried out after about 2-3 months of regular cultivations in the appropriate levels of MTX.

All five clones were grown in DMEM (Gibco, Grand Island, NY) supplemented with 5% dialyzed FBS (dFBS), 1% of 100X glutamine-penicillin-streptomycin stock (Irvine Scientific, Santa Ana, CA) and 1% of 100X non-essential amino acids stock (Gibco, Grand Island, NY). The medium for dhfr$^-$ cells was additionally supplemented with 1% of 100X hypoxanthine-thymidine (HT) stock (Gibco, Grand Island, NY) whereas that for 100 nM cells, 1 μM

cells and 10 μM cells was supplemented with 100 nM, 1 μM and 10 μM MTX respectively.

Cells were maintained as monolayer cultures in T 75 flasks in a humidified 5% CO_2 incubator at 37°C. They were passaged every 3-5 days upon reaching confluence. Total cell counts were monitored using a Coulter counter as well as a hemocytometer. Viability was measured using the trypan blue exclusion method. The kinetics of growth and HBsAg secretion were studied in the presence as well as in the absence of methotrexate. Each experiment was carried out in duplicate flasks. Samples were taken each day over a period of 6-7 days to monitor cell count, floater count, glucose, lactate, NH_3, glutamine and the secreted HbsAg titer. Unused supernatant was frozen at -20°C for future use.

In another set of experiments, dose-response curves or MTX toxicity curves were generated by growing each clone in various increasing concentrations of MTX and measuring the average specific growth rate for each clone at each concentration of MTX at the end of four days.

Analytical methods

Glucose and lactate were measured using a glucose-lactate analyzer (YSI, Yellow Springs, OH). NH_3 concentration in the spent medium was measured using an ammonia kit (Sigma Chemical Co., St. Louis, MO). Glutamine concentration was measured using a two-step assay as used by McQueen.[11] Briefly, the culture supernatant was treated with glutaminase (Sigma Chemical Co.,

St. Louis, MO) in 0.1M sodium acetate for 20 minutes during which time the reaction went to completion releasing 1 mole NH_4^+ per mole of glutamine consumed as confirmed using standard solutions. This glutaminase-treated supernatant was then analyzed for NH_3 using the above mentioned kit and compared with untreated supernatant diluted in a similar way. The difference in the NH_4^+ concentration corresponds to the concentration of glutamine in the medium.

HbsAg assay

The concentration of HbsAg in the supernatant was measured using the Auzyme Monoclonal ELISA Kit (Abbott Labs., North Chicago, IL). A slight modification of the standard protocol was used in our assay. Instead of using the human plasma-derived positive control, we first determined the concentration of HbsAg in a *S. cerevisiae*-derived purified HBsAg preparation. This recombinant HbsAg was then used as a positive control as well as a standard for determination of HbsAg concentration in CHO cell supernatants.

Flow cytometric assay for intracellular DHFR content

These experiments were carried out using the protocol described by Schimke et al.[5] Briefly, late exponential cultures of cells growing in T 25 flasks were incubated with 30 μM MTX-F (fluorescein methotrexate)(Molecular Probes Inc., Eugene, OR) in presence of 30 μM GTH (glycine-thymidine-hypoxanthine)(required to counteract the toxic effect of MTX-F) for a period of 24 hours at 37°C in

a 5% CO_2 incubator. A duplicate flask of cells treated in the same way except the incubation with MTX-F was used as a control for the detection of autofluorescence from cells. At the end of 24 hours, the medium containing MTX-F was aspirated, and cells were washed once with PBS and incubated with MTX-F-free medium under the same conditions as before for 15 minutes to allow for efflux of any unbound MTX-F. The medium was aspirated, cells washed once with PBS, trypsinized and centrifuged at 1000 rpm. The resulting cell pellet was resuspended at a concentration of 1 x 10^6 cells/ml in ice-cold PBS and stored on ice until analysis.

The experiments were performed on a Cytofluorograph 50H (Ortho Diagnostic Systems Inc., Westwood, MA) using an argon laser (Lexel Corp., Fremont, CA) operated at 488 nm and 200 mw. The FITC fluorescence was measured using a 520 nm bandpass filter. A total of 10,000 cells were analyzed by gating on the scatter, and the resulting histograms were acquired and stored using a Model 2140 data handling system (Ortho Diagnostic Systems Inc., Westwood, MA). The data were transferred to a PC and analyzed using the *multi2d* software (Phoenix Flow Systems, La Jolla, CA). The log mean channel number of unlabeled cells was subtracted from that of the labeled cells in order to correct for autofluorescence. The relative DHFR activity for each clone is reported as the net mean log green fluorescence channel number of that clone normalized with the net mean log green fluorescence channel number of the parental clone.

Southern Hybridization

Most of the protocols used in these experiments are a combination of protocols described in Maniatis[10] and Gene screen Plus (NEN Research Products, Boston, MA). Late log-phase cultures of all clones were used to isolate genomic DNA using standard techniques. The DNA was stored in aliquots at -20°C after determining the concentration using OD_{260} measurements. For the experiments, 10 μg each of dhfr$^-$ and parental DNA and 1 μg each of 100 nM, 1μM and 10 μM DNA were digested with excess *EcoR*I for a period of 20-24 hours at 37°C and loaded on a 0.7% agarose gel containing ethidium bromide. Electrophoresis was conducted in 0.5 X TBE for 8 hours at 40-50 volts. The gel was photographed using Polaroid film 665 (positive and negative) and the negative was analyzed by densitometry to determine the relative amounts of DNA loaded in each lane (and hence each clone). The DNA was transferred to a Nylon Gene Screen Plus membrane (NEN Research Products, Boston, MA) in the presence of 10 X SSC solution for 18-24 hours.

Two probes were used in these hybridizations, namely, HbsAg and *dhfr*. Plasmids pSV4ST-HBV and pMg1 containing the HbsAg and *dhfr* genes, respectively, were provided by Jeff Browne and Dora Delgado (Amgen Inc., Thousand Oaks, CA). *E. coli* HB 101 was transformed with these plasmids using the $CaCl_2$ procedure.[10] Minipreps were performed in order to isolate adequate amounts of both plasmids. Plasmid pSV4ST-HBV was digested with *Sal*I whereas plasmid pMg1 was subjected to double digestion with *EcoR*I and *Pst*I for 4 hours at 37°C. These digested plasmids were loaded on a 0.7% agarose

gel and electrophoresed in 1 X TAE for 3 hours at 75 volts. The probes consisting of the HBsAg and *dhfr* genes were purified from the gel using Geneclean kit (Bio 101, La Jolla, CA).

Pre-hybridization of the nylon membrane was performed in a water bath at 42°C for 2 hours. The pre-hybridization mixture consisted of 10% dextran sulfate, 1% SDS, 50% deionized formamide and 200 μg/ml denatured salmon sperm DNA. 50 ng of denatured DNA probe was labeled with [α-^{32}P] dCTP (Amersham Corp., Arlington Heights, IL) using the Random Prime DNA Labeling kit (Boehringer Mannheim, Indianapolis, IN). The labeled probe was purified from the unincorporated radiolabeled nucleotides using Nensorb purification cartridge (Du Pont Co., Wilmington, DE). At the end of pre-hybridization, denatured radiolabeled probe was added to the bag and hybridization was carried out for 16-24 hours in a 42°C water bath. Standard washing protocols were used to remove non-specific hybridization. Autoradiography was performed by exposing the membrane to an X-ray film at -70°C for 24 hours. The resulting autoradiogram was scanned using a laser densitometer (Pharmacia LKB Biotechnology, Piacataway, NJ) and the bands quantitated. The gene copy numbers in each clone were determined after normalizing with respect to the amount of DNA loaded for each clone. The relative gene copy numbers were determined by normalizing the value obtained above by the parental value.

Karyotype analysis

The slide preparation and subsequent solid staining for karyotype analysis was carried out using a slight modification a protocol by Worton et al.[16]. All

steps were the same except the incubation with colcemid in order to induce a metaphase arrest. These cells were incubated with 0.5 μg/ml colcemid for longer time periods (5-8 hours for dhfr$^-$, parental and 100 nM cells; 16-20 hours for 1 μM and 10 μM cells) in order to obtain a high mitotic index. The slides were then stained with Giemsa stain and observed under an Olympus microscope using a 100X objective.

2.4 RESULTS

Flow cytometry

The first issue that we addressed was to determine whether clones adapted to higher MTX concentrations exhibited elevated levels of intracellular DHFR. The flow cytometric assay described earlier was used to determine the intracellular DHFR content on a single-cell basis. As described earlier, the relative DHFR activity was calculated for each clone including the dhfr$^-$ clone. Based on the labeling protocol employed, single-cell green fluorescence is expected to be greater in cells with greater DHFR activity. Table I summarizes the relative DHFR activity (which is proportional to the intracellular DHFR content) and the standard deviation (which reflects the heterogeneity in the population) for all the clones. As expected the dhfr$^-$ cells exhibited zero relative DHFR activity. The relative DHFR activity increases with MTX concentrations upto 1 μM. The 1 μM clone exhibits the highest relative DHFR activity. Surprisingly, the 10 μM clone had lower relative DHFR activity as compared to the 1 μM

clone. More on this anomalous behavior has been discussed in the Discussion section.

There is a clear trend in the heterogeneity of intracellular DHFR activity as a function of MTX levels. Figure 2 shows that the parental unamplified clone has a population which is quite homogeneous with respect to intracellular DHFR content, whereas the amplified clones are much more heterogeneous with respect to the DHFR content. This is probably indicative of greater heterogeneity with respect to the gene copy number on a single-cell basis. A small fraction of parental cells exhibited higher green fluorescence, but this was a direct consequence of that subpopulation having a larger cell size as is evident from the broad tail in forward scatter (data not shown). Furthermore, this phenomenon was not caused by treatment of these cells with MTX-F since the unlabeled parental cells also showed this bimodal distribution. Such a bimodal distribution of forward scatter was not observed in the amplified clones (data not shown). Moreover, the forward scatter data did not exhibit the same heterogeneity that was observed in the green fluorescence of the amplified clones. Hence, green fluorescence heterogeneity for these clones is not a consequence of cell size heterogeneity.

Southern Hybridization

The relative *dhfr* and HbsAg gene copy number in different clones was determined using southern hybridizations as described in Materials and Methods. Since the hybridization pattern of the *dhfr* probe mimicked that of the HbsAg

probe, only the autoradiogram of HbsAg hybridization is shown in Figure 3. The bands for the parental line are slightly displaced to those of the amplified lines because the amount of DNA loaded for the parental line is ten times that loaded for the amplified lines. The autoradiogram showed a number of bands which were quantitated using densitometry. The total area under the peak was measured and expressed as absorbance units millimeters (AU.mm). This value was normalized with respect to the amount of DNA loaded in each lane to obtain a value proportional to the copy number for each clone. This was further normalized with respect to the peak area of parental cells. These normalized copy numbers of both *dhfr* and HbsAg genes are shown in Table II. The dhfr$^-$ clone tested negative for both *dhfr* and HbsAg genes;the DNA from this clone did not hybridize to either of the probes. A good degree of coamplification of *dhfr* and HbsAg genes is evident from data in Table II lending support to the idea that both vectors have been integrated into a chromosome in close proximity to each other. Furthermore, the gene copy number increase with MTX level upto 1 μM. This shows that the amplified clones exhibit higher intracellular DHFR activity as a result of *dhfr* gene amplification. However, we see that even though the 1 μM clone has the highest cloned gene copy number, the 10 μM and 100 nM clone have almost same number of copies of both genes. A possible explanation for this behavior is suggested in the Discussion section.

Kinetics of growth

As described in Materials and Methods, batch experiments were carried out over a period of 6-7 days for studying the kinetics of growth and product

formation. Since the behavior of amplified clones did not vary significantly with or without MTX especially during the course of the batch culture, only data for cells grown with MTX is presented here. Figure 4 shows the batch growth curves for all clones except dhfr$^-$ and parental grown in the presence of MTX. The dhfr$^-$ clone exhibits the highest growth rate whereas the 1 μM clone exhibits slowest growth characteristics. Table III summarizes specific growth rates calculated during exponential phase of growth (first 4 days of the batch culture) for each clone. It is clear that specific growth rates are inversely proportional to the concentration of methotrexate in the medium (and, hence to the cloned gene copy number) except for the 10 μM clone which showed a growth rate that is lower than that of the 100 nM clone but higher than that of 1 μM clone. This was unexpected since with increased MTX in the culture medium (and a corresponding supposed increase in the cloned gene copy number), the 10 μM cells were expected to be under greater metabolic stress than the 1 μM clone and hence were expected to exhibit a lower specific growth rate. More on this anamolous behavior is discussed in future sections. An interesting trend was observed in the Coulter counter measurements for cell size distribution and total cell counts (data not shown). The mean cell size of amplified clones (13-16 μm) was much higher compared to dhfr$^-$ and parental cells (10-12 μm). This was independent of the phase of growth. The larger cell size of the amplified clones could be attributed to a number of factors such as increased amounts of intracellular MTX, elevated levels of intracellular HbsAg or some other factor, but no further experiments were carried out to confirm

or reject either of these possibilities. The dhfr$^-$ and parental cells were also much more homogeneous in size compared to the amplified clones.

A dose-response experiment was performed for each clone as described in Materials and Methods. The data for each clone except the dhfr$^-$ was fit using standard Monod-type inhibition kinetics in order to calculate K_i, the inhibition constant, and μ_{max} as follows:

$$\mu = \frac{\mu_{max} K_i}{[I] + K_i}, \tag{1}$$

μ_{max} for each clone was obtained from experimental data as the specific growth rate in presence of the corresponding concentration of MTX;e.g., for 100 nM cells, μ_{max} is the specific growth rate of 100 nM cells growing in the presence of 100 nM MTX and so on. K_i was obtained by fitting the experimental data to the form in Equation 1 using Mathematica software. The K_i and μ_{max} values so obtained are summarized in Table IV. The dhfr$^-$ clone did not grow at all when cultivated without HT supplement in the medium. It also showed the same poor growth characteristics for a range of MTX concentrations, confirming the fact that it was indeed a dhfr$^-$ clone. The dose response curves for the parental, 100 nM, 1 μM and 10 μM clones are shown in Figures 5A, 5B, 5C, and 5D, respectively.

Kinetics of HbsAg secretion

Samples collected and frozen from the batch experiments were analyzed for HbsAg concentration using ELISA. Since HbsAg secretion kinetics did not vary

significantly with or without MTX, only data for cells grown in the presence of MTX are shown in Figure 6 and 7. As expected the product titers increase with MTX levels up to 1 μM and hence with cloned gene dosage. The same trend was observed for the specific HbsAg secretion rates for all clones. The 10 μM clone exhibits similar secretion levels as the 100 nM clone throughout the batch culture. The difference in the specific HbsAg secretion rate of the 1 and 10 μM clones is even more dramatic. The specific secretion rate of the 1 μM clone is almost two times that of 10 μM clone.This anamolous behavior behavior of the 10 μM clone behavior was further investigated by means of Southern hybridization experiments, the results of which have been discussed in the earlier section. The dhfr$^-$ clone tested negative for HbsAg using this assay.

The supernantant samples were also analyzed for various residual nutrient as well as secreted by-product metabolite concentrations using protocols described in the Materials and Methods section. The specific uptake rates of glucose and glutamine as well as specific production rates of lactate and ammonia have been shown in Figures 8-15. These results show that the specific metabolite consumption and production rates do not vary significantly among the various clones although the 1 μM clone exhibited the highest values among the various clones studied.

Karyotype analysis

These experiments were carried out with the aim of examining the chromosome number and appearance of double minute chromosomes in the parental

and amplified clones. Within the limits of resolution of our microscope, the solid staining of chromosomes did not show any significant differences between the clones (data not shown). Thus, these experiments did not reveal any new information that could shed some light on the anomalous behavior of the 10 μM clone.

2.5 DISCUSSION AND CONCLUSIONS

We have investigated the kinetics of cell growth and product formation in a batch culture for various clones as a function of cloned gene dosage. Amplified clones adapted to increasing concentrations of MTX exhibit higher relative DHFR activity as determined by flow cytometry. They also are more heterogeneous with respect to the DHFR distribution in the cell population. The higher DHFR activity in amplified clones was due to increased *dhfr* gene copies resulting from *dhfr* gene amplification. Southern hybridizations showed a good correlation between the number of copies of *dhfr* and HbsAg genes in all clones demonstrating coamplification of these genes and thus strongly suggesting their proximity in the site of integration into a chromosome of the cell. This amplification of HbsAg genes results in increased productivity of amplified clones as determined from batch culture experiments.

Our batch culture experiments showed that the specific growth rate decreases monotonically with increased cloned gene dosage. Assuming the integration of *dhfr* and HbsAg genes into the chromosome is stable in presence of selection levels of MTX, these differences may be ascribed to reduction in

host cell biosynthetic activity for native cellular components due to redirection of cellular resources towards cloned gene and cloned gene product synthesis, processing and secretion. It is also possible that disruption of chromosomal DNA by the vector may be responsible for some of these effects, although the preliminary karyotyping analysis did not show any large disruption of host chromosomes for the parental or amplified clones. The HbsAg titers as well as the specific HbsAg secretion rate increased with cloned gene dosage. Consumption rates of metabolites such as glucose and glutamine and production rates of lactate and NH_3 did not vary significantly between the clones indicating that the observed physiological differences were not due to nutrient limitations or by-product accumulation.

Properties of the 10 μM clone were consistent with its content of amplified cloned genes, but the amplified *dhfr* content was lower than expected relative to other clones selected at lower MTX concentrations. We speculate that the 10 μM clone has acquired resistance to this high level of MTX by some mechanism other than *dhfr* gene amplification. Other mechanisms of acquiring resistance to MTX such as reduced inward MTX transport[14] and altered affinity to DHFR[3,4] have been shown to occur in CHO cells. Recently, Barsoum[1] has observed that as MTX concentration increases above 1 μM, clones owing their resistance to MTX-resistant DHFR mutations and to decreased cellular uptake of MTX arise with greater frequency. It is possible that, during selection of clones resistant to 10 μM MTX starting from the 1 μM clone, the cells acquired one or more spontaneous mutations resulting in one or both of

the two phenotypes mentioned above. This would enable the 10 μM cells to survive higher concentrations of MTX used for the dose-response experiments discussed earlier. Since the main focus of our work was to investigate the effect of cloned gene dosage on recombinant CHO cell physiology, the 10 μM clone was not used for the experiments discussed in the following chapter.

2.6 ACKNOWLEDGMENTS

We would like to thank a number of people at Amgen for helping during different stages of the project including Jeff Browne and Gerry Trail for providing recombinant clones, Dora Delgado for providing plasmid DNA containing HbsAg and dhfr genes, and Keith Langley for providing purified HbsAg derived from *S. cerevisiae*. This research was supported by the National Science Foundation (Grant No. BCS-8805636) and by a grant from Amgen, Inc..

2.7 REFERENCES

1. **Barsoum, J., 1990**. Introduction of Stable High-Copy-Number DNA into Chinese Hamster Ovary Cells by Electroporation. *DNA and Cell Biology*, **9(4)**: 293-300.

2. **Da Silva, N.A. 1988**. Host Plasmid Interactions and Regulation of Cloned Gene Expression in Recombinant Cells, Ph.D. Thesis, California Institute of Technology, Pasadena, CA.

3. **Flintoff, W.E., Davidson, S.V., Siminovitch, L. 1976**. Isolation and partial characterization of three methotrexate-resistant phenotypes from Chinese hamster ovary cells. *Somatic Cell Genetics*, **2**: 245-261.

4. **Haber, D.A., Beverley, S.M., Kiely, M.L., Schimke, R.T. 1981**. Properties of an altered dihydrofolate reductase encoded by amplified genes in cultured fibroblasts. *J. Biol. Chem.*, **256**: 9501-9510.

5. **Kaufman, R.J., Bertino, J.R., Schimke, R.T. 1978**. Quantitation of Dihydrofolate Reductase in Individual Parental and Methotrexate-resistant Murine cells : Use of Fluorescence Activated Cell Sorter. *J. Biol. Chem.*, **253**: 5852-5860.

6. **Kaufman, R.J., Schimke, R.T. 1981**. Amplification and Loss of Dihydrofolate Reductase Genes in a Chinese Hamster Ovary Cell Line. *Mol. Cell. Biol.*, **1**: 1069-1076.

7. **Kaufman, R.J., Sharp, P.A. 1982**. Amplification and Expression of Sequences Cotransfected with a Modular Dihydrofolate Reductase Complementary DNA gene. *J. Mol. Biol.*, **159**: 601-621.

8. **Kaufman, R.J., Wasley, L.C., Spliliotes, A.J., Gossels, S.D., Latt, S.A., Larsen, G.R., Kay, R.M. 1985**. Coamplification and Coexpression of Human Tissue-Type Plasminogen Activator and Murine Dihydrofolate Reductase Sequences in Chinese Hamster Ovary Cells. *Mol. Cell. Biol.*, **5**: 1750-1759.

9. **Lin, F.K., Suggs, S., Lin, C.H., Browne, J.K., Smalling, R., Egrie, J.C., Chen,K.K., Fox, G.M., Martin, F., Satbinsky, Z., Badrawi, S.M., Lai, P.H., Goldwasser, E. 1985**. Cloning and expression of the human erythropoietin gene. *Proc. Nat. Acad. Sci.*, **82** :7580-7584.

10. **Maniatis, T., Sambrook, J., Fritsch, E.F. 1989**. *Molecular Cloning: A Laboratory Manual*, **Vol. 1-3**, Cold Spring Harbor Laboratory Press, NY.

11. **McQueen, A. 1989**. Ammonium ion effects on Hybridoma cell physiology, Ph.D. Thesis, California Institute of Technology, Pasadena, CA.

12. **Schimke, R.T. 1984**. Gene amplification, Drug resistance, and Cancer. *Cancer Research*, **44**: 1735-1742.

13. **Seo, J.H., Bailey, J.E. 1985**. Effect of recombinant plasmid content on growth properties and cloned gene product formation in *E. Coli. Biotech. Bioeng.*, **27**: 1668-1674.

14. **Sirotnak, F.M., Moccio, D.M., Kelleher, L.E., Goutas, L.J. 1981**. Relative frequency and kinetic properties of transport-defective phenotypes among methotrexate-resistant L1210 clonal cell lines derived *in vivo*. *Cancer Research*, **41**: 4447-4452.

15. **Subramani, S., Mulligan, R., Berg, P. 1981**. Expression of the Mouse Dihydrofolate Reductase Complimentary Deoxyribonucleic Acid in Simian Virus 40 Vectors. *Mol. Cell. Biol.*, **1**: 854-864.

16. **Worton, R.G., Duff, C. 1979**. Karyotyping. *Methods in Enzymology*, **Vol. LVIII**: 322-345.

2.8 TABLES

Table I. Comparison of intracellular DHFR content in different clones. The DHFR activity is obtained by subtracting the mean log green fluorescence channel number of unlabeled cells from the mean log green fluorescence channel number of labeled cells. The relative DHFR activity is calculated by normalizing the DHFR activity of each clone by the DHFR activity of the parental clone. The standard deviation value which reflects the heterogeneity in cell population with respect to the intracellular DHFR content is shown only for the labeled cells.

Clone	Relative DHFR activity	Standard deviation
$dhfr^-$	0	8.8
Parental	1	9.3
100 nM	14	21.8
1 μM	19	22.7
10 μM	16	23

Table II. Relative *dhfr* and HbsAg gene copy number in different clones. All values have been normalized with respect to the respective parental gene copy number.

Clone	HbsAg	*dhfr*
Parental	1	1
100 nM	21	20
1 μM	40	39
10 μM	18	20

Table III. Comparison of specific growth rate in different clones. These were calculated for each clone during the exponential phase of the batch culture.

Clone	Specific growth rate, hr^{-1}
$dhfr^-$	0.039
Parental	0.030
100 nM	0.023
1 μM	0.008
10 μM	0.019

Table IV. Dose response characteristics of different clones. Each clone was grown in the presence of increasing concentration of MTX with resulting average specific growth rate as shown in Figure 5 A, B, C, and D. These data were fit by Equation (1) to obtain these parameters.

Clone	μ_{max}, hr^{-1}	K_i, nM
Parental	0.024	13
100 nM	0.022	828
1 μM	0.010	2150
10 μM	0.013	21700

2.9 FIGURES

Figure 1. Schematic diagram of biosynthetic reaction catalyzed by dihydrofolate reductase (DHFR).

Figure 2. Green fluorescence histograms of labeled cells: Parental (A), 100 nM (B), 1 μM (C) and 10 μM (D) clones. The relative DHFR activity (net mean log green fluorescence) and corresponding standard deviation values are summarized in Table I. The numbers 1 and 2 represent the regions used for data analysis.

Figure 3. Southern hybridization pattern of *Eco*RI digested genomic DNA from various clones probed with ^{32}P labeled HbsAg probe. The dhfr$^-$ clone showed no hybridization to the HbsAg probe. 10 μg DNA of dhfr$^-$ and parental cells and 1 μg each of DNA from the 100 nM, 1 μM and 10 μM clones were used in this experiment.

Figure 4. Cell number of various clones in a batch culture. Clones are as follows: ($\diamond$) dhfr$^-$, ($\bullet$) Parental, ($\bigcirc$) 100 nM, ($\blacksquare$) 10 μM, ($\square$) 1 μM. All clones were inoculated at 1 x 10^5 cells/ml and growth rates were calculated for the exponential phase (first four days of culture).

Figure 5. Dose-response curves for Parental (A), 100 nM (B), 1 μM (C) and 10 μM clones. The dhfr$^-$ clone did not survive any MTX concentration or any medium without HT supplement. Lines were calculated from Equation (1) using the parameters listed in Table IV.

Figure 6. Secreted HbsAg concentration in a batch culture experiment. Clones are as follows: (●) Parental, (○) 100 nM, (■) 10 μM, (□) 1 μM.

Figure 7. Specific HbsAg secretion rate in a batch culture experiment. Clones are as follows: (●) Parental, (○) 100 nM, (■) 10 μM, (□) 1 μM.

Figure 8. Extracellular glucose concentration in a batch culture experiment. Clones are as follows: (◇) dhfr$^-$, (●) Parental, (○) 100 nM, (■) 10 μM, (□) 1 μM.

Figure 9. Specific glucose consumption rate in a batch culture experiment. Clones are as follows: (◇) dhfr$^-$, (●) Parental, (○) 100 nM, (■) 10 μM, (□) 1 μM.

Figure 10. Extracellular lactate concentration in a batch culture experiment. Clones are as follows: (◇) dhfr$^-$, (●) Parental, (○) 100 nM, (■) 10 μM, (□) 1 μM.

Figure 11. Specific lactate production rate in a batch culture experiment. Clones are as follows: (◇) dhfr$^-$, (●) Parental, (○) 100 nM, (■) 10 μM, (□) 1 μM.

Figure 12. Extracellular glutamine concentration in a batch culture experiment. Clones are as follows: (◇) dhfr$^-$, (●) Parental, (○) 100 nM, (■) 10 μM, (□) 1 μM.

Figure 13. Specific glutamine consumption rate in a batch culture experiment. Clones are as follows: (◇) dhfr$^-$, (●) Parental, (○) 100 nM, (■) 10 μM, (□) 1 μM.

Figure 14. Extracellular NH_3 concentration in a batch culture experiment. Clones are as follows: (◇) dhfr$^-$, (●) Parental, (○) 100 nM, (■) 10 μM, (□) 1 μM.

Figure 15. Specific NH_3 production rate in a batch culture experiment. Clones are as follows: (◇) dhfr$^-$, (●) Parental, (○) 100 nM, (■) 10 μM, (□) 1 μM.

Figure 1

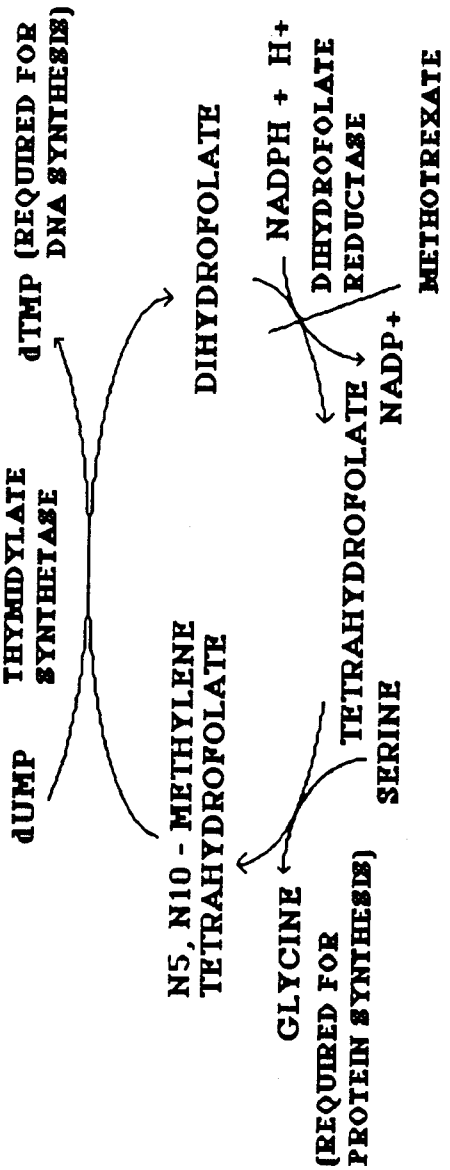

dUMP
THYMIDYLATE SYNTHETASE
dTMP (REQUIRED FOR DNA SYNTHESIS)
DIHYDROFOLATE
NADPH + H+
DIHYDROFOLATE REDUCTASE
NADP+
METHOTREXATE
N5, N10 - METHYLENE TETRAHYDROFOLATE
TETRAHYDROFOLATE
SERINE
GLYCINE
(REQUIRED FOR PROTEIN SYNTHESIS)

Figure 2

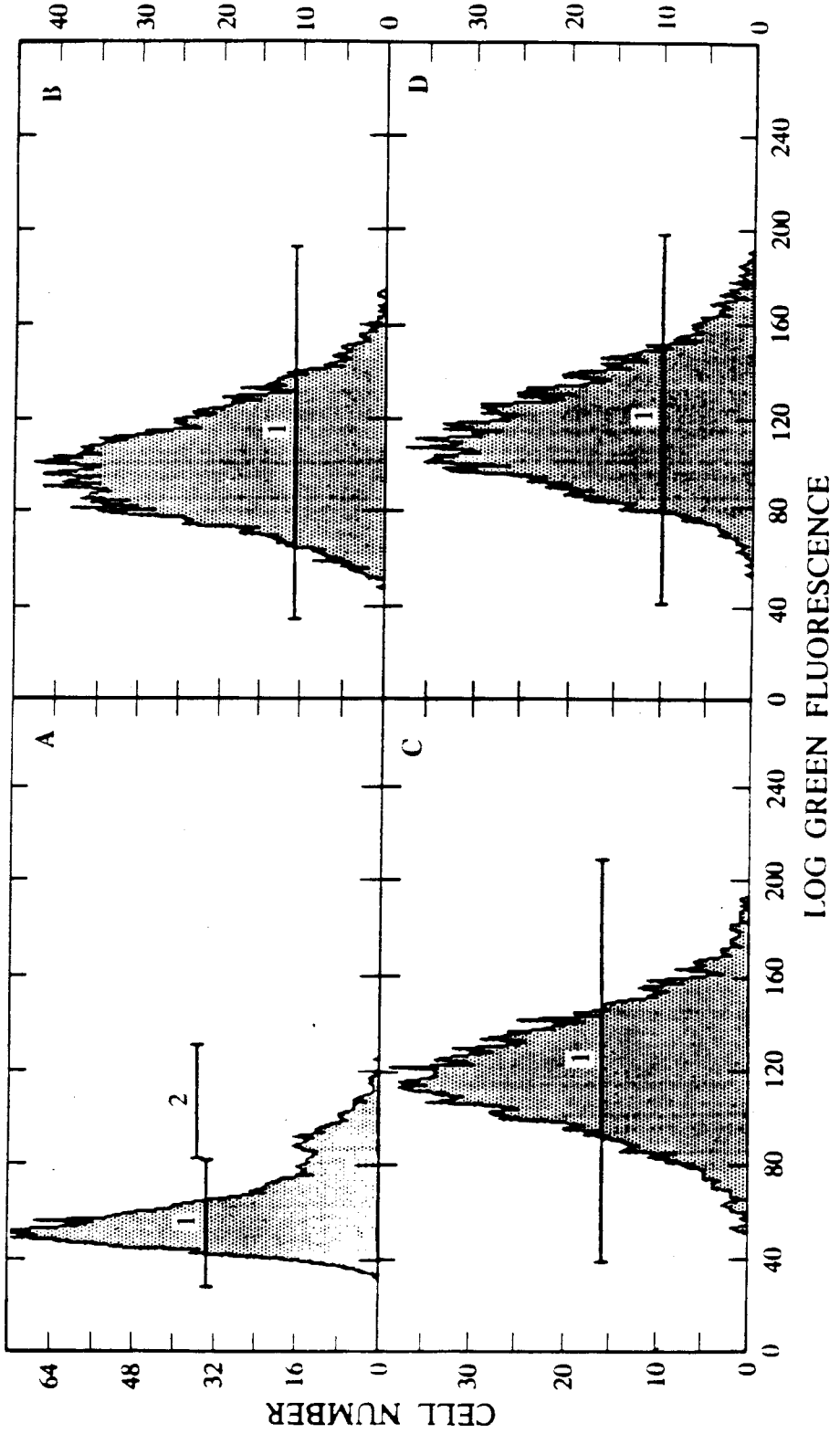

A
B
C
D
1
2
CELL NUMBER
LOG GREEN FLUORESCENCE
0
16
32
48
64
10
20
30
40
80
120
160
200
240

Figure 3

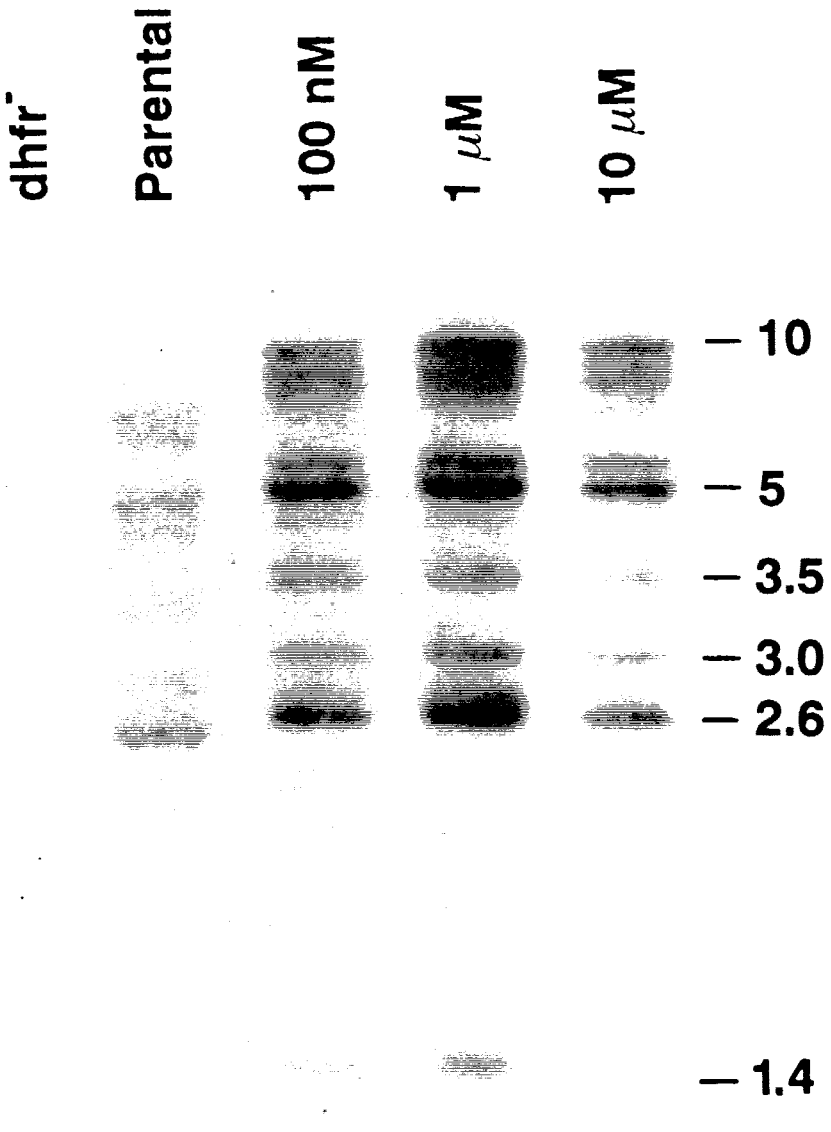


Figure 4

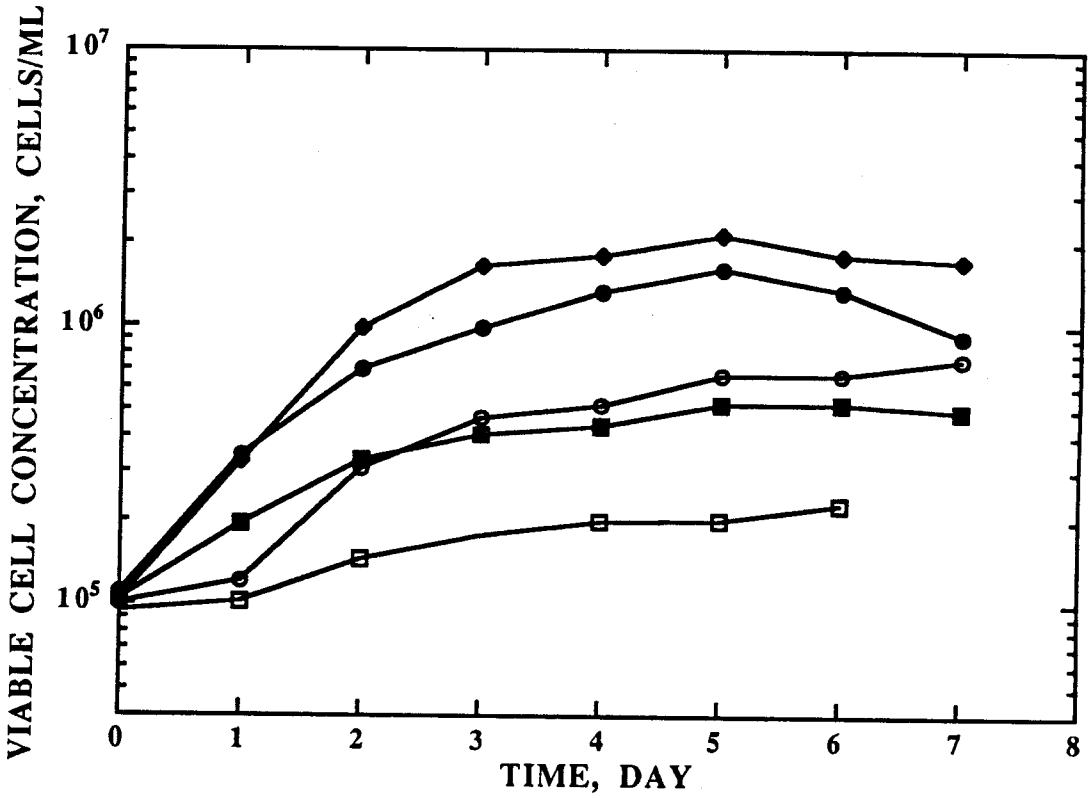

VIABLE CELL CONCENTRATION, CELLS/ML
10^7
10^6
10^5
0
1
2
3
4
5
6
7
8
TIME, DAY

Figure 5

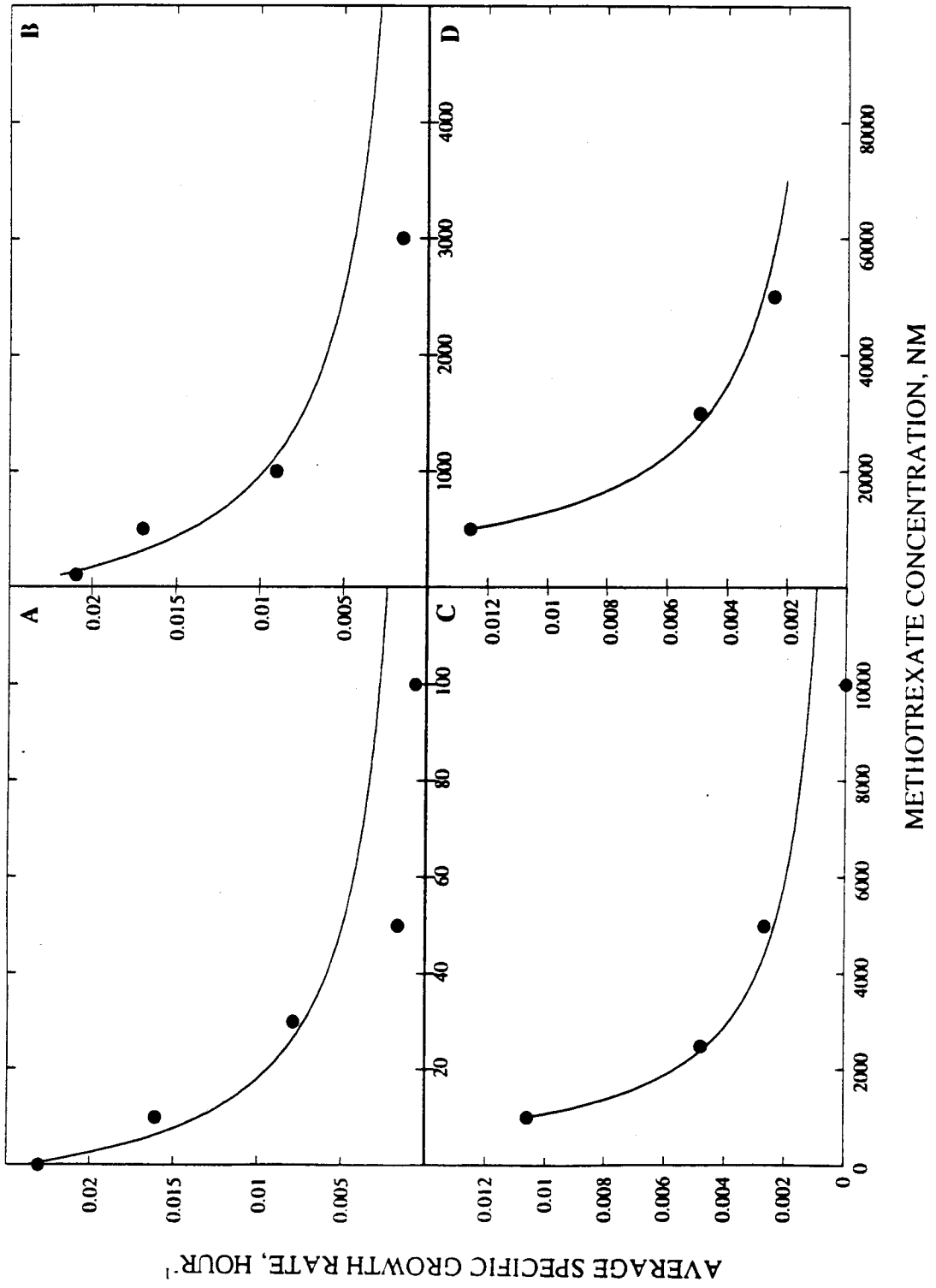

A
B
C
D
AVERAGE SPECIFIC GROWTH RATE, HOUR-1
METHOTREXATE CONCENTRATION, NM

Figure 6

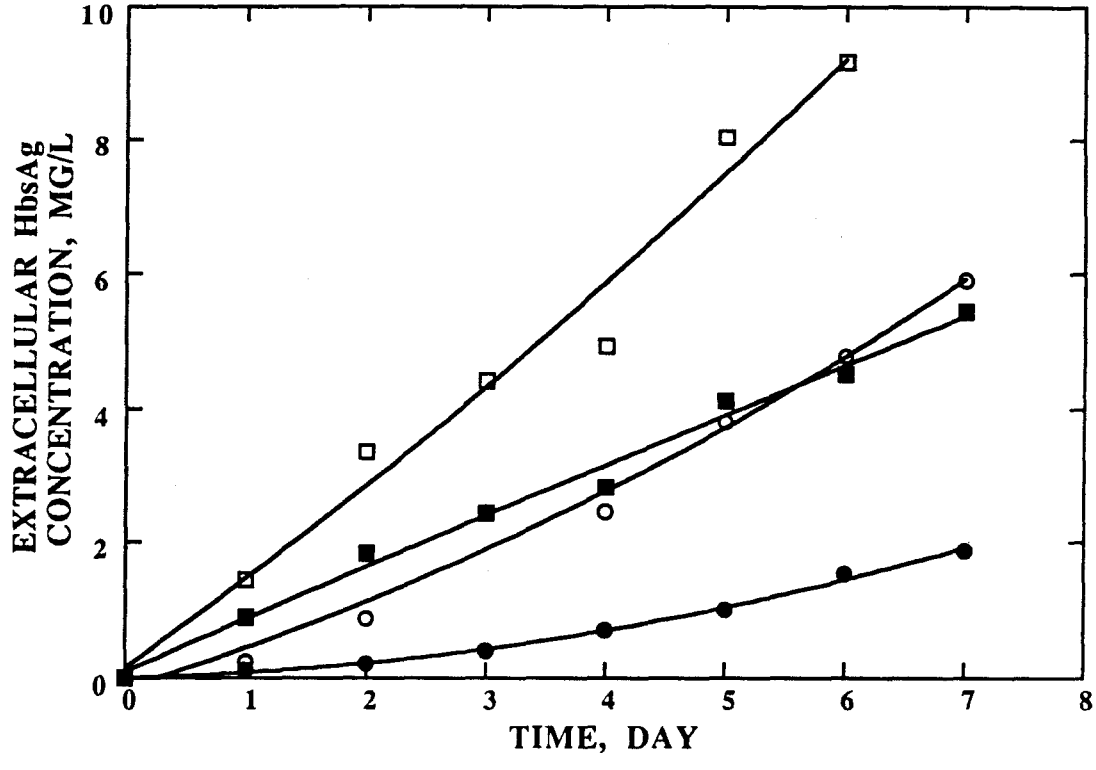

EXTRACELLULAR HbsAg
CONCENTRATION, MG/L
10
8
6
4
2
0
0
1
2
3
4
5
6
7
8
TIME, DAY

Figure 7

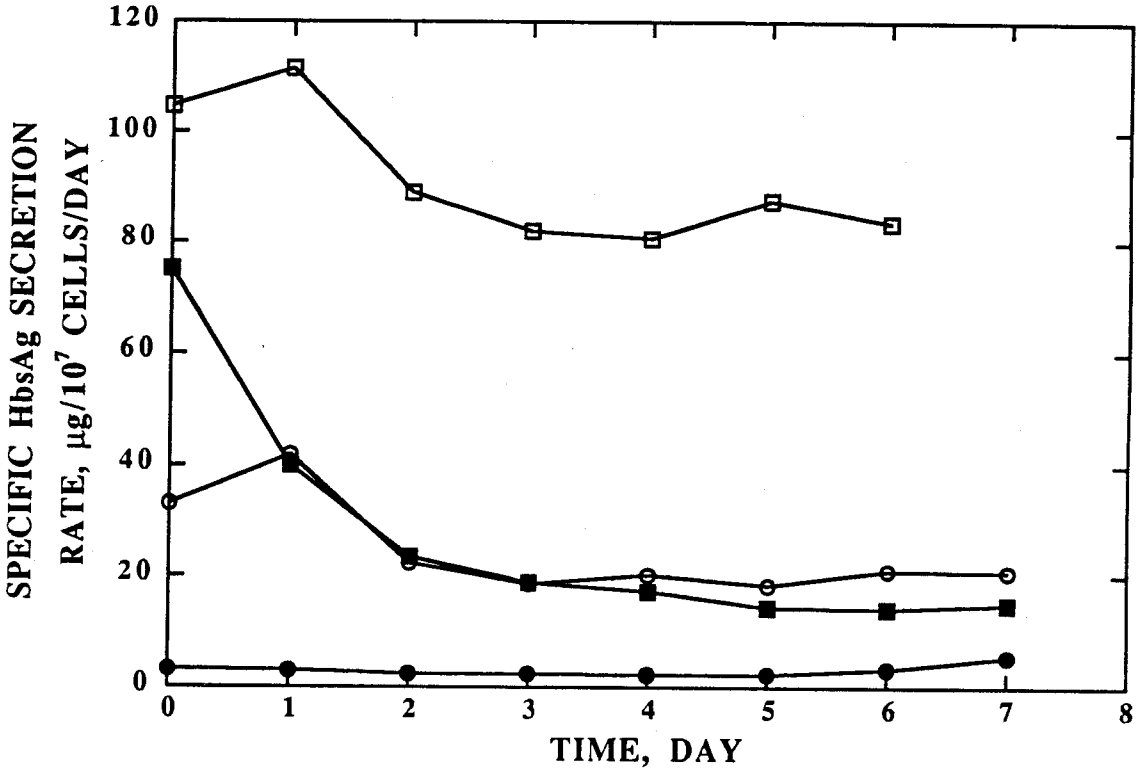

SPECIFIC HbsAg SECRETION RATE, μg/10^7 CELLS/DAY
120
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
TIME, DAY

Figure 8

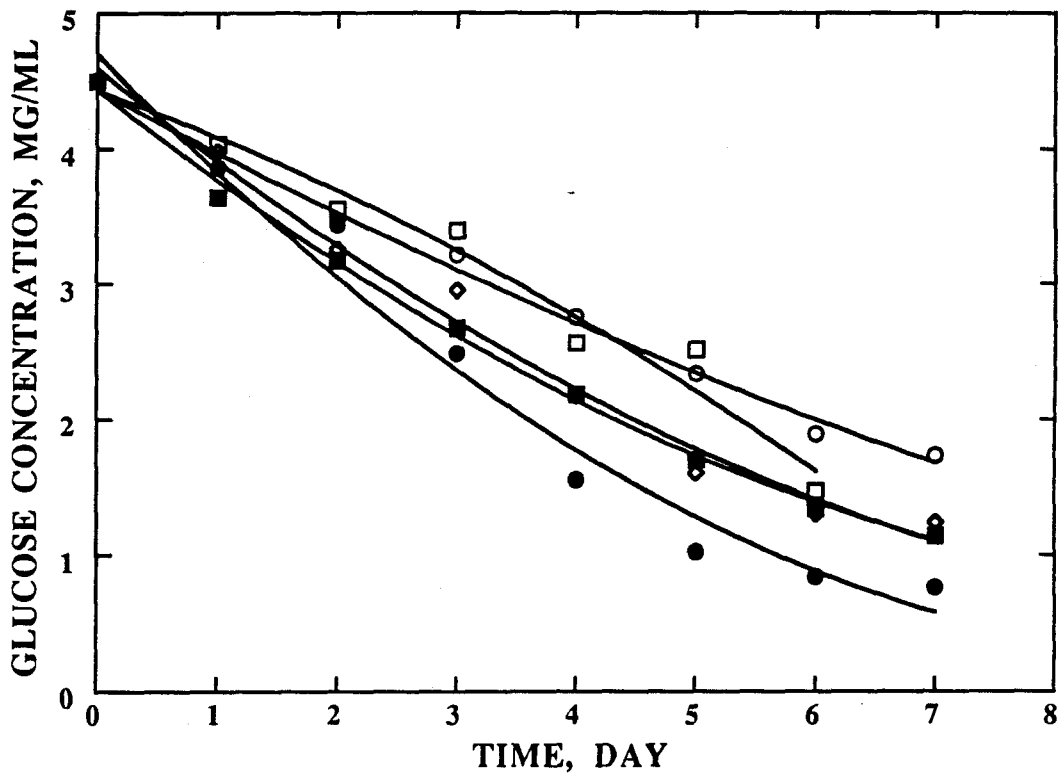


Figure 9

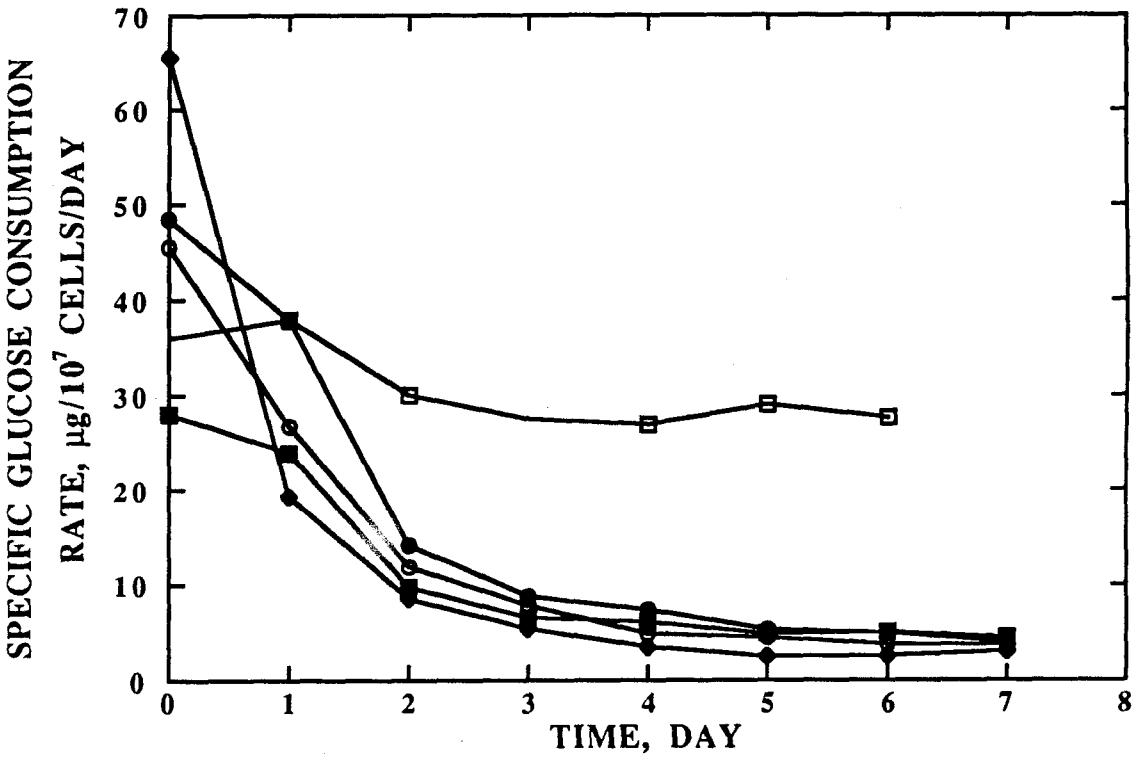

SPECIFIC GLUCOSE CONSUMPTION
RATE, μg/10⁷ CELLS/DAY
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
TIME, DAY

Figure 10

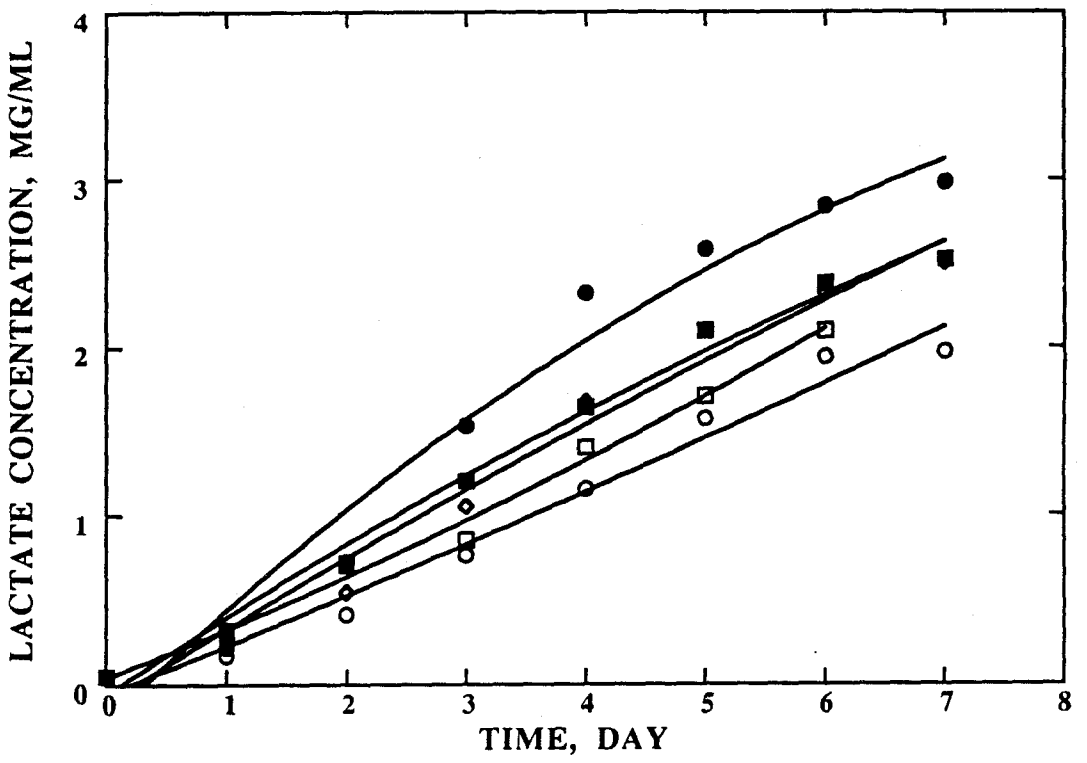


Figure 11

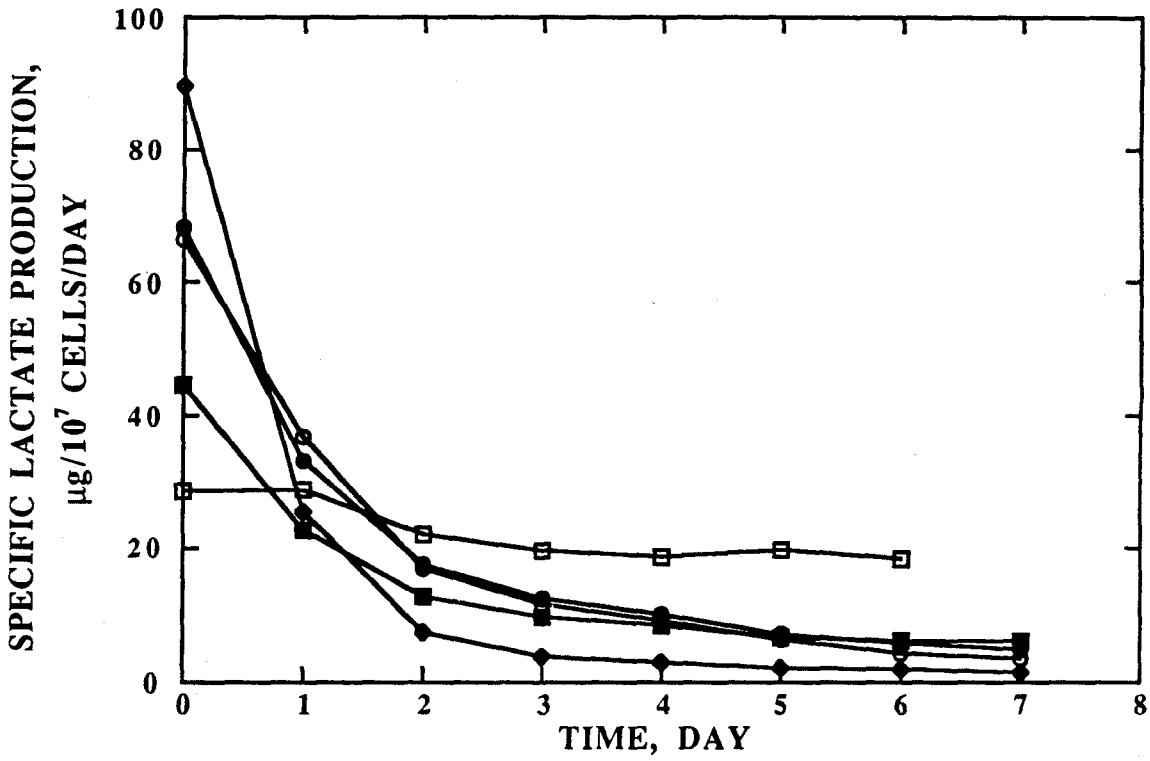

SPECIFIC LACTATE PRODUCTION,
μg/10⁷ CELLS/DAY
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
TIME, DAY

Figure 12

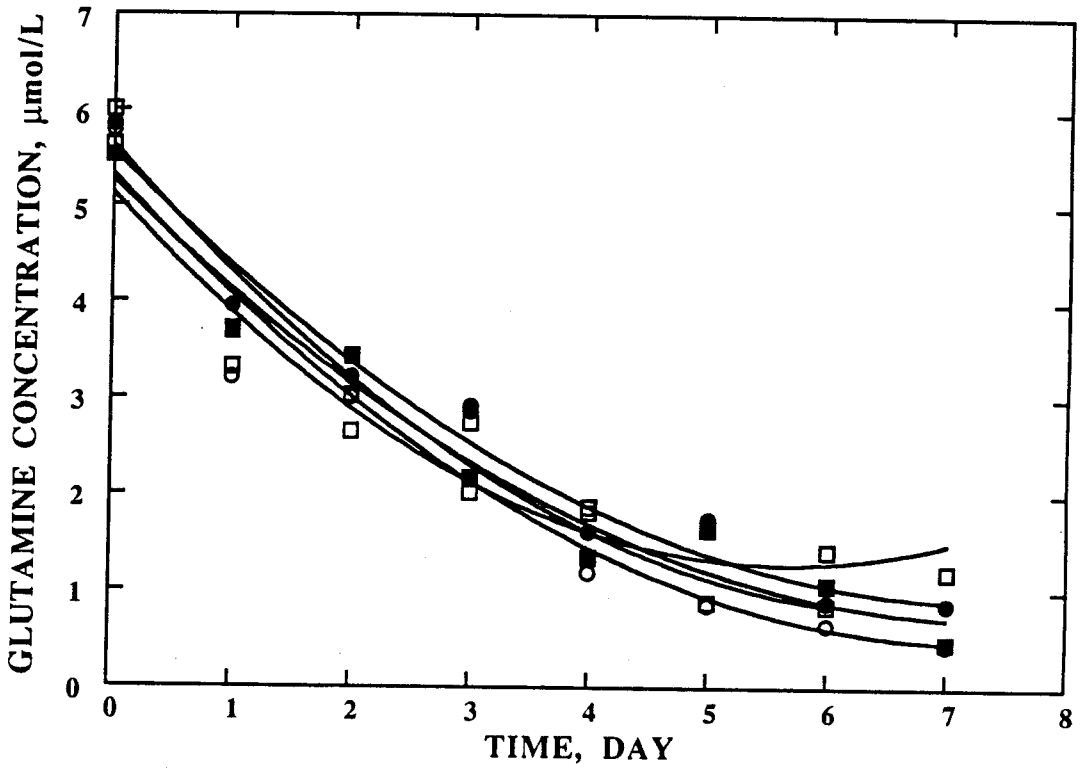

GLUTAMINE CONCENTRATION, µmol/L
0
1
2
3
4
5
6
7
0
1
2
3
4
5
6
7
8
TIME, DAY

Figure 13

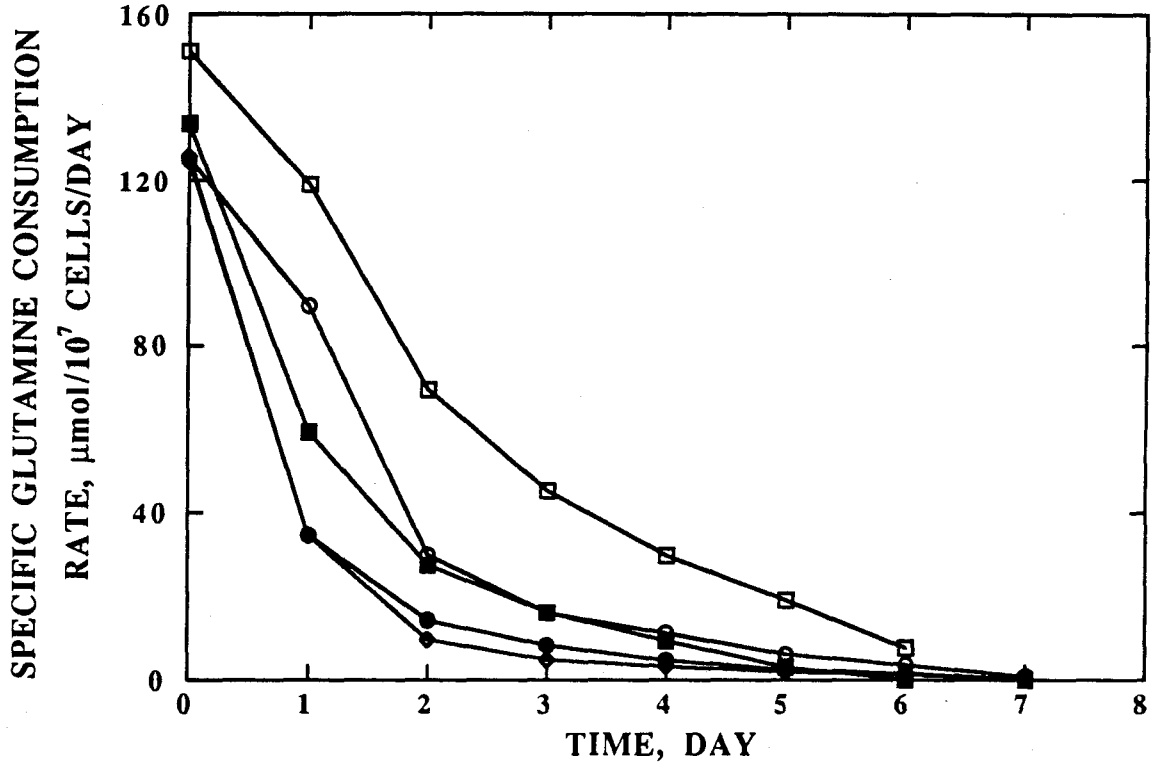

SPECIFIC GLUTAMINE CONSUMPTION
RATE, µmol/10⁷ CELLS/DAY
160
120
80
40
0
0
1
2
3
4
5
6
7
8
TIME, DAY

Figure 14

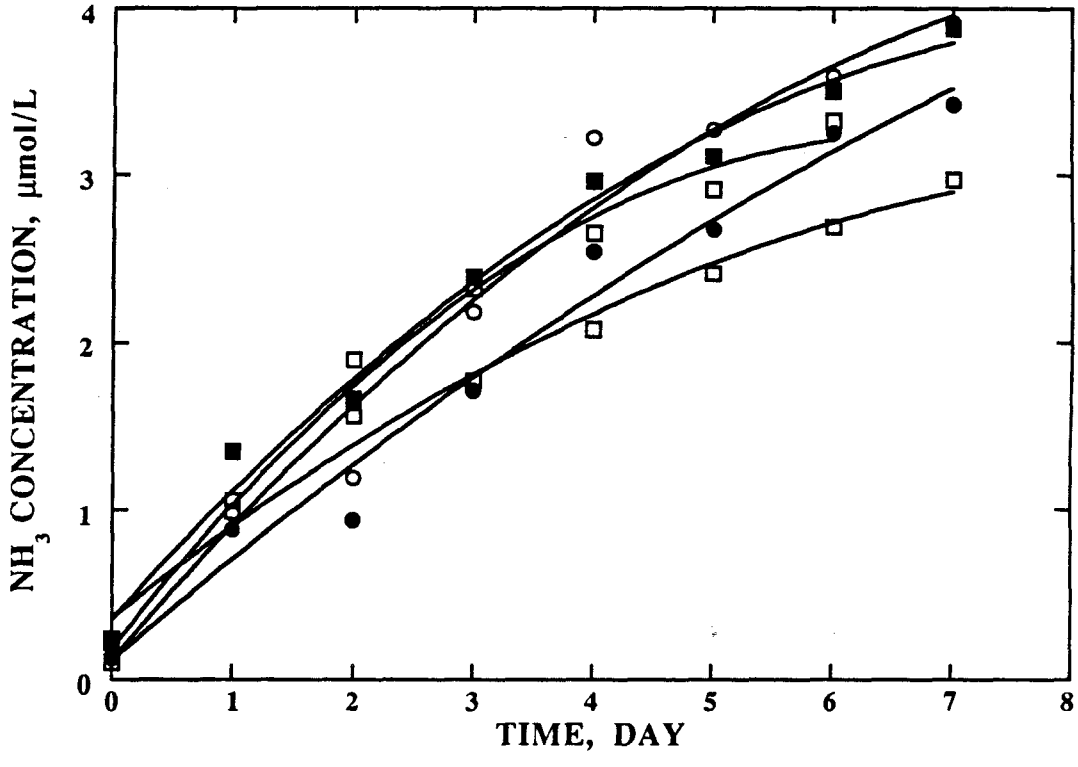
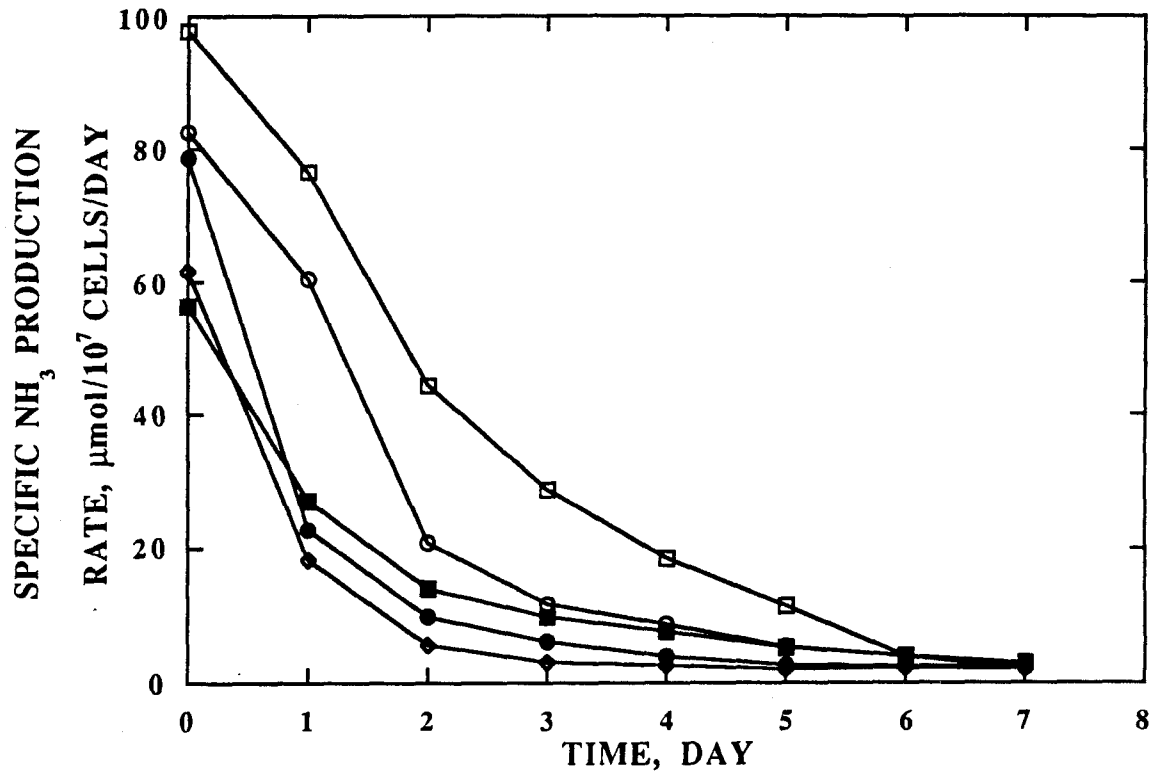


Figure 15


SPECIFIC NH3 PRODUCTION
RATE, μmol/10^7 CELLS/DAY
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
TIME, DAY

CHAPTER 3

Effect of cloned gene dosage on HbsAg synthesis and secretion

3.1 SUMMARY

In this chapter we have investigated the effect of cloned gene dosage on some key steps involved in the synthesis, processing, and secretion of HbsAg.

Results obtained using Southern and Northern hybridization experiments show that the relative mRNA level increases monotonically with relative gene copy number. Pulse-chase experiments show that the efficiency of HbsAg secretion (defined here as the fraction of total initial HbsAg that is secreted into the extracellular medium at the end of a 23.5 h chase) decreases, overall efficiency of HbsAg expression (amount of HbsAg secreted into the medium/gene copy number) decreases, and also that the intracellular HbsAg degradation increases with increasing HbsAg gene copy number. Comparisons between the relative gene copy number, relative mRNA level, relative amount of total initial HbsAg (0 h chase) indicate that neither transcription nor translation seems to be limiting at high copy numbers. Some post-translational step causes a bottleneck in the overall process of HbsAg synthesis and secretion at high cloned gene copy number. Some possible steps that we think may be responsible for this bottleneck at high cloned gene dosage are either the availability of lipid components for proper assembly of HbsAg lipoprotein particle, and/or the availability of glycosylation enzymes required for proper glycosylation of HbsAg, and/or the availability of sufficient amounts of intracellular ATP required for rapid transit of HbsAg through the ER, and finally, increased proteolysis of intracellular HbsAg, especially for the highest amplified clone.

3.2 INTRODUCTION

Previous studies with *E. coli* have shown that maximum specific growth rate and the overall efficiency of foreign plasmid gene expression (β-lactamase specific activity/plasmid content) decreased significantly with increasing plasmid content.[12] Similar detrimental effects of high cloned gene expression have also been observed in *S. cerevisiae* (Baker's Yeast).[1] These effects presumably occur due to competition between the cellular DNA and cloned gene product DNA and also among their respective RNA's for the cells's limited pool of resources of enzymes, transcription factors, ribosomes and energy among others.

The normal process of HbsAg synthesis and secretion occurs in a number of sequential steps as follows: transcription of HbsAg genes, translation of the resulting mRNAs in association with membrane-bound polyribosomes in the rough endoplasmic reticulum, secretion of the HbsAg lipoprotein particle into the lumen of the ER, post-translational modifications such as glycosylation in the ER followed by transport to the Golgi complex, further glycosylation in the Golgi and finally secretion. In this chapter we have investigated the effect of cloned gene dosage on some key steps involved in the synthesis and secretion of HbsAg. Specifically, we have focused our attention on the processes of transcription of HbsAg genes, translation of the resulting mRNA and post-translational processing and secretion of HbsAg. The aim of these studies was to identify possible rate-limiting steps in this multistep process that may arise due to overexpression of HbsAg resulting from HbsAg gene amplification.

The relative gene copy numbers in different clones have been determined using Southern hybridization as discussed in the earlier chapter. Relative HbsAg mRNA levels in different clones were measured using Northern hybridization. Pulse-chase experiments followed by immunoprecipitation were used to determine HbsAg synthesis rate, intracellular HbsAg degradation, and the temporal nature of secretion of HbsAg into the extracellular medium.

3.3 MATERIALS AND METHODS

Cell culture

The same five clones described in the previous chapter, namely, dhfr$^-$, parental, 100 nM, 1 μM and the 10 μM cells were used in these experiments.

For routine passages, cells were maintained as monolayer cultures in T 75 flasks in a humidified 5% CO_2 incubator at 37°C. They were sub-cultured every 3-5 days upon reaching confluence. Total cell counts were monitored using a Coulter counter as well as a hemocytometer. Viability was measured using the trypan blue exclusion method.

Northern Hybridization

For Northern analysis, total cell RNA was isolated from late-log phase cultures of all clones using the protocol described by Maniatis et al.[7] Extreme care was taken during the entire procedure in order to minimize RNA degradation by endogenous and exogenous RNAase contaminants. After quantitation of the

amounts of RNA obtained for each clone by OD_{260} measurements, the RNA was stored in 100% ethanol at -70°C. For the experiment, known amounts of total cell RNA from dhfr$^-$, parental, 100 nM, 1 μM and 10 μM clones were loaded on a 0.8% agarose formaldehyde gel along with 1 μl (0.5 mg/ml) ethidium bromide. Electrophoresis was conducted at 50-60 volts for 8 hours. Capillary transfer and formaldehyde reversal were carried out per Gene Screen Plus (NEN Research Products, Boston MA). Pre-hybridization and hybridization were done essentially in the same manner as described for Southerns. Autoradiography was performed by exposing the membrane to an X-ray film for 48 hours at -70 °C. The relative mRNA contents were determined by quantitating the bands using densitometry and normalizing first with respect to the amount of total RNA loaded for each clone and subsequently with respect to the value so obtained for the parental clone.

Pulse-Chase method

Cells were grown until mid-exponential phase in 35 mm tissue culture dishes. The medium was aspirated and cells were washed two times with methionine-free medium. Cells were then incubated for 30 minutes at 37 °C in a 5% CO_2 incubator in 2 ml methionine-free medium. The medium was then aspirated and replaced by 1 ml methionine-free medium containing 100 μCi of L-[^{35}S]-methionine (1037 Curies/mmol, 10 μCi/μl) (Amersham Corp., Arlington Heights, IL). These cells were incubated at 37 °C in a 5% CO_2 incubator

for the desired pulse time. For experiments involving the determination of HbsAg synthesis rate, pulse times of 1 h, 2 h and 4 h were used in parental and 1 μM cells. In these experiments the supernatant and cell extracts were processed and analyzed by immunoprecipitation immediately following the end of the various pulse periods. The other set of pulse-chase experiments was carried out with parental, 100 nM and 1 μM cells. A constant pulse of 3 h was used for all samples. At the end of the pulse, medium was aspirated and discarded as radioactive waste. The cells were washed two times with complete medium containing 200 mM methionine and then incubated in 1 ml of the same medium for the desired chase times. Various chase times ranging from 0 to 24 h were used in these experiments since HbsAg is known to be secreted with a half life of 4-5 hours in various mammalian cells[9]. At the end of the chase, medium was aspirated and transferred to a tube. 250 μl of this extracellular medium was diluted 1:1 in lysis buffer[13] (0.1 M Tris-Cl, pH 8.0, 0.1 M NaCl, 0.1 M EDTA, 2% Triton X-100, 0.1% SDS) and stored on ice. Cells were washed two times with ice-cold PBS and incubated with 500 μl lysis buffer for 20 minutes on ice. The cell extract was centrifuged at 12000 rpm for 5 minutes at 4°C and supernatant transferred to a new tube on ice. 250 μl each of cell homogenate and diluted extracellular medium were added to tubes containing 10 μl anti-HbsAg monoclonal antibody (Zymed Labs. Inc., South San Francisco, CA) in 500 μl Tris-Triton buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 2% Triton X-100, 0.1 mM EDTA, pH 8.0). These tubes were mixed gently by inversion and incubated overnight at 4°C on a slow rotater. The following morning,

the immunoprecipitation mixture was processed according to the protocol by Licari et al.[5] Briefly, the immunoprecipitation mixture was incubated with 50 μl Protein A Sepharose for 1 h at 4°C on a slow rotater and then the Protein A Sepharose-immunoprecipitated complex was recovered as a pellet by centrifugation. This complex was further treated by a series of washes with Tris-Triton buffer, 10 mM Tris-Cl, pH 8.0 and 0.1% SDS in that order. The washed pellet was then resupended in 40 μl sample buffer and denatured by boiling. 5 μl of this sample was used in scintillation counting and the rest was stored at -20°C until use in SDS-PAGE. A 12.5% polyacrylamide gel was used to electrophorese the protein sample after which it was sequentially soaked in fixer and fluor and dried at 80°C in a slab gel dryer. The dried gel was exposed to an X-ray film for 7-15 days, developed and the resulting autoradiogram was subjected to densitometry for quantitation of the HbsAg bands. Total cells at the beginning of each chase point were used to normalize the amount of protein present in each sample. In addition to this, the extracellular medium HbsAg amounts were normalized to take into account the variable volume of the extracellular medium at the end of each chase.

3.4 RESULTS

Northern Hybridization

Figure 1 shows the autoradiogram obtained as a result of hybridization of HbsAg probe to the total RNA isolated from cells as described earlier. Although the bands were not sharp due to partial degradation of the mRNA,

quantitation of the main band corresponding to the major transcript was possible using scanning densitometry. Table I shows the HbsAg message level normalized by that for the parental clone. These results indicate that the relative mRNA levels increase monotonically with relative gene copy number.

Pulse-Chase and Immunoprecipitation

As described in Materials and Methods, HbsAg synthesis rates were determined for parental and 1 μM clones using relatively short pulse times. The relative amounts of HbsAg synthesized at the end of each pulse time were quantitated using scanning densitometry and expressed as AU.mm. Since the longest pulse was much shorter than the doubling time of these cells, no normalization with respect to the cell number was necessary for different pulse times. Furthermore, no significant protein was detected in the extracellular medium for both clones. Figure 2 shows the relative amount of HbsAg present in the cell extract supernatant plotted versus pulse time. For both clones these data are well fit by straight lines;the slope of each line indicates zero-order net synthesis rate. Since HbsAg is a secreted protein, the net rate of intracellular accumulation equals the synthesis rate minus the sum of degradation and secretion rates. After normalizing the data for the two clones by the total number of cells at the end of the 4 h chase, the net synthesis rate expressed as AU.mm/10^6 cells for the amplified 1 μM clone was found to be about 20 times higher than that calculated for the unamplified parental clone.

In the above experiments no chases were performed. In order to study the pattern of HbsAg degradation, kinetics of HbsAg secretion and the efficiency of HbsAg secretion (defined here as the fraction of total initial protein that is secreted into the extracellular medium at the end of the longest chase time of 23.5 h), pulse-chase experiments were carried out as described earlier. Since the amounts of HbsAg secreted into the extracellular medium were relatively small and since only a fraction of the total medium was used for immunoprecipitation, the bands on the autoradiograms were quite faint, especially for smaller chase times such as 0 and 5.5 h. This was expected based on the earlier results from the synthesis rate experiments. However, very distinct bands were obtained for HbsAg in the extracellular medium for chase times of 9.5 and 23.5 h. No such problem was encountered for any of the cell extract samples for any clone.

Figure 3 shows a typical autoradiogram for a pulse-chase experiment with 100 nM cells. Two distinct bands were observed in the extracellular medium (EM) as well as cell extract (CE). The smaller band which migrates through the same distance in both EM and CE samples corresponds to a polypeptide of about 20 Kd. The size of the bigger band, however, differs in the EM and CE samples. This band corresponds to a polypeptide of about 25 and 23 Kd in the EM and CE, respectively. The final normalized values of the total amount of HbsAg present in the cell extract supernatant at 0 and 23.5 h are presented along with the amount of HbsAg present in the extracellular medium at the end of 23.5 h chase in Table II. The percentage secreted HbsAg is the same as the efficiency of HbsAg secretion. The percentage degraded HbsAg was calculated

as the amount of HbsAg unaccounted for at the end of the 23.5 h chase divided by the total initial protein (0 h chase).

3.5 DISCUSSION AND CONCLUSIONS

The aim of this study was to investigate the influence of cloned gene dosage on synthesis and secretion of HbsAg. The process of transfection of mammalian cells with a cloning vector followed by its integration into the host cell chromosome can have many effects ranging from silence to lethality by disrupting a function essential for viability. This process can lead to an alteration of native cell functions at many levels such as host cell DNA replication and transcription, RNA and protein function, protein processing, cellular energy and intermediary metabolism among others. Many of these changes can and do occur simultaneously. This issue is further complicated in mammalian cells by the fact that clones resulting from a transfection can differ widely in their growth characteristics and productivity, presumably due to different sites of integration and their subsequent consequences on host cell function. Since all of our cell populations were cloned and since the hybridization pattern of parental and amplified clones obtained from Southerns is similar, "site of integration" is not expected to be significant when comparing physiological characteristics of these clones.

Results from Southerns discussed in the previous chapter showed that the relative gene copy number increases with MTX levels upto 1 μM. A similar trend was observed with mRNA levels which were obtained using Northern

hybridizations. Figures 4 and 5 show the relationship of the relative HbsAg mRNA level with the corresponding relative HbsAg gene copy number and the average specific HbsAg secretion rate for parental, 100 nM and 1 μM cells respectively. It is clear that the relationship between DNA and mRNA is qualitatively different from that between mRNA and protein. The relative mRNA content increases monotonically with gene copy number but a much more rapid increase was observed in 1 μM cells compared to 100 nM cells. As discussed earlier, the Southern hybridizations showed that multiple copies of HbsAg gene have been integrated into the CHO chromosomes at different sites and each integrant has undergone further rounds of amplification upon MTX treatment. The transcriptional activity of a particular gene or a set of genes depends on a number of factors such as proximity to promoter/enhancer and other regulatory elements. Hence, all sites on a particular chromosome/s may not be transcribed with the same efficiency. Since the original vector used in the transfection does not contain any regulated promoter, the expression of HbsAg gene is essentially constitutive and depends on the site of integration into the chromosome. Thus, it is possible that we observe a rapid increase in mRNA copy number when gene copy number increases because sites of active chromosomal rearrangements (in this case *dhfr* and HbsAg gene amplification) are also sites of high transcriptional activity. The results from Figure 4 indicate that transcription does not get saturated at high cloned gene dosage and hence, is not the rate limiting step in the process of HbsAg synthesis and secretion. On the other hand, the relatively linear relationship between mRNA and average

secretion rate suggests overburdening of resources required for translational, post-translational and secretory apparatus, although the cellular machinery involved in the post-transcriptional processing of HbsAg has not been saturated. These experiments formed the basis for our experiments involved with investigation of intracellular HbsAg synthesis, degradation and secretion into the extracellular medium.

The pulse-chase experiments provide useful insights into the reasons for reduced efficiency of cloned gene expression in highly amplified clones. Figures 6 and 7 show the relationship between the relative mRNA level with the total initial amount of intracellular HbsAg and the amount of HbsAg secreted into the medium at the end of the longest chase time of 23.5 respectively. Clearly, the intracellular HbsAg amount increases almost linearly with mRNA levels for the three clones whereas Figure 7 shows that the amount of HbsAg secreted into the medium plateaus off for the 1 μM clone which has the highest relative cloned gene copy number and relative mRNA levels. Thus these results indicate that the overall efficiency of HbsAg expression (defined as relative amount of HbsAg secreted into the medium, [AU.mm/10^6 cells] / relative HbsAg gene copy number) decreases with increasing gene dosage (Figure 8). Combining the results from Figure 4 and 6, we conclude that neither transcription, nor translation gets saturated at high cloned gene dosage and hence these steps do not cause bottleneck(s) in the overall HbsAg synthesis and secretion pathway. However, Figures 6 and 7 show that some step after translation is probably rate-limiting at high cloned gene dosage. Results presented in Table VI clearly show

that there is no significant degradation of HbsAg in parental and 100 nM cells. However, while almost 83% of the initial amount of HbsAg is secreted by the parental cells at the end of 24 h, in 100 nM cells only 55% of the initial amount is secreted. This indicates that even at these moderate levels of amplification, some post-translational limitation is beginning to set in. This behavior is even more pronounced in 1 μM cells. The overall secretion efficiency of 1 μM cells based on the total initial HbsAg (0 h chase) is about 18%. Comparing the total initial amount of labeled HbsAg (0 h chase) with the amounts of HbsAg remaining in the cell extract and the extracellular medium at the end of 23.5 h chase suggests that more than half of the initial HbsAg cannot be accounted for and has probably undergone degradation. It is very unlikely that this degradation occurs after the protein is secreted into the extracellular medium. Within the first 2.5 h, the intracellular HbsAg level dropped to half its initial value (data not shown). It would be almost impossible for these cells to process and secrete such a large amount of HbsAg in such a short time, especially since data for 100 nM cells have suggested the possibility of a rate-limiting step in the secretory pathway at lower levels of intracellular HbsAg. These results further indicate that some post-translational secretion step is rate limiting and that intracellular proteases alleviate the overburdening of this pathway by degrading more than half of the increased HbsAg protein synthesized as a result of HbsAg gene amplification.

The 20 Kd polypeptide probably is the unglycosylated HbsAg whereas the 23 and 25 Kd polypeptides are presumably its glycosylated derivatives based on

earlier findings.[9] Patzer et al.[9] have shown that the intracellular glycosylated HbsAg has high mannose residues whereas the glycosylated HbsAg secreted into the EM has terminal sialic acid residues on its oligosaccharide chain. In our case, too, this could explain the difference in molecular weight of the larger band present in the EM and CE. Though no detailed experiments have been performed to study the differences in glycosylation patterns of the 23 and 25 Kd HbsAg that are found in the CE and EM, respectively, our results are in good qualitative agreement with earlier results. Thus, these cells seem to secrete both glycosylated and unglycosylated HbsAg, but the intracellular processing of HbsAg to the complex sialic acid form does not occur until just prior to secretion. We have observed a difference in proportion of amounts of glycosylated:unglycosylated HbsAg present in the cell extracts (1.5-2.5 : 1) v/s supernatant (1-1.3 : 1). It is possible, that the reasons for this proportionality difference may be a consequence of the post-translational bottleneck. It has been found that HbsAg polypeptide has one site for N-linked glycosylation at its Asn-146 residue. Furthermore, this glycosylaton does affect either antigenicity or immunogenicity of HbsAg.[6,8]

We think that there are four potential candidate steps that may be responsible for the reduced secretion efficiency and overall expression efficiency at high cloned gene dosage. The first one could be due to saturation of the glycosylation apparatus such that there is an insufficient availability of enzymes required for the proper glycosylation of HbsAg. This would result in an underglycosylated form of intracellular HbsAg. The second possible candidate for a post-translational rate-limiting step is the availability of sufficient

ER membrane required for the proper assembly of the 22 nm HbsAg lipoprotein particle. Under normal circumstances, the HbsAg polypeptide monomers are initially synthesized as integral membrane proteins which aggregate and undergo a conformational change. The ER membrane then invaginates to form the lipoprotein complex which gets extruded into the lumen of the ER.[13] Due to HbsAg gene amplification, cells synthesize increased amounts of HbsAg polypeptide monomers. It is possible then, that at high HbsAg cloned gene dosage, the lipid biosynthetic pathway for ER membrane synthesis gets saturated as a result of which proper assembly of HbsAg lipoprotein particles does not occur in the ER. Recent findings by Dorner and coworkers have shown that underglycosylated or misfolded proteins are found to be associated with BiP (heavy chain immunoglobulin binding protein) or GRP78, a resident protein of the endoplasmic reticulum (ER).[2,3] This could also explain the difference in proportion of glycosylated:unglycosylated HbsAg in cell extracts as compared to that in the supernatant. Furthermore, dissociation from GRP78 may be a primary ATP-dependent step, *in vivo*.[4] Both the above mentioned steps may lead to the formation of a complex between GRP78 and the incompletely assembled or incompletely glycosylated HbsAg lipoprotein particle and prevent the transition of HbsAg through the ER. Insufficient availability of ATP could then be a third potential candidate step responsible for the bottleneck. The fourth possibility may be increased proteolysis of excess HbsAg at high cloned gene dosage, a phenomena which we have indeed observed for the 1 μM cells in our pulse-chase experiments. However, this phenomena was not observed

for the moderately amplified clone, the 100 nM cells, which exhibit a reduced secretion efficiency as well as reduced overall expression efficiency compared to the parental unamplified clone.

In summary, we found that increased gene copy number resulted in lower specific growth rate, reduced secretion efficiency, increased intracellular degradation of the excess HbsAg and reduced overall efficiency of HbsAg expression. This was especially true for 1 μM cells which synthesize much more HbsAg than the cell's post-translational pathway can handle before getting saturated. Our results indicate that neither transcription, nor translation gets saturated at high gene dosage. Some step in the post-translational secretory pathway seems to cause a bottleneck in the overall pathway of HbsAg synthesis and secretion. These results suggest that the observed physiological differences between the parental unamplified clone and the amplified clones may be due to a reduction in host cell biosynthetic activity for native cellular components because of redirection of cellular resources towards cloned gene and cloned gene product synthesis, processing and secretion. Our results show that it may not be optimal to operate the cells at very high cloned gene dosage situation since the deleterious effects of high copy number such as poor growth, poor secretion efficiency and intracellular degradation may outweigh the positive benefits of increased productivity. These factors must be considered in concert with the process economics in order to evaluate and optimize the cloned gene expression from a recombinant cell line using a particular production process.

3.6 ACKNOWLEDGMENTS

This research was supported by the National Science Foundation (Grant No. BCS-8805636) and by a grant from Amgen, Inc..

3.7 REFERENCES

1. **Da Silva, N.A. 1988.** Host Plasmid Interactions and Regulation of Cloned Gene Expression in Recombinant Cells, Ph.D. Thesis, California Institute of Technology, Pasadena, CA.

2. **Dorner A.J., Bole, D.G., Kaufman, R.J. 1987.** The relationship of N-linked glycosylation and heavy chain-binding protein association with secretion of glycoproteins. *J. Cell Biol.*, **105**: 2665-2674.

3. **Dorner A.J., Krane, M.G., Kaufman, R.J. 1988.** Reduction of endogenous GRP78 levels improves secretion of a heterologous protein in CHO cells. *Mol. Cell Biol.*, **105**: 4063-4070.

4. **Dorner A.J., Wasley, L.C., Kaufman, R.J. 1990.** Protein dissociation from GRP78 and secretion are blocked by depletion of cellular ATP levels. *Proc. Natl. Acad. Sci.*, **87**: 7429-7432.

5. **Licari, P., Bailey, J.E. 1991.** Factors influencing recombinant protein yields in an insect cell-baculovirus expression system: Multiplicity of infection and intracellular degradation. *Biotech. Bioeng.*, **37**: 238-246.

6. **Machida, A., Kishimoto, S., Ohnuma, H., Miyamoto, H., Baba, K., Oda, K., Nakamura, T., Funatsu, G., Miyakawa, Y., Mayumi, M. 1982.** A glycopeptide containing 15 amino acid residues derived

from Hepatitis B surface antigen particles: Demonstration of immunogenicity to raise anti-HBs in mice. *Mol. Immunol.*, **19**: 1087-1093.

7. **Maniatis, T., Sambrook, J., Fritsch, E.F. 1989**. *Molecular Cloning: A Laboratory Manual*, **Vol. 1-3**, Cold Spring Harbor Laboratory Press, NY.

8. **Neurath, A.R., Strick, N., Oleszco, W.R. 1981**. Localization of Hepatitis B surface antigen determinant deduced from results of chemical modification. *J. Virol. Methods*, **3**: 115-125.

9. **Patzer, E.J., Nakamura, G.R., Yaffe, A. 1984**. Intracellular transport and secretion of Hepatitis B Surface Antigen in mammalian cells. *J. Virol.*, **51**: 346-353.

10. **Peretti, S.W. 1987**. Theoretical modeling and experimental investigation of host-plasmid interactions in recombinant *Escherichia coli*, Ph.D. Thesis, California Institute of Technology, Pasadena, CA.

11. **Wood, T.K., Peretti, S.W. 1990**. Depression of protein synthetic capacity due to cloned gene expression in *E. coli*. *Biotech Bioeng.*, **36**: 865-878.

12. **Seo, J.H., Bailey, J.E. 1985**. Effect of recombinant plasmid content on growth properties and cloned gene product formation in *E. Coli*. *Biotech. Bioeng.*, **27**: 1668-1674.

13. **Simon, K., Lingappa, V.R., Ganem, D. 1988**. Secreted Hepatitis B Surface Antigen polypeptides are derived from a transmembrane precursor. *J. Cell Biol.*, **107**: 2163-2168.

3.8 TABLES

Table I. Relative HbsAg mRNA levels in different clones. All values have been normalized with respect to the parental HbsAg mRNA level.

Clone	HbsAg
Parental	1
100 nM	11
1 μM	51
10 μM	10

Table II. Comparison of total intracellular HbsAg, % secreted and %degraded as a function of gene copy number. The % values are calculated with respect to the total initial amount of HbsAg (0 h chase).

Clone	Relative amount of HbsAg AU*mm/ 10^6 cells			% Secreted HbsAg	%Degraded HbsAg
	Supernatant	Cell extract			
	23.5 hr	0 hr	23.5 hr		
Parental	1.9	2.2	0.4	83	-
100 nM	7.9	14.4	6.4	55	-
1 μM	10.0	55.8	7.7	18	68

3.9 FIGURES

Figure 1. Northern hybridization for detection of HbsAg mRNA level in different clones. The dhfr$^-$ clone showed no hybridization to the HbsAg probe.

Figure 2. Net synthesis rate of HbsAg obtained for parental (○) and 1 μM (●) cells by plotting the amount of HbsAg labeled versus pulse time and determining the slope of the resulting straight line.

Figure 3. Typical autoradiogram of pulse-chase experiment with 100 nM cells. The numbers indicate the chase time. EM and CE designate extracellular medium and cell extract, respectively.

Figure 4. Relationship between relative HbsAg mRNA level with relative HbsAg gene copy number for different clones.

Figure 5. Relationship between relative HbsAg mRNA level with average HbsAg secretion rate determined from batch culture experiments for different clones.

Figure 6. Relationship between relative HbsAg mRNA level with total initial intracellular HbsAg for different clones.

Figure 7. Relationship between relative HbsAg mRNA level with amount of HbsAg secreted into the medium by the end of the longest chase time of 23.5 h by different clones.

Figure 8. Relationship between relative HbsAg gene copy number with overall efficiency of HbsAg expression (amount of HbsAg secreted into the medium [AU.mm/10^6 cells]/relative gene copy number)

Figure 1

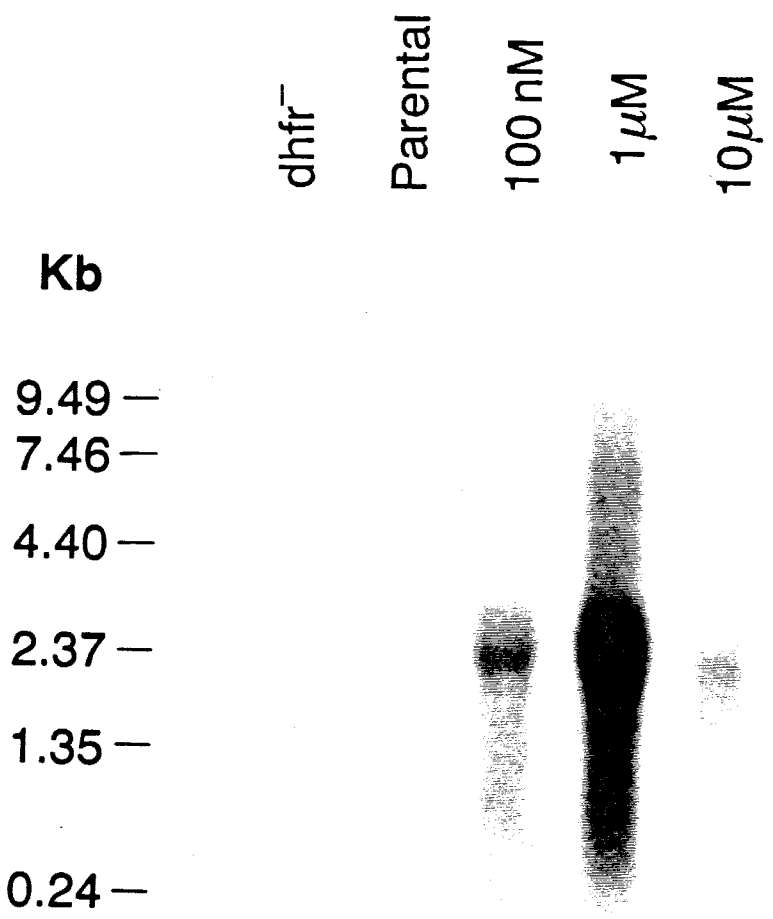


Figure 2

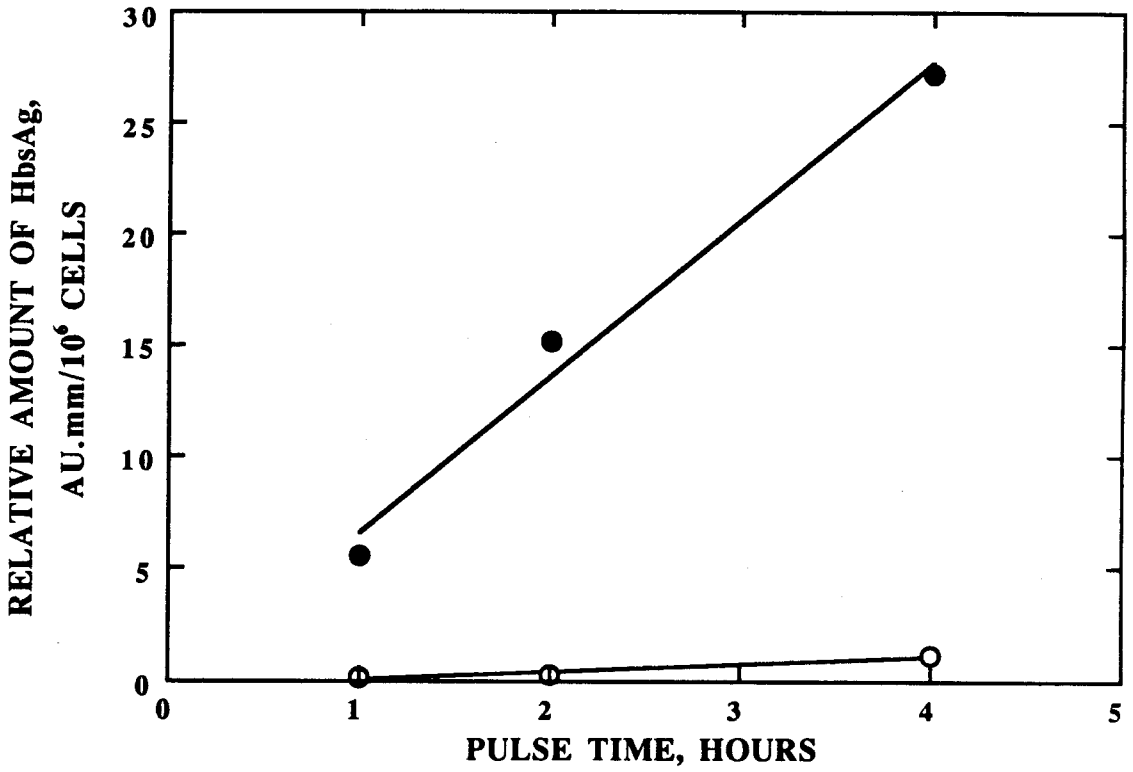

RELATIVE AMOUNT OF HbsAg,
AU.mm/10⁶ CELLS
30
25
20
15
10
5
0
0
1
2
3
4
5
PULSE TIME, HOURS

Figure 3

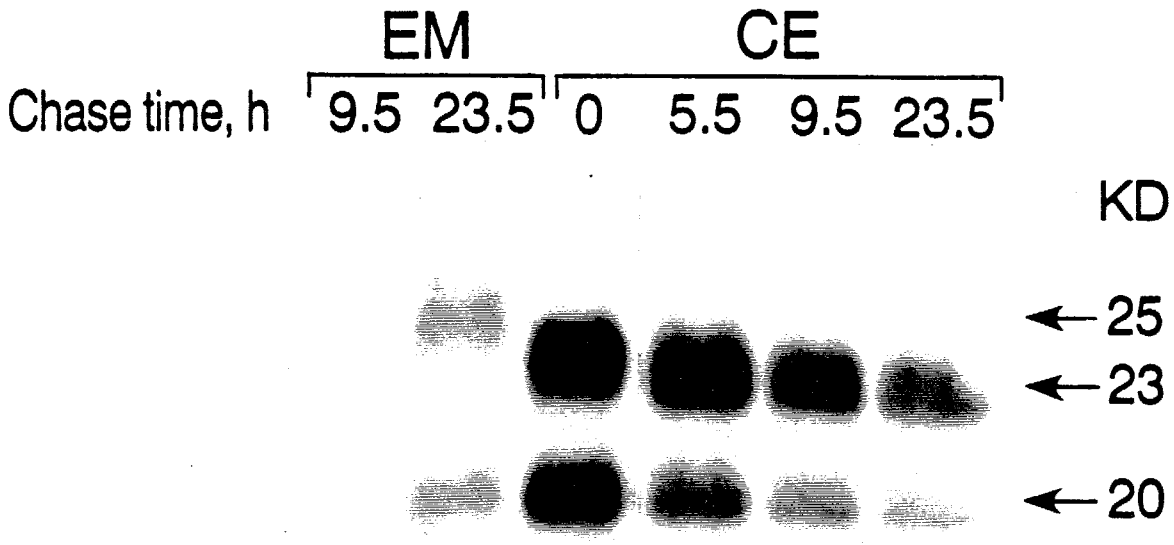


Figure 4

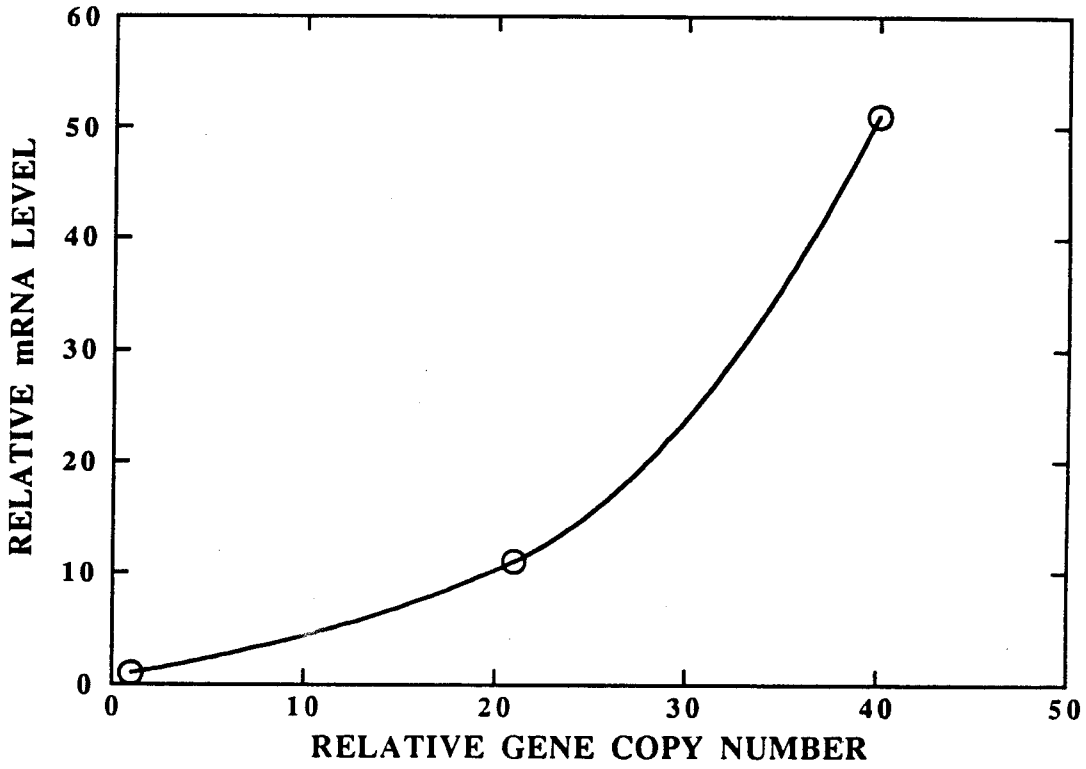

RELATIVE mRNA LEVEL
60
50
40
30
20
10
0
0
10
20
30
40
50
RELATIVE GENE COPY NUMBER

Figure 5

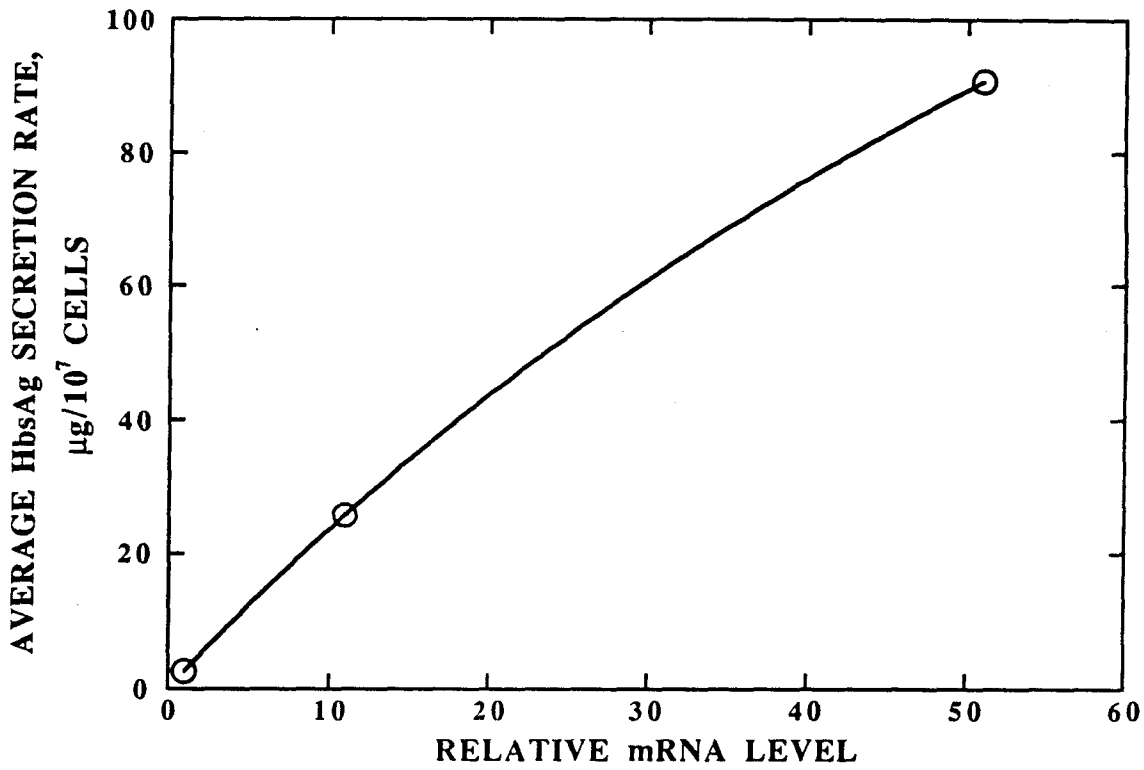

AVERAGE HbsAg SECRETION RATE, µg/10^7 CELLS
100
80
60
40
20
0
0
10
20
30
40
50
60
RELATIVE mRNA LEVEL

Figure 6

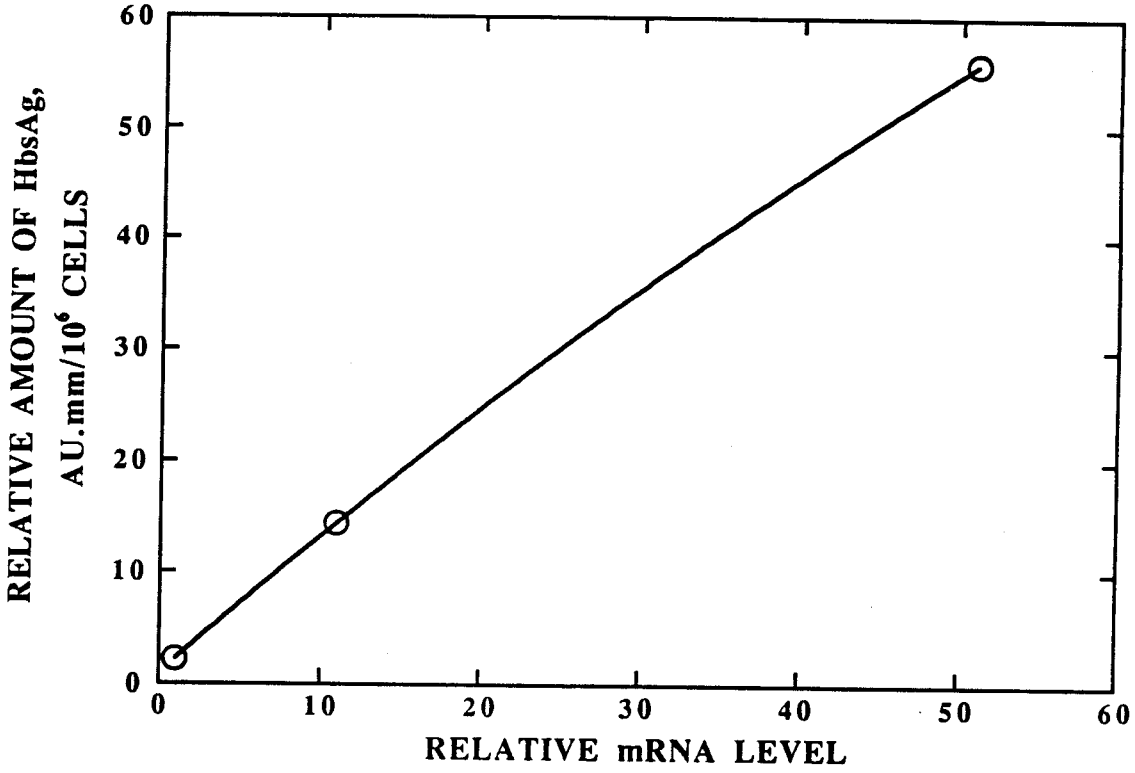

RELATIVE AMOUNT OF HbsAg, AU.mm/10⁶ CELLS
0
10
20
30
40
50
60
RELATIVE mRNA LEVEL

Figure 7

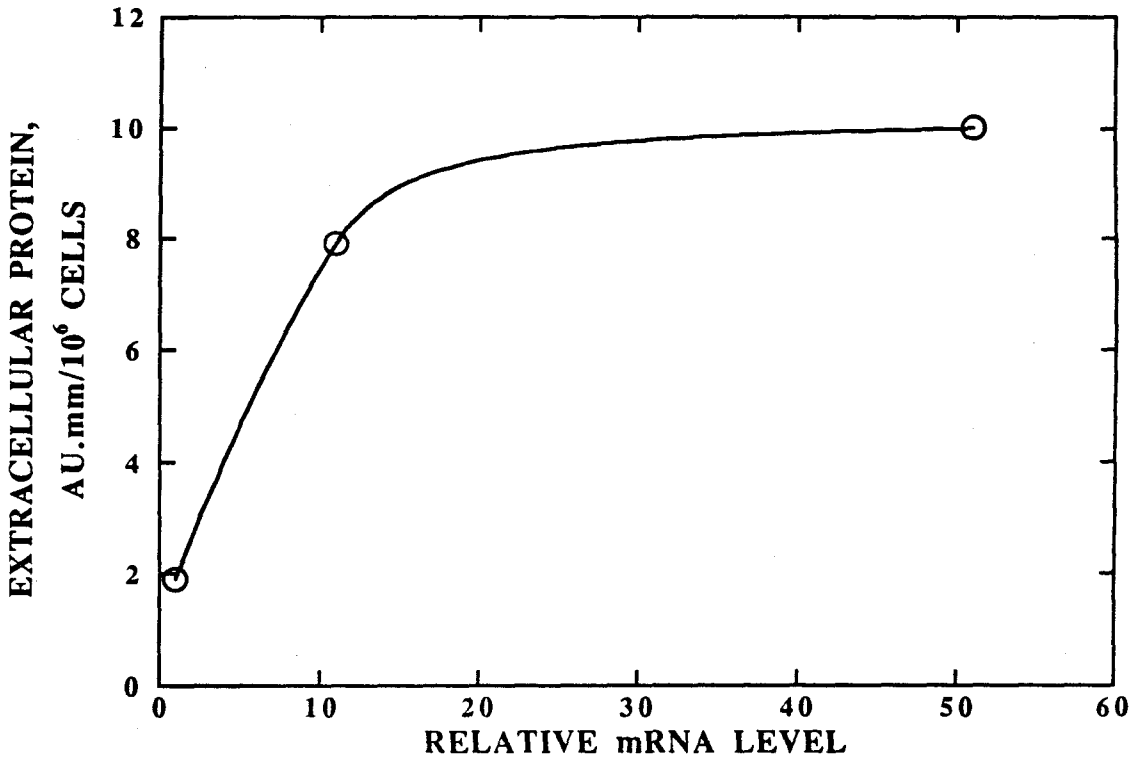
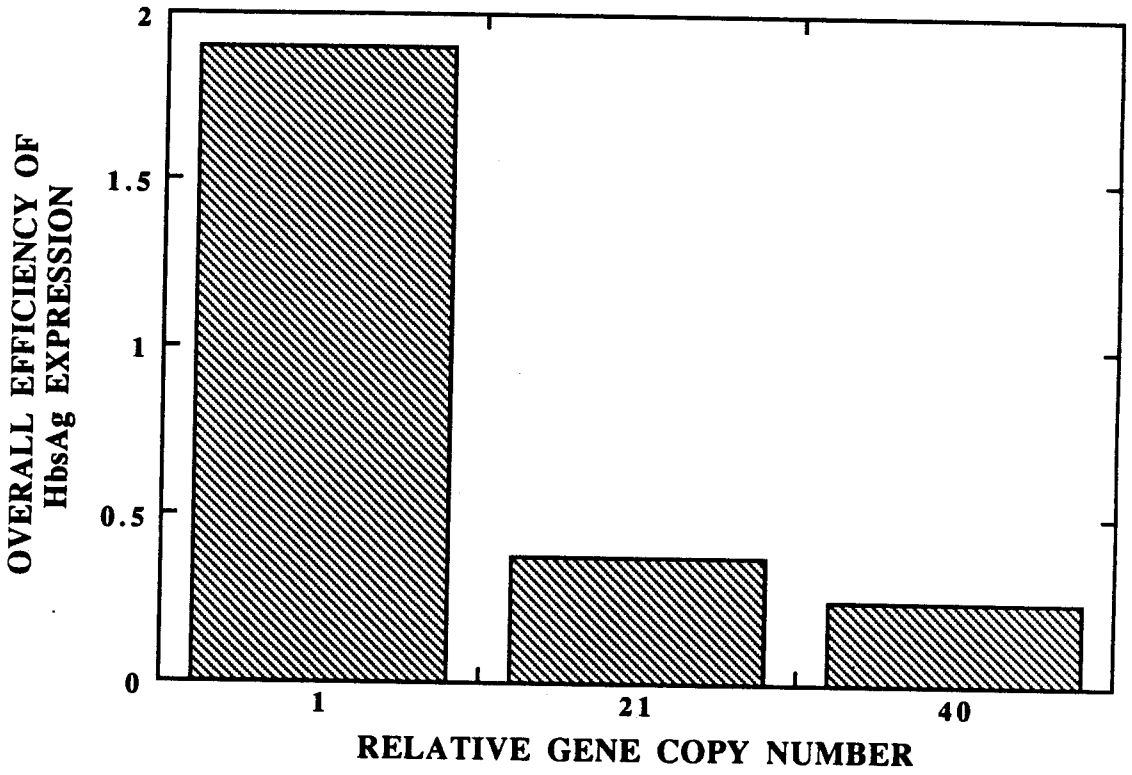

EXTRACELLULAR PROTEIN,
AU.mm/10^6 CELLS
12
10
8
6
4
2
0
0
10
20
30
40
50
60
RELATIVE mRNA LEVEL

Figure 8


OVERALL EFFICIENCY OF HbsAg EXPRESSION
2
1.5
1
0.5
0
1
21
40
RELATIVE GENE COPY NUMBER

CHAPTER 4

NMR spectroscopy of recombinant CHO cells using a continuous flow packed bed reactor system

4.1 SUMMARY

Several clones of CHO cells, including recombinant cell lines expressing Hepatitis B surface antigen, were grown in macroporous collagen microspheres. These provided sufficient cell density in a packed bed recirculation system for ^{31}P nuclear magnetic resonance spectroscopic estimation of metabolite concentration. Intracellular nucleoside triphosphate as well as nucleoside tri- plus diphosphate levels were higher in cells expressing HbsAg as compared to dhfr$^-$ cell line. In general, levels of all the phosphorylated metabolites detected using our reactor set-up were higher in cells expressing HbsAg as compared to the dhfr$^-$ cell line. Since earlier batch culture experiments as well as these experiments show that specific growth rate decreases with relative HbsAg gene copy number, we speculate that the available cellular resources are being redirected towards the HbsAg synthesis and secretion in these clones. Using this system, ^{13}C NMR did not show resonances corresponding to any metabolite other than glucose and lactate.

4.2 INTRODUCTION

Currently, a large number of proteins required for therapeutic and diagnostic use are being produced using mammalian cell culture. Environmental optimization of the productivity of mammalian cells depends upon improved fundamental understanding of cell-environment interactions. This in turn requires experimental access to intracellular composition under well-defined environmental conditions. Nuclear magnetic resonance (NMR) spectroscopy allows estimation of intracellular pH, levels of phosphorylated intermediates, and information about carbon fluxes through various metabolic pathways provided sufficiently high density can be achieved. Maintaining such high cell densities within an NMR spectrometer, however, presents challenges in provision of adequate nutrient and oxygen supply and removal of toxic end-products.

Many investigators have addressed these requirements by retaining cells within the NMR probe using different immobilization techniques such as Cytodex microcarriers[7] (Pharmacia) or Vitafiber hollow fiber units[6] (Amicon) among others. Some of the methods of perfusing such high density cultures have been described by Gillies et al.[5] and Egan[3] recently. Fernandez et al.[4] have demonstrated the use of an immobilized cell membrane reactor to monitor the metabolism of hybridoma cells using NMR.

In this paper, we demonstrate the application of a new continuous-flow NMR system to study the intracellular metabolism under conditions which mimics the *in vivo* cellular environment. ^{31}P and ^{13}C NMR studies were conducted in a packed bed reactor (packing:collagen microspheres) containing

perfused Chinese hamster ovary (CHO) cells producing recombinant Hepatitis B Surface antigen (HBsAg). As mentioned in the earlier chapter, introduction of a DNA vector for the purposes of cloned gene expression can perturb among other things, the cellular energy and intermediary metabolism. We were primarily interested in determining the intracellular levels of important phosphorylated metabolites including those of NTP which is a good indicator of cellular energetic state. These studies were carried out using various CHO clones adapted to different concentrations of methotrexate (MTX). Resistance to higher MTX concentrations is provided by increased dihydrofolate reductase (DHFR) levels which, based on the cell line's construction, should and indeed have been correlated with larger HbsAg gene dosage in the preceding chapters.

4.3 MATERIALS AND METHODS

Packed bed reactor set-up

A 20 mm NMR tube was modified to form a packed bed reactor as shown in Figure 1. A hollow glass tube passing through the center of the teflon cap covering the open end of the NMR tube serves to anchor two glass frits (pore size 100 μm) which form the upper and lower barriers of the packed bed reactor. This tube also serves to deliver conditioned feed medium to the bottom of the NMR tube below the lower frit. Two smaller hollow glass tubes positioned above the upper glass frit act as level controllers by removing the spent medium coming out of the upper frit after passing through the packed

bed of microspheres containing the cells. The collagen microspheres (350-800 μm size) were donated generously by Verax Corporation[8]. Cells were grown as per standard protocol supplied by Verax and then loaded in the modified NMR tube to form the packed bed. In this packed system the microspheres are retained at the same time allowing a free unrestricted passage of medium components. This packed bed reactor was then connected to a bioreactor by means of a recirculation loop. In order to minimize any exchange of gases in the recirculation loop norprene tubing was used for all tubing requirements. The dissolved oxygen concentration, pH and temperature were controlled in the bioreactor which served as a reservoir for the medium.

Cell culture and packed bed reactor operation

Four of the five clones mentioned in the earlier chapters were used in these experiments. They are the dhfr$^-$ cells, parental unamplified cells, 100 nM cells and the 10 μM cells.

All four clones were grown as before in DMEM (Gibco) supplemented with 5% dialyzed FBS (dFBS), 1% of 100X glutamine-penicillin-streptomycin stock (Irvine Scientific) and 1% of 100X non-essential amino acids stock (Gibco). The medium for dhfr$^-$ cells was additionally supplemented with 1% of 100X hypoxanthine-thymidine (HT) stock (Gibco) whereas that for 100 nM cells and 10 μM cells was supplemented with 100 nM and 10 μM MTX respectively.

Cells were regularly maintained as monolayer cultures in T 75 flasks at 37°C in a humidified 5% CO_2 incubator. The cells were inoculated at 1 x

10^5 cells/ml and passaged every 3-5 days upon reaching confluence. For NMR experiments, high density cultures were obtained using the standard protocol for growing cells on Verax microspheres. Briefly, 5-8 ml cytomax microspheres each were added to five 250 ml erlenmeyer flasks and pretreated with 50 ml medium containing 5% dFBS overnight at 37°C. These flasks were kept stationary and upright during this pretreatment period. The following day, each flask was inoculated with exponential phase cultures from T 75 flasks at a density of 1 x 10^6 cells/ml beads in 50 ml of culture medium. These flasks were placed on an orbital shaker inside a humidified CO_2 incubator at 37°C. For the first 24 hours following inoculation, the flasks were kept stationary with their caps loosened. On day 1, 25 ml of medium was exchanged with fresh medium and the rotary shaker was activated at 100 rpm. On day 3, 50 ml of medium was exchanged with fresh medium. From day 5-14, cell growth was allowed to proceed with regular medium changes in order to maintain a uniform pH near 7.2 and prevent nutrient depletion and/or end-product inhibition. Prior to the NMR experiment, viability and cell count was measured using the standard collagenase disruption procedure. Briefly, 0.25 ml bead sample was put in a preweighed tube. The weight of the tube with beads was measured after removing all medium from the tube. 1 ml collagenase solution (1 mg/ml in PBS) was added to the tube containing the microsphere sample and the tube was sealed with parafilm and placed in a 37°C waterbath for 1-2 hours. Cell counts and viability analysis was done using appropriate dilutions of this disrupted collagen cell suspension sample. The microspheres from these five flasks were

pooled and loaded into the modified NMR tube under sterile conditions. Prior to connecting the NMR tube to the recirculation loop, the loop was primed with medium from the reservoir in order to minimize the introduction of air bubbles into the packed bed. This primed loop was then connected to the NMR tube again under sterile conditions. The modified NMR tube was manually lowered inside the magnet of the spectrometer. In all NMR experiments the total reservoir volume was approximately 600 ml. The reservoir was supplemented with 10% D_2O which provided the deuterium lock signal for the spectrometer. The medium flow rate was maintained at 5-10 ml/min during the course of an experiment. ^{31}P and ^{13}C spectrum were then acquired under these continuous-flow conditions.

NMR spectroscopy

NMR spectroscopy was performed on Bruker 300AM wide bore system equipped with a 20mm broadband probe. Undecoupled ^{31}P NMR spectra were collected in blocks of 1200 scans each of which entailed approximately 15 minutes of acquisition time. Each scan consisted of a 40^o ($20\mu s$) radio frequency pulse followed by an 8K signal acquisition with a 200ms recycle delay. The total recycle time was 700ms. Methylene diphosphoric acid (MDP) was used as an internal standard. MDP resonates at 18.59 ppm downfield from 85% phosphoric acid which is assigned to zero ppm. Broad band decoupled (3 watts) (3 watts) ^{13}C spectra were acquired in 20 minute blocks of 16K data points with 1200 scans, a 37^o pulse, and a 200ms recycle delay. ^{13}C experiments involved

the substitution of 1-^{13}C labeled glucose (Sigma) for the normally nonenriched glucose at 25 mM initial concentration. The spectra were collected in small increment time blocks (15, 40 min) in order to monitor any small time dependent changes in the observed metabolites. These individual blocks were added together to increase the signal to noise ratio for the smaller metabolite peaks for data analysis. The resultant FID was exponentially multiplied (LB=10, 15), Fourier transformed, and spline baseline corrected before peak areas were measured for quantitation.

Since these spectra were not acquired under fully relaxed conditions, correction factors were necessary to obtain relative metabolite concentrations from the observed peak areas. Correction factors were calculated for each peak based upon the ratio of the observed spectrum to the one that was acquired under fully relaxed conditions (recycle delay of 10 seconds). This ratio, which is slightly different for each resonance because of differing T_1 values, allows the extrapolation to fully relaxed peak areas for quantitation.

4.4 RESULTS AND DISCUSSION

As described earlier, four clones were used in this study. Other experiments have shown that specific growth rate is inversely proportional to the concentration of methotrexate in the medium (hence, to the amplification in DHFR and HbsAg genes). In addition, the specific HBsAg secretion was found to increase with gene copy number (discussed in earlier chapters). Concentrations of intracellular metabolites as a function of methotrexate selection level

were obtained using ^{31}P experiments as described in Materials and Methods. For all four clones the cell density on the day of the experiment was between $3 - 8 \times 10^7$ cells/ml-beads. Average specific growth rates of various clones grown using Verax microspheres is summarized in Table 1.

Typical ^{31}P spectra exhibited resonances due to extracellular inorganic phosphate (P_i), sugar phosphates, nucleotide di- and tri-phosphates (NTP + NDP) and pyridine nucleotides designated by NAD(H). All experiments were carried out over a period of 4-5 hours during which the spectrum exhibited a stable behavior. The viability of cells before and after the experiment was found to be around 90%. One experiment was carried out with dhfr$^-$ cells over a period of 15-20 hours in order to acquire ^{13}C spectrum. The ^{31}P spectrum at the end of the experiment was extremely similar to another taken earlier in the run. The final viability in this experiment was found to be 70-75%.

Cells also showed almost no dependence on the dissolved oxygen concentration. During one run air was supplied constantly to the reservoir throughout the run without any DO control. During a second run with the same clone the DO was controlled at a constant value of 50% air saturation. The ^{31}P spectra obtained under these experimental conditions were essentially identical. For all subsequent experiments, the DO in the reservoir was controlled at 50% air saturation.

A typical ^{31}P spectrum for each 15 minute block and that averaged over a period of three hours for the 100 nM clone is shown in Figures 2A and 2B respectively. The relative concentrations of each ^{31}P metabolites detected

was calculated as described in Materials and Methods. These relative concentrations were then normalized with respect to the average cell concentration during the experiment. While the peaks from NTP-γ and NTP-α contain contributions from both NTP and NDP, the NTP-β peak is due to triphosphates alone. Hence, this peak was used as the major indicator of cell energetics of the various clones. Table 2 summarizes the normalized relative NTP-β and NTP + NDP levels for each clone. Figure 3 shows the relative concentration of various phosphorylated metabolites for the clones studied.

The results presented above show that intracellular NTP-β level increases in the transformed cells relative to the dhfr$^-$ cells. Furthermore, most of the phosphorylated metabolites observed in a typical ^{31}P spectrum were present at higher levels in transformed cells which express HbsAg as compared to the dhfr$^-$ cells. Unfortunately, we do not have similar data for the 1 μM clone which has the highest cloned gene dosage among the clones studied. The decline in NTP-β level in the 10 μM clone does not fit the trend observed in other clones; the 10 μM clone has also exhibited anamolous behavior in other experiments. The bases for these trends have been further investigated using other experiments which have been discussed elsewhere in this thesis. Using this system, ^{13}C NMR did not show resonances corresponding to any metabolite other than glucose and lactate.

Recently Dorner et al.[2] have reported findings suggesting the role of intracellular ATP pools in facilitating the secretion of certain heterologous secretory proteins. From our results, it seems possible that the higher NTP and higher

intracellular intermediary metabolite levels in the transformed cells producing HbsAg may be getting utilized in the production of the cloned gene product, in this case, HbsAg.

4.5 CONCLUSIONS

We have demonstrated the feasibility of studying the intracellular metabolism of perfused recombinant CHO cells in a continuous-flow packed bed under conditions which mimic the *in vivo* cellular environment. Since the microspheres used in this study also facilitate the growth of anchorage-independent cells such as hybridomas this reactor configuration can be used for studying the intracellular metabolism of various other cell lines that can be grown in culture.

Our results indicate that transformed cells producing HbsAg exhibit higher levels of intracellular metabolites including sugar phosphates and NTP as compared to dhfr$^-$ cells. We speculate that these increased levels of intracellular metabolites may be getting shunted off towards the synthesis and secretion of HbsAg instead of being utilized by the cell for the production of precursors required for various biosynthetic reactions. These experiments show that cell energetics and intermediary metabolism do get altered in cells resulting from transfection of the wild type host cells with a DNA vector for purposes of cloned gene expression.

4.6 ACKNOWLEDGMENTS

We would like to thank Amgen for donating various CHO cell line clones used in this experiment and Margaret Worden of Verax Corporation for supplying the unweighted collagen microspheres and the relevant protocols for growing CHO cells on these microspheres.

4.7 REFERENCES

1. **Browne J.K.** (personal communication), Amgen Center, Thousand Oaks, CA 91320-1789.

2. **Dorner, A.J., Wasley, L.C. and Kaufman, R.J., 1990**. Protein dissociation from GRP78 and secretion are blocked by depletion of cellular ATP levels. *Mol. Cell. Biol.*, **87**, 7429-7432.

3. **Egan, W., 1987** in *Phosphorus NMR in Biology*, (ed. C.T. Burt), **Vol. 1**, 135, CRC Press, Boca Raton, Florida.

4. **Fernandez, E.J., Mancuso, A., and Clark, D.S., 1988**. NMR spectroscopy studies of hybridoma metabolism in a simple membrane bioreactor. *Biotech. Prog.*, **4(3)**, 173-183 (1988).

5. **Gillies, R.J., Chresand, T.J., Drury, D.D., and Dale, B.E., 1986**. Design and application of bioreactors for analyses of mammalian cells by NMR. *Rev. Magn. Reson. Med.*, **1**, 155.

6. **Gonzalez-Mendez, R., Wemmer, D., Hahn, G., Wade-Jardetzky, N., and Jardetzky, O.,1982**. Continuous-flow culture for NMR of mammalian cells. *Biochim. Biophys. Acta.*, **720**, 274-280.

7. **Ugurbil, K., Guernsey, D.L., Brown, T.R., Glynn, P., Tobker, N., and Edelman, I.S., 1981**. ^{31}P studies of intact anchorage-dependent fibroblasts. *Proc. Natl. Acad. Sci.*, **78**, 4843-4847.

8. **Verax Corporation**, Etna Road, HC-61, Box 6, Lebanon, NH 03766.

4.8 TABLES

Table I. Comparison of growth rates of various clones grown on Verax microspheres. These growth rates were calculated based on the initial and final cell numbers during 2 weeks of culture.

Clone	Specific growth rate, hr^{-1}
$Dhfr^-$	0.013
Parental	0.012
100 nM	0.009
10 μM	0.008

Table II. Comparison of relative NTP-β and (NTP + NDP) concentrations in various clones. All values have been normalized with respect to the average cell number present in the packed bed during the experiment.

Clone	Relative NTP-β concentration	Relative NTP + NDP concentration
$Dhfr^-$	16.91	61.23
Parental	18.93	77.45
100 nM	27.63	116.6
10 μM	14.21	73.1

4.9 FIGURES

Figure 1. Schematic of the entire packed bed reactor set-up. All operations with the packed bed reactor were carried out in a sterile laminar flow hood. The connections between the recirculation loop and the NMR tube were made under sterile conditions using 100% ethanol.

Figure 2. NMR spectrum for 100 nM cells growing in collagen microspheres in a packed bed reactor. ^{31}P spectrum at 121.5 MHz recorded using 40^o pulse and 0.7 s total recycle time. (A) a 15 minute spectrum (NS=1200) and (B) spectrum averaged over a period of 3 hours. Spectra were exponentially multiplied with LB=10 Hz and spline baseline corrected. Chemical shifts are referenced to methylene diphosphoric acid (MDP) which resonates at 18.59 ppm downfield to 85% phosphoric acid which is assigned to 0 ppm. The most commonly observed phosphorylated metabolites have been labeled in the 3 hour spectrum.

Figure 3. Time sequence of 40 minute blocks of ^{13}C spectra using $dhfr^-$ cells. Over the entire duration of the experiment, only two significant peaks were observed in the NMR spectra, namely, glucose and lactate.

Figure 4. Relative concentrations of various phosphorylated metabolites in different clones. The various clones are as follows: (■) $dhfr^-$, (▨) Parental, (▨) 100 nM, (□) 10 μM. These numbers were calculated as described in the Materials and Methods.

Figure 1

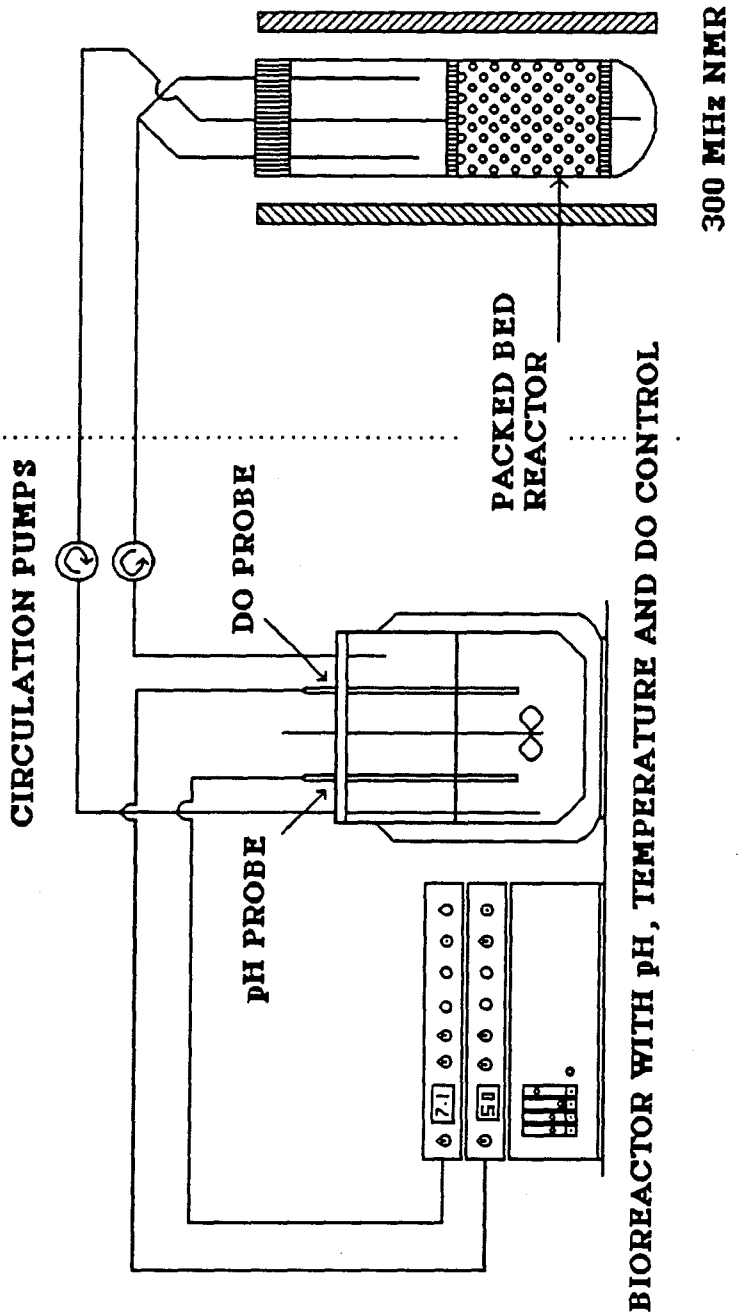

300 MHz NMR
PACKED BED REACTOR
CIRCULATION PUMPS
DO PROBE
pH PROBE
BIOREACTOR WITH pH, TEMPERATURE AND DO CONTROL

Figure 2

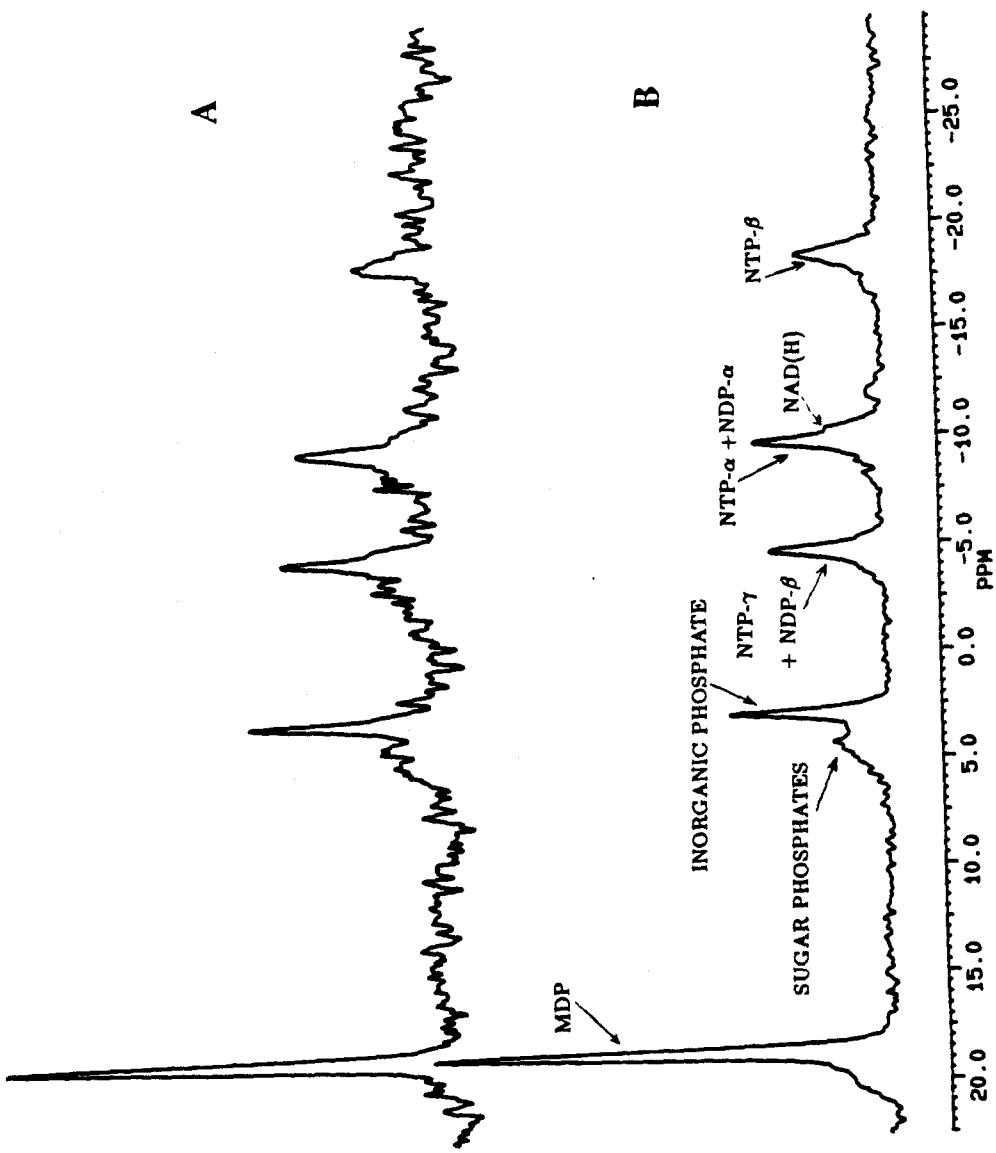

A
B
MDP
INORGANIC PHOSPHATE
SUGAR PHOSPHATES
NTP-γ
+ NDP-β
NTP-α +NDP-α
NAD(H)
NTP-β
20.0
15.0
10.0
5.0
0.0
-5.0
-10.0
-15.0
-20.0
-25.0
PPM

Figure 3

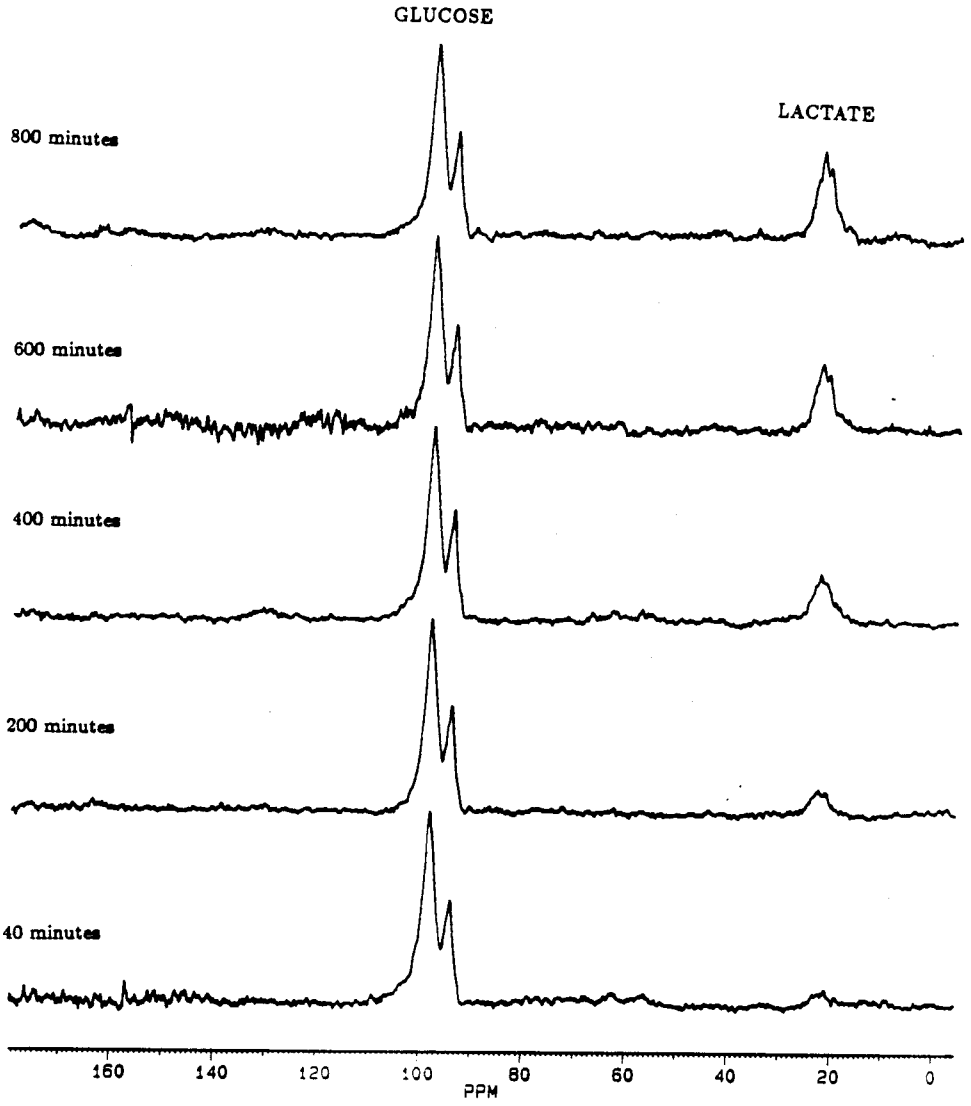
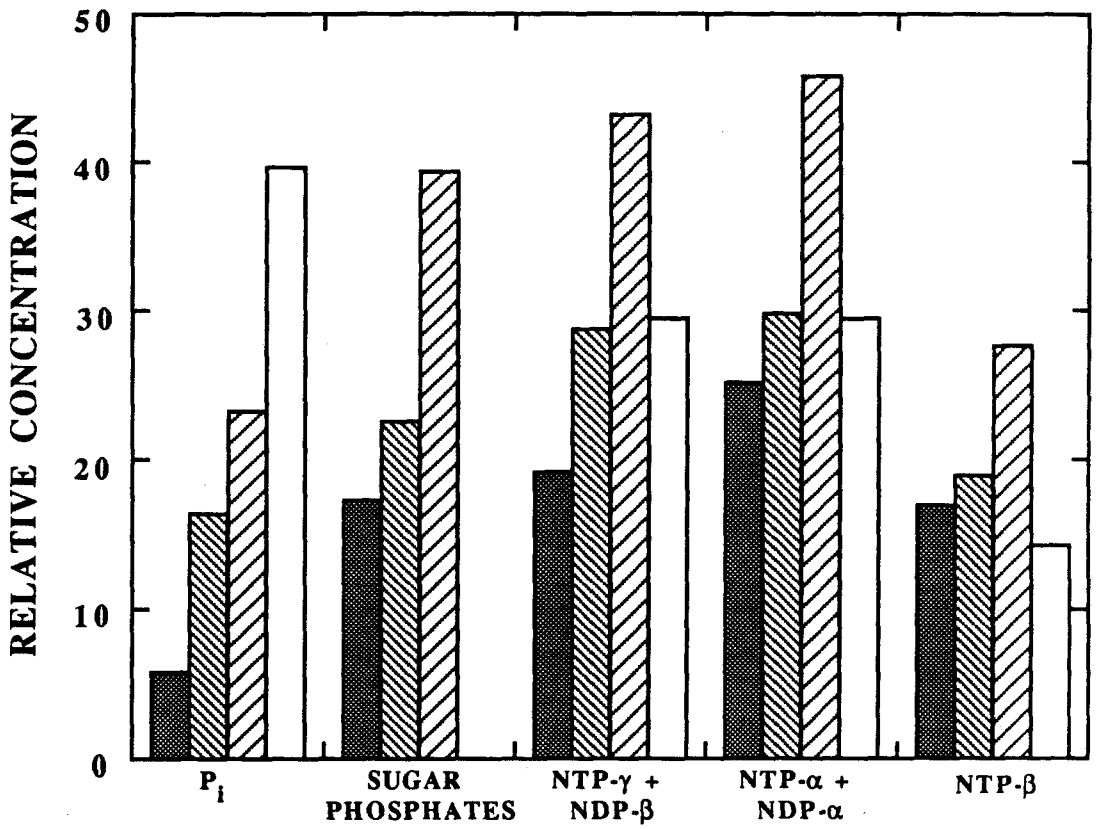

GLUCOSE
LACTATE
800 minutes
600 minutes
400 minutes
200 minutes
40 minutes
160
140
120
100
80
60
40
20
0
PPM

Figure 4


RELATIVE CONCENTRATION
50
40
30
20
10
0
P_i
SUGAR PHOSPHATES
NTP-γ + NDP-β
NTP-α + NDP-α
NTP-β

CHAPTER 5

Effect of VHb expression on growth and specific productivity of recombinant CHO cells expressing tPA

5.1 SUMMARY

Using the eukaryotic expression vector pMSG, we have cloned the *Vitreoscilla* hemoglobin gene (VHb) in CHO cells producing tPA. VHb expression was obtained by induction of the MMTV promoter with dexamethasone, a glucocorticoid hormone. Batch culture experiments show that VHb-expressing cells exhibit a lower specific growth rate than parental CHO-tPA cells. However, these VHb-expressing cells show higher specific tPA production rate compared to the CHO-tPA cells throughout the batch culture.

5.2 INTRODUCTION

Recently, a gene coding for a bacterial hemoglobin that is naturally expressed under poorly oxygenated environments[5,13] was isolated from the aerobic bacterium *Vitreoscilla* and cloned into *E. coli*.[4] Under oxygen-limited conditions, expression of *Vitreoscilla* hemoglobin (VHb) resulted in faster growth, considerably higher cell densities, increased respiration rates and increased protein synthesis in fed-batch fermentations.[4,6,7] One hypothesis proposed to explain these results was that the intracellular expression of VHb improved the overall efficiency of oxygen-limited ATP production by increasing the proton translocation efficiency. Preliminary results from some current experiments in our laboratory are consistent with this hypothesis. This VHb gene has been cloned in a variety of other microorganisms such as *Streptomyces lividans* and *Streptomyces coelicolor* and similar effects have been reported.[9]

Over the last decade, great progress has been made in the development of a number of mammalian expression systems for production of heterologous proteins. Many studies with a wide range of hosts as well as different heterologous proteins have shown that one of the main obstacles of obtaining high level expression of these proteins occurs because of bottlenecks present in the post-translational secretory pathway. Recent papers by Dorner and coworkers[1,2,3] have shown that a number of heterologous secretory proteins expressed by recombinant CHO cells interact to various degrees with BiP (immunoglobulin heavy chain binding protein) or GRP78 (glucose regulated protein of molecular weight 78Kd), a resident protein of the endoplasmic reticulum (ER). *In*

vitro, dissociation from GRP78 requires ATP.[12] Results from Dorner and co-workers[3] indicate that secretion of GRP78-associated proteins may be more sensitive to perturbation of intracellular ATP levels by CCCP treatment than unassociated proteins. *In vivo* dissociation from GRP78 may be a primary ATP-dependent step in transport from the ER.

The possibility that expression of *Vitreoscilla* hemoglobin in recombinant CHO cells may accelerate a limiting step in post-translational processing of product was the motivation for this research. Our goals in this work were two-fold: first, to express VHb in CHO cells producing tPA using some suitable vector, and, second, to investigate the effect of VHb expression on cell growth and tPA production.

We have used pMSG, a commercially available, commonly used eukaryotic expression vector.[8,11] We cloned the VHb structural gene in the multicloning site of pMSG present downstream of the MMTV promoter which is inducible by dexamethasone, a glucocorticoid hormone. The resulting construct, designated pMSG-VHb was used to transfect CHO cells producing tPA. The presence of VHb gene in the recombinant transfectants has been confirmed by Southern hybridization, and the inducible nature of VHb protein expression demonstrated using SDS-PAGE followed by Western blots. The effect of VHb expression on cell growth and tPA productivity of these cells was investigated in batch culture experiments.

5.3 MATERIALS AND METHODS

Bacterial strains and plasmid vectors

Escherichia coli strain HB101 was used for all routine bacterial culture work including minipreps and maxipreps for isolation of plasmid DNA. The vector pMSG was obtained from Pharmacia LKB Biotechnology (Piscataway, NJ). The source of the *Vitreoscilla* hemoglobin structural gene was pRED302 (Chaitan Khosla, personal communication), a derivative of pRED2.[5] All restriction enzymes used in this study were obtained from Boehringer Mannheim (Indianapolis, IN). In order to have an in-house stock of pMSG, *E. coli* HB101 were transformed with pMSG using the standard $CaCl_2$ protocol from Maniatis.[10] A 10 ml overnight culture was used in the miniprep for isolation of pMSG plasmid DNA. The authenticity of this plasmid was confirmed by performing several restriction digestions of the original and the HB101-derived pMSG plasmid DNA.

Construction of pMSG-VHb

A frozen stock of HB101-pRED302 was plated out on an LB-ampicillin agar plate. The following day, a 10 ml overnight culture was started from a single colony. A miniprep was carried out on this overnight culture for pRED302 isolation. pRED302 was digested with *XbaI* and *SspI* and the digested sample was run on a 0.7% agarose gel. This resulted in 3 fragments of approximate sizes of 2.5 kb, 0.56 kb and 0.54 kb. One of the smaller fragments

contains the VHb structural gene. Test digestions were carried out with these two fragments with *Mlu*I and *Bsu36*I since these sites are present in the VHb structural gene. Electrophoresis of these digested samples on a 2% agarose gel confirmed that the 0.54 kb band contains the VHb structural gene. This band was eluted and purified from the gel using the GeneClean Kit (Bio 101, La Jolla, CA), resuspended in 30 ml TE (pH 8.0) and stored at -20°C. Part of this purified fragment was used for construction of pMSG-VHb and the remaining sample was frozen down at -20°C for use in Southern hybridization experiments. pMSG was sequentially digested with *Nhe*I and *Sma*I with an intermediate phenol:chloroform extraction and ethanol precipitation step. The digested vector was run on a 0.7% agarose gel and the larger fragment was purified using GeneClean Kit, resuspended in 20 ml TE (pH 8.0) and stored at -20°C. An overnight blunt-sticky end ligation reaction was carried out at 8-15°C for cloning the *Xba*I/*Ssp*I VHb fragment into the *Nhe*I/*Sma*I digested pMSG. This vector was named pMSG-VHb. The following day, competent HB101 were transformed separately with no plasmid, pMSG and pMSG-VHb respectively and spread on LB-ampicillin plates. Nine colonies were picked from the pMSG-VHb plate as potential transformants for further analysis. Minipreps were carried out on overnight cultures of all these potential transformants for pMSG-VHb plasmid DNA isolation. Vectors pMSG and pMSG-VHb were digested with different restriction enzymes such as *BamH*I, *Hind*III, *Sal*I and *Mlu*I for verifying the authenticity of the new construct. These samples when run on a 0.7% agarose gel confirmed the presence of VHb insert in the multicloning site of pMSG. A maxiprep was carried out using two 100 ml cultures

of one of the pMSG-VHb transformants for large-scale plasmid DNA isolation. The DNA was extracted with phenol:chloroform, ethanol precipitated, and the pellet was resuspended in 1 ml TE (pH 8.0) and stored at -20°C until further use in CHO cell transfection.

Cell culture and CHO cell transfection

Chinese hamster ovary (CHO) cells producing tPA (ATCC 9606) were obtained from ATCC (Bethesda, Maryland). These cells were grown routinely in non-selection medium containing DMEM (high glucose) (GIBCO, Grand Island, NY) supplemented with 1X penicillin-streptomycin-glutamine (Irvine Scientific, Irvine, CA) solution and 5% dialyzed FBS (GIBCO, Grand Island, NY) in a 5% CO_2 humidified incubator at 37°C. 100 X 20 mm tissue culture dishes were used in all the experiments. The selection medium for transfected cells contains 25 mg/l mycophenolic acid (Gibco, Grand Island, NY), 1 X HAT (Gibco, Grand Island, NY) and 250 mg/l xanthine(Sigma, St. Louis, MO) in addition to the non-selection medium components. During regular cultures, cells were passaged every 2-3 days upon semi-confluency. Total cell counts and the cell size distribution were monitored by the Coulter counter. Cell viability was determined using the trypan blue exclusion method. The CHO cells producing tPA were tranfected with pMSG-VHb using the standard calcium phosphate procedure described in Maniatis[10]. Briefly, one 100 mm tissue culture dish with 10 ml non-selection medium was inoculated with 1 x 10^6 cells 24 hours prior to transfection. 20 μg pMSG-VHb DNA was digested

overnight with *EcoRI* at 37°C. This linearized vector DNA was subjected to phenol:chloroform extraction and ethanol precipitation and the resulting pellet was resuspended in an appropriate volume of 0.1 X TE (pH 8.0). This was combined with 20 μg of carrier DNA and the calcium phosphate-DNA precipitate was formed according to the standard protocol. 1 ml of this precipitate was added to the 100 mm dish containing cells. These cells were incubated for 24 hours at 37°C after which the medium was aspirated and fresh non-selection medium added in order to facilitate the expression of XGPRT. Cells were then diluted 1 : 8 in selection medium and allowed to grow in this medium. The medium was replaced every two days and single colonies began to appear in about two weeks. About 40 colonies resistant to selection pressure were picked using cotton tips wetted with trypsin and transferred to 24-well tissue culture plates. These colonies were expanded to 60 and 100 mm dishes upon reaching confluency in the wells. All except 3 clones were frozen down at -70°C. The three clones (clones 4, 19 and 30) were gradually expanded to 100 mm tissue culture dishes for further studies. The presence of VHb gene in the CHO cell chromosomes and the inducible expression of VHb protein was confirmed in these transfected clones using Southern hybridization and Western blots respectively which have been discussed later. After these initial confirmatory tests, only the parental CHO-tPA and one VHb expressing clone (clone 30) were used for detailed physiological characterization studies.

Dose-Response Experiments

Cells were inoculated at 4 x 10^5 cells/dish. 24 hours post inoculation, dexamethasone (Sigma, St. Louis, MO) was added to each dish at final concentrations ranging from 0 to 2 μM. Cell growth was monitored every day as described earlier. Cell extracts were prepared for Western blot analysis as described in the following section. In one experiment, the VHb expression was monitored as a function of dexamethasone concentration (0, 0.1, 0.2, 0.5, and 1.0 μM) after 48 hours of induction. In the other experiment, VHb expression was monitored for a constant dexamethasone concentration for induction times of 24, 48, 72, and 96 hours induction period.

SDS-PAGE and Western Blot analysis

Cell extracts were prepared for each sample as per the protocol provided by Exogene Inc. (Monrovia). Briefly, cells were harvested by trypsinization. The cell suspension was centrifuged at 2500 rpm for 10 minutes at 4°C and supernantant was aspirated completely. The resulting cell pellet was resuspended in 40 μl lysis buffer (100 mM Tris pH 8.0, 10 mM NaCl and 10 mM EDTA pH 8.0). This cell suspension was subjected to three freeze-thaw cycles between dry ice-ethanol and 37°C waterbath for 5 minutes each. The resulting suspension was centrifuged at 12000 rpm for 2 minutes at 4°C. The supernatant was transferred to an Eppendorf tube and stored at -20°C until SDS-PAGE was performed. SDS-PAGE was carried out using standard protocols using the Protein

mini-gel apparatus (Biorad, Richmond, CA). The proteins were transferred to a nitrocellulose membrane and VHb detection was performed using a protocol provided by Exogene, Inc. (Monrovia). Briefly, the membrane was incubated for 30 minutes with blocking buffer (PBS containing 1% non-fat dried milk) to prevent non-specific binding. This was followed by a 30 minute incubation with 100 μl rabbit anti-serum to VHb in 50 ml blocking buffer. The membrane was then subjected to two 10 minute washes with PBS. The membrane was then incubated with 50 μl horseradish peroxidase conjugated anti-rabbit antibody in 50 ml blocking buffer for 30 minutes. After two 10 minute washes with PBS, the membrane was then incubated with the horseradish peroxidase substrate 4-chloro-4-naphthol for 30 minutes which resulted in the appearance of bands corresponding to VHb protein. JM101:pRED2[5] (this strain expresses high levels of VHb) cell extract was used as VHb standard.

Southern Hybridization

Genomic DNA was isolated from various clones using standard protocols from Maniatis et al.[10]. 5 μg DNA each of the parental CHO-tPA and the three VHb-CHO clones was digested overnight with *EcoR*1 at 37^{o}C. Agarose gel electrophoresis of this digested DNA was carried out using 0.5 X TBE for six hours at 60 volts. The DNA was then transferred overnight to an Immobilon-S membrane (Millipore, Bedford, MA) using capillary blot technique.[11] The VHb gene probe isolated by miniprep was labeled with biotin using a PolarPlex Kit (Millipore, Bedford, MA). Prehybridization and hybridization was carried

out overnight in a 68°C waterbath according to the PolarPlex protocol. The following day, detection reaction was performed using the Lumigen reagent. A permanent image of the hybridization pattern was obtained by exposing the membrane to an X-ray film for time periods ranging from 10 minutes to 1 hour.

Batch Growth and tPA Production Kinetics

The clones used for this study were the parental CHO-tPA and the VHb expressing CHO-tPA cells (clone 30). For the VHb-expressing cells, dexamethasone concentrations of 0, 0.1 and 0.5 μM were used. These cells were induced on day 1 of the batch culture. All these experiments were carried out using 100X20 tissue culture dishes. Cells were inoculated on day 0 at a density of 4 x 10^5 cells per dish. On each day of the 5 day batch culture experiment, one dish was removed from the incubator for measurements of cell number, tPA titers and other supernatant metabolite concentrations. Total cell count was monitored using a Coulter Counter. Viability was measured by the trypan blue exclusion method using a hemocytometer. Supernatant was frozen at -20°C for tPA and metabolite analysis. tPA production was monitored using an ELISA kit (COALIZA, KabiVitrum, Franklin, OH) according to the standard protocol provided by the manufacturer. tPA concentrations in each sample were calculated by using a calibration curve obtained using standard tPA samples provided in the kit. The total amount of tPA produced each day was calculated by multiplying the concentration of tPA obtained by the volume of supernatant present in each dish. This was done to account for

the progressive reduction in volume of the supernatant due to evaporation of water during the course of the batch culture. On each day, cell extracts were prepared and stored as described earlier for analysis of VHb expression during the batch culture. The day 1 sample from VHb-expressing cells corresponds to the uninduced level of VHb expression whereas the CHO-tPA sample serves as negative control for VHb expression since these cells lack the VHb gene.

5.4 RESULTS

Dose-response experiments

Induction experiments were done with the parental CHO-tPA cell line as well as the three VHb-CHO clones described earlier, namely, clones 30, 4 and 19. The results of Western blots from dose-response experiments for the VHb-CHO clone 30 is shown in Figures 1 and 2. Although it is not very clear in the photograph, the uninduced sample (no dexamethasone) shows a very faint band corresponding to VHb, indicating that the MMTV promoter is slightly leaky under the conditions of our experiment. However we observed a significant induction of the MMTV promoter for dexamethasone concentration ranging from 0 to 2 μM. Furthermore, VHb expression is observed 24 to 96 hours post-induction. Thus, our dose- and time-response experiments clearly show that these CHO cells express VHb in an inducible manner. No VHb expression was obtained for induced or uninduced parental CHO-tPA cells. Similar patterns of VHb expression were observed in the VHb-CHO clones 4 and 19 (data not shown).

Southern hybridization

Figure 3 shows a typical hybridization pattern of *EcoR*I digested genomic DNA from various clones probed with a biotin-labeled VHb gene probe. All VHb-CHO clones show two distinct hybridization bands whereas the parental clone shows one band when probed with the VHb gene. The lower band is present in all four clones, suggesting that this is a fragment of the genomic DNA which exhibits substantial homology with VHb gene. This band could be a hemoglobin gene that is endogenous to CHO cells. At this point, however, we do not have any additional information about the nature of the DNA fragment corresponding to the lower band. The upper band on the other hand is present only in VHb-CHO clones indicating that this band corresponds to the VHb gene present in the vector pMSG-VHb that has been integrated in the CHO cell chromosome as a result of transfection. Thus, we have established the presence of the VHb gene integrated into the chromosome of CHO-VHb cells. Earlier, we have shown that this gene is indeed expressed under appropriate induction conditions resulting in intracellular VHb protein production as detected by Westerns.

Batch culture experiments

Once the cloning of the VHb gene and its expression in CHO cells to give intracellular VHb protein was established, we were interested in determining the effect of VHb expression on cell growth and tPA productivity in batch

cultures. The batch culture experiment was carried out in two independent runs in order to establish reproducibility and consistency of our experiments. The results of these experiments are discussed below. Figures 4 and 5 show the growth curves obtained during these two runs while total tPA amounts for the same two runs are shown in Figures 6 and 7. Figure 8 shows the specific tPA productivity averaged for the two runs, expressed as total tPA produced/10^6 cells. From these results, it is clear that the specific growth rate of VHb-CHO cells is about 20-30% lower than the specific growth rate of the parental CHO-tPA clone. However, this effect does not seem due to VHb expression since the uninduced VHb-CHO cells show almost the same growth characteristics as the induced VHb-CHO cells. This effect is probably due to some unknown host-vector interaction that manifests itself as a result of integration of transfected DNA sequences into the host cell chromosomes. Furthermore, the growth curves were almost the same for both induced as well as uninduced parental CHO-tPA cells upto a dexamethasone concentration of 1 μM. Thus we can rule out with reasonable confidence any significant effect of dexamethasone on the observed cell physiology and productivity.

However, the tPA productivity characteristics are significantly different in the VHb-CHO clone as compared to the parental CHO-tPA clone. The total amount of tPA produced by VHb-CHO cells was either equal to or higher than the total amount of tPA produced by CHO-tPA cells throughout the batch. The most dramatic difference between the parental and VHb-CHO cells was observed in the specific tPA productivity. The per cell tPA production is about 40-100% higher in VHb-CHO cells as compared to the parental CHO-tPA cells.

5.5 DISCUSSION AND CONCLUSION

In this work, we have attempted to influence the productivity of recombinant CHO cells by cloning a gene coding *Vitreoscilla* hemoglobin which has been implicated in increased ATP-production in oxygen-limited *E. coli.* To this end, we have cloned the VHb gene into recombinant CHO cells producing tPA using pMSG vector. VHb expression upon dexamethasone induction has been demonstrated using Western blot analysis. The batch culture experiments show that VHb-CHO cells have slightly lower specific growth rates as compared to the parental CHO-tPA cells. However, both total tPA amounts as well as specific tPA production is higher in VHb-CHO cells throughout the batch.

At this time, we cannot completely exclude the possibility that this enhanced production might be due to some other host-vector interaction. This productivity enhancement effect is observed even for uninduced VHb-CHO cells. However, we have shown that the MMTV promoter to be slightly leaky so that some VHb expression is obtained even in the uninduced cells. It is possible, then, that this low amount of intracellular VHb is enough to cause the beneficial effects that we observe.

We experienced substantial declines in tPA production in clones that had been propagated over a period of 3-4 months. The batch culture experiments described above were done using either, the original clonal population or by starting with fresh cells from a frozen stock of this clonal population. Cells were maintained in culture for about 1-2 months before other experiments were

done. Subsequent quasi-steady state medium replacement experiments (done with the intention of extending the batch culture) showed a large drop in the productivity of VHb-CHO cells without much change in the specific growth rate and VHb expression. During the same period, the parental CHO-tPA cells showed no significant differences in growth and productivity from earlier results. When we repeated the batch culture experiments with VHb-CHO cells that had been maintained in culture for about 3-4 months, the same low tPA productivity was obtained. At this point, we speculated that the VHb-CHO cells had likely changed during the course of 4 months of passage. The batch culture experiment described above done using VHb-CHO cells that had been in culture for 4 months and VHb-CHO cells that were freshly thawed from the original stock confirmed our hypothesis of deterioration of the culture. The freshly thawed VHb-CHO cells and the parental CHO-tPA cells showed growth and productivity patterns that we had obtained in our earlier experiments from the initial clones. The VHb-CHO cells that had been passaged for 4 months had specific growth rate that was lower than parental cells but slightly higher than the freshly thawed VHb-CHO cells. Furthermore, the "old culture" showed the same low tPA productivity characteristics that had been observed earlier.

5.6 ACKNOWLEDGMENTS

We would like to thank Dr. Pauli Kallio for his help in the construction of pMSG-VHb vector. This work was supported by the Advanced Industrial Concepts Division of the US Department of Energy.

5.7 REFERENCES

1. **Dorner A.J., Bole, D.G., Kaufman, R.J. 1987**. The relationship of N-linked glycosylation and heavy chain-binding protein association with secretion of glycoproteins. *J. Cell Biol.*, **105**: 2665-2674.

2. **Dorner A.J., Krane, M.G., Kaufman, R.J. 1988**. Reduction of endogenous GRP78 levels improves secretion of a heterologous protein in CHO cells. *Mol. Cell Biol.*, **105**: 4063-4070.

3. **Dorner A.J., Wasley, L.C., Kaufman, R.J. 1990**. Protein dissociation from GRP78 and secretion are blocked by depletion of cellular ATP levels. *Proc. Natl. Acad. Sci.*, **87**: 7429-7432.

4. **Khosla, C., Bailey, J.E. 1988**. Heterologous expression of a bacterial hemoglobin improves growth properties of recombinant *Escherichia coli*. *Nature*, **331**: 633-635.

5. **Khosla, C., Bailey, J.E. 1988**. The *Vitreoscilla* hemoglobin gene: Molecular cloning, nucleotide sequence and genetic expression in *Escherichia coli*. *Mol. Gen. Genet.*, **214**: 158-161.

6. **Khosla, C., Curtis, J.E., Bydalek, P., Swartz, J.R., Bailey, J.E. 1990**. Expression of recombinant proteins in *Escherichia coli* using an oxygen-responsive promoter. *Bio/Technology*, **8**: 554-558.

7. **Khosla, C., Curtis, J.E., DeModena, J. A., Rinas, U., Bailey, J.E. 1990**. Expression of intracellular hemoglobin improves protein synthesis in oxygen-limited *Escherichia coli*. *Bio/Technology*, **8**: 849-853.

8. **Lee, F., Mulligan, R., Berg, P., Ringold, G. 1981**. Glucocorticoids regulate expression of dihydrofolate reductase cDNA in mouse mammary tumor virus chimeric plasmids. *Nature*, **294**: 228-232.

9. **Magnolo, S.K., Leenutaphong, D.L., DeModena, J. A., Curtis, J.E., Bailey, J.E., Galazzo, J.L., Hughes, D.E. 1991**. Actinorhodin production by *Streptomyces coelicolor* and growth of *Streptomyces lividans* are improved by the expression of a bacterial hemoglobin. *Bio/Technology*, **8**: 849-853.

10. **Maniatis, T., Sambrook, J., Fritsch, E.F. 1989**. *Molecular Cloning: A Laboratory Manual*, **Vol. 1-3**, Cold Spring Harbor Laboratory Press, NY.

11. **Mulligan, R.C., Berg, P. 1981**. Selection of animal cells that express the *Escherichia coli* gene coding for xanthine-guanine phosphoribosyltransferase. *Proc. Natl. Acad. Sci.*, **78**: 2072-2076.

12. **Munro, S., Pelham, H. R. B. 1986**. An hsp70-like protein in the ER: identity with the 78Kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell*, **46**: 291-300.

13. **Wakabayashi, S., Matsubara, H., Webster, D.A. 1986**. Primary sequence of a dimeric bacterial hemoglobin from *Vitreoscilla*. *Nature*, **322**: 481-483.

5.8 FIGURES

Figure 1. Western blot for detection of VHb expression as a function of dexamethasone concentration. Dexamethasone concentrations ranging from 0 to 1 μM were used in this experiment. Cell extracts were prepared as described earlier after 48 hours post-induction.

Figure 2. Western blot for detection of VHb expression as a function of time post-induction. Dexamethasone concentrations of 0.1 and 0.5 μM were used in this experiment. Cell extracts were prepared at 24, 48, 72 and 96 hours post-induction.

Figure 3. Southern hybridization pattern of *EcoR*I digested genomic DNA from parental CHO-tPA clone and three VHb-CHO clones. The lower band present in all four clones probably corresponds to a hemoglobin which is endogenous to CHO cells. The bigger band, however, is present only in VHb-CHO clones and corresponds to the VHb gene that has been integrated into the host cell chromosomes as a result of transfection.

Figure 4. Total cell number/dish in batch culture for run 1. Cells were inoculated at 4 x 10^5 cells/100 mm tissue culture dish. The symbols are as follows: (×) CHO-tPA, (○) VHb-CHO OμM dexamethasone, (□) VHb-CHO O.1μM dexamethasone, (△) VHb-CHO O.5μM dexamethasone.

Figure 5. Total cell number/dish in batch culture for run 2. Cells were inoculated at 4 x 10^5 cells/100 mm tissue culture dish. The symbols are as

follows: (×) CHO-tPA, (○) VHb-CHO OμM dexamethasone, (□) VHb-CHO O.1μM dexamethasone, (△) VHb-CHO O.5μM dexamethasone.

Figure 6. Total tPA production/dish in batch culture for run 1. Cells were inoculated at 4 x 10^5 cells/100 mm tissue culture dish. The symbols are as follows: (×) CHO-tPA, (○) VHb-CHO OμM dexamethasone, (□) VHb-CHO O.1μM dexamethasone, (△) VHb-CHO O.5μM dexamethasone.

Figure 7. Total tPA production/dish in batch culture for run 2. Cells were inoculated at 4 x 10^5 cells/100 mm tissue culture dish. The symbols are as follows: (×) CHO-tPA, (○) VHb-CHO OμM dexamethasone, (□) VHb-CHO O.1μM dexamethasone, (△) VHb-CHO O.5μM dexamethasone.

Figure 8. Specific tPA production in batch culture averaged for run 1 and run 2 . Cells were inoculated at 4 x 10^5 cells/100 mm tissue culture dish. The symbols are as follows: (×) CHO-tPA, (○) VHb-CHO OμM dexamethasone, (□) VHb-CHO O.1μM dexamethasone, (△) VHb-CHO O.5μM dexamethasone. The standard deviation for each time point during the batch culture is shown by the error bars.

Figure 1

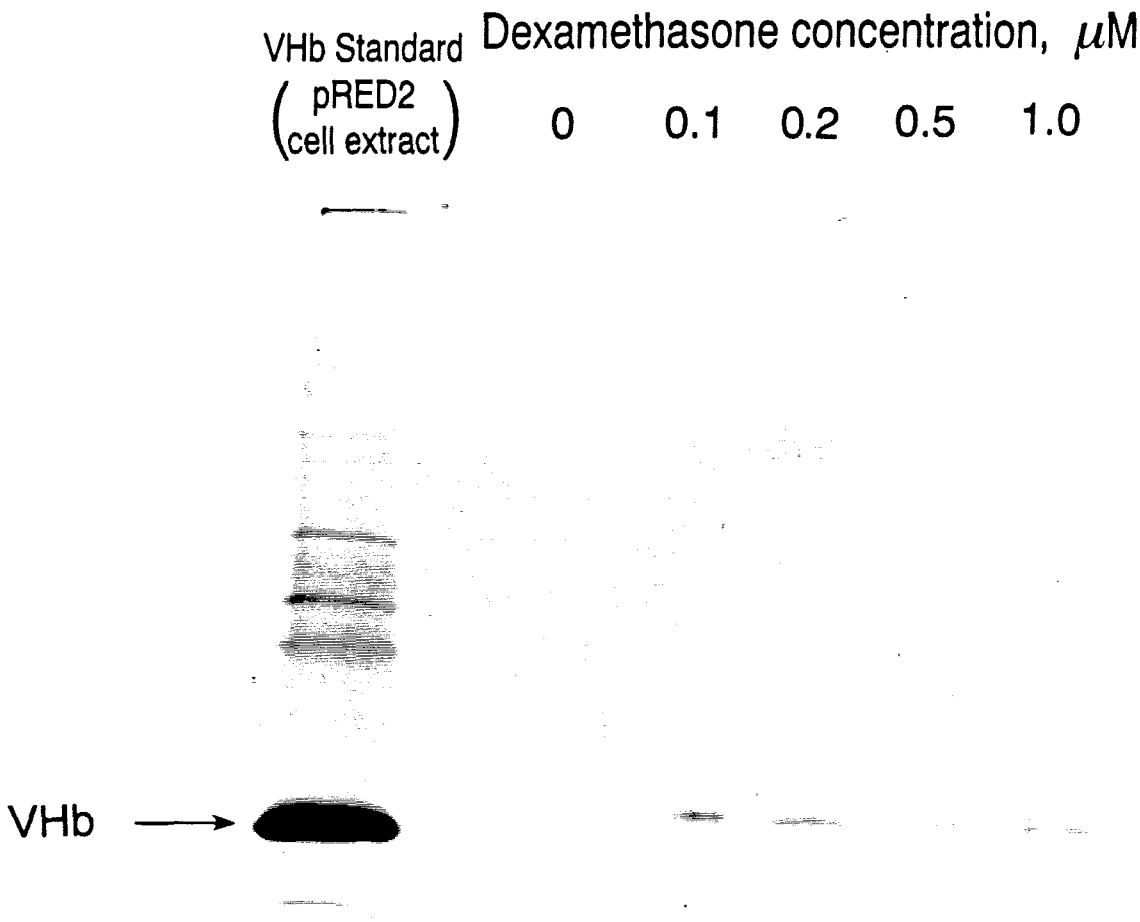

VHb Standard
(pRED2 cell extract)
Dexamethasone concentration, μM
0
0.1
0.2
0.5
1.0
VHb

Figure 2

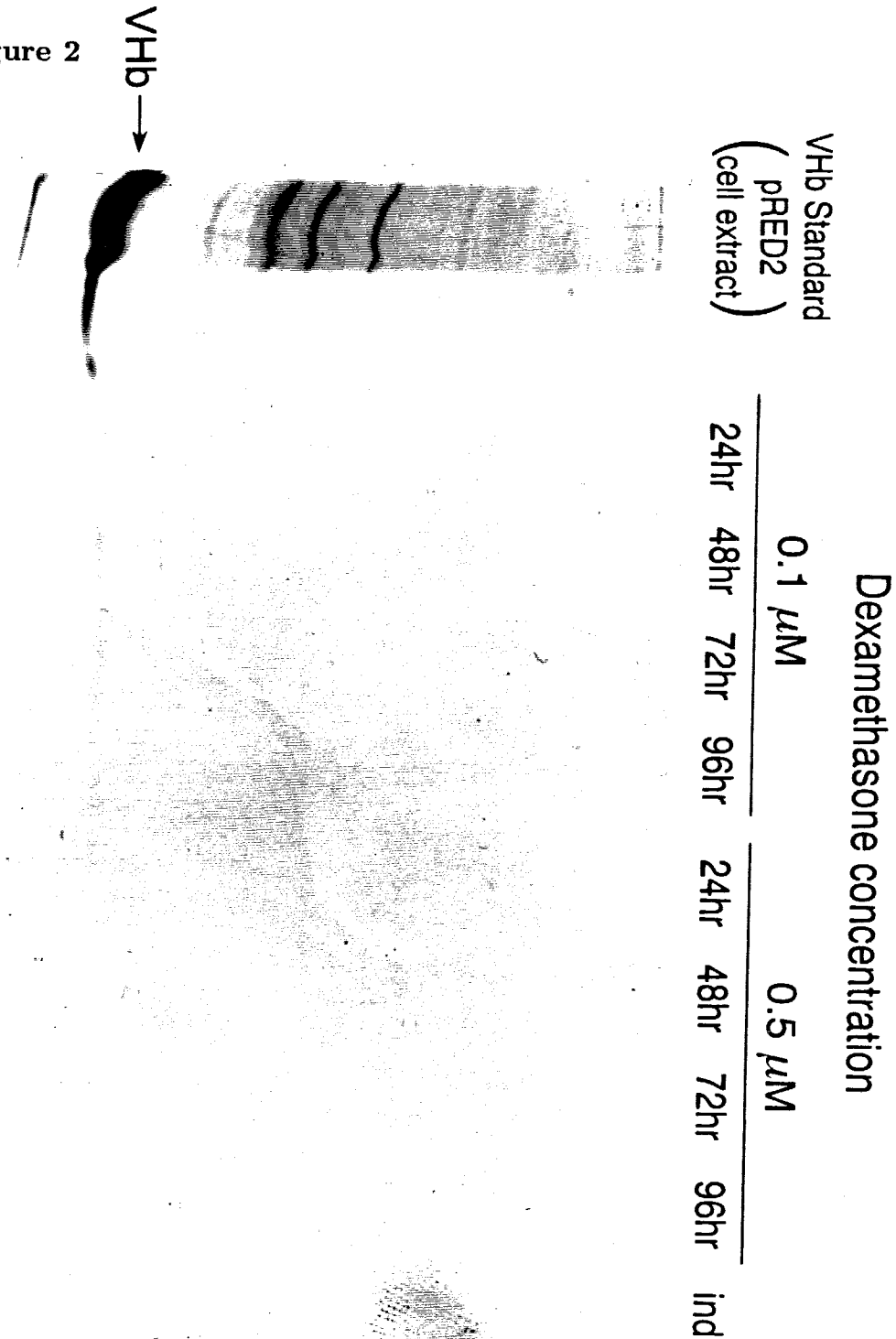

VHb
VHb Standard
(pRED2 cell extract)
Dexamethasone concentration
0.1 μM
0.5 μM
post-induction time
24hr
48hr
72hr
96hr
24hr
48hr
72hr
96hr

Figure 3

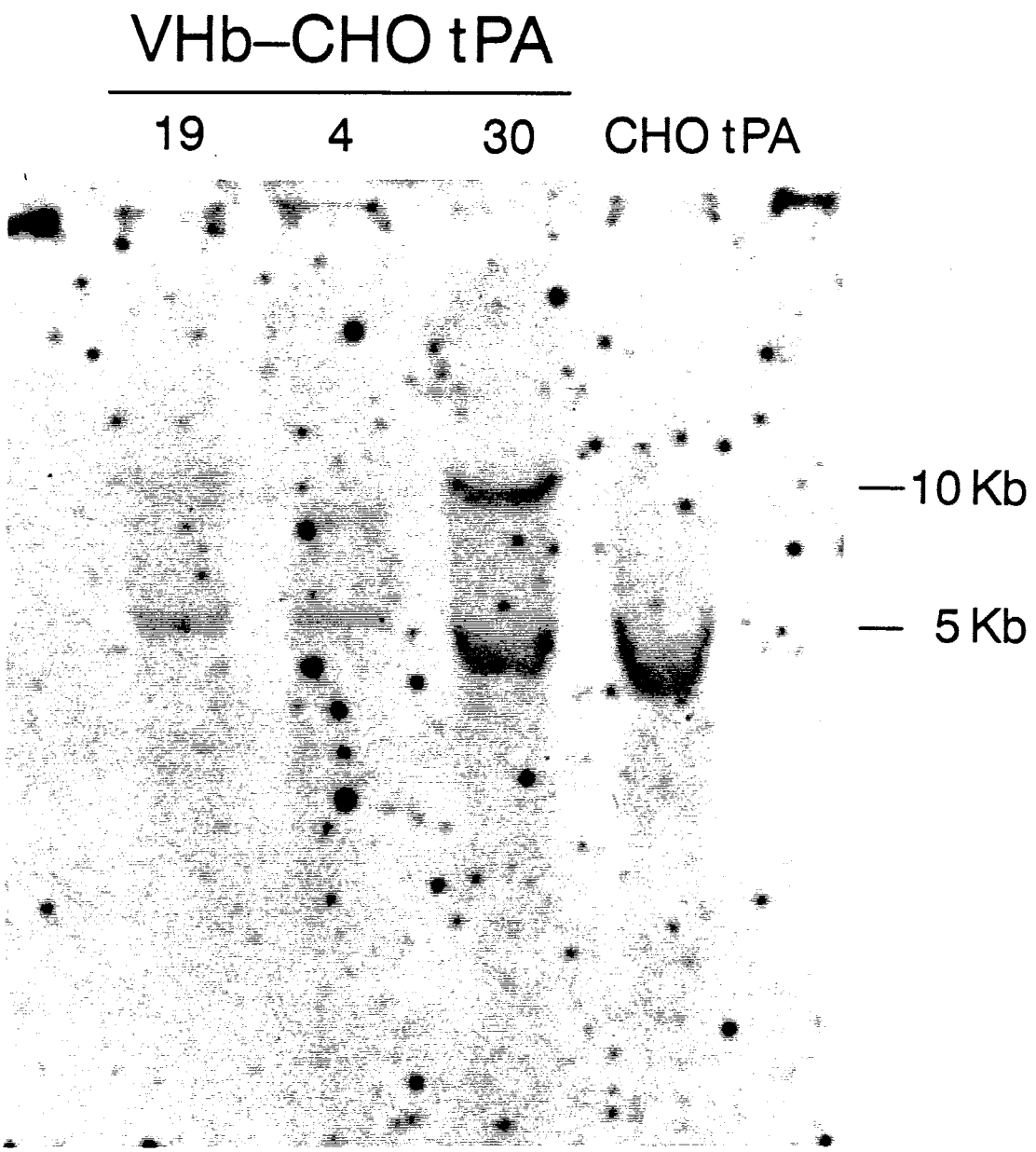


Figure 4

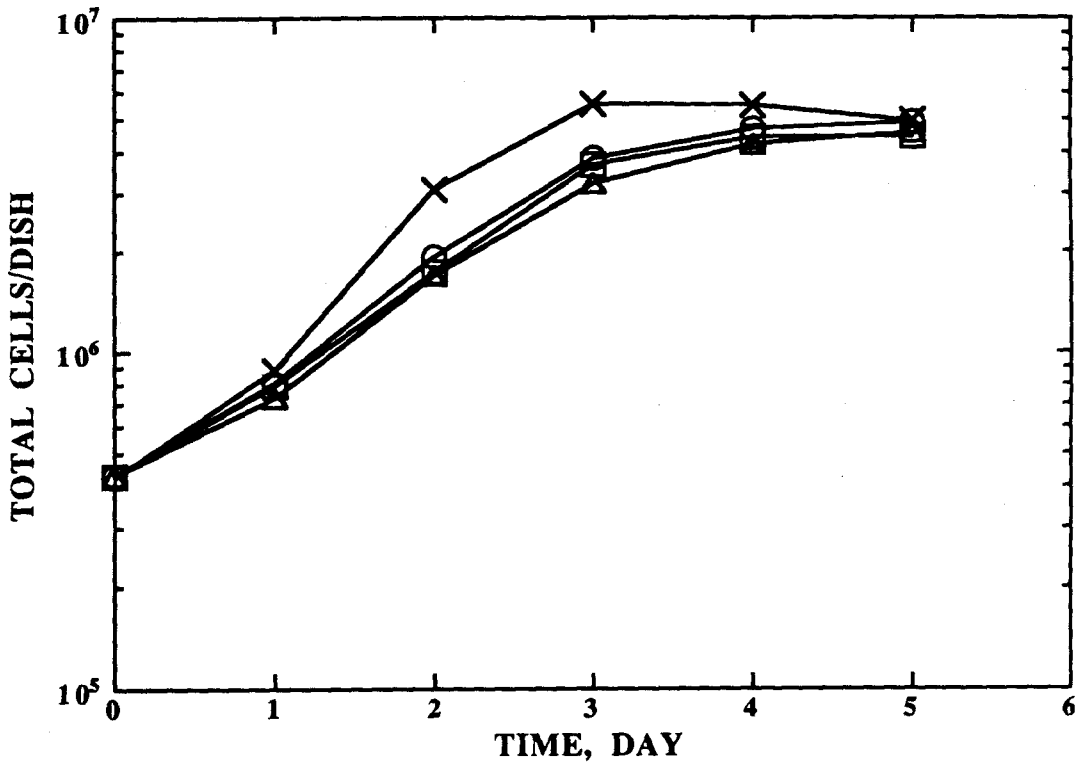


Figure 5

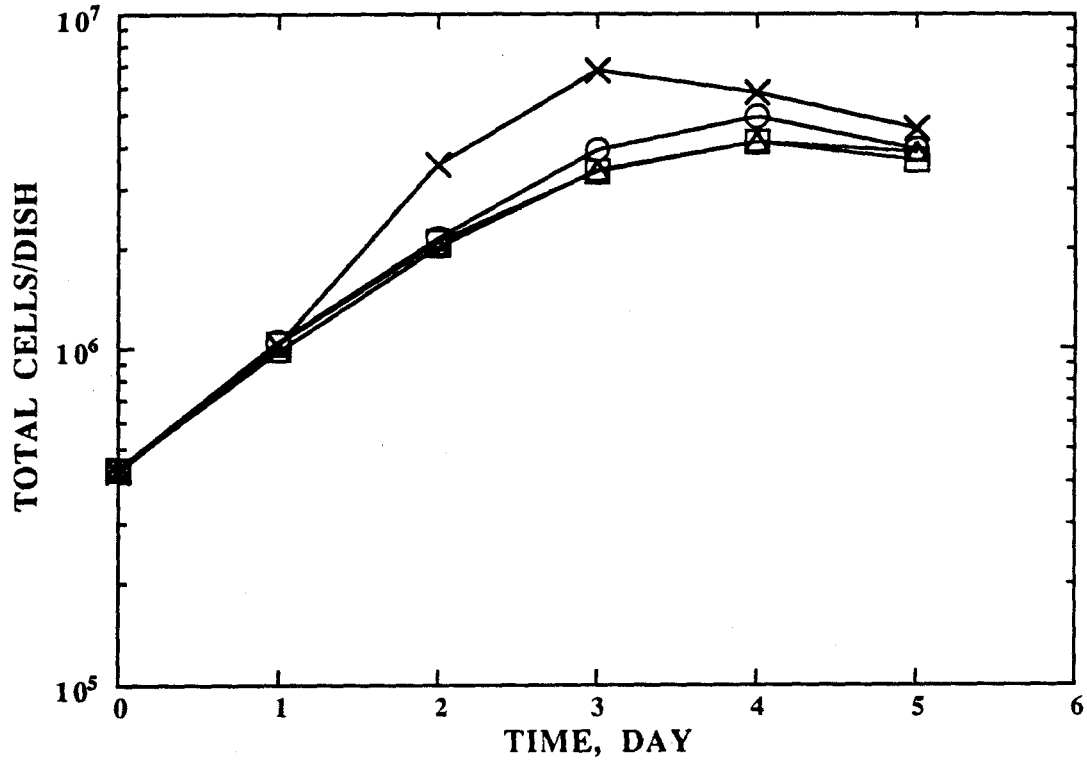


Figure 6

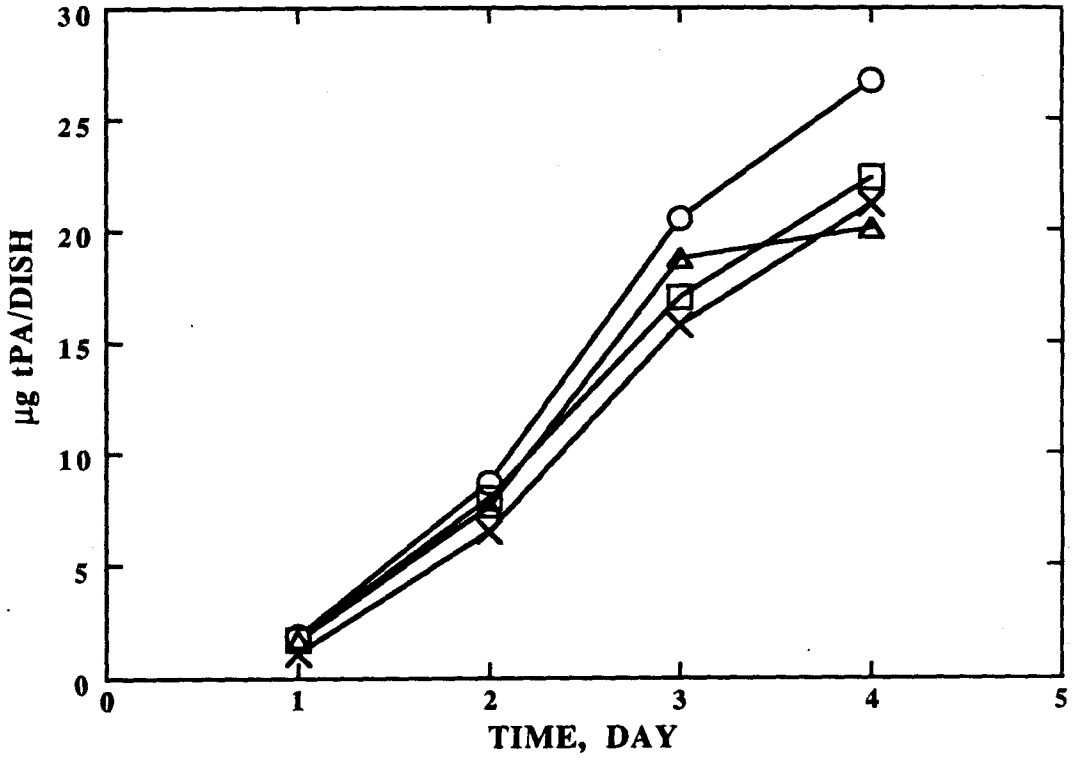


Figure 7

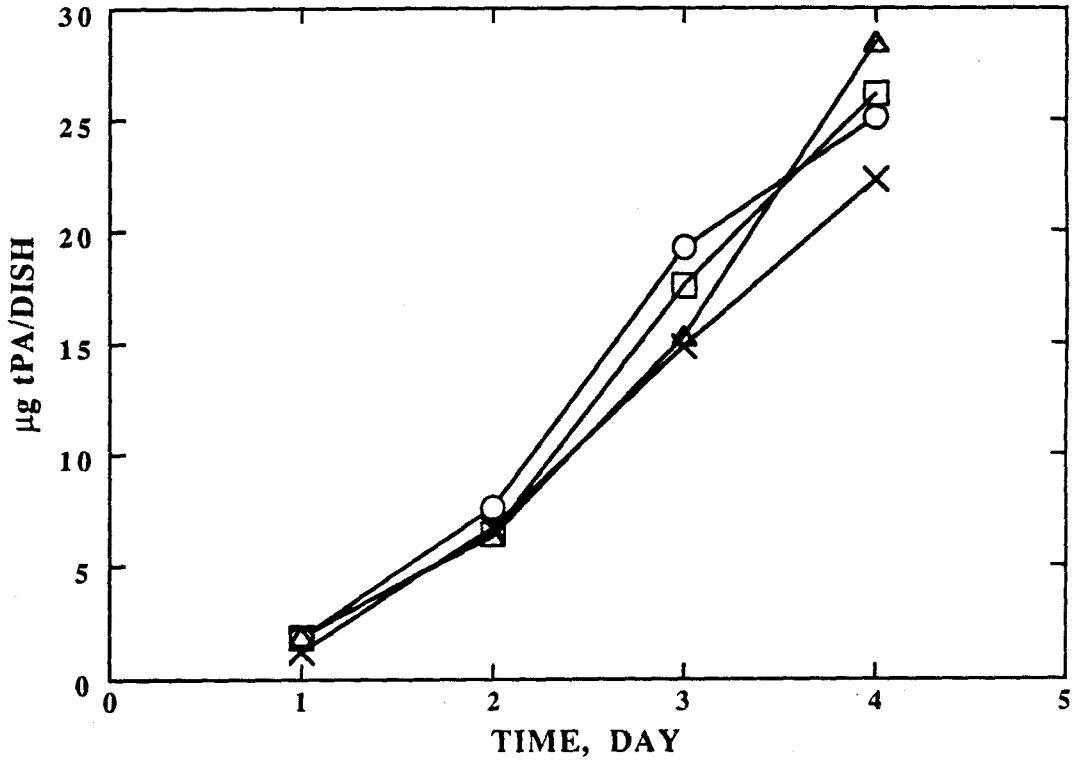
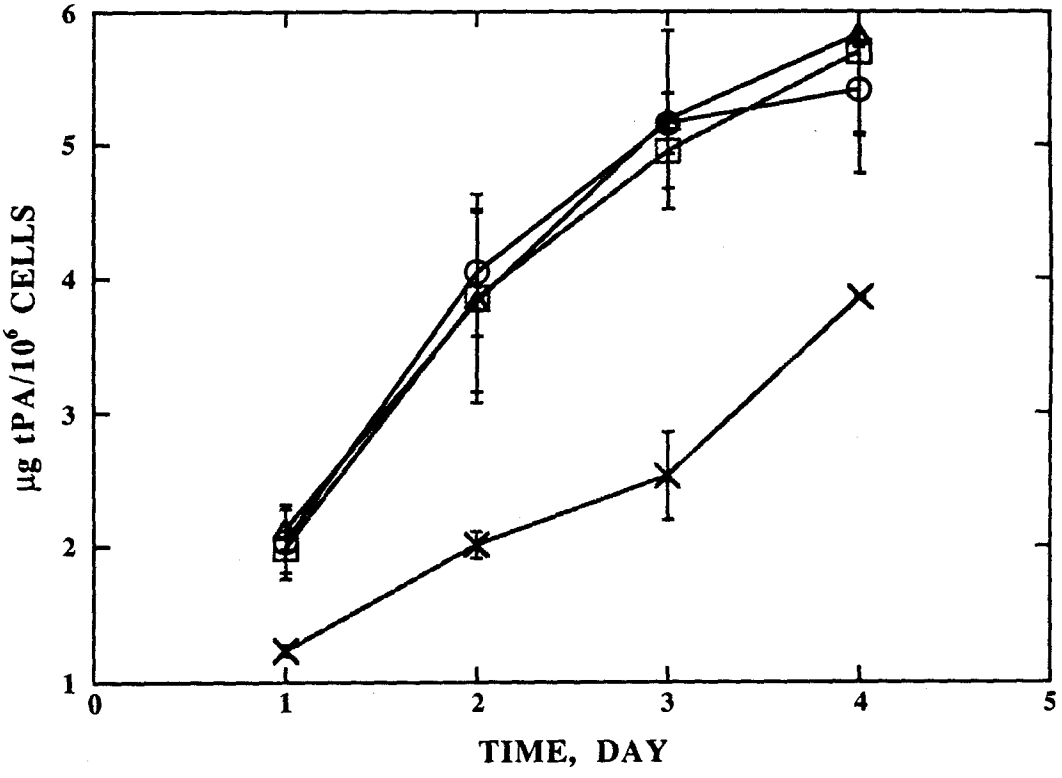


Figure 8



CHAPTER 6

Conclusions

A large number of recombinant proteins used for therapeutic, diagnostic and research purposes are being produced using mammalian cell culture. Bearing in mind, the dramatic impact host-vector interactions can have on yields of cloned protein product, we have focused our work on studying the influence of some genetic factors on the productivity of recombinant CHO cells.

The first issue we have addressed is to determine the effect of cloned gene dosage on recombinant CHO cell physiology. Gene amplification is a routinely used technique to achieve high level expression of heterologous proteins in mammalian cells by increasing the vector copy number. Keeping in mind the various perturbations in native cellular functions that can result following transfection and subsequent genetic rearrangements such as gene amplification, we have utilized a number of experimental techniques to characterize in considerable detail the rates of various intracellular processes in response to increased copy number and consequently increased expression levels. We have used a Chinese hamster ovary (CHO) cell line producing recombinant Hepatitis B surface antigen (HbsAg) for these studies.

Clones adapted to increasing MTX levels exhibit higher intracellular DHFR activity as determined using flow cytometry. These elevated intracellular DHFR levels were shown to be a consequence of *dhfr* gene amplification. This *dhfr* gene amplification resulted in coamplification of HbsAg genes in clones adapted to increasing MTX concentrations. Thus, both *dhfr* and HbsAg gene copy number increases with MTX level in the medium. The flow cytometry experiments also show that the amplified clones exhibit a great deal of heterogeneity in DHFR content as compared to parental cells.

Furthermore, the effect of increased HbsAg gene copy number has been investigated in batch culture experiments. These experiments show that specific growth rate decreases with cloned gene dosage. Secreted HbsAg titers and specific HbsAg secretion rates were found to increase with cloned gene dosage. Specific glucose and glutamine consumption rates as well as specific lactate and NH_3 production rates did not vary significantly between the clones although the 1 μM clone exhibited metabolic rates that were slightly higher than those for the other clones.

We have done a systematic analysis of effect of cloned gene dosage on some important steps involved in HbsAg synthesis and secretory pathway in order to determine if any step becomes saturated at high level of expression of the cloned gene product. Results obtained using Northern hybridization followed by autoradiography and densitometry show that the relative mRNA level increases monotonically with relative gene copy number. Pulse-chase experiments show that the efficiency of HbsAg secretion and overall HbsAg expression efficiency decreases while the intracellular HbsAg degradation increases with increasing gene copy number. Our results also indicate that neither transcription nor translation is limiting at high cloned gene dosage. Some post-translational secretory step causes a bottleneck in the overall process of HbsAg synthesis and secretion at high cloned gene dosage.

We have identified four possible steps which may be responsible for saturating the post-translational apparatus at a high relative gene copy number.

These are either the availability of lipid components for ER membrane formation, and/or the availability of glycosylation enzymes for the proper glycosylation of HbsAg, and/or the availability of sufficient amounts of intracellular ATP required for rapid transit of secretory proteins through ER, and/or increased intracellular proteolysis of excess HbsAg, especially for the highest amplified clone. It would be interesting to investigate in more detail if either of these steps do indeed limit the overall productivity of these cells.

We have demonstrated the feasibility of a novel continuous flow packed bed reactor configuration (packing: collagen microspheres) to study the intracellular metabolism of recombinant CHO cells using ^{31}P NMR spectroscopy. Comparing results obtained for all the clones studied indicate that levels of most phosphorylated metabolites including NTP are higher in cells producing HbsAg. These HbsAg-producing cells, which exhibit lower specific growth rate in the packed bed, may be diverting these higher NTP amounts towards the synthesis and secretion of the cloned gene product, in this case, HbsAg. Thus, our results seem to indicate that energy and intermediary metabolism gets altered in cells expressing a cloned gene product.

The systematic experimental approach that we have used is applicable to other systems besides HbsAg production in recombinant CHO cells. Such an approach can help provide a fundamental understanding of the influence of genetic factors on key cellular processes involved in cloned gene product synthesis . Identification of rate-limiting steps/bottlenecks can provide insights into design of improvements that could alleviate these limitations.

In this connection, we have cloned the *Vitreoscilla* hemoglobin gene (VHb) in CHO cells producing tPA and obtained inducible intracellular expression of VHb. We confirmed the presence of VHb gene in the genomic DNA of these cells by Southern blots and the inducible nature of VHb protein expression using SDS-PAGE followed by Western blots. Batch culture experiments with these cells experiments show that VHb-CHO cells have a slightly lower specific growth rate as compared to the parental CHO-tPA cells. However, specific tPA production is significantly higher in VHb-CHO cells compared to CHO-tPA cells throughout the batch. It is not conclusive whether the effect was due to VHb expression specifically or due to some other unknown host-vector interaction that manifests itself as a result of transfection and subsequent integration of the vector DNA into host cell chromosomes. The effect of increased tPA productivity is observed even for uninduced VHb-CHO cells. However, our observations from Western blot experiments have shown the MMTV promoter to be slightly leaky as a result of which some low level of VHb expression is obtained even in the uninduced cells. It is possible then, that this low amount of intracellular VHb is enough to cause the effects that we observe. However, more detailed experiments are necessary to establish conclusively the role of intracellular VHb on tPA production.

We have also investigated the effect of some growth factors on cell growth and monoclonal antibody productivity in batch hybridoma cultures. Before doing these experiments, the cells were adapted to a serum-free medium using a gradual weaning procedure. We have found that even though the specific

growth rate of cells is not much affected by addition of growth factors, a significant variation is observed in monoclonal antibody production when certain growth factors are added to the medium. These results may indicate that growth factors affect some step in the monoclonal antibody synthesis and secretion pathway without significantly affecting the cell specific growth rate.

APPENDIX A

Effect of growth factors on cell growth and monoclonal antibody production in batch hybridoma cultures

A.1 SUMMARY

Effects of growth factors such as EGF, FGF and IL-2 on cell proliferation and monoclonal antibody production in a hybridoma cell line adapted to a completely defined serum-free medium were determined in batch cultures. The results indicate that the presence of growth factors in the medium enhances the antibody secretion without significantly affecting the growth rate. The specific antibody secretion rate of cells grown in serum-free medium supplemented with growth factors was 35% higher than those grown in serum-free medium alone.

A.2 INTRODUCTION

Hybridomas are important cell lines used in the production of monoclonal antibodies which find widespread applications in areas such as therapy, diagnosis, tumor imaging and drug delivery. The particular hybridoma cell line TIB 131 was chosen as a model system for this study. Proliferation of mammalian cells is controlled by specific macromolecular regulatory factors termed 'growth factors'. Interleukin-2 (IL-2) is an important growth factor which stimulates proliferation in both human and murine T-lymphocytes, or T-cells. Normal B-cells also respond with modest proliferation to sources of IL-2.[5] Since TIB 131 is a B-cell hybridoma and since recombinant IL-2 was commercially available, we investigated IL-2 as a candidate for manipulating growth and antibody production by the hybridoma cell line. Effects of other readily available recombinant growth factors, in particular EGF and FGF, were also determined in the same study.

A.3 MATERIALS AND METHODS

Cell line

TIB 131, a mouse-mouse hybridoma was obtained from the American Type Culture Collection (ATCC; Rockville, MD). It secretes monoclonal antibodies which react with all classes of intermediate filaments. This hybridoma has been derived from a NS-1 mouse myeloma line.[3]

Cell culture

The hybridoma cell line was adapted to a completely defined KSLM serum-free medium.[1] Briefly, the basal medium (RDF) consisted of a 2:1:1 mixture (by volume) of RPMI 1640, HAM F12 and DMEM. This was supplemented with insulin, transferrin, LDL, bovine serum albumin, oleic acid, mercaptoethanol, sodium selenite and aminoethanol.

Instead of direct introduction of the cells into the KSLM serum-free medium, a weaning procedure was adopted. In this procedure the KSLM serum-free medium was supplemented with 5% Equine serum in the first passage. During the subsequent passages, the serum concentration was reduced in a stepwise manner until the cells showed the original growth characteristics in the completely defined KSLM serum-free medium alone. As controls, cells were grown in DMEM supplemented with either 10% Equine serum or 10% FCS.

The cells were maintained regularly in T-flasks at 37°C in a humid atmosphere of 10% CO_2. The cells were subcultured every three days upon reaching the stationary phase (around 2 x 10^6 cells/ml).

Batch culture experiments

In one set of experiments with EGF alone, the cells were grown in serum-free medium supplemented with different EGF concentrations such as 1, 5, 10, 20, 50 and 100 ng/ml. In the second set of experiments, the cells were grown in serum-free medium supplemented with 10 ng/ml EGF, 10 ng/ml FGF, or 20

units/ml Interleukin-2 (IL-2). The cells were adapted to a particular growth factor concentration by cultivating them continuously for over 100 cell doublings in presence of that growth factor concentration. The adapted cells were then used to study the kinetics of growth and product formation as a function of different growth factors. All experiments, including the ones with serum were carried out within the same time span and thus were under comparable conditions. Samples were taken at regular intervals to monitor growth rate and antibody production. Total cell count was determined using a Coulter counter. Viability was obtained using the trypan blue exclusion method.

ELISA for monoclonal antibody detection and quantification

The IgG concentration in the culture medium was determined by using a modification of an ELISA procedure used in this laboratory. [2] In the modified assay, standard mouse IgG solutions (1 to 250 ng/mL) and two dilutions each of the cell-free IgG containing supernatants were prepared in KSLM serum-free medium instead of medium containing serum. An additional step of incubating the 96-well plate with 2% BSA solution at 37°C for 1 hour was carried out in order to prevent non-specific binding between the secreted IgG and the surface of the well. The absorbance of each sample measured at 405 nm using an ELISA reader (SLT Instruments) was proportional to the amount of IgG in the original sample.

A.4 RESULTS AND DISCUSSION

It was anticipated that, being a transformed cell line, TIB 131 could show a reduced or a loss of requirement for specific growth factors. The growth and product secretion kinetics for cells grown in the presence of different growth factors are summarized in Figures 1 and 2. The specific growth rate of cells grown in serum-free medium alone is almost the same as that of cells grown in serum-free medium supplemented with different growth factors or medium supplemented with 10 % FCS.

Significant variation in antibody productivity is evident. The antibody productivity of cells grown in serum-free medium supplemented with different growth factors or medium supplemented with 10 % FCS was found to be around 35% higher than those grown in serum-free medium alone.

It has been observed previously that antibody titer was linearly correlated to the integral of viable murine hybridoma cell concentration, indicating that the specific secretion rate of the viable cells (Strain J4C2) is approximately independent of growth rate or stage of the culture.[4] The kinetics of antibody secretion of cells as a function of medium composition is compared in Figure 3. Analysis of the data in Figure 3 shows an excellent agreement with the earlier model which is reflected in high linear correlation coefficient values of 0.98 or more for all medium compositions studied. The specific antibody secretion rates and specific growth rates for cells grown in medium supplemented with different growth factors or 10 % FCS are shown in Table 1. These data indicate

that the presence of the growth factor in the medium increases the antibody productivity even though the growth rate seems to be relatively unaffected.

In order to investigate whether the enhancement in antibody secretion was dependent on the concentration of growth factor in the medium, we carried out dose-response experiments with EGF as described earlier in the Materials and Methods section. There was no significant difference in the growth rates among different samples as seen in Figure 4. However, once again, the antibody productivity and, consequently, the specific antibody secretion rates were significantly higher in cells grown in serum-free medium supplemented with EGF as compared to cells grown in serum-free medium alone (data not shown).

These results indicate that, under conditions considered here, growth factors play a stimulatory role in the antibody production process without having a significant effect on cell growth.

A.5 ACKNOWLEDGMENTS

This research was supported by the National Science Foundation (Grant No. EET88-05636)

A.6 REFERENCES

1. **Kawamoto, T., Sato, D.J., McClure, D.B. and Sato, G.H.,1986.** Serum-free medium for the growth of NS-1 mouse myeloma cells and the isolation of NS-1 hybridomas. *Methods in Enzymology*, **121**, 266-277.

2. **Meilhoc, E., Wittrup, K.D. and Bailey, J.E.,1990.** Influence of dissolved oxygen concentration on growth, mitochondrial function and antibody production of hybridoma cells in batch culture. *Bioproc. Eng.*, **5**, 263-274.

3. **Pruss, R.M., Mirsky, R., Raff, M.C., Thorpe, R., Dowding, A.J. and Anderton, B.H., 1981.** All classes of intermediate filaments share common antigenic determinant defined by a monoclonal antibody. *Cell*, **27**, 419-428.

4. **Renard, J.M., Spagnoli, R., Mazier, C., Salles, M.F. and Mandine, E., 1988.** Evidence that monoclonal antibody production kinetics is related to the integral of the viable cell curve in batch systems. *Biotech. Lett.*, **10**, 91-96.

5. **Swain, S.L.and Dutton, R.W.,1983** in *Interleukins, Lymphokines, and Cytokines*, Oppenheim, J.J. and Cohen, S. (eds.), Academic Press, New York.

A.7 TABLES

Table I. Specific antibody secretion rates and specific growth rates for cells grown in medium supplemented with different growth factors or 10 % FCS.

Medium composition	Specific growth rate, hr^{-1}	Specific antibody secretion rate, pg/viable cell/hr
KSLM ALONE	0.045	12.9
KSLM + EGF	0.048	15.6
KSLM + FGF	0.049	15.7
KSLM + IL-2	0.046	16.4
DMEM + 10 % FCS	0.048	15.3

A.8 FIGURES

Figure 1. Batch growth kinetics as a function of medium composition. Cells were inoculated at 1 x 10^5 cells/ml. The symbols are as follows: (○) no growth factors, (◇) DMEM + 10% FCS, (△) 10 ng/ml EGF, (□) 10 ng/ml FGF, (×) 20 units/ml IL-2.

Figure 2. Batch monoclonal antibody secretion kinetics as a function of medium composition. Supernatant samples were taken everyday and stored at -20°C for future ELISA analysis. The symbols are as follows: (○) no growth factors, (◇) DMEM + 10% FCS, (△) 10 ng/ml EGF, (□) 10 ng/ml FGF, (×) 20 units/ml IL-2.

Figure 3. Antibody concentration plotted against the integral number of viable cells. The slope of the line gives the specific antibody secretion rate. The symbols are as follows: (○) no growth factors, (◇) DMEM + 10% FCS, (△) 10 ng/ml EGF, (□) 10 ng/ml FGF, (×) 20 units/ml IL-2.

Figure 4. Batch growth kinetics as a function of EGF concentration. The symbols are as follows: (●) no EGF, (○) DMEM + 10% Equine serum, (□) 1 ng/ml EGF, (■) 5 ng/ml EGF, (◇) 10 ng/ml EGF, (◆) 20 ng/ml EGF, (△) 50 ng/ml EGF, (▲) 100 ng/ml EGF.

Figure 1

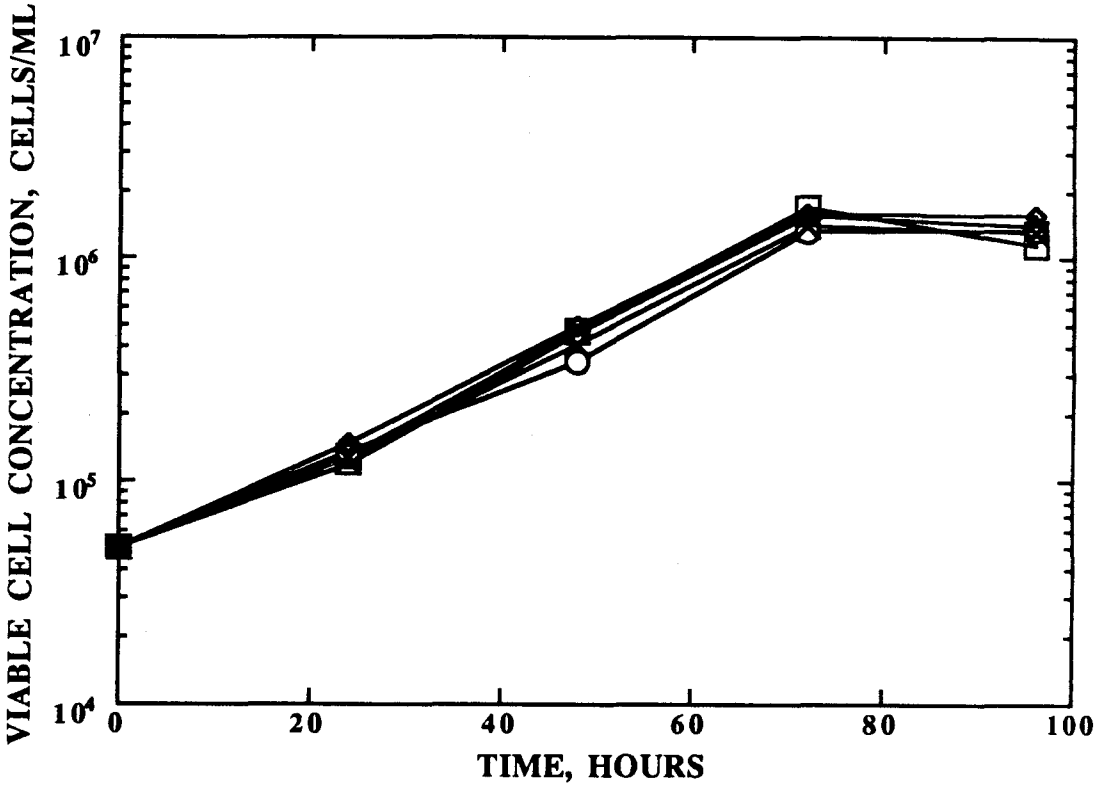

VIABLE CELL CONCENTRATION, CELLS/ML
10^7
10^6
10^5
10^4
0
20
40
60
80
100
TIME, HOURS

Figure 2

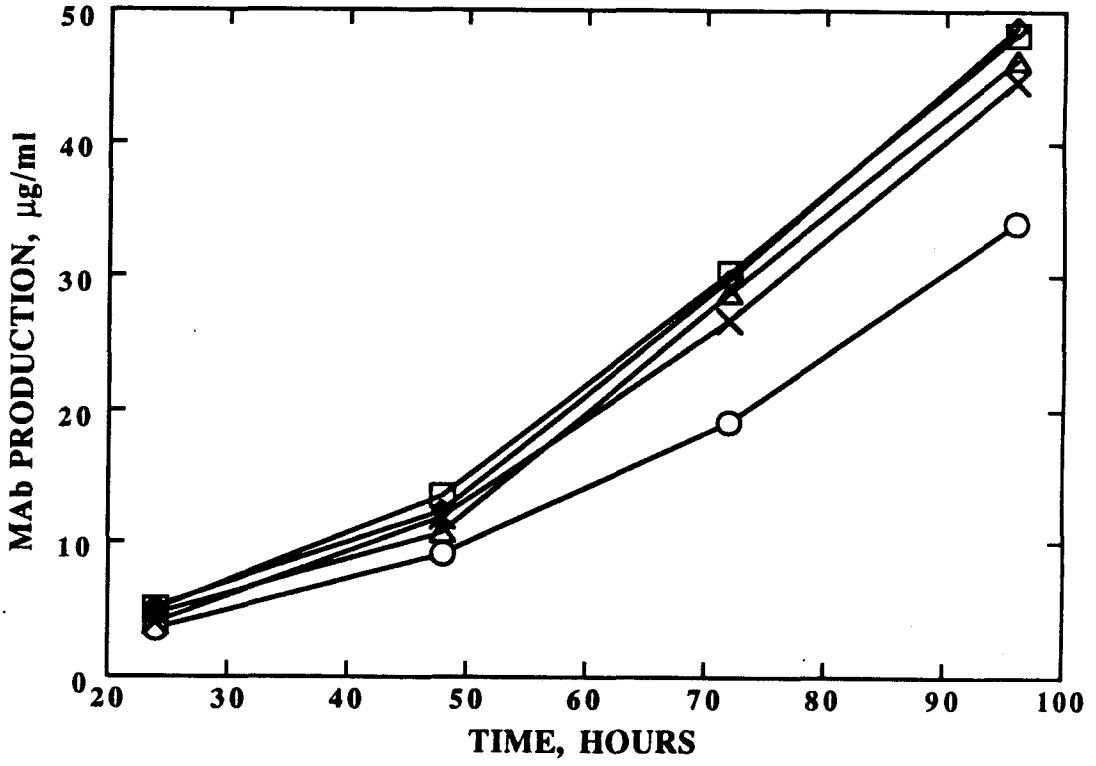

MAb PRODUCTION, µg/ml
0
10
20
30
40
50
20
30
40
50
60
70
80
90
100
TIME, HOURS

Figure 3

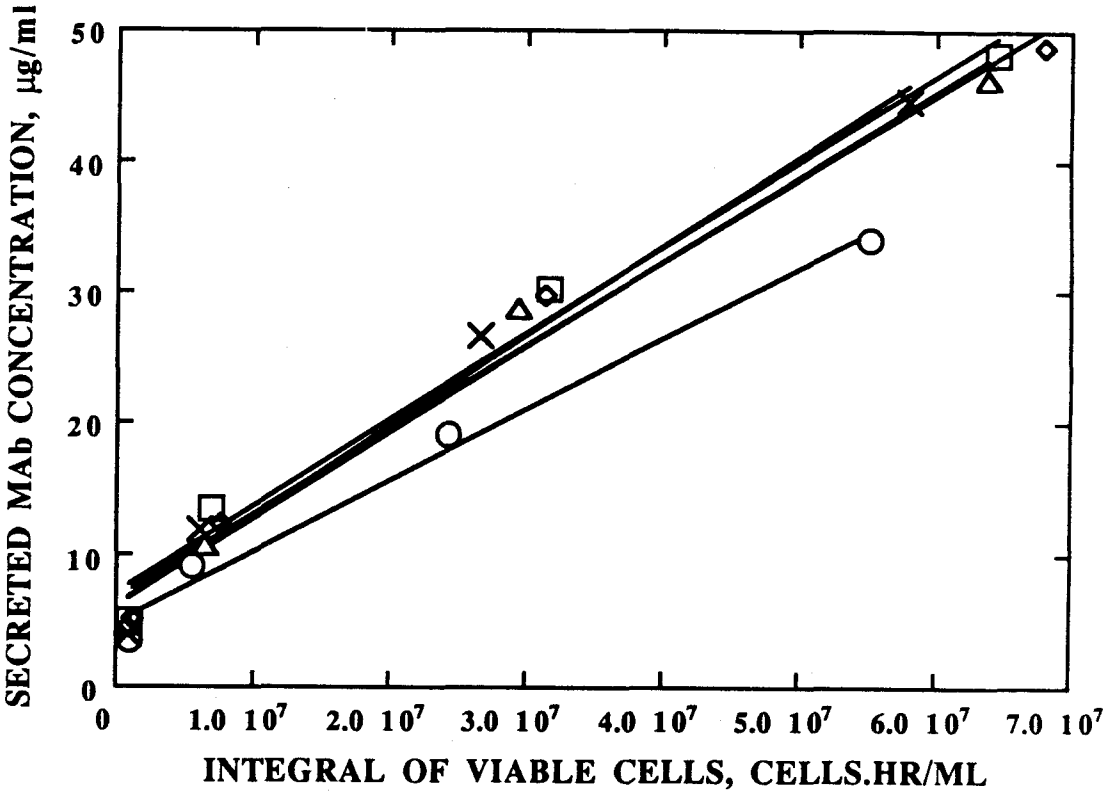
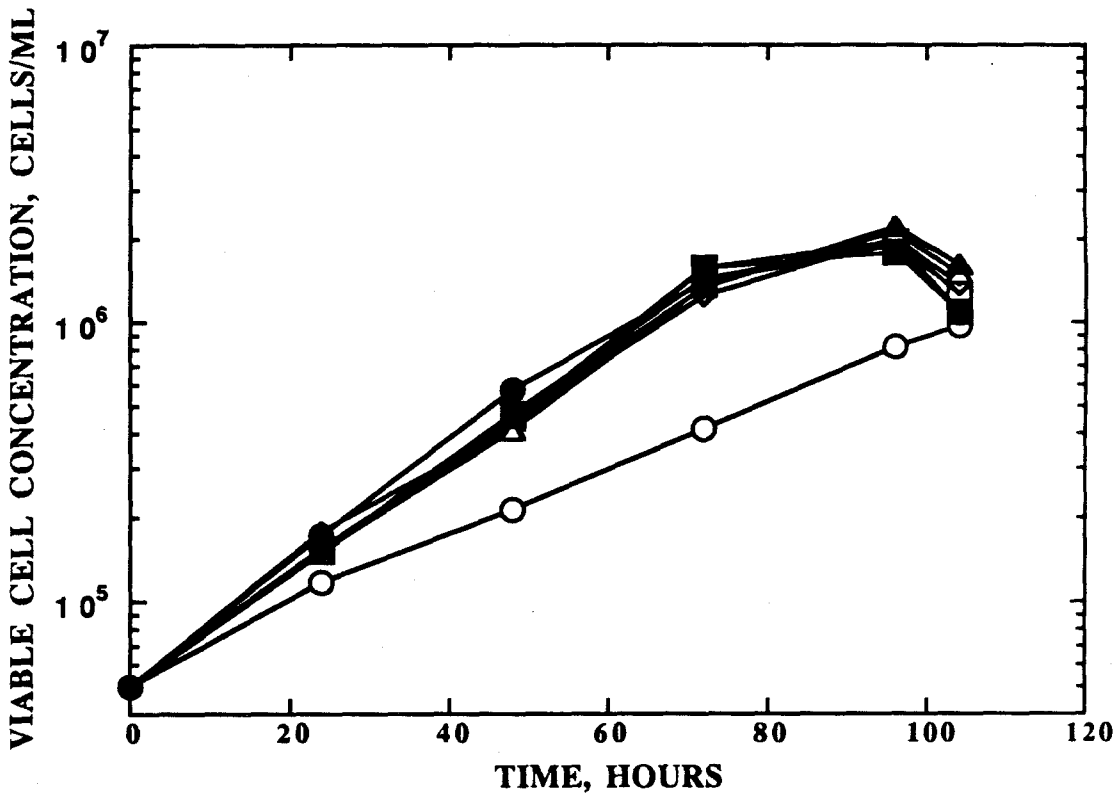

SECRETED MAb CONCENTRATION, µg/ml
50
40
30
20
10
0
0
1.0 10^7
2.0 10^7
3.0 10^7
4.0 10^7
5.0 10^7
6.0 10^7
7.0 10^7
INTEGRAL OF VIABLE CELLS, CELLS.HR/ML

Figure 4


VIABLE CELL CONCENTRATION, CELLS/ML
10^7
10^6
10^5
0
20
40
60
80
100
120
TIME, HOURS