

ABSTRACT

Title of Dissertation: **PROTEIN PRODUCTION DEVELOPMENT
WITH RECOMBINANT VACCINIA VIRUS**

Nicole Aleece Bleckwenn, Ph.D., 2004

Dissertation Directed By: **Professor, William E. Bentley, Department of
Chemical Engineering**

The vaccinia virus expression system was developed into a scaleable recombinant protein production process in perfused mammalian cell culture. Growth of anchorage dependent HeLa cells on microcarriers and the suspension adapted HeLa S3 cell line were studied in bioreactor cultures utilizing the ATF System or hollow fiber filter, respectively, for perfusion. Recombinant vaccinia virus expressing enhanced green fluorescent protein (EGFP) as a model protein was used to study the effects of several process parameters on expression. These included multiplicity of infection (MOI), volume during infection, serum concentration during infection, inducer concentration, timing of inducer addition relative to infection, and dissolved oxygen and temperature during the protein production phase. Increases in protein yield were made as each of these parameters was studied. The microcarrier based system reached 20 mg/l EGFP while the suspension based system achieved 27 mg/l under the conditions found

through experiment. A second virus containing the gene for gp120, an HIV envelope coat protein with complex post-translational modifications, was produced in microcarrier based bioreactor culture with HeLa cells. The protein produced was purified and analyzed for post-translational modifications which found that half of the molecular weight was contributed through N-linked glycans. The reactor culture produced 10.5 mg/l gp120 at 96 hours post infection with an ID₅₀ of 3.1 µg/ml. A survey of expression, using both EGFP and gp120 expressing viruses, was conducted on several mammalian cell lines which may be more appropriate for commercial manufacturing processes. Results varied, depending on the protein produced, with HeLa cells producing the most EGFP and BS-C-1 the most gp120. 293 cells performed fairly well in both cases and their use in other manufacturing processes and ability to grow in serum-free suspension culture lead to a recommendation that they be considered for further process development. These studies have provided insight into the vaccinia virus expression system as a potentially large-scale production method for complex human proteins. Further optimization of the process could continue to increase the yields and potentially bring this viral process into the arena of available technologies for production.

PROTEIN PRODUCTION DEVELOPMENT WITH
RECOMBINANT VACCINIA VIRUS

by

Nicole Aleece Bleckwenn

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Advisory Committee:

Professor William E. Bentley, Chair
Dr. Joseph Shiloach, Special Faculty Member
Professor William A. Weigand
Assistant Professor John Fisher
Professor Siba Samal

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DEDICATION

I would like to dedicate this work to my parents for their endless support and encouragement, without which, I would not be where I am today.

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I would like to acknowledge the hard work and dedication of both of my advisors, Dr. William E. Bentley and Dr. Joseph Shiloach. I would particularly like to thank Dr. Shiloach for his guidance and the opportunities he gave me to learn not only in regards to scientific research, but in all aspects of managing a lab and my career. Through his extra efforts, I have been involved with things not often open to graduate students. Additionally, I would like to thank all the members of Dr. Shiloach and Dr. Bentley's labs for their help and encouragement, particularly Nimish Dalal. Thank you to my family and friends who have provided the foundation onto which I have held to make it through this degree. Thank you all.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
µg	Microgram
6×HIS	6× histidine tag
A ₂₈₀	Absorbance measured at 280 nm
ATF	ATF™ System (Alternating Tangential Flow)
AV	Adenovirus
BCIP/NBT	5-bromo-4-chloro-3-indoyl-phosphate/nitroblue tetrazolium
bp	Base pairs
cc	Cubic centimeters
CHO	Chinese Hamster Ovary
CMV	Cytomegalovirus
CO ₂	Carbon dioxide
d	Day
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DO	Dissolved Oxygen
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EGFP	Enhanced green fluorescent protein
EMC	Encephalomyocarditis
FBS	Fetal bovine serum
g	Gravity
GFP	Green fluorescent protein
<i>gpt</i>	<i>E. coli gpt</i> gene
h	Hour
HA	Hemagglutinin
HIV	Human immunodeficiency virus
hpi	Hours post infection
ID ₅₀	50% Inhibitory dose
IPTG	Isopropyl-β-D-thiogalactopyranoside
k	kilo
kDa	Kilodalton
l	Liter
<i>lac I</i>	<i>E. coli lac</i> repressor gene
<i>lac O</i>	<i>E. coli lac</i> operator
MCS	Multiple cloning site
mg	Milligram
ml	Milliliter
mM	Millimolar
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MVA	Modified vaccinia virus Ankara
nm	Nanometer
<i>P. pastoris</i>	<i>Pichia pastoris</i>

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pfu	Plaque forming units
RNA	Ribonucleic acid
rpm	Revolutions per minute
rt	Room temperature
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
<i>SLO</i>	Stem-loop/ <i>lac O</i>
TK	Thymidine kinase
VOTE	Vaccinia virus/ <i>lac</i> operon/T7 RNA polymerase/EMC leader
WR	Western Reserve

CHAPTER 1: Introduction and Background

1.1 Recombinant Protein Production

Recombinant protein production is a method for generating large quantities of proteins which cannot be obtained from natural sources, especially proteins used as human therapeutics. Generation of these proteins is accomplished by genetically engineering a cell line or vector to introduce the gene for the protein of interest into a cellular system and have those cells use their metabolic machinery to mass produce the protein. The simplest method of production is fermentation with procaryotes such as *Escherichia coli* (*E. coli*). It is attractive due to the short fermentation time and high yield but is limited to simple, unmodified proteins because prokaryotes do not possess the machinery to perform post-translational modifications. Eukaryotic yeasts such as *Pichia pastoris* (*P. pastoris*) or *Saccharomyces cerevisiae* (*S. cerevisiae*) can be used to introduce some post-translational processing, but these often result in proteins with high mannose structures, different from those found on human proteins. Insect cells offer more post-translational processing, but proteins made in these cells are also not produced in the same manner as human proteins due to differences in N-linked glycosylation and sialylation (Fang et al. 2000; Palomares et al. 2003; Percival et al. 1997), without significant metabolic engineering of the host cells (Ailor and Betenbaugh 1999; Ailor et al. 2000; Chang et al. 2003; Hollister et al. 2002; Hollister et al. 1998; Jarvis 2003; Jarvis et al. 1998; Palomares et al. 2003).

Production of complex human proteins with the most appropriate post-translational modifications is best achieved through the use of mammalian or,

specifically, human cell lines. These processing modifications are often required for proper activity of the protein (Delorme et al. 1992; Eckhardt et al. 2002; Li et al. 1993; Lively et al. 1995; Nishikawa et al. 2000; Perrin et al. 1998). Transient DNA transfection can be used to obtain small quantities of proteins in a short period of time in mammalian cell culture, but for larger amounts and long-term production, stable transfection is required. Development of a stable cell line can be long and laborious, increasing the cost of the product. In order to overcome some of these limitations, viral vectors can be used with strong promoter elements to increase the yield of recombinant protein. The viral based insect cell-baculovirus expression system has been shown to produce high levels of recombinant protein (Hu and Bentley 1999; Taticek et al. 2001; Taticek and Shuler 1997). Although viral infection and protein expression is a transient process, it has several advantages. Some viruses can shut-down host functions and protein synthesis in order to devote more resources to viral processes (Broder and Earl 1999; Cacoullos and Bablanian 1993), including recombinant protein production. Also, multiple cell lines can be tested for production characteristics without the long time required for cell line development.

An investigation of the vaccinia virus expression system as a potentially large-scale recombinant protein production method was chosen for this work because we wished to explore a viral expression method that could produce complex human proteins. In order to increase the expression level of recombinant protein in this viral system, a vector utilizing the controllable T7 promoter was utilized and methods to increase the cell density prior to infection were evaluated.

1.2 Background

1.2.1 *Vaccinia Virus*

Vaccinia virus is an orthopoxvirus of the family *Poxviridae*. It is closely related to the virus that causes Smallpox and has traditionally been used as the live viral vaccine against Smallpox. *Vaccinia* has a wide host range that includes most mammalian species as well as humans (Broder and Earl 1999), allowing for infection of many mammalian cell lines. Its use as a gene delivery vector in animal species (Brochier et al. 1991; Hanlon et al. 1998; Kieny et al. 1984; Wiktor et al. 1984) and humans (Cooney et al. 1991; Cooney et al. 1993; Gomella et al. 2001; Graham et al. 1992; Hanke et al. 2002; McAneny et al. 1996; McClain et al. 2000; Zajac et al. 2003) has been under investigation for several years. The genetic material of *vaccinia* is comprised of a linear double stranded DNA molecule, approximately 200,000 bp (Moss 1996), with terminal hairpins and inverted repeats (Fenner et al. 1989). Researchers have found several regions within the genome that can accept interruptions of the sequence with recombinant DNA and still function effectively (Timiryasova et al. 1993). These include the thymidine kinase (TK) gene, the hemagglutinin (HA) gene, and others. Additionally, other researchers have shown that the genome can accept large genetic inserts, up to 25,000 bp of DNA without loss of function (Smith and Moss 1983).

Vaccinia has the ability to shut down host cell protein synthesis after infection and, therefore, force the devotion of resources toward viral proteins and the recombinant protein associated with the virus (Broder and Earl 1999; Cacoullos and Bablanian 1993). It also has the unique feature that it brings its own protein

production machinery into the cell for transcription of viral and recombinant proteins within the cytoplasm (Moss 1996). Because of this, the recombinant gene does not need to enter the nucleus to be transcribed, nor does the mRNA need to be transported out of the nucleus to be translated. These transport phenomena may limit the size of protein which can be produced through transfection methods or other viral systems which do not have this feature (Moss 1996). These unique features of vaccinia virus allow for post-translational modifications that follow the human processing pathway and for large recombinant gene inserts.

Due to the infectivity of vaccinia, there are concerns for workers exposed to the virus. Biosafety Level II precautions must be taken when working with unattenuated strains and workers should be vaccinated. The Western Reserve (WR) strain used in this research is mostly cell associated and therefore large amounts of free virus are not present suspended in the culture medium (Blasco and Moss 1992), but this is still considered an infective strain and precautions must be taken.

1.2.2 Vaccinia/lac Operon/T7 RNA polymerase/EMC leader (VOTE)

Vaccinia virus is available with several different promoter elements that contain natural vaccinia promoters, synthetic promoters, common mammalian promoters such as CMV, or the T7 promoter system. The VOTE vaccinia virus expression system was originally designed by Ward (Ward et al. 1995) and is based on the T7 promoter. It is composed of two elements available from their laboratory. These include the WR strain of vaccinia modified with an insertion into the TK gene, vT7lacOI, and a plasmid, pVOTE, which contains several elements and a multiple cloning site (MCS) for recombinant gene insertion. A diagram of the system,

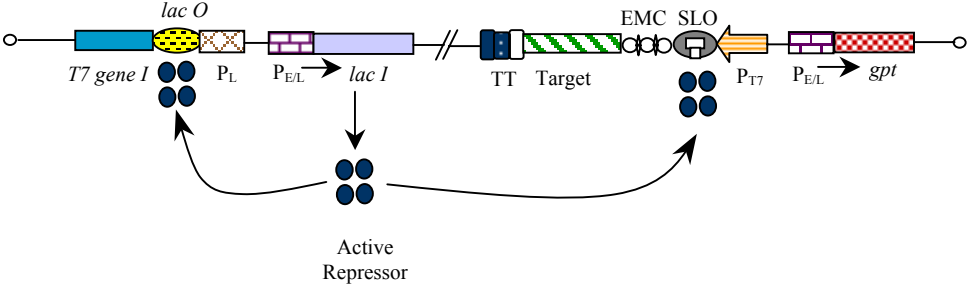
modified from Ward (Ward et al. 1995), is shown in Figure 1-1. The left portion is contained in the segment inserted into the TK region of vT7lacOI. It contains the bacteriophage T7 RNA polymerase gene (*T7 gene 1*) under the control of a late vaccinia promoter. Additionally, there is a *lac O* repressor binding site upstream of the polymerase gene that is used to reduce leaky expression by binding active repressor units and blocking transcription. The *lac* repressor is produced within this same region from the *lac I* gene under a vaccinia early/late promoter. The right side of Figure 1-1 contains the second element of this system, available in a plasmid, pVOTE.1 or pVOTE.2 with differing restriction endonuclease sites in the MCS. It is flanked by left and right sequences of the HA gene to allow homologous recombination when transfected into a vaccinia infected cell. The plasmid is used as a cloning vector where the DNA of the recombinant gene to be produced is inserted. It contains the *E. coli gpt* gene under the vaccinia early/late promoter that allows for selection of recombinant virus. It also has a segment with the T7 promoter followed by a stem-loop/*lacO* (SLO) sequence that will be bound by active repressor molecules when in the uninduced state. This is then followed by an encephalomyocarditis (EMC) leader to enhance translatability and stability, and the gene of interest is cloned immediately downstream of this segment. Additionally, a triple terminator was included in order to stop transcription of open reading frame insertions.

The recombinant gene of interest is cloned into the plasmid vector and then transfected into a cell line infected with vT7lacOI. This results in recombinant virus due to homologous recombination with the HA flanking regions on the plasmid and the HA gene in the vaccinia genome. Recombinant virus must then be isolated under

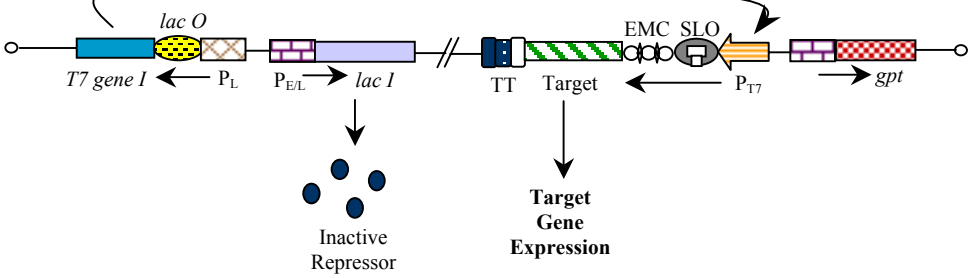
Figure 1-1 VOTE Elements

VOTE gene elements are shown in the uninduced (top) and induced (bottom) state. The left portion is contained within the TK gene of the vaccinia genome and the right portion is contained in the HA gene. The *gpt* gene is controlled by the vaccinia early/late promoter to allow selection of the recombinant gene. In the uninduced state, *lac I* is produced by an early/late promoter and produces repressor monomers that form active repressor tetramer units. These bind to the *lac O* and *SLO* regions to stop transcription of the *T7 gene 1* and the target gene, if leaky expression of T7 polymerase should occur. When induced, the repressor cannot form active units and the *lac O* and *SLO* are unbound. This allows the late vaccinia promoter (P_L) to produce T7 RNA polymerase that then binds to the T7 promoter. The target gene is then transcribed from the *SLO* to the triple terminator (TT). Translation of the target gene mRNA is enhanced by the EMC leader. Figure reproduced with permission (Ward et al. 1995) Copyright (1995) National Academy of Sciences, U.S.A.

Uninduced



Induced



selective conditions for the *gpt* gene by plaque isolation. It is then amplified and purified to make a viral stock.

1.2.3 HeLa Cells

HeLa cells, originally derived from a human cervical adenocarcinoma (Gey et al. 1952; Jones et al. 1971), are available as an anchorage dependent (ATCC CL-2) or a suspension adapted cell line, HeLa S3 (ATCC CL-2.2). These cells have been used extensively in research to produce small quantities of proteins with the vaccinia virus expression system. Due to the base of available literature with these cells, and because they have proven successful in producing various proteins (Davis et al. 1996; Hu et al. 2000; Jun et al. 1996) our initial work focused on them as the host cell line.

1.2.4 Cell Culture

Methods of cell culture for recombinant protein production are available with various reactor configurations. The choice of production system depends largely on the resources available and recombinant product characteristics, particularly the location of expression, intracellular or secreted (Bleckwenn and Shiloach 2004). In small-scale work, tissue culture flasks are the primary method of cell growth for anchorage dependent cells whereas small shake or spinner flasks are the choice for suspension cells. These methods are scaleable to a certain point, but eventually reach size limitations and must be modified for large-scale work. Large-scale anchorage dependent methods include stacked plate systems, packed bed, fluidized bed, hollow fiber, or microcarrier culture in stirred tank bioreactors with suspension culture

usually performed in traditional stirred tank bioreactors (Sambanis and Hu 1993; Zhang et al. 1993).

Microcarrier culture was chosen in this work as the method of growth for the anchorage dependent HeLa cells in a stirred tank bioreactor. Microcarriers are small, usually spherical, beads made of a variety of materials onto which the anchorage dependent cells can attach. The material of construction varies from glass, plastic, polymer, cross-linked dextran and others, with surfaces often coated with collagen, fibronectin, or positively charged to aid in cell attraction. The carriers are designed to be suspended in liquid culture medium with mixing, but most will settle within a few minutes after stopping agitation. By utilizing microcarriers, we can provide a solid support for the cells to attach and grow, but can suspend the cell covered carriers in liquid culture medium and treat the culture in much the same manner as cell suspension culture, with a few modifications.

1.2.5 Bioreactor Technology and Perfusion (Microcarrier and Suspension)

There are four main modes of operation of a bioreactor. These are batch, where all nutrients for growth are present at the beginning of the run and no medium replenishment occurs; fed-batch, where the culture is run in batch mode until nutrients become limiting and then a feed solution is gradually added to the reactor; perfusion, where a cell separation device is used to retain all the cells in the reactor while medium is constantly removed and replaced; or continuous, where culture, including cells and medium, is constantly removed and fresh medium is added to the reactor (Bleckwenn and Shiloach 2004). The mode which results in the highest cell density with cell culture is usually perfusion. This is because it allows for both

addition of nutrients and removal of harmful waste products, unlike batch and fed-batch. Additionally, it is preferred over continuous culture because the slow growth rate of mammalian cells, relative to prokaryotes, makes continuous mode difficult to implement without causing washout (complete removal of all cells from the culture). Microcarrier and cell suspension based culture in a bioreactor can initially be run in batch mode. Eventually, feeding, to supply nutrients for continued growth, and removal of waste products becomes necessary. There are several systems available to aid in the perfusion of culture in a stirred tank bioreactor. These include hollow fiber, spin filter, acoustic filter, and others. These methods generally work well for single cell suspension culture (Woodside et al. 1998), but are often not suitable for work with microcarriers because they can clog and often require pumping the culture through tubing to the device, crushing and damaging the carriers and cells. A system involving alternating tangential flow (ATF) external to the reactor was made available for our use from Refine Technologies (East Hanover, NJ). It contains a screen module to retain the cell-covered microcarriers while removing medium from the culture. It uses the alternating motion of a diaphragm to pull the culture into and push it out of the center of the unit through stainless steel tubing, avoiding pumping the culture through tubing. By alternating the direction of flow of the culture, buildup of microcarriers on the screen module is mostly prevented, reducing clogging problems with this filter. This system was investigated as a means of perfusion in the microcarrier system so the reactor culture could reach high cell density prior to infection.

1.2.6 Enhanced Green Fluorescent Protein

Enhanced green fluorescent protein (EGFP) is a modified version of green fluorescent protein (GFP). GFP was originally cloned from the jellyfish *Aequorea victoria* (Chalfie et al. 1994). It is a naturally fluorescing protein that is non-toxic and requires no co-factors to produce fluorescence (Chalfie et al. 1994; Cramer et al. 1996). It is a fairly stable molecule (Chalfie et al. 1994) and has thus proven very useful in various biological studies as a visible tag. EGFP differs slightly from the native protein in that it has been human codon usage optimized and the wavelength of excitation and emission are different (Clontech Laboratories 2000). EGFP has a maximum excitation wavelength at 488 nm and an emission maximum at 509 nm.

GFP variants have been used in a variety of organisms including *E. coli*, yeast, insect cells, insect larvae, and mammalian cell culture as model proteins to study production conditions (Albano et al. 1998; Cha et al. 1997; Kirsch et al. 2003; Meissner et al. 2001). Previous researchers have also used GFP as fusion proteins with other genes of interest to track protein production (Albano et al. 1998; Cha et al. 1999a; Cha et al. 1999b; Cha et al. 1999c; Cha et al. 2000; Cramer et al. 1996; DeLisa et al. 1999; Laukkanen et al. 1996; Li et al. 2000; Wu et al. 2000). In several of these cases, fluorescence measurements have been shown to correlate well with the expression levels of the fusion protein.

1.2.7 gp120

gp120 is an HIV-1 envelope glycoprotein that has been considered a potential vaccine candidate against HIV infection (Cooney et al. 1991; Cooney et al. 1993; Graham et al. 1992; Hanke et al. 2002). It is approximately 120 kDa with 23 N-

linked glycosylation sites that account for about half the molecular weight and are required for functionality (Li et al. 1993). It was previously produced in a T7 vaccinia virus system in a packed bed reactor (Hu et al. 2000). The production level reached was 2-3 $\mu\text{g}/10^6$ cells. The system required a co-infection of two viruses, one containing the T7 polymerase gene and the other containing gp120 under control of the T7 promoter. It was shown to produce the correctly processed protein.

1.2.8 Project Overview / Summary

The initial research objectives for this project were as follows:

- 1) Construct vaccinia virus strain with the gene for EGFP reporter protein
- 2) Study the growth of anchorage dependent HeLa cells on various microcarriers to achieve high cell density growth
- 3) Define the growth conditions for HeLa cells in bioreactor perfusion culture
- 4) Investigate the infection and expression conditions with EGFP virus
- 5) Construct vaccinia virus strain with the gene for gp120 protein
- 6) Produce gp120 protein in bioreactor culture
- 7) Analyze the post-translational processing and activity of recombinant gp120
- 8) Develop and define a bioreactor production strategy using the recombinant vaccinia virus system with anchorage dependent HeLa cells

Through the course of this research project, the following additional objectives were added:

- 9) Investigate production strategies with the EGFP virus in the suspension adapted HeLa S3 cell line
- 10) Define growth conditions for the HeLa S3 suspension cells in perfused bioreactor culture using a hollow fiber filtration device
- 11) Compare expression levels of EGFP and gp120 proteins with these vaccinia viruses in several cell lines with consideration for regulatory acceptability

The work presented here is divided into several chapters which comprise papers submitted individually for publication. Chapter 2 begins with the construction of a recombinant vaccinia virus, expressing EGFP as a reporter protein. This virus was used to investigate several parameters related to infection for their effect on protein expression in tissue flask HeLa cell culture. These were MOI, volume during infection, and serum concentration during infection. MOI can have a significant effect on expression of recombinant viral protein. A lowered volume during infection may alter the infection kinetics by increasing the chances of a virus particle encountering a cell. Serum proteins may interfere with the binding of virus particles to the cell surface. In addition to these parameters, evaluation of several commercial microcarriers was performed to find the most appropriate microcarrier for growth of anchorage dependent HeLa cells and infection with vaccinia virus.

This work was continued in Chapter 3 with an experiment to determine the best concentration of microcarriers for growth and infection of the cells. More process parameters were studied in this work with the use of the EGFP reporter virus which included length of the infection phase and inducer concentration and timing of its addition relative to infection. These two parameters have a direct effect on the expression of recombinant protein via control of the promoter. Evaluation of MOI was performed for microcarrier culture with the conditions defined for growth of HeLa cells. This was a re-evaluation of this parameter due to differences in the infection kinetics between the static tissue flask culture, studied previously, and the dynamic microcarrier based environment evaluated here. The investigation of microcarrier based spinner flask culture was then extended to evaluate dissolved oxygen (DO) and temperature during the protein production phase for their effect on protein expression. Sufficient oxygen supply may be necessary for optimum expression levels, but high levels could lead to decreased productivity through increases in protease activity or changes in the post-translational modifications of the protein, possibly affecting biological activity. A reduced temperature may play a role in slowing the cytopathic effects from viral infection, potentially leading to a higher overall level of protein production.

Chapter 4 begins with a description of the concerns for scale-up of this process. The issues were each addressed, starting with a description of the bioreactor system and perfusion device used to obtain high cell density prior to infection. A method of in-vessel trypsinization was studied to provide a scaleable alternative to harvesting cells from tissue culture flasks for seeding of the bioreactor. The results

obtained from previous chapters were used to define the production process, but DO and temperature were reevaluated in the bioreactor because these parameters were better isolated in this controlled environment. This chapter concludes with construction of a gp120 expressing virus and production of this protein in bioreactor culture. Examination of the purified protein to determine the extent of post-translational modifications and activity was also performed to verify the correct processing of a complex protein.

The work was further expanded in Chapter 5 to the suspension adapted HeLa S3 cell line. Here, several parameters were revisited with the EGFP reporter virus to determine the best levels at which to produce protein in spinner flask culture. MOI and temperature during production were studied because differences in the way single cells are infected versus microcarrier attached cells and a slightly higher growth rate than the anchorage dependent HeLa cells warranted more study in this suspension system. The results were then translated to the bioreactor scale where growth conditions were defined for perfusion culture using a hollow fiber filter. Infection of the culture for production of EGFP was performed according to the results of the spinner flask experiments and previous work with the anchorage dependent cells.

Chapter 6 then concludes the experimental results portion of this work with an evaluation of several mammalian cell lines for their expression levels of two proteins. EGFP was produced in the cytoplasm of the infected cells and gp120 was produced and secreted into the medium of infected cultures. The expression levels and post-translational modifications were compared between HeLa, BS-C-1, Vero, MRC-5, and 293 cells.

Chapter 7 summarizes the results found from these experiments and describes suggestions for future directions with this expression method.

CHAPTER 2: Exploring Vaccinia Virus as a Tool for Large Scale Recombinant Protein Expression¹

2.1 Summary

A recombinant vaccinia virus was engineered to express enhanced green fluorescent protein (EGFP) under control of the T7 promoter using the VOTE expression system (Ward et al. 1995) in HeLa cells. Infection of HeLa cells with this virus and induction with IPTG demonstrated the utility of this construct for easily measuring protein expression. This construct was used to evaluate several production parameters, specifically, multiplicity of infection (MOI), volume during infection and serum concentration during the infection phase. In static culture, increasing multiplicity of infection was found to increase expression of EGFP up to a plateau around MOI of 1.0. Expression was also shown to increase with decreasing volume during the infection phase. Serum concentration during the infection phase was only marginally significant from 0 to 7.5%. Cytodex[®] 3 microcarriers were found to have the best characteristics for HeLa cell growth. These cells were grown and infected in microcarrier spinner flask culture and the maximum expression was 2.2 $\mu\text{g EGFP}/10^6$ cells at the time of infection, demonstrating the ability of this system to successfully express recombinant proteins at larger scale.

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2.2 Introduction

Production of proteins in recombinant systems is the preferred method for generating human therapeutics and several methods are available. The use of bacteria such as *Escherichia coli* (*E. coli*) is currently the simplest method because of the short fermentation time and high yield. But prokaryotic organisms are often unable to perform post-translational modifications that may be required for complex human protein activity. To produce these proteins, eukaryotic cells such as yeast, insect, or mammalian cells may be utilized. Although systems such as insect cells can produce large quantities of protein, they rarely make identical post-translational modifications to the proteins as are found in human cells (Fang et al. 2000; Percival et al. 1997) without significant metabolic engineering effort (Ailor and Betenbaugh 1999; Ailor et al. 1999; Ailor et al. 2000). A human cell line is likely to achieve authentic processing of human proteins with minimal reengineering.

An alternative mammalian production strategy for use as a bioreactor-scale production method is investigated in this work. This method employs vaccinia virus to introduce the recombinant gene. Vaccinia virus is an orthopoxvirus, of the family *Poxviridae*, which is related to the virus that causes Smallpox. Traditionally, vaccinia has been used as the live viral vaccine agent against Smallpox infection until worldwide eradication of the disease in the 1970's. Vaccinia virus has several interesting characteristics. First, the virus replicates in the cytoplasm of the infected cell and carries all of the necessary machinery for transcription into the cytoplasm of the infected cell (Moss 1996). Therefore, the viral genes (and any recombinant genes the virus carries) are not required to enter the nucleus of the infected cell to be

transcribed. Likewise, there is no need for transport of the mRNA transcripts out of the nucleus for translation. These nuclear transport phenomena are thought to limit the size of a recombinant gene that can be produced by more traditional mammalian cell expression methods such as transfection (Moss 1996). The second feature of interest for vaccinia is the wide host range. This virus can infect most mammalian species including humans (Broder and Earl 1999). Because of this, human cell culture can be used to produce recombinant human proteins that may be more authentically processed because the post-translational processing pathways of the host cell line are used. The infectious nature of vaccinia virus should be taken into consideration, as vaccination of researchers and other employees who may be exposed is necessary. In order to reduce this risk, less infective strains could be used such as the modified vaccinia virus Ankara (MVA), which was found to be less infective and pathogenic to humans due to its long passage history in chick embryo fibroblast cells (Carroll and Moss 1997; Sutter and Moss 1992). These less infective strains could be engineered to produce recombinant proteins in much the same way as the more infective strains, but because most of the available complex expression systems were created with the more common infectious strains, the work described here was carried out using the Western Reserve (WR) strain of vaccinia for which Biosafety Level 2 procedures should be observed.

The production of recombinant protein using this vaccinia system was evaluated with a reporter virus strain carrying the gene for enhanced green fluorescent protein, EGFP. This protein is a modified version of green fluorescent protein originally isolated from the jellyfish, *Aequorea Victoria* (Chalfie et al. 1994). EGFP is

easily visualized within live cells using a fluorescent microscope and can also be quantified using a fluorescent spectrophotometer. Many other researchers have used green fluorescent proteins as reporters to study production in various systems such as *E. coli* (Albano et al. 1998; Cha et al. 2000; DeLisa et al. 1999; Wu et al. 2000), yeast (Li et al. 2000), insect cells (Cha et al. 1999c; Laukkanen et al. 1996), insect larvae (Cha et al. 1999b; Cha et al. 1997), or mammalian cells (Crameri et al. 1996). This makes EGFP an ideal candidate for use as a reporter protein to study various production parameters in this system.

A key element of this production system is the use of the VOTE (vaccinia virus/*lac* operon/T7 RNA polymerase/EMC) vaccinia expression system (Ward et al. 1995). This system allows the use of a single virus that contains the elements necessary for T7 promoter controlled expression of the recombinant gene. Additionally, use of the *lacO* and a stem loop *lacO* (SLO) sequence provide tight control of expression through the addition of the inducing agent isopropyl- β -d-thiogalactopyranoside (IPTG) (Ward et al. 1995). The encephalomyocarditis (EMC) virus RNA leader has also been included to enhance stability and translatability of the recombinant transcripts (Ward et al. 1995). This system has an advantage over previously studied vaccinia production systems in that it requires the use of only a single virus to achieve T7 promoter controlled expression. Previous systems required dual infection wherein one virus contained the T7 RNA polymerase gene and another contained the recombinant gene under control of the T7 promoter (Barrett et al. 1989; Hu et al. 2000). Additionally, the T7 promoter can potentially produce higher levels

of protein than the available native or synthetic promoters for vaccinia (Fuerst et al. 1987; Fuerst et al. 1986).

To achieve high productivity, high cell density prior to virus infection is needed. HeLa cells have been chosen because they are a human continuous cell line that can reach relatively high cell densities. Although a suspension adapted cell line is available, the attachment dependent strain was chosen, due to the nature of the vaccinia strain used, which requires cell-to-cell contact for spread of the virus infection (Blasco and Moss 1992). The attachment dependent nature of this line may enhance infection spread by offering more cell-to-cell contact. Additionally, higher cell densities may be achieved in bioreactor culture with attachment dependent cells adhering to a solid surface, such as microcarriers, than with the suspension adapted cells.

Several methods are available for growth of attachment dependent cells at a large-scale, all of which involve attachment to a solid substrate. Reactor types such as cell factories, hollow fiber, packed bed, or microcarrier suspension culture can be used (Sambanis and Hu). For this work, microcarrier culture has been chosen because one can use a traditional stirred tank vessel without investment in a new type of reactor, and cells can also be easily sampled from the culture for cell density measurements, which can be difficult with other reactor types.

In this work, we describe the implementation of the above components to study several production parameters, specifically multiplicity of infection (MOI, pfu/cell), volume during infection phase and serum concentration during the infection phase, for the expression of recombinant proteins.

2.3 Materials and Methods

2.3.1 Cell Type and Maintenance

HeLa cells (ATCC CCL-2) and BS-C-1 cells (ATCC CCL-26) were grown in tissue culture flasks and passaged every 3-4 days in Dulbecco's modified Eagle's medium with 4.5 g/l glucose (DMEM, Biofluids, Rockville, MD) and 10% fetal bovine serum (FBS, Biofluids).

2.3.2 Cell Growth on Microcarriers

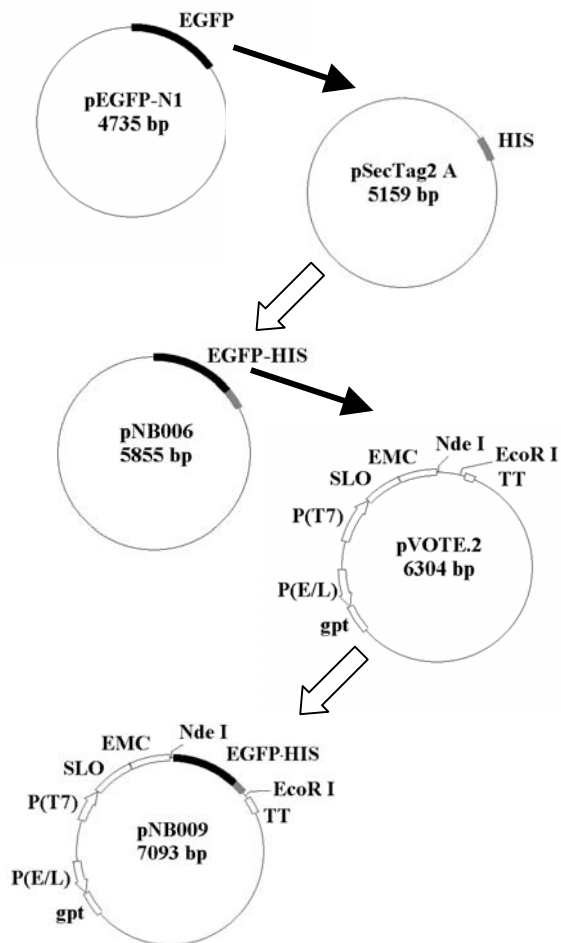
Cell growth on microcarriers in 150 ml spinner flasks (Corning, Acton, MA) was achieved by seeding HeLa cells with microcarriers prepared according to manufacturers' directions. During seeding, a reduced media volume was used and an intermittent stirring protocol was followed, where the agitation was run at 50 rpm for 30 seconds every 20 minutes, for four hours. At the end of the seeding stage, media was added to the final volume and the agitation was set to run at 50 rpm, continuously.

2.3.3 Virus Construction and Stock Preparation

A reporter virus expressing the EGFP gene under control of the T7 promoter was constructed as follows (Figure 2-1). The plasmid pEGFP-N1 was obtained from Clontech (Palo Alto, CA). PCR primers were designed to allow amplification of the EGFP gene with *Acc65* I and *Not* I restriction sites (5'- GAA TTC TGC AGT CGA CGG TAC C-3' and 5'-GTC GCG GCC GCG TAC TTG TAC AGC TCG TCC-3'). This PCR product was then inserted into a pCR[®] 4-TOPO[®] vector (Invitrogen, Carlsbad, CA). A restriction digest using *Acc65* I and *Not* I (NEB, Beverly, MA) was

Figure 2-1 Plasmid Construction

EGFP=enhanced green fluorescent protein gene, HIS=histidine tag gene, gpt=gpt selection gene, P(E/L)= vaccinia 7.5 early/late promoter, P(T7)= T7 promoter, SLO=stem loop *lac O*, EMC= encephalomyocarditis virus RNA leader, TT=triple terminator



performed on this plasmid to isolate the EGFP fragment, which was then inserted into the *Acc65* I and *Not* I digested pSecTag2 A plasmid (Invitrogen). Sequencing of the EGFP insert and 6×histidine tag (6×HIS) region confirmed the correct frame and gene sequence. The resulting plasmid was denoted pNB006.

PCR primers were designed to allow amplification of the EGFP plus 6×HIS from pNB006 with *Nde* I and *EcoR* I restriction sites (5'-CAT ATG GTG AGC AAG GGC GAG-3' and 5'-GGG TTT GAA TTC AAT GAT GAT GAT G-3'). The digested PCR product was then inserted into the pVOTE.2 vector (kindly supplied by B. Moss, NIAID, NIH) at the *Nde* I and *EcoR* I digestion sites. The resulting vector plasmid, denoted pNB009, was checked by sequencing the region containing EGFP and 6×HIS.

A recombinant vaccinia virus was then generated in a monolayer of HeLa cells in a T-25cm² flask, by infecting cells with vT7lacOI (kindly provided by B. Moss, NIAID, NIH) at an MOI of 0.05 and then transfected with 5 µg pNB009 plasmid DNA using Lipofectamine Plus (Invitrogen). Recombinant vaccinia virus was isolated via *gpt* selective pressure and plaque purification was performed using infected BS-C-1 cells with agarose gel overlays (Moss and Earl 2000). The *gpt* gene is contained on the pVOTE.2 plasmid near the recombinant gene for EGFP, and therefore, selection for the *gpt* gene also selects for the recombinant gene. PCR analysis verified the presence of the recombinant virus and absence of vT7lacOI without the insert using the following primers 5'-CGG TGT CTG TAT GAT CTT CTA-3' with 5'-TGA GTG CTT GGT ATA AGG AGC CC-3' (designed by Terri

Shors, NIAID, NIH) and 5'-GCT TTG TTA GCA GCC GGA TC-3' with 5'-GGA AAG AGT CAA ATG GCT CTC C-3'.

Amplified recombinant virus was purified by ultra centrifugation (Beckman Coulter, Palo Alto, CA) at 32,000×g for 80 minutes on a 36% sucrose cushion. The viral pellet was suspended in 10 mM Tris pH 9.0 and aliquots were frozen at -70°C. The recombinant virus is denoted vNB009.

Titer determination was performed by serially diluting trypsinized stock virus and infecting wells of BS-C-1 monolayers in 6-well plates, in duplicate (Moss and Earl 2000). Dilutions from 10⁻⁶ to 10⁻¹¹ were plated and the entire titer procedure was performed three times. After three days of incubation at 37°C, 5% CO₂, the cells were stained with a 37% formaldehyde, 5% ethanol solution containing crystal violet. Plaques were counted and the titer was determined by the average of the three sets.

2.3.4 Analytical Methods

2.3.4.1 Cell Counts and Viability Measurements

Viability assays were performed by 0.4% trypan blue (Sigma, St. Louis, MO) exclusion staining. Cell counts were performed by counting cell suspension samples with a hemacytometer (Baxter Scientific, McGaw Park, IL). Cells from microcarrier samples were prepared for these measurements by centrifuging a sample at 300×g for 1 minute. Aspiration of media and washing of the cell and microcarrier pellet with D-PBS with calcium and magnesium (Invitrogen) followed. The pellets were then resuspended in 1× trypsin-EDTA solution (Gibco, Cat. No. 25200072) and incubated at 37°C for 30 minutes with vortexing every 10 minutes. An equal volume of

DMEM+10% FBS was then added and the resulting cell suspension was used for counting and trypan blue exclusion staining.

2.3.4.2 Glucose and Lactate Measurements

Glucose and lactate were measured from media samples with a YSI 2700 Biochemistry Analyzer (Yellow Springs Instrument Company, Yellow Springs, OH).

2.3.4.3 Fluorescence Measurements

Fluorescence measurements for the MOI evaluation were performed in 96-well black walled plates with cover glass bottoms (Nunc 164588, Naperville IL). A SpectraMax Gemini fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA) was utilized in well-scan mode with 9 spatial readings per well and 6 overall readings per well which were averaged for each well and reported in relative fluorescence units (RFU). The wavelength settings were an excitation of 485 nm and emission of 512 nm with an auto cutoff filter of 495 nm.

Fluorescence measurements for all other experiments were made on samples and compared to standards of rEGFP (Clontech) in either lysis buffer (100 mM Tris pH 7.4 with 1× Complete™ protease inhibitor (Boehringer Mannheim GmbH, Germany) and 0.5% Triton X-100) or DMEM+10% FBS without phenol red. The readings were converted to EGFP expression units of $\mu\text{g}/10^6$ cells at infection using the standard curves and cell count measurements. A SpectraMax Gemini fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA) was utilized with wavelength settings at an excitation of 485 nm and emission of 512 nm with an auto cutoff filter of 495 nm. Ninety-six-well black plates (Fisher Scientific, Pittsburgh, PA) were used to measure both media and pellet samples.

2.3.4.4 Virus Expression Verification

Approximately one million HeLa cells in wells of a 6-well plate were infected at a MOI of 1.0 with vNB009 in 0.5 ml of DMEM without phenol red with 2.5% FBS and 1.0 mM IPTG for one hour with rocking of the plate at 15 minute intervals in a 37°C, 5% CO₂, humidified incubator. At the end of the infection period, the media volume was increased to 2.5 ml (DMEM with 10% FBS and 1 mM IPTG) and incubated for 43 hours. Additionally, one well was left uninfected and another was infected with the recombinant virus, but did not contain the inducing agent IPTG. Visualization of the cells was performed with a fluorescence microscope (DM IRB, Leica, Wetzlar, Germany) equipped with a FITC filter (Chroma, Brattleboro, VT). The images were recorded using digital photography (Kodak MDS 290 system, Fisher Scientific) at 200× magnification.

2.3.4.5 96-well Multiplicity of Infection Evaluation

Confluent HeLa cells were harvested from T-162cm² flasks and counted. The cells were divided into 0.5 ml aliquots with 4.0x10⁶ cells/tube and incubated in serum-free DMEM at 37°C with the appropriate amount of vNB009 virus for infections at MOI 0.0, 0.01, 0.025, 0.05, 0.075, 0.10, 0.25, 0.50, 0.75, 1.0, 2.0, and 5.0. The incubation was performed in the presence of 1.0 mM IPTG for one hour with gentle agitation every 15 minutes. At the end of the incubation, 3.5 ml DMEM+10% FBS with 1.0 mM IPTG was added and 1x10⁵ cells were seeded into eight wells per MOI in four 96-well black walled plates with cover glass bottoms (Nunc 164588, Naperville IL), which were incubated at 37°C, 5% CO₂ in a humidified incubator. At 24, 40, 44, and 48 hours post infection, a plate was removed

and fluorescence measurements made directly on the intact cells and media in the plate.

2.3.4.6 6-well Parameter Evaluations

HeLa cells were harvested from confluent T-162cm² flasks and seeded into 6-well tissue culture plates at a density of 1.25×10^5 cells per well in 2 ml of DMEM+10% FBS without phenol red. They were grown at 37°C, 5% CO₂ in a humidified incubator for two days. Cells were infected with vNB009 recombinant virus (MOI of 0.5, 1.0, 1.5, 2.0, 3.0, or 4.0) prepared in DMEM with appropriate amounts of FBS (0, 2.5, 5.0, or 7.5%). Each well was infected with an appropriate volume (0.5 or 1.5 ml) for one hour with rocking of the plates at 15 minute intervals to prevent drying of the cells. Entire wells were sacrificed for each sample where the cells were scraped and collected into a tube and pelleted by centrifugation (2,500×g, 5 minutes). Supernatant was removed and the pellet was frozen at -70°C.

The pellets were later analyzed for fluorescence by thawing to room temperature and resuspending in 250 µl lysis buffer. The samples were incubated at 37°C for 15 minutes with rigorous vortexing twice and then centrifuged (15,000×g, 5 minutes) to pellet debris. One hundred microliter samples of the supernatant were transferred to 96-well black plates for fluorescence measurements.

2.3.4.7 Spinner Flask Infections

Cells were grown to confluency in T-162cm² tissue culture flasks and counted after harvesting with cell dissociation solution. Cytodex[®] 3 microcarriers were prepared according to manufacturer's instructions and 0.2 g were placed in 125 ml spinner flasks in a reduced volume of 50 ml of DMEM+10% FBS without phenol red.

HeLa cells were then seeded into the flasks by adding 10 ml of 1×10^6 cells/ml. The flasks were placed in an incubator at 37°C , 5% CO_2 , in humidified conditions with stirring at 50 rpm for 30 seconds every 20 minutes for four hours. The volume was then adjusted to 100 ml with DMEM+10% FBS without phenol red, leaving 1×10^5 cell/ml and 2 g/l Cytodex[®] 3 in each flask. Flasks were then incubated at 37°C , 5% CO_2 , in humidified conditions with stirring at 50 rpm. Samples were taken daily for cell count, viability, glucose and lactate. Feeding was performed, as deemed necessary by glucose and lactate measurements, by removing the flasks from the agitation source and allowing the microcarriers with cells to settle. A portion of the media was then removed and replaced with fresh media.

Cells were infected at day five when the cell density was approximately 1 to 1.5×10^6 cells/ml. Infection was then carried out in a reduced volume of 50 ml, which approximates the cell to media volume ratio used for infection in 6-well plates of 0.5 ml/well. The infection was done in serum-free media with 1 mM IPTG at 50 rpm for one hour. The volume was then increased to 100 ml with media and serum was added to a final 10% FBS. Samples were taken at several times where cells on microcarriers were centrifuged ($300 \times g$), supernatant removed to a second tube and both were frozen at -70°C . Cell count and viability measurements were made as previously described around 24 and 48 hours post infection.

Fluorescence measurements were made on both media and pellet samples. Pellets were resuspended in 250 μl lysis buffer. The suspension was then incubated at 37°C for 15 minutes with vigorous vortexing twice. The debris was pelleted by centrifugation ($15,000 \times g$, 5 minutes) and supernatant was used for fluorescence

measurements. Standards of rEGFP were prepared in both media and the lysis solution used above. Media and lysed pellet samples of 100 μ l were transferred to 96-well black plates along with both sets of standards. Measurements of the EGFP fluorescence were made as described previously.

2.4 Results and Discussion

2.4.1 EGFP Expression

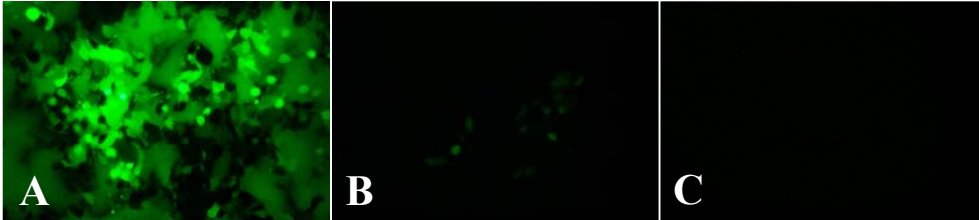
Construction of a recombinant vaccinia virus encoding for the production of EGFP was performed as described in the Materials and Methods section. In brief, the EGFP gene was amplified from pEGFP-N1 (Clontech) and combined with the histidine tag region of pSecTag2.A (Invitrogen). This segment was then inserted into the pVOTE.2 vector (B. Moss, NIAID, NIH) that contains the T7 promoter and *gpt* selection gene (Figure 2-1). This final plasmid, pNB009, was transfected into HeLa cells, which were previously infected with vT7lacOI (B. Moss, NIAID, NIH). The virus vT7lacOI is an engineered vaccinia virus that carries the T7 RNA polymerase and *lac I* genes. The recombinant virus, denoted vNB009, resulting from homologous recombination of the pNB009 plasmid and vT7lacOI was plaque purified under selective pressure for the *gpt* gene, amplified in HeLa cells, and purified by sucrose cushion centrifugation.

EGFP expression was verified by infection of HeLa cell monolayers with vNB009 at an MOI of 1.0 and induction with 1 mM IPTG in 6-well tissue culture plates. Figure 2-2 shows images taken with a fluorescence microscope. It is apparent by the appearance of many green fluorescing cells, Panel A, that expression is indeed

Figure 2-2 Fluorescence Images of Infection with vNB009

Panel A, HeLa cells infected with vNB009 and induced with 1 mM IPTG. Panel B, cells infected with vNB009. Panel C, cells without infection or induction.

Images taken with a Kodak EDAS Digital microscopy system on a Leica DM IRB fluorescent microscope with a FITC filter at 200× magnification.



occurring when the cells are infected with the recombinant virus and induced with IPTG. Cells that are infected but not induced, Panel B, show a very low level of green fluorescence, indicating that expression of EGFP is tightly controlled by the addition of the inducer. Additionally, the absence of green fluorescence in the uninfected cells, Panel C, demonstrates a negligible background fluorescence of the HeLa cells. Thus, EGFP expression using the VOTE system in vaccinia is possible and furthermore is tightly controlled by the presence of the inducing agent.

2.4.2 Multiplicity of Infection Evaluation

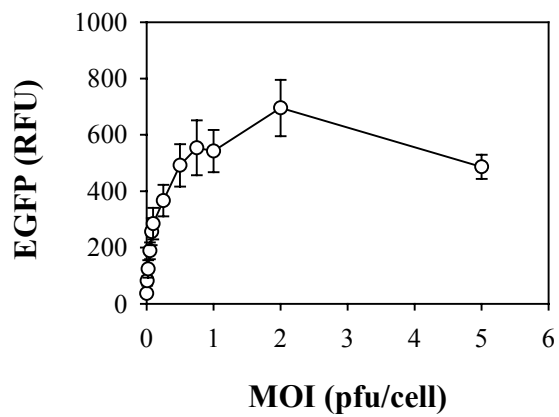
Expression levels of EGFP at varying MOI were examined in 96-well culture plates (Figure 2-3). Several samples were taken during the protein production stage and the maximum level for each MOI was plotted. The data show a sharp increase in the EGFP expression, which levels off upon approaching an MOI of 1.0. This indicates that the expression may not appreciably increase with more than one virus particle per cell, which agrees with what is known about the inability of vaccinia virus to superinfect cells (Christen et al. 1990), such as is found with insect cell baculovirus (MOI>10) (Wang et al. 1993).

2.4.3 Evaluation of MOI, Volume and Serum Concentration During Infection of HeLa Cells In Static Plate Culture

MOI, volume, and serum concentration during the 1 h infection phase were evaluated using a set of factorial experiments which indicated that the best expression in 6-well plates was obtained at a volume of 0.5 ml, a serum concentration of 0% FBS during the infection phase, and a MOI of 1.0. The first experiment conducted was

Figure 2-3 EGFP Expression at Varying MOI

Maximum EGFP expression levels in relative fluorescence units (RFU) obtained from infection and induction of HeLa cells in 96-well plate culture. Eight samples were taken per MOI at several times after infection and the average of the maximum expression points are plotted with standard errors.



arranged as a 2^3 factorial treatment structure that was used to evaluate maximum EGFP production for all combinations of levels for each parameter (Table 2-1). MOI was studied at levels of 0.5 and 1.5, volumes of 0.5 and 1.5 ml and serum concentrations of 2.5 and 5.0% FBS. ANOVA analysis was performed on the maximum expression levels and no interactions among the variables were found, so the effects were considered separately. The analysis showed that EGFP expression was statistically higher at an MOI of 1.5 than at an MOI of 0.5 ($p=0.010$), indicating that an examination of other MOIs would be needed to establish the MOI where maximum expression occurs. Additionally, the expression of EGFP was found to be statistically higher at a volume during the infection phase of 0.5 ml versus 1.5 ml ($p=0.012$). This phenomenon has been seen by other researchers where lowering the volume is believed to enhance infection by increasing the rate of binding (Dee and Shuler 1997b). Because 0.5 ml was the practical lower limit for infection in 6-well plates due to the thin layer of media that can cause the cells to dry out, it was the final level chosen and further evaluation of this parameter in 6-well plates was deemed unnecessary. Recombinant protein expression was not found to be significantly different for the two serum concentrations tested during the infection phase ($p=0.224$). This may have been due to the small range tested. Another evaluation, with a wider range of serum concentration values, was needed to firmly establish the absence of an effect of serum.

A 4^2 factorial treatment structure was designed to test a wider range of both MOI and serum concentration during the infection phase in 6-well plates (Figure 2-4). Serum concentration was tested at 0, 2.5, 5.0 and 7.5% FBS and MOI at 1.0, 2.0, 3.0,

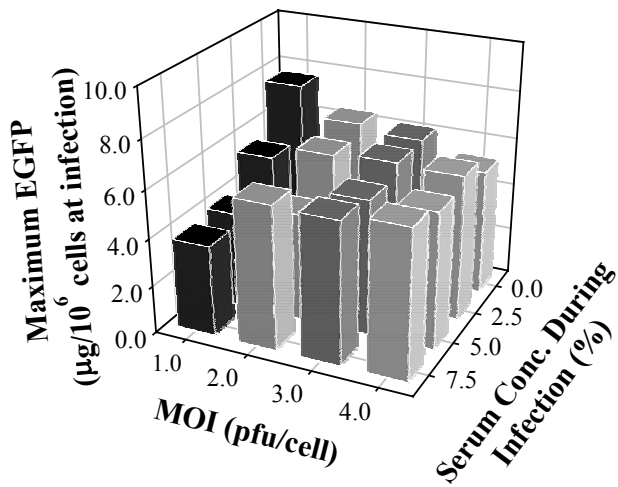
Table 2-1 Evaluation of MOI, Volume and Serum Concentration During Infection Phase

MOI (pfu/cell)	Volume During Infection Phase (ml)	Serum Concentration During Infection Phase (%)	Maximum EGFP ($\mu\text{g}/10^6$ cells at infection)
Uninduced Control			
1.0	0.5	2.5	0.05
0.5	0.5	2.5	3.66
0.5	0.5	5.0	2.88
1.5	0.5	2.5	4.03
1.5	0.5	5.0	4.05
0.5	1.5	2.5	2.20
0.5	1.5	5.0	1.88
1.5	1.5	2.5	3.57
1.5	1.5	5.0	3.04

$SE_p=0.30$

Figure 2-4 Evaluation of MOI and Serum Concentration During Infection Phase

Maximum EGFP expression levels, in duplicate, obtained from infection and induction of HeLa cells in 6-well plates. The pooled standard error obtained from ANOVA analysis of this data was 1.36.



and 4.0. ANOVA analysis was performed on the maximum measured expression levels for each treatment combination. Again, no significant interactions among the variables were found, therefore only main effects were considered. The results show that the effect of serum was marginally significant ($p=0.054$) on the expression of EGFP, which indicates that lower levels of serum can be used during infection. Expression of EGFP was not found to be statistically affected by MOI within the region tested ($p=0.278$). This finding agrees with the results suggested by the 96-well multiplicity of infection evaluation where a plateau of expression appeared to occur around an MOI of 1.0 and more than one infectious virus particle per cell may not significantly increase the level of EGFP expression.

2.4.4 Microcarrier Selection Process

An analysis of several microcarriers was performed, types and some descriptive details are displayed in Table 2-2. The critical parameters for selection were the ability of the cells to attach to the microcarriers under agitation, the cell growth profile, the cell layering characteristics, and the maximum supported cell density.

The first experiment was performed in 6-well, low binding plates on a rotary shaker at 50 rpm. Cells were seeded into plates in the presence of microcarriers and microscopic visualization of the attachment and spreading of the cells was performed. Once the cells were grown to confluency, a cell removal and counting procedure was followed to determine the ability of the microcarriers to allow an easy sampling procedure. Based on these two criteria, four of the microcarriers were deemed unacceptable and were removed from future study (Cultispher-G, Cultispher-S, and

Table 2-2 Microcarrier Characteristics

Microcarrier	Material	Recommended Concentration (g/l)
Biosilon [®] 169159 (Nunc)	Globular plastic	23.5
Biosilon [®] 169191 (Nunc)	Globular plastic	23.5
Cultispher-G (Percell)	Cross-linked gelatin	1.0
Cultispher-S (Percell)	Cross-linked gelatin	1.0
Cytodex [®] 1 (Pharmacia)	Cross-linked dextran, positively charged	1.0
Cytodex [®] 3 (Pharmacia)	Cross-linked dextran, collagen coated	1.0
Cytopore [™] 2 (Pharmacia)	Macroporous, cross-linked dextran, positive charge	1.0
FACT FC102-915 (SoloHill)	Copolymer, charged gelatin coated	20.0
FACT FC102-1521 (SoloHill)	Copolymer, charged gelatin coated	20.0
Gelatin C102-915 (SoloHill)	Copolymer, gelatin coated	20.0
Gelatin C102-1521 (SoloHill)	Copolymer, gelatin coated	20.0
Hillex (SoloHill)	Trimethylamine (semiporous)	20.0

the two Gelatin microcarriers from SoloHill). The other eight microcarriers were retained for further evaluation.

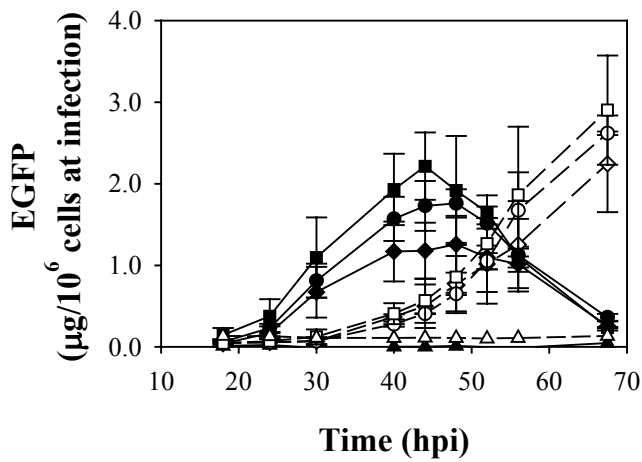
These microcarriers were tested in 125 ml spinner flasks. Flasks were prepared with microcarriers at the concentration recommended by each manufacturer and seeded with 1×10^5 cells/ml. Samples were taken daily for cell count and viability measurements. Three of the microcarriers tested, Cytodex[®] 1, Cytodex[®] 3 and Biosilon[®] 169191 lead to cell densities above 1.0×10^6 cells/ml (data not shown). These three microcarriers were evaluated further at a concentration of 20 g/l, (which was near the upper region of several of the brands tested), and with HeLa cells seeded to a density of 2×10^5 cells/ml. All three microcarriers had exponential growth profiles (data not shown) with maximum cell densities of $3.7 \times 10^6 \pm 0.41 \times 10^6$, $3.0 \times 10^6 \pm 0.13 \times 10^6$ and $3.1 \times 10^6 \pm 0.32 \times 10^6$ for Biosilon[®] 169191, Cytodex[®] 1 and Cytodex[®] 3, respectively. The Biosilon[®] microcarriers were observed to allow the cells to form thick layers on the surface. This was considered an undesirable characteristic because during infection, the inner cells would likely not be infected in the primary infection, and therefore may affect the overall expression kinetics. Based on all these findings, and because the growth of HeLa on Cytodex[®] 3 during growth was slightly higher than on Cytodex[®] 1, Cytodex[®] 3 microcarriers were found to be the most appropriate microcarrier for this work.

2.4.5 Infection and Expression from HeLa cells on Microcarriers

EGFP expression from HeLa cells grown on microcarriers in spinner flasks at varying levels of MOI was examined (Figure 2-5). The evaluated levels of MOI were 0.0, 1.0, 2.0, and 3.0. The volume during infection was reduced to approximately the

Figure 2-5 MOI Effect on EGFP Expression from HeLa Cells Grown on Microcarriers in Spinner Flasks

EGFP expression levels from HeLa cells on Cytodex[®] 3 microcarriers in 125 ml spinner flasks infected with vNB009 and induced with 1 mM IPTG. Solid lines and filled symbols, intracellular EGFP measurements. Dashed lines and open symbols, extracellular EGFP measurements. Triangles, MOI=0.0; diamonds, MOI=1.0; circles, MOI=2.0; squares, MOI=3.0.



same volume ratio (media volume/cell) found best in monolayer culture and the infection was performed in serum-free media. Glucose and lactate were measured throughout the growth and production phases (data not shown). Up to 48 hpi, the levels were kept within an acceptable range for growth by feeding once at 24 hpi. After 48 hpi, most cultures showed a significant amount of cell lysis due to the cytopathic affects of the virus that made feeding the culture unrealistic.

The expression results show that although the errors are fairly wide, maximum expression appears to occur at MOI 3.0. This result was not found to be significantly different from MOI of 1.0 or 2.0 upon ANOVA analysis. The large variance and the difference with the optimum MOI obtained from static culture experiments can be explained by the dynamic nature of the microcarrier system and by the fact that infection was done during late log phase for the microcarriers versus early log phase for the stationary cultures, where infection may be more efficient. Additionally, the method of cell counts on microcarriers may not be the most accurate, particularly at higher cell densities, leading to a higher variability. In the future, other methods of cell counting will be investigated, such as nuclei counts (van Wezel 1973), to minimize this source of variation.

Taking the above into consideration, the results show that both infection and expression occur with this method of production. These parameters will be further studied in spinner flask microcarrier culture to optimize conditions and reduce the controllable sources of variation.

2.5 Conclusions

The method of recombinant protein production described here has shown encouraging results. In particular, lowered volume during the infection phase helped to increase expression. No serum during the infection phase was needed to ensure infection and expression of EGFP. Additionally, an MOI near 1.0 was found to provide adequate infection and higher amounts of virus were found ineffective for increasing protein production in static plate cultures. Future work will be directed at optimizing the conditions of expression in microcarrier cultures, including bioreactor cultures. Other factors that may affect expression such as inducer concentration and timing of inducer addition will also be investigated. To further exploit the versatility of this expression method, studies are underway to evaluate different cell lines such as HEK-293, BHK-21, WI 38, or Vero infected with this vaccinia virus construct. Other protein candidates that require complex post-translational processing are also being engineered into vaccinia constructs with the same promoter and expression elements and will be tested with these cell lines to determine the best expression and processing profile.

CHAPTER 3: Evaluation of Production Parameters with the Vaccinia Virus Expression System Using Microcarrier Attached HeLa Cells²

3.1 Summary

Parameters which affect production of the recombinant reporter protein, EGFP, in the T7 promoter based VOTE vaccinia virus-HeLa cell expression system were examined. Anchorage dependent HeLa cells were grown on 5 g/l Cytodex[®] 3 microcarriers, which were found to provide the optimum conditions for growth and infection. Length of infection phase, inducer concentration and timing of its addition relative to infection were evaluated in 6-well plate cultures. One hour infection with 1.0 mM IPTG added at the time of infection provided a robust process. Examination of the multiplicity of infection (MOI) in the dynamic environment of microcarrier culture indicated a need for an increase in the number of virus particles per cell to 5.0, higher than needed for complete infection in tissue flask culture. Additionally, dissolved oxygen level and temperature during protein production phase were evaluated for their effect on EGFP expression in microcarrier spinner flask culture. Increased dissolved oxygen from 30% to 50%, and decreased temperature from 37°C to 31°C, showed a slight increase in production over the course of the production phase. The level of production achieved with this system reached approximately 17 µg EGFP/10⁶ infected cells.

² Bleckwenn NA, Bentley WE, Shiloach J. 2004. Evaluation of production parameters with the vaccinia virus expression system using microcarrier attached HeLa cells. Submitted 2004.

3.2 Introduction

Vaccinia virus infection of HeLa cell culture has shown potential as a recombinant protein production method (Bleckwenn et al. 2003). The current literature on growth and infection of HeLa cells in large-scale culture is limited (Barrett et al. 1989; Hu et al. 2000). This study investigates microcarrier based culture of HeLa cells in spinner flasks to obtain basic information on how to best cultivate and infect these cells to express recombinant proteins. Microcarriers provide solid support onto which anchorage dependent cells attach and grow (Croughan et al. 2000; Hawboldt et al. 1994; Iyer et al. 1999; Kang et al. 2000), but also the carriers can be suspended in culture medium and treated similarly to suspension culture, which makes this method of cultivation amenable to scale-up in conventional stirred tank bioreactors.

Enhanced green fluorescent protein (EGFP) was used as a model protein with this viral expression system. Green fluorescent protein has previously been used as a model for studying protein production with other expression methods, such as *E. coli* (Cha et al. 1999d; DeLisa et al. 1999), the insect cell or insect larvae-baculovirus system (Cha et al. 1999a; Cha et al. 1999b; Cha et al. 1999c; Cha et al. 1997; Laukkanen et al. 1996), and mammalian cell systems (Durocher et al. 2002; Sen et al. 2003), because its expression levels are easily quantified via fluorescence spectrophotometry. The VOTE vaccinia expression system (Ward et al. 1995) was used to control expression of the EGFP reporter gene, where the gene is introduced into the cytoplasm of the cell via the viral infection and protein is made upon induction with isopropyl- β -D-thiogalactopyranoside (IPTG). The VOTE construct is

a T7 promoter based system (Alexander et al. 1992) where all the control components are present in a single virus, unlike other vaccinia constructs that require dual infection (Fuerst et al. 1987; Hu et al. 2000). Expression is tightly controlled by the addition of the inducing agent, IPTG, allowing for controlled expression of the protein of interest, which is advantageous when the protein to be produced is detrimental to the cell.

Previous work in our lab evaluated several basic infection characteristics with this reporter virus in small-scale tissue flask experiments, which included multiplicity of infection (MOI), and both volume of medium and serum concentration during the infection phase. Additionally, an examination of twelve commercial microcarriers for growth and cell sampling potential found that Cytodex[®] 3 possessed the most appropriate characteristics. These conditions were used to infect microcarrier based spinner flask cultures at MOI from 0 to 3.0 where MOI of 3.0 reached $2.2 \mu\text{g}/10^6$ cells at infection. This maximum was not statistically different from the maximums for MOI 1.0 or 2.0 and an evaluation of a wider range of MOI was suggested (Bleckwenn et al. 2003).

The process parameters that can affect protein yield in a production system are numerous. This study evaluates several key parameters for the establishment of cells and expression of recombinant proteins with the VOTE vaccinia expression system. The parameters evaluated include the concentration of microcarriers for the anchorage dependent HeLa cells, induction conditions, a wider range of multiplicity of infection (MOI) in the microcarrier based system, dissolved oxygen (DO) level

during the protein production phase, and temperature during the protein production phase.

3.3 Materials and Methods

3.3.1 Cell Line Maintenance and Expansion

HeLa cells (ATCC CCL-2) were maintained in tissue culture flasks and were passaged every 3-4 days in Dulbecco's modified Eagle's medium with 4.5 g/l glucose (DMEM, Biosource, Camarillo, CA) and 10% fetal bovine serum (FBS, Biosource). In preparation for seeding well plates or microcarriers for experiments, confluent HeLa cells in multiple T-162 cm² tissue culture flasks were harvested using cell dissociation solution (Hanks' based, Invitrogen, Carlsbad, CA) and counted as described below. Medium used for all experiments was composed of DMEM with 4.5 g/l glucose without phenol red and supplemented with 4 mM L-glutamine (Biosource) and 10% FBS unless otherwise specified.

Cytodex[®] 3 microcarriers (Amersham Biosciences, Piscataway, NJ) were prepared according to manufacturer's instructions which involved washing with PBS, sterilization by autoclaving, and washing again with culture medium. To seed the microcarriers, cells were harvested from tissue culture flasks and combined with Cytodex[®] 3 microcarriers in 250 ml siliconized spinner flasks with paddles (Bellco Glass, Inc., Vineland, NJ), at a final concentration of 1×10^5 cell/ml. An initial reduced volume of 100 ml was used with an agitation profile of 30 seconds on at 50 rpm and 20 minutes off, in a 37°C humidified incubator with 5% CO₂ for four hours. At the end of the four hour seeding phase, medium was added to the final 200 ml

working volume and the agitation was set to a constant 50 rpm. Samples were taken daily for cell count and viability. The cultures were fed as needed by allowing the cell bound microcarriers to settle, removing a portion of the spent medium and replacing it with an equal volume of fresh medium.

3.3.2 Viral Stock Preparation

Construction of the reporter virus, vNB009, containing the gene for EGFP with a C-terminal histidine tag (6×HIS), along with viral purification and titer determination of the stock was described previously (Bleckwenn et al. 2003).

3.3.3 Cell Count and Viability Measurements

A cell suspension sample for cell count and viability determinations from microcarrier cultures was prepared by centrifugation of a 1.0 ml sample at 300×g for 5 minutes. The supernatant was then aspirated and the sample washed with 0.5 ml of D-PBS with calcium and magnesium (Invitrogen). The pellet was then resuspended in 0.5 ml of 1× trypsin-EDTA solution (Invitrogen) and incubated for 30 minutes at 37°C with vortexing every 10 minutes. An equal volume of DMEM+10% FBS was added and the sample was mixed. The resulting solution was used for cell count and viability measurements using 0.4% trypan blue exclusion staining (Sigma, St. Louis, MO) on a hemacytometer.

3.3.4 Nutrient and Metabolite Measurements

Medium samples from experiments were used to measure glucose and lactate with a YSI 2700 Biochemistry Analyzer (Yellow Springs Instrument Co., Yellow Springs, OH).

3.3.5 Fluorescence Analysis of EGFP Expression

Fluorescence measurements were made according to Bleckwenn et al. (2003). Briefly, pellet samples were resuspended and lysed in lysis buffer (100 mM Tris pH 7.4, with 1× Complete protease inhibitor (Boehringer Mannheim GmbH, Germany) and 0.5% Triton X-100). Fluorescence measurements of the supernatant from pellet lysis and culture medium were made in 96-well black plates (Fisher Scientific, Pittsburg, PA) using a SpectraMax Gemini fluorescence spectrophotometer (Molecular Devices, Sunnyvale, CA) with excitation at 485nm, emission at 512 nm and an auto cutoff filter of 495 nm against standards of rEGFP (Clontech BD Biosciences, Palo Alto, CA).

3.4 Results

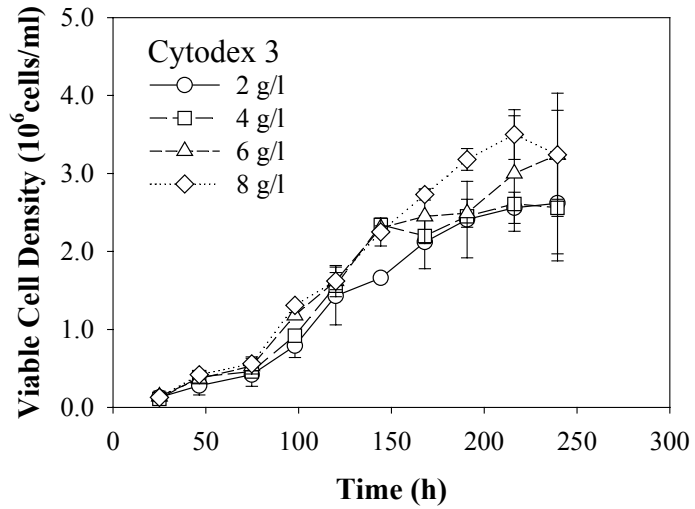
3.4.1 Microcarrier Density Evaluation

The effect of Cytodex[®] 3 microcarrier concentration on viable cell density and coverage of the carriers was initially tested at 5, 10, 15 and 20 g/l in 200 ml spinner flask cultures in duplicate, but did not show significant differences in maximum cell density ($P= 0.64$, data not shown). A lower range of microcarrier concentrations was then tested at 2, 4, 6 and 8 g/l to determine the optimum microcarrier density. Viable cell density as a function of time is shown in Figure 3-1 for these carrier concentrations. The 2 g/l concentration appeared to lag behind the others at about 120 hours after seeding and the carriers were completely confluent upon visual inspection (data not shown). The concentrations of 4, 6, and 8 g/l follow approximately the same growth profile and reached approximately the same

Figure 3-1 Growth of HeLa Cells on Cytodex[®] 3 Microcarriers

Viable cell densities from spinner flask cultures (200 ml) of microcarrier attached

HeLa cells were grown on various concentrations of Cytodex[®] 3 microcarriers.



maximum cell density, around 2.5 to 3.5×10^6 cells/ml. Visual inspection of the coverage of cells on the carriers (data not shown), revealed that a concentration between 4 and 6 g/l appeared to allow single layer growth, while reaching confluency between 150 and 175 hours after seeding.

3.4.2 Effect of Infection Duration on EGFP Production

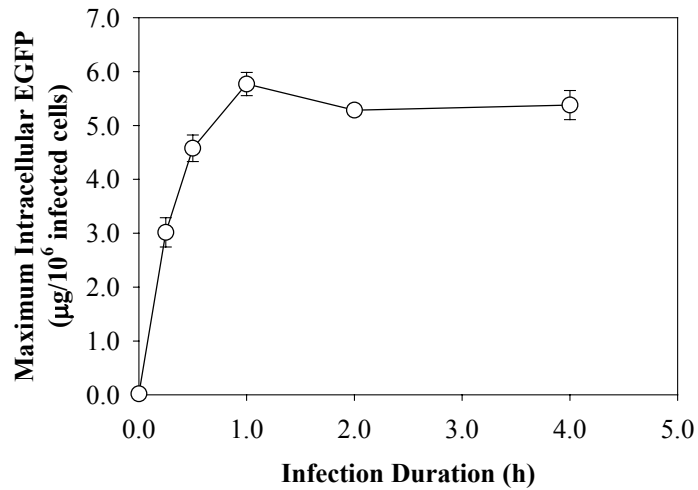
The effect of the length of the infection phase on EGFP production is seen in Figure 3-2. The infection was carried out in a reduced volume of serum-free medium, 0.5 ml, with 1.0 mM IPTG in 6-well plate culture where the cell concentration at infection was $2.00 \pm 0.13 \times 10^6$ cells/well. Virus vNB009 was added to the cells to infect at an MOI of 1.0 . Plates were incubated, for 15 , 30 , 60 , 120 , or 240 minutes, at which time medium was added to bring the volume to 2.5 ml with the serum concentration brought to 10% FBS and inducer to 1.0 mM IPTG, completing the infection phase. Infected cells were incubated for 36 , 42 and 48 hours for each infection length (e.g. 3 samples, 5 infection lengths). The maximum intracellular EGFP expression levels of the three time points sampled for each infection length were averaged for duplicate wells. The results show a minimum infection time of one hour for maximum protein expression.

3.4.3 Effect of IPTG Concentration and Timing of Its Addition Relative to Infection on EGFP Production

A 6×3 factorial treatment structure experiment was performed on HeLa cells in 6-well plates. The concentrations of IPTG inducer tested were 0.0 , 0.1 , 0.5 , 1.0 , 2.0 , and 5.0 mM. Inducer was added to the medium either one hour prior to infection,

Figure 3-2 Effect of Infection Duration on Reporter Protein Expression

Six-well plate cultures of HeLa cells were infected at an MOI of 1.0 for varying amounts of time. After the infection period, fresh medium, FBS, and IPTG were added.



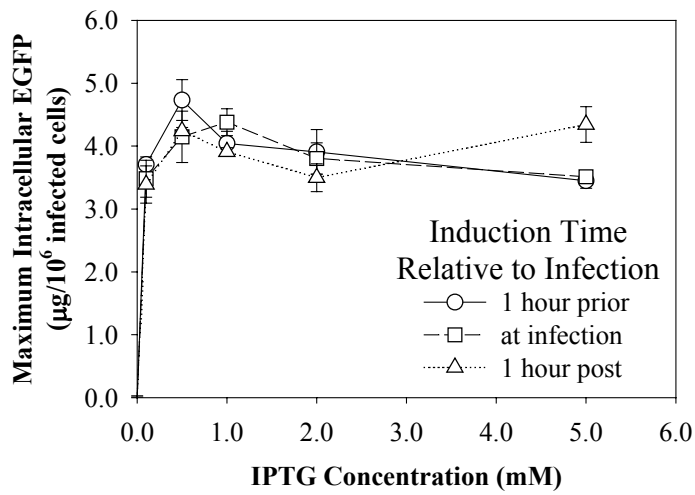
at the time of infection, or one hour after infection. The cells were prepared and infected in the same manner as previously described, at an MOI of 1.0 in a reduced volume of serum-free medium, and for 1 hour as found from Figure 3-2. At the end of the one hour infection phase, the medium was increased and serum added to 10% FBS with the appropriate IPTG level. The maximum intracellular expression levels found for each well were averaged over duplicate wells (Figure 3-3). There was no interaction between the two tested parameters ($P=0.12$) and no significant differences were found between the times evaluated for addition of the inducer ($P=0.82$). However, the differences in IPTG concentration were statistically significant ($P<0.0001$) and maximum expression levels were achieved with IPTG concentrations of 0.5 mM and above.

3.4.4 Multiplicity of Infection in Microcarrier Culture Spinner Flasks

Multiplicity of infection was evaluated in triplicate spinner flask cultures of microcarrier attached HeLa cells. Cultures were infected 5 days after seeding when the cell density was $1.22\pm 0.083\times 10^6$ cells/ml. The cells were washed with serum-free medium, the volume was reduced to 70 ml for infection in serum-free medium containing 1.0 mM IPTG. Trypsinized vNB009 virus was then added to the culture and the flasks were incubated at 37°C, 5% CO₂ at 50 rpm for one hour. At the end of this infection phase, medium, FBS and IPTG were added to a final volume of 200 ml, 10% FBS, and 1.0 mM IPTG. Samples of pellet, containing cells and carriers, and supernatant were taken up to 66 hpi. Medium (100 ml) was exchanged with fresh medium at 24 hpi to maintain the level of glucose above 1.0 g/l and the concentration of lactate below 2.0 g/l. The expression levels of EGFP over the course of the protein

Figure 3-3 Effect of IPTG Concentration and Timing of Its Addition Relative to Infection on Protein Expression

A factorial treatment structure design of experiment was used to determine the effects IPTG concentration and the timing of its addition relative to infection on protein expression. The timing of addition is separated into three separate lines as the concentration of IPTG is plotted along the x-axis.



production phase are depicted in Figure 3-4, where intracellular levels are shown in Panel A and extracellular levels in Panel B. Although there are not significant differences in the maximum intracellular expression levels for MOI 0.5 and above ($P \geq 0.36$), it appears that MOI 5.0 and 10.0 were slightly better in the initial 40 hours, after which 0.5 and 1.0 increased to the same levels. The maximum obtained was $2.6 \mu\text{g}/10^6$ infected cells. The extracellular levels of EGFP began to increase as the intracellular values began to peak and then decline. Their levels were approximately equal for MOI 0.5 to 10.0, but were lower for MOI 0.1.

3.4.5 Effect of Dissolved Oxygen Level on Protein Production

The effect of dissolved oxygen concentration on protein expression was studied in spinner flask cultures infected at MOI 5.0 for one hour with 1.0 mM IPTG added at infection. At the end of the infection phase, medium was added to bring the culture volume to 200 ml with 10% FBS and 1 mM IPTG. Each flask was mixed well and varying amounts of culture (including medium, cells, and microcarriers), were removed to achieve volumes of 50, 100, 150, and 200 ml while maintaining the same microcarrier concentration and cell density. These volumes correspond to surface area to volume (SA/V) ratios of 1.08, 0.54, 0.36, and 0.27 cm^{-1} , respectively, and allow for a rough comparison of changing dissolved oxygen levels. Samples from the cultures for metabolite and protein analysis were taken up to 66 hpi and feeding of the culture was performed at 24 hpi. Figure 3-5 shows the intracellular expression levels. From this data, the maximum level of production was found with an SA/V ratio of 0.54 cm^{-1} at $3.13 \mu\text{g}/10^6$ infected cells. Both higher and lower SA/V ratios show lower rates of production and maximum levels of expression.

Figure 3-4 EGFP Expression vs. MOI in Microcarrier Spinner Flask Cultures

200 ml spinner flask cultures containing HeLa cells attached to microcarriers were infected at various MOI.

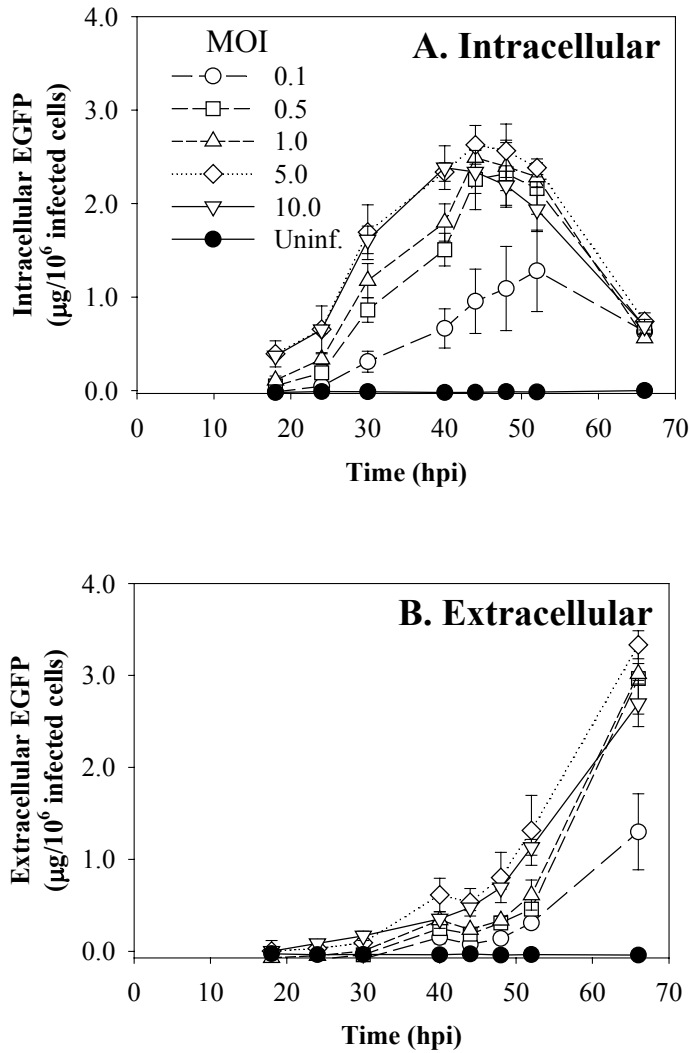
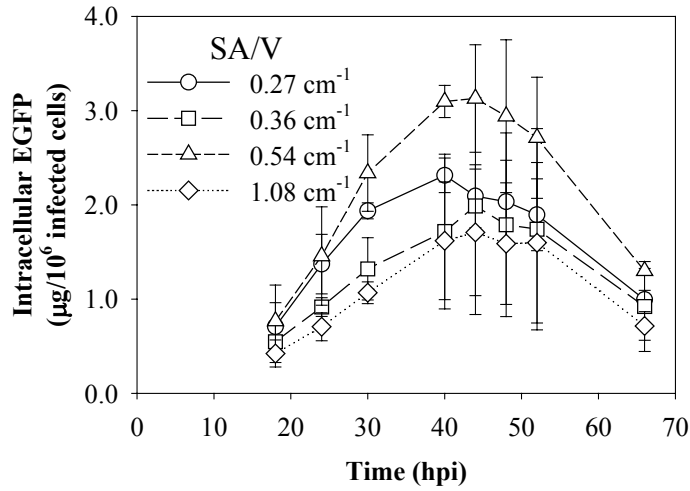


Figure 3-5 Effect of Dissolved Oxygen Level on Protein Production

Different surface area to volume ratios were used to approximate differences in dissolved oxygen level. Cultures of volume 50, 100, 150, and 200 ml were grown in 250 ml spinner flasks.



3.4.6 Effect of Temperature on Protein Production

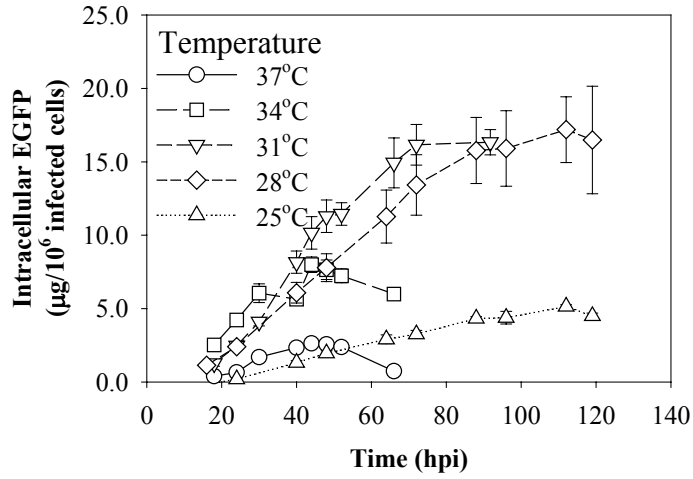
An evaluation of process temperature during the protein production phase was made in spinner flask cultures containing microcarrier attached HeLa cells. Cells were seeded onto microcarriers and infected as before at an MOI of 5.0 with 1.0 mM IPTG, then brought to 200 ml after the one hour infection phase. The temperatures were then adjusted to 25, 28, 31, or 34°C, with 37°C ($\pm 0.5^\circ\text{C}$) tested previously in the dissolved oxygen experiment. The intracellular expression profiles of the reporter protein for the different temperatures followed the same general trend, shown in Figure 3-6. At 34°C maximum expression was achieved ~48 hpi, while the maximum for 31°C occurred ~80 hpi, and later for 28 and 25°C. The highest expression level was achieved at 31°C, at $17.2 \pm 2.24 \mu\text{g}/10^6$ infected cells.

3.5 Discussion

The protein production potential of the HeLa cell-VOTE vaccinia expression system and its initial characterization were established in our lab (Bleckwenn et al. 2003). While the previous study demonstrated to potential for HeLa-VOTE vaccinia, several factors remained unexamined. Specifically, this article describes the systematic evaluation of several key parameters affecting growth, and production: microcarrier concentration, infection time and duration, and inducer concentration and timing of its addition relative to infection. Also, while our previous study identified Cytodex[®] 3 as most promising, the current work found that 5 g/l of these carriers was sufficient in terms of surface area for cell growth and spatial expansion after infection. Since the growth profiles and maximum cell densities were all similar

Figure 3-6 Effect of Temperature on Protein Production

Temperatures were adjusted in microcarrier based spinner flask cultures after the infection phase and protein production levels are compared.



for the microcarrier densities evaluated, visual inspection of cell coverage on the microcarriers, although somewhat subjective, was critical to this evaluation.

Infection parameters influencing protein expression levels were evaluated in 6-well plate cultures which included infection duration, inducer concentration, and timing of inducer addition relative to the time of infection. These small-scale experiments were more amenable than microcarrier spinner flask cultures for testing a larger number of conditions. The length of the infection phase is critical for most viruses to ensure binding of the virus particles and entry into the cells (Chillakuru et al. 1991; Locker et al. 2000; Petricevich et al. 2001; Vanderplasschen et al. 1998; Vanderplasschen and Smith 1999). The infection duration experiment performed here resulted in an optimal length of infection of one hour, which coincides with the value found by other researchers with other vaccinia infection systems (Chillakuru et al. 1991; Hu et al. 2000).

The concentration of the induction agent, IPTG, and the timing of its addition relative to infection in this system both play a critical role in protein expression, as was evident by the results in Figures 3-2 and 3-3. Since these two parameters may be linked and interact with one another, they were tested together in a 6×3 factorial treatment structure experimental design. Interestingly, no interaction was found between inducer concentration and induction time for the ranges tested. Further, no significant difference was found for the timing of inducer addition relative to infection, indicating that the transfer of IPTG to the cells was not limiting for the timescale on which cell cultures run, relative to protein production.

In some cases, certain foreign proteins may be detrimental to the cell and could affect protein production and cell culture health, possibly requiring a delayed induction time to reach maximum expression levels, but this is not the case for EGFP. In addition, the ability to control the time of induction may be beneficial for secondary infection processes (Bentley et al. 1994; Hu and Bentley 2000; Hu and Bentley 2001), where the culture is initially infected at a very low MOI in order to conserve viral stocks. The small number of initially infected cells at low MOIs would serve as virus production factories, rather than protein production factories, to produce the virus particles that will infect the remainder of the culture. To maximize the production of virus particles and conserve resources for the infected cells, induction of protein expression could be delayed until secondary infection of the culture has occurred. The minimum IPTG concentration required for maximum expression showed little difference for concentrations higher than 0.5 mM. Although 0.5 mM IPTG was found to be sufficient, the level of expression drops off significantly below 0.5 mM IPTG, thus, to ensure that maximal levels of protein production are achieved, a value of 1.0 mM was chosen for all future studies. Additionally, for convenience, since the timing of inducer addition did not affect expression, adding the induction agent at the time of infection was chosen for all subsequent work.

Multiplicity of infection is another parameter that can affect protein production. For insect cells, superinfection of the cells (infection with more than one virus particle per cell) can affect the level of protein production (Hu et al. 2003; Licari and Bailey 1991). Cells infected with vaccinia virus are largely incapable of

multiple infection past the first hour of exposure to virus (Christen et al. 1990), and using an MOI greater than 1.0 did not affect the expression in monolayer plate culture (Bleckwenn et al. 2003). The dynamic nature of microcarrier culture, however, may alter the infection kinetics from those seen in plate culture. Similar results have been seen in insect cells in plate versus spinner flask culture (Dalal and Bentley 1999; Dee and Shuler 1997a). Our work with microcarrier based HeLa cell culture found that an MOI of 5.0 yielded the highest intracellular expression of EGFP, and although MOI 10.0 initially followed the same rate of production, the level did not reach the same maximum due to an earlier decline in expression, possibly due to an increased cytopathic effect and cell lysis. Lower MOIs did not appear to have the same rate of expression as MOI 5.0, and therefore, 5.0 pfu/cell was chosen for all microcarrier based work to ensure complete, simultaneous infection and maximal expression of the recombinant protein. Other researchers have also used higher MOIs for vaccinia virus infection in large-scale HeLa cell packed bed (Hu et al. 2000) or Vero cell microcarrier culture (Barrett et al. 1989).

Process parameters such as dissolved oxygen and temperature, may also affect the final yield of protein. Providing an ample supply of oxygen is crucial to maintaining cell health and protein production and may also affect proper post-translational processing as was seen with the insect cell baculovirus system (Donaldson et al. 1999; Zhang et al. 2002b) and other expression systems (e.g. hybridomas, CHO, etc. (Kunkel et al. 2000; Kunkel et al. 1998)). To determine whether supplying more oxygen to the cells would help increase production of the recombinant protein, an evaluation in spinner flask culture was performed. The

dissolved oxygen level was varied by adjusting the surface area to volume ratio while maintaining constant cell and microcarrier densities. The results suggest that increasing DO increases the level of protein production but only to a certain point (e.g. more production obtained at 0.54 cm^{-1}). The level of dissolved oxygen during the production phase can play an important role in the health of cells, particularly while they are burdened from a viral infection. Interestingly, vaccinia virus will shut down host cell protein production in order to dedicate more resources for producing its own proteins and progeny virus (Cacoullos and Bablanian 1993), and in our case, production of the recombinant protein. In utilizing a viral production system, it is necessary to reduce the burdens caused by infection and the cytopathic effects of the virus as much as possible, to allow for more time to produce the recombinant protein before the cells die and lyse. Other researchers using the insect cell baculovirus system found optimum levels of DO for protein production in their respective systems, but some also found that high levels of DO decreased protein production due to an increase in protease activity (Hu and Bentley 1999; Lindsay and Betenbaugh 1992; Naggie and Bentley 1998).

Concerning temperature, most mammalian cell lines grow best at 37°C (Freshney 2000), and significant deviations above this temperature are devastating to the cells. Lower temperatures are tolerable, but the cells tend to grow slower. It has been found that virus systems such as Semliki Forest Virus (SFV) and adenovirus (AV) both produce higher amounts of protein when production occurs at a lowered temperature (Jardon and Garnier 2003; Schlaeger and Lundstrom 1998). Insect cell baculovirus systems have also shown improvement of protein production with

decreased temperatures (Andersen et al. 1996; Gotoh et al. 2004). It was suggested with the SFV system that the lower temperature reduces cytopathic effects of the viral infection and allows the cells to remain healthier and producing protein for a longer period of time, resulting in higher yield. In the present work, temperature was adjusted after the infection phase to ensure that this change had no affect on the kinetics of infection, only on protein production and cytopathic viral effects. Importantly, lowering the temperature to 31°C increased the specific yield six-fold over the 37°C culture in microcarrier based spinner flasks.

It is important to note that dissolved oxygen and temperature are not independent. By changing the SA/V, changes in the soluble carbon dioxide can occur which could affect the culture pH. Additionally, changing the incubation temperature of spinner flasks, without DO control, affects the level of dissolved oxygen. Translating the results obtained from these spinner flask cultures to large-scale culture cannot be made directly because altering these parameters in this way cannot isolate the variable being studied. In a more controlled environment, such as a bioreactor, the result of changing DO and temperature could be tested in isolation from other variables. The conditions found here, therefore, are sufficient to guide further work at the reactor scale.

3.6 Conclusions

In summary, the results of the various parameter experiments shown here suggest that the optimum conditions for establishing the HeLa cells are to seed and grow them on 5 g/l Cytodex[®] 3 microcarriers. Infection parameters were also refined to increase protein production with the VOTE vaccinia expression system which

resulted in an infection at MOI 5.0 for one hour with inducer added at the time of infection at a concentration of 1.0 mM IPTG. By increasing the dissolved oxygen through adjustment of the SA/V, and lowering the temperature of the culture to 31°C, increases in protein production were seen. The maximum level of protein production, under the conditions tested, lead to a yield of 17-18 µg intracellular EGFP/10⁶ infected cells when the culture was infected at the conditions described.

CHAPTER 4: Vaccinia Virus Production of Recombinant Proteins in a Microcarrier Based Mammalian Cell Bioreactor³

4.1 Summary

The HeLa cell-vaccinia virus expression system was evaluated for the production of recombinant proteins (enhanced green fluorescent protein (EGFP) and HIV envelope coat protein gp120) using microcarriers in 1.5 l perfused bioreactor cultures. Perfusion was achieved by use of an alternating tangential flow device (ATF), increasing the length of the exponential phase by 50 hours compared to batch culture and increasing the maximum cell density to 4.4×10^6 cell/ml. A seed train expansion method using cells harvested from microcarrier culture and reseeded onto fresh carriers was developed. Enhanced green fluorescent protein (EGFP) was first used as a model protein to study process parameters affecting protein yield, specifically dissolved oxygen (DO) and temperature during the production phase. The highest level of EGFP, $12 \pm 1.5 \mu\text{g}/10^6$ infected cells, was obtained at 50% DO and 31°C. These setpoints were then used to produce glycoprotein gp120, which was purified and deglycosylated, revealing a significant amount of N-linked glycosylation. Also, biological activity was assayed, revealing an ID₅₀ of 3.1 μg/ml, which is comparable to previous reports.

³ Bleckwenn NA, Golding H, Bentley WE, Shiloach J. 2004. Vaccinia virus production of recombinant proteins in a microcarrier based mammalian cell bioreactor. Submitted 2004.

4.2 Introduction

Recombinant protein expression from cell culture can be accomplished by stable or transient integration of DNA into the host cells. Viral vectors provide one such method of efficient transient expression and have been used widely (e.g. insect cells, insect larvae). The baculovirus has proven to be very effective at producing large quantities of foreign proteins (Bentley et al. 1994; Cha et al. 1999c; Chung et al. 1993; Licari and Bailey 1991; Lindsay and Betenbaugh 1992; Wickham and Nemerow 1993). Generally though, complex proteins made with this system are not processed in the same manner as in mammalian cells, without significantly altering the host cells' post-translational processing machinery (Ailor and Betenbaugh 1999; Ailor et al. 1999; Ailor et al. 2000; Fang et al. 2000; Percival et al. 1997). When proper post-translational modifications are necessary, for example, often for proteins used as therapeutic agents (Eckhardt et al. 2002; Fenouillet and Jones 1996; Li et al. 1993), it may be more advantageous to use a mammalian derived host cell line.

Our lab has postulated that a viral system used with mammalian cell culture, a more complex system with innate capabilities for complex post-translational processing, might also lead to increased levels of protein production compared to stable integration of foreign DNA into the host cell. Vaccinia virus, an orthopoxvirus related to the virus that causes Smallpox, was chosen as the viral vector due to its unique characteristics. This virus has a wide host range (Broder and Earl 1999) and its own transcriptional machinery is brought into the cytoplasm of the infected cell (Moss 1996). This feature allows the recombinant protein to be transcribed in the cytoplasm, eliminating nuclear transport requirements for the recombinant DNA and

mRNA transcripts. Bypassing these potentially rate-limiting transport steps during cell line development and protein production may increase the ability of this system to produce larger proteins than those systems which do not have this feature.

Additionally, it is speculated that the capacity for gene insertions in vaccinia virus is up to 25,000 bp of DNA, exceeding the capacity of other mammalian expression vectors (Moss 1991; Smith and Moss 1983). Comparing short term methods of production, viral vectors are considered more efficient than DNA transfection methods and can be used to infect a large volume of cell culture (Moss 1991).

Recombinant proteins produced with vaccinia can undergo N- and O-linked glycosylation, phosphorylation, myristylation, proteolytic cleavage, polarized membrane and nuclear transport, and secretion (Moss 1991). Researchers have used the vaccinia expression system to infect many cell types for production of proteins including a nerve growth factor (NGF) (Edwards et al. 1988) and Factor VIII (Pavirani et al. 1987), wherein the processing modifications of the cell lines were examined. For both proteins, cell lines were identified which produced and properly processed proteins.

Vaccinia virus has been studied for many years as a method of gene delivery in animal species and humans (Brochier et al. 1991; Cooney et al. 1991; Cooney et al. 1993; Graham et al. 1992; Hanke et al. 2002; Hanlon et al. 1998; Kieny et al. 1984; McAneny et al. 1996; McClain et al. 2000; Moss 1996; Wiktor et al. 1984; Zajac et al. 2003) and as a research tool for protein production in small-scale (Arp et al. 1996; Chakrabarti et al. 1997; Davis et al. 1996). Few researchers have investigated the potential for production of proteins in bioreactors via vaccinia virus infection, with

various promoter elements. A 40 l bioreactor with Vero cells attached to microcarriers was used to produce gp160, the full length form of the HIV-1 envelope coat protein that contains gp120 (Barrett et al. 1989). A packed bed bioreactor (1.6 l) utilizing a T7 based co-infection scenario with HeLa cells was used to produce gp120 previously in our lab (Hu et al. 2000). Both studies achieved production of these HIV envelope proteins at 2-3 $\mu\text{g}/10^6$ cells. In this report recombinant vaccinia virus containing the gene for the reporter protein enhanced green fluorescent protein (EGFP) in an inducible T7 promoter based virus construct called the VOTE system (Ward et al. 1995) was tested for its potential in large-scale production. This protein was produced earlier at 2.2 μg EGFP/ 10^6 cells at infection in spinner flask culture of HeLa cells attached to microcarriers (Bleckwenn et al. 2003), similar to the levels found by Hu and Barrett (Barrett et al. 1989; Hu et al. 2000).

Growth of anchorage dependent mammalian cells at large-scale can be accomplished through the use of special reactors such as stacked plate systems, packed bed or hollow fiber bioreactors or with microcarriers in a standard stirred tank bioreactor (Bleckwenn and Shiloach 2004). Microcarrier cultures were chosen for this work because, once seeded with cells, the carriers are treated similarly to suspension culture. However, media replacement, generation, and harvest of the large number of cells needed for seeding the bioreactor may be difficult.

The work presented here concentrates on defining the proper conditions required for the production of recombinant protein from the HeLa cell-vaccinia system with microcarrier culture in a stirred tank bioreactor. Methods for media replacement, infection, and protein expression were developed using EGFP and were

then used to produce gp120. Production and analysis of these proteins provides new insight on the potential of vaccinia for production of complex human proteins.

4.3 Materials and Methods

4.3.1 Cell Maintenance and Expansion

HeLa cells (ATCC CCL-2) were used for all production experiments. BS-C-1 cells (ATCC CCL-26) were used for titer determination of viral stocks. Both were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose (Biosource, Camarillo, CA) supplemented with 10% fetal bovine serum (FBS, Biosource). The cells were grown in tissue culture flasks and passaged every 3-4 days. Cells used for experiments were prepared by expanding the culture to multiple tissue culture flasks and were harvested using Hanks' based cell dissociation solution (Invitrogen, Carlsbad, CA) and counted as described below. Medium used for all experimental conditions was composed of DMEM with 4.5 g/L glucose without phenol red and supplemented with 4 mM L-glutamine (Biosource) and 10% FBS unless otherwise specified. All bioreactor work included 100 units/ml Penicillin-Streptomycin (PS, Invitrogen, Carlsbad, CA) to reduce the chance of contamination.

4.3.2 Cell Count and Viability Measurements

Cell suspension samples were used to measure cell count and viability using 0.4% Trypan Blue exclusion staining (Sigma, St. Louis, MO) on a hemacytometer. To obtain the suspension samples from the anchorage dependent cells, cells from tissue culture flasks were harvested using cell dissociation solution and a suspension of these cells were then counted. Cells from microcarrier based experiments needed

to be removed from the carriers before counting and this was accomplished by the following trypsinization procedure. A sample of the microcarrier was centrifuged at 300×g for 5 minutes. The supernatant was aspirated and the pellet washed with D-PBS with 0.9 mM calcium chloride and 0.5 mM magnesium chloride. The pellet was then resuspended in 1× trypsin-EDTA solution and incubated at 37°C for 30 minutes with vortexing every 10 minutes. This sample was mixed with an equal volume of DMEM+10% FBS and was then used as the cell suspension sample for counting procedures. A sample of the culture with cells still attached to the carriers was visualized via microscopic observation at 100× magnification to estimate the percent coverage of the cells on the carriers.

4.3.3 Nutrient and Metabolite Measurements

Samples of culture medium were taken from the spinner flasks or bioreactors and glucose and lactate were measured on a YSI 2700 Biochemistry Analyzer (Yellow Springs Instrument Co., Yellow Springs, OH).

4.3.4 Seeding and Growth of HeLa Cells on Microcarriers in Spinner Flasks

Cytodex[®] 3 microcarriers (Amersham Biosciences, Piscataway, NJ) were hydrated and washed in PBS and sterilized for 30 minutes at 121°C. The carriers were then washed in sterile PBS and finally in culture medium. Spinner flask (250 ml Bellco, siliconized) cultures were seeded with 1.0×10^5 cell/ml onto 5 g/l Cytodex[®] 3 microcarriers. The initial volume of medium was reduced to half (100 ml) and an intermittent stirring profile (30 sec on and 20 min off for 4 h) was used to attach the HeLa cells to the microcarriers. After seeding, the volume was restored to 200 ml

and the stirring speed was maintained at 50 rpm in a 5% CO₂, humidified, 37°C incubator. Cultures were grown and monitored daily for cell density, viability, percent coverage of the carriers, glucose and lactate.

The cultures used in microcarrier seeding were prepared and grown as described above with 20×10^6 HeLa cells seeded onto 5 g/l Cytodex[®] 3 in 200 ml of culture. These were grown for five days until almost confluent. The microcarriers were then allowed to settle for five minutes, after which 150 ml of medium was removed. The culture was then washed twice with PBS and again reduced to 50 ml total volume. Trypsin-EDTA solution was added (0.25% (v/v) trypsin and 1 mM EDTA final). The spinner flasks were then returned to agitation at 50 rpm for 20 min. 50 ml medium with 10% serum was then added and the flasks incubated for another 10 minutes. Samples were removed and the freely suspended cells counted. 20×10^6 cells from these suspensions were then seeded into new spinner flasks with 5 g/l Cytodex[®] 3 microcarriers in the standard reduced volume with agitation profile for seeding. These cultures were monitored for cell count, viability and estimation of percent coverage of the carriers.

4.3.5 Seeding Microcarriers with HeLa Cells and Growth in Bioreactor

Cytodex[®] 3 microcarriers (5 g/l) were used for all reactor experiments and HeLa cells were seeded at 1.5×10^5 cells/ml in DMEM+10% FBS into a 2.2 l siliconized bioreactor vessel (Bioflow 3000, New Brunswick Scientific, New Brunswick, NJ). The initial seeding volume was reduced to ~500 ml in the reactor and an agitation profile was programmed to control the pitched-blade impeller at 50 rpm for 1 minute (where the carriers and cells would be well mixed) and 20 minutes

at 15 rpm (where the carriers would settle). This was continued for four hours until the cells were attached to the carriers. At the end of this seeding stage, medium was brought to 1.5 l and the agitation adjusted to a constant 50 rpm. The DO and pH were controlled at 30% (air saturation) and 7.2 by altering the headspace gas composition. Temperature was controlled at 36.5°C. The cells were allowed to grow in batch mode for two days, after which perfusion feeding was used to maintain the glucose above 1.0 g/l and the lactate below 2.0 g/l. Generally, exchanged medium volumes ranged from zero, on the first day of seeding, to half a reactor volume per day, on day five before infection, or up to one reactor volume per day for uninfected cultures. This was accomplished through the use of the ATF™ System (ATF, Refine Technology Inc., East Hanover, NJ) with a mesh screen module and controlling the filtrate pump speed which removed spent medium through the ATF (see Results). That is, fresh medium was added when the level controller signaled to the feed addition pump, maintaining a constant reactor volume.

4.3.6 Infection of Bioreactor Cultures and Protein Production

Viable cell density determinations were made prior to infection to determine the total number of cells in the culture. Medium without serum, but containing 1.0 mM IPTG, was exchanged with the culture medium and the volume reduced to ~400 ml using the ATF. These medium manipulations took ~1 h. The amount of virus needed for infection at an MOI of 5.0 was trypsinized for 30 minutes at 37°C with vortexing every 10 minutes and then diluted in 100 ml of serum-free medium with 1.0 mM IPTG. The diluted virus was added to the reactor and the infection phase, in the lowered volume of serum-free medium, lasted for 1 h. The medium was then brought

to 1.5 l and the serum concentration back to 10% FBS. The ATF was run during and after infection, but without drawing off filtrate until step feeding occurred at either 24 or both 24 and 48 hours post infection (hpi). Samples were taken for analysis. Cell density and viability measurements after infection were not possible because the method of removing the cells from the carriers was too harsh for the fragile, infected cells, resulting in cell lysis and inaccurate determinations. Measurements of EGFP fluorescence were made on culture medium and lysed pellet samples according to Bleckwenn (Bleckwenn et al. 2003).

4.3.7 *gp120 Vaccinia Virus Construction*

Plasmid pTM-DHgp120H (kindly provided by Michael Cho, (Lee et al. 2000)) was used to create the gp120 containing vaccinia virus following methods similar to those described in Bleckwenn (Bleckwenn et al. 2003). Specifically, *Nde* I (New England Biolabs, Beverly, MA) was used to partially digest plasmid pTH-DHgp120H to cleave the plasmid at the beginning of the gp120 gene. The reaction mixture was heated to 65°C to stop the reaction and inactivate *Nde* I, after which the mixture was digested with *Xho* I (New England Biolabs) to completion. Additionally, plasmid pVOTE.2 was cleaved with *Nde* I and *Xho* I in sequential digests. The pVOTE.2 digested plasmid was treated with CIP (New England Biolabs). A 0.9% agarose gel was run with the digest samples and the 1536 bp band from pTM-DHgp120H and 6299 bp band from pVOTE.2 were extracted and purified. A ligation reaction was then performed with these fragments, and the resulting plasmid transformed into competent *E. coli* Top 10F'. Plasmid pNB014 was isolated from culture of this strain and gels of an *Nde* I and *Xho* I digest were performed to verify

fragment sizes. Additionally, the plasmid was used for PCR reactions to verify the gene sequence.

Virus vNB014 containing the gp120 and histidine tag (6×HIS) with the VOTE elements was created by the following method. A T-25cm² flask of confluent HeLa cells was infected at an MOI of 0.05 with vT7lacOI for two hours at 37°C in a 5% CO₂ humidified incubator with periodic rocking. At the end of the infection, 5 µg of pNB014 plasmid, containing the gp120-6×HIS gene, was transfected using Lipofectamine Plus (Invitrogen). The cells were incubated for 3-4 days and then recombinant virus was harvested by scraping, centrifuging, and resuspending in 0.5 ml serum-free medium (DMEM, Biofluids). The suspension was subjected to three cycles of freeze-thaw in a dry ice-ethanol and 37°C waterbath. The virus preparation was then amplified under selective pressure with MPA (25 µg/ml), xanthine (250 µg/ml) and hypoxanthine (15 µg/ml) (all from Sigma, St. Louis, MO) in a flask of HeLa cells and harvested after 3 d infection. The resulting virus was harvested and two rounds of plaque purification on confluent monolayers of HeLa cells with selective agents in Minimal Essential Medium (Invitrogen) +10% FBS and 1% agarose were performed to isolate the recombinant virus (Bleckwenn et al. 2003). Amplification of the virus plaque was performed in 12-well, T-25 cm², and T-162cm² flasks under selective pressure and then finally amplified to ten T-162cm² flasks with no selective agents. The purity of the recombinant virus was verified by infecting 12-well culture of HeLa cells and isolating the DNA. PCR reactions of the resulting DNA were performed with primers 5'–CAT ATG AGA GTG ATG GGG ATC AGG AAG AAT –3' forward and 5'–CTC GAG TTA ATG GTG ATG ATG GTG ATG

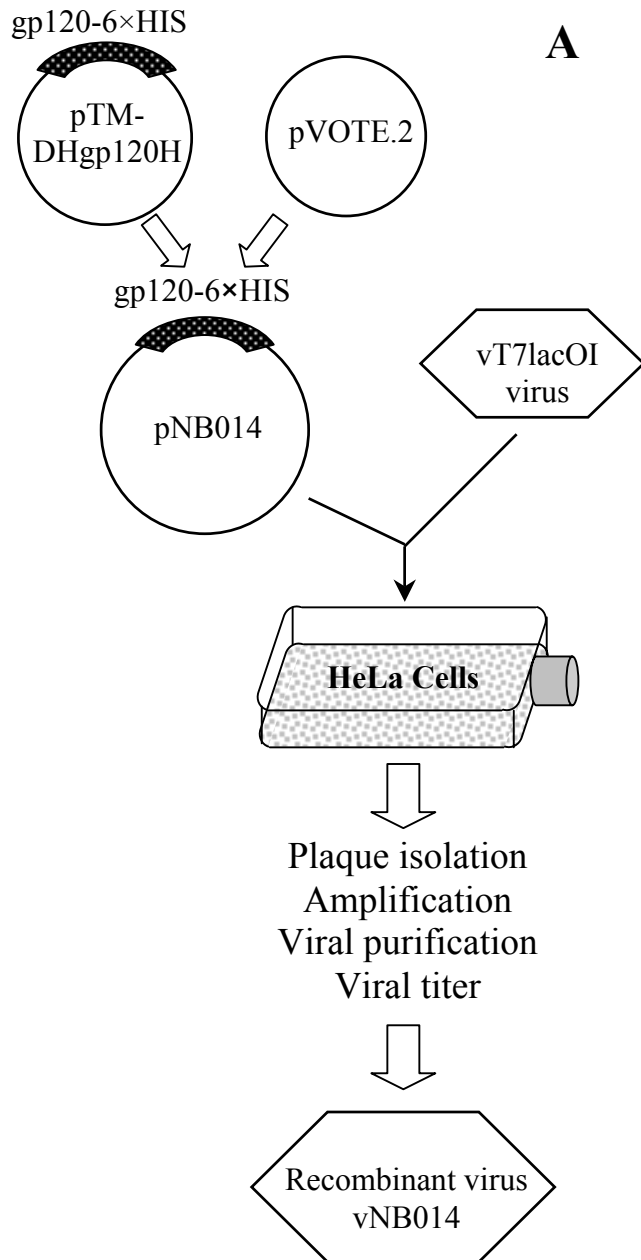
TCT -3' reverse to verify the presence of the gp120-6×HIS gene segment and with primers 5'- CGG TGT CTG TAT GAT CTT CTA C -3' forward and 5'- TGA GTG CTT GGT ATA AGG AGC CC -3' reverse, (designed by Terri Shors NIAID, NIH), to verify the presence of the entire insert containing all of the VOTE promoter control elements. The amplified virus was then purified by 36% sucrose gradient centrifugation and resuspended in buffer (10 mM Tris-Cl, pH 9.0). Titer determination was made in triplicate on confluent monolayers of BS-C-1 cells by serial dilution of the stock and duplicate plating of a range of dilutions. Plaques were counted and the concentration determined to be 1.93×10^{10} pfu/ml. Viral stock vNB014 was frozen at -70°C for storage. Figure 4-1 depicts a schematic representation of the virus construction in Panel A and Western blot verification of gp120 expression in Panel B.

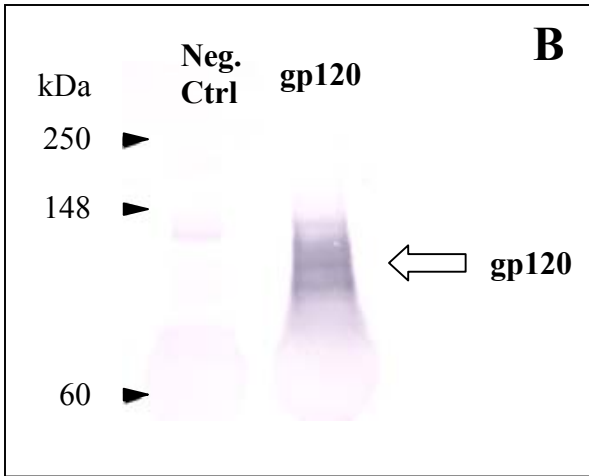
4.3.8 gp120 Protein Purification on Nickel Column

Supernatant from bioreactor cultures were collected at 96 hpi and frozen. Supernatant (200 ml) was thawed to 4°C and NP-40 was added to a concentration of 0.5% to inactivate virus particles and NaH₂PO₄ (20 mM) and NaCl (500 mM) were added. The solution was stirred overnight at 4°C. A Hi-Trap HP 1 ml column (Amersham Biosciences, Piscataway, NJ) was used on an ACTAprime purification unit (Amersham Biosciences). The column was washed with wash buffer (containing 50 mM NaH₂PO₄ 20 mM NaCl and 20 mM imidazole). The protein was then eluted with a 10 ml gradient from 20 to 500 mM imidazole. Fractions (0.5 ml) were collected. The column was then finally washed with wash buffer. Absorbance measurements (A₂₈₀) were made on the elution and wash fractions to determine the

Figure 4-1 gp120 Virus Construction and Expression Verification

pNB014 plasmid was constructed as shown (Panel A) and used for transfection of HeLa cells infected with vT7lacOI to generate recombinant virus containing the gp120 gene. Isolation, amplification, purification, and titering lead to the generation of a viral stock, vNB014 (see Methods). Panel B shows a Western blot of supernatant samples from 6-well plate cultures of HeLa cells infected with either vNB014, containing the gp120 gene in the right lane, or another virus without the gp120 gene as a negative control in the left lane.





location of the protein in 96-well UV-plates (Coring Costar, Acton, MA). The fractions were pooled appropriately and run on reducing SDS-PAGE gels for Western blotting or quantification using SafeStain (Invitrogen).

4.3.9 gp120 Western Blot / N- and O-linked Glycosylation / Biological Activity

Analyses

Analysis of the gp120 protein for vNB014 infected and control cultures was performed by Western blot analysis on supernatant samples, since this protein is secreted into the medium. The reduced samples were run on 4-20% Tris-Glycine SDS-PAGE gels and blotted on nitrocellulose membranes. The membranes were blocked with 5% normal donkey serum (KPL, Gaithersburg, MD) in TTBS at 4°C overnight, then washed with TTBS and incubated with primary antibody (1:2000) for one hour at room temperature. The primary antibody, HIV-1 gp160B Antiserum (HT3) in Goat (Reagent 188), was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH as well as the gp120 standard, HIV-1 gp120 CM (Reagent 2003-CM). The membranes were then washed and incubated with secondary antibody, (1:5000) Donkey Anti-Goat IgG (KPL) for 1 h at rt. They were then washed a final time and developed with BCIP/NBT Phosphatase Substrate (KPL) solution and washed in water and dried. Scans of the Western blots were quantified with the Kodak EDAS system using standards of gp120 for comparison.

Purified gp120 was used for analysis of the glycosylation level of the protein. PNGase treatment to remove N-linked glycans and a series of enzymatic reactions to remove the O-linked glycans were performed using a deglycosylation kit (E-DEGLY

Sigma) and the treated proteins were then run on a reducing SDS-PAGE gel and transferred to nitrocellulose for Western blotting as described above.

A fusion inhibition assay was used to determine the biological activity of the DH12 gp120 envelope protein in the manner previously described in Hu (Hu et al. 2000). Briefly, 1×10^5 PM1 target cells ($CD4^+$, $CXCR4^+$, $CCR5^+$) per well were incubated for 1 hour at 37°C with serial dilutions of the purified gp120 protein from 0.3 to 30 mg/ml in duplicate wells of a 96-well plate. The same number of Tf228 effector cells, expressing BH10/IIIB gp160, were added to each well and the incubation continued at 37°C in a CO_2 humidified incubator another for 3-4 hours. The absence of soluble envelope protein causes fusion of the two cell types and formation of syncytia, which can be inhibited in the presence of soluble gp120 protein. The degree of fusion inhibition was determined for each well, scored after incubation. Plotting the degree of inhibition as a function of gp120 concentration allowed the determination of the 50% inhibitory dose (ID_{50}) from the fusion-inhibition curve (Lee et al. 1997).

4.4 Results

4.4.1 Seeding Cells From Microcarrier to Microcarrier

Small-scale experiments were performed in duplicate to determine the feasibility of seeding cells from already growing microcarrier culture to fresh microcarriers. HeLa cells were grown either in tissue culture flasks, or on 5 g/l Cytodex[®] 3 microcarriers in spinner flasks. Cells were removed from the culture flasks using cell dissociation solution or from the microcarrier culture via

trypsinization inside the spinner flasks and both were seeded into new spinner flasks with freshly prepared microcarriers. Figure 4-2 shows the average growth curves for the two types of cultures seeded.

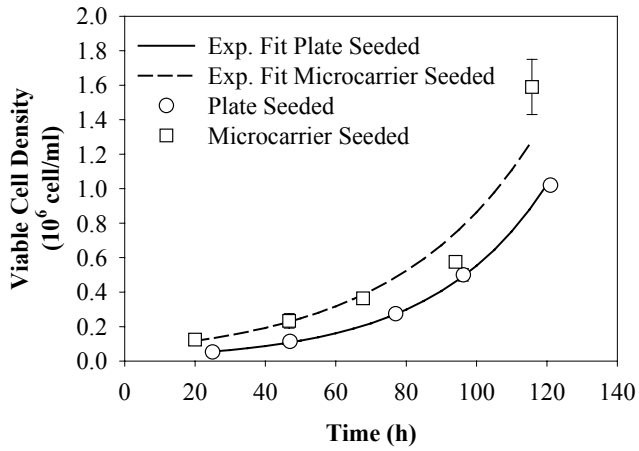
Regression analysis was performed for each flask independently using an exponential equation, $VCD = VCD_0 e^{\mu t}$, to determine the growth rate, μ , and cell density at seeding, VCD_0 . The analysis resulted in R^2 values of 1.0 and 1.0 for the plate seeded cultures and 0.96 and 0.97 for the microcarrier seeded cultures. ANOVA analysis showed no significance difference, $P=0.083$, between the growth rates for these two culture types, 0.031 and 0.025 h^{-1} for plate and microcarrier seeded, respectively. Analysis of the intercepts, or VCD_0 , showed that the plate seeded cultures started with an average VCD of 0.025×10^6 cells/ml versus the microcarrier seeded cultures with VCD 0.071×10^6 cells/ml. These values were statistically different with $P=0.049$.

4.4.2 Growth of Anchorage Dependent HeLa Cells in a Bioreactor

This work utilizes microcarriers in a traditional stirred tank bioreactor for growth of anchorage dependent HeLa cells. To perfuse the culture, the bioreactor was equipped with an alternating tangential flow device (ATF) from Refine Technology, Co. (East Hanover, NJ) The ATF unit is equipped with a nylon mesh membrane with pores of approximately $70 \mu\text{m}$, that allows media and cell debris to pass through, but not microcarriers (Figure 4-3A). The system works by alternatively pulling culture up into the ATF inner chamber via the movement of a diaphragm at the top of the unit. The movement is controlled by alternating air and vacuum cycles. The system cycled about four times per minute for a “slow” pull up of the culture into

Figure 4-2 Seeding Method Evaluation

Cytodex[®] 3 microcarriers were seeded with anchorage dependent HeLa cells harvested either from tissue culture plates (circles) or previous microcarrier culture (squares). Exponential growth curves were fit to the average viable cell densities and are shown by the solid and dashed lines.



the center of the cylindrical mesh filter and a “fast” push of the culture back into the reactor to “self-clean” the mesh membrane and allow sufficient mixing with the contents of the reactor. The filtrate side of the mesh membrane is connected to outlet tubing and a pump draws off spent medium at a controlled rate. The time course of a single pressure-exhaust cycle as seen through the front sight glass of the ATF is shown in Figure 4-3B. Batch growth of HeLa cells without medium exchange was compared to duplicate growth experiments with perfusion via the ATF, results shown in Figure 4-4. Perfusing the culture extended the exponential growth phase by at least 50 hours and lead to a higher maximum viable cell density of 4.4×10^6 versus 1.5×10^6 cells/ml for the batch culture.

4.4.3 Dissolved Oxygen Effect on Protein Production in Bioreactor Culture

The dissolved oxygen level during the protein production phase was tested at two levels (30% and 50%), corresponding to estimates from our previous spinner flask study (Bleckwenn et al. 2004a). Five days after seeding, the culture (1.5 l, HeLa on microcarriers) was infected with vNB009 (with EGFP gene) at an MOI of 5.0 in a reduced volume (~0.4 l) of serum-free medium containing 1.0 mM IPTG. After infection (1 h), serum was reintroduced to 10% FBS and the volume restored to 1.5 l. At this point the DO setpoint was either maintained at 30% or increased to 50% air saturation. The ATF was kept running to prevent drying of cells onto the membrane, but filtrate was not removed for the first day after infection. At 24 hpi, the ATF was used to remove 1 l of spent medium and fresh medium with IPTG was added. Both cultures peaked in expression around 50 hpi, but the 50% DO case produced

Figure 4-3 Reactor Schematic and Culture Motion with ATF™ System

A schematic drawing of the system (Panel A) used for all bioreactor experiments is shown with the ATF components used for perfusion feeding. The motion of the liquid culture medium is seen through the sightglass on the side of the ATF housing (Panel B). The light gray seen in the back of the view above the liquid culture is the cylindrical mesh membrane. As the diaphragm pulls up with vacuum, the culture moves up slowly into the unit in the first five panels, then quickly pushes back into the reactor when the diaphragm extends with air pressure.

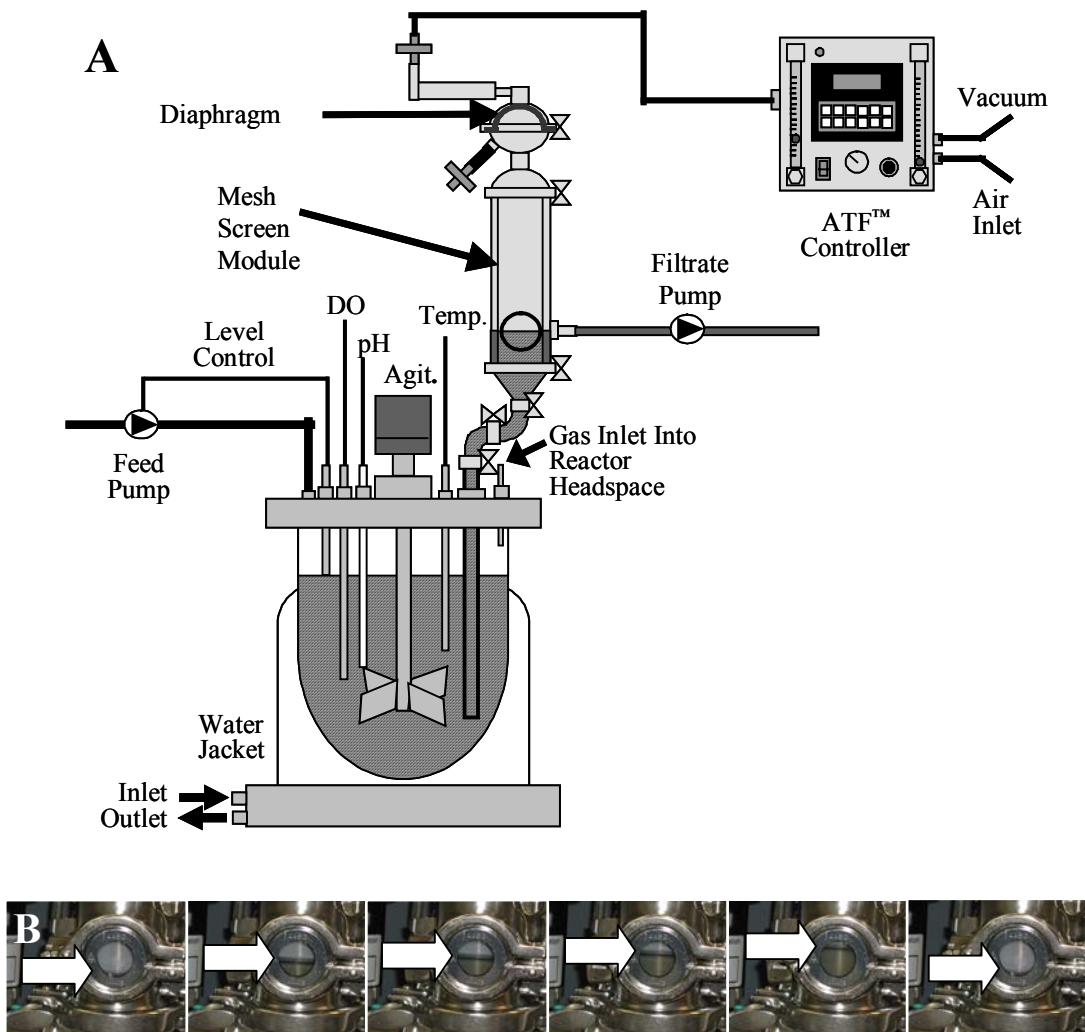
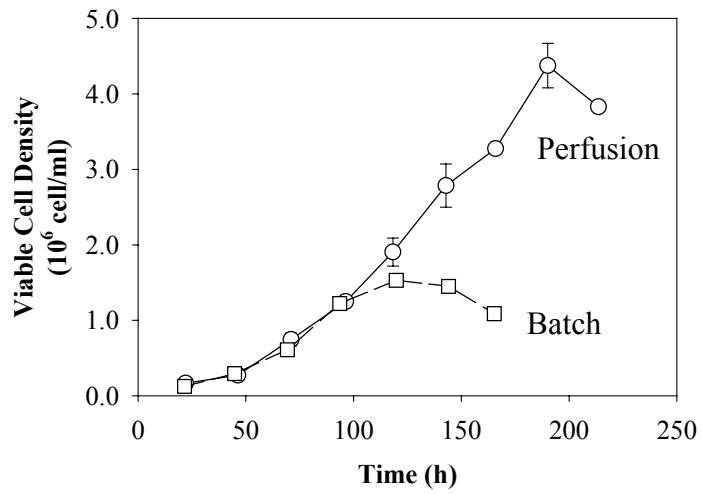


Figure 4-4 Batch Versus Perfusion Growth with ATF™ System

The viable cell densities are plotted over time for bioreactor culture grown with ATF for perfusion feeding (circles) or batch bioreactor culture without feeding (squares).



8.0±0.81 µg/10⁶ infected cells compared to 6.0±1.5 for 30% air saturation (Figure 4-5). Overall, the cultures at 50% DO produced more EGFP than the cultures at 30%.

4.4.4 Temperature Effect on Protein Production in Bioreactor Culture

An evaluation of process temperature during the protein production phase was also made. Our previous study with spinner flasks showed a dramatic increase in production at lower temperatures (31°C). For the bioreactor cultures, cells were prepared and infected in the same manner as described previously, but after the infection phase, the DO was brought to 50% air saturation and the temperature was adjusted to 31°C. In Figure 4-6, the maximum level of EGFP reached for the 31°C culture was 12±1.5 µg/10⁶ infected cells. Over the time course, the level of EGFP remained higher in the 31°C culture than the 36.5°C culture (50% DO data shown in Figure 4-5).

4.4.5 Production of gp120 Protein in Bioreactor Culture

The virus created to produce gp120 was used to infect microcarrier attached HeLa cells in bioreactor culture. The growth, infection, and protein production conditions were chosen based on previous results with the reporter protein, EGFP. HeLa cells were grown on Cytodex[®] 3 microcarriers in a 1.5 l bioreactor utilizing the ATF unit for perfusion. Approximately 3×10⁸ cells were infected with vNB014 at an MOI of 5.0 at ~400 ml (see above) with 1.0 mM IPTG but no serum. After the 1 h infection, the conditions were reset to those of EGFP (31°C, 1.0 mM IPTG, 50% DO). The run profile is depicted in Figures 4-7A and 4-7B. A Western blot of the

Figure 4-5 Effect of Dissolved Oxygen on Protein Production in Bioreactor

Intracellular levels of EGFP are compared for bioreactor runs with the dissolved oxygen at 30% (circles) or 50% (squares) during the protein production phase.

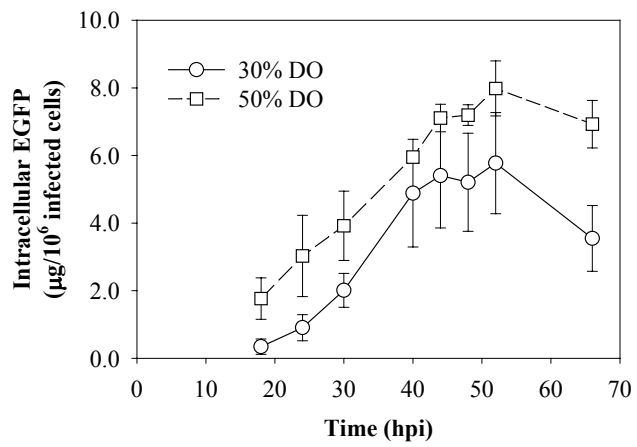


Figure 4-6 Effect of Lowered Temperature During Protein Production in Bioreactor

Intracellular levels of EGFP are shown for bioreactor runs with the temperature lowered to 31°C during the protein production phase.

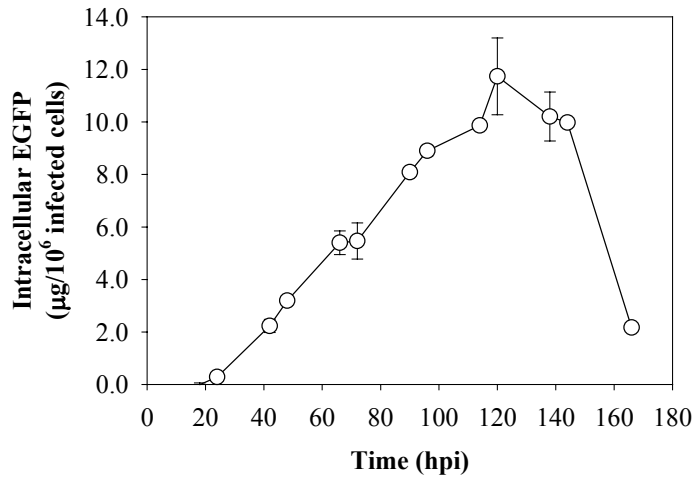
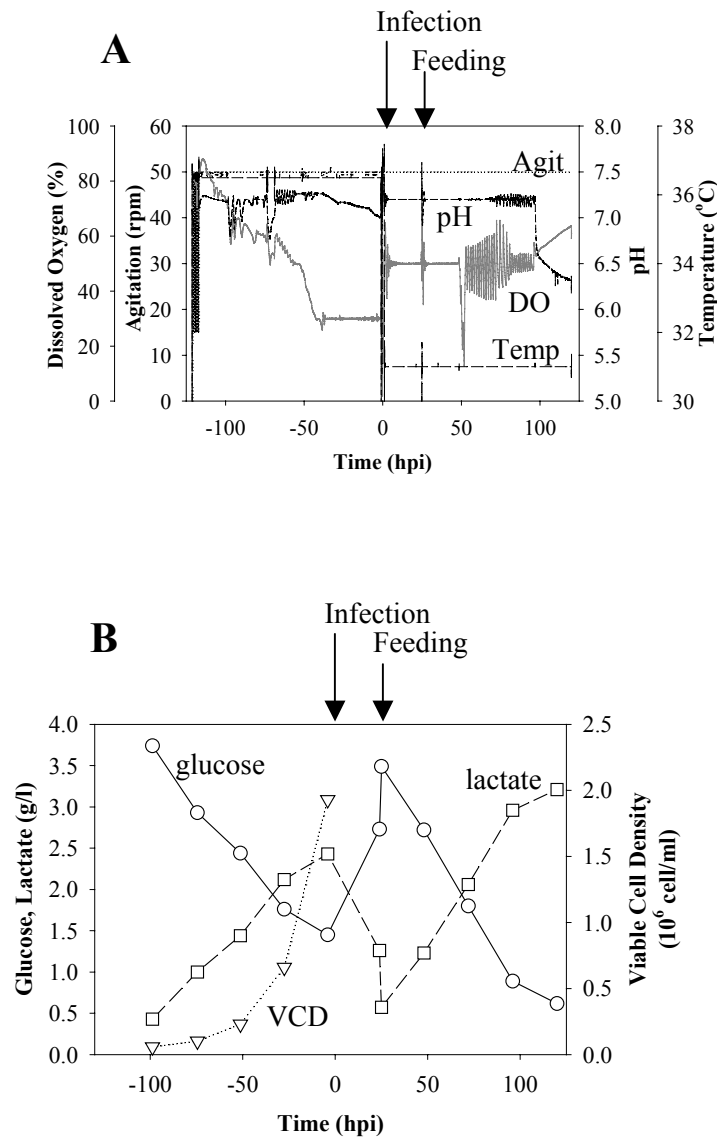
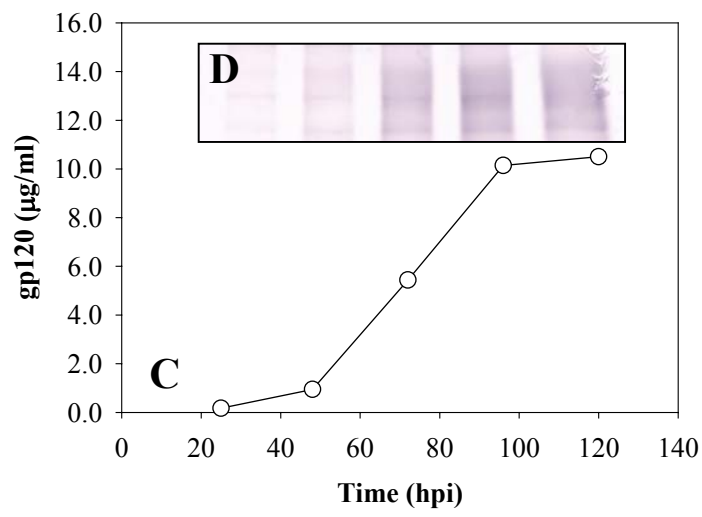


Figure 4-7 gp120 Production in Microcarrier Based Reactor with ATF™

System

Online parameter measurements for the reactor used to produce gp120 protein are shown in Panel A. Panel B shows glucose, lactate, and viable cell density measurements. Arrows indicate medium operations and infection. Panel C shows the expression of gp120 over time, quantified against standards of gp120. Western blot samples corresponding to each timepoint in Panel C are shown inside panel D.





supernatant samples and the corresponding gp120 band quantification are shown in Figures 4-7C and 4-7D. The production run was terminated ~120 hpi when the controlled bioreactor parameters, pH and DO, were observed to vary significantly and visual inspection revealed less than 5% coverage of cells on the carriers. The maximum expression level reached by this culture was 10.5 mg/l at 120 hpi, corresponding to 5.4 $\mu\text{g}/10^6$ infected cells.

4.4.6 Purification of gp120 Protein on IMAC Column

The gp120 was purified via IMAC column (See Methods). Supernatant (200 ml) collected at 96 hpi was treated with NaH_2PO_4 (20 mM) and NaCl (500 mM) and NP-40 (0.5% v/v), to inactivate any virus particles, and then was loaded onto the column (1 ml). The column was washed and the protein was eluted with 10 column volumes of a 20 to 500 mM imidazole gradient (Figure 4-8A). Analysis of the pooled fractions by reducing SDS-PAGE and Western blot with gp160 antibody are seen in Figure 4-8B and 4-8C. The partially purified gp120 was concentrated for further analysis.

4.4.7 Analysis of Produced and Purified gp120 Protein – Glycosylation and Activity

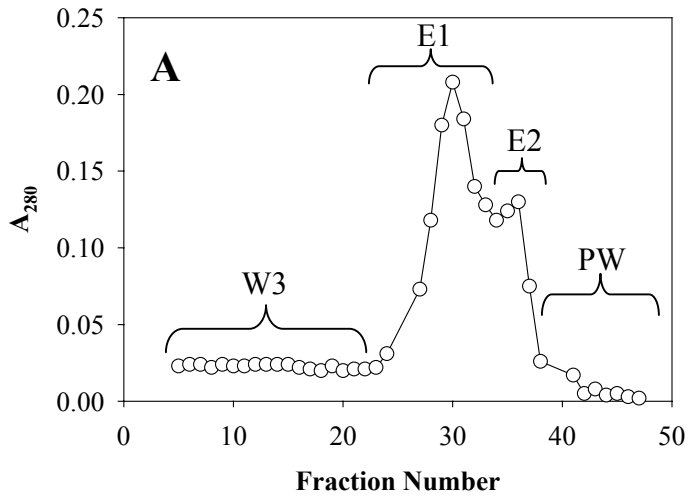
The purified gp120 was subjected to deglycosylation reactions with PNGase, to remove N-linked sugar groups, and a set of enzymatic reactions to remove the O-linked sugar groups. Comparing the results seen in Figure 4-9 for the purified protein and the standard gp120, a drop in the molecular weight of the protein upon PNGase digestion revealed proteins around 60 kDa for both cases. The additional removal of

Figure 4-8 Purification of gp120-6×HIS Protein from 96 hpi Culture

Supernatant

A_{280} measurements of collected fractions are shown in Panel A. Panel B and C show reducing SDS-PAGE and Western blot analysis of purification samples, respectively.

PP= Prior to purification supernatant treated with salts and NP-40 but prior to loading on column, FT= Flow through, W1, W2, W3= Pooled wash fractions, E1, E2= Pooled elution fractions, PW= Pooled post wash



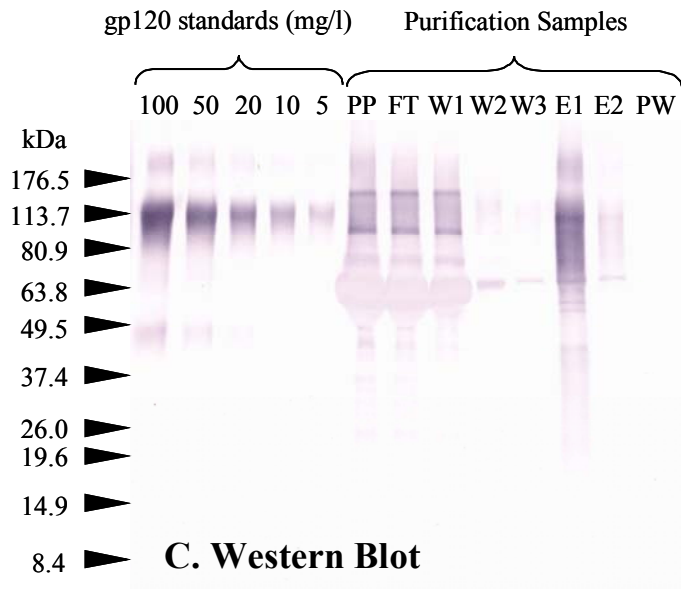
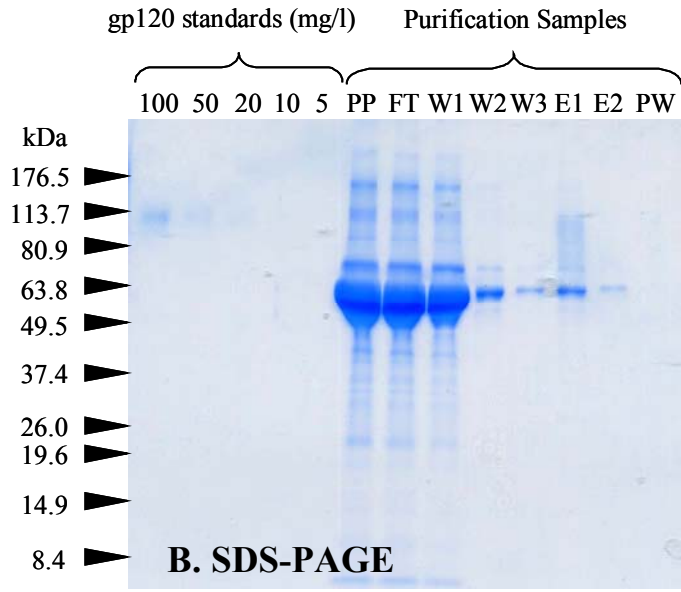


Figure 4-9 Western Blot Analysis of Deglycosylated gp120 Samples

Purified gp120 and gp120 standard were subjected to deglycosylation reactions then analyzed by Western blot. U= Undigested sample, N= PNGase treatment to remove N-linked glycans, N/O= Enzymatic treatment to remove both N- and O-linked glycans. The two arrows at the right show the approximate locations of the full length gp120, at the top, and the deglycosylated form at about 60 kDa, bottom arrow.

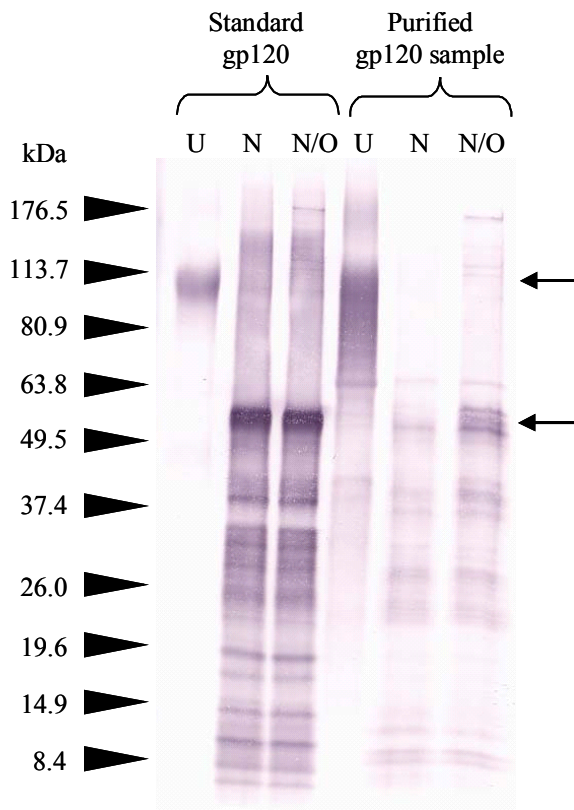
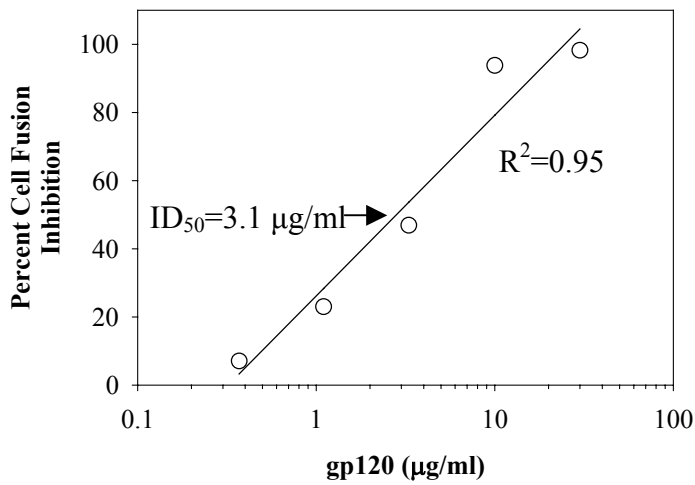


Figure 4-10 Fusion-Inhibition Assay for Biological Activity of Purified gp120

Percent cell fusion inhibition of cells expressing HIV receptors CXCR5 and CCR5 with cells expressing HIV envelope are plotted for varying concentrations of purified gp120 produced from bioreactor culture. The ID₅₀ dose is calculated from the regression line and shown at the location of the arrow.



the O-linked sugar groups does not further reduce the molecular weight. An activity assay was performed to verify the function of the produced protein. Figure 4-10 shows the fusion-inhibition curve for the gp120 produced and purified as above. The activity of gp120 (50% inhibitory dose, ID₅₀) was calculated to be 3.1 µg/ml.

4.5 Discussion

The growth and infection of anchorage dependent cells in large-scale bioreactor cultures present some technical difficulties. A solid support, onto which the anchorage dependent cells can attach, must be provided and medium manipulations for viral infection must be technically manageable, including complete exchange of medium without removing the cells, and the ability to control the bioreactor conditions at a lower volume during the infection phase. This lowered volume has been shown to be beneficial to protein expression in plate culture (Bleckwenn et al. 2003). While several systems that support these tasks are available, our work has focused on the stirred tank reactor with microcarriers because once the carriers are seeded with cells, they can be treated in largely the same manner as cells in suspension culture. A few issues that arise when utilizing microcarrier cultures are the method of aeration, the method of cell separation for perfusion feeding, and the ability to achieve the proper seed train of anchorage dependent cells (Bleckwenn and Shiloach 2004).

The perfusion methods currently used for suspension culture (hollow fiber, acoustic filter, spin filter, and inclined settler) are not suitable for microcarrier culture mainly because they require pumping through tubing, which can clog and damage the cells/carriers (Bleckwenn and Shiloach 2004). Although it is possible to

replace the media by allowing the microcarriers to settle at the spinner flask scale, this operation mode is not practical for large-scale bioreactors. To achieve media separation, we implemented the ATF system which operates by transferring the cell covered carriers directly to the unit through stainless steel tubing. The action of a diaphragm, in response to alternating vacuum and air pressure, pulls the culture to the ATF and pushes it back to the reactor. The ATF houses a mesh screen filter which allows the passage of cell debris and medium to the filtrate line, but maintains the attached cells and carriers in the reactor. Spent medium is removed at a controlled rate and fresh medium can be added to the reactor. The ATF can also be used to retain the cells during medium exchange and volume reduction in preparation for infection. This method of feeding worked well with the Cytodex[®] 3 microcarriers and HeLa cells used in our work. It was able to handle the operations of daily feeding and 3× volume reduction during infection procedures without clogging or loss of function. Compared to batch culture, this perfusion method increased the cell density at infection, thus increasing the volumetric productivity.

It might be interesting to see whether the ATF is capable of selecting viable cells. That is, it was shown for both hybridoma and CHO cell cultures that the use of an inclined settler could preferentially select the smaller nonviable cells for removal from the bioreactor (Batt et al. 1990; Searles et al. 1994). The ATF, with its mesh screen with pore size around 70 μm, should allow passage of any cells not attached to microcarriers. Because the cells are anchorage dependent, it is likely that the most viable cells are attached to carriers and will be retained in the reactor, whereas, nonviable cells will detach and could be selectively removed by this cell separation

method. After infection, cells will begin to detach, but are significantly larger than uninfected cells due to the formation of large syncytia, and we speculate that they might be retained in the reactor.

Obtaining the large number of anchorage dependent cells needed for seeding a bioreactor becomes a significant issue as the scale increases. For spinner flasks or small-scale bioreactor culture, it is possible to harvest the needed cells from multiple tissue culture flasks but this approach is not practical for large bioreactors. To overcome this problem, researchers have previously investigated the potential of different cell lines to be harvested from the microcarrier and then reseeded onto fresh carriers (Barrett et al. 1989). Interestingly, other researchers have found that Vero cells could be seeded from microcarrier to microcarrier by adding fresh microcarriers to a culture of confluent carriers and repeating the agitation profile, with no trypsinization of the cells (Wang and Fan 1999). Upon comparison of HeLa cultures seeded from harvested tissue culture plates or harvested from carriers and reseeded onto fresh carriers, we found that there was no significant decline in growth rate or cell density achieved five days after seeding, when infection would occur. The culture seeded from carrier culture, appeared to seed onto the carriers better, increasing the initial cell density and thereby maintaining a slightly higher cell density than the tissue culture plate seeded culture over the five days tested. This phenomenon suggests a possible adaptation to attachment of the cells on the carriers which may be passed on to the next stage in the seed train.

In addition to increasing cell density, our work focused on two parameters that affect protein yield, dissolved oxygen and temperature. The level of dissolved oxygen

in bioreactor culture can contribute to high yields of protein (Kunkel et al. 2000; Lin et al. 1993; Lin and Miller 1992). Some researchers have found optimum levels of DO increased protein production, but that high levels decreased production due to a concurrent increase in proteases in the culture, (Hu and Bentley 1999; Naggie and Bentley 1998; Scott et al. 1992; Wang et al. 1996). In our expression system increasing the DO from 30% to 50% during the protein production phase resulted in a higher maximum level of intracellular EGFP. Although these results were only minimally different, the higher trend in expression for the 50% DO case, maintained throughout the course of the production phase, suggests that expression may benefit from increasing DO.

Most mammalian cell lines grow best at 37°C, and significant deviations above this temperature are devastating to the cells (Freshney 2000). Lower temperatures are tolerable, but the cells grow slower. Interestingly, Schlaeger and Lundstrom (Schlaeger and Lundstrom 1998) found higher yield at lowered temperature (33°C) with Semliki Forest Virus (SFV) infection. They suggested that a reduction in the cytopathic effects from the viral infection may increase the life of the cells and lead to a longer protein production phase, thereby increasing protein yield. In the present work, temperature was adjusted after the infection phase to ensure that this change had no affect on the kinetics of infection, only on protein production and cytopathic viral effects. Lowering the temperature to 31°C increased specific yield fifty percent over the 36.5°C culture yielding about 20 mg/l intracellular EGFP. This modest increase was not as dramatic as that seen in the previous study in spinner flask culture (Bleckwenn et al. 2004a). In this case, lowering the temperature to 31°C also

slows the process and draws out protein production over a longer period of time, with the maximum occurring about 75 hours after the maximum for the culture at 36.5°C.

Once the previous experiments had lead to a defined culture process with the model protein, EGFP, a virus containing the gene for gp120, a secreted HIV-1 envelope coat protein with significant post-translational modification, was used to infect bioreactor culture and verify the production and processing capabilities of this system. Maximum expression occurred at 120 hpi with a level reaching 10.5 mg/l, corresponding to 5.4 $\mu\text{g}/10^6$ infected cells which is about double the amount seen previously with the vaccinia system (Barrett et al. 1989; Bleckwenn et al. 2003; Hu et al. 2000). The protein was purified using an IMAC column and was treated with enzymes to remove N-linked or both N- and O-linked sugar groups. The results revealed that the molecular weight of the protein drops to 60 kDa upon N-linked sugar group removal, but does not drop further on O-linked sugar group removal. This suggests that the majority of the glycosylation modifications are N-linked for this protein. Additionally, the molecular weight of this treated protein was approximately the same as the theoretical amino acid weight for the protein with no post-translational modifications (~58 kDa). A fusion-inhibition assay was used to determine the biological activity of the purified gp120 which resulted in an ID_{50} of 3.1 $\mu\text{g}/\text{ml}$, similar to that seen by Hu et al (Hu et al. 2000). This shows that the protein was active in binding the receptors CXCR5 and CCR5 and blocking fusion with cells expressing HIV envelope.

4.6 Conclusions

The utility of the VOTE vaccinia virus expression system for producing protein in mammalian cells has been established. Several issues relating to the practical operations of growing and infecting anchorage dependent cells have been addressed through the use of the ATF System for perfusion and medium manipulations, which can be scaled up through the use of larger units. Large-scale considerations for seed train manipulations were evaluated and shown to be reasonable by the ability of these cells to be seeded from previous microcarrier cultures onto fresh carriers. The controllable process parameters of dissolved oxygen and temperature have been studied for their effect on recombinant protein production during the production phase and lead to increases in protein yield, albeit more conditions and replicates might be warranted for locating an optimum. These results indicate that, with proper optimization, the vaccinia virus-HeLa expression system can yield high levels of recombinant protein. The system could be further optimized by adjusting other process parameters such as pH or altering medium components. With the basic process parameters defined, a complex protein of commercial interest, gp120, was then produced in bioreactor culture. The protein was purified for post-translational modification and biological activity analyses, both proved that the protein was glycosylated and active.

CHAPTER 5: Perfusion of Mammalian Cell Suspension Bioreactor Culture and Reduced Temperature During Production to Increase Protein Yield with the Vaccinia Virus Expression System⁴

5.1 Summary

Adaptation of the vaccinia virus expression system to HeLa S3 suspension bioreactor culture for the production of recombinant protein was conducted. Evaluation of a hollow fiber device for perfusion of suspension culture demonstrated its potential for increased cell density prior to infection and lengthened exponential growth phase. The hollow fiber was also used for medium manipulations prior to infection. Two process parameters, multiplicity of infection (MOI) and temperature during the protein production phase, were evaluated to determine their effect on expression of the reporter protein, EGFP. An MOI of 1.0 was sufficient for infection and lead to the highest level of intracellular EGFP expression. Reducing the temperature to 34°C during the protein production phase increased production of the protein two-fold compared to 37°C in spinner flask culture. Scaling up the process to a 1.5 liter bioreactor with hollow fiber perfusion, lead to an overall production level of 10.44 µg EGFP/10⁶ infected cells, or 27 mg EGFP per liter.

⁴ Bleckwenn NA, Bentley WE, Shiloach J. 2004. Perfusion of mammalian cell suspension bioreactor culture and reduced temperature during production to increase protein yield with the vaccinia virus expression system. Submitted 2004.

5.2 Introduction

Vaccinia vaccinia virus infection has been described previously as a method to obtain small quantities of proteins in HeLa S3 cultures (Hooper et al. 1993; Jun et al. 1996) and as a gene delivery vector (Cooney et al. 1991; Cooney et al. 1993; Gomella et al. 2001; Graham et al. 1992; Hanke et al. 2002; Hanlon et al. 1998; McAneny et al. 1996; McClain et al. 2000; Wiktor et al. 1984; Zajac et al. 2003). To begin the process of scaling up this viral expression method for the production of large amounts of recombinant proteins, evaluation of cell growth, infection, and protein production are required.

A recombinant vaccinia virus expressing enhanced green fluorescent protein (EGFP) controlled by the T7 promoter based VOTE expression system (Ward et al. 1995) was previously constructed (Bleckwenn et al. 2003). Infection of microcarrier attached HeLa cells grown in spinner flasks with the recombinant vaccinia virus produced 2.2 μg EGFP/ 10^6 cells at infection (Bleckwenn et al. 2003). An optimization of several of the infection and process parameters such as inducer concentration, multiplicity of infection, dissolved oxygen (DO), and temperature lead to an increase in the production to about 17 μg EGFP/ 10^6 infected cells in spinner flask cultures (Bleckwenn et al. 2004a). Protein levels from bioreactors have been lower (e.g. 2-3 μg / 10^6 cells for HIV gp160 and gp120 (Barrett et al. 1989; Hu et al. 2000)) so we were interested in making process modifications that would increase this yield.

That is, to assess the potential of this system, growth strategies implementing hollow fiber perfusion and other production parameters such as multiplicity of

infection (MOI) and temperature were evaluated. Medium replacement via perfusion can dramatically boost cell density and yield (Chen et al. 1992; Kyung et al. 1994; Zhang et al. 1993). The MOI also plays an important role in expression. Interestingly, previous work showed that the best MOI was different for tissue flask and microcarrier cultures (Bleckwenn et al. 2003; Bleckwenn et al. 2004a). This was not unique to vaccinia, differences between tissue plate culture and suspension culture were shown in baculovirus/insect cell studies (Dee and Shuler 1997a). Further, lowered temperature during the protein production phase was shown to be beneficial for both the Semliki Forest Virus expression system (Schlaeger and Lundstrom 1998) and the vaccinia virus expression system in microcarrier based cultures (Bleckwenn et al. 2004a). This may have been due to a reduced cytopathic effect from the virus at lowered temperatures, allowing the cells to remain intact and producing protein for a longer period of time, although this was not shown.

Several methods are available for perfusion of suspension culture including hollow fiber, spin filter, and acoustic filter (Bleckwenn and Shiloach 2004). The present viral expression method requires that the cell separation device enable rapid medium exchanges, such as during the infection process when the serum concentration and volume are reduced creating a more effective infection environment. The hollow fiber system is relatively simple, requiring only a filter cartridge, tubing, and peristaltic pumps. Hollow fiber cartridges have been successfully used by other laboratories for perfusion of mammalian cell cultures (Chen et al. 1992; Kyung et al. 1994; Zhang et al. 1993) and can handle large rates of

exchange if sized properly. This work describes scale-up evaluation of the HeLa S3-vaccinia protein expression method in a hollow fiber perfusion culture.

5.3 Materials and Methods

5.3.1 Cell Maintenance and Expansion

HeLa S3 cells (ATCC CCL-2.2) were maintained in 200 ml spinner flasks in Dulbecco's Modified Eagle's Medium (DMEM, Biosource, Camarillo, CA) with 4.5 g/l glucose, without calcium, supplemented with 4 mM glutamine (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS, Biosource). The cultures were passaged every 3-4 days, reducing the cell density to approximately 2×10^5 cell/ml while retaining at least 10-20% conditioned medium in the new culture. Cultures were expanded into multiple 1000 ml spinner flasks to obtain the number of cells needed for seeding bioreactor cultures.

5.3.2 Cell Density and Viability Measurements

Samples of cell suspension were taken from cultures and a portion diluted in PBS and mixed with 0.4% trypan blue solution (Sigma, St. Louis, MO) for viability staining. The stained solution was then counted using a hemacytometer to determine total cell density and viability.

5.3.3 Metabolite Measurements

Glucose and lactate measurements were made on supernatant samples from spinner flask or bioreactor cultures using a YSI 2700 Biochemistry Analyzer (Yellow Springs Instrument Co., Yellow Springs, OH).

5.3.4 Spinner Flask Seeding and Growth

Spinner flask cultures containing 200 ml of culture in 250 ml Bellco spinner flasks with stir bar and paddle (Bellco Glass, Inc., Vineland, NJ) were seeded with 1.0×10^5 cell/ml. Agitation speed was set at 50 rpm in a 5% CO₂, humidified, 37°C incubator. Cultures were monitored daily for cell density, viability, glucose and lactate.

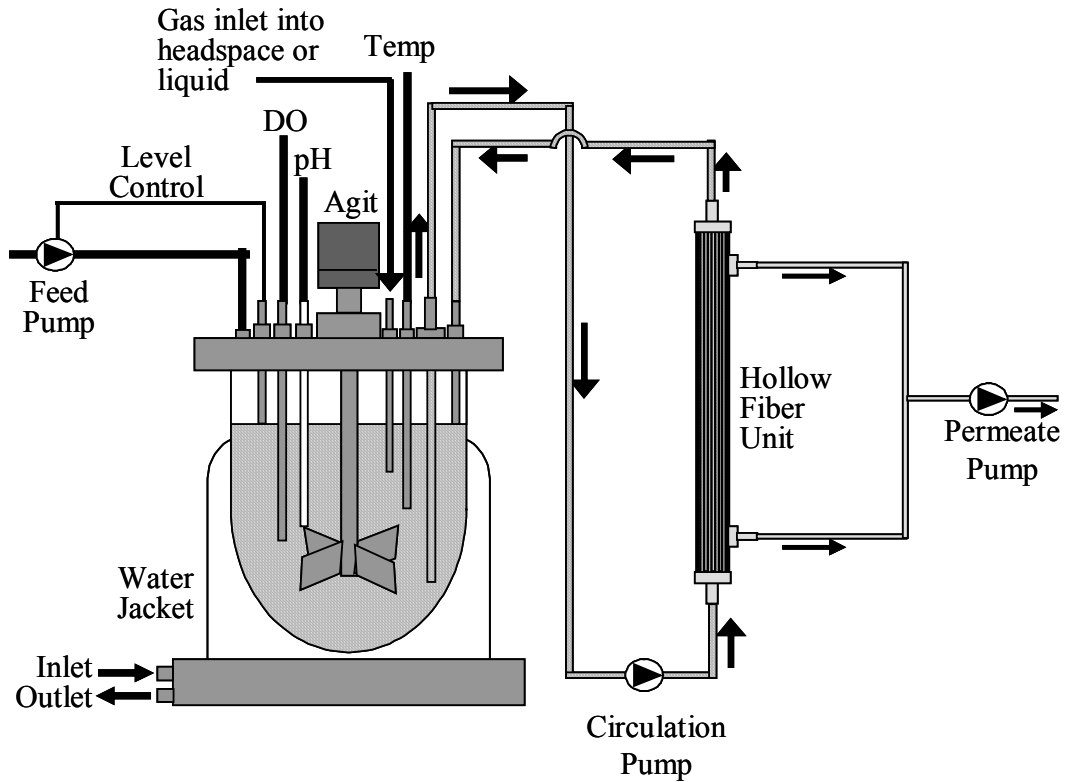
5.3.5 Suspension Bioreactor Seeding and Growth

HeLa S3 cells were seeded into a 2.2 l bioreactor (New Brunswick Scientific, New Brunswick, NJ) with a working volume of 1.5 l using the same medium as was used for spinner flask culture with the addition of 100 units/ml Penicillin and 100 µg/ml Streptomycin (Invitrogen) to reduce the chance of contamination.

Approximately 10-20% of the final reactor volume was conditioned culture medium from the seeding spinner flasks to ensure growth of the culture. Cells were seeded to a density of 2×10^5 cell/ml and grown at 36.5°C, 30% DO based on air saturation at 36.5°C, and pH 7.2. Perfusion feeding was achieved for this culture by using a hollow fiber cartridge (A/G Technology Corp., Needham, MA) with 0.45 µm pores and 1.0 mm inner diameter capillaries through which the culture flowed. Culture was circulated at a rate of about 15 reactor volumes per day and medium exchanged up to 1.5 reactor volumes per day via level controlled addition of fresh feed. Aeration was achieved through headspace gassing at 1.0 l/min until day 3-4 when the aeration was switched to sparging into the culture at 0.3 l/min and 0.1% Pluronic F-68 was added to reduce foaming and shear effects. A schematic of the reactor setup is shown in Figure 5-1 which details the arrangement of reactor components and hollow fiber

Figure 5-1 Suspension Bioreactor Schematic with Hollow Fiber Unit for Perfusion

A schematic diagram of the cell suspension based bioreactor with the hollow fiber unit for perfusion is shown with the probes and control sources for the online parameters.



perfusion unit. Samples were taken daily to determine cell density, viability, and glucose and lactate concentrations.

5.3.6 Virus Preparation

Virus containing the gene for EGFP, vNB009, was constructed as described previously (Bleckwenn et al. 2003). A portion of the viral stock was incubated 1:1 with 1× trypsin at 37°C for 30 minutes with vigorous vortexing every 10 minutes, to break up clumps. This preparation was then used to infect spinner flask or bioreactor culture at the appropriate MOI.

5.3.7 Infection

Spinner flask cultures of HeLa S3 cells in exponential phase were mixed to obtain a uniform population for infection experiments. 20×10^6 cells were centrifuged at $300 \times g$ for 5 minutes for each experimental condition. Cells were resuspended in approximately 20 ml of their conditioned medium. They were transferred to fresh Bellco 250 ml spinner flasks with stir bar and paddle. Medium was brought to 70 ml with serum-free medium and inducer was added to 1.0 mM IPTG. Virus vNB009 was added to infect at the appropriate MOI and the flasks were returned to the incubator for one hour of infection. Medium was then brought up to 200ml with 10% FBS and 1.0 mM IPTG. The cultures were incubated and samples taken daily for cell count, viability, glucose, lactate, and protein determination. Feeding was performed to keep glucose above 1.0 g/l and lactate below 2.0 g/l at either 24 hpi or both 24 and 48 hpi by centrifuging half of the culture at $300 \times g$ for 5 minutes to pellet the cells,

aspirating the medium and resuspending them in fresh medium with IPTG. This mixture was then returned to the spinner flask and the culture incubated.

5.3.8 Production in Bioreactors

Five days after seeding 2×10^5 cell/ml into the bioreactor, viable cell density measurement was made on the culture to determine the total number of cells. Medium without serum or Pluronic F-68 but containing 1.0 mM IPTG was connected to the reactor through the feed addition pump. A hollow fiber cartridge was used to perform exchange of the culture supernatant to this serum-free induction medium and to reduce the volume in the reactor to about 400 ml. This exchange and reduction process took about five hours to perform. Trypsinized virus vNB009 was diluted in 100 ml of serum-free medium and added to the reactor to infect at an MOI of 1.0. The infection phase lasted one hour in this reduced serum and volume environment, after which the medium volume was brought back to 1.5 liters and serum and Pluronic F-68 reintroduced to 10% and 0.1%, respectively. The temperature and DO setpoints were also adjusted at this time to either 34°C or 36.5°C and 50%, respectively. The culture was gradually fed using the hollow fiber for exchange and level control for addition of one liter of media between 24 and 48 hpi. Samples were taken periodically and analyzed for glucose, lactate, cell density, viability, and recombinant protein expression.

5.4 Results

5.4.1 *Effect of MOI on EGFP Production with Suspension HeLa S3 Cells*

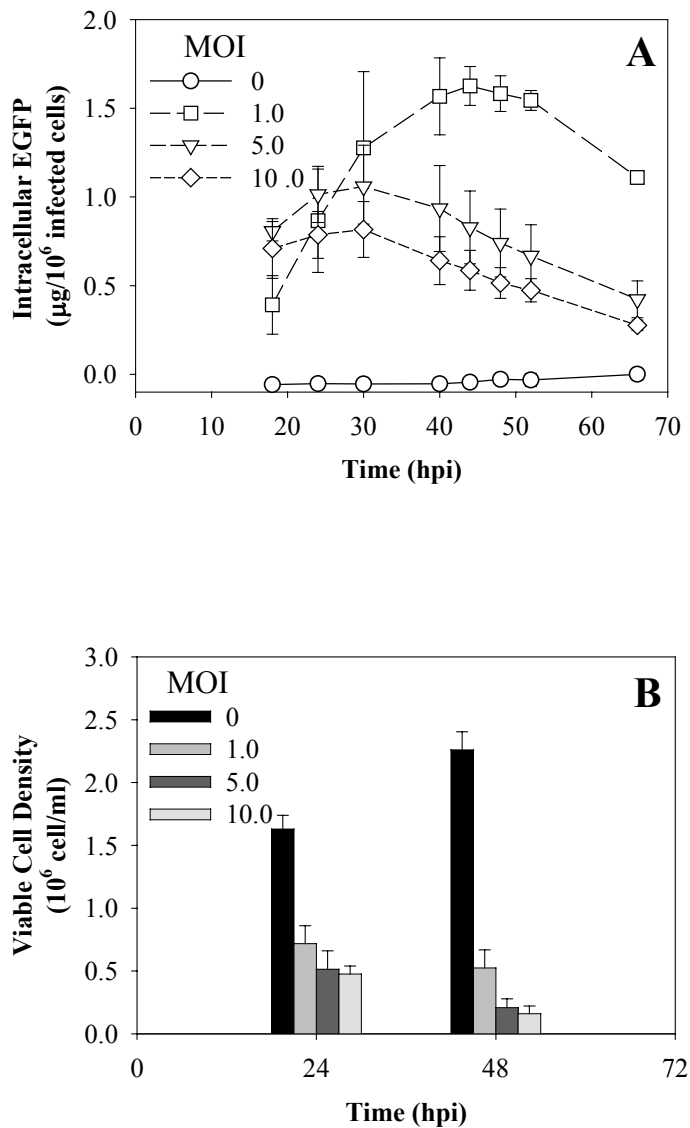
As noted earlier, the effects of MOI might change depending on scale and reactor configuration. Here, we first evaluated the MOI in spinner flasks. Spinner flask cultures with suspension HeLa S3 were used to determine the effect of MOI on the recombinant protein yield. Cultures (200 ml) were infected with vaccinia virus carrying the EGFP reporter gene, vNB009, at MOI 0.0, 1.0, 5.0, and 10.0 pfu/cell. The resulting intracellular EGFP expression values are shown in Figure 5-2A. MOI 1.0 had the highest expression level at $1.6 \pm 0.11 \mu\text{g}/10^6$ infected cells occurring ~44 hpi. This value was statistically higher than that for MOI 10.0, $P=0.017$, but not MOI 5, $P=0.053$, where both MOI 5.0 and 10.0 had maximums occurring ~14 hpi earlier than MOI 1.0. Viable cell densities measured at 24 and 48 hpi are shown in Figure 5-2B, showing slightly higher cell densities at 24 and 48 hpi for MOI 1.0 as compared to MOI 5.0 or 10.0, although these were not statistically different, $P \geq 0.084$. In all three cases, the intracellular EGFP levels drop after reaching their maximums.

5.4.2 *Effect of Temperature on EGFP Expression in HeLa S3 Suspension Culture*

Lowering the culture temperature in a viral expression system may increase the protein yield. Here, we evaluate temperature reduction in spinner flask cultures containing 10^6 HeLa S3 cells/ml infected at an MOI of 1.0 with the recombinant virus carrying the EGFP reporter gene. After infection, the flasks were incubated at 25, 28, 31, 34, and $37^\circ\text{C} \pm 0.5^\circ\text{C}$ (37°C culture results were from previous MOI experiment at MOI 1.0) and the intracellular EGFP was determined. The expression profiles are

Figure 5-2 Effect of Multiplicity of Infection on Production in Spinner Flask Cultures

The intracellular EGFP levels are shown in Panel A for MOI = 0 (circle), 1.0 (square), 5.0 (triangle), and 10.0 (diamond). The viable cell densities at 24 and 48 hpi are shown in Panel B for MOI = 0 (black), 1.0 (medium gray), 5.0 (dark gray), and 10.0 (light gray).



shown in Figure 5-3A. Maximum EGFP levels were seen with 34°C at 44 hpi, 31°C at 52 hpi, and 28°C at 96 hpi. The EGFP levels achieved were 3.28 ± 0.080 , 2.57 ± 0.36 , and $3.4 \pm 0.29 \mu\text{g}/10^6$ infected cells, respectively, all within error of each other, $P \geq 0.057$. All three of these temperatures resulted in maximum EGFP levels above 37°C and 25°C with statistical significance, $P \leq 0.0065$. The percent viability is plotted in Figure 5-3B which shows that 25°C and 28°C maintain higher cell viabilities than temperatures above 30°C.

5.4.3 Perfusion Growth of HeLa S3 Suspension Cells

The growth profile of HeLa S3 suspension cells was determined by growing the cells using perfusion mode in a bioreactor equipped with a hollow fiber membrane cartridge. The total cells density and the percent viability are shown in Figure 5-4. Exponential growth of the HeLa S3 cells was maintained for about seven days in this system with a maximum viable cell density of 1.3×10^7 cells/ml.

5.4.4 Production of EGFP in HeLa S3 Suspension Bioreactor with Hollow Fiber Perfusion

EGFP was produced in perfused HeLa S3 bioreactor culture under the conditions determined above for the MOI and temperature and compared to a single production run at 36.5°C. The results for the runs at 34°C are shown in Figures 5-5A to 5-5D and the comparison of expression in Figure 5-6. Infection was performed in the bioreactor at a cell concentration of $2.9 \pm 0.37 \times 10^6$ cell/ml, using an MOI of 1.0, and adjusting the temperature during production to either 34°C or 36.5°C. Figures 5-5A to 5-5C described the overall production process at 34°C with the online

Figure 5-3 Effect of Temperature on Production in Spinner Flask Cultures

The intracellular EGFP levels are shown in Panel A and viabilities in Panel B for temperatures during the production phase of 37°C (square, data from Figure 5-2A), 34°C (circle), 31°C (downward pointing triangle), 28°C (diamond), and 25°C (upward pointing triangle).

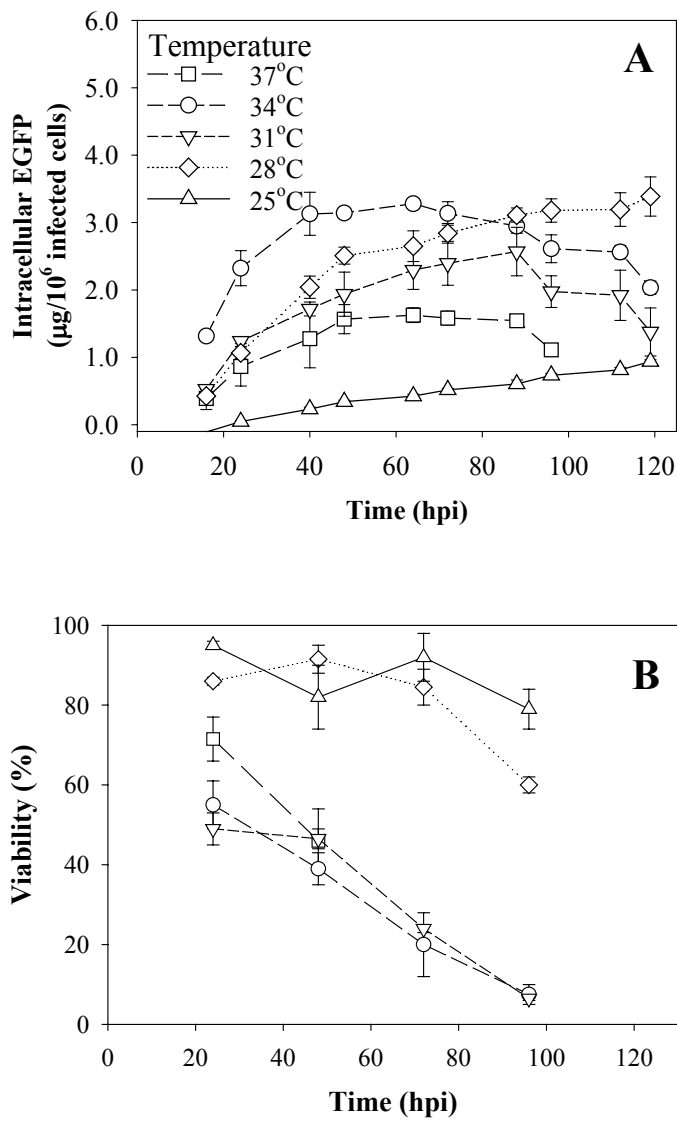
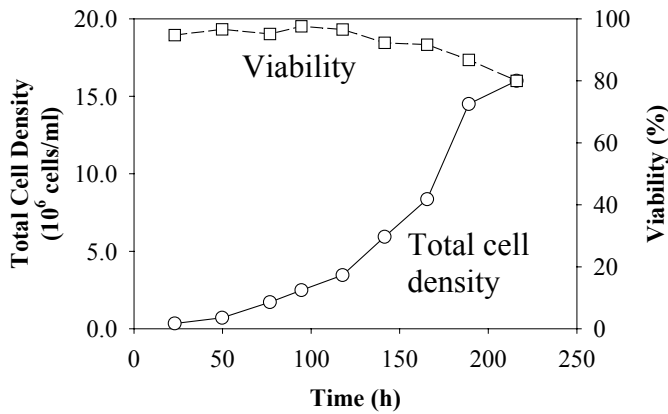


Figure 5-4 Growth of HeLa S3 Cells in Suspension Bioreactor Culture with Hollow Fiber Perfusion Device

Total cell density (circle) and percent viability (square) are shown over the course of a single bioreactor run growing HeLa S3 suspension cells while utilizing a hollow fiber cartridge for perfusion of the culture. Error in measurement is less than 12% for total cell density, except for one anomalous measurement at 189 hours with 27%, and 2.5% for viability.



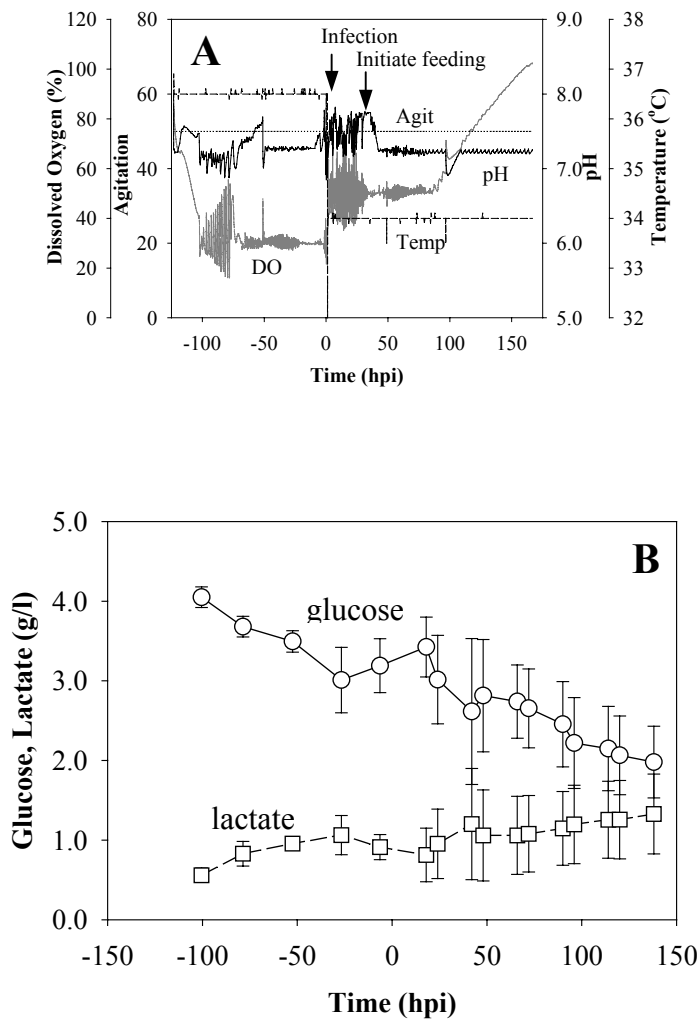
parameters for a typical production run in 5-5A, the average glucose and lactate values in 5-5B and the average total cell density and percent viability in 5-5C. Intracellular and extracellular levels of EGFP are shown in Figure 5-6 for both temperatures. The maximum intracellular EGFP was achieved for the production run at 34°C, ~120 hpi at $10.44 \pm 0.018 \mu\text{g}/10^6$ infected cells. This corresponds to 27 mg/l, which is higher than the level obtained at 36.5°C (at $3.5 \mu\text{g}/10^6$ infected cells). In both cases, the intracellular level of EGFP drops after reaching the maximum value, and the extracellular concentration increases over time.

5.5 Discussion

Vaccinia virus infection of HeLa S3 cells has been used to produce small quantities of proteins in spinner flask cultures (Hooper et al. 1993; Jun et al. 1996). Scale-up of the process for production in bioreactor culture requires an examination of critical process parameters such as multiplicity of infection, temperature, and cell density. Multiplicity of infection, in other viral systems, has been shown to affect protein yield (Blasey et al. 1997; Gotoh et al. 2002; Hu and Bentley 2001). In this work, the optimum MOI was found to be 1.0, indicating that only one infectious virus particle was needed per cell to achieve maximum expression. This is less than the MOI of 5.0 that was found optimum for the anchorage dependent HeLa cell line in microcarrier based culture (Bleckwenn et al. 2004a). The infection kinetics are certainly different for the cell suspension based system than in the microcarrier based system. A similar phenomenon was described previously for baculovirus infection where suspension cultures had higher attachment rates for the virus than monolayer cultures (Dalal and Bentley 1999; Dee and Shuler 1997a). Initially, it was thought

Figure 5-5 Production Run at 34°C

The online parameters measured through the data acquisition program are plotted in Panel A for a typical run from seeding, through infection, to harvest, with arrows showing the onset of infection and initiation of feeding after infection. Average glucose (circle) and lactate (square) values are shown in Panel B over the course of the two production runs. Average total cell density (circle) and percent viability (square) values are shown in Panel C over the course of the two production runs.



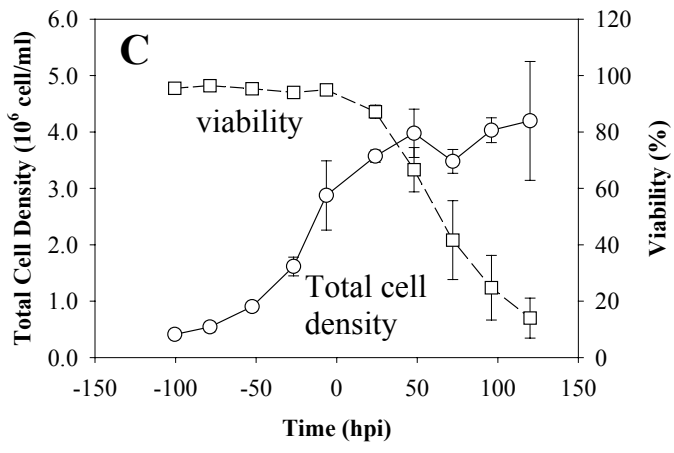
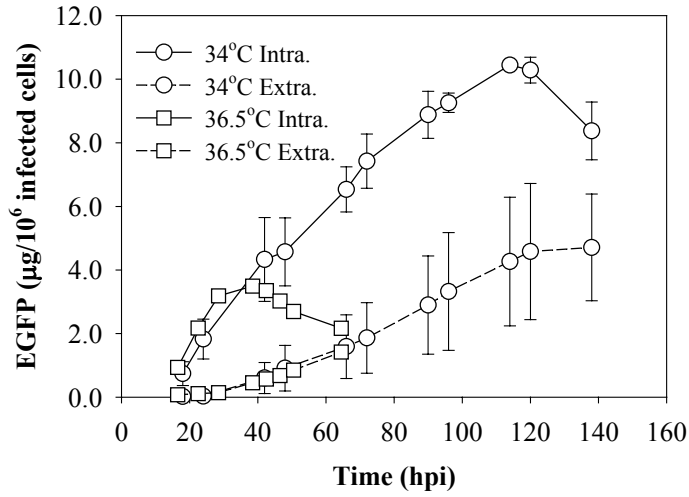


Figure 5-6 Production of EGFP in HeLa S3 Suspension Bioreactor

Average intracellular (circle with solid line) and extracellular (circle with dashed line) levels of EGFP for the average of two production runs at 34°C and intracellular (square with solid line) and extracellular (square with dashed line) levels of EGFP for a single production run at 36.5°C are shown.



that the infection in the vaccinia infected suspension system might be less efficient than the microcarrier based system because, in order to maintain cell health, a portion of conditioned medium containing serum was carried over into the infection medium. Previous results with the anchorage dependent HeLa cells showed that the removal of serum during infection lead to the highest level of expression (Bleckwenn et al. 2003). However, it appeared that the level of serum was reduced sufficiently enough to not interfere with infection, which agreed with the results of Bleckwenn that a low level of serum, 2.5%, did not dramatically lower the expression. In the present system, MOIs of 5.0 and 10.0 appeared to increase cell death over MOI 1.0, as indicated by the lower viable cell densities reported at 24 and 48 hpi, but these results were not shown to be statistically significant. This lower viable cell density may partially explain the lower expression at these higher MOI, due to a smaller number of cells available for protein production.

Several researchers have modeled virus systems to gain an understanding about the limiting factors for viral infection processes. Dee et al (Dee and Shuler 1997a) developed a model for baculovirus infected insect cells which could be generalized to other acid-dependent enveloped viruses. They found that binding of the virus to the cell receptor was the rate limiting step. Chilakuru modeled the infection of vaccinia virus, finding that the ionic composition of the medium and presence of serum proteins played an important role in adsorption kinetics and cells infected during exponential phase yielded more virus particles than those infected in lag or stationary phase (Chillakuru et al. 1991). These results suggest that other parameters, such as medium composition, may affect protein expression via the

amount of virus binding. They also support our goal of increasing infection efficiency by obtaining a high density of cells in exponential growth phase prior to infection.

Lowering culture temperature in viral systems can lower the cytopathic effect and increase protein yield. Temperatures of 34°C, in the Semliki Forest Virus (SFV) system, (Schlaeger and Lundstrom 1998) and 31°C, in the microcarrier based vaccinia system (Bleckwenn et al. 2004a) increased protein production compared to 37°C cultures. In the HeLa S3 suspension cells it was found that cultures grown at 28°C after infection produced the largest amount of intracellular EGFP, within error of 31°C and 34°C, but because this level of production was reached much later, a temperature of 34°C after the infection phase was used for further experiments. The viability of the cultures over time decreased more rapidly for temperatures above 30°C than for those below, indicating decreased cytopathic effect at lower temperatures, leading to slower cell death. The experiments performed in spinner flasks where the cultures were incubated at lower temperatures not only affected the temperature of the culture, but also the level of dissolved gases such as oxygen and carbon dioxide, thereby, potentially changing the culture pH. Without isolating temperature as the only changed parameter, it is likely that the results obtained from these spinner flasks were not due to temperature alone.

Of particular importance to a viral infection system is the achievement of high cell density of exponentially growing cells, which will very likely increase the volumetric productivity. It is particularly important to have healthy, exponentially growing cells for efficient infection with vaccinia virus (Chillakuru et al. 1991). A

hollow fiber perfusion method was implemented to generate high cell density cultures and to perform the media replacement and volume reduction needed for efficient infection. The maximum viable cell density reached for HeLa S3 cells grown in a bioreactor, with a hollow fiber cartridge used for perfusion, was 1.3×10^7 cell/ml, several times higher than what was achieved in batch spinner flask culture. This increase in maximum cell density was also accompanied by an exponential growth phase of about seven days. The growth profile provided a timeframe in which to infect the cells from approximately 100-175 h post seeding. The medium manipulations required just prior to infection, to reduce the serum concentration and lower the volume in the reactor while maintaining the total number of cells, was easily performed with the hollow fiber and could be scaled up with larger hollow fiber units as the size of the bioreactor increases.

EGFP reporter protein was produced in 1.5 liter bioreactor culture using the conditions defined by the spinner flask infection experiments and the growth profile of HeLa S3 using hollow fiber perfusion feeding. The expression results indicate that the production of EGFP in this perfusion based suspension system attained $10.44 \pm 0.018 \mu\text{g}/10^6$ infected cells. This was three times the level seen when the production temperature was maintained at 36.5°C . It is also important to notice that the maximum level of expression for the bioreactor system occurred about 50-60 hours later than the spinner culture at the same temperature, possibly due to controlled environment and the cells' physiological state. The dramatic increase in production at lowered temperature in bioreactor cultures, where temperature was controlled

separately from dissolved gases, indicated that temperature plays a significant role in protein yield with this expression method.

5.6 Conclusions

The HeLa S3-vaccinia virus expression system has been used for research purposes to make small quantities of proteins. Through an investigation of some key parameters and evaluation of a hollow fiber perfusion method in order to increase cell density, the production capacity of this expression method was increased to the bioreactor scale at 1.5 liters, and theoretically could be scaled further with increased reactor and hollow fiber cartridge size. This system could be further optimized for production purposes by altering other process parameters such as pH or altering medium components. These results provide a starting point for further process improvements to this method of, laboratory to pilot scale, protein production.

CHAPTER 6: Comparison of Protein Expression in Multiple Mammalian Cell Lines Utilizing the Vaccinia Virus Expression System⁵

6.1 Summary

Production of recombinant proteins with the vaccinia virus expression system in five mammalian cell lines (HeLa, BS-C-1, Vero, MRC-5, and 293) was investigated for protein yield and proper post-translational modifications. Regulatory acceptance of the host cell line was taken into consideration, as well as relevant process knowledge for ease of scale-up with the particular cell type. Two proteins were expressed, enhanced green fluorescent protein (EGFP) in the cytoplasm, and gp120 secreted into the culture medium. Because gp120 contained a significant amount of post-translational modification, this protein, produced by the different cell lines, was further analyzed by PNGase digestion for N-linked glycosylation modifications. HeLa cells produced the most EGFP at 17.2 µg/well with BS-C-1 and 293 following. BS-C-1 produced the most gp120 at 28.2 µg/ml with 293 and Vero following. MRC-5 had a very high productivity on a per cell basis, but low cell density and slow growth rate made the overall production low. Based on these results and overall process considerations, 293 cells are recommended for further production process optimization in a serum-free suspension system.

⁵ Bleckwenn NA, Bentley WE, Shiloach J. 2004. Comparison of protein expression in multiple mammalian cell lines utilizing the vaccinia virus expression system. Submitted 2004.

6.2 Introduction

The vaccinia virus expression system has been used to produce protein with HeLa (Bleckwenn et al. 2003; Bleckwenn et al. 2004a; Hu et al. 2000) and HeLa S3 (Jun et al. 1996) cells. Although these results were promising for a manufacturing process based on vaccinia virus infection, concern remained that HeLa cells, and the HeLa S3 suspension line derived from them, were inappropriate for manufacture of therapeutic proteins. These cells were originally isolated from a human cervical adenocarcinoma (Gey et al. 1952; Jones et al. 1971) and are known to cause cancer when injected into mice. Other researchers produced protein using vaccinia virus with Vero cells attached to microcarriers in 40 l culture (Barrett et al. 1989), but only at $\sim 2\text{-}3 \mu\text{g gp160}/10^6$ cells. Because of this low level of expression and concerns about HeLa as host cells, we carried out an investigation of other cell lines, evaluating protein production capability, including several that might be more acceptable to the regulatory authorities as a production cell line using the vaccinia expression system.

The HeLa cell line used in previous work was used as a point of comparison. By testing this line under the same conditions as the others, it was possible to make direct comparisons between the cell lines at the chosen conditions of infection and expression. This was important because some work had already been done to optimize the HeLa cell system with vaccinia, indicating that optimization with each cell line could improve production. The cells chosen for comparison were BS-C-1, MRC-5, Vero and 293. BS-C-1 cells have been used extensively with vaccinia virus in plaque assays in order to make titer determinations because they form tight monolayers on tissue culture plates and infection results in distinct plaque formation

(Moss and Earl 2000). The cells originated from African green monkey kidney cells (Hopps 1963) and their historical use with vaccinia virus suggests they may be useful as a point of comparison to more appropriate cell lines for the purpose of recombinant protein production.

MRC-5 cells are currently used by BioReliance Corp. (Rockville, MD) in the manufacture of clinical, and potentially commercial, supplies of vaccinia virus for a Smallpox vaccine. This cell line was derived from human lung fibroblasts (Jacobs et al. 1970). The cells are anchorage dependent, but have a fairly slow growth rate. Due to the established manufacturing process with these cells for vaccine production, they have already been through some of the regulatory hurdles of the approval process.

Vero cells are also an anchorage dependent cell line, like BS-C-1. They originated from African green monkey kidney cells (Yasumura and Kawakita 1962) and were used previously by Barrett (Barrett et al. 1989) to produce HIV-1 gp160 in a 40 liter microcarrier based bioreactor utilizing vaccinia virus. These cells are fairly easily grown and have a faster growth rate than MRC-5 cells (bi-weekly split ratio 1:3 to 1:6 versus 1:2 to 1:5 recommended by ATCC Rockville, MD). They have already been shown to produce recombinant proteins via vaccinia virus. Also, the limited culture life of these cells, compared to continuous cell lines, makes them appealing for use in commercial manufacturing processes due to the reduced level of concern over cancer causing components from the host cell passing along to the final product. Hence, Vero cells were also tested.

293 cells were derived from human embryonic kidney cells (Graham et al. 1977) that are not considered immortal. This cell line was also tested for protein

production. These cells have been used for clinical production of biologic products (Farson et al. 1999; Habib et al. 1999) which makes them desirable due to their history of use in clinical evaluation. Additionally, there are several commercially available media for adaptation of these cells to serum-free suspension growth. Utilizing a defined serum-free medium makes this cell line amenable to large-scale production.

Previous surveys of cell lines for recombinant protein production using another virus-mediated expression system have proven successful in that cell lines with superior glycosylation characteristics, growth rate, and product yield have been found (Davis et al. 1993; Hink et al. 1991; Rhiel et al. 1997; Wickham et al. 1992; Zhang et al. 2002a; Zhang et al. 2002b). We tested protein production of two proteins, enhanced green fluorescent protein (EGFP), which is expressed in the cytoplasm, and the HIV-1 envelope coat glycoprotein, gp120, which is secreted into the culture medium and contains a significant amount of N-linked glycans. In summary, this article describes a production comparison of EGFP and gp120 in the HeLa, BS-C-1, Vero, MRC-5 and 293 cell lines using the vaccinia virus expression system.

6.3 Materials and Methods

6.3.1 Cell Line Growth and Maintenance

HeLa (ATCC CCL-2), BS-C-1 (ATCC CCL-26), Vero (ATCC CCL-81), MRC-5 (ATCC CCL-171), and 293 (ATCC CRL-1573) were all grown in Dulbecco's Modified Essential Medium (DMEM, Biosource, Camarillo, CA)

supplemented with 10% fetal bovine serum (FBS) in 75-cm² tissue culture flasks and passaged every 3-4 days. Vero cells were used within 25 passages, MRC-5 within 12 passages, and 293 within 25 passages of thawing for experiments.

6.3.2 Virus Preparation

Viruses vNB009, producing EGFP, and vNB014, producing gp120, were constructed as described previously (Bleckwenn et al. 2003; Bleckwenn et al. 2004b). Purified viral stocks were used for all experiments where a portion of the stock was incubated with an equivalent volume of 1× trypsin to separate clumps. This mixture was incubated at 37°C for 30 minutes with vigorous vortexing every 10 minutes. The trypsinized stock was then diluted and used to infect cultures at the appropriate multiplicity of infection (MOI).

6.3.3 6-Well Plate Infection and Expression

Wells of 6-well plates were seeded 2-3 days prior to use in experiments at densities which would result in wells just prior to confluent for experiment. They were grown in DMEM+10% FBS. For infection, one 6-well plate for each cell line was sacrificed for cell count determination. Six wells were prepared, with cells removed and resuspended, to determine the number of cells per well. Specifically, the densities used for infection were HeLa at 1.6×10^6 , BS-C-1 at 3.4×10^6 , Vero at 8.3×10^5 , MRC-5 at 1.7×10^5 , and 293 at 1.2×10^6 cells/well. The remaining plates were then used for infection and assumed to have the same cell densities for each cell line. The cells were washed with DMEM without serum and either infected for 1 hour in the presence of 1.0 mM IPTG at an MOI of 1.0 with vNB009, vNB014, or mock

infected. Plates were sacrificed for samples at 24, 48, and 72 hpi. Two wells with each virus or uninfected control were harvested at each timepoint. Cells were scraped using the plunger of a 1 cc syringe. The medium and cells were collected and centrifuged for 5 minutes at 1000×g. Supernatant medium was removed to a separate tube and both pellet and medium samples were saved for each well.

Pictures of cells for each condition were taken with a microscope using 100× magnification and a Kodak MDS90 documentation system. Additionally, fluorescence photography of the vNB009 infected cells was also performed using a FITC filter.

6.3.4 EGFP Analysis

Analysis of EGFP expression in the vNB009 and mock infected cells was performed by resuspending the cell pellets for those samples in lysis buffer (100 mM Tris pH 7.4, with 1× Complete protease inhibitor (Boehringer Mannheim GmbH, Germany) and 0.5% Triton X-100) and incubating for 30 minutes at 37°C. Fluorescence intensity was measured on 100 µl samples in 96-well optically black plates using a fluorescence spectrophotometer with excitation 485 nm and emission 512 nm with an auto cutoff filter at 495nm. Samples of supernatant were compared against standards of rEGFP in medium to determine extracellular EGFP. Readings from the lysed pellet samples were compared against standards of recombinant EGFP (Clontech) in lysis solution to determine intracellular expression levels of EGFP.

6.3.5 *gp120 Analysis*

Analysis of the gp120 protein was performed by Western blot after running samples on reducing SDS-PAGE gels. Primary antibody, HIV-1 gp160B Antiserum (HT3) in Goat (Reagent 188), from DAIDS, NIAID, NIH produced under contract by Repligen, was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH as well as the gp120 standard, HIV-1 gp120 CM (Reagent 2003-CM), used in the Western blots. Scans of the Western blots were quantified with Kodak EDAS system

6.3.6 *gp120 Purification and N-linked Glycan Analysis*

Culture supernatants were collected and purified using a Ni-NTA spin purification kit (Qiagen). Briefly, a 10× salt and NP-40 solution was added to the supernatants to a final concentration of 0.5% NP-40, 50 mM NaH₂PO₄ and 300 mM NaCl, mixed and incubated overnight at 4°C to inactivate virus. Imidazole was added to 10 mM and the pH adjusted to 8.0. The Ni-NTA resin column was equilibrated with lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0) and two aliquots of 500 µl supernatant were cycled through the column twice to bind the protein, centrifuged at 100×g for 2 minutes each time. The column was washed four times with 600 µl wash buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0). The protein was then eluted for each sample in two 100 µl fractions with elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM Imidazole, pH 8.0), centrifuging at 100×g for 1 minute each time. The two elutions for each cell line supernatant were combined and concentrated (Microcon YM-10, 10 kDa cutoff, 5 min, 14,000×g) resulting in reduction of half the volume. Concentrated eluates (100

μl) were then dialyzed (Amika Dispo-Biodialyzer) into 10 mM Tris at pH 7.0.

Deglycosylation reactions were performed with PNGase F (Sigma E-DEGLY) in pH 7.0 buffer for 24 hours at 37°C. Portions of these reactions were used as samples for reducing SDS-PAGE gel and Western blot analysis.

6.4 Results

6.4.1 Comparison of EGFP Production in Five Cell Lines

Six-well plate cultures of HeLa, BS-C-1, Vero, MRC-5, and 293 cells were infected with vNB009 vaccinia virus, containing the gene for EGFP, at an MOI of 1.0 and induced (1.0 mM IPTG) to express EGFP. Cell pellets were analyzed for EGFP expression (Figure 6-1). The HeLa cell line expressed more EGFP on a per cell basis (Figure 6-1A), $10.9 \mu\text{g}/10^6$ infected cells, and overall (Figure 6-1B), reaching $17.2 \mu\text{g}/\text{well}$, than any of the other lines tested. On a per cell basis, MRC-5 cells reached about half the specific production as the HeLa cells but because the cell density was lower, due to their larger size and slower growth rate, the overall production was low. BS-C-1 cells produced $2.6\times$ less EGFP/ml and the 293 about $8\times$ less than HeLa (Figure 6-1B). Visualization of the cells was performed using standard light and fluorescence microscopy and images are shown in Figure 6-2. Evident in these photos are the extent of infection between the various cell lines and the relative EGFP expression levels. All the cell lines showed approximately the same degree of cytopathic effect from the viral infection, based on the rounding appearance and detachment from the culture plates. Note the apparent differences in the protein expression level agree well with the quantitative results of Figure 6-1.

Figure 6-1 EGFP Expression in Five Cell Lines

Intracellular EGFP expression profiles are shown for the five cell lines tested: HeLa (circle), BS-C-1 (square), Vero (upward pointing triangle), MRC-5 (downward pointing triangle), and 293 (diamond). Results are presented on a per cell basis, Panel A, and in terms of overall production per well, Panel B.

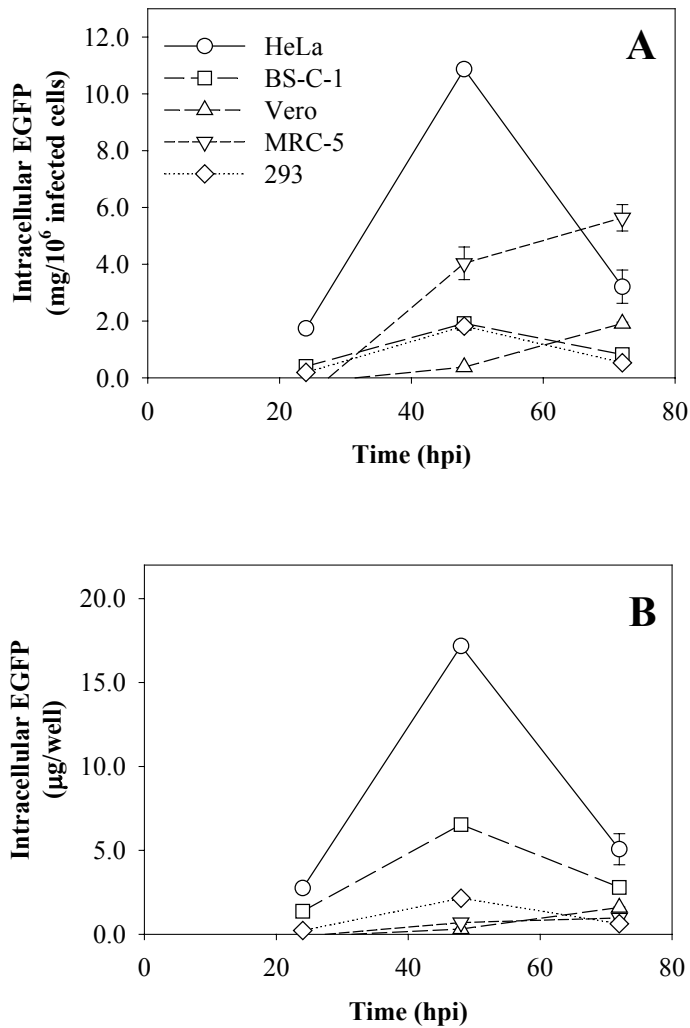
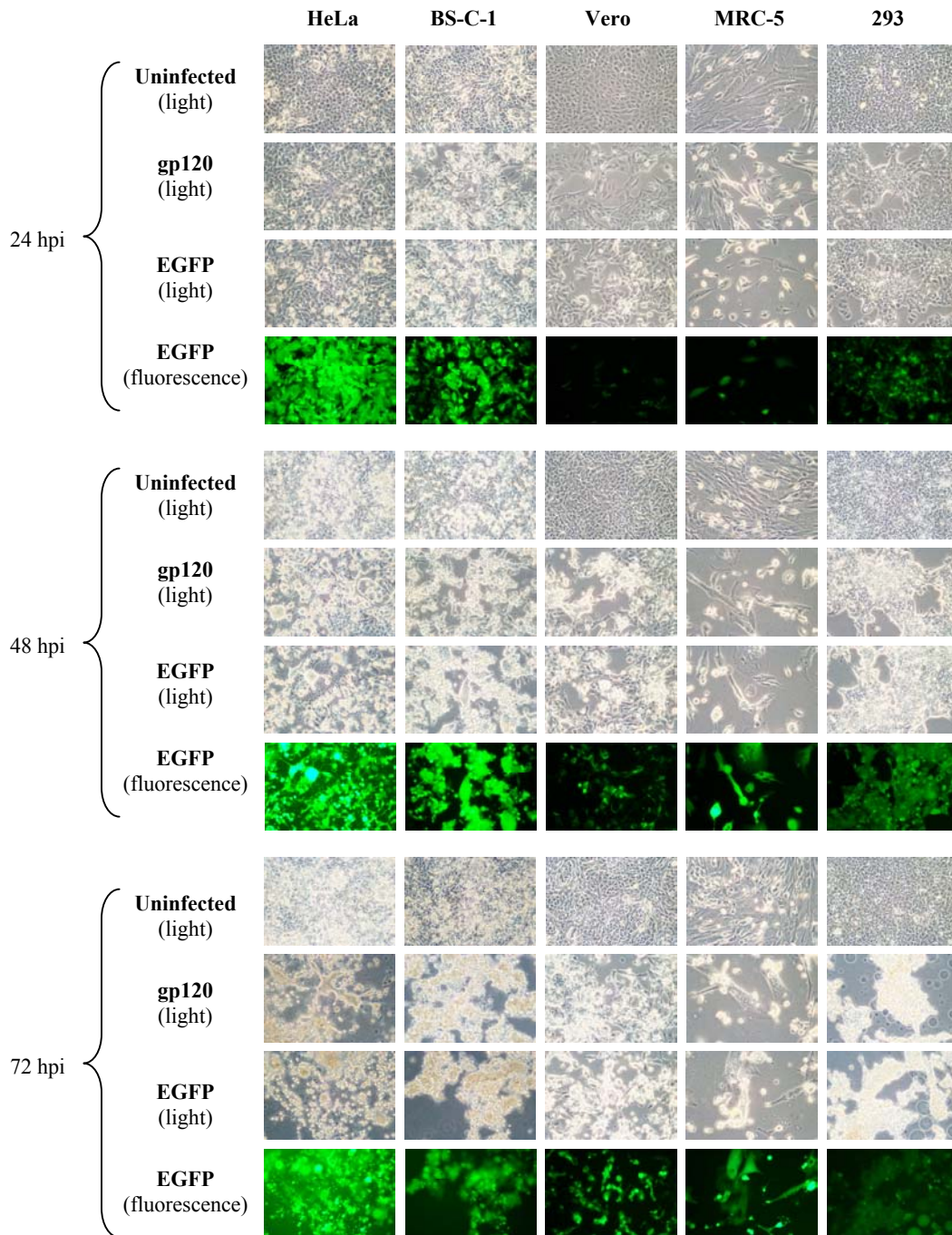


Figure 6-2 Microscopic Images of Cell Lines

Pictures of the various cells lines are shown infected with EGFP or gp120 producing virus, or mock infected under 100× magnification at 24, 48, and 72 hpi. Fluorescence images are shown for EGFP expressing cultures only because this protein is only present in these cultures and the others do not contain any fluorescing proteins.



6.4.2 Comparison of gp120 Production in Five Cell Lines

The infection protocol used for EGFP production was also performed with all five cell lines using vaccinia virus vNB014 to produce gp120 protein. The gp120 production was followed by Western blot analysis (as opposed to EGFP fluorescence). Figure 6-3 shows the quantified Western results on a per cell basis (Panel A), and the overall production (Panel B). On a per cell basis, MRC-5 cells outperformed the others, reaching $142 \mu\text{g}/10^6$ infected cells, but as before, due to the lower cell density and slow growth rate they did not perform as well on an overall basis. Vero and 293 cells were next with 2 to 3-fold less gp120. The overall production results showed that BS-C-1 was superior at $28.2 \mu\text{g}/\text{ml}$ ($19.9 \mu\text{g}/\text{ml}$ 293, $16.8 \mu\text{g}/\text{ml}$ Vero, $9.75 \mu\text{g}/\text{ml}$ MRC-5, and $6.76 \mu\text{g}/\text{ml}$ HeLa).

6.4.3 Initial gp120 Glycan Analysis

Figure 6-4A depicts a Western blot comparison of each of these cell lines at 72 hpi. There was a difference in the appearance of the bands, where 293 and Vero produced gp120 with slightly sharper bands than the other cell lines. Purified gp120 from all five cell lines was subjected to PNGase F treatment to remove the N-linked glycans from the protein. Previous results in HeLa cells revealed the N-linked glycans contributed about half the molecular weight of the protein (Hu et al. 2000). SDS-PAGE and Western blot analysis of the results of these digestions are shown in Figure 6-4B. The PNGase treatment trimmed the proteins down to approximately 60 kDa. Note, the PNGase F digested bands appeared significantly sharper than the undigested bands for all cell lines.

Figure 6-3 gp120 Expression in Five Cell Lines

Secreted gp120 expression profiles are shown for the five cell lines tested:

HeLa (circle), BS-C-1 (square), Vero (upward pointing triangle), MRC-5 (downward pointing triangle), and 293 (diamond). Results are presented on a per cell basis, Panel A, and in terms of overall production per milliliter of medium, Panel B.

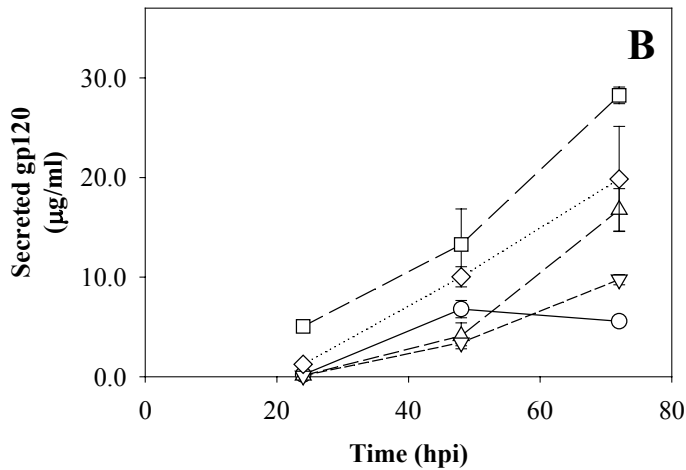
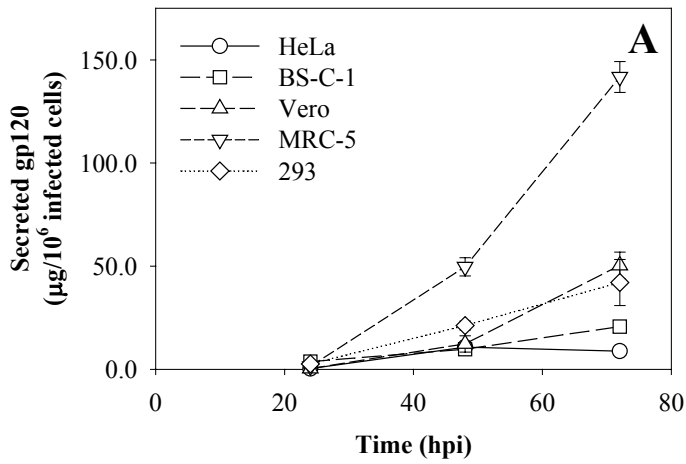
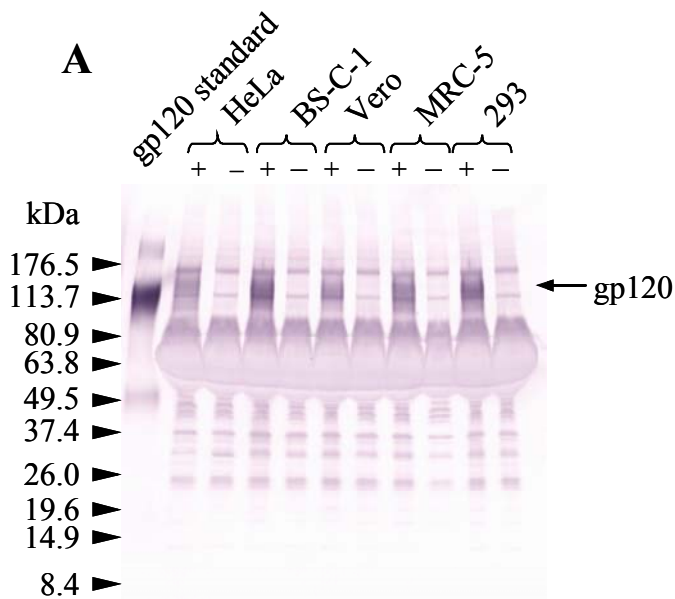
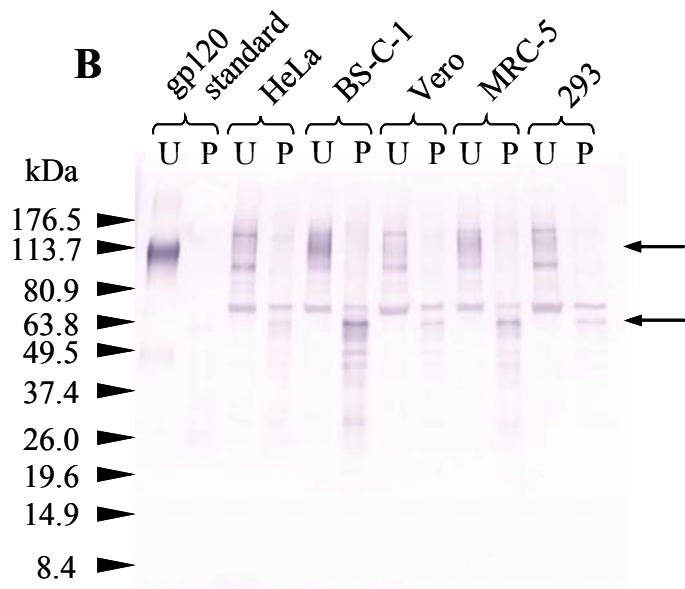


Figure 6-4 Western Blot Analysis of gp120 Production at 72 hpi

Western blot analysis is shown in Panel A for the 72 hpi supernatant samples from cultures infected with virus expressing gp120 protein (+) for each cell line, next to supernatants from cultures infected with virus expressing EGFP (-), as negative controls. The left most lane shows gp120 standard. Western blot analysis is shown in Panel B for the concentrated, purified, supernatant samples taken at 72 hpi from cultures infected with virus expressing gp120 protein for each cell line. Each sample is either untreated (U) or treated with PNGase (P) to remove N-linked glycans from the protein. gp120 standard, untreated and treated, is shown in the left most lanes. The upper arrow on the right indicates the position of the undigested protein, whereas the lower arrow on the right indicates the position of the PNGase digested gp120.





Note, microscopy images of the infected cells are also shown in Figure 6-2. The degree of cytopathic effect (as evident by cell rounding and detachment from the culture plate) from this virus among the cell lines was roughly equivalent, and showed approximately the same level of infection, similar to the results of EGFP.

6.5 Discussion

Production of recombinant proteins with the vaccinia virus expression system is advantageous because multiple cell lines can be screened for levels of expression and proper post-translational modifications without having to generate multiple recombinant cell lines. Additionally, vaccinia virus may be able to produce very large proteins (the genome is fairly large, 200,000 bp). That is, other researchers have speculated that vaccinia can accommodate an insert up to 25,000 bp without hindering the viral and protein production processes (Smith and Moss 1983).

Our results have demonstrated that vaccinia can produce both intracellular and secreted proteins in several cell lines, some of which have been used for clinical production processes. Interestingly, our results (which were not optimized) revealed that peak production was not consistent under these conditions, BS-C-1 cells were best in terms of gp120 but HeLa were best for EGFP. This may be due, in part, to the location of expression, (e.g. EGFP was produced intracellularly and gp120 was secreted). Also, because EGFP does not require post-translational modifications, protein expression is not limited by these processing pathways. Differences in protein structure via varying glycosylation patterns were also revealed after comparing the tightness of the Western bands prior to and upon PNGase F

digestion. The tightness is affected either by the heterogeneity of the amino acid sequence as a result of truncation or because of heterogeneity of post-translational modifications. The protein bands appear to span a molecular weight range of about 90 to 120 kDa. If the width of the expression bands were caused by truncation of the amino acid structure of the protein, the bands would also appear with a range covering 50 kDa upon deglycosylation analysis. But upon treatment with PNGase, the width of the bands drops to only about a 10 kDa span at 60 kDa molecular weight, which is close to the theoretical molecular weight calculated for the amino acid sequence without any post-translational processing of 58 kDa. This suggests heterogeneity in the glycosylation patterns of the gp120 produced by these cell lines. Although defined and consistent heterogeneities of glycosylation structure are somewhat expected and may be accepted in production processes, often, only one particular structure is the active form. It is desirable to produce only the active form in order to increase potency of the compound and reduce dosage.

6.6 Conclusions

In summary, the vaccinia virus expression system has been shown to produce both intracellular and secreted proteins in various cell lines. Complex post-translational modifications were compared among the cell lines tested and could further be evaluated in other mammalian cell lines. The results from overall expression of both gp120 and EGFP, as well as other process considerations, suggested that investigation and optimization of a 293 based process, where the host cells could be adapted to serum-free suspension growth, was warranted for further advancement of this expression method for production purposes.

CHAPTER 7: Conclusions and Future Directions

7.1 Concluding Remarks

The vaccinia virus expression system offers a method of recombinant protein production for complex proteins of significant size. The work presented here has evaluated several aspects of the infection and production process and the ability to scale these up to bioreactor culture. The overall objective to develop a recombinant protein production process was achieved, but has left room for further improvements. The work was performed with incremental improvements to the understanding of the process and the overall results of what was discovered follows.

Chapter 2 focused on the construction of the reporter protein virus and an initial investigation of infection and expression conditions in small-scale experiments. Six-well plate HeLa cultures were used to study multiplicity of infection (MOI), volume during infection, and serum concentration during infection for their effect on EGFP expression level. Maximum expression was achieved at an MOI of 1.0 with a 0.5 ml infection volume in serum-free medium. Additionally, twelve commercial microcarriers were evaluated for attachment and growth characteristics with HeLa cells. Cytodex[®] 3 were found to be the most appropriate and were used to support growth of HeLa cells in spinner flask culture which, after infection with the reporter virus, produced 2.2 µg EGFP/10⁶ cells at infection.

Chapter 3 delved deeper into the infection and expression conditions through investigation of other parameters and infection of microcarrier based culture in spinner flasks. Length of the infection phase, inducer concentration and timing of its

addition relative to infection were evaluated in 6-well plate HeLa cultures. One hour infection with 1.0 mM IPTG added at the time of infection provided a robust process. Evaluation of the best concentration of Cytodex[®] 3 microcarriers for growth of HeLa was performed and found 5 g/l to provide sufficient surface area for growth. MOI was reevaluated in the dynamic microcarrier culture environment which indicated a need for an increased number of virus particles per cell to 5.0, higher than that determined in Chapter 2 for plate culture. Dissolved oxygen level and temperature during the protein production phase were evaluated for their effect on EGFP expression in microcarrier spinner flask culture. Increasing dissolved oxygen to 50%, and decreasing temperature to 31°C, showed an increase in production over the course of the production phase. The level of production achieved with this system reached ~17 µg EGFP/10⁶ infected cells.

Chapter 4 expanded the process into perfused bioreactor culture and examined the effects of scaling up this viral process. Perfusion growth of HeLa cells on microcarriers was studied with an alternating tangential flow device (ATF) which increased the length of the exponential phase and the maximum cell density, to 4.4×10⁶ cell/ml, compared to batch culture. Concerns about seed train expansion were addressed by demonstrating that an in-flask trypsinization procedure could be used to provide the cells for seeding. Dissolved oxygen and temperature were revisited in the large-scale environment because these parameters could be better understood in a system which isolates and separately controls these parameters. A process where the DO and temperature were adjusted after infection to 50% and 31°C, respectively, produced the highest level of EGFP, 12±1.5 µg/10⁶ infected cells.

The defined process was then used to produce gp120, an HIV-1 envelope coat protein with complex post-translational modifications. The purified protein was digested for glycosylation analysis, showing a significant amount of N-linked glycans and biological activity was determined by a fusion-inhibition assay resulting in an ID₅₀ of 3.1 µg/ml.

Chapter 5 describes an investigation of the HeLa S3 suspension adapted cell line for production of recombinant proteins with vaccinia virus. Cell suspension processes are more common and easier to implement in most bioprocess laboratories. A hollow fiber device was used for perfusion feeding of the bioreactor culture, in much the same way as the ATF was used for microcarrier culture. Two process parameters were reevaluated in suspension culture in spinner flasks to determine if their effect varied from the microcarrier based system. These were MOI and temperature. Single cell suspension culture was thought to have different infection kinetics than microcarrier based culture. The growth rate of HeLa S3 was somewhat higher than anchorage dependent HeLa cells, potentially altering the balance of reduced cytopathic effect from lowered temperature with lowered overall processing. An MOI of 1.0 lead to the highest level of intracellular expression of EGFP while reducing the temperature to 34°C during the protein production phase also increased production of the protein two-fold over the 37°C culture. Scaling up the process to bioreactor culture with hollow fiber perfusion, lead to an overall production level of 10.44 µg EGFP/10⁶ infected cells, or 27 mg EGFP/l.

The HeLa cell line is potentially inappropriate for regulatory approvable processes due to its ability to cause tumors when injected into mice. Therefore,

Chapter 6 evaluated the production of both EGFP and gp120 protein, produced in the cytoplasm and secreted into the medium, respectively, with five mammalian cell lines (HeLa, BS-C-1, Vero, MRC-5, and 293). HeLa cells produced the most EGFP at 17.2 µg/well with BS-C-1 and 293 following. BS-C-1 produced the most gp120 at 28.2 µg/ml with 293 and Vero following. MRC-5 had a very high productivity on a per cell basis, but low cell density and slow growth rate made the overall production low. Significant amounts of N-linked glycosylation modifications were found on the gp120 protein produced, based on PNGase digestion. These results and consideration of other processing factors lead to a recommendation for further production process optimization in a serum-free suspension system using 293 cells.

The work reported here demonstrates the capacity for the VOTE vaccinia virus expression system in production of recombinant proteins. Various infection and expression related parameters were studied to continually increase production levels with this virus. Additionally, some concerns raised by other researchers during presentations of this work about transfer of the process and suitability of manufacturing were addressed in Chapters 5 and 6. Overall, we were able to achieve production of both intracellular and complex secreted proteins by this method and showed the appropriate processing and activity of the gp120 produced.

7.2 Future Directions

7.2.1 Scale-up of Microcarrier and Suspension Processes

Future research could be aimed at further increasing the size of bioreactor system for production. Both microcarrier and suspension based bioreactor systems

should be able to increase in size through the use of larger bioreactor vessels. The perfusion methods should also be scaleable with larger ATF or hollow fiber systems. Particular care will need to be taken during the infection process to ensure that controllable process parameters are not affected by the reduction in volume, as this will depend on the location of the various probes in the bioreactor and their ability to take accurate measurements during these medium manipulations. These changes may also affect the process because they may take longer to perform at large-scale, and this will need to be taken into consideration when planning the infection and feeding timing.

7.2.2 293 Serum-Free Suspension Growth and Vaccinia Virus Infection

Some investigation of the growth of 293 cells in a serum-free suspension environment with a commercially available medium has already been performed in our lab, reaching 1.1×10^7 viable cell/ml. This work could be utilized and infection conditions reevaluated with this cell line in serum-free suspension culture. It may be necessary to either use DMEM without serum as the infection medium or contact the supplier of the serum-free medium to remove any serum like components and Pluronic F-68 from the medium for the infection phase, as these may prove to interfere with infection in the same manner as serum. Bioreactor growth should be similar to other suspension processes such as the HeLa S3 and the results obtained from those experiences provide a starting point for optimization of a 293 based process.

7.2.3 *CHO Cell Vaccinia Infection*

One industrially significant cell line that is an exception to the wide host range of wild-type vaccinia virus is the Chinese Hamster Ovary (CHO) cell line, commonly used for manufacturing processes. Some work has been done by other researchers to overcome this host range restriction of vaccinia. This was accomplished by inserting the cowpox *hr* (*host range*) gene into a recombinant vaccinia virus and resulted in permissive infection and production of recombinant chloramphenicol acetyltransferase (RamseyEwing and Moss 1996). This vaccinia expression system was not inducible although it was controlled by the T7 promoter through which the T7 polymerase was produced via a vaccinia promoter. Construction of a recombinant vaccinia with the VOTE, inducible, expression system containing a multiple cloning site would bring this virus construct in line with that used for the work described here. An investigation and optimization of a vaccinia based production process with the CHO cell line infected with this VOTE-CHO construct could prove more useful than other cell lines because of the broad knowledge available for CHO based processes.

7.2.4 *Manipulation of the Vaccinia Genome*

A newly emerging area of study with vaccinia virus is looking into the change of regulation of genes upon vaccinia virus infection (Guerra et al. 2003). By utilizing the results obtained from these types of studies, researchers may be able to remove non-essential genes, thereby increasing the capacity for larger inserts. There is also the potential to find genes which can be controlled to reduce the infectivity or cytopathic effects of the virus, leading to safer viral vectors that are permissive to commercial cell lines, longer production capability, and higher yields. An interesting

possibility would be to genetically engineer the promoters controlling the genes regulating cytopathic effect or infection, thereby allowing inducible control of these processes. This could result in a process where infection is only allowed during the prescribed period to allow generation of viral stocks or for the initial infection of a protein production process. Through control of the cytopathic effects, which may be linked to infectivity, we could allow infection early in the culture, and then turn off the genes causing death in the cells while still allowing the viral and cellular machinery the opportunity to produce and process more protein. This concept would be similar to using baculovirus to infect mammalian cell culture where the virus will infect and produce protein, but not cause cell death (Condreay et al. 1999; Hu et al. 2003).

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