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CHARACTERIZATION AND OPTIMIZATION OF THE PRODUCTION OF ADENO-ASSOCIATED VIRAL VECTORS USING A BACULOVIRUS EXPRESSION VECTOR/INSECT CELL SYSTEM

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THÈSE PRÉSENTÉE EN VUE DE L'OBTENTION
DU DIPLÔME DE PHILOSOPHIÆ DOCTOR (Ph.D.)
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Cette thèse intitulée:

CHARACTERIZATION AND OPTIMIZATION OF THE PRODUCTION OF ADENO-ASSOCIATED VIRAL VECTORS USING A BACULOVIRUS EXPRESSION VECTOR/INSECT CELL SYSTEM

présentée par: AUCOIN Marc Gordon

en vue de l'obtention du diplôme de: Philosophiæ Doctor

a été dûment acceptée par le jury d'examen constitué de:

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DEDICATION

To the fall armyworm.

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ABSTRACT

Adeno-associated viruses exist as a class of virus that have shown promising characteristics as gene therapy vectors. They have further been shown in some instances to also illicit a strong immune response against more threatening viruses like the human immunodeficiency virus. All this interest has led to seek ways to optimally produce these vectors. In mammalian cells, these vectors have been produced in a number of cell culture platforms including HEK293 and HeLa cells. They have been produced using adenovirus vectors, herpes vectors and/or plasmids. Although recent improvements in the ability to produce these vectors at a reasonable scale in suspension by transient transfection have been reported, their production in insect cells using baculoviruses still promises to be one of the most efficient and scalable platforms to achieve the goal of producing enough material for clinical trials. The system was originally described in a seminal paper on the subject in 2002 (Urabe et al., 2002). Starting with what was known at the time, the system was designed so that the Rep78 protein was attenuated and that both the Rep52 protein and the capsid proteins were highly expressed. This was achieved by putting the former under a truncated insect cell immediate early promoter and the latter under insect cell polyhedrin promoters. With an adjustment to the genetic sequence of the capsid proteins, Urabe et al. (2002) were able to mimic the proper stoichiometry of the viral particle proteins. The combination of three baculovectors containing the Rep genes, the Cap gene and the transgene of interest showed excellent results with expression levels and per cell synthesis surpassing what was generally achieved in adherent cell culture using transfection.

To optimize the cellular and volumetric production a detailed characterization of the system was undertaken. To gauge the effect of each baculovirus vector used for the production of AAV vectors, the quantity of each, the ratio between them

and the time at which they were added, were investigated. The highest titers were achieved when BacRep and BacCap were added at high multiplicities of infection. Manipulating expression levels with the multiplicity of infection is difficult since there is inherent competition between the baculoviruses. This is believed to be the reason why offsetting the ratio of BacRep to BacCap always led to lower production of AAV vectors. Furthermore, the highest AAV production was achieved when all three baculoviruses infected the cell at relatively the same time, otherwise a significant reduction occurred. To minimize the amount of total baculovirus added to the system, BacITR, which only supplies the AAV vector genome, could be added at lower multiplicities of infection without significantly affecting the production of AAV vectors.

It was also found that using high MOIs of BacRep may be problematic in the long run. Instabilities with this baculovirus construct that are atypical of passaging other recombinant baculoviruses resulted in a significant decrease in expression levels over fewer passages. It has been speculated that the instability ensues from the palindromic sequence resulting from the head-to-head orientation of the Rep genes (Kohlbrenner et al., 2005a). The use of low passage BacRep minimizes this effect. An alternate four-baculovirus system developed by Kohlbrenner et al. (2005a) increases the stability by separating the two Rep genes into two baculoviruses. Unlike the triple infection system, it was found, in this work, that capsids are likely the limiting factor when using the quadruple infection system.

The total number of capsids produced always significantly exceeded the number of functional AAV vectors and vector genomes. This implied that there was a potential to increase the efficiency of genome encapsidation. To try and address this limitation using culture parameters, the temperature of the process was modulated. Increasing the temperature during the production phase of the process from 27°C

to 30°C increased replication proteins early in the culture, which is thought to be a key reason for the observed increase in SS progeny DNA, $\sim$ two-fold increase in DNAse resistant particles and $\sim$ three-fold increase in functional vectors produced. The most significant replication protein increase was Rep78, which may suggest that the attenuated expression of the Rep78, designed because of its detrimental effects in mammalian cells, may be over-attenuated in the insect cell system.

To fully exploit the insect cell system, the maximum density at which AAV could be produced was investigated. Without adding nutrients during the culture, the synthesis per cell significantly decreased beyond $4x10^6$ cells/ml. Medium renewal at the time of infection allowed production at $\sim$ $1x10^7$ cells/ml although the synthesis per cell was only a fraction of the maximum achieved at lower cell densities. Supplementing the media using a nutrient cocktail increased the specific production but did not achieve the maximum level seen at lower cell densities. Resuspending cells from the early exponential phase, in fresh media, to higher cell densities, maintained specific production levels up to $\sim$ $1x10^7$ cells/ml. Cell "age" therefore played a role in achieving the highest production levels. The strategies developed in this work yield $\sim 6.5x10^{12}$ functional AAV particles/L of cell culture.

CONDENSÉ

L'Organisation Mondiale de la Santé (OMS) estime que plus d'un million de personnes souffrent de maladies monogéniques. Pour combattre les effets de ces maladies, la protéine codée par le gène déficient doit être administrée de façon routinière ou bien le gène déficient doit être corrigé pour que la protéine soit produite par la cellule.

Pour corriger les gènes déficients, des véhicules permettant de livrer l'information génétique au sein des cellules cibles sont requis. Un moyen efficace de livrer cette information repose sur l'utilisation de vecteurs viraux. La thérapie génique est un secteur important dont l'essor a été considérablement ralenti à cause d'accidents survenus durant les programmes d'évaluation clinique. Deux événements ont en particulier marqué l'histoire de la thérapie génique. Le premier était la mort de Jesse Gelsinger, qui était directement liée à l'administration d'un vecteur adénovirus. Le deuxième incident s'est manifesté comme effet secondaire des traitements utilisant des vecteurs rétroviraux: deux enfants qui participaient à un essai clinique visant à traiter la maladie du déficit immunitaire combiné sévère lié au chromosome X, ont développé une forme de leucémie.

Malgré ces échecs, les résultats expérimentaux continuent à démontrer un avenir prometteur pour les vecteurs viraux, et la Chine a récemment autorisé la première commercialisation d'un vecteur viral comme agent thérapeutique. Les vecteurs dérivés du virus adéno-associé (AAV) sont reconnus comme étant très prometteurs avec plusieurs essais cliniques en cours. Entre autres, des essais cliniques en Phase I ont été initiés récemment pour le traitement de la dystrophie musculaire de Duchenne; des succès en Phase I pour le traitement de la maladie de Parkinson nécessite la poursuite d'essais cliniques en Phase II; et des essais cliniques en Phase III existent pour le traitement du cancer de la prostate.

Ce petit virus (20 à 26 nm) possède un génome à ADN linéaire simple-brin qui comprend deux cadres ouverts de lecture, pour les gènes des protéines de réplication (Rep) et le gène des protéines structurales (Cap), entre deux séquences palindromiques inversées identiques (ITR). Les gènes des protéines de réplication codent pour quatre protéines: Rep78, Rep68, Rep 52 et Rep40. Le gène des protéines structurales (Cap) code pour trois protéines qui forment la capside virale: VP1, VP2 et VP3. Les ITRs sont les seules composantes génomiques qui doivent être fournis en *cis*. Ceci veut dire que les deux cadres ouverts de lecture peuvent être remplacés par un transgène d'intérêt pour créer un vecteur viral. Considéré non-pathogénique, ce petit vecteur ne cause pas de réponses immunitaires significatives une fois administré.

Le AAV est un virus qui se réplique seulement en présence de virus assistants comme l'adénovirus ou l'herpès, sinon il s'intègre dans le chromosome de la cellule hôte pour persister sous forme d'infection latente. Les méthodes traditionnelles de production se servaient de la transfection de cellules mammifères adhérentes par des plasmides qui contenaient les séquences Rep et Cap, et le génome du vecteur AAV, suivi par une infection d'adénovirus. Des avancées dans le domaine ont pu éliminer l'utilisation d'un adénovirus de type sauvage en le remplaçant par seulement quelques séquences adénovirales qui pouvaient être livrées par transfection. Malgré des succès avec les AAV produits par la transfection, cette méthode reste limitée par l'efficacité de la transfection à grande échelle. Avec le but de trouver des moyens plus adéquats pour la mise à l'échelle, de nombreux groupes ont travaillé pour créer des lignées stables, mais l'établissement de ces lignées s'avère difficile.

La production de vecteurs AAV dans les cellules d'insecte a le potentiel de produire assez de matériel pour répondre au besoin des cliniciens. Les cellules d'insecte, contrairement à la culture de cellule mammifère, sont libres de pathogènes humains, n'exigent pas l'usage de sérum animal et sont facilement cultivées en suspension. De

plus, la preuve a largement été faite que la technologie utilisant les cellules d'insecte était une plate-forme utilisable à grande échelle. Les cellules d'insecte ont été considérablement exploitées, en conjonction avec le système de vecteur d'expression baculovirus (BEV), pour la production transitoire de protéines recombinantes. Dans ce système, un virus recombinant d'insecte est "fabriqué" pour coder une ou plusieurs protéines d'intérêt. Ce vecteur est alors utilisé pour livrer le ou les gènes étrangers à des cellules dans lesquelles le niveau de production de ces protéines recombinantes peut atteindre jusqu'à 50% des protéines cellulaires totales. En fait, le système BEV a été exploité avec succès pour la production d'une vaste gamme de produits à hautes valeurs commerciales, incluant la production de vaccins, et les vecteurs AAV (Urabe et al., 2002; Meghrous et al., 2005).

De nombreux travaux ont été réalisés pour étudier la configuration de bioréacteurs et l'optimisation des paramètres fondamentaux des cultures de cellules d'insecte, mais en terme de dynamique de procédé, le système BEV est un des systèmes les moins étudiés. Les stratégies pour augmenter les concentrations de cellules viables et la production de protéines exigent l'optimisation de la livraison de virus, les niveaux de nutriments ainsi que le temps de récolte du produit.

Le travail présenté dans cette thèse est centré sur la caractérisation de la production du vecteur AAV dans les cellules d'insecte avec le système BEV. Pour la production, les gènes pour les protéines de réplication AAV et les protéines structurales AAV ont été mis sous le contrôle de promoteurs de gènes d'insecte et clonés dans des vecteurs baculovirus (Urabe et al., 2002). Plus précisément, le gène pour la protéine Rep52, sous le contrôle du promoteur de la polyhédrine et le gène pour la protéine Rep78, sous le contrôle d'un promoteur tronqué IE1, sont contenus dans un premier baculovirus, BacRep. Le gène pour les protéines structurales, sous le contrôle du promoteur de la polyhédrine, est contenu dans un deuxième baculovirus, BacCap. Le

transgène encadré des ITR AAV est contenu dans un troisième baculovirus, BacITR.

Le but ultime de cette recherche était de comprendre comment atteindre des rendements de produit plus élevés non seulement en terme de productions spécifique (par cellule) mais aussi en terme volumétrique (par unité de volume de culture). Pour caractériser ce système, la synthèse des protéines de réplication, les protéines structurales, les capsides formées, les génomes encapsidés et les particules actives, qui ont la capacité de transduire des cellules mammifères, ont été examinées.

L'importance de la multiplicité d'infection (MOI) dans les systèmes BEV a été démontrée à plusieurs reprises. La MOI, simplement dit, est le ratio entre le nombre de virus et le nombre de cellule lorsque le virus est ajouté à la culture. Quand la MOI totale est haute ($>> 1$), elle provoque une infection synchrone, où la population complète devient infectée et les cellules cessent de croître. Quand la MOI totale est basse ($<< 1$), elle provoque une infection non-synchrone, ce qui crée des populations de cellules infectées et non-infectées. Meghrous et al. (2005) ont exploré des stratégies non-synchrones sans succès pour la production de AAV par tri-infection. Dans cette thèse, pour mieux saisir les phénomènes d'interaction entre les protéines et les virus, des conditions provocant des infections synchrones sont utilisées. Avec le système tri-infection utilisé, la MOI de chaque type de virus peut être optimisée. Pour explorer comment la MOI de chaque virus et le ratio entre ces derniers peuvent influencer la production du AAV, des plans expérimentaux factoriels ont été conçus pour des infections de cultures à $2x10^6$ cellules/ml. Pour s'assurer que chaque cellule était atteinte des trois baculovirus, la quantité minimale pour chacun des trois virus pouvait elle-même causer une infection synchrone si utilisée seule. La quantité maximale a été établie par des contraintes pratiques, dont le volume de solution virale qui devait être ajoutée à la culture. Un titre maximal de particules actives est obtenu quand un équilibre existe entre les quantités de baculovirus codant pour

les protéines AAV. De plus, le titre augmente avec une augmentation de ces deux virus jusqu'à un point de saturation. Le baculovirus contenant le génome du vecteur AAV n'influence pas le titre d'une manière appréciable s'il est présent dans la cellule.

Comme on peut optimiser la quantité de chaque baculovirus ajouté à la culture, le moment de l'ajout peut aussi être optimisé. En gardant l'idée originale d'une infection synchrone à $2x10^6$ cellules/ml, des stratégies de délai d'addition ont été explorées. L'importance de l'ajout du BacRep dès le début de l'infection est ressorti comme indication principale. Un délai de 12 h a contribué à la diminution du titre du vecteur AAV par trois ordres de grandeurs. Tout comme l'étude sur la MOI, l'effet du délai de l'ajout du BacITR était le moins prononcé.

Le système BEV est un système lytique pour les cellules. Cependant avec la production des vecteurs AAV, les quantités de capsides AAV ont pu être corrélées à la viabilité de la cellule. Or, la surproduction de capsides a un effet néfaste sur la cellule. Plus il y a de capsides, plus la viabilité est basse. De plus, quand la viabilité reste élevé, il y a une augmentation dans le titre du AAV si le temps de récolte est décalé. Cependant, les titres maximaux sont généralement accompagnés d'une perte de viabilité.

L'équilibre est délicat. Le nombre de capsides doit être élevé mais ceci doit être accompagné de niveaux élevés de protéines de réplication car il existe aussi une corrélation entre les niveaux de Rep52 et la production de vecteurs AAV actifs. De plus, le BacRep bigénique, qui comprend les gènes pour le Rep78 et le Rep52, s'est avéré instable. Ces instabilités se sont manifestées aux cours de quelques passages de virus en forme de réduction dans les niveaux d'expression. Cette perte d'expression est probablement due à une recombinaison homologue entre les deux gènes qui sont placés tête-à-tête produisant une séquence pratiquement palindromique. Aux cours

des passages, la population de virus perd son uniformité et devient très hétérogène. Cependant, la minimisation du nombre de passages réduit cet effet. Il est estimé que suffisamment de vecteur AAV peut être produit pour de grands essais cliniques avec l'utilisation de baculovirus amplifié jusqu'au deuxième passage, cependant ceci reste une limitation du système par tri-infection.

Une autre solution potentielle est l'utilisation de deux BacRep monogénique qui codent pour chacune des deux protéines de réplication: BacRep52 et BacRep78 (Kohlbrenner et al., 2005a). En utilisant les mêmes stratégies que pour le système tri-infection, qui démontrait un besoin d'un certain équilibre entre les quantités de baculovirus codant pour les protéines AAV (et non pour le génome du vecteur), les deux BacRep monogéniques ont été ajoutés à des concentrations équivalentes à celui du BacCap. Ceci n'a pas donné d'aussi bons résultats que le système tri-infection. Quand la MOI totale a été abaissée en réduisant la MOI des BacRep de moitié pour que la MOI totale de la quadruple infection soit pareille à celui de la tri-infection, une amélioration du titre AAV a été observée sans toute fois atteindre les mêmes niveaux que pour la tri-infection. Une différence majeure observée entre les deux systèmes était liée au niveau de capsides produites. Avec la quadruple infection, les niveaux étaient plus faibles. Quand une combinaison du BacRep bigénique et un des deux BacRep monogéniques a été utilisée, que ce soit le BacRep52 ou le BacRep78, le titre du AAV produit est resté inchangé. Ces observations mènent à la conclusion que les capsides sont la composante limitante dans le système de quadruple infection.

Même s'il y a besoin de sur-exprimer les capsides pour obtenir des titres optimaux de AAV, il existe une marge de manoeuvre pour optimiser l'encapsidation (le processus d'insertion du génome viral dans la capside). L'écart entre le nombre de particule virale et le nombre de vecteur actif est de plusieurs ordres de grandeurs. Pour diminuer cet écart, il a été postulé que les conditions environnementales, dont la

température de la culture, pouvaient jouer un rôle. L'hypothèse première était qu'en abaissant la température, la production de protéines structurales ralentirait, ce qui donnerait plus de temps pour le processus d'encapsidation. Pour essayer d'isoler l'effet de la température sur la production de protéines (et non sur l'absorption du baculovirus et l'acheminement au noyau), le moment du changement de température a été considéré comme un deuxième facteur essentiel. Cette étude, avec le système tri-infection, a en effet permis d'augmenter le degré d'encapsidation, non à cause d'une baisse de température mais à cause d'une hausse de température. Le titre de vecteurs AAV actifs a augmenté avec une hausse de 27°C à 30°C mais a diminué si la température était augmentée à 33°C. De plus, les meilleurs résultats des cultures qui ont subit une diminution de température, n'ont jamais atteint les niveaux de production à 30°C même en retardant la récolte jusqu'à 120 hpi. Le meilleur moment pour changer la température était entre -24 hpi jusqu'à 6 hpi. Une augmentation de la température à 30°C au-delà de 6 hpi, augmente le titre AAV actif mais pas autant que si la température était augmentée auparavant. La production à 30°C entraîne des niveaux de Rep78 supérieures aux productions à 24°C ou 27°C, et l'expression du Rep52 est observée plus tôt. Quoiqu'il y ait une augmentation en protéines de réplication, le nombre total de particules virales ne change pas significativement, ce qui rend l'écart entre les particules AAV totales et les particules actives plus petit.

La production spécifique a été optimisée par rapport à la MOI et à la température de la culture, à une densité cellulaire de $2x10^6$ cellules/ml. Pour prendre avantage du potentiel qu'offre la culture de cellules d'insecte, la production de vecteurs AAV à des densités cellulaires plus élevées a été étudiée en vu d'optimiser la production volumétrique. Meghrous et al. (2005) ont démontré des limitations avec la production de AAV à 27°C (et une récolte à 72 hpi) à des densités cellulaires supérieures à $1\text{-}2x10^6$ cellules/ml. Le milieu de culture par contre, devrait être assez riche en nutriments pour maintenir la production spécifique jusqu'à $3\text{-}4x10^6$ cellules/ml. Des

recherches subséquentes par Chahal et al. (2007) ont indiqué des problématiques de récupération ou de sous-estimation de titre viral causées par un effet relié à la densité cellulaire. Pour éliminer ces effets, les culots cellulaires ont été resuspendus dans un tampon, enrichi de sel, à une densité cellulaire de l'ordre de 7-8x10^6 cellules/ml.

La production spécifique commence à baisser au-delà de 2x10^6 cellules/ml mais des niveaux acceptables sont maintenus jusqu'à 3-4x10^6 cellules/ml, ce qui est plus en accord avec la littérature. À haute densité cellulaire, sans ajout de nutriments ou de renouvellement de milieu, le milieu devient épuisé en sérine ce qui semble entraîner une perte totale (détectable) de production de vecteurs AAV. En renouvelant le milieu au temps d'infection, une partie de la production est récupérée, mais le milieu devient maintenant épuisé en cystine. Un cocktail riche en nutriments conçu pour la production à haute densité (Bédard et al., 1994, 1997) a été utilisé pour éviter l'épuisement du milieu. Même avec l'enrichissement du milieu, la production spécifique a diminué. Pour vérifier si "l'âge" de la cellule pouvait jouer un rôle dans la diminution de la production spécifique, des cellules du début de la phase exponentielle, où les titres maximaux ont été réalisés auparavant, ont été concentrées à différentes densités cellulaires. Cette stratégie a permis de maintenir la production spécifique jusqu'à 1x10^7 cellules/ml. Au delà de cette concentration cellulaire, le milieu devient épuisé de cystine, tout comme les expériences avec le renouvellement de milieu.

Les protéines de réplications sont essentielles à la formation des vecteurs AAV actifs. Les douze premières heures après l'infection sont critiques. Si un des trois virus est manquant, le titre est affaibli. De plus, c'est dans ces douze premières heures où l'effet de la température est le plus haut, probablement dû à une augmentation de protéines de réplication et un enchaînement plus rapide d'événements viraux. L'instabilité du BacRep complique la mise à l'échelle du procédé tri-infection,

mais les effets de l'instabilité de ce baculovirus peuvent être minimisés en purifiant par plaque et en minimisant le nombre de passages utilisés durant l'amplification du virus. L'utilisation de constructions baculovirales plus stables, incluant celles utilisées dans le système avec quadruple infection, seront nécessaires si plus de 10^{15} vecteurs AAV actifs sont requis. Cependant l'optimisation future de ce dernier devra adresser la production "sous-optimale" de capsides. Avec la tri-infection, la production à haute densité ($1x10^7$ cellules/ml) est possible. Il faudra pousser plus loin l'analyse des besoins nutritionnels post-infection pour produire à des densités encore plus élevées. De plus, des stratégies de production en mode de perfusion pourraient s'avérer utiles dans ce cas.

TABLE OF CONTENTS

LIST OF FIGURES

NOMENCLATURE

$Ad2$:	adenovirus serotype 2
$Ad5$:	adenovirus serotype 5
AAV:	adeno-associated virus
$BEVS$:	baculovirus expression vector system
BHK:	Syrian hamster kidney cell line
$\beta - Gal$:	β-galactosidase
CCI:	cell concentration at infection
CLP:	core-like particles
$\%CV$:	% coefficient of variation or relative standard deviation
$p\triangle IE1$:	truncated immediate early 1 promoter
DRP:	DNase-resistant particles
ETU:	enhanced transduction unit
F/T:	freeze/thaw
GFP:	green fluorescent protein
$HEK293$:	human embryonic kidney cell line
$HeLa$:	cell line derived from cervical cancer cells
HIV:	human immunodeficiency virus
HSV:	herpes simplex virus
hpi:	hours post-infection
HPV:	human papilloma virus
IVP:	infectious viral particles
$LacZ$:	gene for β-galactosidase
MOI:	multiplicity of infection (viruses/cell)
OUR:	oxygen uptake rate
$SEAP$:	secreted-alkaline phosphatase
TOI:	time of infection

VG: viral genomes

VLP: virus-like particle

VP: viral particles

WT: wild type

LIST OF TABLES

LIST OF APPENDICES

INTRODUCTION

The delay between bench-scale discovery and commercialization can be in the order of decades for bio-pharmaceutical products. Still, to satisfy regulatory agencies that oversee the approval of therapeutics, the process by which the therapeutic is made needs to be "locked in" early on. It is therefore crucial to integrate manufacturing concerns at early stages to address potential problems, and to seek ways that make the process economically viable.

Although China was the first to approve an adenovirus vector for a commercial gene therapy application, other parts of the world are more hesitant to give their consent for the commercialization of gene therapy. The death of Jesse Gelsinger in 1999, who developed a severe inflammatory response to a first generation adenovirus vector, and a least three cases of early childhood leukemia in patients who were being treated for X-linked severe combined immunodeficiency disease (X-SCID or "bubble boy disease") using a retrovirus vector, are among the concerns with the use of these viral vectors.

Viral vectors, however, remain the most prominent vehicles for gene delivery and gene therapy protocols. Among these, recombinant adeno-associated viruses (AAV) are being heavily studied. AAV vectors are derived from a non-pathogenic "replication-dependant" human virus. No significant immunological responses against transduced cells or the vector-derived gene product have been reported. Their ability to transduce both dividing and non-dividing cells and their stable long-term gene expression make them attractive alternatives to other gene delivery systems used for gene therapy. The broad applicability of AAV vectors strengthens the need to investigate their production. Furthermore, examples of the promising future of AAV vectors include: Phase III clinical trials for the treatment of prostate cancer; potential Phase

II trials for the treatment of Parkinson's disease after 9 out of 12 patients of a Phase I trial reported improved movement after being administered the vector (HealthDay News); the first human gene therapy trial in the United States for Duchenne muscular dystrophy (DMD), initiated in 2006; and bench-scale successes of AAV as a vaccine against HIV and HPV.

Currently, AAV is produced predominantly through the transfection of mammalian cells or through the use of transformed mammalian cells stably expressing AAV genes. The transfection method generally consists of co-transfection of HEK293 cells with multiple plasmids and is appropriate to generate and test different AAV vectors; however, it has been limited by the inability to achieve high transfection efficiencies of mammalian cells in suspension. This traditional method has relied primarily on production in roller bottles, petri dishes or cell factories, which are conventional means of culturing adherent cells. These means, however, are not amenable to scale-up. Generation of stable cell lines is suitable for large-scale production of AAV and has focused mainly on the transformation of HeLa cells; however, the generation of such cell lines is tedious and time-consuming. A major drawback cited for the limited amount of human gene therapy clinical trials based on AAV is the shortcomings of large-scale production based on the aforementioned methods. It has been estimated that up to 10^{15} AAV particles could be needed for a human clinical trial.

Aspects that need to be considered when choosing a production method include the efficiency of generating high-titer preparations, amenability to scale-up, and the ability to be free of pathogenic components or replicative competent adeno-associated virus. The baculovirus/insect cell system, which has been used for the production of a variety of virus-like particles, has the potential to resolve the above issues. The seminal work by Urabe et al. (2002) proved that functional AAV vectors could be produced at high titers in insect cells and Meghrous et al. (2005) further demonstrated that production of AAV could be maintained in a 20 L bioreactor. Insect

cells are an interesting "factory" for producing functional AAV particles since they can be cultured in suspension at various production scales and have been previously shown to grow to high cell densities. Another advantage of this method includes the elimination of traditional helper viruses.

To produce functional AAV particles using the baculovirus/insect cell system requires that the genes for AAV replication proteins and structural proteins be delivered to the cell and be under the control of insect cell promoters for efficient expression. Furthermore, the AAV vector genome containing the transgene of interest must also be delivered to the cell.

The driving hypothesis behind many of the objectives set for this work was that the formation of functional AAV particles, for a given cell density at infection, was primarily dependant on the distribution and replication of the baculoviruses and not only on the level of AAV Rep and Cap gene expression. In this system, there is an inherent competition between AAV synthesis and the synthesis of the three baculoviruses. The consistent production of functional particles was therefore believed to be dependant in part on factors such as the multiplicity of infection (the ratio of baculoviruses to cells at infection), the ratio between the three baculoviruses and the order of infection. It was believed these factors would dictate if and when the transgenes for the AAV would be expressed in the insect cell. A second hypothesis which was investigated was that high volumetric yields were limited by low specific productivities at high cell densities because of limiting nutrient conditions and not as a result of a change in the dynamics associated with the interaction of the three baculoviruses and the cells.

The overall objective of this research project was to characterize the production process to help in the optimization of the volumetric and cell specific production of this viral vector in insect cells. More specifically, this work aimed to: 1. detail the

dynamics of the triple-infection and quadruple infection, as well as the production of functional vectors in terms of virus capsid proteins, replication proteins, assembled viral particles and functional particles; 2. investigate the effect of various factors including the multiplicity of infection, ratio between the baculoviruses, and the time of infection; and 3. identify limiting conditions for the production of functional particles when infecting the cells at increasing cell densities.

In a first part, a detailed literature review broken down into two chapters describe the state of the art of AAV production and the state of the art of baculovirus/insect cell technology, the technology of central focus in this thesis. The first chapter was submitted as a review article entitled: *Critical assessment of current adeno-associated viral vector production and quantification methods* to the scientific journal *Biotechnology Advances.* Following these two chapters, a detailed study on the quantity and ratios of baculoviruses that need to be used for the optimal production of AAV using three baculovirus vectors was undertaken. This was the object of a second publication entitled: *Production of adeno-associated viral vectors in insect cells using triple infection: optimization of baculovirus concentration ratios* published in the journal *Biotechnology and Bioengineering.* A second baculovirus/insect cell system consisting of four baculovirus vectors is the subject of another study in Chapter 4, that emphasizes the balance required between the synthesis of the replication and structural proteins for optimal production. Chapter 5 details work on the manipulation of culture temperature to improve the production of AAV through environmental conditions that favor the assembly of complete vectors. This study was the subject of a third publication entitled: *Improving AAV vector yield in insect cells by modulating the temperature after infection* also published in the journal *Biotechnology and Bioengineering.* The final chapter of this thesis deals with the production of AAV vectors in high cell density insect cell cultures.

CHAPTER 1

CRITICAL ASSESSMENT OF CURRENT ADENO-ASSOCIATED VIRAL VECTOR PRODUCTION AND QUANTIFICATION METHODS

The goal of this thesis is to characterize the production of adeno-associated viral vectors in insect cells in order to develop strategies that will "optimize" the overall process. Key to the characterization of the process is the quantification of the adeno-associated viral vectors themselves. This chapter will allow the reader to understand the different methods used to quantify adeno-associated viral vectors and will allow an objective evaluation of the insect cell platform for the production of adeno-associated viral vectors. It has been submitted as a review article entitled: "**Critical assessment of current adeno-associated viral vector production and quantification methods**", to ***Biotechnology Advances.***

Critical assessment of current adeno-associated viral vector production and quantification methods

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1.1 Abstract

Adeno-associated viral vectors have emerged as one of the most studied vectors for gene therapy. Numerous production methods have been described, each with their advantages and disadvantages. A challenge in assessing the current state of the art exists in comparing yields from one production system to the next due to the wide variety of quantification techniques. In this review, AAV vector production methods are summarized and the yields of the different processes are standardized to the number of harvested cells. Titers are further streamlined into five categories: transduction units, enhanced transduction units, infectious particles, DNase-resistant particles and total particles, and the importance of each type of measure is discussed.

Keywords: review, production, quantification, adeno-associated virus, ELISA, PCR, infectious assay, replication assay, transduction assay

1.2 Introduction

Adeno-associated viruses (AAV) are small replication-dependent viruses that preferentially undergo latent infections. To complete their lytic life cycle, wild-type AAV rely on the presence of other viruses, most commonly adenoviruses or herpes viruses. AAV particles contain a single stranded DNA genome consisting of approximately 4.7 kb. This genome has two open reading frames (ORFs) with genes encoding for structural proteins that make up the capsid and non-structural proteins needed for replication and formation of progeny virus. The two ORFs are flanked by inverted terminal repeats (ITRs) that are necessary for proper encapsidation of the viral genome and serve as the origin of replication among other functions. For the construction of a gene therapy vector, the ITRs are the only components that need to be supplied in *cis*. The two ORFs containing the Rep and Cap genes can be

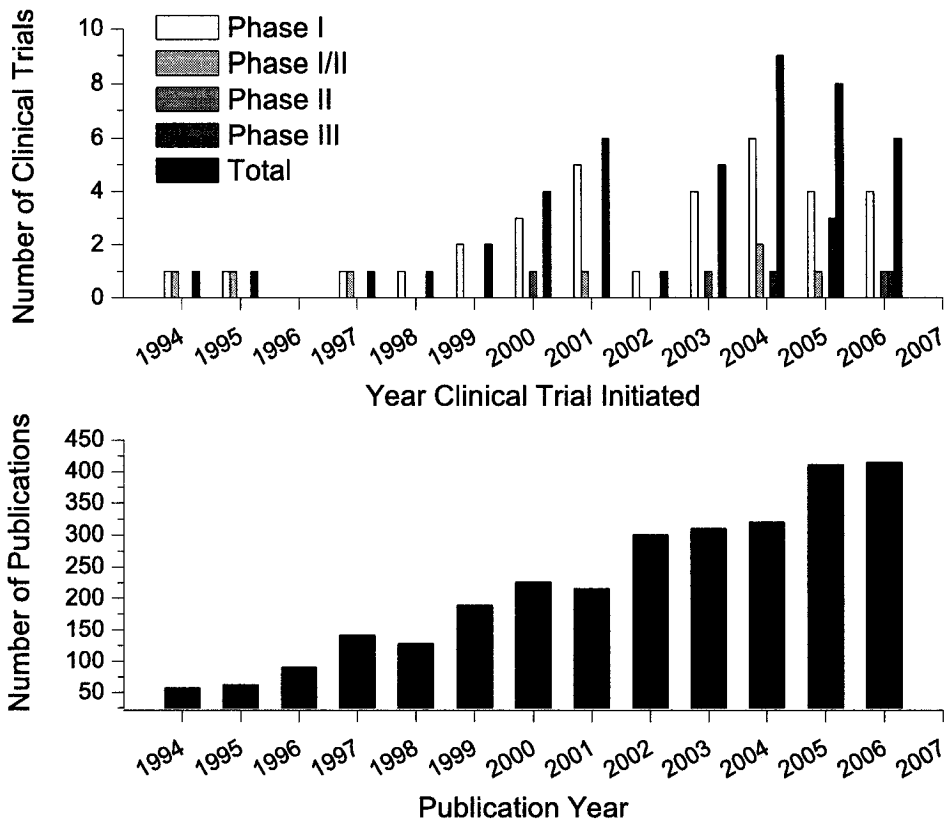

Figure 1.1 Overview of the status of AAV as of January 2007 in terms of clinical trials and publications. Data for clinical trial status were obtained from the Journal of Gene Medicine (Edelstein, 2007). Publication data are the result of bibliographic searches on adeno-associated virus or AAV in the PubMed electronic database.

removed from the genome and supplied in *trans*, allowing for an insert of approximately equivalent length (Grieger and Samulski, 2005). Despite its small carrying capacity, vectors based on adeno-associated virus are promising candidates for viral gene therapy. The absence of significant host reaction, persistent transgene expression levels after administration and the ability to deliver genes to a variety of tissues are among the reasons for the interest in this class of vectors.

To date, almost 50 clinical trials have been undertaken worldwide. These include

mostly Phase I and II trials; however, trials for the treatment of prostate cancer are in Phase III (Edelstein, 2007). The increase in clinical trials, as seen in Figure 1.1, only partially reflects the growing need for both increases in production capacity and efficiency. Interest in AAV is also evidenced by the steady increase in the number of publications over the last decade (Figure 1.1). It has been estimated that between 10^3 and 10^5 DNA-containing AAV particles (herein referred to as DNase-resistant particles (DRP)) per cell will be needed for efficient *in vivo* gene transfer (reviewed by Clark, 2002). If the correlation of dose to body weight holds between species, up to 10^{14} DRPs will be needed for human clinical doses (Grimm and Kleinschmidt, 1999). According to a dose escalation study reported by Kay et al. (2000), the initial cohort was given $2x10^{11}$ DRPs/kg. Doses, however, are not only based on the mass of the patient, but also on the target tissue. It has been estimated that only $1x10^8$ DRPs would be needed for retinal gene therapy, while $1x10^{14}$ DRPs would be needed for gene therapy targeting muscle or liver (Matthews et al., 2002). Dosages, however, are often based on animal data and this practice may actually overestimate the amount required to achieve therapeutic results. Still, a significant amount of material will be needed to support the development of AAV for approved use in the treatment of diseases. It is realistic to assume that 10^{15} DRPs could be needed for large animal studies or for human clinical trials as suggested by Urabe et al. (2002).

This review serves multiple purposes. Chief among these, it aims to identify current production capacity by reviewing the wide range of methods used to generate adeno-associated vectors. To achieve this goal, methods to characterize this vector are surveyed to establish a basis for comparison. Based on the current state of the art, prospective avenues are discussed.

1.3 Adeno-associated vector production

The production of adeno-associated viral vectors has been studied extensively, although most reports are based on adherent cell lines on 10-15 cm plates. A mid to large-scale production is generally reported as 20 to 50 of these plates. A wide variety of methods to produce these vectors exist. Of these, three categories are most prominent, even though most methods overlap with each other: production by transfection, infection, or the use of stable cell lines.

Transfection of cells followed by infection, usually with a helper virus, was established early on for the production of AAV viral vectors. A spin on this approach was to supply helper functions on a plasmid and to supply the vector sequence using rAAV itself (Drittanti et al., 2001). A marked improvement in the evolution of AAV vector production occurred when the helper function could be supplied by a plasmid, thus eliminating the need for any viral infection. This reduced concerns surrounding replication competent AAV and pathogenic adenovirus contamination. While there have been great strides in transfection technology, equal advances have arisen in the use of viral vectors for gene delivery. This is one of the reasons for the second category of production methods, which are based on infection of cells with recombinant viruses. The primary sources include vectors based on herpes virus, adenovirus and baculovirus. The final method is based on stable cell lines. These can be further subdivided into two types: packaging and producer cell lines. Packaging cell lines contain the rep and cap gene sequences while producer cell lines also contain the AAV vector genome. Production of AAV vectors in these cell lines generally occurs after infection or transfection of these stable cell lines with the required helper functions. Stable cell lines have been made using HeLa, BHK and 293 cells; however most have been derived from HeLa cells.

Reporting vector titers has been complicated by the wide array of titration methods,

but has also suffered from a lack of reference material and a standard basis for comparison. In some instances the values reported have been normalized to the volume of liquid in the final concentrated solution, to the volume of liquid in the cell culture or even to the volume of liquid added as the lysis buffer. Others have been normalized to the number of cells used, while others were normalized to the number of producing cells (cells that were known to be infected or transfected).

One way to compare all these different production systems is to normalize yields to the total number of cells at harvest. The resulting per cell titers of different production strategies are summarized in Table 1.1. Table 1.1 is sorted based on the production strategies, followed by the cell line used, the transgene used and the year of publication. Titers have been broken down into four categories: VP is defined as the number of total particles; DRP is defined as packaged vector genomes and refers to the sample preparation (DNase-Resistant Particles); IP is defined as infectious particles determined through an infection/replication assay; ETU is defined as the expression of a transgene in the presence of helper function; and TU is defined as the expression of a transgene without the supply of any complimentary function. The definition of transduction in this paper is broader than the conventional definition of a transfer of genetic material to a cell, and implies gene expression as well.

Table 1.1: Summary of processes and yields for AAV-2 (unless otherwise specified) vector production.

METHOD	CELL LINE	GENE	YIELD	REFERENCE
Transfection				
Primarily Transfection + Infection				
Double plasmid transfection using Lipofectin/Integrin targeting peptide/DNA (LID) complexes followed by herpes simplex virus type 1 (HSV-1) derived virus (glycoprotein H and thymidinkinase deleted mutant of HSV-1 strain SC16) infection (PS1, MOI=20)	BHK	GFP	660 ETU[1]/cell	Feudner et al. (2001)
Single plasmid transfection mediated by Lipofectamine + recombinant HSV-1 infection (d27.1-rc, MOI=10)	HEK293	GFP	380 IP[2]/cell	Conway et al. (1999)
Double plasmid transfection by calcium phosphate precipitation and infection with adenovirus (dl 309, MOI=2)	HEK293	LacZ	220 ETU[3]/cell[4]	Li et al. (1997)
Double plasmid transfection using the calcium phosphate precipitation method followed by rAAV infection (MOI=100-300)	HEK293	LacZ	1200-2800 DRP/cell	Urabe et al. (2000)
Single plasmid transfection by calcium phosphate precipitation + infection with herpes simplex vector (AAV type 5)	HEK293	LacZ	1200-9000 DRP/cell	Wustner et al. (2002)
Transfection Only				
Double plasmid transfection using calcium phosphate precipitation	HEK293	GFP	500 IP[5]/cell[6] 150 IP[5]/cell[7]	Grimm et al. (1998)

Table 1.1: Summary of processes and yields for AAV vector production (continued)

METHOD	CELL LINE	GENE	YIELD	REFERENCE
Double plasmid transfection mediated by Lipofectamine	HEK293	GFP	13600 DRP/cell 135 ETU[8]/cell	Collaco et al. (1999)
Triple plasmid transfection using calcium phosphate precipitation method	HEK293	GFP	18000 DRP/cell[6] 4680 DRP/cell	Liu et al. (2003)
Triple plasmid transfection using PEI complex-forming method	HEK293	GFP	$\leq$13894 VP/cell $\leq$452 IP[9]/cell	Park et al. (2006)
Triple plasmid transfection using PEI complex-forming method	HEK293	GFP	30500 DRP/cell 30-100 ETU[21]/cell	Durocher et al. (2007)
Double plasmid transfection by calcium phosphate precipitation	HEK293	LacZ	120 ETU[10]/cell	Matsushita et al. (1998)
Triple plasmid transfection using calcium phosphate precipitation	HEK293	LacZ	940000 DRP/cell 1100-1200 ETU[3]/cell	Xiao et al. (1998)
Quadruple plasmid transfection using calcium phosphate precipitation method	HEK293	LacZ	200000 - 400000 DRP/cell	Ogasawara et al. (1999)
Triple plasmid transfection using PEI complex-forming method	HEK293	LacZ	33114 VP/cell 10850 DRP/cell 124 IP[11]/cell 49-80 ETU[12]/cell	Drittanti et al. (2001)
Triple plasmid transfection using calcium phosphate precipitation method	HEK293	LacZ	$\leq$16700 DRP/cell	Feng et al. (2007)
Double plasmid transfection using the FreeStyle 293 Expression System (lipid mediated, Invitrogen) (AAV type 5)	HEK293F[13]	GFP	32400 DRP/cell 10-18 TU/cell[14]	Smith et al. (2003)
Triple plasmid transfection using PEI complex-forming method	HeLa	GFP	$\sim$4500 DRP/cell $\sim$10 TU[15]/cell	Reed et al. (2006)

Table 1.1: Summary of processes and yields for AAV vector production (continued)

METHOD	CELL LINE	GENE	YIELD	REFERENCE
Infection				
Double herpes simplex vector infection	B130[16]	GFP	~53000 VP/cell ~0.06 TU[17]/cell	Booth et al. (2004)
Double herpes simplex vector infection	BHK	GFP	~155000 VP/cell ~40 TU[17]/cell	Booth et al. (2004)
Triple adenovirus infection	HEK293	GFP	52000 - 300000 DRP/cell 200-600 ETU[23]/cell	Zhang and Li (2001)
Dual infection with rAAV vector and pseudorabies (swine herpesvirus) vector	HEK293	GFP	0.08 TU[18]/cell	Shiau et al. (2005)
Dual baculovirus vector and adenovirus infection	HEK293	LacZ	~0.33 ETU[19]/cell[20]	Sollerbrant et al. (2001)
Dual infection with rAAV vector and herpes simplex vector (AAV type 5)	HEK293	LacZ	1000 - 11000 DRP/cell	Wustner et al. (2002)
Triple baculovirus vector infection	High Five (*T. ni*)	GFP	~225 ETU[21]/cell	Meghrous et al. (2005)
Triple baculovirus vector infection	Sf9	GFP	45000 DRP/cell 33 TU/cell	Urabe et al. (2002)
Quadruple baculovirus vector infection	Sf9	GFP	~50000 DRP/cell	Kohlbrenner et al. (2005)
Triple baculovirus vector infection	Sf9	GFP	210000 VP/cell 253 ETU[21]/cell	Meghrous et al. (2005)
Triple baculovirus vector infection	Sf9	GFP	300000 VP/cell 70 ETU[21]/cell	Aucoin et al. (2006)

Table 1.1: Summary of processes and yields for AAV vector production (continued)

METHOD	CELL LINE	GENE	YIELD	REFERENCE
Triple baculovirus vector infection (for the production of AAV-5 vectors	Sf9	GFP	70000 VP/cell 20000 DRP/cell	Urabe et al. (2006)
Triple baculovirus vector infection	Sf9	GFP	710000 VP/cell 12000 DRP/cell 141 ETU[21]/cell	Aucoin et al. (2007)
Triple baculovirus vector infection	Sf9	LacZ	300000 VP/cell 2 ETU[21]/cell	Aucoin et al. (2006)
Triple baculovirus vector infection	Sf9	SEAP	300000 VP/cell 0.02 ETU[21]/cell	Aucoin et al. (2006)
Stable Cell Line + Infection/Stable Cell Line + Transfection/ Stable Cell Line				
Stable cell line expressing rep/cap genes + infection with adenovirus (MOI=10) and an adenovirus-AAV hybrid vector (MOI=10)	A549 (K209)	GFP	262-378 TU[22]/cell	Gao et al. (2002)
Stable cell line containing rep, cap and vector + infection with adenovirus (Adts149, MOI=100)	A549 (SP53.26)	GFP	3500 ETU[23]/cell	Farson et al. (2004)
Stable cell line containing rep, cap and vector + infection with adenovirus (Adts149, MOI=10)	A549 (SY69)	GFP	210000 DRP/cell 660 ETU[24]/cell	Farson et al. (2004)

Table 1.1: Summary of processes and yields for AAV vector production (continued)

METHOD	CELL LINE	GENE	YIELD	REFERENCE
Recombinant herpes simplex virus infection (d27.1-rc) of proviral cell line containing the hGFP-encoding gene (hgfp) and neomycin resistance gene inserted between AAV-2 ITRs	HEK293 (GFP-92)	GFP	338-480 ETU[2]/cell	Conway et al. (1999)
Stable cell line containing rep/cap genes and rAAV vector sequence infected with an adenovirus vector (Ad-Cre, an adenovirus carrying the Cre recombinase gene of P1 phage, MOI=5)	HEK293 (293-GFP145)	GFP	137600 DRP/cell[4] 1720 ETU[25]/cell[4]	Qiao et al. (2002b)
Stable cell line expressing AAV rep-cap genes + transfection of AAV vector plasmid + infection with Ad.dl324 (an E1-deleted Ad5) (MOI=10)	HEK293 (HEK293RC21)	LacZ	200 DRP/cell[26] 3.7 IP[27]/cell[26] 0.43 ETU[12]/cell[26]	Chadeuf et al. (2000)
Stable cell line expressing AAV rep-cap genes + transfection of AAV vector plasmid and pAdc helper plasmid	HEK293 (HEK293RC21)	LacZ	$\leq$0.5 DRP/cell[26] 0.0032 IP[27]/cell[26] 0.002 ETU[12]/cell[26]	Chadeuf et al. (2000)
Antisense mediated regulation of rep and cap expression in a stable cell line + infection with two adenovirus vector (Ad-Cre, MOI=1 and AVC2Cap, MOI=0.1) and an AAV vector	HEK293 (293CR)	LacZ	2900 DRP/cell[28]	Okada et al. (2001)

Table 1.1: Summary of processes and yields for AAV vector production (continued)

METHOD	CELL LINE	GENE	YIELD	REFERENCE
Antisense mediated regulation of rep and cap expression in a stable cell line + transfection of AAV vector plasmid + infection with two adenovirus vector (Ad-Cre, MOI=1 and AVC2Cap, MOI=0.1)	HEK293 (293CR)	LacZ	60000 DRP/cell[28]	Okada et al. (2001)
Stable cell line expressing Rep/Cap genes of AAV-2, VA RNA, E2A and E4 of Ad5 + transfection of AAV vector plasmid by calcium phosphate precipitation method	HEK293T[29] (293T R/C.VA.E2A.E4 no.3)	LacZ	30-640 TU[15]/cell	Nakamura et al. (2004)
Stable cell line containing rep/cap + infection with adenovirus (sub100r, a temperature sensitive virus with mutation in E2b, MOI= 10) and an adenovirus-AAV hybrid vector (MOI=10)	HeLa (B50)	GFP	590000 DRP/cell 78 TU[22]/cell	Gao et al. (1998)
Stable producer cell line with drug inducible adenovirus genes	HeLa (GFP-155-XX6)	GFP	6 ETU[25]/cell[4]	Qiao et al. (2002a)
Stable cell line expressing AAV rep-cap genes and AAV vector sequence + replication-defective HSV-1 (HSVΔUL30) (MOI = 5)	HeLa (HeRC32AAVhALD15)	Human adrenoleukodystrophy	33333 VP/cell 8333 DRP/cell 113 IP[27]/cell	Toublanc et al. (2004)
Stable cell line expressing AAV rep-cap genes + transfection of AAV vector plasmid and pAdc helper plasmid	HeLa (HeRC32)	LacZ	0.5 DRP/cell[26] 0.0034 IP[27]/cell[26] 0.0007 ETU[12]/cell[26]	Chadeuf et al. (2000)

Table 1.1: Summary of processes and yields for AAV vector production (continued)

METHOD	CELL LINE	GENE	YIELD	REFERENCE
Stable cell line expressing AAV rep-cap genes + transfection of AAV vector plasmid + infection with Ad5 (MOI=50)	HeLa (HeLaRC32)	LacZ	669 DRP/cell[26] 6 IP[27]/cell[26] 1.7 ETU[12]/cell[26]	Chadeuf et al. (2000)
Stable cell line containing vector, rep and cap + infection by adenovirus	HeLa (H44)	LacZ	Upto 36 IP[30]/cell	Clark et al. (1995)
Stable cell line containing rep/cap genes and rAAV vector sequence infected with an adenovirus vector (MOI=10)	HeLa (TtetA2Rluc c.49)	Luciferase	5300 DRP/cell 102 ETU[8]/cell	Inoue and Russell (1998)

Nomenclature

TU	Transduction Units	no co-infection with helper virus
ETU	Enhanced Transduction Units	transduction with helper virus
IP	Infectious Particles	number of particles that are able to replicate in the presence of rep, cap and helper functions
DRP	DNase Resistant Particles	genomes detected after DNase treatment
VP	Viral Particles	total particles by ELISA (A20 antibody) or by electron microscopy

Notes

[1]Co-infection of HeLa cells with adenovirus 5(Ad5, MOI=50)
[2]Co-infection of C12 cells, a HeLa derived cell line containing AAV-2 rep and cap genes, with adenovirus (MOI=20)
[3]Co-infection of HEK293 cells with adenovirus (Ad5 dl309, MOI=1)
[4]Assuming $5x10^6$ cells per 10 cm plate
[5]Co-infection of HeLa cells with Ad5 (MOI=100) and AAV-2 (MOI=10)
[6]Per transfected cells
[7]Assuming highest transfection efficiency gave highest AAV yield
[8]Co-infection of HeLa cells with adenovirus (MOI=10)
[9]Co-infection of HeLaRC32 cells, HeLa derived cells containing AAV-2 rep and cap genes with Ad5 (MOI=20)
[10]Co-infection of HEK293 cells with adenovirus 2 (Ad2, MOI=50)
[11]Co-infection of HeLaRC32 cells with Ad5 (MOI=100)

[12]Co-infection of HEK293 cells with Ad.dl324 (an E1-deleted Ad5, MOI=10)
[13]HEK293F are a clone of HEK293 cells adapted in FreeStyle^{TM} 293 Expression Medium
[14]Infection of COS-1 cells
[15]Infection of HeLa cells
[16]B130 is a neomycin resistant BHK-derived cell line that constitutively expresses the HSV-1 ICP27 protein
[17]Infection of HEK293T cells, HEK293 cells that express the Large-T antigen of SV40
[18]Infection of HEK293 cells
[19]Co-infection of HEK293 cells with vAd-CO1, a control adenovirus lacking the E and E3 regions (MOI=4)
[20]Assuming 1500000 cells in 500μl culture volume per 60mm dish
[21]Co-infection HEK293E, HEK293 cells that express the Epstein Barr Nuclear Antigen, with Ad5 (MOI>10)
[22]Infection of 84-31 cells, HEK293 cells stably expressing Ad-E4
[23]Co-infection of HEK293 with adenovirus
[24]Co-infection of HuH7 cells with adenovirus (Add*l*309, MOI=10)
[25]Co-infection of HeLa cells with Ad5 (MOI=1)
[26]Assumes 1x10^7 cells/15 cm plate
[27]Co-infection of HeLaRC32 with adenovirus (MOI=50)
[28]Assuming 2x10^5 cells per 3.5 cm dish
[29]HEK293 cells that express the Large-T antigen of SV40
[30]Co-infection of C12 cells with adenovirus

Although cell factories and roller bottles still figure prominently in the technologies used for generating AAV, other processes, that are more amenable to scale-up and intensification, will be sought to meet the anticipated needs in an economical and viable fashion. The current state of the art reflects this notion with reports of AAV production using suspension cultures (Smith et al., 2003; Farson et al., 2004; Meghrous et al., 2005; Park et al., 2006; Feng et al., 2007; Durocher et al., 2007). As can be seen from the production table, transfection in cells other than HEK293 cells has been limited; however, recent work by Reed et al. (2006), has demonstrated the efficient transfection of HeLa cells using polyethyleneimine (PEI), which has been touted as a scaleable, cost-effective transfection platform. This promises to open up this avenue of production for studies at larger scale. The problem with packaging cell lines, as reported by Farson et al. (2004), is that they are difficult to make. Out of 1296 clones screened for being AAV rep/cap positive, only one contained the genes. The difficulty in making stable cell lines renders this approach inflexible. Booth et al. (2004) have presented a high yielding dual herpesvirus system that holds a lot of promise, however, it still remains to be seen if the system is amenable to a serum-free suspension process. Although still not widely accepted, the baculovirus/insect cell system should not be discounted as a platform for the production of AAV. It has been shown that on a per cell basis, Sf9 cells produce as much as mammalian cell lines if not more. The distinct advantage of the baculovirus/insect cell system is its ability to be cultivated in suspension in a high cell density environment, leading to volumetric yields that are greater than mammalian cell based platforms.

1.4 Quantification of AAV vectors

Thirteen years after the first clinical trial was initiated, there are still no standards for measuring AAV nor is there any standard reference material. This remains a major

hurdle in assessing the wide variety of methods available to produce AAV. AAV-2 is the most studied and best characterized of all adeno-associated vectors, which is why it is often used as a model for the production of AAV vectors. Establishing a single golden standard based on AAV-2 would be beneficial for benchmarking and process optimization, however, the push towards other serotypes/pseudotypes/variants that offer enhanced characteristics for therapeutic applications has taken the focus away from this goal.

What the field is lacking in standardization, it makes up for in the methods used to quantify the AAV vectors produced. Generally, quantification of AAV vectors is based on the number of packaged genomes, as evidenced by the estimates of future vector requirements. Although this value does not adequately describe the vector, the process, or the preparation, it remains one of the only truly comparative means to describe the vector.

1.4.1 Capsid/particle analysis

Exploitation of the viral shell is at the core of viral gene delivery. Adeno-associated viruses are small (20-26 nm Ø), non-enveloped icosahedral viruses, first identified in electron micrographs as contaminants in adenovirus preparations. Their capsids are made up of 3 viral proteins (VP1, VP2 and VP3), which are translated from alternatively spliced forms of the p40 transcript. VP1, the largest of the three, has been implicated in the infectivity of the virus (Girod et al., 2002). VP3, the smallest of the three, is the most abundant structural protein in the capsids and is responsible for tissue tropism. VP2 was shown to be necessary for capsid assembly in studies done in insect cells (Ruffing et al., 1992), however, virus composed of only VP3 have been recently produced (Warrington et al., 2004). The exact function that VP2 plays remains unelucidated. AAV-2 capsid proteins can be visualized by Western blot using

commercially available antibodies that recognize either VP1, VP1 and VP2, or all three, VP1, VP2 and VP3. Upon densitometric analysis of protein bands resolved by Western blot, structural protein ratios of VP1:VP2:VP3 have been observed to fluctuate from 1:1:8 (reviewed by Kronenberg et al., 2001) to 1:1:20 (Girod et al., 2002). In crude preparations, we have observed ratios that average from 1:1:5 to 1:1:20 (unpublished).

An A20 antibody exists that recognizes a specific surface epitope of assembled AAV-2 capsids (Witsuba et al., 1997) and an enzyme-linked immunosorbent assay (ELISA) has been developed (Grimm et al., 1999). Calibration of the ELISA was done using both electron microscopy and protein content assays on purified capsid preparations. Protein content was equated to capsid titers by assuming that a capsid is made up of 60 subunits having a uniform stoichiometry of structural proteins (VP1:VP2:VP3 = 1:1:10); one particle is estimated to have a molecular weight around 6.5×10^{-9} ng. Depending on where the sample is situated on the calibration curve, the coefficient of variation (CV) could vary between 3 and 67% (Grimm et al., 1999). In our lab, the particle titer for each sample is determined using the commercially available ELISA kit, based on the aforementioned work, using several serial dilutions that fall within the calibration range. Using this approach the average coefficient of variation for sample titers is less than 25%, with values, however, ranging from 0 to 88% (n=100).

Given that capsid proteins were considered a limiting factor in early AAV vector production, and that vector assembly is still the rate limiting step in current production methods, the capsid titer should figure more prominently as a characterization method. But as can be seen in Table 1.1, the number of groups which report this value are few and far between. This measurement is seen by our group as being quite important in view of optimizing AAV production processes. In a study on the production of AAV vectors in insect cells it was found that the number of total

particles detected via this method was correlated with the decline in cell viability (Aucoin et al., 2006). The ELISA allows reliable detection from various steps in the production process and does not require highly purified material, although it may be influenced by the level of salt in the sample to be measured (Grimm et al., 1999). Furthermore, for scaleable purification schemes that do not rely on gradient centrifugation but on chromatographic methods (Auricchio et al., 2001; Zolotukhin et al., 2002; Kaludov et al., 2002; Chahal et al., 2007; Zolotukhin et al., 1999), which do not separate empty and filled capsids, determining the number of empty particles remaining in the final preparation is critical. In fact, by reducing the number of empty particles, it has been shown that the potency of the vector increases (Urabe et al., 2006). Recently, ELISAs for AAV-1/6, AAV-4 and AAV-5 have been developed (Kuck et al., 2007).

Although methods to detect specific serotypes using affinity capture methods have been developed, they do not provide much flexibility. Because capsids play a broad role including host-immune response, antibody neutralization, and virus-cell interaction, there is a significant trend towards capsid modifications to improve AAV as a vector for gene delivery (Girod et al., 1999; Wu et al., 2000; Hauck et al., 2003; Perabo et al., 2006; Maheshri et al., 2006). Unlike affinity-based methods, ion-exchange chromatography presents itself as a method that may be transferable to multiple serotypes/variants because it is based on generic physical properties. A cation-exchange analytical HPLC method for the quantification of AAV-2 vectors has been developed (Debelak et al., 2000). The use of HPLC as an analytical method has yet to be adopted as a standard method of quantification. This is likely owing to the low intrinsic absorbance of intact AAV particles. When analyzing crude samples, the AAV signal tends to be drowned out due to higher intrinsic absorbance of the other components in the sample. Still, in order to get a reliable signal, according to Debelak et al. (2000), only $1x10^{11}$ DRP/ml of purified material are required. Un-

fortunately, their study did not assess the number of empty particles in the injected samples; the number of total particles needed to detect a significant signal may be higher since the method does not resolve empty and genome containing particles. Nonetheless, cation-exchange chromatography is a method that may be used with multiple serotypes since it is based on generic physical properties. Given the low coefficient of variation (9%) from the measurements of various solutions with different concentrations, HPLC still holds promise. In the work done in our labs, the advantage of HPLC methods for the detection of viral particles has been proven (Transfiguracion et al., 2001, 2004).

Generally, from those that report total particles, transfection processes yield on average mid 10^4 VP/cell. Booth et al. (2004) have shown the ability to increase this value to above 10^5 VP/cell in BHK cells through the herpes simplex derived infection process (see Table 1.1). In insect cells, using the baculovirus expression system, the total number of particles approaches 10^6 VP/cell (see Table 1.1). The latter; however, has led to the speculation of an inefficient encapsidation process of the genome (Aucoin et al., 2007).

1.4.2 Genome analysis: packaged genomes

When speaking of quantities of particles in terms of vector genomes, most often this titer refers to the number of DNase-resistant particles and the terms are used interchangeably. This implies that exogenous DNA has been enzymatically degraded and any remaining intact DNA must be protected within the viral capsid. The reasoning behind this type of assay is that transduction of cells will not occur if the transgene is not present, therefore, the detection of the vector genome should be a good indicator of the number of functional particles, without needing to rely on the expression of said transgene (further discussion on this point will follow in the next

section). The latter however assumes that only viral capsids can protect the viral DNA from digestion; it has been suggested that viral genomes can be protected from enzymatic digestion when bound to replication proteins, cellular proteins or even when only a portion of the viral DNA is contained within the particle (Grimm et al., 1999). This raises the question of how well this measure represents the transduction ability of the vector. The number of packaged genomes may not correlate well with the immunogenicity of the vector either, which is more readily related to the properties of the capsids. Still, this value is the most commonly reported measure of AAV titers.

According to Gao et al. (2000) AAV titers determined by slot/dot-blot hybridization can vary 5- to 10-fold in both inter-assay and intra-assay even though this was regarded as one of the main methods used to quantify DNA. It has been reported that the formulation of the solution to be tested influences the reading, with magnesium ions inhibiting DNA from binding to nitrocellulose and nylon membranes (Kube and Srivastava, 1997). Furthermore when compared to a PCR method (reviewed by Gao et al., 2000), a linear correlation could not be achieved and variations between 2 and 10-fold were reported. PCR has been reported to be a more robust and accurate way to assay AAV genomes and has also been pursued as a quicker way to detect AAV vectors (Clark et al., 1999). Surprisingly, when values obtained from real time PCR were compared to values obtained by dot-blot, the values were always within a factor of 2. This may support the reports that the variability of the dot-blot assay depends on the quality of the starting material.

Real time PCR for AAV-2 has also been reported by Rohr et al. (2002). In their study, the average intra-assay coefficient of variation was 4% and the average inter-assay coefficient of variation was 48%. Veldwijk et al. (2002) have reported an average intra-assay coefficient of variation of 4% and an average inter-assay coefficient of variation of 9% for their real time PCR based method; however, the low CVs

could be a result of using highly purified and concentrated stocks. Most recently reliable detection of AAV from crude lysates has been achieved with real time PCR (Mayginnes et al., 2006). Using DNase and alkali treatments, results from the PCR-based method were correlated with transduction assays relying on GFP expression (R^2=0.887). It should be noted that when crude lysate samples were used, proper dilutions were needed to avoid gross under and over-estimations of the titers, which occurred due to inhibiting components in the cell lysates or spent media and to samples containing little to no AAV DNA, respectively. This method, however, avoided the use of sample purification steps like spin columns, which can cause yield reduction. Samples with as little as $1x10^8$ DRP/ml could be analyzed confidently using this method. This advance is highly relevant to those wishing to study and optimize the process for improved packaging of the viral genomes, which remains one of the limiting steps in AAV production.

Another method that can be used to estimate the number of packaged genomes is through indirect observation of a sample using electron microscopy. Empty particles are characterized by low electron densities in the core of the particle (Ruffing et al., 1992; Clark et al., 1995; Grimm et al., 1998; Kaludov et al., 2002; Qiao et al., 2002b). This estimation, however, is complicated by possible intermediate particles that appear neither empty nor full, as reported by Grimm et al. (1998).

Finally, for highly purified material, a spectrophotometric method has been established to determine the number of capsids and the number of genomes from the A_{260}/A_{280} ratio (Sommer et al., 2003). This method requires the denaturation of particles prior to analysis because of significant light scatter resulting from intact particles. Although this method is sensitive to impurities and to buffer formulations, it is probably the simplest method available to measure AAV particles.

Though there are reports of per cell DRP production approaching 10^6 for methods

based on transfection and stable cell lines (see Table 1.1), the majority of methods report levels on the order of mid 10^4. The most recent reports on AAV production in suspension cultures, which are most likely the type of production method that will be used in the future to meet clinical needs, have shown the ability to maintain DRP titers around 10^4, independently proving the scalability of AAV productions.

1.4.3 Vector functionality: transduction and infection assays

The most important criterion for a gene therapy vector is its ability to transduce specific cells: those that need gene correction. Unfortunately, cells that are to be targeted in humans are not often cultured in labs; therefore, transduction of the desired cells is generally not tested as part of a routine assay. It is for this reason that commonly available cell lines are generally used to assess the quality of the vectors. Not all vectors, however, will be able to transduce the same cell line with the same level of efficiency, highlighting the inherent drawback of assessing the quality of the vector based on the expression of the transgene. This section attempts to capture the wide array of methodologies used to describe the quality of AAV vectors.

These assays have been referred to as dilution replication assays, end-point dilution assays, fluorescent cell assays, gene transfer assays, infectious center assays, infectivity titrations, modified fluorescent focus assays, single cell fluorescence assays, replication center assays, serial dilution replication assays, transduction assays and tissue-culture infected dose at 50% ($TCID_{50}$) measurements. Consequently vector quality titers have been reported in transduction units, infectious units, ($TCID_{50}$), infectious particles, infectious viral particles, replication centers, replication competent particles and replicative titers. These measurements, however, are not as unique and distinct as their nomenclature may suggest. The root of these assays seek to estimate the ability of the vector to deliver the transgene of interest and can be segregated

into two broad categories: transduction/expression assays or infection/replication assays.

1.4.3.1 Transduction/expression assays

Theoretically, the evaluation of transduction efficiency would be best represented by lone AAV addition to a non-complimenting cell line and measuring the expression of the transgene. This type of assay, however, is limited by the levels of detectable expression, which are a function of many biological properties of the capsid, the transgene and promoter, and the target cell line. With reporter genes for β-galactosidase, GFP, SEAP, and luciferase under strong promoters such as the cytomegalovirus (CMV) promoter, detecting transgene expression is relatively easy. For therapeutic transgenes with tissue specific promoters, detection may not be so straight forward. The transduction of cells is further hampered by the ability of the target cells to provide an environment propitious for the conversion of the vector genome to a double stranded transcriptional template, which is considered to be the rate limiting step in gene expression from AAV vectors (Ferrari et al., 1996; Fisher et al., 1996). Clark et al. (1996) found that levels of gene expression differed if their AAV vector, containing the LacZ gene, was applied to HEK293, HeLa, or human foreskin fibroblast cells. Two approaches have been suggested for establishing the transduction ability of the vector. The first involves applying serially diluted vector preparations of unknown titer, while the second determines the transduction levels given a known quantity of vector genomes (Urabe et al., 2002). Rohr et al. (2002) evaluated the intra-assay and inter-assay coefficients of variation for transduction of HeLa cells based on the former approach. Using GFP as the reporter gene, an average intra-assay coefficient of variation of 6% and an average inter-assay coefficient of variation of 10% were obtained. Transduction levels established using the above approaches generally lead to large discrepancies between the number of DRPs and the number of transducing

particles, as seen from Table 1.1.

To enhance the rate of double strand synthesis, helper virus, most commonly adenovirus (Adv) or herpes virus, or changes in the extra-cellular environment due to heat shock, exposure to genotoxic agents such as UV irradiation, γ-irradiation, certain chemical carcinogens or metabolic inhibitors, can be used (reviewed by Berns and Giraud, 1996). Clark et al. (1996) found that while there were differences in transduction levels between cell lines when only the AAV vectors were supplied to the cells, upon addition of a helper adenovirus, the difference in transduction levels between cell lines was muted. For this reason, it is believed that the ability of the vector to transduce a cell is not likely due to problems associated with entry or trafficking to the nucleus, but in the specific ability of each cell type to convert the single stranded genome into a double stranded template. The addition of a helper virus not only levels the playing field, but enhances gene expression levels. Increases in the transduction efficiency of up to three orders of magnitude have been observed (reviewed by Grimm et al., 1999). In our lab, evaluating AAV titers by detecting GFP expression in HEK293E cells co-infected with wild-type adenovirus type 5 has proven useful for process optimization as a simple and reliable measure with average coefficients of variation of $\sim$21% (n=430). This is consistent with reports from other labs, which have reported coefficients of variation between 25 and 30% for a similar assay (Jenny et al., 2005).

We have also observed in our work, that given the same cell line, type and quantity of helper virus, incubation period, production method and similar levels of capsids, the level of gene expression can vary by approximately three orders of magnitude depending on the transgene (Aucoin et al., 2006). This example further serves to highlight that different assay conditions may also influence the observable titers. Differences in functional titers between vectors with different transgenes have been reported by many others including Nam et al. (2004) who observed differences in

yields of AAV-LacZ, AAV-GFP and AAV-GAD65.

Not all transduction assays reported in the literature that use helper virus, use the same type or MOI. We have seen in our work that varying the MOI of helper wild-type adenovirus type 5 from MOIs of 10 to 500 (number of infectious particles per cell, which corresponds to 100 to 5000 total particles per cell) did not significantly affect the levels of gene expression (unpublished data), therefore attenuating the effect of this factor. This is also consistent with work by Zhen et al. (2004) who used an infection/replication assay (see next section) and observed increases in AAV genome copy number for increasing adenovirus input up to 50 particles per cell with no significant increase thereafter. There are still groups that have reported titers while co-infecting with helper virus at MOIs of 1 (see Notes of Table 1.1), which may have resulted in: 1. an underestimation of the enhanced transduction titer, and 2. more variability in these titers. Zhen et al. (2004) also explored the use of adenovirus type 2 instead of 5, which resulted in a three fold reduction in the number of replicated genomes, indicating a possible dependence on the type of helper virus used.

1.4.3.2 Infection/replication assays

Infection/replication assays differ from the transduction/expression assays previously described because transgene expression is not necessarily needed. In these assays, determining whether the transgene was trafficked to the nucleus is key in assessing the quality of the vector. There is a large body of work surrounding this approach, and many believe that knowing whether the vector was able to bind to the surface of the cell, was internalized and was delivered to the nucleus is a better indicator of how the vector will perform *in vivo*. These assays yield an end-point dilution titer based on the detection of vector genomes or expression of a reporter gene. It

is expected that values obtained from this type of assay will be significantly higher than transduction/expression assays since it generally does not rely on transgene expression. Additionally replication of the genome should amplify the signal for detection.

Unlike the transduction/expression assays described in the previous section, infection/replication assays generally use HeLa derived target cells and require AAV Rep genes, AAV Cap genes and adenovirus helper functions, so that AAV vectors can replicate. The AAV Rep and Cap genes can be supplied by plasmid transfection, wild-type AAV (Grimm et al., 1998) or by infection with a recombinant adenovirus (Zhang and Li, 2001). Clark et al. (1995) was one of the first to use a HeLa-derived packaging cell line (cells containing the AAV Rep and Cap genes) to circumvent the need to co-infect cells with wild-type AAV or supplying the genes by transfection. Now there exists a number of such cell lines used for titration including the C12 (Clark et al., 1995, 1996), C37 (Atkinson et al., 1998), HeLaRC32 (Salvetti et al., 1998) and D7-4 (Zhen et al., 2004) lines.

Clark et al. (1995, 1996) showed that the sensitivity of the assay could be enhanced close to ten-fold using PCR to amplify the genome sequences before detecting the genomes by Southern blot. Atkinson et al. (1998) developed a ninety-six well plate detection scheme to resolve vector genomes by direct hybridization. In a study by Drittanti et al. (2000), real-time PCR detection of genomes increased the sensitivity a hundred-fold over dot-blot hybridization. Zhen et al. (2004) increased the sensitivity of this type of assay by establishing a linear correlation between the number of input genomes and the number of genomes amplified in their packaging cell line. The coefficient of variation for this method is as high as 48%; however, in light of the variability of biological assays, this is still considered acceptable. Furthermore this method can be applied to lysates or other crude material though there may be issues in recovery/underestimations depending on the "cleanliness" of the starting

material even with the high dilutions required for the endpoint assay. Certain types of aggregation/affiliation seem hard to reverse once formed (Chahal et al., 2007).

GFP expression has also been used to detect replicated AAV vectors (Zolotukhin et al., 1999; Veldwijk et al., 2002). Zolotukhin et al. (1999) showed through a comparison between infectious particles estimated by detection of replicated genomes or fluorescence expression, that the two methods showed little difference in the measured titer.

The most consistent method to quantify and determine functionality of AAV vectors is most likely based on the infection/replication assay; however, a single packaging cell line should be made available for use. The drawbacks of this method may be with pseudotypes/variants that do not use AAV-2 inverted terminal repeats. Recently, Mohiuddin et al. (2005) developed a replication assay that did not require a packaging cell line and avoided the use of adenovirus. The assay was designed in such a way that it could be tailored to a number of different serotypes/pseudotypes/variants using an approach where one cell line does not fit all. In this assay, a recombinant replication deficient herpesvirus containing the AAV rep/cap genes was used to deliver the complimenting AAV functions for replication of the AAV vector. The key to the success of this methodology is the use of cell lines that are permissive to HSV and AAV infection.

Recently, Zhen et al. (2004) have shown, using an infectious assay with increased sensitivity, that the titers approach the theoretical limit (~30% of the packaged genomes) of what could be detected. Although this is a positive result, it suggests that the number of packaged vector genomes may be an adequate titer value and that a biological assay may not be as informative as the community believes.

1.4.4 Relationship between transduction units: enhanced transduction units: infectious units: packaged genomes: total particles

By the simple nature of the various quantification methods that exist, accurately determining the ratios between total particles, particles that contain genomes and the number of particles that are able to transduce cells is prone to a large variability. Given the uncertainty associated with each measure (as well as the inherent sensitivity of detection of each assay) and the lack of standardization, it is difficult to compare different methods with regards to these ratios. The calculation of these ratios, however, when assessing methods within a same lab with a certain degree of consistency, can lead to valuable information. It is with this in mind that the following section is discussed.

It is believed that a comparative analysis of a secondary assay and the number of genome containing particles can be used to assess the efficiency of packaging. Clark et al. (1999) have shown that larger constructs had larger DRP:IP ratios, which showed potential limitations in the proper packaging of genomes. In the seminal paper on the use of ELISA as a method to quantify the total number of capsids by Grimm et al. (1999), it was reported that on average, using a transfection protocol to generate AAV vectors, the ratio of the total number of VP to DRP was 16; however, the results ranged from 5 to 76. This led to the conclusion that overexpression of capsids could lead to their underutilization and that packaging of genomes is in fact the limiting step. Furthermore, when they compared the total particles with the number of vectors that were able to transduce cells, the average ratio was closer to $5x10^4$ to $1x10^5$, and could range up to $3.63x10^5$. Recently, Bleker et al. (2006) reported that changes in capsid proteins could lead to changes in the interaction between rep proteins and the capsid, resulting in different packaging abilities. With the increased push towards AAV variants with modified capsids (Maheshri et al., 2006;

Perabo et al., 2006), the enumeration of total particles should be considered in determining the need to develop adequate scalable separation strategies for minimizing the number of empty particles.

In the system developed by Urabe et al. (2002), as discussed in the section on VP quantification, there is no lack of capsids produced because the Cap gene is driven by the strong insect polyhedrin promoter. The number of capsids produced has been shown to affect the viability of the insect cells (Aucoin et al., 2006), which could lead to AAV losses in the supernatant. Noting that the number of genomes is approximately 10 to 100 fold lower than the total number of capsids detected by ELISA, a greater utilization of the capsids through a greater degree of encapsidation would be beneficial. To be able to make an assessment on encapsidation efficiency, however, there must be a certain degree of consistency between the various measures. Rohr et al. (2002) have reported a good correlation (R^2=0.72) between the number of genomes detected by real-time PCR and the number of infectious particles, giving an average ratio of 253; however, the standard deviation was 241. Veldwijk et al. (2002) have also shown a good correlation between the log of the transduction units and the log of PCR quantification having a Pearson correlation coefficient (r) of 0.89. This linear regression, however, masks the true variability in the ratios; although the average genome:transduction unit ratio was $\sim$ 7000, the actual range spanned from 200 to 34000. Nonetheless, the correlation predicted the transduction units in the study to within one order of magnitude. More recently, work done by Zhen et al. (2004) has shown that a more consistent number could be established between the infectious titer and the genome measurement when both measurements used Q-PCR. The average ratio between the two titers was approximately 10 for purified material, a result that is in part due to the increased sensitivity in detection of the infectious particle assay.

A broad overview of Table 1.1 shows that cells can produce $\sim$10-100 TU/cell, $\sim$100-

1000 ETU/cell, ~100-1000 IP/cell, ~1000-100000 DRP/cell and ~10000-1000000 VP/cell. By lowering the ratio between the various measures (given a set method of detection), the therapeutic index may be improved, assuming that by reducing the ratio between measures there is a reduction in defective vectors. Lowering the ratio may also be an indicator of packaging efficiency, which is still thought to be the limiting step in AAV vector production.

1.5 Concluding Remarks

The advances and diversity in successful AAV production methods ensure a certain amount of competitiveness that will drive the optimization of these processes in terms of cost, stability, flexibility and robustness. Standardization of quantification and qualification methods is crucial, and must play a larger role in order for a true optimization to take place.

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CHAPTER 2

BACULOVIRUS EXPRESSION VECTOR SYSTEM (BEVS): FROM PROTEINS TO VIRUS-LIKE PARTICLES AND VIRAL VECTORS

2.1 Introduction

In the previous chapter, a range of production methods for AAV vectors was presented. Given the variability of the techniques used for the quantification of AAV, not one process is significantly better than the others based on the cell specific production. Each process has its own advantages and disadvantages. This chapter is a review of baculovirus-insect cell technology, the focus technology of this thesis, which has as advantage the potential for high cell density cultivation.

The baculovirus/insect cell system is known for its ability to produce high levels of biologically active recombinant proteins due in part to the type of post-translational modifications achieved in insect cells. The latter, the large DNA insert capacity and the capability of multiple gene expression has given this system a unique niche in the gamut of expression systems. Primarily, this system has been used in vaccine development, for the production of therapeutic and diagnostic agents and structure/function studies (Friesen, 1997) but is now being considered as a feasible alternative for large scale production of proteins including the production of vaccines.

This chapter will briefly review baculoviruses, their use and their behavior as an expression vector. It will also highlight the depth of work that has been accomplished in insect cells with regards to virus-like particle production and how the production of viral vectors, such as adeno-associated viral vectors, is a natural extension of this branch of study. This chapter will also describe the major works done in the field

of baculovirus-insect cell technology that serve as the basis for this thesis. Finally, this chapter will examine the most recent advances in the area that can be used to further improve the production of AAV vectors beyond the scope of this thesis.

2.2 Baculoviruses

Baculoviruses are a well defined family that infect a wide range of lepidopteran (butterfly and moth) species. They are large rod-shaped (30 to 60 nm by 250 to 300 nm) enveloped viruses having double stranded circular DNA that have in particularity two morphologically distinct forms of infectious particles: occluded virus and budded virus (non-occluded virus). Occluded virus, which are responsible for horizontal transmission (insect to insect), consist of enveloped virions within a crystaline protein matrix. Budded virus, which are responsible for cell to cell infection, consist of single virion enveloped by a plasma membrane. The baculovirus family has been divided into two genera, granuloviruses and nucleopolyhedroviruses, and differ mainly in the number of virions within an occluded virus particle, with granuloviruses having only one virion while nucleopolyhedroviruses having 20 or more (as reviewed by Friesen, 1997). The recombinant baculoviruses used within this thesis, *Autographa californica* nuclear polyhedrosis virus (AcMNPV) are part of the latter and are the best characterized of all baculoviruses; for these reasons and for sake of brevity, all subsequent descriptions will be based on this representative member.

Through genetic manipulation, baculoviruses have evolved from simple insect viruses to versatile biotechnological tools that are able to deliver genetic material to insect and mammalian cells. Reviews on the varied uses and successes of baculovirus-based expression systems have been published recently (Hu, 2005; Kost et al., 2005). As expression vectors, the occluded form of the virus is non-essential. Since the gene coding for the major protein (polyhedrin) of the occlusion matrix does not affect

the propagation of budded virus, necessary for efficient use in cell culture, it can be replaced with a gene of interest. Under the control of the native polyhedrin (polh) promoter the transgene can be highly expressed (at least 25% and some have reported close to 50% of all cellular protein, as reviewed by Jarvis (1997), Maranga et al. (2002a) and Wickham et al. (1992a)). Although early vectors relied primarily on the strong polh promoter to drive transgene expression, there exists other promoters including, but not exclusively, the p10 and IE1 insect promoters, that have been used to either moderate levels or to control the temporal expression. Most recently, Dalal et al. (2005) reported the use of the Early-to-Late insect cell promoter with the reporter protein GFP, which allowed the expression of the reporter transgene to be detected 18 hours earlier than what was observed when the transgene was under the control of the polh promoter.

2.2.1 Replication cycle

Understanding the baculovirus replication cycle can shed some light on the development of these viruses as vectors and provides insight into the use of various promoters to control the temporal expression of proteins. The latter is also key in the development of process strategies and a concern that cannot be neglected in trying to simulate a baculovirus/insect cell culture. The work done on characterizing the replication cycle, as reviewed by Friesen (1997), has been based on the synchronous infection of cells in cell culture, making it directly applicable to the work in this thesis. For a more thorough examination of the events that occur in the baculovirus replication cycle, the reader is pointed to Friesen (1997) and Miller (1997).

Budded virus enters a cell primarily through receptor-mediated endocytosis but also by membrane fusion. The viral envelope fuses with the endosome as the environment acidifies and the nucleocapsid is released to travel to the nucleus where its

genomic material is uncoated and the infection cycle is initiated. This process can take as little as 15 minutes from the time of virus inoculation (reviewed by Friesen, 1997). The baculovirus infection cycle consists of different phases upon which each subsequent phase depends on the products of the former in a sort of cascade control mechanism. The infection cycle can be divided into three phases: early (0 to 6-9 hours post-infection), late (6-12 hours post-infection) and very late (18 to 76 hours post-infection). In the early phase, transcriptional activators, apoptotic suppressors and DNA replication factors are expressed. Early viral gene products block progression of the cell cycle in S and G_2/M phases. As transcription of early genes peak and starts to decline, the onset and acceleration of late viral transcription, which is mediated by a virus-encoded RNA polymerase, takes place. The late phase is characterized by viral DNA replication and the production of budded virus. The very late phase is accompanied by the high expression of occlusion-specific genes. Among these occlusion-specific genes, those under the control of the polyhedrin (polh) promoter are highly transcribed. In this stage of the infection cycle, nucleocapsids start accumulating in the nucleus, acquire an envelope, and in the wild-type system, are embedded in the polyhedrin matrix. Figure 2.1 shows an insect cell in very late phase (72 hpi) with recombinant baculoviruses within the cell and budding through the plasma membrane.

2.3 Protein, Virus-Like Particle and Viral Vector Production using Baculovirus Expression Vectors and Insect Cells

The BEVS has been used to generate hundreds of different proteins, a number of which have been scaled up to 100L (Elias et al., 2003). Of interest, in the context of this thesis, is the work done on the production of virus-like particles. Virus-like particles (VLPs) resemble naturally occurring viruses without the nucleic acid

Figure 2.1 Electron microscopy of infected insect cell (72hpi). Bb = budding baculovirus. Bn = internal baculovirus.

content. These particles cannot self-replicate, making them ideal candidates for antigens or immunogens. VLPs produced in insect cells have been the subject of nearly two decades of research for studying viral assembly, virus-cell interaction and for the development of vaccines. They span the entire spectrum of viruses including RNA, double-stranded RNA, single- and double-stranded DNA, enveloped and non-enveloped viruses. The wide variety of virus-like particles produced in insect cells are included in Table 2.1 grouped by virus family. An extended list including relevant references to each virus-like particle studied is presented in Appendix I.

Table 2.1: Virus-like particles produced in insect cells

Type of Virus	Reference
Astroviridae	Human astrovirus serotype 1
Birnaviridae	Infectious bursal disease virus (IBDV)
Bunyaviridae	Hantaan virus
Caliciviridae	Hawaii human calicivirus
	Hepatitis E
	Human enteric caliciviridae (SRSV)
	Human calicivirus MX virus (Mexican strain)
	Norwalk virus
	Porcine enteric calicivirus
	Rabbit hemorrhagic disease virus (RHDV)
	Toronto virus
Coronaviridae	Human Coronavirus - SARS
Flaviviridae	Hepatitis C
Hepadnaviridae	Hepatitis B
Nodaviridae	Flock house virus
Orthomyxoviridae	Influenza virus
Papovaviridae	B-lymphotropic papovavirus
	Bovine papillomavirus (type 1)
	Cottontail rabbit papillomavirus
	Equine papillomavirus (type 1)
	Human papillomavirus (type 6, 11, 16, 33)
	Murine polyomavirus
	Simian virus (type 40)

Table 2.1: Virus-like particles produced in insect cells (continued)

Virus Family	Virus Type
	JC virus
Parvoviridae	Adeno-associated virus
	Aleutian mink disease parvovirus
	B19
	Canine parvovirus
	Mink enteritis parvovirus
	Parvovirus minute virus of mice
	Porcine parvovirus
Picornaviridae	Enterovirus (type 71)
	Poliovirus
Reoviridae	Bluetongue virus
	Epizootic hemorrhagic disease virus
	Rice dwarf virus (RDV)
	Rotavirus
Retroviridae	Feline immunodeficiency virus
	Human immunodeficiency virus (type 1, 2)
	Murine leukaemia virus
	Rous sarcoma virus
Totiviridae	Leishmania RNA virus

The most recent reviews on VLPs have highlighted the ability to produce various types of particles and their efficiency to elicit immunogenic responses in animal and human trials (Casal, 2001; Noad and Roy, 2003) as well as addressing issues surrounding the processing of these particles (Maranga et al., 2002a). Since then, studies have extended the list of VLPs made in insect cells (included in Table 2.1), addressed scale-up issues and have examined strategies for large-scale production, including simulating cultures infected at low multiplicities of infection. Furthermore, the production of viral vectors, which is a natural extension of virus-like particle production, has been achieved in insect cells (Urabe et al., 2002) and serves as the

basis of this thesis work.

The main difference in the production of a viral vector is the incorporation of genetic material within the virus-like particle. This, however, is not a recent find. Forstova et al. (1995) sought to demonstrate the ability to package exogenous DNA into a polyomavirus VLP made up of only the major structural protein, VP1, after initial experiments seemed to show DNA associated with VLPs composed of three structural proteins (Forstova et al., 1993). Particles were made in insect cells and in a subsequent step, after the purification of the particles, linear polyoma DNA fragments or plasmids containing various genes (p43, CAT), were incubated with the particles in a packaging step. The drawback of this method is the low level of actual encapsidation as seen in the low levels of protection against DNase for linear (5-10%) and supercoiled plasmid DNA (30%). A similar approach was taken by Touze and Coursaget (1998) using papillomavirus-like particles that were produced in insect cells using the HPV-16 L1 gene. The packaging of plasmid DNA was shown to decrease with the size of the DNA with 36% being encapsidated for a plasmid of 5 kbp, 19% for a 7.2 kbp plasmid and 0% for a 10.3 kbp plasmid.

Pawlita et al. (1996) noted in the production of B-lymphotropic papovavirus (LPV)-like particles (based on the major structural protein VP1) that 65% of the particles were consistent in appearance with LPV virions. Further analysis revealed a 4.5 kb linear double stranded DNA protected by the VLP, which contained sequences homologous to Sf9 cell DNA, baculovirus genome and to portions of the sequence coding for VP1. The encapsidation of cellular Sf9 DNA of $\sim$ 5 kb has been reported in VP1-based murine polyomavirus as well (Gillock et al., 1997). In the study conducted by Pawlita et al. (1996), the filled particles represented 10% of the purified VP1 particles. This is a relatively high frequency for non-selective random encapsidation as it approaches values obtained for the encapsidation of AAV genomes in insect cells (Urabe et al., 2006; Aucoin et al., 2007b). They further describe an encapsidation

process not unlike the maturation process described for viruses in general; viral particles lacked the association with DNA or contained smaller DNA fragments at the beginning of the process and as the process progressed a greater proportion of VLPs contained larger DNA fragments. They also showed that the process took up to 5 days post-infection to reach maximum encapsidation.

Hepatitis C RNA transcripts from cDNA have been shown to incorporate into Hepatitis C VLPs (Baumert et al., 1998) when produced in insect cells. An aspect that stands out from this study is the selective nature of the incorporation of Hepatitis C RNA over transcripts of other viruses. This result leads to speculation upon appropriate genetic sequences that could be used for generating a proper Hepatitis C viral vector.

Upon the finding that Papovaviridae VLPs could be used to package foreign DNA (An et al., 1999; Forstova et al., 1995; Gillock et al., 1997; Goldmann et al., 1999), the use of VLPs to package other molecules, such as propidium iodide, has been investigated (Goldmann et al., 2000). The packaging method suggested by Goldmann et al. (1999, 2000) involves a dissociation/reassociation step of the capsid proteins after the purification of particles produced in Sf9 cells.

The production of a true viral vector, namely an adeno-associated viral vector based on serotype 2 (Urabe et al., 2002), requires functional replication proteins for the encapsidation of the viral vector genome. Three baculoviruses have been used to deliver the gene for the structural proteins, the genes for the non-structural (replication) proteins and the vector genome. The gene for the structural proteins (cap gene) and the gene for one of the replication proteins (Rep52 gene) are under the polyhedrin promoter in two separate baculoviruses. The baculovirus containing the gene for Rep52 also contains the gene for the second replication protein, Rep78; however, the gene for Rep78 is under a truncated promoter of the immediate-early

1 gene (ΔIE-1) of *Orgyia pseudotsugata* nuclear polyhedrosis virus (Urabe et al., 2002). This was done to ensure a weaker expression of this protein, which, in mammalian cells, has been shown to inhibit the formation of infectious particles when overexpressed (Li et al., 1997a). Another baculovirus is used to supply the AAV vector genome containing the native AAV inverted terminal repeat sequences, which are necessary for the proper encapsidation of the viral genome. Key to the success of this system is the functionality of the AAV replication proteins in insect cells, which allow both the excision of the AAV vector genome from the baculovirus genome, and the replication and accumulation of AAV vector genomes in insect cells.

2.3.1 Factors influencing BEVS/insect cells production

Once the ability to express the desired proteins in an insect cell line has been achieved, a number of factors can then be considered for the optimal synthesis of the desired product. The following sections, highlight some of the most important factors that have been investigated for the production of foreign protein, with an emphasis on what has been used for the production of virus-like particles and AAV.

2.3.1.1 Cell choice

Cells derived from *Spodoptera frugiperda* (Sf9, Sf21, Sf158) and *Trichoplusia ni* (High Five^{TM}, Hi5, Tn5) cells have been used for VLP production. Choice of cell line can affect the specific and volumetric production. Higher specific titers have been achieved for the production of VLPs (Jiang et al., 1998; Wang et al., 2000) and AAV (Meghrous et al., 2005) in *Trichoplusia ni* cells (*T. ni*) compared to *Spodoptera frugiperda* (Sf) cells. It has been suggested that this may be because *T. ni* cells are larger and therefore have a higher carrying capacity. *T. ni* cells, however, are not

cultured to as high of a cell density as Sf cells; therefore, on a volumetric basis, *T. ni* cells do not necessarily result in the highest yields. When producing AAV vectors, the overall yield at the end of the culture on a volumetric basis was similar for both Sf and *T. ni* cells (Meghrous et al., 2005).

T. ni derived cells have also been shown to produce more secreted and glycosylated product compared to Sf9 cells, which leads to differences in where product accumulates. In a recent study of the production of Hepatitis E virus-like particles, particles were found in the supernatant for infected Tn5 cultures but not in Sf9 infected cultures (Li et al., 2005).

Choice of cell line needs to be considered in terms of eventual scale-up of cell cultures in bioreactors and for process intensification through high cell density cultivation. Sparging and agitation, which are needed to maintain levels of dissolved oxygen, lead to significant shear stress for the cells. *T. ni* cells have been reported to be more susceptible to shear stress then Sf9 cells (reviewed by Maranga et al., 2002a) and recently, differences within Sf clonal isolates have been observed (Maranga et al., 2004). Sf21 cells have been found to withstand more than twice as much shear stress compared to Sf9 cells (2.2 N/m^2 for Sf21 cells compared to 1 N/m^2 for Sf9 cells).

Groups reporting the use of *T. ni* cells for production (Jiang et al., 1998; Lerch and Friesen, 1992; Li et al., 2005, 1997b; Meghrous et al., 2005; Sico et al., 2002; Wang et al., 2000) have used Sf cells to propagate their baculovirus stocks. In our lab, it has been observed that the propagation of baculovirus in *T. ni* cells resulted in a lower proportion of infectious baculovirus particles being produced compared to Sf9 cells (unpublished results). The production of viral stocks are therefore routinely done using Sf cells. A consequence of choosing *T. ni* cells as the production cell line is the need to maintain a second cell line for baculovirus propagation.

2.4 Culture Conditions

Recently a body of work focusing primarily on the processes behind making VLPs has started to accumulate, with work on bluetongue virus (Maranga et al., 2002a; Zheng et al., 1999), rotavirus (Jiang et al., 1998; Palomares et al., 2002, 1999; Park et al., 2004), human (Maranga et al., 2002a; Tsao et al., 1996) and porcine (Cruz et al., 2002; Maranga et al., 2003, 2004, 2002b) parvoviruses, human immunodeficiency virus (Cruz et al., 1998b, 1999), and infectious bursal disease virus (Hu and Bentley, 2001, 2000; Hu et al., 1999) being reported. These works focus less on the construction of the baculovirus vectors used but on the bioreactors, culture conditions and infection. These productions have been done at various scales from shake or spinner flasks to bioreactors varying from 2 to 50 L. Maranga et al. (2004) have recently shown a successful scale up based on the engineering principle of maintaining constant shear stress. This was achieved by calculating the maximum impeller shear stress at 2 and 25 L scales. The main parameter that was used to make sure the maximum shear stress was not exceeded was the stirrer speed.

Culture conditions such as process temperature have ranged from 25°C to 29°C, with most cultures being done at either 27°C or 28°C. For the production of VLPs, it has been reported that the effect of temperature is negligible when a proper dissolved oxygen concentration during the culture is maintained (Tsao et al., 1996). The effect of oxygen concentration has been studied through adjustments in the aeration rates and the impeller rotational speed for the production of VLPs (Cruz et al., 1998b). Using three different dissolved oxygen concentrations (10, 25 and 50% of oxygen saturation), optimal product titer and quality occurred at 25%. In another study based on the production of rotavirus VLPs, changing the dissolved oxygen concentration from 20 to 50% did not alter the growth of the cells but the effect on VLP production was not studied (Park et al., 2004). This latter finding is consistent

with studies on respiration of insect cells where DO has been shown to be of no significant consequence on the viable cell density or the viability between 5-100% of oxygen saturation (Kamen et al., 1996). The effect of oxygen concentration though, has also been suggested to be a function of other factors including the multiplicity of infection (Gotoh et al., 2002), in which case maintaining low dissolved oxygen levels, but above the threshold limit, gave the best results.

Although oxygen concentrations have been deemed an important factor on the outcome of the culture, the optimal oxygen concentration is in fact dependent on many other factors, including the medium and the bioreactor. The effect of oxygen concentration can be further complicated at high cell densities when additional measures need to be taken to ensure a proper dissolved oxygen concentration ie. sparging and possibly higher agitation rates. It is therefore believed that oxygen levels need to be directly linked to the needs of the cells. To achieve this link, the specific consumption rates have been calculated, and has further been used to successfully predict viable cell concentrations (Kamen et al., 1996).

2.5 Infection

Infection is a defining moment in the process whereby the production phase starts. It consists of the virus binding to the cell, being internalized and routed either to the nucleus, to continue a productive infection, or to the lysosomes to be destroyed. Because infection involves the addition of extra material in the reactor, it is one of the major steps in the overall process. It is also important since it creates populations of cells: those that are infected and those that are not. How much and when are the primary questions when discussing infection. These are defined by the multiplicity of infection (MOI), the number of viruses put into the system per cell, and the time of infection (TOI), when virus is added to the system. TOI, however, has yet to find

an exact definition and generally reflects the condition of the cells or the cell density.

2.5.1 Multiplicity of infection

The multiplicity of infection is defined as the number of infectious virions per cell initially added to the cell culture. Normalizing the number of viruses to the number of cells is expected to create a dimensionless parameter that is able to describe the system regardless of the actual concentrations of each used. An aspect that this ratio does not account for is the volume in which the contact of these two species takes place; therefore, it is possible that the micro-environment differs when different cell densities are used. Although the use of cell concentrations and virus concentrations would be more appropriate to describe the system at infection, the MOI remains a convenient and simplifying concept, albeit somewhat controversial. In a recent study on the process involved in making porcine parvovirus VLPs, Maranga et al. (2004) showed that when using low cell densities of infection and across a small range (0.6-1.5 x 10^6 cells/ml) the concept of MOI held regardless of the cell density at infection.

MOIs between 10^{-4} (Zheng et al., 1999) to 10^5 (French et al., 1990) have been studied for the production of VLPs in insect cells with the majority of the studies using MOIs between 1 and 50. For small scale studies, the use of high MOIs is commonplace, generating product rapidly in a reliable manner. However, when considering scaling the process, a number of process related issues surround the use of large MOIs. High MOIs will require generation of large viral stocks, hence, scale-up of the process will need to consider both vector stock propagation as well the production step. Multiple rounds of virus amplification can result in the deleterious "passage effect", a decrease in the quality of the virus due to inherent instabilities. Certain baculovirus constructs have been shown to be more prone to instability. Pijlman et al. (2003) showed

that there could be spontaneous excision of vector sequences from bacmid-derived baculovirus vectors but also showed, that the addition of homologous regions, which act as origins of DNA replication, could improve the stability of the vector (Pijlman et al., 2004). These findings are both alarming and encouraging since not all processes seem to be amenable to the use of low MOIs. Another practical consideration in the use of high MOIs is that generally culture supernatant, after filtration, with or without conditioning (addition of 1% FBS for stability and storage), is used as vector stock. When high MOIs are used, either a large volume or concentration of the virus, a time consuming process, is necessary. The more volume used, the more spent medium is added to the system unless the baculovirus is reconstituted in fresh medium after concentration. This can cause the growth/infection medium to be less than optimal, rendering possible limiting conditions unless supplements are added. Characterization and minimization of viral stock solutions to be added are definite considerations that need to be addressed when looking to maximize the yield of the process.

Low MOIs have not only been investigated to reduce the burden of generating large amounts of vector stock but also as a mean to maintain consistent vector quality. It has been sugested that less defective interfering particles (DIPs) are made when low MOIs are used because low MOIs enhance the probability of only having one virus in an infected cell. Having at most one virus per cell ensures no complementary viruses present that will aid in the propagation of DIPs. Strategies using low MOIs for protein production have led to comparable product titers given appropriate infection and harvest strategies (Maranga et al., 2003; Wong et al., 1996; Zheng et al., 1999). It has been suggested, however, that this may be the case solely for simple proteins or products that are insensitive to proteolysis (Radford et al., 1997a; Zheng et al., 1999). In any case, to produce maximal titers with an asynchronous infection (at low MOIs), it has been reported that the cell density at infection should be much

lower, allowing the density to reach the peak cell density (see section 2.5.2) of the system by the time all cells are infected with the virus (Wong et al., 1996). This has been further probed through culture simulation (Gotoh et al., 2004b) where similar conclusions were drawn. There remains a large interest in production at low MOI, and new strategies continue to be reported (Zhang and Merchuk, 2004; Zhang et al., 2005; Enden et al., 2005).

2.5.1.1 Co-infection

It has been reported that a minimum MOI of 3 is needed for the synchronous infection of cells (Nielsen, 2000) and can be explained theoretically by considering the infection process as a random event capable of being described by a Poisson distribution (Belyaev et al., 1995; Gotoh et al., 2004b; Hu and Bentley, 2001; Kamen et al., 1996; Licari and Bailey, 1992; Palomares et al., 2002; Tsao et al., 1996). Equation 2.1 defines the probability of a cell being infected by j viruses using the form of a Poisson distribution having the MOI as mean. It can then be seen that at an MOI of 3, the probability of a cell not being infected is 0.05.

$$P(j) = \frac{e^{-MOI} * MOI^{j}}{j!} \tag{2.1}$$

The importance of interpreting infection as a random event is of particular importance when multiple genes need to be expressed for the protein/particle to form. Roy and her co-workers have investigated the production of Bluetongue core-like and virus-like particles that have consisted of up to 5 structural proteins (Belyaev et al., 1995; Belyaev and Roy, 1993; French et al., 1990; French and Roy, 1990; Hyatt et al., 1993; Le Blois et al., 1991; Loudon and Roy, 1991). This group has argued that as the number of viruses increase the proportion of cells that are infected with

an equal ratio of viruses decreases (Belyaev et al., 1995). They have also shown that their co-infection experiments have led to mixtures of core-like particles and VLPs (double shelled) whereas when a single multiple gene baculovirus was used, most particles were double shelled (Belyaev and Roy, 1993). This lack of control of the distribution of viruses among the cells has led others to move towards dual or multiple gene baculovirus vectors (An et al., 1999; Brown et al., 1991; Weyer and Possee, 1991). Co-infection may be a necessary strategy though, as large constructs containing multiple genes are inherently unstable and therefore currently not amenable to process scale-up.

The use of multiple baculoviruses is a common occurrence and over 30% of the publications reviewed have used multiple baculovirus vectors to produce VLPs. These include VLPs based on bluetongue virus (French et al., 1990; Roy et al., 1994), human parvovirus B19 (Kajigaya et al., 1991; Tsao et al., 1996), rotavirus (Conner et al., 1996; Crawford et al., 1994; Jiang et al., 1998; Palomares et al., 2002, 1999; Sabara et al., 1991), adeno-associated virus (Hoque et al., 1999; Ruffing et al., 1992), poliovirus (Brautigam et al., 1993), human immunodeficiency virus (Zhao et al., 1994), SV40 (Kosukegawa et al., 1996), infectious bursal disease virus (Hu and Bentley, 2001; Hu et al., 1999), coronavirus (Ho et al., 2004) and enterovirus (Hu et al., 2003a), to cite a few. Multiple baculoviruses give rise to additional degrees of freedom in the optimization of such systems, which include the MOIs of each baculovirus. Many groups have made chimeric particles with varying compositions by varying the MOI ratio of the different baculoviruses in the system (Crawford et al., 1994; Hu and Bentley, 2001; Hu et al., 1999, 2003a; Palomares et al., 1999, 2002; Tsao et al., 1996). Although, the average composition of the VLPs at harvest is an average of a cell population having a distribution of cells carrying particles with various ratios due to the distribution of viruses in the cells, it has been shown that the average compositions are consistent over different experiments. It has also been

shown that similar results can be obtained with low multiplicities of infection for the production of VLPs of various compositions through co-infection (Tsao et al., 1996). One can consider the following: if each infected cell can produce 100-200 copies of progeny virus within 24 hours, infection of a subset of cells with a low MOI (0.1) can in turn infect the cells, at an appropriate cell density, at an MOI of ~10 upon secondary infection. In this scenario, the cells used during the primary infection are not VLP producing cells and only represent a small portion of the cells in culture. For systems needing co-infection, strategies must ensure that cells receive all the required viruses. The ability of a cell to be infected has been described to decrease as a function of time once the cell has been initially infected (Hu and Bentley, 2001, 2000). In co-infection systems, this may be important if intermediate MOIs are used, because all cells may be infected but not necessarily with all the viruses needed to produce the desired particles.

Another aspect, which renders the concept of MOI non-trivial, is the differences in the foreign sequence the baculovector carries. Depending on the strength of the promoter, the size of the products and the type of sequence carried, the rates and levels of productions will differ. Kohlbrenner et al. (2005a) reported increases in AAV titer obtained when increasing MOIs of one of the three baculoviruses used, the one containing the genes for AAV replication proteins, a finding similar to a report by Meghrous et al. (2005). Kohlbrenner et al. (2005a) suggested that the different transgenes compete for available transcription factors, with those under strong promoters being favored while those under the control of weaker promoters being further attenuated by the presence of the others. Similarly, Vieira et al. (2005) have shown differences in DNA replication (lower rates of DNA synthesis) between the use of a triple monocistronic baculovirus and a single tricistronic baculovirus. What remains unclear, however, is whether the reduction occured due to the burden of more virus (3x as much) or due to competition between the three viruses.

It has also been reported that the choice of MOI could be influenced by the composition of the medium. Most work that have dealt with VLPs, of which the majority were focussed on the construction of the appropriate baculoviruses, supplement the media with 1 to 10% serum. Fetal bovine serum has been shown to decrease the binding rate of baculovirus while augmenting the cell infection rate (Maranga et al., 2002c). This study, along with earlier reports by Wickham et al. (1992b), leads to the plausibility of reducing the MOI when supplementing the medium with a serum-like component that can reduce the number of effective binding sites (that do not lead to infection), thus augmenting the extracellular virus concentration, which is thought to be a driving force in the actual infection process. Although serum is a good source of vitamins and growth factors, it is made up of undefined compounds. Despite being shown that serum and heat treated serum are beneficial (Maranga et al., 2002c; Petricevich et al., 2001), studies that focus on process considerations, avoid the use of serum. The work on serum free medium was pioneered in the late eighties (Maiorella et al., 1988) and overcomes the inherent variability in serum lots, the possibility of serum carrying animal derived contaminants and the problems associated with downstream processing of material containing serum.

Although the concept of the MOI is not novel, as can be seen in this section, the MOI remains a critical factor in the optimization of BEV systems. As a concluding remark on multiplicities of infection, whether choosing high or low MOIs, the reliability of the assay of the viral stock is critical to ensure system reproducibility. Plaque forming units are generally the basis of MOIs and are the result of plaque assays or end-point dilution assays, which have been reported to have a coefficient of variation of 10-50% (Nielsen, 1992; Tsao et al., 1996) and 45% (Nielsen, 1992), respectively. These assays, however, require a lot of time and are operator dependant. A method that is fast and reliable based on staining viral DNA with SYBR Green (nucleic acid stain) and that helps corroborate plaque assays has been reported (Shen et al., 2002). It has

also been used to help characterize the quality of the baculovirus stocks (Jorio et al., 2006a,b). This method, however reliable, consistently yields higher titers than that obtained by plaque assay and a correction factor needs to be estimated to relate the two values. Lo and Chao (2004) developed a PCR quantification method for baculovirus, however, it is likely that this method will suffer the same drawbacks as that developed by Shen et al. (2002). A flow cytometric method that looks at staining both the baculovirus DNA and protein has been reported and the values obtained are more consistent with those obtained by plaque assay (Stoffel et al., 2005; Stoffel and Rowlen, 2005) and may be a method that could be employed for those that have access to the appropriate instrumentation. Mena et al. (2002) reported a biologic-based method that relies on the viability of infected cells; the drawback of this methodology is the six days required for the assay. Recently, however, a similar method that relies on growth cessation but that reduces the assay time to a day has been reported (Pouliquen et al., 2006). Reliability and consistency are key for proper evaluation of factors such as the multiplicity of infection.

2.5.2 Time of infection (TOI)

Although time of infection is commonly used throughout literature on viral systems, the wording is very misleading since it refers to the moment when virus is added to the system and not to an actual time, which would be an arbitrary measure without a defined point of reference that is not associated with time. Since time of infection refers to the moment virus is added to the system, and not to infection per se, this moment needs definition. The broadest way that has been used to describe the TOI is based on the position on the cell growth curve, which have been broken down into early-, mid-, late-exponential phases and even stationary phases. This definition however, is too vague to achieve reproducible strategies.

Generally, in batch culture, specific production (units per cell) will decline if virus is added above a peak cell density (Caron et al., 1990) and has been reported to be anywhere between 30 and 70% of the maximum cell density (reviewed by Elias et al., 2000). Even lower relative cell densities at infection, equivalent to $\sim$ 10% - 20% of the maximum cell density, have been reported to achieve maximum specific production of rotavirus VLPs and AAV viral vectors (Park et al., 2004; Meghrous et al., 2005). Therefore, one characteristic that can define the moment virus is added to the system is the cell concentration at infection (CCI). This in fact has been adopted by some instead of using TOI (Maranga et al., 2002b), and as mentioned in the section on the multiplicity of infection, gives the MOI more meaning. The CCI, however, is not enough to characterize the moment virus is added to the system because growth and production are dependant on the composition of the medium. Different media can support growth to different maximum cell densities. Two recent reports, not focussed on media effects, exemplify these differences. Aucoin et al. (2007b) reported that SF-900 II media (Gibco BRL) allowed growth beyond $10x10^6$ cells/ml when inoculated at $0.5x10^6$ cells/ml; while Lecina et al. (2006) reported that cells grown in IPL-41 (HyClone) supplemented with yeast extract and lactalbumin hydrolysate, reached a maximum density of $3x10^6$ cells/ml when inoculated at $0.3x10^6$ cells/ml. Therefore, to maximize the volumetric production, selection of appropriate culture medium is criticial.

To circumvent the "weakening" of the media that occurs during the growth period, batch and replacement mode of operation (also known as sequential batch operation) has been used, whereby the medium is removed prior to the addition of virus and replaced with fresh medium. This has been shown to improve the production of AAV viral vectors allowing the cell density to be increased 3 fold without compromising the cell specific productivity (Meghrous et al., 2005); however, the amenability to large scale is not easily achievable without appropriate equipment. Furthermore, the

fresh medium added for the "infection" period need not necessarily be the same as the one used during the growth period. Growth and infection have been studied in different media (Hoque et al., 1999), which reflects the understanding that the metabolism pre- and post-infection is different for the cells. In fact, studies on substrate limitations have shown that the order of nutrient depletion in uninfected and infected cultures are not the same i.e. the consumption patterns pre- and post infection change (Radford et al., 1997b). From a process perspective, using two different media is not necessarily an approach that is desired.

Zheng et al. (1999) have used a simple addition of medium (dilution of cells with fresh medium), prior to infection with low multiplicities of infection, to allow the cells to be in an optimal environment for the production of bluetongue virus-like particles and avoid any lag period introduced by the addition of the baculovirus. The renewal of medium has also led to the culturing of insect cells in perfusion mode (Deutschmann and Jager, 1994; Hu et al., 2003b; Zhang et al., 1998). Feeding strategies such as fed-batch mode of operation, whether it be a continuous feed or one-time addition of nutrient supplements have been used to move the optimal "time of infection"; this overcomes nutrient limitations associated with cultivating cells to high densities and improves the production of recombinant proteins (Bédard et al., 1994, 1997; Chan et al., 1998, 2002; Chiou et al., 2000; Deutschmann and Jager, 1994; Elias et al., 2000; Garnier et al., 1996; Hu et al., 2003b; Nguyen et al., 1993; Zhang et al., 1998). Maranga et al. (2003) supplemented their medium with glutamine, which was being depleted in culture, and were able to achieve improved VLP titers when infection took place between 4 and 5 x 10^6 cells/ml but not higher. This is similar to other reports producing protein (expoxide hydrolase) which have supplemented their medium with glucose and glutamine (Wang et al., 1993). Glutamine, however, has also been reported to be non-essential for the culture of Sf-9 cells or the production of protein as long as the medium contains a source of ammonium ions (Ohman et al.,

1996), and therefore must be examined carefully if one considers it the limiting nutrient. Depletion of cysteine has been shown to correlate with decreased production of recombinant products (Radford et al., 1997b) and along with glutamine decrease significantly over the course of the culture (Jang et al., 2000).

The selective feeding of glutamine has also been unable to alleviate limitations pointing to multiple potential limiting nutrients (Caron et al., 1990; Wang et al., 1993). Supplement cocktails have included glucose, amino acids, yeastolate, lipids, vitamins, and trace elements (Bédard et al., 1994, 1997; Chan et al., 1998, 2002; Elias et al., 2000); however in some cases, the addition of other specific compounds such as 20-hydroxyecdysone, an insect hormone, has yielded increased product formation (Chan et al., 2002). Addition of cholesterol has been reported to decrease baculovirus infection but only when added prior to infection. Addition 24 h after infection did not seem to affect the infection process (Maranga et al., 2002c). Achieving optimal results requires balancing the chemical composition of the supplements and the physiological state of the cell. This can lead to complex feeding strategies, which, if not automated, will less likely be employed for an industrial process. Medium and feed optimization for the production of VLPs or viral vectors in insect cells have not been reported in the literature surveyed, but are intimately linked to defining the optimal time of infection.

Media composition and feeding strategies, just like the cell densities, are only indicators of how the cell will respond to infection, treating the population of cells as one unit. The composition of the medium will however, affect the condition of the cells, changing the distribution of the population. Generally, the cell population will maintain a relatively constant distribution of cells in each cell cycle phase. As a reminder to the reader, the natural cell cycle involves the cell progressing through a number of phases for replication. In the G0-G1 phase, cells grow, carry out normal metabolism and the organelles duplicate; in the S phase, DNA replication takes place; in the G2

phase, cells grows further and prepare for mitosis; and in the M phase, duplicated chromosomes are separated into two nuclei and the entire cell splits into two daughter cells. The cell cycle of the *Spodoptera frugiperda*, generally takes close to a day, as evidenced by the doubling time of these cells. The distribution of cells among these phases, however, can be forced to change depending on the composition of the medium/cell density. Studies on the cell cycle have shown that late in cultures or limiting yeastolate in KBM10 media results in an accumulation of cells in the G2/M phase (Calles et al., 2006; Doverskog et al., 2000). Saito et al. (2002) and Kioukia et al. (1995) have suggested that infection occurring in the G1 or S phases allow increased viral product. This may be because of the shorter time it takes the cell to reach the point in their cell cycle at which they are arrested. Saito et al. (2002) showed that the cell cycle progresses after infection until the S or G2/M phase. As can be seen, the TOI, just like the MOI, although basic concepts, are far from trivial.

To add to the complexity, co-infection systems allow for additional degrees of freedom as the time at which individual virus stocks are added does not have to be done simultaneously. This has been studied by Palomares for the production of viral like particles of various composition (Palomares et al., 1999, 2002). This phenomena however, has also been indirectly studied by Hyatt et al. (1993), Tsao et al. (1996), Hu and Bentley (2001), Park et al. (2004) and Meghrous et al. (2005). In these latter studies, MOIs of one of the viruses was below 1 while the other viruses had MOIs greater then 1. If the genetic material provided by the baculovirus put into the system at low MOI is required, there will be a lag time present for it to be delivered to all cells by a secondary infection. This has not always proven to be fruitful. In the work done by Meghrous et al. (2005), such a strategy has lead to less than optimal yields of functional AAV vectors being produced. However, Hyatt et al. (1993) have shown that optimal results occurred when the baculovirus containing the sequence for non-structural proteins were used at MOIs less than 1, for the production of bluetongue

virus-like particles. It has also been reported for the production of rotavirus that using a baculovirus coding for VP7 at an MOI less than 1 and baculovirus coding for VP2 and VP6 at MOIs greater or equal to 1 yielded an optimal condition (Park et al., 2004).

2.6 Production dynamics, monitoring and time of harvest

Process monitoring of insect cell culture growth and infection have been studied through oxygen uptake rate (Kamen et al., 1996), carbon dioxide evolution rates (Kamen et al., 1996; Zeiser et al., 2000), and relative permitivity measurements (Zeiser et al., 1999). Few reports, though, have used these tools to control actions taken on the culture. Palomares et al. (2004) has suggested the use of OUR to indicate when cultures should be supplemented with glucose after infection. Lecina et al. (2006) has also suggested the use of OUR, widening the scope of applicability and using it to determine appropriate time of infection, of feeding and of harvest. Still, VLP formation can be quite complex and the elucidation of true synthesis dynamics have not been generally achieved, and actions, such as feeding, infection or harvest have not been based on on-line measurements except for the aforementioned studies.

Details on cell culture and infection process in early reports of VLP synthesis in insect cells are sparse focussing more on the construction of baculovirus vectors containing genes for viral structural proteins. Certain details, such as the dynamics of protein/capsid expression were not formally investigated and for the most part were described in terms of protein accumulation. These studies, however, can still shed some light into the process dynamics that need to be considered for larger scale productions.

For example, Luo et al. (1990) showed the evolution of gag protein expression and showed that accumulation did not occur within the insect cells. Secretion of the particles resulted in an accumulation in the supernatant as early as one day post-infection. It was suggested that the budding process closely resembled that of HIV-1 and SIV gag particles. The continued increase in accumulation over the 3 day monitoring period also seemed to indicate gag protein stability, leading to a maximum harvest on the third day. In a more recent report, Pr55gag particles (HIV-1-like particles) have been shown to be susceptible to proteolytic degradation (Cruz et al., 1999), therefore requiring definition of an optimal harvesting time.

In a study on a co-infection system to produce rotavirus-like particles, it was shown that particles were also secreted in the supernatant (Sabara et al., 1991). To achieve a deeper understanding of the system, cells infected with a single type of virus was investigated prior to looking at the co-infection system. When cells were infected with only one baculovirus expressing the VP6 protein, it was shown that the protein was cell associated, with little or no protein observed in the supernatant fraction. However, when looking at the expression of VP7 after 48 hpi, the protein was primarily found in the supernatant. When co-infection took place with baculoviruses coding for VP6 and VP7, after 24 hours, the proteins were primarily detected in the supernatant with little or none associated with the cell pellet. This may be interpreted as an indication that no cells (or very few) were solely infected by the baculovirus used for the expression of VP6, hence confirming a uniform co-infection process. Charpilienne et al. (2001) produced double layered rotavirus particles having either GFP or DsRed fused to the VP2 inner core structural protein. The localization of the fluorescent protein allowed the exterior of the particle to be indistinguishable from VLPs produced without the fluorescent add-on. The fluorescent nature of the particles is readily amenable to on-line monitoring of large scale cultures of rotavirus-VLP. Recently, using rotavirus VP6 and VP2-GFP-fusion proteins, the synergistic

or dependant behavior of multiple protein expression was visually confirmed (Mena et al., 2006).

By describing the dynamics of protein production, maximum protein accumulation can be determined and the peak can serve as a potential optimal time of harvest. In a recent study on the production of porcine parvovirus-like particles, the optimal harvest time was reported to be highly dependent on the MOI, and would not have been observed if monitoring of the production process was not undertaken (Maranga et al., 2003). Following the production of particles is still quite time consuming and is not easily done using on-line measurements. Meghrous et al. (2005) were able to follow expression of GFP and capacitance of cultured insect cells; however, neither were correlated with the production of AAV.

Fusion of fluorescent proteins to capsid proteins has proven useful for rotavirus process development as previously mentioned. Gilbert et al. (2004) fused a fluorescent protein to the N-terminal region of the canine parvovirus VP2, which has led to the formation of fluorescent particles. However, the particles formed in this study were not only composed of the VP2 fusion protein but also of VP2 moieties that had the EGFP cleaved off. It has not been determined if a minimum amount of "unaltered" VP2 is necessary for the assembly of the virus-like particle. Caballero et al. (2004) showed that they were able to produce astro-VLPs with a truncated form of the ORF2, which codes for the structural proteins. Addition of the GFP gene at the end of this ORF led to structural proteins attached to GFP, similar to the strategy used by Charpilienne et al. (2001), and the formation of fluorescent VLPs.

Further work on the incorporation of fluorescent molecules may help further the development of larger scale processes, including downstream processing (Mena et al., 2005). However, the assembly of the particles must not be compromised by such an alteration. Advances in this area with respect to the assembly of viral vectors need

to be investigated especially with regards to the encapsidation process. As long as the behavior of the synthesis of these particles remains unaltered, these new chimeric fluorescent particles will greatly help future process optimization.

2.7 Models describing the baculovirus/insect cell system and VLP formation

The modeling of the baculovirus/insect cell system involves describing the dynamics of uninfected cell growth, the binding and infection of the cells by the virus(es), the infection cycle and the production of foreign proteins in infected cells as previously suggested by Power and Nielsen (1996). Most of the models existing are segregated yet unstructured; however, structured but unsegregated models have also been reported (Jang et al., 2000; Sanderson et al., 1999). In other words, most groups looking at modeling this type of system acknowledge that upon virus addition:

1. populations will form eg. a population of infected cells and a population of non-infected cells; and that

2. infection events (baculovirus replication cycle) are temporally regulated.

Prior to virus addition, insect cell growth follows the typical dynamics described by the Monod equation. The primary drawback of this approach is the selection of the limiting nutrient, which is to date still unknown. Some have used either an undefined nutrient having an arbitrary initial concentration (Power et al., 1994), glucose (Bentley et al., 1994) or glutamine (Hu and Bentley, 2000, 2001). Licari and Bailey (1992) formulated their growth model independent of substrate consumption by making the growth of cells in a monolayer a function of free surface area. Others, have used the specific growth rate in an exponential equation to formulate a model

for cell growth independent of substrate concentration (Dee and Shuler, 1997a; Power et al., 1992). The inherent problem with the latter is the lack of an upper bound on growth other than the infection of cells (which is modeled as stopping growth). The simulation of growth holds well for all the above approaches but does not offer any additional specific insight to this system with regards to recombinant protein synthesis.

When it comes to the addition of virus, the distribution of the virus across the cell population becomes important. As previously mentioned in the section on the multiplicity of infection, the infection process considered as a random event can be described by a generic form of the Poisson distribution function (Hu and Bentley, 2000, 2001; Licari and Bailey, 1992) with the ratio of total virus to total cells as mean (Eq. 2.1). This however, presumes that the infection process is instantaneous and that all cells have equal opportunity to be infected.

Dee and Shuler have described a quantitative model for the trafficking of baculovirus in insect cells which considers multiple mechanistic steps in the infection process, unlike most which clump the processes together, including attachment, internalization, endosomal fusion and nuclear accumulation (Dee and Shuler, 1997b,a). Some of the key findings in their work include the proportion of internalized baculovirus that is degraded (50%) and the mean times for the various trafficking steps. Their model, however, does not provide a description of recombinant protein synthesis as a result of the genomic material reaching the nucleus. Their model uses the Poisson distribution to describe the distribution of virus in cells after calculating the attachment of the virus to the cell in a previous step using a binding rate constant. This differs from the approach of Hu and Bentley (Equation 2.2) which couples the attachment and the distribution, using an efficiency constant within the Poisson distribution formulation.

$$P(t,j) = \frac{e^{-\alpha * \frac{V(t)}{N_o(t)}} * \left(\alpha * \frac{V(t)}{N_o(t)}\right)^j}{j!} \tag{2.2}$$

P(t,j) is the probability of a cell being infected with j viruses at time t, α is the infection efficiency factor for uninfected cells, V(t) is the extra-cellular virus concentration at time t, and N_o(t) is the non-infected cell concentration at time t.

Another approach uses an empirical formulation to describe the attachment kinetics (Power et al., 1994) whereby the binding rate does not equal the infection rate since each insect cell is capable of binding multiple viruses. In this model, the infection rate is proportional to the binding rate through the fraction of non-infected cells to the number of total virus binding cells. A probabilistic equation based on a Poisson distribution describing the availability of entry sites on a cell surface has been proposed by (de Gooijer et al., 1992):

$$P_o = e^{-\frac{v_{tot}}{BC}} \tag{2.3}$$

where P_o is the probability that an entry site remains empty, v_{tot} is total number of virions, B is the number of entry sites on a cell and C is the number of non-infected cells. In this scenario, the probability of a particular virus binding to the cell is proportional to the fraction of that type of virus to the total number of viruses and the probability that a site does not remain empty (1-P_o). Although this formulation of the model was for differentiating baculoviruses based on their activity (defective, abortive or infectious baculoviruses), this type of reasoning could be applied to co-infection schemes. Still, no matter the approaches that have been used to describe the binding/infection of a cell, a prevailing methodology describes the rate of virus change to be proportional to the amount of virus present (Dee and Shuler, 1997b; Power et al., 1992; Zhang and Merchuk, 2004).

Most models developed ignore secondary infection (infection of already infected cells) with only a few accounting for this phenomena (Dee and Shuler, 1997a; Hu and Bentley, 2000, 2001; Sanderson et al., 1999). A major reason for ignoring this step is to simplify already quite complicated models. Dee and Shuler, however, have shown the ability of cells to uptake virus up to 24 hpi, albeit decreased from the original time of infection. This is accounted for in the Hu and Bentley models using an attenuation factor based on the time post-infection.

Once a productive viral infection is accounted for, there is a need to account for the temporal distribution of events thereafter. Tracking the infected cell mathematically is generally done using a variable, τ, for the time post-infection of individual cells. The onset and cessation of specific events, such as baculovirus replication and protein synthesis are then related to τ and not time. Furthermore, the onset and cessation of events, such as recombinant protein synthesis, have to be estimated. Hu and Bentley (2001, 2000) believed these events were a function of the number of viruses in the cell. Gotoh et al. (2004b) believed that these events were not necessarily only a function of viruses and therefore speculated that the number of infected cells that were producing virus or protein at any point could be more adequately represented using a Weibull distribution (Gotoh et al., 2004b). Although both methods adequately describe the cultures, both approaches remain speculative.

Models describing product formation have linked the production to: the number of infected cells (Bentley et al., 1994), the number of viruses within the cell, the culture time and the time post-infection (Licari and Bailey, 1992; Power et al., 1994) as well as substrate depletion (Hu and Bentley, 2001, 2000). The use of a model to describe particle composition as a result of co-infection has also been achieved (Tsao et al., 1996). The models of Hu and Bentley further consider the formation of VLPs as the result of the interaction of the recombinant proteins produced within the insect cells (Hu and Bentley, 2001, 2000). Recently, a model of rotavirus-like particle production

based on viral DNA replication and transcription has been reported (Roldao et al., 2006, 2007). This study built upon work done by Rosinski et al. (2002), who looked at the replication of baculoviruses using real-time PCR and was able to characterize the replication kinetics in a biphasic manner.

Given the need to assess an appropriate harvest time, prediction of product formation rates and degradation rates are also important. For product that accumulates in the cell, it is also important to estimate when the product is released in the medium (through leakage or cell lysis). To this end, Haas and Nielsen (2005) developed a model that relates product release to cell viability.

2.8 Summary

There is a need for the advancement of vaccine production and gene therapy to not only find ways of delivering the genes of interest to the cells but also to produce these products efficiently at larger scale. There is a considerable amount of work being done at developing the baculovectors; however, incorporating production strategies to facilitate the manufacturing of these products for clinical applications is still in its infancy. Finally, there is a definite need, for the appropriate progression, to further delve into the limitations associated with the process i.e. vector stock characterization, development of rational feeding, infection and operating strategies.

CHAPTER 3

PRODUCTION OF ADENO-ASSOCIATED VIRAL VECTORS IN INSECT CELLS USING TRIPLE INFECTION: OPTIMIZATION OF BACULOVIRUS CONCENTRATION RATIOS

In the previous chapter, the amount of virus added to cells was said to be a significant factor that will highly influence the trajectory and outcome of the culture. In the following chapter, the amount of baculovirus that needs to be added to produce an optimal quantity of functional AAV is explored.

3.1 Potential outcomes

The production of AAV in insect cells, as developed by Urabe et al. (2002), requires three different baculovectors. Two of these vectors, contain the AAV components that can be supplied in *trans* for the production of a functional AAV vector. These components are the genes for the AAV replication proteins and the AAV structural proteins. The third baculovirus contains the *cis* components necessary for the synthesis of a functional AAV vector, namely the transgene of interest flanked by AAV inverted terminal repeats. These three baculoviruses are herein referred to as BacRep, for the baculovirus containing the genes for the replication proteins; BacCap, for the baculovirus containing the gene for the structural proteins; and BacITR for the baculovirus containing the AAV vector genome.

Before exploring the quantity of virus that needs to be added to the culture, it is important to understand what each of these vectors will supply and how they are expected to interact with the other vectors. In other words, if only one cell were to be

mixed with only one of each of these baculovectors, what are the potential outcomes? Figure 3.1 schematically depicts the potential outcomes. It should be understood that if an infection does occur, then the cell will arrest in the S and G_2/M phases shortly thereafter and will cease to divide. Also, in all cases of baculovirus infection, the baculovirus will replicate and produce progeny baculovirus. Therefore, in terms of infection outcomes described in Figure 3.1, only the effect of the foreign genes were considered.

Only those cells, which have been infected by all three types of virus will produce the desired product. Cells that are not infected will be rare at high MOIs (>3). Therefore, realistically there are seven outcomes to contend with.

Once the different outcomes are understood, it then becomes important to understand how multiple viruses of each type can be distributed over a population of cells. The infection process is said to follow a distribution of rare events i.e. the Poisson distribution. The form of the Poisson distribution, however, has varied amongst research groups. Most basically, it is thought that the number of viruses per cell will vary according to a Poisson distribution with the mean equivalent to the MOI, the ratio of the total number of viruses to the total number of cells.

This assumes, however, that all cells have the same capacity to take up virus, i.e. all cells are the same. One characteristic of the cells that is known to differ for each cell, is the cell diameter or cell size. For this reason, it was of interest to probe how virus would be distributed assuming that virus binds to cellular receptors whose quantity is proportional to the surface area. This approach is similar to an earlier approach used by de Gooijer et al. (1992). The probability of a virus attaching to a unit surface area can therefore be described by Equation 3.1.

$$P = 1 - e^{-\frac{Virus}{SurfaceArea}} \tag{3.1}$$

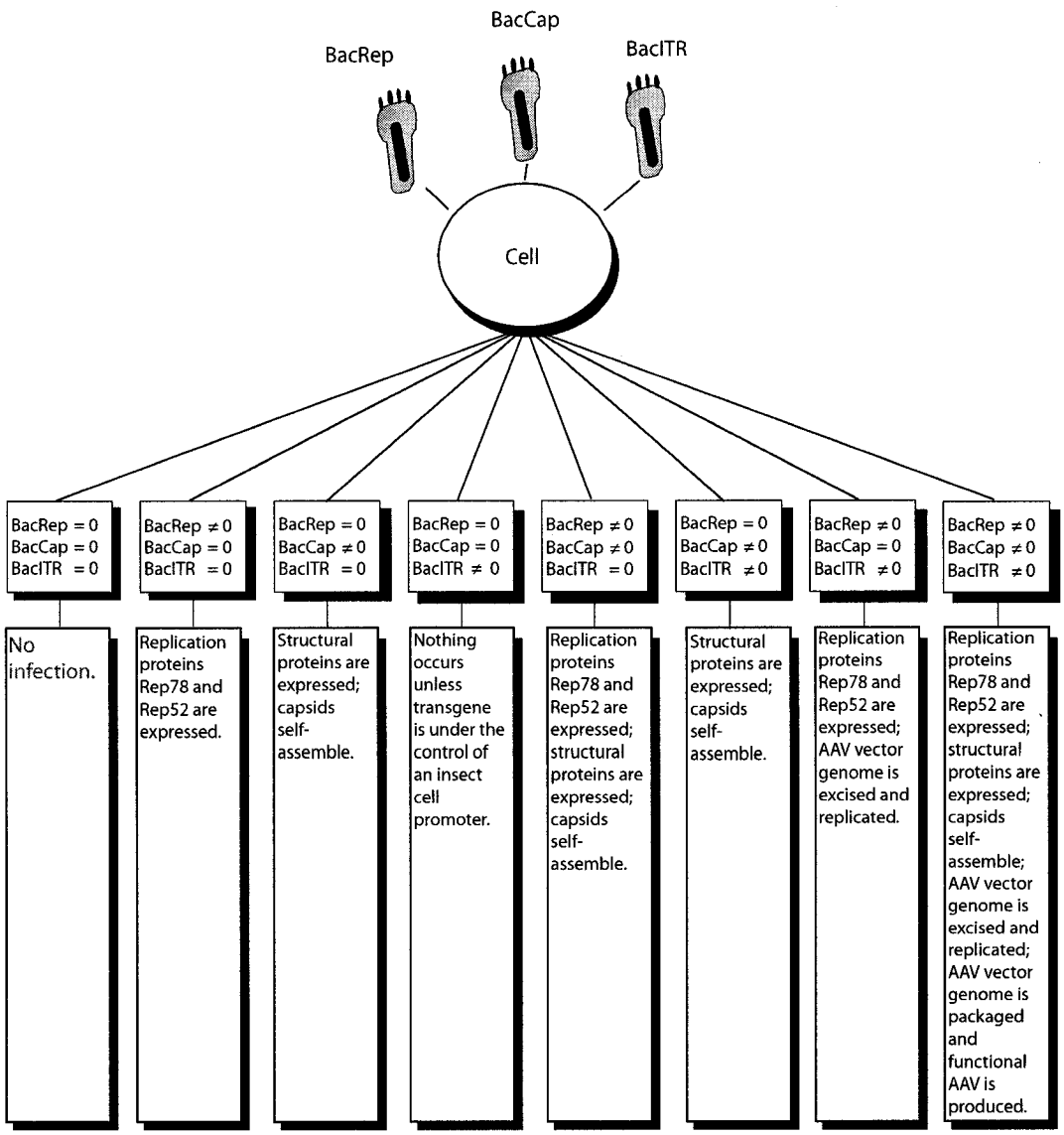

Figure 3.1 Potential populations when infecting with 3 different viruses.

Figure 3.2 A & B show two different histograms of typical cell size distributions of equivalent number of cells. These histograms can be approximated by a normal distribution to ease the analysis. Figure 3.2 C, compares the distributions shown in Figure 3.2 A & B and adds a third population that has a similar mean cell diameter but that has a greater variance, to accentuate the effect of varying cell size distribution. Figure 3.2 C also shows how the cell size distribution then affects the distribution in terms of available surface area (dotted lines) and how virus (MOI=10) that has been distributed based on surface area (dashed lines) is distributed among cells with different size diameters.

Comparing the distribution of MOIs across the cell population (Figure 3.3), it can be seen that the Poisson distribution as defined in Equation 2.1, is a good approximation even if virus was distributed based on available surface area. Of interest to note, is that if normality holds and the cell size distribution becomes broader, the MOI distribution tends to shift to the left resulting in a significant change in the number of cells having lower MOIs. This should be an aspect to consider when striving to get a number of different viruses in each cell.

3.2 Production of adeno-associated viral vectors in insect cells using triple infection: optimization of baculovirus concentration ratios

The following is an exploratory study of the effect the quantity of each baculovirus virus has on the production of AAV vectors entitled "**Production of adeno-associated viral vectors in insect cells using triple infection: optimization of baculovirus concentration ratios**", which has been published in the scientific journal, ***Biotechnology and Bioengineering***. The goal of this paper was to understand the effects of the multiplicity of infection keeping in mind the hypothesis that every cell needs to be infected by all three viruses to produce an

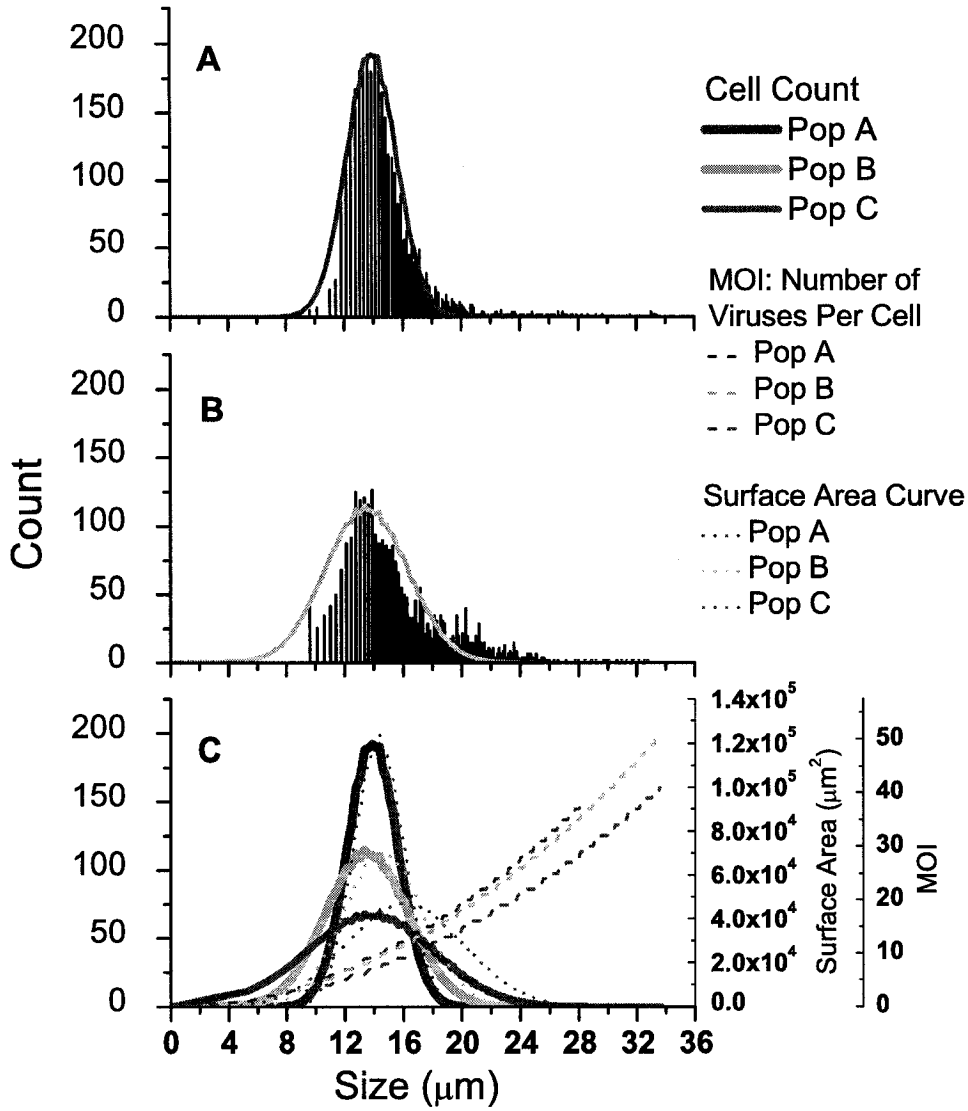

Figure 3.2 A and B. Cell size distribution of typical cell populations at around $2x10^6$ cells/ml and their normal approximations. C. Cell size distribution approximations of three different populations, distribution of surface area and average MOI per cell of a certain diameter when virus is distributed based on available surface area.

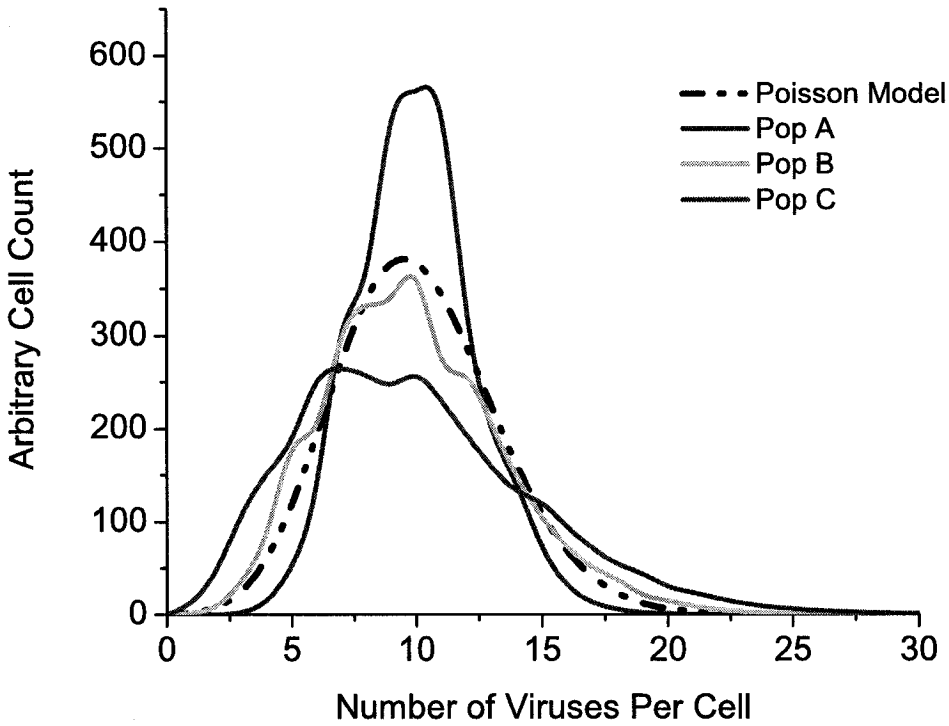

Figure 3.3 MOI distribution among cells of different populations (defined in Figure 3.4). Poisson Model curve refers to distribution obtained using Equation 2.1.

adeno-associated vector. A new combination of viruses, which maximizes product yield while increasing the ratio of infectious to total particles produced, is described. Discussion of baculovirus load and protein expression are discussed with regards to producing optimal quantities of functional AAV. The additional degree of freedom in terms of optimization with regards to infection timing has also been addressed. Effects on the viability of the cell due to the total number of AAV capsids are reported. Furthermore, experiments producing vectors with different transgenes are examined. The results are significant towards further understanding the formation of an active vector irrespective of the transgene of interest and towards conditions that could optimize the process.

It should be noted that in the following manuscript, what has been previously defined as enhanced transduction units (ETU) in Chapter 1, is referred to as infectious viral particles.

Production of adeno-associated viral vectors in insect cells using triple infection: optimization of baculovirus concentration ratios

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3.2.1 Abstract

The production of viral vectors or virus-like particles for gene therapy or vaccinations using the baculovirus expression system is gaining in popularity. Recently, reports of a viral vector based on adeno-associated virus (AAV) produced in insect cells using

the baculovirus expression vector system have been published. This system requires the triple infection of cells with baculovirus vectors containing the AAV gene for replication proteins (BacRep), the AAV gene for structural proteins (BacCap), and the AAV vector genome (BacITR). A statistical approach was used to investigate the multiplicities of infection of the three baculoviruses and the results were extended to the production of AAVs containing various transgenes. Highest AAV yields were obtained when BacRep and BacCap, the baculovirus vectors containing genes that code for proteins necessary for the formation of the AAV vector, were added in equal amounts at high multiplicities of infection. These combinations also resulted in the closest ratios of infectious to total AAV particles produced. Overexpression of the AAV structural proteins led to the production of empty AAV capsids, which is believed to overload the cellular machinery, preventing proper encapsidation of the AAV vector transgene, and decreased the viability of the insect cells. Delaying the input of BacCap, to reduce the amount of capsids produced, resulted in lower infectious AAV titers then when all three baculoviruses were put into the system at the same time. The amount of BacITR added to the system can be less than the other two without loss of AAV yield.

Keywords: multiplicity of infection; time of infection; baculovirus; insect cells; adeno-associated virus

3.2.2 Introduction

The baculovirus/insect cell system is a proven platform for the scalable generation of recombinant proteins. Still, the production of viral vectors (other than producing baculovirus as a vector) in insect cells is in its infancy. One of the first reports of viral vector production in insect cells involved the production of recombinant AAV-2 (Urabe et al., 2002). In this system, all the structural proteins are supplied by one

baculovirus, while two other baculoviruses supply the transgene of interest flanked by AAV-ITRs and the gene that codes for the replication proteins, respectively. The AAV vectors produced in insect cells and studied thus far have mostly used GFP as the transgene of interest. With a surge of interest in this system as a means to produce AAV particles (Chen et al., 2004; DiMattia et al., 2005; Kohlbrenner et al., 2004, 2005a; Lane et al., 2005; Urabe et al., 2004, 2006), establishing the effect of process parameters surrounding culture infection is crucial.

Of the various parameters, the multiplicity of infection (MOI), which describes the ratio of infectious baculovirus to the number of viable cells at the time of virus addition, is of utmost importance. From a process perspective, the quantity of virus used dictates the preparation of the viral stocks and can affect the process involved in generating the recombinant product (yield, time of harvest, etc.). The initial amount of baculovirus used to infect cells and produce proteins can have varied results. Some have shown that the only effect of varying the initial MOI (10^{-1} - 10) of baculovirus is on the timing of recombinant protein and baculovirus production and not on the amount of accumulated product (Radford et al., 1997); while others, studying the production of virus-like particles (VLPs) have shown that lower MOIs resulted in lower maximum product yields (Maranga et al., 2003).

Single and multiple protein expression from baculovirus infection can lead to the formation of virus-like particles, which are often sought for vaccines (Maranga et al., 2003; Sico et al., 2002; Zheng et al., 1999). Zheng et al. (1999) have shown that upon a proper feeding/infection strategy, MOIs as low as 0.0001 PFU cell^{-1} could be used to produce bluetongue core-like particles. Co-infection has been used for the production of virus-like particles, in which, various baculoviruses code for various structural proteins (Hu and Bentley, 2001; Hu et al., 2003; Jiang et al., 1998; Palomares et al., 2002, 1999; Tsao et al., 1996). These groups showed that they could alter the composition of their virus-like particles, which self-assemble if various

amounts of the different baculoviruses, each coding for different structural proteins, are used. Meghrous et al. (2005) showed that changing the ratios between the three baculovirus MOIs used altered the infectious AAV titer. The issue of co-infection systems adds another dimension to the question of optimal MOI with additional degrees of freedom. What MOI should be considered foremost: individual MOIs or the overall MOI? Should the viruses be added simultaneously, or can the delay of one of the viruses allow a better overall formation of the desired product? In the case of producing rotavirus-like particles, Palomares et al. (2002, 1999) showed that the ratio of proteins synthesized and assembled into VLPs could be manipulated, similar to varying initial MOIs, by delaying the infection by 3 or 6 h.

In the following study, the production of bioactive adeno-associated viral vectors is examined with respect to the initial individual multiplicities of infection and expanded on the initial report by Meghrous et al. (2005). This paper details the effect of varying individual multiplicities and the added freedom co-infection offers for the optimization of the process. A focus on individual multiplicities of infection greater than one to achieve the greatest number of cells infected by all three viruses is used. Further to using AAV-GFP as the model system for the production of AAV vectors, the production of AAV-SEAP and AAV-LacZ is investigated as two additional systems for the generalization of the findings.

3.2.3 Materials and Methods

3.2.3.1 Cells and medium

Spodoptera frugiperda (Sf9) cells were grown in serum-free SF-900 II (Gibco BRL, Burlington, Ont., Canada). Cells were routinely maintained in 125 mL plastic shake flasks (Corning GlassWorks, Corning, NY) with a working volume of 20 mL at

27°C and agitated at 110 rpm. Cells were subcultured twice per week to maintain the cell density between 0.5 and $5x10^6$ cells/mL. Cell densities were assessed using a haemocytometer. Cell viability was determined via the trypan blue exclusion method. HEK293 EBNA cells (used for testing the infectious nature of the AAV produced) were maintained in HSFM medium (Gibco BRL) supplemented with 1% bovine calf serum, 10 mM HEPES, and 50 mg/mL of G-418 at 37°C in a 5% CO_2 atmosphere. Cells were grown in plastic shake flasks. Stock cells were routinely passaged every 3 days and diluted appropriately to maintain the cells in exponential growth (between 0.2 and $1x10^6$ cells/mL).

3.2.3.2 Baculovirus viral stocks

BacITRGFP, which has the transgene for green fluorescent protein (GFP) under both the p10 insect and cytomegalovirus (CMV) mammalian promoters between AAV ITRs was kindly provided by Dr. R.M. Kotin from the National Institutes of Health (Bethesda, MD) and has been described previously (Urabe et al., 2002). BacITRLacZ and BacITRSEAP were kindly provided by Avigen (Alameda, CA) and Généthon (France), respectively. LacZ and SEAP genes are only under the cytomegalovirus (CMV) mammalian promoter. BacRep and BacCap, which contain the gene sequences for the replication proteins and structural proteins were either supplied by Dr. Kotin or made at the Biotechnology Research Institute of the National Research Council of Canada (BRI-NRC) by Dr. Jamal Meghrous starting from plasmids supplied by Dr. Kotin. The baculoviruses were amplified in Sf9 insect cells grown in suspension in SF-900 II medium. Sf9 cells in log phase growth were centrifuged at 290g for 5 min and resuspended in fresh media at a cell density of $2x10^6$ cells/mL. Cells were infected at an MOI of 1 and the budded viruses were harvested 72 hpi. Baculoviruses were recovered in the supernatant, after centrifugation at 2,600g for 15 min, which was then filtered using a 0.22 mM (pore size) filter. One

percent BCS was added to the baculovirus stocks before being stored at 4°C. Titers of the viruses were determined by flow cytometry (Shen et al., 2002) prior to use.

3.2.3.3 Design of experiments

A full factorial experiment (2^3) was designed and analyzed using Design-Expert Version 6.0.6 statistical software (Minneapolis, MN). Replicates of the corner points and three center points were included in the design. An additional run without infection was done as negative control. The individual multiplicities of infection of the three baculovirus vectors were chosen as the factors (1 and 9 were chosen as the lower and upper levels and 5 as the centerpoint). The volume of non-concentrated viral stock that could be used established the upper bound of MOI chosen in this study. Cells were grown to approximately 2.5 million cells/mL before being aliquoted into twenty 125 mL shake flasks. The cell density was chosen such that operation would be within non-limiting nutrient conditions. To avoid confounding dilution effects with virus effects, each baculovirus vector solution added to shake flasks were topped to 10 mL with spent medium (medium removed from cells grown to 2.5 million cells/mL).

A second full factorial experiment (3x2) was designed to further corroborate results from the first factorial experiment, and to generalize the results. The factors investigated were the MOIs of BacITRtransgene (3 and 10) and the transgene (gene for GFP, gene for β-galactosidase, and the gene for SEAP). The range of MOIs tested in this second series of experiments was established based on the results of the first set of experiments. To reduce any potential negative effect of adding a large volume of virus, the viral stocks for this set of experiments were concentrated by ultracentrifugation. Briefly, vector stocks were spun at 43,000 rpm (215,000g) for 65 min at 4°C in a Ti-45 rotor using an L8-70M Beckman ultracentrifuge (Beckman Instruments,

Palo Alto, CA). The supernatant was discarded while the pellet was resuspended in fresh medium and filtered through a 0.8/0.2 μm Supor membrane syringe filter (Pall Corporation, Ann Arbor, MI). Each baculovirus vector solution added to shake flasks were topped to 3 mL with fresh medium. Cells were grown to approximately 2.0 million cells/mL before being aliquoted into twenty 125 mL shake flasks. The initial cell density of the mother culture is slightly lower than in the first set of experiments accounting for the smaller volume of viral stock being added due to the concentration.

To further evaluate the added degrees of freedom given by a multi-vector system, the time of addition was investigated. Briefly, at 2.0 million cells/mL cells were infected with two of the three viruses. The third virus was either added at 6 or 12 h later. The individual MOIs chosen for BacITRGFP, BacRep, and BacCap were 3, 10, and 10, respectively.

3.2.3.4 Characterization assays

3.2.3.4.1 Gene transfer assays for infectious viral particle (IVP) titers

Recombinant AAV-2 production was determined by transducing HEK293 EBNA cells in HSFM medium containing 1% BCS and 10 mM HEPES. Sf9 cell culture samples were subjected to three freeze/thaw cycles. Cell lysates were heated at 60°C for 15 min to inactivate the baculovirus. HEK293 EBNA cells were infected with wild-type adenovirus (ATCC number VR-1516), at an MOI of >50, prior to aliquoting cells in a multiwell plate.

SEAP. Fifty microliter of HEK 293 EBNA cells at a density of 0.5×10^6 cells/mL were added to each well in a 96-well plate. To each of these wells, 50 μL of diluted sample (10^{-1} - 10^{-8}) was added. The plate was incubated (37°C, 5% CO_2) on a rocker table for 24 h. Fifty microliter of a PNPP in diethanolamine buffer solution was added

and left to incubate for 30 min in the dark. The reaction was stopped with 50 μL of 3 N NaOH and the absorbance was read at 405 nm using a plate reader. Wells were scored as being positive or negative.

β-Galactosidase. Fifty microliter of HEK293 EBNA cells at a density of 0.5x10^6 cells/mL were added to each well in a 96-well plate. To each of these wells, 50 μL of diluted sample (10^{-1} - 10^{-8}) was added. The plate was incubated (37°C, 5% CO_2) on a rocker table for 24 h. Fifty microliter of an X-gal working solution was added and left to incubate for an additional 24 h (37°C, 5% CO_2) on a rocker table. Blue cells were counted in each well using an optical microscope.

GFP. HEK293 EBNA cells (0.5x10^6) were plated in each well of a 12-well culture plate and infected with serial dilutions of cell lysate in HSFM medium to get between 2% and 30% of the cells transduced. The plates were incubated at 37°C for 24 h on a circular shaker rotating at 100 rpm. Cells were harvested, resuspended in 1mL of PBS buffer and were fixed with 2% formaldehyde. Cells only infected with adenovirus were used as negative control. For each sample, the percentage of fluorescent cells was determined by flow cytometry using a minimum of 10,000 cells.

3.2.3.4.2 Flow cytometry. Analysis of the infected Sf9 and HEK293 EBNA cells were performed with the Coulter EPICS^{TM} XL-MCL flow cytometer (Beckman-Coulter, FL) equipped with a 15-mW air-cooled argon-ion 488 nm laser. Green fluorescence of the GFP was detected using a 550 nm long-pass dichroic filter and a 525 nm band-pass filter. Light scatter values were measured on a linear scale of 1,024 channels and fluorescence intensities on a logarithmic scale. A minimum of 10,000 events was collected using list-mode format for each experiment. Flow cytometric data were analyzed using the EXPO32^{TM} software package. Flow cytometry calibration was conducted using standard fluorospheres (Coulter, Miami, FL).

3.2.3.4.3 ELISA for total viral particle (VP) concentration. The total viral vector particles were assessed using cell lysate and a commercially available ELISA kit (cat# PRATV, ARP American Research Products, Inc., Belmont, MA). This kit is based on a sandwich ELISA technique and uses a mouse monoclonal antibody (A20) specific for a conformational epitope on assembled AAV particles.

3.2.3.4.4 Western blot analysis of viral proteins. The antibody used to detect the Rep proteins is a monoclonal antibody to adeno-associated virus Rep 78, 68, 52, and 40 (cat# MAB689P). The antibody used to detect the Cap (VP) proteins is a monoclonal antibody to adeno-associated virus VP1, 2, and 3 (cat# MAB659P). Both were purchased from Maine Biotechnology Services (Portland, ME).

3.2.3.4.5 Real-time polymerase chain reaction. Cell culture broth was freeze/thawed three times before being subjected to 0.3 N NaOH and heated for 30 min at 65°C. The samples were then passed through a MicroSpin^{TM} G-25 column (Amersham Biosciences, Little Chalfont Buckinghamshire, England). The PCR conditions were as follows: 94°C for 600 s, then 40 times 94°C for 60 s, 55°C for 60 s, and 72°C for 60 s. The primers used were for CMV (CAAGTACGCCCCCTATTGAC and AAGTCCCGTTGTTGATTTTGGTG).

3.2.4 Results

3.2.4.1 Baculovirus stocks

The generation of viral stocks (BacITR, BacCap, and BacRep) required the amplification of baculoviruses over several passages. It was observed in our work that the level of the major replication protein (Rep52) decreased with increasing passage

number when the same amount of baculovirus vector was used to infect the cultures (Figure 3.4). When used with the other two viruses, BacCap and BacITRGFP (both P3), the overall amount of replication protein was reduced; however, the decrease between passages 2 and 4 went from 15:1 when infecting the cells alone to 3:1 when infecting the cells with the combination of the three viruses. The most abrupt change occurred between passage 2 and 3 for both scenarios. As well as obtaining decreased amounts of replication protein, it was observed that the infectious AAV titer also decreased when the passage number of the BacRep stock increased. The infectious particle titer decreased by a factor of 3 when the passage of the BacRep vector stock was varied between 2 and 4. Due to the size of the experiments undertaken for this study, the use of P3 viruses was required to assure consistency within the experiments.

3.2.4.2 Effects of individual MOI on process dynamics

The initial MOIs of BacITRGFP, BacCap, and BacRep were varied from 1 to 9 to create a factorial design of experiment that could be used to study the effect of these three viruses on the production of AAV under synchronous infection conditions. Characterization of the process dynamics was done on each condition in the factorial design. Synchronous infection was observed with cell growth cessation within 12 hpi. Cultures were allowed to continue up until 216 hpi (Figure 3.5). Beyond 96 hpi, AAV infectious particle titers remained relatively constant in the culture demonstrating a stability of the product.

Marked decreases in viability occurred between 24 and 48 hpi. Cultures infected with high levels of BacCap and low levels of the other two baculoviruses resulted in the greatest apparent decrease. Those cultures with the highest retention of viability in that time period were those cultures infected with low levels of BacCap.

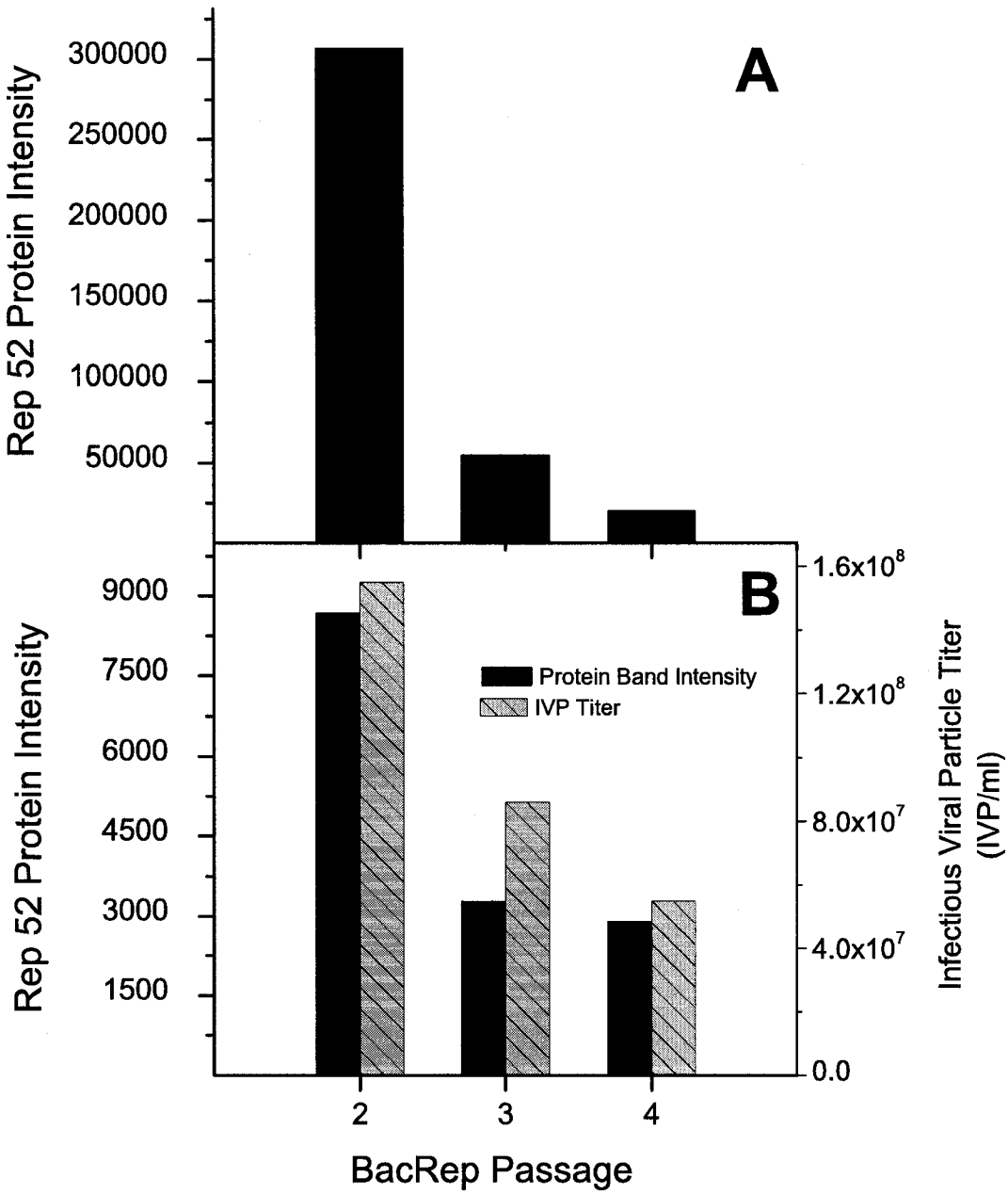

Figure 3.4 Baculovirus passage effect. A: Rep52 protein band intensity at harvest (72 hpi) of insect cell culture infected only with BacRep. B: Rep52 protein band intensity and infectious AAV particle titer at harvest (72 hpi) of insect cell culture infected with BacRep, BacCap, and BacITRGFP.

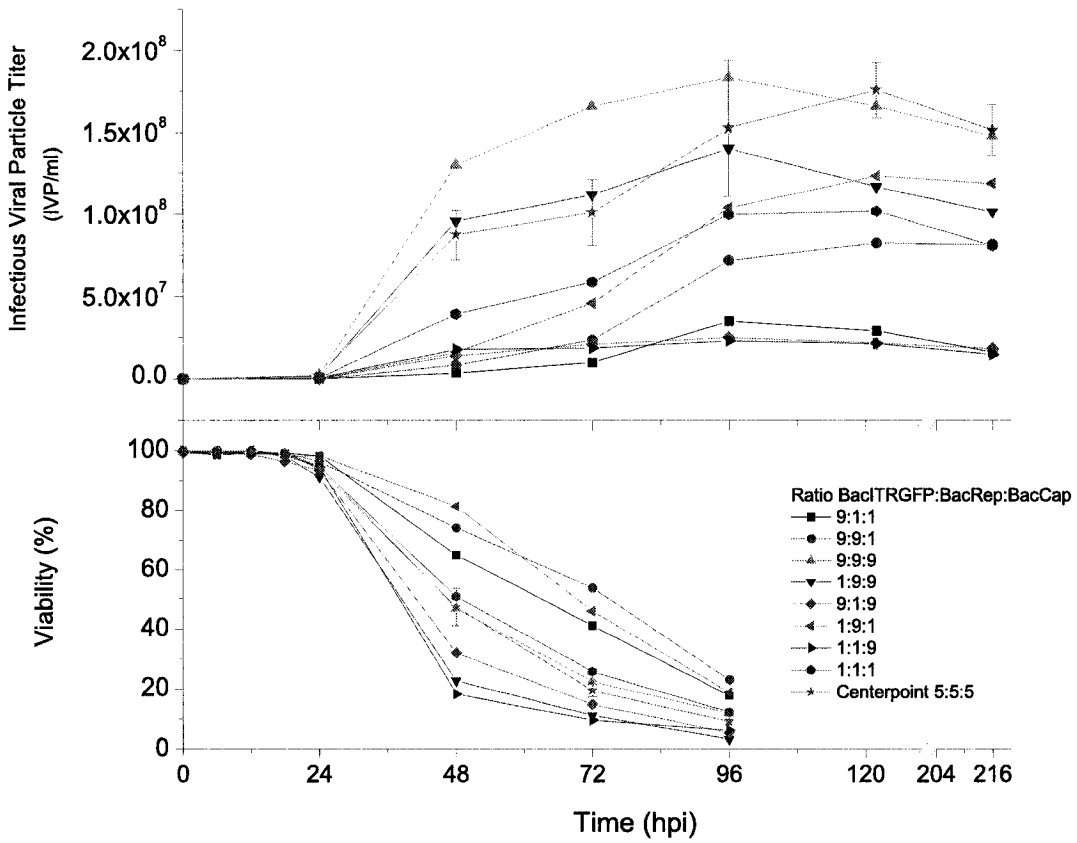

Figure 3.5 A: AAV IVP dynamics associated with cultivation/infection of Sf9 insect cells. B: Dynamics associated with the viability of the cells. Each curve represents the average of two independent cultures with exception of the center-point. Error bars represent the standard deviation (n=3) of independent cultures.

3.2.4.3 Characterization of the system at 72 hpi

Figure 3.6 describes the responses obtained at 72 hpi for the various conditions tested. The treatments (MOI of BacITR:BacRep:BacCap) are ordered in decreasing infectious particle titer (left to right). From the genome analysis, it can be observed that the highest level of CMV promoters was detected when the level of BacRep was high and the other two baculoviruses were set to low levels. The lowest concentration of genomes occurred when the BacRep level was low and the BacCap level was high. These maximum and minimum concentrations of genomes do not coincide with the highest and lowest concentrations of Rep52 detected. The reader should note that the genome analysis in this study was a measure done on the culture broth that included signal from both BacITR and AAV genomes, which both contain the CMV sequence. The concentration of total viral particles remained relatively steady for systems with equal amounts of BacRep and BacCap, and either decreased with high BacRep and low BacCap or increased with low BacRep and high BacCap. The lowest viability observed occurred when a high BacCap MOI and a low BacRep and BacITRGFP were used. The highest infectious AAV particle titer was obtained when the highest initial MOIs of each baculovirus were used. The second highest titer observed occurred when a high MOI of BacRep and BacCap and a low MOI of BacITRGFP was used. The smallest titer was obtained when a high MOI was used for BacITRGFP and low MOIs of BacRep and BacCap.

Table 3.1 summarizes the significant factor effects, based on the analysis of variance of a number of system responses. Responses were transformed as necessary to make ANOVA valid, that is, residuals were checked for being normally distributed with a constant variance. Only factors and interactions that were significant or that kept hierarchical consistency were kept in the analysis. From this table it can be seen that the responses greatly depend on the combination of MOI chosen, as evidenced

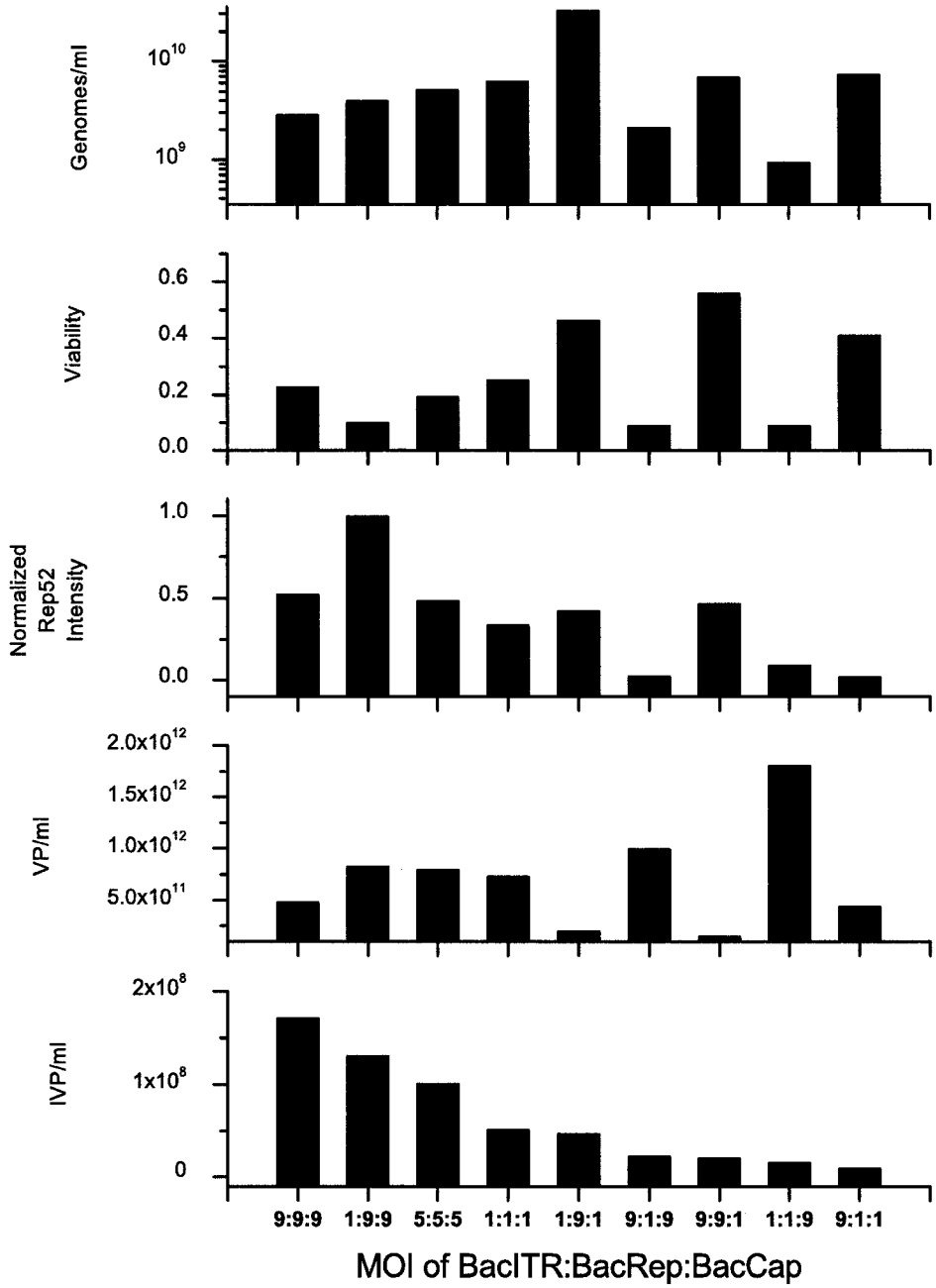

Figure 3.6 Responses at 72 hpi obtained for the various MOI combinations tested. From top: genomes/mL=concentration of CMV sequences detected in culture broth; viability=fraction of insect cells remaining uncolored after trypan blue exposure; Rep52 intensity=band intensity detected by Western blot associated with the replication protein Rep52; VP/mL=AAVcapsids concentration detected by ELISA; IVP/ml=infectiousAAVparticle titer determined by gene transfer assay.

Table 3.1 Summary of the significant effects (95% confidence level)

	BacITR(A)	BacRep(B)	BacCap(C)	AB	AC	BC	ABC
CMV Genomes			1↓	2↓			
Insect Cell Viability	3↑	2↑	1↓			4↓	
Rep52 protein (band intensity)	3↓	1↑	4↑			2↑	
Total AAV particle (VP) concentration	3↓	2↓	1↑				
Infectious AAV particle (IVP) titer	6↓	1↑	3↑	5↑	4↑	2↑	

Effects are numbered such that 1 has the greatest effect (either positive or negative).

by the number of two-factor interactions. The only exception was the production of viral particles. When BacCap was increased, the amount of viral particles produced increased no matter the levels of the other two viruses; however, high levels of BacRep and BacITRGFP levels decreased the amount of viral particles produced. From Figure 3.7, the highest levels of capsids coincided with the lowest levels of viral replication proteins.

3.2.4.3.1 Two-factor interactions. Factor interactions occur when the effect of one factor on the response depends on the level of another factor. An interaction effect existed between BacITRGFP and BacRep on the concentration of genomes detected. When low levels of BacITRGFP were added, a significant change in the levels of genomes was detected when the level of BacRep was changed, while at high levels of BacITRGFP, the number of genomes detected did not differ greatly as the level of BacRep was changed. High levels of BacRep with low amounts of BacITRGFP yielded greater concentrations of genomes than when low levels of BacRep were used.

When looking at viability as a system response, it was observed that there was a significant interaction effect between the levels of BacCap and BacRep. Changing the level of BacCap when BacRep was kept high resulted in a greater change in viability than when BacRep was kept at a low level. The highest viability occurred when BacRep was at a high level and BacCap was at a low level.

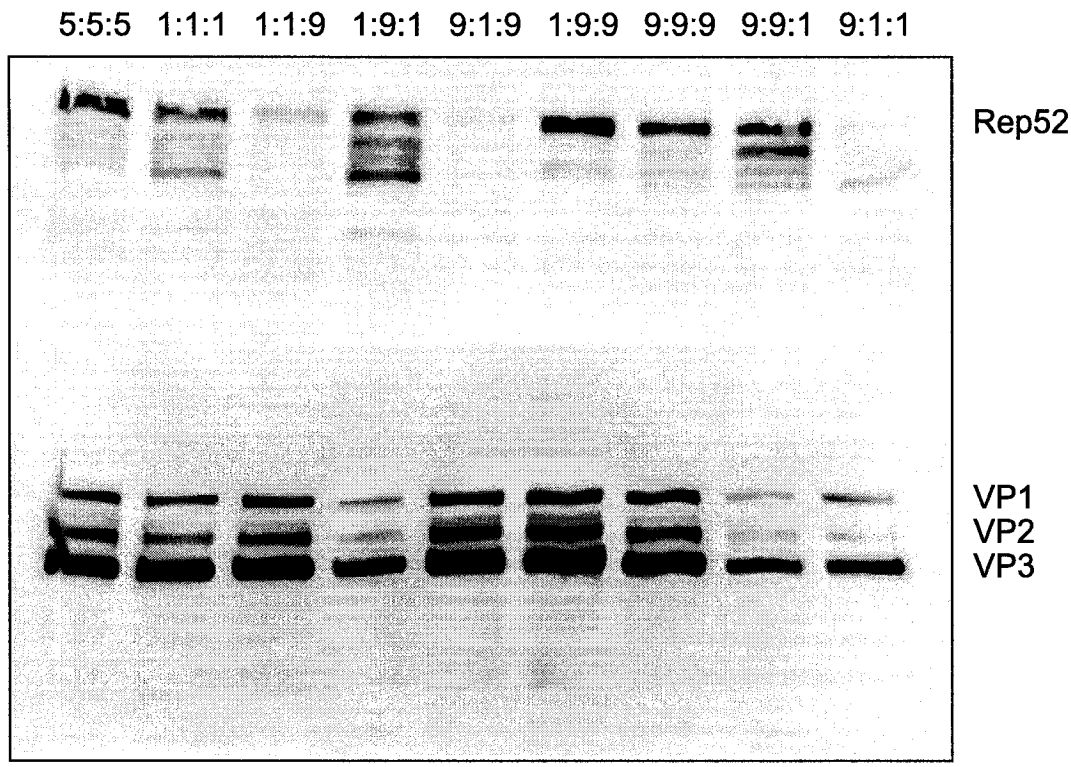

Figure 3.7 Western analysis of AAV replication and structural proteins in the culture broth at 72 hpi. Lanes identified by the MOI of the three baculoviruses used (BacITR:BacRep:BacCap).

A marked change in the level of Rep52 occurred when BacRep was at a high level and BacCap was increased from a low to a high level. The level of Rep52 detected increased when higher amounts of BacCap were added to the system. This may seem counter intuitive; however, when looking at the Western Blots (Fig. 3.7) various patterns appeared. Rep52 was readily detected for all samples; however, the bands below Rep52 are unknown and are thought to be degradation products or improperly spliced versions of the replication proteins, which is why they were detected by Western analysis. The lanes displaying the unknown products were of cultures having a high MOI of BacRep and a low MOI of BacCap.

There were significant interactions with all components when analyzing the infectious

AAV titer. The most significant interaction effect is between BacRep and BacCap (Table 3.1). This is due to a marked increase in the level of IVP produced when BacRep was at a high level and BacCap was increased from low to high. The other two significant interactions are with BacITRGFP. Increases in IVP titer were greater when the level of BacITRGFP was high and the level of BacRep was increased from low to high. Similarly the increase in IVP titer was greater when the level of BacITRGFP was high and the level of BacCap was increased from low to high. When BacCap is kept low, increasing BacITRGFP results in a decrease in IVP titer.

3.2.4.4 Transgene generalization

As a generalization experiment, it was sought to probe further the role of the BacITRtransgene level on the production of bioactive AAV. It was therefore chosen to vary the MOIs between 3 and 10 at the same time as varying the BacITR construct. Two additional reporter genes were examined: LacZ, which codes for β-galactosidase and the gene for secreted alkaline phosphatase (SEAP). In this set of experiments increasing the overall baculoviral load, that is, increasing BacITRtransgene from 3 to 10, decreased the viability of the cell regardless of the transgene (Fig. 3.8). The transgene, however, also seemed to play a role in the viability of the cell. These changes in viability do not seem linked to different levels in capsids produced (Fig. 3.9). The effect of varying the MOI of the BacITRtransgene between 3 and 10 on the production of bioactive AAV was not significant (even at a 65% confidence level) regardless of the transgene used (Fig. 3.9).

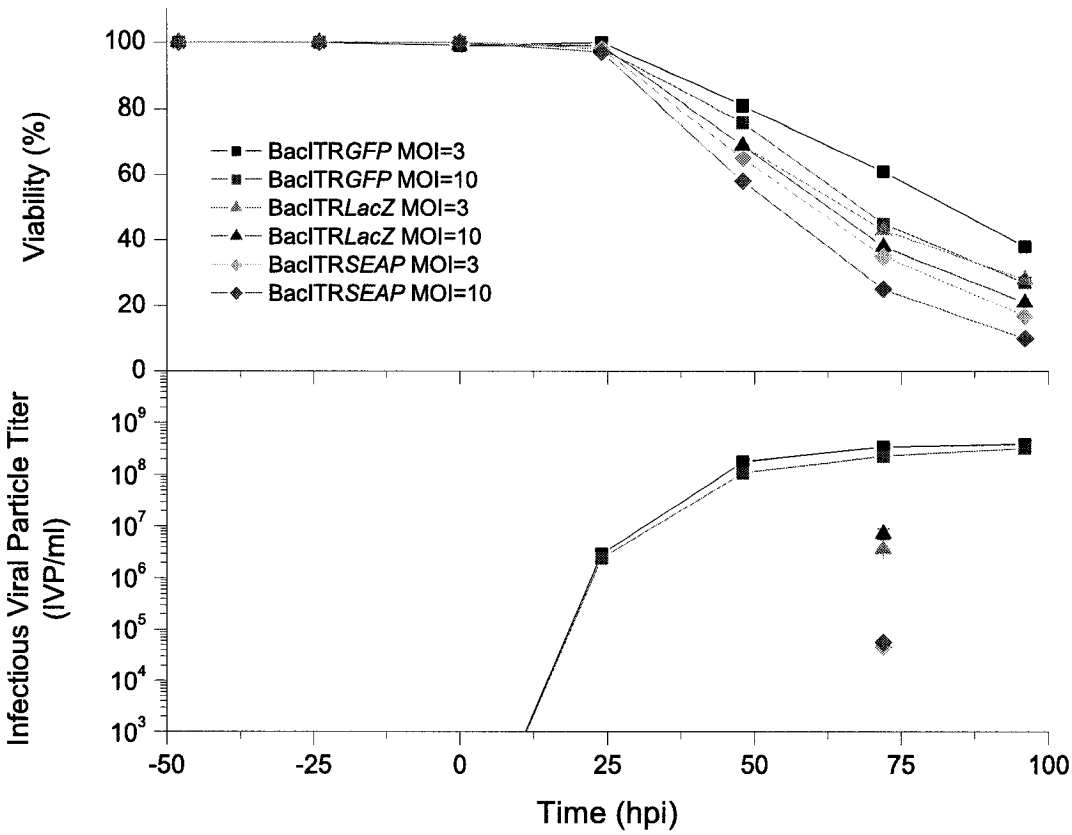

Figure 3.8 A: Dynamics of infectious AAV with various transgenes produced in Sf9 insect cells. B: Dynamics associated with the viability of the insect cells. Each curve represents the average of two independent cultures.

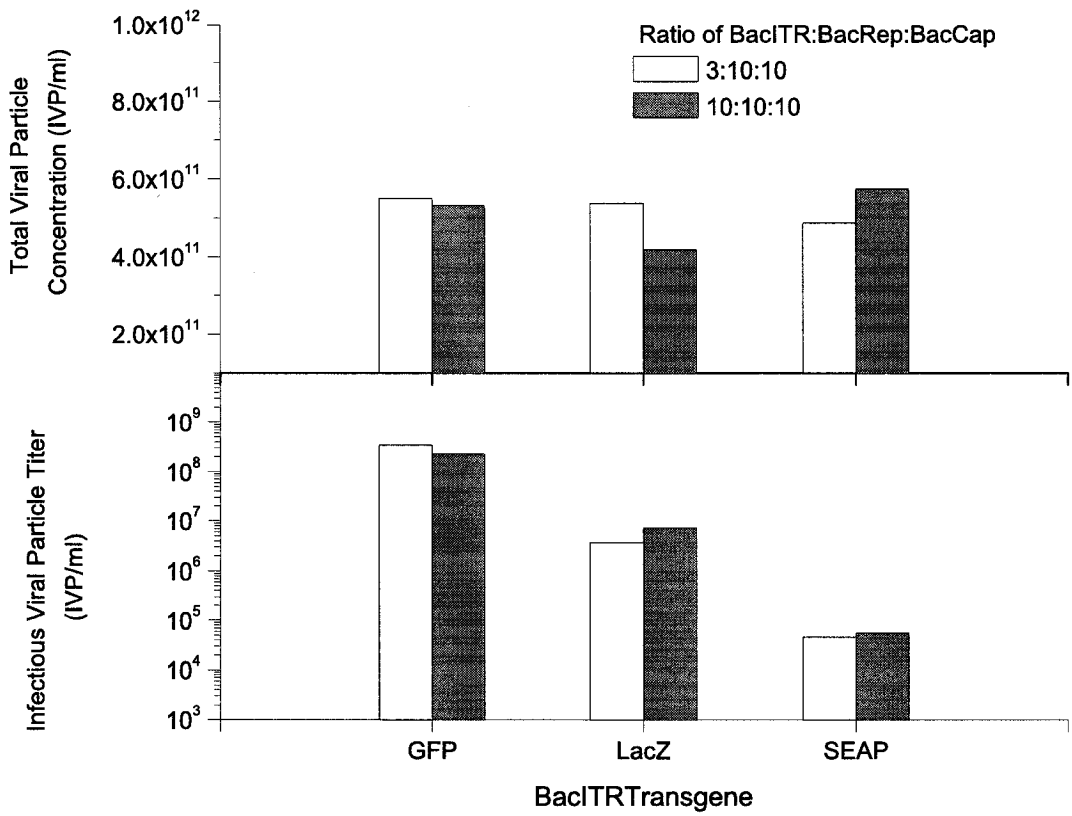

Figure 3.9 AAV particle concentrations and AAV infectious viral particle titers at 72 hpi for AAV vectors with various transgenes.

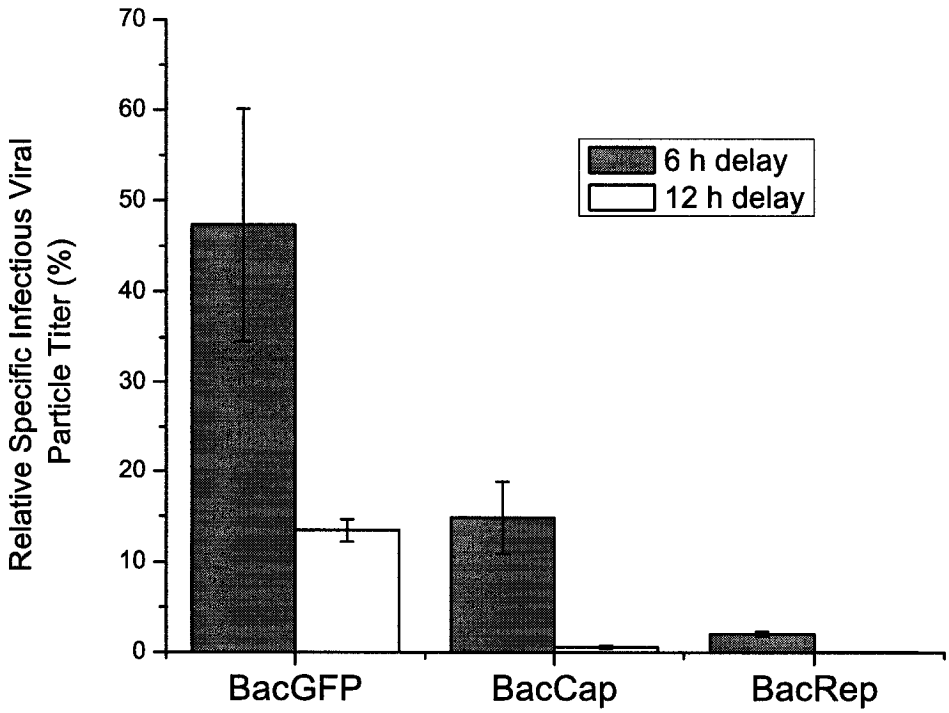

Figure 3.10 Effect of delaying one of the three viruses by 6 or 12 h on the cell specific production at 72 hpi.

3.2.4.5 Relative time of infection: delay in the addition of virus

As can be seen by Figure 3.10, we have found no evidence to support the use of a delay between the baculovirus infections. However, it should be noted that delaying certain baculoviruses affect the titer more profoundly then others. Delaying BacITRGFP up to 12 h still allows a significant amount of AAV to be formed while delaying BacCap and BacRep reduces the infectious viral particle titer by up to 4 orders of magnitude. Delaying the addition of BacCap is less detrimental to the system then delaying BacRep.

3.2.5 Discussion

3.2.5.1 Baculovirus stocks

An important consideration when looking at the ability to scale-up a process such as the one described within this article is the quality of the starting material. Generating large quantities of material requires the amplification of cells and viral stocks. Although it has been previously shown that similar titers at various scales of operation can be achieved (Meghrous et al., 2005), the baculovirus passage was not specified and is assumed to be of the same passage. Recently Kohlbrenner et al. (2005b) showed that the quantity of viral protein (structural or non-structural) decreased when infecting a culture with an equivalent amount of BacRep or BacCap of increasing passage number up to passage 5. The "passage effect", defined as the production of progeny baculovirus variants with potential atypical morphogenisis and/or lower virulence due to a sequence of repeated serial passages, has been well described and was previously reviewed by Krell (1996). These variants are often referred to as defective interfering particles, and for the sake of clarity will be referred to as defective interfering baculovirus particles (DIBP). Wickham et al. (1991) related the MOI to the production of DIBP and showed the observed effect of various quantities of DIBP on the production of foreign protein in various cell lines. They showed, however, that in Sf9 cells the effect was the least prominent. This problem was encountered at high passage number inoculums, however, the passage number was never specified. Researchers have shown that deletions within the viral DNA could occur within 10 passages (Pijlman et al. (2003) and as reviewed by Krell (1996)). In this system, we see that stability of the viral constructs may be even more sensitive. Within the first few passages of BacRep, a reduction in the synthesis of replication proteins, which is ultimately detrimental to the production of bioactive AAV, is observed. Kohlbrenner et al. (2005b) suggested that the reason

for the instability in the BacRep construct was due to the palindromic orientation of the Rep52 and Rep78 sequences. To correct this instability, the two genes were put into separate baculovirus vectors (BacRep52 and BacRep78). Although stability of the protein expression was shown over multiple passages, the quadruple infection strategy gave lower yields of infectious particles (Kohlbrenner et al., 2005b). Further refinement in the genetic makeup of the baculoviruses, such as the inclusion or the increase in density of homologous regions, may improve the stability of the baculoviral constructs as suggested by Pijlman et al. (2004). Furthermore, from a process perspective, the number of different baculoviruses used should not be increased if there is no need. However, given that this system is based on triple infection, there is a need to consider the question of infection strategies; having more than one baculovirus vector, what is the proper amount of each to put in to the system to obtain optimal results?

3.2.5.2 Process dynamics: changes due to multiplicities of infection

Published reports on the production of AAV in insect cells have described that the harvest of the culture should occur after the third day post-infection (~72 hpi) (Kohlbrenner et al., 2005b; Meghrous et al., 2005; Urabe et al., 2002). The reason for this optimal harvest time was not justified based on the maximum amount of AAV produced. For Meghrous et al. (2005), the optimal harvest time was related to the time at which AAV went from being mostly a cell-associated product to being released in the supernatant. The "optimal" process was dictated by a constraint linked to the downstream processing of the harvest material, which is facilitated by the recovery and treatment of only the cell pellet. The data obtained in our experiment (Fig. 3.5) corroborated initial findings by Meghrous et al. (2005) that the titer of infectious particles increases significantly (95% confidence level) until 96 hpi. From further studies, when using high MOIs (~10:~10:~10), the percent of AAV

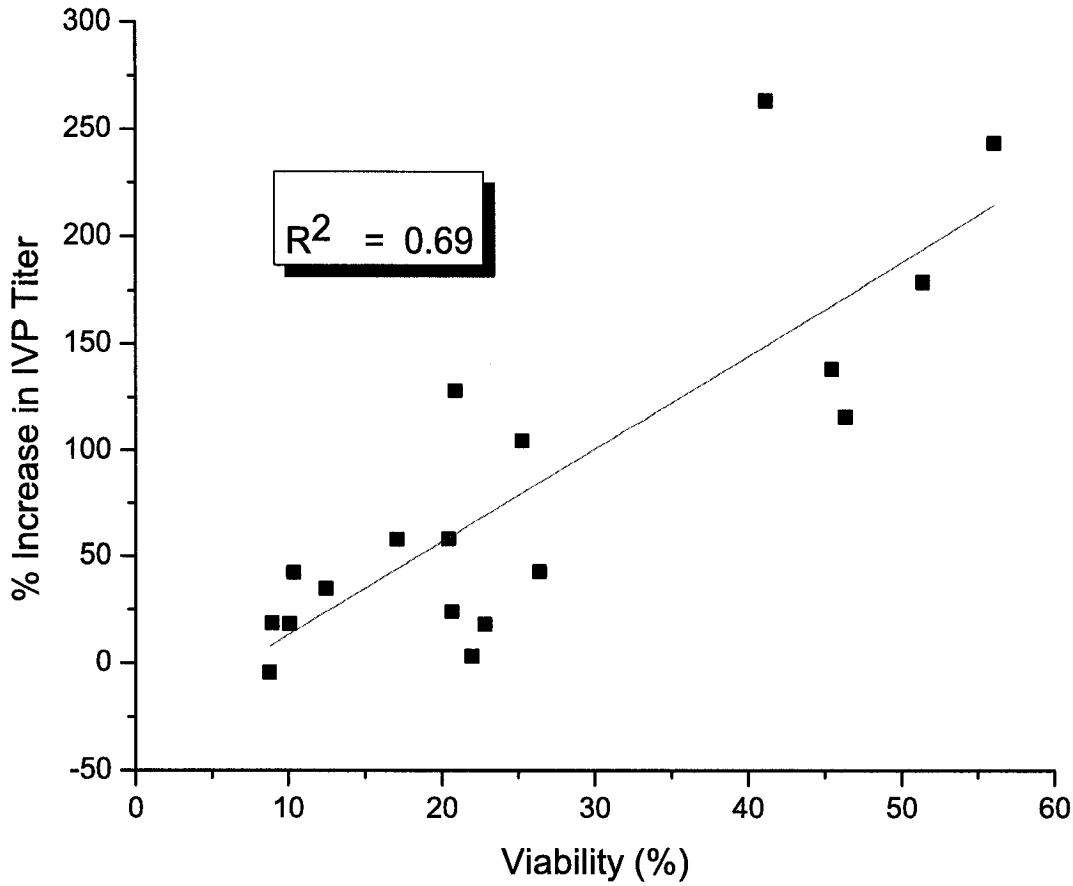

Figure 3.11 Relationship between the viability of infected insect cells at 72 hpi and the increase in AAV infectious particle (IVP) titer between 72 and 96 hpi.

associated with the cell dropped from ~60% to ~40% between 72 and 96 hpi (data not shown). If only the pellet is considered, at high MOIs, the optimal harvest time obtained in this work was 72 hpi (the gain in titer from the increase in production time was smaller than the loss of particles from the pellet due to cell leakage or lysis). However, for other MOI combinations, which resulted in significantly less product than in the optimal conditions, increases up to 260% in IVP titer occurred between 72 and 96 hpi. The extent to which the IVP titers increased was correlated to the viability of the cells at 72 hpi (Fig. 3.11).

3.2.5.3 Cell viability and viral particle concentration

The viability of the cells is a measure of the health/strength of the cell/factory. As was previously shown in Figure 3.11, the extent to which the cell could increase the amount of AAV produced beyond 72 hpi was correlated to the state of the cell, that is, the viability of the cell. It has been previously reported that only 2% - 5% of the produced viral genomes eventually bud out of the cell (Haas, 2004) with a significant portion of viral DNA accumulating within the cell. The intracellular accumulation of the baculovirus leads to the lytic nature of the system, and is generally the predominant cause of the drop in cell viability in the latter part of the culture. Cell viability, however, is influenced by many factors including, but not exclusively, shear stress and medium composition. In this system, it was found that the amount of capsids produced after 72 hpi was correlated not only to the viability at 72 hpi but also to the rate of viability decrease after 24 hpi (Fig. 3.12). Cell viabilities and viral particles, however, did not correlate to the titer of infectious particles in the system. This phenomena is similar to what has been observed in mammalian cells (Grimm et al., 1999) whereby overexpression of viral structural proteins did not lead to similar increases in infectious titers. In our system, overexpression of capsids led to the rapid decline in the viability of the cells. We have also observed variation in the viabilities depending on the type of transgene used (Fig. 3.9). To date, these changes in viability remain unexplained, but emphasize the complexity of using viability as an indicator for levels of production.

3.2.5.4 Viral particles (VP), replication protein (Rep52), and infectious viral particles (IVP)

The ratio of VP:IVP is an important parameter that needs to be optimized at the same time as maximizing the IVP titer. Our data suggests that overproduction

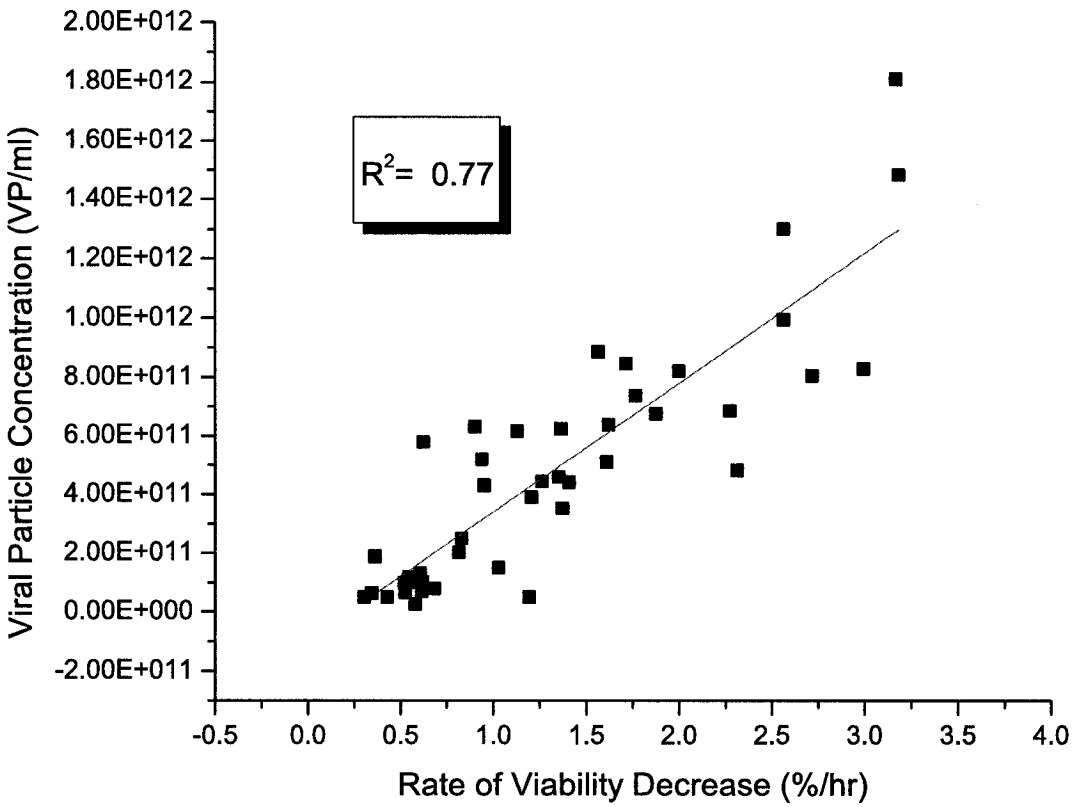

Figure 3.12 Relationship between the AAV particle concentration at 72 hpi and the rate of insect cell viability decrease between 24 and 48 hpi.

of capsids leads to decreases in cellular viability, reducing the opportunity for vector maturation. From an application's perspective, the generation of excess empty particles requires the development of downstream processing schemes, which has the ability to discriminate between empty and full particles. Ultracentrifugation in iodixanol or cesium chloride gradients have been used for this purpose; however, are not easily amenable to large scale. Therefore, it was important for us to investigate what helped reduce this ratio in the upstream phase of the process.

Rep78 or Rep68 are necessary for DNA replication and have been found to be sufficient by themselves for both the replication of the DNA and the formation of infectious particles (Holscher et al., 1995; Ni et al., 1994). Even though the formation of infectious particles does not need the presence of either of the small replication proteins (Rep52 or Rep40), the presence of Rep52 can dramatically increase the titer of infectious particles when Rep78 or 68 is also present (Holscher et al., 1995). Rep52 and Rep40 have been reported necessary for the efficient accumulation of progeny single stranded DNA but have not been implied in DNA replication (Chejanovsky and Carter, 1989). This may explain why there was only a weak relationship between the Rep52 intensity and IVP observed (Fig. 3.13); however, Figure 3.13 also shows a relationship between the amount of Rep52 protein and the ratio of viral particles to infectious viral particles. From this figure, it could be concluded that beyond a certain level of replication proteins, there was little or no benefit, or that the limiting reagent was no longer that of the precursor replication proteins (Rep52). It was recognized in the early 80s that AAV capsids are readily formed into particles but the encapsidation of the genome or "packaging process" was a slow process requiring several hours (Myers and Carter, 1980). This packaging process leads to the model of AAV DNA being packaged into preformed capsids. King et al. (2001) have described the necessity of the helicase function of the smaller replication proteins (Rep52/40) for the translocation of full-length genomes from the capsid surface to the inside.

What we have failed to detect in this study is whether the early expression of the larger replication protein and the later expression of viral particles leads to lower levels of association between the vector genome and the capsids, thus creating the limiting step. In a recent study by Urabe et al. (2006), delaying the expression of the larger replication protein (Rep78) by using a truncated late insect cell promoter (Δp10) doubled the number of vector genomes per cell.

In this study, which was primarily driven by the desire to understand the effect and the interaction of the baculoviruses on the production of AAV vector, it was also shown that high BacRep levels affected the amount of capsids produced. It is believed that there is a point at which there are sub-optimal levels of capsids for the encapsidation process as suggested by the unknown bands found in the Western blots (Fig. 3.7). This could be due to the concentration of viral particles within the cell or because of a distribution problem among the cells; however, the reason remains unclear. The pattern observed in our Westerns has been previously observed for the production of rAAV in mammalian cells (Grimm et al., 1998; Urabe et al., 2006; Wistuba et al., 1995) but never in the context of varying plasmid/virus concentrations. At high levels of BacRep and low levels of BacCap, it is believed that the amount of replication proteins does not balance the level of capsids resulting in the degradation of excess replication proteins. These degradation products may have been the reason for lower titers as they may compete with the complete Rep52 molecules. At 72 hpi an overproduction of Rep52 did not lead to higher infectious AAV titers.

The level of BacRep increases the level of infectious particles, most probably due to the increases in replication proteins in the system. The ratio of viral particles to infectious viral particles is decreased, however, not only due to the encapsidation of genomes, which is accomplished in the presence of replication proteins, but also due to the competition for resources, which limits the amount of structural proteins

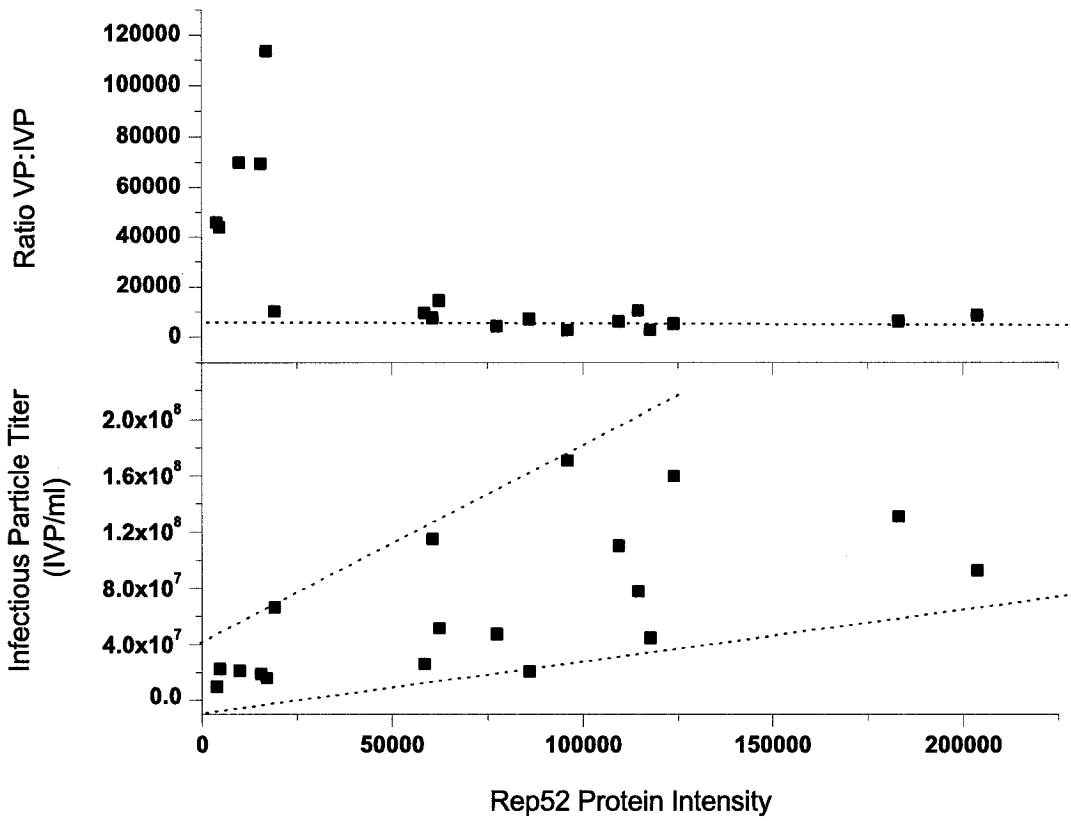

Figure 3.13 Relationship between Rep52 intensity and: (top) the ratio of AAV particles to infectious AAV vectors; (bottom) infectious AAV vectors titer.

produced.

3.2.5.5 BacITRtransgene

An unexpected result relating to the effect of varying the MOIs of the baculoviruses was the high titer of infectious AAV particles obtained with the combination 1:9:9 BacITRGFP:BacRep:BacCap. This suggested to us that there might be a possible minimum level of BacITRGFP needed for the production, and beyond this minimum, there would be no clear advantage of adding more. This is important for producing vector stocks. From an industrial perspective, if one of the viruses can be reduced it

could result in savings in terms of cost and stability of the virus/system. A recent study (Kohlbrenner et al., 2005b), has shown the instability of the vector containing the transgene; therefore, knowing that there is no clear advantage of increasing the amount of BacITRGFP added to the system on the production of AAV, a greater stability of the entire system may be achieved. It was believed that the lower bound on MOI chosen is slightly below the threshold value (one active virus per cell). This was probable, as it has been previously reported that an MOI of 3 is needed for the synchronous infection of cells (Nielsen, 2000). Upon investigating the lower bound in the generalization experiment by setting it to 3, it was revealed that the level of BacITRtransgene no longer produced a significant effect. This result also points towards the importance of the replication proteins which are not only needed for the replication of the vector genome, but also for the excision and sequestration of single stranded progeny DNA. These results are further supported by the delay experiment, whereby addition of BacITRtransgene has less of a negative impact than the addition of BacRep.

Furthermore, among the responses monitored, the production of GFP was readily measured through the detection of fluorescence in cells by flow cytometry. It has been hypothesized by others that competition for transcription factors exists, with strong promoters attenuating weaker ones. GFP is under the p10 promoter in this system, and although making some analysis easier, may be detrimental to the system by using up available resources. In our work we have found that adjusting the level of BacITRtransgene did not significantly affect the level of bioactive AAV produced. In fact, no correlation between the level of cells expressing GFP and the infectious viral particle titer was obtained. We have seen some evidence to support an inverse correlation between the levels of GFP and the viral particle concentration, which supports the notion of competition of transcription factors. In Figure 3.9, systems without the p10 promoter (AAV-LacZ and AAV-SEAP) seemed to be favored by

higher quantities of BacITRtransgene in terms of infectious particle titer, however these results are not statistically significant.

3.2.5.6 Relative time of infection: delay in the addition of virus

The hypothesis behind the delay of virus addition was that capsid overproduction could be limited by delaying the addition of BacCap. Decrease in baculovirus from the supernatant occurs up to 12 hpi before the onset of baculovirus budding from the cells, even though the majority of virus is taken up within the first hour after addition (data not shown). It was therefore decided to limit the delay to 12 hpi. Furthermore, since baculovirus late gene expression, which coincides with the production of baculoviral polymerase, starts between 6 and 12 h post-infection, 6 hpi was chosen as a second time point.

The replication proteins are essential for the replication of the AAV vector genome and its encapsidation. The expression of the larger replication protein (Rep78) is driven by a truncated promoter of the immediate-early 1 gene of *Orgyia pseudotsugata* nuclear polyhedrosis virus (ΔIE1) (Urabe et al., 2002), which is thought to depend on cellular polymerases available in the early stages of infection. It is believed that this may be the reason why the later addition of BacRep affected the production of AAV more drastically than the other two components.

The effect of delaying the addition of BacCap was greater than anticipated with only a fraction of the AAV infectious particle titer obtained compared to when all three viruses were added at the same time. A shorter delay may have reduced the effect, however, in light of these observations, higher infectious titers are not thought possible with a delay strategy.

3.2.6 Conclusions

Future optimization will need to address the stability of the viruses as our results point to high baculovirus multiplicities of infection for achieving the highest AAV titers with the lowest ratio of AAV VP:IVP. It is believed that the system studied was operating under conditions where enough of the individual components were produced but that the assembly was the limiting event. Equal amounts of all three baculoviruses was not needed; less BacITR may be adequate for producing bioactive AAV and has been shown for multiple vectors (BacITRSEAP, BacITRLacZ, BacITRGFP). There was a need, however, to maintain a balance between BacRep and BacCap. It was also beneficial to use high MOIs of these two baculovectors. High values of capsids correlated well with low viabilities. Low levels of capsids also related to higher levels of degraded (improperly spliced) Rep52, which could point to the under-utilization of proteins. Replication proteins were needed for producing infectious AAV and were related to the increase in genomes, and correlated to the packaging of the genome by decreasing the ratio of AAV capsids to infectious AAV particles.

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3.3 Complementing results: additional evaluation of the multiplicity of infection

3.3.1 Utility of principal component analysis when analyzing DOEs with multiple outputs

To gain a better perspective on how all the responses were interrelated, a Principal Component Analysis of the responses at 72 hpi, from the full factorial DOE on

the multiplicity of infection (2^3), was undertaken. Principal Component Analysis (PCA) is a technique used to reduce large complex data sets to a simpler lower dimension data set consisting of principal components. This is achieved through a linear transformation of the original data set. For an excellent tutorial on this method and on partial least-squared methods, the reader is pointed to works by Shlens (2005) and Geladi and Kowalski (1986). The reason PCA was chosen was because of its ability to identify redundancy or correlation among sets of measurements and variables. To achieve this goal, both, PCA and partial least squares discriminant analysis (PLS-DA), were carried out using the statistical software package, Simca-P+ 10.0 (Umetrics, NJ, USA).

Two principal components were found to be significant by cross-validation. These components describe 75% of the variation in the original data, with PC1 describing 50% and PC2 describing 25% of the variability respectively. The scores plots (Figure 3.14) show three important sets of groupings. The first grouping is the duplicate and triplicate runs; these are closely grouped, indicating a highly reproducible system.

The second grouping is for experiments with high and low levels of BacRep (Figure 3.14 A), with those at low levels (X:1:X) concentrated in the bottom right hand quadrant and those with high levels (X:5or9:X) concentrated on a diagonal through the three other quadrants. Using PLS-DA on these two groupings shows that they differ most in the levels of Rep52, Ratio of VP:IVP, the number of baculoviruses detected and the titers of IVP and VP. This grouping indicates the importance of BacRep, which affects both measures of the goals of this research: the IVP titer and the ratio of VP to IVP. The loading plot from the PCA analysis (Figure 3.15) shows that the level of Rep52 in the system is highly correlated to the level of infectious viral particles and inversely correlated to the ratio of VP to IVP.

The third grouping is for those with high and low levels of BacCap (Figure 3.14 B),

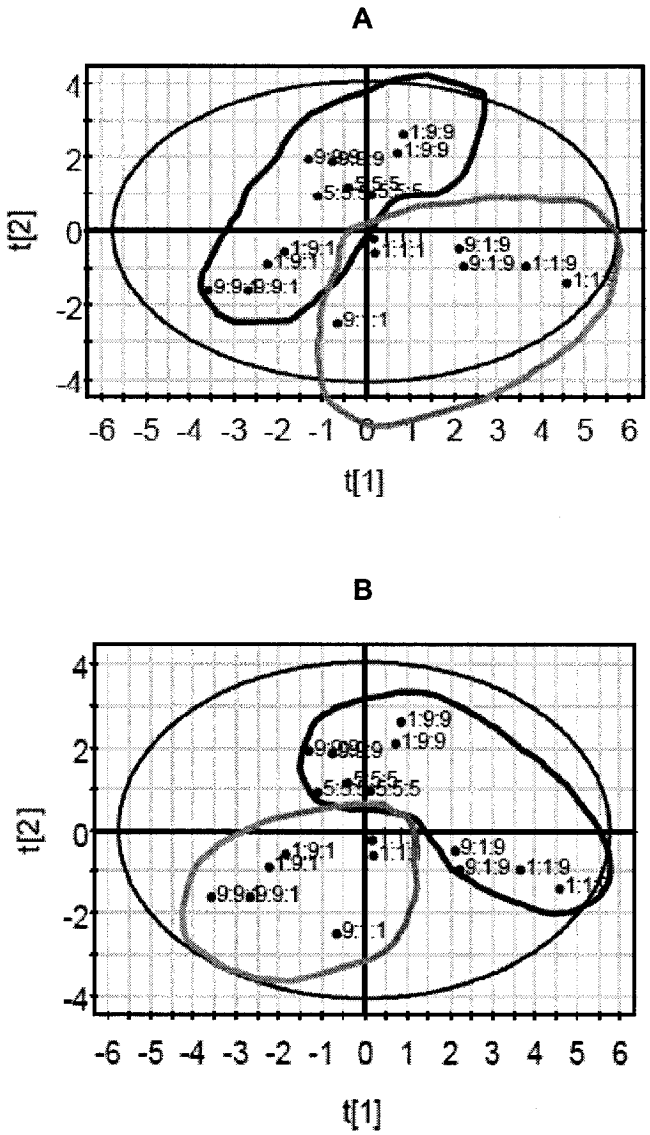

Figure 3.14 Scores plots of PCA analysis using observations at 72 hpi. Samples are identified by the ratio of initial multiplicities of infection of BacITR:BacRep:BacCap. (A) Grouping of high (black) and low (grey) levels of BacRep. (B) Groupings of high (black) and low (grey) levels of BacCap.

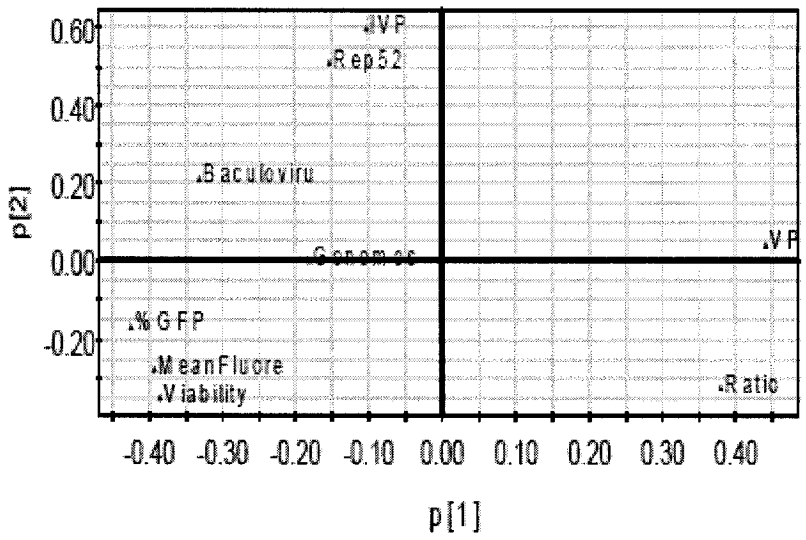

Figure 3.15 Loading plot of PCA analysis for infectious viral particles (IVP), capsids (VP), ratio of VP:IVP (Ratio), replication protein intensity (Rep52), CMV promoter sequences (Genomes), baculovirus titer in the supernatant (Baculoviru), % cell expressing GFP (% GFP), mean fluorescence of Sf9 cells (Mean Fluore), and the viability of the cells (Viability).

with those at low levels (X:X:1) concentrated in the bottom left hand quadrant and those with high levels (X:X:5or9) concentrated on a diagonal through the three other quadrants. Using PLS-DA on these two groupings shows that they differ most in the levels of viability, fluorescence and VP. VPs and fluorescence appear in opposite quadrants indicating they are inversely correlated and that there is competition for resources within the cell. This competition, however, is beneficial to the production of functional AAV, since the overproduction of capsids leads to low cell viability. From the loading plot (Figure 3.15), it can be seen that the amount of VP produced is inversely correlated with the viability of the cells.

The significance of these relationships were explored in Section 3.2; however, it can be seen that analysis based on principal components can allow quick visual representations of the relationships between experiments, conditions and variables.

3.3.2 Overall Multiplicity of Infection

The results obtained in the DOE presented in Section 3.2, point to a maxima in functional titer at a corner point of the design space. This, obviously, does not allow one to conclude that a global maximum was found but that only a constrained maximum was obtained. Although practically, MOIs of ten were chosen as upper limits for each vector, it was of interest to see if the addition of more BacRep and BacCap would result in even higher functional titers.

Two sets of cultures were used to investigate this question. As control, cells were infected at $2x10^6$ cells/ml with BacRep, BacCap and BacITRGFP at MOIs of 10,10 and 3, respectively. The other set of cultures were also infected at $2x10^6$ cells/ml but with BacRep, BacCap and BacITRGFP at MOIs of 30,30 and 3, respectively. As can be seen by 3.16, the addition of more BacRep and BacCap does not improve the functional titer. This is consistent with a previous report that showed a saturation in the number of functional AAV particles that could be synthesized above an overall MOI of ~15-30 (Meghrous et al., 2005). These results reinforce the selection of MOIs described in Section 3.2.

3.3.3 Production strategy: combining asynchronous and synchronous infection

Although work by Meghrous et al. (2005) suggested that low MOI strategies resulted in poorer titers, the results presented previously in this chapter can be used to refine this assessment. The multiplicity of infection study resulted in two points that could be used to develop a novel production strategy. These two points are:

1. BacITR does not need to be supplied in equal quantities to BacRep and Bac-

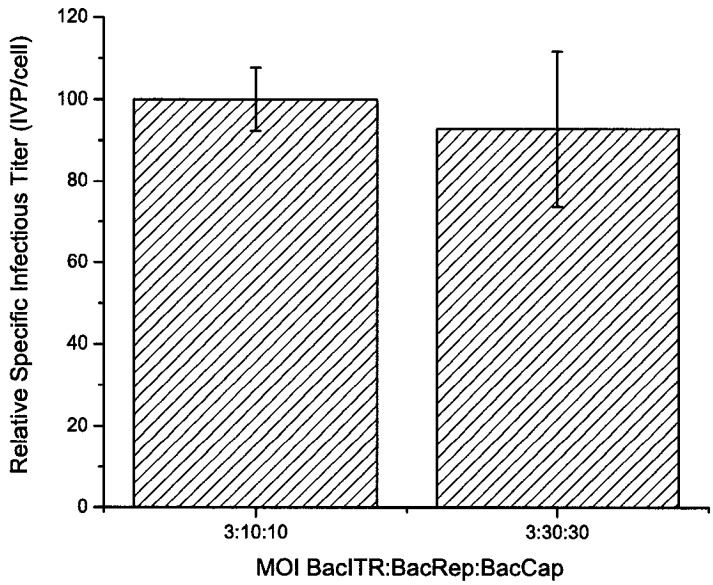

Figure 3.16 Effect of increasing the MOI of BacRep and BacCap beyond 10 on the production of functional AAV vectors.

Cap, and in fact can be supplied in a lesser amount; and

2. BacITR affects production of functional AAV the least when supplied a number of hours after synchronously infecting the cells with BacRep and BacCap.

Since there exists a moderate amount of flexibility in the amount of BacITR added to the system, it is likely that both the amplification of BacITR and production of functional AAV could be combined. This would achieve a process whereby only a minimal amount of this vector would be used (asynchronous infection).

To evaluate this strategy, a short Matlab program was developed to simulate this process (Appendix II). Simulations were based on the models developed by Hu and Bentley (2000) for cell growth, infection and production of viral progeny. For simplicity, it was assumed that reinfection of cells was negligible and therefore omitted. This assumption is considered reasonable since the quantity of virus initially and the number of infected cells will be much smaller than the number of total cells. To make sure that the simulation adequately described the system at hand, model parameters were calibrated using one of the BacITRGFP amplification runs. For the amplification, cells were inoculated at $\sim 0.5 \times 10^6$ cells/ml and infected with BacITRGFP (MOI=0.01) when the cells reached 1×10^6 cells/ml. As can be seen in Figure 3.17, there is a point during the amplification of the single virus where a sharp decrease in the number of uninfected cells occur. Prior to this point, less than 10% of the culture is infected with virus. Adding BacRep and BacCap within the defined operating window, should allow for a synchronous infection of the remaining 90% of the culture, ensuring that this portion of the culture will receive all three viruses, as prescribed from the study on the multiplicity of infection (section 3.2). The robustness of the process relies on the size of the operating window, which is why this strategy relies on the amplification of BacITR and not the two other vectors. It is believed, however, that the excision of the AAV vector genome from the baculovirus

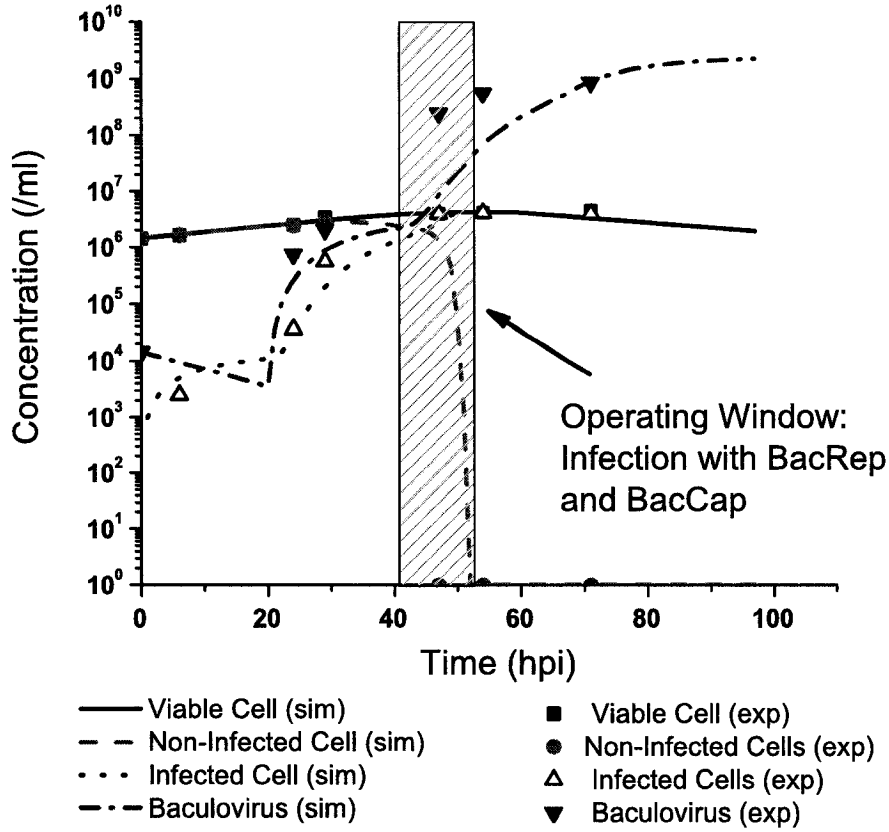

Figure 3.17 Simulation of BacITRGFP amplification in insect cells. ~1x10^6 cells/ml were infected with an MOI=0.01.

backbone may limit the replication of the BacITR, therefore addition of BacRep too early may cause lower vector yields if not all cells are infected with BacITR. The ability to simulate the culture will aid in choosing an appropriate initial MOI for the optimal cell concentration at infection for the production of functional AAV and help define an appropriate operating window for synchronous infection.

CHAPTER 4

QUADRUPLE INFECTION: USE OF TWO MONOCISTRONIC BACREPS TO SUPPLY AAV REP GENES

In the previous chapter, the stability of the BacRep vector was put into question and the issue of baculovirus competition for resources was suggested. In this chapter, work done on a BEV system consisting of four baculovirus vectors for the production of AAV vectors (Kohlbrenner et al., 2005a) was investigated.

Baculovirus vectors have been shown to suffer from the presence of defective and abortive forms of the virus. Some have even tried to account for their presence in the development of infection strategies (de Gooijer et al., 1992). Serial passaging of the virus has been well documented as resulting in the propagation of defective particles and is known as the "passage effect" (reviewed by Krell, 1996). To reduce this "passage effect", propagation and amplification of the virus is done by infecting cultures at low MOIs (<0.1). This is to ensure that defective particles, that would otherwise be able to replicate in the presence of complimentary components brought in by a functional baculovirus, are not propagated. Kohlbrenner et al. (2005a) have reported, however, that even with serial passaging at an MOI of 0.1, reduction of the expression of replication proteins occurs over only a few passages, similar to the findings reported by Aucoin et al. (2006).

Driven by the hypothesis that the head-to-head orientation of the two rep genes (Urabe et al., 2002), which made a near perfect palindromic sequence, heightened the chances of homologous recombination and deletion of the rep sequences, Kohlbrenner et al. (2005a) sought to put the two rep genes in seperate baculoviruses. Upon repeated passages of these new monocistronic (one gene) BacReps (BacRep52 and

BacRep78), it was shown that compared to the bicistronic (two gene) vector, expression levels were maintained, thus inferring greater vector stability. Side by side, the bicistronic BacRep vector allowed a 30% greater yield using P2 vectors compared to the use of the two monicistronic vectors (Kohlbrenner et al., 2005a); however, it was also shown that from P2 to P3, the reduction in Rep expression from the bicistronic BacRep caused a 50% reduction in AAV vector yield (Aucoin et al., 2006). Therefore, if late passage vectors need to be used, it is expected that the use of the monocistronic vectors should fare better and allow for higher functional AAV titers. The following study explores the quadruple infection as an alternative to the triple infection system.

4.1 Materials and Methods

Cells, medium and culture conditions were identical to those described previously (Aucoin et al., 2006). BacITRGFP, BacRep, and BacCap, kindly provided by Dr. R.M. Kotin from the National Institutes of Health (Bethesda, MD), BacITRLacZ kindly provided by Avigen (Alameda, CA) and BacITRSEAP kindly provided by Généthon (France), were prepared as previously described (Aucoin et al., 2006). BacRep52 and BacRep78, contain genes for Rep52 and Rep78 in two separate baculoviruses and were kindly supplied by Dr. S. Zolotukhin (University of Florida, FL).

Briefly, BacRep52 and BacRep78 were amplified 2 passages directly from the stocks received form Dr. S. Zolotukhin. As the stability of these vectors was reported to be improved, plaque purification was not performed on these vectors. To reduce any potential negative effect of adding a large volume of virus, the viral stocks for this set of experiments were concentrated by ultracentrifugation. Vector stocks were spun at 43,000 rpm (215,000g) for 65 min at 4°C in a Ti-45 rotor using an L8-70M

Beckman ultracentrifuge (Beckman Instruments, Palo Alto, CA). The supernatant was discarded while the pellet was resuspended in fresh medium and filtered through a 0.8/0.2 μm Supor membrane syringe filter (Pall Corporation, Ann Arbor, MI). Each baculovirus vector solution added to shake flasks were topped to 3 mL with fresh medium. Cells were always cultured in larger volumes to approximately 2.0 million cells/mL before being aliquoted into 125 mL shake flasks.

4.1.1 Design of experiments

A mirror full factorial experiment (3x2) was designed to investigate the effect of using the 2 monocistronic baculoviruses to supply the replication protein transgenes instead of supplying them on a single baculovirus (mirror to the (3x2) experiments described by Aucoin et al. (2006)). Both BacRep vectors and BacCap were used at an MOI of 10 each. The other factors investigated simultaneously were the MOIs of Bac-ITRtransgene (3 and 10) and the transgene (gene for GFP, gene for β-galactosidase, and the gene for SEAP).

A second set of cultures were conducted to test if the overall burden of an additional 10 vectors per cell affected the production of AAV. In this set of cultures the MOI of both BacRep52 and BacRep78 vectors were reduced by half (MOI=5 each) so that they would be equivalent to the optimal MOI of BacRep previously reported (Aucoin et al., 2006). For all cultures in this second set, BacCap was used at an MOI of 10 and BacITRGFP at an MOI of 3.

A third set of cultures aiming at modifying the amount of replication proteins made in the cell utilized a combination of the bicistronic BacRep vector and either BacRep52 or BacRep78.

All cultures in the three sets of experiments were done at least in duplicate. As nega-

tive controls, cells were infected with one of the two monocistronic vectors (MOI=10), BacCap (MOI=10) and BacITRGFP (MOI=3).

4.1.2 Characterization assays

The characterization assays in this study were described previously (Aucoin et al., 2006) and were used without any modification.

4.2 Results

Infection of cells with a bicistronic BacRep vector (MOI=10), BacCap (MOI=10) and BacITRGFP (MOI=3) was considered to be the positive control and was used to compare to the negative controls described in the materials and methods. As reported previously, functional AAV titers are not correlated to the viability of the cells, which were similar for all three controls, albeit marginally higher for those containing the monocistronic vectors (Figure 4.1). The differences, may be in part attributed to the smaller quantities of AAV particles (VP) produced for these systems.

As can be seen in Figure 4.1, when using only BacRep52 as the supplier of AAV Rep genes to the system, no functional AAV was detected, Rep52 was heavily expressed and the expression of viral particles was suppressed. When only using BacRep78, Rep78 is readily detected (data not shown) as well as the expression of Rep52. The quantitiy of Rep52 detected, was surprisingly similar if not greater than what was detected in the positive control. Since both replication proteins were present it is not surprising that functional AAV was also detected; however, only a fraction of what was obtained for the positive control was produced (~1%). It is not clear why, if the replication proteins were supplied in great enough quantity, the production of

functional AAV vectors was so poor; however, there was a significant reduction in the number of AAV particles (VP) detected.

4.2.1 Effect of substituting BacRep (MOI=10) with BacRep52 (MOI=10) and BacRep78 (MOI=10)

The effect of substituting the bicistronic Rep vector with two monocistronic vectors was investigated within a factorial design of experiment that also investigated the multiplicity of infection of the BacITR. Each condition was investigated for the production of three different AAV-2 vectors: AAVGFP, AAVLacZ and AAVSEAP.

As a first indication of culture performance, the cell viability at harvest (72hpi) were compared (Figure 4.2). Only for the production of AAVLacz was there a demonstrable difference between the use of the monocistronic and bicistronic vectors, with the use of the monocistronic vector having lower cell viabilities at harvest. Most cultures had viabilities between 50 and 60%, however those cultures producing AAVSEAP resulted in a wider range of cell viabilities, with some cultures being harvested with viabilites between 20 and 30%. The viabilities of cells producing AAVSEAP, however, reacted the same way whether the bicistronic BacRep vector or the two monocistronic vectors were used.

As can be seen in Figure 4.3, the levels of AAV vector based on their transduction potential, differed greatly depending on the vector transgene, regardless of the use of the monocistronic or bicistronic BacRep vectors. These differences are attributed to the ability to adequately detect the reporter gene, therefore only vectors with the same transgene can be adequately compared with one another. Within each set of vectors, the production levels were comparable (similar orders of magnitude). Overall, certain trends appeared with regard to functional titers. For example, lowering the BacITRGFP MOI for both the monocistronic and bicistronic systems,

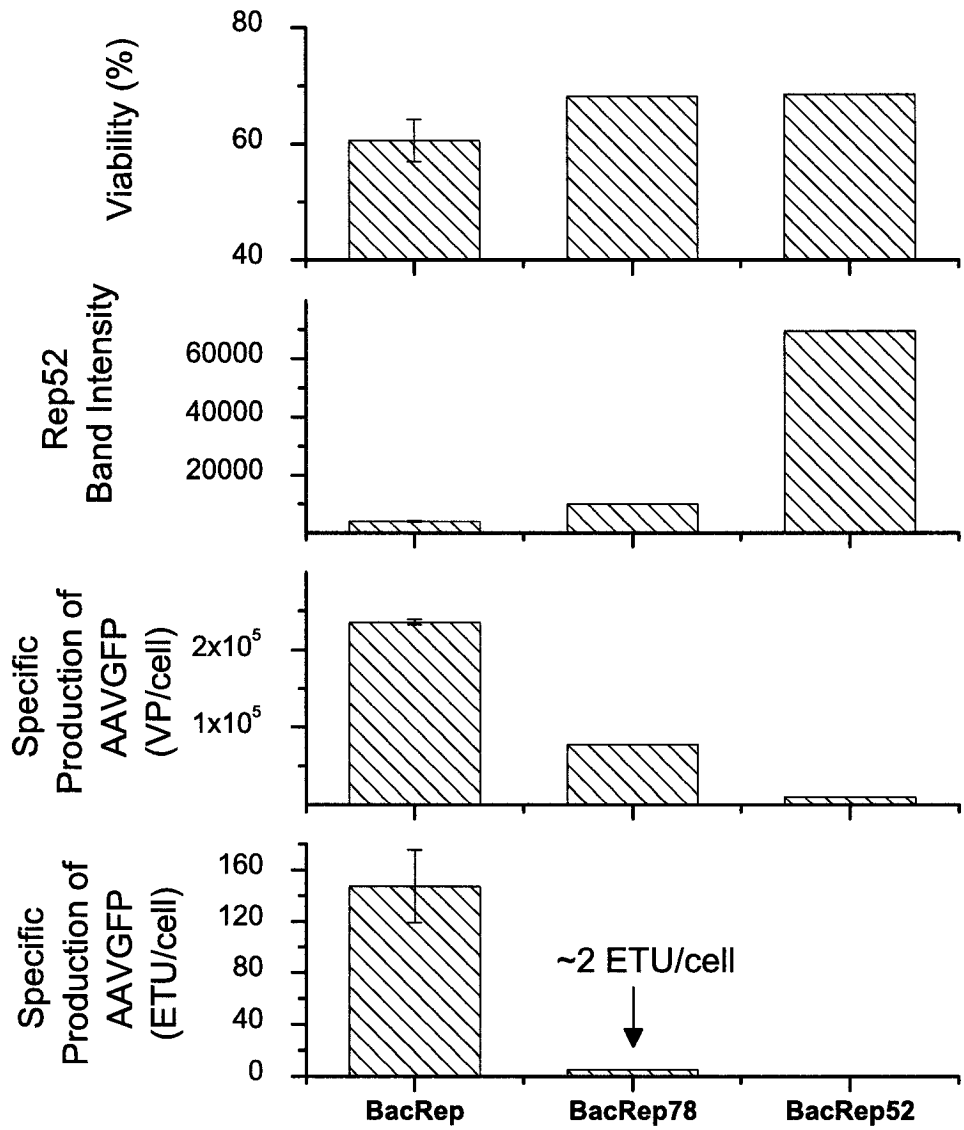

Figure 4.1 Analysis of positive and negative controls for the production of AAV with monocistronic BacRep vectors at 72 hpi. BacRep (bicistronic BacRep vector, MOI=10), BacRep78 (monocistronic BacRep vector containing the gene for AAV Rep78, MOI=10), BacRep52 (monocistronic BacRep vector containing the gene for AAV Rep52, MOI=10). All cultures co-infected with BacCap (MOI=10) and Bac-ITRGFP (MOI=3).

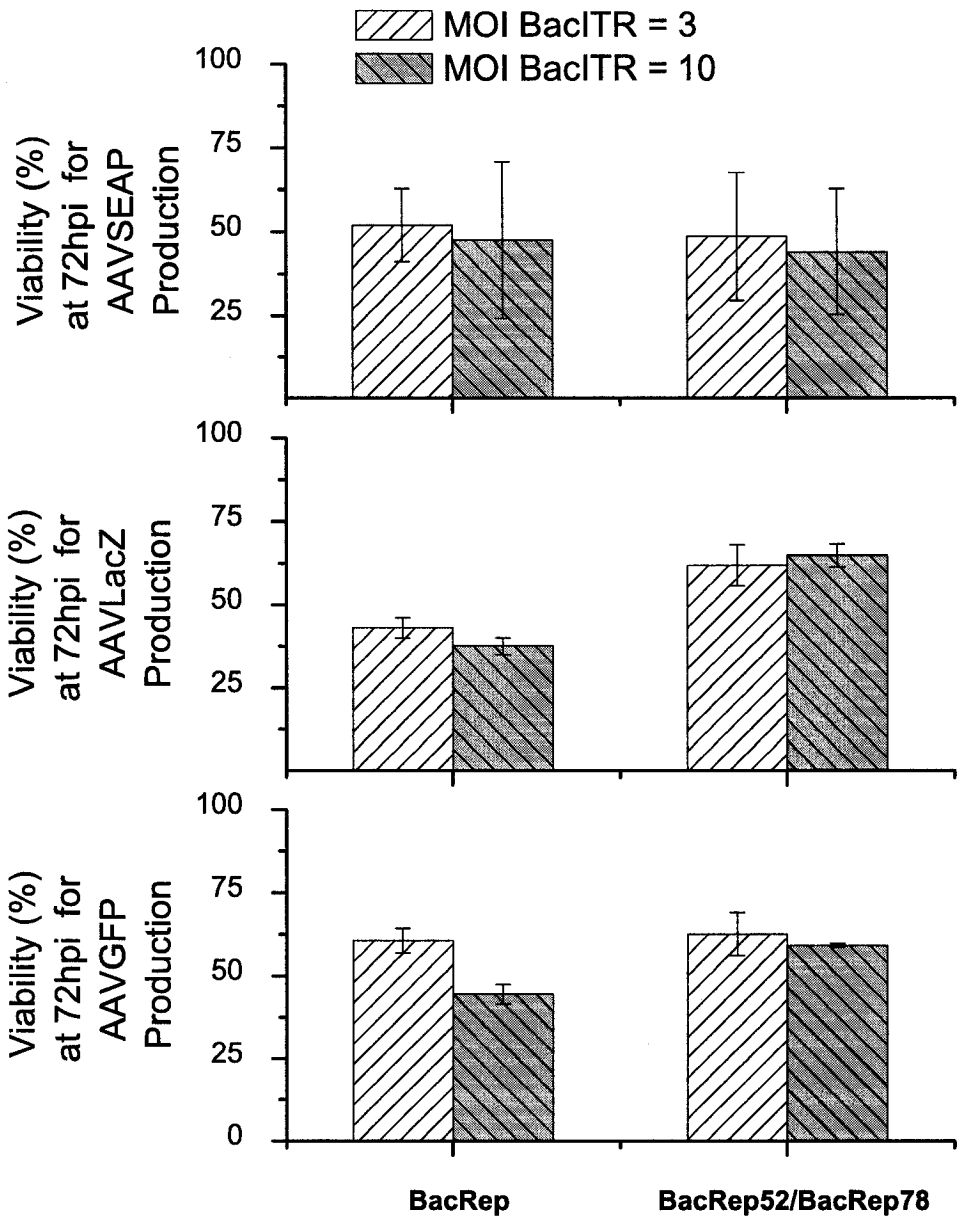

Figure 4.2 Viability comparison for AAV vectors produced at 27°C using triple infection or quadruple infection. MOI of BacCap for all experiments was 10. MOI of BacRep was 10. MOI of BacRep52 and BacRep 78 were both 10. The above analysis was done for a harvest at 72 hpi. Error bars represent the range of responses from duplicate cultures.

resulted in slightly higher titers, while the opposite occurred for BacITRLacZ and BacITRSEAP. Earlier, it was suggested that this may be due to the fact that with the BacITRGFP construct, GFP is under the p10 promoter, which allows GFP production in the insect cells, unlike the two other constructs whereby the AAV transgene is not expressed in insect cells. It is believed that the production of GFP reduced the expression of other products under the control of very late promoters (Aucoin et al., 2006). Regardless of the use of the two monocistronic vectors or the single bicistronic vector, this explanation remains possible.

Differences in functional AAV titers between systems using monocistronic and bicistronic vectors were most noticeable in the production of AAVGFP and AAVLacZ, with the bicistronic system producing ~3-4 times more functional vector. In fact, when looking at the production of AAV capsids (Figure 4.4), a similar observation can be made. Cultures producing AAVGFP and AAVLacZ resulted in discernable differences in the amount of AAV particles produced, with the bicistronic system producing ~5-6 times more capsids than the monocistronic system. Interestingly, the lower levels of functional vector were consistent with lower levels of AAV capsid production (Figure 4.4) and higher levels of Rep52 production (Figure 4.5) for these productions.

AAVSEAP production was the most variable, with the widest range of values for enhanced transduction titers, capsid titers and Rep52 protein levels. The reason for the variability among duplicate cultures of AAVSEAP seems to be linked to the BacITRSEAP, which is the only aspect that differs from the rest of the experiments. Beyond this link, the reason for this variability remains unknown.

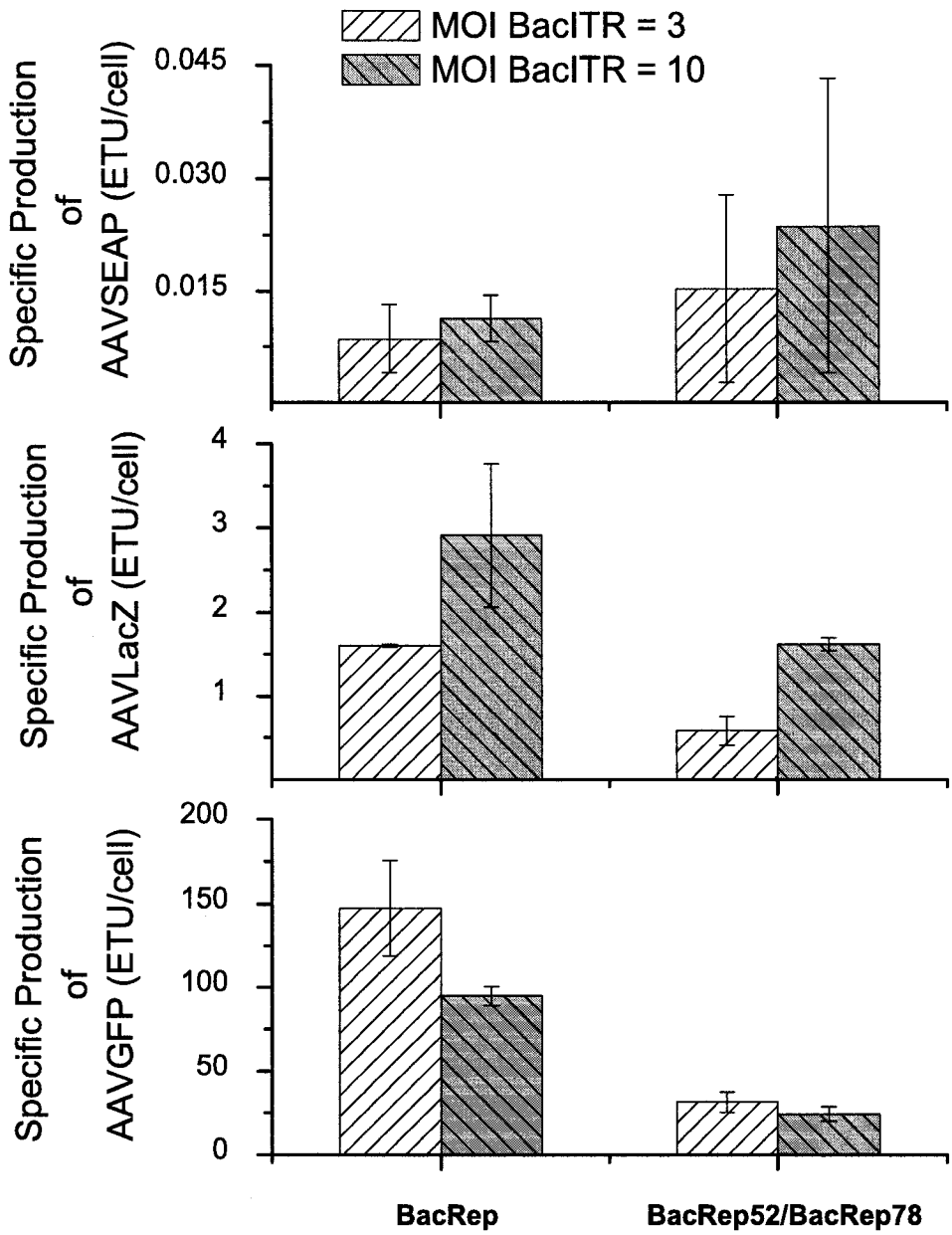

Figure 4.3 Viability comparison of AAV vectors produced at 27°C using triple infection or quadruple infection. MOI of BacCap for all experiments was 10. MOI of BacRep was 10. MOI of BacRep52 and BacRep78 were both 10. The above analysis was done for a harvest at 72 hpi. Error bars represent the range of responses from duplicate cultures.

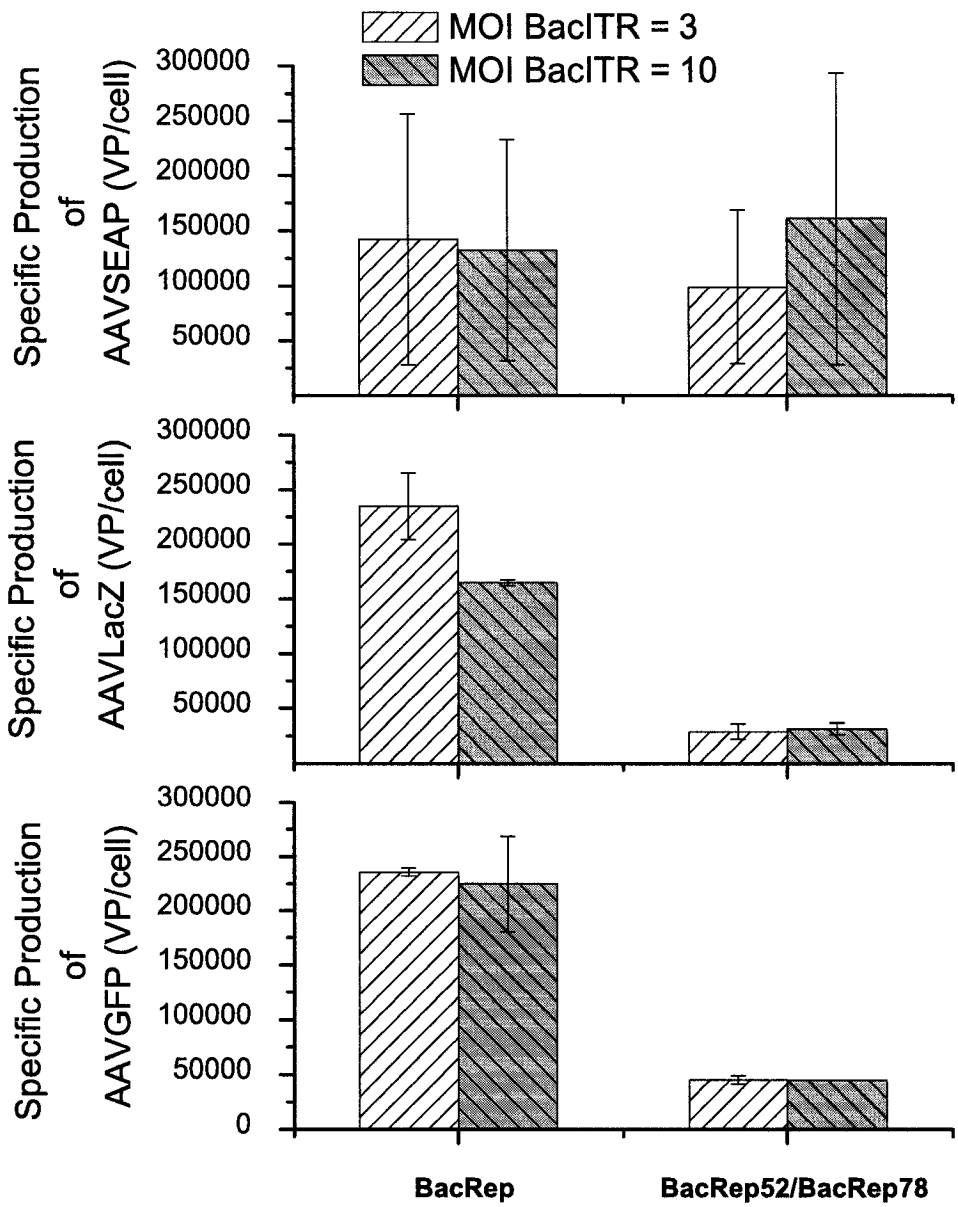

Figure 4.4 AAV viral particle titer comparison of AAV vectors produced at 27°C using triple infection or quadruple infection. MOI of BacCap for all experiments was 10. MOI of BacRep was 10. MOI of BacRep52 and BacRep78 were both 10. The above analysis was done for a harvest at 72 hpi. Error bars represent the range of responses from duplicate cultures.

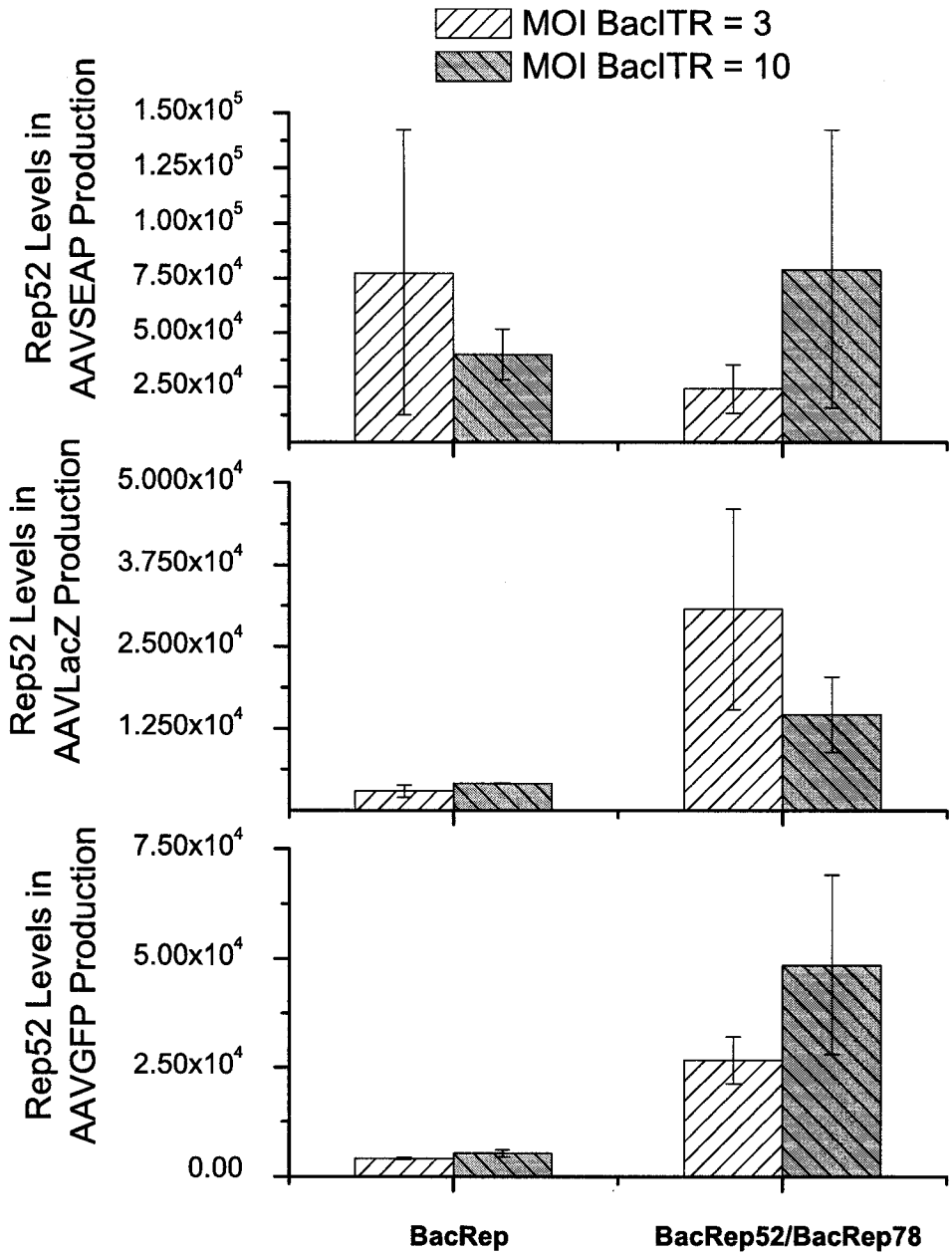

Figure 4.5 Rep52 production level comparison for AAV vectors produced at 27°C using triple infection or quadruple infection. MOI of BacCap for all experiments was 10. MOI of BacRep was 10. MOI of BacRep52 and BacRep78 were both 10. The above analysis was done for a harvest at 72 hpi. Error bars represent the range of responses from duplicate cultures.

4.2.2 Effect of partially or completely substituting BacRep (MOI=10) with BacRep52 (MOI=5) and BacRep78 (MOI=5)

To probe whether the overall baculovirus load or too much replication protein were the cause of lower functional AAV titers, the two monocistronic BacReps were supplied at lower MOIs and or used in combination with the bicistronic BacRep. Comparing these new cultures to a positive control (BacRep, MOI=10, BacCap, MOI=10, BacITRGFP, MOI=3), it was seen that a marginal improvement was obtained when lowering the MOIs from 10 to 5, however still only ~40% of the functional AAV titer that was achieved using the bicistronic vector (Figure 4.6).

Combining one of the monocistronic BacRep vectors (BacRep52 or BacRep78) with the bicistronic BacRep vector (each at MOIs of 5), both resulted in approximately 75% of the functional AAV titer achieveved using only the bicistronic vector at an MOI of 10. Furthermore the yield of the cultures containing the monocistronic vectors did not increase as much as the control culture over time, as seen by a reduction in the relative enhanced transduction titers beyond 72 hpi (Figure 4.6).

4.2.3 GFP monitoring

Although, GFP is not directly linked to the production of functional AAV, it is still monitored for additional insight on the process. In Figure 4.7, it can be seen that at 24 hpi, the number of cells that have detectable levels of GFP are lower for the culture with the two monocistronic BacReps as compared with the three other cultures. The main difference between this culture and the rest is the overall baculovirus load (10 additional baculoviruses per cell). It has not been ascertained whether the difference is caused by the overall load on cells or due to a different distribution of the viruses over the cell population. Given that the error bars of duplicate cultures are so small,

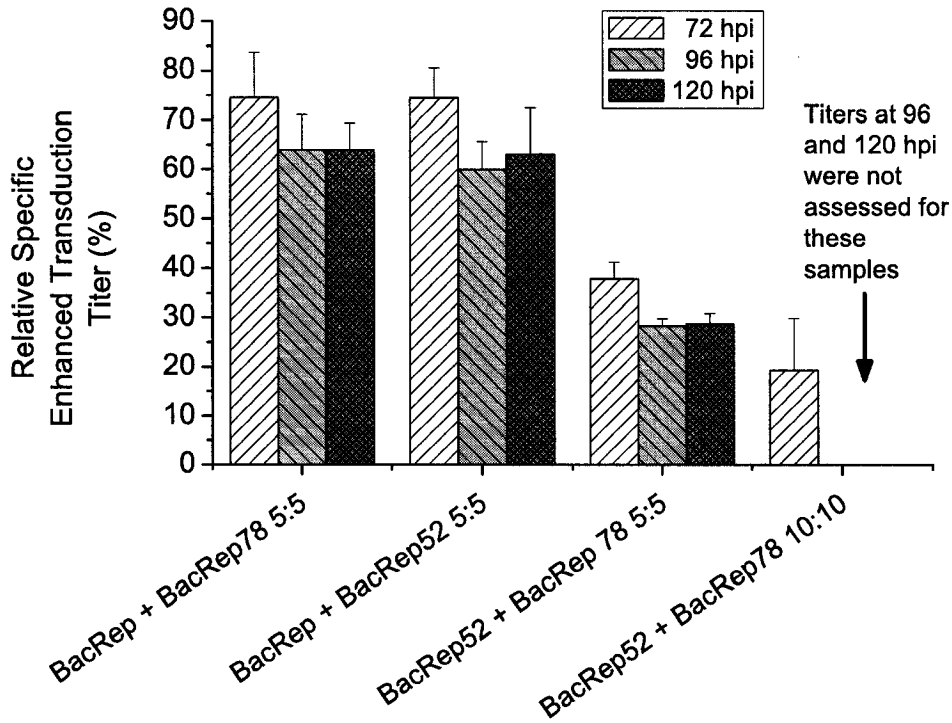

Figure 4.6 Enhanced transduction titers at various times post infection for AAVGFP production using either the bicistronic BacRep vector at an MOI of 10; both monocistronic BacRep vectors (BacRep52 and BacRep78) at an MOI of 5 each; or using a combination of the bicistronic BacRep and one of the two monocistronic BacRep vectors, each at an MOI of 5. BacCap and BacITRGFP were supplied at MOIs of 10 and 3, respectively. Error bars represent the range of responses from duplicate cultures.

the varying distributions are not likely to be the cause.

The mean fluorescence profiles are comparable for both the dual monocistronic and bicistronic systems, albeit the dual monocistronic system is somewhat lower beyond 24 hpi. The lower mean fluorescence is consistent with the elevated levels of replication protein (Figure 4.5) and the diminished levels of capsids (Figure 4.4) previously seen. The negative controls, however, give completely different profiles then the complete systems. Cultures having only BacRep52 (and BacCap and BacITRGFP), had very low mean intensities even though in terms of the number of cells expressing GFP are very similar to the other 3-vector systems. Cultures having only BacRep78 (and BacCap and BacITRGFP), had very high mean intensities and the total number of cells with detectable levels of GFP were greater than all other systems.

When BacRep52 and BacRep78 were added together, but at reduced MOIs, the number of cells expressing GFP at 24 hpi, was similar to the control culture using the bicistronic vector (Figure 4.8). Beyond 24 hpi, however, those cultures infected with BacRep52 had lower amounts of detectable GFP. As seen in Figure 4.7, cultures infected with only BacRep78 or in combination with the bicistronic vector (Figure 4.8), always gave the highest number of cells with detectable GFP and also the highest levels of GFP.

4.3 Discussion

There is strong evidence to support that there is an inherent instability with the original set of baculovectors (Kohlbrenner et al., 2005a; Aucoin et al., 2006). While most would like to avoid co-infecting with multiple monocistronic vectors as evidenced by recent reports on the production of rotavirus VLPs (Vieira et al., 2005; Roldao et al., 2006), separating the two AAV rep genes into two seperate baculoviruses, may be

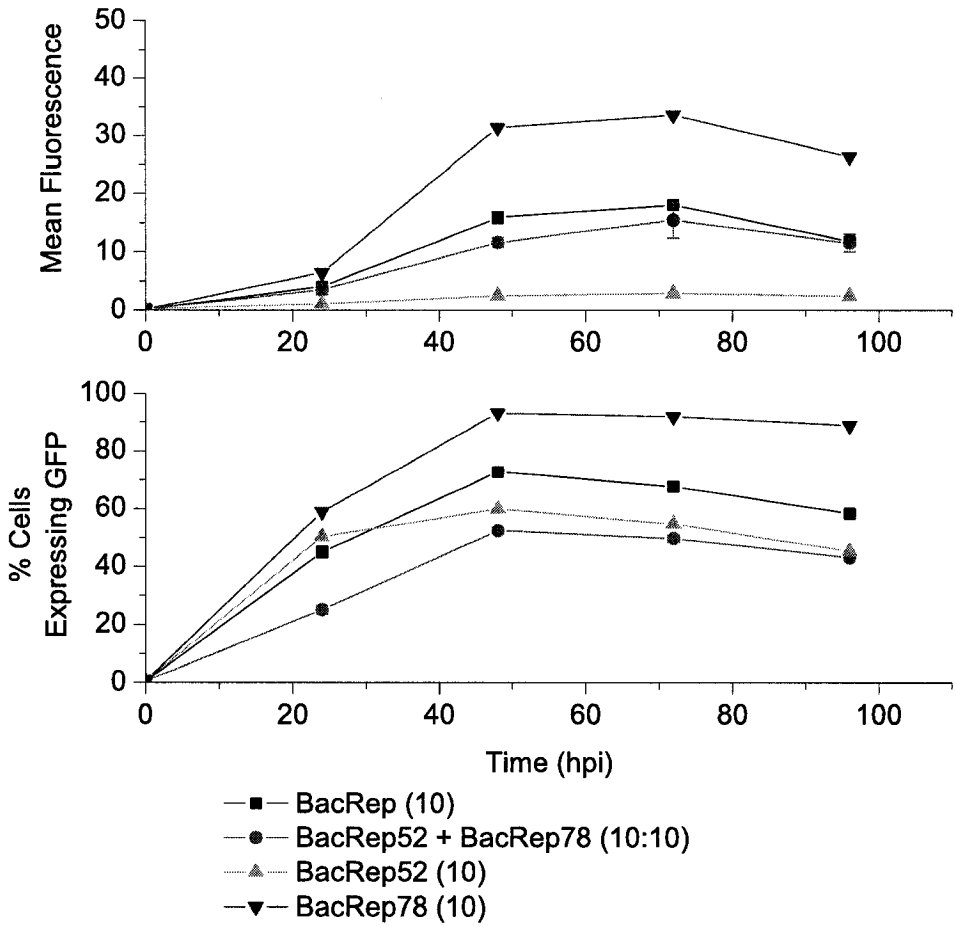

Figure 4.7 Dynamics of GFP expression in insect cells using either the bicistronic BacRep vector at an MOI of 10; both monocistronic BacRep vectors (BacRep52 and BacRep78) at an MOI of 10 each; or using only of the two monocistronic BacRep vectors at an MOI of 10. BacCap and BacITRGFP were supplied at MOIs of 10 and 3, respectively. Error bars represent the range of responses from duplicate cultures.

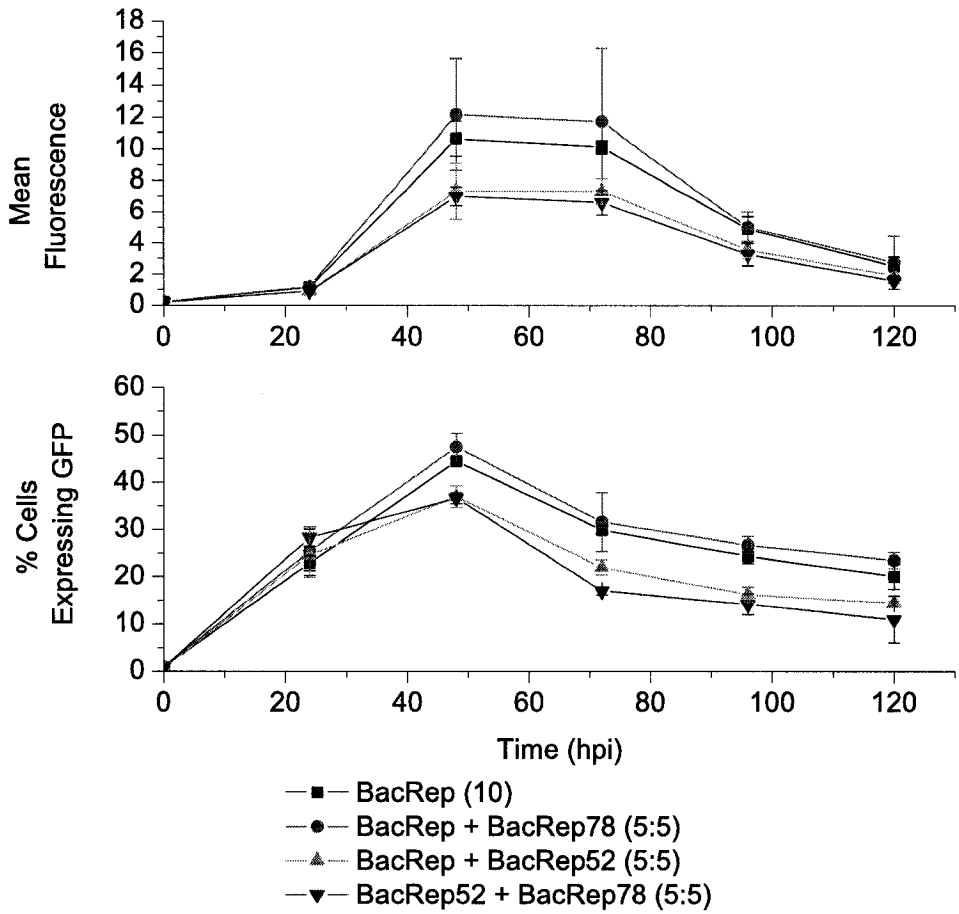

Figure 4.8 Dynamics of GFP expression in insect cells using either the bicistronic BacRep vector at an MOI of 10; both monocistronic BacRep vectors (BacRep52 and BacRep78) at an MOI of 5 each; or using a combination of the bicistronic BacRep and one of the two monocistronic BacRep vectors, each at an MOI of 5. BacCap and BacITRGFP were supplied at MOIs of 10 and 3, respectively. Error bars represent the range of responses from duplicate cultures.

the simplest way to guarantee the stability of the viral stocks (Kohlbrenner et al., 2005a). This does not, however, guarantee that the overall yield will be increased. One of the first questions that arose with substituting BacRep with two baculovectors, was the question of how much of the two new vectors should be used. It is understood that adding a fourth vector doubles the number of potential outcomes for a cell (2^3 vs 2^4) as described in Chapter 3, therefore the choice is not trivial. In the previous chapter on the effect of varying the multiplicity of infection, those vectors that were coding for AAV proteins needed to be added in similar quantities. It was suggested that the reason for this may be that offsetting the initial amount of virus would lead to a greater discrepency in the amount of vector that can be transcribed in the very late phase of infection. Therefore, the initial approach was to try and balance baculovirus vectors containing the Rep and Cap genes by setting them equal to one another.

In this short study, it could be seen that the overall yield of functional AAV vectors was compromised by the use of the two monocistronic BacRep vectors, BacRep52 and BacRep78. The cause for this decrease in functional titer is not clearly evident. The main differences between the two types of cultures are the high expression of Rep52 and the reduced quantity of total viral particles produced. Although it is possible that the overexpression of AAV Rep78 has a similar effect in insect cells as reported in mammalian cells, whereby overexpression of this protein causes a decrease in capsid production (Li et al., 1997a), the reduction of capsid production is even more pronounced in the negative control when only BacRep52 is used (and no Rep78 protein expression was detected). As was reported previously (Aucoin et al., 2006), the quantity of baculoviruses need to be carefully balanced or there is a shift in the predominant expression of one protein. The same balance that was needed, as proposed ealier (Aucoin et al., 2006), was not achieved. Although, the results may be due to discrepencies in the original baculovirus determination count, the values

obtained were consistent with other material tested, therefore the balance has to be made with the strength of the viral promoters.

The data presented in this chapter suggests that the viral polyhedrin promoter driving the Rep52 expression from the BacRep52 construct drowns out the expression of other proteins driven from very late promoters. This is not only seen with the reduction of capsids being expressed but also in the expression of GFP (Figures 4.7 and 4.8). Higher GFP expression levels when only BacRep78 is used is most likely due in part to the absence of competing very late prometers. It is hypothesized that a second reason for the very high expression of GFP seen in Figures 4.7 may be due to AAV genome replication. Rep78, under a truncated $\triangle$IE1 promoter, is presumed to excise and replicate the AAV vector. Because the AAV vector genome also contains the GFP under the p10 promoter, it is possible that GFP expression also ensues from AAV replicate forms.

With the new monocistronic vectors, AAV capsids may be below the threshold number and is the limiting component in the synthesis of functional AAV vectors. This is supported by the lack of any difference between the combinations of the bicistronic and monocistronic BacRep vectors, which suggests enough Rep52 or Rep78 are present and that increasing their expression does not help the synthesis. This notion is also supported by the use of only the BacRep78 vector to deliver Rep genes. In that negative control, Rep52 levels were actually greater than what was observed in the positive control (Figure 4.1) and yet only 1% of the functional AAV titers were produced. The only other difference measured was in the number of capsids produced, which were lower when the monocistronic BacRep78 vector was used. Surprisingly, even though the number of capsids may limit the overall production, similar VP:ETU ratios were achieved for both the dual monocistronic and bicistronic systems, suggesting that the levels of replication proteins are not the only factor affecting the packaging efficiency.

4.4 Concluding Remarks

The quadruple infection system for the production of AAV remains a viable alternative for the production of AAV, with enhanced stability in the baculovirus constructs. However, in terms of larger scale productions, the process would then require the propagation of four viral stocks. To optimize this system, further investigation will be needed to ensure that the expression of capsids is not the limiting factor.

CHAPTER 5

IMPROVING AAV VECTOR YIELD IN INSECT CELLS BY MODULATING THE TEMPERATURE AFTER INFECTION

This chapter presents the journal article "**Improving AAV vector yield in insect cells by modulating the temperature after infection**". This article was published in ***Biotechnology and Bioengineering***.

It is believed that packaging of vector genomes within empty viral particles in the baculovirus/insect cell system is an inefficient process. The study done for this paper tries to address this particular subject. Previous studies on temperature have shown that temperature can have an effect on the production of protein; however, for the number of studies done, there is no real consensus. The majority of studies tend to favor lower temperatures for better protein quality. This is the first time that temperature has been used to affect the production of a viral vector in insect cells. It is also the first time that temperature has been used to modulate recombinant protein synthesis under the control of an immediate early promoter (Rep78 in this case) and, to the authors knowledge, the first time that the time at which the temperature switch occurs has been considered as a factor. This study not only addresses a concern with the production of AAV vectors in insect cells but it also adds to the understanding of the formation of AAV vectors in insect cells. Increased levels of replication protein, which have been reported to inhibit vector production in mammalian cells are concomitant to higher vector yields in insect cells, highlighting a difference between the two systems. It should be noted that in the following manuscript, what has been previously defined as enhanced transduction units (ETU) in Chapter 1, is referred to as infectious viral particles.

Improving AAV vector yield in insect cells by modulating the temperature after infection

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5.1 Abstract

Vectors based on adeno-associated viruses (AAV) are sought for therapeutic gene delivery because of their ability to transduce a variety of tissues with no significant immunological response. Production using the baculovirus expression vector (BEV)/insect cell system has the potential to meet the needs for pre-clinical and clinical trials. In this co-infection system, three baculoviruses are used to produce the AAV vector. A strategy aimed at increasing encapsidation/maturation of the viral vector involved varying the temperature over the course of the process. Cultures were subjected to temperature changes at various times pre- and post-infection (up to 24 hours post-infection). It was found that raising the culture temperature to 30°C at the time of infection nearly tripled the infectious titer. In fact, increasing the temperature to 30°C at any time in the process investigated resulted in an increase in titer. Also, raising the culture to 33°C or lowering the temperature to 24° or 21°C resulted in lower titers. The rise in infectious titer was also confirmed by an increase in DNase resistant particles (DRPs). Varying the temperature, however, did not affect the total amount of capsids significantly. Therefore increasing the culture temperature resulted in better encapsidation as determined by the ratio of capsids to DRPs to infectious particles. It is believed that an increase in early proteins and

possibly a quicker cascade of baculovirus infection events resulted in this increased packaging efficiency.

Keywords: insect cells, baculovirus, adeno-associated virus, temperature

5.2 Introduction

Adeno-associated virus (AAV) is currently one of the most promising candidates as a vector for gene therapy. It is considered non-pathogenic in humans and is able to provide long-term gene expression without inducing a significant immune response. It is currently being investigated as a potential HIV vaccine, and a treatment for cystic fibrosis, muscular dystrophy and hemophilia B. AAV vectors are also currently in Phase 3 clinical trials for the treatment of prostate cancer. Although AAV results are very promising at bench-scale, the limited number of human clinical trials is due to the challenges associated with large-scale production. Currently AAV vectors are produced predominantly through transfection of AAV genes into mammalian cells or through the use of mammalian cells that stably express AAV gene products. Both of these methods have major drawbacks. Most transfection protocols result in an inefficient delivery of the genes to the cell and often use adherent cell systems, which result in poor volumetric yields. Although stable cell lines are amenable to production in suspension, which allows for an increase in volumetric yield, they are difficult to obtain. An alternative bench scale method, developed by Urabe et al. (2002), uses insect cells to produce the desired AAV vector.

Production of AAV vectors in insect cells results in a significant difference between the number of total particles detected and the number of infectious particles, on the order of 10000 (Aucoin et al., 2006; Chahal et al., 2007). This discrepancy has not been reported in competing systems, such as the production of AAV in mammalian

cells; however, the number of particles detected through ELISA at the production stage, i.e. not purified by ultracentrifugation, has not been extensively studied by others. Nonetheless, it was thought that the rate of capsid production, which has been shown to affect cell viability (Aucoin et al., 2006), negatively impacted the packaging of AAV genomes in the insect cell system. Since temperature is known to affect rates of reaction, it was hypothesized that changing the culture temperature could affect the production of AAV vectors in a positive manner.

The effect of temperature on insect cells has been extensively studied (Andersen et al., 1996; Cain et al., 1999; Donaldson et al., 1999; Gotoh et al., 2004; Hara et al., 1993; Lynn, 2002; Reuveny et al., 1993; Shao-Hua et al., 1998; Zhang et al., 1994). Generally, insect cells are cultured at either 27°C or 28°C, but have been reported to grow best at temperatures anywhere between 27 and 30°C (Shuler et al., 1991). In fact, production of heat shock proteins in Sf9 cells as a response to elevated temperatures has not been observed below 37°C (Huhtala et al., 2005). In some instances, lowering temperature to 24°C or even 20°C has been shown to yield better protein processing conditions in insect cells (BTI Tn5B1-4 and Sf9), without being detrimental to the amount of product produced (Donaldson et al. (1999), Cain et al. (1999), respectively); however other reports, specifically on the production of β-galactosidase, have yielded conflicting results. Reuveny et al. (1993) reported optimal temperatures between 25 and 27°C, while Hara et al. (1993) reported a gain factor of 1.7 in β-galactosidase activity when produced at 30°C compared to production at 27°C. In TN-368 cells, greater quantities of polyhedra were produced at 25°C and 32°C compared to cultures at 28°C (Hink and Strauss, 1976). Temperature cycling between temperatures below 28°C and 28°C every 12 hours has led to higher post-infection viabilities but has not been studied in terms of recombinant protein production; higher viabilities of the infected insect cells resulted in larger quantities of baculovirus being produced (Shao-Hua et al., 1998). The effect of temperature

on oxygen consumption in the BEV/insect cell system has also been studied (Gotoh et al., 2004). They showed that although lower maximum cell densities and lower growth rates were recorded at 20°C compared to 28°C, equivalent extracellular concentrations of their product, GFPuv, were obtained. For the production of virus-like particles (VLPs), it has been reported that the effect of temperature can be misinterpreted in spinner flasks cultures; although cell growth is slower at 25°C, an increase in the production of VLP proteins has been observed compared to cultures done at 27°C or 29°C (Tsao et al., 1996). This decrease in production at higher temperatures, however, has been linked to oxygen depletion and lactate accumulation in the medium due to high rates of cell growth in a spinner flask. Maintaining a proper dissolved oxygen concentration during the culture is essential and, in the aforementioned study, was easily addressed in a bioreactor therefore removing any effect of temperature (Tsao et al., 1996).

Donaldson et al. (1999) hypothesized that by lowering the temperature during infection, the desired recombinant protein being produced could spend more time in the endoplasmic reticulum and Golgi apparatus before being secreted into the medium. Cain et al. (1999) found that the transport and localization of their recombinant glycoprotein differed significantly if cells were cultured at 20°C versus 27°C. At higher temperatures the protein remained in the cytoplasm, while lower temperatures encouraged cell surface localization. Reuveny et al. (1993) observed a similar effect of temperature on product transport; however, unlike Cain et al. (1999), higher temperatures led to higher amounts of products being secreted by the cells.

It is believed, in a simplified manner that the production of AAV, using the three baculovirus system originally developed by Urabe et al. (2002), commences in the early phase of the baculovirus life cycle with the synthesis of Rep78 protein, which is under a truncated immediate early 1 promoter (ΔIE1) of a similar lepidopteran virus. Rep78 is implicated in the nicking and rescue of the AAV viral genome in

mammalian cells (Li et al., 2003; Zhou et al., 1999) and is thought to function similarly in this system. The excision, amplification and accumulation of various AAV DNA replicative forms is thought to occur through the attachment of the Rep78 protein to the AAV inverted terminal repeats, which are within a recombinant baculovirus containing the vector genome (BacITR). When the very late phase of the baculovirus life cycle is initiated, Rep52, and the capsid proteins VP1, VP2, VP3, which are under the control of the polyhedrin promoter (polh), are produced. VP1, VP2 and VP3 self-assemble spontaneously to form empty capsids (Urabe et al., 2002), and are presumed to be devoid of DNA. Rep52 allows the accumulation of single stranded progeny DNA and are implicated in the packaging of the genome into the capsid (Timpe et al., 2005; Zhou and Muzyczka, 1998). Also in the very late phase, GFP, which is under the control of a p10 promoter, is produced. The assembly of AAV vectors cannot commence until the very late phase is initiated since significant accumulation of single stranded progeny AAV DNA is assumed to start only in the presence of Rep52, which like the capsids are expressed in the very late phase of infection. GFP is, therefore, a good indicator of when AAV assembly could commence given that all the components are present.

In order not to affect the cells prematurely, an appropriate "switching time" was deemed as important as the temperature itself. Once a baculovirus has entered the cell, the nucleocapsid must travel to the nucleus before the infection cycle is initiated. The baculovirus infection cycle consists of different phases whereby each subsequent phase depends on the products of the former in a sort of cascade control mechanism (Friesen, 1997). The infection cycle can be divided into three phases: early (0 to 6-9 hours post-infection), late (6-12 hours post-infection) and very late (18 + hours post-infection). It was therefore decided to investigate what effect would be incurred if the temperature were switched before infection (24-36 hours before infection), at infection (0 hpi), towards the end of the early phase (6 hpi), towards the end of the

late phase (12 hpi) and during the very late phase (24 hpi). It was also thought that, by 6 hpi, we would segregate phenomena associated with protein synthesis and not due to the adsorption and viral entry steps.

5.3 Materials and Methods

5.3.1 Cells and medium

Spodoptera frugiperda (Sf9) cells were grown in serum-free SF-900 II (GIBCO BRL, Burlington, ON, Canada) at 27°C for maintenance, plaque assays and AAV production. Cells were routinely maintained in 125 ml plastic shake flasks (Corning Glass Works, Corning, NY, USA) with a working volume of 20 ml and agitated at 110 rpm. Cell densities were assessed using a haemocytometer. Cell viability was determined via the trypan blue exclusion method.

HEK293 EBNA cells (used for testing the infectious nature of the AAV produced) were maintained in HSFM medium (GIBCO BRL, Burlington, ON, Canada) supplemented with 1% bovine calf serum, 10 mM HEPES and 50 g/mL of G-418 at 37°C in a 5% CO_2 atmosphere. Cells were grown in plastic shake flasks. Stock cells were routinely passaged every 3 days and diluted appropriately to maintain the cells in exponential growth (between 0.2 and 1 x 10^6 cells/ml).

5.3.2 Baculovirus viral stocks

BacITRGFP, which has the transgene for green fluorescent protein (GFP) under both the p10 insect and cytomegalovirus (CMV) mammalian promoters between AAV ITRs was kindly provided by Dr. R.M. Kotin from the National Institutes of Health (Bethesda, MD, USA) and has been described previously (Urabe et al.,

2002). Kotin also supplied BacRep and BacCap, which contain the gene sequences for the replication proteins and structural proteins, respectively. Passage 3 virus stocks were concentrated ~10X by ultracentrifugation (145 000 g for 65 min at 4°C). Titers were assessed by plaque assay and were verified by flow cytometry prior to use (Shen et al., 2002). The individual multiplicities of infection of the three baculovirus vectors were chosen based on previous work (Aucoin et al., 2006).

5.3.3 Design of experiments

A 2L flask having a working volume of 500 ml was inoculated with 0.5 million cells/ml and designated the mother culture. Cells were grown at 27°C and agitated at 110 rpm. At 1 million cells/ml, 25 ml aliquots were transferred from the mother culture to 125 ml shake flask and grown at 21°C, 24°C, 30°C, 33°C. When the mother culture reached ~2 million cells/ml, the remaining culture was transferred to 125 ml shake flasks. Cells were subjected to triple infection at ~2 million cells/ml by BacITRGFP, BacRep and BacCap with MOIs of 3, 10 and 10 respectively. Following the addition of virus, the cultures were subsequently incubated at 21°C, 24°C, 27°C, 30°C or 33°C. At 6 hpi, 12 hpi and 24 hpi, selected cultures incubated at 27°C were then incubated at a temperature of 21°C, 24°C, 30°C or 33°C. Samples were collected every 24 hours from seeding to 144 hpi. Experimental conditions were tested at least in duplicate. The experimental conditions are summarized in Table 5.1.

5.3.4 Gene transfer assays for infectious viral particle (IVP) titers

Recombinant AAV-2 production was determined by transducing HEK293 EBNA cells. Sf9 cell culture samples were subjected to three freeze/thaw cycles. Cell lysates were heated at 60°C for 15 min to inactivate the baculovirus. HEK293 EBNA cells

Table 5.1 Summary of experimental conditions studied

	Time of switch (hpi)				
Temperature after Switch	-24	0	6	12	24
21	X	X	X	X	X
24	X	X	X	X	X
27	Control				
30	X	X	X	X	X
33	X	X	X	X	X

were infected with wild type adenovirus (ATCC number VR-1516), at an MOI of 10, prior to aliquoting cells in a multiwell plate. 0.5 x 10^6 HEK293 EBNA cells were plated in each well of a 12-well culture plate and infected with serial dilutions of cell lysate in HSFM medium to get between 2 and 30% of the cells transduced. The plates were incubated at 37°C for 24 h on a circular shaker rotating at 110 rpm. Cells were harvested, resuspended in 1mL of PBS buffer and were fixed with 2% formaldehyde. Cells only infected with adenovirus were used as negative control. For each sample, the percentage of fluorescent cells was determined by flow cytometry using a minimum of 10,000 cells.

5.3.5 GFP expression in insect cells

Cells removed from culture were centrifuged at 300 g for 3 min. The supernatant was removed and the cells were resuspended in 2% para-formaldehyde solution in DPBS. Cells were then analyzed by flow cytometry and analyzed for GFP expression.

5.3.6 Flow cytometry

Analysis of the GFP expression in infected Sf9 and HEK293 EBNA cells was done using a Coulter EPICS^{TM} XL-MCL flow cytometer (Beckman-Coulter, Fl) equipped

with a 15-mW air-cooled argon-ion 488 nm laser. Green fluorescence of the GFP was detected using a 550 nm long-pass dichroic filter and a 525 nm band-pass filter. Flow cytometric data were analyzed using the EXPO32^{TM} software package.

5.3.7 Western blot analysis of viral proteins

Frozen culture samples were thawed, reduced with β-mercaptoethanol and heated to 100°C before being applied to a 4-15% Tris-HCl Bio-Rad Polyacrylamide Ready Gel (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario). After transfer onto a nitrocellulose membrane (Amersham Hybond-ECL, GE Healthcare Bio-Sciences Inc., Baie d'Urf, Qubec) the membranes were probed using Rep or Cap antibodies. The antibody used to detect the Rep proteins is a monoclonal antibody to adeno-associated virus Rep 78, 68, 52 and 40 (cat# MAB689P). The antibody used to detect the Cap (VP) proteins is a monoclonal antibody to adeno-associated virus VP1, 2, and 3 (cat# MAB659P). Both were purchased from Maine Biotechnology Services (Portland, ME, USA). Bands were analyzed using KODAK software (Kodak Digital Science, Eastman Kodak, New Haven, CT).

5.3.8 ELISA for total viral particle (VP) concentration

The total viral vector particles were assessed using cell lysates and a commercially available ELISA kit (cat# PRATV, ARP American Research Products, Inc., Belmont, MA, USA). This kit is based on a sandwich ELISA technique and uses a mouse monoclonal antibody (A20) specific for a conformational epitope on assembled AAV particles.

5.3.9 Dot Blots for DNase resistant particles

DNase resistant AAV particles were assessed based on a method developed by (Wu et al., 2000). Briefly, culture broth was combined with DNase1 (50 U) in an appropriate buffer, mixed and incubated for 1 hr at 37°C. DNase1 was then inactivated by incubating the sample in a 75°C water bath for 20 min. 10% SDS was added to make a final concentration 0.5% and 30 μg of Proteinase K was added. The solution was mixed and incubated for 2 hrs at 37°C. A phenol/chloroform extraction was performed. The mixture was shaken to form an emulsion and then centrifuged at maximum speed (~16000 x g) for 3 min. The aqueous phase was recovered. To this solution, 1/10 volume of 3M sodium acetate (pH 5.2) was added with 1 μL of glycogen and 1 volume of isopropanol. The mixture was mixed and incubated for 1 hr at -20°C. After the incubation period the sample was centrifuged for 30 min at ~6000 x g at room temperature. The supernatant was removed and discarded. The pellet was washed with 70% EtOH before being dried for 10 min at 37°C. The pellet was resuspended in TE buffer (10mM TRIS and 1mM EDTA, pH 8) and stored at -20°C. The resuspended sample was combined with 10N NaOH and 0.5M EDTA before being applied to a nylon membrane (positively charged, Roche) under vacuum. DNA was fixed to the membrane by exposing the membrane to UV light for 5 minutes before being probed.

5.3.10 DIG DNA labeling and detection

DNA was probed using a DIG DNA labeling and detection kit (Roche Diagnostics Canada, Laval, Quebec, Canada). The sequence for the CMV promoter was used to make the DIG-labeled DNA probe.

Table 5.2 Characterstics of non-infected Sf9 cells cultured in SF-900II media used as negative controls

	Growth Rate (h-1)		
Temp	Experimental	Literature (Reuveny et al., 1993)	Maximum Cell Density
21	0.0140+/-0.0005	0.017 (22°C)	$1.07x10^7$ cells/ml (plateau not reached)
24	0.0221+/-0.0028	0.019 (25°C)	$1.48x10^7$ cells/ml
27	0.0271+/-0.0006	0.028	$1.35x10^7$ cells/ml+/- $1.36x10^6$ cells/ml (n=4)
30	0.0388+/-0.0005	0.047	$1.34x10^7$ cells/ml
33	0.0324+/-0.0017	0.034 (35°C)	$1.17x10^7$ cells/ml

5.4 Results

5.4.1 Non-infected control cultures

Cultures that were not infected were switched to different temperatures at densities of around $1x10^6$ cells/ml and their progression was followed. Table 5.2 shows that the temperature did not affect significantly the maximum cell density obtained, however the growth rate was significantly affected as expected. SF-900II supported high cellular densities, which is more or less independent of the temperature of incubation, when the temperature was changed within the same passage.

5.4.2 Harvest and analysis at 72 hpi

Samples harvested at 72 hpi, which has previously been shown to be an optimal harvest time for AAV vectors in insect cells (Aucoin et al., 2006; Meghrous et al., 2005), were analyzed for infectious vector particles, DNase resistant particles and total particles (capsids). The infectious AAV vector particles were quantified from the culture broth and the results are shown in Figure 5.1. Generally, when the temperature of the culture was lowered, greater quantities of infectious AAV particles were observed for cultures maintained at 27°C longer. All cultures subjected to a

temperature lower than 27°C gave titers lower than what were obtained when the culture was maintained at 27°C. When the temperature of the culture was raised to 30°C, the best results occurred when the culture was maintained at 30°C the longest. All cultures subjected to a temperature of 30°C gave titers higher than what were obtained when the culture was maintained at 27°C. For cultures switched to a temperature of 33°C, no matter the timing of the switch, none produced as much infectious AAV as the control culture kept at 27°C. Unlike cultures switched to temperatures below 27°C, the impact on the infectious AAV titer was smaller when the culture was switched to 33°C closer to the time of infection.

Since those cultures, which were incubated at some point at 21°C or 33°C, did not show any benefit to the production of infectious AAV, analysis of the capsids was limited to cultures incubated at temperatures between 24°C and 30°C. Unlike the number of infectious particles, the number of capsids produced did not differ significantly as seen in Figure 5.2. In fact, the largest capsid production at 72 hpi generally occurred when cultures were maintained at 27°C.

Samples switched to different temperatures at the time of infection (0 hpi) were further analyzed for DNase resistant particles. Figure 5.3 shows that the number of DNase resistant particles was also increased (>1.5X) when the temperature at infection was increased. The relationship between the number of infectious AAV particles (IVP), the number of DNase resistant particles (DRP) and capsids (VP) is summarized in Table 5.3. At 27°C, the average ratio of DRP/IVP was 103 with a standard deviation of 26 (n=4). The differences observed in the ratios of DRP/IVP for the cultures switched to 24°C or 30°C (Table 5.3) are not statistically significant. The average ratio of VP/IVP at harvest for those cultures kept at 27°C was 11026 with a standard deviation of 1336 (n=4). When comparing this ratio to those obtained from the cultures switched to 24°C or 30°C (Table 5.3), it can be seen that the values significantly decrease when switched to 30°C.

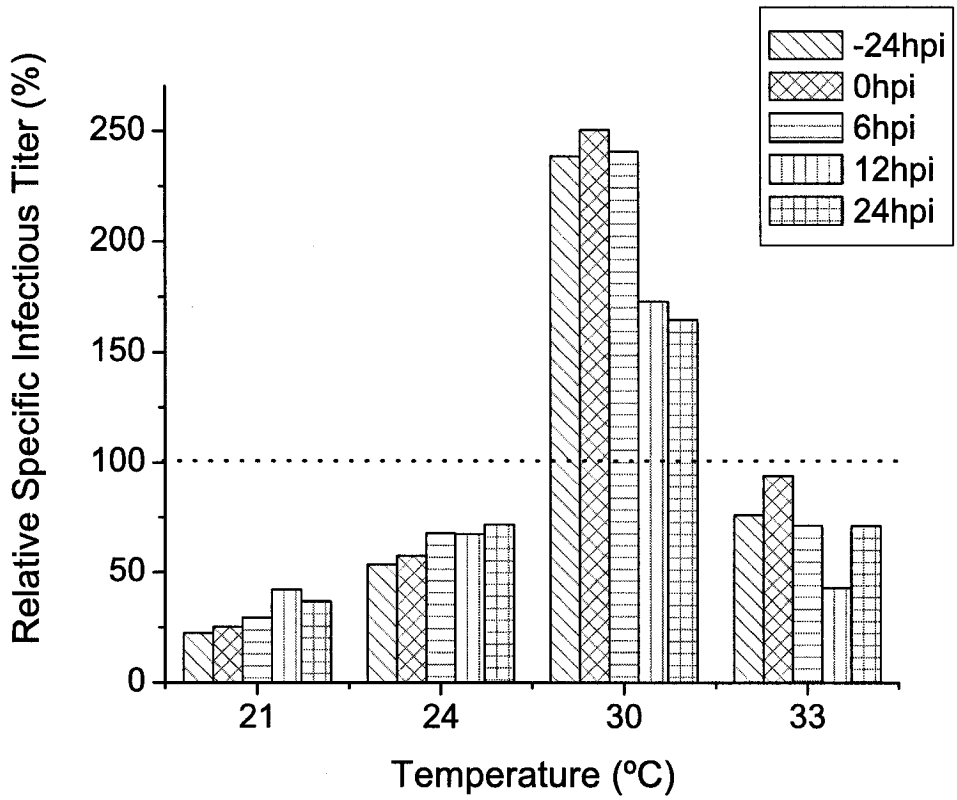

Figure 5.1 Average specific infectious AAV titer at 72 hpi of cultures switched to different temperatures at different times post-infection relative to cultures maintained at 27°C.

Table 5.3 Relationship between infectious AAV particles, DNase-resistant AAV particles and AAV total particles harvested at 72 hpi for cultures either kept at 27°C or switched to a different temperature at t=0 hpi. Ratios obtained for the cultures kept at 27°C are of 4 independent cultures while ratios obtained for cultures switched to 24°C or 30°C are of duplicate cultures. The average %CV is an average coefficient of variation for each type of measure.

Temp Post-Infection	IVP	DRP	VP
Average %CV	21	7	25
24	1	90	17289
27	1	103	11026
30	1	79	4612

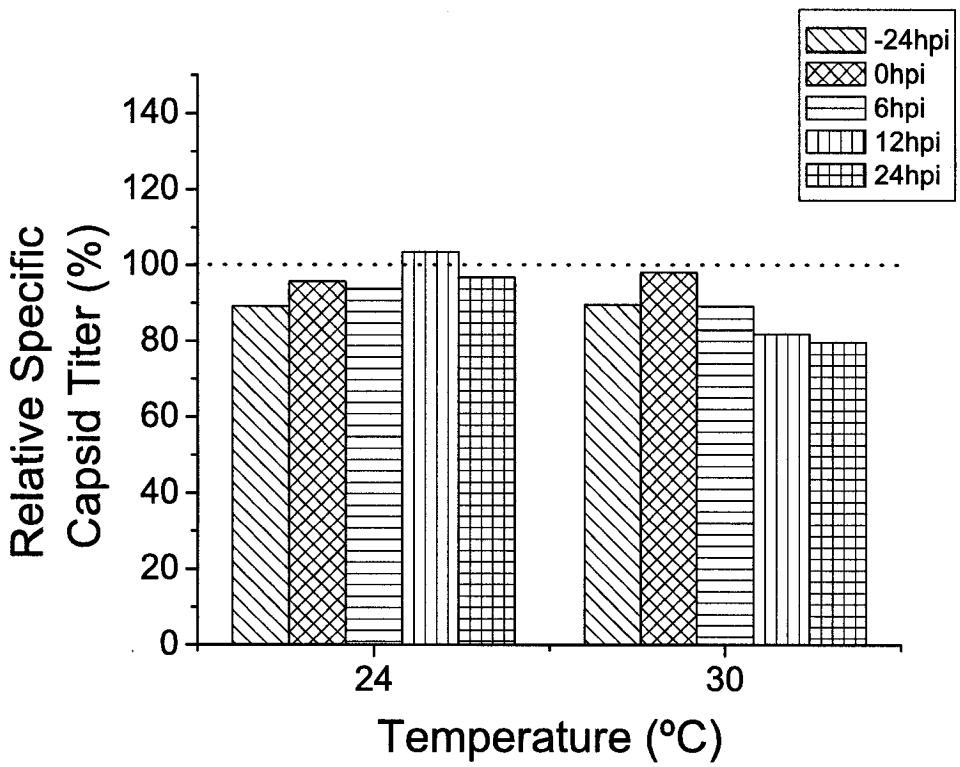

Figure 5.2 Average specific AAV capsid titer at 72 hpi of cultures switched to different temperatures at different times post-infection relative to cultures maintained at 27°C.

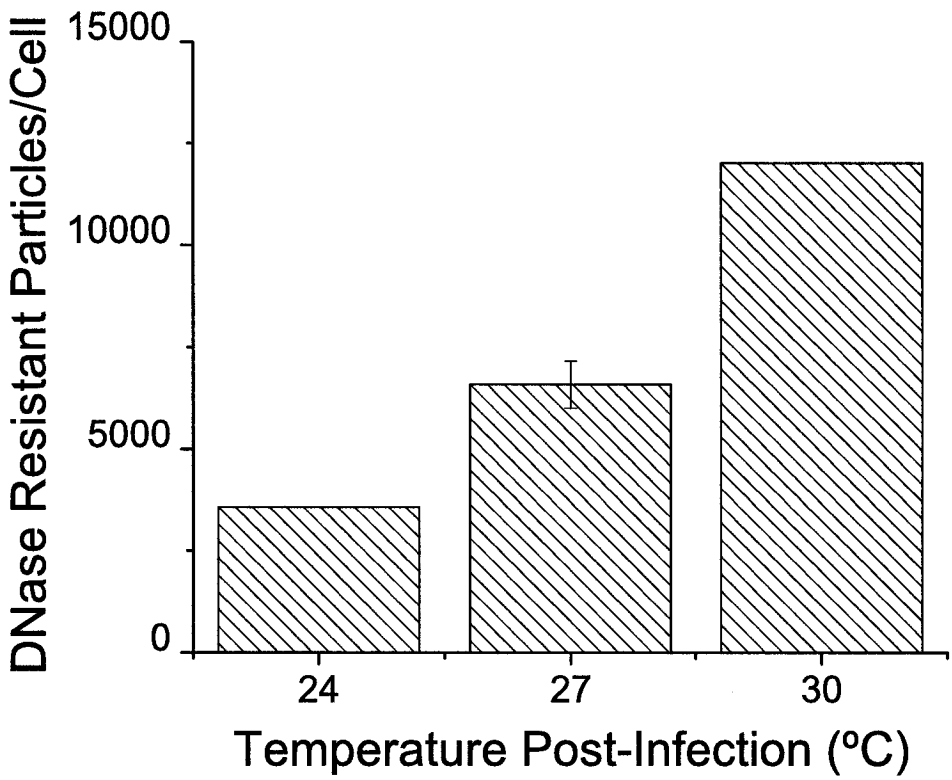

Figure 5.3 Average cell specific DNase Resistant Particle concentration for cultures switched to different temperatures at t=0hpi.

5.4.3 Analysis of system dynamics

The earliest indicator that differentiated the cultures was the dynamics associated with the viability of the cells. The earlier the temperature was reduced to 24°C or 21°C in the life of the culture (from a temperature of 27°C), the longer the viability was maintained (Figure 5.4). Viability was best maintained when the culture was switched to 21°C at the time of infection (0 hpi). When the temperature of the culture was raised from the initial temperature of 27°C to 30°C, the opposite occurred. The later the temperature was raised, the longer the viability could be maintained. These trends, however, were less apparent when the temperature was raised to 33°C.

To further probe why more infectious particles were produced by switching the temperature to 30°C, the dynamics of various components were evaluated for the cultures switched to 24°C, 27°C or 30°C at the time of infection (0 hpi).

Infectious AAV vectors can be detected as early as 24 hpi for cultures switched to 30°C at the time of infection (0 hpi) and begin to level beyond 48 hpi (Figure 5.5). For cultures kept at 27°C or switched to 24°C, again at the time of infection (0 hpi), infectious AAV vectors were detected by 48 hpi. Furthermore, titers achieved in cultures switched to 30°C at the time of infection (0 hpi), were never reached by cultures kept at 27°C or switched to 24°C even after 120 hpi.

At 24hpi, the level of Rep78 protein reached in the culture switched to 30°C was much higher than what was achieved in either of the two other cultures, nearly doubling the maximum intensity detected for the culture kept at 27°C (Figure 5.6). Furthermore, the onset of the production of Rep52 was detected earlier for cultures brought up to 30°C (24 hpi vs 48 hpi). At 48hpi, where the maximum Rep52 protein is detected for the culture at 30°C, the level nearly doubled that of the other two cultures. Although levels of Rep52 protein at 72 hpi for cultures kept at 27°C surpassed the maximum

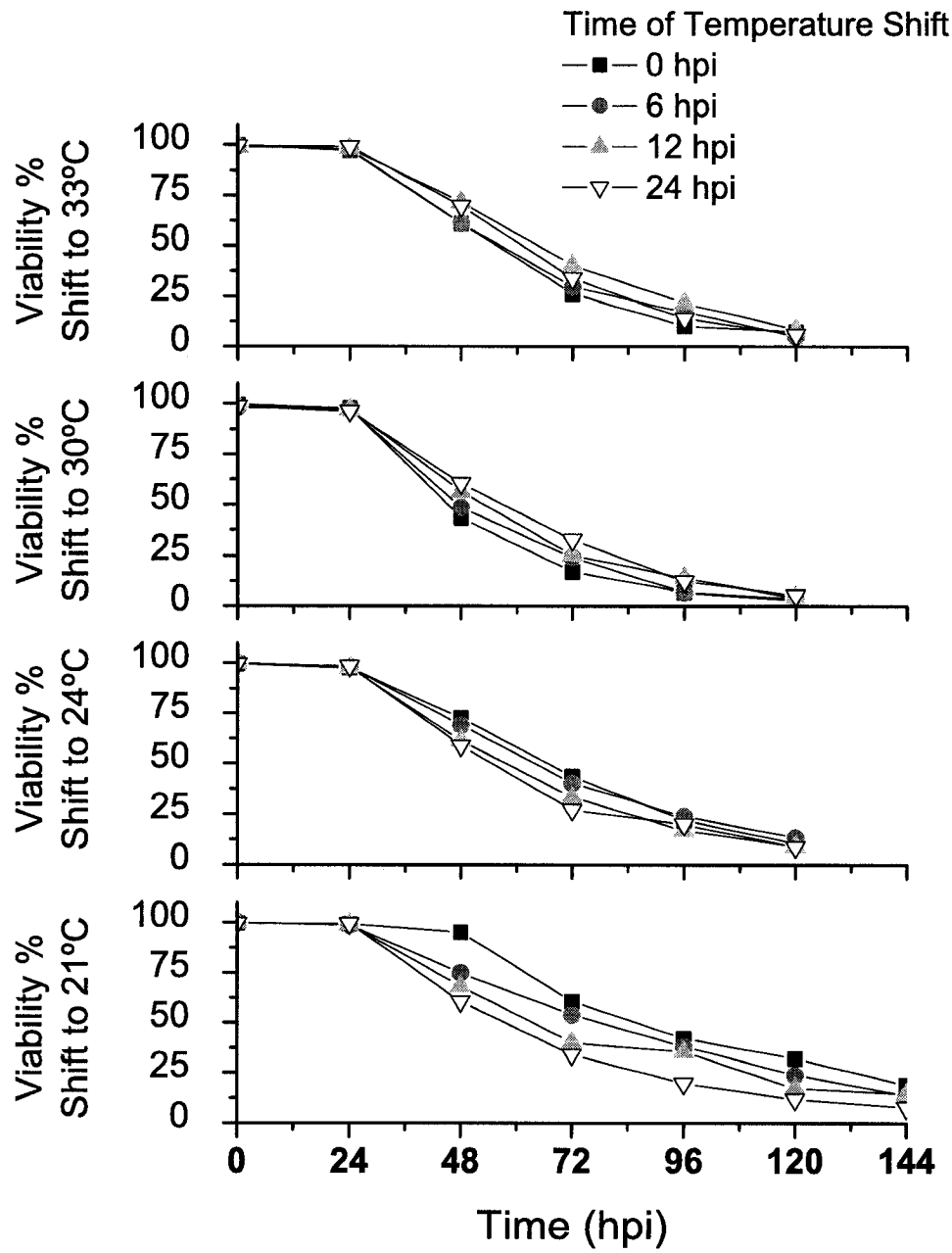

Figure 5.4 Dynamics of the average viability of cells exposed to various temperatures at times post-infection.

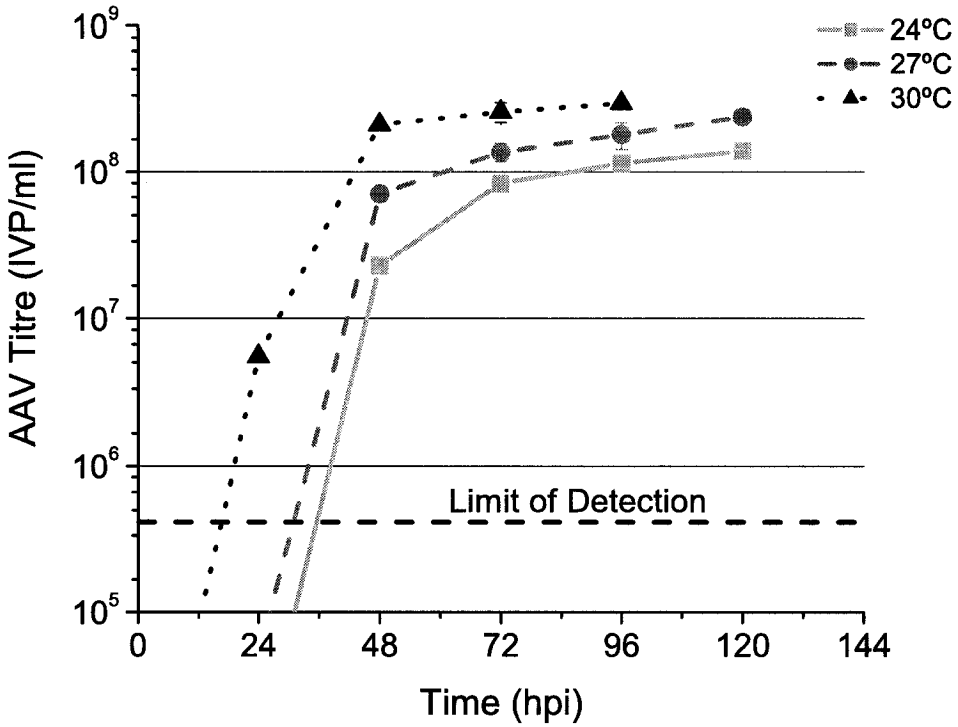

Figure 5.5 Dynamics of infectious AAV titer for cultures switched to different temperatures at t=0hpi.

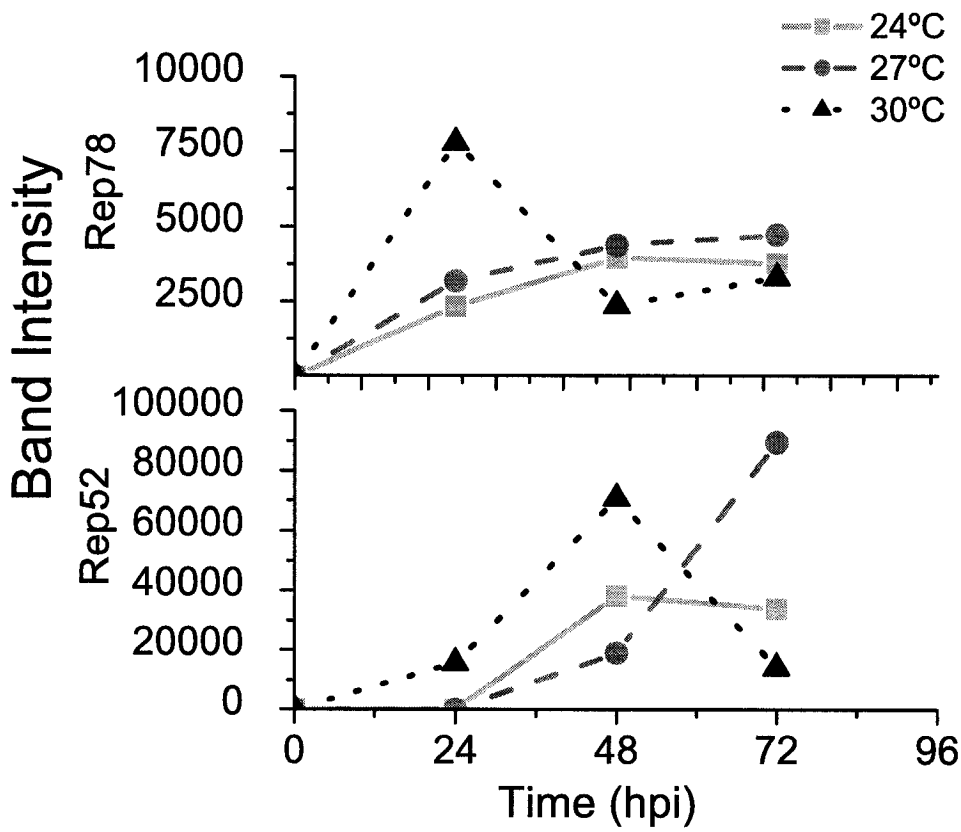

Figure 5.6 Dynamics of replication proteins and progeny DNA, detected through Western and Southern blotting, respectively, and quantified by densitometry, of cultures switched to different temperatures at t=0hpi.

achieved in cultures switched to 30°C, which occurred at 48 hpi, Rep78 levels never surpassed those achieved for cultures switched to 30°C.

The number of cells expressing GFP was the same for cultures kept at 27°C or switched to 24°C or 30°C, after 48 hpi (Figure 5.7). At 24 hpi, however, there was significant difference with cultures switched to 30°C at infection showing almost twice as many cells expressing GFP over cultures kept at 27°C and close to five times as many cells expressing GFP than cultures switched to 24°C. Furthermore, the mean

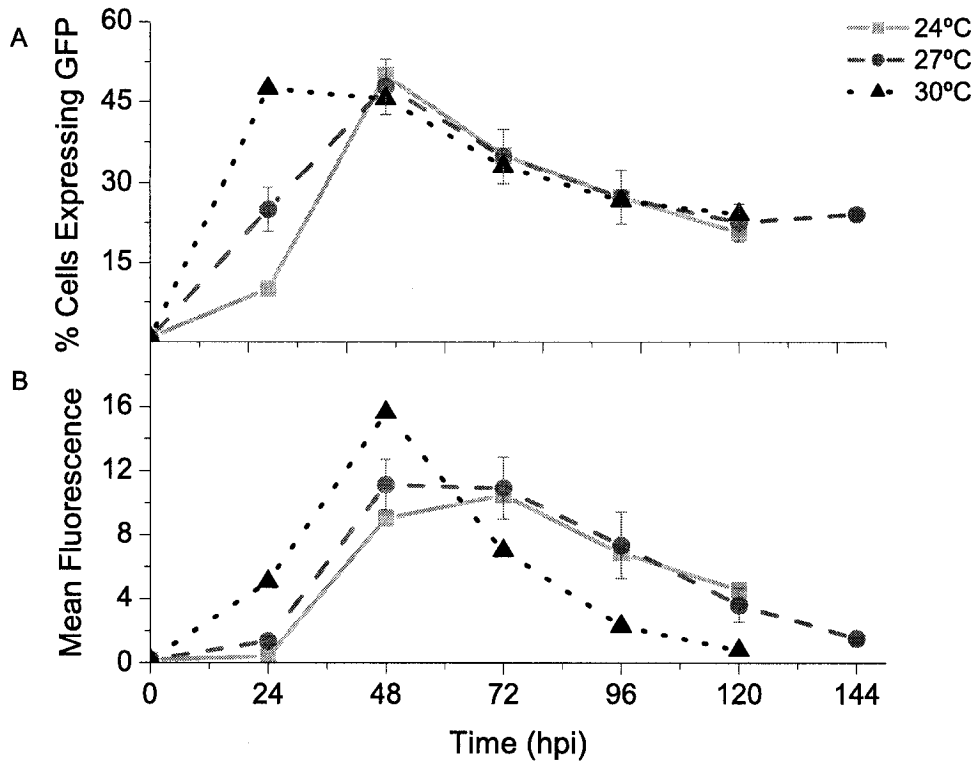

Figure 5.7 Dynamics of GFP production for cultures switched to different temperatures at t=0hpi. A) % cells expressing GFP B) mean fluorescence of cells.

intensity of the GFP recorded was close to 4 times greater than in cultures kept at 27°C and 13 times greater than those cultures switched to 24°C, again at 24 hpi. At 48 hpi, the mean intensity difference was reduced and cultures switched to 30°C were only approximately one and a half times greater then cultures kept at 27°C and close to two times that of cultures switched to 24°C.

5.5 Discussion

5.5.1 Temperature shift and effect

Based on the available literature it was believed that manipulating the temperature during the infection would affect the synthesis of recombinant AAV vectors. It was assumed that lowering the temperature would allow for reduced capsid loads (and hence higher cellular viabilities), better processing of the precursor elements: rep proteins, AAV vector DNA and AAV vector capsids, and allow for the proper maturation of the AAV vector.

Temperature, however, has also been linked to the process of infection. Virus movement in culture is governed by Brownian motion (reviewed by Petricevich et al., 2001); therefore, an increase in temperature should increase the movement of virus in the culture allowing for more collisions between virus and cells. Since the rate of infection is limited by the binding efficiency of baculovirus to cells (Dee and Shuler, 1997), temperature should play a prominent role in the infection process. It was therefore believed that the adsorption of virus, which leads to a productive infection, and synthesis of viral components could both be affected by temperature but in different ways. It was this hypothesis that led us to investigate the effect of the "switching" time since there was a desire to segregate the effect of the temperature shift on production and not on infection. A caveat associated with this strategy, however, is that temperature has been shown to affect the time at which a baculovirus transitions from "life" cycle phases. In all instances studied by Cain et al. (1999) and Donaldson et al. (1999), temperature not only played on the quality of the material produced, but also shifted the time of harvest. At lower temperatures, harvest occurred at later times post-infection (Cain et al., 1999; Donaldson et al., 1999). Reuveny et al. (1993) observed earlier production as the temperature increased from

22°C to 27°C.

In this particular study, it has been observed that raising the temperature to 30°C was beneficial to the production of AAV vectors (Figure 5.1). More important though, was that the time at which the temperature of incubation was changed was also significant. When examining those cultures that were switched to different temperatures, those switched after the 6-12hpi generally gave a different response than those switched at the time of infection. For cultures exposed to a temperature of 33°C (Figure 5.1), for example, it is interesting to note that the highest results occurred when the temperature at infection was raised to 33°C and that subjecting the culture to a temperature of 33°C thereafter was more detrimental. This suggests that two mechanisms of action were responsible for the changes in specific production. In a first part, around the time of infection, an increase in temperature seemed to have a beneficial effect; however, later, during the infection, an increase in temperature negatively impacted the production of AAV particles. Two conclusions that may be drawn are that the time switches selected did isolate two separate phenomena and that the maturation process was significant between 24 and 72 hpi. It is, however, hard to isolate whether the temperature effect between 0 and 6 hpi was a result of higher degree of productive infection (binding, uptake and trafficking of baculoviruses), or solely due to greater rates of protein synthesis.

Although a decrease in temperature allowed for the viabilities to be maintained for longer periods of time (Figure 5.4), the concentrations of AAV were not recovered over the extended period of time (Figure 5.5). Even with lower temperatures, the baculovirus loads and the production of capsids caused an unavoidable decrease in viability. This points to a relatively rapid process, which needs to occur early in the infection. We believe that the rate of production of capsids may influence the viability of the cells, probably due to a local accumulation, which may also be the reason for better encapsidation. It has been suspected and shown by others that temperature

affects the localization of proteins within the cell and affects the transport of the proteins within the cell.

Figure 5.6 shows an important difference between the cultures exposed to different temperatures: the level of Rep78 protein was much greater for the culture done at 30°C than those done at 27°C or 24°C. Furthermore, the appearance of Rep52 occurred at 24hpi and remained significantly higher at 48hpi. The appearance of Rep52 might be related to the earlier onset of the very late phase since a similar observation was made with the appearance of GFP; however, it should be noted that since the genetic sequence coding for Rep52 is embedded within the sequence for Rep78, the expression of Rep52 cannot be isolated from the expression of Rep78 (Kohlbrenner et al., 2005). Furthermore, Rep52 alone has been shown to be weakly correlated to the level of IVP produced (Aucoin et al., 2006). The greater quantity of Rep78 might, however, be more strongly correlated with higher levels of IVP, by allowing a greater quantity of genomes to be excised and transformed into AAV replicative forms. Although excessive levels of Rep78 have inhibitory effects on rAAV yield in mammalian cells (Grimm et al., 1998), optimal levels in insect cells have yet to be established. In this system the replication of the genome could in part be associated with the level of GFP detected. An increased quantity of genomes at early times post-infection (24hpi) for cultures carried out at 30°C was assumed based on the fluorescence measurements done on the insect cells. The greater percentage of cells expressing GFP at 24 hpi was also a sign of higher degree of productive infections.

It is very likely that temperature will affect more than a single phenomenon, and yet, as shown in Figure 5.8, when the infectious AAV vector concentration was plotted versus temperature, it resulted in linear trends between 24°C and 30°C. Raising the temperature resulted in very significant changes in level of infectious AAV produced at 48hpi. Furthermore, the lines seemed to converge around 31°C at

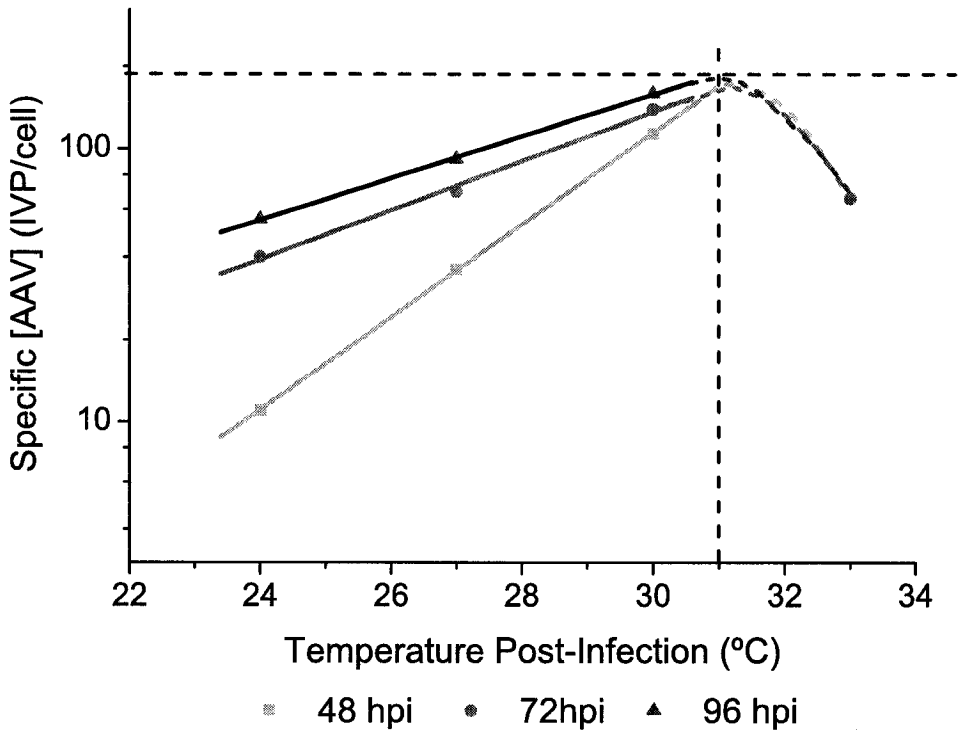

Figure 5.8 Temperature effect on the production of infectious AAV particles at various times post-infection.

a maximum IVP/cell value of ~175 IVP/cell. The maximum level; however, should be dictated by the DRP or VP/cell values, which were around 10000 and 1000000 respectively (Table 5.3), and not by the IVP/cell value. Figure 5.8 also suggests that a temperature slightly higher than 30°C could yield higher titers, especially at 48 hpi. However, from the drop observed at 33°C, one may not want to work close to the apex, where the process stability may be highly influenced by small variations in temperature.

Finally, switching temperatures before or after infection did not produce better results than switching the temperature at the time of virus addition (0 hpi). This is a convenient result since it is easiest to switch the temperatures at the time of

infection. Furthermore, since there is a benefit to switching the temperature at any time post infection, when switching the temperature to 30°C, the change in temperature does not have to be abrupt, therefore quite amenable to a temperature that is ramped up in a bioreactor. Switching the temperature to 30°C at the time of infection also allows the time of harvest to be 24 hours earlier than when kept at 27°C.

5.6 Acknowledgements

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CHAPTER 6

A STUDY ON HOW TO INCREASE THE CELL DENSITY AT INFECTION WITHOUT DECREASING THE SPECIFIC PRODUCTION OF AAV VECTORS

Previous chapters focussed primarily on optimizing the quantity of baculovirus used at low cell concentrations (~2x10^6 cells/ml) for maximal per cell AAV vector production. This chapter deals with aspects surrounding the enhanced production of AAV vectors in insect cells cultured at higher cell densities, taking advantage of the full potential of insect cell culture. With appropriate feeding strategies, Elias et al. (2000) were able to maintain the specific production levels of β-galactosidase up to 14x10^6 cells/ml (33% of the maximum cell density achieved). Similar strategies were applied for the production of AAV in our lab, however, the specific production decreased to $\sim$ 22% of the maximum specific production (unpublished). Furthermore, when investigating the peak cell density for the production of AAV at 27°C, Meghrous et al. (2005) found that the specific production decreased at cell densities lower than what has been previously reported for single protein production also in SF-900 II medium (Wong et al., 1996; Tsao et al., 1996). For a more detailed review of cell density as a factor in the production of recombinant protein, the reader can refer back to section 2.5.2. The goal of this study was to identify conditions that may have caused the limitations observed by Meghrous et al., and to devise a strategy to maximize the volumetric production of functional AAV.

6.1 Materials and Methods

6.1.1 Cells and medium

Spodoptera frugiperda (Sf9) and HEK293 EBNA cells were maintained in serum-free SF-900 II at 28°C and serum supplemented HSFM media at 37°C (GIBCO BRL, Burlington, ON, Canada) for AAV production and quantification, respectively. Further details on the cell culture are found elsewhere (Aucoin et al., 2006, 2007a,b). Cell densities were assessed using a haemocytometer and Coulter Counter Multisizer. Cell viability was determined via the trypan blue exclusion method. The cell size distribution of the cell population was also recorded using the Coulter Counter Multisizer, by monitoring particles having sizes between 9 and 30 μm.

6.1.2 Baculovirus viral stocks

BacITRGFP, which has the transgene for green fluorescent protein (GFP) under both the p10 insect and cytomegalovirus (CMV) mammalian promoters between AAV ITRs was kindly provided by Dr. R.M. Kotin from the National Institutes of Health (Bethesda, MD, USA) and has been described previously (Urabe et al., 2002). Dr. Kotin also supplied BacRep and BacCap, which contain the gene sequences for the replication proteins and structural proteins. Viruses were plaque purified using a standard procedure and 5 viral colonies were randomly selected. All five colonies were amplified to P1, quantified, and assessed for their ability to either produce replication proteins, capsid proteins or infectious AAV vectors. The highest producers were amplified to P2 by infecting cells in their logarithmic growth phase at $1x10^6$ cells/ml with an MOI of 0.01. Supernatants were collected ~48 hpi and filtered through a 0.2 μm filter after being centrifuged at 300 g for 15 minutes. The baculovirus stock solution was kept at 4°C in the dark. P2 stocks were concentrated

~10X by ultracentrifugation (average 145 000 g for 65 min at 4°C), resupended in fresh SF-900 II medium and stored until use at 4°C. Titers of the viruses were determined by flow cytometry (Shen et al., 2002) and verified by plaque assay prior to use.

6.1.3 Infection/production strategies

The multiplicities of infection used were based on previously established conditions (Aucoin et al., 2006) and kept constant for the different cell densities. After baculovirus addition, cultures were maintained at 30°C for the production of AAV vectors unless otherwise specified (Aucoin et al., 2007b).

6.1.4 Experimental design

6.1.4.1 Controls

Duplicate control cultures (40 ml working volume in a plastic 250 ml shake flask), which were infected at ~2x10^6 cells/ml and kept at 28°C for the production period, were ran in parallel as a baseline production.

6.1.4.2 Infection of cells at various cell densities

600 ml of Sf9 cell culture was initiated by inoculating cells in exponential growth phase in a 2 L flask at 0.5x10^6 cells/ml for growth at 28°C. When the cells reached the required densities (~1,~2,~4,~8-10 million cells/ml), 32 ml of cells were removed and added to a plastic 250 ml shake flask. 8 ml of virus solution, composed of the various vectors needed and fresh media, were added and the cultures were incubated

thereafter at 30°C. 2 ml samples were taken at 0, 24, 48 and 72 hpi for analysis.

6.1.4.3 Infection of cells at 4 and 8-10x10^6 cells/ml in fresh media

600 ml of Sf9 cell culture was initiated by inoculating cells in exponential growth phase in a 2 L flask at 0.5x10^6 cells/ml and then grown at 28°C. When the cells reached the required densities (~4,~8-10x10^6 cells/ml), 32 ml of cells were removed, centrifuged at 600 x g for 15 min at room temperature and resupended in fresh media containing appropriate quantities of baculovirus and added to a plastic 250 ml shake flask. The cultures were incubated thereafter at 30°C. 2 ml samples were taken at 0, 24, 48 and 72 hpi for different analyses.

6.1.4.4 Infection of cells at 4 and 8-10x10^6 cells/ml that have been fed a nutrient cocktail supplement

The nutrient cocktail used was previously described by Bédard et al. (1994, 1997) to alleviate nutrient limitations at high cell densities. Composition of the cocktail is further described in Appendix III.

600 ml of Sf9 cell culture was initiated by inoculating cells in exponential growth phase in a 2L flask at 0.5x10^6 cells/ml and then grown at 28°C. At 2x10^6 cells/ml, 32 ml aliquots were transferred to 250 ml shake flasks and a volume of cocktail equivalent to 2% of the volume was added. At 4x10^6 cells/ml a volume of cocktail equivalent to 4% of the volume of culture was added. Following the cocktail addition, the cells were either kept at 28°C and cultivated for an extra day or an appropriate quantity of viral solution topped with fresh media was added (8 ml total) and the cells were incubated thereafter at 30°C. Cells that were not infected were left to grow to 8x10^6 cells/ml. A volume of cocktail equivalent to ~6-8% of the volume

of the culture was added prior to the addition of an appropriate quantity of virus solution topped with fresh media (8 ml) and the cells were incubated thereafter at 30°C. Samples were taken at 0, 24, 48 and 72 hpi.

6.1.4.5 Growth and infection of equivalent age cells at various densities in fresh media

4 x 600 ml of Sf9 cell culture were initiated by inoculating cells in exponential growth phase in 4 x 2 L shake flasks at $0.5x10^6$ cells/ml and then grown at 28°C. At $2x10^6$ cells/ml, the mother cultures were combined, centrifuged at 600 x g for 15 min at room temperature and then resuspended to get appropriate cell densities (1.0, 2.0, 4.0 and 8.0 and $10.0x10^6$ cells/ml). The cells were resuspended in either fresh media (negative control) or fresh media containing appropriate quantities of baculovirus. Samples were taken at 0, 6, 12, 24, 48 and 72 hpi.

6.1.4.6 Culture sampling procedure experiment

For all flasks ~2.25 ml per sampling point were taken from each shake flask. 1.5 ml were frozen at -80°C to be analyzed at a later date. 85 μl were set aside for cell counts, cell size distribution and viability determination. 0.5 ml samples were centrifuged (Hereaus Pico microcentrifuge) at 2000 rpm for 10 min. Supernatants (approx: 500 μl) were transferred to an Ultrafree-MC 30000 NMWL Filter Unit from Millipore and centrifuged at 8000 rpm for a minimum of 20 min while the pellets were resuspended in a 50 mM TRIS buffer containing 2 mM $MgCl_2$ and 37.5 mM $MgSO_4$. The resulting permeates from the Ultrafree-MC 30000 NMWL Filter Unit were either frozen at -80°C for subsequent amino acid/metabolite analysis or stored at 4°C for analysis of the osmolality. A second set of 0.5 ml samples were centrifuged

at 2000 rpm for 3 min. The supernatants were kept for baculovirus quantification at a later date while the pellets were fixed by first adding 500 μl of cold PBS followed by the addition of 500 μl of cold 4% formaldehyde solution. The fixed cells were stored at 4°C for a minimum of 30 min before being analyzed by flow cytometry for GFP fluorescence.

6.1.4.7 Cell cycle analysis

Cells from a mother culture inoculated at $0.5x10^6$ cells/ml were sampled at different points during their growth (1, 2, 4, and 8-10 million cells), centrifuged (5 min, 400 x g, 4°C) and washed with PBS containing 5 mM EDTA. The cells were then fixed with the slow addition of cold 100% anhydrous ethanol (kept at -20°C). Cells were kept at -20°C until analyzed. On the day of analysis, cells were centrifuged (5 min, 600 x g, 4°C) and washed with PBS containing 5 mM EDTA before being stained using a propidium iodide solution (PBS + propidium iodide 50 mg/ml + RNase A (DNase free) 20 mg/ml) and incubated at 37°C for 30 min. Cells were filtered through a 60μm mesh, and analyzed by flow cytometry making sure that the flow rate of the cells though the cytometer did not exceed 500 events per sec.

6.1.4.8 Analytical

The analysis of amino acids in the medium were measured by analytical HPLC as previously reported (Kamen et al., 1991). Both an internal and external control were used to normalize the results. Although duplicate measurements were not performed, samples from duplicate cultures were analyzed and averaged. Glucose, lactate and ammonia were measured using a Kodak Biolyzer (Kodak, New Haven, Connecticut). Upon repeated analysis of a control sample (n=8), the coefficient of

variation on the measurement was less than 10% for all components measured. The largest CV was associated with the measure of cystine, which gave a %CV of ~8. Therefore, components whose values did not vary by more than 25% in any of the cultures examined were not deemed to provide significant information on potential limitations. Osmolality was measured using a commercial osmometer.

6.1.4.9 Production and consumption rates

The specific production and consumption rates were calculated by dividing the difference in the quantity of metabolites by the average cell density corresponding to the time interval, multiplied by the time interval (trapezoidal rule).

6.1.4.10 Functional AAV titration

AAV was detected by gene transfer assay (transduction/expression assay) and described elsewhere (Aucoin et al., 2006, 2007a,b). Briefly, samples were subjected to three freeze/thaw (F/T) cycles to ensure cell lysis and heated at 60°C for 15 min to inactivate the baculovirus. HEK293 EBNA cells in HSFM medium containing 1% BCS and 10 mM HEPES were transduced with AAV in the presence of excess wild-type Ad5 (ATCC number VR-1516, MOI$\geq$10). Analysis of the infected HEK293 EBNA cells was performed with a Coulter EPICS^{TM} XL-MCL flow cytometer (Beckman-Coulter, Fl). Green fluorescence from the GFP was detected and the flow cytometric data were analyzed using the EXPO32^{TM} software package.

Werstern blots of AAV rep and cap proteins were done as previously described (Aucoin et al., 2006, 2007a,b) with commercial antibodies obtained from Maine Biotechnology Services (Portland, ME).

6.2 Results

6.2.1 Baculovirus selection

To ensure the highest possible yields, viruses were plaque purified and amplified to attain the desired amount of virus needed for the complete set of experiments in a minimal amount of passages. As specified in the methods section (section 6.1.2), five random plaques from each baculovirus vector purification were selected and amplified to P1 for screening purposes. Western blot analysis of protein expression from P1 amplified BacRep and BacCap showed a significant heterogeneity in the BacRep stock that was not apparent in the BacCap stock (Figure 6.1). The highest expressing BacRep and BacCap were chosen, and amplified to P2. P1 BacITRGFP, amplified from plaque isolates, was screened by the amount of functional AAV that could be produced in the presence of BacRep (P3, MOI=10) and BacCap (P3, MOI=10), when it was added at an MOI of 3. All 5 BacITRGFP were compared to a control culture infected with BacRep (P3, MOI=10), BacCap (P3, MOI=10), and BacITRGFP (P3, MOI=3) that were used in previous experiments. Variation between functional yields were observed (Figure 6.2); the isolate that gave the highest specific titers was chosen for subsequent use.

6.2.2 Cell size distribution and cell cycle analysis

In order to apply the MOI generically, with the assumption that the actual cell concentration did not affect the number of virus particles needed at higher cell densities, it was considered important to ascertain whether the cells at various cell densities had common characteristics. This was done by analyzing the cell size distribution and the cell cycle distribution of cells grown to increasing cell densities.

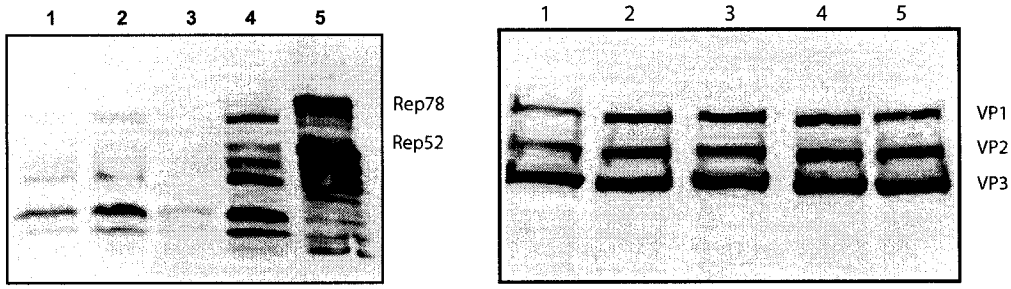

Figure 6.1 Western blot of (A) replication proteins from P1 amplified BacReps; and (B) VP proteins from P1 amplified BacCaps. Both series consisted of infecting insect cells with MOIs of 10.

As can be seen in Figure 6.3, when the cell size distributions were normalized to the total number of cells in each sample, no noticeable differences could be detected. Therefore, given that the virus could be distributed based on the availability of receptors, which could be assumed to be proportional to the surface area of a cell (see Chapter 3), it was not expected that this would account for differences that would be seen at different cell densities.

When investigating the proportion of cells in various cell cycle phases, it was observed that the proportion of cells in each phase did not change significantly for cells grown up to $1x10^7$ cells/ml (Figure 6.4). Most of the cells were equally distributed between G_1 and the G_2/M phases with only approximately 20% of the cells in the S phase. A small population of polyploidal cells also existed in all samples. The proportion of these cells remained constant ($\sim$ 5% of all cells) for the four cell densities examined (data not shown).

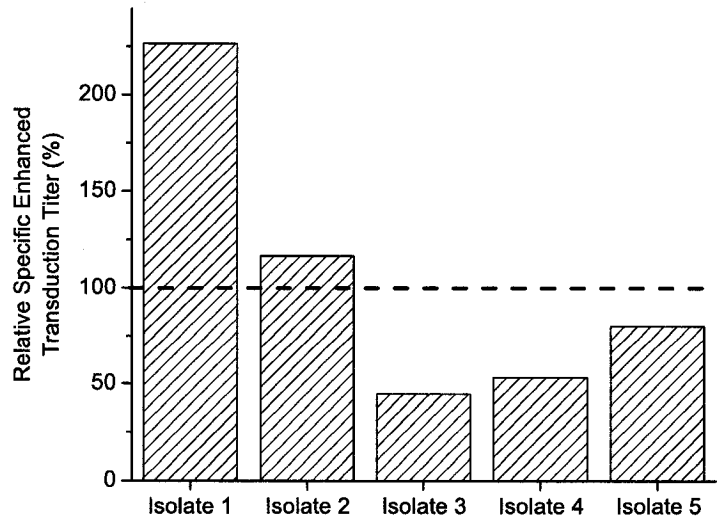

Figure 6.2 Screening of P1 BacITRGFP amplified from various isolates obtained from plaque purification for the production of functional AAV vectors. Insect cells were infected with P3 BacRep (MOI=10) and P3 BacCap (MOI=10). 100% mark refers to a control production using insect cells infected with P3 BacRep (MOI=10), P3 BacCap (MOI=10) and P3 BacITRGFP (MOI=3).

6.2.3 Recovery

To determine the maximum production of AAV vectors (and not only the amount associated with the cell pellet), samples of culture broth (cells and medium) harvested at 48hpi, were analyzed as previously reported (Aucoin et al., 2006, 2007b). To minimize effects associated with the number of cells in culture, a second set of samples were centrifuged and the cell pellets were resuspended to ~$7x10^6$ cells/mL in a salt enriched TRIS buffer (Chahal et al., 2007). Enhanced transduction titers for each sample preparation were compared in Figure 6.5. As can be seen, at high cell densities, if the buffer was not used, enhanced transduction units were underestimated up to sixty times. All subsequent analysis of AAV productions were done

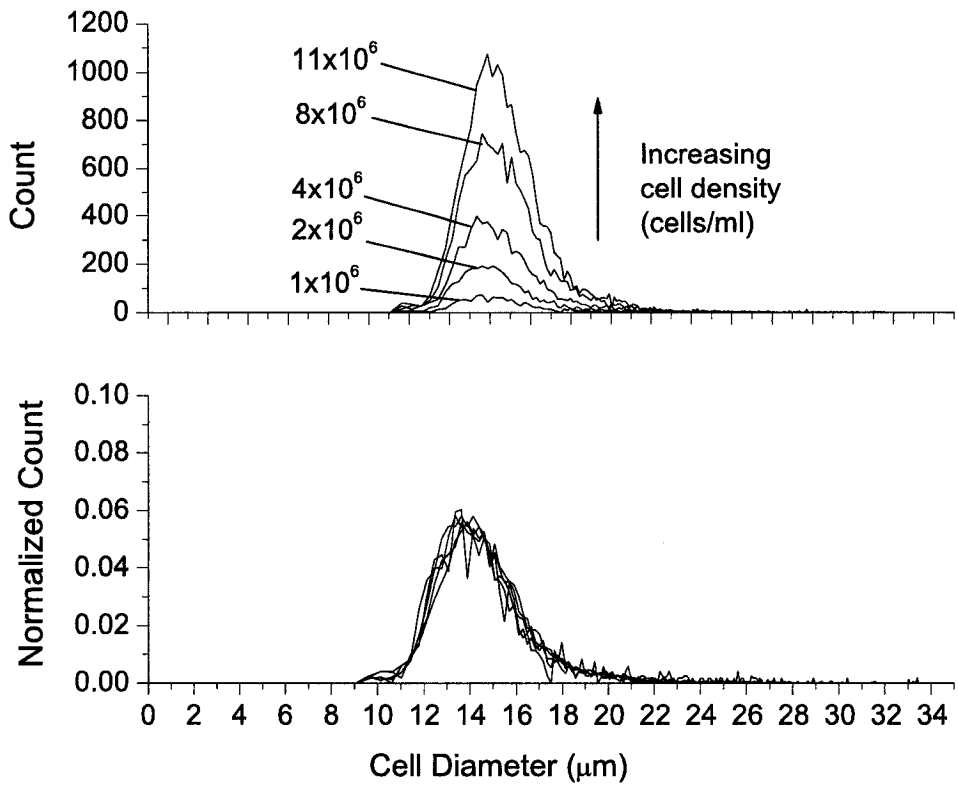

Figure 6.3 Cell size distribution of cells prior to infection for different cell densities. To normalize the distributions (bottom plot), cell counts were divided by the total number of cells counted for each sample.

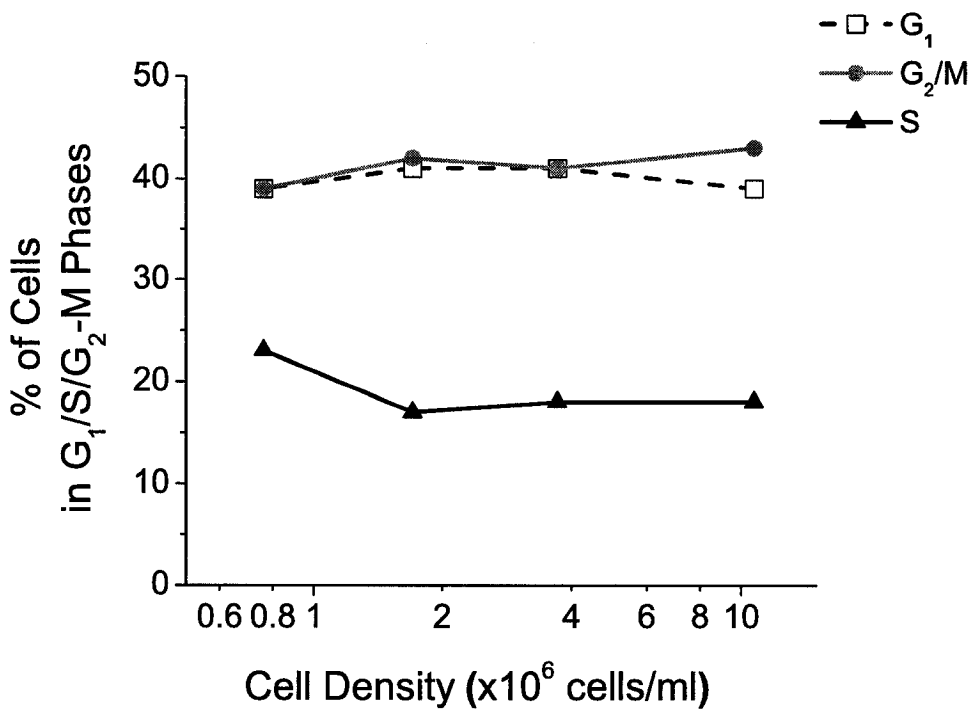

Figure 6.4 Cell cycle distribution of cells grown to various cell densities. Cells were inoculated at an initial cell density of 0.5×10^6 cells/ml.

$$\%Increase = \frac{ETU_{ResuspendedPellet} - ETU_{CellBroth}}{ETU_{CellBroth}} * 100\%$$

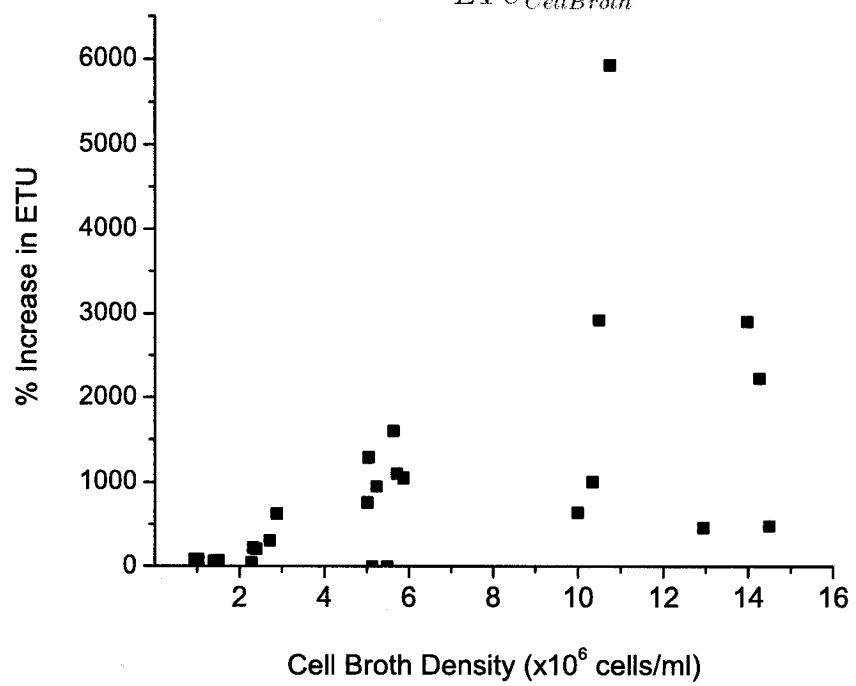

Figure 6.5 Comparison of AAV titers obtained from enhanced transduction assay when either cell broth or cell pellets resuspended in high salt buffer (50mM TRIS, 2 mM $MgCl_2$, 37.5 mM $MgSO_4$) to a density around $7x10^6$ cells/ml were used. X-axis is the cell density of the cell broth, which is also the cell density at harvest. Both types of samples were subjected to 3X F/T and 15min at 65°C.

using the results obtained from pellets resuspended in the TRIS buffer.

6.2.4 Positive control

As a positive control, cells were inoculated at $0.5x10^6$ cells/ml, grown to $2x10^6$ cells/ml and infected, all at 28°C. Cells were harvested 48 hpi and resuspended in the buffer described previously. When compared to an equivalent culture where the temperature was increased to 30°C after addition of virus ie. for the produc-

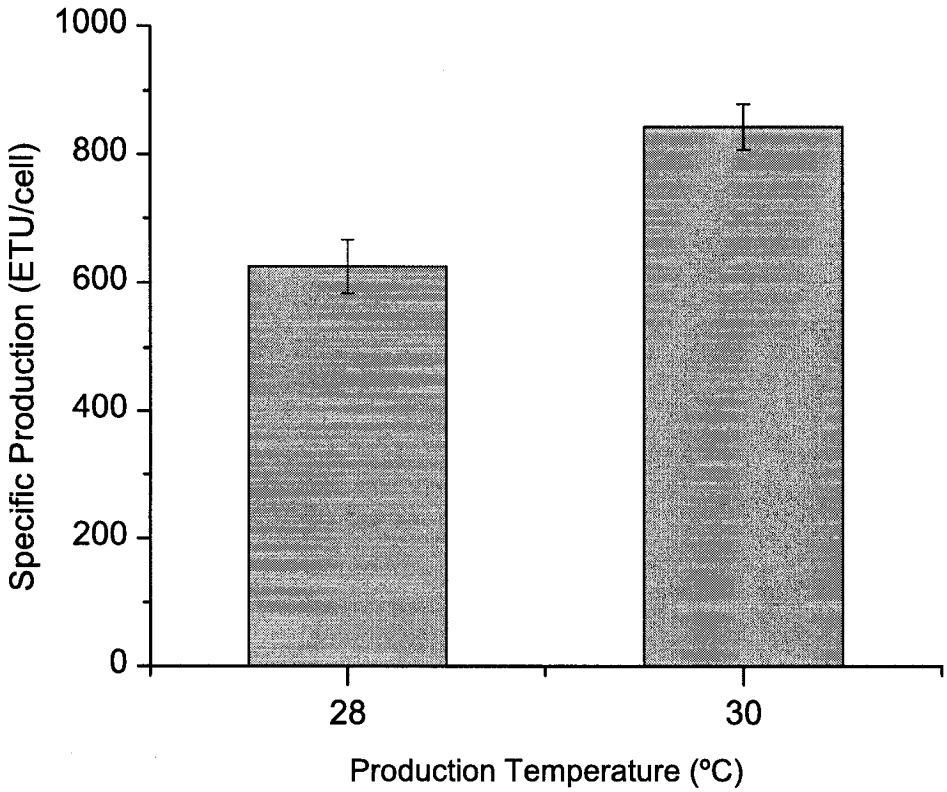

Figure 6.6 Comparison of AAV enhanced transduction titers for production at either 28°C or 30°C. Cells were infected at $2x10^6$ cells/ml with BacRep (MOI=10), BacCap (MOI=10) and BacITR (MOI=3). Error bars represent the range of titers obtained from duplicate cultures.

tion period, it can be seen that increased AAV vector production resulted with the increased temperature (Figure 6.6).

6.2.5 Peak cell density

A "peak cell density" for the production of functional AAV vectors having a growth phase at 28°C and a production phase carried out at 30°C was assessed in SF-900 II media. Figure 6.7 suggests that the peak cell density occurred beyond ~$2x10^6$

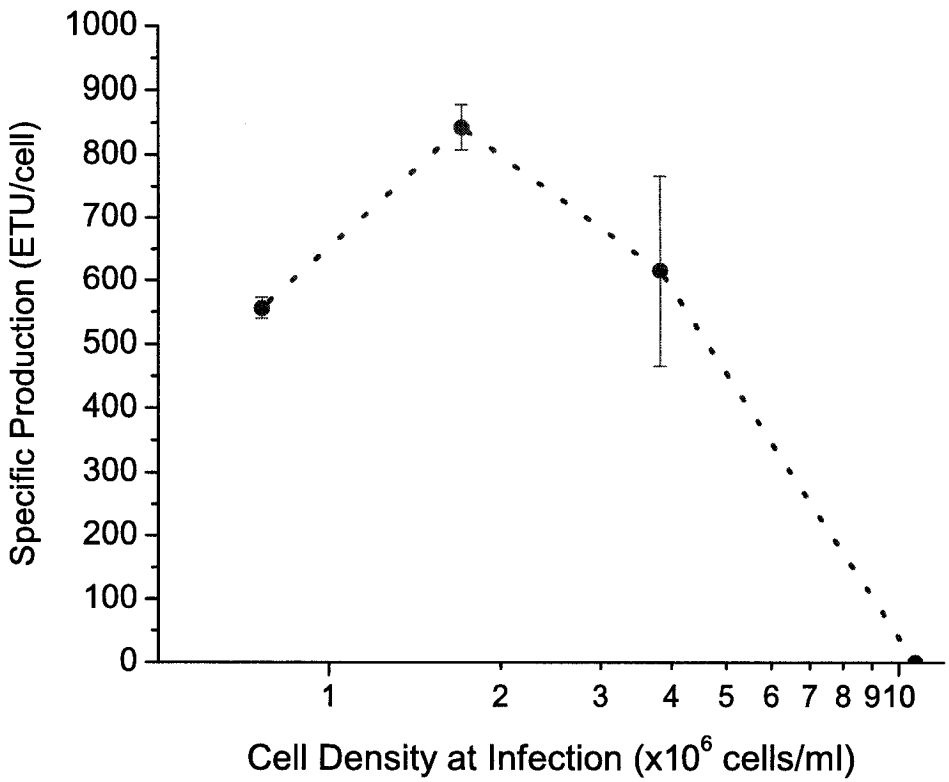

Figure 6.7 Cells grown at 28°C to the desired cell density of infection followed by the addition of virus and incubation at 30°C for the production period. Error bars represent the range of titers obtained from duplicate cultures.

cells/ml and possibly even beyond $3x10^6$ cells/ml, given the range obtained in the duplicate cultures at $4x10^6$ cells/ml. To investigate potential limitations beyond this density, amino acid, glucose, lactate and ammonia concentrations were measured. It is thought that even though the nutrients measured may not show complete depletion, that the consumption/production dynamics may indicate limiting conditions.

When looking at the various profiles it can be seen that serine was reduced below the limit of detection within 24 hpi for the culture infected at $\sim 1x10^7$ cells/ml (Figure 6.8). In the same period, leucine and glutamine were significantly reduced. Cystine

levels for cultures infected at $4x10^6$ cells/ml or $1x10^7$ cells/ml were comparable at 24 hpi, suggesting that the limitation in serine may have resulted in a change in cystine consumption. Beyond 24 hpi, cystine and tyrosine levels increase, which suggests that after infection there is some biological change that allows this increase. By 24 hpi cell growth had ceased and the infection process was well underway. A significant increase in the rate of ammonia synthesis was observed for all cultures after infection.

6.2.6 Effect of Medium Exchange

To investigate whether supplying increased nutrient levels at the time of infection could alleviate the limitations observed in the production of AAV, cells were centrifuged and resuspended in fresh media containing the virus. As can be seen in Figure 6.9, when cells were infected $\sim 4x10^6$ cells/ml, the medium exchange may have slightly lowered the titer, although overlapping error bars negates the significance of this difference. It is hypothesized that resuspending the cells in media containing virus may be unduly stressful for the cells, yet this remains to be confirmed. Still, it is not expected that results between cells grown to $4x10^6$ cells/ml with or without medium exchange prior to infection to be very different, as there is no presumed limitation at this point (Figure 6.9). At much higher densities ($\sim 1.1x10^7$ cells/ml), production of functional AAV is restored, albeit only at a fraction of the maximum specific production ($\sim$10%).

When examining the nutrient/metabolite profiles for the cultures that underwent a medium exchange prior to infection (Figure 6.10), it can be seen that serine no longer dropped below the detection limit prior to harvest at 48 hpi. However, cystine dropped below the level of detection between 24 and 48 hpi.

The medium exchange increased the nutrient levels as desired; however, it also increased the specific rates of consumption (between 0 and 24 hpi) of most nutrients.

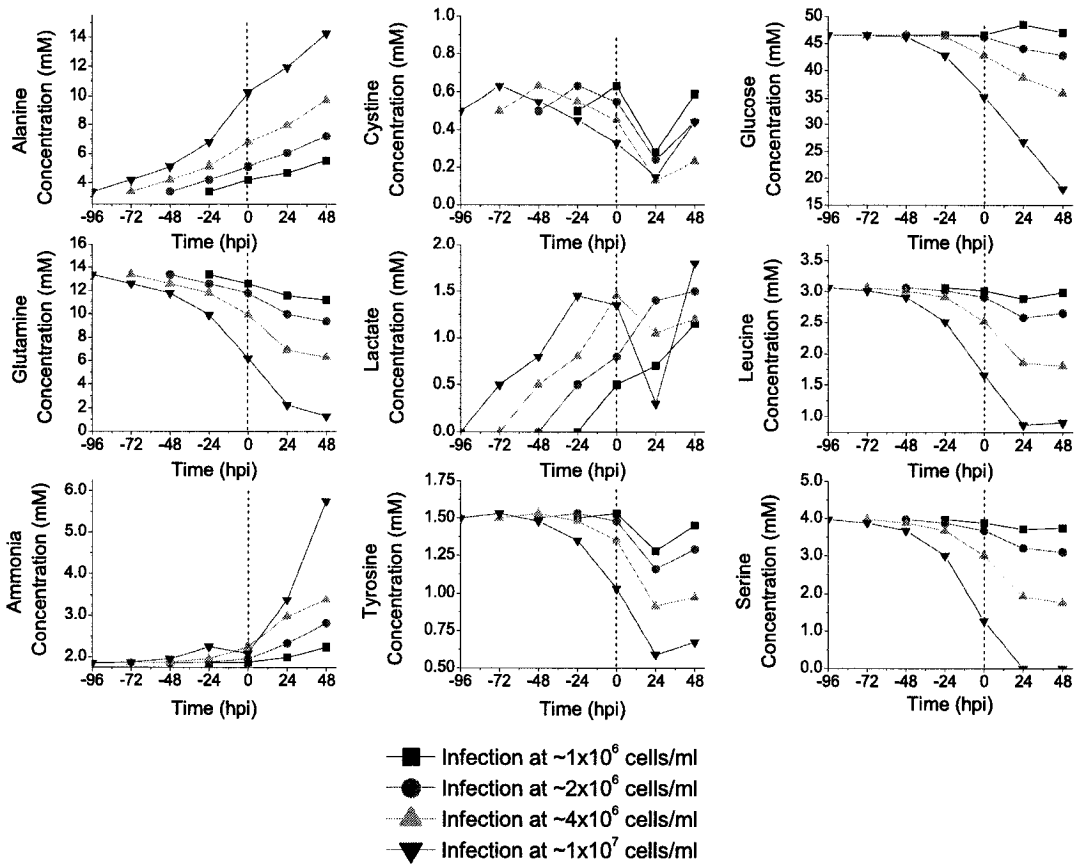

Figure 6.8 Average dynamic profile of nutrients and metabolites of cultures infected at various cell densities. Nutrients and metabolites shown change by at least 25% for one of the conditions tested.

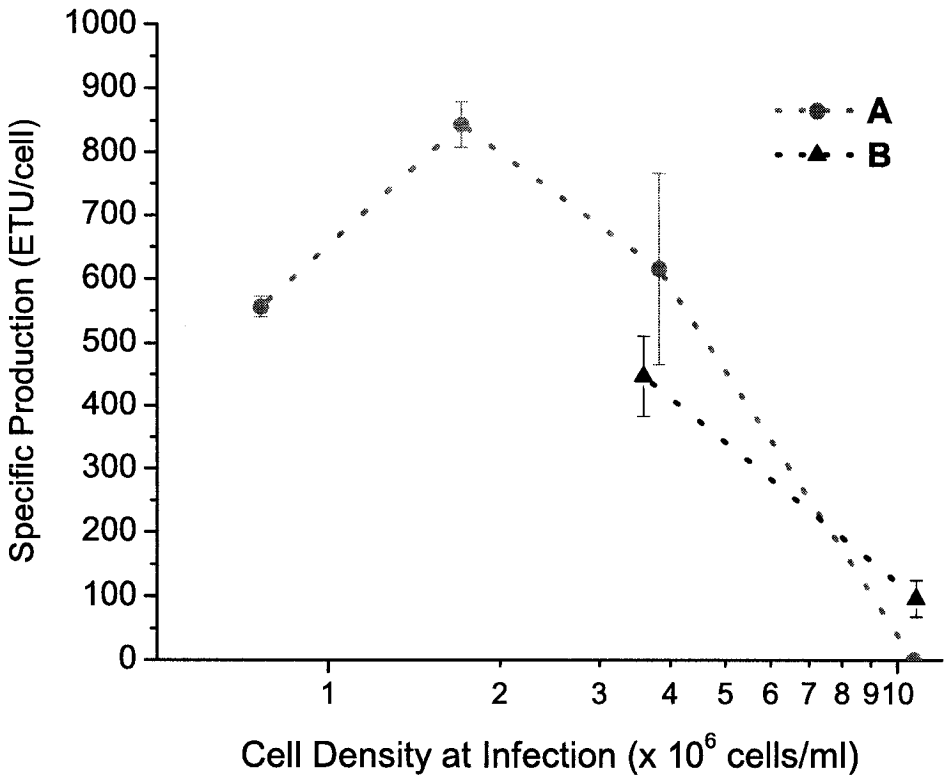

Figure 6.9 Comparison of AAV enhanced transduction titers for A: cells grown at 28°C to the desired cell density of infection followed by the addition of virus and incubated at 30°C for the production period; and B: cells grown at 28°C to the desired cell density of infection, centrifuged and resuspended in fresh SF-900 II medium containing virus, and incubated at 30°C for the production period. Error bars represent the range of titers obtained from duplicate cultures.

The increase in consumption/production rates were seen whether the exchange occurred at 4 or 11x10^6 cells/ml (Figure 6.11), with the exception of serine and leucine which remained similar for both cultures with and without media exchange at 4x10^6 cells/ml. It was initially thought that the increase in rate were due solely to the increase in nutrient/metabolite concentrations; however, upon further investigation, a correlation could not be established between the two. These observations suggest that medium exchange does not guarantee that enough nutrients will be available during the production, in part due to the increased consumption rates.

6.2.7 Effect of exponential feed

Previous work by Bédard et al. (1997) established a complex feed that could help alleviate nutrient limitations. Using that formulation, it was decided to attempt to preempt nutrient limitations by maintaining a more consistent level of nutrients throughout the culture. A slow addition from the point at which the peak cell density started to decrease (Figure 6.7) consisted of a volume of cocktail proportional to the cell density. As can be seen in Figure 6.12, although the osmolality was kept below 400 mOsm, the average cell diameter of the cells increased significantly before infection for cells grown to ~8x10^6 cells/ml.

In terms of production of functional AAV, feeding cells that were then infected at 4x10^6 cells/ml did not increase the production compared to those cells that did not receive the feed. Similar to exchanging the medium at 4x10^6 cells/ml, this was not expected to yield a dramatic increase as there was no perceived limitation at this point (Figure 6.13). When cells were grown to 8-10x10^6 cells/ml while being fed, 50-60% of the maximum specific production was achieved.

Although there was production beyond 4x10^6 cells/ml, there was still a decrease in the specific production. Prior to infection, during the feeding regimen, certain

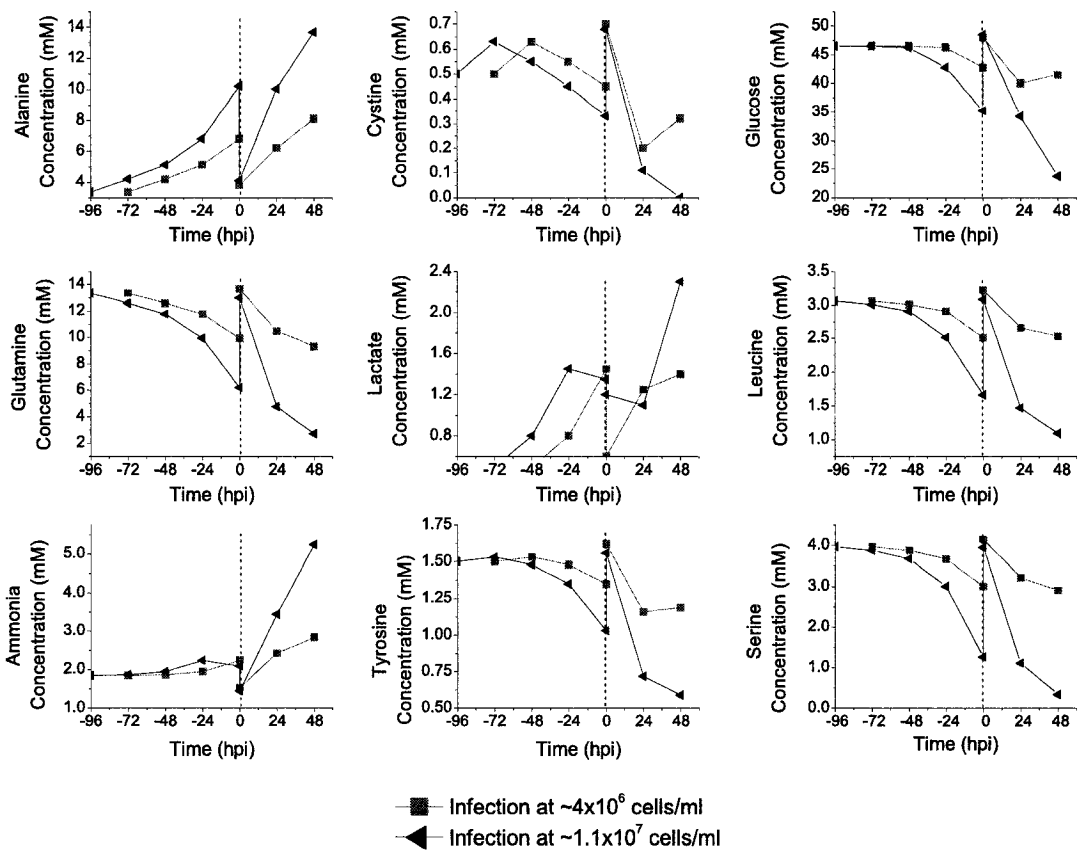

Figure 6.10 Average dynamic profile of nutrients and metabolites of cultures that underwent a medium exchange at the time of infection. Nutrients and metabolites shown change by at least 25% for one of the conditions tested.

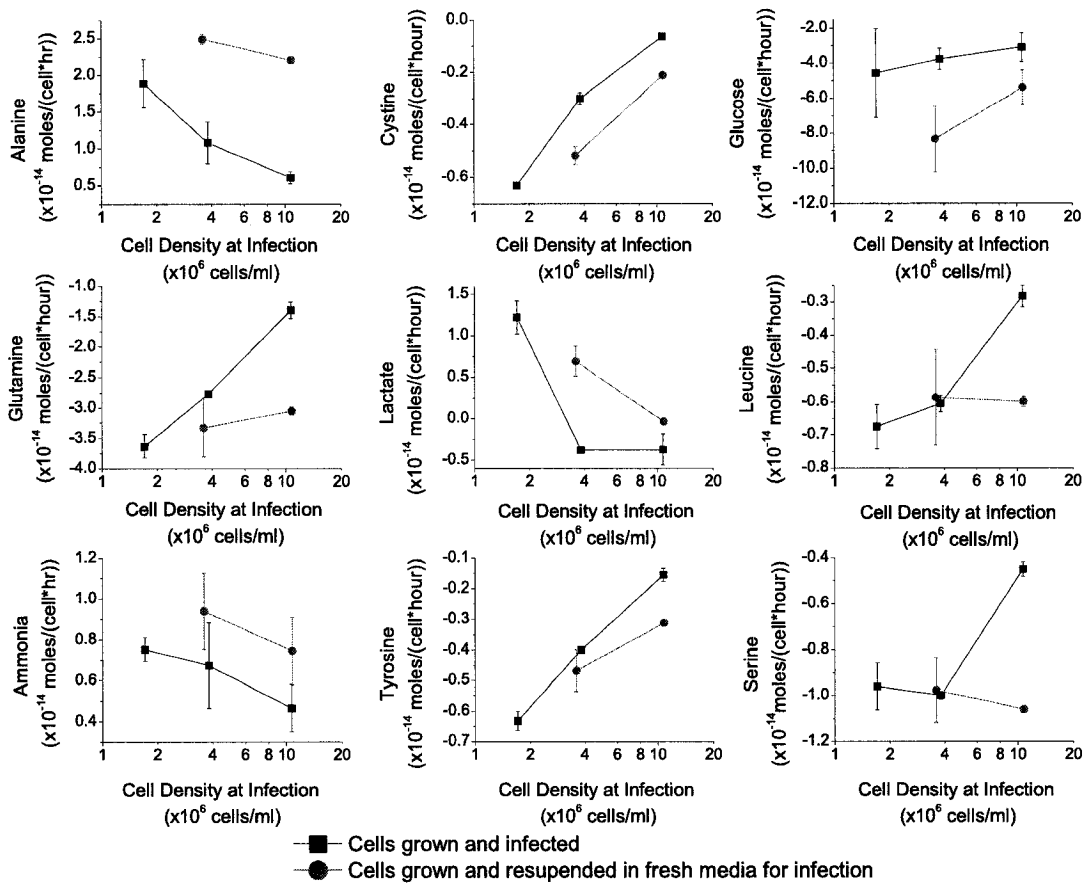

Figure 6.11 Specific production and consumption rates for the first 24 hpi. Error bars represent the range of values for duplicate cultures.

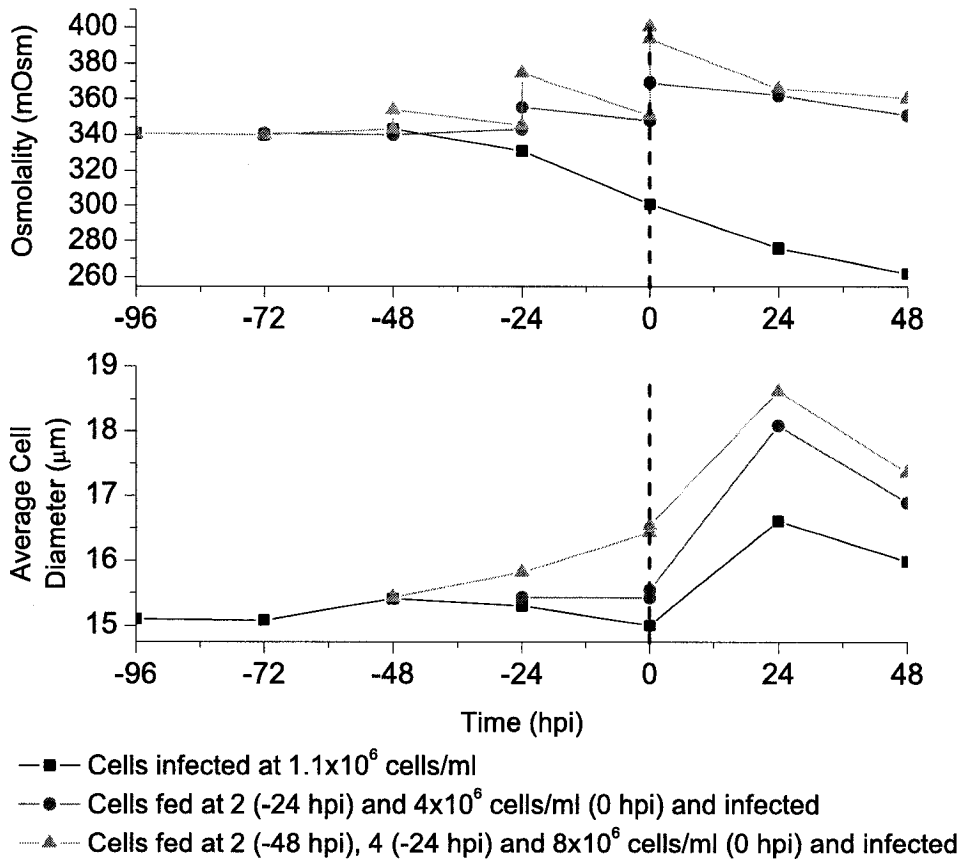

Figure 6.12 Osmolality and average cell diameter profiles of cultures fed a concentrated nutrient cocktail during the cell growth period.

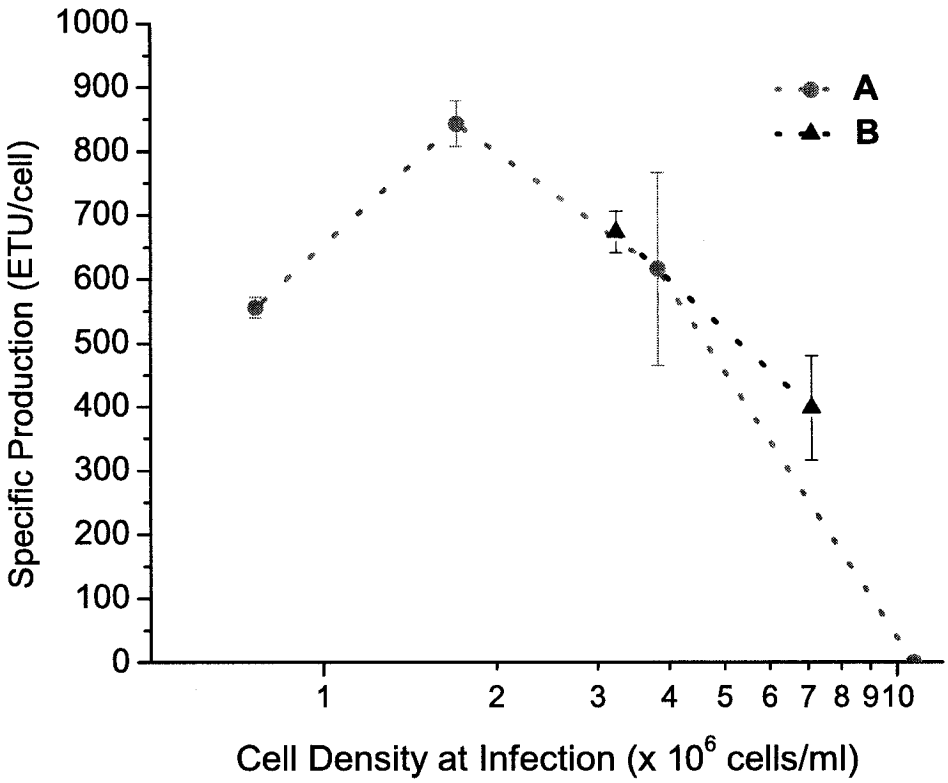

Figure 6.13 Comparison of AAV enhanced transduction titers for A: cells grown at 28°C to the desired cell density of infection followed by the addition of virus and incubation at 30°C for the production period; and B: cells grown at 28°C to the desired cell density of infection, with addition of concentrated nutrient solution at $2x10^6$ cells/ml and $4x10^6$ cells/ml for cultures infected at $4x10^6$ cells/ml or at $2x10^6$ cells/ml and $4x10^6$ cells/ml and $8x10^6$ cells/ml for cultures infected at $8x10^6$ cells/ml. Cells were incubated at 30°C for the production period. Error bars represent the range of titers obtained from duplicate cultures.

nutrients accumulated in the media including cystine, while others, like serine, were consumed rapidly (Figure 6.14). After the addition of virus (and switching the culture to 30°C), the rate of cystine consumption increased, but no measured nutrients were depleted before harvest. Therefore, exhaustion of the measured nutrients may not be the only reason for the reduction in specific AAV functional titer.

6.2.8 Effect of concentrating cells from early exponential growth

In order to assess whether the state of the cell played a role in the production of functional AAV, cells of equivalent "age" were resuspended to various cell densities and infected. Cells were inoculated at $0.5x10^6$ cells/ml and grown to $2x10^6$ cells/ml, which was the target cell "age", given that the maximum specific productions were recorded at this density (Figure 6.7). As can be seen in Figure 6.15, specific production was maintained up to ~9-$10x10^6$ cells/ml, and rapidly declined to ~50% of the maximum specific production at around $1.1x10^7$ cells/ml.

Similar to the cultures that underwent a medium exchange, cultures that were infected ~$1.1x10^7$ cells/ml, had cystine concentrations that were no longer detectable at 48 hpi (Figure 6.16). In addition to the 9 nutrient/metabolites shown in Figure 6.16, threonine was reduced by 25% and 34% beyond the time of infection for cells infected at ~9-$10x10^6$ cells/ml and ~$1.1x10^7$ cells/ml, respectively. Unlike the cultures grown to appropriate densities and then infected, those cultures that had low "age" cells showed more consistent production/consumption rates with increasing cell densities (Figure 6.17). Although the rate of ammonia and lactate synthesis for the "low age" cells were greater than those of "high age" cells (without medium renewal) in the first 24hpi, differences emerged beyond 24 hpi. Alanine did not reach the same elevated concentrations when using "low age" cells compared to "high age" cells (Figure 6.16 vs Figures 6.8 and 6.10). Also, beyond 24 hpi, the rate of ammonia

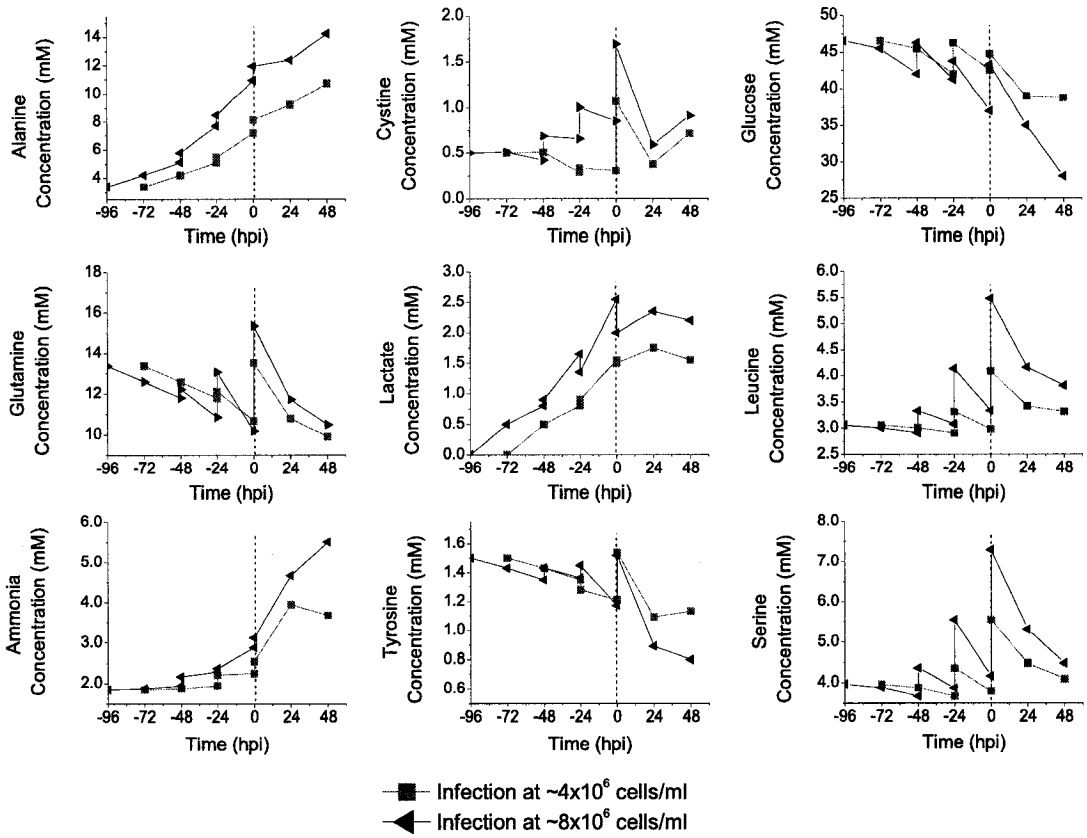

Figure 6.14 Average dynamic profile of nutrients and metabolites of cultures that were fed a nutrient cocktail prior to infection. Nutrients and metabolites shown changed by at least 25% for one of the conditions tested.

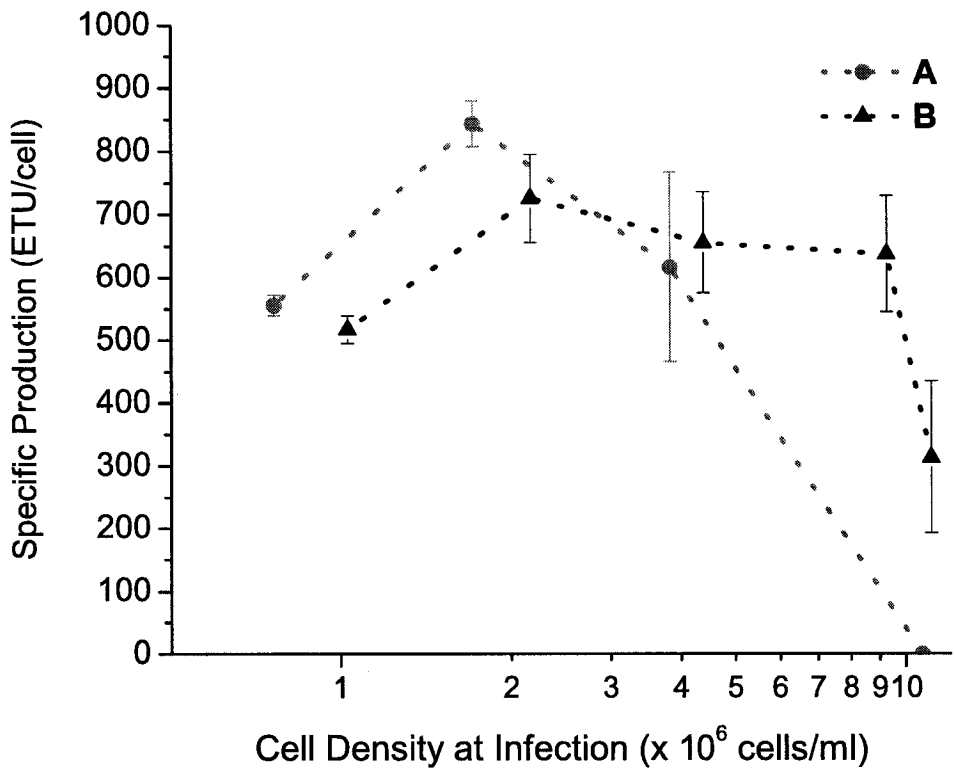

Figure 6.15 Comparison of AAV enhanced transduction titers for A: cells grown at 28°C to the desired cell density of infection followed by the addition of virus and incubation at 30°C for the production period; and B: cells grown at 28°C to $2x10^6$, centrifuged and resuspended to various cell densities in fresh medium containing virus before incubation at 30°C for the production period. Error bars represent the range of titers obtained from duplicate cultures.

synthesis dropped significantly for "low age" cells while the rate remained practically unchanged for "high age" cells. Furthermore, cells grown to $\sim 1.1 \text{x} 10^7$ seemed to have switched from production to consumption of lactate earlier than other cultures.

6.3 Discussion

6.3.1 Vector stability, selection, and production temperature

To study the production of AAV vectors in high cell density insect cell cultures, conditions that were previously shown to promote high specific productions were used. Among these, previous work by Kohlbrenner et al. (2005a) and Aucoin et al. (2006) that suggested the use of low passage baculovirus vectors, was deemed essential. Therefore, baculovirus vectors were plaque purified, screened and amplified only up to a second passage using low multiplicities of infection. The screening, however, has led to further concerns about the stability of the BacRep vector, which showed a huge variability in protein expression from the different isolates. This has actually been previously reported by Kohlbrenner et al. (2005a); however, in their work, 6 out of the ten clonal isolates displayed very similar levels of rep protein expression, and only 4 with lower and varying levels of rep protein expression. To minimize the heterogeneity, amplified over sequential passages, stocks need to be, if the biology of the construct is not modified, purified and screened rigorously before use. Unlike the BacRep isolates, the BacCap isolates yielded similar protein expression patterns for all examined. BacITR isolates showed some variation in their ability to produce functional AAV but not to the same extent as what was observed with the BacRep construct.

Selection, amplification at low MOIs (0.01) and production at 28°C, one degree higher then what was used in previous reports (Urabe et al., 2002; Aucoin et al.,

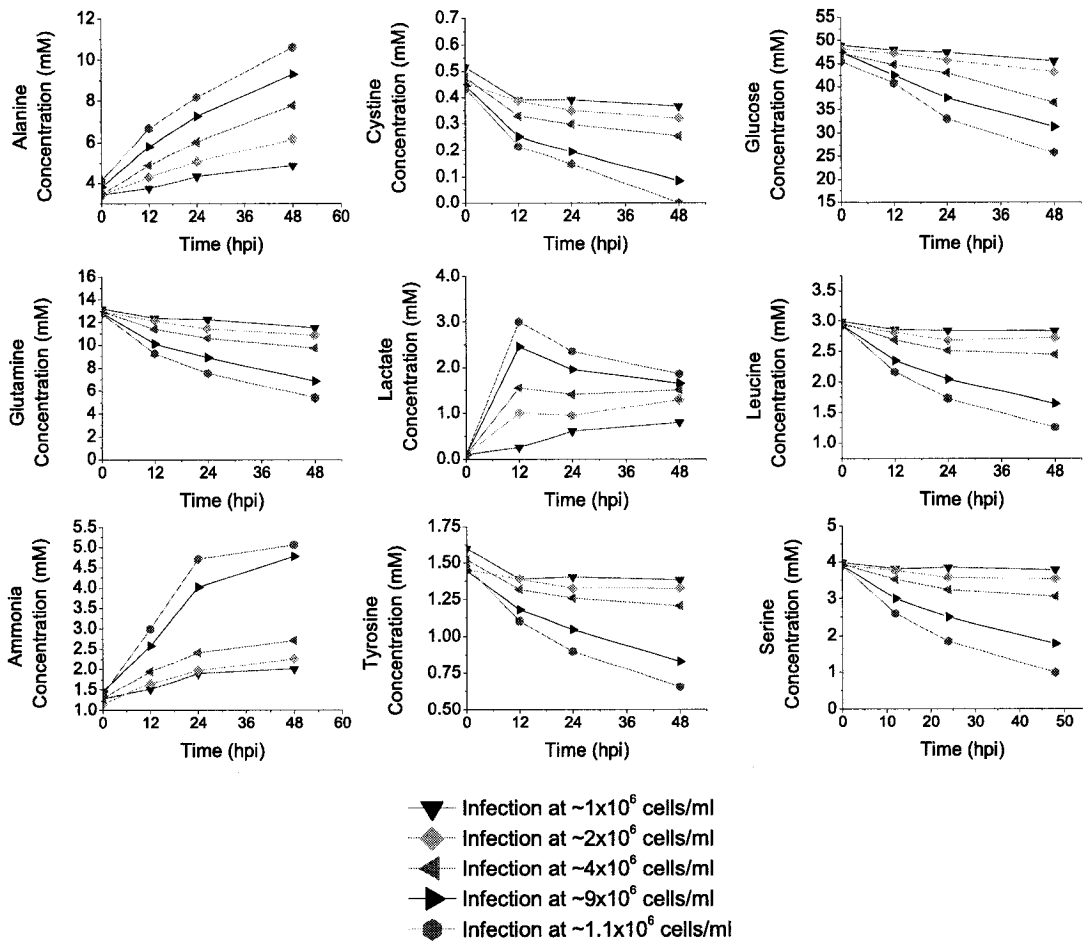

Figure 6.16 Average dynamic profile of nutrients and metabolites post-infection of cells that were concentrated to different cell densities before being infected. Nutrients and metabolites shown changed by at least 25% for one of the conditions tested.

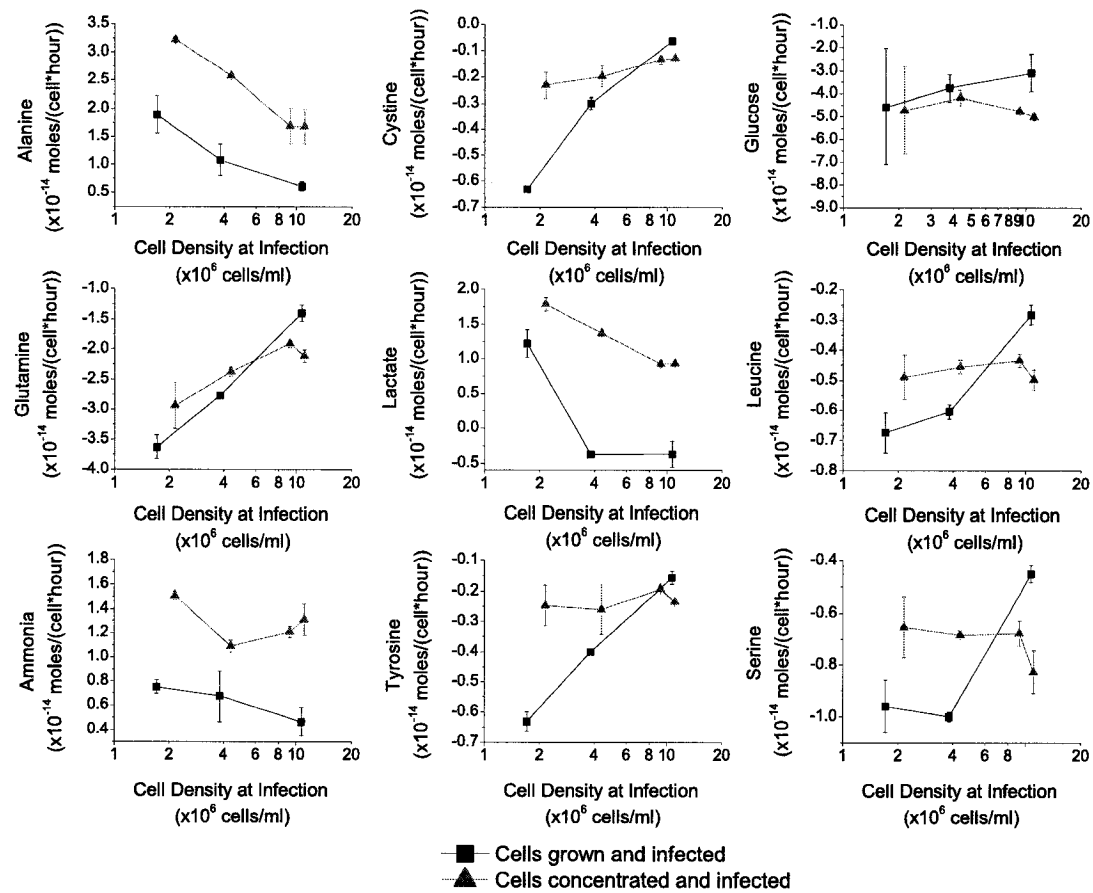

Figure 6.17 Comparison of specific consumption and production rates for the first 24 hpi. Negative values imply consumption and positive values imply production.

2006), increased the specific production of functional AAV ~5-10 times over what was previously reported (Aucoin et al., 2006, 2007b). Increasing the temperature to 30°C resulted in a further increase in the production as previously reported (Aucoin et al., 2007b), however, not to the same extent. The smaller increase may be due in part to an increase in production at 28°C, to a smaller change in temperature, or due to differences in the quality of the viruses used.

6.3.2 Functional AAV recovery

According to Chahal et al. (2007), when harvested insect cells are concentrated to 20x10^6 cells/ml prior to freeze/thaw/heat treatments, less functional AAV are detected then when the cells are concentrated to 10x10^6 cells/ml, indicating a cell density effect on functional AAV recovery. To investigate how this could lead to the misinterpretation of the peak cell density for the production of functional AAV, two sets of samples were taken and analyzed. The first was simply the culture broth. The second was the cell pellet resuspended to a constant density in a high salt buffer. The recovery/infectious assay was compromised at high cell densities when kept in culture media. Titers were highly underestimated at higher cell densities if kept as culture broth, which could in fact lead to improper conclusions about the limitations of the system. It is suspected that the early work by Meghrous et al. (2005) may have suffered from this underestimation, since the present work suggests a higher peak cell density; however, because titers were regained after medium exchange in that earlier study, detection problems could not have been the sole reason for the low peak cell densities previously observed.

6.3.3 Production at high cell densities

In this study, the peak cell density for the production of AAV at 30°C was higher than what was observed for productions kept at 27°C (Meghrous et al., 2005), and is more consistent with the production of other proteins in SF-900 II medium (Bédard et al., 1994; Wong et al., 1996; Tsao et al., 1996; Radford et al., 1997b). Although being higher, decreased production was still observed at high cell densities.

An analysis of the media in terms of amino acids and glucose at the moment of virus addition did not reveal limitations in the measured nutrients at that point. This, however, does not preclude the possibility that other components such as vitamins, hormones, lipids and/or trace elements were limiting. Yeastolate is a good source of many of these unmeasured components being a complex mixture of amino acids, peptides, polysaccharides, vitamins and minerals (reviewed by Shen et al., 2007). Calles et al. (2006) have shown that limitations in yeastolate leads to the synchronization of cells in the G_2/M phase. In this study cells were similarly distributed in each cell cycle phase for the range of densities studied (Figure 6.4), therefore was not indicative of any limitation. The onset of the stationary phase is also a sign of nutrient depletion, however, in this work the growth rate was approximately constant up to $\sim 1 \times 10^7$ cells/ml (data not shown). The cell size, which has also been suggested to be related to the state of the cell (Meneses-Acosta et al., 2001), when normalized to the cell density, had similar distributions for the various cell densities examined (Figure 6.3). The low levels of lactate suggested that oxygen was not limiting for the cells (reviewed by Bédard and Kamen, 1998; Ikonomou et al., 2003) and since ammonia has been reported to be tolerated up to 10 mM (reviewed by Bédard and Kamen, 1998), the levels detected at the time of virus addition (<2.5 mM) was not considered inhibitory. Alanine accumulation has also been shown not to be detrimental to protein production up to 40 mM (Bédard et al., 1994), there-

fore, although alanine accumulated up to 10 mM, it was also not considered to be inhibitory at the point of virus addition. Of the potential indicators examined, none readily pointed to detrimental conditions before the production phase began, which led to the hypothesis that conditions that limited the production of functional AAV may have occurred beyond the point of virus addition, during the production phase at 30°C.

Of all the nutrients/metabolites measured, only a small subset changed significantly ($\geq$25%) in the 48 hpi that was used to produce functional AAV; these changes (those that were greater than 25%) were generally observed only for cultures infected at densities greater than $4x10^6$ cells/ml. The amino acids that changed less than 25% were considered to be in non-limiting quantities and were, therefore, not presented in this chapter.

As of 24 hpi, serine levels for the culture infected at $\sim 1.1x10^7$ cells/ml, were reduced below the limit of detection and therefore assumed to be exhausted. Although Sf-9 cells have been reported to grow in the absence of serine (Tremblay et al., 1992), it is readily consumed in both non-infected and infected cultures (Radford et al., 1997b; Tom et al., 1995). Serine is used for energy production (Drews et al., 1995) but has also been shown to be a significant "one-carbon" donor to both purines and thymidine (Tremblay et al., 1992). Generally, it has not been reported to be the first amino acid to be exhausted, and in the work done by Radford et al. (1997b), cystine was the first amino acid to be depleted for both uninfected cultures and cultures infected at $\sim 5x10^6$ cells/ml. Although the study presented here used the same medium, SF-900 II, the levels of serine detected were half of what were described by Radford et al. (1997b), with initial concentrations of 4 mM instead of 6-10 mM. This observation may explain in part why serine was the first amino acid to be exhausted. Speculatively, the fact that a DNA viral vector is being produced, and extensive viral genomic replication occurs (especially in the first 24 hours), this may in part

explain an increased need for serine.

Medium renewal is thought to positively affect the culture either by increasing the level of limiting nutrients or by removing inhibitory by-products. In this study, when medium was renewed prior to infection, serine was no longer depleted prior to harvest; however, cystine was exhausted sometime between 24 and 48 hpi. Since the depletion occurred later in the culture, a significant amount of functional AAV was still produced, albeit only at a fraction of the maximum specific production. Although early work suggested that insect cells were auxotrophic on cysteine, these assertions have been reversed by work done by Doverskog et al. (1998) that showed that cells could be adapted to cysteine/cystine free medium. Cysteine, however is an essential amino acid used in the synthesis of protein and glutathione, and although the cell is able to produce it from methionine (and serine), its biosynthesis is down-regulated late in culture (Doverskog et al., 1998). It has also been reported that in culture, once cystine is depleted, consumption of all other amino acids ceases (Radford et al., 1997b). Furthermore, the specific uptake rate and consumption of cystine and other nutrients have been found to be dependent on the level of cystine in the medium. This is consistent with our observation of increased consumption rate of cystine upon medium renewal.

It is generally accepted that Sf9 cultures are not limited by inhibiting components, which is why there has been a significant amount of work done on fed-batch insect cell cultures. In fact, Bédard et al. (1997) have shown, using a cocktail of nutrients to supplement the medium, that specific production could be maintained beyond a cell density of 11.5×10^6 cells/ml. Although the culture is not known to be significantly inhibited by the addition of supplementary nutrients, the overall osmolality of the medium has been reported to negatively impact production levels (Elias et al., 2000). To maintain the osmolality below 400 mOsm, increase nutrient levels for the production phase and mimic the semi-continuous feeding regimen described by

Elias et al. (2000), a pseudo-exponential feed consisting of pulses (since the experiments were done in shake flask) was added to cells during the growth phase. An essential element of the cocktail used is the yeastolate ultrafiltrate, which has been shown to significantly enhance production levels at high cell densities when used to supplement SF-900 II (Bédard et al., 1994). Although there was production of functional AAV, the specific production was still only ~50% of the maximum. It has been hypothesized that the time of cocktail addition may be just as important as the addition itself. It may be possible that the feed, which also contained lipids, vitamins and other trace elements, was started too early and caused an inhibition early in the culture. On-line monitoring tools have the potential to help identify and predict key times for nutrient addition, yet there remains many challenges in the use of existing indicators. Furthermore, there may exist potential to reduce the number of components in the cocktail; but this has been the existing challenge for nearly two decades. For example cultures fed only "limiting" amino acids became limited in yeastolate components (other than amino acids) (Kim and Park, 1999). Furthermore, yeastolate is a complex mixture, whose consistency is variable from lot to lot. This makes it more difficult to simplify feed mixtures that contain yeastolate. Recent progress in identifying the growth-promoting components of yeastolate suggest that the effect may be due to large peptides (Shen et al., 2007); however, more work needs to be done in this area.

Whether it be due to the cell "age", cell density or simply the nutrient levels, the consumption rates post-infection decreased for increasing cell densities. To further assess and segregate the effect of cell density on the production of functional AAV, cells of the same "age" were resuspended in equivalent media at various cell densities. Similar to the previous experiment on medium renewal, cultures infected at ~1.1×10^7 cells/ml in this experiment, also depleted cystine between 24 and 48 hpi. Unlike the earlier study with medium renewal, concentration of the cells resulted in ~3.5 times

more functional AAV. Although it may be due to later depletion of cystine (sometime between 24 and 48 hpi), the capacity of maintaining the specific production up to an infection density of ~9x10^6 cells/ml suggests that indeed there is an unidentified cellular state that changes over time. Calles et al. (2006) recently reported that the addition of conditioned media to low passage cells could improve their proliferation, while the same quantity of conditioned media negatively impacted the production of recombinant protein (by up to 50%). It is therefore plausible that the conditioning of the medium results in changes in the cells that cause them to be less productive. Many questions remain, but one that seems to be the most relevant is at what point do the medium or the cell become conditioned? With doubling times of less than a day, the operational window to target a cell density between 4 and 10x10^6 in batch or fed-batch operation is small, which therefore requires greater control over the cells. One way that this control may be achieved, as well as reducing the "cell age" at infection, could be through a semi-perfusion scheme, whereby media is removed from the reactor thereby concentrating the cells as they grow.

Concluding remarks

Although the stability of the BacRep vector remains an issue, the volumetric production of AAV using insect cells is greater than other available systems. The work presented here suggests that "low age" cells or cells that have not been "conditioned", can maintain the specific production of AAV up to ~1x10^7 cells/ml. This translates to ~6.5x10^{12} functional AAV particles/1L bioreactor. Although it is feasible to perform medium renewal at larger scale, this mode of operation increases the likelihood of contamination and requires specialized equipment. Therefore, processes that rely on cell retention devices that could allow the *in situ* concentration or perfusion should be further studied for the production of AAV.

OVERALL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

The production of AAV vectors in insect cells has, as do all other production methods, its advantages and its disadvantages. The high titers achieved and the scalability of the system, however, favor the continued study of this system to bring it to the forefront of production methods.

Unlike methods that rely on transient transfection, which require the cultivation of *E. coli* for the production of plasmid DNA in a first step, the use of the baculovirus/insect cell system can rely on a single cell line and single vector system for both vector stock preparation and AAV production. The use of the baculovirus/insect cell system also foregoes the use of components from pathogenic viruses, often required in mammalian based systems.

To fully characterize the process, the baculovirus/insect cell system was analyzed for AAV replication proteins, structural proteins, assembled capsids, DNase-resistant particles, and functional particles. Functional particles, assessed by an enhanced transduction assay, were given the most weight in this work. Enhanced transduction assays often underestimate the true number of functional particles, however, the consistency (low %CV), relative ease of the assay and cost, were most appropriate for the large number of samples analyzed.

Of all components involved in the synthesis of an AAV vector, the replication proteins play the most important role. In this work, it is still not clear whether all four AAV replication proteins (Rep78, Rep68, Rep52 and Rep40) are produced. Rep68 and Rep40, which are translated from spliced Rep78 and Rep52 mRNA respectively, were generally not observed on SDS-PAGE gels. Differences in temporal expression between Rep78 and Rep52 occur since Rep78 is under an early stage promoter

while Rep52 is under a very late stage promoter. This results in low levels of Rep78 expression observed at harvest. Most Rep78 bands were unquantifiable at 72 hpi. On the other hand, Rep52 was always readily detected. A problem with Rep52 expression, however, was that upon increased expression multiple bands appeared below the 52 kDa molecular weight marker (of which one may or may not be Rep40). It has been hypothesized that these products may be the result of degradation of replication proteins that lack association with an AAV capsid, since increased viral particles decrease the appearance of these smaller proteins. Since Rep52 was consistently quantifiable at harvest, its level was compared to other measurements. Levels of Rep52 expression are correlated to the number of functional AAV vectors, albeit weakly. Overexpression of replication proteins was observed when using the two monocistronic BacRep vectors to supply the replication protein genes, and when the bicistronic BacRep vector and the BacCap vectors were not added in equal concentrations. This results in lower functional AAV titers.

As with the production of other proteins using the BEVS, the multiplicity of infection was shown to be a critical parameter. Varying the baculovirus ratios can affect the functional AAV vector yield by an order of magnitude (a factor of 10). Increasing the amount of BacRep and BacCap added to the system increases the production of functional AAV vectors as long as both are increased. Above individual MOIs of 10, the cellular machinery is thought to be overwhelmed, and there is an observed saturation in the number of functional AAV produced. When two monocistronic BacRep vectors are used (quadruple infection) the balance is more complicated and is not achieved by using equal MOIs of each BacRep vector and BacCap vector. Maintaining the overall baculovirus load by reducing the quantity of each monocistronic BacRep vector by half improves the production, but further improvement is expected with increased capsid production. Further optimization of the concentrations of monocistronic BacRep vectors used could be done to improve

vector production if this system is chosen.

Overexpression of capsids is needed for the highest functional AAV titers, but not at the expense of the replication proteins. In fact, production of functional AAV is sustained as long as the cell remains viable, however, capsid synthesis is correlated to the viability of the insect cells. Therefore, the synthesis of functional AAV has to be a rapid process so that as many functional vectors can be made before the cells become non-viable. Increasing the temperature at the time of infection from 27°C to 30°C, increases expression of Rep78 and allows an earlier on-set of Rep52 protein expression. These increases accompanied increases in functional AAV synthesis, without sacrificing the number of capsids synthesized significantly. Furthermore, although increasing the temperature to 30°C at any point before 24 hpi results in increased functional vector, the greatest increases occur when the temperature is increased before 6 hpi.

The early synthesis of replication proteins is important to the production of functional AAV vectors. Adding both BacCap and BacITR to the cell culture in quantities that cause a synchronous infection of the culture and delaying the addition of BacRep causes a dramatic drop in the production of functional AAV. Delaying the addition by twelve hours results in a decrease of more than three orders of magnitude in functional AAV. On the other hand, a 12 hour delay in the addition of BacITR lowers the functional titer by less than one order of magnitude. The concentration of BacITR at the moment of infection affects the production the least. This is believed to be due to the fact that the BacITR serves only as a vector to transport the AAV vector genome to the cell. It is the replication proteins, or more precisely Rep78, that is needed for the excision from the baculovirus genome and for the subsequent replication of the AAV vector genome. This is why little effect is seen when varying the MOI of BacITR above a minimum threshold.

Although a delay in the addition was shown to yield lower results, the production of functional AAV suggests that given an appropriate indicator within the first 12 hours after the addition of virus, the culture can be "saved" to a certain extent by further addition of baculovirus vectors. In this study, however, no such indicator was observed. Granted, regulatory agencies may not even allow this type of feedback control for material geared towards human testing, for larger scale productions, contingency strategies to maximize output when routine operation go amiss are needed. Similarly, when viabilities are high at the time of harvest, increased titers can be achieved by extending the duration of the infection period, although highest yields are generally accompanied with rapid decreases in viability.

The volumetric production of functional AAV can further be increased by increasing the cell density at infection; however there exists some limitations. Cells grown to very high cell densities before infection have lower production of functional AAV per cell. These lower productions are accompanied by depletion of serine. Renewal of the medium by replacing spent media with fresh media at the time of infection, increases the specific production but does not completely restore the specific production to levels achieved at low CCI. Although serine is not depleted after medium renewal, cysteine levels are. The cysteine consumption rates increase with medium renewal but are not as high as when the cells are at a lower cell density. This may be an indication that the state of the cell is different at higher cell densities, although other markers such as the proportion of cells in each cell cycle phase and the cell size distribution did not resolve these differences. Furthermore, feeding the cells alleviated the depletion of nutrients but did not completely restore the specific production. Using cells from the early exponential phase, concentrating and resuspending them in fresh media restored the specific production of functional AAV up to a cell density of $\sim 1 \times 10^7$ cells per ml. Beyond this point, specific production of functional AAV decreased and was accompanied by the depletion of cysteine.

The sustained production suggests that the MOI is independent of the cell density as long as the cells are similar. Production at high cell densities, therefore, also require a proportional increase in baculovirus added to the system. This increase will require the concentration of viral stocks to minimize the volume of spent media added. At the scale used in this work, ultracentrifugation is a practical, quick and efficient method to concentrate the viral stocks; however, at much larger scale, other methods such as tangential flow filtration will need to be explored and characterized for efficient use of high cell density cultures. The concentration step does lengthen the overall process especially with the preparation of three vector stocks.

The use of monocistronic BacRep vectors would further require a fourth baculovirus stock, however, it is currently the only method that ensures stability in the BacRep vectors over multiple passages. Depending on the scale of production desired, the bicistronic BacRep may not be suitable. The BacRep instabilities manifest themselves in a significant passage effect that results in a reduction in replication protein expression upon serial passaging. The reduction in replication protein expression over multiple passages is correlated to a reduction in functional AAV titer. Furthermore, the decrease in replication protein expression is most likely due to an increasing heterogeneity in the virus population. This was observed upon plaque purification of the viral stock.

The effect is minimized when a minimal amount of passaging is used. The importance of minimizing the number of passages for the baculovirus stocks, has led to the estimation of the number of AAV vectors that could be produced using P2 baculoviruses. Generally, in the work done for this thesis, plaque purified material was amplified to 6 ml (P1). 6 ml of P1 can be amplified to 100 L using an MOI of 0.01 (the MOI used for virus amplification in this thesis). 100 L of baculovirus stock is enough to allow either a 1000 L production at a cell density of $2x10^6$ cells/ml or a 200 L production at $1x10^7$ cells/ml. These productions would result in $\sim 1x10^{15}$

ETU (functional AAV vectors) or $1x10^{17}$ DRP, which would be sufficient material to supply large clinical trials. It is estimated a campaign to produce this amount of material (given the appropriate equipment), would take approximately 4 months after an initial screening and selection of the baculoviruses to be used. The time line is based on sequential operation and could be reduced if certain operations were ran in parallel such as the baculovirus stock generation.

On the molecular biology side of this system, improving the bicistronic BacRep construct is needed. Recent reports have suggested the incorporation of homologous region sequences could enhance the stability of Bac-to-Bac constructs. It would also be of interest to modify the construct to increase the expression of Rep78. In this thesis, increasing the temperature to 30°C resulted in increased Rep78 expression, which suggests that the quantity of Rep78 may be suboptimal, given the level of capsids produced. In a first attempt to explore this idea, the truncated immediate early promoter used to control Rep78 was replaced with a non-truncated version (in collaboration with Gilles St-Laurent and Yves Durocher of the Biotechnology Research Institute). Preliminary results indicate similar AAV production levels, however, a detailed examination of the baculovirus or expression levels have not been completed. This area has the potential to reduce the gap between the number of total particles and the number of functional particles produced.

On the process side, there are two areas that are directly linked to the work at hand. The first is the combination of asynchronous and synchronous infection strategies. The preparation of the three viral stocks, which include amplification and concentration steps is not trivial. Although complete asynchronous infection has been shown by others to yield lower overall yields, it is believed that a combination of asynchronous/synchronous infection strategies could be used. BacITR promises to be the ideal candidate for such a strategy because of the minimal impact it has shown through studies of its multiplicity of infection and its relative time of infection. This

would reduce the pre-production stages by allowing the amplification of BacITR to be done within the same culture as the production of AAV. It would also eliminate concentration of this vector stock. The second area that could be advanced is the high cell density production of AAV. Selective nutrient addition post-infection and the use of perfusion strategies is thought to be key in achieving production beyond 10^7 cell/ml.

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APPENDIX I

SUMMARY OF VLPS MADE IN INSECT CELLS

Table I.1: Virus-like particles produced in insect cells

TYPE OF VIRUS	REFERENCE
Astroviridae	
Human astrovirus serotype 1	Caballero et al. (2004)
Birnaviridae	
Infectious bursal disease virus (IBDV)	Hu and Bentley (2000)
	Hu and Bentley (2001)
	Hu et al. (1999)
	Kibenge et al. (1999)
	Martinez-Torrecuadrada et al. (2000)
	Wang et al. (2000)
Bunyaviridae	
Hantaan virus	Betenbaugh et al. (1995)
Caliciviridae	
Hawaii human calicivirus	Green et al. (1997)
Hepatitis E	Li et al. (2005)
	Li et al. (1997b)
	Li et al. (2000)
	Xing et al. (1999)
Human enteric caliciviridae (SRSV)	Dingle et al. (1995)
Human calicivirus MX virus (Mexican strain)	Jiang et al. (1995)
Norwalk virus	Ball et al. (1998)
	Bertolotti-Ciarlet et al. (2002)
	Jiang et al. (1992)
	White et al. (1997)
Porcine enteric calicivirus	Guo et al. (2001)
Rabbit hemorrhagic disease virus (RHDV)	Laurent et al. (1994)
	Plana-Duran et al. (1996)
Toronto virus	Leite et al. (1996)

Table I.1: (continued)

Type of Virus	Reference
Coronaviridae	
Human Coronavirus - SARS	Ho et al. (2004)
	Mortola and Roy (2004)
Flaviviridae	
Hepatitis C	Baumert et al. (1998)
	Baumert et al. (1999)
	Baumert et al. (2000)
	Choi et al. (2004)
	Xiang et al. (2002)
	Zhao et al. (2004)
Hepadnaviridae	
Hepatitis B	Kang et al. (1987)
	Takehara et al. (1988)
Nodaviridae	
Flock house virus	Fisher et al. (1993)
Orthomyxoviridae	
Influenza virus	Galarza et al. (2005)
	Latham and Galarza (2001)
	Pushko et al. (2005)
Papovaviridae	
B-lymphotropic papovavirus	Pawlita et al. (1996)
Bovine papillomavirus (type 1)	Kirnbauer et al. (1992)
Cottontail rabbit papillomavirus	Breitburd et al. (1995)
	Kirnbauer et al. (1993)
Equine papillomavirus (type 1)	Ghim et al. (2004)
Human papillomavirus (type 6, 11, 16, 33)	Christensen et al. (2001)
	Christensen et al. (1994b)
	Fang et al. (2000)
	Kirnbauer et al. (1992)
	Kirnbauer et al. (1993)
	Rose et al. (1993)
	Sadeyen et al. (2003)
	Touze and Coursaget (1998)

Table I.1: (continued)

Type of Virus	Reference
	Volpers et al. (1994)
Murine polyomavirus	An et al. (1999)
	Delos et al. (1993)
	Forstova et al. (1995)
	Forstova et al. (1993)
	Gillock et al. (1997)
	Montross et al. (1991)
Simian virus (type 40)	Kosukegawa et al. (1996)
JC virus	Chang et al. (1997)
	Goldmann et al. (1999)
	Goldmann et al. (2000)
Parvoviridae	
Adeno-associated virus	Aucoin et al. (2007a)
	DiMattia et al. (2005)
	Hoque et al. (1999)
	Lane et al. (2005)
	Ruffing et al. (1992)
Aleutian mink disease parvovirus	Christensen et al. (1993)
B19	Brown et al. (1991)
	Heegaard et al. (2002)
	Kajigaya et al. (1991)
	Rosenfeld et al. (1992)
	Sico et al. (2002)
	Tsao et al. (1996)
Canine parvovirus	Gilbert et al. (2004)
	Lopez de Turiso et al. (1992)
	Saliki et al. (1992)
Mink enteritis parvovirus	Christensen et al. (1994a)
Parvovirus minute virus of mice	Hernando et al. (2000)
Porcine parvovirus	Maranga et al. (2003)
	Maranga et al. (2004)
	Maranga et al. (2002b)
	Maranga et al. (2002c)
	Martinez et al. (1992)

Table I.1: (continued)

Type of Virus	Reference
	Rueda et al. (2001)
Picornaviridae	
Enterovirus (type 71)	Hu et al. (2003a)
Poliovirus	Brautigam et al. (1993)
	Urakawa et al. (1989)
Reoviridae	
Bluetongue virus	Belyaev et al. (1995)
	Belyaev and Roy (1993)
	French et al. (1990)
	French and Roy (1990)
	Hyatt et al. (1993)
	Le Blois et al. (1991)
	Loudon and Roy (1991)
	Roy et al. (1994)
	Zheng et al. (1999)
Epizootic hemorrhagic disease virus	Le Blois et al. (1991)
Rice dwarf virus (RDV)	Hagiwara et al. (2003)
Rotavirus	Charpilienne et al. (2001)
	Conner et al. (1996)
	Crawford et al. (1994)
	Frenchick et al. (1992)
	Jiang et al. (1998)
	Kim et al. (2002)
	Labbe et al. (1991)
	Mena et al. (2005)
	O'Neal et al. (1997)
	Palomares et al. (1999)
	Palomares et al. (2002)
	Park et al. (2004)
	Redmond et al. (1993)
	Sabara et al. (1991)
	Vieira et al. (2005)

Table I.1: (continued)

Type of Virus	Reference
Retroviridae	
Feline immunodeficiency virus	Morikawa et al. (1991)
Human immunodeficiency virus (type 1, 2)	Adamson et al. (2003b)
	Ako-Adjei et al. (2005)
	Cruz et al. (1998a)
	Cruz et al. (1999)
	Cruz et al. (1998b)
	Gheysen et al. (1989)
	Hong and Boulanger (1993)
	Kang (1995)
	Lerch and Friesen (1992)
	Luo et al. (1992)
	Luo et al. (1990)
	Tobin et al. (1997)
	Zhao et al. (1994)
Murine leukaemia virus	Adamson et al. (2003a)
Rous sarcoma virus	Ako-Adjei et al. (2005)
Totiviridae	
Leishmania RNA virus	Cadd and Patterson (1994)
	Ro et al. (2004)

APPENDIX II

MATLAB CODE

Italicized text are comments in the code and should be preceded by % in Matlab.

LowMOI.m

The purpose of this code is to simulate a baculovirus infection that starts with the addition of a small quantity of BacITR and to determine when would it be optimal to add BacRep and BacCap for the production of AAV. The code is based on the work done by Hu and Bentley.

```
clear all
close all

global vmax jhigh tauvhigh tauvlow delta endtime umax Ks Yxs kd td deltatau kdinf al

Experimental values upon which calibration of the model will be done

CellTime=   [0 6 24 29 47 54 71];
CellTotal=  [1441176 1662941 2475882 3407058 3960588 4161764 4499411];
Inf=        [0 2494 36395 557054 3942765 4107661 3947333];
VirTime =   [0 24 29 47 54 71];
VirReal=    [14411 744555 2074383 246827765 551427009 880852228];

Parameters from literature
```

umax=0.033; *(h^{-1})*

Yxs=8.46e5; *(cells/mM glutamine) (Power et al., 1994; Hu and Bentley, 2001)*

Ks=3; *Monod constant (mM) (Radford et al., 1997b)*

kd=0.0008; *cell death constant pre-infection (h^{-1}) (Hu and Bentley, 2001)*

kdinf=0.02; *cell death constant post-infection (h^{-1}) (Hu and Bentley, 2001)*

al=0.07; *attachment coefficient (this work)*

jhigh=20; *number of viruses above which the metabolic burden does not change (Hu and Bentley, 2001)*

tauvlow=12; *number of hours post-infection (individual times of infection) when a cell will start to produce progeny virions (h)*

tauvhigh=20; *number of hours post-infection (individual times of infection) that a cell will continue to produce progeny virions*

kv=100; *rate at which baculovirus is produced in the cell (pfu/cell/hr)*

deltatau=36; *(h) (Hu and Bentley, 2000))*

vmax=300; *(Hu and Bentley, 2001)*

td=60; *time when death will occur as a function of the initial MOI (Hu and Bentley, 2000)*

Initial conditions (to be modified according to process chosen)

```
delta=1;                    time step
endtime=96;                 when culture simulation ends
Xinf(1)=0;
Xnon(1)=CellTotal(1);
Xtotal(1)=Xinf(1)+Xnon(1);
Bac(1)=VirReal(1);
BacAds(1)=1;                to initiate adsorption a first time step post infection
Xfree(1)=Xnon(1);
Deadinf(1)=0;
Xdead(1)=0;
S(1)=13;                    mM
```

Start of overall loop (calculations for every time step post infection)

```
for t=1:delta:endtime+1
waitbar(t);
Time(t)=t-1;
DynMOI=dMOI(al,Bac(t),Xnon(t));
if DynMOI>2000
Xnon(t)=1;
else
Xnon(t)=Xnon(t);
end

if Xnon(t)>=1
DynMOI=dMOI(al,Bac(t),Xnon(t));
[amin, amax]=Bacminmax(DynMOI);
Probinf=0;
for a=amin:amax
P=Prob(a,al,Bac(t),Xnon(t));
Probinf=Probinf+P*Xnon(t);
end
dXnon(t)=(umax*S(t)/(S(t)+Ks)-kd)*Xnon(t)-Probinf;
else
dXnon(t)=0;
end
```

Bac adsorption

```
if t==1
z=2;
else
z=t;
end
if BacAds(z-1)>=1e-10
```

```
DynMOI=dMOI(al,Bac(t),Xnon(t));
[amin, amax]=Bacminmax(DynMOI);
Probinfa(t)=0;
for a=amin:amax
Pnow=Prob(a,al,Bac(t),Xnon(t));
Probinfa(t)=Probinfa(t)+a*Pnow*Xnon(t);
end
BacAds(t)=Probinfa(t);
else
BacAds(t)=0;
end
```

Bac Production

```
if Time(t)>=tauvlow
if BacProd(t-1)¿=0
BacTerm1=0;
for timepostinf=tauvlow:tauvhigh+deltatau
if t>timepostinf
PriorMOI=dMOI(al,Bac(t-timepostinf),Xnon(t-timepostinf));
[aminprior, amaxprior]=Bacminmax(PriorMOI);
for art=aminprior:amaxprior
Pprior=Prob(art,al,Bac(t-timepostinf), Xnon(t-timepostinf));
if (Time-timepostinf)¿=0
if art>jhigh
if art>=1
BacTerm1= BacTerm1 + effectbud(art, kv)*(deltatau-timepostinf+tauvlow)...
/deltatau*S(t)/(Ks+S(t))*Pprior*Xnoninf(t-timepostinf);
else
BacProd(t)=0;
end
else
```

```
BacProd(t)=0;
end
end
end
end
end timepostinf
end
BacTerm2=0;
for bart=1:jhigh
tauvlowprime=tauv(bart);
efb=effectbud(bart, kv);
for timepostinf=tauvlowprime:tauvlowprime+deltatau
if t>timepostinf
Pprior=Prob(bart,al,Bac(t-timepostinf),Xnon(t-timepostinf));
BacTerm2=BacTerm2+efb*(deltatau-timepostinf+tauvlowprime)/deltatau*S(t)...
/(Ks+S(t))*Pprior*Xnon(t-timepostinf);
end
end


end
BacProd(t)=BacTerm1+BacTerm2;
else
BacProd(t)=0.1;
end


dBac(t)=BacProd(t)-BacAds(t);


if t<td
dDeadinf(t)=0;
else
dDeadinf(t)=kdinf*Xinf(t);
end
```

```
if t<0
dXinfGrow(t)=umax*S(t)/(S(t)+Ks)*Xinf(t);  to allow some growth post-infection if necessary
else
dXinfGrow(t)=0;
end


Term=0;
if Xnon(t)>Xnon(1)*2e-5
DynMOI=dMOI(al,Bac(t),Xnon(t));
[jmin, jmax]=Bacminmax(DynMOI);
for just=jmin:jmax
Pnow=Prob(just,al,Bac(t),Xnon(t));
Term=Term+Pnow*Xnon(t);
end
else
Term=0;
end


dXinf(t)=Term-dDeadinf(t)+dXinfGrow(t);


Xinf(t+1)=Xinf(t)+dXinf(t); Deadinf(t+1)=Deadinf(t)+dDeadinf(t);


if t<td
dXdead(t)=kd*(Xnon(t)+Xinf(t));
else
dXdead(t)=kd*Xnon(t)+dDeadinf(t);
end
```

```
Xdead(t+1)=Xdead(t)+dXdead(t);
qs = umax/Yxs*S(t)/(Ks+S(t));


dSdt(t)=-qs*(Xnon(t)+Xinf(t));
if S(t)<0
S(t)=0;
else
S(t)=S(t);
end


if Time(t)¿1
if S(t-1)>=0
S(t+1)=S(t)+dSdt(t);
else
S(t+1)=0;
end
else
S(t+1)=S(1);
end


Xnon(t+1)=Xnon(t)+dXnon(t);


Bac(t+1)=Bac(t)+dBac(t);
if Bac(t+1)<=0
Bac(t+1)=1e-14;
else
Bac(t+1)=Bac(t+1);
end
Xtotal(t+1)=Xinf(t+1)+Xnon(t+1);
end  End of main loop for timepost infection
```

```
Time(t+1)=t;
```

Bacminmax.m

```
function [min, max]=Bacminmax(DynMOI)


if DynMOI<20
min=0;
elseif DynMOI>=20 & DynMOI<500
min=round(DynMOI-20);
elseif DynMOI>=500
min=0;
end


if DynMOI<2.5
max=6;
elseif DynMOI>=2.5 & DynMOI<5
max=12;
elseif DynMOI>=5 & DynMOI<500
max=round(DynMOI+20);
elseif DynMOI>=500
max=0;
end
```

dMOI.m

```
function dynMOI=dMOI(al,Vi,Ni)
dynMOI=al*Vi/Ni;
```

effectbud.m

this function caculates the rate of budding which is dependant on the number of "effective viruses"

```
function efbtemp=effectbud(vir, kv)
```

```
global vmax
if vir<=vmax
efbtemp=kv*log10(vir+1)
else
efbtemp=log10(vmax);
end
```

Prob.m

```
function Pinf=Prob(a,al,BacITR,Xnoninf)
DynMOIITR=al*BacITR/Xnoninf;
if a==0
Pinf=0;
else
Pinf=exp(a*log(DynMOIITR)-DynMOIITR-gammaln(a+1));
end
```

tauv.m

```
function tv=tauv(a)
global jhigh tauvhigh tauvlow
if a<=jhigh
tv=tauvhigh-round((tauvhigh-tauvlow)/(jhigh-1)*(a-1));
else
tv=tauvlow;
end
```

APPENDIX III

FED-BATCH/COCKTAIL FORMULATION

Table III.1 Cocktail Composition*

Component	Volumetric Composition (mL)
Amino Acid Concentrate	135
Tyrosine Solution (1.39 g L-Tyrosine Disodium Salt in 50 ml H_2O)	7.5
Vitamin/Trace Element Solution	8.75
Glucose Concentrate (500g/L)	15
Lipid Emulsion	22
Yeastolate	56.25

*for a 2.5L culture

Table III.2 Amino Acid Concentrate Composition

Component	Concentration in g/L
Leucine	3.279
Isoleucine	2.521
Arginine	7.839
Glutamate	6.696
Tryptophan	0.715
Glycine	0.711
Histidine	1.552
Lysine	4.211
Methionine	2.238
Phenylalanine	1.552
Serine	8.504
Threonine	3.573
Valine	4.099
Proline	4.604
Cysteine	5.636
Aspartate	1.646
Glutamine	26.298

Table III.3 Vitamin/Trace Element Solution

Component	Concentration (mg/L)
Thiamine HCl/2H2O	0.082
Riboflavin	0.08
D-Ca Pantothenate	0.008
Pyridoxine HCl	0.4
para-aminobenzoic acid	0.32
Nicotinic acid	0.16
I-Inositol	0.4
Biotin	0.16
Choline chloride	20
Vitamin B12	0.24
Folic Acid	0.087
Molybdic acid ammonium salt	0.04
Cobalt Chloride hexahydrate	0.05
Cupric chloride	0.16
Manganese chloride	0.02
Zinc chloride	0.04
$FeSO_4 \cdot H_2O$	0.55
Aspartic acid	0.356

Table III.4 Lipid Emulsion

Component	Volumetric Composition (mg/ml ethanol)
Cholesterol	4.5
α-tocopherol acetate	2
Cod liver oil fatty acid methyl esters	10
Tween 80	25