An Engineering Analysis of the Insect Cell - Baculovirus Expression System

Thesis by

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ABSTRACT

Baculovirus expression vectors are routinely used for the expression of heterologous proteins, affording the user relatively high product yields and a variety of post-translational modifications. The insect cell-baculovirus system utilizing the *Autographa californica* multicapsid nuclear polyhedrosis virus has been studied here with regard to product yield and product quality. Product yield was demonstrated to be affected by both the time of infection and the multiplicity of infection. Through experimental results and population modeling of infected cultures, the product yield was maximized by infecting in the early-exponential growth phase with a low multiplicity of infection.

The importance of intracellular protein degradation on cloned protein yield and product quality in this system was also studied. Intracellular degradation was observed throughout the infection; however, when compared to the synthesis rate, degradation was found to have an insignificant effect on system productivity. A population of discrete ß-galactosidase fragments was observed in protein-radiolabelling experiments. The synthesis rate of these fragments compared to the degradation rate of the complete ß-galactosidase molecule suggested that they are probably not a result of intracellular protein degradation. Although proteolytic origin cannot be excluded, transcript hybridization analysis suggests that premature transcript termination may be the cause. It is believed that after premature transcription termination, the transcript is translated to yield the fragmented polypeptides. Other evidence using different recombinant viruses, all expressing related forms of ß-galactosidase, are consistent with the hypothesis of premature transcription termination. Although the synthesis of ß-galactosidase was most thoroughly studied, transcript heterogeneity and protein heterogeneity were observed for a variety of recombinant viruses with the heterologous gene under the control of the polyhedrin promoter.

When expressing a heterologous glycoprotein, the insect cell-baculovirus expression system produces a heterogeneous population of glycoforms different from those of authentic mammalian hosts. Four different cell lines that can be used as hosts for baculovirus expression were analyzed for the existence of endogenous exoglycosidases. Cells derived from *Spodoptera frugiperda*, *Trichoplusia ni*, *Bombyx mori*, and *Malacosoma disstria* contained N-acetyl-ß-glucosaminidase, N-acetyl-ß-galactosaminidase, ß-galactosidase, and sialidase activities. These endogenous activities were also observed in the medium from uninfected and wild-type baculovirus-infected cultures. Lectin analysis of insect cell glycoproteins indicates that oligosaccharides formed in these hosts are susceptible to the exoglycosidase activities identified.

On the basis of reports that insect cells lack terminal glycosyltransferases, mammalian ß1,4-galactosyltransferase and α2,6-sialyltransferase were cloned into baculovirus expression vectors. The degree of altered glycosylation resulting from the expression of these enzymes in insect cells, alone or together, will require more sensitive carbohydrate analysis than that provided by lectins.

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CHAPTER I

Introduction

1.1 The Insect Cell - Baculovirus Expression System

In addition to their role as potential biological control agents, baculoviruses have proven to be efficient vectors for the expression of heterologous genes. Nuclear polyhedrosis viruses (NPVs), a subgroup of Baculoviridae (Matthews, 1982), are pathogens with hosts belonging primarily to the Lepidopteran family (Granados and Federici, 1986). A common NPV for the expression of foreign genes is *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV). This virus consists of a 128 kb double-stranded, supercoiled, circular piece of DNA embedded in an occlusion body (OB; Cochran *et al.*, 1982; Smith and Summers, 1978; Smith and Summers, 1979).

The replication strategy of NPVs is unusual in that these viruses possess a biphasic life cycle (Volkman *et al.*, 1976). The first phase is the synthesis of nonoccluded virions capable of budding to the extracellular space. The second phase occurs later in the infection process when virions are embedded within OBs, composed primarily of polyhedrin, a 29 kDa structural protein produced in abundance at this late stage of infection (Vlak and Rohrmann, 1985). In nature, OBs serve to protect the virus from environmental factors and are important in the horizontal transmission of the virus. The nonoccluded virus, unable to exist in the environment, is important in sustaining the infection of the host (Fraser, 1986).

The promoter for the polyhedrin gene has been utilized for the expression of prokaryotic and eucaryotic proteins. Large quantities of recombinant protein may be obtained by replacing the nonessential polyhedrin structural gene with the gene of interest (Smith *et al.*, 1983a; Smith *et al.*, 1983b). After manipulating the virus in this fashion, occlusion bodies are no longer formed. The lack of OB formation is not detrimental to the utility of the system; *in vitro* cultivation is dependent only on the nonoccluded, extracellular form of the virus (Fraser, 1986). The most common insect cell host used for

baculovirus infection in cell culture is derived from *Spodoptera frugiperda* (fall armyworm). *S. frugiperda* cells are amenable to both stationary and suspension culture.

A myriad of recombinant proteins have been expressed in this system, affording a picture, though somewhat incomplete, of the advantages and limitations of this system. The benefits of the baculovirus expression vector systems include high, yet regulated, expression of heterologous genes by use of the strong polyhedrin promoter, the ability to clone large DNA inserts that are due to the expandable nucleocapsid (Pennock *et al.*, 1984), and the occurrence of a broad range of post-translational modifications. Recent reviews outline the variety of proteins produced as well as the modifications that have been observed in the insect cell-baculovirus system (Luckow, 1991; Luckow and Summers, 1988; Miller, 1988).

Protein and RNA processing that have been observed in this system include signal recognition resulting in both secretion (Smith *et al.*, 1983b; Smith *et al.*, 1985; Maeda *et al.*, 1985; Bustos *et al.*, 1987) and localization (Jeang *et al.*, 1987; Matsuura *et al.*, 1986; Coelingh *et al.*, 1987), intron recognition and proper splicing (Jeang *et al.*, 1987), assembly of multimeric proteins, and covalent post-translational modifications including proper disulfide linkages (Estes *et al.*, 1987; St. Angelo *et al.*, 1987), phosphorylation (Jeang *et al.*, 1987; Ollo and Maniatis, 1987; Miyamoto *et al.*, 1985), and glycosylation (Luckow and Summers, 1988; Jeang *et al.*, 1987; Jarvis and Summers, 1989). Although O-linked glycosylation is complete in these systems (Jeang *et al.*, 1987), N-linked glycosylation is variable (Luckow and Summers, 1988; Jeang *et al.*, 1987; Jarvis and Summers, 1989). The advantage of this expression system compared to bacterial systems is the array of post-translational processing that occurs in a manner similar to mammalian systems. In comparison to mammalian expression systems, although the post-translational processes are not identical, baculovirus expression vectors produce considerably greater quantities of cloned proteins with similar biological activity.

1.2 Thesis Motivation

The proper host used in the expression of a heterologous protein may be judged by a number of criteria. Being able to produce an abundance of biologically active proteins, the baculovirus system provides benefits characteristic of both mammalian and bacterial systems. After successful expression of a cloned gene, basic biochemical engineering studies are essential to an efficient bioprocess. Operational variables should be optimized to produce the greatest yields possible, and the quality of the product should be assessed. Although these factors may be influenced by the protein product itself, they are largely attributable to the expression system. Baculovirus expression vectors are routinely used for the expression of cloned proteins; however, the system itself is poorly understood from a biochemical engineering perspective. Little information is provided in the literature regarding basic characterization of the operating system and commonalties of protein products produced in the system. To evaluate the potential of this system, a more thorough analysis of the insect cell-baculovirus expression system is required.

The insect cell-baculovirus system provides the biochemical engineer with a formidable task in designing an operating strategy that in addition to maximizing yields allows reproducibility. Since this system is a multicomponent system, a number of variables not associated with routine animal cell culture exist. Parameters that affect product yield in this system have not been clearly identified or studied. For instance, what is the influence of the infection time and the multiplicity of infection on product yield? In view of the fact that this is a multi-parameter system, mathematical modeling will prove helpful in designing an optimal operating strategy.

Product yield may also be influenced by intracellular protein degradation. Since this system is based on the viral infection of insect cells, it is possible that a cellular stress response is induced at the time of infection. Since foreign protein degradation is often associated with stress response ("heat-shock response"), heterologous protein yields may

be decreased because of degradation. Heterologous protein production in the insect cell-baculovirus expression system culminates in cell lysis. If a stress response is induced, cellular proteases will ultimately be released into the medium. The scenario of heterologous product and cellular proteases coexisting in the abiotic phase for extended periods of time suggests the need for operating strategies other than simple batch processes. In addition to the potential for reduced protein yields, degradation of a cloned protein may also result in product heterogeneity.

Another important concern in the selection of a host for the expression of heterologous glycoproteins is the degree of glycosylation that occurs in the host. Although conflicting reports have been presented regarding the extent of glycosylation of cloned proteins expressed using baculovirus expression vectors, glycoform heterogeneity greater than that occurring in other expression systems is observed (Davidson and Castellino, 1991; Davidson *et al.*, 1990). Although glycoform characterization of proteins produced in this system is in its infancy, possible reasons for the heterogeneity have yet to be investigated. One can envision a degree of heterogeneity arising from oligosaccharide degradation or from the temporal effects of infection. Furthermore, it has been accepted that insect cells lack terminal glycosyltransferases, e.g., α2,6-sialyltransferase. Recent work in mammalian cell lines has demonstrated the ability to use heterologous glycosyltransferases to alter species glycosylation (Lee *et al.*, 1989; Ernst *et al.*, 1989; Larsen *et al.*, 1989). Although similar work has not been done with insect cells, the possibility exists that a similar strategy will prove fruitful.

Although preliminary studies suggest baculovirus expression vectors are ideal tools for the expression of eucaryotic proteins, further characterization of the system is necessary. Product yield and quality and the means to affect these, need to be investigated prior to evaluating this system as a tool of industrial significance.

1.3 Thesis Scope

The objective of this study is to develop an understanding of the insect cell-baculovirus system from a biochemical engineering perspective. The effect of infection time and the multiplicity of infection (MOI) on protein yield are studied in Chapter 2. Although early reports suggest that product yields are not dependent on the MOI or the time of infection (provided infection occurs in the exponential growth phase), in-depth analysis indicates otherwise. A systematic study of these two variables and their importance in determining product yields is presented. The effect of intracellular degradation on protein yields is also discussed in this chapter. A recombinant baculovirus encoding *Escherichia coli* ß-galactosidase is used in these studies.

The experimental results obtained in Chapter 2 regarding the effect of MOI and time of infection on protein yields suggested the utility of mathematical modeling in describing observed trends and in proposing more efficient operating strategies. To model this system effectively, a population model accounting for infection at different times in the growth curve by multiple virions was developed and is presented in Chapter 3. This model provides a mathematical justification to the intuitive arguments presented in the Discussion of Chapter 2. Although the model was developed based on experimental results from a single protein product, it is likely applicable to other proteins synthesized in this system, provided the parameters are adjusted accordingly.

In protein degradation studies, it was observed that heterologous proteins expressed in this system contain a high degree of size heterogeneity. The fragmented proteins produced were originally thought to emanate from intracellular protein degradation. The results presented in Chapter 2 and those presented in Chapter 4 suggest that intracellular protein degradation is not responsible for the size heterogeneity observed. Further characterization of the system suggests that premature transcription termination may be the cause of size heterogeneity. Although unequivocal proof has not

been obtained, the high degree of heterogeneity produced is an important observation and necessitates size-exclusion steps in the downstream purification.

Studies as to possible reasons for glycoform heterogeneity are presented in Chapter 5. A number of exoglycosidases were demonstrated to exist in insect cells susceptible to baculovirus infection. Although *Spodoptera frugiperda* cells are the most commonly used baculovirus hosts, three other cell lines susceptible to baculovirus infection were obtained and analyzed for exoglycosidase activity. All cell lines investigated contained sialidase, ß-galactosidase, and N-acetyl-ß-hexosaminidase activityies. In addition, the activities of these exoglycosidases were present in the medium of uninfected cells and in the medium of wild-type AcMNPV-infected cells.

Chapter 6 investigates means of altering the terminal glycosylation produced in insect cells by the heterologous expression of mammalian glycosyltransferases. Specifically, a rat liver cDNA encoding α2,6-sialyltransferase was expressed using a baculovirus and a transient alteration in glycoforms emanating from this activity was investigated. Lectin-binding results suggesting successful alteration of glycosylation were later determined to originate from the association of serum proteins contained in the medium with cell membranes, despite excessive washing. After adapting the cells to protein-free medium, lectin-binding assays proved limited in detecting altered glycosylation. Similar studies were completed with a mammalian ß-1,4-galactosyltransferase gene. This work demonstrates that altered glycosylation from the expression of mammalian α2,6-sialyltransferase or ß1,4-galactosyltransferase, alone or together, will require more sensitive carbohydrate analysis.

1.4 References

Bustos, M., Luckow, V. A., Griffing, L., Summers, M. D., and Hall, T. C. 1987. Expression, glycosylation, and secretion of phaseolin in a baculovirus system. *Plant Mol. Biol.* **10**: 475.

Cochran, M. A., Carstens, E. B., Eaton, B. T., Faulkner, P. 1982. Molecular cloning and physical mapping of restriction endonuclease fragments of *Autographa californica* nuclear polyhedrosis virus DNA. *J. Virol.* **41**: 940.

Coelingh, L. L., Murphy, B. R., Collins, P. L., Lebacq-Verheyden, A. M. and Battey, J. F. 1987. Expression of biologically active and antigenically authentic parainfluenza type 3 hemagglutinin-neuraminidase glycoprotein by a recombinant baculovirus. *Virol.* **160**: 465.

Davidson, D. J., Castellino, F. J. 1991. Asparagine-linked oligosaccharide processing in Lepidopteran insect cells. Temporal dependence of the nature of the oligosaccharides assembled on asparagine-289 of recombinant human plasminogen produced in baculovirus vector infected *Spodoptera frugiperda* cells. *Biochem.* **30**: 6167.

Davidson, D. J., Fraser, M. J., Castellino, F. J. 1990. Oligosaccharide processing in the expression of human plasminogen cDNA by Lepidopteran insect (*Spodoptera frugiperda*) cells. *Biochem.* **29**: 5584.

Ernst, L. K., Rajan, V. P., Larsen, R. D., Ruff, M. M., and Lowe, J. B. 1989. Stable expression of blood group H determinants and GDP-L-fucose:ß-D-galactose 2-α-L-

fucosyltransferase in mouse cells after transfection with human DNA. *J. Biol. Chem.* **264**: 3436.

Estes, M. K., Crawford, S. E., Penaranda, M. E., Burns, J., Chan, W., Ericson, B., Smith, G. E., and Summers, M. D. 1987. Synthesis and immunogenicity of rotavirus major capsid antigen using a baculovirus expression system. *J. Virol.* **61**: 1488.

Fraser, M. J. 1986. Transposon mutagenesis of baculoviruses: transposon shuttling and implication for speciation. *Ann. Entomol. Soc. Am.* **79**: 773.

Granados, R. R., and Federici, B. A. 1986. The Biology of Baculoviruses. Vol. 1. CRC Press, Boca Raton, Fla.

Jarvis, D. L. and Summers, M. D. 1989. Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. *Mol. Cell. Biol.* **9**: 214.

Jeang, K., Giam, C., Nerenberg, and M., Khoury, G. 1987. Abundant synthesis of functional human T-cell leukemia-virus type-I P40X-protein in eucaryotic cells by using a baculovirus expression vector. *J. Virol.* **61**: 708.

Jeang, K., Holmgren-Konig, M., and Khoury, G. 1987. A baculovirus vector can express intron-containing genes. *J. Virol.* **61**: 1761.

Larson, R. D., Rajan, V. P., Ruff, M. M., Kukowska-Latallo, J., Cummings, R. D., and Lowe, J. B. 1989. Isolation of a cDNA encoding a murine UDPgalactose:ß-D-galactosyl-

1,4-N-acetyl-D-glucosaminide α-1,3-galactoslytransferase: expression cloning by gene transfer. *Proc. Natl. Acad. Sci. U.S.A.* **86**: 8227.

Lee, E. U., Roth, J., and Paulson, J. C. 1989. Alteration of terminal glycosylation sequences on N-linked oligosaccharides of Chinese hamster ovary cells by expression of ß-galactoside α2,6-sialyltransferase. *J. Biol. Chem.* **264**: 13848.

Luckow, V. A. 1991. Cloning and expression of heterologous genes in insect cells with baculovirus vectors, *In* A. Prokop, R. K. Bajpai, and C. S. Ho (eds.), Recombinant DNA Technology and Applications. McGraw-Hill, Inc., NY, pp. 97-152.

Luckow, V. A., Summers, M. D. 1988. Trends in the development of baculovirus expression vectors. *BioTechnol.* **6**: 47.

Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T., Saeki, I., and Furusawa, M. 1985. Production of human α-interferon in silkworm using a baculovirus vector. *Nature (London).* **315**: 592.

Matsuura, Y., Possee, R. D., and Bishop, D. 1986. Expression of the s-coded genes of lymphocytic choriomeningitis arenavirus using a baculovirus vector. *J. Gen. Virol.* **67**: 1515.

Matthews, R. E. F. 1982. Classification of nomenclature of viruses. Fourth Report of the International Committee on Taxonomy of Viruses, *Intervirol.* **17**: 52.

Miller, L. K. 1988. Baculoviruses as gene expression vectors. *Ann. Rev. Microbiol.* **42**: 177.

Miyamoto, C., Smith, G. E., Farrell-Towi, J., Chizzonitte, R., Summers, M. D., and Ju, G. 1985. Production of human c-myc protein in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.* **5**: 2860.

Ollo, R., and Maniatis, T. 1987. Drosophila Kruppel gene product produced in baculovirus expression system is a nuclear phosphoprotein that binds to DNA. *Proc. Nat. Acad. Sci. U.S.A.* **84**: 5700.

Pennock, G. D., Shoemaker, C., and Miller, L. K. 1984. Strong and regulated expression of *E. coli* ß-galactosidase in insect cells using a baculovirus vector. *Mol. Cell. Biol.* **4**: 399.

Smith, G. E., Ju, G., Ericson, B., Moschera, J., Lahm, H., Chizzonette, R. and Summers, M. D. 1985. Modification and secretion of human interleukin 2 produced in insect cells by a baculovirus expression vector. *Proc. Nat. Acad. Sci. U.S.A.* **82**: 8404.

Smith, G. E., Fraser, M. J., Summers, M. D. 1983a. Molecular engineering of the *Autographa californica* nuclear polyhedrosis virus genome: deletion mutations within the polyhedrin gene. *J. Virol.* **46**: 584.

Smith, G. E., and Summers, M. D. 1978. Analysis of baculovirus genomes with restriction endonucleases. *Virol.* **89**: 517.

Smith, G. E. and Summers, M. D. 1979. Restriction maps of five *Autographa californica* MNPV variants, *Trichoplusia ni* MNPV, and *Galleria mellonella* MNPV DNAs with endonucleases SmaI, KpnI, BamHI, SacI, XhoI, and *Eco*RI. *J. Virol.* **30**: 828.

Smith, G. E., Summers, M. D., and Fraser, M. J. 1983b. Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.* **3**: 2156.

St. Angelo, C., Smith, G. E., Summers, M. D., and Krug, R. M. 1987. Two of the three influenza viral polymerase proteins expressed by using baculovirus vectors form a complex in insect cells. *J. Virol.* **61**: 361.

Vlak, J. M., and Rohrmann, G. F. 1985. The nature of polyhedrin, *In* K. Sherman and K. Maramorosc (eds.), Viral Insecticides for Biological Control. Academic Press, NY, pp. 489-542.

Volkman, L. E., Summers, M. D., and Hsieh, C. H. 1976. Occluded and nonoccluded nuclear polyhedrosis virus growth in *Trichoplusia ni*: comparative infectivity, and *in vitro* growth studies. *J. Virol.* **19**: 820.

CHAPTER II

Factors Influencing Recombinant Protein Yields in an Insect Cell - Baculovirus Expression System: Multiplicity of Infection and Intracellular Protein Degradation

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2.1 Summary

The insect cell (Sf9)-baculovirus (AcMNPV) expression system was employed for the synthesis of ß-galactosidase, a model heterologous protein. In the recombinant virus studied, the *lacZ* gene is fused to a portion of the polyhedrin structural gene and is under the control of the polyhedrin promoter. The effect of the multiplicity of infection (MOI) on product titer was determined by infecting cells with MOI values ranging from 0 to 100 and monitoring the production of ß-galactosidase with time. The relationship between final product titer and MOI was dependent on the growth phase of the cells prior to infection. The final product titer from cells infected in the early-exponential phase was relatively independent of MOI. However, in late-exponential phase there was a logarithmic relationship between the final ß-galactosidase titer and the MOI used, with the highest MOI studied resulting in greatest protein synthesis.

The synthesis and degradation rates of ß-galactosidase were investigated by a pulse-chase technique using L-[^{35}S]-methionine. At 24 hours post-infection the degradation rate is of the same order of magnitude as the synthesis rate. However, the synthesis rate of ß-galactosidase increases dramatically at 96 hours post-infection. During this later period, the degradation rate is negligible. Although degradation of recombinant protein occurs in this system, degradation activity declines as infection proceeds and is insignificant late in infection when recombinant protein expression is intense.

2.2 Introduction

Multiplicity of infection (MOI) is defined as the number of plaque-forming units (PFU) per cell that is added at the time of infection. Virus titer (PFU/ml) is usually determined by the end-point dilution method (1). The MOI and the time of infection are two parameters that are easily manipulated and that may be important in optimizing heterologous protein yields in baculovirus expression vectors. The effect of MOI on recombinant protein synthesis in Sf9 cells growing in sparged bioreactors has been studied by Murhammer and Goochee (2). They reported relatively constant ß-galactosidase production for MOI values ranging from 1 to 10 with cells infected in the mid- to late-exponential phase. For example, in studies with airlift bioreactors containing 0.2% Pluronic F-68 and 20 ppm antifoam C, product titers at MOI values of 1 and 5 were 0.145 and 0.165 mg, respectively. In studies with a recombinant baculovirus expressing human macrophage colony stimulating factor, Maiorella *et al.* (3) reported that product yields were relatively insensitive to the multiplicity of infection in the range of 0.5 to 10. In this study, cells were infected at a high cell density (approximately $2x10^6$ cells/ml) during exponential growth. The authors stated that a dramatic decrease in product yields was observed if cells were infected during the stationary phase.

Cell growth was demonstrated to be dependent on MOI for cells grown in ExCell 400, a serum-free medium (4). In this study total cell counts were observed to be a function of MOI in the range of 1 to 10; cultures infected at high MOIs exhibited a more rapid decrease in total cell counts during infection. Schopf *et al.* (5) recently compared MOI values of 1 and 10 for both wild-type and recombinant AcMNPV (coding for ß-galactosidase)-infected cells. In this study, cell viability, cell size, ß-galactosidase activity, oxygen consumption, DNA distribution and respiratory activity were compared. In all of the studies, the MOI did not have a dramatic effect in the cells infected with the recombinant virus.

In addition to the multiplicity of infection, intracellular protein degradation may be an important factor influencing heterologous protein yields in the insect cell-baculovirus system. There is evidence that protein degradation exists at all cell levels, encompassing both prokaryotes and eucaryotes (6). Degradation in mammalian cells and bacterial cells has attracted considerable attention in recent years; however, little work has been done concerning proteolysis in insect cells or the insect cell-baculovirus system.

2.3 Materials and Methods

Cell culture. *Spodoptera frugiperda* IPL-Sf9, a continuous cell line, was obtained from the ATCC (#CRL-1711). Cells were cultured in modified TNM-FH medium [Grace's medium (Gibco) supplemented with 3.3 g/l Difco yeastolate, 3.3 g/l lactalbumin hydrolysate, 0.35 g/l $NaHCO_3$, 10% fetal bovine serum (Hyclone), 5000 units/l penicillin, and 5 mg/l streptomycin (Sigma)]. Cultures grew as monolayers in stationary, 25 ml T-flasks maintained at 27°C. Cells were passaged by flushing the monolayer off the surface of the T-flask with a Pasteur pipette. Total cell counts and cell size were determined by a Coulter Counter (Coulter Electronics, Inc.); viable cell counts were determined via trypan blue exclusion.

Virus stocks and infection. Wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and recombinant virus encoding a fusion ß-galactosidase under the control of the polyhedrin promoter (ß-gal AcMNPV) were obtained from Michael Lochrie (California Institute of Technology). The fusion protein contains 11 amino acids of the polyhedrin protein and lacks 7 amino acids of the native ß-galactosidase.

Infection began when the medium was aspirated off the monolayer culture and virus stock solution was added (designated time 0 post-infection). The amount of virus added depended on the multiplicity of infection used. After addition of viral stock, cultures were placed on a rocking platform for 1 hour, while maintained at 27°C. Cells were removed from the platform, and after one additional hour at 27°C, the medium containing virus was replaced with fresh medium. This step prevented the ß-galactosidase introduced into the culture by the viral stock from affecting the enzyme titers (an attribute important at high MOIs).

ß-galactosidase assay. ß-galactosidase activity was assayed by using O-nitrophenyl-ß-D galactoside (ONPG) as a substrate. The conversion to O-nitrophenyl was recorded by monitoring the absorption at 420 nm with time (extinction coefficient = 4.5 ml/μmole-cm). 0.3 ml of ONPG were added to 0.3 ml of sample and 2.4 ml of Z-buffer (16.1 g/l Na_2HPO_4-$7H_2O$, 5.5 g/l NaH_2PO_4-H_2O, 0.75 g/l KCl, 0.246 g/l $MgSO_4$-$7H_2O$, 2.7 ml/l ß-mercaptoethanol) at 37°C. Optical density at 37°C was recorded after a lag time of 2 minutes.

Samples for the determination of extracellular ß-galactosidase were made by removing an aliquot of supernatant from the culture and centrifuging at 1000 g for 5 minutes to remove cellular debris. Samples for total ß-galactosidase assays were obtained by resuspending the monolayer with a Pasteur pipette, removing a culture sample, sonicating for 3 min, and subsequent centrifugating at 1000 g for 7 min.

Pulse-chase method. Cells were grown to a cell density of 10^6 cells/ml and infected as noted above. The pulse-chase experiment commenced at the desired time post-infection. Cells were gently resuspended and centrifuged at 500 g for 5 minutes. The supernatant was removed and the cells were washed twice with methionine-free medium (containing 2.5% fetal bovine serum). Cells were then resuspended in the methionine-free medium to a density of 10^6/ml. 250 μl of cell suspension and 50 μCi of L-[^{35}S]-methionine (1037 Curies/mmol, 10μCi/μl) were added to glass tubes maintained at 27°C. The cells were then incubated at 27°C for the desired pulse time. If a chase was required, 1 ml warmed, complete medium and 3 μl of methionine solution (30 mg/ml) were added. The pulse or chase was terminated by adding 2 ml of ice-cold, complete medium and placing on ice. Then the culture was centrifuged at 1000 g for 5 minutes and the supernatant removed. The resulting cell pellet was washed 1 time with cold PBS^+ (PBS containing Ca^{+2} and Mg^{+2}). After washing, 250 μl of lysis buffer (93% PBS^+, 2% 50 mM EDTA, 1% NP-40, 0.6% DOC, and 0.1% SDS) and 50 μl of Protein A-Sepharose

were added. The sample was placed on ice for 20 minutes, and subsequently centrifuged for 45 minutes in an Eppendorf microcentrifuge. 150 µl of supernatant was then transferred to Eppendorf tubes containing 35 µl antiserum to ß-galactosidase in 1 ml tris-triton buffer (50 mM Tris-Cl, 0.15 M NaCl, 0.1 mM EDTA, and 2% Triton X-100). Samples were mixed 4 times by gentle inversion and then incubated overnight on a rotor at 4°C. The following morning 50 µl of Protein A-Sepharose (pre-equilibrated in 10 mM Tris-Cl) were added and the samples were mixed by gentle inversion. Samples were returned to the rotor for 1 hour at 4°C. After incubation the samples were centrifuged for 1 minute at room temperature and the supernatant removed. The pellet was washed 5 times with ice-cold tris-triton buffer, 1 time with ice-cold 10 mM tris-HCl, and 2 times with ice-cold 0.1% SDS. The resulting pellet was resuspended in 55 µl sample buffer, vortexed for 10 seconds, boiled for 5 minutes, and vortexed again for 10 seconds. The sample was then centrifuged for 5 minutes at room temperature, and 45 µl of the supernatant were removed. Of this, 10 µl were used in scintillation counting and the remainder used in SDS-PAGE. Duplicate samples were run for all pulse-chase times investigated.

2.4 Results

ß-galactosidase is an intracellular protein in the insect cell-baculovirus system. However, as the time post-infection increases, ß-galactosidase is released into the medium. This "secretion" appears to be due to the cell membranes losing integrity, becoming leaky and ultimately lysing. Figure 1 is a characteristic time trajectory of intracellular and extracellular product concentrations (per unit volume of culture) over the life span of the system. The maximum net-synthesis rate is attained at about 50 hours post-infection, while the intracellular ß-galactosidase concentration peaks at ≈110 hours post-infection (p.i.). These data are for a culture infected with an MOI of 10 at 8.6×10^5 viable cells/ml.

The effect of multiplicity of infection. The effect of multiplicity of infection on the Sf9-AcMNPV system was determined by infecting cells at different growth phases with the ß-galactosidase virus at a variety of MOIs, ranging from 0 to 100. Final ß-galactosidase activities as a function of MOI are presented for cells infected in the early (4.3×10^5 cells/ml) and late-exponential phase (1.2×10^6 cells/ml) in Figure 2; Figure 3 is a characteristic growth curve indicating the time of infection for these experiments.

With infection during the late-exponential growth phase, both cell density (Figure 4) and product formation are strongly dependent on MOI. The higher the MOI used, the more rapid is the decrease in cell viability and the greater are the product yields. The relationship between MOI and final product yield is logarithmic in nature.

Cultures infected during the early-exponential growth phase show a similar relationship between cell viability and MOI. The higher the MOI, the more rapid the decrease in cell viability (Figure 5). Although the growth characteristics are similar, cells infected early in the exponential growth phase did not exhibit this logarithmic relationship between final ß-galactosidase titer and the multiplicity of infection. A

relatively steady value was obtained for MOI values ranging from 0.1 to 100; a slight decrease is observed for MOI values greater than 1. The final product titer for cells infected in the early-exponential phase is greater than that obtained for cells infected in the late-exponential phase for MOI values up to 10. However, the maximum yield of recombinant protein occurred in cells infected in the late-exponential phase with an MOI of 100.

The effect of the multiplicity of infection on transient heterologous protein synthesis for cells infected during the mid- to late-exponential phase was investigated by radiolabelling with L-[^{35}S]-methionine. In Figure 6 total scintillation counts of immunoprecipitated culture lysates are plotted against MOI on a logarithmic scale for samples at 24 hours p.i., pulsed for 60 minutes. Again, synthesis rate is approximately proportional to the logarithm of the multiplicity of infection for MOI values of one and greater for cells infected in the mid- to late-exponential growth phase.

Total scintillation counts performed on immunoprecipitated culture lysates are presented here as a means to analyze trends. Although these data include radioactivity incorporated into any polypeptide that immunoprecipitated in addition to the ß-galactosidase, the data are a valid reflection of the labelled, heterologous protein levels in the culture. This conclusion is based on the fact that the immunoprecipitation is very specific; the antiserum against ß-galactosidase is not cross-reacting significantly with other proteins, be they viral, cellular, or serum-associated. Two controls were used to demonstrate the IgG specificity. One control consisted of cells infected with the wild-type virus, and the other control consisted of cells that were not infected. These controls, as well as cells infected with the ß-gal AcMNPV, were radiolabelled for 40 minutes; the resulting autoradiogram is presented in Figure 7. Little or no cross-reactivity is observed. The antiserum specificity is high since it is raised in mice against *E. coli* ß-galactosidase. In addition, the lysate is "pre-cleared" with Protein A-Sepharose prior to the immunoprecipitation.

Intracellular protein degradation. Cell counts for radiolabelling-immunoprecipitation experiments were made by using a Coulter Counter. The counts include infected and noninfected cells but exclude lysed cells. Such counts do, however, include many cells deemed nonviable by trypan blue exclusion. The reason for using total cell counts in normalization of the data is that a cell deemed nonviable by trypan blue exclusion has not been demonstrated to be inactive in viral activity. By definition, the termination of a lytic viral life cycle is cell lysis. Release of large quantities of ß-galactosidase into the medium prior to cell lysis is evidence that the cellular membrane is compromised by the infection process. Secretion by the insect cell secretion apparatus is not believed to be the mode of transport to the extracellular space, since ß-galactosidase activity does not appear in the medium until well after synthesis has begun (Figure 1). ß-galactosidase leakage may be an indication that trypan blue exclusion is not a valid indication that viral processes have terminated. (In the preceding section trypan blue exclusion was used as an indication of the health of the culture; i.e., the cells' ability to divide. It was not intended to distinguish between the activity or inactivity of viral-directed, intracellular processes.)

Radiolabelling-immunoprecipitation experiments were completed at 24, 48, 72, 96, and 120 hours post-infection to understand the temporal nature of heterologous protein synthesis. All samples were infected with an MOI of 10 and originated from the same cell culture and virus stock. Figure 8 depicts the total scintillation counts of immunoprecipitated culture lysates obtained after a 10-minute pulse during the infection period. The data are normalized per 10^6 cells. These results indicate a highly regulated temporal event, with the maximum net synthesis rate occurring at 4 days post-infection. This period of maximal net synthesis is relatively short-lived; a rapid rise and fall is noticed between 3 and 5 days.

A simplified model describing intracellular synthesis and degradation of a protein may be written as

$$\frac{dE}{dt} = K_S - K_d E - \frac{1}{N}\frac{dN}{dt} E,$$

where E is the quantity of the protein of interest (indicated by enzyme activity in this case) on a per-cell basis (units/cell), t is time, K_S is the zero-order rate constant of enzyme synthesis (units/cell-time), K_d is the first-order rate constant of degradation ($time^{-1}$), and N is the number of cells per unit volume of culture. The rate of protein synthesis is dependent on several factors, including the amount of mRNA, tRNA, amino acids and the number of ribosomes available in the cell. The zero-order rate constant of synthesis is a simplification that lumps these components into one parameter. A model accounting for the different factors that are included in K_S is difficult, since their values are often unknown.

The term $\frac{1}{N}\frac{dN}{dt} E$ accounts for the dilution of intracellular protein that is due to cell division. In this system cell division is not solely the result of growth but is also dependent on infection. In the pulse-chase experiments presented, this term is relatively insignificant, since the duration of the chase times studied are considerably less than both the cell doubling time and the time scale on which the virus affects the cell.

The net synthesis rates and the degradation rates at 24 and 96 hours post-infection were analyzed. At each of these times, several immunoprecipitation experiments were completed using various pulse-chase times. All of the data presented are normalized by 10^5 total cells per sample and are expressed in units of AU*mm/10^5 cells-minute. The units AU*mm, absorbance units*millimeters, represent the area under a peak obtained from densitometry. To iterate, normalization is based on total cells, including cells encompassing a broad range of times post-infection as well as cells that are not infected.

Typical data for the net synthesis of ß-galactosidase at 24 hours post-infection are presented in Figure 9. The net synthesis rate constant, $K_{s(net)}$, obtained from these data is 4.5×10^{-3} AU*mm/(10^5 cells-min). It should be emphasized that the *net* rate of synthesis observed equals synthesis rate minus degradation rate. The synthesis rate is actually equal to or greater than that observed. At 96 hours post-infection, $K_{s(net)}$ was observed to be 1.1 AU*mm/(10^5 cells-min), indicating a greater than 200-fold increase in the net synthesis rate when compared to the net synthesis rate at 24 hours post-infection.

Similar experiments were completed to determine the degradation rates throughout the infection life cycle. Data for degradation at 24 hours p.i. are presented in Figures 9 and 10. From Figure 10 the first-order degradation constant, K_d, of ß-galactosidase in this system was calculated to be 9.4×10^{-3} min^{-1} (based on 10^5 cells), corresponding to a half-life of 74 minutes. The similarity between the degradation rate and the net synthesis rate at 24 hours post-infection is most clearly evident in a display of the pulse and chase data in one figure (with degradation data plotted against an inverted time scale). Figure 9 demonstrates that the degradation rate and the net synthesis rate are of the same order of magnitude. Pulse-chase studies later in the infection period indicate that, when the net synthesis rate increased rapidly, the degradation rate decreased relative to the degradation rate observed at 24 hours post-infection. No degradation was observed at 96 hours post-infection with the chase times studied ($\leq$ 20 minutes). It is possible that degradation is occurring and may be observed for chase times greater than 20 minutes. However, a degradation rate on this order is of little significance when compared to the rapid net synthesis rate observed.

2.5 Discussion

The effect of multiplicity of infection. The final product concentration as a function of the MOI is highly dependent on the growth phase of the cells. Cell density, transient product titers, and final product titers were demonstrated to be strongly dependent on the multiplicity of infection with cells infected in the late-exponential growth phase. The logarithmic relation between the product concentration and the MOI suggests that as the MOI increases, the system becomes saturated; i.e., a point is reached at which an increase in MOI does not result in a proportional increase in the final product levels.

If the cells are infected in the early-exponential growth phase, the variation in product titer is reduced. This relative insensitivity to MOI may be explained by considering the population dynamics of the culture. If upon infection a subpopulation of cells are infected, the viral life cycle will progress in these cells. Later in the viral life cycle, nonoccluded, infectious virions will be released from these cells into the extracellular space. These secondary virions are then able to infect cells that were not originally infected and that remain in the exponential growth phase. At extremely high MOI values, the cells may be synchronously infected (the primary infection), and at lower MOIs only a subpopulation may be infected. However, because of the early infection time, a secondary infection will occur in the later case, thus minimizing the difference in final product titers. The slight decrease in final ß-galactosidase titers observed for MOI values greater than 1 in cultures infected early is the result of the mass, synchronous infection. The few cells that are uninfected will produce fewer cell progeny (relative to a low MOI) to be infected by the secondary virions. Thus, the total number of cells infected with a high MOI will be less than the total number of cells infected with a low MOI, leading to a slight decrease in final product titers. These hypotheses are supported by Figures 2 and 5.

Examination of Figure 2 indicates that maximum heterologous protein synthesis is obtained by infecting cells in the late-exponential phase with a very high multiplicity of infection (100 in the data presented). However, it is more feasible to infect the culture earlier in the exponential phase with a lower MOI (e.g., 0.1) and to obtain final product concentrations of the same order of magnitude. By utilizing a lower MOI and relying on secondary infection of the culture, the amount of stock virus needed will be substantially reduced. Studies with animal viruses indicate that a high MOI results in an increase in the production of defective viral progeny in infected cells (7). Although this has not been observed with wild-type or recombinant AcMNPV, the lower MOI may prove important in maintaining the viability of virus progeny.

The dependence of final product titer on MOI at later times post-infection does not contradict the work of Murhammer and Goochee (2), Maiorella *et al.* (3), and Schopf *et al.* (5). The differences observed in this study and the previous studies may be explained by the importance of the cell density and growth phase of the cells at the time of infection. Murhammer and Goochee (2) infected at a cell density of $8x10^5$ cells/ml. Furthermore a slight difference in product titers was observed in their results. Maiorella *et al.* (3) used a new medium formulation (lacking serum) that resulted in different growth characteristics. Although they infected at a cell density on the order of $2x10^6$, the maximum cell density obtainable in this media was $4\text{-}5x10^6$. In addition, the recombinant virus was different and may have affected the cells differently.

Intracellular protein degradation. From studies with mammalian cells, it appears that all proteins are subjected to some form of degradation (8,9). It is well documented that in eucaryotic cells there is a continual turnover of proteins (8,10). In eucaryotes, the half-life of a cytosolic protein is dependent on the nature of the amino terminal residue (11). For example, a methionine residue in this position indicates a relatively stable protein ($t_{1/2} > 20$ hours), while an arginine residue is a signal for rapid

degradation ($t_{1/2} \approx 2$ minutes) (11). These simple markers are recognized by ubiquitin, an 8.5 kDa protein present in all eucaryotic cells (12). The ubiquitin covalently attaches to the protein to be degraded. Although the mechanism by which this protein induces proteolysis is unknown, ubiquitinated proteins are selectively degraded (12). A similar protein has yet to be isolated in prokaryotes. Other factors influence degradation rates, including temperature, nutrient deprivation, composition of the growth medium, and growth phase (6).

Although hydrolysis of native proteins occurs, proteolysis is often aimed at "abnormal proteins" (6). Abnormal proteins consist of proteins that are foreign to the cell, including recombinant proteins, mutated proteins, proteins incorporating exogenously-supplied amino acid analogs, and proteins resulting from transcriptional and translational errors. Examples in both bacterial and mammalian cell systems have demonstrated the ability of a cell to identify a protein as foreign and target it for degradation (12,13,14). It has been suggested that some proteins are degraded as rapidly as they are synthesized (6).

Intracellular degradation of heterologous protein in the insect cell-baculovirus expression system is likely given the cell-virus interactions. Shortly after infection cells are synthesizing foreign proteins, and it is plausible that some mechanism of defense is activated by the cell. In addition to considering the possibility that the cell is targeting foreign proteins for proteolytic action, degradative action by the virus may exist. Viral-encoded proteases may be responsible for the turnover of normal viral proteins, and may be acting inadvertently on the ß-galactosidase. Alternatively, viral-encoded proteases may be able to recognize nonviral proteins and target them for hydrolysis.

The data presented here suggest that the intracellular degradation witnessed at early times post-infection is primarily the response of a healthy, viable cell that is responding to a stressful, highly disrupted state created by intracellular viral activities. However, as cellular processes are compromised for the viral activities, the degradation rate becomes negligible. ß-galactosidase degradation occurs on a much slower time scale

relative to the time scale of synthesis at later times when polyhedrin promoter activity is maximal.

Endogenous protease activity responsible for the dissolution of inclusion bodies in a nuclear polyhedrosis virus was first demonstrated in the silkworm, *Bombyx mori* (15). Likewise, the AcMNPV occlusion bodies contain a protease responsible for occlusion protein degradation (16). This enzyme requires an alkaline environment for activity, the net effect of which is the release of several virions (15). The alkaline protease has been demonstrated to be absent in occlusion bodies propagated *in vitro* (16,17). On the basis of this evidence and the fact that *in vitro* cultivation occurs below pH 7, the alkaline protease is not a likely cause of heterologous protein degradation witnessed at early times post-infection.

The immunoprecipitation technique does not quantify ß-galactosidase released to the extracellular medium because of active secretion, leakage, or cell lysis. As a result; the net synthesis rates may be greater than those reported. Furthermore, the error introduced from protein release to the extracellular space should be greater at later times post-infection when transport predominantly occurs (Figure 1).

Table 1 itemizes the synthesis and degradation parameters obtained in this study to allow comparison of net synthesis rates observed from pulse-chase studies with net synthesis rates based on intracellular enzyme activity (Figure 1). To compare radiolabelling data with that of enzyme activity in the bulk, the ratio of net synthesis rates at 96 hours p.i. and 24 hours p.i. was calculated for each method. This dimensionless quantity, $[\frac{dE}{dT}(96 \text{ h.p.i.})]/[\frac{dE}{dT}(24 \text{ h.p.i.})]$, is 150 for the pulse-chase data and 160 for the enzyme activity data of Figure 1. These values indicate consistency between these two methods. This is expected since cells were infected at the same cell density and MOI (8.6×10^5/ml and 10, respectively) in both experiments.

In the wild-type system polyhedrin production begins at 24 hours and continues until cell death. The data presented here may be explained if after the primary infection a

distribution of populations exists, one population composed of infected cells and another containing uninfected cells. Nonoccluded virus from infected cells is observed between 36 and 72 hours post-infection, thus allowing a secondary infection of the culture to occur. Depending upon the original distribution of infected and uninfected cells, several time trajectories of product synthesis may be hypothesized. The data presented in Figure 1 and in the radiolabelling-immunoprecipitation experiments suggest that the primary infection affected a small population of the cells and that the secondary infection involved the majority of the population. Several factors will affect the time trajectory of protein synthesis in this system, including the time of infection, the number of cells, the MOI, the conditions of the primary infection, and the passage number of the virus.

Results presented here for recombinant virus pertain specifically to the virus studied and its heterologous gene product, ß-galactosidase. Trends may well differ for other constructs expressing other proteins.

2.6 Acknowledgments

We would like to thank Michael Lochrie for supplying wild-type baculovirus and ß-Gal AcMNPV. This research was supported by the National Science Foundation (Grant No. CBT-8721973) and by a grant for Predoctoral Training in Biotechnology from the National Institute of General Medical Sciences, National Research Service Award 1 T32 GM 08346-01 from the Pharmacology Sciences Program.

2.7 References

1. Summers, M. D. and Smith, G. E. (1985). A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures. Texas Agricultural Experiment Station Bulletin No. 1555.

2. Murhammer, D. W. and Goochee, C. F. (1988). *Biotechnol.* **6**: 1411.

3. Maiorella, B., Inlow, D., Shauger, A., and Harano, D. (1988). *Biotechnol.* **6**: 1406.

4. Godwin, G., Belisle, B., DeGiovanni, A., Kohler, J., Gong, T., and Wojchowski, D. (1989). *In Vitro.* **25**: 19A.

5. Schopf, B., Howaldt, M. W., and Bailey, J. E. (1990) *J. of Biotechnol.* **15**: 169.

6. Goldberg, A. L. and Dice, J. F. (1974). *Ann. Rev. Biochem.* **43**: 835.

7. Strauss, E. G., Strauss, J. H., and Levine, A. J. (1990). *Virus Evolution.* in Fields Virology. Raven Press, NY.

8. Shimke, R. T. (1970). *Mammalian Protein Metab.* **4**: 177.

9. Rechcigl, M. (1971). Enzyme Synthesis and Degradation in Mammalian Systems. University Park Press, Baltimore.

10. Berlin, C. M. and Shimke, R. T. (1965). *Mol. Pharmacol.* **1**: 149.

11. Bachmair, A., Finley, D., and Varshavsky, D. (1986). *Science.* **234**: 179.

12. Finley, D. and Varshavsky, D. (1985). *Trends Biochem. Sci.* **10**: 343.

13. Platt, T., Miller, J., and Weber, K. (1970). *Nature.* **228**: 1154.

14. Lin, S. and Zabin, I. (1972). *J. Biol. Chem.* **247**: 2205.

15. Yamafuji, K., Yoshihara, F. and Hirayama,K. (1957). *Enzymologia.* **19**: 53.

16. Wood, H. A. (1980). *Virol.* **103**: 392.

17. Zummes, N. and Faulkner, P. (1979). *J. of Invert. Path.* **33**: 383.

2.8 Tables

Table 1. Synthesis and degradation rate constants for the production of ß-galactosidase in the Sf9-360-lac AcMNPV system.

From pulse-chase experiments:					From Figure 1:
Time (hours p.i.)	Ks(net) ($AU*mm/10^5$ cells-min)	Kd (min^{-1})	Total Cells (cells/ml)	Ks(net)*N (AU*mm/ml-min)	Ks(net) (units/ml-hr)
24	$4.4x10^{-3}$	$9.4x10^{-3}$	$1.7x10^6$	$7.5x10^{-2}$	$1.7x10^{-3}$
96	1.1	not observed	$1.0x10^6$	11	$2.7x10^{-1}$

2.9 Figures

Figure 1. The production of ß-galactosidase (activity units/ml culture) as a function of time post-infection (hours p.i.). Δ indicates intracellular ß-galactosidase activity and ● indicates extracellular ß-galactosidase activity.

Figure 2. The production of ß-galactosidase (activity units/ml culture) as a function of the multiplicity of infection on a logarithmic scale. □ indicates cells infected in the early-exponential growth phase (4.3×10^5 cells/ml) and ● indicates cells infected in the mid- to late-exponential growth phase (1.1×10^6 cells/ml).

Figure 3. The growth of Sf9 cells in TNM-FH media. Viable cells/ml on a logarithmic scale are plotted against time (hours). The arrows (4.3×10^5 and 1.2×10^6 cells/ml) correspond to the infection times described in Figure 2.

Figure 4. Viable cell density of cultures infected with different MOIs. Viable cells (10^6 cells/ml) are plotted against time (days post-infection). MOI values used are: ○ 0, ● 0.01, □ 0.1, ■ 1.0, Δ 10., ▲ 100. Infection began at 1.1×10^6 cells/ml ("late-exponential" phase).

Figure 5. Viable cell density of cultures infected with different MOIs. Viable cells (10^6 cells/ml) are plotted against time (days post-infection). MOI values used are: ● 0.01, □ 0.1, ■ 1.0, Δ 10., ▲ 100. Infection began at 5.5×10^5 cells/ml ("early-exponential" phase).

Figure 6. The effect of MOI on ^{35}S incorporation in ß-galactosidase at 24 hours p.i. Total scintillation counts (normalized per cell) are plotted against the multiplicity of infection on a logarithmic scale for a pulse time of 60 minutes.

Figure 7. An SDS-polyacrylamide gel autoradiogram used to analyze cross-reactivity with the antiserum. The lanes indicated are immunoprecipitate from cells that were not infected (2 and 3); cells infected with the wild-type AcMNPV (4 and 5); and cells infected with ß-gal AcMNPV (1 and 6). ß-galactosidase is indicated by the arrow.

Figure 8. The effect of time post-infection on ^{35}S incorporation in ß-galactosidase. Total scintillation counts (normalized per 10^6 cells) are plotted against time post-infection (days). This data are based on a 10-minute pulse time for all data points.

Figure 9. The net synthesis and degradation of ß-galactosidase at 24 hours post-infection. The relative quantities of L-[^{35}S]-methionine incorporated (AU*mm/10^5 cell) are plotted against time (minutes). The net synthesis data are indicated by ▲ and correspond to the upper time scale (pulse time). The degradation data are indicated by □ and correspond to the lower time scale (chase time).

Figure 10. Degradation of ß-galactosidase at 24 hours post-infection. The logarithm of the relative quantities of L-[^{35}S]-methionine incorporated (AU*mm/10^5 cell) is plotted against the chase time (minutes).

Figure 1.

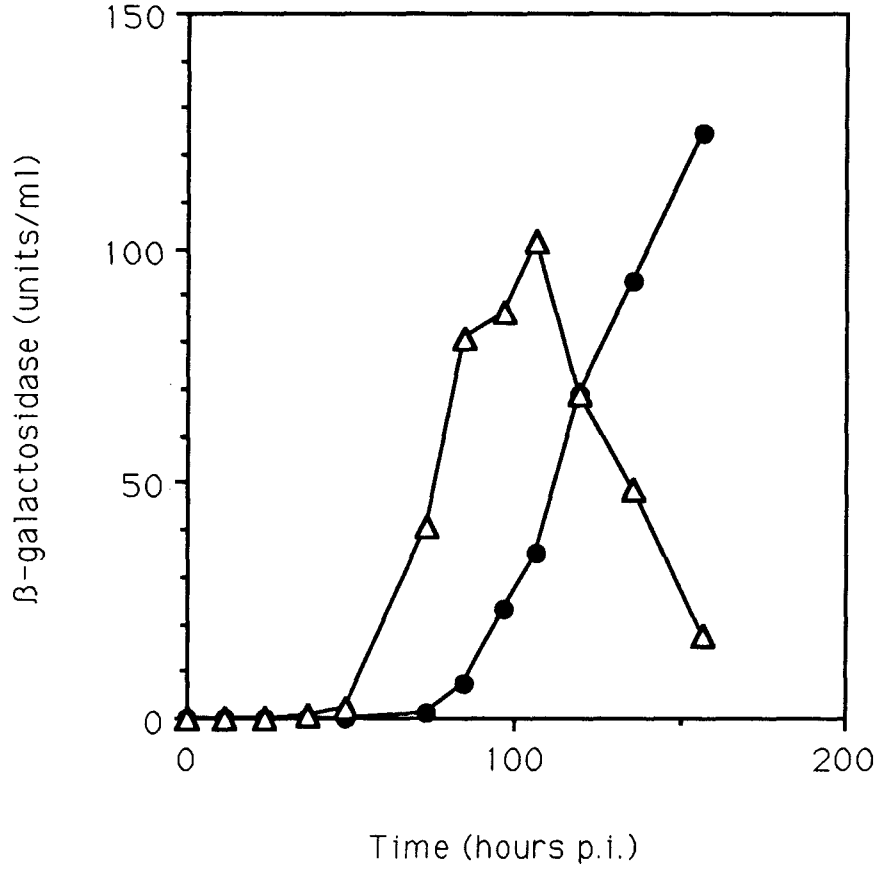


Figure 2.

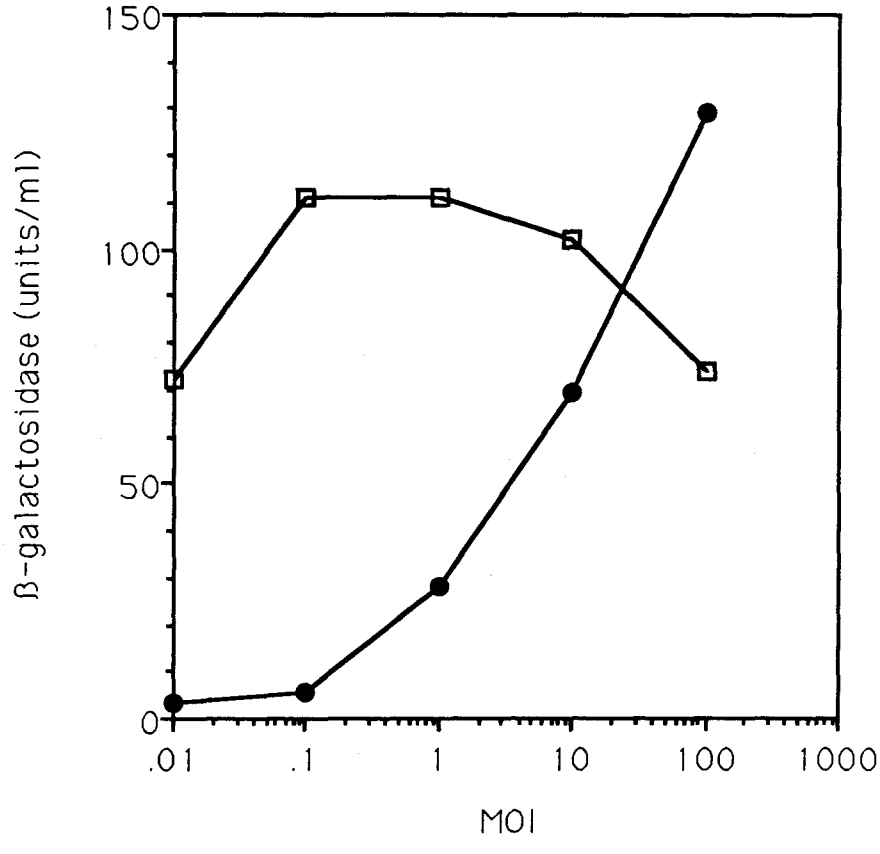


Figure 3.

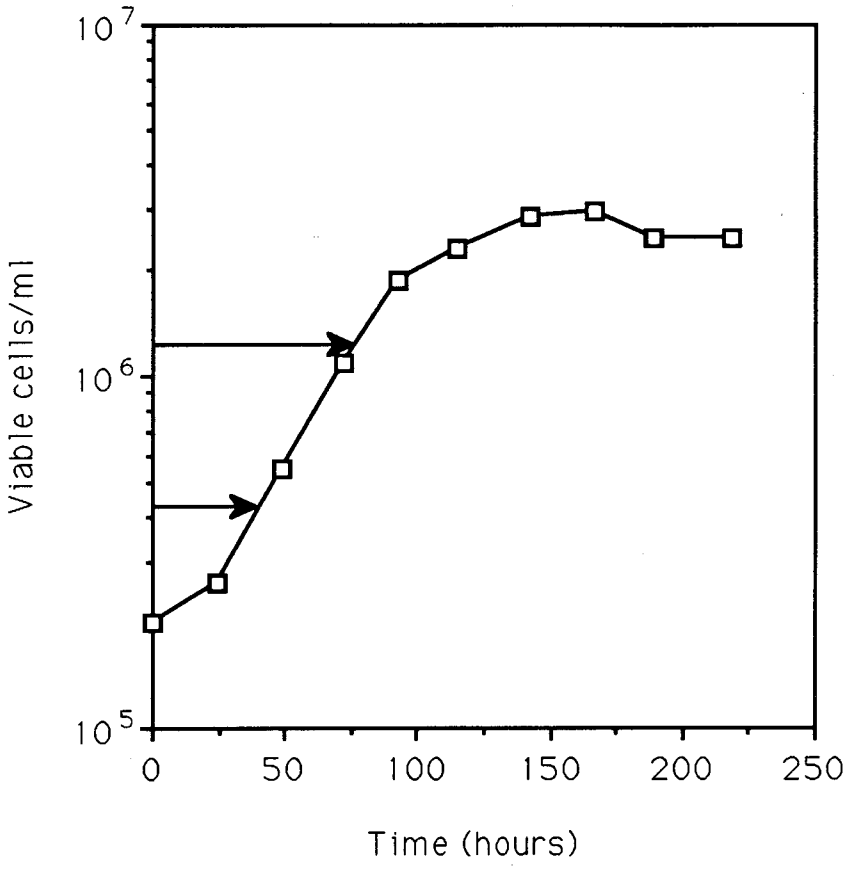


Figure 4.

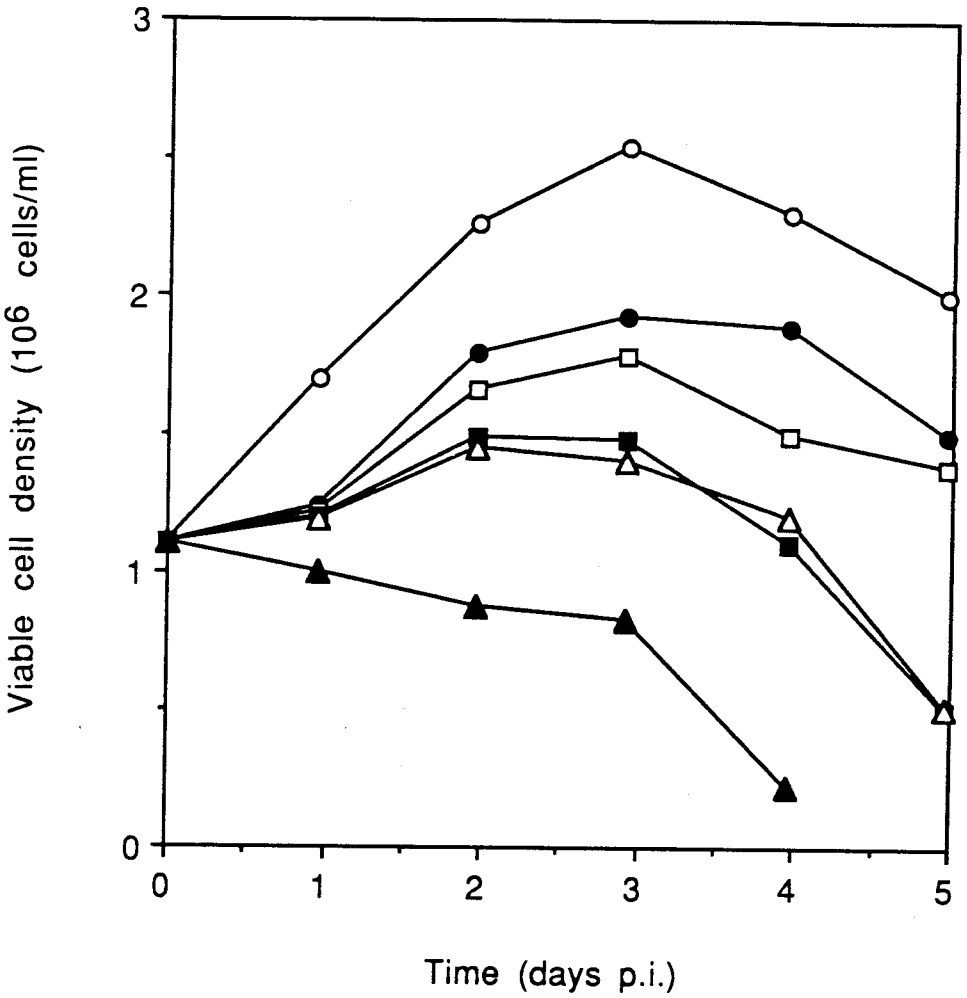


Figure 5.

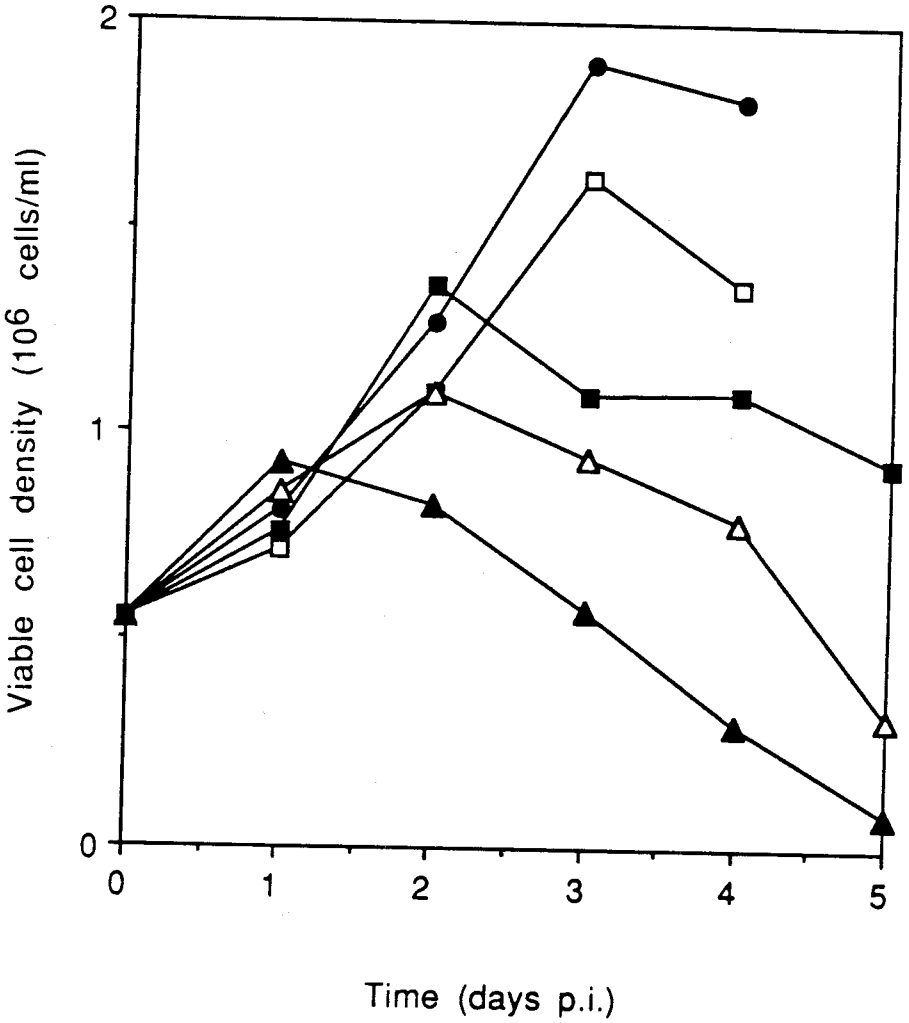


Figure 6.

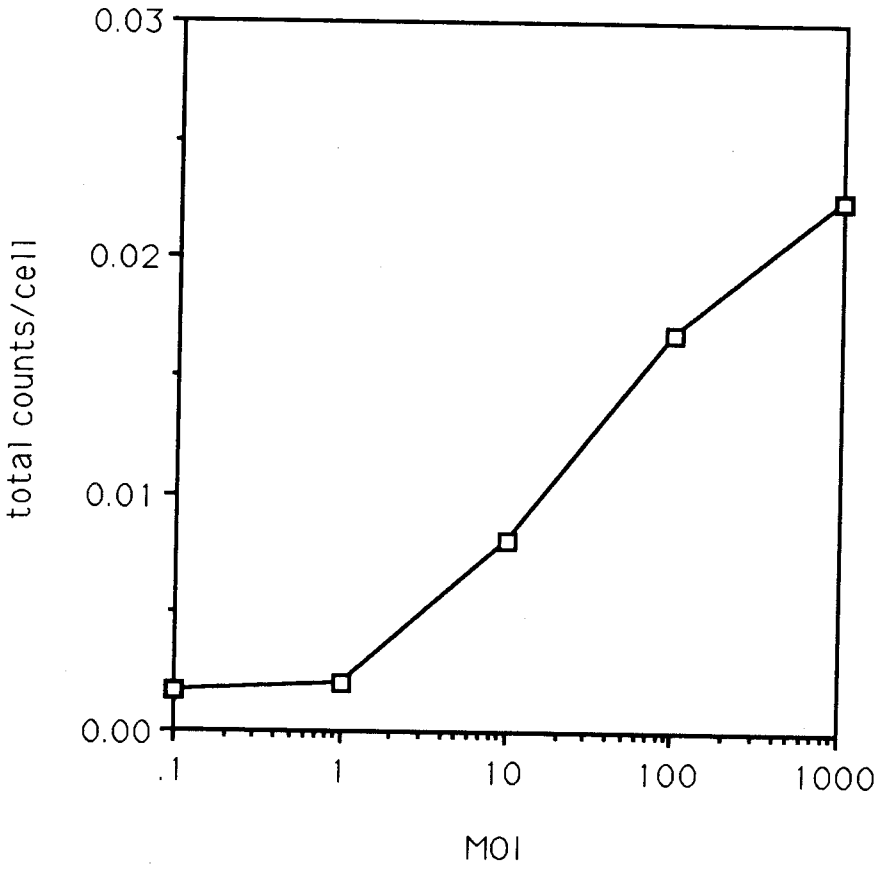


Figure 7.

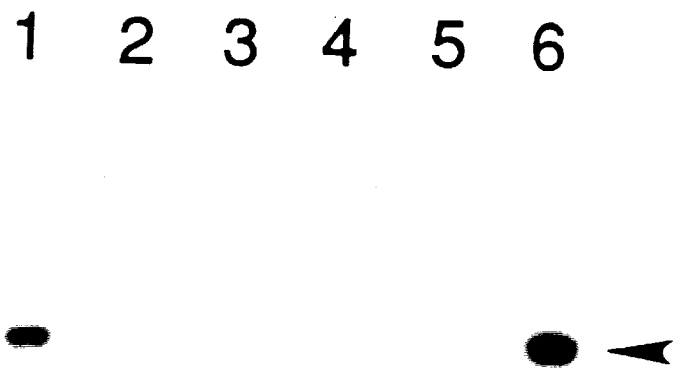


Figure 8.

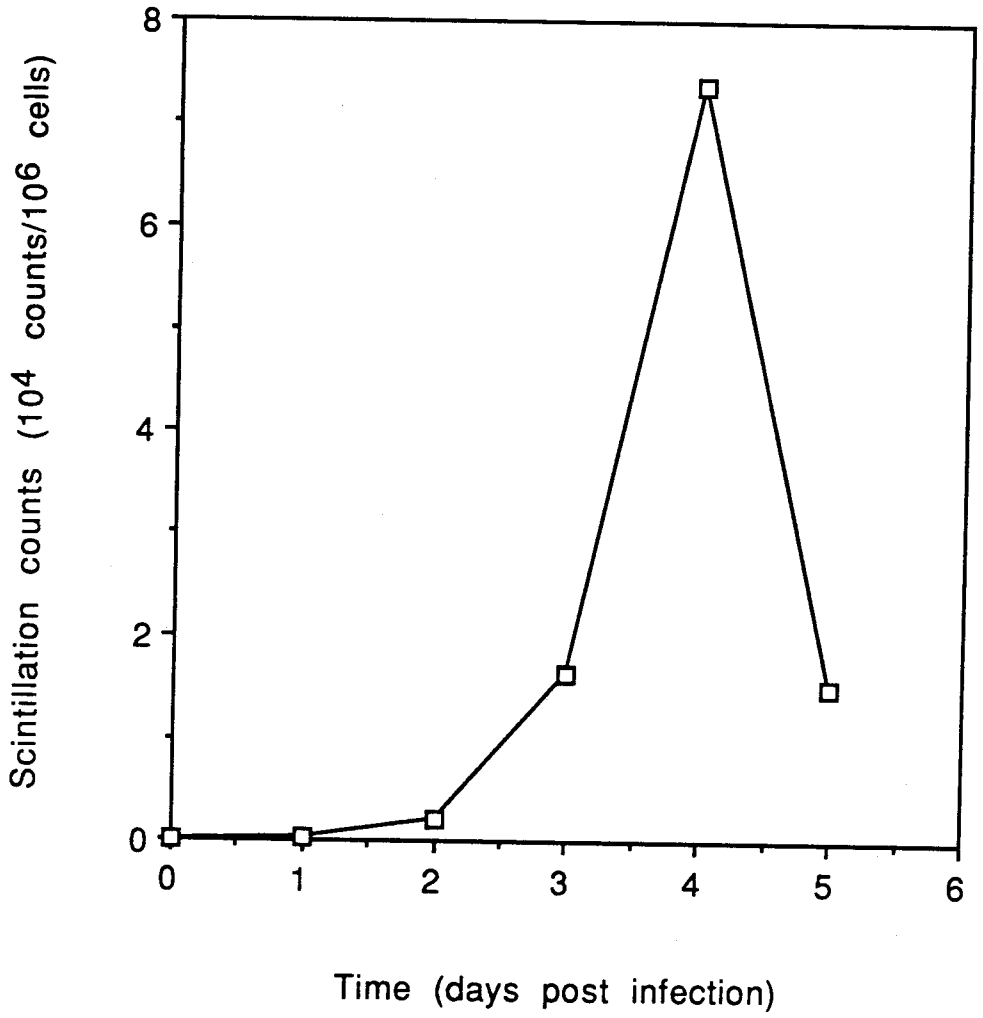

Scintillation counts (10⁴ counts/10⁶ cells)
8
6
4
2
0
0
1
2
3
4
5
6
Time (days post infection)

Figure 9.

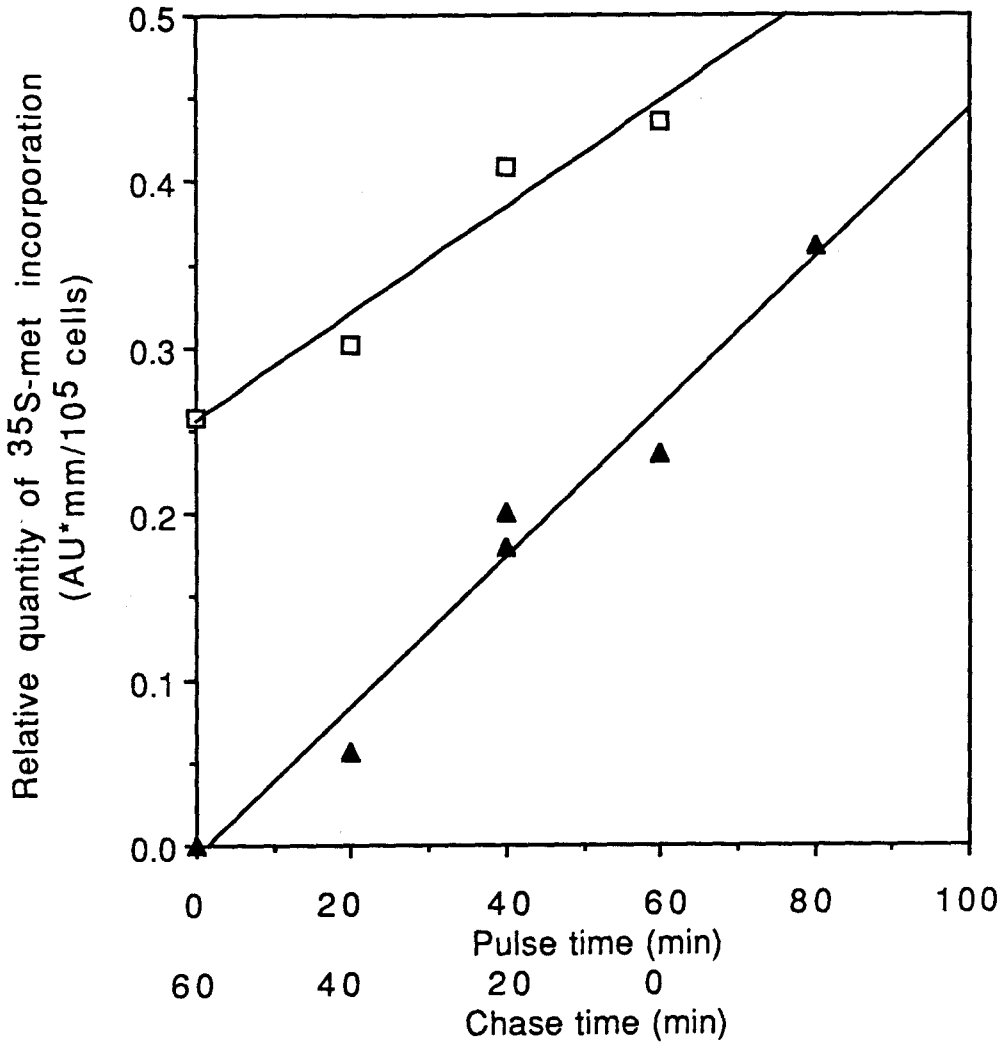
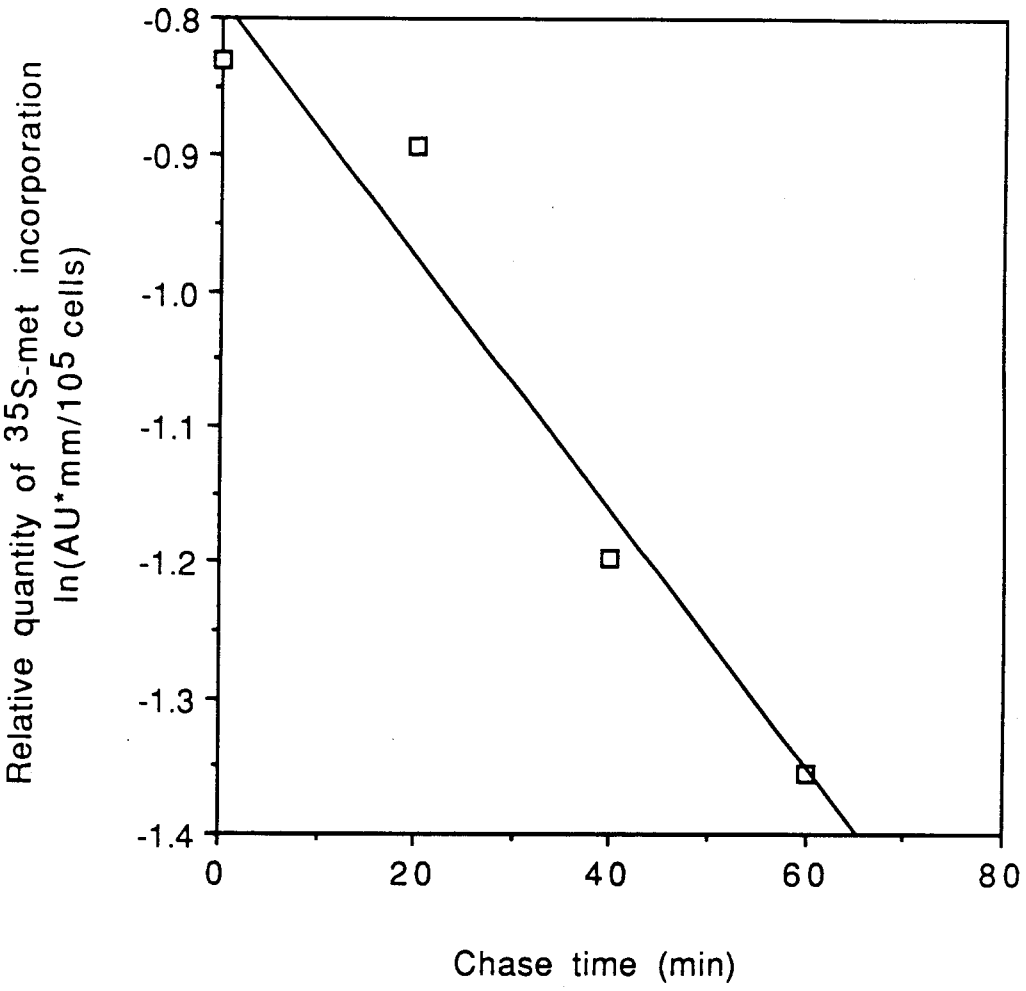

Relative quantity of 35S-met incorporation (AU*mm/10^5 cells)
0.5
0.4
0.3
0.2
0.1
0.0
0
20
40
60
80
100
Pulse time (min)
60
40
20
0
Chase time (min)

Figure 10.


-0.8
-0.9
-1.0
-1.1
-1.2
-1.3
-1.4
0
20
40
60
80
Relative quantity of 35S-met incorporation
ln(AU*mm/10^5 cells)
Chase time (min)

CHAPTER III

Modeling the Population Dynamics of Baculovirus - Infected Insect Cells: Optimizing Infection Strategies for Enhanced Recombinant Protein Yields

Source: Licari, P. and Bailey, J. E. 1992. Biotech. and Bioeng. **39**: 432-441.

3.1 Summary

The insect cell-baculovirus model presented here is capable of simulating cell population dynamics, extracellular virion densities, and heterologous product titers in reasonable agreement with experimental data for a wide range of multiplicities of infection (MOI) and times of infection. The model accounts for the infection of a single cell by multiple virions and the consequences on the time course of infection. The probability of infection by more than one virion was approximated using the Poisson distribution, which proved to be a refinement over second-order kinetics. The model tracks initiation and duration of important events in the progression of infected cell development (virus replication, recombinant protein synthesis, and cell lysis) for subpopulations delineated by the time and extent of their initial infection. The model suggests infection strategies, weighing the importance of MOI and infection time. Maximum product titers result from infection in early-exponential growth phase with low MOI.

3.2 Introduction

From a biochemical engineering perspective, the insect cell-baculovirus expression system poses a unique challenge. In addition to optimizing cell culture procedures, one must optimize the infection strategy. Two parameters important for efficient production of recombinant proteins are the time of infection and the multiplicity of infection (MOI). It has been demonstrated that product yields are dependent on the time in a batch cultivation of cells at which baculovirus is introduced and infection occurs (7). For example, product yield decreases if infection occurs late in the exponential growth phase. The MOI is defined as the amount of virus, in terms of plaque-forming units (PFU), that is added per cell at the time of infection. Depending upon the time of infection, the MOI has been reported to influence product yields (7). If infection takes place early in the exponential growth phase, product yields are relatively independent of MOI. A high MOI results in greater product yields if initial infection occurs in the late-exponential phase. To optimize the production of recombinant protein, a quantitative description of the effects of time of infection and multiplicity of infection on product expression is desirable.

This paper describes a mathematical model developed to follow the progression of an infected cell culture from initial infection to the final state of unilateral cell death. The motivation for this study was multifaceted: to understand and simulate the trends observed with MOI and time of infection, and to determine optimum operating strategies for the infection of cultured insect cells by baculovirus. The model presented here describes an experimental system well; the experimental system consisted of *Spodoptera frugiperda* cells infected with recombinant AcMNPV encoding *Escherichia coli* ß-galactosidase.

3.3 Mathematical Model

Mathematical modeling has been used to describe the regulation of species populations by various factors, including limited resources, predation, and disease (1,2,5,8,10,11,12,13,18,19,21). Although early host-pathogen models assumed that pathogens are relatively short-lived outside the host (9), extensions that include pathogens capable of existing in the environment for extended periods, e.g., wild-type baculovirus, have recently been formulated (6). The common goal of these models is to describe the effects of the pathogen on host abundance and to determine equilibrium or stability criteria. The proposed model differs from ecological population models in several aspects. First, the population we are concerned with is one of nonmotile insect cells, and not one of free roaming, multicellular organisms. In addition, the liquid environment in which the cells are studied is a confined environment with an efficient transfer medium for transport of secondary virions. The focus of this model differs from other population models in that we are interested primarily in a secondary product of the infection and not with the steady-state populations of cells and/or virions.

A detailed model of baculovirus replication was outlined by Shuler *et al.* (14). Beginning with virion attachment, the pathways of virus replication on a molecular scale were pictorially represented. De Gooijer *et al.* (3) published a model describing the synthesis of baculovirus in insect cell cultures. This model was developed for reactor-design purposes, specifically for the continuous production of insect cells and baculovirus. The proposed model describing baculovirus-insect cell population dynamics complements this reactor design model by relating the fundamental aspects of cell-virus dynamics to the macroscopic characteristics of the culture as a whole.

Cell growth and infection. The initial infection of a culture of insect cells is a random event dependent on the number of cells present and the number of virions

introduced into the culture at the time of infection. The addition of virus to a culture will produce two populations: infected and uninfected cells. The population of infected cells is composed of several subpopulations, each demarcated by the number of virions that attached and entered the cells at the time of their initial infection. It is important to distinguish these subpopulations, since the rate of the infection process is dependent on the number of virions that initially infect a cell (unpublished observation).

To develop a dynamic model, the cell population is characterized by a frequency function $n(t,\tau,NV)$ where $n(t,\tau,NV)dt$ is defined as the number of cells/ml in the time interval (t, t+dt) that were infected with NV virions at time $t-\tau$. The value of τ ranges from 0 to τ_L, the time post-infection at which cell lysis occurs, where $\tau_L = \tau_L(NV)$. By defining t_{INF} as the time of initial infection, the time post-infection of the culture is $(t - t_{INF})$. The number concentration of healthy, uninfected cells at time t is indicated by $N_o(t)$ (cells/ml). The differential equation describing the uninfected cell density is

$$\frac{dN_o(t)}{dt} = \mu(t) \cdot N_o(t) - \sum_{NV=1}^{NV_{MAX}} n(t,0,NV), \qquad (1)$$

where $\mu(t)$ is the specific growth rate (h-1) at time t. The term $\sum_{NV=1}^{NV_{MAX}} n(t,0,NV)$ accounts for infection and will be discussed further below. Although this model is easily extended to include several means of cell cultivation and infection, cell growth was considered here to occur in a stationary T-flask. In the stationary flask the exponential growth rate is dependent upon cell density, which determines the remaining space available for daughter cells ("free area"). Possible nutrient limitation that may exist at higher cell densities is neglected. Accounting for the effect of diminished free area on growth, the specific growth rate is written

$$\mu(t) = \mu_{MAX} \cdot A^*(t), \tag{2}$$

where μ_{MAX} is the maximum specific growth rate (h^{-1}) and A^* is a dimensionless, free area of the flask. A^* is defined here as

$$A^*(t) = \left[\frac{KY - N_o(t) - \sum_{NV=1}^{NV_{MAX}} \int_{t_{INF}}^{t} n(\theta,0,NV)\, d\theta}{KY} \right], \tag{3}$$

where KY is the carrying capacity (i.e., the maximum final cell density corresponding to a confluent monolayer) of the culture flask (cells/ml). After a cell is infected by baculovirus, the capacity to divide is diminished, and at some later time, cell division ceases. With the baculovirus studied, this time appears to occur shortly after infection. In this model it is assumed that cells cease to divide immediately after infection.

The multiplicity of infection, MOI, expressed in units of virions/cell is defined as

$$MOI = \frac{V_{EX}(t_{INF})}{N_o(t_{INF})}, \tag{4}$$

where $V_{EX}(t_{INF})$ is the extracellular virion concentration (virions/ml of culture) and $N_o(t_{INF})$ is the density of uninfected cells, both at the time of initial infection. The infection process is a complex chain of events that commences when a virion encounters a cell. For infection to occur the virion must first attach to a cell, possibly by binding to a host cell receptor. After attachment, the virion enters the cell, and activities essential for its propagation commence. In this model, attachment and entrance into the cell are considered to occur at the same instant. Since the precise means of attachment are unknown, and attachment and entry into the cell are rapid, not distinguishing between the

events is justified considering the scope and purpose of this model. In addition, it is assumed that all viruses, once attached to a cell, will infect that cell.

A second-order equation may be used to describe infection in which the frequency of virion-cell encounters is the product of the virion and cell densities. Then, the rate of accumulation of cells infected by a single virion (N_1) is

$$\frac{dN_1(t)}{dt} = K_1 \cdot N_o(t) \cdot V_{EX}(t), \qquad (5)$$

where K_1 is a second-order rate constant. This equation is analogous to that used in one host-one pathogen models first described by Lotka (8) and Volterra (18). In simulations using this equation, the model results were qualitatively different from the experimental results at MOI values greater than unity. Modeled product titers were observed to saturate at high MOI, in contrast to the experimental data.

These results suggested that an approach accounting for the influence of multiple virions infecting a single cell was necessary. The distribution of virions over the anchored cells is closely analogous to the distribution of yeast cells in a hemacytometer, a situation well described by a Poisson distribution (16). According to this analogy, the mean and variance, λ, of the Poisson distribution should be equal to the ratio of the number of virions exposed to the cells divided by the number of cells anchored on the surface of the tissue flask. This quantity is substantially smaller than the MOI because the numerator in the definition of MOI contains all virions in the entire culture volume. At the time of initial infection, medium containing virions is allowed to come in contact with the monolayer of cells for a relatively short period (see Materials and Methods). Assay of the virion density in the decanted medium after this infection interval shows negligible depletion of virus (data not shown). Under such conditions, using a different medium volume at the same initial virion density changes the MOI but does not change the outcome of the infection stage of the experiment. To account for this system dependence,

a proportionality factor, α, was introduced such that $\lambda=\alpha \cdot V_{EX}(t)/N_O(t)$ where $0 \leq \alpha \leq 1$. Regardless of the MOI, α should remain constant for all initial infections, since the same physical system and the same medium volume are used. Thus, at any time, the probability of NV virions infecting a cell, P(NV,t), is calculated from

$$P(NV,t) = \frac{\exp\left\{-\alpha \cdot \left[\frac{V_{EX}(t)}{N_o(t)}\right]\right\} \cdot \left\{\alpha \cdot \left[\frac{V_{EX}(t)}{N_o(t)}\right]\right\}^{NV}}{(NV)!} \qquad NV=0,1, ..., NV_{MAX}-1. \tag{6}$$

P(0,t) is the probability that a susceptible host is not infected at time t. An upper limit on the number of virions able to infect one cell, NV_{MAX}, has been imposed. The probability of a cell's being successfully infected with NV_{MAX} virions is

$$P(NV_{MAX},t) = 1.0 - \sum_{NV=0}^{NV_{MAX}-1} P(NV,t). \tag{7}$$

The upper limit imposed in this analysis is a reasonable assumption when one considers the limited capacity of a single cell. The upper limit may be a result of an effective saturation of cellular machinery influenced by the virus when the number of infecting virions becomes sufficiently large. Given the work by Wickham *et al.*, it is unlikely that NV_{MAX} is limited by receptor density (20). In operational terms, this assumption presumes that a cell infected with $(NV_{MAX}+1)$ virions behaves no differently than a cell infected with NV_{MAX} virions. After calculating the values of P(NV,t), the value of n(t,0,NV)dt is

$$n(t,0,NV)dt = N_O(t) \cdot P(NV,t) \qquad NV=1, ..., NV_{MAX}. \tag{8}$$

Since cells do not divide after infection, the evolution equation from t to t+Δt for infected cells is

$$n(t+\Delta t,\tau+\Delta t,NV) = n(t,\tau,NV)\,. \qquad (9)$$

Three time points in the infection process are important from a modeling perspective: the time post-infection of extracellular virion synthesis (τ_V), the time post-infection of recombinant protein synthesis (τ_P), and the time post-infection of cell lysis (τ_L). The times at which these events occur are affected by the number of virions infecting a given cell at a specific time (unpublished observation). Although determining the precise dependence of these times on NV is difficult, it is reasonable to assume that each of the three time points will occur earlier for larger NV. On the basis of experimental data, maximum and minimum times for τ_V, τ_P, and τ_L are estimated. In the absence of any experimental information regarding the relationship between values of τ and NV, a two-point, linear interpolation of the available data was assumed:

$$\tau_i(NV) = \tau_{i\,MAX} - \left(\frac{\tau_{i\,MAX} - \tau_{i\,MIN}}{NV_{MAX} - 1}\right)(NV - 1)\,, \qquad (10)$$

where i indicates the subscripts V, P, and L cited previously.

After initial infection occurs the virus stock solution is removed and replaced with fresh medium (see Materials and Methods Section). Secondary infection of healthy, uninfected cells is dependent on the synthesis of extracellular virions by infected cells. It is assumed on a single-cell basis that secondary virion production is linear, beginning at τ_V and continuing for a time $\Delta\tau_V$. Furthermore, it is assumed that the total number of virions produced per infected cell is relatively independent of NV and that a maximum number of virions may be produced per cell prior to cell lysis, defined here as ß (virions/cell). The rate of virion synthesis, K_V, is a function of NV and is defined as

$$K_V = 0 \qquad \tau < \tau_V(NV)$$

$$K_V(t,\tau,NV) = \left(\frac{\beta}{\Delta\tau_V}\right) A^*(t) \qquad \tau_V(NV) \le \tau < \tau_V(NV)+\Delta\tau_V \,. \tag{11}$$

$A^*(t)$, which is equal to $\mu(t)/\mu_{MAX}$ from Eq. 2, serves as a measure of the health of the culture and its ability to synthesize viral products. The processes of virion synthesis and budding are not distinguished in this analysis; i.e., they are assumed to occur simultaneously. The density of free virions at time t is a function of the quantity being synthesized by infected cells and the amount infecting uninfected cells (for simplicity, binding to previously infected cells is neglected). The differential equation describing the change in free-virion density with time is

$$\frac{dV_{EX}(t)}{dt} = \sum_{NV=1}^{NV_{MAX}} \int_{\tau_V(NV)}^{\tau_V(NV)+\Delta\tau_V(NV)} K_V(t,\theta,NV)\cdot n(t,\theta,NV)\, d\theta - \sum_{NV=1}^{NV_{MAX}} NV\cdot n(t,0,NV) \,. \tag{12}$$

The total number concentration of cells infected at any given time, $N_{INF}(t)$, is equal to all cells previously infected and not yet lysed, which can be evaluated using

$$N_{INF}(t) = \sum_{NV=1}^{NV_{MAX}} \int_{t-\tau_L(NV)}^{t} n(\theta,0,NV)\, d\theta \,. \tag{13}$$

The *in vitro* infection of insect cells by baculovirus is invariably lethal, culminating in cell lysis. The total number concentration of lysed cells at any given time, $N_{LYS}(t)$, may be determined by

$$N_{LYS}(t) = \sum_{NV=1}^{NV_{MAX}} \int_{t_{INF}+\tau_L(NV)}^{t} n(\theta,\tau_L(NV),NV)\, d\theta. \quad (14)$$

Product synthesis. Previous studies (7) of the synthesis and degradation of recombinant protein in the Sf9-AcMNPV system showed that although intracellular protein degradation occurs, it does not significantly reduce the amount of product formed late in infection when recombinant protein expression is greatest. Since final product yields are relatively unaffected by degradation, the net synthesis of heterologous protein is considered to be a zero-order reaction with rate K_S. K_S is a function of t, τ, and NV. It is assumed that a maximum quantity of cloned protein may be produced per cell prior to cell lysis and that this quantity is independent of the number of virions to infect a cell. This constant is designated ζ and has units of enzyme activity units per cell. Although the value of NV does not affect the quantity of protein produced, it is important in defining the time interval during which recombinant protein is produced. $K_S(t,\tau,NV)$ is given by

$$K_S = 0 \qquad 0 \le \tau < \tau_P(NV)$$

$$K_S(t,\tau,NV) = \left(\frac{\zeta}{\tau_L(NV) - \tau_P(NV)}\right) A^*(t) \qquad \tau_P(NV) \le t < \tau_L(NV). \quad (15)$$

$A^*(t)$ is used in Eq. 15 for the same reasons as in Eq. 11; recombinant protein production is greatest when the uninfected cells are dividing at the maximum, specific growth rate. When the cells are in stationary phase, product synthesis is negligible.

This model was developed on the basis of studies with a baculovirus encoding *E. coli* ß-galactosidase gene under the polyhedrin promoter. This protein is an intracellular protein in *E. coli* and in infected insect cells. Although not secreted, ß-galactosidase is present post-infection in the extracellular space of a recombinant baculovirus-*S.*

frugiperda culture prior to cell lysis, indicating leakage from the cell. The leakage process occurs relatively late after the onset of heterologous protein synthesis, suggesting that transfer from the cell is not by a normal protein-export mechanism but is an artifact of an unhealthy cell. A proportionality constant, K_{LK}, is used to describe the leakage of product from the cell. The quantity of product released to the abiotic phase is proportional to the driving force ($[P]_{biotic}$ - $[P]_{abiotic}$), where $[P]_{biotic}$ is in units of enzyme units/cell volume and $[P]_{abiotic}$ is expressed as enzyme units per culture volume. Since the biotic volume is a small fraction of the abiotic volume and protein is synthesized in the biotic volume, $[P]_{biotic} >> [P]_{abiotic}$ so ($[P]_{biotic}$ - $[P]_{abiotic}$) may be approximated by $[P]_{biotic}$. The time post-infection that leakage begins is defined as τ_{LK}. τ_{LK} is a function of NV, such that the higher NV, the lower the value of τ_{LK}. In this model, the values of $\tau_{LK}(NV)$ are calculated in the same manner as τ_V, τ_P, and τ_L (Eq. 10). With appropriate bounds on the time interval, $K_{LK}(\tau)$ is defined as

$$\begin{aligned} K_{LK} &= 0 && 0 \le \tau < \tau_{LK}(NV) \\ K_{LK} &= K^{o}_{LK} && \tau_{LK}(NV) \le \tau < \tau_{L}(NV). \end{aligned} \qquad (16)$$

Defining $p_i(t,\tau,NV)$ as the intracellular product concentration (activity units/cell) of cells in the state (t,τ,NV), the rate of intracellular product accumulation is

$$\frac{dp_i(t,\tau,NV)}{dt} = K_S(t,\tau,NV) - K_{LK}(\tau) \cdot p_i(t,\tau,NV). \qquad (17)$$

The total quantity of intracellular protein at any time provides a means of direct comparison with experimental results. This quantity, $P_{INT}(t)$ (enzyme activity units/ml of culture), is calculated by

$$P_{INT}(t) = \sum_{NV=1}^{NV_{MAX}} \int_{\tau_P(NV)}^{\tau_L(NV)} p_i(t,\theta,NV) \cdot n(t,\theta,NV)\, d\theta. \tag{18}$$

The quantity of product in the abiotic phase at time t is composed of product released because of leakage and product released from the cell at lysis. This quantity is calculated by

$$P_{EXT}(t) = \sum_{NV=1}^{NV_{MAX}} \int_{t_{INF}+\tau_L(NV)}^{t} p_i(\theta,\tau_L(NV),NV) \cdot n(\theta,\tau_L(NV),NV)\, d\theta$$
$$+ \sum_{NV=1}^{NV_{MAX}} \int_{t_{INF}+\tau_{LK}(NV)}^{t} \int_{\tau_{LK}(NV)}^{\tau_L(NV)} K_{LK}(\alpha) \cdot p_i(\theta,\alpha,NV) \cdot n(\theta,\alpha,NV)\, d\alpha\, d\theta. \tag{19}$$

Numerical solution of the model was accomplished by approximating derivatives using a first-order difference approximation (Euler method) and evaluating integrals by a first-order summation approximation with a Δt of one hour. These calculations were indistinguishable from others employing a smaller Δt.

3.4 Materials and Methods

Cell culture. *Spodoptera frugiperda* IPL-Sf9, a continuous cell line, was obtained from the ATCC (#CRL-1711). Cells were cultured in modified TNM-FH medium consisting of Grace's medium (Gibco) supplemented with 3.3 g/l Difco yeastolate, 3.3 g/l lactalbumin hydrolysate, 0.35 g/l $NaHCO_3$, 10% fetal bovine serum (Hyclone), 5000 units/l penicillin, and 5 mg/l streptomycin (Sigma). Cultures grew as monolayers in stationary T-flasks maintained at 27°C. Cells were passaged by flushing the monolayer off the surface of the T-flask with a Pasteur pipette. Total cell counts were determined by a Coulter Counter (Coulter Electronics, Inc.), and viable cell counts were determined by trypan blue exclusion (4).

Virus stocks and infection. Recombinant AcMNPV virus encoding a fusion polyhedrin-ß-galactosidase protein under the control of the polyhedrin promoter (constructed using the transfer vector pAc360 (17) and designated 360-lac) was obtained from Michael Lochrie (California Institute of Technology). The fusion protein contains a segment of the polyhedrin protein fused with ß-galactosidase (Mr 120 kDa).

Infection began when medium was aspirated off the monolayer culture and virus stock solution was added (designated time 0 post-infection). After addition of viral stock, cultures were placed on a rocking platform for 1 hour, while maintained at 27°C. Cells were removed from the platform, and after one additional hour at 27°C, the medium containing virus was replaced with fresh medium. This step prevented any ß-galactosidase introduced with the viral stock from affecting enzyme titers. Mock-infection of cells was used as a control in all experiments: Cultures were treated identically to the infected cells, with the exception that fresh medium was added at the time of infection. All assays were performed on individual T-flasks at different times

post-infection from a bank of flasks treated identically during cell inoculation and infection.

Final-state virus titers were determined using the end-point dilution method by the addition of 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal) to the medium (17). Samples for virus titer determination were made by removing an aliquot from the T-flask and centrifuging at 1000 g for 7 minutes to remove cellular debris.

ß-galactosidase assay. ß-galactosidase activity was assayed using O-nitrophenyl-ß-D galactoside (ONPG) as a substrate. The conversion to O-nitrophenyl was recorded by monitoring the absorption at 420 nm with time (extinction coefficient = 4.5 ml/µmol-cm). 0.3 ml of ONPG (10 g/l) were added to 0.3 ml of sample and 2.4 ml of Z-buffer (16.1 g/l Na_2HPO_4-$7H_2O$, 5.5 g/l NaH_2PO_4-H_2O, 0.75 g/l KCl, 0.246 g/l $MgSO_4$-$7H_2O$, 2.7 ml/l ß-mercaptoethanol) at 37°C. Optical density at 37°C was recorded after a lag time of 2 minutes.

Samples for the determination of extracellular ß-galactosidase were made by removing an aliquot of supernatant from the culture and centrifuging at 1000 g for 5 minutes. Samples for total ß-galactosidase assays were obtained by resuspending the monolayer with a Pasteur pipette, removing a culture sample, sonicating for 3 min, and subsequently centrifugating at 1000 g for 7 min.

3.5 Results and Discussion

Estimation of parameters from experimental studies. The maximum specific growth rate and the final cell density used in the growth equation (μ_{MAX} and KY) are readily obtained from a growth curve of uninfected Sf9 cells (Figure 1). The values of μ_{MAX} and KY derived from Figure 1 and used in the model are listed in Table 1. Theoretically, estimation of α based on initial infection studies is possible. However, differences in virus titers were unreliable because of difficulties in accurately determining virus titers. Such studies did demonstrate that the value of α is very small; i.e., it is closer to zero than to unity. A value of 0.04 was assigned for all times in the infection process. Although somewhat arbitrary, the value of this parameter and other parameters was not changed to simulate different cases. Conceptually, the small value of α is expected as discussed above. There are at present insufficient experimental data to determine the maximum number of virions that can infect a cell or the point at which the cellular machinery is saturated. Because of the lack of experimental information regarding NV_{MAX}, the influence of this parameter on the behavior of the model was investigated in simulations; for NV_{MAX} values of 5 and 10, results were not significantly different. Consequently, a value of NV_{MAX}=5 will be used in all calculations reported.

Upper and lower bounds on the times important in the infection process are determined from data on the time trajectories of cell growth, virion production, and product accumulation. Minimum and maximum values for τ_V, τ_P, τ_{LK}, and τ_L are listed in Table 2. The period of product synthesis and product leakage begins at a characteristic time and continues until cell lysis. The time interval during which virion synthesis occurs is relatively short-lived and appears to be independent of MOI (unpublished observation). $\Delta\tau_V$ is estimated to be 25 hours. ß (virions/cell) and ζ (activity units/cell) are determined by dividing the final concentration of virions or product by the cell density actively synthesizing it. For example, a final virus titer of 6×10^8 PFU/ml was assayed for cells

infected in the early- to mid-exponential growth phase with an initial MOI of 1 (see experimental data of Fig. 4). The actively dividing population at the time of virion synthesis was between $5x10^5$ and $1x10^6$ cells/ml. Using an average cell density of $7.5x10^5$ cells/ml, ß is estimated to be 800 PFU/cell. The value of ζ was based on the final product titers for cells infected in the early-exponential growth phase (Fig. 6). Calculations analogous to those used to calculate ß suggested a range of $1.2x10^{-4}$ to $2.0x10^{-4}$ units/cell. The values of ß and ζ used in all simulations are listed in Table 1. All of the milestone points and rates were estimated from experiments chosen to facilitate parameter estimation. These parameters were then fixed, and the model was employed to simulate cultures with different MOI and initial infection times.

Growth of uninfected and infected cells. Figure 1 compares an experimental growth curve of uninfected cells with that calculated by the model. Time zero in the model is defined as the time of onset of exponential growth of uninfected cells in experimental studies. This time is somewhat later than the experimental time of cell inoculation in the flask because of the time required for attachment and adaptation of the cells. Considering the simplicity of the growth equation, the growth-phase trajectory and the stationary phase maximum cell density are calculated quite well. Agreement between modeled growth and experimental growth is important because the nondimensional quantity $\mu(t)/\mu MAX$ is used as a measure of the cellular activity in other parts of the model.

Experimental growth curves for cells infected with MOIs ranging from 0.01 to 100 are presented in Figure 2A. In these experiments cells were infected in the early- to mid-exponential growth phase (corresponding to 25 hours in Figure 1). As expected, cell death is more rapid for cultures infected with higher MOI. Prior to comparing these data with model simulations, the inadequacy of the trypan blue dye-exclusion technique in discerning uninfected cells from infected cells should be addressed. Uninfected cells and

cells at an early stage of infection cannot be distinguished by dye exclusion. It is only at some later time after infection that the cellular membrane is sufficiently compromised and dye penetrates. Although direct comparison cannot be made, the model trajectories of uninfected cell densities (Figure 2B) are qualitatively similar to experimental trajectories for the same conditions (Figure 2A). The eventual decline of uninfected cells is less rapid in Figure 2A because the experimental estimation of uninfected cells includes unstained, infected cells. Examination of Figure 2 demonstrates the importance of the initial MOI on the population dynamics. At an MOI of 100, the great majority of cells are immediately infected, leaving few viable cells to divide. For low MOIs (0.01 to 1) few cells are initially infected and the cell population continues to increase. For all MOIs a precipitous decrease in uninfected cells is observed at some point in time, an inevitable consequence of the synthesis of viral progeny.

The model calculates time trajectories of various subpopulations that wax and wane following initial infection. Figure 3 shows trajectories of uninfected cells, infected cells, and lysed cells for a culture infected with an MOI of 0.01 at 25 hours. The ability to observe these different cell populations in simulations provides insight into the influence of different operating strategies.

Synthesis of progeny virus. Synthesis of virions and the extracellular concentration of virions as a function of time may be simulated with this model. Figure 4 presents the calculated, extracellular virion density as a function of time for several MOIs (corresponding to ca. 25 hours in Figure 1). If one is concerned with maximizing virion production, these results indicate that an MOI of 1 yields the greatest density of virions for an infection occurring at this time. Experimental values of final virus titers for MOIs of 0.1, 1, and 10 are also presented on Figure 4. General agreement between the modeling results and the experimental data is demonstrated for the final virus titers.

Production of ß-galactosidase. A central objective of this modeling study is to investigate the influence of the MOI and the time of infection on cloned protein production. The time trajectories of intracellular and extracellular ß-galactosidase concentrations are presented for two representative cases in Figure 5. Figure 5A (MOI=10, t_{INF}=0 h) and Figure 5B (MOI=0.1, t_{INF}=65 h) represent extremely different operating strategies. Although minor differences exist in the time trajectories, general agreement between the model and experimental data is demonstrated over a wide range of conditions. Leakage of ß-galactosidase out of the cell and trypan blue penetration both increase as the time post-infection increases. However, establishing an experimental correlation between the two was not possible.

Final product titers were determined for cultures infected in the early- and late-exponential phases for MOI values ranging from 0.01 to 100. The experimental data and the model simulations for identical conditions are presented in Figure 6. Model results for cells infected early in the exponential phase (25 h) are very similar to the experimental data. Qualitative agreement exists between model and experiment for cells infected in the late-exponential phase (75 h), although model estimates are too high for low MOI.

Insight into the trends observed in Figure 6 is gained by analyzing the simulated time trajectories of uninfected cells, infected cells, and lysed cells. Infection of a culture in the early-exponential growth phase tends to minimize the effect of MOI on final product titer because progeny virions are produced early enough to encounter uninfected cells still in the exponential growth phase and capable of product synthesis. Product yield decreases for cultures infected in the early-exponential growth phase with low MOIs (0.01) and high MOIs (100). By infecting at a very low MOI, few cells are initially infected, and by the time sufficient progeny virions are synthesized for mass infection, the cell density has approached confluence. The reason for the decrease at high MOI values is the mass, synchronous initial infection. By initially infecting the vast majority of

the cells, few cells are left to divide, and the total number of cells which synthesize product is reduced.

Product titers are strongly dependent on the MOI used for cultures infected in the late-exponential growth phase, with higher MOIs resulting in higher titers. Cells infected late in the exponential growth phase have a reduced capacity to divide and to synthesize protein, as indicated by a reduced value of $\mu(t)/\mu_{MAX}$. Infection with a low MOI results in a large proportion of uninfected cells that continue to divide. By the time progeny virions are produced from the infected population, the uninfected cells have approached stationary phase and have a severely reduced capacity for protein synthesis. An initial mass infection of cells is necessary to optimize product yields when infecting a culture in the late-exponential growth phase.

Many simulations were conducted to map the effect of time of infection on final product yields for MOI values ranging from 0.01 to 100 (Figure 7). For each MOI there is an optimum time of infection (and vice versa). Furthermore, maximum titers are obtained with early infection times and low MOIs. In addition to maximizing titers, this strategy prevents the synthesis of defective progeny that may result from use of high MOI (23). Given a culture of cells at a specified time in the growth curve, the optimum MOI to be used in the infection strategy can be obtained from Figure 7B.

3.6 Acknowledgments

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3.7 Nomenclature

$A^*(t)$	nondimensionalized free area
K_1	second-order rate constant in Eq. 5
$K_{LK}(\tau)$	first-order rate constant of protein leakage (h^{-1})
$K_S(t,\tau,NV)$	zero-order rate constant of protein synthesis (units/cell·h)
$K_V(t,\tau,NV)$	zero-order rate constant of virion synthesis (virions/cell·h)
KY	carrying capacity of culture flask (cells/ml)
MOI	multiplicity of infection (virions/cell)
$N_o(t)$	uninfected cell density (cells/ml)
$N_1(t)$	infected cell density of Eq. 5 (cells/ml)
$n(t,\tau,NV)dt$	infected cell density (cells/ml)
$N_{INF}(t)$	total infected cell density (cells/ml)
$N_{LYS}(t)$	lysed cell density (cells/ml)
NV	number of virions to infect a cell initially
NV_{MAX}	maximum number of virions to infect a single cell
$P_{EXT}(t)$	extracellular product concentration (activity units/ml)
$P_{INT}(t)$	intracellular product concentration (activity units/ml)
$p_i(t,\tau,NV)$	intracellular product concentration (activity units/cell)
P(NV,t)	probability of NV virions infecting a single cell
t	time (h)
t_{INF}	time of initial infection (h)
$V_{EX}(t)$	extracellular virion concentration (virions/ml)

Greek Letters

α	proportionality constant in Eq. 6
β	number of virions produced per cell (virions/cell)
τ	time post-infection (h)
τ_{iMIN}	minimum time post-infection for event i to commence (h)
τ_{iMAX}	maximum time post-infection for event i to commence (h)
$\tau_{LK}(NV)$	time post-infection of onset of product leakage (h)
$\tau_{L}(NV)$	time post-infection of cell lysis (h)
$\tau_{P}(NV)$	time post-infection of onset of product synthesis (h)
$\tau_{V}(NV)$	time post-infection of onset of virion synthesis (h)
μ	specific growth rate (h^{-1})
μ_{MAX}	maximum specific growth rate (h^{-1})
ζ	total product produced per cell (activity units/cell)

3.8 References

1. Beddington, J. R., Free, C. A, Lawton, J. H. 1975. Dynamical complexity in predator-prey models framed in difference equations. *Nature (London).* **255**: 58-60.

2. Chesson, P. L., Murdoch, W. W. 1986. Aggregation of risk: relationships amongst host-parasitoid models. *Am. Nat.* **127**: 696-715.

3. de Gooijer, C. D., van Lier, F. L. J., van den End, E. J., Vlak, J. M., Tramper, J. 1989. A model for baculovirus production with continuous insect cell cultures. *Appl. Microbiol. Biotech.* **30**: 497-501.

4. Freshney, R. I. 1983. Culture of Animal Cells. Alan R. Liss, Inc., NY.

5. Hassell, M. P., May, R. M. 1973. Stability in insect host-parasite models. *J. Anim. Ecol.* **42**: 693-726.

6. Hochberg, M. E., Hassell, M. P., May, R. M. 1990. The dynamics of host-parasitoid-pathogen interactions. *Am. Nat.* **135**: 74-94.

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

8. Lotka, A. J. 1925. Elements of Physical Biology. Williams & Wilkins, Baltimore, MD.

9. May, R. M. 1985. Regulation of populations with nonoverlapping generations by microparasites: a purely chaotic system. *Am. Nat.* **125**: 573-584.

10. May, R. M. 1978. Host-parasitoid systems in patchy environments: a phenomenological model. *J. Anim. Ecol.* **47**: 833-844.

11. May, R. M., Hassell, M. P. 1981. The dynamics of multiparasitoid-host interactions. *Am. Nat.* **117**: 234-261.

12. Murdoch, W. W., Oaten, A. 1975. Predation and population stability. *Adv. Ecol. Res.* **9**: 2-131.

13. Nicholson, A. J. 1933. The balance of animal populations. *J. Anim. Ecol.* **2**: 132-178.

14. Shuler, M. L., Cho, T., Wickham, T., Ogonah, O., Kool, O., Hammer, D. A., Granados, R. R. Wood, H. A. 1991. Bioreactor development for production of viral pesticides or heterologous proteins in insect cell cultures. *Biochemical Engin.* **589**: 399-422.

15. Strauss, E. G., Strauss, J. H., Levine, A. J. 1990. Virus Evolution. *In* B. N. Fields and D. M. Knipe (eds.), Fields Virology. Raven Press, NY.

16. "Student." 1907. On the error of counting with a haemacytometer. *Biometrika.* **5**: 351-360.

17. Summers, M. D., Smith, G. E. 1987. A manual of methods for baculovirus vectors and insect cell culture procedure. Texas Agricultural Experiment Station Bulletin No. 1555.

18. Volterra, V. 1931. Variations and fluctuations in the number of individuals in animal species living together. *In* Animal Ecology, with Special Reference to Insects. R.N. Chapman (ed), McGraw-Hill, NY. pp. 409-448.

19. Watt, K. E. F. 1959. A mathematical model for the effect of densities of attacked and attacking species on the number attacked. *Can. Entom.* **91**: 129-144.

20. Wickham, T. J., Granados, R. R., Wood, H. A. 1990. General Analysis of Receptor Mediated Viral Attachment to Cell Surfaces. *Biophys. J.* **58**: 1501-1516.

21. Young, S. Y., Yearian, W. C. 1986. Soil application of Pseudoplusia NPV: persistence and incidence of infection in soybean looper caged in soybean. *J. Invert. Pathol.* **8**: 860-864.

3.9 Tables

Table 1. Values of parameters used in model.

Parameter	Value
μMAX	0.035 h^{-1}
KY	$2.6x10^6$ cells/ml
ß	800 virions/cell
ζ	$1.8x10^{-4}$
α	0.04
K_{LK}	0.05
NV_{MAX}	5

Table 2. Lower and upper bounds on time points (h) describing the infection process.

Parameter	Minimum (h)	Maximum (h)
τV	20	30
τP	25	35
τLK	30	60
τL	50	120

3.10 Figures

Figure 1. The growth of uninfected Sf9 cells in TNM-FH medium. Viable cells/ml on a logarithmic scale are plotted against time (hours). □ indicates experimental data, and the line without symbols was generated using the model.

Figure 2. A. Unstained cell density of cultures infected with different MOIs. Cell densities (10^6 cells/ml) are plotted against time (hours post-infection). MOI values used are: ● 0.01, □ 0.1, ▲ 1.0, ○ 10., ■ 100. Infection began at $5.5x10^5$ cells/ml (early-exponential phase). B. Uninfected cell density of cultures infected with different MOIs as calculated from the model. Uninfected cell densities (10^6 cells/ml) are plotted against time (hours post-infection). MOI values used are: ● 0.01, □ 0.1, ▲ 1.0, ○ 10., ■ 100. Infection began at 50 hours or $5.5x10^5$ cells/ml.

Figure 3. Population cell densities (10^6/ml) as calculated from the model for a culture infected with an MOI of 0.01 and an infection time of 25 hours. Uninfected cell density (▲), infected cell density (□), and lysed cell density (●) are plotted against time (hours post-infection).

Figure 4. Virion density (ml^{-1}) as a function of time post-infection for different MOIs as calculated from the model. Virion density for MOI of 0.01 (●), 0.1 (□), 1.0 (▲), 10 (○) and 100 (■) are plotted against hours post-infection. Final virus titers as determined from experiment are indicated by the corresponding larger symbols containing a line.

Figure 5. The production of ß-galactosidase (activity units/ml culture) as a function of time post-infection (hours p.i.) for a culture infected at (A) 0 hours ($2.5x10^5$ cells/ml)

with an MOI of 10 and (B) 65 hours ($1x10^6$ cells/ml) with an MOI of 0.1. Experimental data: ● intracellular ß-galactosidase activity and ■ extracellular ß-galactosidase activity. Model results: ○ intracellular ß-galactosidase activity and □ extracellular ß-galactosidase activity.

Figure 6. Final-state ß-galactosidase titers (activity units/ml culture) as a function of the multiplicity of infection on a logarithmic scale. For cells infected in the early-exponential growth phase, □ indicates experimental data and ■ indicates model simulations. For cells infected in the late-exponential growth phase, ○ indicates experimental data and ● indicates results obtained from the model.

Figure 7. A. Final-state ß-galactosidase titers (activity units/ml) as a function of both the time of infection (hours post-cell inoculation) and the multiplicity of infection. B: Contour plot for optimizing infection strategies. Domains of the time of initial infection (h) and MOI corresponding to different ranges of final ß-galactosidase titers are shown. The legend to the right indicates final product titers (activity units/ml).

Figure 1.

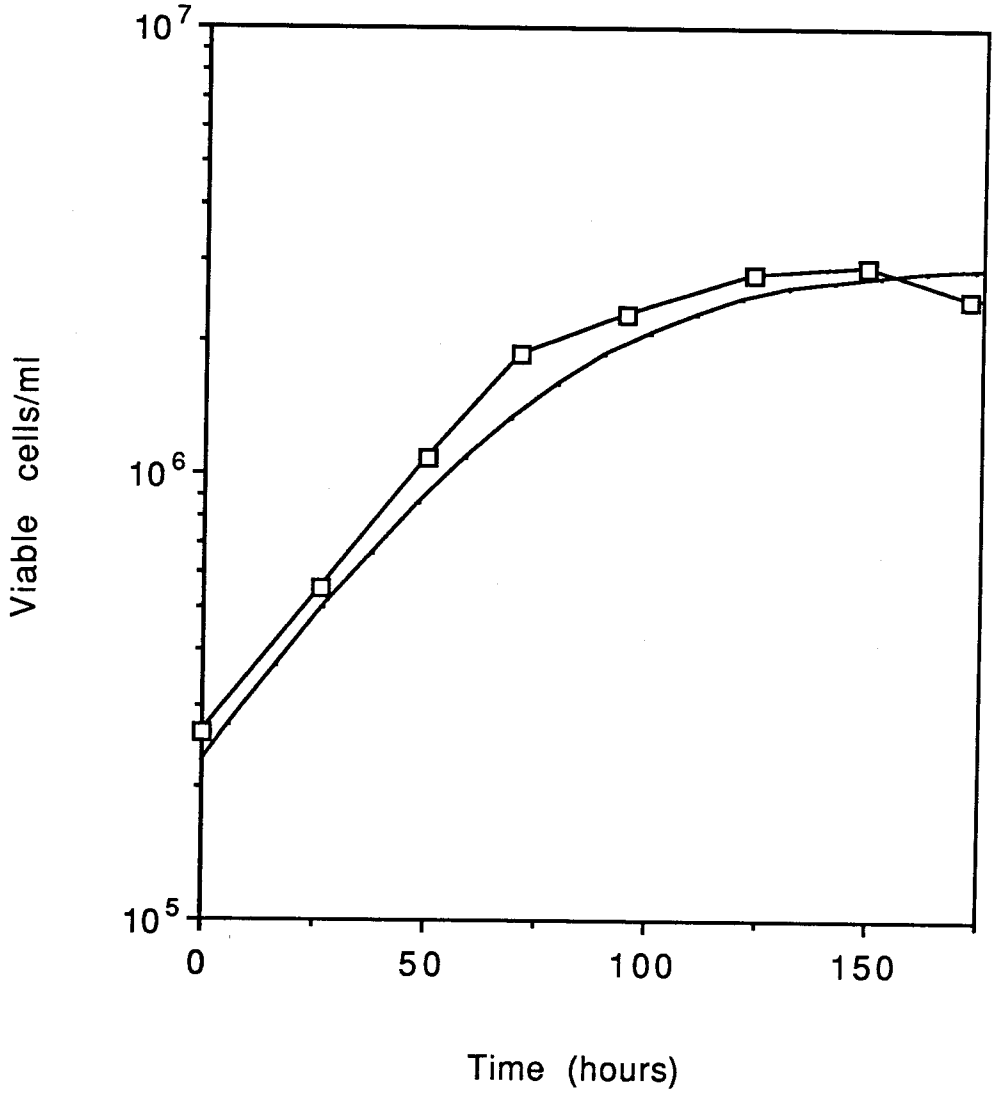


Figure 2.

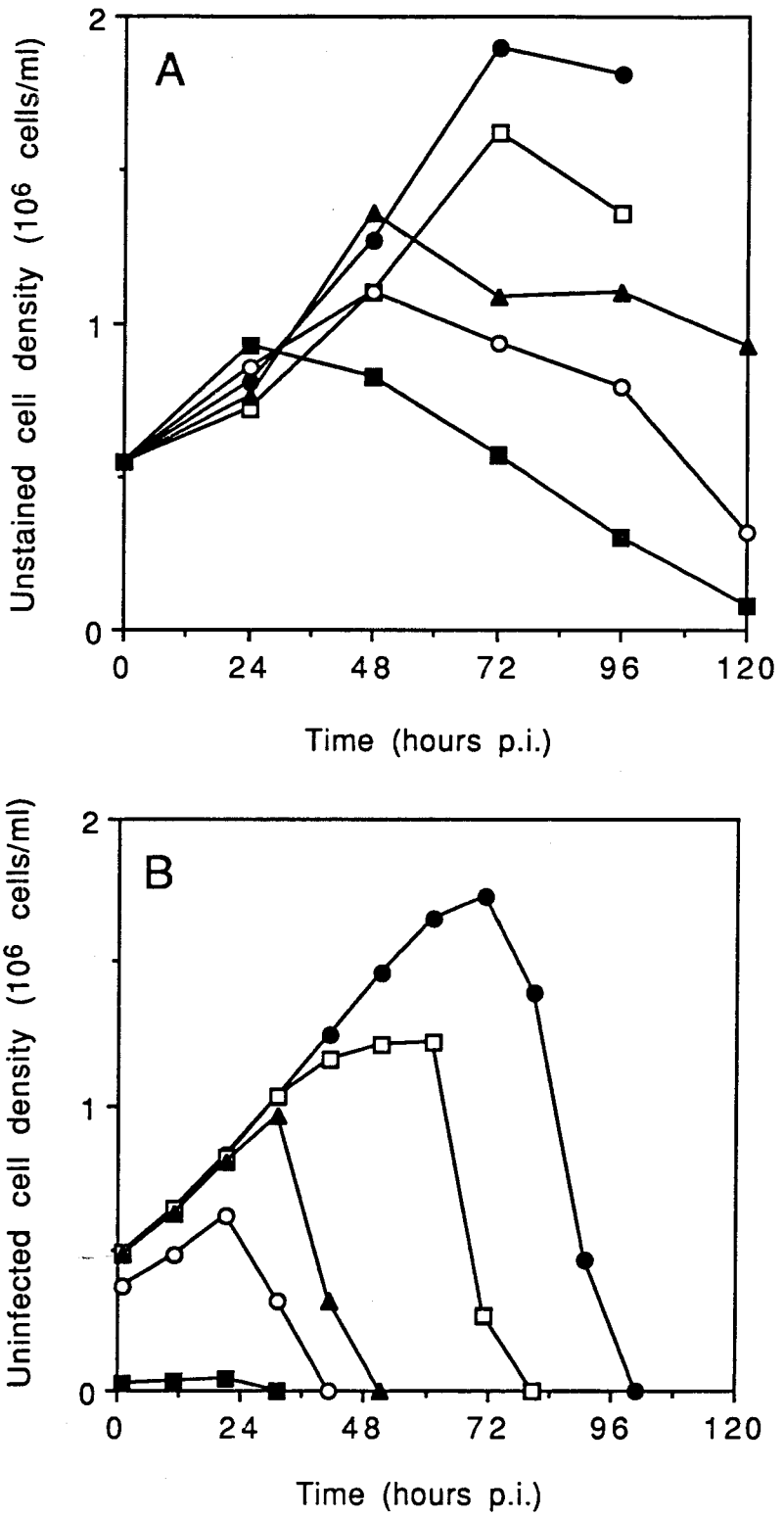

A
Unstained cell density (10^6 cells/ml)
2
1
0
0
24
48
72
96
120
Time (hours p.i.)
B
Uninfected cell density (10^6 cells/ml)
2
1
0
0
24
48
72
96
120
Time (hours p.i.)

Figure 3.

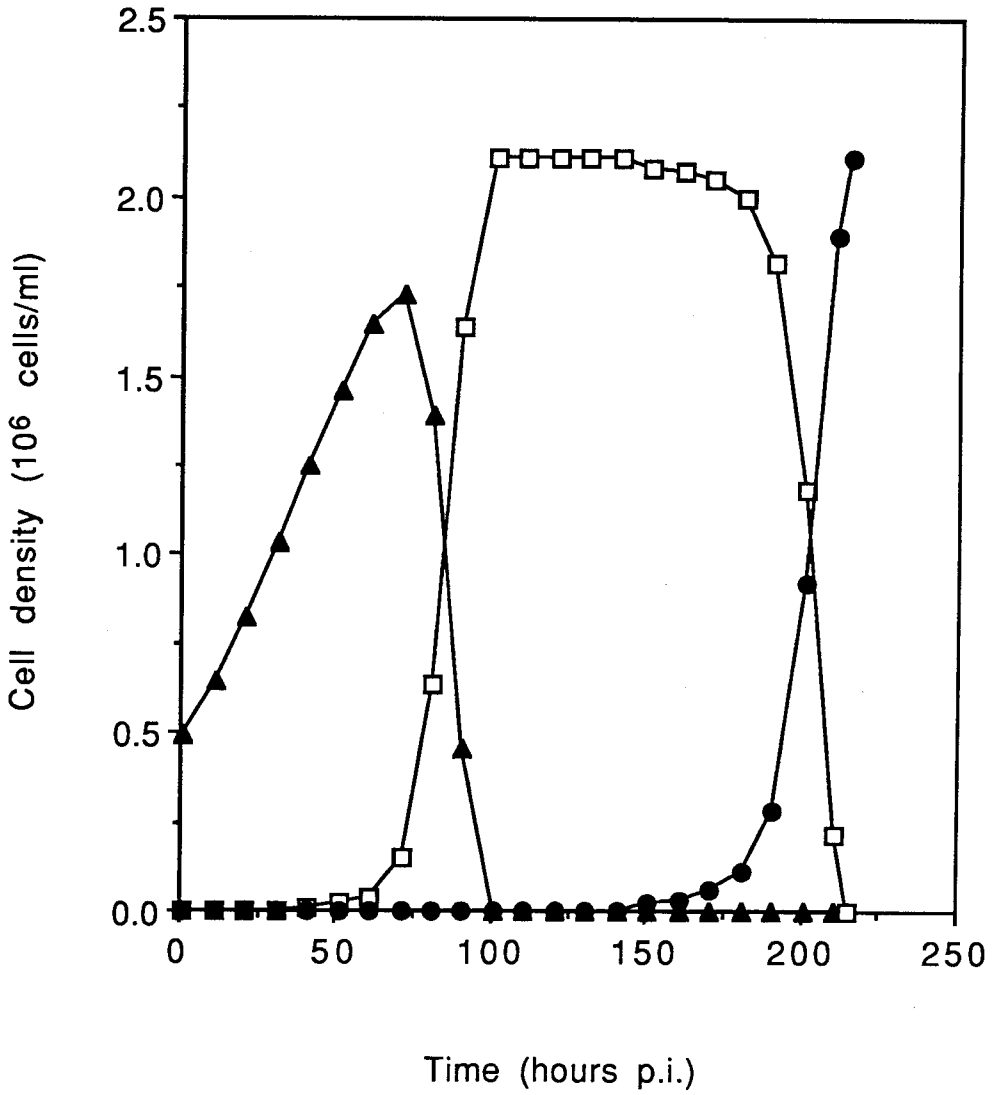

2.5
2.0
1.5
1.0
0.5
0.0
Cell density (10⁶ cells/ml)
0
50
100
150
200
250
Time (hours p.i.)

Figure 4.

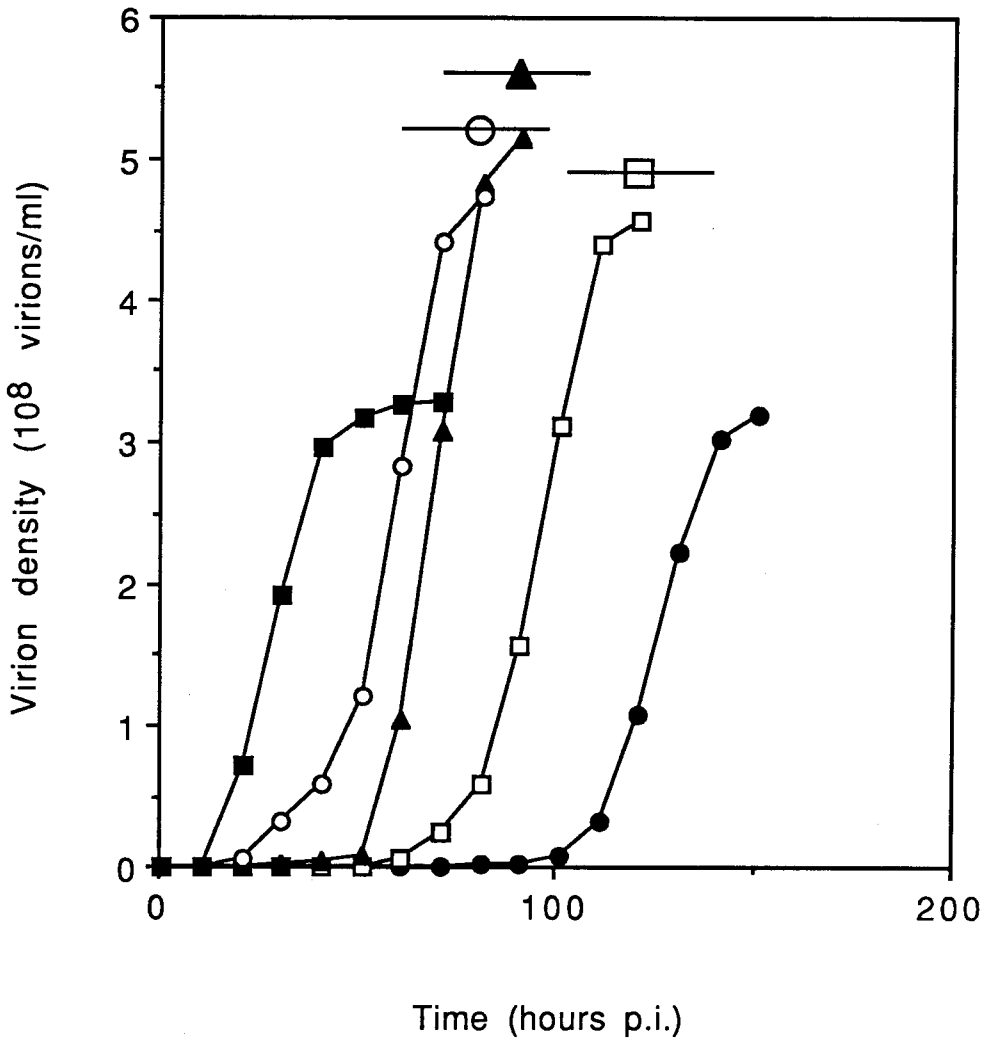
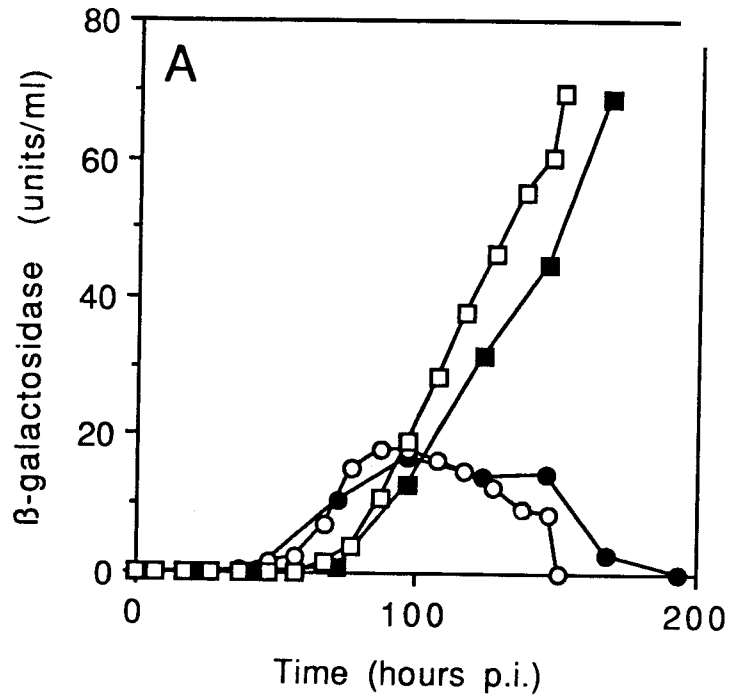

6
5
4
3
2
1
0
0
100
200
Virion density (10^8 virions/ml)
Time (hours p.i.)

Figure 5.


A
ß-galactosidase (units/ml)
80
60
40
20
0
0
100
200
Time (hours p.i.)

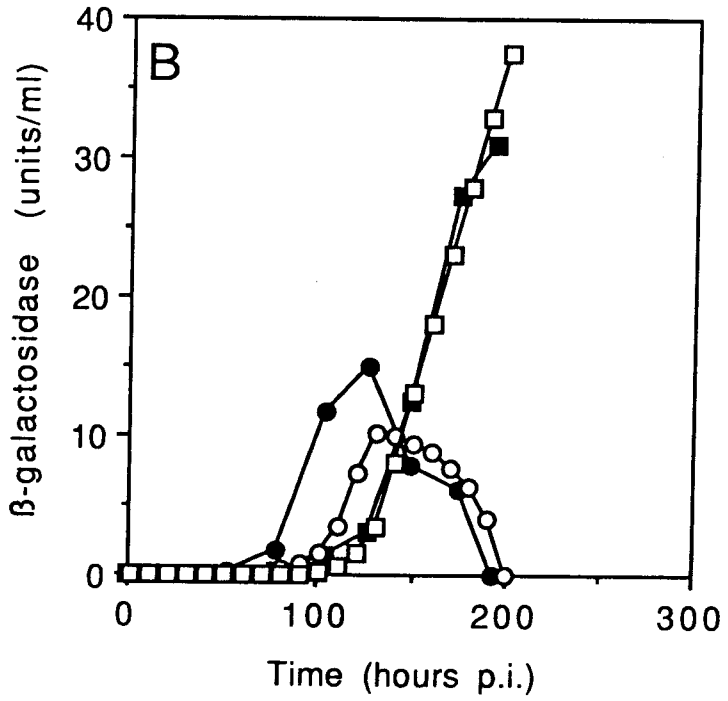

B
ß-galactosidase (units/ml)
40
30
20
10
0
0
100
200
300
Time (hours p.i.)

Figure 6.

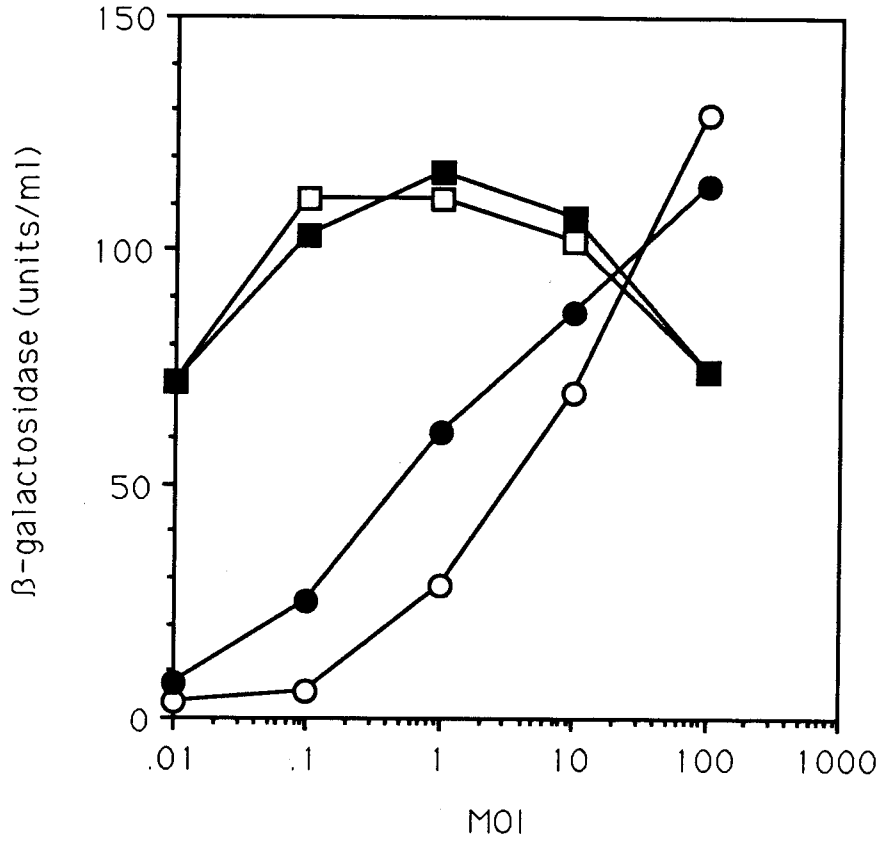
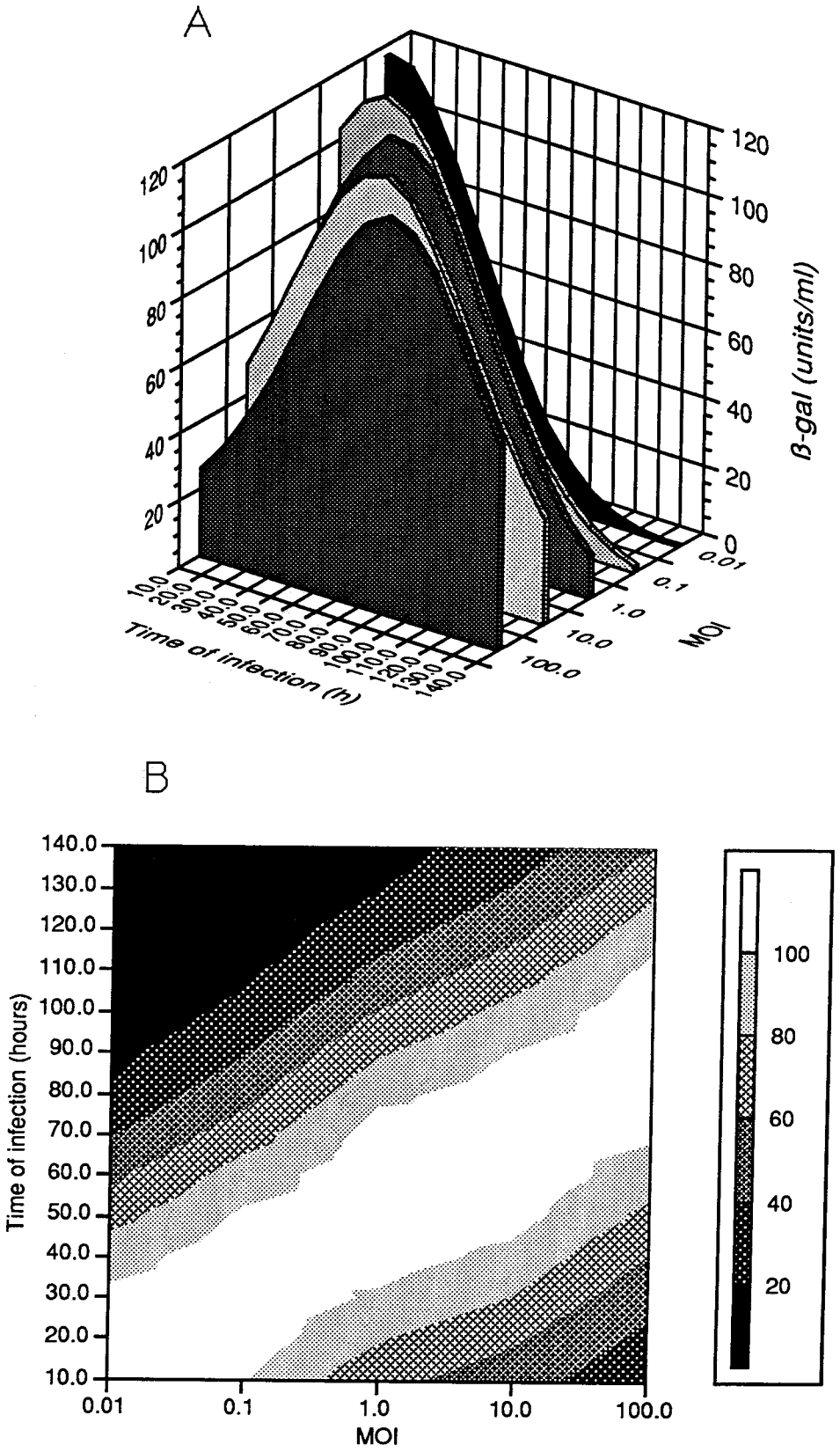


Figure 7.


A
120
100
80
60
40
20
0
β-gal (units/ml)
Time of infection (h)
MOI
0.01
0.1
1.0
10.0
100.0
B
140.0
130.0
120.0
110.0
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
Time of infection (hours)
0.01
0.1
1.0
10.0
100.0
MOI
100
80
60
40
20

CHAPTER IV

Production of a Discrete, Heterogeneous Population of ß-Galactosidase Polypeptides Using Baculovirus Expression Vectors

Source: Licari, P. and Bailey, J. E. 1992. Biotech. and Bioeng. **39**: 932-944.

4.1 Summary

Gel electrophoresis analysis of immunoprecipitated ß-galactosidase and polyhedrin-ß-galactosidase expressed in *Spodoptera frugiperda* cells infected with recombinant *Autographa californica* nuclear polyhedrosis virus revealed the existence of a population of discrete ß-galactosidase polypeptides. Several of the polypeptides observed in the fusion protein expression experiments exhibit a consistent pattern of slightly greater molecular weight when compared to the nonfusion ß-galactosidase, which is compatible with the hypothesis that these fusion protein fragments retain the N-terminal polyhedrin residues. Pulse-chase experiments showed that overall ß-galactosidase degradation occurred at a negligible rate compared to the synthesis rate at 96 hours post-infection, yet the fragments are observed for short pulse times. Degradation of several different ß-galactosidase polypeptides was observed 24 hours post-infection. RNA hybridization analysis of *lacZ* transcripts shows significant heterogeneity, which may result from premature transcription termination. Although a proteolytic origin cannot be excluded, the data assembled suggest that premature termination of transcription or translation is the likely cause for the heterogeneous population of immunoreactive peptides observed. Many discrete forms of ß-galactosidase polypeptides were also observed in studies with *E. coli*, indicating that production of these heterogeneous forms is not a consequence of heterologous expression of the enzyme.

4.2 Introduction

Recombinant proteins produced using baculovirus expression vectors are expressed as fusion or nonfusion proteins, with the former containing a segment of the polyhedrin protein. Although recombinant proteins may be expressed in quantities exceeding 0.5 mg/ml of culture, the levels are significantly less than that of wild-type polyhedrin (1 mg/ml) (22). Some factors influencing the extent of expression for genes under the control of the late polyhedrin promoter have been reported (23,24,18,36).

Another baculovirus gene expressed late in the infection life cycle is the p10 gene (12). The p10 protein (M_r 10 kDa) is associated with the fibrous structures found in the cytoplasm and nucleus of infected cells (25,46,48,49). Like the polyhedrin gene, the p10 gene is not necessary for the infectivity and the replication of nonoccluded virus (4,30,50). The promoter of this gene has recently been utilized for the expression of heterologous genes, specifically the *Escherichia coli lacZ* gene (50). This construct resulted in expression of a fusion protein (p10-ß-galactosidase) with a mass of 121 kDa.

Proper proteolytic processing of signal peptides in the insect cell-baculovirus expression system has been observed for several proteins synthesized using the AcMNPV expression system. Human IL-2 (45), α–interferon (26), glucocerebrosidase (27), and mouse IL-3 (32) have been analyzed and show proper signal sequence cleavage. Other proteins produced using the baculovirus expression system have been observed to undergo proteolytic cleavages that are different from those in mammalian cells. Rusche and coworkers expressed the human immunodeficiency virus envelope gene in a baculovirus expression vector (41). The resulting 160 kDa protein was not cleaved to yield glycoproteins gp120 and gp41. Possee demonstrated that human influenza virus hemagglutinin (HA) was plasma-membrane bound, indicating signal peptide cleavage (38). However, the proteolytic cleavage of precursor HA to the two fragments HA1 and HA2 was not observed. This is in contrast to studies by Kuroda *et al.* (18), in which

influenza (fowl plague) virus hemagglutinin was properly cleaved in the AcMNPV expression system to yield HA1 and HA2. Differences in proteolytic processing of baculovirus-expressed human gastrin-releasing peptide precursor and the human lung cell line protein have been reported (19). Although cleavage of the prepro-hormone appeared similar, when mapped it was different from the processing that occurred in lung cells. These data suggest novel enzymatic cleavage that had not been described in mammalian cells.

An alkaline protease, most likely associated with the digestive juices of the insect and not the virus itself (33), is implicated in the dissolution of matrix protein and is responsible for virion release from the occlusion body (5,6,17,51). Studies indicate that the protein behaves as a serine protease and requires an alkaline environment for activity (5). The alkaline protease is absent in occlusion bodies propagated *in vitro* (51). Furthermore, enzymatic activity is not expected in *in vitro* cell culture since cultivation of cells occurs at pH values below 7.

Synthesis and degradation rates of ß-galactosidase expressed using the AcMNPV system have been reported previously (21). In that study degradation of heterologous protein was observed early in the infection process (24 hours post-infection). However, later in the infection process when the synthesis rate of ß-galactosidase increased dramatically, negligible degradation was observed. The authors concluded that the process of intracellular protein degradation is not an important factor in determining the final yield of cloned ß-galactosidase. Product heterogeneity that was due to degradation was not addressed in that work. Jarvis and coworkers (13) have observed many different immunoreactive forms of ß-galactosidase expressed from the *lacZ* gene under the control of the polyhedrin promoter. On the other hand, using the IE1 promoter, an immediate early viral gene promoter, no heterogeneous ß-galactosidase polypeptides were observed. A stable transformant containing this construct produced significantly lower quantities of ß-galactosidase than baculovirus-infected cells.

This work investigates the populations of ß-galactosidase polypeptides and transcripts that are produced by expression of *lacZ* cloned into baculovirus behind different promoters. These data will be compared with a similar analysis of ß-galactosidase produced in wild-type *Escherichia coli*. Pulse-chase experiments are employed to characterize ß-galactosidase degradation rates in the baculovirus expression system.

4.3 Materials and Methods

Insect cell culture. *Spodoptera frugiperda* IPL-Sf9, a continuous cell line, was obtained from the ATCC (#CRL-1711). Cells were cultured in modified TNM-FH medium [Grace's medium (Gibco) supplemented with 3.3 g/l Difco yeastolate, 3.3 g/l lactalbumin hydrolysate, 0.35 g/l $NaHCO_3$, 10% fetal bovine serum (Hyclone), 5000 units/l penicillin, and 5 mg/l streptomycin (Sigma)]. Cultures grew as monolayers in stationary T-flasks maintained at 27°C. Cells were passaged by flushing the monolayer off the surface of the T-flask with a Pasteur pipette. Total cell counts were determined by a Coulter Counter (Coulter Electronics, Inc.).

Virus stocks and infection. Wild-type *Autographa californica* nuclear polyhedrosis virus (AcMNPV) and recombinant AcMNPV virus encoding a fusion polyhedrin-ß-galactosidase protein under the control of the polyhedrin promoter (constructed using the transfer vector pAc360 (22) and designated 360-lac) were obtained from Michael Lochrie (California Institute of Technology). The fusion protein contains a segment of the polyhedrin protein fused with ß-galactosidase (M_r 120 kDa). Another construct containing the *lacZ* structural gene under the control of the polyhedrin promoter (941-lac) was obtained from Dr. Max Summers (Texas A&M, 22). The resulting enzyme is a nonfusion protein with a mass of approximately 110 kDa. A virus containing a p10'-'*lacZ* protein fusion under the control of the p10 promoter (p10-lac) was obtained from Dr. J. M. Vlak (Agricultural University, The Netherlands, 49). This construct results in a protein containing the N terminus of the p10 protein (M_r 5000) fused to ß-galactosidase (M_r 116,000).

A recombinant baculovirus with a human ß1,4-galactosyltransferase gene under the control of the polyhedrin promoter was constructed and purified, using techniques described elsewhere (47). A nonfusion transfer vector, pVL1392, was purchased from

InVitrogen (San Diego, CA). The ß1,4-galactosyltransferase gene was obtained from Dr. M. Fukuda (La Jolla Cancer Research Foundation). The gene was inserted into the *EcoR*I site of the transfer vector. The recombinant virus was designated GT-AcMNPV.

Infection began when medium was aspirated off the monolayer culture and virus stock solution was added (designated time 0 post-infection). Unless noted otherwise, a multiplicity of infection (MOI) of 10 was used for all experiments. After addition of viral stock, cultures were placed on a rocking platform for 1 hour at 27°C. Cells were removed from the platform, and after one additional hour at 27°C, the medium containing virus was replaced with fresh medium. Mock-infection of cells was used as a control in several experiments: Cultures were treated identically to the infected cells, except that fresh medium was added at the time of infection instead of virus stock.

Bacterial strains and medium. *E. coli* strain MG1655, a wild-type strain, was grown in minimal medium supplemented with a low sulfur amino acid mixture (31).

Radiolabelling-immunoprecipitation method (insect cells). *S. frugiperda* cells were grown to a cell density of 10^6 cells/ml and infected as noted above. The radiolabelling-immunoprecipitation experiment commenced at the indicated time post-infection. Cells were gently resuspended and centrifuged at 250 g for 5 minutes. The supernatant was removed and the cells were washed twice with methionine-free medium (containing 2.5% fetal bovine serum). Cells were then resuspended in the methionine-free medium to a density of 10^6/ml. 250 µl of cell suspension and 50 µCi of L-[^{35}S]-methionine (1037 Ci/mmol, 10µCi/µl) were added to glass tubes maintained at 27°C. The cells were then incubated at 27°C for the desired time. If a chase was required, 1 ml of warm complete medium and 3 µl of methionine (30 mg/ml) were added for the desired chase time. The reaction was quenched by adding 2 ml of ice-cold, complete medium and placing on ice. The culture was then centrifuged at 1500 g for 5 minutes and the

supernatant removed. The resulting cell pellet was washed once with ice-cold PBS^+ (PBS containing Ca^{+2} and Mg^{+2}). After washing, 250 µl of lysis buffer (93% PBS^+, 2% 50 mM EDTA, 1% NP-40, 0.6% DOC, and 0.1% SDS) and 50 µl of Protein A-Sepharose were added. The sample was placed on ice for 20 minutes, and subsequently centrifuged for 45 minutes in an Eppendorf microcentrifuge. 150 µl of supernatant was then transferred to Eppendorf tubes containing 35 µl antiserum to ß-galactosidase (Sigma) in 1 ml Tris-Triton buffer (50 mM Tris-Cl, 0.15 M NaCl, 0.1 mM EDTA, and 2% Triton X-100). Samples were mixed 4 times by gentle inversion and then incubated overnight on a rotor at 4°C. The following morning 50 µl of Protein A-Sepharose (pre-equilibrated in 10 mM Tris-Cl) were added and the samples mixed by gentle inversion. Samples were returned to the rotor for 1 hour at 4°C. After incubation the samples were centrifuged for 1 minute at room temperature and the supernatant removed. The pellet was washed 5 times with ice-cold Tris-Triton buffer, once with ice-cold 10 mM Tris-HCl, and 2 times with ice-cold 0.1% SDS. The resulting pellet was resuspended in 55 µl sample buffer [18% glycerol (v/v), 9% ß-mercaptoethanol (v/v), 5% SDS (w/v), 0.01% bromophenol blue (w/v)], vortexed for 10 seconds, boiled for 5 minutes, and vortexed again for 10 seconds. The sample was then centrifuged for 5 minutes at room temperature and 45 µl of supernatant were removed.

Variations in the above procedure include the use of 50 µg/ml monoclonal antibodies to ß-galactosidase (Boehringer Mannheim) in place of the antiserum, and the addition of 30 µg/ml APMSF [(4-amidinophenyl)-methane-sulfonyl-fluoride], 0.4 mg/ml $EDTA\text{-}Na_2$, 0.5 µg/ml leupeptin, and 0.7 µg/ml pepstatin (Boehringer Mannheim) to the lysis buffer. These changes are noted when implemented into the above protocol.

Radiolabelling-immunoprecipitation method (*E. coli*). Cultures for radiolabelling consisted of 5 ml fresh medium inoculated with 100 µl of an overnight culture. These were grown to an optical density of A_{600} 0.4 prior to labelling. Cells were

induced by the addition of 1mM isopropylthiogalactoside (IPTG). Cells were labelled with 10 μCi of L-[^{35}S]-methionine (1037 Ci/mmol, 10μCi/μl) per 1 ml of culture. Labelling was stopped by the addition of trichloroacetic acid to a final concentration of 5%. After spinning in a microfuge for 15 minutes, the pellet was washed with ice-cold acetone and dried. The pellet was resuspended in 70 μl of boiling buffer (10 mM Tris-Cl, pH 7.5, 1 mM EDTA, 1% SDS) and boiled for 5 minutes. After centrifugation in a microfuge, the supernatant was saved for immunoprecipitation as outlined above for the insect cells.

Western blot analysis. Samples were run on 7.5% SDS-PAGE gel at 15-20 mAmp. Gels and nitrocellulose membranes were soaked in electroblotting buffer (2.4 g/l Tris base, 11.2 g/l glycine, 200 ml/l methanol) for 30 minutes. Transfer of protein from gel to membrane occurred in a Transphor electroblot apparatus (Hoeffer TE52) run at 1.1 Amps for 45 minutes. After transfer, the membrane was rinsed in 1X PBS and then placed in 100 ml of blocking buffer [100 ml PBS, 1 g Carnation Instant Nonfat Dry Milk (D. A. Johnson, J. W. Gautsch, J. R. Sportsman, and J. H. Elder, 1984, Gene Anal. Tech. **1**: 3)] for 30 minutes. The blocking buffer was then drained from the membrane and 100 ml of ß-galactosidase antiserum (Sigma) diluted in blocking buffer were added. After soaking the membrane in this solution for 30 minutes, it was rinsed in 1X PBS for 10 minutes. Goat anti-mouse IgG peroxidase conjugate (Sigma) was then diluted in 100 ml of blocking buffer and added to the membrane for 30 minutes. After incubation in the anti-IgG solution, the membrane was rinsed two times in 1X PBS. 100 ml DAB substrate-developing solution (50 mg 3,3-diaminobenzidine, 2 ml 1% $CoCl_2$ in H_2O, 98 ml PBS, and 0.1 ml 30% H_2O_2) were then added. The reaction was stopped by rinsing thoroughly with distilled water.

Elution of protein from SDS-polyacrylamide gel. The gel slice containing the protein of interest was isolated and macerated with a razor blade. 3 volumes of elution buffer (10 mM NH_4HCO_3, 0.1% SDS, 30 μg/ml APMSF, 0.4 mg/ml EDTA-Na_2, 0.5 μg/ml leupeptin, and 0.7 μg/ml pepstatin) were added to the homogenized gel. The protein was eluted for 24 hours at room temperature with gentle agitation. The sample was centrifuged for 5 minutes in a microfuge. 1 ml of Tris-Triton buffer was added to the supernatant, and an immunoprecipitation was performed as described above.

RNA analysis. Total RNA was obtained by treatment with guanidine thiocyanate and subsequent centrifugation on a cesium chloride gradient. Polyadenylated RNA was purified by affinity chromatography on oligo(dT)-cellulose. Both of the protocols used in the RNA extraction were from Sambrook *et al.* (42). The 2779 nt *EcoRI*/*Acc*1 fragment of the *lacZ* gene in pCO1 plasmid (16) was used as a probe in *lacZ* hybridizations. This fragment contains the 5' end of the *lacZ* gene. DNA probes were isolated from a 1.4% agarose gel and then purified using GeneClean (Bio 101, Inc.). Probes were radiolabelled by random primed labelling with [^{32}P]dCTP (Boehringer Mannheim). Labelled DNA was purified from unincorporated, radioactive nucleotides using NENSORB 20 cartridges (Dupont).

Gel electrophoresis and hybridization of RNA. Approximately 30 μg of total RNA and poly(A)$^+$ RNA were dissolved in 30 μl H_2O. 103 μl of formaldehyde-denaturing buffer (129 μl 10X MOPS, 226 μl 37% formaldehyde, and 645 μl formamide) were added to each sample. Samples were heated at 65^{o}C for 15 minutes and immediately transferred to ice. 27 μl of loading buffer (50% glycerol, 1 mM EDTA (pH 8), 0.25% bromophenol blue, and 0.25% xylene cyanol FF) were added and the sample was loaded on an agarose gel containing 1X formaldehyde running buffer [0.02 M MOPS (pH 7.0), 8 mM sodium acetate, 1 mM EDTA (pH 8.0)] and 2.2 M formaldehyde. The gel

was run at 4 V/cm. RNA was transferred from the agarose gel to a nitrocellulose membrane in 10X SSC. After transfer (≈36 hours) the nitrocellulose membrane was dried at 80°C for 1 hour. 20 ml of prehybridization solution (10 ml formamide, 2 ml 10% SDS, 4 ml 5 M NaCl, 2 g dextran sulfate, 100 μg/ml denatured salmon sperm, and water to 20 ml) were added to the membrane and allowed to incubate at 42°C for 2 hours. The DNA probe was then added to the membrane and incubated overnight at 42°C. RNA blots were washed in 2X SSC at room temperature for 5 minutes and in 2X SSC, 1% SDS at 60°C for 30 minutes.

4.4 Results

Expression of *lacZ* produces a discrete population of polypeptides with size heterogeneity. Radiolabelling-immunoprecipitation experiments were done for cells infected with the 360-lac virus with an MOI of 10 at various times post-infection (p.i.). An autoradiogram of ß-galactosidase immunoprecipitate from a culture at 96 hours p.i. is presented in Figure 1A. Several bands that are smaller than the ß-galactosidase fusion protein are observed, ranging in molecular weight from about 25 kDa to 120 kDa. This array of discrete polypeptides was synthesized from 24 hours p.i. until cell lysis, without significant differences (data not shown). The dynamics of net synthesis for different polypeptides appearing in Figure 1A is not directly evident since samples were prepared with different dilutions so as to load equal amounts of radioactivity per lane. Figure 1B is a graphical presentation of Figure 1A that accounts for the dilution factors used. The quantity of ß-galactosidase and each polypeptide studied is increasing with radiolabelling time, although with different synthesis rates. It is interesting to note that the relative accumulation rates of some of these polypeptides is different for different labelling times. For example, comparison of bands at ≈97 kDa and ≈64 kDa (fragments f and h) with bands at ≈55 kDa (fragments d and e) for short labelling times is different for longer labelling times.

The presence of numerous polypeptides in addition to the complete ß-galactosidase protein in Figure 1 may be due to a lack of specificity in the immunoprecipitation; that is, the antiserum against ß-galactosidase may be cross-reacting with other proteins, be they viral, cellular, or serum-associated. Two controls were run to disprove this hypothesis. One control consisted of cells infected with the wild-type virus, and the other control consisted of mock-infected cells. These controls as well as cells infected with the 360-lac virus and 941-lac virus were pulsed for 10 minutes; the resulting autoradiogram is presented in Figure 2. There appears to be no cross-reactivity

with the ß-galactosidase antiserum in the controls studied. Furthermore, immunoprecipitations using monoclonal antibodies to *E. coli* ß-galactosidase were done for 360- and 941-lac virus-infected cultures. No differences in the fragmentation pattern or relative quantity of the polypeptides were observed when compared with immunoprecipitate using antiserum (data not shown).

A Western blot using the immunoprecipitate from a radiolabelling experiment was done for further evidence that the observed fragments were ß-galactosidase and not proteins that co-precipitate with the protein of interest (data not shown). Several bands from this Western blot correlate with bands from a companion autoradiogram from the same gel, indicating that the polypeptides are ß-galactosidase fragments and not co-precipitated proteins. A one-to-one correspondence could not be made between the bands from the Western blot and the autoradiogram because of a sensitivity difference in the two methods.

In an effort to determine if these fragments were produced at any step in the immunoprecipitation procedure, uninfected, wild-type baculovirus-infected, and 360-lac infected cells were washed in phosphate buffered saline and immediately lysed in SDS-PAGE sample buffer. Because of the concentrations of SDS and ß-mercaptoethanol employed, no protease activity was expected, once the sample buffer was added to the cell pellet. These lysates were subsequently run on a denaturing gel, and a Western analysis was performed using monoclonal antibodies to ß-galactosidase (Figure 3). The Western blot indicates that the fragments are ß-galactosidase and that they are produced only in the 360-lac AcMNPV-infected cells and not in uninfected or wild-type baculovirus-infected cells. This experiment further demonstrates that the fragments are produced intracellularly and are not formed during cell lysis or at any other step in the immunoprecipitation experiment.

Intracellular degradation of ß-galactosidase. A simplified model used to describe intracellular protein synthesis and degradation has been previously applied to the baculovirus-insect cell system studied here (21).

$$\frac{dE}{dt} = K_S - K_dE - \frac{1}{N}\frac{dN}{dt}E ,$$

where E is the quantity of the protein of interest on a per cell basis (activity units/cell), t is time, K_S is the zero-order rate constant of synthesis of that protein (units/cell-time), K_d is the first-order rate constant of degradation ($time^{-1}$), and N is the number of cells per unit volume of culture. The rate of protein synthesis is dependent on several factors, including the amount of mRNA, tRNA, amino acids and the number of ribosomes available in the cell. The zero-order rate constant of synthesis is a simplification that lumps the effects of interactions of these components into one parameter. The term $\frac{1}{N}\frac{dN}{dt}E$ accounts for the dilution of intracellular protein that is due to cell division. In the pulse-chase experiments presented, this term is relatively insignificant since the duration of the chase times studied is considerably less than both the cell doubling time and the time scale on which the virus affects the cell. Values for $K_{S(net)}$ at 24 and 96 hours post-infection and for K_d at 24 hours post-infection for 360-lac infected cells have been published (21), where $K_{S(net)}$ describes the net synthesis of protein. Degradation of ß-galactosidase in that study was not observed at 96 hours post-infection; chase times on the order of 20 minutes were insufficient to detect degradation. By decreasing the pulse time and studying chase times ranging from 1 to 2 hours, degradation was observed (Figure 4). From Figure 4 a value of K_d at 96 hours post-infection for 360-lac infected cells may be estimated to be 5.9×10^{-3} min^{-1}. The values of $K_{S(net)}$ and K_d from this work and previous work are listed in Table 1. It should be emphasized that as in the previous study (21), this analysis is based upon the single, radiolabelled band on the

SDS-PAGE gels with molecular weight corresponding to the intact polyhedrin-ß-galactosidase fusion protein.

Degradation of some of the predominating fragments were observed at 24 hours post-infection in 360-lac infected cells. In Figure 5 all bands become fainter with increasing chase time at 24 hours post-infection, indicating degradation of ß-galactosidase and each of the observed ß-galactosidase peptides. The densitometry data for three individual fragments are shown in Figure 5B.

A comparison of the 360-lac and the 941-lac viruses. A comparison of ß-galactosidase polypeptides from fusion and nonfusion ß-galactosidase, both produced in the insect cell-baculovirus system, was made by radiolabelling and immunoprecipitation. The immunoprecipitate from each culture was run on a polyacrylamide gradient gel (6% to 14% acrylamide); the resulting autoradiogram is presented in Figure 6. For most polypeptides observed in the 360-lac virus infected culture there is a corresponding polypeptide for the 941-lac virus-infected culture. Although the bands correspond, there is an incremental mass difference between corresponding fragments produced by the 360-lac and 941-lac constructs. This is likely due to the existence of the polyhedrin portion attached to the fusion protein. The fusion/nonfusion size difference becomes more evident as the fragments become smaller. Resolution of the corresponding fusion and nonfusion fragments may be enhanced for smaller polypeptides since the polyhedrin portion is a larger percentage of the total mass. This trend is also observed in Figure 2, lane 1 where immunoprecipitate from both 360-lac and 941-lac infected cells was simultaneously loaded into a single lane. In this figure, the size difference is clear for the smaller fragments.

Heterogeneous gene product is not polyhedrin promoter specific. The phenomenon of heterogeneous ß-galactosidase expression is not specific to the

polyhedrin promoter as demonstrated by studies with the p10 promoter-*lacZ* gene virus (p10-lac). Radiolabelling-immunoprecipitation studies of Sf9 cells infected with the p10-lac virus and cells infected with the 360-lac virus are presented in Figure 7. The existence of several ß-galactosidase polypeptides, ranging in size from 25 kDa to 120 kDa, are observed with the p10-lac virus-infected cells. This indicates that similar, though not identical, processes arise in the expression of ß-galactosidase under the control of the p10 promoter. Several bands observed for cells infected with the 360-lac virus are also observed for cells infected with the p10-lac virus. The corresponding polypeptides associated with the 360-lac virus are slightly larger than those for the p10-lac virus because of the difference in the recombinant gene-fusion constructs.

The existence of variable length RNAs transcribed from the *lacZ* gene. The transcripts produced in the 360-lac and 941-lac viruses were examined by hybridization to the 2.8 kb *EcoR*1/*Acc*1 *lacZ* probe described in Materials and Methods. Mock-infected cells and cells infected with the wild-type virus were used as controls in the Northern hybridizations. The data for total RNA are presented in Figure 8. Several distinct RNAs are observed that are smaller than the corresponding, complete messages for both the 360-lac and 941-lac virus-infected cells. No hybridization to the *lacZ* probe occurred in the control lanes containing total RNA from mock-infected cells and wild-type virus-infected cells. Hybridizations with polyadenylated RNA showed a similar trend; however, RNA degradation was greater (data not shown). It is common in Northern hybridizations to observe smeared signals indicative of RNA degradation. Distinct bands, as observed here, are not a common result of RNA degradation; however, degradation cannot be ruled out in these studies.

Radiolabelling studies of ß-galactosidase in *E. coli*. In an effort to clarify the influence of the expression system on the appearance of multiple ß-galactosidase

fragments, synthesis of ß-galactosidase in its native host, *E. coli*, was studied. *E. coli* MG1655 cells induced with 1 mM IPTG were radiolabelled for 2 min and 30 min. The results are presented in lanes 2 and 3 (30 min pulse) and lane 4 (2 min pulse) of Figure 9. A number of the fragments observed in the baculovirus system are produced in *E. coli*. A second surprising trend is the approximate invariance of the relative quantity of each particular ß-galactosidase fragment for the 2-minute pulse compared to the 30-minute pulse.

Cross-reactivity with other *E. coli* proteins was analyzed by radiolabelling uninduced cells. A comparison of lanes 1 and 2 of Figure 9 demonstrates that a few of the proteins are cross-reacting; however, the majority appear to be ß-galactosidase fragments. Two of the three major fragments are not present in the uninduced cells. Those fragments that correlate between uninduced and induced *E. coli* are not observed in the baculovirus-produced ß-galactosidase.

In labelling experiments with *E. coli*, the addition of a protease-inhibitor cocktail containing APMSF (an inhibitor of serine proteases), EDTA (an inhibitor of metalloproteases), leupeptin (an inhibitor of serine and cysteine proteases), and pepstatin (an inhibitor of aspartate proteases) to the boiling and immunoprecipitation buffers strongly suggests that the production of these fragments is not a consequence of proteolysis during the radiolabelling-immunoprecipitation protocol (Figure 9). In addition, the duration of several steps in the immunoprecipitation protocol for infected Sf9 cells was varied in an effort to observe any changes in the quantity of fragments present. No variation in relative abundance of the fragments was observed. Radiolabelled ß-galactosidase was passively eluted from a gel, subjected to the immunoprecipitation protocol, and subsequently run on a denaturing gel (Figure 9). The lack of the characteristic fragments indicates that the immunoprecipitation procedure is not responsible for protein fragmentation. Furthermore, the purified radiolabelled ß-galactosidase was added to unlabelled, infected insect cells prior to cell lysis and

subjected to the complete immunoprecipitation procedure. No fragments were observed (data not shown); one should note that the protease inhibitors used in elution of labelled ß-galactosidase remained in the solution that was added to infected cells. This experiment, coupled with the total lysate Western (Figure 3), demonstrates that the processes that give rise to a heterogeneous population of ß-galactosidase polypeptides occur in the cell prior to lysis. The passive elution experiment was necessary to obtain purified ß-galactosidase; commercial sources of this protein (from Sigma and Boehringer Mannheim) contain the characteristic array of polypeptides demonstrated here.

Variable length transcripts from a galactosyltransferase gene under the control of the polyhedrin promoter. Although in-depth protein studies have not been done on ß1,4-galactosyltransferase produced using the baculovirus described in the Materials and Methods Section, RNA hybridization with a ß1,4-galactosyltransferase probe indicates similar behavior to that observed for the 360-lac virus (Figure 10). The predominating band greater than 2.3 kb corresponds to the full-length message. The two transcripts of length greater than the major transcript are due to transcription readthrough, a process known to occur in the wild-type system. Two additional transcripts that are shorter than the complete transcript are also present. These bands are too short to be complete transcripts, irrespective of the extent of polyadenylation.

4.5 Discussion

Numerous studies have demonstrated that there is a continual turnover of proteins in eucaryotic cells (39,44). In addition to the degradation of native cellular proteins, selective proteolysis of abnormal proteins occurs (9,10,37). Abnormal proteins include mutated proteins (insertions, deletions, nonsense, and missense mutations), proteins containing amino acid analogs, recombinant proteins, and proteins resulting from errors in transcription or translation. Many stressful environments, such as a shift to elevated temperature, increase the quantity of nonnative protein forms present in the cell. In response to the sudden increase of abnormal protein content, cells synthesize elevated levels of stress proteins, often referred to as heat-shock proteins. Several factors are known to induce a stress response, including temperature shifts (20,43,53), addition of ethanol (8), and viral infection (15,52).

The baculovirus expression system is a likely candidate for heterologous protein degradation. The potential for degradation is important in practice not only from the point of view concerned with yield but also in regard to product heterogeneity. Studies presented here for the *lacZ* gene downstream of the polyhedrin promoter demonstrate that although degradation exists, the rate is relatively insignificant. A comparison of the values of $K_{s(net)}$, K_d, and the ratio $K_d/K_{s(net)}$ at 24 hours post-infection and 96 hours post-infection demonstrates the relatively insignificant effect of degradation on yield (Table 1). Furthermore, the ß-galactosidase fragments produced in the baculovirus system are observed in the wild-type bacterial host. These facts indicate that the baculovirus expression system is not flawed by high degradation or the synthesis of heterogeneous gene product for the case of ß-galactosidase. A large deviation exists between the final concentration of polyhedrin produced by infection of wild-type virus compared to final levels of other recombinant proteins expressed under the control of the polyhedrin promoter. Polyhedrin is expressed to a concentration of approximately 1 mg/ml, whereas

ß-galactosidase expression is roughly half of that value. The difference between wild-type and recombinant protein expression is unlikely due to the degradation of heterologous protein for the present cases of *E. coli* ß-galactosidase and the polyhedrin-ß-galactosidase fusion protein.

This study raises further questions that do not directly pertain to the baculovirus expression system but are important nonetheless. The data presented suggest that these fragments are not degradation products, although unequivocal proof of this has not been provided. If the fragments are degradation products, it suggests that a similar proteolytic system exists in *E. coli* and in the *S. frugiperda*-AcMNPV system. Careful comparison of a polyhedrin-*lacZ* fusion construct with direct *lacZ* expression demonstrates a consistent size difference between the polypeptides obtained in these two cases. The most likely cause of this consistent size difference is the extension of the fusion construct by an amino-terminal segment of polyhedrin. The data suggest that the polypeptides produced from the fusion-expression virus contain a common amino terminus. Various mechanisms of protein degradation may be hypothesized that are consistent with this data. One can envision specific endoproteases that cleave the whole protein at particular sites and in a subsequent step the fragment containing the original carboxy terminus is rapidly degraded. In this scenario, a site-specific cleavage may be coupled to the N-end rule; i.e., the first proteolytic cleavage results in the formation of a new amino-terminus with a more destabilizing residue. A similar model has been suggested by Bachmair *et al.* (1). An alternative mechanism may involve exoproteases. Exoproteases may begin degrading at the carboxy termini of molecules and continue until a resistant portion of the protein is reached. These "resistant" portions are less susceptible to proteolysis but still may be degraded eventually (as indicated by the multiple fragments produced). Such specific proteolytic sites or resistant spots may be demarcated by sequence and/or conformation.

Another feasible interpretation of the data is that these fragments are not a secondary product of the complete protein but originate because of premature

transcription termination or premature translation termination. Pausing and/or premature transcript termination has been observed for the epidermal growth factor (EGF) receptor primary transcript in human carcinoma nuclei (11). In that study it was postulated that blocks to RNA elongation are a regulation mechanism in gene expression of EGF receptors. The authors suggested that an elongation block may prevent overloading of RNA polymerase II complex for a gene under the control of a highly active promoter. Other studies with *c-myc* and hsp70 genes have described cellular regulation of RNA elongation (29,34,40). The Northern hybridization data presented for both the *lacZ* and ß1,4-galactosyltransferase transcripts suggest premature transcript termination. Because of the low quantity and likely short half-life of the aborted transcripts, the quality of the hybridization data is not unequivocal proof of this mechanism. In addition, AcMNPV transcription often occurs from both strands of the viral genome, including transcription of the polyhedrin region (35). Since the DNA probe used in this Northern hybridization was double-stranded, these data are not conclusive proof that these shorter transcripts are templates for translation. Several reasons for transcriptional termination may be hypothesized. The transcription of the *lacZ* gene may be occurring so rapidly that the RNA polymerase frequently falls off the DNA template. A stall that is due to RNA secondary structure may facilitate the disruption of the RNA polymerase-template complex. The rapidity of the transcriptional process may lead to promiscuity of the RNA polymerase and other transcriptional factors. Promiscuous transcription may result in the misreading of signals important in termination. For instance, the sequence of two consecutive lysines found in the *lacZ* gene, AAAAAA, may be misinterpreted as the polyadenylation sequence AAUAAA.

Premature translation termination has been implicated in the regulation of overlapping genes (3). In studies of the phage X174 lysis gene (3), expression of the gene appeared to be regulated by a frameshift-restart mechanism (14). A premature termination codon may arise because of a point mutation or an induced reading frameshift. The latter

may be caused by a "frameshift" sequence, a sequence believed responsible for frameshifts (7,14). All of the data presented here are consistent with such a mechanism.

Premature translation termination has been observed in *E. coli* during amino acid starvation (2,12). During amino acid starvation the majority of proteins synthesized were of low molecular weight, and in induced cultures, fragments of ß-galactosidase were observed. These results were attributed to the premature release of ribosomes from the messenger RNA. It was suggested that the movement of ribosomes may be arrested when a codon requiring the incorporation of a missing amino acid is encountered. If this hypothesis is correct, a high molecular weight protein such as ß-galactosidase is more susceptible to premature translation termination. If one utilizes a medium containing an excess of all required amino acids, this premature termination is not expected to occur. However, under the action of a strong promoter, the intracellular pool of amino acids may temporarily become deficient in one or more of the required amino acids. The results of this scenario would be analogous to the starvation studies in *E. coli* discussed above and would be consistent with the data presented here. If this is the case, the use of strong promoters and translation initiation sites may generally give rise to multiple product forms that are due to a limitation imposed by the cell, i.e., a limited intracellular pool of charged tRNAs and/or amino acids.

Both the premature transcription termination and the premature translation termination hypotheses are supported by the comparison of fusion and nonfusion constructs in the baculovirus system, where a common N-terminus is suggested for the former case. Regardless of the mechanism resulting in protein fragmentation, the importance of the protein or transcript itself is evident. Sequence and conformation may be instrumental in directing the fragmentation process. Comparison between the *E. coli* -produced and the Sf9-produced ß-galactosidase demonstrates this. Similarities among the polypeptides produced using the p10 promoter and those produced from the polyhedrin promoter (Figure 7) are further evidence that specific sites in the gene,

message, or protein are responsible for production of particular ß-galactosidase fragments. Although a one-to-one correspondence between polypeptides synthesized using the two different viral promoters cannot be made, several correlations do exist. Those polypeptides that show a correlation demonstrate a slight size difference that is similar to that observed with fusion and nonfusion ß-galactosidase produced using the polyhedrin promoter. It is not known why the ß-galactosidase peptide heterogeneity for expression by the p10 promoter was not identical to that observed with the *lacZ* gene under the control of the polyhedrin promoter. Promoter strength may be a variable leading to such differences, as may differences in the cellular state at different stages of the viral-infection process.

ß-galactosidase was studied as a model protein for several reasons, most importantly because a number of degradation studies of this protein in other host cells have been conducted. ß-galactosidase is a large protein (≈120 kDa) that is not actively secreted by cells and is easy to assay. All of these attributes were useful in studying the degradation process. To date, the fact that this protein may be especially susceptible to fragmentation has not been noted. The existence of these fragments and uncertainty about the basis for their production should be carefully considered in any future studies of degradation using ß-galactosidase as a model protein. However unique a model system may prove to be, the observed behavior forewarns one of the possible behavior of other genes expressed in the same system, as was observed at the RNA level with ß1,4-galactosyltransferase.

4.6 Acknowledgments

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4.7 References

1. Bachmair, A., Finley, D., and Varshavsky, A. 1986. *Science.* **234**: 179-186.

2. Brunschede, H., and Bremer, H. 1971. *J. Mol. Biol.* **57**: 35-57.

3. Buckley, K. J., and Hayashi, M. 1987. *J. Mol. Biol.* **198**: 599-607.

4. Cochran, M. A., Carstens, E. B., Eaton, B. T., and Faulkner, P. 1982. *J. Virol.* **41**: 940-946.

5. Eppstein, D. A., and Thoma, J. A. 1975. *Biochem. and Biophys. Res. Commun.* **62**: 478-484.

6. Eppstein, D. A., Thoma, J. A., Scott, H. A., and Young, S. Y. 1975. *Virol.* **67**: 591-594.

7. Fox, T. D., and Weis-Brummer, B. 1980. *Nature (London).* **288**: 60-63.

8. Goff, S. A., and Goldberg, A. L. 1985. *Cell.* **41**: 587-595.

9. Goldberg, A. L. 1972. *Proc. Natl. Acad. Sci. USA* **69**: 422-426.

10. Goldberg, A., and St. John, A. C. 1976. *Ann. Rev. Biochem.* **45**: 747-803.

11. Haley, J. D., and Waterfield, M. D. 1991. *J. Biol. Chem.* **266**: 1746-1753.

12. Hansen, M. T., Bennett, P. M., and von Meyenburg, K. 1973. *J. Mol. Biol.* **77**: 589-604.

13. Jarvis, D. L., Fleming, J. G. Kovacs, G. R., Summers, M. D., and Guarino, L. A. 1990. *BioTechnol.* **8**: 950-955.

14. Kastelein, R. A., Remaut, E., Fiers, W., and van Duin, J. 1982. *Nature (London).* **295**: 35-41.

15. Khandjian, E. W., and Turler, H. 1983. *Mol. Cell. Biol.* **3**: 1-8.

16. Khosla, C., and Bailey, J. E. 1989. *J. Bact.* **171**: 5995-6004.

17. Koslov, E. A., Sirdorova, N. M., and Serebryani, S. B. 1975. *J. Invert. Path.* **25**: 97-101.

18. Kuroda, K., Hauser, C., Rott, R., Klenk, H., and Doerfler, W. 1986. *EMBO.* **5**: 1359-1365.

19. Lebacq-Verheyden, A., Kasprzyk, P. G., Raum, M. G., Van Wyke Coelingh, K., Lebacq, J. A., and Battey, J. F. 1988. *Mol. and Cell. Biol.* **8**: 3129-3135.

20. Lemaux, P. G., Herendeen, S. L., Bloch, P. L., and Neidhardt, F. 1978. *Cell.* **13**: 427-434.

21. Licari, P., and Bailey, J. E. 1991. *Biotech. and Bioeng.* **37**: 238-246.

22. Luckow, V. A., and Summers, M. D. 1988. *BioTechnol.* **6**: 47-55.

23. Luckow, V. A., and Summers, M. D. 1988. *Virol.* **167**: 56-71.

24. Luckow, V. A., and Summers, M. D. 1989. *Virol.* **170**: 31-39.

25. MacKinnon, E. A., Henderson, J. F., Stoltz, D. B., and Faulkner, P. 1974. *J. Ultrastruct. Res.* **49**: 419-435.

26. Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T., Saeki, Y., Sato, Y., and Furusawa, M. 1985. *Nature (London).* **315**: 592-594.

27. Martin, B. M., Tsuji, S., LaMarca, M. E., Maysac, K., Eliason, W., and Ginns, E. I. 1988. *DNA.* **7**: 99-106.

28. Matsuura, Y., Possee, R. D., Overton, H. A., and Bishop, D. H. L. 1987. *J. Gen. Virol.* **68**: 1233-1250.

29. Miller, H., Asselin, C., Dufort, D., Yang, J., Gupta, K., Marcu, K., and Nepveu, A. 1989. *Mol. Cell. Biol.* **9**: 5340-5349.

30. Miller, D. W., Safer, P., and Miller, L. K. 1986. An insect baculovirus host-vector system for high level expression of foreign genes, p. 277-299. *In* J. K. Setlow and A. Hollaender (eds.), Genetic Engineering: Principles and Methods. Plenum Press, NY.

31. Minkley, E. G., and Ippen-Ihler, K. 1977. *J. Bact.* **129**: 1613-1622.

32. Miyajima, A., Schreurs, J., Otsu, K., Kondo, A., Arai, K., and Maeda, S. 1987. *Gene.* **58**: 273-281.

33. Nagata, M., and Tanada, Y. 1983. *Arch. Virol.* **76**: 245-256.

34. Nepveu, A., Levine, R. A., Campisi, J., Greenberg, M. E., Ziff, E. B., and Marcu, K. B. 1987. *Oncogene.* **1**: 243-250.

35. Ooi, B. G., and Miller, L. K. 1990. *J. Virol.* **64**: 3126-3129.

36. Ooi, B. G., Rankin, C., and Miller, L. K. 1990. *J. Mol. Biol.* **210**: 721-736.

37. Pine, M. J. 1966. *J. Bact.* **92**: 847-850.

38. Possee, R. D. 1986. *Virus Res.* **5**: 43-59.

39. Rechcigl, M. 1971. Enzyme Synthesis and Degradation in Mammalian Systems. University Park Press, Baltimore.

40. Rouguie, A. E., and Lis, J. T. 1988. *Cell.* **54**: 795-804.

41. Rusche, J. R., Lynn, D. L., Robert-Guroff, M., Langlois, A. J., Lyerly, H. K., Carson, H., Krohn, K., Ranki, A., Gallo, R., Bolognesi, D. P., Putney, S. D., and Matthews, T. J. 1988. *Proc. Natl. Acad. Sci. USA.* **84**: 6924-6928.

42. Sambrook, J., Fritsch, E., and Maniatis, T. 1989. Molecular Cloning, a Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY.

43. Schlesinger, M., Ashburner, M., and Tissieres, A. 1982. Heat Shock: from Bacteria to Man. Cold Spring Harbor Press, Cold Spring Harbor, NY.

44. Shimke, R. T. 1970. *Mammalian Protein Metab.* **4**: 177.

45. Smith, G. E., Grace, J., Ericson, B. L., Moschera, J., Lahm, H. W., Chizzonite, R., and Summers, M. D. 1985. *Proc. Natl. Acad. Sci. USA* **82**: 8404-8408.

46. Summers, M. D. 1991. ACS Symposium Series. **453**: 237-251.

47. Summers, M. D. and Arnott, H. J. 1969. *J. Ultrastruct. Res.* **28**: 462-480.

48. Summers, M. D., and Smith, G. E. 1985. A manual of methods for baculovirus vectors and insect cell culture procedures. Texas Agriculutural Experiment Station, Bulletin No. 1555.

49. Tanada, Y., and Hess, R. T. 1984. The cytopathology of baculovirus infection in insects, pp. 517-556. *In* R. C. Kung and H. Arai (eds.) Insect Ultrastructure, Vol. 2. Plenum Press, NY.

50. Van der Wilk, F., van Lent, J. W. M., and Vlak, J. M. 1987. *J. Gen. Virol.* **68**: 2615-2623.

51. Vlak, J. M., Klinkenberg, F. A., Zaal, K., Usmany, M., Klinge-Roode, E. C., Geervlient, J. B., Roosien, J., and van Lent, J. 1988. *J. Gen. Virol.* **69**: 765-776.

52. Wood, H. A. 1980. *Virol.* **103**: 392-399.

53. Wu, B. J., Hirst, H. C., Jones, N. C., and Morimoto, R. I. 1986. *Mol. Cell. Biol.* **6**: 2994-2999.

54. Yamamori, T., Ito, K., Nakamura, Y., and Yura, T. 1978. *J. Bact.* **134**: 1133-1140.

4.8 Tables

Table 1. The synthesis and degradation of ß-galactosidase in the Sf9-360-lac AcMNPV system. The zero-order rate constant of net enzyme synthesis ($K_{S(net)}$), the first-order rate constant of degradation (K_d), and the ratio of $K_d/K_{S(net)}$ are tabulated for 24 and 96 hours post-infection. The units AU*mm (absorbance units·millimeters) represent the peak area obtained from densitometry. [a] data from Reference 21.

Time (hours p.i.)	**Ks(net)** (AU*mm/10^5 cells-min)	**Kd** (min^{-1})	**Kd/Ks(net)** 10^5 cells/(AU*mm)
24	$4.4x10^{-3}$ [a]	$9.4x10^{-3}$ [a]	2.1
96	1.1 [a]	$5.9x10^{-3}$	$5.4x10^{-3}$

4.9 Figures

Figure 1. The synthesis of several polypeptides smaller than ß-galactosidase. **A.** Radiolabelled immunoprecipitates from Sf9 cells infected with the 360-lac virus were run on a 7% SDS-polyacrylamide gel. The samples are from a radiolabelling-immunoprecipitation experiment in which infected cells at 96 hours post-infection were radiolabelled for 2-7 minutes prior to immunoprecipitation. Replicate samples were run for each time investigated. Lane number, radiolabelling times, and the dilution (v:v) used in sample preparation are as follows: lanes 1 and 2: 2 minutes, diluted 1:7, lanes 3 and 4: 4 minutes, diluted 1:13, lanes 5 and 6: 7 minutes, diluted 1:26. The polyhedrin-ß-galactosidase fusion protein is indicated on the right. Molecular weight standards are indicated on the left in kilodaltons. **B.** Densitometry analysis of Figure 1A. Absorbance units*millimeters (AU*mm) are plotted as a function of radiolabelling time for 8 polypeptides: fragment a (□); fragment b (○); fragment c (■); fragment d (●); fragment e (△); fragment f (▲); fragment g (+); fragment h (X).

Figure 2. An analysis of cross-reactivity with the antiserum used in ß-galactosidase immunoprecipitations. Cells were infected with 360-lac virus, 941-lac virus, wild-type AcMNPV, and other cells were mock-infected. At 24 hours post-infection the cells were radiolabelled for 10 minutes, and then the ß-galactosidase was immunoprecipitated. The lanes indicated are immunoprecipitate from cells that were infected with 360-lac virus and cells infected with 941-lac virus simultaneously loaded into a single lane (lane 1), mock-infected cells (lanes 2 and 3); cells infected with the wild-type AcMNPV (lanes 4 and 5); cells infected with 360-lac AcMNPV (lane 6). The complete ß-galactosidase protein is indicated by the arrow. Molecular weight standards are indicated on the left in kilodaltons.

Figure 3. Western blot analysis of total-cell lysate indicating ß-galactosidase protein and polypeptides. Samples are from total-cell lysate of uninfected cells (lane 2), wild-type baculovirus-infected cells (lane 3) and 360-lac AcMNPV-infected cells (lanes 1 and 4). An MOI of 10 was used and the cells were lysed late in the infection process. The polyhedrin-ß-galactosidase fusion protein is indicated.

Figure 4. Degradation of ß-galactosidase at 96 hours post-infection. Samples are from cells infected with the 360-lac recombinant virus. The logarithm of the relative quantities of L-[^{35}S]-methionine incorporated into the complete ß-galactosidase protein (AU·mm/10^5 cells) is plotted versus the chase time (minutes).

Figure 5. Degradation of ß-galactosidase and the accompanying polypeptides. 360-lac infected cells (24 hours p.i.) were radiolabelled for 46 minutes. **A.** Subsequent chase of the label was done with samples for 0 minutes (lane 1); 20 minutes (lanes 2 and 3); 40 minutes (lane 4); 60 minutes (lane 5); 80 minutes (lane 6). The autoradiogram is from a 7% acrylamide gel. The polyhedrin-ß-galactosidase protein is indicated by the arrow. Molecular weight standards are indicated on the left in kilodaltons. **B.** Densitometry analysis of the initial degradation rate for ß-galactosidase and accompanying polypeptides. The relative quantities of L-[^{35}S]-methionine incorporated (AU*mm/µl sample) is plotted against the chase time (minutes). AU*mm (absorbance units*millimeters) is the area obtained under the densitometry curve. For ß-galactosidase, the units are (AU*mm/µl sample)x0.25. The proteins analyzed were ß-galactosidase (■), polypeptide A (□), polypeptide B (●), and polypeptide C (○), as indicated in part A.

Figure 6. Incremental size difference between corresponding fragments produced by the 360-lac and 941-lac constructs. The autoradiogram is from a 6% to 14% acrylamide gradient gel on which immunoprecipitates were run. Lanes 1 and 2 are the nonfusion

protein and peptides (941-lac virus), while lanes 3 and 4 are the fusion protein and peptides (360-lac virus). ß-galactosidase and the polyhedrin-ß-galactosidase fusion protein are indicated on the right by the arrow. Molecular weight standards are indicated on the left in kilodaltons.

Figure 7. Discrete ß-galactosidase fragments are produced from the *lacZ* gene under the control of the p10 promoter. The autoradiogram is from a 7% SDS-polyacrylamide gel. Samples indicated are from a radiolabelling-immunoprecipitation experiment in which infected cells were labelled for 10 minutes. Lane 1 corresponds to cells infected with the p10-lac virus, and lane 2 corresponds to cells infected with the 360-lac virus. The ß-galactosidase fusion proteins are indicated by the arrow on the right. Molecular weight standards are indicated on the left in kilodaltons.

Figure 8. Northern blot analysis of polyhedrin promoter-*lacZ* gene transcription. Total RNA was fractionated on a 1.3% agarose gel and was probed with the *lacZ* gene as described in the Materials and Methods Section. Lane 1 corresponds to RNA from cells infected with the 941-lac virus; lane 2 corresponds to RNA from cells infected with the 360-lac virus; lane 3 corresponds to RNA from cells infected with the wild-type virus, lane 4 corresponds to RNA from mock-infected cells. One wash for 30 minutes was done in 2X SSC and 0.1% SDS at 60°C. The positions of molecular weight standards are indicated on the left (in kilobases).

Figure 9. Radiolabelled immunoprecipitates from *E. coli* (lanes 1-4) and 360-lac infected Sf9 cells run on a 7% SDS-polyacrylamide gel. Lane 1: uninduced, 30-minute label period, lane 2: induced, 30-minute label period, lane 3: induced, 30-minute label period, protease inhibitor cocktail used, lane 4: induced, 2-minute label period, lane 5: Sf9 cells infected with 941-lac virus, 30-minute label period. Lane 6: radiolabelled ß-galactosidase

eluted from an SDS-polyacrylamide gel and subsequently subjected to the immunoprecipitation protocol.

Figure 10. Northern blot analysis of polyhedrin promoter-ß1,4-galactosyltransferase gene transcription. Total RNA was fractionated on a 1.2% agarose gel and probed with the complete ß1,4-galactosyltransferase gene as described in the Materials and Methods Section. Lane 2 corresponds to RNA from uninfected cells, lane 3 corresponds to RNA from wild-type baculovirus-infected cells, and lanes 1 and 4 correspond to RNA from cells infected with the GT-AcMNPV virus cells. One wash for 30 minutes was done in 2X SSC and 0.1% SDS at 60°C.

Figure 1A.

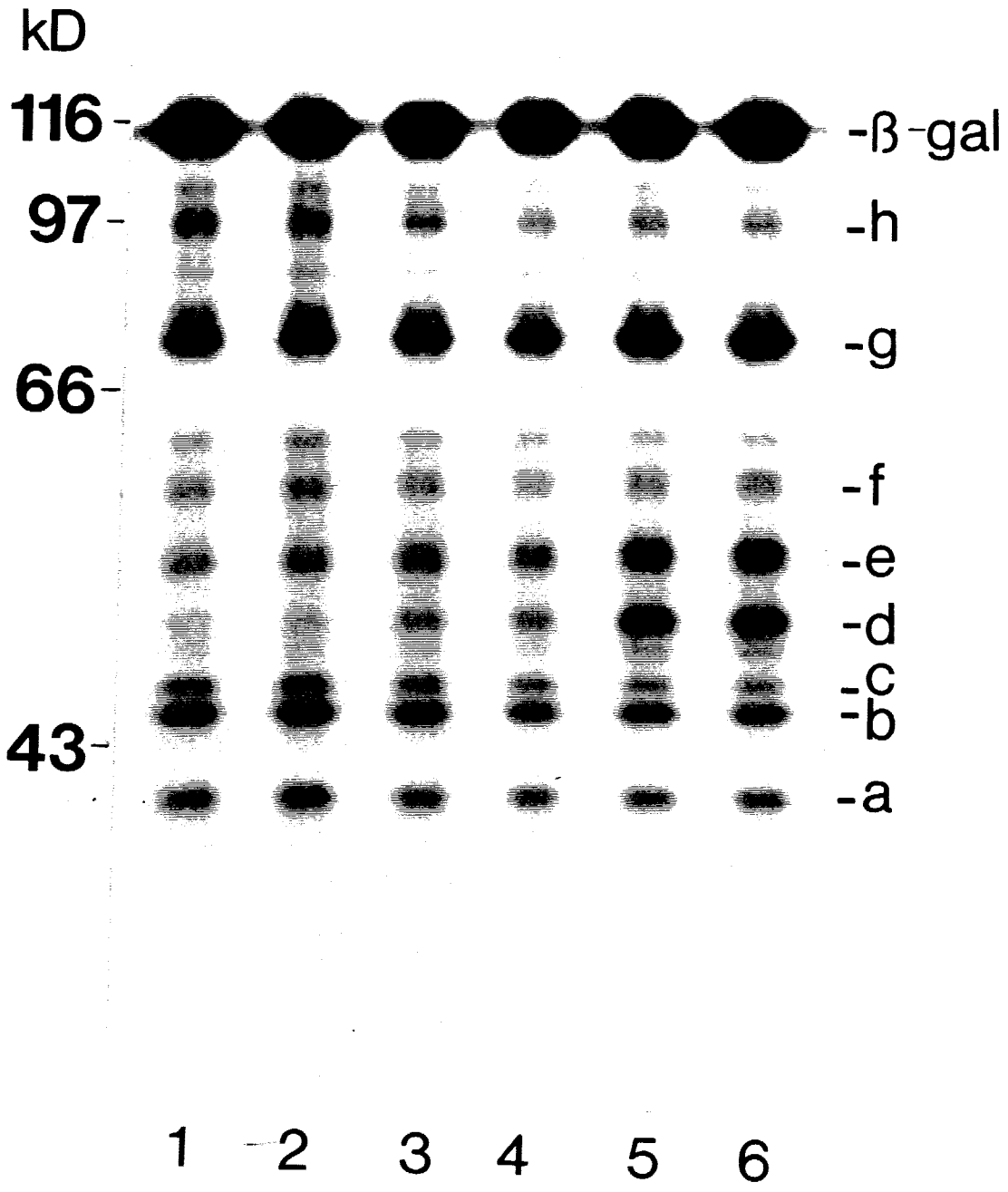
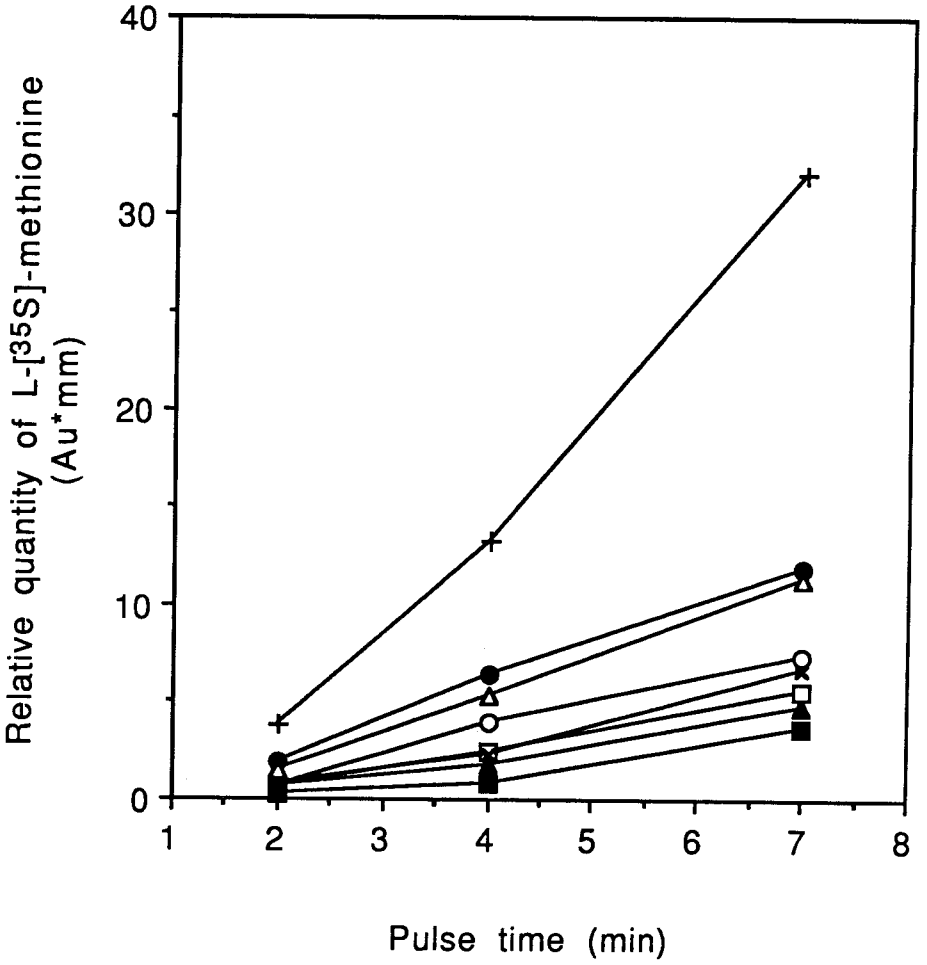


Figure 1B.


Relative quantity of L-[35S]-methionine (Au*mm)
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30
20
10
0
1
2
3
4
5
6
7
8
Pulse time (min)

Figure 2.

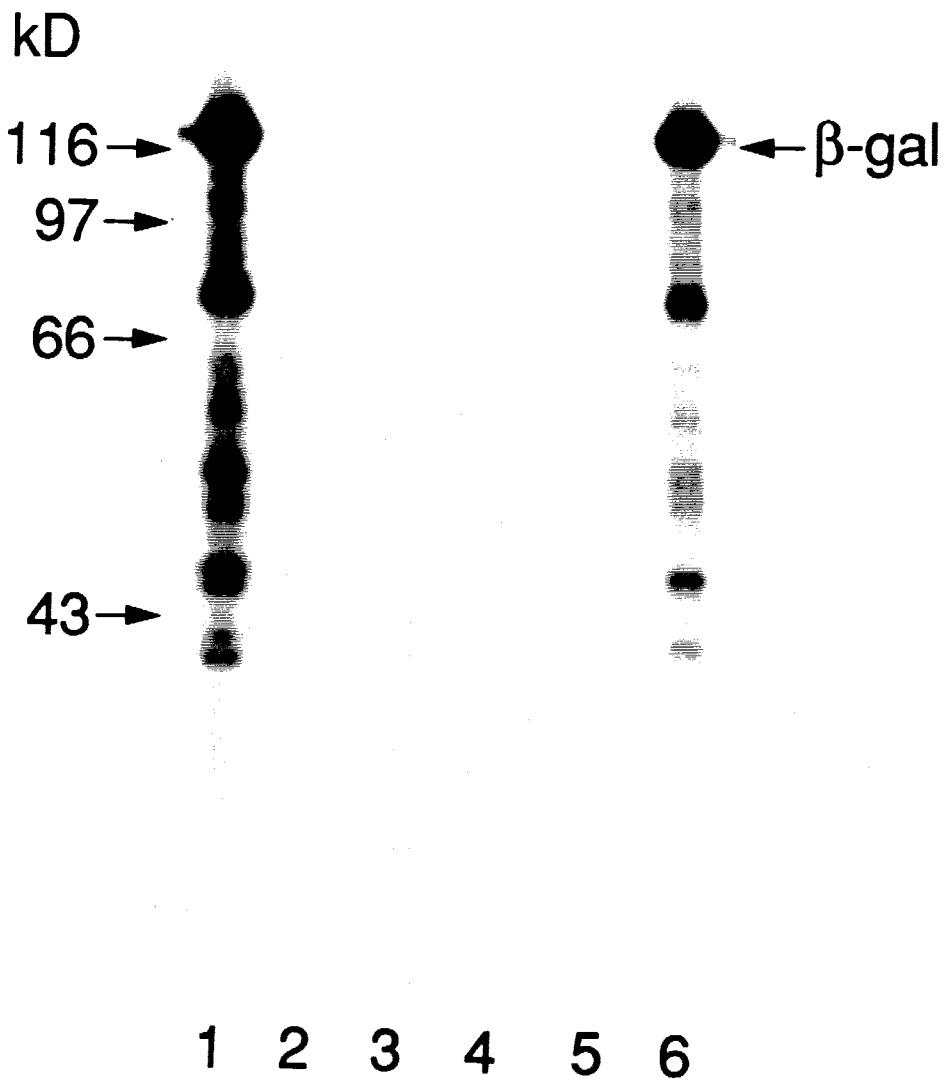


Figure 3.

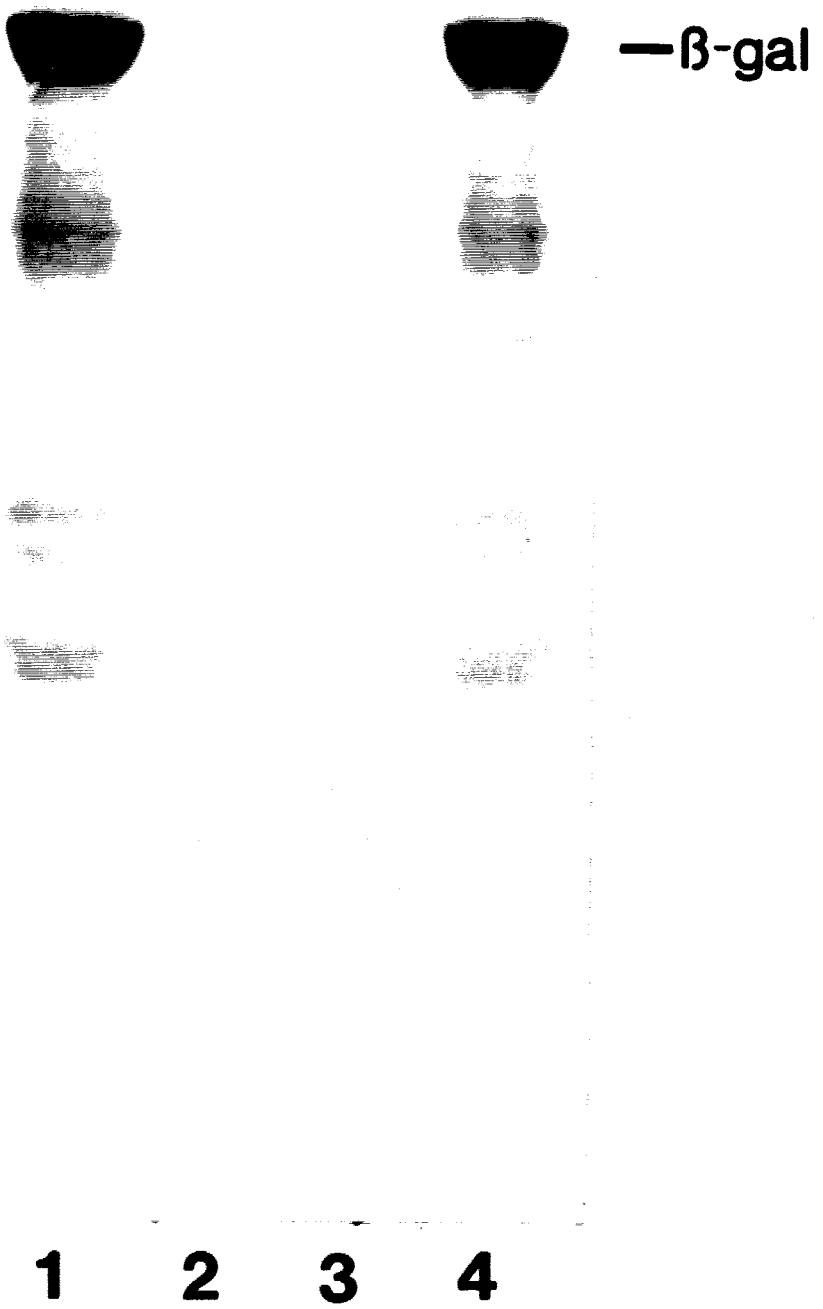


Figure 4.

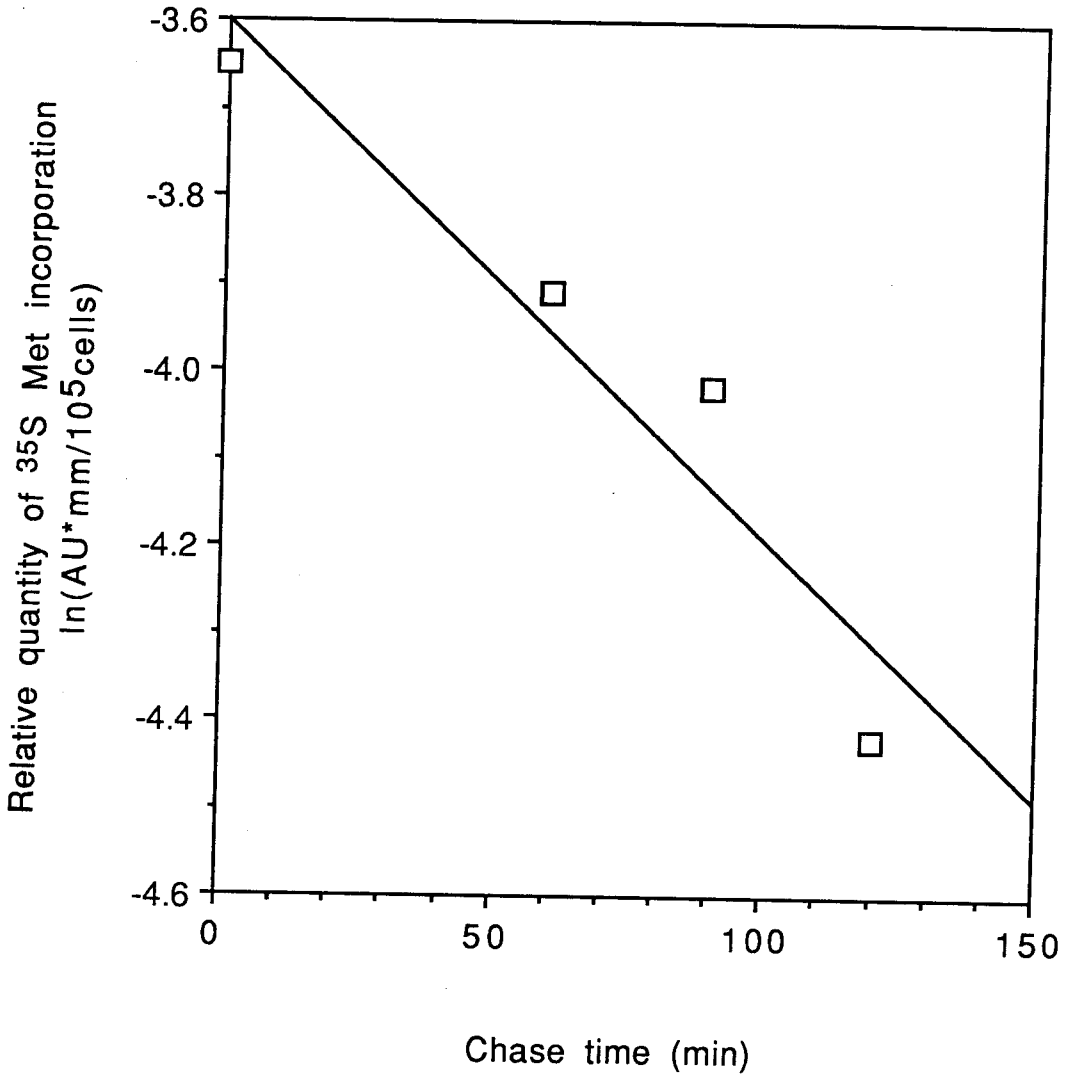

-3.6
-3.8
-4.0
-4.2
-4.4
-4.6
0
50
100
150
Relative quantity of 35S Met incorporation
ln(AU*mm/10^5cells)
Chase time (min)

Figure 5A.

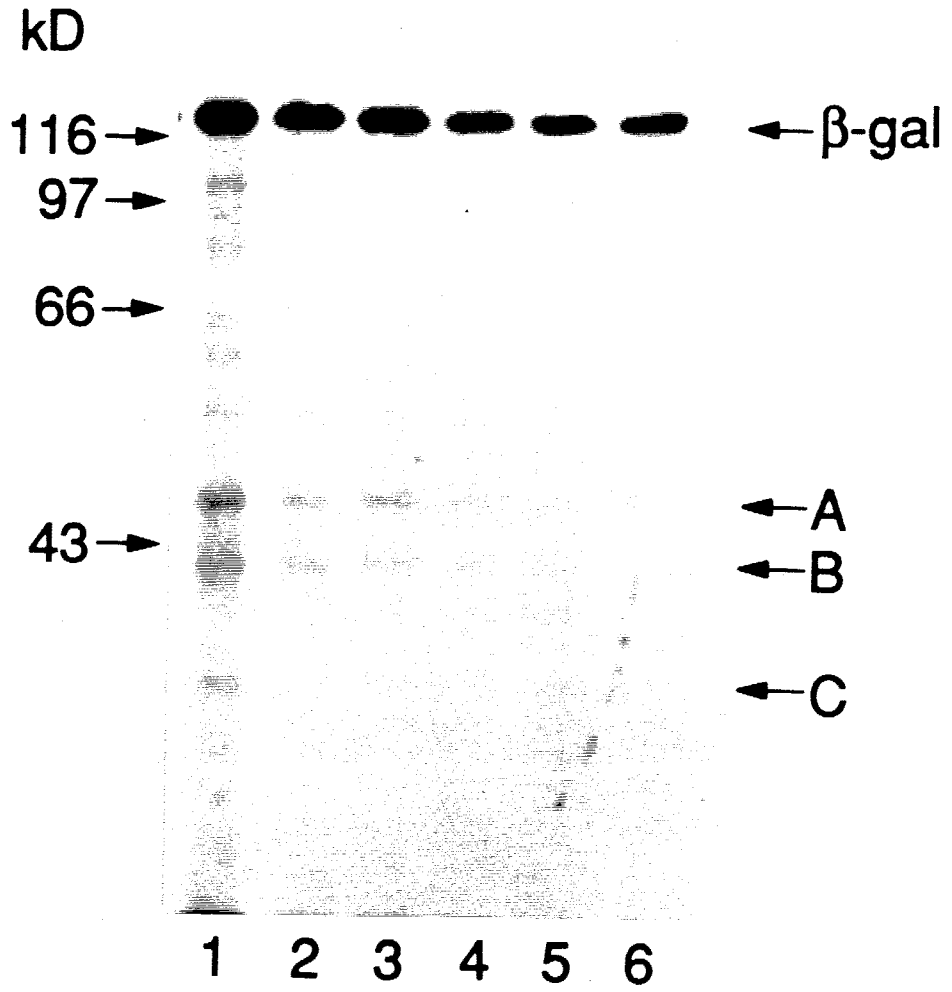
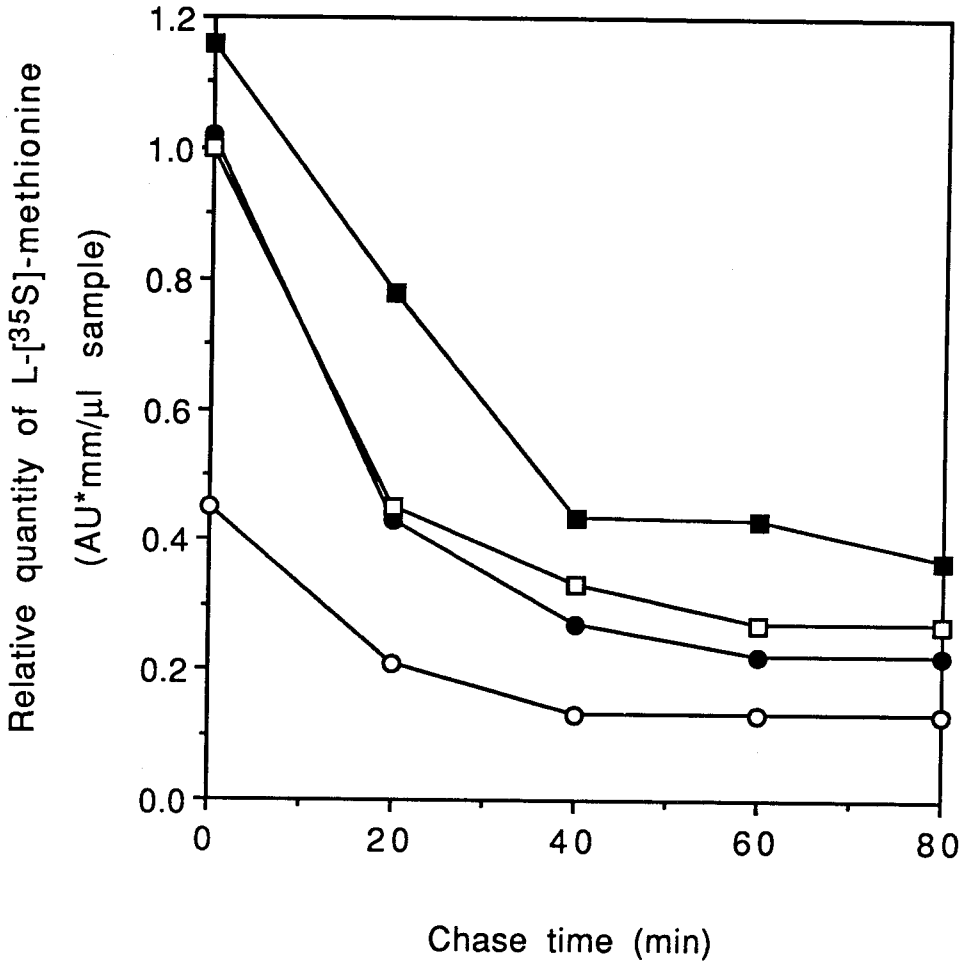


Figure 5B.


Relative quantity of L-[35S]-methionine
(AU*mm/µl sample)
1.2
1.0
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0.6
0.4
0.2
0.0
0
20
40
60
80
Chase time (min)

Figure 6.

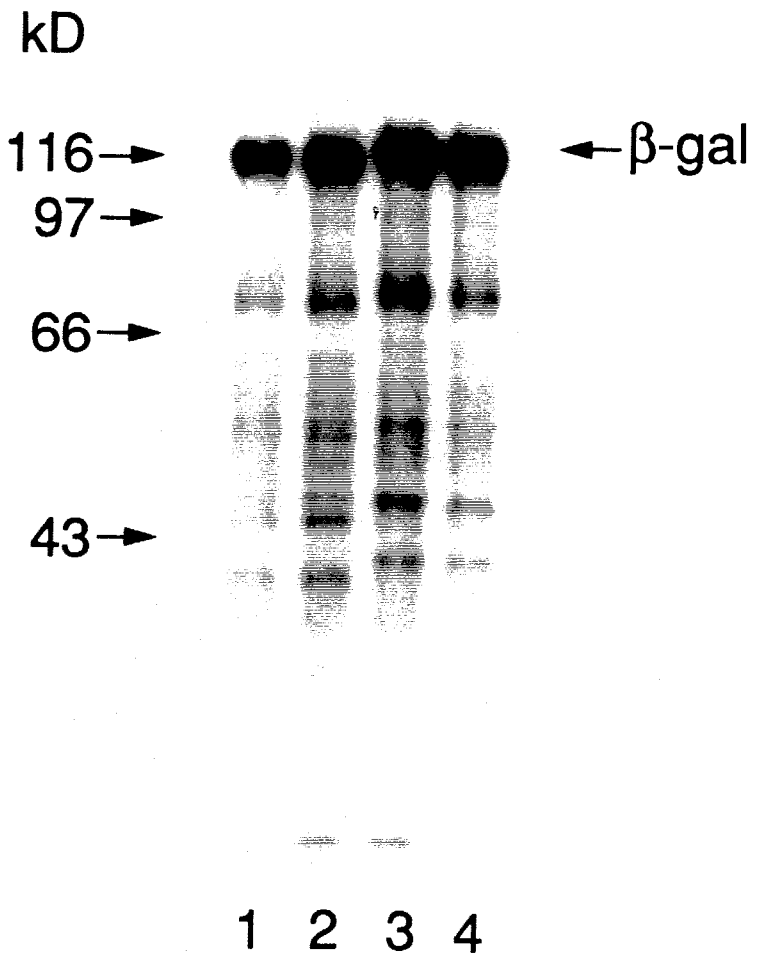


Figure 7.

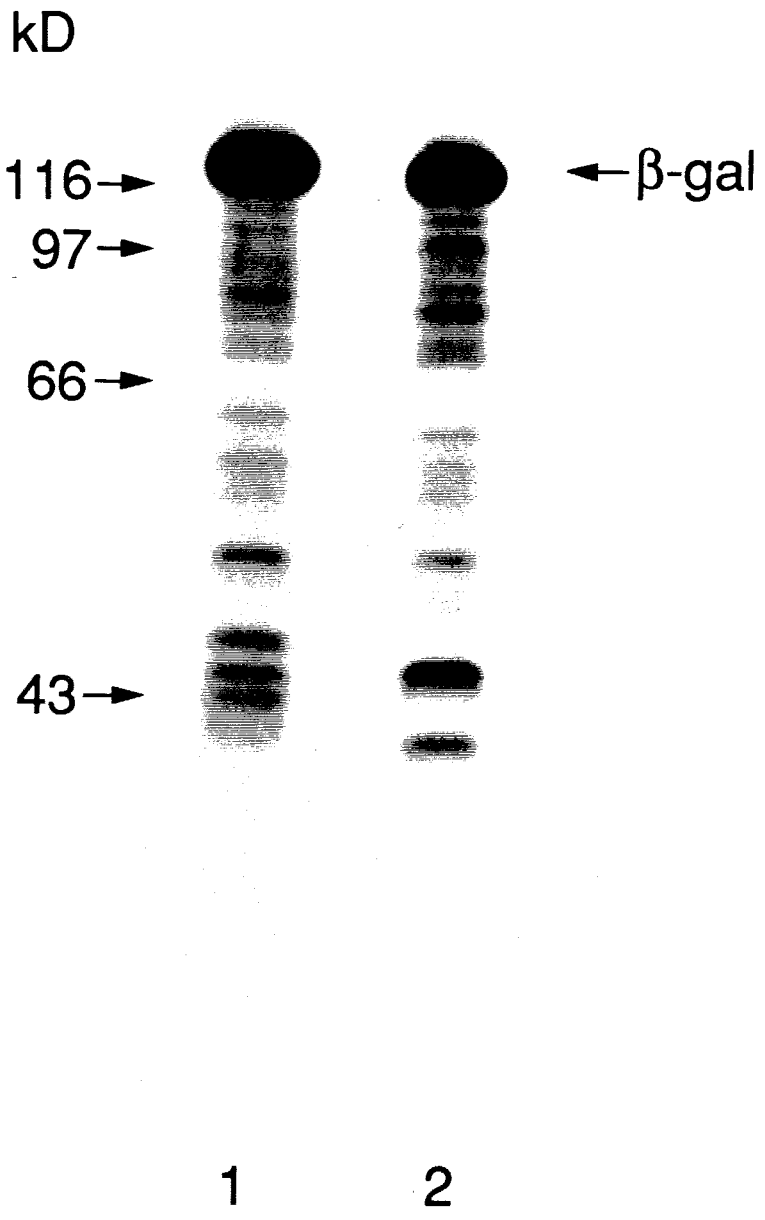


Figure 8.

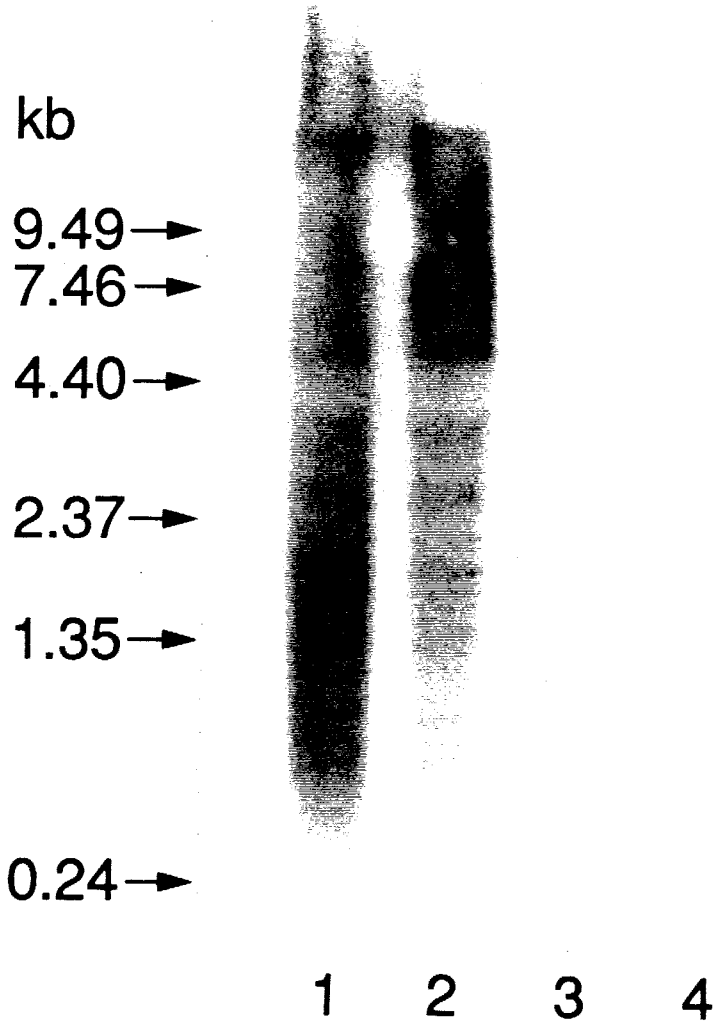


Figure 9.

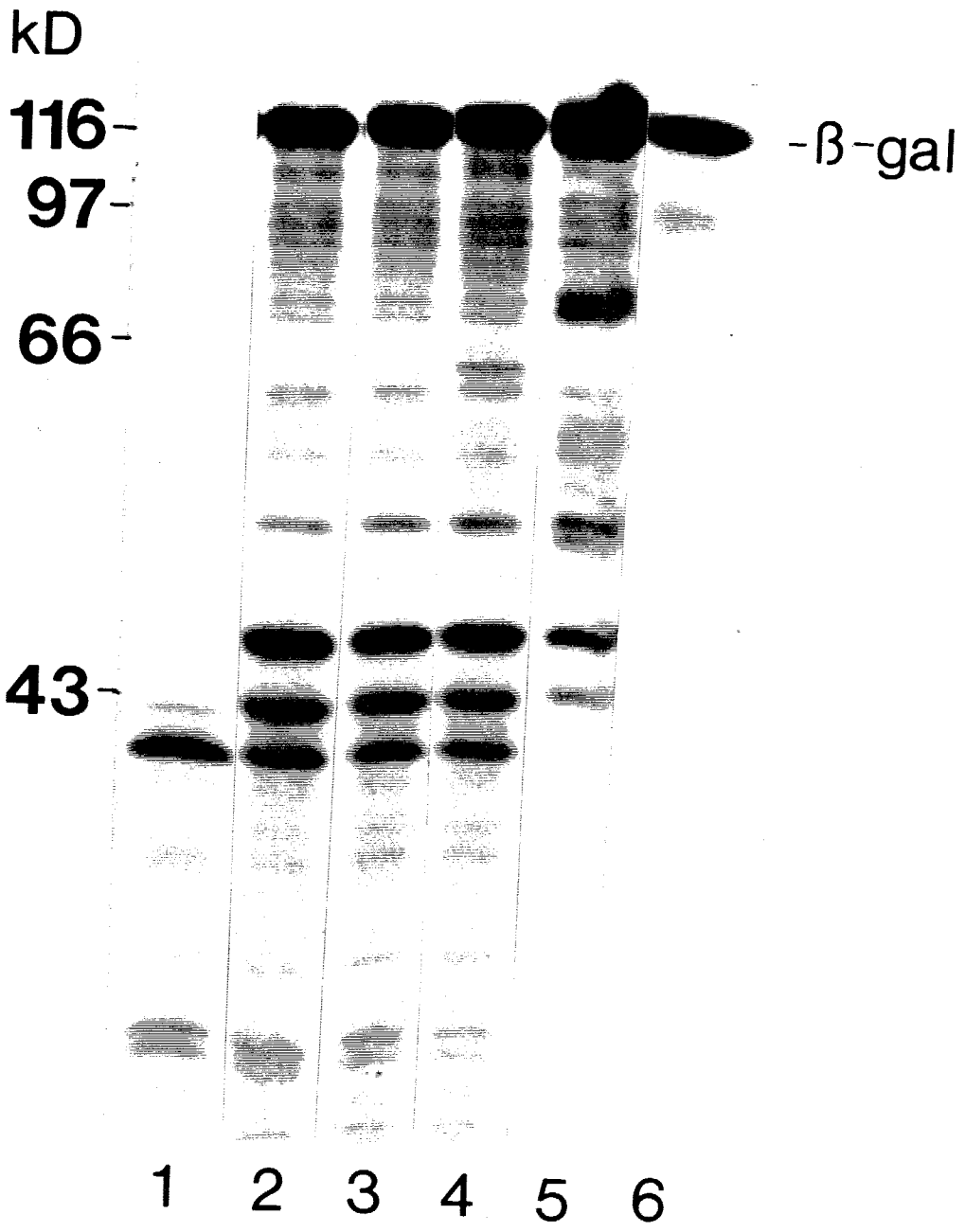
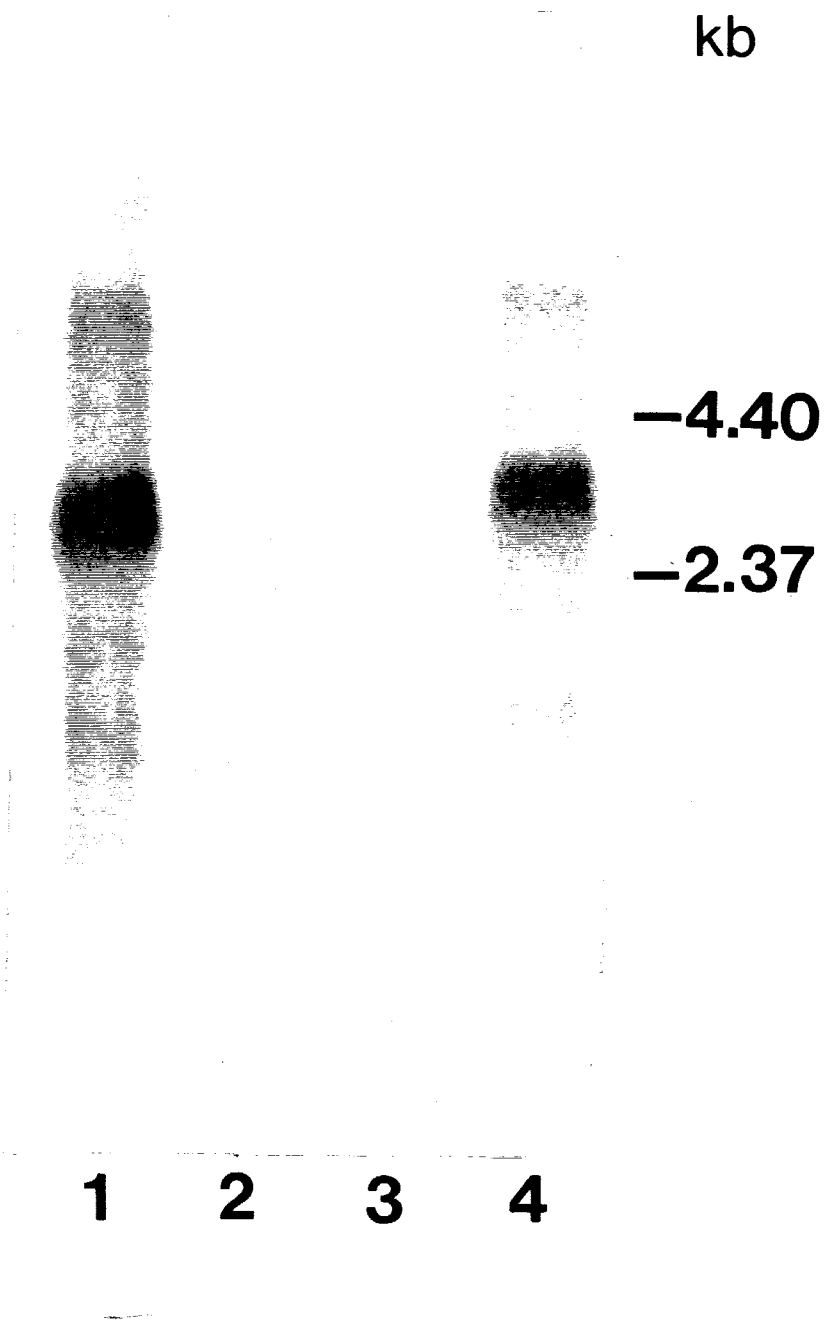


Figure 10.



CHAPTER V

Insect Cell Hosts for Baculovirus Expression Vectors Contain Endogenous Exoglycosidase Activity

The material contained in this chapter has been submitted for publication in *Proceedings of the National Academy of Sciences.*

5.1 Summary

Four different insect cell lines that can be used as hosts for baculovirus infection were assayed for the presence of endogenous exoglycosidases. All four cell lines, derived from *Spodoptera frugiperda*, *Trichoplusia ni*, *Bombyx mori*, or *Malacosoma disstria*, contained N-acetyl-ß-glucosaminidase, N-acetyl-ß-galactosaminidase, ß-galactosidase, and sialidase activities. The exoglycosidases were cell-associated, but activity was also observed in the medium from uninfected and wild-type baculovirus-infected cells. Oligosaccharide analysis of cellular glycoproteins using lectins recognizing Galß1,3GalNAc, Galß1,4GlcNAc, and NeuAcα2,6Gal demonstrated that only Galß1,3GalNAc was present. The demonstration that these cells contain exoglycosidases raises the possibility that the oligosaccharides of baculovirus-expressed glycoproteins are subject to enzymatic degradation.

5.2 Introduction

The insect cell-baculovirus expression system is a popular means of expressing recombinant proteins (1,2,3). By replacing the baculovirus polyhedrin structural gene with the gene of interest, recombinant proteins can be produced in high yields relative to other eucaryotic expression systems. Synthesis of heterologous proteins begins at approximately 24 hours post-infection and continues until approximately 4-5 days post-infection, when the cell lyses. A number of Lepidopteran insects and cell lines derived from these insects are susceptible to baculovirus infection and can be used as hosts for heterologous protein expression. Four examples are insect cell lines derived from *Spodoptera frugiperda* (fall armyworm), *Trichoplusia ni* (cabbage looper), *Bombyx mori* (silkworm), and *Malacosoma disstria* (forest tent caterpillar).

The insect cell-baculovirus system is a promising tool for the expression of heterologous glycoproteins because insect cells are capable of both N- and O- linked glycosylation. However, glycoproteins produced in mammalian systems are usually slightly larger than the same proteins expressed using the baculovirus expression system. This size difference has been attributed to differences in the structures of the oligosaccharide side chains on these proteins. Most evidence suggests that insect cells can add oligomannosidic N-linked oligosaccharides to newly synthesized glycoproteins and convert them to endo-ß-N-acetyl-D-glucosaminidase H (endo H)-resistant structures, but they cannot convert them to the complex structures found in higher eucaryotes (4-7). Similarly, O-linked oligosaccharides synthesized in insect cells appear to lack the terminal sialic acid residues that are often found in higher organisms (8). Based upon these findings, most investigators believe that insect cells lack the enzymes required for the latter steps in N- and O-linked oligosaccharide processing. However, several recent reports from one laboratory have contradicted this conclusion and suggested that insect

cells are capable of terminal N-glycosylation events, including the addition of sialic acid (9-11). Presently, there is no explanation for the discrepancy in these results.

Exoglycosidases are enzymes responsible for the hydrolysis of an O-glycosidic bond in a nonreducing terminal monosaccharide. N-acetyl-ß-glucosaminidase and N-acetyl-ß-galactosaminidase hydrolyze the glycosidic bond of the corresponding N-acetyl-ß-hexosamine when it occupies a terminal position on an oligosaccharide side-chain. These two different activities have been attributed to a single enzyme originally found in ram testes extract (12). This lack of specificity is reflected in the preferred name, N-acetyl-ß-hexosaminidase. Common sources of N-acetyl-ß-hexosaminidase include ram testes (13), jack bean meal (14), and *Streptococcus pneumoniae* (15-16). Other exoglycosidases are ß-galactosidase, which hydrolyzes the glycosidic bond of terminal ß-galactoside residues, and sialidase, which hydrolyzes the glycosidic bond of terminal sialic acids. The specificity of exoglycosidases depends on the cell type from which they are isolated. For example, ß-galactosidase isolated from jack bean meal cleaves ß1,4 linkages faster than ß1,3 linkages (17), whereas ß-galactosidase isolated from bovine testes preferably cleaves ß1,3 linkages (18). Sialidase isolated from *Clostridium perfringens* favors the hydrolysis of α2,3 linkages (19), whereas *Arthrobacter ureafaciens* sialidase prefers α2,6 linkages (20). In spite of these preferences, both sialidases can hydrolyze α2,3, α2,6, and α2,8 linkages. Besides their monosaccharide and glycosidic bond specificities, the activity of exoglycosidases depends on the precise structure of the oligosaccharide or the glycoprotein to which it is attached ("aglycon specificity," 21). Exoglycosidases have been observed in mammalian cell lines; recently sialidase was observed in Chinese hamster ovary cells (Goochee, C. F., and Gramer, M. J. (1992) Glycobiology Keystone Symposium, *J. Cell. Biochem.* Supplement 16D, 154).

Endogenous exoglycosidases in baculovirus-infected insect cells could remove terminal sugars during the isolation of foreign glycoproteins and complicate the subsequent analysis of their oligosaccharide structures. This may be one factor that

contributes to the contradictory results obtained in structural studies on different foreign glycoproteins by different workers, as discussed above. The purpose of this study was to test this hypothesis by assaying four different insect cell lines for the presence of endogenous exoglycosidases, including N-acetyl-ß-glucosaminidase, N-acetyl-ß-galactosaminidase, ß-galactosidase, and sialidase. The results of these studies showed that all four of these cell lines contain substantial levels of N-acetyl-ß-hexosaminidase and sialidase activities, as well as low levels of ß-galactosidase activity.

5.3 Materials and Methods

Cell lines, culture conditions, harvesting procedure. *S. frugiperda* (Sf9) cells were obtained from the ATCC (No. CRL-1711); *T. ni* (TN-368), *B. mori*, and *M. disstria* (MD108) cell lines were originally obtained from Dr. Max Summers. The Sf9 and TN-368 cell lines are derived from ovarian tissue, the *B. mori* cell line from embryos, and MD108 from larval hemocytes. All cells were cultured at 27°C in stationary T-25 flasks containing 4.5 ml of TNM-FH medium (22) supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), 5000 units/L penicillin, and 5 mg/L streptomycin (Gibco BRL, Grand Island, NY). All cell lines were also adapted to a protein-free medium, Insect-Xpress (Whittaker Bioproducts, Walkersville, MD). Unless otherwise noted, experiments were performed using cells grown in TNM-FH.

Cells in the late exponential growth phase were harvested by centrifugation at 1000 g for 5 minutes. The supernatant was saved and the cell pellet was washed twice with 1 ml of phosphate buffered saline (PBS). Cells were resuspended in PBS, and subjected to three rounds of freeze-thaw and mechanical homogenization. Homogenates were then used directly in exoglycosidase assays. Total protein content of samples was determined using the Bio-Rad Protein Assay (Bio-Rad, Richmond, CA), an assay based on the Bradford colorimetric procedure (23).

Infection conditions. Cells in the exponential growth phase were infected with wild-type *Autographa californica* multicapsid nuclear polyhedrosis virus (AcMNPV) at a multiplicity of infection of 10 plaque-forming units/cell. Infected culture samples were centrifuged in a microfuge to remove cellular debris, and the supernatant was subsequently stored at -20°C.

N-acetyl-ß-hexosaminidase activity assay. p-nitrophenyl-N-acetyl-ß-D-glucosaminide and p-nitrophenyl-N-acetyl-ß-D-galactosaminide (Sigma, St. Louis, MO, 24) were dissolved in H_2O at a concentration of 1 mg/ml. Further dilutions were made in H_2O as required. The substrate was preincubated at the appropriate temperature prior to the beginning of the assay. 1 ml of substrate solution and the appropriate volume of cell lysate or supernatant were added to microcuvettes. The absorbance at 495 nm was read in 2 min intervals for 20 min at constant temperature. pH values were monitored before and after absorbance readings to assure a constant pH (5.5). It should be noted that water was used instead of buffer because the high protein content of samples tended to cause precipitation; activity was also observed using McIlvaine buffer (0.05 M sodium phosphate, 0.025 M citric acid, pH 5.5, 25). The rate of hydrolysis is expressed in terms of µmol p-nitrophenyl released per minute.

ß-galactosidase activity assay. ß-galactosidase activity was determined using O-nitrophenyl-ß-galactoside (Sigma) and resorufin-ß-galactoside (Boehringer Mannheim, Indianapolis, IN, 26). The assay employing O-nitrophenyl-ß-galactoside has been described elsewhere (27). Resorufin-ß-galactoside was dissolved in dimethylsulfoxide (25 mM). This solution was diluted in PBS to a concentration of 0.05 mM. For spectrophotometric studies, the appropriate volume of cell lysate was added to 1 ml of substrate solution. For spectrofluorometric studies, the appropriate volume of cell lysate was added to 3 ml of substrate solution. The release of resorufin (7-hydroxy-2-phenoxazone) was monitored via spectrophotometry and spectrofluorometry as indicated (absorbance maximum at 572 nm and emission maximum at 583 nm, extinction coefficient at 572 nm = 66 $mmol^{-1}$ x 1 x cm^{-1}). ß-galactosidase purified from bovine testes was used as a standard to allow conversion of fluorometric readings to activity units. One activity unit is defined as the amount of ß-galactosidase required to hydrolyze 1 µmol of resorufin-ß-galactoside within 1 min at pH 4.3 and 37°C.

Sialidase activity assay. Cell lysate or supernatant was added to 5 µl of 50 mM 2'(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (Boehringer Mannheim) and 25 µl H_2O (28). Following incubation at 37°C for 1 hour, 20 µl of the reaction mixture were diluted with 1 ml H_2O. The sample was then excited at 365 nm and emission read at 448 nm. Emission readings were converted to activity units by comparison with sialidase from *Arthrobacter ureafaciens* (Oxford Glycosystems, Rosedale, NY). One activity unit is defined as the amount of sialidase required to hydrolyze 1 µmole of N-acetylneuraminosyl-D-lactose per min at pH 5 and 25°C.

Sialidase activity also was determined using fetuin (Boehringer Mannheim) as a substrate. 100 µg of fetuin was incubated with cell lysate at 37°C for 18 hours. NeuAc content was then determined by high pH anion exchange chromatography (HPAEC) as described below.

High pH anion exchange chromatography. The HPAEC system consisted of a Dionex BIO LC liquid chromatography unit, a Carbopac PA1 column (4 mm x 250 mm), and a pulsed electrochemical detector operating in the pulsed amperometric detection mode (Dionex Corp., Sunnyvale, CA). Samples were prepared as described in the text and centrifuged for 1 minute in a microcentrifuge immediately prior to analysis. 20 µl of sample were injected into the column and eluted with a constant concentration of NaOH (100 mM) and a linear gradient of sodium acetate (50 to 180 mM) over 20 minutes with a flow rate of 1 ml/min at room temperature. NeuAc was identified by its co-elution with N-acetylneuraminic acid standard (Dionex).

Lectin analysis. Total cellular proteins were fractionated on an 8.7% SDS-polyacrylamide gel and transferred to nitrocellulose in a Hoefer Blotting apparatus. Oligosaccharide moieties were detected by using a Glycan Differentiation Kit

(Boehringer Mannheim). This method involves binding digoxigenin-conjugated lectin to glycoprotein, followed by immunodetection of digoxigenin.

5.4 Results

N-Acetyl-ß-hexosaminidase activity. The hydrolysis of p-nitrophenyl-N-acetyl-ß-D-glucosaminide and p-nitrophenyl-N-acetyl-ß-D-galactosaminide in the presence of increasing amounts of *S. frugiperda* cell lysate were investigated at 27°C and 37°C (Figure 1). Under the specified conditions, the p-nitrophenyl group was detectably hydrolyzed from p-nitrophenyl-N-acetyl-ß-D-glucosaminide, but not from p-nitrophenyl-N-acetyl-ß-D-galactosaminide. The rate of p-nitrophenyl-N-acetyl-ß-D-glucosaminide hydrolysis increased with a corresponding increase in total cellular protein. The p-nitrophenyl group was hydrolyzed more slowly at 27°C than at 37°C, but even at the lower temperature, there was significant activity. The negative rate observed for the p-nitrophenyl-N-acetyl-ß-D-galactosaminide substrate is due to cell suspensions settling in the cuvette. (These experiments were carried out with cell lysate, resulting in a turbid solution that settled with time. After separating the soluble and insoluble fractions by centrifugation, exoglycosidase activity was predominantly located in the soluble lysate fraction). Hydrolysis of p-nitrophenyl-N-acetyl-ß-D-galactosaminide was observed with prolonged incubation at 37°C (data not shown).

The activity of N-acetyl-ß-glucosaminidase from *S. frugiperda* cells followed Michaelis-Menten kinetics as indicated by a Lineweaver-Burk plot (Figure 2). The V_{MAX} and K_m for hydrolysis of p-nitrophenyl-N-acetyl-ß-D-glucosaminide were calculated to be 2.7×10^{-4} µmol/min and 0.13 µM, respectively, under the specified conditions (37°C, 58 µg *S. frugiperda* protein). Similar behavior was observed with extracts from *T. ni*, *B. mori*, and *M. disstria* cells. Table 1 shows the results obtained for the hydrolysis of p-nitrophenyl-N-acetyl-ß-D-glucosaminide at 37°C with the different cell lines. To allow direct comparisons, specific activities based on total cellular protein are presented; the *T. ni* cell lysate demonstrated the greatest N-acetyl-ß-glucosaminidase specific activity.

Surprisingly, fresh TNM-FH with 10% serum, which had never been exposed to insect cells, had some endogenous N-acetyl-ß-glucosaminidase activity (Table 2). However, this activity was increased significantly by incubation with any one of the four different insect cell lines. The cell-specific nature of the observed exoglycosidase activity was demonstrated by adapting each of these cell lines to Insect-Xpress medium, a protein-free medium that contained no detectable N-acetyl-ß-glucosaminidase activity (Table 2). Cell extracts (data not shown) and the cell-free supernatant fraction (Table 2) from all four cell lines grown in Insect-Xpress medium contained significant levels of N-acetyl-ß-glucosaminidase activity.

N-acetyl-ß-glucosaminidase activity in the supernatant of *S. frugiperda* cells infected with wild-type AcMNPV is presented in Figure 3 as a function of time post-infection. Throughout the infection, the level of extracellular N-acetyl-ß-glucosaminidase activity was always considerably greater than the activity present in fresh TNM-FH medium containing 10% fetal bovine serum. A dramatic rise in extracellular N-acetyl-ß-glucosaminidase activity was observed during the first 30 hours of infection. The elevated levels of extracellular N-acetyl-ß-glucosaminidase activity compared to fresh medium indicated a release of the exoglycosidase from the cells into the medium.

ß-Galactosidase activity. The presence of ß-galactosidase activity was investigated by using two different, artificial substrates, O-nitrophenyl-ß-galactoside and resorufin-ß-galactoside. No ß-galactosidase activity could be detected with O-nitrophenyl-ß-galactoside as the substrate. However, resorufin-ß-galactoside provided different results. In the presence of ß-galactosidase, resorufin-ß-galactoside is cleaved to yield ß-galactoside and resorufin. Resorufin may be detected by spectrophotometric techniques or spectrofluorometric techniques, the latter being more sensitive. Using conditions similar to those used in the N-acetyl-ß-glucosaminidase studies, no ß-galactosidase activity was detected spectrophotometrically at 27°C or 37°C (Table 3).

These data can be compared to those in Table 1, as equal protein concentrations (30 μg/ml) were used and the response is based on a change in concentration over the same time scale. By using fluorometry and increasing the protein concentration to 170 μg/ml, a characteristic color response was detected, indicating a relatively low, but measurable, ß-galactosidase activity. By the same fluorescence assay, ß-galactosidase activity was detected in all cell lines at 37°C; activity was also detected at 27°C for all cell lines except *S. frugiperda* (Table 3). A very high ß-galactosidase activity was observed in fresh TNM-FH medium that was not exposed to cells (Table 4). Supernatants from the four cell lines grown in TNM-FH had a slightly lower activity than the fresh medium, perhaps due to ß-galactosidase degradation during the time of the cultivation. Similar supernatant analysis with cells cultivated in protein-free medium indicated a zero basal level of activity in fresh Insect-Xpress medium and an elevated level in medium removed from healthy cell cultures (Table 4). Furthermore, cells cultivated in Insect-Xpress hydrolyzed the resorufin-ß-galactoside, indicating that the cellular activity presented in Table 3 is not an artifact of residual serum-containing medium associated with the cells.

Sialidase activity. Varying quantities of *S. frugiperda* cell lysate were incubated with 2'(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid as described in the Materials and Methods Section. A linear response of sialidase activity was observed with increasing quantities of cell extract (Figure 4). On a per cell basis, this correlates to approximately 4 μUnits per uninfected cell. Activity was not observed in fresh TNM-FH medium that was not exposed to cells (Table 5). Sialidase activity was demonstrated to be present in the extracellular medium of uninfected cells as well as wild-type AcMNPV-infected cells (data not shown).

Fetuin, a sialylated glycoprotein, was incubated with lysed, uninfected *S. frugiperda* cells for 18 hours. After incubation, the sample was analyzed by HPAEC for free N-acetylneuraminic acid (Figure 5). The peak area corresponding to NeuAc was 4

times greater for the sample containing fetuin (Panel C) than the control sample containing an equal volume of H_2O and cells (Panel B). No NeuAc was observed for the control consisting of fetuin in PBS (data not shown).

T. ni, *B. mori*, and *M. disstria* cell lysates were also assayed for sialidase activity. Each had detectable sialidase activity at 37°C when 2'(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid was used as the substrate (Table 5). *T. ni*, *B. mori*, and *M. disstria* cells contained more activity per mg of total protein than *S. frugiperda* cells. There was little difference in the levels of sialidase activity detected in *S. frugiperda* cells at 27°C and 37°C. All cell lines also exhibited sialidase activity when cultured in protein-free medium (data not shown).

Lectin analysis. The glycosylation of native cellular proteins was investigated by using lectin-binding assays (Figure 6). Peanut agglutinin (PNA), a lectin specific for the Galß1,3GalNAc linkage, bound selectively to several large proteins in lysates from each of the four different cell lines. By contrast, *Datura stramonium* agglutinin (DSA), a lectin specific for Galß1,4GlcNAc, showed little or no binding, although a faint band was observed at approximately 30 kDa. *Sambucus nigra* agglutinin (SNA), with a specificity for NeuAcα2,6Gal, provided similar results. This suggests that the 30 kDa protein contains terminal NeuAcα2,6Galß1,4GlcNAc, which is not thought to exist in insect cells. The molecular weight of this protein produced in *T. ni* cultures differs from that obtained with the other cell lines. Nonspecific binding of both DSA and SNA to this 30 kDa protein have not been ruled out. It should be emphasized that the Galß1,3GalNAc moiety detected by PNA likely belongs to an O-linked oligosaccharide whereas carbohydrates recognized by DSA and SNA are present on N-linked oligosaccharides. Studies with all four cell lines infected with wild-type AcMNPV provided similar results with these 3 lectins (data not shown).

5.5 Discussion

This study has demonstrated that four different insect cell lines, derived from *Spodoptera frugiperda*, *Trichoplusia ni*, *Bombyx mori*, and *Malacosoma disstria*, contain significant levels of endogenous N-acetyl-ß-hexosaminidase and sialidase activities and low levels of ß-galactosidase activity. Interestingly, exoglycosidase activities were detected in TNM-FH medium supplemented with 10% serum, which had never been exposed to these cells. However, elevated levels of N-acetyl-ß-hexosaminidase and sialidase activity were observed in the supernatant from insect cell cultures. Further, the cell-specific nature of the exoglycosidase activity was documented by growing the cells in protein-free medium, which lacked detectable exoglycosidase activity. Insect cell exoglycosidases were detected both in cell extracts and in the extracellular medium. The extracellular activity probably results from specific or nonspecific secretion of exoglycosidases. Baculovirus infection induced a dramatic increase in the levels of extracellular exoglycosidase activity. Presumably, this reflects a loss of membrane integrity and cell lysis due to the viral infection. Alternatively, it may result from a virus-induced effect on the secretion of these enzymes.

It is important to recognize the limitations of artificial substrates in the analysis of exoglycosidases. Although providing the researcher with a product that is relatively easy to assay, the hydrolysis of artificial substrates is not identical to that of natural substrates. For most artificial substrates, the rate of hydrolysis is greater than the rate of hydrolysis with natural oligosaccharides or glycoproteins (25). However, even with natural substrates, rates are dependent on the nature and structure of the substrate. The usefulness of 2'(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid for predicting behavior with natural substrates with *S. frugiperda* cells has been demonstrated here in experiments employing fetuin as the substrate. It should be emphasized that the hydrolysis of resorufin-ß-galactoside and lectin studies demonstrating the existence of the

Galß1,3GalNAc moiety are not contradictory. Lectin studies with PNA indicating the presence of the Galß1,3GalNAc linkage should not be construed as evidence for the lack of ß-galactosidase, since this exoglycosidase isolated from a variety of sources has demonstrated a dramatic linkage specificity (29). With regard to the p-nitrophenyl-substrates, the results for the two different substrates presented in Figure 1 provide an interesting control. The only structural difference between these substrates is the position of the hydroxyl group at C-5. The difference in the hydrolysis rate of the two compounds suggests enzymatic recognition of the N-acetyl-ß-hexosamine and not the p-nitrophenyl moiety. Similar behavior has been observed for other N-acetyl-ß-hexosaminidases. Ram testes N-acetyl-ß-hexosaminidase hydrolyzed p-nitrophenyl-N-acetyl-ß-D-glucosaminide approximately 7 times faster than p-nitrophenyl-N-acetyl-ß-D-galactosaminide (12).

The exoglycosidase activities present in insect cells susceptible to baculovirus infection may have a strong influence on the final structures of the oligosaccharides attached to glycoproteins expressed in the baculovirus system. These enzymes could remove terminal sugars before or during the isolation of newly synthesized foreign glycoproteins. It is tempting to speculate that exoglycosidases may remove terminal sugars in intracellular compartments, thereby serving as regulators of oligosaccharide structure in insect cell glycosylation pathways. However, it is not necessary to speculate on the functions of these enzymes to see how they could complicate the subsequent structural analysis of oligosaccharide side-chains attached to insect cell-derived glycoproteins. Exoglycosidases, which may be more or less active on different glycoproteins, could be an important factor that contributes to the contradictory results reported in structural studies on different foreign glycoproteins. We conclude that the presence of exoglycosidase activities should be taken into consideration in interpreting these studies and that measures should be taken to minimize the potential effects of endogenous exoglycosidases in studies on insect cell-derived oligosaccharide structures.

5.6 Acknowledgments

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5.7 Nomenclature

AcMNPV	*Autographa californica* multicapsid nuclear polyhedrosis virus
GlcNAc	N-acetyl-ß-glucosamine
GalNAc	N-acetyl-ß-galactosamine
Gal	galactose
NeuAc	N-acetylneuraminic acid
ONPG	O-nitrophenyl-ß-galactoside
PBS	phosphate buffered saline
SDS	sodium dodecyl sulfate
pNP-GlcNAc	p-nitrophenyl-N-acetyl-ß-D-glucosaminide
pNP-GalNAc	p-nitrophenyl-N-acetyl-ß-D-galactosaminide
PNA	peanut agglutinin
DSA	*Datura stramonium* agglutinin
SNA	*Sambucus nigra* agglutinin
HPAEC	high pH anion exchange chromatography
nd	not detected

5.8 References

1. Luckow, V. A. (1991) in *Recombinant DNA Technology and Applications,* eds. Prokop, A., Bajpai, R. K., & Ho, C. S. (McGraw-Hill, Inc., NY) pp. 97-152.

2. Luckow, V. A., & Summers, M. D. (1988) Trends in the development of baculovirus expression vectors. *Biotechnol.* **6**, 47-55.

3. Miller, L. K. (1988) Baculoviruses as gene expression vectors. *Ann. Rev. Microbiol.* **42**, 177-199.

4. Butters, T. D., Hughes, R. C., & Vischer, P. (1981) Steps in the biosynthesis of mosquito cell glycoproteins and the effects of tunicamycin. *Biochim. Biophys. Acta.* **640**, 672-686.

5. Hsieh, P., & Robbins, P. W. (1984) Regulation of asparagine-linked oligosaccharide processing: oligosaccharide processing in *Aedes albopictus* mosquito cells. *J. Biol. Chem.* **259**, 2375-2382.

6. Jarvis, D. L., & Summers, M. D. (1989) Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. *Mol. Cell. Biol.* **9**, 214-223.

7. Kuroda, K., Geyer, H., Geyer, R., Doerfler, W., & Klenk, H. D. (1990) The oligosaccharides of influenza virus hemagglutin expressed in insect cells by a baculovirus vector. *Virol.* **174**, 418-429.

8. Thomsen, D. R., Post, L. E., & Elhammer, A. P. (1990) Structure of O-glycosidically-linked oligosaccharides synthesized by the insect cell line Sf9. *J. Cell. Biochem.* **43**, 67-79.

9. Davidson, D. J., Fraser, M. J., & Castellino, F. J. (1990) Oligosaccharide processing in the expression of human plasminogen cDNA by Lepidopteran insect (*Spodoptera frugiperda*) cells. *Biochem.* **29**, 5584-5590.

10. Davidson, D. J., & Castellino, F. J. (1991) Asparagine-linked oligosaccharide processing in Lepidopteran insect cells. Temporal dependence of the nature of the oligosaccharides assembled on asparagine-289 of recombinant human plasminogen produced in baculovirus vector infected *Spodoptera frugiperda* cells. *Biochem.* **30**, 6167-6174.

11. Davidson, D. J., Bretthauer, R. K., & Castellino, F. J. (1991) α-mannosidase catalyzed trimming of high mannose glycans in noninfected and baculovirus-infected *Spodoptera frugiperda* cells (IPLB-SF-21AE). A possible contributing regulatory mechanism for assembly of complex-type oligosaccharides in infected cells. *Biochem.* **30**, 9811-9815.

12. Woolen, J. W., Heyworth, R., & Walker, P. G. (1961) Studies on glucosaminidase. *Biochem. J.* **78**, 111-116.

13. Woolen, J. W., Walker, P. G., & Heyworth, R. (1961) Studies on glucosaminidase. *Biochem. J.* **79**, 294-298.

14. Li, S., & Li, Y. (1970) Studies on the glycosidases of jack bean meal. *J. Biol. Chem.* **245**, 5153-5160.

15. Hughes, R. C., & Jeanloz, R. W. (1964) The extracellular glycosidases of *Diplococcus pneumoniae*. 1. Purification and properties of a ß-N-acetylglucosaminidase. Action on the α1-acid glycoprotein of human plasma. *Biochem.* **3**, 1535-1543.

16. Hughes, R. C., & Jeanloz, R. W. (1964) The extracellular glycosidases of *Diplococcus pneumoniae*. 2. Purification and properties of a ß-N-acetylglucosaminidase. Action on a derivative of the α1-acid glycoprotein of human plasma. *Biochem.* **3**, 1543-1548.

17. Arakawa, M., Ogato, S., Muramatsu, T., & Kobato, A. (1974) ß-galactosidases from jack bean meal and almond emulsion. *J. Biochem.* **75**, 707-714.

18. Li, S., Mazzotta, M. Y., Chien, S., & Li, Y. (1975) Isolation and characterization of jack bean ß-galactosidase. *J. Biol. Chem.* **250**, 6786-6791.

19. Corfield, A. P., Veh, R. W., Wember, M., Michalski, J. C., & Schauer, R. (1981) The release of N-acetyl- and N-glycolloyl-neuraminic acid from soluble complex carbohydrates and erythrocytes by bacterial, viral and mammalian sialidases. *Biochem. J.* **197**, 293-299.

20. Uchida, Y., Tsukada, Y., & Sugimori, T. (1979) Enzymatic properties of neuraminidases. *J. Biochem.* **86**, 1573-1585.

21. Kobata, A. (1979) Use of endo- and exoglycosidases for structural studies of glycoconjugates. *Anal. Biochem.* **100**, 1-14.

22. Hink, W. F. (1970) Established insect cell line from the cabbage looper *Trichoplusia ni*. *Nature* **226**, 466-467.

23. Bradford, M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

24. Borooah, J., Leaback, D. H., & Walker, P. G. (1961) Studies on glucosaminidase. *Biochem. J.* **78**, 106-110.

25. Montreuil, J., Bouquelet, S., Debray, H., Fournet, B., Spik, G., & Strecker, G. (1987) in *Carbohydrate Analysis,* eds. Chaplin, M. F., & Kennedy, J. F. (IRL Press, Oxford, U.K.) pp. 143-204

26. Hofmann, J., & Sernetz, M. (1984) Immobilized enzyme kinetics analyzed by flow-through microfluorimetry. *Anal. Chim. Acta.* **163**, 67-72.

27. Licari, P., & Bailey, J. E. (1991) Factors influencing recombinant protein yeilds in an insect cell-baculovirus expression system: multiplicity of infection and intracellular protein degradation. *Biotechnol. Bioeng.* **37**, 238-246.

28. Miller, R. W., Lee, R. T., Lee, Y. C., Reynolds, L. W., & Uchida, Y. (1980) The synthesis of 4-methylumbelliferyl α-ketoside of N-acetylneuraminic acid and its use in a fluorometric assay for neuraminidase. *Anal. Biochem.* **101**, 166-174.

29. DiCioccio, R. A., Klock, P. J., Barlow, J. J., & Matta, K. L. (1980) Rapid procedures for determination of endo-N-acetyl-α-D-galactosaminidase in *Clostridium perfringens*, and of the substrate specificity of exo-ß-D-galactosidases. *Carb. Res.* **81**, 315-322.

5.9 Tables

Table 1. Rate of hydrolysis of p-nitrophenyl from p-nitrophenyl-N-acetyl-ß-D-glucosaminide (pNP-GlcNAc) and p-nitrophenyl-N-acetyl-ß-D-galactosaminide (pNP-GalNAc) at 37°C. Specific activity is expressed in units of µmol p-nitrophenyl released per minute per µg total cellular protein. Approximately equal quantities of total protein were used for each cell line (30 µg/ml) with a substrate concentration of 1 mg/ml. nd indicates that a response was not detected.

Cell Line	Specific Activity	
	pNP-GlcNAc (µmol/min-µg)x10^{-5}	pNP-GalNAc (µmol/min-µg)
S. frugiperda	0.7	nd
T. ni	1.3	nd
B. mori	0.9	nd
M. disstria	0.5	nd

Table 2. Rate of hydrolysis of p-nitrophenyl from p-nitrophenyl-N-acetyl-ß-D-glucosaminide in the presence of cell culture supernatant. 100 µl of clarified supernatant were added to 1 ml of 1 mg/ml p-nitrophenyl-N-acetyl-ß-D-glucosaminide and the rate of hydrolysis monitored at 37°C. For reference, N-acetyl-ß-glucosaminidase activity of fresh TNM-FH medium and fresh Insect-Xpress (protein-free medium) are also listed.

Supernatant derived from:		**Activity** (µmol/min-µl)x10^{-6}
Cell Line	**Medium**	
S. frugiperda	TNM-FH	3.8
T. ni	TNM-FH	1.5
B. mori	TNM-FH	5.9
M. disstria	TNM-FH	3.0
No cells	TNM-FH	0.9
S. frugiperda	Insect-Xpress	0.9
T. ni	Insect-Xpress	0.5
B. mori	Insect-Xpress	2.8
M. disstria	Insect-Xpress	4.9
No cells	Insect-Xpress	0.0

Table 3. ß-Galactosidase activity as measured using resorufin-ß-galactoside as a substrate at 27°C and 37°C. The rate of substrate hydrolysis was detected by spectrophotometry and spectrofluorometry as indicated. For the spectrophotometric response, total protein concentrations equal to those of Table 1 (30 µg/ml) were used to allow comparison. Approximately equal quantities of total protein were used for each cell line in the spectrofluorometry studies (170 µg/ml). nd indicates that a response was not detected.

Cell Line	Spectrophotometry Response		Fluorometry (mUnits/ug)x10^{-3}	
	27°C	37°C	27°C	37°C
S. frugiperda	nd	nd	nd	1.5
T. ni	nd	nd	0.8	1.3
B. mori	nd	nd	1.4	2.8
M. disstria	nd	nd	1.5	2.9

Table 4. ß-galactosidase activity in cell culture supernatants. Clarified supernatant was added to 3 ml of 0.05 mM resorufin-ß-galactoside and the rate of hydrolysis monitored at 37°C. For reference, ß-galactosidase activity of fresh TNM-FH medium and fresh Insect-Xpress (protein-free medium) are also listed.

Supernatant derived from:		**Activity (mUnits/µl)x10^{-3}**
Cell Line	**Medium**	
S. frugiperda	TNM-FH	1.5
T. ni	TNM-FH	2.3
B. mori	TNM-FH	1.4
M. disstria	TNM-FH	2.0
No cells	TNM-FH	2.5
S. frugiperda	Insect-Xpress	0.07
T. ni	Insect-Xpress	0.11
B. mori	Insect-Xpress	0.07
M. disstria	Insect-Xpress	0.25
No cells	Insect-Xpress	0.00

Table 5. Sialidase activity as measured using 2'(4-methylumbelliferyl)-α–D-N-NeuAc. Specific activity is expressed in mUnits per μg total cellular protein. Emission at 448 nm was detected after 1-hour incubation at the indicated temperature.

Cell Line	T (°C)	Specific Activity (mUnits/μg)
S. frugiperda	27	0.07
S. frugiperda	37	0.08
T. ni	37	0.14
B. mori	37	0.12
M. disstria	37	0.14
TNM-FH	37	0.00

5.10 Figures

Figure 1. *S. frugiperda* cell lysate contains substantial levels of N-acetyl-ß-glucosaminidase, but not N-acetyl-ß-galactosaminidase activity. The plot shows the rate of p-nitrophenyl hydrolysis (µmol p-nitrophenyl released per min) from p-nitrophenyl-N-acetyl-ß-D-glucosaminide and p-nitrophenyl-N-acetyl-ß-D-galactosaminide as a function of total cellular protein (µg) from *S. frugiperda* cells at 27°C and 37°C. Symbols correspond to: p-nitrophenyl-N-acetyl-ß-D-glucosaminide hydrolysis at 37°C (●) and 27°C (○) and p-nitrophenyl-N-acetyl-ß-D-galactosaminide at 37°C (■) and 27°C (□).

Figure 2. Lineweaver-Burk plot for hydrolysis of p-nitrophenyl-N-acetyl-ß-D-glucosaminide using 58 µg total protein/ml from *S. frugiperda* cells. Inverse rate, 1/V (min/µmol) is plotted as a function of the inverse substrate concentration, 1/[S] (mM^{-1}). Approximately 58 µg/ml of total cellular protein was incubated with substrate at 37°C.

Figure 3. N-acetyl-ß-glucosaminidase in the supernatant of wild-type baculovirus-infected *S. frugiperda* cells at different times post-infection (hours). 100 µl of clarified supernatant was added to 1 ml of 1 mg/ml p-nitrophenyl-N-acetyl-ß-D-glucosaminide and the rate of hydrolysis monitored at 37°C. For reference, N-acetyl-ß-glucosaminidase activity of fresh TNM-FH medium is indicated by the horizontal line.

Figure 4. Sialidase activity in *S. frugiperda* cell lysate. Sialidase activity (mUnits) was assayed by incubation with the substrate 2'(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid and plotted as a function of total cellular protein (µg). The cell lysate was prepared as described in the Materials and Methods prior to incubation with the substrate for 1 hour at 37°C.

Figure 5. Removal of NeuAc from fetuin incubated with cell lysate. Pulsed amperometric response (μCoulombs) is plotted as a function of time (min). N-acetylneuraminic acid standard (1 nmol) elutes at approximately 15 min in Chromatogram A. Chromatogram B is from total uninfected cell lysate (approximately 1 x 10^6 cells in 100 μl H_2O) and indicates a small peak eluting at the same time as the NeuAc standard. Chromatogram C is from 100 μg fetuin incubated with an equal volume of cell lysate as in Chromatogram B. The area of the peak corresponding to NeuAc in Chromatogram C is approximately 4 times that of the peak area for Chromatogram B. Elution conditions were constant 100 mM NaOH and a gradient of sodium acetate from 50 to 180 mM over 20 minutes with a flow rate of 1 ml/min.

Figure 6. Lectin analysis of uninfected cellular protein. Total protein was fractionated on an 8.7% SDS-polyacrylamide gel, transferred to nitrocellulose and probed with the indicated lectins. The lectins are noted at the top of each panel. The letter designations at the bottom of each panel refer to *S. frugiperda* (S), *T. ni* (T), *B. mori* (B), and *M. disstria* (M). A molecular weight ladder is shown on the left.

Figure 1.

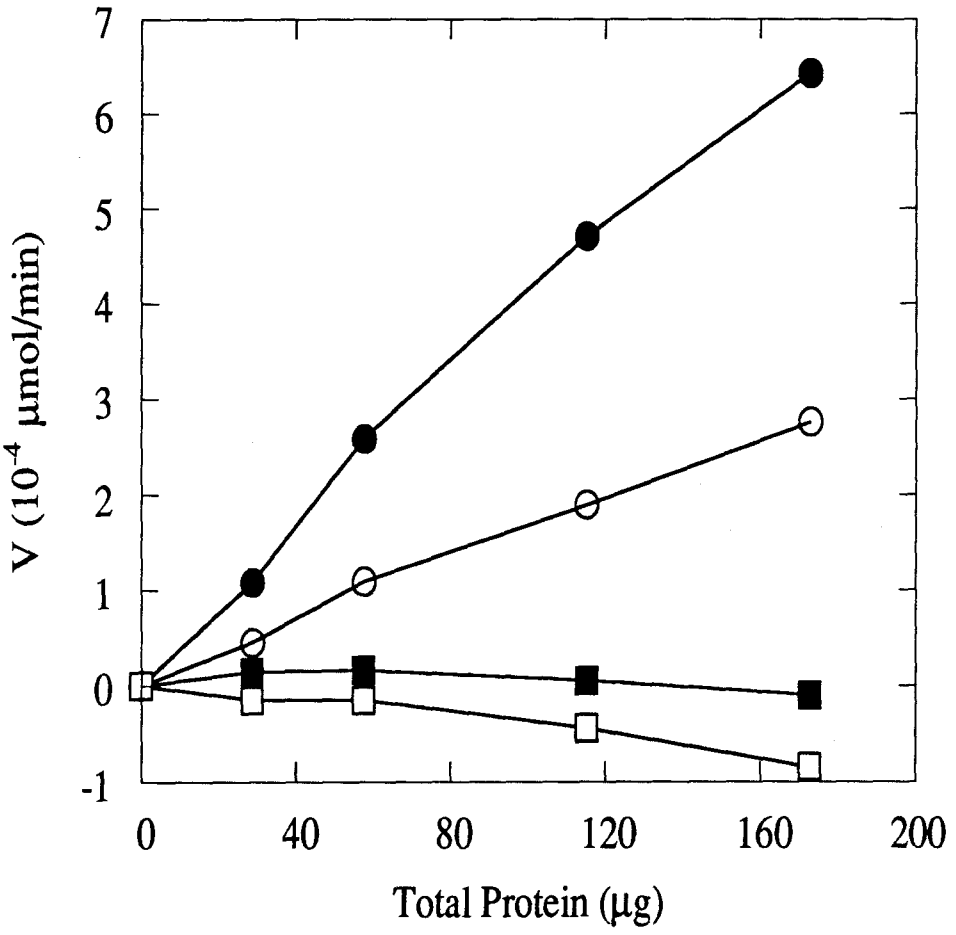


Figure 2.

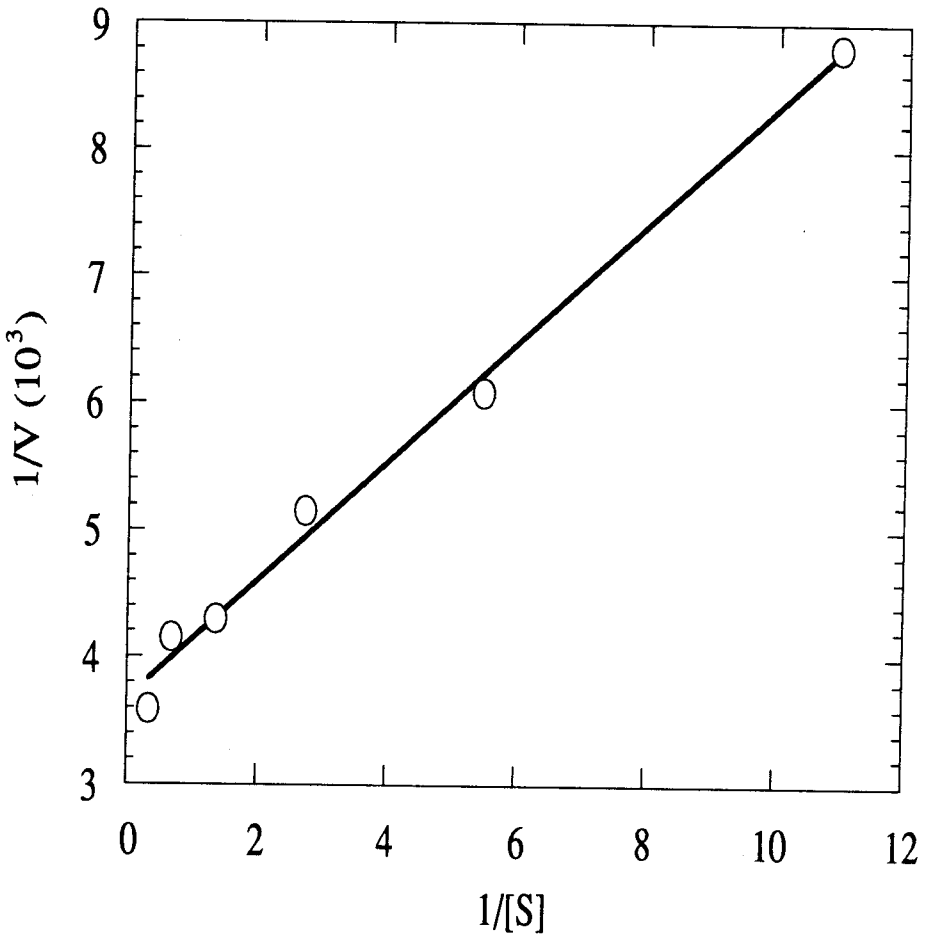


Figure 3.

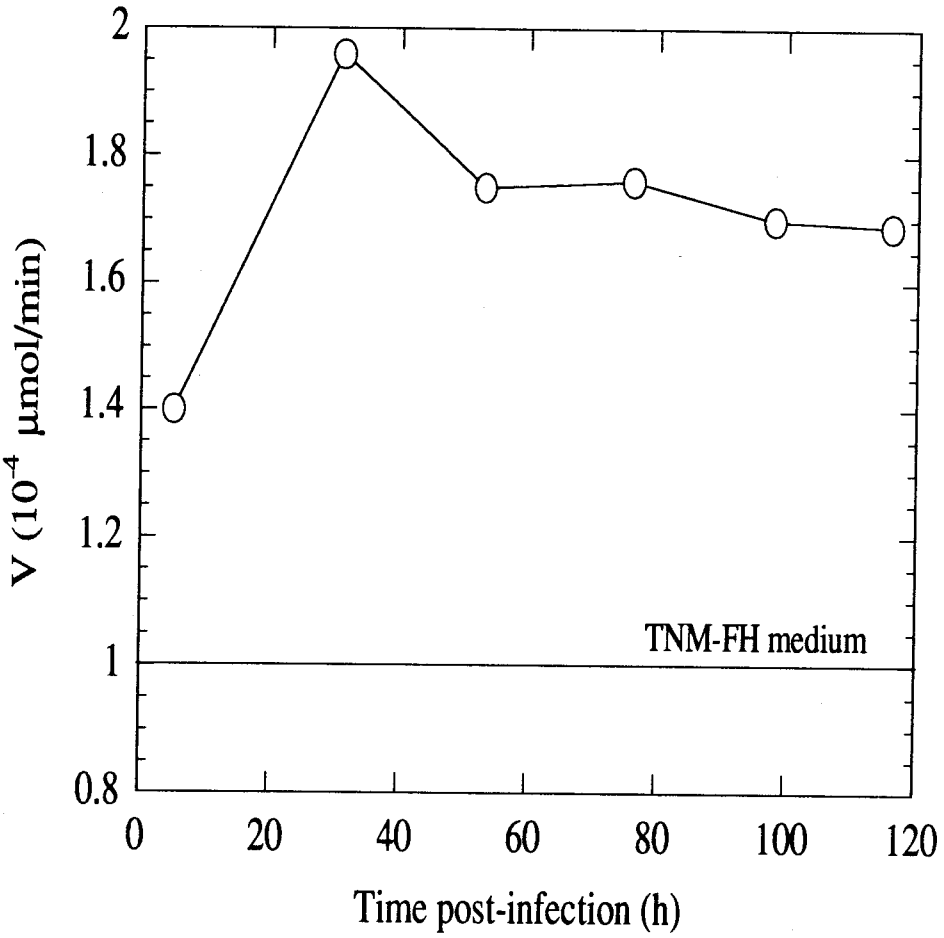

2
1.8
1.6
1.4
1.2
1
0.8
V (10^{-4} µmol/min)
TNM-FH medium
0
20
40
60
80
100
120
Time post-infection (h)

Figure 4.

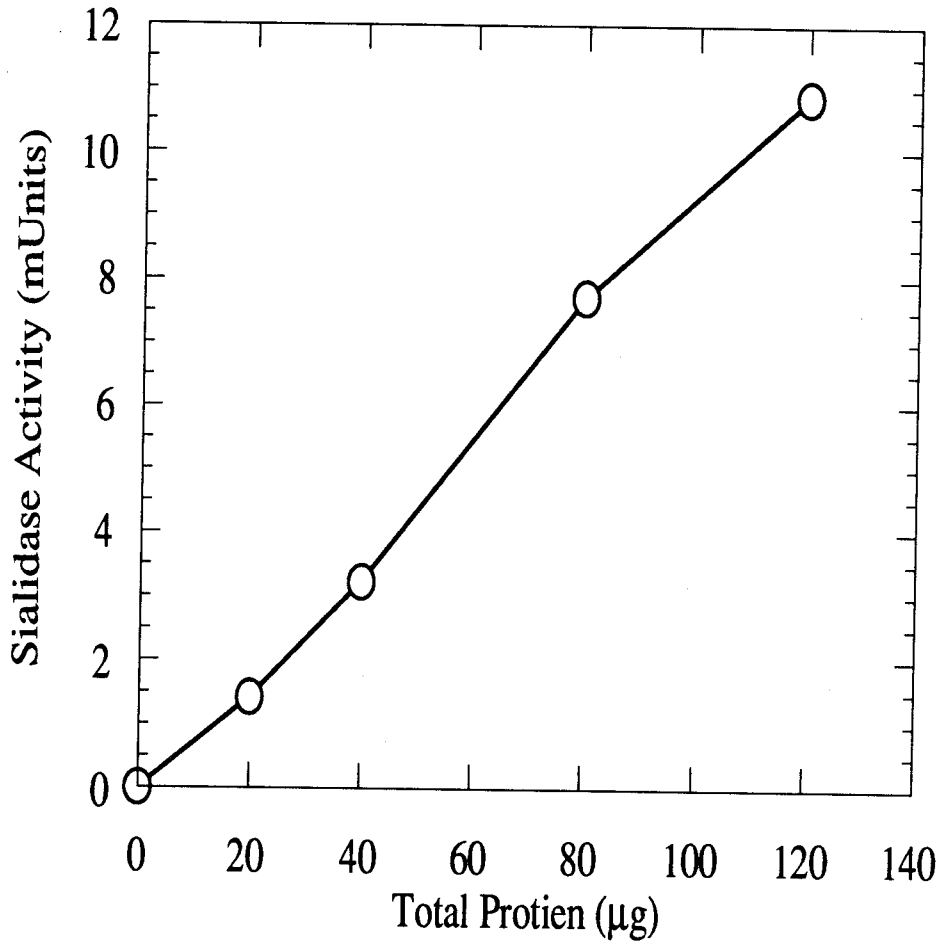

Sialidase Activity (mUnits)
12
10
8
6
4
2
0
0
20
40
60
80
100
120
140
Total Protien (µg)

Figure 5.

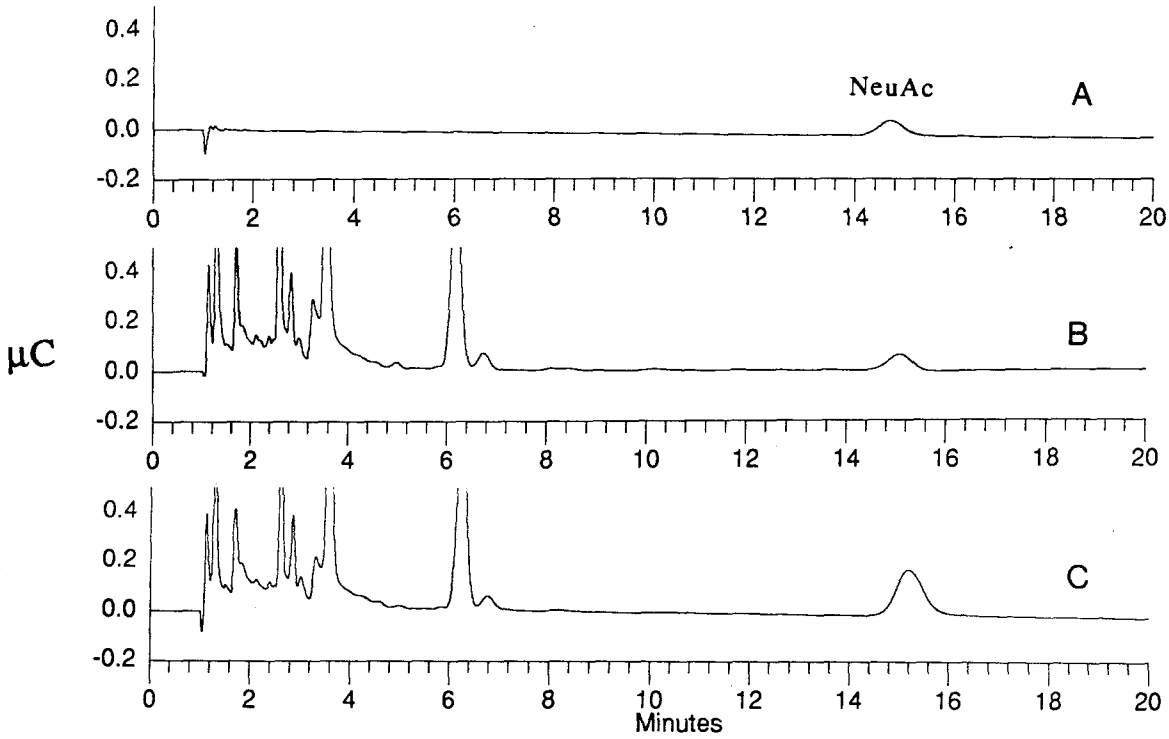
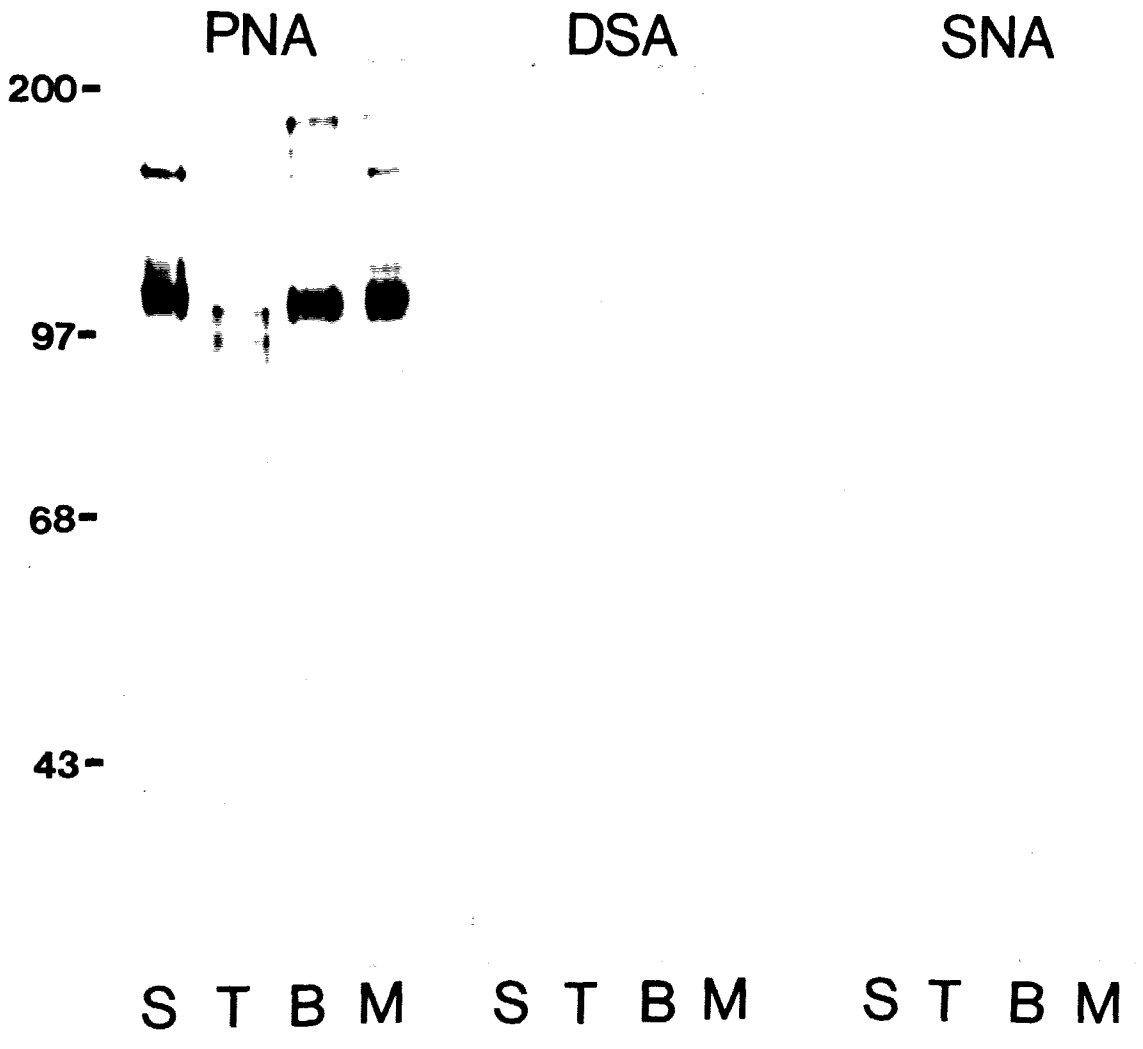


Figure 6.



CHAPTER VI

Developments in Altering Glycosylation in the Insect Cell - Baculovirus Expression System via the Expression of Mammalian Glycosyltransferases

6.1 Summary

A recombinant baculovirus (ST-AcMNPV) containing a rat liver ß-galactoside α2,6-sialyltransferase gene under the control of the polyhedrin promoter was constructed. *Spodoptera frugiperda* cells infected with this virus produced ß-galactoside α2,6-sialyltransferase DNA, appropriate-sized RNA transcripts, and the protein while mock-infected and wild-type AcMNPV-infected cells did not. Activity of α2,6-sialyltransferase synthesized in ST-AcMNPV-infected cultures was demonstrated by the *in vitro* sialylation of asialofetuin. Studies with FITC-labelled lectin specific for the NeuAcα2,6Gal linkage indicated that a variety of cellular and/or viral proteins associated with the cell surface that are not ordinarily sialylated were sialylated *in vivo* in ST-AcMNPV-infected cells but not in controls when cells were cultured in serum-containing medium. After adapting *S. frugiperda* cells to protein-free medium and infecting with ST-AcMNPV lectin-binding was greatly reduced. Further analysis indicated that recombinant virus-infected cells in serum-containing medium bind to serum glycoproteins. These serum glycoproteins were inadvertently measured, resulting in increased lectin-binding. Mammalian ß1,4-galactosyltransferase was also cloned into a baculovirus expression vector (GT-AcMNPV). Cultures grown in protein-free medium and infected with GT-AcMNPV alone or in combination with ST-AcMNPV did not demonstrate altered glycosylation by lectin-binding; more sensitive carbohydrate analysis is required to quantitate altered glycosylation from the introduction of these two enzymes.

6.2 Introduction

Several proteins that have been expressed using recombinant baculovirus, including tPA (Steiner *et al.*, 1988; Furlong *et al.*, 1988; Jarvis and Summers, 1989), interferon-α (Maeda *et al.*, 1985), interferon-ß2 (Matsuura *et al.*, 1989), human granulocyte-macrophage colony-stimulating factor (Chiou and Wu, 1990), and erythropoietin (Quelle *et al.*, 1989), are secreted glycoproteins in their native hosts. These proteins have been glycosylated and successfully secreted in the AcMNPV-*S. frugiperda* expression system. Infected insect cells are not so greatly compromised by baculovirus infection that the cells are incapable of accomplishing many of the post-translational processes characteristic of higher eucaryotic cells.

N- and O-linked glycosylation of numerous proteins have been demonstrated to occur in baculovirus-infected insect cells (see Fraser, 1989; Luckow and Summers, 1988 for review). N-linked glycosylation in infected *S. frugiperda* cells is reported to be different from that in mammalian systems. A number of glycoproteins produced in baculovirus-infected insect cells are similar to their authentic counterparts produced in mammalian cells in terms of immunogenicity, activity, and localization/secretion (Fraser, 1989; Luckow and Summers, 1988). However, size differences are often observed between glycoproteins produced in insect cells and mammalian cells; this difference has often been attributed to a fundamental difference in the glycosylation pattern. Until recently it was believed that high mannose oligosaccharides were the only type of oligosaccharides produced in insect cells (Butters *et al.*, 1981a; Butters *et al.*, 1981b; Ryan *et al.*, 1985). Butters *et al.* detected negligible levels of N-acetylglucosaminyl-, galactosyl- and sialyltransferases in the insect cell line *Aedes aegypti* (1981a).

Studies with tPA demonstrate that infected *Spodoptera frugiperda* cells are capable of producing an endoglycosidase-H-resistant form, an indication of the existence of complex oligosaccharides (Jarvis and Summers, 1989). In the same study it was

demonstrated that core glycosylation is necessary for secretion of tPA into the medium. Comparative studies with tPA and interferon-ß produced in infected insect cells using recombinant baculoviruses demonstrated that N-glycosylation may or may not be required for the transport of glycoproteins through the secretory pathway (Jarvis *et al.*, 1989). In contrast to tPA, interferon-ß was secreted to the extracellular space in the presence of tunicamycin, an inhibitor of N-linked glycosylation. It was also observed that terminal processing of the oligosaccharide is not required for transport for all of the proteins studied. Jarvis *et al.* (1990) observed two proteins that immunoprecipitated with nonglycosylated precursors of glycoproteins. It was suggested that these proteins are analogs to the mammalian immunoglobulin heavy chain binding/glucose regulated protein (BiP/GRP78) and that binding of these proteins to glycoprotein precursors acts to block the transport of nonglycosylated glycoproteins. Many forms of complex N-linked oligosaccharides were observed in a recent study of a recombinant AcMNPV-*S. frugiperda* system expressing a modified human plasminogen (Davidson and Castellino, 1990, 1991). The relative amounts of complex glycoforms observed were highly dependent on the time when medium replacements occurred during the cultivation. Glycoforms with complex glycosylation were present at very low levels (ca. 4%) at an early stage of the cultivation, yet were the predominant form late in infection; detection and identification of these glycoforms was done by high-pH anion-exchange chromatography (HPAEC). It has been reported that baculovirus infection leads to increased α−mannosidase activity, resulting in a shift from high-mannose oligosaccharides to complex-type oligosaccharides (Davidson *et al.*, 1991).

Inability of a given cell line to conduct particular reactions within the glycosylation reaction network may arise from absence of the necessary precursor or absence of the necessary substrate. If the necessary substrates are known to be present (or have not been shown conclusively to be absent), lack of the corresponding enzymatic activity may be the reason for the absence of the particular type of oligosaccharide

modification. Installation of that activity by cloning the gene encoding that activity into the cell line is a feasible and already demonstrated strategy for expanding the repertoire of glycosylation activities of a particular host. For example, Ernst *et al.* (1989) transfected mouse cells deficient in α1,2-fucosyltransferase (α1,2-FT) activity with human DNA containing the α1,2-FT gene. Transfected cells expressing active α1,2-FT enzyme were demonstrated to produce terminal Fucα1,2Gal linkages on surface glycoconjugates. Alteration of the terminal sequences of N-linked oligosaccharides in Chinese hamster ovary (CHO) cells was accomplished by expression of cloned ß-galactoside α2,6-sialyltransferase (Gal α2,6-ST) cDNA (Lee *et al.*, 1989). Wild-type CHO cells produce sugar chains terminating with only the NeuAcα2,3Gal linkage due to the absence of Gal α2,6-ST (Takeuchi *et al.*, 1988). By expression of Gal α2,6-ST in CHO cells, terminal sugar residues containing α2,6-NeuAc were produced. Gal α2,6-ST enzyme activity was demonstrated to be localized throughout the Golgi apparatus. Altered glycosylation phenotypes of CHO cells were also demonstrated by expressing cDNA encoding α1,3-galactosyltransferase (α1,3-GT; Smith *et al.*, 1990). Terminal α1,3-galactosyl residues were present on the recombinant strain expressing α1,3-GT but were not observed on the parental line. These examples demonstrate the ability to produce cultured cells capable of synthesizing carbohydrate groups with modified terminal residues through the introduction of cloned glycosyltransferases.

Gal α2,6-ST catalyzes the transfer of N-acetylneuraminic acid to the appropriate acceptor oligosaccharide, Galß1,4GlcNAc (Beyer *et al.*, 1981; Kornfeld and Kornfeld, 1985). One study has demonstrated the transfer of N-acetylneuraminic acid to terminal mannose residues in the presence of Gal α2,6-ST (van Pelt *et al.*, 1989). This enzyme is localized in different components of the Golgi apparatus, depending upon the cell type (Roth *et al.*, 1985; Roth *et al.*, 1986). Purification of rat liver Gal α2,6-ST to homogeneity (Weinstein *et al.*, 1982a) enabled detailed enzymatic characterization of the Gal α2,6-ST and its affinity for different acceptor glycoproteins (Weinstein *et al.*,

1982b). A cDNA clone of rat liver Gal α2,6-ST was obtained by Weinstein *et al.* (1987) allowing analysis of the protein primary structure. In addition, cloning of the cDNA provided the means for heterologous expression of Gal α2,6-ST in CHO cells discussed previously (Lee *et al.*, 1989).

On the premise of providing sialylation rarely observed in baculovirus expression systems, a recombinant baculovirus containing the rat liver ß-galactoside α2,6-sialyltransferase gene under the control of the polyhedrin promoter was constructed in this study. Infection of *Spodoptera frugiperda* cells with this virus resulted in the transient expression of active ß-galactoside α2,6-sialyltransferase as demonstrated by *in vitro* studies. It was observed that relatively late in infection, lectin specific for the NeuAcα2,6Gal linkage produced by α2,6-sialyltransferase bound to cellular and/or viral proteins associated with the cellular membrane when cells were cultivated in serum containing medium. Similar studies using cells cultivated in protein-free medium demonstrated that in the previous studies, serum proteins were sticking to cells late in infection, and these glycoproteins were responsible for a large proportion of the bound lectin. Lectin analysis using DSA, a lectin specific for the Galß1,4GlcNAc, demonstrated that the accepting oligosaccharide for α2,6-ST may be in relatively low quantity. In an effort to correct this deficiency, ß1,4-galactosyltransferase was expressed in insect cells by a baculovirus expression vector. Altered glycosylation from ß1,4-GT, alone or co-expressed with α2,6-ST was not observed with lectin-binding assays; more sensitive oligosaccharide analysis will be required to determine the exact effect of these heterologous enzymes in altering insect cell glycosylation.

6.3 Materials and Methods

Cells, wild-type virus, and infection. *Spodoptera frugiperda* IPL-Sf9 cells were obtained from the ATCC (No. CRL-1711; Rockville, MD). Cells grew as anchored monolayers in stationary T-25 flasks containing TNM-FH medium (Hink, 1970) with 10% FBS (Hyclone, Logan, Utah), 5000 units/L penicillin, and 5 mg/L streptomycin (Gibco BRL, Grand Island, NY) at 27°C. Wild-type AcMNPV virus was obtained from Dr. M. Summers (Texas A&M, College Station). Cultures were infected with a multiplicity of infection (MOI) of 10 during the mid-exponential growth phase. MOI is defined as the number of plaque-forming units per cell added at the time of infection. Cells were resuspended for sampling or transfer by gently flushing medium over the attached monolayer with a Pasteur pipette. Controls in all experiments consisted of mock-infected cells (cells treated identically during initial infection except that medium without virus was used at the time of infection) and wild-type AcMNPV-infected cells.

S. frugiperda cells were adapted to protein-free medium (Insect Xpress, Whittaker Bioproducts, Walkersville, MD). Culture and infection conditions for cultures grown in protein-free medium were identical to cultures grown in TNM-FH.

Plasmid construction, transfection, and purification. A nonfusion baculovirus transfer vector, pVL1392, was purchased from Invitrogen (San Diego, CA). The α2,6-sialyltransferase cDNA was supplied by Dr. J. C. Paulson (Cytel Corp., LaJolla, CA) in a Bluescript plasmid. The internal *EcoR*I site of the gene was previously mutated while maintaining the original amino acid sequence (Lee *et al.*, 1989). The 1.6 kb α2,6-sialyltransferase gene was removed from the Bluescript plasmid by *EcoR*I digestion and ligated into the *EcoR*I site of pVL1392; this vector is designated pVL1392-ST. Correct orientation of the gene was validated by subsequent digestions of the transfer vector with *EcoR*V, *Xho*I, and *BspM*I. Sf9 cells were co-transfected with 2 μg pVL1392-ST and 1 μg

wild-type viral DNA, using techniques described in detail elsewhere (Summers and Smith, 1987). After selecting several occlusion negative plaques, recombinant viruses were identified by DNA dot hybridization. Three rounds of plaque purification were completed after initial DNA dot hybridizations (Summers and Smith, 1987).

Similar techniques were used in the construction of a recombinant virus encoding mammalian ß1,4-galactosyltransferase (Chapter 4).

DNA and RNA isolation and hybridizations. Cells at 72 hours post-infection were lysed with 5M guanidinium thiocyanate in 0.05M Tris, 0.05 M EDTA, and 0.1M N-laurylsarcosine. After shearing the solution with a 20 Gauge hypodermic needle, 1.7 ml of the lysate were added to 2.1 ml CsTFA (Pharmacia LKB, Piscataway, NJ) containing 60 µg ethidium bromide. The samples were spun at 42 K RPM for 30 hours in a Beckman SW-50.1 rotor. DNA and RNA were removed from the gradients as separate entities. Precipitation of nucleic acids via isopropanol was followed by 3 washes with 70% ethanol. Samples were then dissolved in TE. Equal quantities of DNA were dot-blotted onto nitrocellulose. Hybridization of radiolabelled probes to immobilized nucleic acids was performed as described (Sambrook *et al.*, 1989). RNA was electrophoresed on a 1.2% agarose gel containing formaldehyde, transferred to nitrocellulose, and hybridized to radiolabelled probe as described elsewhere (Sambrook *et al.*, 1989). 0.24-9.5 kb RNA ladder was purchased from Gibco BRL.

The 1.6 kb cDNA encoding Gal α2,6-ST was labelled with ^{32}P-CTP by the method of random nucleotide priming (Feinberg and Vogelstein, 1983; 1984). After labelling and probe purification in Nensorb 20 cartridges (NEN, Reston, VA) specific activity of probe was 0.5-2 x 10^9 CPM/µg DNA. After hybridization at 42°C overnight, membranes were washed in 2X SSC at room temperature for 5 minutes, 2X SSC, 1% SDS at 60°C for 1 hour, and 0.1X SSC at room temperature for 30 minutes prior to exposure.

Radiolabelling and immunoprecipitation analysis. The radiolabelling and immunoprecipitation procedure has been described in detail elsewhere (Licari and Bailey, 1991). Briefly, cells at 63 hours post-infection were labelled with 50 μCi L-[^{35}S]-methionine (1037 Ci/mmol, ≈10 μCi/μl) for 20 minutes. After cell lysis, antibody to Gal α2,6-ST was allowed to bind for 12 hours. Removal of the antibody-antigen complex was by Protein A-Sepharose. After denaturation in SDS-PAGE buffer, immunoprecipitate was run on a 10% SDS-polyacrylamide gel at 20 mAMPS, soaked in Autofluor Image Enhancer (National Diagnostics, Manville, NJ) containing 10% glycerol for 1 hour, and dried prior to exposure at -70°C. Affinity-purified rabbit antibodies to α2,6-sialyltransferase were provided by Dr. J. C. Paulson (Cytel Corp., LaJolla, CA).

α2,6-sialyltransferase activity assay. The assay described by Gross *et al.* (1988, 1990) was used to assay for α2,6-sialyltransferase. CMP-9-fluoresceinyl-NeuAc, asialofetuin, and rat liver α2,6-sialyltransferase were purchased by Boehringer Mannheim (Indianapolis, IN). Following resuspension of cells, cultures to be assayed were centrifuged at 1500 g for 5 min. Supernatant was decanted and frozen at -20°C. The cell pellet was washed with 1 ml PBS and resuspended in 0.5 ml PBS. After cell lysis by sonication, cell extract was separated from cell debris by centrifugation in a microcentrifuge for 5 min. Samples containing 10 μl cell extract or culture supernatant, 2 μl CMP-9-fluoresceinyl-NeuAc (0.4 mg/ml), 25 μl asialofetuin (1 mg/ml), and 37 μl sample buffer (100 mM sodium cacodylate, pH 6, 0.2% (v/v) Triton X-100, and 2 mg/ml BSA) were incubated at 37°C for 3 hours. After incubation, the fluorescent-labelled fetuin was separated from the donor CMP-9-fluoresceinyl-NeuAc by the use of Centricon-10 microconcentrators, 10,000 MW cutoff (Amicon, Beverly, MA) spinning at 6,000 RPM in a Beckmen JA-14 rotor. Residual CMP-9-fluoresceinyl-NeuAc was removed by washing with 6 ml of PBS in the microconcentrator. Fluorescent labelled

fetuin was diluted to a final volume of 1 ml with PBS, excited at 490 nm; emission fluorescence was measured at 520 nm in a Shimadzu fluorometer. Emission readings were converted to activity units by comparison with rat liver α2,6-sialyltransferase. One unit is defined as the activity that transfers 1 μmol of N-acetylneuraminic acid from CMP-NeuAc to asialo-α_1-acid glycoprotein in 1 min at 37°C.

Fluorescence microscopy. Cells were labelled with FITC-SNA (EY Laboratories, San Mateo, CA) as described by Lee *et al.* (1989). 1 x 10^6 cells were resuspended, centrifuged at 250 g, washed twice with PBS, and resuspended in 250 μl of PBS. Cells were incubated with 40 μg/ml FITC-SNA at 30°C for 30 minutes. Fetuin in varying concentrations was added as a competitor in some studies. Unbound lectin was removed by washing once in 1 ml PBS and a subsequent wash in 1 ml distilled H_2O. Cells resuspended in 200 μl distilled H_2O were mounted with an equal volume of mounting solution (100 mM Tris, pH 9.0, 90% (v/v) glycerol, 0.1% paraphenylenediamine) and analyzed via phase-contrast and fluorescent microscopy (excitation at 488 nm) using a Zeiss confocal microscope.

Cells to be treated with sialidase were fixed for 20 minutes at room temperature in a 4% solution of paraformaldehyde in PBS at approximately 10^6 cells/ml. After fixing, cells were washed 3X in 1 ml PBS, and resuspended in 200 μl blocking buffer (3% BSA in PBS) for 45 minutes. 0.1 unit (10 μl) of *A. ureafaciens* sialidase and 27.5 μl 5X reaction buffer (500 mM sodium acetate) were added to half of the sample (approximately 10^6 cells in 100 μl of blocking buffer), while 10 μl of H_2O and 27.5 μl reaction buffer were added to the other half of the sample. The suspensions were incubated at 37°C for 18 hours. Cells were then washed 3X with 1 ml PBS per wash, incubated with FITC-SNA, washed, and mounted as described above.

6.4 Results

Expression of active α2,6-sialyltransferase. The transfer vector construction used for the insertion of the Gal α2,6-ST gene into the baculovirus genome is shown in Figure 1. The 1.6 kb gene was inserted downstream of the polyhedrin promoter in the *EcoR*I site of the transfer vector pVL1392. Correct orientation was demonstrated by a single digestion with *EcoR*V and a double digestion with *Xho*I and *BspM*I. Transcription and polyadenylation signals are provided by the flanking polyhedrin DNA. Translation start and stop sites are contained on the Gal α2,6-ST cDNA. Co-transfection of transfer vector DNA and wild-type baculovirus DNA produced occlusion-negative virus. Prior to plaque purification, cellular DNA from cultures infected with several occlusion-negative plaques were analyzed by dot-blot techniques. Controls consisted of total DNA from mock-infected and wild-type AcMNPV-infected cells. Several positive clones were then subjected to three rounds of plaque purification each. Positive clones were amplified in T-25 flasks. One clone, designated ST-AcMNPV, was used for further studies.

Figure 2 is a dot blot of DNA from mock-infected, wild-type AcMNPV-infected, and ST-AcMNPV-infected cells. At 72 hours post-infection total DNA was harvested; approximately 2 μg of each DNA sample were blotted to nitrocellulose and hybridized to radiolabelled Gal α2,6-ST DNA as described in the Materials and Methods Section. Controls consisted of 1 μg of Gal α2,6-ST DNA and transfer vector pVL1392 DNA (lacking the Gal α2,6-ST gene). The ST-AcMNPV virus sample is a clear positive, indicating that the Gal α2,6-ST gene was successfully transferred to the baculovirus genome. The mock-infected and wild-type AcMNPV-infected cells, as well as the transfer vector DNA, show no hybridization to the probe.

At 72 hours post-infection total RNA was isolated from cells infected with ST-AcMNPV and the appropriate controls. After separation of equal quantities of the RNA on a formaldehyde gel and transfer to nitrocellulose, Northern hybridization indicated a

transcript of approximately 2.3 kb in the lane corresponding to cells infected with the ST-AcMNPV virus; no hybridization occurred with the RNA from mock-infected and wild-type AcMNPV-infected cells. The size of the observed transcript is in general agreement with published literature for Gal α2,6-ST transcription in CHO cells (Lee *et al.*, 1989).

Mock-infected, wild-type AcMNPV-infected, and ST-AcMNPV-infected cells were radiolabelled for 20 minutes with L-[^{35}S]-methionine. After immunoprecipitation with antibodies to α2,6-sialyltransferase, samples were run on a 10% SDS-polyacrylamide gel. The quantity of sample loaded was based on normalization by the total number of cells that were radiolabelled. This study demonstrated the existence of a protein with an approximate molecular weight of 46 kDa present only in the lane corresponding to ST-AcMNPV-infected cells (Figure 4). This molecular weight corresponds to the molecular weight observed for sialyltransferase produced in CHO cells (Lee *et al.*, 1989) and that found in liver hepatocytes (Roth *et al.*, 1985; Weinstein *et al.*, 1987). Several smaller proteins were precipitated in the lane from ST-AcMNPV-infected cells. These proteins are most likely fragments of ß-galactoside α2,6-sialyltransferase; they may be degradation products or may result from premature transcription or translation termination (Licari and Bailey, 1992). Some proteins were precipitated with the Gal α2,6-ST antibody in the mock-infected and wild-type AcMNPV-infected samples; however, these proteins are present at much lower levels than proteins precipitated from the ST-AcMNPV-infected cells. Specifically, a band with the approximate molecular weight of Gal α2,6-ST is present in the lane corresponding to mock-infected cells. Since the antibodies used in this experiment were affinity-purified, structural homology to rat liver Gal α2,6-ST is suggested. Prior to immunoprecipitation, cell lysate was "pre-cleared" with Protein A-Sepharose to remove any extraneous binding to the precipitating matrix.

Mock-infected, wild-type AcMNPV-infected, and ST-AcMNPV-infected cultures were analyzed for α2,6-sialyltransferase activity. Since degradation of ß-galactosidase

expressed in the baculovirus-*S. frugiperda* system has been demonstrated to be insignificant (Licari and Bailey, 1991), samples were taken after cell lysis to ensure maximum heterologous protein accumulation. Cell pellets resuspended in PBS were sonicated and then centrifuged. The cell pellet fraction did not demonstrate any noticeable activity difference between samples and controls (PBS). However, α2,6-sialyltransferase activity well above the baseline, as defined by fresh medium, was observed for the supernatant from the ST-AcMNPV-infected cells (Figure 5). The supernatant from ST-AcMNPV-infected cells was approximately 0.8 to 1.0 units/l compared to values on the order of 0.1 units/l for mock-infected cells, wild-type AcMNPV-infected cells, and medium. No significant differences between mock-infected cells, wild-type AcMNPV-infected cells, and the fresh medium were observed. This assay proved inadequate for the assay of intracellular α2,6-sialyltransferase on unlysed cells; a limitation believed to result from both the degradation of substrate and the endogenous sialidase associated with cells (Chapter 5).

SNA binding to host cell proteins. The existence of N-acetylneuraminic acid in *Spodoptera frugiperda* cells was demonstrated in Chapter 5 (Figure 5). The lectin *Sambucus nigra* agglutinin (SNA) is known to be highly selective for binding to NeuAcα2,6Gal (Broekaert *et al.*, 1984; Shibuya *et al.*, 1987a, 1987b). Figure 6 shows confocal microscope images of *S. frugiperda* cells cultured in TNM-FH medium, which are mock-infected, infected with wild-type virus, and infected with the recombinant Gal α2,6-ST virus. After exposure of each sample to SNA conjugated with the fluorochrome fluorescein (FITC-SNA), almost all cells in the culture infected with Gal α2,6-ST recombinant virus exhibit intense fluorescence indicative of a high density of bound SNA on the cell, while the controls are almost completely negative. The addition of fetuin as a competitor was also added to ST-AcMNPV-infected cells during the FITC-SNA assay. At high concentrations of fetuin (1-2.5 mg/ml) binding of FITC-SNA to ST-AcMNPV-

infected cells was decreased, indicating that the lectin is not irreversibly cross-reacting with an unidentified moiety (data not shown).

In order to determine if the lectin was binding to a moiety other than NeuAc, cells were fixed with paraformaldehyde, blocked in a solution of 3% BSA, and treated with sialidase for 18 hours prior to incubation with FITC-SNA. Figure 7 demonstrates that cells treated with sialidase for this period do not bind the conjugated lectin, whereas cells that are not treated bind the lectin strongly.

Degree of SNA binding is affected by medium. Cells cultured in protein-free medium and infected with ST-AcMNPV did not bind SNA to the degree that was observed for ST-AcMNPV-infected cells cultured in serum-containing medium. This discrepancy in behavior between cells cultured in TNM-FH and cells cultured in protein-free medium suggested that either the serum-containing medium provides an excess of a particular component that facilitates the sialylation reaction or that serum proteins are binding to cells late in infection, thus amplifying SNA binding. Cells cultured and infected in protein-free medium were harvested very late in infection (8 days post-infection, viability = 0%) and placed in TNM-FH medium for 4 hours. These cells were then washed in PBS and incubated with FITC-SNA as described in the Materials and Methods Section. Cells that were placed in TNM-FH exhibited stronger SNA binding than cells that were not placed in TNM-FH (data not shown). Since the viability of cells was 0%, it appears that glycoproteins from the medium adhere to cells. Furthermore, this abundant SNA lectin-binding attributed to serum proteins was observed for cells cultured in serum-containing medium and infected with different recombinant virions that do not encode α2,6-ST (e.g., 360-lac AcMNPV previously discussed).

Expression of mammalian ß1,4-galactosyltransferase. Mammalian ß1,4-galactosyltransferase (ß1,4-GT) was expressed using the baculovirus expression system

(cloning and analysis techniques were analogous to those presented for the ST-AcMNPV). DNA, RNA, and activity analysis from cultures infected with GT-AcMNPV indicated that infected cultures produce active ß1,4-GT. DSA, a lectin specific for the Galß1,4GlcNAc linkage product of the ß1,4-GT enzyme, was used to assay for an increase in Galß1,4GlcNAc linkages. Cultures grown in protein-free medium and infected with GT-AcMNPV did not demonstrate a binding difference to DSA when compared to uninfected cells. Furthermore, cultures grown in protein-free medium and co-infected with GT-AcMNPV and ST-AcMNPV did not demonstrate a difference in insect cell glycosylation detectable by DSA or SNA lectin-binding when compared with cultures infected with only ST-AcMNPV.

6.5 Discussion

Successful insertion and expression of the Gal α2,6-ST gene into the baculovirus genome was proven by DNA and RNA hybridization, radiolabelling-immunoprecipitation studies with the protein product, and *in vitro* activity studies. FITC-labelled SNA was demonstrated to bind to ST-AcMNPV-infected cells late in infection when the cells were cultured in serum-containing medium. Studies with cells cultured in protein-free medium demonstrated that previously observed SNA binding was largely due to serum proteins adhering to infected cells and not entirely due to altered insect cell glycosylation. Lectin analysis of total cell lysate with DSA, a lectin specific for Galß1,4GlcNAc, demonstrated that the accepting oligosaccharide for α2,6-ST may be present in limiting quantities (Chapter 5). ß1,4-GT was cloned and expressed in insect cells in hopes of amplifying acceptor oligosaccharides to α2,6-ST. Expression of ß1,4-GT, alone or in combination with α2,6-ST, did not result in any visible differences from cells infected with only ST-AcMNPV virus. To date, analysis of insect cell cultures infected with these virions is severely limited by the lectin-based techniques used to detect altered glycosylation. Other techniques, e.g., HPAEC, should prove fruitful in discerning differences in insect cell glycosylation resulting from the expression of these two heterologous genes.

The NeuAcα2,6Gal linkage made by α2,6-ST and the Galß1,4GlcNAc made by ß1,4-GT have been detected only in a plasminogen variant expressed in *S. frugiperda* cells infected with recombinant baculovirus expressing a plasminogen variant (Davidson and Castellino, 1991). It was the goal of this work to synthesize the NeuAcα2,6Galß1,4GlcNAc linkage under more general conditions by the overexpression of Gal α2,6-ST and ß1,4-GT. It is interesting to note that no hybridization to the Gal α2,6-ST DNA probe was observed for DNA or RNA from uninfected or wild-type baculovirus-infected cells, yet a cellular protein demonstrated selective binding of the

affinity-purified antibodies to Gal α2,6-ST. Whether the demonstrated structural homology of this protein is indicative of any residual sialyltransferase activity of the host cell line was not explicitly addressed in this work. DNA and RNA hybridization to the ß1,4-GT DNA probe demonstrated no significant homology under low stringency conditions (Chapter 4, Figure 10). Antibodies to ß1,4-GT were not available to perform similar protein-antibody binding studies.

Other researchers who have expressed cloned transferases in heterologous systems, and indeed practitioners of metabolic engineering in more general contexts, have carefully articulated the difficulty of proving conclusively cause-and-effect relationships between the introduction of a particular gene and the manifestation of a corresponding activity. It is difficult if not impossible to disprove categorically that the particular genetic engineering manipulation that was undertaken did not cause some change in the system, which resulted in expression of an endogenous activity akin to that which was inserted by genetic engineering. Further, expression of other endogenous activities coupled to the particular process of interest may also be influenced by the genetic engineering operation. Therefore, consistent use of controls is important to minimize the possibility of fortuitous results that arise to some extent independently of the particular genetic manipulation that was undertaken. As previously discussed, an important control in studies with baculovirus infection is insect cells infected with a recombinant virus encoding a gene other than that of interest. Lectin-binding differences observed here for wild-type and recombinant baculovirus infection is believed to originate from a cellular morphology difference late in infection. Late in infection, wild-type baculovirus-infected cells disseminate because of polyhedra accumulation, whereas recombinant AcMNPV-infected cells tend to condense into small particles, thus providing a surface for lectin-binding that is visible through microscopy. This work also demonstrates that the importance of other factors (e.g, the medium) must be investigated when studying altered glycosylation. Results presented here demonstrate the benefits of protein-free medium as

well as the necessity for developing more sophisticated techniques for carbohydrate analysis.

6.6 Acknowledgments

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6.7 Nomenclature

Gal α2,6-ST	ß-galactoside α2,6-sialyltransferase
SNA	*Sambucus nigra* agglutinin
FITC-SNA	fluorescein isothiocyanate-SNA
NeuAc	N-acetylneuraminic acid
Gal	galactose
GlcNAc	N-acetylglucosamine
R	oligosaccharide chain
Sf	*Spodoptera frugiperda*
AcMNPV	*Autographa californica* nuclear polyhedrosis virus
ST-AcMNPV	recombinant virus containing the Gal α2,6-ST gene
MOI	multiplicity of infection
CHO cells	Chinese hamster ovary cells
α1,2-FT	α1,2-fucosyltransferase
α1,3-GT	α1,3-galactosyltransferase
tPA	tissue plasminogen activator
HPAEC	high pH anion exchange chromatography
PBS	phosphate buffered saline
SSC	sodium chloride, sodium citrate buffer
SDS	sodium dodecyl sulfate
TE	tris-ethylenediaminetetraacetic acid solution

6.8 References

Beyer, T. A., Saddler, J. E., Rearick, J. I., Paulson, J. C., and Hill, R. L. (1981) Glycosyltransferases and their use in assessing oligosaccharide structure and structure-function relationships. *Adv. Enzymol.*, 52, 23-175.

Broekaert, W. F., Nsinba-Lubaki, M., Peeters, B., and Peumana, W. J. (1984) A lectin from elderberry (*Sambucus nigra* L.) bark. *Biochem. J.*, 221, 163-169.

Butters, T. D., Hughes, R. C., and Vischer, P. (1981a) Steps in the biosynthesis of mosquito cell membrane glycoproteins and the effects of tunicamycin. *Biochim. et Biophys. Acta*, 640, 672-686.

Butters, T. D., and Hughes, R. C. (1981b) Isolation and characterization of mosquito cell membrane glycoproteins. *Biochim. et Biophys. Acta*, 640, 655-671.

Chiou, C. J., and Wu, M. C. (1990) Expression of human granulocyte-macrophage colony-stimulating factor gene in insect cells by a baculovirus vector. *FEBS Letters*, 259, 249-253.

Davidson, D. J., and Castellino, F. J. (1990) Oligosaccharide processing in the expression of human plasminogen cDNA by lepidopteran Insect (*Spodoptera frugiperda*) cells. *Biochem.*, 29, 5584-5590.

Davidson, D. J., Bretthauer, R. K., and Castellino, F. J. (1991) α-mannosidase-catalyzed trimming of high-mannose glycans in noninfected and baculovirus-infected *Spodoptera frugiperda* cells. *Biochem.*, 30, 9811-9815.

Davidson, D. J., and Castellino, F. J. (1991) Asparagine-linked oligosaccharide processing in lepidopteran insect cells. Temporal dependence of the nature of the oligosaccharides assembled on asparigine-289 of recombinant human plasminogen produced in baculovirus vector infected *Spodoptera frugiperda* (IPLB-SF-21AE) cells. *Biochem.*, 30, 6167-6174.

Ernst, L. K., Rajan, V. P., Larsen, R. D., Ruff, M. M., and Lowe, J. B. (1989) Stable expression of blood group H determinants and GDP-L-fucose:ß-D-galactose 2-α-L-fucosyltransferase in mouse cells after transfection with human DNA. *J. Biol. Chem.*, 264, 3436-3447.

Feinberg, A. P., and Vogelstein, B. (1983) A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, 132, 6-13.

Feinberg, A. P., and Vogelstein, B. (1984) Addendum: a technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.*, 137, 266-267.

Fraser, M. J. (1989) Expression of eucaryotic genes in insect cell cultures. *In Vitro Cell. & Devel. Biol.*, 25, 225-235.

Furlong, A. M., Thomsen, D. R., Marotti, K. R., Post, L. E., and Sharma, S. K. (1988) Active human tissue plasminogen activator secreted from insect cells using a baculovirus vector. *Biotech. and Appl. Biochem.*, 10, 454-464.

Gross, H. J., and Brossmer, R. (1988) Enzymatic introduction of a fluorescent sialic acid into oligosaccharide. *Eur. J. Biochem.*, 177, 583-589.

Gross, H. J., Sticher, U., and Brossmer, R. (1990) A highly sensitive fluorometric assay for sialyltransferase activity using CMP-9-fluoresceinyl-NeuAc as donor. *Anal. Biochem.*, 186, 127-134.

Hink, W. F. (1970) Established insect cell line from the cabbage looper *Trichoplusia ni*. *Nature*, 226, 466-467.

Jarvis, D. L., Fleming, J. G. W., Kovacs, G. R., Summers, M. D., and Guarino, L. A. (1990) Use of early baculovirus promoters for continuous expression and efficient processing of foreign gene products in stably transformed lepidopteran cells. *Bio/Technol.*, 8, 950-955.

Jarvis, D. L., Oker Bloom, C., and Summers, M. D. (1989) Role of glycosylation in the transport of recombinant glycoproteins through the secretory pathway of lepidopteran insect cells. *J. of Cell. Biochem.*, 42, 181-191.

Jarvis, D. L., and Summers, M. D. (1989). Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected insect cells. *Mol. Cell. Biol.*, 9, 214-223.

Kornfeld, R., and Kornfeld, S. (1985) Assembly of asparagine-linked oligosaccharides. *Ann. Rev. Biochem.*, 54, 631-664.

Lee, E. U., Roth, J., and Paulson, J. C. (1989) Alteration of terminal glycosylation sequences on N-linked oligosaccharides of Chinese hamster ovary cells by expression of ß-galactoside α2,6-sialyltransferase. *J. Biol. Chem.*, 264, 13848-13855.

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

Licari, P., and Bailey, J. E. (1992) Production of a discrete, heterogeneous population of ß-galactosidase polypeptides using baculovirus expression vectors. *Biotech. and Bioeng.*, 39, 932-944.

Luckow, V. A., and Summers, M. D. (1988) Trends in the development of baculovirus expression vectors. *Bio/Technol.*, 6, 47-55.

Maeda, S., Kawai, T., Obinata, M., Fujiwara, H., Horiuchi, T., Saeki, I., and Furusawa, M. (1985) Production of human α–interferon in silkworm using a baculovirus vector. *Nature*, 315, 592-593.

Matsuura, Y, Tatsumi, M., Enami, K., Morikawa, S., Yamazaki, S., and Kohase, M. (1989) Expression of IL-6/IFN-ß2 in a baculovirus system and its biological function. *Annals. N.Y. Acad. Sci.*, 557, 122-129.

Meyers, R. W., Lee, R. T., Lee, Y. C., Thomas, G. H., Reynolds, L. W., and Uchida, Y. (1980) The synthesis of 4-methylumbelliferyl α–ketoside of N-acetylneuraminic acid and its use in a fluorometric assay for sialidase. *Anal. Biochem.* 101, 166-174.

Quelle, F. W., Casiake, L. F., Burkert, R. E., and Wojchowski, D. M. (1989) High-level expression and purification of a recombinant human erythropoietin produced using a baculovirus vector. *Blood*, 74, 652-657.

Roth, J., Taatjes, D. J., Lucocq, J. M., Weistein, J., and Paulson, J. C. (1985) Demonstration of an extensive trans-tubular network continuous with the Golgi apparatus stack that may function in glycosylation. *Cell*, 43, 287-295.

Roth, J., Taatjes, D. J., Weinstein, J., Paulson, J. C., Greenwell, P., Watkins, W. M. (1986) Differential subcompartmentation of terminal glycosylation in the Golgi apparatus of intestinal absorptive and goblet cells. *J. Biol. Chem.*, 261, 14307-14312.

Ryan, R. O., Anderson, D. R., Grimes, W. J., and Law, J. H. (1985) Arylphorin from *Manduca sexta*: carbohydrate structure and immunological studies. Arch. Biochem. and Biophys., 243, 115-124.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning*. Cold Spring Harbor Laboratory Press, NY.

Shibuya, N., Goldstein, I. J., Broekaert, W. F., Nsimba-Lubaki, M., and Peeters, B. (1987a) The elderberry (*Sambucus nigra* L) bark lectin recognizes the Neu5Ac(α–2-6)Gal GalNAc sequence. *J. Biol. Chem.*, 262, 1596-1601.

Shibuya, N., Goldstein, I. J., Broekaert, W. F., Nsimba-Lubaki, M., Peeters, B., and Peumans, W. J. (1987b) Fractionation of sialylated oligosaccharides, glycopeptides, and glycoproteins on immobilized elderberry (*Sambucus nigra* L) bark lectin. *Arch. Biochem. Biophys.*, 254, 1-8.

Smith, D. F., Larsen, R. D.,Mattox, S., Lowe, J. B., and Cummings, R. D. (1990) Transfer and expression of a murine UDP-gal:ß-D-gal-α1,3-galactosyltransferase gene in transfected Chinese hamster ovary cells. *J. Biol. Chem.*, 265, 6225-6234.

Steiner, H., Pohl, S., Gunne, H., Hellers, M., Elhammer, A., and Hansson, L. (1988) Human tissue-type plasminogen activator synthesized by using a baculovirus vector in insect cells compared with human plasminogen activator produced in mouse cells. *Gene*, 73, 449-457.

Summers, M. D., and Smith, G. E. (1987) *A manual of methods for baculovirus vectors and insect cell culture procedure.* Texas Agricultural Experiment Station Bulletin No. 1555.

Takeuchi, M., Takasaki, S., Miyazaki, H., Kato, T., Hoshi, S., Kochibe, N., and Kobata, A. (1988) Comparative study of the asparagine-linked sugar chains of human erythropoietins purified from urine and the culture medium of recombinant Chinese hamster ovary cells. *J. Biol. Chem.*, 263, 3657-3663.

van Pelt, J., Dorland, L., Duran, M., Hokke, C. H., Kamerling, J. P., and Vliegenthart, F. G. (1989) Transfer of sialic acid in α2,-6 linkage to mannose in Manß1-4GlcNAc and Manß1-4GlcNAcß1-4GlcNAc by the action of Galß1-4GlcNAca2-6-sialyltransferase. *FEBS Letters*, 256, 179-184.

Vlak, J. M., and Rohrmann, G. F. (1985) The nature of polyhedrin. In K. Sherman and K. Maramorosc (eds.) P. 489-542. *Viral Insecticides for Biological Control.* Academic Press, NY.

Weinstein, J., de Souza-e-Silva, U., and Paulson, J. C. (1982a) Purification of a Galß1-4GlcNAcα2-6sialyltransferase and a Galß1-3(4)GlcNAcα2-3sialyltransferase to homogeneity from rat liver. *J. Biol. Chem.*, 257, 13835-13845.

Weinstein, J., de Souza-e-Silva, U., and Paulson, J. C. (1982b) Sialylation of glycoprotein oligosaccharides N-linked to asparagine. *J. Biol. Chem.*, 257, 13845-13853.

Weinstein, J., Lee, E. U., McEntee, K., Lai, P. H., and Paulson, J. C. (1987) Primary structure of ß-galactoside α2,6-sialyltransferase. *J. Biol. Chem.*, 262, 17735-17743.

6.9 Figures

1. Baculovirus transfer vector containing the ß-galactoside α2,6-ST gene. The 1.6 kb gene encoding Gal α2,6-ST (st, unfilled region) was inserted into the *EcoR*I site of the polylinker contained on the pVL1392 transfer vector, downstream of the polyhedrin promoter. Sequences in gray (ph) are polyhedrin DNA containing transcription start and polyadenylation signals. Single lines indicate native baculovirus sequences needed for recombination. The black region indicates pUC8 DNA containing the *bla* gene for ampicillin resistance.

2. DNA dot blot indicating presence of the Gal α2,6-ST gene in ST-AcMNPV-infected cells. DNA from mock-infected cells (position 2A), wild-type AcMNPV-infected cells (3A), ST-AcMNPV-infected cells (4A) was probed with radiolabelled Gal α2,6-ST DNA. Hybridization of the probe occurred only with ST-AcMNPV-infected cells. Position 1B contains Gal α2,6-ST DNA as a positive control and position 1A contains pVL1392 DNA without the Gal α2,6-ST gene.

3. The presence of Gal α2,6-ST transcripts in ST-AcMNPV-infected cells. Total RNA from ST-AcMNPV-infected cells (lane 1), wild-type AcMNPV-infected cells (lane 2), and mock-infected cells (lane 3) was run on a 1.2% agarose gel, blotted, and subjected to hybridization with radiolabelled Gal α2,6-ST DNA. The only positive band indicates an approximate 2.3 kb transcript present in lane 1, corresponding to RNA from cells infected with ST-AcMNPV.

4. Immunoprecipitation of radiolabelled Gal α2,6-ST from ST-AcMNPV-infected cells. Mock-infected cells (lane 1), wild-type AcMNPV-infected cells (lane 2), and ST-AcMNPV-infected cells (lane 3) were labelled with L-[^{35}S]-methionine for 20 minutes

and subjected to immunoprecipitation with affinity-purified antibodies to Gal α2,6-ST. The band at approximately 46 kDa in lane 3 corresponds to Gal α2,6-ST from cells infected with ST-AcMNPV.

5. Sialyltransferase activity in the supernatant of Sf9 cells infected with ST-AcMNPV. Gal α2,6-sialyltransferase activity (units/l of supernatant) is depicted for mock-infected cells (Mock), wild-type AcMNPV-infected cells (WT), ST-AcMNPV-infected cells (ST), and for TNM-FH medium (TNMFH). Infected cultures were allowed to lyse prior to sampling.

6. Binding of FITC-SNA to Sf9 cells infected with ST-AcMNPV. Panels A, C, and E show phase-contrast microscopy while B, D, and F show the corresponding views in florescence microscopy for cells incubated with FITC labelled SNA. Mock-infected cells (panels A and B) and wild-type AcMNPV-infected cells (panels C and D) show no fluorescence, while cells infected with the ST-AcMNPV virus (panels E and F) are fluorescent. Cells were infected with an MOI of 10 and analyzed at 87 hours post-infection. All samples are at the same magnification; a size bar is located in the lower right-hand corner of each panel. Panel C is darker than A and E because the polyhedra produced in wild-type baculovirus-infected cells are more refractive, and the conditions to view these cells differ from other, less refractive cells.

7. Binding of FITC-SNA to ST-AcMNPV-infected cells is eliminated by pre-treatment of cells with sialidase. Panels A and C show phase-contrast microscopy, while B and D show the corresponding views in fluorescence microscopy of the same samples (A and C, respectively) for ST-AcMNPV-infected cells incubated with FITC labelled SNA. Control cells not treated with sialidase (panels A and B) are fluorescent, while cells treated with sialidase (panels C and D) are not fluorescent. Cells were infected

with an MOI of 10 and analyzed at 96 hours post-infection. All samples are at the same magnification; a size bar is located in the lower, right-hand corner of each panel.

Figure 1.

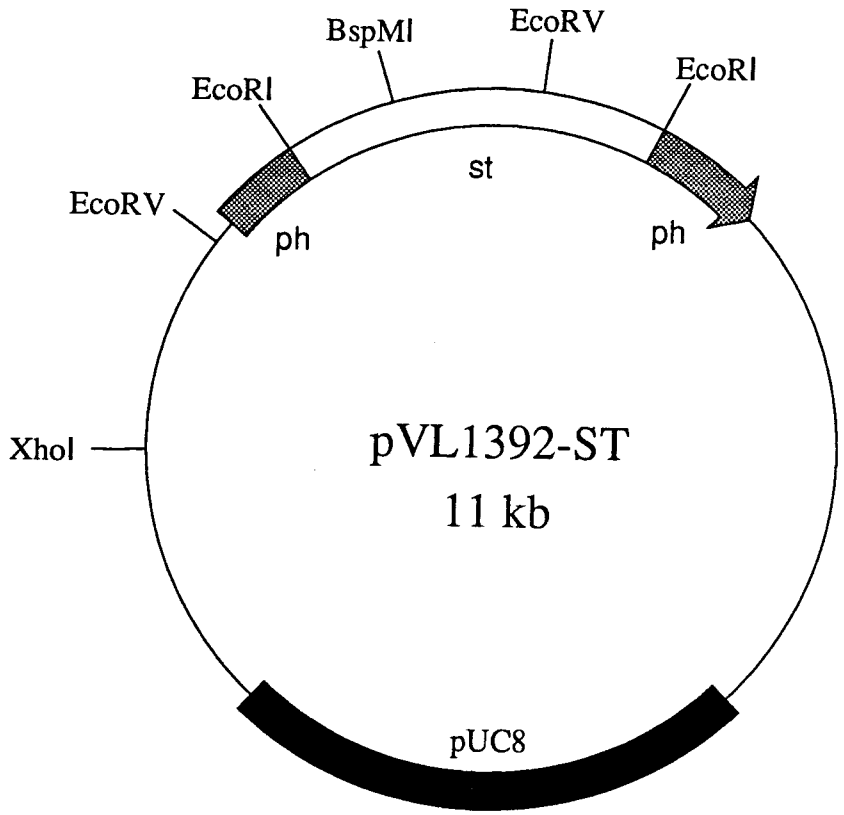

BspMI
EcoRV
EcoRI
EcoRI
st
EcoRV
ph
ph
XhoI
pVL1392-ST
11 kb
pUC8

Figure 2.

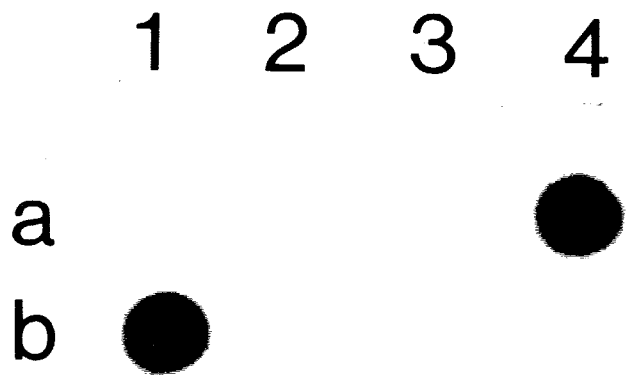


Figure 3.

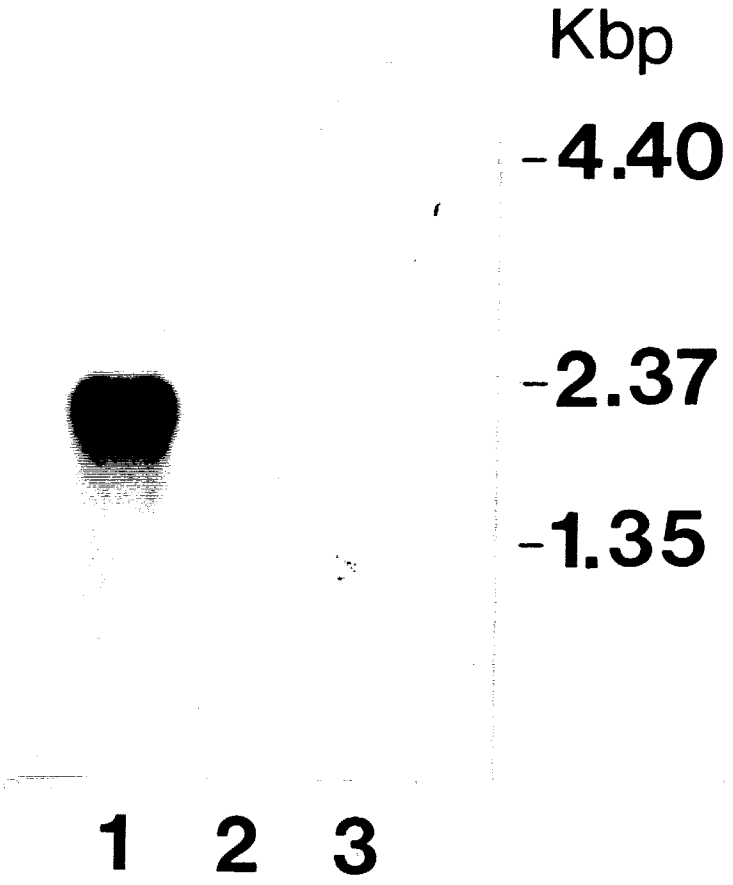


Figure 4.

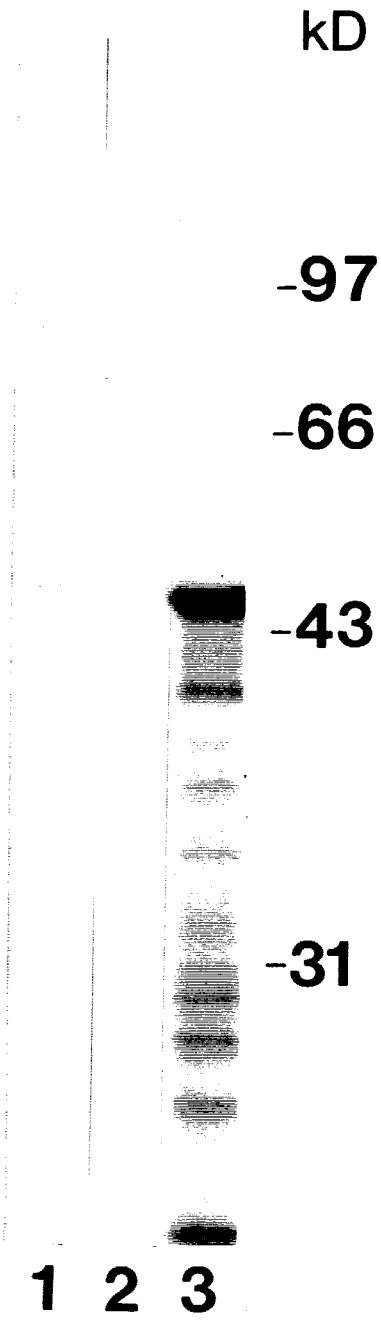


Figure 5.

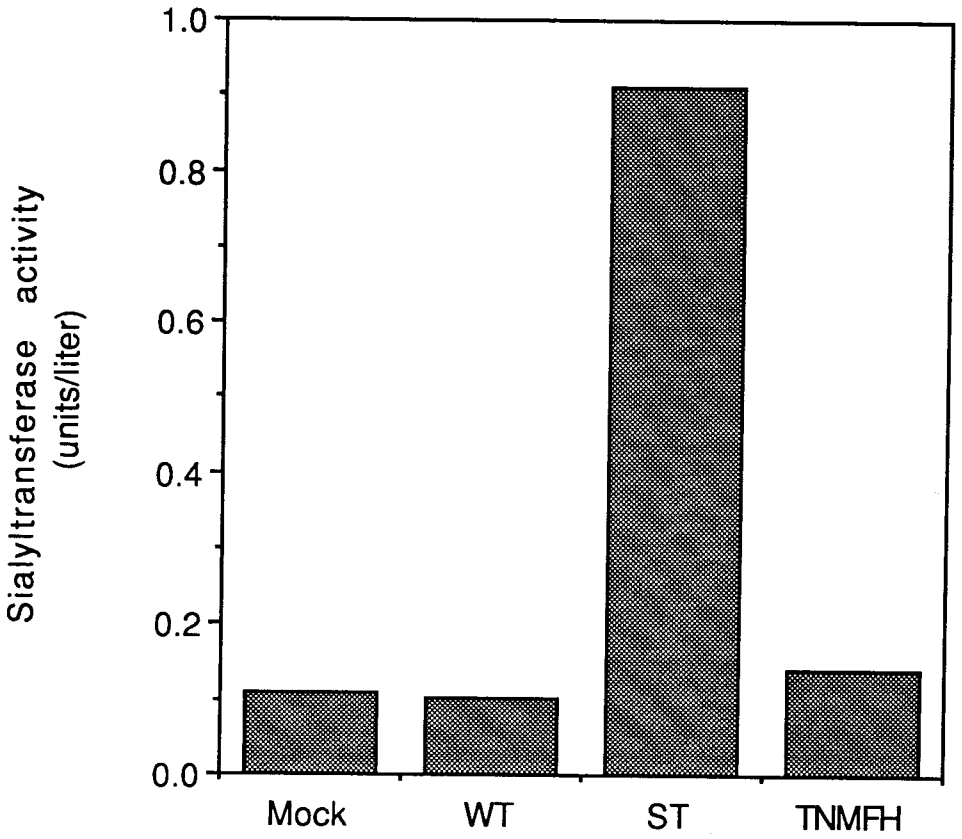

Sialyltransferase activity
(units/liter)
1.0
0.8
0.6
0.4
0.2
0.0
Mock
WT
ST
TNMFH

Figure 6.

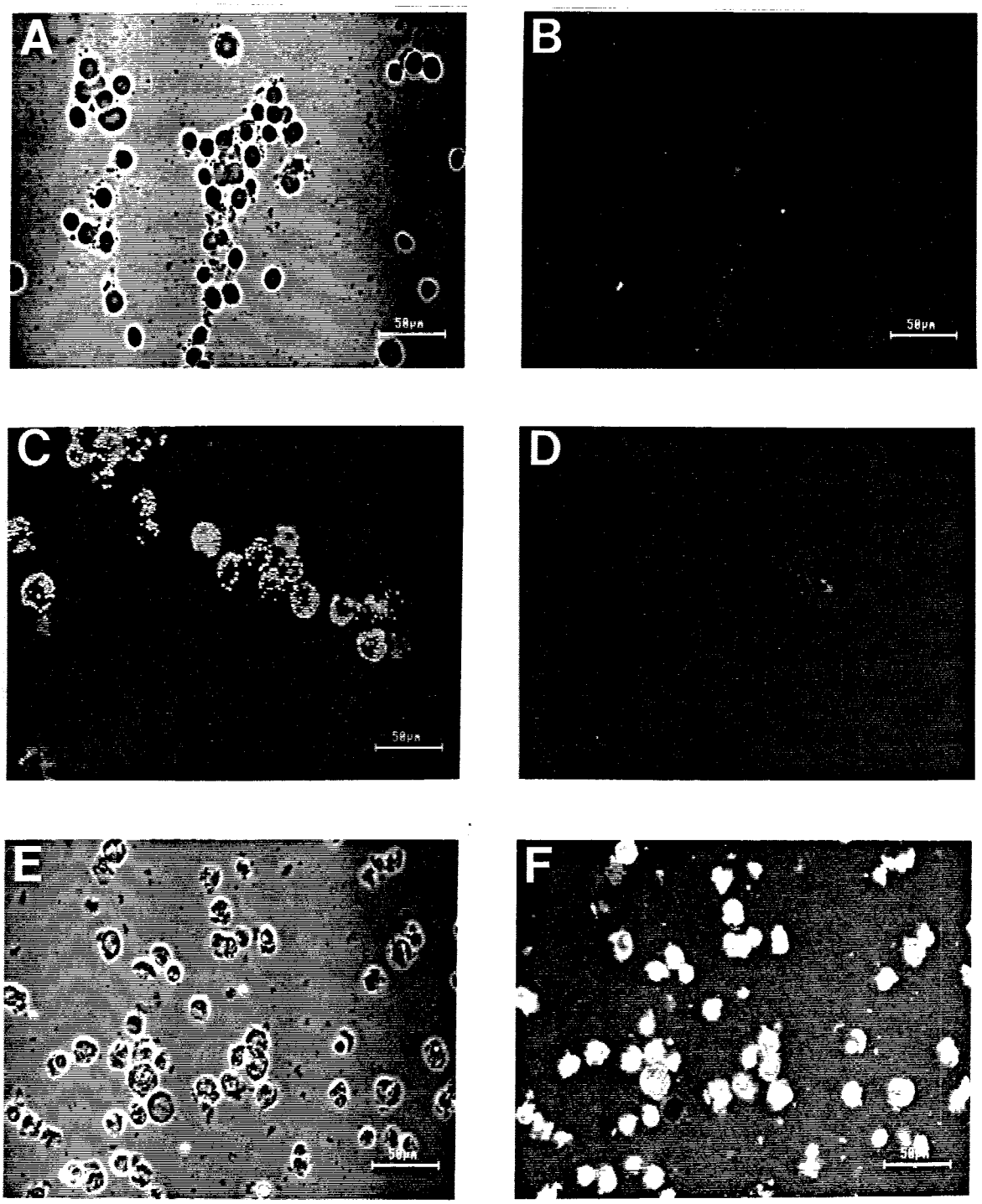
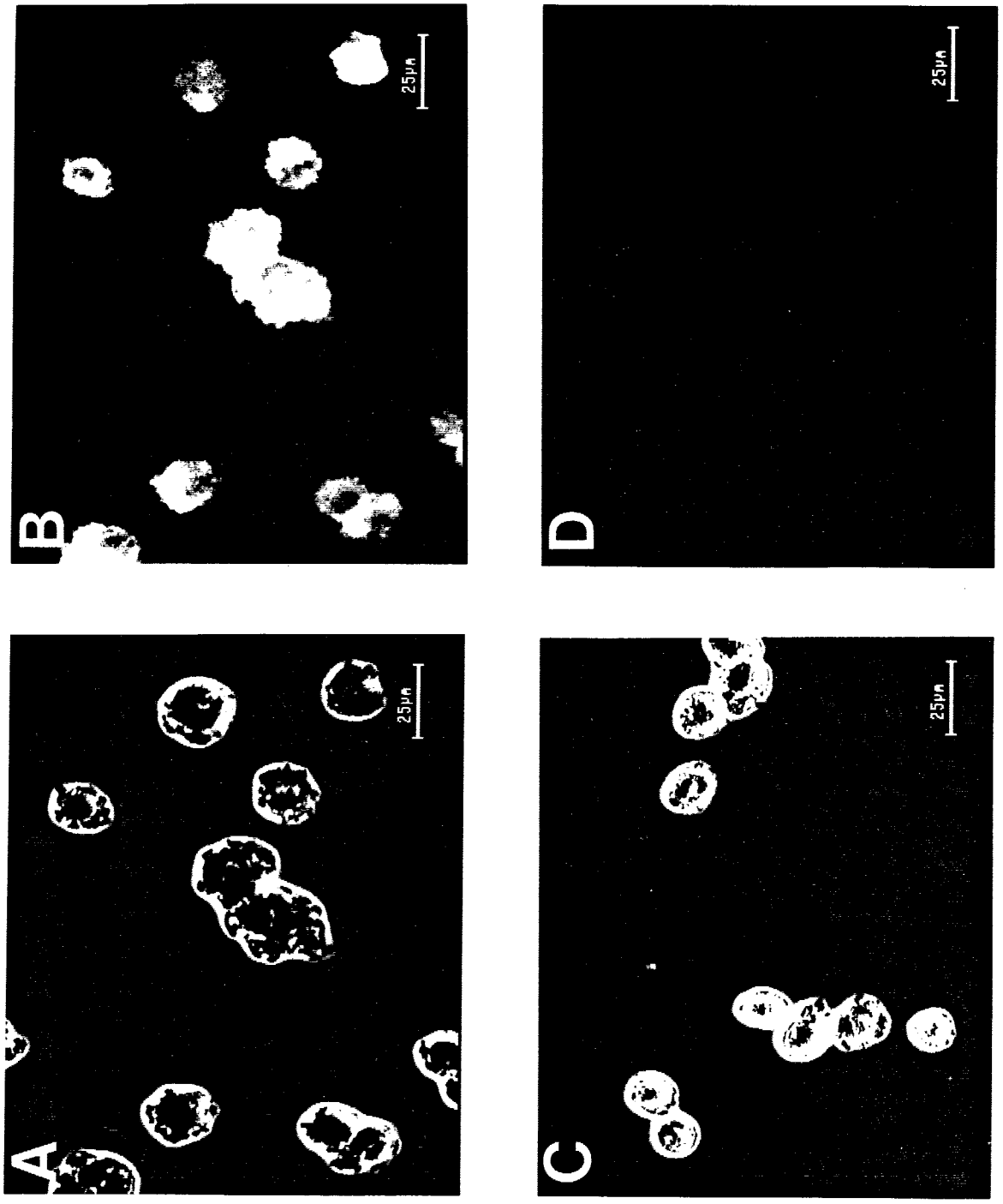


Figure 7.



CHAPTER VII

Conclusions

7.1 Implications for the Insect Cell - Baculovirus Expression System

The goal of this work was to develop a greater understanding of the insect cell-baculovirus expression system from a biochemical engineering perspective. Although this thesis encompasses a variety of topics, they are intimately related by their importance in determining product yield and product quality.

An investigation of operating strategies demonstrated that product yield is dependent both on the time of infection and the multiplicity of infection. Product yields from cultures infected in the late-exponential growth phase were sensitive to MOI, with lower MOIs resulting in decreased yields. Product yields from cultures infected in the early-exponential growth phase were relatively resistant to changes in MOI. Cultures infected in the early-exponential growth phase yielded maximal titers that could be realized only by using very high MOIs when infecting cultures in the late-exponential growth phase.

A population model accounting for infection by multiple virions agreed with the experimental results regarding the effects of infection time and the multiplicity of infection. This model simulated behavior resulting from infection at any point in the growth curve over MOI values ranging from 0 to 100. A Poisson distribution was used to calculate the probability of infection of a cell by more than one virion. Important time points in the infection process, such as the onset of recombinant protein synthesis and the time of cell lysis, were calculated as a function of the time of infection and the number of virions to infect a cell. These calculations not only simulate experimental, final-state product titers well but were in general agreement with cell population dynamics, extracellular virion densities, and product titers throughout the infection.

The intracellular degradation of ß-galactosidase produced in this system was studied by the use of pulse-chase techniques involving protein radiolabelling and immunoprecipitation. In these studies degradation was shown not to affect final product

yields. Early in infection (24 hours post-infection) the degradation rate was of the same order of magnitude as the synthesis rate; however, at later times in the infection process when synthesis increased dramatically, degradation was insignificant. Although yield was not affected by intracellular protein degradation, an array of different sized ß-galactosidase polypeptides were observed. Further analysis of these polypeptides indicated that the fragments do not appear to be the result of intracellular protein degradation. In addition, the fragments were shown not to be an artifact of the experimental technique. There was strong evidence to suggest that premature transcriptional termination was occurring, and that these truncated transcripts were being translated to yield polypeptide fragments. In studies with the *lacZ* gene under the control of the baculovirus p10 promoter, similar ß-galactosidase fragments were also observed. Degradation or premature translation termination have not been completely ruled out by the experimental data. Regardless of the precise origin, baculovirus expression vectors do produce a wide array of fragmented proteins. Although the recombinant virus encoding the *lacZ* gene under the control of the polyhedrin promoter was studied most thoroughly, transcript heterogeneity was observed in Northern blots from a culture infected with a recombinant virus encoding ß1,4-galactosyltransferase. Protein heterogeneity was also observed for a number of proteins produced in this system, including α2,6-sialyltransferase described in Chapter 6.

Heterogeneity of a different type, namely, glycoform heterogeneity, was addressed in Chapter 5. Four cell lines susceptible to baculovirus infection were analyzed for the existence of endogenous exoglycosidases. All four cell lines derived from *Spodoptera frugiperda*, *Trichoplusia ni*, *Bombyx mori*, and *Malacosoma disstria* contained N-acetyl-ß-hexosaminidase, ß-galactosidase, and sialidase activities. Exoglycosidase activities were present in the medium from uninfected cells and in the medium from wild-type AcMNPV-infected cells. Lectin analysis of cellular glycoproteins indicated that endogenous exoglycosidases likely contribute to the degradation of

oligosaccharides on foreign glycoproteins expressed with recombinant baculoviruses in these hosts.

Attempts were made to alter the terminal glycosylation of insect cells by the heterologous expression of glycosyltransferases. Recombinant virions encoding mammalian α2,6-sialyltransferase and ß1,4-galactosyltransferase were constructed. After determining if active protein was synthesized, carbohydrate analysis was completed using lectins. A high degree of lectin-binding was observed that was due to the binding of serum-associated proteins to cells infected with recombinant viruses. More sensitive carbohydrate analysis is required to determine altered glycosylation properly when insect cells are infected with either or both of these recombinant virions.

Baculovirus expression vectors are indeed capable of producing abundant quantities of foreign protein. However, one must be concerned with the quality of the protein being produced. The size heterogeneity observed is an important observation but is easily overcome during the downstream purification process. Glycoform heterogeneity or glycoform deficiencies (e.g., the lack of terminal sialic acid) resulting from baculovirus expression poses a more perplexing obstacle. Purification to isolate specific glycoforms is not likely feasible because of subtle differences involved between glycoprotein species. *In vitro* glycosylation poses problems associated with the tertiary structure of the protein. The importance of glycoform heterogeneity will need to be evaluated for each individual protein.

If the insect cell-baculovirus system is used as an industrial organism, the greatest difficulty will be in maintaining a reproducible process that produces a consistent, well characterized population of recombinant protein.

7.2 Recommendations for Future Work

To consider baculovirus expression vectors as viable hosts for the expression of glycoproteins, a more fundamental understanding of the nature of glycosylation that occurs in insect cells, both uninfected and baculovirus-infected, is required. Only after we understand the insect cell glycosylation pathway, enzymes existing in insect cells, and how the enzymes are affected by viral infection will we be able to engineer the resulting glycoforms by successful manipulation of the cell. This will involve complete oligosaccharide mapping of insect cell glycoproteins as well as recombinant proteins produced under the control of the polyhedrin promoter. Furthermore, because of the lytic nature of the virus, the extent of glycosylation is likely a transient phenomenon because of disruption by infection of the normal cellular state (Davidson and Castellino, 1991; Davidson *et al.*, 1990). For example, important differences may exist between an oligosaccharide synthesized at 24 hours post-infection and an oligosaccharide synthesized at 96 hours post-infection. Provided a secreted glycoprotein is produced, one could envision a harvesting procedure to minimize glycoform heterogeneity arising from temporal changes in the repertoire of oligosaccharide-processing enzymes. If the protein of interest is not secreted but remains associated with the cell, protein harvesting may need to occur earlier in the infection process, thus compromising yield in order to obtain a more homogeneous population of protein. Different operating strategies that maximize product quality need to be developed.

Although glycosidases are present in other cell lines used for the expression of heterologous glycoproteins, they may not come into contact with the protein product as readily as glycosidases in the insect cell-baculovirus system. Results presented in Chapter 5 demonstrate that although these enzymes exist in the medium of uninfected cells, elevated levels are witnessed after viral infection. Work addressing means to minimize residence time of the protein in the medium or the addition of glycosidase inhibitors

should be completed if this system is to be considered a viable means of glycoprotein expression. Continued work identifying endo- and exoglycosidases is necessary. By understanding exactly what glycosidases and glycosyltransferases are present in the insect cell host, unique solutions to the heterogeneity problem may arise. For example, if we are able to impart ß1,4-galactosyltransferase activity in the insect cell host such that the Galß1,4GlcNAc linkage is produced, protection against N-acetyl-ß-glucosaminidase is afforded if ß-galactosidase activity is negligible. From the data presented here, it is likely that solutions to existing problems will involve both alternative cultivation techniques and genetic manipulation. As the scientific community continues to learn more about the importance of glycosylation, ideal hosts may not be judged by the quantity, but instead the quality, of glycoprotein which they produce. Continued effort to understand glycosylation of the insect cell-baculovirus system is necessary if this system is to be evaluated favorably.

A number of specific genetic engineering avenues towards more desirable glycosylation are suggested by the work presented here. The lack of a high degree of glycoform modification from the expression of ß1,4-galactosyltransferase and α2,6-sialyltransferase, alone or together, suggests that the accepting oligosaccharide is present in limiting amount. Lectin analysis with wheat germ agglutinin (WGA), a lectin specific for N-acetylglucosamine, indicates that terminal GlcNAc residues are absent or exist in very low quantities. Provided the proper accepting oligosaccharides exist, heterologous expression of an N-acetylglucosaminyltransferase gene may create the proper substrate for the galactosyltransferase and the sialyltransferase. A number of N-acetylglucosaminyltransferases existing in mammalian cells demonstrate a high degree of specificity towards the accepting oligosaccharide (Schachter *et al.*, 1983; Brockhausen *et al.*, 1988). This type of genetic manipulation should be done after complete characterization of the existing oligosaccharide structures. It is also feasible that the cells contain N-acetylglucosaminyltransferase activity, but because of the presence of N-

acetyl-ß-glucosaminidase, the product is not observed. An alternative approach would be gene disruption of glycosidases that interfere with attaining the desired glycoforms.

The work involving genetic manipulations presented here has relied solely on the transient expression of mammalian glycosyltransferase through baculovirus infection. Since baculovirus infection results in cell death, it is important to develop a means of stable and constitutive expression. Although constitutive expression has been reported (Jarvis *et al.*, 1990), the promoter controlling the constitutive gene is turned off when the resulting cell line is infected with a baculovirus (D. Jarvis, personal communication). To date, the ability to create a stably transformed cell line susceptible to baculovirus infection has not been realized and represents an important unknown technology for this system.

The strong polyhedrin promoter may be a source of product heterogeneity. Promoter strength has been discussed in Chapter 3 as a possible source of the protein fragments observed. Studies pertaining to the effect of promoter strength on product quality remain relatively uncharted territory. One example of adverse consequences resulting from overexpression was observed during the production of bovine somatotropin in *E. coli* (Bogosian *et al.*, 1989). In this study, during the high-level synthesis of the cloned protein, norleucine was synthesized and incorporated into the protein in place of methionine residues. Higher eucaryotic hosts are used to express cloned genes because of intricate post-translational modifications that are desired. It is foreseeable that in an effort to maximize product yields, the flux of protein through a particular pathway exceeds the limitations of that pathway. In the realm of glycobiology, the importance of synthesis rate on glycoform heterogeneity has not been investigated. However, one can envision the synthesis rate of a cloned protein saturating the glycosylation pathway, resulting in increased glycoform heterogeneity. It is likely that an optimal expression rate will account for both product yield and quality. Although pertinent, studies involving the effect of promoter strength on product quality are poorly

suited to baculovirus expression vectors because of cell disruption and ultimate cell lysis that occurs because of infection.

Given the results presented here, the insect cell-baculovirus expression system currently offers an arduous challenge to those wishing to produce well-characterized eucaryotic proteins consistently.

7.3 References

Bogosian, G., Violand, B. N., Dorward-King, E. J., Workman, W. E., Jung, P. E., and Kane, J. F. 1989. Biosynthesis and incorporation into protein of norleucine by *Escherichia coli*. *J. Biol. Chem.* **264**: 531.

Brockhausen, I., Carver, J. P., and Schachter, H. 1988. Control of glycoprotein synthesis. The use of oligosaccharide substrates and HPLC to study the sequential pathway for N-acetylglucosaminyltransferases I, II, III, IV, V, and VI in the biosynthesis of highly branched N-glycans by hen oviduct membranes. *Biochem. Cell Biol.* **66**: 1134.

Davidson, D. J., Castellino, F. J. 1991. Asparagine-linked oligosaccharide processing in Lepidopteran insect cells. Temporal dependence of the nature of the oligosaccharides assembled on asparagine-289 of recombinant human plasminogen produced in baculovirus vector infected *Spodoptera frugiperda* cells. *Biochem.* **30**: 6167.

Davidson, D. J., Fraser, M. J., and Castellino, F. J. 1990. Oligosaccharide processing in the expression of human plasminogen cDNA by Lepidopteran insect (*Spodoptera frugiperda*) cells. *Biochem.* **29**: 5584.

Jarvis, D. L., Fleming, J. G. W., Kovacs, G. R., Summers, M. D., and Guarino, L. A. 1990. Use of early baculovirus promoters for continuous expression and efficient processing of foreign gene products in stably transformed lepidopteran cells. *BioTechnol.* **8**: 950.

Schachter, H., Narasimhan, S., Gleeson, P., and Vella, G. 1983. Control of branching during the biosynthesis of asparagine-linked oligosaccharides. *Can. J. Biochem. Cell Biol.* **61**: 1049.