THE UNIVERSITY OF CALGARY

The Development of Stably Transformed Lepidopteran Insect Cell Technology for Both the Expression of Recombinant Proteins and the Generation of Baculovirus Artificial Chromosomes

by

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ABSTRACT

A primary goal of the biotechnology industry is the synthesis of large quantities of recombinant protein to be used as human therapeutics, subunit vaccines, drug discovery targets, and basic research tools. Recombinant proteins, to date, have largely been produced using genetically engineered bacteria, yeast, baculoviruses, and mammalian cells, yet each systems suffers from severe production disadvantages. Our present work has focused on the development of transformed insect cell expression technology with characteristics that are superior to other expression systems, particularly the baculovirus expression system.

An expression cassette and protocols for continuous, high-level expression of secreted glycoproteins from transformed insect cell lines was developed in this dissertation. The cassette utilizes silkmoth cytoplasmic actin gene promoter to drive foreign gene expression, and also contains the *ie-1* transactivator gene and the HR3 enhancer region of the *Bombyx mori* nuclear polyhedrosis virus to stimulate gene expression. Using an antibiotic resistance selection scheme, a cloned Bm5 cell line (silkmoth) transformed with the expression cassette containing the secreted glycoprotein juvenile hormone esterase (JHE) as a reporter gene, produced 210 µg/mL in stirred suspension culture, and 150 µg/mL in serum-free medium in static culture. The baculovirus expression system (AcNPV infected Sf21 cells) could only produce 4 µg/mL active JHE in static cultures. This cell line exhibited stable recombinant protein expression for over 4 months, and lepidopteran insect cells other than Bm5 cells were also shown to be equally efficient for producing recombinant proteins with this expression cassette.

For the expression of intracellular proteins from genetically engineered organisms, extra yield-reducing steps for purification are required when the protein of interest cannot be naturally secreted into an extracellular environment. We therefore generated DNA coding for a secretion module - a fusion protein that contains JHE at the N-terminus to supply all the signals necessary to "piggy-back" an intracellular protein into an extracellular environment. This resulted in the efficient secretion two intracellular proteins from transfected insect cells. An intra-protein histidine tag allowed purification of the fusion protein using metal chelate affinity chromatography under non-denaturing conditions, and an intra-

protein enteropeptidase cleavage site was recognized for liberation of the intracellular protein from the secretion module.

In an effort to further evaluate the potential of the transformed insect cell system developed in this thesis for the expression of recombinant proteins, collaborations were established with other research groups at the University of Calgary to express proteins in insect cells that could not be produced efficiently in other protein expression systems. Four secreted proteins [human tissue plasminogen activator (t-PA), human granulocyte-macrophage colony-stimulating factor (GM-CSF), a soluble isoform of the alpha subunit of the human granulocyte-macrophage colony-stimulating factor receptor (solGMrα), and a non-glycosylated form of bovine transferrin (ngbTF)], one G-protein coupled membrane receptor [rat protease activated receptor 2 (rPAR-2)], two ion exchangers [native bovine retinal rod Na^+-Ca^2+K^+ exchanger (bNCKX) and a modified bovine retinal rod Na^+-Ca^2+K^+ exchanger (bNCKXdd)], and a secreted intracellular protein [*Bombyx mori* chorion factor 1 (BmCF1)] were successfully expressed. Whenever possible, direct comparisons of expression levels or biological activity were made with other expression systems including transformed mammalian cells, baculovirus, and *Pichia pastoris* (yeast). These comparisons were found to favor the use transformed insect cells over other systems for recombinant protein expression.

Finally, transformed insect cell expression technology was shown to be a useful research tool in insect biology and for the study of baculoviruses. Stably transformed insect cell lines were used to create baculovirus artificial chromosomes (BVACs). BVACs can potentially be used *in vitro* for basic research and large-scale recombinant protein production, or *in vivo* to generate transgenic insects for study and biopesticide industry-related applications. Our approach was to inactivate a single baculovirus gene rendering a baculovirus as an infectious, yet harmless, self-replicating extra-chromosomal entity that can carry useful genes of scientific or commercial value into lepidopteran insect cells. Rescuing insect cell lines were generated to make BVACs and infectious BVAC inocula. It appears, however, that the successful generation of pure BVACs was hampered by recombination events where the virulence was regained by the BVACs from the rescuing cell line. Research is still in progress to correct this problem.

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NOMENCLATURE

ABP1	maize auxin-binding protein
AcNPV	*Autographa califomica* nuclear polyhedrosis virus
Apo A-I	apolipoprotein A-I
bcl-2	B-cell lymphoma proto-oncogene product
bGH	bovine growth hormone
BmNPV	*Bombyx mori* nuclear polyhedrosis virus
BmCF1	*Bombyx mori* chorion factor 1
bNCKX	bovine retinal rod Na^{+}-Ca^{2}+K^{+} exchanger
bNCKXdd	modified bovine retinal rod Na^{+}-Ca^{2}+K^{+} exchanger
BVAC	Baculovirus artificial chromosome
C9	complement protein C9
cat	chloramphenicol acetyl-transferase gene
CAT	chloramphenicol acetyl-transferase protein
c-Myb	*c-Myb* proto-oncogene product
COX-2	cyclooxygenase
DSPA	vampire bat plasminogen activator
FBS	fetal bovine serum
GABA	γ-aminobutyric acid
G-CSF	granulocyte colony stimulating factor
GM-CSF	granulocyte-macrophage colony stimulating factor
gp120	HIV glycoprotein 120
HbsAg	hepatitis B virus surface antigen
hIl-5	human interleukin 5
HIV	human immunodefficiency virus
HR3	enhancer region of BmNPV
H-ras	oncogenic GTP binding protein
huB_2AR	human β-andrenergic receptor
hu-LIF	leukemia inhibitory factor

ie-1	immediate early gene of BmNPV
IE-1	protein product of the immediate early gene of BmNPV
IFN	interferon
IgG	immunoglobulin class G
Il	interleukin
jhe	juvenile hormone esterase gene
JHE	juvenile hormone esterase protein
LacZ	gene encoding β-galactosidase
lef-8	gene encoding BmNPV late expression factor 8
LEF-8	BmNPV late expression factor 8
MCP-1	monocyte chemoattractant protein-1
ngbTf	non-glycosylated form of bovine transferrin
NNOS	neuronal nitric oxide synthase
p53	tumor suppressor protein
PAC	puromycin acteyl-transferase
PAI	plasminogen activator inhibitor
rPAR-2	rat protease activated receptor 2
scFv	single chain Fv antibody
SCG10	neruon-specific growth associated protein
solGMrα	soluble isoform of the α subunit of the human granulocyte-macrophage colony stimulating receptor
TAP	tick anticaogulant peptide
Tf	transferrin
u-PA	urokinase plasminogen activator
t-PA	tissue plasminogen activator

CHAPTER 1
Introduction

1.1 General Introduction to Recombinant Protein Expression

A primary goal of the biotechnology industry is the synthesis of large quantities of native protein, required for use as human therapeutics, subunit vaccines (both human and animal), drug discovery targets, and basic research tools. Proteins produced using genetic engineering techniques are known as recombinant proteins.

Some recombinant proteins represent the next generation of drugs for the treatment of a variety of genetic and acquired human diseases. Proteins are superior to conventional drugs due to their specificity and that they are natural, endogenous molecules, having evolved with the human body. Currently the most prominent recombinant protein is human insulin, which is utilized for the treatment of type II diabetes. Prior to its approval for use in 1982, insulin for diabetics was purified from the pancreas of pigs. Since then, approximately 25 other recombinant proteins have been approved for use as human therapeutics in the United States by the Food and Drug Administration (see Table 1.1), while a further 180 cytokines, antibodies, hormones, and enzymes were being tested in clinical trials in the United States in 1996 (Genetic and Engineering News, September 15, 1996). This number is expected to increase dramatically soon, when the discovery of novel genes by the human genome project and the deciphering of their role *in vivo* will lead to an avalanche in the development of novel protein treatments for human diseases. The current and projected market value of selected products is shown in Table 1.2 (Genetic and Engineering News, 1994).

There are 5 recombinant protein subunit vaccines currently approved for use in the U.S., and approximately 50 other candidates were being tested in human clinical trials in the U.S. in 1996 against cancer, AIDS, whooping cough, genital herpes, multiple sclerosis and other ailments. Subunit vaccines differ from conventional vaccines because they only contain a small protein piece (subunit) of an offending pathogen, as opposed to an attenuated or killed whole organism, yet they are still sufficient to elicit a protective immune

Product Name	Protein	Company	Treatment (Date of First U.S. Approval)
Actimmune	IFN-γ2b	Genentech	chronic granulomatous disease (1990)
Activase	t-PA	Genentech	acute myocardial infarction (1987), acute pulmonary embolism (1990) ischemic stroke (1996)
Alferon N	IFN-αn3	Interferon	genital warts (1989)
Betaseron	IFN-β1b	Chiron	multiple sclerosis (1993)
Cerezyme	glucocerebrosdiase	Genzyme	Gaucher's disease (1994)
Epogen	erythropoeitin	Amgen	anemia (1989)
Humatrope	somatatropin (hGH)	Eli Lilly	human growth hormone deficiency (1987)
Humulin	insulin	Eli Lily	diabetes (1982)
Intron A	IFN-α2b	Schering-Plough	hairy cell leukemia (1986) genital warts(1988), Kaposi's sarcoma (1988), hepatitis B (1992)
Leukine	GM-CSF	Immunex	bone marrow transplanation (1991)
Neupogen	G-CSF	Amgen	neutropenia (1991), bone marrow transplanation (1991)
Proleuin	IL-2	Chiron	renal cell carcinoma (1992)
Pulomozyme	DNase	Genentech	cystic fibrosis (1993)
Recombinate	antihemophilic factor	Baxter	hemophilia A (1992)

Table 1.1: Summary of some of the recombinant proteins approved for use as therapeutic medicines in the United States by the U.S. Food and Drug Administration (Source: Genetic and Engineering News, September 15,1996).

Recombinant Protein	1994 Market	2004 Market (Est.)
	($U.S. millions)	
Cardiovascular		
EPO	1430	3100
t-PA	260	420
Blood factors	35	70
Others	0	30
Cancer		
CSF's	870	2580
Interferons	835	2830
Interleukins	40	80
Others	0	350
Hormones/Growth Factors		
hGH	235	510
Insulin	600	980
Others	0	600
Vaccines		
HIV	0	900
Hepatitis B	650	1250
Herpes	0	250
Others	0	350
TOTAL	5080	15800

Table 1.2: Product sales for various recombinant proteins in the U.S. in 1994 and estimated product sales for 2004 (Source: Genetic and Engineering News, October 15,1995).

response against the pathogen. Hepatitis B subunit vaccines, that consist of either the hepatitis B virus surface antigen or core antigen, had a 1994 market value of US$650 million in the United States, which is projected to be US$1,250 million by 2004 (Genetic and Engineering News, October 15, 1995). There is also a strong market for animal subunit vaccines against those pathogenic species causing bovine viral diarrhea, foot-and-mouth disease, blue-tongue, and bovine herpes.

Other recombinant proteins are needed for drug discovery programs, that follow after the identification of the gene products responsible for a disease such as obesity (*fat*, *tubby* and *obese* genes), cystic fibrosis (CFTR) and breast cancer (BRCA1 and BRCA2). Currently there are over 450 known protein drug targets of an estimated 3,000 to 10,000 potential protein drug targets in the human genome (Drews, 1996). One approach, referred to as the "structure-based approach to drug discovery", first ascertains the target protein's 3-dimensional molecular structure, and molecular modeling is then used to design small synthetic ligands that bind the protein's active site, either as an agonist or antagonist. For this application, milligram quantities of pure recombinant target protein are required for structural analysis by x-ray crystallography and nuclear magnetic resonance spectroscopy.

At present, perhaps the biggest demand for recombinant proteins exists in the area of basic scientific research. Proteins are fundamental to all living species, and, due to an explosion in technology for the detection and cloning of genes, novel proteins are reported daily. To characterize such novel proteins for basic research, milligram quantities of recombinant protein are needed per study.

Due to their complexity, recombinant proteins are impossible to synthesize chemically. Instead, molecular biologists view organisms as potential protein factories, where the thousands of intricate biochemical reactions necessary for producing a single protein molecule are naturally present and can be harnessed to produce a recombinant protein via genetic engineering techniques. This area, known as protein expression, is still in its infancy, and thus, a major objective of this thesis is to combine molecular biology and chemical engineering to develop methods of producing recombinant proteins superior to those currently available. The standard organisms and techniques currently used for protein expression are reviewed in the following sections.

1.2 Prokaryotic and Lower Eukaryotic Expression Systems

1.2.1 Bacterial Expression of Recombinant Proteins

The study of *Escherichia coli* in the 1960's and 1970's made it the best understood organism in nature, and thus the initial attempts to express cloned genes for the production of recombinant naturally used *E. coli* as the host organism. In 1982, *E. coli* was the first organism approved by the U.S. F.D.A. to produce a recombinant protein (human insulin) destined for *in vivo* human use. The key features that continue to make *E. coli* useful for protein expression are that it is easy to manipulate genetically and it grows quickly (20-60 min doubling time) on inexpensive media to high cell densities.

Genes, whose protein products are required, are cloned next to a suitable promoter in a plasmid vector which is used to transform the bacteria. Once a plasmid is inside the bacteria, transcription of the foreign gene can be induced by activating the promoter. Common promoters include the *trp* promoter, which is active in the absence of the amino acid tryptophan, or the *lac* promoter, which is activated by the presence of lactose or lactose analogs such as isopropyl-B-D-thiogalactose (IPTG).

Thousands of recombinant proteins have been expressed successfully in *E. coli*, some of which are listed in Table 1.3. Generally, expression levels are high and in the range of 10 to 1,000 mg/L, however expression in bacteria has its shortfalls. Numerous yield-reducing steps are required to purify an over-expressed protein; these include harvesting of the cells, lysis of the cells, and isolating the desired protein from thousands of other bacterial proteins. *E. coli* does not have a secretion system, and therefore normally secreted heterologous proteins accumulate within the cytoplasm and often form insoluble aggregates in the cytoplasm known as "inclusion bodies". Recovery of the active protein from inclusion bodies requires re-solubilization and re-folding steps, which does not necessarily restore the proteins full activity. Furthermore, proteolysis of the protein product often occurs in *E. coli*, which contains dozens of endoproteases (Goldberg and Goff, 1986).

Although extremely successful for producing many proteins for use as antigens and in research, expression in *E. coli* is not useful when post-translational modifications are required for the application of the protein. *E. coli* lack the enzymes and cellular compartments required for glycosylation, signal peptide cleavage, and intron splicing from

Protein	Promoter	Culture Size	Expression Level (mg/L)	Reference
IL-1α	λP_L	10 L	49	Hsuing et al., 1986
IL-1β	λP_L	10 L	354	Hsuing et al., 1986
c-Myb	T7	2-5 mL	30	Yasakuma et al., 1995
p53	T7	2-5 mL	100	Yasakuma et al., 1995
scFv*	T7	10 mL	20	Wickert et al., 1995
SCG10	λP_L	1 L	10	Antonsson et al., 1997
NNOS	*tac*	1 L	20-24	Roman et al., 1995
apoA-I*	*tac*	1 L	30-70	Schmidt et al., 1995

Table 1.3: Summary of expression levels of various recombinant proteins produced in *E. coli.* *Reported for other expression systems in this thesis.

genomic DNAs.

1.2.2 Expression of Recombinant Proteins in Yeast

Like bacteria, yeast species, such as *Saccharomyces cerevisae* and *Pichia pastoris*, are convenient hosts for the expression of recombinant proteins. They can be easily manipulated genetically with plasmid vectors, they grow quickly relative to both mammalian and insect cell lines (4-5 h versus 15-50 h), they grow on inexpensive media, and large-scale fermentation technology is well established owing to its use in the baking and brewing industry. Furthermore, recombinant yeast are eukaryotic cells which can be used to express recombinant proteins that require post-translational modifications, such as limited signal peptide cleavage, simple glycosylation, acetylation, phosphorylation, myristylation, pamitylation and carboxylation (Marino, 1991).

Once transformed with an expression plasmid, heterologous proteins can be expressed in *S. cerevisiae* using a constitutive promoter such as the alcohol dehydrogenase promoter I, or an inducible promoter such as GAL1 which is activated by the presence of galactose in the culture medium. Proteins can be expressed intra-cellularly, typically at levels of 2 to 5% of total cell protein (0.4-1.0 mg/L at a cell concentration of 50 g dry weight/L), but proteolysis and recovery may reduce yields significantly. Furthermore, the cytoplasm is associated with reducing conditions, so that the isolated protein may require denaturation and re-folding steps to regain full biological activity.

For secreted proteins, yeasts are eukaryotic and do have the desirable capacity for limited secretion, simplifying the recovery of an over-expressed product. However, yields are much lower than for those proteins expressed intracellularly (Table 1.4), and a protein to be secreted must contain yeast signal peptide sequences derived from yeast proteins such as invertase or the mating pheromone alpha-factor; therefore extra cloning steps are required to create a chimeric gene for heterologous protein secretion.

For glycoproteins, *S. cerevisae* and *P. pastoris* have the ability to perform simple N-linked and O-linked glycosylation. The inability to perform complex N-linked glycosylation (Figure 1.1) on a recombinant glycoprotein can affect the functionality of the protein (Rademacher and Parekh, 1988), its antigenic properties (Feizi and Childs, 1987),

Protein	Species	Expression Level (mg/L)	Culture Conditions	Reference
hu-c9*	*S. cerevisae*	0.1	shake flask	Tomlinson et al., 1993
HbsAg	*S. cerevisae*	50-100	1600 L fermenter	Stephanne et al., 1990
huGM-CSF	*S. cerevisae*	24.5	n.r.	Ernst et al., 1987
HbsAg	*P. pastoris*	375	240 L fermenter	Cregg et al., 1987
TAP	*P. pastoris*	1700	15 L fermenter	Laroche et al., 1994
scFv*	*P. pastoris*	100	n.r.	Ridder et al., 1995
huMCP	*P. pastoris*	100	n.r.	Beall et al., 1998
Enterokinase	*P. pastoris*	6.5	shake flask	Vozzu et al., 1996

Table 1.4: Summary of expression levels of various recombinant proteins produced in yeast. *Reported for other expression systems in this thesis.

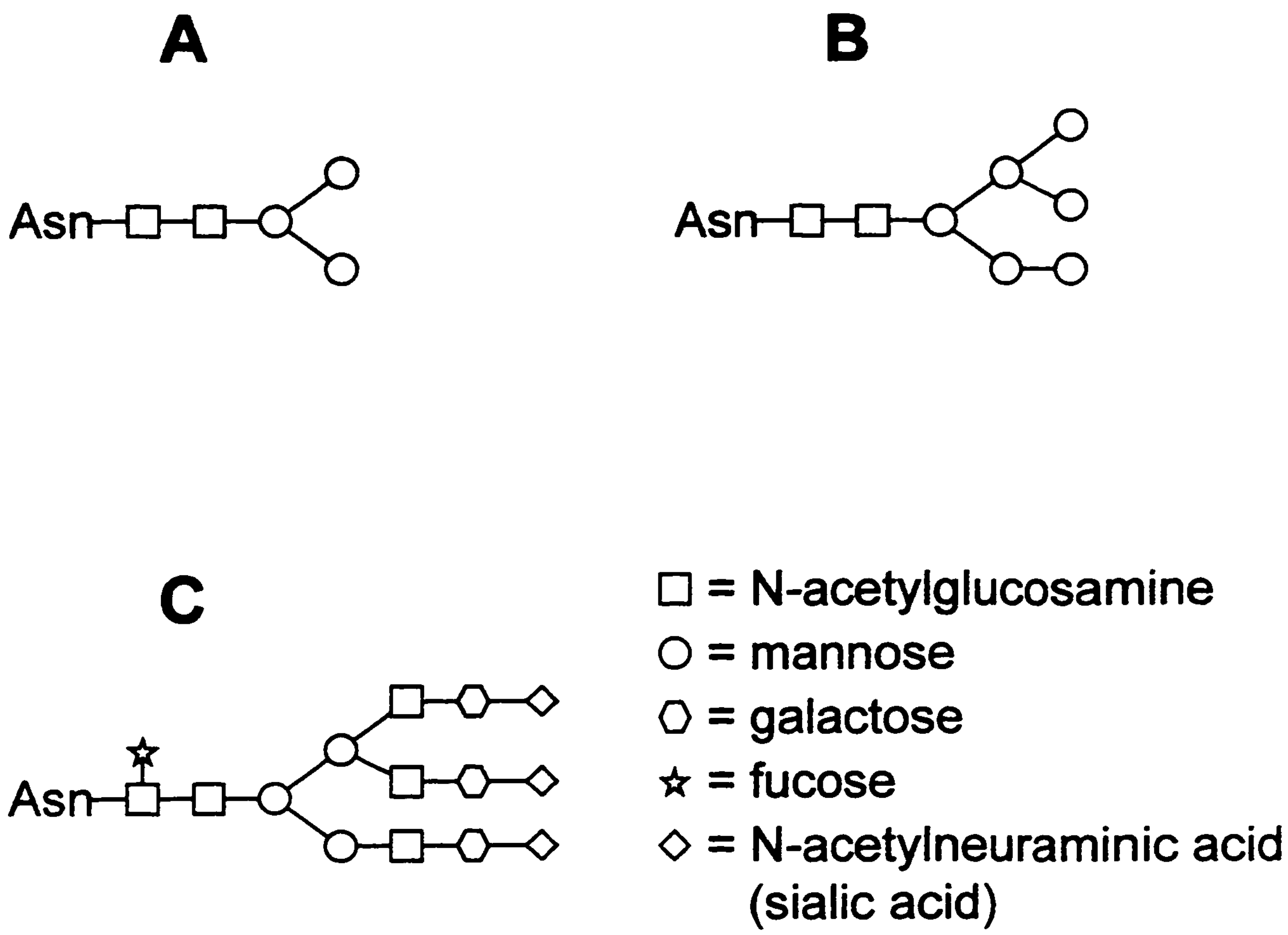


Figure 1.1: Common N–linked oligosaccharide structures found on glycoproteins. Each oligosaccharide contains a core region (shown in A) added *en bloc* in the endoplasmic reticulum, which can be modified in the golgi apparatus to an oligomannose structure (shown in B) or a complex structure (shown in C). The complex structure typically contains the core plus a terminal region consisting of branched trisaccharides (N-acetylglucosamine-galactose-sialic acid) linked to the core mannoses. In addition, a fucose residue may be added, usually to the core N-acetylglucosamine attached to asparagine.

allergenic properties (Altmann et al., 1993, Ma et al., 1995), thermal solubility and stability (West, 1986) and the *in vivo* half-life (Matsumoto et al., 1995; Grossmann et al., 1997). In yeasts, N-linked carbohydrate moieties are generally hyperglycosylated with mannose (more than 50 mannose residues can be added to the core glycosylation structure; Herscovics and Orlean, 1993), which can compromise the efficacy of recombinant proteins such as the hepatitis B vaccine (Kniskern et al., 1994).

1.3 Higher Eukaryotic Expression Systems

An overview of three different, yet most widely used higher eukaryotic expression systems is presented in this section to illustrate the molecular biology, mechanics, advantages, and shortcoming in current protein expression technology.

1.3.1 Baculovirus Expression System

The baculovirus/insect cell expression system is currently one of the industrial workhorses for the rapid generation of recombinant proteins. Baculoviridae are a large family of invertebrate-specific viruses, characterized by a circular, double-stranded DNA genome of 80 to 220 kbp in length contained within an enveloped rod-shaped virion (Miller, 1988). They can infect 600 species of arthropods (Iatrou et al., 1994), however one sub-family, the nuclear polyhedrosis virus (NPV), mainly infects lepidopteran insects and has the unique characteristic of producing large proteinaceous occlusion bodies (OBs) in infected cells in the very late stages of infection. In nature, these OBs serve to protect the mature virions embedded within them from damaging environment effects such as chemicals, ultraviolet radiation, and temperature changes, until they can be ingested by uninfected insects. An ingested OB becomes soluble in the alkaline pH of the insect midgut and the virions are released to propagate the NPV infection in the insect tissues (Figure 1.2).

The baculovirus expression system exploits two features of OBs: first, that the amount of polyhedrin protein contained in an OB is large and produced in a short time period, indicating that the polyhedrin promoter is an extremely powerful driver of gene expression; and second, that although the OB is necessary in nature, individual virions can

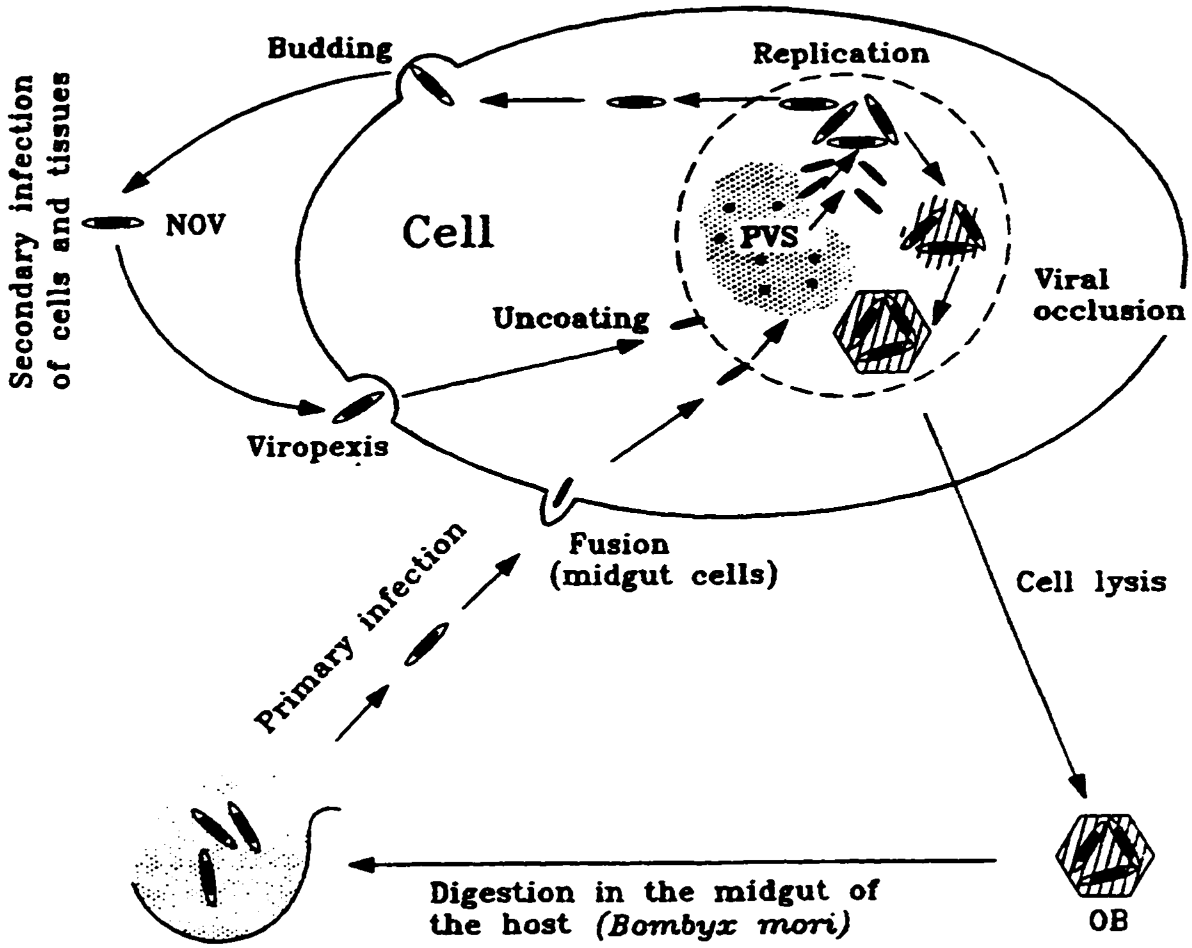


Figure 1.2: The life cycle of the *Bombyx mori* nuclear polyhedrosis virus (BmNPV) infecting a *Bombyx mori* (silkworm) cell. Following primary infection and the formation of the precursor of virogenic stroma (PVS), two populations of viruses result - nonoccluded virus (NOV) bud from the plasma membrane in the secondary infection cycle, and occluded virus (OB) accumulate in the nucleus in the late phase of infection (Reproduced from Zhang, 1993).

survive and propagate infection without the protection of an OB in a benevolent environment, such as *in vitro* insect cell culture. Therefore, replacement of the polyhedrin gene with a heterologous gene sequence by genetic engineering resulted in a recombinant NPV which produced large amounts of the heterologous protein *in vitro* from the polyhedrin gene promoter in the very late stage of infection, without affecting the viral life cycle (Smith et al., 1983).

Over 500 recombinant proteins have now been produced using this system (Patterson et al., 1995), that mainly employs the *Autographa californica* NPV for laboratory-scale or larger scale production. The major advantage of this type of expression system is the high level of recombinant proteins that can be obtained through the use of strong late- or very late-phase promoters of the basic protein (Hill-Perkins and Possee, 1990), polyhedrin (Smith et al., 1983), or p10 (Vlak et al., 1988) genes. A sample of reported expression levels is shown in Table 1.5, and these reveal that recombinant protein concentrations greater than 100 mg/L are usually feasible for some classes of recombinant proteins. In addition, this system is popular due to the short development time; a pure recombinant virus can be obtained in an average of 4-5 weeks versus 3-5 months required for generating stably transformed, amplified mammalian cell lines (Coleman et al., 1997).

The NPVs themselves are also safe. Although they may have the ability to gain entry into certain mammalian cell types such as human, mouse and rabbit hepatocytes (Hofmann et al., 1995), they have been shown to be unable to replicate in mammals (Tjia et al., 1982). Furthermore, their *in vitro* cultured lepidopteran insect cell hosts are safe because they are not neoplastically transformed, in contrast to transformed mammalian cells that potentially harbor cancer causing DNA elements, and insect cells are also maintained at only 25-28°C and therefore are less likely to harbor pathogens compatible with human workers and patients than mammalian cell lines.

Normal higher eukaryotic post-translational modifications have been reported using this system such as signal peptide cleavage, O-glycosylation, phosphorylation, mistrylation, prenylation, acylation, amidation, and carboxymethylation (O'Reilly et al., 1992). Considerable attention has been focused on the N-glycosylation capabilities of this system, particularly for the production of proteins destined for *in vivo* use in humans. Most of the

Protein	Baculovirus Cell line	Promoter	Expression Level (mg/L)	Culture Conditions	Reference
Secreted					
JHE	AcNPV/Sf21	polyhedrin	12	6-well plate	Bonning and Hammock, 1995
JHE	AcNPV/Sf21	p10	13	6-well plate	Bonning and Hammock, 1995
JHE	AcNPV/Sf21	basic protein	16	6-well plate	Bonning and Hammock, 1995
JHE	AcNPV/Hi5	polyhedrin	32	shake flask	Bonning and Hammock, 1995
JHE	ACNPV/Sf21	polyhedrin	22	spinner flask	Bonning and Hammock, 1995
hu-LIF*	AcNPV/Sf21	polyhedrin	12	roller bottle	Geisse et al., 1996
Apo A1*	AcNPV/Sf21	polyhedrin	40-50	1 L reactor	Sorci-Thomas et al., 1996
hu-prorenin*	AcNPV/Sf9	polyhedrin	0.5	23 L reactor	Mathews et al., 1996
hu-C9*	AcNPV/Sf21	polyhedrin	0.7	shake flask	Tomlinson et al, 1993
huIL-5*	AcNPV/Sf9	polyhedrin	5-15	3 L reactor	Brown et al., 1995
HIV gp120	ACNPV/sf9	polyhedrin	10-15	5 L reactor	Murphy et al., 1993
Intracellular					
CAT	AcNPV/Sf21	polyhedrin	>100	T-flask	Luckow and Summers, 1988
CAT	BmNPV/Bm5	polyhedrin	250	2-stage 1.5 L reactor	Zhang et al., 1993
B-gal*	AcNPV/Hi5	polyhedrin	>500	Shake flask	Wickham et al., 1992
Membrane					
G proteins	AcNPV/Sf21	polyhedrin	15	T-flask	Labrecque et al., 1992

Table 1.5: Expression of various recombinant proteins produced by the baculovirus expression system.

*Reported for other expression systems in this thesis.

evidence suggests oligomannose type carbohydrate moieties on baculovirus expressed glycoproteins (Grabenhorst et al., 1993; James et al., 1995; Jarvis et al., 1995), although complex glycosylation has been reported for some proteins (Davidson et al., 1990; Davis and Wood, 1995; Ogonah et al., 1996).

The baculovirus system does suffer from disadvantages that mainly relate to the fact that this is a transient expression system and the host cells are killed and lysed in each infection cycle. The process is inherently batchwise, or at best semi-continuous (Zhang et al., 1994), and not suited to more effective bioreactor configurations such as continuous-perfusion systems (Trampler et al., 1994). Useful promoters for foreign gene expression are active only in the late or very late-phase of virus infection when most of the host cell machinery is compromised. Therefore intron splicing machinery, although present (Iatrou et al., 1989), is inefficient (Sumathy et al., 1997), secretion pathways are compromised (Jarvis et al., 1989) limiting expression levels of secreted proteins (see Table 1.5 for comparison), glycosylation may be incomplete (Stoltenberg et al., 1996; Geisse et al., 1997), and complex glycosylation events are rare, occasionally resulting in an extremely short half-life when a protein is injected *in vivo* (Grossman et al., 1997). In addition, the lysis of host cells by the virus and release of host proteins complicates the purification process, and may also result in some proteolysis of the over-expressed protein (Copeland et al., 1991). Finally, for the expression of biologically active membrane proteins and ion channels, only a short window of opportunity exists to study their physiological properties before viral induced plasma membrane disruption occurs.

1.3.2 Stably Transformed Mammalian Cells

Mammalian expression systems employ a variety of different cell lines, expression cassettes, and antibiotic resistance markers to generate stably transformed cell lines over-expressing a recombinant protein in *in vitro* cultures. The advantages of stably transformed mammalian cell lines over the baculovirus expression system relate to the cytopathic effects caused by viral infection. They include the maintenance of cell cultures in perpetuity for continuous production, expression from both cDNAs and intron-containing genomic genes, performing essential post-translational modifications in a stable cellular environment, providing an intact plasma membrane for physiologically functional membrane proteins, and increased secretion efficiency due to a fully functional secretory pathway for simpler purification schemes and lower risk of proteolysis by intracellular proteases.

The most common hosts used for the establishment of stably transformed mammalian cell lines include Chinese hamster ovary (CHO) cells, baby hamster kidney cells (BHK) cells, human embryonic kidney (HEK) 293 cells, mouse L-cells, and myeloma cell lines like NSO and Sp2/0 cell lines. Most of the above-mentioned cell lines require a surface for growth and are serum dependant and therefore unsuitable for large scale production of recombinant proteins. However, considerable research efforts have been devoted to adapting mammalian cell lines to suspension culture and developing serum free medium (Berg et al., 1993; Gu et al., 1996). Mammalian cell lines, such as CHO cells, can now be grown on a large scale in suspension culture, up to 8,000 L (Keen and Rapson, 1995), and at least one sub-clone of CHO cells (CHO K1) has been succesfully adapted to protein-free medium (Zang et al., 1995).

A variety of expression plasmids are available that employ different combinations of promoter, enhancer, intron, and polyadenlyation sequences. Promoters of the following genes are frequently employed: Simian Virus (SV40) early genes, human cytomegalovirus (HCMV) major immediate early gene, the Rous Sarcoma virus (RSV) long terminal repeat (LTR) and the mouse mammary tumor virus (MMTV) LTR. Promoters are frequently complemented by transcriptional enhancer sequences such as the HTLV-1 LTR enhancer, the major immediate early gene enhancer of the HCMV, the SV40 early enhancer and the RSV LTR enhancer. Certain transcription termination and polyadenylation signals can prolong the mRNA half-life leading to higher recombinant protein expression levels; the bovine growth hormone terminator, the SV40 early and late genes terminators, the human B-globin gene terminator, and SV40 t antigen terminator have been employed. The presence of introns in over-expressed messages has also been found to stimulate protein expression by an unknown mechanism (Brinster et al., 1988; Peticleric et al., 1995), and thus some expression cassettes employ introns from the SV40 late genes (VP1), SV40 early genes, or synthetic introns such as the adenovirus splice donor/immunoglobulin G splice acceptor (Peticleric et al., 1995). Finally, protein expression levels can be further stimulated by the presence of transcription activators in cell lines to boost mRNA levels, including the transactivation of the herpes simplex virus (HSV)-1 immediate early gene promoters by the HSV protein VP16 (Hippenmeyer and Highkin, 1993) and transactivation of the HCMV immediate early gene promoter by the protein products from the adenovirus E1A early gene (Cockett et al., 1991).

Since stable chromosomal integration and expression of foreign genes is a relatively inefficient process, a variety of selection markers are available and are cloned into an expression cassette. This encourages mammalian cells to integrate the expression cassette into their genome to generate stably transformed cell lines overexpressing the desired recombinant protein. A selection marker is a gene co-expressed with the desired gene to allow a cell's survival in the presence of a lethal drug in the culture medium. *E. coli* hygromycin-B-phosphotransferase is a protein that allows growth in the presence of the hygromycin-B - a drug which normally inhibits protein synthesis by disrupting protein translocation and promoting mistranslation (Gritz and Davies, 1983). Aminoglycoside phosphotransferase is a bacterial protein that allows growth in G418 - a neomycin analog that interferes with ribosome function and blocks protein synthesis (Southern and Berg, 1982). Dihydrofolate reductase (DHFR) is an enzyme necessary for purine biosynthesis and is inhibited by the drug methotrexate (MTX; Simonsen and Levinson, 1983). Increasing the concentration of MTX with this marker seems to select not only for those cells that express higher levels of DHFR, but also express higher levels of the desired recombinant protein. The DHFR selection marker is known as an 'amplifiable' selection marker because gradually increasing the MTX concentration over a period of 2 to 5 months selects for those cells that can spontaneously increase the integrated expression cassette copy number to several hundred copies/cell and provide more DHFR for survival (Kaufman et al., 1986). A corresponding increase in the desired gene expression level occurs due to the copy number increase of the expression cassette and its guaranteed position in transcriptionally active regions of the chromosomes. A typical expression cassette used to generate stable mammalian cell lines is described in Figure 1.3.

Transformed mammalian cell lines have been able to express a variety of secreted proteins, some of which are summarized in Table 1.6. Expression levels are in the order of

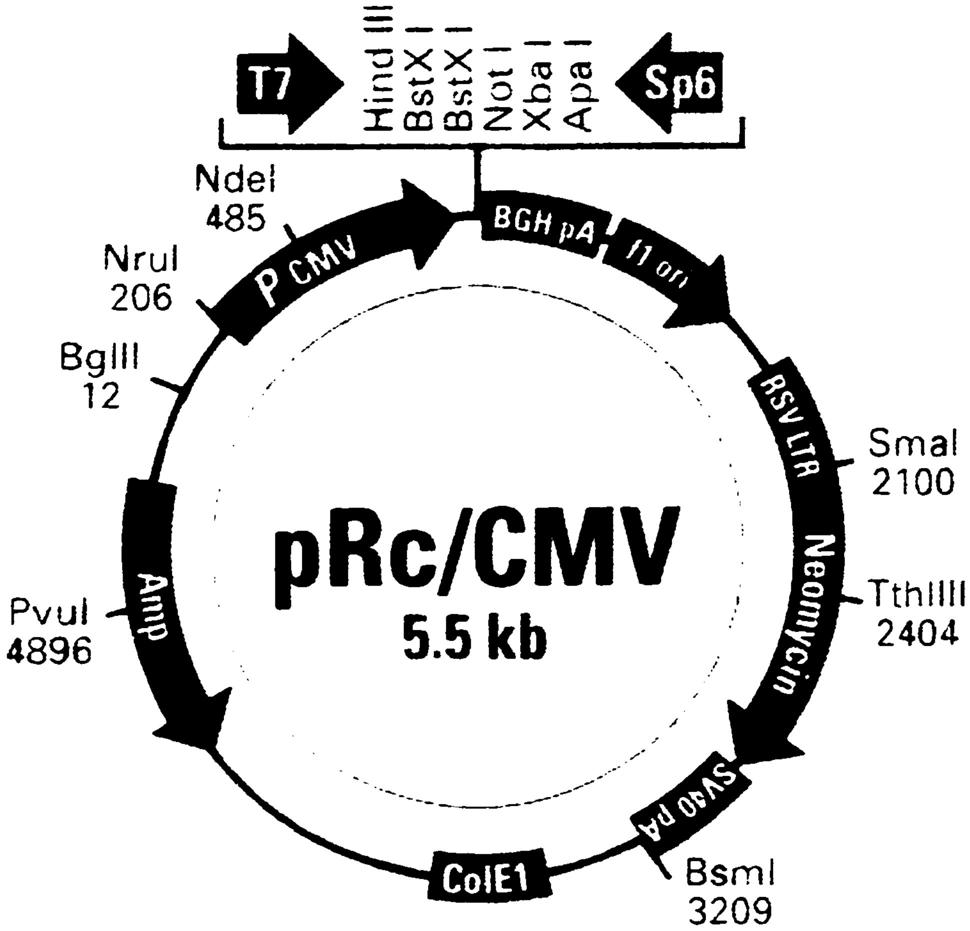


Figure 1.3: A typical eukaryotic expression cassette for producing recombinant proteins from stably transformed mammalian cells. This cassette contains the enhancer/promoter sequences of the human cytomegalovirus immediate early gene (P_{CMV}), a multiple cloning site for insertion of the desired gene of interest, and polyadenylation and transcription termination sequences from the bovine growth hormone gene (BGH pA). Integration of the expression cassette into the chromosomes of host mammalian cell lines is encouraged in the presence of neomycin antibiotic analogs by the expression of the neomycin resistance gene (Neomycin) from the enhancer/promoter sequence of the Rous sarcoma virus long terminal repeat (RSV LTR), with polyadenlyation signal and transcription termination sequences of SV40 late genes. The plasmid also contains the ampicillin gene (Amp) and the ColE1 origin of replication for selection and maintenance in *E. coli* (Reproduced from Invitrogen 1996 catalogue).

Protein	Host Cell	Promoter	Expression Level (mg/L)	Culture Conditions	References
hu-LIF*	CHO	AdMLP/ Sv40 enhancer	11-17	roller bottle	Geisse et al., 1996
hu-LIF*	Sp2/O	IgG L kappa/ Ig heavy enhancer	19-25	roller bottle	Geisse et al., 1996
Apo A1*	CHO	metallothionine	20-30	t-flask	Schmidt et al., 1997
hu Tf half molecule	BHK	metallothionine	55-120	roller bottle	Mason et al. 1991
ICAM-1	BHK	HSV IE175/ VP16 transactivator	105	t-flask	Warren et al., 1994
bGH	BHK	HSV IE175/ VP16 transactivator	4	t-flask	Hippenmeyer and Highkin, 1993
IgG kappa light chain	CHO	CMV promoter-enhancer	74	2.5 L reactor	Zang et al., 1995
u-PA	CHO	CMV promoter-enhancer	118	2.5 L reactor	Zang et al., 1995
DSPA alpha1	CHO	SV40 late	60	spinner flask	Petri et al., 1995
hu IFN alpha 2b	NSO	CMV promoter-enhancer	120	t-flask	Rossmann et al., 1996
huKGF	CHO	SV40 early	3	3.0 L reactor	Hsu et al., 1998
hu-prorenin*	CHO	RSV	10	23 L reactor	Mathews et al., 1996

Table 1.6: Expression levels of various recombinant proteins produced from stably transformed mammalian cells. *Reported for other expression systems in this thesis.

2 to 120 mg/L. Reports of continuous over-expression of intracellular proteins in mammalian cells are rare, because transformed cells offer few advantages over other systems for this class of proteins; (i) purification of an intracellular protein require steps to harvest and lyse cells, and would be complicated by the presence of other intracellular proteins; (ii) expression levels are low for intracellular proteins, presumably due to a cytotoxic accumulation within a cell that selects against high expressors in the transformation process; and, (iii) glycosylation of intracellular proteins rarely occurs and so lower eukaryotic or prokaryotic systems may be more suitable.

One major advantage of mammalian cells over other eukaryotic expression systems is their ability to perform complex glycosylation, a particularly important feature of proteins destined for *in vivo* human use. However, complex glycosylation in most mammalian cell lines used for recombinant glycoprotein synthesis can result in heterogeneous carbohydrate structures, that resemble, but are certainly not identical to the authentic protein produced in human tissue. For example, CHO, BHK, and mouse cell lines express α1,-3-galactosyltransferase, an enzyme that is not synthesized in humans, and results in a carbohydrate moiety on recombinant proteins that is the target of over 1% of human serum IgG (Hamedeh et al., 1992), probably because this moiety is present on enteric bacteria. In addition, the glycosylation pattern in mammalian cells can be affected by environmental conditions including the variation in batch culture (Goochee et al., 1990), the use of serum or serum-free media (Gawlitzek et al., 1995), cell growth rate (Hahn and Goochee, 1992), pH (Borys et al, 1993), and nutrient limitations (Gawlitzek et al., 1995).

Some disadvantages of stably transformed mammalian cell expression systems include the time it takes to generate stable, amplified cell lines overexpressing a recombinant protein (3-5 months; Coleman et al., 1997), the process is labor intensive, the transformed cell lines are not necessarily genetically stable (Weidle et al., 1988; Pallavicini et al., 1990), and the system is effective for only secreted and membrane proteins. Actually, for the study of biologically active membrane proteins of human or mammalian origin, mammalian cell lines often contain background levels of endogenous membrane proteins that may interfere with biological assays, requiring researchers to use cell lines of non-mammalian origin for this purpose.

1.3.4 Transient Expression in COS Cells

The transient expression of recombinant proteins in COS cells represents a compromise between viral expression systems, such as the baculovirus expression system, and transformed mammalian cells. In this system, a genetically modified African green monkey kidney cell line (CV-1) known as COS cells, can support limited replication of expression plasmids containing the SV40 origin replication. This allows rapid and high level expression of a recombinant protein for several days following transfection of COS cells with an expression plasmid, without impairing the host cells, and does offer some of the post-translational advantages of mammalian cell hosts including the potential for complex glycosylation.

To originally generate the COS cell line, CV-1 cells were transformed with an origin-defective SV40 virus that was integrated into the cell chromosomal DNA (Gluzman, 1981). COS cells express the SV40 large T-antigen, which is the only protein required *in trans* for SV40 replication, but cannot actually produce viral particles. However, transfection of COS cells with an SV40 origin-containing expression plasmid leads to extrachromosomal replication of the plasmid from 10,000 to 100,000 copies/cell (Mellon et al., 1981). The plasmid replication peaks at about 48 h post-transfection, whereupon the copy number gradually declines and the cells lose viability and die, presumably due to a cytotoxic effect from high levels of extrachromosomal replicating DNA (Gerard and Gluzman, 1985). This transient situation is sufficiently effective for a high level of transcription and translation of a desired gene under control of a suitable eukaryotic promoter, starting approximately 24 h post-transfection, reaching a maximum 72 h post-transfection, and continuing for 5-10 days (Edwards and Arrufo, 1993).

Clearly the major advantage of using this system is the short time period required to obtain recombinant protein. By subjecting a large number of cells to transfection, in the order of 10^8 cells per/batch in roller bottles or on microcarriers, multiple harvests of spent culture medium can lead to the cumulative production of several milligrams of recombinant protein in 5 days (Ridder et al., 1995). A sample of recombinant proteins expressed using this system, and their expression levels is shown in Table 1.7, which is generally in the order of 1.0 mg/L.

Protein	Promoter	Expression Level (mg/L)	Culture Conditions	Reference
C9*	SV40 late	0.1	static	Tomlinson et al., 1993
Hu-LIF*	pXMT-3	4-5	roller bottle	Geisse et al., 1996
scFv*	AdMLP	1-2	roller bottle	Ridder et al., 1995

Table 1.7: Expression levels of various recombinant proteins transiently produced by COS cells. *Reported for other expression systems in this thesis.

This system is sufficient for the rapid production of recombinant proteins on a research-scale. Obviously it is not suited to large-scale production due to the cost and technical difficulties of transfecting excessive numbers of cells. Although these cells may be capable of some complex glycosylation, shown by the appearance of sialic acid (Goelz et al., 1990), they do not express all the enzymes necessary for authentic glycosylation of human proteins. For example, COS cells do not express α-(1,3)fucosyltransferase which is needed to transfer fucose to sialyl or asialyl precursors (Goelz et al., 1990). Furthermore, proteins tend to be underglycosylated in COS cells (Aruffo and Seed, 1987; Geisse et al., 1997), suggested to be due to overburdening of the host cell glycosylation machinery with protein expressed from high copy numbers of expression plasmids.

1.4 Scope of Thesis

From the above review, it is clear that many different protein expression systems exist, each having their own advantages and disadvantages. To improve on this, our research is focused on the establishment of a stably transformed insect cell expression package, that is proven effective for the expression of most classes of biologically active recombinant proteins. The starting point for this technology is the Ph.D. thesis of Dr. Maolong Lu (Lu,1996). This type of transformed insect cell expression technology is also potentially useful as a research tool in the study of the molecular biology of insects. To achieve these research goals, the following items were addressed in this dissertation:

(1) Construction of a plasmid vector for the high level expression of recombinant proteins in transfected lepidopteran insect cells,

(2) Development of protocols for the generation of stably transformed insect cell lines,

(3) Evaluation of the capacity of stably transformed insect cell lines to produce secreted proteins on a research and large-scale,

(4) Genetic characterization of stably transformed insect cell lines,

(5) Extension of this expression system to the production of cytoplasmic and nuclear factors,

(6) Demonstration of the expression of biologically active membrane proteins in transformed insect cells,

(7) Establishment of a stably transformed packaging cell line for the generation of baculovirus artificial chromosomes (BVACS), and

(8) Generation of baculovirus artificial chromosomes for future use as protein expression vectors and for the creation of transgenic lepidopteran insects.

CHAPTER 2

General Materials And Methods

2.1 Plasmid DNA Preparation

2.1.1 Quick Miniprep

A single colony of *E. coli* HB101 (Boyer and Roulland-Dossoix, 1969) transformed with a pBluescript SK+ (pBs; Stratagene) based recombinant plasmid was inoculated into 2 mL LB medium containing 100 µg/mL of ampicillin and incubated at 37°C overnight. One hundred microliters of bacterial culture was pelleted at 3,000 rpm for 1 min in a benchtop centrifuge. The pellet was resuspended in 25 µL of ddH_2O and vortexed vigorously with an equal volume of phenol. After centrifuging for 2 min, 15 µL of the supernatant was mixed with 2.5 µL of 6X DNA dye [0.25% bromophenol blue, 0.25% xylene cyanol FF and 40% (w/v) glycerol]. The mixture was analyzed on a 1% agarose gel for supercoiled plasmid DNA. Supercoiled plasmid DNA could be easily discriminated from RNA and genomic DNA, and those plamids containing successfully ligated DNA inserts migrated more slowly than plasmids without an insert.

2.1.2 Miniprep

One and a half mL of an overnight bacterial culture was pelleted at 6,000 rpm for 5 min in a benchtop centrifuge and resuspended in 100 µL solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, and 10 mM EDTA pH 8.0). Then 200 µL of freshly prepared solution II (0.2 M NaOH and 1% SDS) were added and mixed gently to cause cell lysis and denature nucleic acid. After 5 min incubation on ice, 150 µL of solution III (90 µL of 3 M potassium acetate, 17.25 µL of glacial acetic acid, and 47.25 µL ddH_2O) mixed well and incubated on ice for 5 min for DNA renaturation and protein-nucleic acid complexes to precipitate. After 5 min spin at 14,000 rpm in a microcentrifuge to pellet debris, the supernatant was transferred to a fresh tube, and the aqueous phase containing the nucleic acid was extracted with 500 µL phenol to remove residual protein, followed by an extraction with 500 µL of chloroform:isoamly alchohol (95:5) to remove phenol. Nucleic acid consisting of plasmid DNA and bacterial RNA was precipitated with 1 mL of 95% ethanol, and pelleted by centrifuging at 14,000 rpm. The pellet was rinsed with 70% ethanol and dissolved in 50 µL ddH_2O containing 20 µg/mL DNAse-free RNAse.

2.1.3 Large-Scale DNA Preparation

A single colony was incubated 8 h in 2 mL of LB containing 100 µg/mL ampicillin and inoculated into 250 mL of terrific broth containing 100 µg/mL ampicillin and incubated overnight. Cells were pelleted by centrifugation at 4,500 rpm for 10 min in a Sorval GS3 rotor. The pellet was resuspended with 5 mL of solution I (as per minipreps) and incubated for 10 min with 1 mL of 10 mM Tris-HCl pH 8.0 containing 100 µg/mL hen egg white lysozyme. The cells were lysed and nucleic acid denatured for 10 min by adding 10 mL freshly prepared solution II, and DNA renatured by adding 7.5 mL of solution III and incubating on ice for 20 min. After centrifuging at 8,000 rpm in a SS23 rotor, the supernatant was mixed well with 0.6 volumes of isopropanol and stored at room temperature for 10 min. The nucleic acid from the supernatant was precipitated by centrifuging at 8,000 rpm for 10 min in a SS34 rotor and dissolved in 3 mL of TE (10 mM Tris-HCl pH 8.0 and 1 mM EDTA pH 8.0).

To further purify the plasmid DNA, 3.3 g of cesium chloride and 200 µL of 10 mg/mL ethidium bromide were added. The sample was spun at 8,000 rpm in an SS34 rotor and the clear supernatant was loaded into a 3.9 mL ultracentrifuge tube (Beckman) and centrifuged at 10,000 rpm for at least 5 h at 20°C in a TL-100 benchtop ultracentrifuge (Beckman) equipped with a TLN-100 rotor. After centrifugation, the band containing supercoiled plasmid DNA was recovered using a 1 mL syringe and a 21-gauge needle. Typically 0.5 mL of solution was collected. The ethidium bromide in the solution was removed by extraction several times with 1 mL of n-butanol saturated with 4mM NaCl and 10mM EDTA until the solution was completely colorless. The solution was diluted with 3 volumes of ddH_2O and plasmid DNA was precipitated with 2.5 volumes of 95% ethanol. After centrifuging at 10,000 rpm for 20 min in a SS34 rotor, plasmid DNA was dissolved in water and precipitated twice with 0.25 M mmonium acetate and 2.5 volumes of 95% ethanol. Finally the pellet was rinsed with 70% ethanol, dissolved in ddH_2O, and the DNA concentration was determined using a Beckman spectrophotometer.

2.2 DNA Manipulation

2.2.1 Preparation of Competent cells

E. coli strain HB101 was streaked onto a LB plate and incubated at 37°C for 15 h. A single colony was inoculated into 2mL of LB and cultured in at 37°C for 8 h. The culture was inoculated into 100 mL of LB and shaken vigorously until the OD_{600} reached 0.3 to 0.5. The culture was chilled on ice for 10 min and the cells were recovered by centrifugation at 4,000 rpm for 10 min in a sorval GS3 rotor. The pellet was resuspended in 50 mL of ice-cold 0.1 M $MgCl_2$ and stored on ice for 20 min. The cells were again pelleted and resuspended in 5 mL 0.1 M $CaCl_2$ and incubated on ice for 1 h. The suspension was mixed with 1.15 mL of 80% glycerol and 100 µL aliquots were rapidly frozen on dry ice and stored at -70°C for later use.

2.2.2 Purification of DNA Fragments

To isolate a DNA fragment, a restriction enzyme digested DNA sample or PCR sample was loaded onto an agarose gel and the fragments resolved by electrophoresis. A gel slice containing the desired DNA band was cut out and sealed in 8,000 MWCO dialysis tubing with 500 µL ddH_2O. The tubing was placed in an electrophoresis tank and electrophoresis continued for 15-60 min to elute the DNA fragment from the gel. The solution containing the fragment was collected, extracted with 500 µL of both phenol and chloroform:isoamyl alchohol (95:5), and precipitated with 0.25 M ammonium acetate, 2.5 volumes of 95% ethanol, and 10 µg yeast tRNA carrier. The nucleic acid was pelleted by centrifugation at 14,000 rpm for 10 min, rinsed with 70% ethanol, and resuspended in 20 µL ddH_2O.

2.2.3 Ligation

To ligate a DNA fragment into a plasmid vector, a 20 µL ligation mixture was prepared that contained 50-200 ng linearized vector, a 5-fold molar excess of insert DNA, 1 mM ATP, 50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 1 mM DTT, 5% (w/v) PEG 8000, and

1 unit of T4 DNA ligase (Life Technologies). For ligation of cohesive termini, the ligation mixture was incubated at 16°C for 2-16 h, while for ligation of blunt termini the ligation mixture was incubated at 20°C for 16 h.

2.2.4 Transformation

Ten microliters of ligation mixture was gently mixed with 100 µL freshly thawed competent cells and incubated on ice for 30 min. The sample was heat shocked for 2 min at 42°C, then mixed with 900 µL of LB, and incubated at 37°C for 30 min. The cells were pelleted by centrifugation at 6,000 rpm, resuspended in 100 µL of fresh LB and spread on a LB agar plate containing 100 µg/mL ampicillin. The plate was incubated overnight at 37°C for colonies to form.

2.2.5 Identification of Recombinant Clones

Pre-screening of individual plasmid DNAs presumed to contain a successfully ligated insert was done using quick minipreps of several colonies. The verification of the plasmid DNAs containing an insert was then undertaken by sequencing or the restriction enzyme digestion pattern of miniprep DNA.

2.2.6 Polymerase Chain Reaction (PCR)

To amplify DNA a fragment from a plasmid or virus template, the polymerase chain reaction was employed. Each 100 µL reaction contained 20 mM Tris-HCl pH 8.0, 10 mM NaCl, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.2 mM each of the four deoxynucleotide triphosphates, 1 unit of *Pfu* DNA polymerase (Stratagene), 5 ng of plasmid template or 20 ng of viral DNA, and 200 pmol of each primer. The amplification conditions were 30 cycles of (1) denaturation at 94°C for 1 min, (2) annealing at 40-55°C (depending on the primer pairs), and (3) extension at 72°C for 2.5 min per kilobase.

2.2.7 Sequencing

Sequencing of plasmid DNA was done by PCR using flourescent dideoxy-nucleotides. A 10 µL solution containing 1 µg plasmid template, 50 nmol of primer, 4 µL of

MIX (Perkin-Elmer) was subjected to 30 PCR cycles consisting of denaturing at 96°C for 30 s, annealing at 50°C for 30 s, and product extension at 60°C for 4 min. The product was precipitated with 2.5 volumes of 95% ethanol, 0.25 M ammonium acetate, and 10 µg yeast tRNA, and the dried pellet was transferred to the University of Calgary Core Sequencing Facility for analysis using acrylamide gel electrophoresis.

2.3 Cell Culture

2.3.1 Cell Lines

Three lepidopteran cell lines were used in this study. Bm5 cells were established from the ovarian tissue of the domesticated silkmoth *Bombyx mori* (Grace, 1967). Sf21 cells were established from the pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda* (Vaughn et al., 1977), and BTI-TN-5B1-4 cells (commonly referred to as High Five™ cells) were established from egg cell homogenates of the cabbage looper *Trichoplusi ni* (Granados et al., 1994)

2.3.2 Culture Media

The lepidopteran insect cells lines were routinely sub-cultured in IPL-41 insect medium (Life Technologies) supplemented with 2.6 g/L tryptose phosphate broth (Difco), 0.35 g/L $NaHCO_3$, 0.069 mg/L $ZnSO_4.7H_2O$, 7.59 mg/L $AlK(SO_4)_2.12H_2O$ and 10% fetal bovine serum (JRH Biosciences). The osmotic pressure was adjusted to 370 mOsm with 9.0 g/L sucrose, and pH adjusted to 6.2 with 10 M NaOH prior to sterile filtering through 0.2 µm filter units (Nalgene). For growth in serum-free media (SFM), two commercial formulations using IPL-41 basal media, EC400 and EC401 (JRH Biosciences) containing less than 10 mg/L protein, were used. No antibiotics were used in media for routine subculturing, however 50 µg/mL gentamycin sulphate (Life Technologies) was used in media for most experiments.

2.3.3 Culture Maintenance

The lepidopteran cell lines were maintained in CO_2 free incubators at 28°C. Cells were subcultured weekly in 25 cm^2 T-flasks at a dilution factor of 1:5 with fresh media.

To preserve cell lines as frozen stocks, the cryopreservant dimethyl sulfoxide (DMSO; Sigma) was used. A freezing medium was prepared that contained either 90% FBS and 10% DMSO for those cells maintained in serum-containing medium, or 50% SFM, 40% conditioned SFM and 10% DMSO for those cells maintained in serum-free medium. Cells for freezing were removed from one 25 cm^2 T-flask and centrifuged at 150g for 5 min. The supernatant was poured off, and the cells resuspended in 5 mL freezing medium at a cell density of approximately 2 x 10^6 viable cells/mL. One milliliter aliquots of this cell suspension were transferred to cryovials (Nunc) which were placed inside a styrofoam box at -70°C to cool slowly overnight. The vials were subsequently transferred to a liquid nitrogen container for long term storage.

To recover frozen cell lines, one cryovial was removed from liquid nitrogen and rapidly thawed in a 28°C water bath. The cells were then placed in a 25 cm^2 T-flask with 4 mL fresh media, allowed to adhere for 5 h at 28°C, followed by replacement of the culture medium containing DMSO and dead cells with 5 mL fresh media.

The trypan blue exclusion method (Fresney, 1987) was used to estimate the cell density and viability of cell cultures. This method is based on the fact that viable cells are impermeable to trypan blue, whereas dead cells are permeable to the dye. Usually a cell culture sample was diluted 1:3 with 0.1% trypan blue in phosphate buffered saline (PBS; 10 mM KH_2PO_4, 2 mM NaH_2PO_4, 140 mM NaCl, 40 mM KCl), and samples were counted at least twice in a hemocytometer.

2.3.4 Transfection

For transfer of foreign DNA into cultured insect cells, a cationic liposome forming compound was used (Lipofectin). These positively charged liposomes are attracted to both DNA, which is negatively charged, and cell membranes which are composed mostly of lipids themselves. Insect cells were prepared for transfection by diluting them in fresh medium to a density of 5 x 10^5 viable cells/mL, and transferring 2 mL of the cell suspension to each well of a 6-well tissue culture plate (35 mm diameter, Falcon), to allow adherence overnight. A transfection solution was prepared that contained 30 µg/mL Lipofectin (Life Technologies) and 6 µg/mL total plasmid DNA in basal IPL-41. The lipid and DNA were diluted separately in basal IPL-41 before being combined, and the transfection solution was incubated on ice for 15 min. The cells were then washed twice with 1 mL basal IPL-41 and incubated at 28 °C with 0.55 mL transfection solution per well. After 5 h transfection, cells were rinsed once with basal IPL-41 and 2 mL complete medium was added to the well. Samples were taken for analysis 2 or 3 days following transfection.

2.3.5 Infection

Tissue culture cells for baculovirus infection were seeded into 6-well tissue culture plates at a density of 10^6 viable cells per well to allow adherence overnight. The cell monolayers were incubated for 1-2 h at room temperature with 1 mL medium containing baculovirus diluted to a multiplicity of infection of 5 units/cell. Following adsorption, the infected cells were rinsed twice with fresh medium, 2 mL of fresh medium was added to each well, and the plates were returned to the 28°C incubator. Time 0.0 h was defined as the end of the adsorption period.

2.3.6 Spinner Flask Culture

For cell growth in stirred suspension culture, 125 mL spinner flasks (Corning) were used with a working volume of 100 mL. Spinner flasks were placed on Cellgro multi-stirrer plates (Thermolyne) located inside a humidified 28°C incubator. The agitation rate of the magnetic stirrer paddle inside each spinner flask was controlled by magnetic stirrers on the plate. The stirrer speed was normally set at 60 rpm. For insect cell characterization in suspension culture, cells from a T-flask or spinner flask were diluted to 10^5 viable cells/mL in 100 mL fresh medium and transferred into a fresh spinner flask.

2.4 Nucleic Acid Detection

2.4.1 Extraction of Wild Type BmNPV DNA from Occluded Virus

The procedure was modified from Iatrou et al. (1985). *Bombyx mori* 4th instar larvae were infected with 20 µL of tissue culture supernatant containing budded BmNPV by injection into the hemolymph through the larval footpads. Ten days post infection the larvae were homogenized with a mortar and pestle in 5 mL of ddH_2O and the mixture was filtered through glasswool. The filtrate containing occlusion bodies was centrifuged at 5,000 rpm in a benchtop centrifuge for 5 min and rinsed 2 times in PBS. The pellet was resuspended in 1 mL of 10 mM Tris-HCl pH 7.8 and 0.4% SDS and rocked gently for 2 h prior to being centrifuged at 110,000g (24,000 rpm in a Beckman SW27 rotor) for 4 h at 15°C on a 30 mL cushion of 65% (w/v) sucrose in 10 mM Tris-HCl pH 7.8 and 10 mM EDTA. The pellet containing pure occlusion bodies was suspended in 1 mL of 0.25 M Tris-HCl pH 7.8 and centrifuged 2 times at 3,000 rpm. The final pellet was stored at -20°C for later use.

To purify viral DNA, occlusion bodies were dissolved in a buffer containing 0.1 M Na_2CO_3, 10 mM EDTA and 0.1 M NaCl pH 10.8 and gently rocked for 1 h at room temperature. Following this, the solution volume was increased by 50% with ddH_2O and the solution finally made 1% with respect to SDS. After the insoluble matrix was removed by centrifugation, the supernatant was extracted 3 times with phenol and chloroform. Viral DNA in the aqueous phase was precipitated in 0.25 M ammonium acetate and 2.5 volumes of 95% ethanol. The DNA was pelleted by centrifugation at 14,000 rpm, and the pellet rinsed with 70% ethanol. The DNA was resuspended in 10 mM Tris HCL pH 8 and 0.1 mM EDTA (TE) and its concentration determined by spectrophotometry. The quality of the DNA was verified both by its ability to be digested with restriction enzymes, and the absence of protein, genomic DNA and RNA when visualized on a 1% agarose gel following electrophoresis. Typically, 1 µg of viral DNA per 10^8 occlusion bodies was recovered.

2.4.2 Isolation of Nucleic Acid from Bm5 cells

One million tissue culture cells were pelleted from culture supernatant and washed 3 times with 1 mL PBS. The cells were resuspended in 500 µL lysis buffer (100 mM-Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 0.2 M NaCl, and 100 µg/mL proteinase K) and incubated overnight at 37°C. The sample was then extracted with 2 cycles of phenol and chloroform, and precipitated with 0.25 M ammonium acetate and 2.5 volumes of ethanol. The nucleic acid was pelleted by centrifugation at 14000 rpm for 10 min, resuspended in TE containing 1 µg/mL RNAse and incubated at 37°C for 1 h. The sample was further extracted with 2 cycles of phenol and chloroform and the aqueous phase containing nucleic acid was precipitated with 0.25 M ammonium acetate and 2.5 volumes of ethanol. The DNA was pelleted by centrifugation at 14,000 rpm for 10 min and the pellet rinsed with 70% ethanol and prior to dissolving in 100 µL TE.

2.4.3 Preparation of Radiolabelled ^{32}P Probes

Radioactively labeled probes were generated by random oligonucleotide labeling (Feinberg and Vogelstein, 1983) of the relevant restriction fragment in the presence of α-^{32}P-dCTP. Thirty two microliter of solution containing 0.1- 0.5 µg of DNA was boiled for 5 min, and cooled rapidly on ice. The following components were added: 10 µL of 5x OLB buffer (see below), 2 µL of 10 mg/mL BSA, 5 µL of α-^{32}P-dCTP (NEN, 3,000 Ci/mmol, 10 mCi/mL) and 1 µL (2-3 units) of large fragment of DNA polymerase I. The mixture was incubated for 1 h at 37 °C, and DNA precipitated by adding 100 µL of 95% ethanol, and 5 µg of yeast tRNA carrier. The pellet containing the probe purified from free nucleotides was resuspended in 50 µL ddH_2O. The probe specific activity was usually 2.5×10^8 cpm/µg.

The 5x OLB buffer is solution A: Solution B: Solution C (100:250:150). Solution A contains 1 mL solution O (1.25 M Tris-HCl pH 8 amd 0.125 M $MgCl_2$), 18 µL beta-mercaptoethanol, 5 µL each of 0.1 M sATP, dTTP, dGTP (previously dissolved in 3 mM Tris-HCl pH 7.0 and 0.2 mM EDTA). Solution B contains 2 M Hepes pH 6.6 adjusted with 4 M NaOH. Solution C contains random hexanucleotides (Life Technologies) in 3 mM Tris-HCl pH 7.0 and 0.2 mM EDTA at 90 OD/mL.

2.4.4 Southern Hybridization

Digested DNA fragments were resolved by electrophoresis in 0.8 % (w/v) agarose gel with EtBr. Following photography, the gel was denatured twice for 20 min each in 0.4 M NaOH and 1 M NaCl, and neutralized twice for 30 min each in 1 M Tris-HCl pH 7.4 and 1.5 M NaCl. The DNA fragments were transferred with 10x SSC (1.5 m NaCl and 0.15 M tri-sodium citrate; adjusted to pH 7.0 with 10 M NaOH) to a Hybond N^+ nylon membrane. After transfer overnight, the membrane was rinsed in 4x SSC and baked at 80°C for 1 h.

The membrane was prehybridized for at least 2 h in a hybridization mixture containing 0.3 M NaCl, 50 mM sodium phosphate pH 7.0, 5x Denhardt's (Denhardt, 1966), 10% Dextran sulphate, 1% SDS, 5 mM EDTA and 2.5 mg/mL total yeast RNA. The probe was denatured by boiling for 5 min, and hybridization was carried out at 63°C for 12-16 h with 5 x 10^5 cpm ^{32}P-labeled probe/mL of hybridization solution. Following hybridization, the membrane was washed at 63°C with 2x and 0.1x SSC containing 0.1% SDS twice for 20 min each time. Finally, the membrane was exposed to autoradiograph film at -20°C.

2.4.5 Dot Blot Hybridization

Supernatant or cell samples for dor blot hybridization were loaded into a dot blot vacuum manifold, and a vacuum was applied to draw the sample containing DNA, cells, or virus, onto a nylon membrane (Hybond N^+). The membrane was removed and laid on 35 MM Whatman paper soaked with 0.5 M NaOH to lyse cells, expose and denature DNA and hydrolyse RNA for 5 min. This was repeated and the membrane was neutralized on 35 MM Whatman paper soaked with 0.5 M Tris-Cl pH 7.5 twice for 5 min each. The membrane was then baked for 1 h at 80°C to crosslink DNA to the membrane and the hybridization with a α-^{32}P-dCTP labelled probe was performed as described in a Southern Hybridization.

2.5 Recombinant Protein Detection

2.5.1 Soluble Protein Extraction

Transfected cells were harvested 48-60 h post-transfection, pelleted at 3,000 rpm for 5 min in a microcentrifuge and washed 3 times with 1 mL of PBS. The cell pellet was resuspended in 100 µL of 0.25 M Tris-Cl pH 7.8, and the cells were lysed by freeze-thawing three times. The debris was pelleted by centrifugation for 5 min at 14,000 rpm, and the supernatant containing the soluble protein was transferred to a new eppendorf tube. Protein concentration was determined by the Bradford assay (Bradford, 1976) using Biorad protein assay reagent and bovine serum albumin (BSA) as a standard.

2.5.2 Preparation of Total Cell Extracts for SDS-Page

Transfected cells were counted 48-60 h post-transfection, pelleted at 3,000 rpm for 5 min in a microcentrifuge and washed 3 times with 1 mL of PBS. Aliquots containing 2.5 x 10^4 - 1.0 x 10^5 viable cells were pelleted and resuspended in 15 µL ddH_2O and 15 µL 2X SDS-Page sample buffer. The viscosity of the samples was reduced by mild sonication for

10 s to shear nucleic acid.

2.5.3 Western Blot Analysis

Sample aliquots containing recombinant proteins were resolved by electrophoresis in a SDS-containing 8-12% acrylamide (SDS-PAGE), and electroblotted onto nitrocellulose Hybond-ECL membrane (Amersham) overnight at 30 V in the cold. After the transfer, the filter was blocked for 1 h at room temperature in 50 mL PBS-0.1% Tween-20 (PBST) containing 10% (w/v) skim milk powder (PBSTM). The filter was incubated for 1 h at room temperature with 5 mL PBST containing species 1 (e.g.: rabbit) primary antibody recognizing the antigen. The filter was washed twice for 15 min with PBST, and incubated 1 h with 5 mL PBSTM containing horseraddish peroxidase-conjugated species 2 anti-species 1 (e.g. goat anti-rabbit) IgG. After washing twice with PBST, the filter was incubated with ECL chemiluminescent substrate (Amersham) according to the supplier's instructions and exposed to X-ray film.

2.5.4 Immunofluorescent Labelling

Five million cells in cell culture media were pelleted by centrifugation at 200g , 4°C, for five minutes and washed once in 10 mL of cold PBS. Cells were gently resuspended in 1 mL 2% paraformaldehyde fixative, 0.5x PBS, 0.1% Triton X-100 permeant, and incubated at 4°C for 30 min. The cells were pelleted, washed twice in 10 mL of PBS and resuspended in 250 µL of primary antibody diluted in PBS. After 1 h incubation at 4°C, the cells were washed twice in 10 mL of PBS, resuspended in 250 µL of fluorescein isocyanate-conjugated secondary antibody diluted in PBS and again incubated for 1 h at 4°C. Finally, the cells were washed in 10 mL of PBS and resuspended in 100 µL of PBS prior to visualization using a fluorescent microscope.

2.5.5 Immunofluorescent Labeling of Live cells for FACs Analysis and Sorting

For immunofluorescent labeling of membrane proteins on live cells, fixation using paraformaldehde solution was not necessary. After washing 5 x 10^6 tissue culture in sterile PBS, the cells were labeled with primary and secondary antibody as described in Section

2.5.4. The labeled cells were then analyzed by FACscan (Becton-Dickinson) which recorded forward scatter (FSC), side scatter (SSC) and green fluorescence (FL1). Live labeled cells were sorted using a FACsorter (Becton-Dickinson) located in the University of Calgary Flow Cytometry Facility.

2.5.6 CAT Assays

The soluble proteins from transfected or transformed cells were extracted and quantified as described in section 2.5.1. The desired amount of protein was brought to 100 µL with 0.25 M Tric-HCl pH 7.8 and mixed with 20 µL of 8 mM acetyl-CoA and 20 µL of 1 mM ^{14}C-chloramphenicol. The reaction was incubated at 37 °C for 1 h and stopped by extraction with 500 µL ice-cold ethyl acetate. The organic phase was transferred to a new tube, lyophilized and dissolved in 15 µL of ethyl acetate. The solution was spotted on a thin layer chromotography plate and resolved with chloroform:methanol (95:5). The TLC plate was exposed to X-ray film overnight at room temperature.

2.5.7 JHE Assays

JHE activities were determined using the partition method (Hammock and Sparks, 1977; Philpott and Hammock, 1990). Supernatant was diluted in 300 µL of 0.2 M sodium phosphate buffer (pH = 7.4) and incubated in for 15 min at 30 °C with a mixture of tritiated juvenile hormone III (JH III; NEN, 13 Ci/mmol) and unlabelled JH III substrate (Sigma Chemicals) at a total concentration of 5×10^{-6} M. Juvenile hormone acid was separated from the substrate following the addition of 150 µL methanol:water:ammonium hydroxide (10:9:1) and extraction with 450 µL trichloroethylene. Two-hundred-microliter aliquots of the aqueous phase (containing JH acid) were counted in a liquid scintillation counter and compared with the equivalent amount of unreacted substrate, to determine the amount of JH converted to JH acid. Sampes were assayed at least in duplicate. Calculated JHE activities nmol JH III hydrolysed/min/mL are reported as JHE concentrations (mg/L) based on the reported specific activity of purified JHE as 1,400 nmol JH III hydrolysed per min per mg (Philpott and Hammock, 1990).

2.5.8 LacZ Staining

The presence of β-galactosidase in cells and animal tissue samples is confirmed when a blue precipitate forms in β-galactosidase staining assays. It was found that unfixed cultured cells could be stained, even in the presence of 50% cell culture medium, while tissue should be initially fixed in several milliliters of 3.7% formaldehyde in PBS. After washing with PBS, a staining solution [0.1 mL of 0.5 M potassium ferrocyanide, 0.1 mL of 0.5 M potassium ferricyanide, 20 μL of 1 M $MgCl_2$, 0.25 mL of 40 mg/mL X-galactosidase, 1 mL of 10X PBS, and 8.53 mL ddH_2O] was added to the samples and incubated at room temperature for at least 4 h until a blue precipitate formed.

CHAPTER 3
Development of Transformed Insect Cell Expression Technology

3.0 Summary

In this chapter, an expression cassette and protocols for continuous, high-level expression of secreted glycoproteins by transformed lepidopteran insect cells were developed. The expression cassette utilizes the promoter of the silkmoth cytoplasmic actin gene to drive expression of foreign gene sequences, and also contains the *ie-1* transactivator gene and the HR3 enhancer region of BmNPV to stimulate gene expression. Using an antibiotic resistance selection scheme, a cloned Bm5 cell line (silkmoth) transformed with the expression cassette containing the secreted glycoprotein juvenile hormone esterase (JHE) as a reporter gene, produced 120 µg/mL active JHE in batch static culture, 210 µg/mL in stirred suspension culture, and 150 µg/mL in serum-free medium in static culture. This compares favourably with the baculovirus expression system (AcNPV infected Sf21 cells), that could only produce 4 µg/mL active JHE in static cultures. This cell line exhibited stable recombinant protein expression for over 4 months, and lepidopteran insect cells other than Bm5 cells were also shown to be equally efficient for producing recombinant proteins with this expression cassette. Finally, the potential for a super-inducible expression cassette is demonstrated.

3.1 Introduction to Expression from Stably Transformed Insect Cell Lines

The production of recombinant proteins in stably transformed insect cells provides advantages of both the baculovirus expression system and transformed mammalian cells for the production of secreted and membrane proteins (Sections 1.3.1 and 1.3.2). Insect cell lines are safe to humans, introns can be spliced from expressed genomic DNAs, and lysis does not occur therefore limiting proteolysis and facilitating purification of secreted proteins. Furthermore, expression from transformed cells is continuous, insect cells can perform most essential post-translational modifications as efficiently as mammalian cells, membrane proteins can be expressed in a stable physiological environment, most insect cell lines can grow well in serum-free medium to high cell densities, and many insect cell lines, particularly of lepidopteran origin, are already well characterized in large-scale suspension culture due to their role as hosts in the baculovirus expression system.

To date the *Drosophila melanogaster* (from the insect order Dipthera) expression system is widely used. This was developed by SmithKline-Beecham Pharmaceuticals and has been recently made commercially available through the Invitrogen Corporation. In this system, an expression vector is introduced into the *D. melanogaster* Schneider 2 cell line (S2 cells; Schneider, 1972) by co-transfection with a second plasmid conferring resistance to the *E. coli* hygromycin B, and stably transformed polyclonal cell lines result after approximately three weeks of hygromycin B selection (Johanson et al., 1989). Recombinant protein expression is induced from the *Drosophila* metallothionein promoter by the addition of a heavy metal such as copper sulphate. S2 cells can grow in serum-free medium in suspension culture to cell densities up to 15 x 10^6 cells/mL, however S2 cells are substantially smaller than Sf21 or Bm5 cells, and secrete large amounts of endogenous contaminating proteins (Dr. Allan Shatzman, SmithKline-Beecham, PA, personal communication). Enzymes, membrane receptors, ion channels, viral antigens and monoclonal antibodies have been successfully produced using this system, however expression levels are generally lower than those from transformed mammalian cells (Table 3.1).

In parallel with the development of the *Drosophila* expression system, a transformed lepidopteran insect cell expression system was developed by Dr. Don Jarvis at Texas A&M

Protein	Expression Level	Reference
Secreted Glycoprotein		
sol hIL-5*	22 mg/L	Johansen et al., 1995
IgG_1	>1 mg/L	Kirkpatrick et al., 1995
gp120	5-35 mg/L	Ivey-Hoyle et al., 1991
gp120	2 mg/L	Culp et al., 1991
Cox-2	12 mg/L	Percival et al., 1997
Ion Channel		
GABA receptor*	$3.5x10^4$ sites/cell	Millar et al., 1994
Membrane Receptor		
hIL-5Rα	$1x10^6$ sites/cell	Johansen et al., 1995
Intracellular		
Dopamine β-hydroxylase	>16 mg/L	Li et al., 1996
H-ras	0.2-0.5% cell protein	Johansen et al., 1989

Table 3.1: Expression levels of various proteins produced from the *Drosophila* expression system. *Reported for other expression systems in this thesis.

University (Jarvis et al., 1990) and is currently marketed by Novagen. To generate stably transformed cell lines, Sf9 cells (Summers and Smith, 1987) are initially co-transfected with a neomycin resistance plasmid and an expression vector employing the immediate early gene promoter (IE1; Guarino and Summers, 1987) of the *Autographa californica* nuclear polyhedrosis virus (AcNPV), followed by selection and isolation of G418 resistant clones over a period of 4 weeks following transfection (Jarvis and Guarino, 1995). This system has been used to express intracellular enzymes, membrane receptors, ion channels, and secreted proteins (Table 3.2), however expression levels are low compared to the baculovirus or transformed mammalian expression systems. Recently, a selection marker that uses puromycin actetyl-transferase to confer resistance to puromycin in mammalian cells (Vara et al., 1986) was reported to function in lepidopteran insect cells (McLachlin and Miller, 1997). This marker appears to allow selection of stably transformed Sf21 cells in less than one week because puromycin acts within hours, whereas Sf9/Sf21 cells continue to divide for a short time in the presence of lethal doses of G418 (Jarvis and Guarino, 1995). Furthermore, the use puromycin is significantly cheaper than G418.

In this chapter, a novel transformed insect cell expression system is described. This system maintains the advantages of transformed cell technology and exceeds the glycoprotein expression levels obtained from all other expression systems including the baculovirus expression system, transformed mammalian cells, as well as the other transformed insect cell expression systems. In this new system, lepidopteran insect cells, such as silkmoth Bm5 cells (Grace 1967), are co-transfected with an expression vector and a plasmid conferring resistance to growth inhibition by hygromycin B (HmB), followed by selection and cloning in HmB. The key to high expression lies in the employment of three genetic elements in the expression cassette. These are, the promoter of the cytoplasmic actin gene of the silkmoth *Bombyx mori* (Mounier and Prudhomme, 1986; Johnson et al., 1992) that is used to drive foreign gene expression; a complete copy of the immediate early gene (*ie-1*) of BmNPV, whose protein product, IE-1, is a transcription factor capable of stimulating the *in vitro* rate of transcription of the actin promoter by up to 100-fold (Lu et al., 1996); and the homologous repeat 3 (HR3) region of the nuclear polyhedrosis virus of *B. mori* (BmNPV) that acts as a transcriptional enhancer of the silkmoth actin promoter *in vitro*

Protein	Expression Level	Reference
Secreted		
t-PA*	1 mg/L	Jarvis et al., 1990
Ion Channel		
GABA*	n.r.	Joyce et al., 1993 Huybrechts et al., 1993
Intracellular		
B-gal*	0.04-2 mg/L	Jarvis et al., 1990
ABP1	n.r.	Henderson et al., 1995
p35	n.r.	Cartier et al., 1994
bcl-2	n.r.	Cartier et al., 1994
Membrane Receptor		
hB_2AR	$3.5x10^4$ sites/cell	Kleymann et al., 1993

Table 3.2: Expression levels of various recombinant proteins produced in transformed lepidopteran cells. *Reported for other expression systems in this thesis.

by two orders of magnitude (Lu et al., 1997). Linkage of the *ie-1* gene and the HR3 element with the actin gene promoter results in a stimulation of foreign gene expression directed by the actin promoter by over 1,000-fold (Lu et al., 1997), providing the motivation for developing this system.

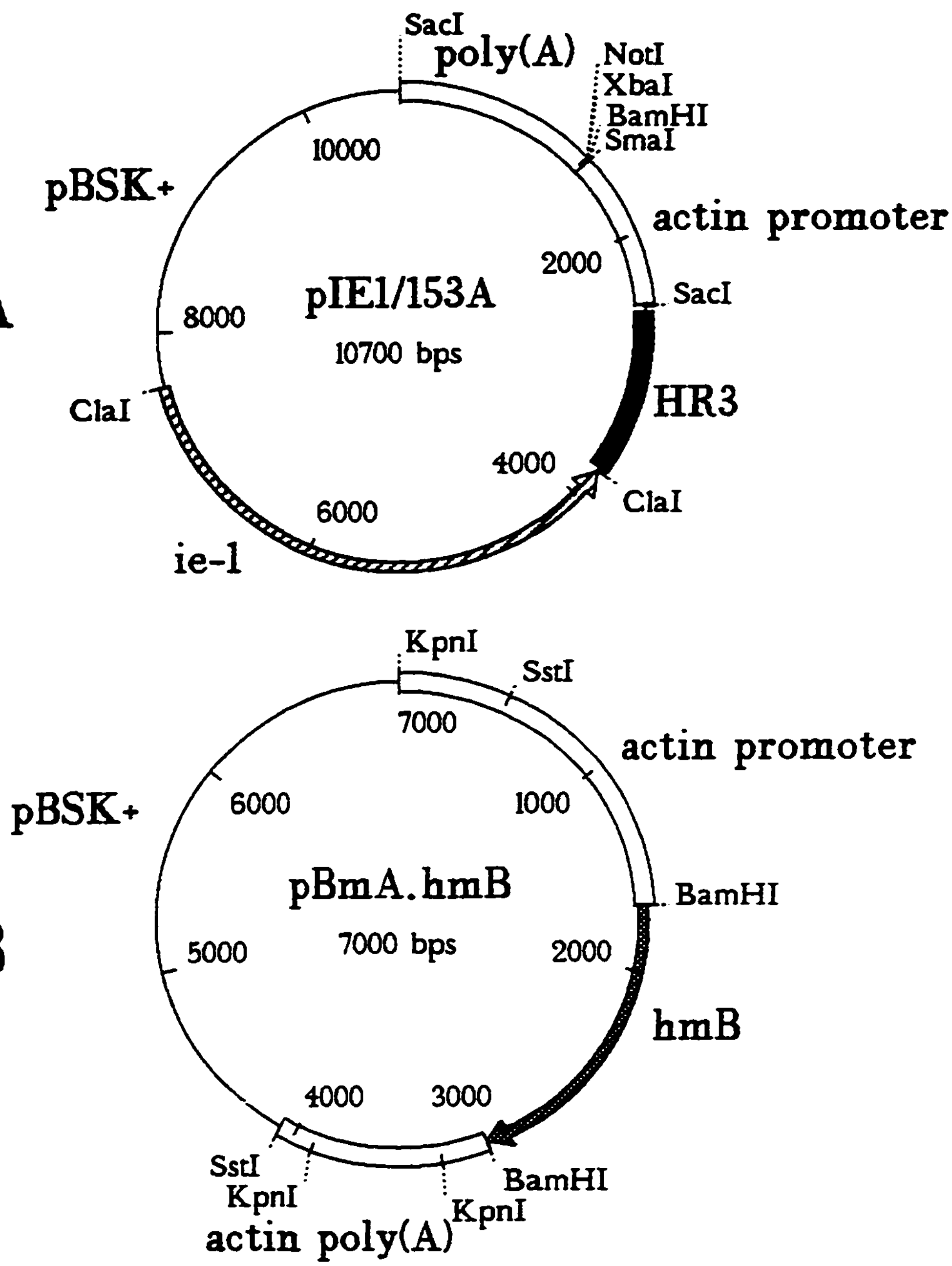
To demonstrate the efficacy of this new system, a naturally secreted insect glycoprotein, juvenile hormone esterase (JHE), was used as a reporter protein. The cDNA for JHE was isolated from the moth *Heliothis virescens* (Hanzlik et al, 1989), and encodes for a mature polypeptide with a calculated molecular mass of 61 kDa. Favourable characteristics for the use of JHE as a reporter protein include its stability at room temperature (Ward et al, 1992), the fact that its activity can be rapidly and accurately detected using a commercially available radiolabelled substrate (Hammock and Roe, 1985), and expression levels obtained using the BES have been previously reported and can be used for comparison (Bonning et al, 1994; Bonning and Hammock, 1995).

3.2 Materials and Methods

3.2.1 Plasmid Constructions

The expression cassette pIE1/153A (Figure 3.1A) was constructed as follows. A 3.8 kb *ClaI* fragment from pBmIE1 (Lu et al, 1996) containing the *ie-1* gene was cloned into the *ClaI* site of the plasmid p153 (Lu et al., 1997) containing the HR3 element of BmNPV, to yield the plasmid pIE1/153. Unwanted cloning sites in remaining pBSK+ polylinker of pIE1/153 were removed by a *SacII* and *BamHI* digest, followed by blunt ending with T4 DNA polymerase and self ligation to yield the vector pIE1/153'. Next, a 2.2 kb *SacI* fragment isolated from pBmA (Johnson et al., 1992) and containing the actin cassette was ligated into the unique *SacI* site of pIE1/153' to form the vector pIE1/153A. The plasmid pIE1/153A.gpf+ was generated as follows. The plasmid pBs.GFP+ (Ha et al., 1996) was linearized with *HindIII*, blunt ended with T4 DNA polymerase, digested again with *NotI*, and the 0.74 kb fragment containing the green fluorescence protein open reading frame was isolated and cloned into the unique *SmaI/NotI* sites of pIE1/153A. To generate the expression cassette pIE1/153A.jhe(kk), *NotI* linkers were ligated to the 1.8 kb *EcoRI* fragment from pAcUW21-KK (Bonning et al., 1995), containing a modified version of the juvenile hormone

A



B

Figure 3.1: Plasmid vectors used for the generation of insect cell lines overexpressing recombinant proteins. (A) The plasmid pIE1/153A.JHE contains the actin promoter, actin polyadenylation, and transcription signals (unfilled blocks), the BmNPV HR3 region (black block), and the BmNPV *ie-1* gene (striped arrow). (B) The plasmid pBmA.HmB contains the actin cassette and the hygromicin B resistance gene (dotted region). Both plasmid use a Bluescript SK+ plasmid backbone and arrows indicate the direction of transcription.

esterase (JHE) cDNA, and this fragment was digested with *NotI* and cloned into the *NotI* site of pBSK+ to yield the plasmid pjhe(kk). The 1.8 kb *NotI* fragment was then isolated from pjhe(kk) and cloned into the *NotI* site in the actin cassette of the expression vector pIE1/153A to yield pIE1/153A.jhe(kk). Plasmid pBmA.hmB (Figure 3.1B) was generated by inserting a 1.4 kb *Bam*HI fragment containing the *E. coli* hygromycin-B-phosphotransferase gene from pT676 (Giordano and McAllister, 1990) into the *BamHI* site of pBmA.

3.2.2 Stable Cell Transformation

To obtain stably transformed cell lines, Bm5 cells were seeded into 6-well culture plates (35 mm diameter) at a density of 5 x 10^5cells/mL (2 mL per well), and transfected for 5 h with 0.55 mL of transfection solution containing 30 µg/mL lipofectin (Life Technologies) and 6 µg/mL total plasmid DNA in basal IPL-41 medium. Forty eight hours after transfection, the culture medium was replaced with fresh medium containing 0.25 mg/mL HmB (Boehringer-Mannheim). Heterogeneous (polyclonal) populations of transformed cells expressing recombinant protein were obtained by weekly subculturing in 6-well plates in the presence of HmB. If the cell density during selection dropped below 10^4 cells/mL, 50% conditioned medium was used to support cell growth. Cloned cell lines were isolated by limiting dilution in the presence of 50% conditioned medium.

3.2.3 Expression of Recombinant Proteins from Stably Transformed Cells

To assess recombinant protein production from stably transformed Bm5 cells in static cultures, cells were seeded into 6-well plates at a cell density of 5 x 10^5cells/mL in 2 mL of fresh medium. In suspension culture, transformed Bm5 cells from T-flasks were inoculated at a density of 1 x 10^5 viable cells/mL into spinner flasks with an initial volume of 100 mL.

3.2.4 Baculovirus Expression

To assess JHE production in baculovirus infected Sf21 cells, cells were seeded into 6-well plates at a cell density of 1 x 10^6viable cells per well, allowed to adhere and infected with supernatant containing AcJHE-KK (Bonning et al., 1997) or AcNoSPJHE (Dr. Bruce

Hammock, U.C. Davis, unpublished) at a multiplicity of infection of 5. Following infection for 1 h, 2 mL of fresh media was added to each well and aliquots withdrawn at various time points for analysis.

3.2.5 Flow Cytometry

Transfected cell populations that were over 90% viable and expressed GFP^{+} were suspended in PBS and directly analyzed by a FACscan (Becton-Dickinson), which recorded forward scatter (FSC), side scatter (SSC) and green fluorescence (FL1). The FACs settings for insect cells were: FSC (E00, linear), SSC (level = 360, linear scale, amplification = 1.0, threshold = 48), and FL1 (level = 300, log scale). From this data, cells were first isolated from debris by their FSC versus SSC characteristics. The transfection efficiency was determined by subtracting those cells displaying background FL1 (control cells transfected with the vector pIE1/153A) from the total cell population.

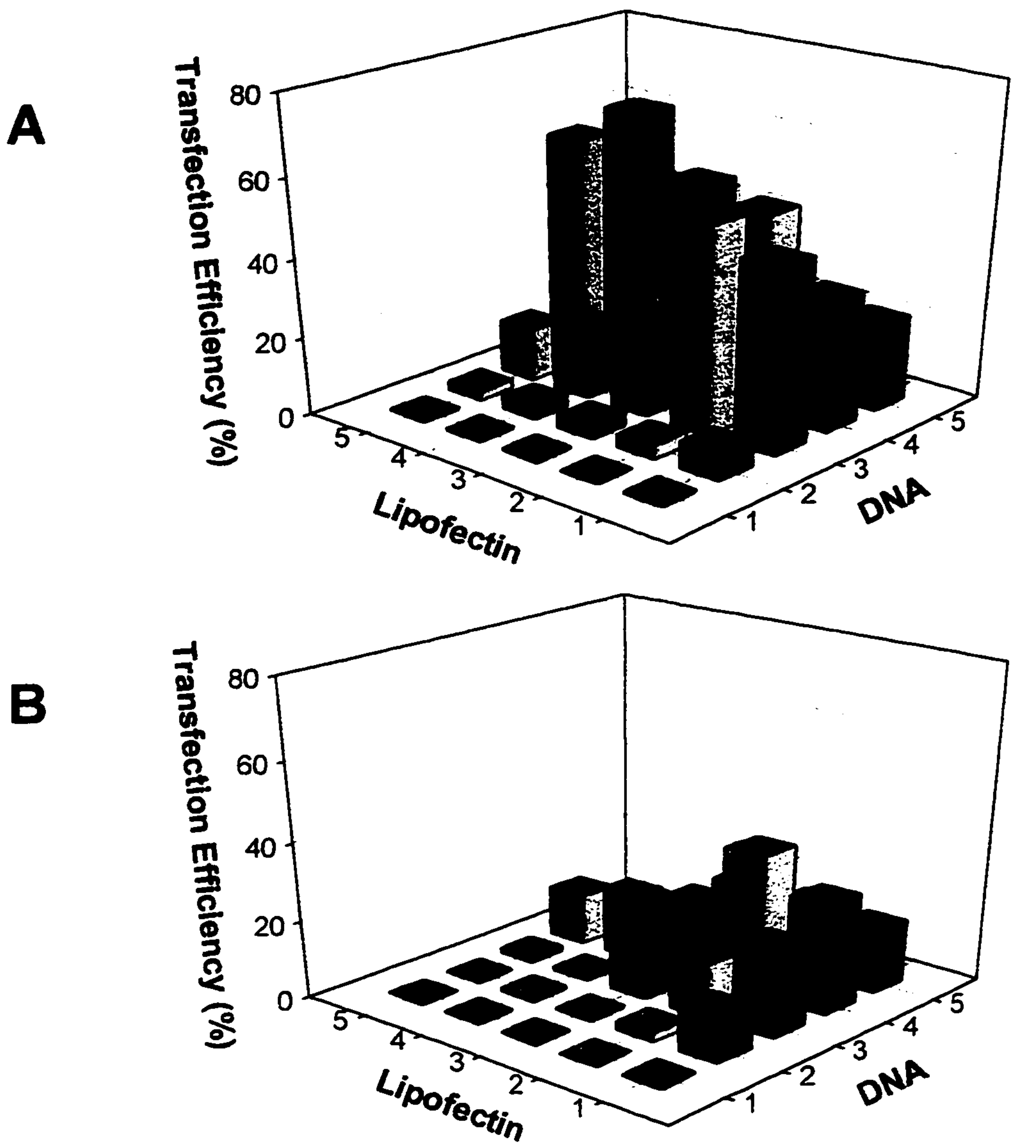
3.3 Results

3.3.1 Optimisation of Transfection Conditions with GFP+

Transfection conditions for cells maintained in serum-containing and serum-free medium were optimized by varying the concentration of Lipofectin (1-100 µg/mL) and plasmid DNA (1-20 µg/mL) in the transfection solution. Green fluorescence protein was a convenient reporter gene in the expression cassette pIE1/153A.GFP+ because it could be directly detected by flow cytometry without any treatment of the cells. Three days post-transfection, cells were analyzed for the expression of GFP+. A summary of the optimization results are shown for cells maintained in serum-containing or serum-free medium in Figure 3.2. From these results, superior transfection efficiencies occured when using 30 µg/mL Lipofectin and 2.5-10 µg/mL plasmid DNA. Furthermore, transfections were more successful when cells maintained in serum-containing medium were used.

3.3.2 The Expression Plasmid pIE1/153A Stimulates Expression from the Basic Actin Cassette

To verify that super-activation of transgene expression resulted from the presence



CODE	1	2	3	4	5
Lipofectin (µg/mL)	1	5	10	30	100
DNA (µg/mL)	1	2.5	5	10	20

Figure 3.2: Optimization of transfection conditions of cells maintained in (A) serum-containing, and (B) serum-free medium. The transfection efficiency was determined by FACs analysis of pIE1/153A.gfp+ transfected cells at varying amounts of DNA and lipofectin in the transfection solution.

of BmIE1 and HR3 in the expression cassette pIE1/153A, Bm5 cells were transfected with either pBmA.jhe(KK) (Lu et al., 1995), pIE1/153A.jhe(kk), or co-transfected with pBmA.jhe(kk) and pBmIE1 (Lu et al., 1996). As shown in Table 3.3, the JHE activity in the medium 3 days following transfection of cells with pIE1/153A.jhe(kk) was over 1,000-fold higher than that obtained from cells transfected with the basic expression vector pBmA.jhe(kk).

3.3.3 Protein Expression in Transformed Cells can be Maximised by a High Ratio of Expression Plasmid to Antibiotic Resistance Plasmid

Bm5 silkworm cells were co-transfected with pIE1/153A.jhe(kk) and pBmA.HmB plasmids at molar ratios of 500:1, 100:1, 25:1, 5:1, 1:1 to simulate a single expression plasmid containing the selection marker, and 1:25. Each population was subcultured weekly in the presence of 0.25 mg/mL hygromycin B and the viable cell density of each population was monitored. Table 3.4 shows that a stably transformed population of cells was obtained after only 3 weeks selection for those cells transfected at a molar ratio of expression plasmid to antibiotic selection plasmid of 1:25, while 6 weeks was required for cells transfected at 100:1. A transformed population could not be obtained for cells transfected at a ratio of 500:1.

After 6 weeks selection, each surviving polyclonal population was seeded into 25 cm^2 T-flasks, allowed to grow for 7 days, and the supernatant assayed for JHE activity. The selected populations are polyclonal because they contain a mixture of expressing and non-expressing clones, and therefore the measured expression levels represent the average of each population. Control cells transfected only with pIE1/153A.jhe(kk) and subcultured in the absence of Hm B had very low JHE activity (< 0.6 µg/mL). It was found that a higher level of JHE was obtained from the population initially co-transfected at a molar ratio of expression plasmid to Hm B resistance plasmid of 100:1; the JHE concentration was 30 µg/mL at 100:1 compared to 4 µg/mL at 1:25. Dot blot hybridisations of genomic DNA also revealed that the integrated copy numbers of the *jhe*, *ie-1*, and *Hm B* genes were influenced by the ratio of expression plasmid to antibiotic resistance plasmid present in the initial transfection (Table 3.5).

Transfection Plasmid	Average JHE Activity	Relative JHE Activity
pBSK+	0 (3)	0
pBmA.JHE (kk)	1.18×10^{-3} (5)	1
pBmA.jhe(kk)+pBmIE1	0.114 (5)	96.9
pIE1/153A.jhe(kk)	1.37 (3)	1,160

Table 3.3: Verification that the expression plasmid, pIE1/153A, provides superior transgene expression than the basic actin cassette, pBmA, or the presence of the transcriptional activator IE-1, pBmIE1, using JHE as a reporter gene. Units of activity are expressed in nmoles of JH-III hydrolyzed per minute per mL of tissue culture medium at 25 °C and numbers in parentheses indicate time of repeat transfections for each vector combination.

Plasmid Ratio	Cell Survival in Selective Medium (weeks) 1	2	3	4	5	6
1:25	+++	++	+++[a]	+++[a]	+++[a]	+++
1:1	+++	++	+++[a]	+++[a]	+++[a]	+++
5:1	+++	+	+++[a]	+++[a]	+++[a]	+++
25:1	+++	+	+	+	+++[a]	+++
100:1	+++	+	-[b]	-[b]	++	+++
500:1	+++	+	-[b]	-[b]	-[b]	-
control	+++	+	-[b]	-[b]	-[b]	-

Table 3.4: Survival of populations of Bm5 cells in 0.25 mg/mL hygromycin B after transfection at different molar ratios of expression plasmid to hygromycin B resistance plasmid (+++ = > 5×10^5 viable cells/mL, ++ = 10^5 to 5×10^5 viable cells/mL, + = 10^4 to 10^5 viable cells/mL, - = <10^4 viable cells/mL, [a]cells were diluted when subcultured, [b]50% conditioned medium was used).

Plasmid Ratio[a]	[JHE] (µg/mL)	*jhe*	*ie-1* (copies/genome)[b]	*HmB*
control	<0.5	0	0	0
1:25	4	1-4	2-8	27-108
1:1	16	2-8	3-12	2-8
5:1	22	4-16	5-20	1-4
25:1	25	7-28	14-56	1-4
100:1	30	17-68	38-152	0.5-2
JHE#1724	59-90	11-44	25-100	0.5-2

Table 3.5: Expression levels obtained from polyclonal transformed populations, transfected at [a]different molar ratios of expression plasmid to hygromycin B resistance plasmid, cultured in 25 cm^2 T-flasks for 7 days. [b]Copies per haploid genome-tetraploid genome of *jhe*, *ie-1*, and *HmB* genes in the polyclonal populations.

3.3.4 Isolation of Clones Over-Expressing JHE

Although a polyclonal population may be sufficiently productive for some applications, improved expression levels were obtained using limiting dilution cloning. Forty-eight clones were amplified in 24-well plates to generate sufficient recombinant protein to assay for high producers. The distribution of JHE concentrations obtained after 10 days growth reveals the degree of heterogeneity in the transformed population (Figure 3.3); the co-transfection using a 100:1 molar ratio of expression plasmid to HmB resistance plasmid, followed by antibiotic selection and cloning, yielded approximately 50% high producers, 25% low producers, and 25% expressing virtually no JHE enzyme at all. One clone was selected and subcloned again as clone JHE#1724. This clone produced 59 to 90 µg/mL active JHE after 7 days and contained approximately 44 *jhe* copies per tetraploid genome.

3.3.5 JHE Production in Static and Suspension Cultures of Clone JHE#1724

To assess the level of recombinant protein expression in clone JHE#1724, cells were seeded into 6-well plates and samples were taken every 2 days for 14 days. Cell densities and JHE concentrations are shown in Figure 3.3. A maximum viable cell density of 2.1 x 10^6 viable cells/mL was reached after 8 days and JHE accumulated to 120 µg/mL after 14 days.

Clone JHE#1724 was also grown in suspension in 100 mL spinner flasks. Here cells grew to 3.7 x 10^6 viable cells/mL after 14 days and produced 210 µg/mL active JHE after 25 days (Figure 3.5). The specific productivities during the growth phases of static and suspension culture were calculated to be 10 and 14 µg/(10^6 viable cells.day), respectively (Table 3.6).

3.3.6 Stability of Clone JHE#1724 Over-Expressing JHE

The ability of clone JHE#1724 to retain its capacity for high-level JHE expression was monitored in serum-containing medium in the presence and absence of antibiotic selective pressure. Each week cells were subcultured at an initial density of 2.5 x 10^5 viable cells/mL in 100% fresh medium, and the supernatants assayed for JHE activity after 7 days growth. After 4 months, no significant decline in JHE concentration was observed in the presence or absence of 0.25 mg/mL HmB (Figure 3.6, Panels A and B respectively). After

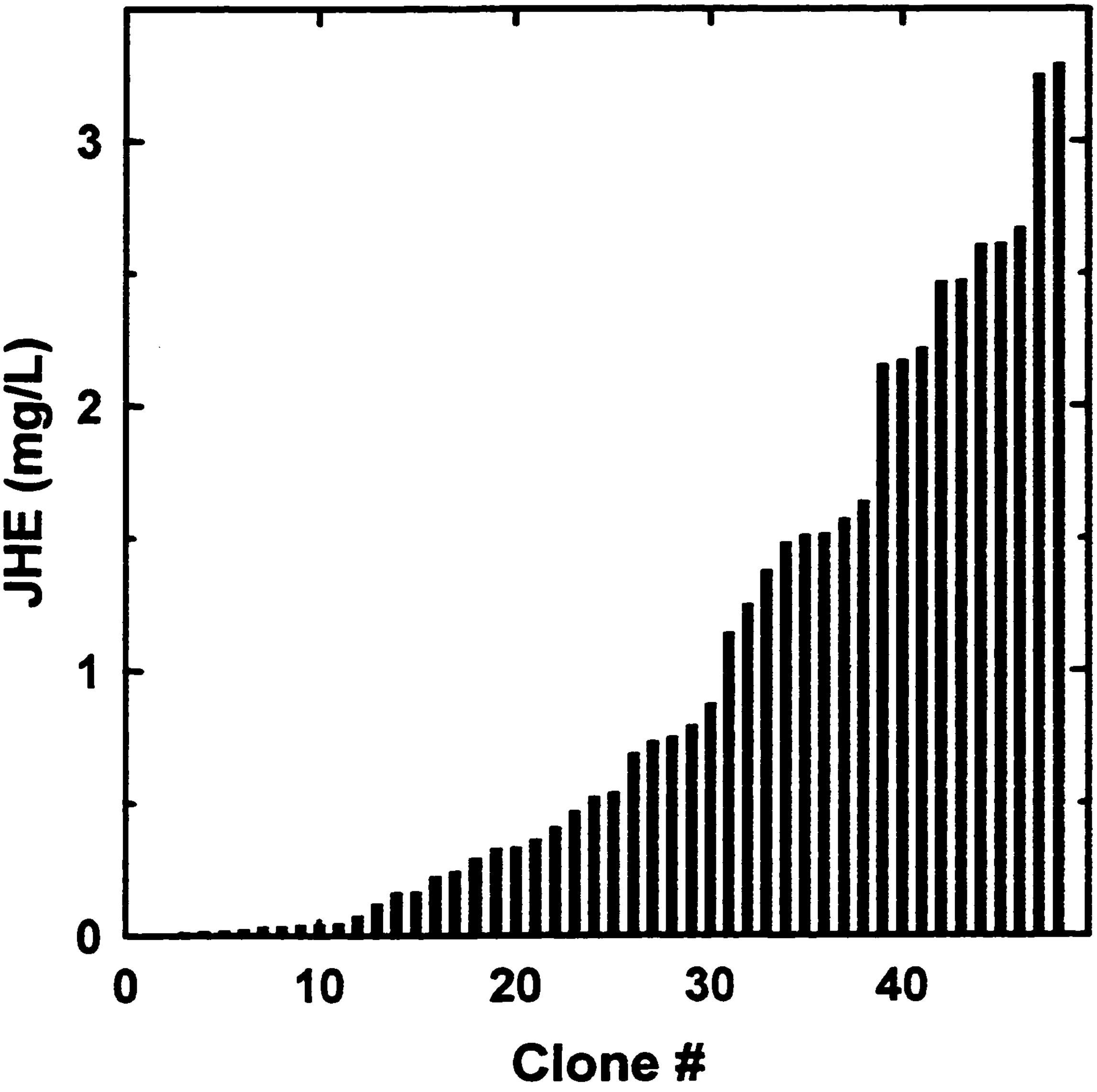


Figure 3.3: Distribution of JHE levels in the supernatant of 48 clones isolated from a heterogeneous transformed population initially transfected at a 100:1 ratio of expression plasmid to antibiotic resistance plasmid. The average JHE concentration from all clones was 0.95 mg/L after 10 days growth. Experiments were carried out in 24-well plates.

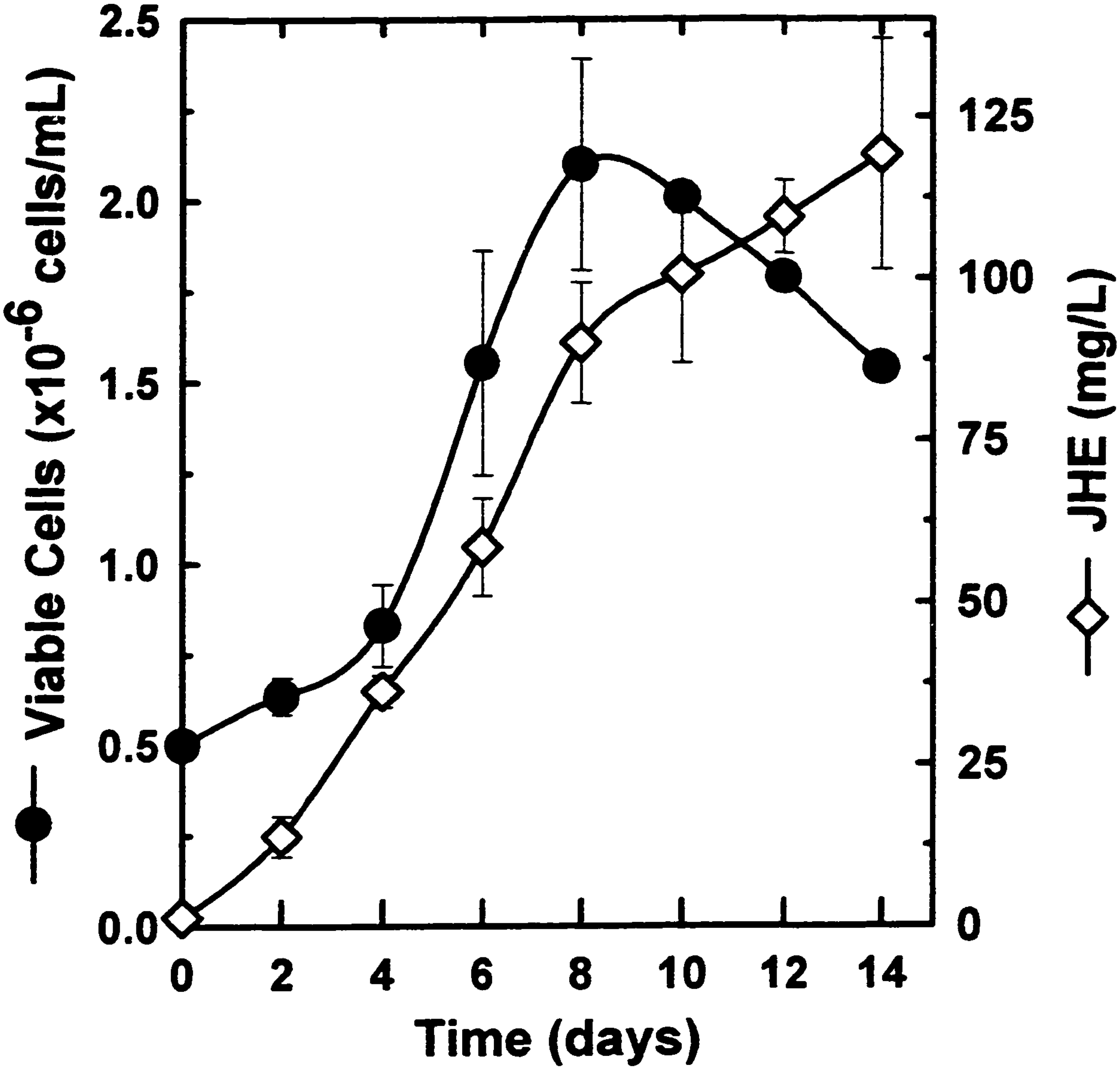


Figure 3.4: Batch production of JHE by clone JHE#1724 in 6-well plates in serum-containing medium.

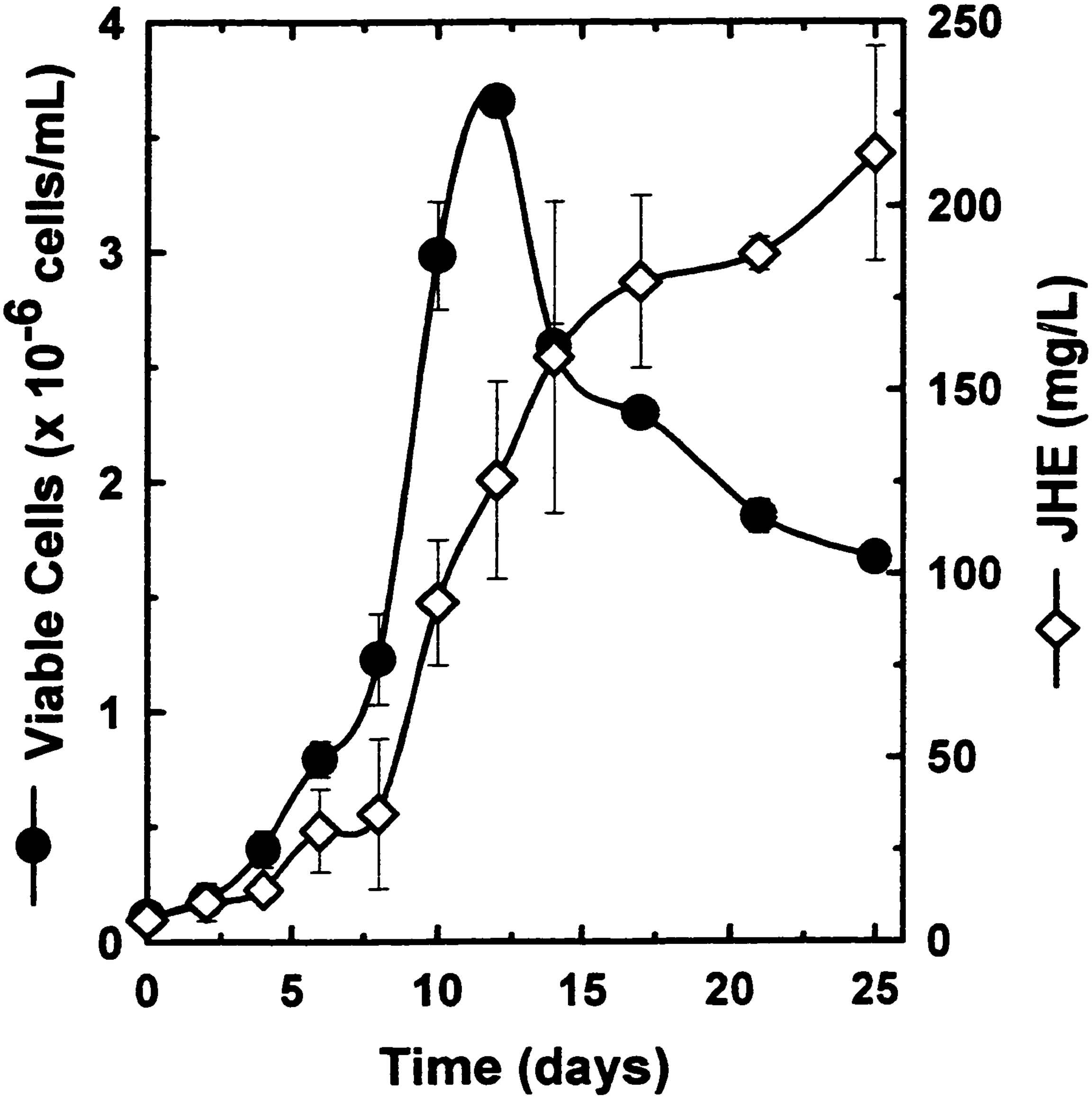


Figure 3.5: Batch production of JHE by clone #1724 in a 100 mL spinner flask in serum-containing medium. The medium in spinner culture was supplemented with 0.2 g/L glutamine and 1.0 g/L glucose

Expression System	Culture Conditions	$X_{v,max}$ (cells/mL)	t_d (h)	$[JHE]_{max}$ (μg/mL)	q_p[a]
Transformed Cells					
Bm5#1724	6-well/FBS	$2.1x10^6$	88	119	10.3
Bm5#1724	6-well/SFM	$1.9x10^6$	111	154	10.5
Bm5#1724	S-flask/FBS	$3.7x10^6$	59	214	13.5
Baculovirus					
AcNoSpJHE	6-well/FBS	$5x10^5$	-	75[b]	34[c]
AcJHE-KK	6-well/FBS	$5x10^5$	-	4	1[c]

Table 3.6: Comparison of JHE expression levels obtained from stably transformed Bm5 cells and two baculoviruses. [a]Units are μg JHE/(10^6 viable cells.day), [b]the JHE expressed by this virus was not secreted, [c]the specific productivity was estimated over 4 days post-infection.

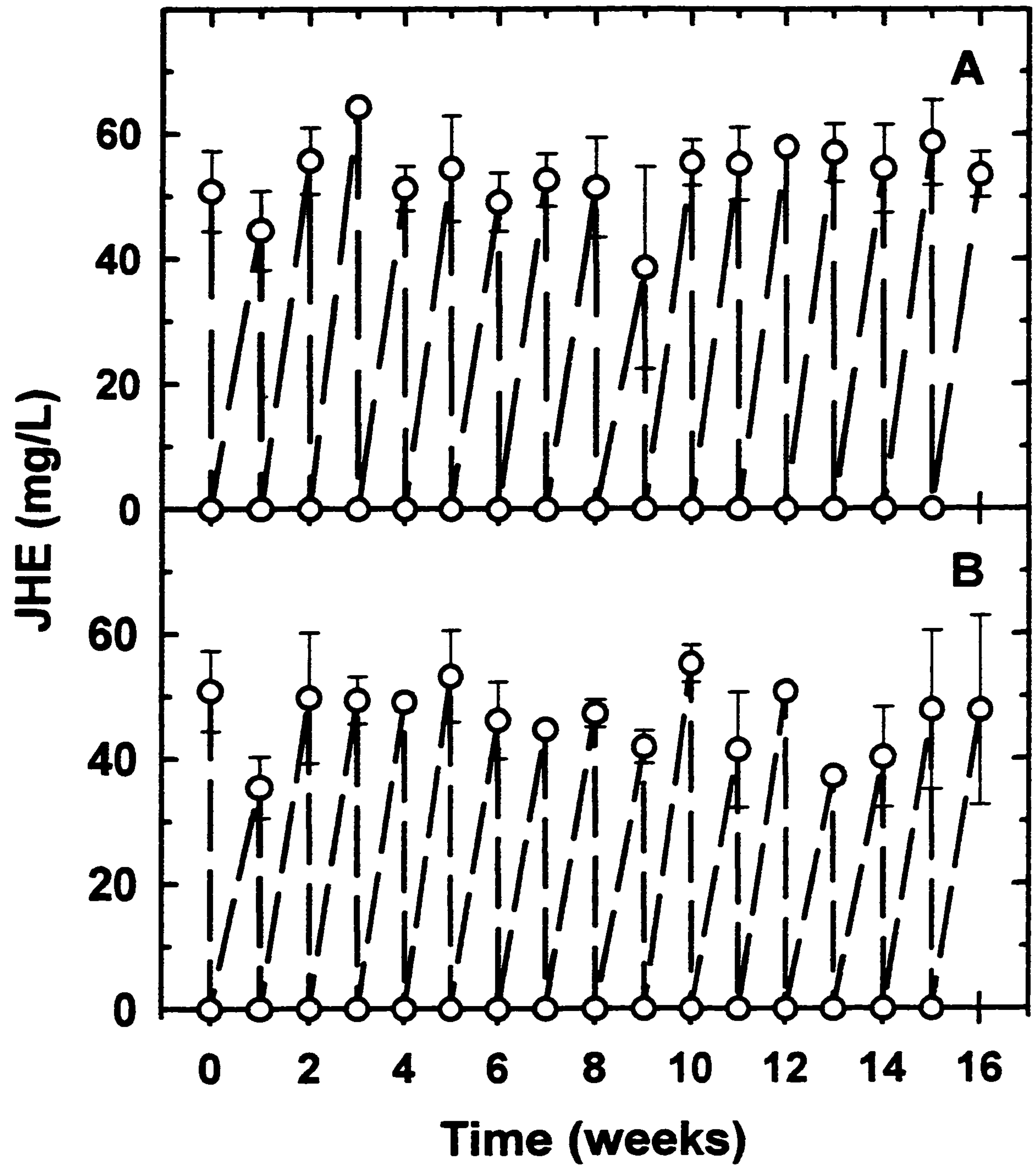


Figure 3.6: Stability of JHE expression from clone #1724 over 16 weeks determined by subculturing experiments. Subculturing was carried out in 25 cm^2 T-flasks using 5 mL of serum-containing medium in (A) the presence, or (B) the absence of 0.25 mg/mL. hygromycin B.

12 months subculturing in 0.25 mg/mL HmB, no significant decline in JHE expression from clone JHE#1724 occurred (data not shown). Transformed cells could also be frozen and recovered from cryogenic storage without loss of expression.

3.3.7 High Expression Levels are Maintained in Serum-Free Medium

Clone #1724 was gradually adapted to EC-400 serum-free medium, in the presence of HmB, over a period of three months. Following adaption, cells were seeded into 6-well plates and samples taken every day for 14 days. As shown in Figure 3.7, clone #1724 reached a maximum cell density of 1.9 x 10^6viable cells/mL after 10 days and produced 150 µg/mL of active JHE after 14 days in culture. This corresponds to an average growth phase specific productivity of 11 µg/(10^6 viable cells.day).

To verify the expression levels of JHE in serum free medium, 20 µL aliquots of each sample were resolved by gel electrophoresis and stained with Coomassie blue (Figure 3.8A). Coomassie blue staining of polyacrylamide gels has an average minimum detection limit of 0.3 µg (in Current Protocols in Molecular Biology). A distinct protein band having an apparent molecular mass of 65 kDa and increasing with time to several micrograms per 20 µL aliquot is clearly evident. The polypeptide in this band was confirmed to be JHE by Western blotting (Figure 3.8B).

3.3.8 Comparison to a Recombinant AcNPV Expressing JHE in Sf21 Cells

A direct comparison of the expression capability of clone #1724 with the BES was made in static cultures. *Spodoptera frugiperda* (Sf21) cells were infected with two recombinant *A. californica* NPVs over-expressing JHE under control of the basic protein promoter. One baculovirus, AcJHE-KK, contained the authentic *jhe* ORF (Bonning et al., 1997), and the other AcNoSpJHE contained a modified *jhe* ORF; the secretion signal peptide from the native *jhe* ORF had been removed and replaced with a synthetic translation start codon (B. Hammock, unpublished). Analysis of supernatant samples taken daily from the two baculovirus infected culture systems revealed that active secreted JHE accumulated to a maximum of only 4 µg/mL after 6 days and the non-secreted form accumulated to a maximum of 68 µg/mL after 5 days (Figure 3.9). Western blot analysis of

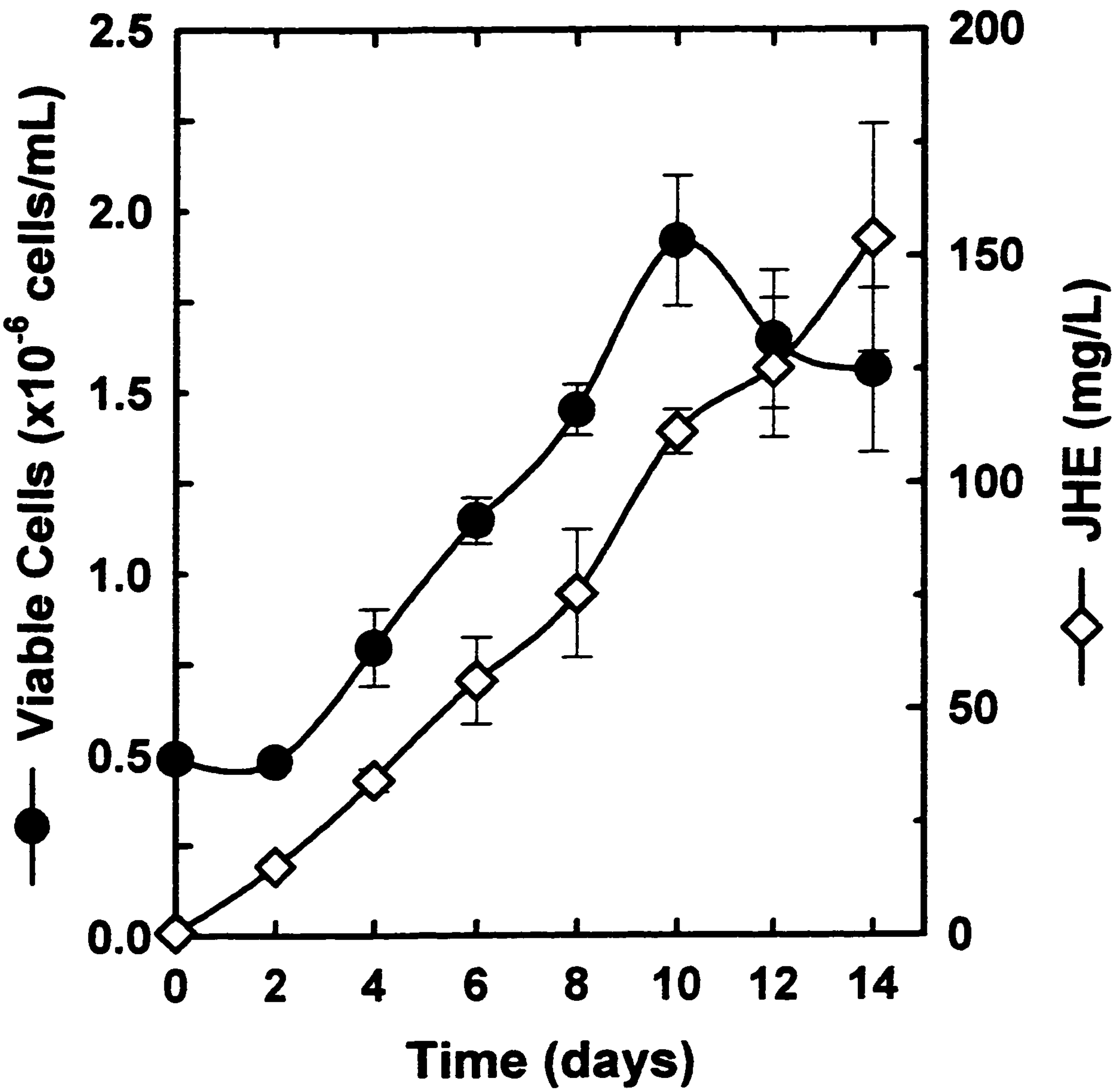


Figure 3.7: Batch production of JHE by clone JHE#1724 grown in EC400 serum-free medium in 6-well plates over a 14 day period.

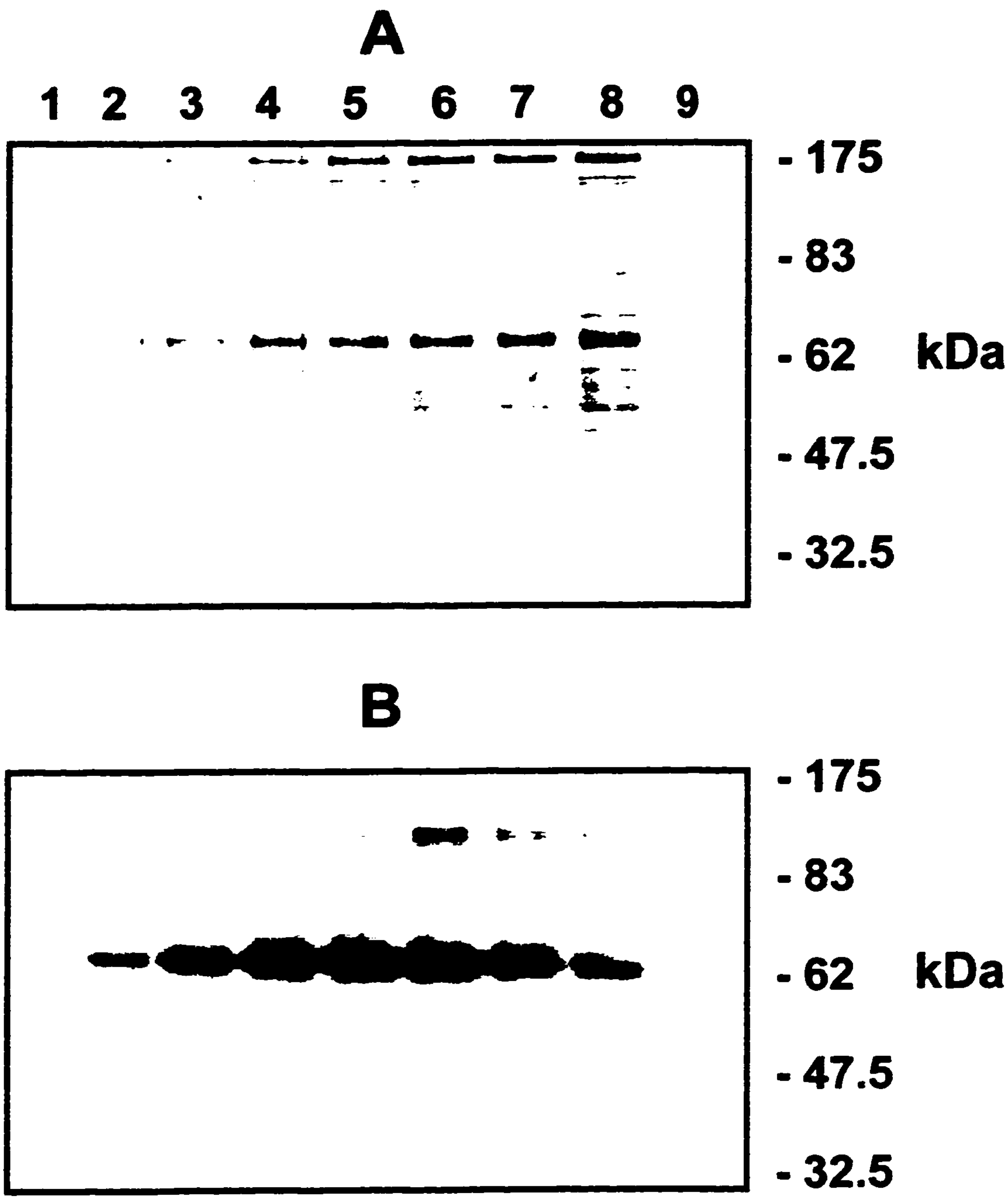


Figure 3.8: (A) Coomassie stained SDS-Page gel of 20 µL samples collected from the batch experiment in EC400. Lanes 1 to 8 show a continuous increase in JHE at approximately 65 kDa, every 2 days from 0 to 14 days. The control in lane 9 is day 6 conditioned medium collected from Bm5 cells subcultured in EC400 in a T-flask. (B) Western blot of the gel shown in (A) probed with a rabbit polyclonal antibody recognizing JHE at approximately 65 kDa (note that in lanes 7 and 8 an air bubble caused incomplete transfer of proteins from the acrylamide gel to the nitrocellulose membrane).

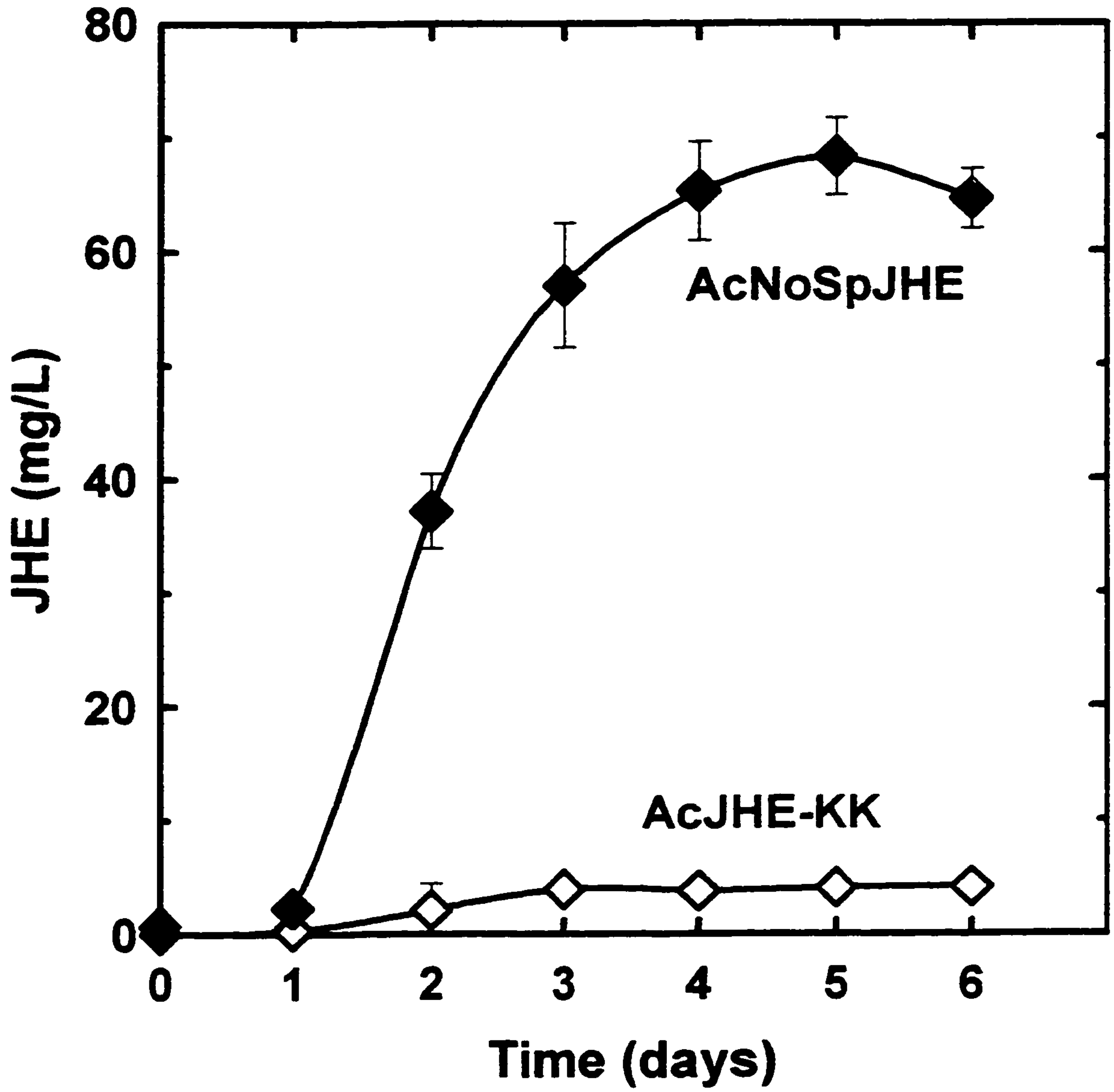


Figure 3.9: Batch production of JHE by AcJHE-KK or AcNoSpJHE-infected Sf21 cells in serum-containing medium in 6-well plates over a 6 day period. Note that JHE produced by AcNoSpJHE was not directed through the secretory pathway.

a day 6 aliquot of cell culture medium and comparison with samples taken from the JHE#1724 static culture confirmed the differences in JHE production obtained on the basis of JHE activity assays (Fig. 3.10A).

The calculated maximum specific JHE productivies were 1.0 μg/(10^6 infected cells.day) by AcJHE-KK and 34 μg/(10^6 infected cells.day) by AcNoSpJHE, which is significantly higher than the 10 μg/(10^6 viable cells.day) obtained from clone #1724 in static culture. However, it is worth noting that, in contrast to the situation with the transformed cell line which continued secreting recombinant JHE into the medium over a period of 2 weeks, the secretion of JHE by baculovirus-infected cells reached a maximum value at 3-5 days post-infection and did not increase thereafter. Furthermore, the non-secreted form of JHE produced by the leaderless virus AcNoSpJHE was not expected to be effectively glycosylated, since it was no longer directed to the secretory pathway where glycosylation occurs, and therefore would not represent the authentic glycoprotein. However, exposure of the baculovirus infected cells and transformed cells to an inhibitor of N-linked glycosylation, tunicamycin (Tkacz and Lampen, 1975), revealed a decrease in the apparent molecular mass of JHE in all cases (Figure 3.10B). The unexpected N-linked glycosylation of the non-secreted form of JHE presumably occurred in the cytoplasm by N-glycosyl-transferase enzyme that had leaked from the damaged secretory pathway in the virus infected host cells.

3.3.9 Recombinant Protein Expression in Transfected and Transformed Other Lepidopteran Insect Cell Lines

The silkmoth cytoplasmic actin gene promoter, the *ie-1* gene of BmNPV and the BmNPV HR3 enhancer were previously shown to function in other lepidopteran insect cell lines including *S. frugiperda* Sf21 and *Choristoneura fumiferana* Cf1 cells (Lu at al., 1996). To examine the production potential of other cell lines with the silkmoth expression cassette, we compared the transient expression levels of JHE obtained from Bm5 cells with those obtained from *Trichoplusia ni* BTI-TN-5B1-4 (High Five™) and Sf21 cells, which are known to have the capacity for complex glycosylation required for the biological activity of some recombinant glycoproteins (Davis and Wood, 1995).

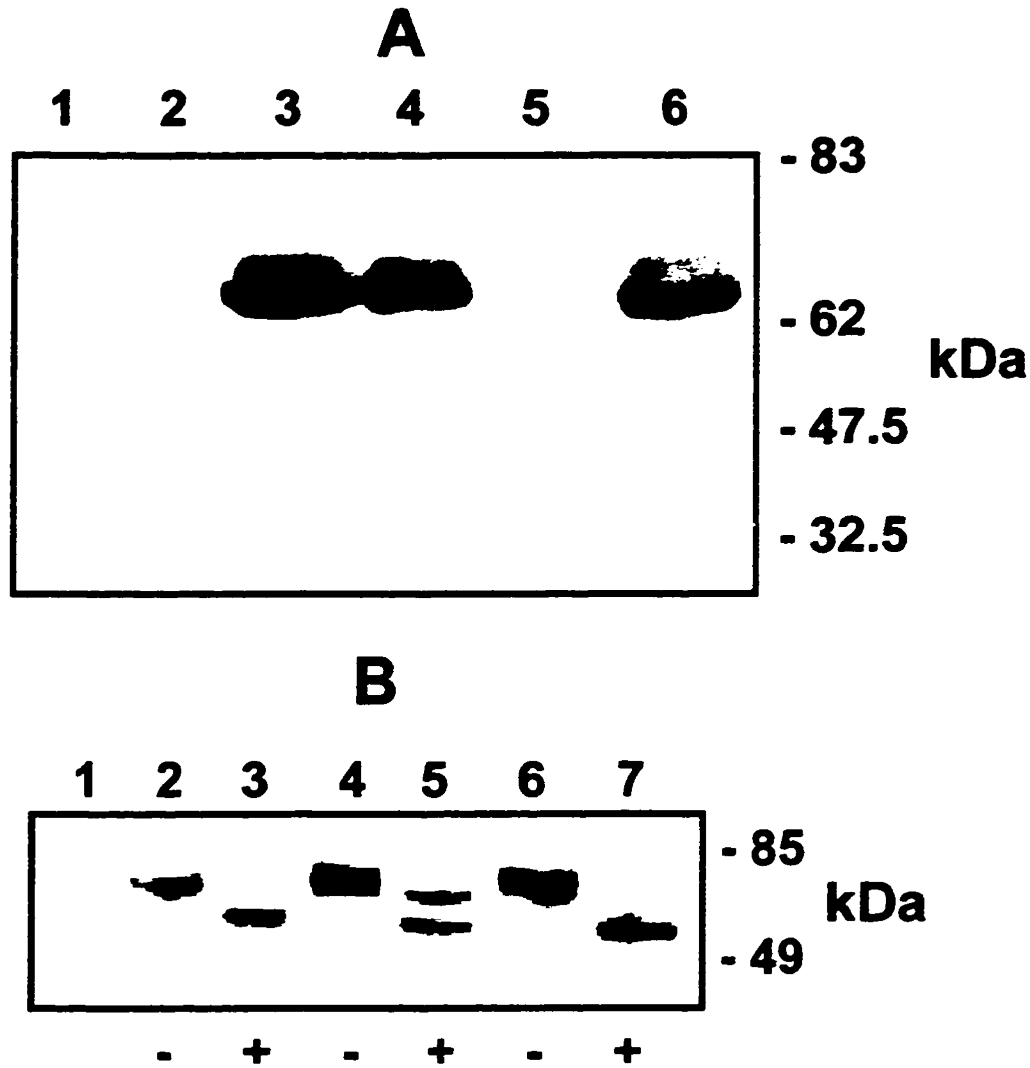


Figure 3.10: (A) Western blot of 5 µL supernatant to confirm relative JHE expression levels in static cultures. Lane 1 contains IPL-41 + 10% FBS. Lane 2 contains EC400 medium. Lane 3 contains day 14 supernatant from JHE#1724 in IPL-41+10% FBS. Lane 4 contains day 14 supernatant from JHE#1724 adapted to EC400. Lane 5 contains day 6 supernatant from AcJHE-KK infected Sf21 cells in IPL-41+10% FBS. Lane 6 contains day 6 supernatant form AcNoSpJHE infected Sf21 cells in IPL-41 + 10% FBS. (B) Western blot analysis of JHE produced by AcJHE-KK-infected Sf21 cells (lanes 2 and 3), or AcNoSpJHE (lanes 4 and 5) or clone JHE#1724 maintained with (+) or without (-) 1.0 µg/mL tunicamycin. Each lane was loaded with sample aliquots containing equal JHE activity. Lane 1 contains control IPL-41 medium.

Following transfection of the cells with pIE1/153A.jhe, culture samples were taken at 60 h post-transfection for cell counts and JHE assays. Differences in the transfection efficiencies among the three cell lines were established by transfecting cells in parallel with a vector expressing green fluorescence protein (pIE1/153A.gfp⁺), and determining the fraction of fluorescent cells in each population by flow cytometry at 60 h post-transfection. Substantial differences in the transfection efficiency were found between cell lines (Table 3.7), and this was also reflected in the corresponding JHE expression levels. The data presented in Table 3.7 also revealed that transfected High Five™ cells had a slightly higher specific productivity than Bm5 cells and that the specific productivity in Sf21 cells is about 7-fold lower than those of Bm5 and High Five™ cells.

Sf-21, Bm5 and Hi5 cells were also stably transformed following a co-transfection with the plasmids pIE1/153A.jhe(kk) and pBmA.hmB, and subculturing in the presence of various concentrations of hygromycin B. The survival of each cell line is shown in Table 3.8, and reveals that higher antibiotic concentrations can be used for both High Five™ and Sf21 cells and that stably transformed polyclonal populations can be obtained in a shorter time period (3 weeks for High Five™ and Sf21 cells versus 5 weeks for Bm5 cells). Polyclonal cell lines were seeded it 5×10^5 cells/mL in T-flasks and the viable cell densities and JHE concentration was measured at the end of 7 days. Table 3.9 reveals that High Five™ cells were more productive than Bm5 cells and Sf21 cells in this experiment.

3.3.10 Potential for a High Level Inducible Expression Cassette

For those target proteins that may be toxic to the host cell, even a low levels of expression, an inducible expression system can be used. In such systems, low level transcription from a basal promoter is suddenly enhanced several hundred-fold by physiological changes. For example, the heat shock promoter is induced by a step increase in temperature, and the metallothionine promoter is induced by the addition of heavy metals. An ecdysone (hormone) inducible expression vector using β-galactosidase reporter gene, pMK43.2, was previously reported to function in *Drosophila* S2 cells that were stably transformed to express the ecdysone receptor (Koell et al., 1991). This expression vector was shown to function in Bm5 cells, which constitutively express an endogenous ecdysone

Cell Line	Transfection Efficiency (%)	JHE (µg/mL)	Final Cell Density (viable cells/mL)	Specific Productivity[a]
Bm5	29.8	5.9	1.65×10^6	5.1
Sf21	8.1	0.25	1.45×10^6	0.8
High Five™	61.5	11.5	1.05×10^6	5.9

Table 3.7: Comparison of the *transient* expression of JHE from different insect cell lines at 60 h post-transfection with pIE1/153A.jhe(kk). The transfection efficiency was estimated by FACs analysis of cells transfected with pIE1/153A.GFP+ for green fluorescence protein detection under identical transfection conditions. Experiments were performed in duplicate. [a]Expressed in µg/(10^6 viable transfected cell.day).

Cell Line	Selection (mg/mL HmB)	Survival in Selective Medium (weeks) 1	2	3	4	5
Bm5	0.5	++	+	-[a]	+	+++
Sf21	1.0	++	++	+++[b]	+++[b]	+++[b]
High Five™	1.0	++	++	+++[b]	+++[b]	+++[b]

Table 3.8: Survival of Bm5, Sf21, and High Five™ cells during the selection of stably transformed polyclonal populations at the predetermined maximum HmB concentration for each cell type (+++ = > 5×10^5 viable cells/mL, ++ = 10^5 to 5×10^5 viable cells/mL, + = 10^4 to 10^5 viable cells/mL, - = $<10^4$ viable cells/mL, [a]cells were diluted when subcultured, [b]50% conditioned medium was used).

Polyclonal Cell Line	Selection (mg/mL HmB)	JHE (µg/mL)	Final Cell Density (viable cells/mL)	Specific Productivity[a]
Bm5	0.5	27	2.6×10^6	3.0
Sf21	1.0	16	4.0×10^6	1.1
High Five™	1.0	43	1.9×10^6	5.1

Table 3.9: Specific JHE productivity from *transformed* polyclonal populations of Bm5, Sf21, and High Five™ cells expressing JHE after 7 days growth. [a]Units are µg/(10^6 viable cells.day).

receptor (Swevers et al., 1995). This expression vector is shown in Figure 3.11.

So far the BmNPV transcription factor IE-1 has only been shown to stimulate expression from one cellular promoter, namely actin (Lu et al., 1996). To address whether IE-1 would stimulate other cellular promoters, such as the *Drosophila* alchohol dehydrogenase promoter (ADH), and could augment the induction of an EcRE- ADH hybrid promoter, Bm5 cells were transfected with pMK43.2 or co-transfected with pBmIE1 and pMK43.2 and 1 µM ecdysone was supplied to some transfected samples 24 h post-transfection. Figure 3.12 qualitatively show no LacZ expression from pMK43.2 in the absence of ecdysone, minor lacZ expression from pMK43.2 in the presence of IE-1 and absence of ecdysone, significant induction of LacZ expression from pMK43.2 in the presence of ecdysone, and super induction of LacZ ecpression from pMK43.2 in the presence of both ecdysone and IE-1.

3.4 Discussion

A novel lepidopteran insect cell protein expression system has been developed that circumvents many of the problems associated with the baculovirus expression system. A stably transformed insect cell line was able to continuously express the secreted glycoprotein juvenile hormone esterase at high levels, and the concentration of active JHE obtained was found to be significantly greater than that obtained from the baculovirus expression system.

One Bm5 derived clone, JHE#1724 over-expressing JHE, produced 210 µg/mL active JHE in simple batch suspension culture and 150 µg/mL in serum-free static cultures. Considering that Bm5 cells are well adapted for growth in large-scale suspension cultures (Zhang et al., 1994) and that minor modifications (culture medium fortification and alternative bioreactor configurations) should result in further improvements in expression levels, the value of this new expression system is obvious. Although direct comparisons for additional proteins have yet to be made, the expression levels obtained by the system appear superior to other insect (reviewed in Section 3.1) and mammalian expression systems used for production of recombinant glycoproteins. Briefly, some recent reports regarding high output mammalian expression systems for secreted glycoproteins include

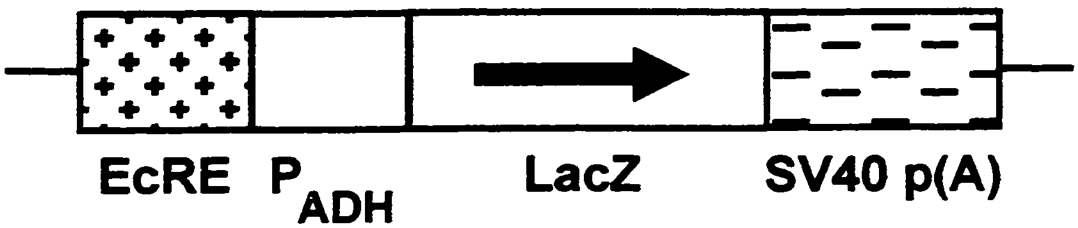


Figure 3.11: Schematic of plasmid pMK43.2. Transcription of the reporter gene, β-galactosidase, from the alchohol dehydrogenase basal promoter (P_{ADH}) is induced by the hormone ecdysone acting through the ecdysone response element (EcRE).

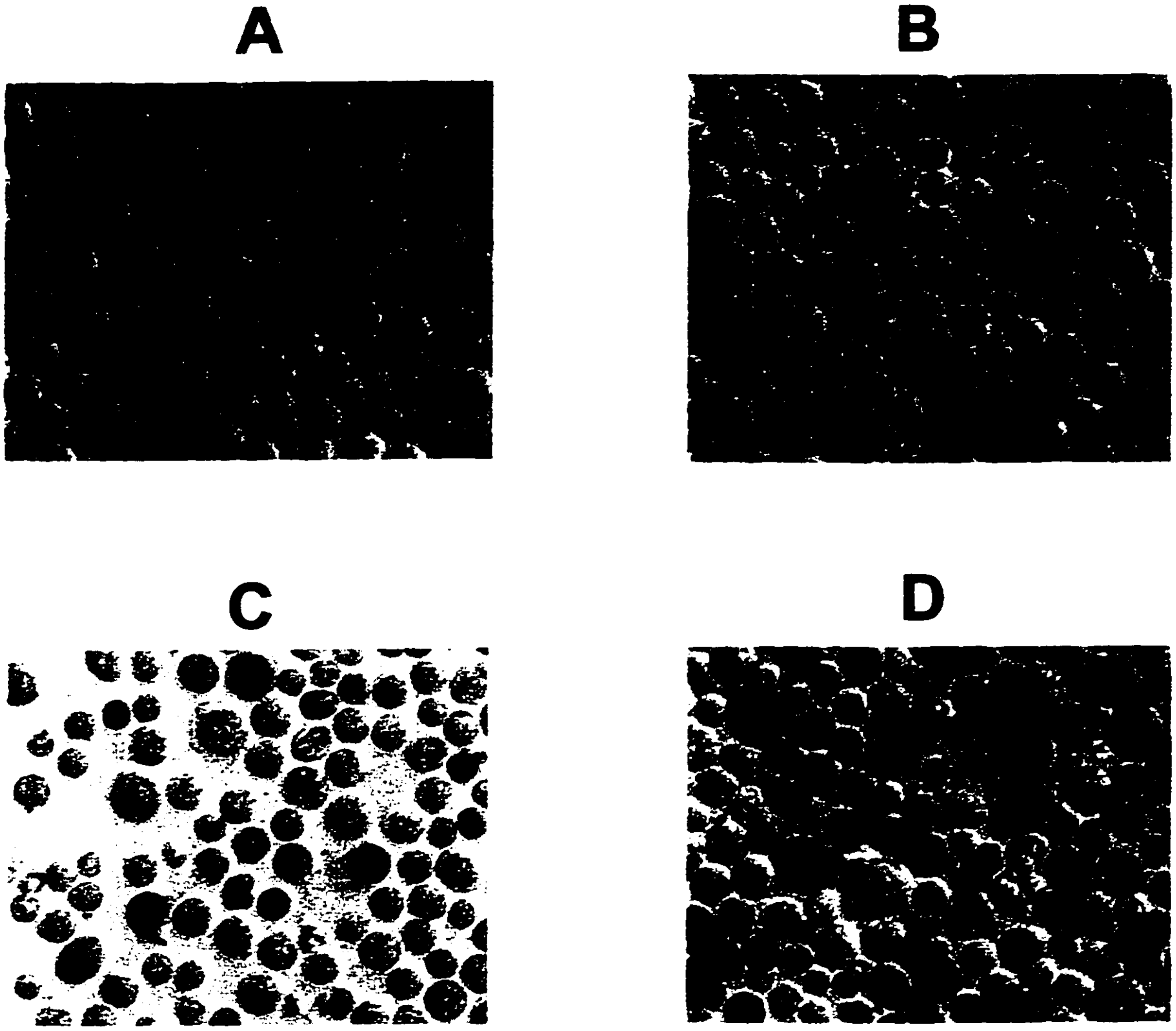


Figure 3.12: β-galactosidase staining assays of Bm5 cells transfected with pMK43.2 (A and C) or co-transfected with pMK43.2 and pBmIE1 (B and D) in the absence (A and B) or presence (C and D) of 1 μM ecdsyone.

the BHK-21/VP16 system (105 µg/mL of secreted ICAM in static cultures; Warren et al., 1994), recombinant NS0 cells (120 µg/mL IFNα2b in static cultures; Rossmann et al, 1996) and recombinant CHO cells (118 µg/mL IgG light chain in protein free medium in a 2.5 L bioreactor; Zang et al., 1995).

Our experiments also suggest that alternative lepidopteran insect cell lines, such as High Five™, may be used more effectively than Bm5 cells for recombinant protein production. High Five™ cells required approximately 50% less time to produce stably transformed cell lines, were more productive, and reportedly grow well in suspension culture (Dee et al., 1997) . Furthermore these cells have some capacity for complex glycosylation (Davis and Wood, 1995).

Finally, it was demonstrated the IE-1 protein can stimulate recombinant protein expression even in an inducible system. Previously, the IE-1 protein has been shown to modulate a number of baculovirus genes (Guarino and Summers, 1986; Carson et al., 1991; Kovacs et al., 1991), but only one cellular gene (Lu et al., 1996). The transactivation of the ADH promoter, even in the absence of induction by ecdysone, suggests that the mode of action of IE-1 may be through interactions with different cellular transcription factors of RNA polymerase II promoters. Thus IE-1 is probably a useful auxiliary protein in any type of lepidopteran expression system.

CHAPTER 4

Characterization of a Stably Transformed Bm5 Cell Line Over-Expressing Human Tissue Plasminogen Activator

4.0 Summary

Tissue plasminogen activator (t-PA) is a complex and valuable serine protease employed as a therapeutic agent for the degradation of blood clots. Stable cell transformation was used to generate a cloned *Bombyx mori* insect cell line (Bm5) over-expressing human t-PA. This cell line expressed 135 mg/L single chain t-PA in serum-free medium in static culture with a maximum specific activity of 120 IU/µg. In serum-containing medium, this cell line expressed 160 mg/L of combined single t-PA, two chain t-PA and a higher molecular weight SDS-stable t-PA complex in suspension culture with a maximum specific activity of 255 IU/µg. Approximately 100 copies of the t-PA gene were randomly integrated into each Bm5 cell. It was also established that the native human t-PA signal peptide is recognized equally efficiently as a *Bombyx mori* specific signal peptide for the secretion of t-PA from Bm5 cells. Finally stably transformed polyclonal populations of Bm5, High Five™ and Sf21 cells expressing t-PA were generated and compared for relative t-PA expression.

4.1 Introduction to Tissue Plasminogen Activator

Tissue plasminogen activator (t-PA) is a serine protease that converts inactive plasminogen into plasmin, an active protease whose function is to degrade the fibrin network of a blood clot (Wun and Capuano, 1986). t-PA is normally secreted by endothelial cells in blood vessel walls in response to venous occlusions, infusion with vasoactive compounds, and physical exercize. Because t-PA has a major role in vascular fibrinolysis, recombinant t-PA was approved by the US Food and Drug Administration for the treatment of acute mycardial infarction in 1987. Produced by Genentech and marketed as Activase, heart attack patients may have a survival advantage if administered Activase within three hours of a heart attack. Activase was also approved for treatment of acute pulmonary embolism in 1990, and ischemic stroke in 1996. Activase treatments cost US$2200 per 100 mg dose with the 1997 U.S. market for Activase being US$250 million (Nature Biotechnology, 14 August, 1996), and projected to be US$420 million by 2001. Activase is produced using transformed mammalian expression technology in CHO cells.

In Chapter 3, the insect glycoprotein juvenile hormone esterase was used as a reporter gene to demonstrate that expression levels in batch cultures of approximately 200 mg/L active protein could be obtained from stably transformed *Bombyx mori* (Bm5) insect cells. However, it is anticipated that expression levels and the biological activity of a recombinant protein produced using this expression system may vary with each protein. Factors that could influence the expression level and biological activity include the size of the protein, its stability, the extent of post-translational processing, the species of origin, and its resistance to proteases. Therefore, to determine whether the high expression levels obtained for JHE using this expression system are not restricted to one secreted glycoprotein, human t-PA cDNA was used as a second reporter gene. t-PA is a complex polypeptide chain of 562 amino acids whose post-translational modifications include the removal of a 35 amino acid preprosequence (Pennica et al., 1983), the formation of 17 intrachain disulphide bonds, O-glycosylation (Harris et al., 1991), and N-glycosylation at 2 (type II) or 3 (type I) of 4 potential sites (Pohl et al., 1984). t-PA is naturally secreted by endothelial cells as a single chain molecule (sct-PA) of 65-68 kDa, that is cleaved after Arg^{275} by plasmin or other serine proteases (Pohl et al., 1984, Ichinose et al., 1984) present in blood plasma into two 30-35 kDa chains (tct-PA) that remain connected by a disulphide bond (Wallen et al., 1982). tct-PA has a higher enzymatic activity than sct-PA (Boose et al., 1989).

The advantages of using t-PA to test this system include the fact that it is a complex molecule and extensively modified post-translationally, it is of non-insect origin, it is used *in vivo* as a therapeutic agent, and methods to detect both the protein and its biological activity are commercially available. Furthermore t-PA has been expressed in a variety of organisms for comparison at levels of 0.1 to 450 mg/L (see Table 4.1), and is known to be poorly expressed and inefficiently processed in the baculovirus-insect cell expression system (Jarvis and Summers, 1989).

In this chapter we have also addressed whether secreted proteins of non-insect origin could be expressed more efficiently from Bm5 insect cells when an insect-specific signal peptide is employed. This is based on the findings that the secretion of heterologous proteins from baculovirus-infected insect cells can be enhanced by replacing the native signal peptides with insect-specific signal peptides such as those from honeybee prepromellitin (Tessier et al., 1991), baculovirus ecdysteroid UDP glucosyltransferase (Murphy et al., 1993), and baculovirus envelope glycoprotein gp67 (Murphy et al., 1993). We therefore synthesized *Bombyx mori* chorion protein signal peptide coding for the optimal recognition in Bm5 cells and to test the secretion efficiency of human t-PA from Bm5 tissue culture cells. In nature, large amounts of chorion polypeptides are efficiently secreted from relatively few follicular cells in the formation of an eggshell around a developing oocyte in a lepidopteran insect.

4.2 Materials and Methods

4.2.1 Plasmid Constructions

The plasmid pIE1/153A.t-PA was constructed by inserting a 1.9 kbp *BamHI* fragment from pVL941-t-PA (kindly provided by Drs. Max Summers and Don Jarvis, Texas A & M) into the unique *BamHI* site of pIE1/153A.

DNA coding for the *Bombyx mori* L.12B chorion protein signal peptide (Spoerel et al., 1986) was generated by synthesizing the following oligonucleotides 1 and 2 (Table 4.2)

Host Organism	Expression Level (mg/L)	Reference
Mammalian Cells		
CHO	33.5	Datar et al., 1993
CHO	13	Parekh et al., 1989
Bowes melanoma	0.45	Datar et al., 1993
C127	16	Parekh et al., 1989
AVI2-664	40-64	Berg et al., 1993
BHK/VP16	10	Hippenmeyer et al., 1994
Baculovirus		
AcNPV/Sf9	1.0	Jarvis et al., 1989
AcNPV/Sf9	2.5	Steiner et al., 1988
Insect Cells		
Transformed Sf9	1.0	Jarvis et al, 1990
Transformed Bm5	135-165	This work
Yeast		
Saccharomyces cerevisae	0.0065[1]	Lemontt et al., 1985
Saccharomyces cerevisae	100[1]	Martegani et al., 1992
Bacteria		
E.coli	460[1,2]	Datar et al., 1993
Fungus		
Aspergillus nidulans	0.1	Upshall et el., 1987

Table 4.1: Summary of the published expression levels of tissue plasminogen activator obtained from a variety of recombinant protein expression systems ([1]not secreted; [2]due to the formation of inclusion bodies, only 5% active t-PA could be recovered).

Primer	Sequence
1)	5'-AAAAAGGATCCAAAATGGCCGCT AAACTCATTCTCTTCGTCTTCGTCTGCGCCACCGCCCTCGTG-3'
2)	5'-AAAAAATCTAGAAAA**G/C**CATG**C/GC/A**TAAGACGGACTGGGCCACGAGGGCG-3'
3)	5'-GAAAGGATCCGCATGCAGGAAATCCATGCCCG-3'
4)	5'-CCCTTCTAGATCACGGTCGCATGTTGTC-3'
5)	5'-GAAAGGATCCATGGGAGCCAGATCTTACCAAG-3'

Table 4.2: List of oligonucleotide synthesized for generating DNA constructs to investigate the effect of the *Bomyx mori* chorion signal peptide on human t-PA expression.

which were annealed, end-filled with klenow enzyme, digested with *BamHI* and *XbaI*, and ligated into pBluescript SK+ (Stratagene). Due to degeneracies in one oligonucleotide, two versions of the signal peptide coding were created, pSP1 or pSP16, that either had a *NcoI or SphI* restriction endonuclease site respectively at their 3' end, for in-frame fusion with the methionine codon on the 5' end of a heterologous gene. For attachment of the pro.t-PA open reading frame to the chorion signal peptide the PCR primers 3 and 4 were synthesized (Table 4.2). PCR amplification using *Pfu* polymerase and the plasmid pVL941-t-PA as a template yielded a 1.6 kbp product that was digested with *SphI* and *XbaI* and inserted into the unique *SphI/XbaI* sites of pSP16 to yield pSP16.pro.t-PA. A *BamHI/NotI* digestion of pSP16.pro.t-PA released a 1.7 kbp product containing the chimeric gene that was ligated into the unique *BamHI/NotI* sites of pIE1/153A to yield pIE1/153A.SP16.pro.t-PA. To attach the mature t-PA open reading frame to the chorion signal peptide, PCR amplification using *Pfu* polymerase, primers 4 and 5 (Table 4.2), and the plasmid pVL941-t-PA as a template yielded a 1.6 kbp product that was digested with *NcoI* and *XbaI* and inserted into the unique *NcoI/XbaI* sites of pSP1 to yield pSP1.t-PA. A *BamHI/NotI* digestion of pSP1.t-PA released a 1.6 kbp product containing the chimeric gene that was ligated into the unique *BamHI/NotI* sites of pIE1/153A to yield pIE1/153A.SP1.t-PA. For expression of a truncated form of t-PA that contains pre-tPA with a synthetic translation start codon, the 1.6 kbp PCR product using primers 3 and 4 (Table 4.2) and pVL941-t-PA as a template was digested with *BamHI* and *NotI* and ligated into the unique *BamHI/NotI* sites of pIE1/153A to yield pIE1/153A.t-PA(-SP).

4.2.2 Genomic DNA Analysis

Total cellular DNA was extracted from tissue culture cells as described previously (Skeiky et al., 1991). For Southern analysis, total cellular DNA and pIE1/153A.t-PA plasmid DNA was digested with *NotI*, resolved by electrophoresis on a 0.8% (w/v) agarose gel and transferred to a nylon membrane (Skeiky et al., 1991). For dot blot analysis, aliquots of *NotI* digested total cellular DNA and pIE1/153A.t-PA plasmid standards were spotted onto Hybond N+ membrane and treated as described previously (Lu et al., 1996). Membranes were hybridized to the randomly labeled t-PA DNA probe (Fotaki and Iatrou, 1988).

4.2.3 t-PA Detection

For Western blots goat-anti-t-PA (1:1000 dilution; American Diagnostica) and horseraddish-peroxidase conjugated rabbit-anti-goat (1:1000 dilution; Jackson Immunochemicals) were used as the primary and secondary antibodies respectively. A single chain t-PA antigen standard was used for quantitation (American Diagnostica).

To measure the biological activity of t-PA, the Spectrolyse indirect enzymatic assay kit (American Diagnostica) was used. In this assay, samples of cell culture supernatant containing t-PA were added to a mixture containing plasminogen, fibrin, and a plasmin substrate (Spectrozyme PL). First t-PA in the sample converts plasminogen to plasmin in the presence of fibrin, and plasmin then cleaves Spectrozyme PL to generate a yellow colored solution. The absorbance of the reaction solution at 405 nm is a quantitative measure of the t-PA activity in the sample. Standards of Bowes melanoma tct-PA of known specific activity were used (American Diagnostica). Samples were assayed at least in duplicates.

To detect relative amounts of immunoreactive t-PA material in cell culture samples, an indirect enzyme linked immunosorbent assay (ELISA) was used. Fifty microliter aliquots of cell culture samples and controls, diluted in cold 0.1 M bicarbonate buffer (pH 9.6), were loaded into 96-well ELISA plates and incubated overnight at 4°C. The plate was washed three times with 150 µL/well phosphate buffered saline containing 0.05% tween-20 (PBST) and blocked with 100 µL/well PBST/3% bovine serum albumin (PBST-BSA) for 1 h at room temperature. The plate was then incubated at 37°C with 50 µL/well PBST-BSA containing goat anti-human t-PA (1:1000 dilution; American Diagnostica) and washed three times with PBST. A second incubation at 37°C was carried out with 50 µL/well PBST-BSA containing rabbit anti-goat IgG (1:1000 dilution; Jackson Immunochemicals). After washing, 100 µL of citrate-phosphate buffer (pH 5) containing 3 mg/mL *o*-phenylenediamine and 0.2 µl/mL H_2O_2 was added to each well. The plate was incubated at room temperature for a few minutes for colour to develop, whereupon the reaction was stopped with 50 µL 1 M HCl, and the absorbance at 490 nM was recorded in each well with a ELISA plate reader. Samples were diluted to be in a linear range and assayed in quadriplicate.

4.3 Results

4.3.1 The Native Human t-PA Signal Peptide Functions as Efficiently as an Insect Specific Signal Peptide for Heterologous Protein Secretion from Bm5 Cells

The L.12B chorion signal peptide was attached to both the pro.t-PA and mature t-PA open reading frames to test whether a *Bombyx mori* specific signal peptide would function more efficiently than the native human signal peptide for the secretion of a heterologous proteins from the *Bombyx mori* Bm5 insect cell line.

The levels of t-PA protein expressed and processed under the influence of the chorion signal peptide were compared using transfection assays with the expression constructs shown in Figure 4.1. The plasmid pIE1/153A.t-PA contains the native prepro-t-PA gene; the plasmid pIE1/153A.SP16.pro-tPA contains the chorion signal peptide, the 12 amino acid pro- region and the 527 amino acid mature t-PA ORF; the plasmid pIE1/153A.SP1.tPA contains the chorion signal peptide and the 527 amino acid mature t-PA ORF; and the control plasmid pIE1/153A.tPA(-SP) contains the 527 mature t-PA ORF without a signal peptide, and with a synthetic methionine translation start codon. Sixty hours following transfection of Bm5 cells maintained in serum-containing medium, cells and culture supernatants were harvested and compared using Western blots, t-PA activity assays, and dot blot hybridizations. The Western blot in Figure 4.2A reveals that the t-PA expressed from insect cells exists as doublets of tct-PA of approximately 35 kDa, due to cleavage of sct-PA by serine proteases present in fetal bovine serum. The blot also reveals that the native human signal peptide is superior to both the chorion-pro-mature t-PA and the chorion-mature t-PA chimeras in its ability to secrete t-PA. Quantitative analysis using t-PA activity assays (Figure 4.2B) confirms these relative expression levels.

To ensure that equivalent amounts of plasmid DNA were delivered to the cells in each transfection, cells were analyzed by dot blot hybridization. Figure 4.2C reveals that a 50% lower transfection efficiency resulted with the plasmid pIE1/153A.SP16.pro-tPA than the other plasmids. With this in mind, it is concluded that human pro-tPA can be secreted from Bm5 cells using the native human signal peptide sequence at least as efficiently as the silkmoth chorion signal peptide.

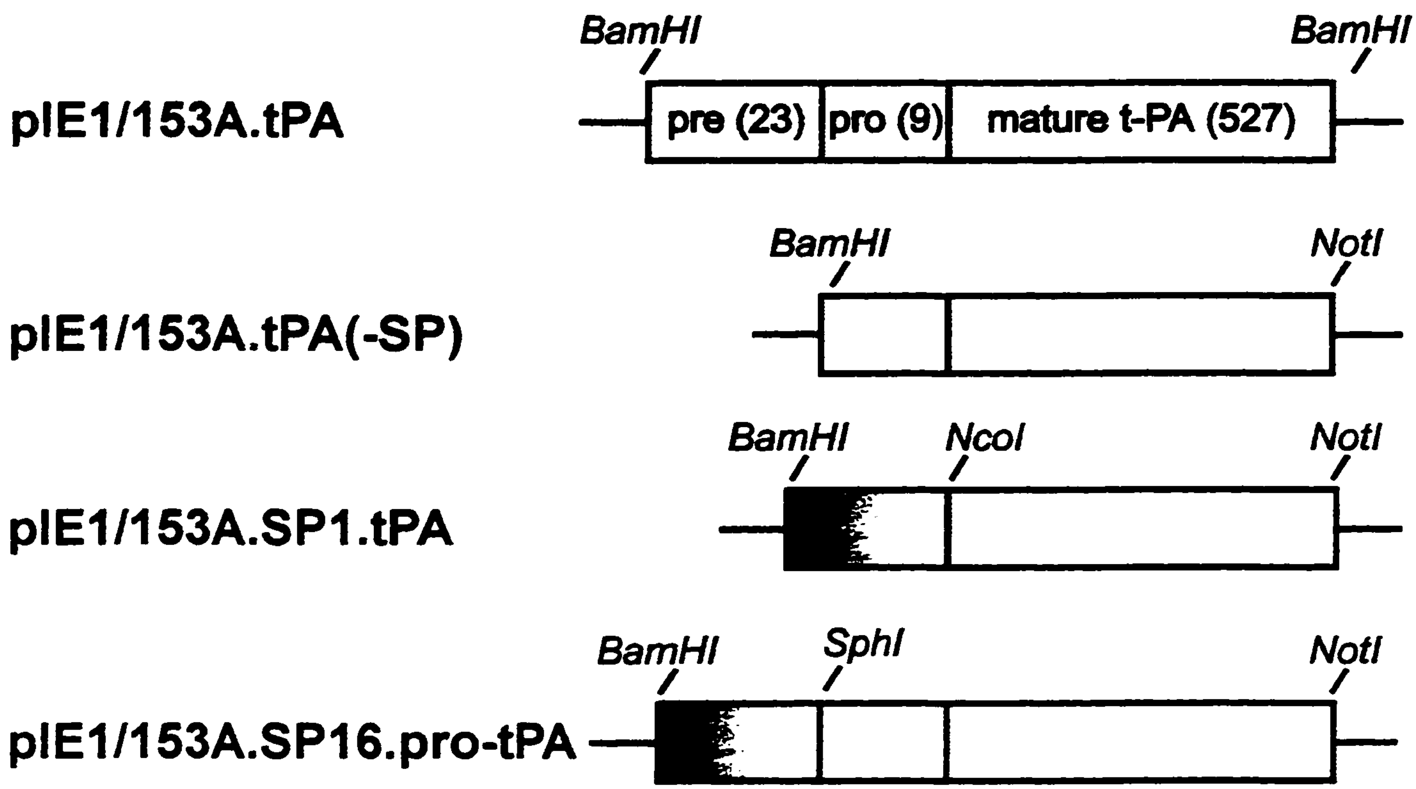


Figure 4.1: Constructs generated to test the secretion efficiency of human t-PA using the *Bombyx mori* chorion signal peptide from transfected Bm5 cells. The native human t-PA protein contains a 23 Amino acid pre region, a 9 amino acid pro- region and a 527 Amino acid mature open reading frame. The shaded box represents the chorion signal peptide.

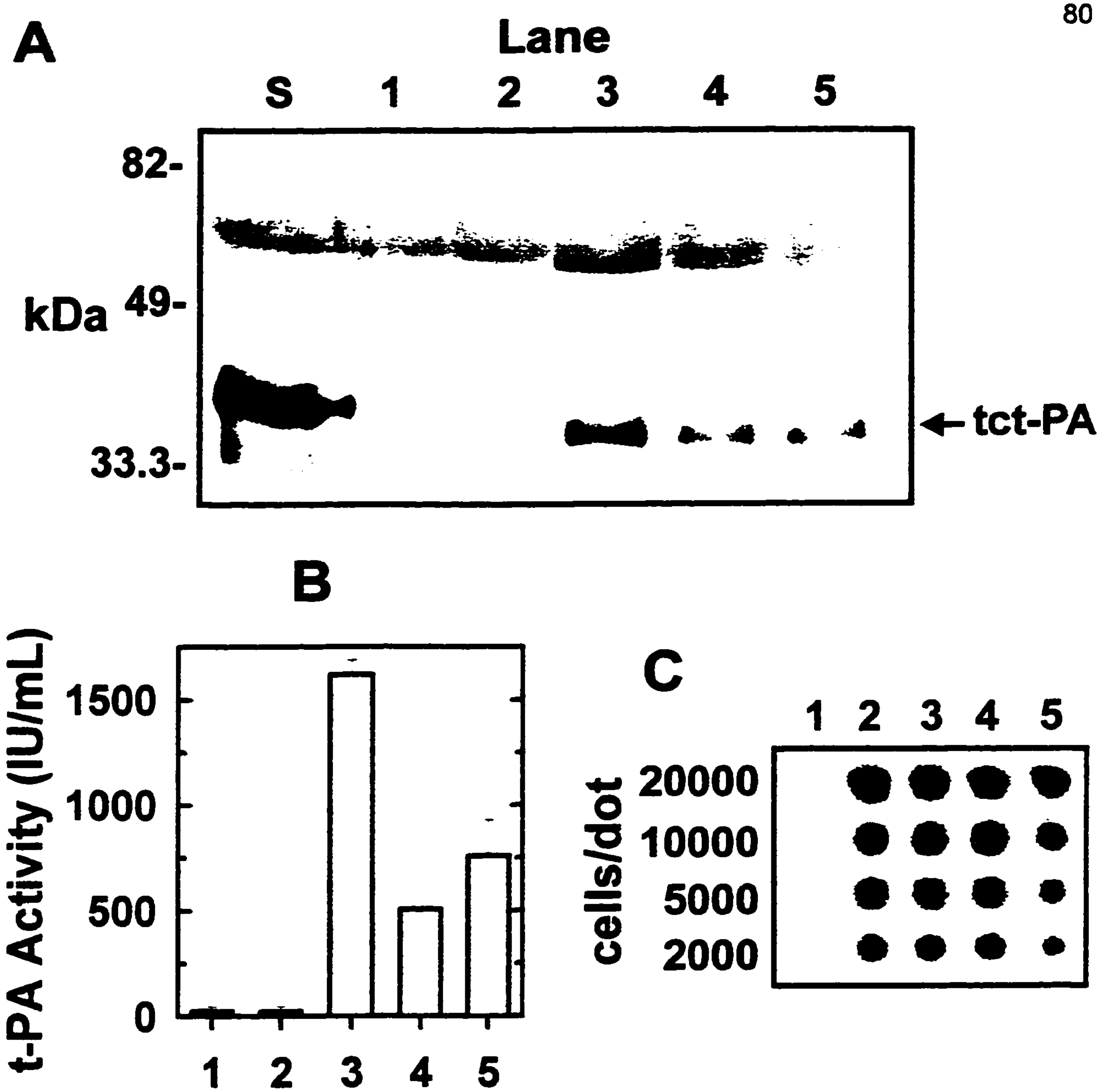


Figure 4.2: Transfection results to compare the secretion efficiency of the native human t-PA signal peptide with the *Bombyx mori* specific chorion signal peptide. Bm5 cells were transfected with the expression plasmids shown in Figure 4.1 and samples taken 60 h post-transfection for analysis. Samples 1 to 5 correspond to the plasmids pIE1/153A, pIE1/153A.tPA(-sp), pIE1/153A.tPA, pIE1/153A.SP1.tPA, and pIE1/153A.SP16.pro.tPA respectively. A) Western analysis of 20 μL aliquots of supernatant to compare the expression of t-PA. Samples were resolved by SDS-PAGE and probed with a goat anti-t-PA antibody. The lane marked *S* contains a tct-PA standard from Bowes melanoma. B) Tissue plasminogen activator activity measured in the supernatants of the corresponding samples analyzed in A). Bars represent standard deviations in sample measurements. C) Comparison of transfection efficiency by dot blot hybridization of samples analyzed in A).

4.3.2 Generation of a Cloned Insect Cell Line Over-Expressing Human t-pa

To generate an insect cell line over-expressing human t-PA, Bm5 cells were co-transfected with the plasmids pIE1/153A.t-PA and pBmA.HmB at a molar ratio of 100:1, and subcultured in 0.25 mg/mL hygromycin B over a 5 week period to obtain a polyclonal population. Thirty-six clones were isolated using limiting dilution cloning, and their supernatant screened by Western blotting. Compared to the polyclonal population, 6 clones expressed more t-PA, 20 clones expressed less, and 10 clones expressed little or no t-PA (data not shown). One clone, Bm5.t-PA#2, was selected for characterization.

4.3.3 Characterization of pIE1/153A.t-PA Integration into Bm5 cells

DNA isolated from Bm5.tPA#2 was subject to Southern analysis to determine if the expression plasmid was actually integrated into the Bm5 genome or maintained as a self-replicating extra-chromosomal array. In a previous report, the transformation of an *Aedes albopictus* (mosquito) insect cell line using hygromycin B selection resulted in self-replicating extrachromosomal arrays of up to 60,000 copies of plasmid, and plasmids could be rescued by transformations of *E. coli* with undigested total cellular DNA (Monroe et al., 1992). The DNA isolated from Bm5.tPA#2 and a plasmid pIE1/153A.t-PA DNA standard were digested with *NotI* to linearize plasmid DNA. If extra-chromosomal, Southern hybridization using a ^{32}P-labeled t-PA probe would reveal a signal at 13 kbp, equal to linearized plasmid DNA. However the signals from Southern blot in Figure 4.3A contain numerous discrete bands of varying molecular weight, indicating that the plasmid is randomly integrated into the Bm5 genome and not maintained as an extra-chromosomal element. Integration was confirmed when plasmid rescue by transformation of *E. coli* with total undigested cellular DNA was unsuccessful.

Dot blot hybridization of DNA isolated from Bm4.tPA#2 and plasmid DNA standards digested with *NotI* reveals that there are approximately 25 copies of the t-PA gene per normal haploid genome (Figure 4.3B). However, Bm5 cells have over 100 chromosomes, (Hink, 1968), compared to 28 in the normal *Bombyx mori* haploid genome. Thus there are approximately 100 copies of the t-PA gene/cell.

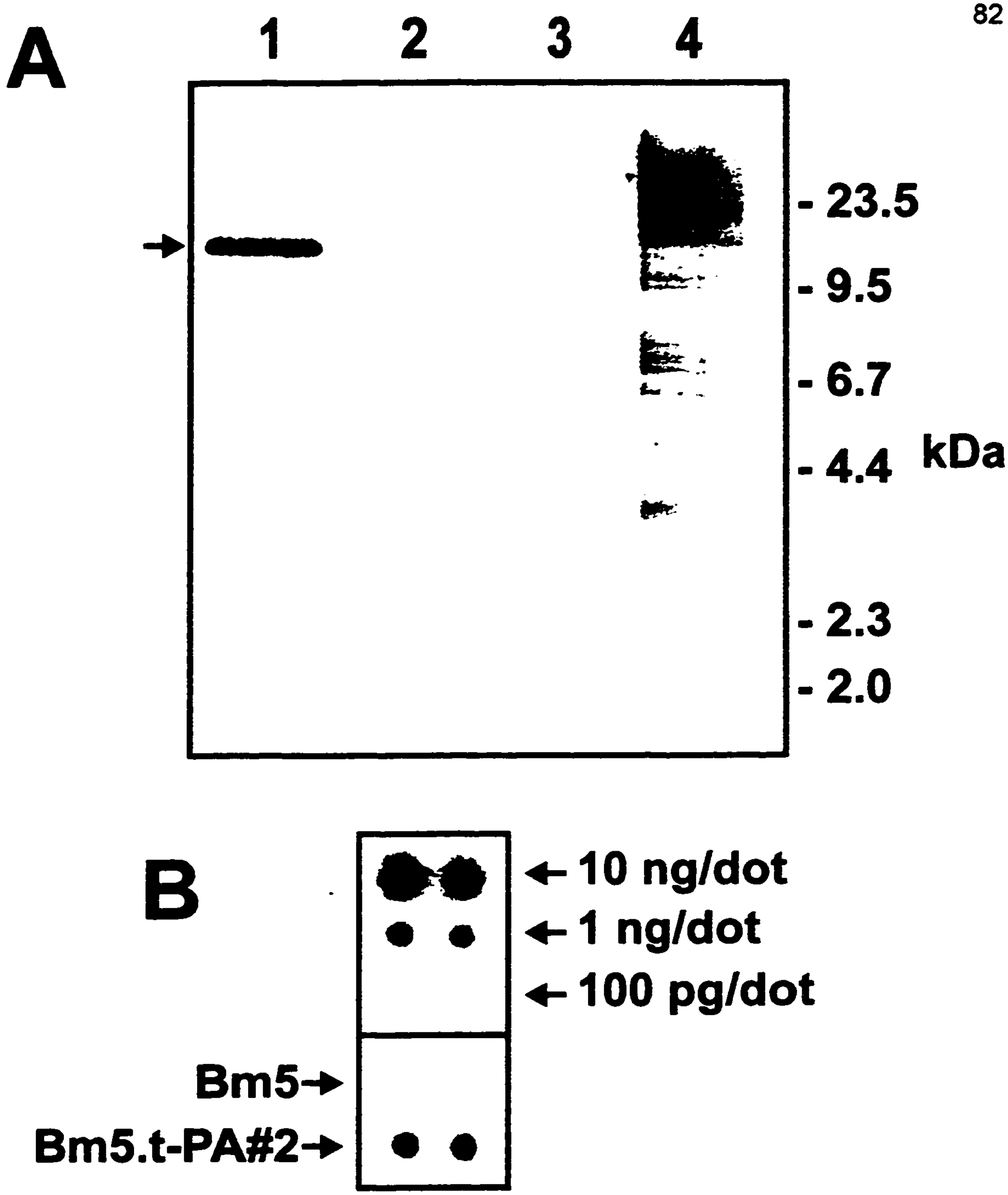


Figure 4.3: A) Southern analysis of total cellular DNA isolated from the stably transformed Bm5 clone, tPA#2, over-expressing t-PA. Five microgram samples of cellular DNA and 1 ng of pIE1/153A.tPA was digested with *NotI*, resolved by agarose gel electrophoresis, transferred to Hybond N+ membrane and hybridized with a random primed ^{32}P-labeled t-PA probe. B) Estimation of t-PA copy number by dot blot hybridization of 5 μg of *NotI* digested total cellular DNA isolated from tPA#2 cells and control Bm5 cells with a random primed ^{32}P-labeled t-PA probe. A standard of *NotI* digested pIE1/153A.tPA plasmid was used for comparison.

4.3.4 Expression of Biologically Active t-PA in Bm5 cells in Serum-Containing Medium

The cell line Bm5.tPA#2 was grown in stirred suspension culture in 100 mL spinner flasks in culture medium containing 10% fetal bovine serum. Figure 4.4A shows that cell line grew from 10^5 viable cells/mL to a maximum of 3.4×10^6 viable cells/mL by day 14. The biological activity of the t-PA produced in this system was measured and reached a maximum value 24,500±5,000 IU/mL by day 12 and declined to 17,900±5500 IU/mL by day 22. Western blot analysis (Figure 4.4B) revealed that the t-PA produced was a mixture of sct-PA, tct-PA, and a higher molecular mass immunoreactive species with a molecular mass of approximately 110 kDa. The higher molecular weight species is presumed to be a SDS-stable complex of t-PA and plasminogen activator inhibitor (PAI) present in serum (Sprengers and Kluft, 1987). An accurate determination of the molecular mass of sc-tPA could not be made due to co-migration of sct-PA with bovine serum albumin present in FBS. The concentrations of the three immunoreactive t-PA species at day 12 and 21 were determined by densitometric scanning of the autoradiogram and comparison to the sct-PA standard, and are summarized in Table 4.3. In total, 96 µg/mL of t-PA was produced by day 12 and 160 µg/mL was produced by day 21. The specific activity was approximately 255 IU/µg on day 12, declining to 112 IU/µg by day 22. The specific activity of pure t-PA produced from Bowes melanoma is 580 IU/µg when measured by a fibrin clot assay (International reference standard tPA preparation 83/517, World Health Organization).

4.3.5 Expression of Biologically Active t-PA in Bm5 cells in Serum-Free Medium

The cell line Bm5.tPA#2 was adapted to EC400 serum-free medium and the growth and t-PA expression was characterized in static cuture over a 14 day period. Here cells grew from 5×10^5 to 2×10^6 viable cells/mL after 8 days and produced approximately 16,000 IU/mL t-PA by day 14 (Figure 4.5A). Western blotting (Figure 4.5B) revealed that the t-pA species expressed in serum-free medium is only sct-PA with an apparent molecular weight of 62 kDa, which is approximately 10% lower than the Bowes melanoma sct-PA standard, presumably due to glycosylation differences. Densitometric scanning of the autotradiogram indicated that the t-PA accumulated to 75 µg/mL after 8 days and 135

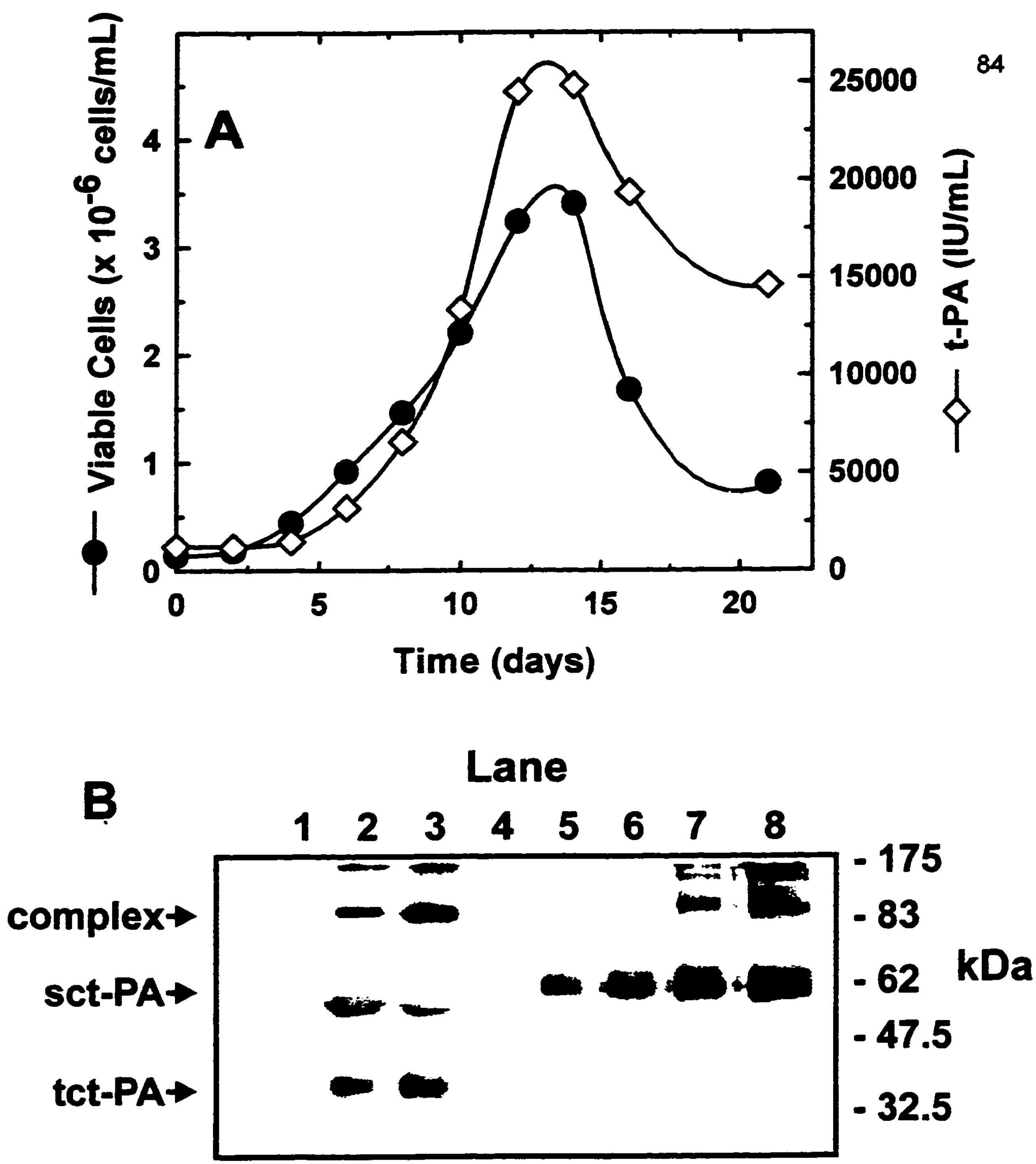


Figure 4.4: A) Batch production of t-PA from stably transformed Bm5 cells in a 100 mL spinner flask over a 21 day period in serum-containing medium. Bars represent standard deviations in sample measurements. B) Western analysis of 6 μL culture supernatant samples from A), and comparison to a single chain t-PA standard. Lane 1 contains control IPL-41 + 10% FBS, lane 2 contains day 12 supernatant, lane 3 contains day 21 supernatant, and lanes 4 to 8 contain 0, 0.25, 0.5, 0.75 and 1.0 μg sc-tPA. standard respectively.

Day	t-PA Activity (IU/mL)	t-PA Concentration (μg/mL)				Average Specific Activity (IU/μg)
		tc-tPA	sc-tPA	110 kDa t-PA Complex	TOTAL t-PA	
12	24,500	28	43	25	96	255
21	17,900	55	40	65	160	112

Table 4.3: Comparison of the average specific activity and distribution of t-PA species present at two time points in a batch suspension culture of Bm5.tPA#2 cells grown in serum-containing medium. The various concentrations were determined by densitometric scanning of the autoradiogram shown in Figure 4.4 and comparison to the sct-PA standards.

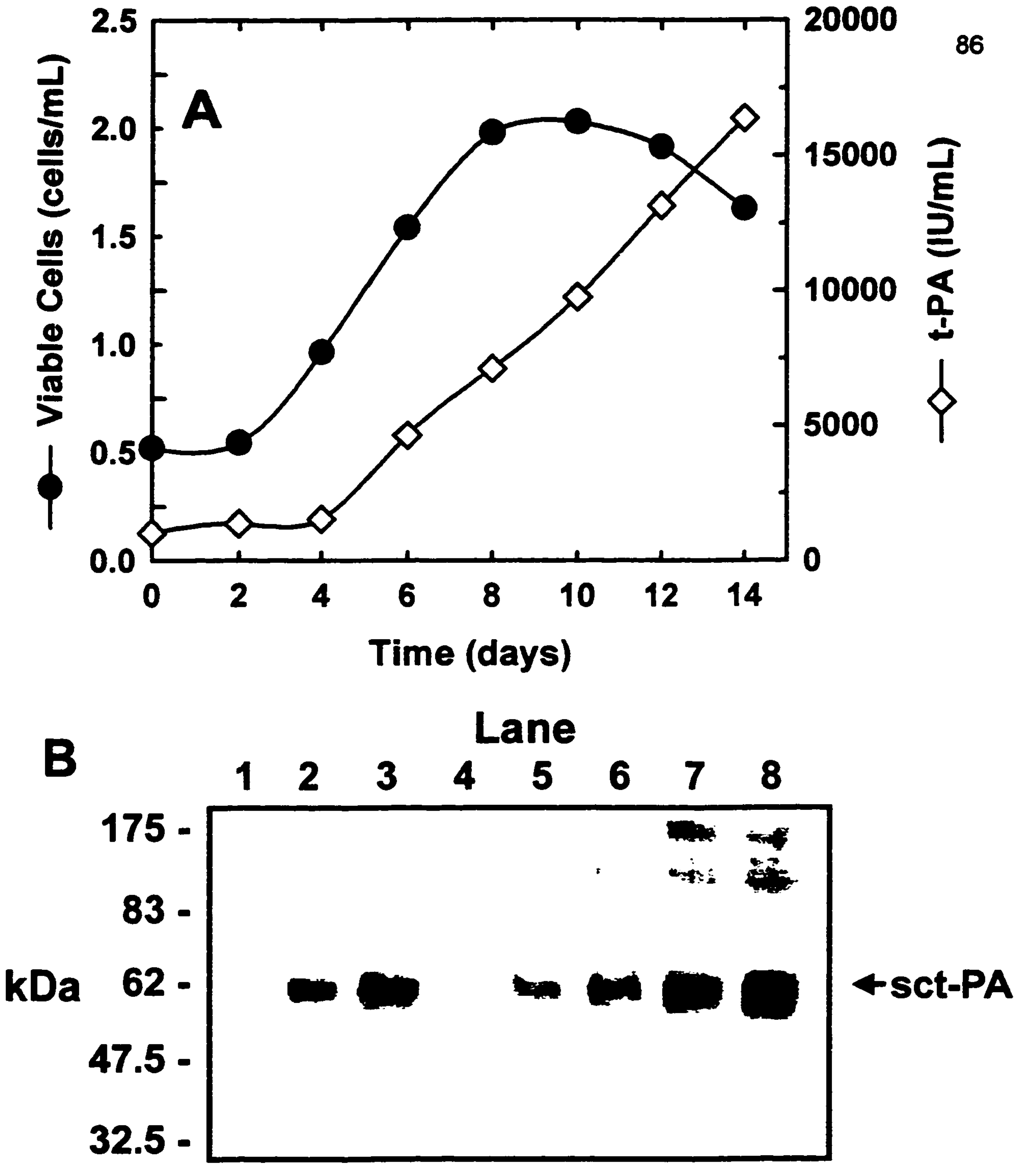


Figure 4.5: A) Batch production of sct-PA from stably transformed Bm5 cells in static culture over a 14 day period in serum-free medium. Bars represent standard deviations of sample measurements. B) Western analysis of 4 µL culture supernatant samples from A), and comparison to a single chain t-PA standard. Lane 1 contains control IPL-41 + 10% FBS, lane 2 contains day 8 supernatant, lane 3 contains day 14 supernatant, and lanes 4 to 8 contain 0, 0.25, 0.5, 0.75 and 1.0 µg sc-tPA standard respectively.

μg/mL after 14 days. The specific activity of the sct-PA was estimated to be 100 to 120 IU/μg.

4.3.6 Expression of Biologically Active t-PA in other Lepidopteran Insect Cell Lines

Stably transformed polyclonal populations of Bm5, High Five, and Sf21 cells over-expressing t-PA were generated by transfecting these cells with pIE1/153A.t-PA and pBmA.HmB at a molar ratio of 100:1, followed by selection using 0.5 mg/mL, 2.0 mg/mL and 1.0 mg/mL of hygromycin B selective pressure respectively. The amount of t-PA produced by each population represents the average production for the distribution of clones. The growth and production of t-PA by each population was characterized by seeding cells into 6-well plates and measuring the viable cell density and relative t-PA levels after 8 days. High Five cells produced more immunoreactive t-PA than both Bm5 cells and Sf21 cells, and were transformed in a shorter time period (Table 4.4).

4.4 Discussion

In this Chapter, a stably transformed Bm5 insect cell line over-expressing recombinant human t-PA was characterized. It was demonstrated that the production of a recombinant protein of human origin, that is extensively modified post-translation and used as a therapeutic agent, is feasible using stably transformed insect cells.

The use of signal peptides of insect origin for the secretion of heterologous proteins from lepidopteran cell lines has been investigated previously for proteins expressed using baculovirus vectors. Because baculoviruses are known to compromise the secretory pathway of infected host cells (Jarvis et al., 1989), it was possible that the issue of signal peptide recognition would be clearer in uninfected lepidopteran cells. The choice of the chorion signal peptide was rationalized on the large amounts of chorion polypeptides that are secreted from relatively few follicular cells during the formation of an eggshell around a developing oocyte in a lepidopteran insect. By transfecting Bm5 cells with expression plasmids, it was observed that the secretion of human t-PA using the native human prepro-sequences was no less efficient than than that directed by the silkmoth chorion signal peptide. These results are in agreement with those of Jarvis and co-workers (Jarvis et al.,

Cell Line	[Hm B] (mg/mL)	Transformation Time[1] (weeks)	Initial Cell Density (viable cells/mL)	Final Cell Density[2] (viable cells/mL)	Relative t-PA Produced[2,3]
Bm5	0.5	6	0.45×10^6	2.2×10^6	1
Sf21	1	3	0.55×10^6	3.4×10^6	0.6
High Five™	2	3	0.55×10^6	1.6×10^6	1.8

[1] Time to obtained stably transformed population following transfection.
[2] After 8 days growth in a 6-well plate.
[3] Determined by ELISA

Table 4.4: Comparison of t-PA production from stably transformed polyclonal populations of Bm5, Sf21, and High Five™ cells. Cells were seeded into 6-well plates at an initial cell density of 5×10^5 viable cells/mL, and allowed to grow for 8 days. The final viable cell density and relative amount of immunoreactive t-PA produced was compared.

1993), who found that secretion of native human prepro-t-PA in baculovirus-infected Sf9 insect cells was not affected by potentially better signal peptides of insect origin including that from mellitin, a native secreted protein of the hymenopteran honeybee; cecropin B, a native secreted protein of the lepidopteran insect *Hyalophora cecropia*; or 64k, a major structural glycoprotein of the baculovirus AcNPV.

Expression levels of human t-PA obtained from transformed Bm5 cells are high and in the same range as those reported in Chapter 3 using juvenile hormone esterase. Western blotting confirmed that transformed Bm5 cell lines were able to produce considerably more quantities of t-PA in both serum-containing (160 µg/mL) and serum-free medium (135 µg/mL) than reports using the baculovirus and transformed mammalian or insect cell lines (Table 4.1). Quantitations using Western blotting were preferred over ELISA, which cannot necessarily distinguish intact proteins from immunoreactive fragments or complexes. In serum-containing medium, the specific activity of the t-PA produced reached a maximum of 255 IU/µg, significantly lower than the t-PA produced by the Bowes melanoma cell line (580 IU/µg), and declined to 112 IU/µg by the end of the culture period. The stability of t-PA is fairly high from -20 to 37°C (Gaffney and Curtis, 1985) and the Western blot in Figure 4B did not reveal significant degradation. Thus it is likely that plasminogen activator inhibitors naturally present in fetal bovine serum, including PAI-1 (Sprengers and Kluft, 1987), α_2-antiplamsin, α_2-antitrypsin, and C_1 inhibitor (Collen and Lijnen, 1986), contributed to the low specific t-PA activity (IU/µg) of the t-PA expressed by insect cells and a decline in t-PA actvitiy in the culture supernatant (IU/mL) with time. In serum-free conditions, sct-PA activity in the supernatant did continue to increase throughout the culture period and the specific activity was consistent (95-120 IU/µg), although significantly lower than that produced by Bowes melanoma. Single chain t-PA has been reported to have a lower specific activity than tc-tPA using the Spectrozyme PL indirect chromogenic assay (Boose et al., 1989) that was used in this study. Furthermore, the species of tPA produced from Bm5 cells appears underglycosylated when compared to Bowes melanoma derived sct-PA, and glycosylation has also been reported to affect enzymatic activity of recombinant t-PAs using a similar indirect chromogenic assay (Parekh et al., 1989). In contrast, the expression of sct-PA from baculovirus infected Sf9 cells was reported with a specific activity of 1250 IU/µg (Steiner et al., 1988).

Finally, it was shown that High Five^{TM} and Sf21 cells can also be stably transformed to express t-PA. High Five^{TM} cells produced more immunoreactive tPA than both Bm5 cells and Sf21 cells, and were transformed in a shorter time period. High Five^{TM} cells grow well in suspension culture (Dee et al., 1997) with the reported capacity for complex glycosylation (Davis and Wood, 1995). Aside from employing alternative insect cell lines to Bm5 cells, further improvements in recombinant protein yields are expected through the use of more effective bioreactor configurations or the utilization of lepidopteran culture media that can elevate the maximum viable cell densities.

CHAPTER 5
Employment of a Secreted Protein in a Module for the Secretion and Purification of Intracellular Proteins

5.0 Summary

In the expression of recombinant polypeptides from genetically engineered organisms, extra yield-reducing steps for purification are required when the polypeptide of interest cannot be naturally secreted into an extracellular environment. In this chapter, the ability of an insect-specific signal peptide derived from the *Bombyx mori* L.12B chorion protein to direct the secretion of three intracellular proteins [chloramphenicol acetyl transferase (CAT), *Bombyx mori* chorion factor 1 (BmCF1)] expressed from transfected insect cells was examined. While this signal peptide functioned efficiently as a chimera with a normally secreted protein, juvenile hormone esterase (JHE), chimeras of the chorion signal peptide fused to these two intracellular proteins were not secreted, suggesting that additional signals were required for successful passage through the secretory pathway. We therefore generated DNA coding for a secretion module - a fusion protein that contains JHE at the N-terminus, followed by a spacer region containing both a histidine tag and an enteropeptidase cleavage site, and the desired intracellular protein attached to the C-terminus. The presence of JHE supplied all the signals necessary to "piggy-back" the intracellular protein into an extracellular environment. This resulted in the efficient secretion of both CAT and BmCF1 from transfected insect cells. The intra-protein histidine tag allowed purification of the fusion protein using metal chelate affinity chromatography under non-denaturing conditions, and the intra-protein enteropeptidase cleavage site was recognized for liberation of the intracellular protein from the secretion module.

5.1 Introduction to Secretion Systems

Cellular systems for over-expressing recombinant proteins allow simpler production and purification schemes when the desired protein can be secreted rather than expressed intracellularly. Most intracellular proteins including cytoplasmic proteins, nuclear factors and protein subunits are currently over-expressed inside prokaryotic cells or lower eukaryotic cells, while expression of intracellular proteins from animal cells is largely accomplished using lytic viral systems, such as the baculovirus, rather than stably transformed mammalian or insect cell lines. These systems can be problematic when inclusion bodies of insoluble protein form (Datar et al., 1993), proteolysis occurs (Copeland and Wang, 1991), and the desired protein has to be isolated from other intracellular proteins. Recombinant protein purification often requires employment of denaturing conditions, followed by post-purification refolding steps to restore the biological activity, as shown by the example in Table 5.1. Alternatively, animal cell lines of mammalian or insect origin have advanced secretory pathways that can potentially be harnessed to secrete overexpressed proteins, that are normally intracellular, into culture medium. Recovery of the protein would then neither involve harming the cells, allowing continuous over batchwise production, nor be complicated by the presence of other intracellular proteins, and could be performed under non-denaturing conditions.

Secreted proteins possess an N-terminal signal peptide of 15 to 30 amino that directs a nascent polypeptide to the secretory pathway of eukaryotic cells or to the cytoplasmic membrane of bacterial cells (von Heijne, 1988). Attempts to secrete intracellular proteins from animal cells by attaching various signal peptide coding in frame to the 5' end of a gene of an intracellular protein have occasionally resulted in the successful secretion of the resulting chimeric protein (Mroczkowski et al, 1994; Laukkanen et al., 1996), while other attempts have been unsuccessful (Tessier et al., 1991; Danoff and Shields, 1993; Martens et al, 1995). Additional auxiliary signals presumably exist within a secretion competent protein for efficient passage through the secretory pathway.

In previous chapters, an effective transformed insect cell expression system for the high level production of secreted proteins was established. This chapter describes attempts to extend this expression system to allow continuous secretion of intracellular proteins using

Secreted t-PA Protein	
Cell Line	CHO
Fermentation Mode	Batch, 5-7 days
Product Concentration	33.5 mg/L
Recovery Operations	Microfiltration Ultrafiltration Affinity Chromotography Gel Chromotography
Downstream Purification Steps	5
Overall Process Yield	47% (16.7 mg/L)
Non-Secreted t-PA Protein	
Strain	*E. coli* K12
Plasmid	pXL130
Fermentation Mode	Batch, 1-2 days
Product Concentration	460 mg/L
Recovery Operations	Centrifugation Ultrafiltration Solubilization Cleavage Re-folding Affinity Chromotography Gel Chromotography
Downstream Purification Steps	16
Overall Process Yield	2.8% (13 mg/L)

Table 5.1: Process purification data for the expression of t-PA either as a secreted protein from CHO cells or a non-secreted protein in *E. coli* (Datar et al., 1993). There is a clear process advantage when the recombinant protein can be secreted.

two approaches: first, by fusing insect specific signal peptide coding from the silkmoth *Bombyx mori* chorion protein (eggshell) in-frame to the 5' end of the desired intracellular protein coding, and second, by fusing the full length gene of juvenile hormone esterase (JHE; Hanzlik et al, 1989), a naturally secreted insect glycoprotein, to the 5' end of the desired intracellular protein coding. If a signal peptide alone proves insufficient for the secretion of intracellular proteins, the latter approach should provide the additional hypothetical coding required for successful passage through the secretory pathway. To demonstrate this secretion module, we have used the bacterial cytoplasmic protein chloramphenicol acetyl transferase (CAT) and the insect nuclear factor *Bombyx mori* chorion factor 1 (BmCF1) as examples of intracellular proteins.

5.2 Materials and Methods

Cell culture, transfections, JHE assays, CAT assays and Western blots have been described previously. For the detection of CAT by Western blot, a rabbit polyclonal anti-CAT antibody (5 Prime - 3 Prime, Inc; 1:1000 dilution) was used as the primary antibody. For detection of BmCFI by Western blot, a mouse monoclonal anti-USP (the drosophila homolog of BmCFI) was used as the primary antibody (provided by Dr F.C Kafatos, EMBL Heidelberg, Germany; 1:30 dilution).

5.2.1 Plasmid Constructions

For the attachment of the truncated *jhe* open reading frame to the chorion signal peptide, the PCR primers 1 and 2 were synthesized (Table 4.2). PCR amplification using *Pfu* polymerase and the plasmid pIE1/153A.jhe (kk) template yielded a 1.8 kbp product that was digested with *NotI* and partially with *SphI* and and ligated in-frame into the unique *SphI/NotI* sites of psp16 to yield psp16.jhe. A complete *NotI*/partial *BamHI* digest of psp16.jhe released a 1.7 kbp fragment containing the chimeric gene that was ligated into the *BamHI/NotI* sites of the expression cassette pIE1/153A to yield pIE1/153A.sp16.jhe. For expression of a truncated form of JHE that contains no signal peptide, psp16.jhe was digested with *NotI* and partially with *NcoI* and the 1.5 kbp product was cloned into the expression cassette to yield the plasmid pIE1/153A.Δjhe. The truncated *jhe* gene contains

1)	5'-CGTGGCGCATGCAAAATTCGCGCAGCGTGG-3'
2)	5'-CGATGGTGATGACCTGACCGTC-3'
3)	5'-GGGCTACCATGGAGAAAAAAATCACTGG-3'
4)	5'-GGGTGCTCTAGAATTTCTGCCATTCATCC-3'
5)	5'-TGTGGGCATGCAGAGCGTGGCGAAG-3'
8)	5'-CGACATTCAAATCTAGAATAAGTCCCCCTAC-3'
7)	5'-AAAAAGGATCCAAAATGGCCGCTAAACTCATTCTCTTCGTCTTCGTCTGCGCCACCGCCCTCGTG-3'
8)	5'-AAAAAATCTAGAAAA**G/C**CATG**C/GC/A**TAAGACGGACTGGGCCACGAGGGCG-3'
9)	5'-AAAAGGATCCATGACTTCACACGTACTCGC-3'
10)	5'-AAAA GGA TCC TTC AAG CGG GCT TCT ACT G-3'
11)	5'-GGGCTACCATGGAGAAAAAAATCACTGG-3'
12)	5'-GGGTGCTCTAGAATTTCTGCCATTCATCC-3'

Table 5.2: Summary of oligonucleotides synthesized for the generation of DNA constructs needed in this chapter. Bold indicates degeneracies.

a synthetic translation start codon.

For attachment of the *cat* open reading frame to the chorion signal peptide, the PCR primers 3 and 4 were synthesized (Table 5.2). PCR amplification using *Pfu* polymerase and the plasmid pIE1/153A.cat template yielded a 0.7 kbp product that was digested with *NcoI* and *XbaI* and ligated in-frame into the unique *NcoI/XbaI* sites of pSP5 to yield pSP5.cat. A *BamHI/NotI* digest of pSP5.cat released a 0.8 kbp fragment containing the chimeric gene that was ligated into the *BamHI/NotI* sites of the expression cassette pIE1/153A to yield pIE1/153A.SP16.cat.

The plasmid pIE1/153.BmCFI was generated by ligating a 3.8 kbp *NotI* fragment of pBmCF1 (Tzertzinis et al., 1994) containing the *BmCF1* open reading frame into the unique *NotI* site of pIE1/153A to yield the plasmid pIE1/153A.BmCF1. For attachment of the BmCF1 open reading frame to the chorion signal peptide, the PCR primers 5 and 6 were synthesized (Table 4.2). PCR amplification using *Pfu* polymerase and the plasmid pIE1/153A.BmCF1 template, yielded a 1.4 kbp product that was digested with *SphI* and *XbaI* and ligated in-frame into the unique *SphI/XbaI* sites of psp16 to yield psp16.BmCF1. A *BamHI/NotI* digest of psp16.BmCF1 released a 1.5 kbp fragment containing the chimeric gene that was ligated into the *BamHI/NotI* sites of the expression cassette pIE1/153A to yield pIE1/153A.sp16.BmCF1.

The plasmid pIE1/153A.jhe.6H.EP.cat was generated in several steps. First oligonucleotides 7 and 8 were synthesized to code for the spacer region in Figure 5.6 These oligonucleotides were annealed together, endfilled by mutually primed synthesis with *klenow* enzyme, double digested with *BamHI* and either *NcoI* or *SphI*, and ligated into pBluescript-SK+ to yield p6H.EP(*NcoI*) or p6H.EP(*SphI*). Then digestion of pSP5.cat with *NcoI* and *NotI* yielded a 0.7 kb product containing the *cat* open reading frame that was ligated in-frame into the *NcoI/NotI* sites of pHisEP(*NcoI*) to yield p6H.EP.cat. Next, PCR amplification using the primers 9 and 10 (Table 5.2), *Pfu* polymerase and the plasmid pIE1/153A.jhe(kk) template yielded a 1.6 kb product containing the *jhe* open reading frame (with no stop codon) that was partially digested with *BamHI* and ligated in-frame and in correct orientation into the unique *BamHI* site of p6H.EP.cat to yield pJHE.6H.EP.cat. A partial

BamHI/complete *NotI* digestion of pjhe.HisEP.cat released a 2.5 kbp fragment containing the coding for the fusion protein that was ligated into the unique *BamHI/NotI* sites of pIE1/153A to yield pIE1/153A.jhe.6H.EP.cat.

The plasmid pIE1/153A.jhe.6H.EP.BmCF1 was generated in several steps. Digestion of psp16BmCF1 with *SphI* and *NotI* yielded a 1.4 kb product containing the *BmCF1* open reading frame that was ligated in-frame into the unique *SphI/NotI* sites of pHisEP (*SphI*) to yield p6H.EP.BmCF1. Then a partial *BamHI* digestion of pjhe.6H.EP.cat released a 1.4 kbp fragment containing the JHE open reading frame that was ligated into the unique *BamHI* site of p6H.EP.BmCF1 to yield the plasmid pjhe.HisEP.BmCF1. A partial *BamHI*/complete *NotI* digestion of pjhe.HisEP.BmCF1 released a 3.2 kbp fragment containing the coding for the fusion protein that was ligated into the unique *BamHI/NotI* sites of pIE1/153A to yield pIE1/153A.jhe.6H.EP.BmCF1.

5.3 Results

5.3.1 Expression of an Intracellular Expressed Protein, CAT, in Transformed Insect Cells is Inferior to the Baculovirus Expression System

To assess the ability of our expression system to over-express an intracellular protein, chloramphenicol acetyl transferase (CAT) was cloned into the *BamHI* sites of the expression cassette to form the plasmid pIE1/153A.cat. A polyclonal population of transformed Bm5 cells over-expressing CAT was obtained following transfection with pIE1/153A.cat and antibiotic selection. Cells from this transformed population were seeded into 25 cm^2 T-flasks at 5×10^5 cells/mL and produced a total of 1.5 µg/mL CAT (0.8 µg/mL intracellular CAT and 0.7 µg/mL extracellular CAT) after 7 days growth. For comparative purposes a recombinant baulovirus, BmNPV.p95.CAT (Schmiel et al, 1993), was used to infect Bm5 cells seeded at 5×10^5 cells/mL in 6 well plates at a moi of approximately 5. CAT accumulated to a maximum of 49 µg/mL by 7 days post infection, substantially higher than the transformed population (Figure 5.1). Attempts to obtain a highly productive clone from the polyclonal population were unsuccessful due to the low frequency of high expressors (from 48 clones tested most produced little or no CAT, while the best clone expressed only 3-fold more CAT than the heterogeneous population). It is presumed that a cell becomes

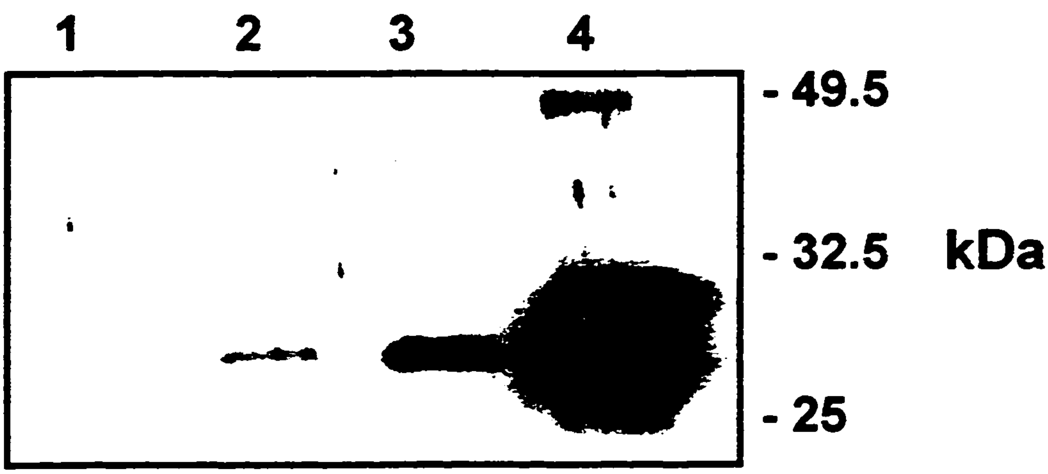


Figure 5.1: Western blot of 20 μL samples of culture medium comparing the expression of an intracellular protein, CAT, from pIE1/153A.cat transfected (lane 2) and stably transformed polyclonal Bm5 cells (lane 3) with baculovirus (BmNPV.p94.cat) infected Bm5 cells (lane 4). Lane 1 contains control IPL-41 + 10% FBS medium. The transformed cell expression levels of CAT (28 kDa) are inferior to the baculovirus expression system.

saturated with intracellular protein, restricting the expression levels. Thus a method to secrete intracellular proteins from transformed cells would not only facilitate purification but also improve expression levels.

5.3.2 A Signal Peptide Derived from a Silkmoth Chorion Protein Functions Efficiently for the Secretion of a Secretion Competent Polypeptide

In the formation of an eggshell around a developing oocyte in the lepidopteran insect *Bombyx mori*, large amounts of chorion polypeptides are efficiently secreted from relatively few follicular cells. We therefore synthesized chorion signal peptide coding for the optimal recognition and secretion of heterologous proteins by lepidopteran tissue culture cells, using our insect cell expression system.

To test the secretion efficiency of this signal peptide, its coding sequence was fused to the 5' end of a truncated JHE gene that lacks its native signal peptide coding, and this chimeric gene was cloned into the expression cassette pIE1/153A to yield the plasmid pIE1/153A.sp16jhe (Figure 5.2). Both the truncated *jhe* gene (with a synthetic translation start codon) and the native, full length *jhe* gene were also cloned into the expression cassette to yield the control plasmids pIE1/153A.Δjhe and pIE1/153A.jhe respectively. JHE is a convenient reporter protein (Bonning and Hammock, 1995) which can be detected with a sensitive radioactive assay (Hammock and Sparks, 1977) or antibodies. Therefore transient expression assays (transfections) were used to compare the expression of the levels of secretion of the chimeric protein with the native, full length protein. Sixty hours following the transfection of Bm5 cells, JHE activity assays revealed that both the full length and chimeric protein were secreted at similar levels in the culture supernatant (Figure 5.3A). Western blotting of both transfected cells and supernatants confirmed both the relative activities of secreted JHE and that the chorion signal peptide was also efficiently cleaved off prior to secretion (Figure 5.3B). Thus the chorion signal peptide can function in an identical manner as the native JHE signal peptide.

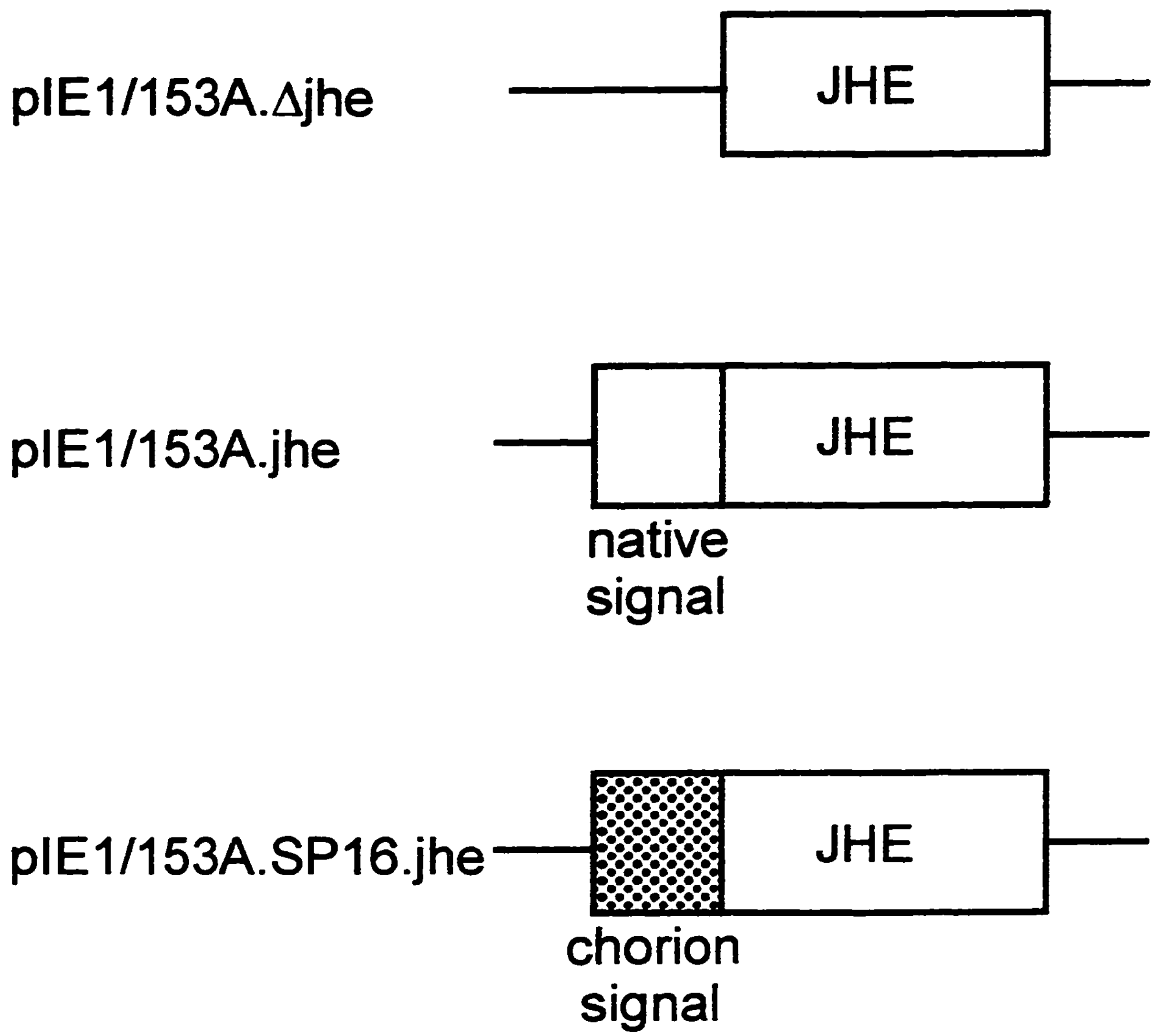


Figure 5.2: DNA constructs used to compare the function and efficiency of the *Bombyx mori* L.12B chorion signal peptide with the native JHE signal peptide for the secretion of JHE from transfected Bm5 cells.

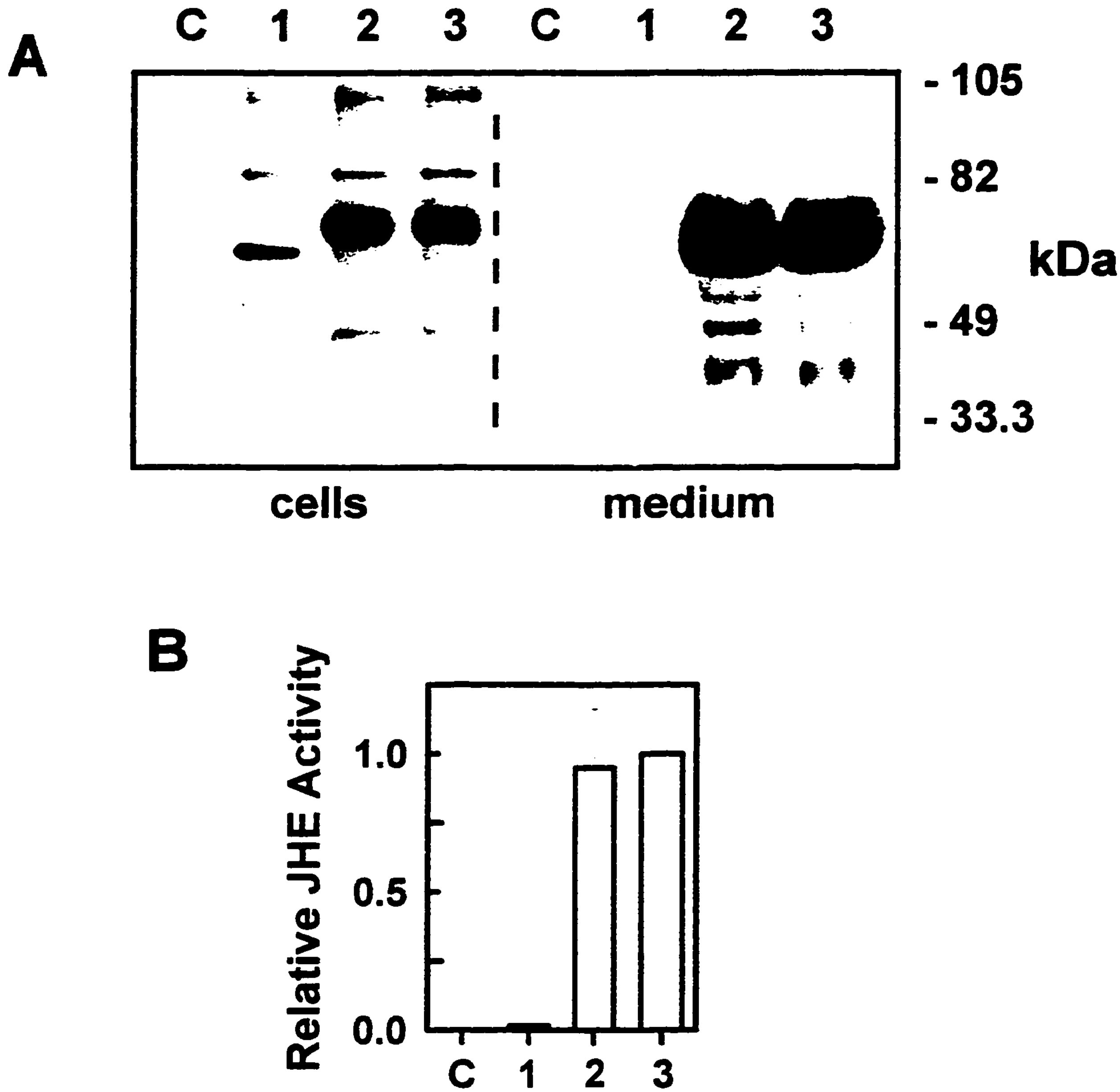


Figure 5.3: A) Western blot of 50,000 cells and 20 µL of culture supernatants of Bm5 cells transfected with the constructs shown in Figure 4.2 to compare the function and secretion efficiency of JHE using either the chorion signal peptide or the native JHE signal peptide. B) JHE assays of the samples in shown in A) confirm that the chorion signal peptide is equally efficient as the native JHE signal peptide for the secretion of heterologous proteins from transfected Bm5 cells. Plasmids 1 is pIE1/153A.Δjhe, 2 is pIE1/153A.jhe, 3 is pIE1/153A.sp16jhe, and C is pBSK+.

5.3.3 The Chorion Signal Peptide Fails to Secrete a Bacterial Intracellular Protein (CAT) or an Insect Nuclear Factor (BmCF1)

To test whether the chorion signal peptide could be used to secrete an intracellular protein, the chorion signal peptide sequence was fused to the 5'-end of the chloramphenical acetyl transferase (*cat*) open reading frame (ORF) and this chimeric gene was inserted into pIE1/153A to yield pIE1/153.SP5cat (Figure 5.4A). The native *cat* gene was also cloned into the expression cassette to yield the control plasmid pIE1/153A.cat (Figure 5.4A). Sixty hours after transfection of Bm5 cells with these plasmids, Western blot analysis revealed that both native CAT, and a heavier protein corresponding to the chimeric CAT were expressed inside the cells (Figure 5.4B). However, only small amounts of the chimeric CAT protein were present in the supernatant compared to the native CAT protein, which naturally leaks from transfected animal cells (Bunker and Moore, 1988). In addition, the species of CAT faintly detected in the culture supernatant retained the signal peptide coding.

To ensure that the failure to secrete CAT using the chorion signal peptide was not merely an anomaly of the CAT protein, the chorion signal peptide sequence was also fused to the 5'- coding for *Bombyx mori* chorion factor 1 (BmCF1), a nuclear factor broadly expressed in silkmoth tissue (Tzertzinis et al., 1994), and inserted into the expression cassette to yield the plasmid pIE1/153A.sp16BmCF1 (Figure 5.5A). The native *BmCF1* was also cloned into the expression cassette to yield the control plasmid pIE1/153A.BmCF1 (Figure 5.5A). Western blot analysis of Bm5 cells 60 h post-transfection revealed that both the chimeric and native forms of BmCF1 were overexpressed inside the cells; however, BmCF1 was not directed into the supernatant by the chorion signal peptide (Figure 5.5B).

5.3.4 A Juvenile Hormone Esterase Fusion Protein can Efficiently Secrete CAT

Having shown that attachment of the chorion signal peptide sequence was insufficient for the secretion of CAT and BmCF1, but sufficient for the secretion of JHE, it was concluded that additional intramolecular signals present on secretion competent proteins are required for passage through the secretory pathway. Therefore two secretion modules were generated in Bluescript (Figure 5.6A), coding for a fusion protein that contains the full length *JHE* gene on the 5' end, followed by a spacer region coding for six

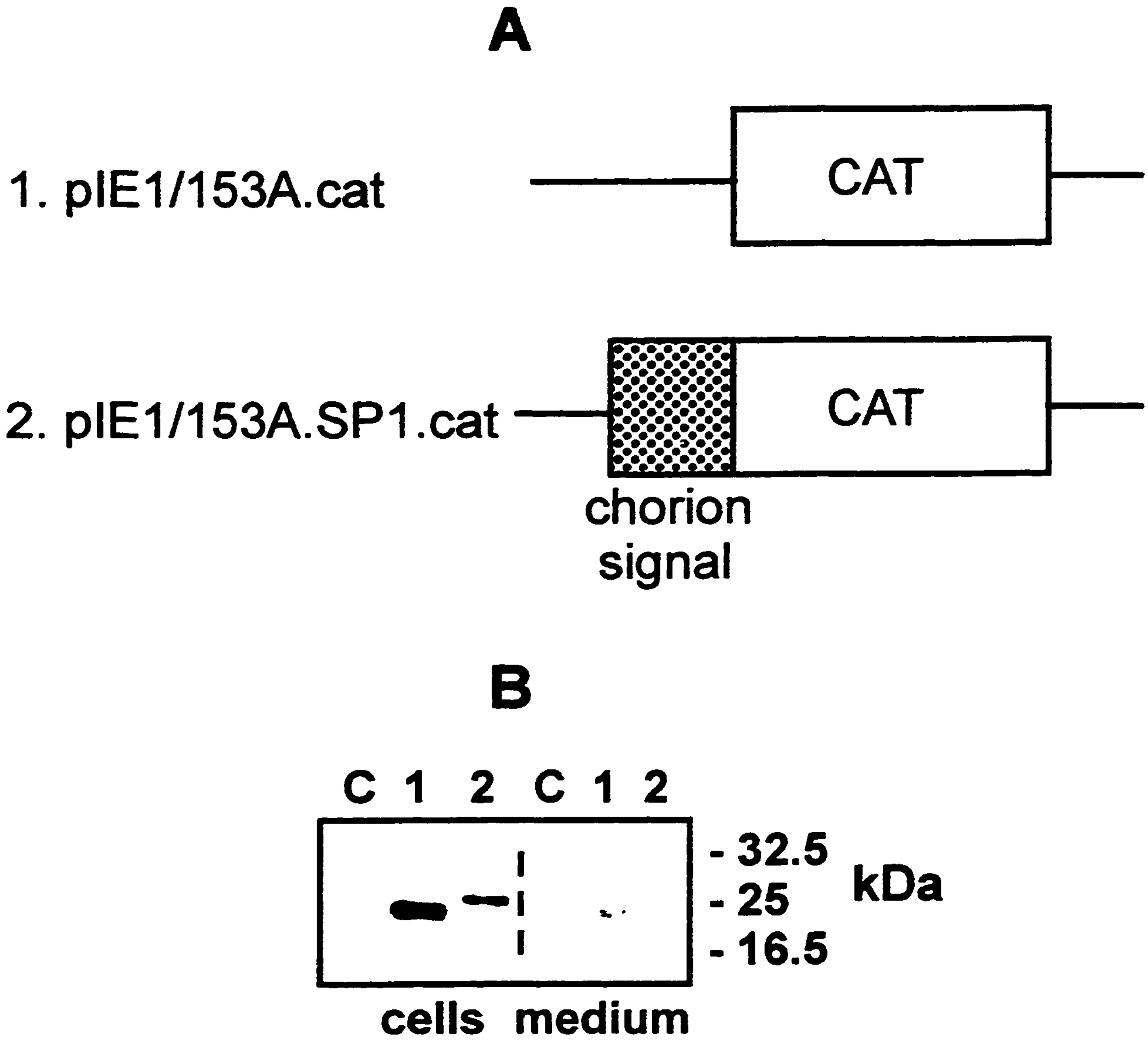


Figure 5.4: A) DNA constructs used to test the ability of the chorion signal peptide to secrete a bacterial cytoplasmic protein, CAT, from transfected Bm5 cells. B) Western blot of 50,000 cells and 20 µL culture supernatant of Bm5 cells transfected with the constructs shown in A). The results suggest that the chorion signal peptide failed to secrete the CAT protein. The control plasmid is pBSK+.

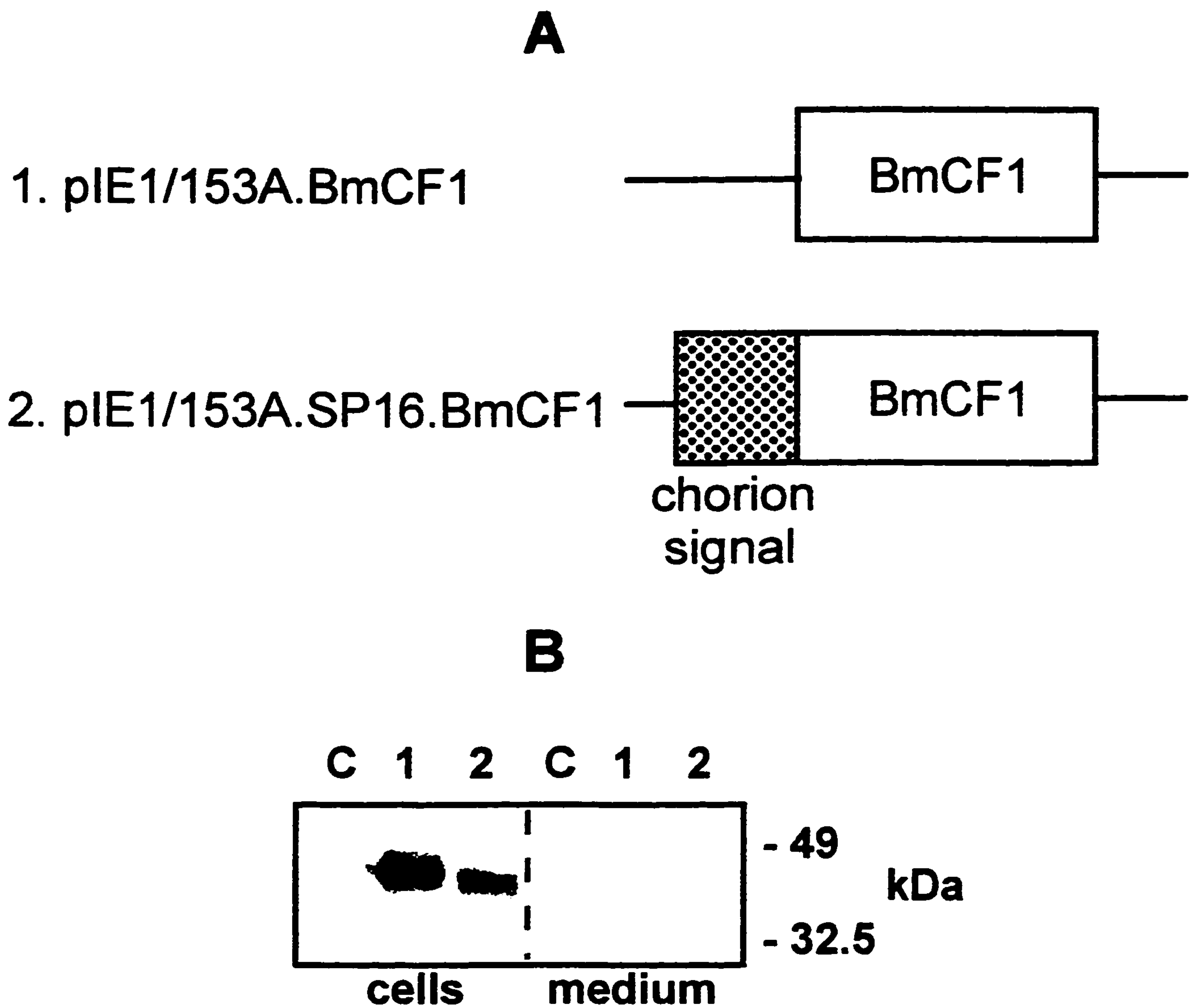


Figure 5.5: A) DNA constructs used to test the ability of the chorion signal peptide to secrete a *Bombyx mori* nuclear factor, BmCF1, from transfected Bm5 cells. B) Western blot of 50,000 cells and 20 µL culture supernatant of Bm5 cells transfected with the constructs shown in A). The results suggest that the chorion signal peptide failed to secrete the BmCF1 protein. The control plasmid is pBSK+.

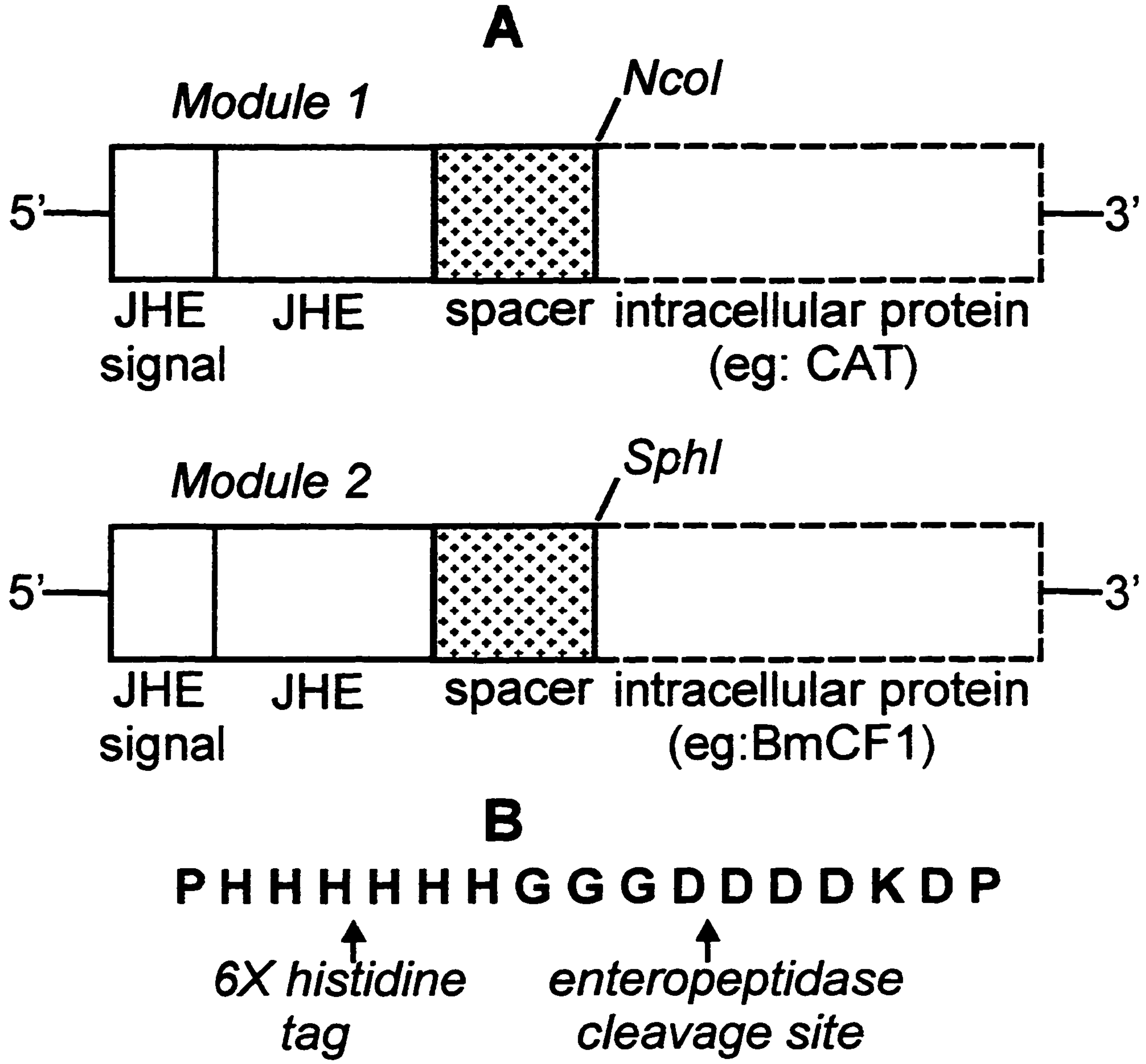


Figure 5.6: A) DNA diagram of two secretion modules constructed to test the secretion of intracellular proteins such as CAT or BmCF1. Each module contains the complete *jhe* ORF coding with a deleted translation stop codon, a spacer region, and restriction endonuclease cleavage sites for the in-frame attachment of the intracellular protein. B) Amino acid coding for the spacer region that contains 6 histidine residues for metal chelate affinity chromotagraphy, a 12 Å spacer region of three glycine residues, an enteropeptide recognition and cleavage site, and flanking proline hinge residues to encourage the spacer to form its own domain.

histidine residues and an enteropeptidase cleavage site (Figure 5.6B), and either a *NcoI* or *SphI* restriction endonuclease site for in-frame attachment of the desired intracellular protein to the 3'- end of the spacer. When expressed, the JHE protein should provide the signal peptide and other signals required for progression of the fusion protein through the secretory pathway.

The ORF of *cat* was attached to the 3' end of secretion module 1 via the *NcoI* site, and the chimeric gene was cloned into the expression cassette to yield the plasmid pIE1/153A.jhe.6H.EP.cat. Transfection assays were used to evaluate the expression and secretion of CAT using this module. Sixty hours post-transfection of Bm5 cells, supernatants were analyzed by Western blotting and revealed that the expressed JHE-CAT fusion protein was secreted into culture supernatant (Figure 5.7). JHE activity assays revealed that the JHE-CAT fusion protein was expressed at a 75% lower level than the full length JHE expressed alone.

To address whether fusion protein retained CAT activity, CAT assays were performed on culture supernatants. Despite the presence of CAT in the supernatant as detected the Western blot in Figure 5.7A, it was found to be inactive (Figure 5.7B; lane 3). Examination of the CAT ORF revealed the presence of one potential N-linked glycosylation sites and cryptic glycosylation of this site in the endoplasmic reticulum of the secretory pathway has been suggested to interfere with the biological activity of CAT (Patel et al., 1992). To test this, the N-linked glycosylation inhibitor tunicamycin (Schwartz and Datema, 1980) was added to culture supernatant following transfection with the plasmid pIE1/153A.jhe.6H.EP.cat at a concentration of 1.0 µg/mL. CAT assays of culture supernatant revealed that the CAT activity was indeed restored in the presence of tunicamycin (Figure 5.7B, lane 4), indicating that the glycosylation either blocked the active/binding site of the CAT enzyme or caused its misfolding. However, it was observed that tunicamycin does depress the growth and protein expression level in transfected Bm5 cells at a concentration of 1.0 µg/mL (data not shown).

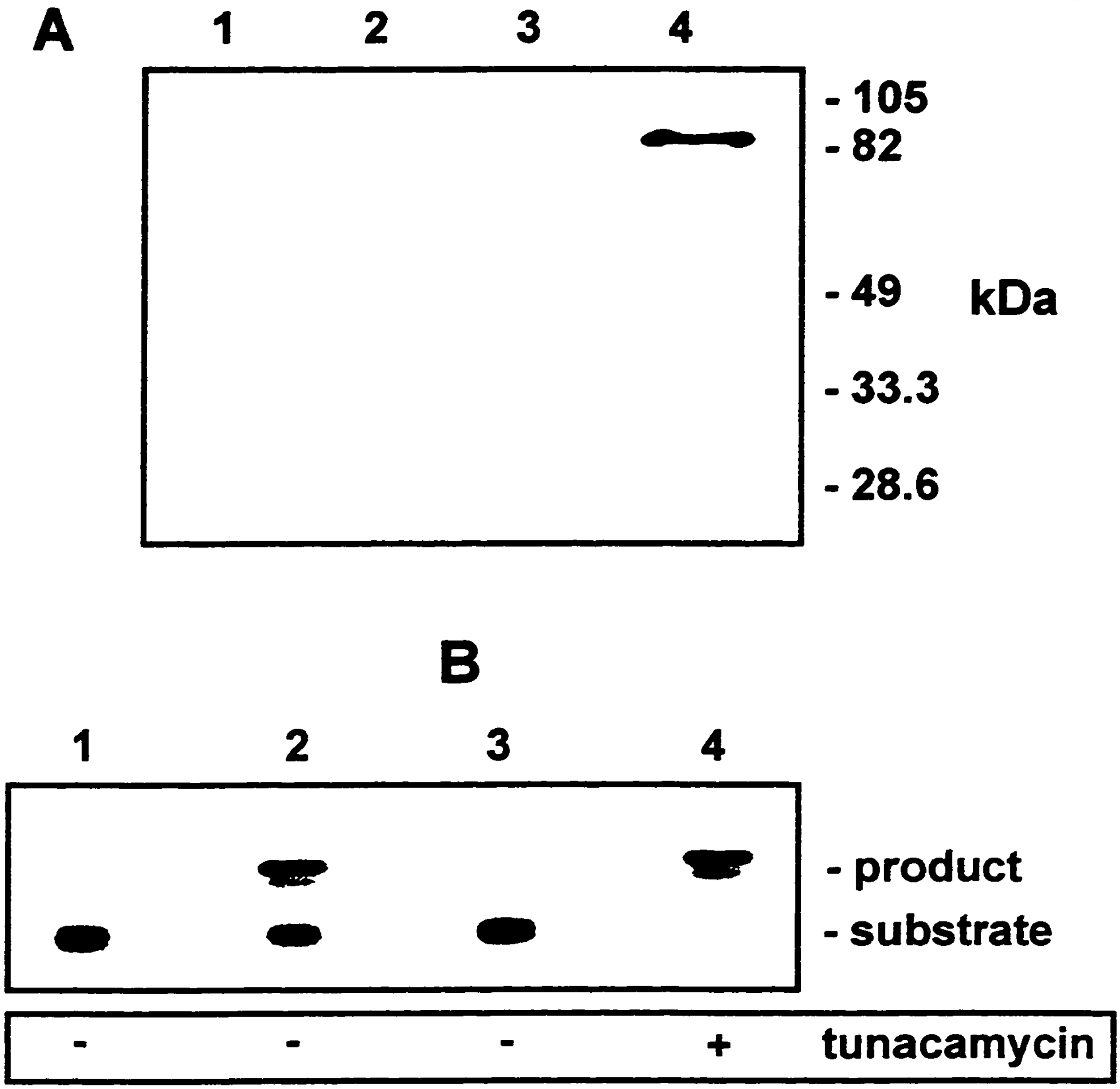


Figure 5.7: A) Western blot of 20 µL of transfected Bm5 cell culture supernatants to demonstrate the ability of secretion Module 1 to secrete CAT. Samples were probed with an antibody recognizing CAT. B) CAT assays of transfected Bm5 cell culture supernatants to test whether the JHE-CAT fusion protein secreted using secretion Module 1 was biologically active. Only when cryptic glycosylation was inhibited by the presence of 1.0 µg/mL of tunicamycin in the medium was the secreted CAT biologically active.

5.3.5 The Histidine Tag can Facilitate the Purification of JHE-CAT Fusion and Enteropeptidase can Liberate BmCF1 from the Fusion Protein

To demonstrate the function of the spacer region for purification of the fusion protein under native conditions, insect cell culture supernatant samples containing the JHE.6H.EP.CAT fusion protein were subjected to nickel chelate affinity chromotography. Table 5.3 reveals that over 80% of the purified fusion protein could be recovered, as determined by JHE assays. Supernatant samples were also digested with a crude enteropeptidase preparation and reveal that the CAT protein could be succesfully liberated from the fusion protein (Figure 5.8).

5.3.6 Efficient Secretion of a Nuclear Factor from Bm5 Cells

Finally, to demonstrate that the successful secretion of CAT was not protein specific, BmCF1, a nuclear factor that contains a nuclear localization signal, was attached to the fusion module and cloned into the expression cassette to yield the plasmid pIE1/153A.jhe.6H.EP.BmCF1, and transfected into Bm5 cells. Western blot analysis of transfection supernatants revealed that BmCF1 was succesfully secreted as a fusion protein despite the presence of a nuclear localization signal (Figure 5.9). JHE activity assays revealed that the JHE-BmCF1 fusion protein was expressed at a 67% lower level than the full length JHE expressed alone.

5.4 Discussion

In this chapter, it was shown that the mere attachment of a signal peptide to the N-terminus of a non-secretion competent protein did not result in its secretion for two intracellular proteins tested. Only when a full length naturally secreted protein was fused to the N-terminus of these intracellular proteins, CAT or BmCF1 were efficiently secreted into the culture supernatant. Thus a naturally secreted protein possesses other intramolecular signals, apart from a signal peptide, that are necessary for efficient passage through the secretory pathway. In those reports where intracellular proteins have been secreted successfully using a signal peptide, such intramolecular signals may be naturally present and sufficient for their secretion.

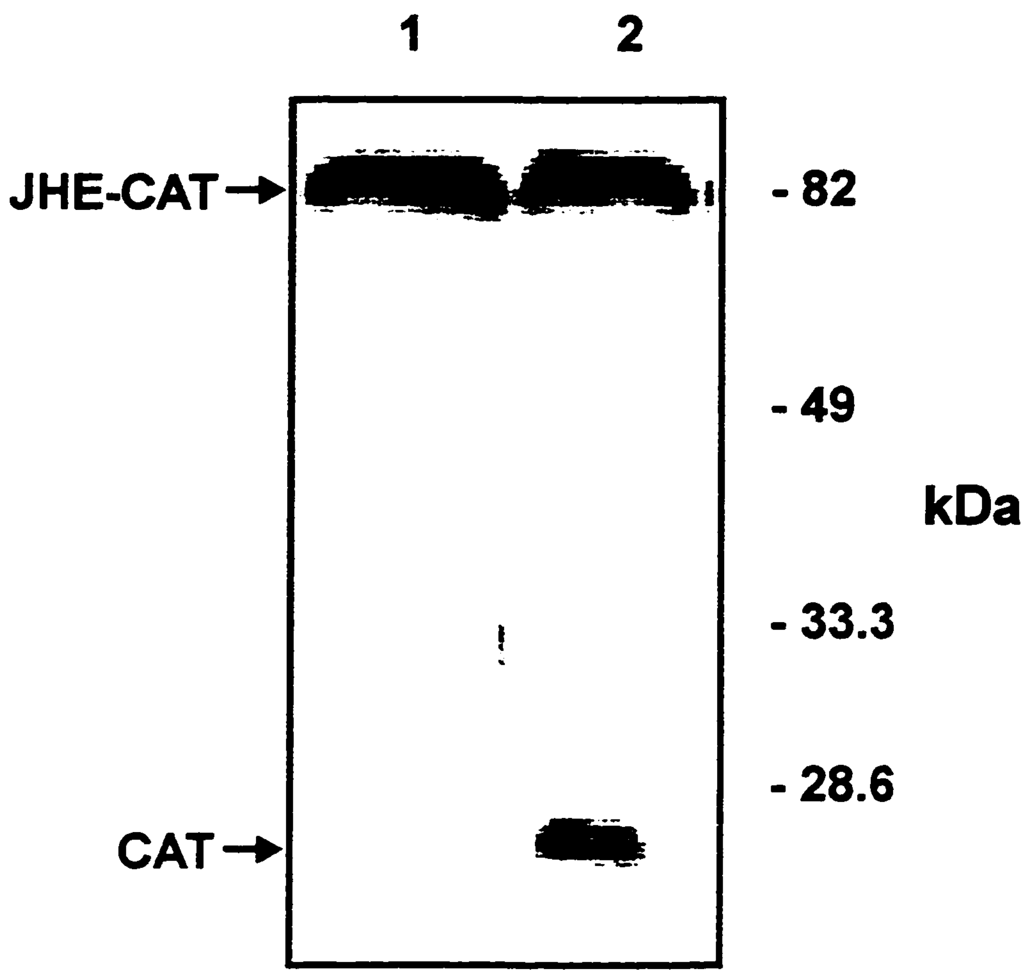


Figure 5.8: Demonstration that the enteropeptidase cleavage site present in the spacer region of the JHE-CAT fusion protein is recognized for the liberation of the CAT molecule. The 90+ kDa fusion protein (lane 1) was partially digested to release CAT at approximately 25 kDa after 36 h digestion with an enteropeptidase extract from calf stomach at 37 ℃ (lane 2).

Sample	Volume (mL)	JHE Activity (U/mL)	Yield (%)
Original	15	1.2	100
Flow Through	15	0.14	12
Wash	15	0.001	0.1
Eluate Fraction 1	0.2	0	0
Eluate Fraction 2	0.2	0.44	0.5
Eluate Fraction 3	0.2	21.2	24.2
Eluate Fraction 4	0.2	41.4	47
Eluate Fraction 5	0.2	7.2	8.3
Eluate Fraction 6	0.2	1.5	1.7

Table 5.3: Demonstration that the JHE-CAT fusion protein can be efficiently purified and concentrated by nickel chelate affinity chromatography under non-denaturing conditions. The 6x histidine tag in the spacer region between JHE and CAT in the fusion protein binds to the Ni^{2+}-agarose matrix in the purification column. The fusion protein is then eluted with 1 M imidazole buffer which competes for Ni^{2+} binding.

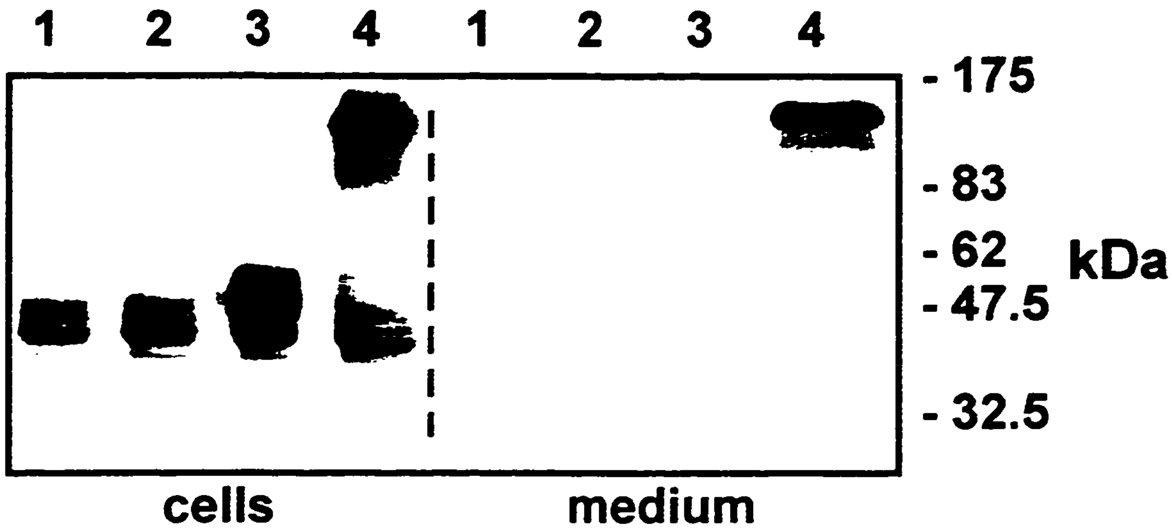


Figure 5.9: Western blot of 50,000 cells and 20 µL aliquots of supernatants of transfected Bm5 cell culture to demonstrate the ability of secretion Module 2 to secrete BmCF1. Samples were probed with an antibody recognizing BmCF1. Plasmid 1 is pBSK+, 2 is pIE1/153A.jhe, 3 is PIE1/153A.BmCF1, and 4 is pIE1/153A.jhe.His.BmCF1.

In Chapter 3 expression levels of 200 mg/L of active JHE were obtained from stably transformed insect cells. Transient expression levels of JHE-CAT or JHE-BmCF1 fusion proteins were found to be approximately 25% to 35% of JHE expressed alone. Assuming the fusion proteins had not depressed the measured JHE activity, extrapolated expression levels using the secretion modules are anticipated to reach 50 to 65 mg/L of JHE from transformed cell lines while the concentration of the intracellular protein in the secretion molecule will vary depending on its molecular mass. It should also be noted that cryptic glycosylation may influence the biological activity when some intracellular proteins are directed through the secretory pathway.

Purification using the histidine tag was relatively efficient. In the non-optimized, small-scale one step purification scheme used, over 80% of the intracellular protein CAT was recovered.

In many cases, the presence of an N-terminal or C-terminal fusion protein may not interfere with the application. For the secretion module presented here, we tried to reduce potential interference by both separating of JHE and the desired intracellular fusion protein with a spacer region, and encouraging the spacer to form a unique domain due to the placement of proline hinge residues at each end of its ends. For those cases where the attachment of JHE to the N-terminus of the desired intracellular protein does interfere with its biological activity or activity, despite this spacer region, the enteropeptidase cleavage site located just upstream of the start of the desired intracellular protein allows for its liberation and purification under non-denaturing conditions. A large-scale purification scheme involving four steps is proposed; the desired 6x His tagged proteins would be initially isolated in the first pass of cell culture supernatant over a Ni^{2+}-affinity column; elution, followed by digestion with a His-tagged enteropeptidase, and a second pass over a Ni^{2+}-affinity column would result in the removal of the secretion module, the undigested fusion protein, and the His-tagged enetropeptidase. Only pure intracellular protein should be finally present in the flow-through.

In conclusion we have demonstrated a method where intracellular proteins can be efficiently secreted from an insect cell line.

CHAPTER 6

EXPRESSION OF SEVEN DIFFERENT RECOMBINANT PROTEINS USING TRANSFORMED INSECT CELL TECHNOLOGY

6.0 Summary

In an effort to further evaluate the potential of the transformed insect cell system developed in this thesis for the expression of recombinant proteins, collaborations were established with other research groups at the University of Calgary to express proteins in insect cells that could not be produced efficiently in other protein expression systems. Three secreted proteins [human granulocyte-macrophage colony-stimulating factor (GM-CSF), a soluble isoform of the alpha subunit of the human granulocyte-macrophage colony-stimulating factor receptor (solGMrα), and a non-glycosylated form of bovine transferrin (ngbTF)], one G-protein coupled membrane receptor [rat protease activated receptor 2 (rPAR-2)], two ion exchangers [native bovine retinal rod Na^{+}-Ca^{2}+K^{+} exchanger (bNCKX) and a modified bovine retinal rod Na^{+}-Ca^{2}+K^{+} exchanger (bNCKXdd)], and a secreted intracellular protein [*Bombyx mori* chorion factor 1 (BmCF1)] were successfully expressed. Whenever possible, direct comparisons of expression levels or biological activity were made with other expression systems including transformed CHO, KNRK, and BHK-21 cells, baculovirus, and *Pichia pastoris*. These comparisons were found to favor the use transformed insect cells over other systems for recombinant protein expression.

6.1 Expression of a Soluble Isoform of the Alpha Subunit of the Granulocyte-Macrophage-Colony Stimulating Factor Receptor by Transformed Bm5 Cells and Transient Expression of GM-CSF from Transfected Bm5 and High Five™ Cells (Collaboration with Dr. Chris Brown's Laboratory, Department of Medicine, University of Calgary)

6.1.1 Introduction

Granulocyte-macrophage colony-stimulating (GM-CSF) factor is a soluble glycoprotein cytokine involved in a number of physiological processes including hematopoiesis and inflammation. GM-CSF was approved for use as a human therapeutic agent following bone marrow transplantation in 1991.

GM-CSF mediates its activity through a high affinity cell surface receptor that consists of an unknown stoichiometric proportion of alpha subunits (GMrα) and beta subunits (GMrβ). Dr. Brown's laboratory has established that soluble forms of recombinant GMrα and GMrβ (solGMrα and solGMrβ), created by removing the transmembrane domains of these proteins, can be used in a solution-phase model to study GM-CSF receptor binding.

Milligram quantities of purified GM-CSF, solGMrα, and solGMrβ are required to determine both the stoichiometric ratio of solGMrα:solGMrβ:GM-CSF molecules in the assembled receptor complex and the 3-D crystallographic structure of the complex. Insufficient quantities of GM-CSF, solGMrα, solGMrβ were produced from transformed mammalian cell lines in Dr. Brown's laboratory, and thus insect cell lines were generated to obtain higher yields. In this section, a direct comparison of the expression of solGMrα from a BHK cell line transformed with a mammalian expression cassette utilizing the human cytomegalovirus (CMV) enhancer-promoter system was made with our novel insect cell expression system. The transient expression of GM-CSF from transfected Bm5 and High Five™ cells was also compared.

6.1.2 Materials and Methods

Plasmids pIE1/153A.solGMRα and pRc/CMV.solGMRα were obtained by polymerase chain reaction (PCR) amplification of a 0.98 kb fragment from plasmid pZEMGMRsol (Brown et al., 1995) using two primers, 5'-ATACAGTCAAGCTTAGCACCATGCTTCTCCTGGTGAC-3' (forward) and 5'-CTATCAGGAACCAAATTCAATGGCTTCACTCCA-3' (reverse). The PCR-amplified fragment contained the first 317 amino acids of the solGMRα open reading frame (ORF; Gearing *et al.*, 1989), and a termination codon that was provided by the reverse primer. This PCR fragment was first cloned into pCR-Script (Invitrogen) and a 1.0 kb *Bam*HI/*Not*I fragment containing the solGMRα ORF was excised from this plasmid and subcloned into the unique *Bam*HI/*Not*I cloning sites of plasmids pIE1/153A and pRc/CMV (Invitrogen) to yield the expression vectors pIE1/153A.solGMRα and pRc/CMV.solGMRα, respectively. The plasmid pIE1/153A.GMCSF was generated by PCR amplifciation of a 450 bp fragment from the plasmid pRC/CMV.GMCSF using two primers, 5'-GAAGGATCCGATGTGGCTGCAGAGCC-3' (forward) and 5'-GAAATCTAGACTCACTCCTGGACTGGC-3' (reverse). The PCR-amplified fragment containing the GM-CSF ORF was digested with *BamHI* and *XbaI* and ligated into the unique *BamHI/XbaI* sites of pIE1/153A to form pIE1/153A.GMCSF.

The stably transformed BHK cell line over-expressing solGMRα was obtained by transfecting BHK cells with plasmid pRc/CMV.solGMRα DNA using the calcium-phosphate method (Graham and Van der Eb, 1973), selecting with 300 μg/mL of G418 (a neomycin analog; Life Technologies), and screening colonies picked from a 60 mm diameter culture dish. For expression of solGMRα by the BHK clone, cells were detached from a T-flask by a trypsin treatment and seeded at a density of 1 x 10^5 viable cells/mL into a new 75 cm^2 T-flask containing 10 mL fresh medium. One milliliter samples of supernatant were taken daily for analysis and replaced with 1 mL fresh medium.

To perform soluble GMRα binding assays on culture supernatants have been described in detail (Brown et al., 1995). ^{125}I-labeled GM-CSF was incubated with supernatant containing solGMRα in the presence or absence of a 100-fold excess of unlabeled solGMRα. Ligand-receptor complexes were precipitated with PEG6000 and specifically bound GM-CSF was determined by measuring radioactivity from ^{125}I labeled GM-CSF in the pellet. Samples were assayed 4 times.

To detect solGMrα by Western blot, a rabbit anti-GMrα antibody and a horseraddish peroxidase-conjugated goat anti-rabbit IgG (Jackson Immunochemicals) were used as the primary and secondary antibodies respectively. To detect GM-CSF by Western blotting, a mouse anti-GM-CSF antibody (Chemicon) and a horseraddish peroxidase-conjugated goat anti-mouse IgM (Vector Laboratories) were used as the primary and secondary antibodies respectively.

6.1.3 solGMrα can be Expressed at Higher Levels from Transformed Bm5 Cells than Transformed BHK Cells

A cloned Bm5 cell line over-expressing a soluble isoform of the alpha subunit of the human granulocyte-macrophage colony stimulating factor receptor (solGMRα; Brown et al., 1995) was obtained following transformation with DNA from plasmids pIE1/153A.solGMRα and pBmA.hmB. The expression levels of this cell line were compared with those of a transformed BHK clone over-expressing the same protein from a mammalian expression cassette employing the enhancer-promoter of the human cytomegalovirus immediate early gene. Radioactive GM-CSF was used as a ligand for receptor binding assays (Brown et al., 1995) to determine the relative amounts of active solGMRα present in static culture supernatants. Bm5 cells grew to a maximum of 2.1 x 10^6 viable cells/mL by day 10 while BHK cells reached confluence at 1.0 x 10^6 viable cells/mL by day 4. Figure 6.1A reveals that approximately 5 times more GM-CSF was bound by the culture supernatant of the transformed Bm5 cells at day 12 than by the culture medium of the BHK clone at day 5. A Western blot to confirm the relative amounts of solGMRα reveals that significantly more than 5-fold solGMRα was actually produced by transformed Bm5 cells than BHK cells (Figure 6.1B). solGMRα has a predicted non-glycosylated molecular mass of 40 kDa, but contains 11 potential N-linked glycosylation sites. The solGMRα produced by Bm5 cells has a molecular mass of approximately 55 kDa. The molecular mass of solGMRα produced by BHK-21 cells has a molecular mass of 55 to 60 kDa (Brown et al., 1995) and has slightly more mass than that produced by Bm5 cells.

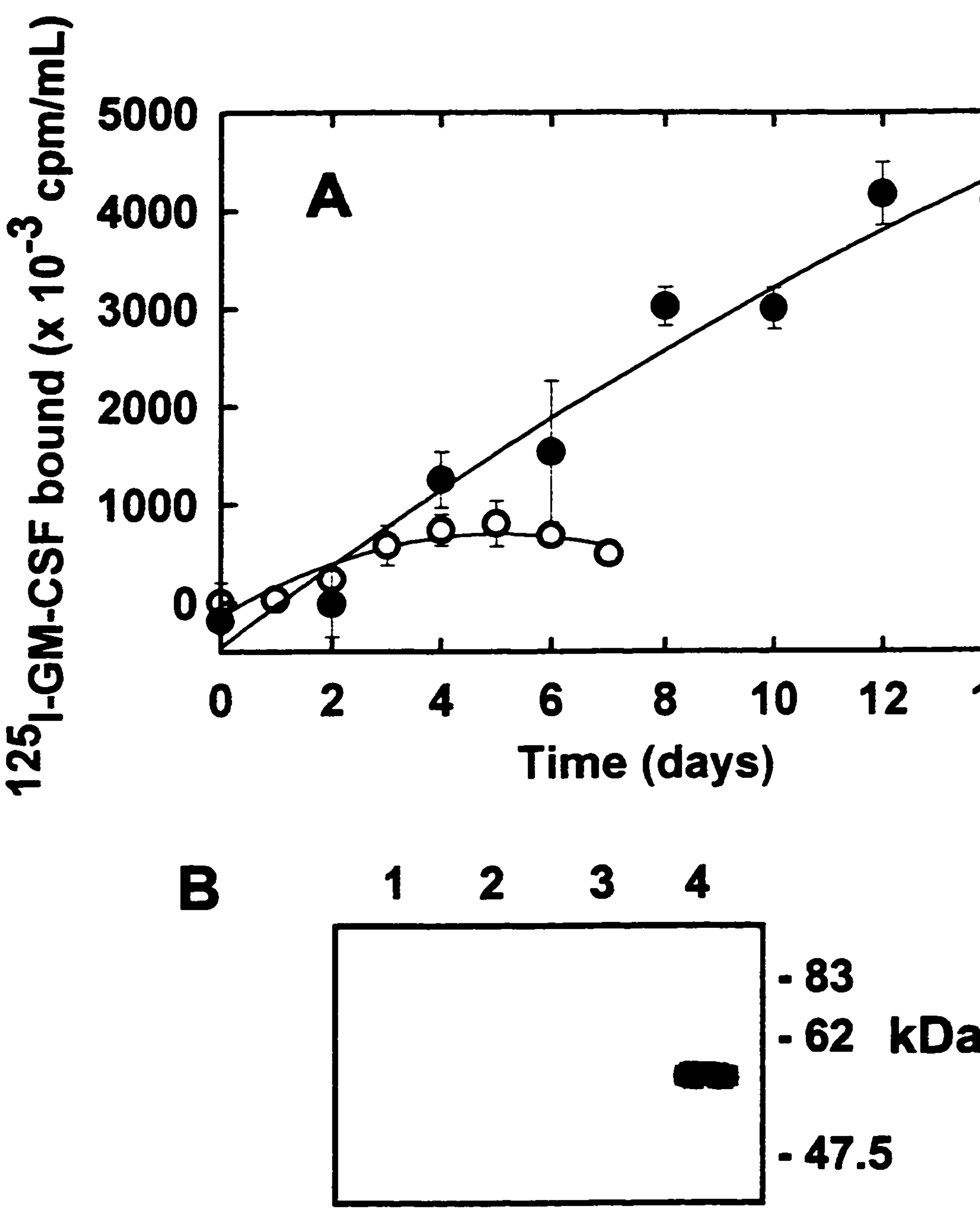


Figure 6.1: A) Batch production of active solGMRα from over-expressing clones of transformed Bm5 (filled circles) and BHK (open circles) cells grown in static culture as determined by receptor binding assays of culture supernatants. The amount of ^{125}I-labelled GM-CSF specifically bound by each culture supernatant is indicated in cpm/mL. B) Western analysis of culture supernatant to determine the relative expression levels of solGMRα. Lane 1 contains 40 μL of control conditioned medium from BHK-21 cells, lane 2 contains 40 μL of day 5 supernatant from transformed BHK-21 cells, lane 3 contains 4 μL of control conditioned medium from Bm5 cells, and lane 4 contains 4 μL of day 10 supernatant from transformed Bm5 cells.

6.1.4 GM-CSF can be Expressed from Transfected High Five™ Cells at a Higher Levels than Bm5 Cells

Bm5 and High Five™ cells were transfected with the plasmid pIE1/153A.GM-CSF and supernatant harvested 3 days post-transfection for analysis. The Western blot in Figure 6.2 indicates that GM-CSF was expressed from both Bm5 and High Five™ cells mostly as a 25 kDa protein, with several lower molecular mass forms present between 18 and 25 kDa that presumably correspond to varying levels of glycosylation. Native human GM-CSF is a heterogeneously glycosylated molecule containing 4 N-linked and 1 O-linked potential glycosylation sites with an apparent molecular mass of 23 to 29 kDa, depending on the extent of glycosylation (Gasson et al., 1997). The recombinant GM-CSF standard, purified from recombinant yeast, has a molecular mass of approximately 18 kDa. The Western blot also reveals that significantly more GM-CSF can be produced by High Five™ cells than Bm5 cells, and quantitation by ELISA reveals that the expression levels were 19.0±2.8 µg/mL and 5.8±0.9 µg/mL of GM-CSF respectively.

6.1.5 Conclusions

Functional solGMRα was succesfully expressed by transformed Bm5 cells. A comparison of expression of solGMRα from BHK cells and Bm5 cells revealed that greater than 5-fold more solGMRα could be produced by the transformed insect cells.

The transient expression levels of GM-CSF from High Five™ and Bm5 cells were 19.0±2.8 µg/mL and 5.8±0.9 µg/mL respectively after 4 days. These levels of expression are similar to those transfection results reported in Section 3.3.9 using JHE as a reporter protein, which were 11.5 µg/mL and 5.9 µg/mL respectively after 3 days. Note that it was also reported in Section 3.3.9 that transfected High Five™ cells typically have a 2-fold higher transfection efficiency than Bm5 cells.

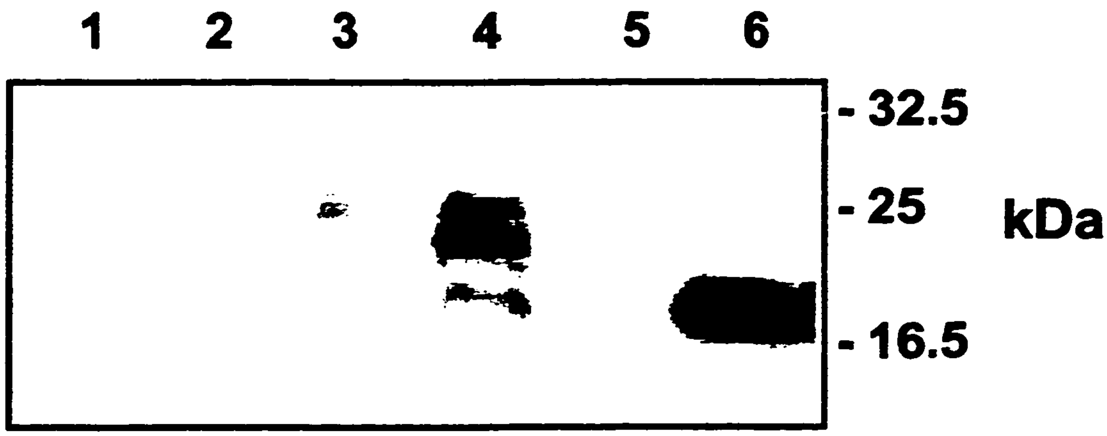


Figure 6.2: Western analysis of 20 μL of culture supernatant to compare the transient expression levels of GM-CSF from Bm5 and High Five™ cells transfected with the expression plasmid pIE1/153A.GMCSF. Supernatant was collected 3 days post-transfection for analysis. Sample loadings are as follows: lane 1, Bm5 control conditioned medium; lane 2, High Five™ control conditioned medium; lane 3, transfected Bm5 cells; lane 4, High Five™ transfected cells; lane 4, 0 μg recombinant yeast derived GM-CSF standard; lane 5, 0.25 μg recombinant yeast derived GM-CSF standard. Samples in lane 3 and 4 were quantified by ELISA and found to be 5.8±0.9 μg/mL and 19.0±2.8 μg/mL respectively.

6.2 Functional Expression of Native and a Modified Bovine Retinal Rod Na^+-Ca^2+K^+ Exchanger by Transformed High Five™ Cells (Collaboration with Dr. Paul Schnetkamp's Laboratory, Department of Medical Biochemistry, University of Calgary)

6.2.1 Introduction

Normally a large concentration gradient of free Ca^{2+} exists between the cytosol of a cell ($\leq 10^{-7}$ M) and both the extracellular fluid ($\sim 10^{-3}$ M) and endoplasmic reticulum (ER). Physiological cues, including odors, electrical impulses and light, transiently open Ca^{2+} channels in the plasma or ER membranes to increase the cytosolic Ca^{2+} and trigger Ca^{2+}-responsive proteins. For this signaling mechanism to work repeatedly, the resting concentration of Ca^{2+} must be kept low by Ca^{2+} pumps in the plasma membrane. The retinal rod sodium-calcium-potassium exchanger (NCKX) utilizes both an inward sodium gradient and an outward potassium gradient to extrude Ca^{2+} that enters rod photoreceptors via the cGMP-gated and light sensitive channels.

The study of bovine retinal rod exchanger (bNCKX; Reilander et al., 1992) in Dr. Schnetkamp's laboratory has been hampered by the lack of functional recombinant protein expression in various expression systems including the baculovirus expression system and transformed mammalian cell lines. However, they hypothesize that an intracellular loop regulates function only in retinal rod cells and may be responsible for lack of Na^+-Ca^{2+}-K^+ exchanging function when this protein is expressed in heterologous expression systems.

To test this hypothesis, the bNCKX gene was engineered into bNCXKdd, where the large N-terminal extracellular region was replaced with that from the bovine cardiac Ca^{2+}-Na^+ exchanger (bNCX) and intracellular cytosolic loop was deleted. Both bNCKX and bNCXKdd were expressed in stably transformed and cloned High Five™ cells (using the pIE1/153A expression vector) and compared to a transformed and cloned CHO cell line over-expressing bNCXKdd. This collaboration provided an opportunity to test whether our novel transformed insect cell expression system could express a functional ion exchanger.

6.2.2 Materials and Methods

To facilitate the cloning of several genes from Dr. Schnetkamp's laboratory into the pIE1/153A expression vector, the multiple cloning site was modified from the following sequence containing 4 unique cloning sites

...**CCCGGG GGATCC** ACTAGT **TCTAGA** T **GCGGCCGC**...

to

...**CCCGGG GGATCC** ACTAGTTCTAGGGAA

GCGGCCGC AA **CCTAGG** AA **TCTAGA**...

in the expression vector pIE1/153A5 containing an additional *AvrII* restriction site. A *NotI/ClaI* digest of the mammalian expression vector pRc/CMV.bNCKX released a 3500 bp fragment containing the *b*NCKX gene that was ligated with a *ClaI-XbaI* linker, digested with *XbaI*, and cloned into the *NotI/XbaI* site of pIE1/153A5 to form pIE1/153A5.bNCKX. To generate the plasmid pIE1/153A5.bNCKXdd, the 1900 bp fragment from a *NotI/XbaI* digest of the baculovirus expression vector VL1392.bNCKXdd was cloned into the unique *NotI/XbaI* sites of pIE1/153A5.

For Western blotting, a mouse anti-cardiac Na^+-Ca^{2+} exchanger IgM antibody (Affinity Bioreagents Inc.) and a horseraddish peroxidase-conjugated goat anti-mouse IgM (Amersham) were used as the primary and secondary antibodies respectively.

To study functional expression of NCKX exchangers, *reverse* Na^+-Ca^{2+} exchange experiments were performed. Cells were first loaded with sodium in buffer (150 mM NaCl. 20 mM Hepes pH 7.4, 0.1 mM EDTA) for 20 min in the presence of 4 μM monesin (a sodium ionophore), followed by washing and incubation for various time intervals with $^{45}Ca^{2+}$ in the presence of either extracellular K^+ or a Na^+ control. Calcium uptake was measured when samples were rapidly filtered over borosilicate glass fiber filters and the filter containing cells was detected in a scintillation counter for radioactive $^{45}Ca^{2+}$.

6.2.3 Functional Modified Bovine Rod NCKX can be Expressed in High Five™ cells but not in CHO cells

Following standard transformation protocols, 24 High Five™ clones over-expressing exchangers were screened by Western blotting (bNCXK) or Northern blotting (bNCKXdd) to identify high producing clones. One clone over-expressing each protein was selected and verified for expression of specific mRNA and protein by Northern and Western blotting respectively. Whenever possible, a comparison of expression levels with CHO cells was made. The expression of actual protein in the Western blot shown in Figure 6.3A reveals that more bNCKXdd protein was produced in High Five™ cells than CHO cells.

Only High Five™ cells over-expressing bNCKXdd were able to take up calcium in a K^+ dependant manner (Figure 6.3B), confirming the hypothesis that the deletion of the large intracellular cytosolic loop would provide the Na^+-Ca^{2+}-K^+ exchanging function to the protein. However, reverse Na^+-Ca^{2+} exchange could still not be detected in stably transformed CHO cells over-expressing bNCKXdd.

6.2.4 Conclusions

Three conclusions can be made from experimental results. Firstly, that the intracellular cytosolic loop is responsible for the lack of Na^+-Ca^{2+}-K^+ exchanging function of native bNCKX when expressed in heterologous expression systems. Second, the amount of bNCKXdd produced by the High Five™ cell line was superior than that produced by the CHO cell line. Finally, the results suggest that High Five™ cells may be extremely useful for studying functional aspects of ion exchangers, in view of the significant background of Na-Ca exchange observed in sodium-loaded HEK293 cells (data not shown) or the lack of function observed here in CHO cells. It is possible that pH differences in lepidopteran and mammalian growth media (pH 6.2 versus 7.2) may provide a selection advantage to lepidopteran cells when over-expressing a potentially toxic protein, such as an ion exchanger, because the native bNCKX functions optimally above pH 7.0 and does not function well at pH 6.0 (Schnetkamp, 1992).

6.3 Expression of a Non-Glycosylated Form of Bovine Transferrin (ngbTf) from Transformed High Five™ Cells (Collaboration with Dr. Tony Schryver's Laboratory, Department of Microbiology and Infectious Diseases, University of Calgary)

6.3.1 Introduction

Iron is an essential element for biological systems, yet it is insoluble in aerobic aqueous systems. Vertebrates bind extracellular iron for distribution to tissues with a serum

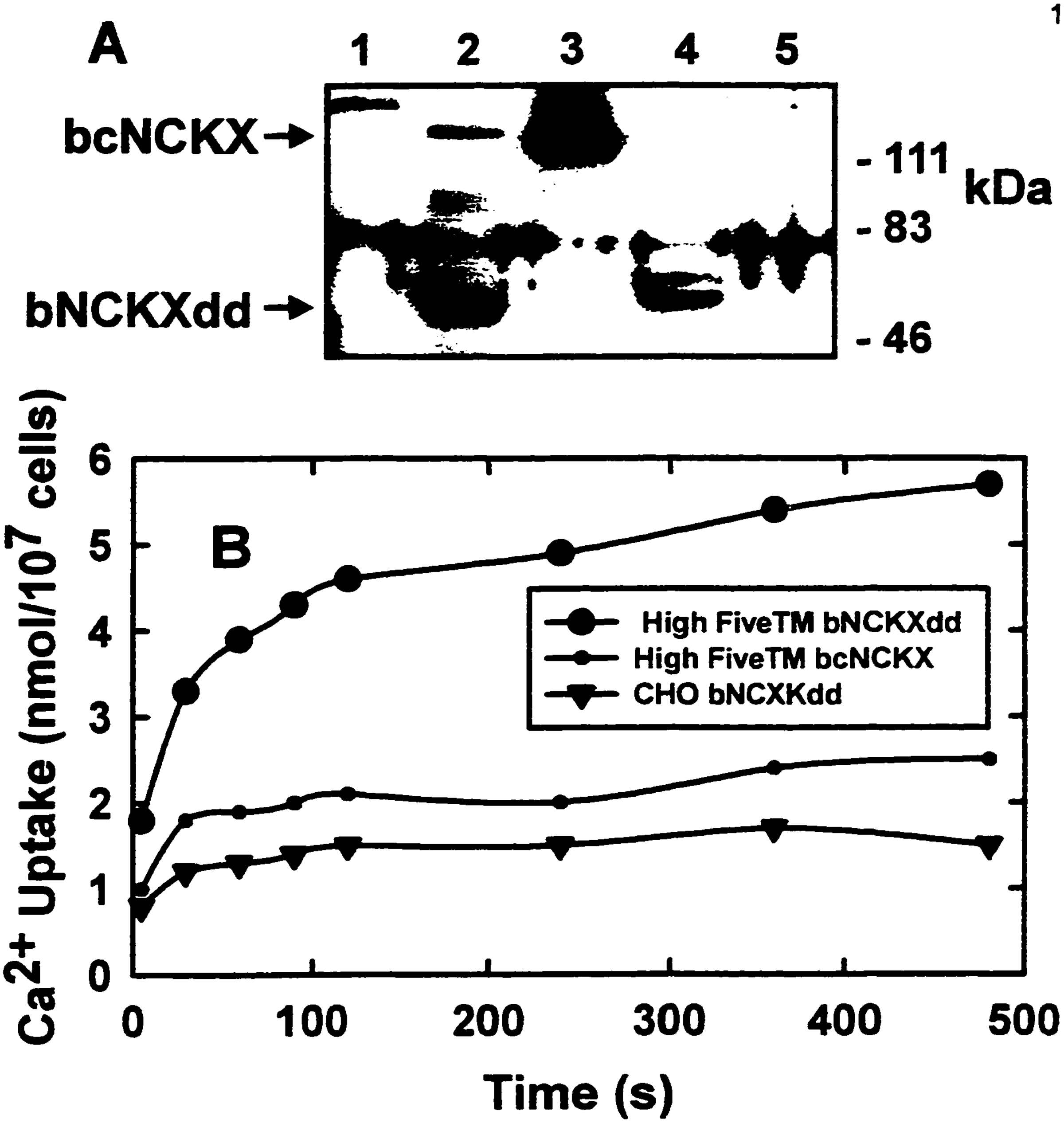


Figure 6.3: A) Western analysis of 5 x 10^4 cells probed for expression of bNCKXdd. The sample loadings are as follows: lane 1, control High Five™ cells; lane 2, transformed High Five™ cells expressing bNCKXdd; lane 3, a positive control of High Five™ cells expressing bovine cardiac NCKX (bcNCKX); lane 4, transformed CHO cells expressing bNCKXdd; lane 5, control CHO cells. B) Reverse Ca^{2+} exchange experiments in the presence of K^{+} to evaluate the Ca^{2+} uptake function of bcNCKX and bNCKXdd expressed by High Five™ or CHO clones. Only High Five™ cells expressing bNCKXdd were able to take up Ca^{2+}.

protein, transferrin (Tf). The binding of iron by transferrin makes it unavailable to potential bacterial pathogens; however, researchers in Dr. Schryver's laboratory at the University of Calgary have discovered that certain bacterial pathogens have evolved to express transferrin receptors to counter this iron restriction in the host by directly binding Tf as a first step in their iron acquisition process. It is speculated that disruption to this iron binding mechanism by pathogens may prevent diseases in humans such as bacterial meningitis by *Haemophilus influenzae* and *Neisseria meningitidis*, gonorrhea by *Neisseria gonorrhoeae*, otitis media by *moraxella catarrhalis*, or vetinary diseases such as *Pasteurella hemolytica* in cattle. Co-crystallization of bovine or human transferrin with the transferrin receptors of these bacterial pathogens will allow researchers to identify sites of interaction between the two proteins for potential drug target sites.

Dr. Schryver's laboratory requires milligram quantities of purified non-glycosylated human and bovine transferrin for crystallization studies, and sufficient material could not be obtained from the baculovirus expression system and recombinant *Pichia pastoris*. In this section, the expression of a non-glycosylation form of bovine transferrin from transformed insect cells is described and compared to both the baculovirus and *Pichia pastoris* expression systems.

6.3.2 Materials and Methods

A 2,200 bp *BamHI/NotI* fragment from pF-ngbTf (Dr. Schryver's laboratory) containing the non-glycosylated form of bovine transferrin was cloned into the unique *BamHI/NotI* sites of pIE1/153A to form pIE1/153A.ngbTf. For insertion of ngbTf into the *Pichia pastoris* expression vector, a 2100 bp *XhoI/NotI* fragment from pGEM-ngbTf (Dr. Schryver's laboratory), containing the ngbTf without the native bTf signal peptide, was cloned in-frame and downstream of the yeast prepro-α-factor signal peptide in pPIC9 (Invitrogen) to form pPIC9-ngbTf-1.

Clones of High Five cells over-expressing ngbTF were isolated as follows. Following generation of a stably transformed polyclonal population of High Five™ cells using routine methods, cells were sparsely seeded into 100 diameter cell culture plates in 25 mL of 50% conditioned culture medium. After 10 days growth, adherent colonies were picked with a

10 μL pipette tip and expanded into 6-well plates. At one point, serum-containing medium was exchanged with serum-free medium and the clones were allowed to produce ngbTf for 7 days to identify high producers by Western blotting.

6.3.3 Non-Glycosylated Bovine Transferrin can be expressed at higher Levels from High Five™ cells than *Pichia Pastoris* and Baculovirus

The polyclonal transformed population and one High Five™ clone, ngbTf#14, were subcultured and grown for 7 days in serum-free medium, and the day 7 supernatant was analyzed by Western blot for relative ngbTF production. Day 4 supernatant from a recombinant baculovirus expressing ngbTf from infected Sf9 cells and day 4 supernatant from a transformed *Pichia pastoris* clone overexpessing ngbTF was included in the Western blot for comparative purposes. Figure 6.4A clearly shows that the polyclonal and cloned High Five™ cells produced about 5-fold more ngbTf than baculovirus-infected Sf9 cells, while Figure 6.4B shows that polyclonal population of transformed High Five™ cells produced approximately 10-fold more ngbTF than the *Pichia pastoris* expression systems.

6.3.4 Conclusions

The expression levels of the recombinant ngbTF from the transformed insect cell expression system developed in this thesis are superior to both the baculovirus and yeast (*Pichia pastoris*) expression systems.

6.4 Expression of a Protease-Activated Receptor from Transformed High Five Cells (Collaboration with Dr. Hollenberg's Laboratory, Department of Medicine, University of Calgary)

6.4.1 Introduction

A proteinase-activated receptor, known as rPAR-2, was recently cloned from rat aorta tissue (Hollenberg et al., 1996). rPAR-2 belongs to a large family of cell surface receptors that span the plasma membrane seven times and couple to heterotrimeric G-proteins (Nysted et al., 1995). According to the current "tethered ligand" model, rPAR-2

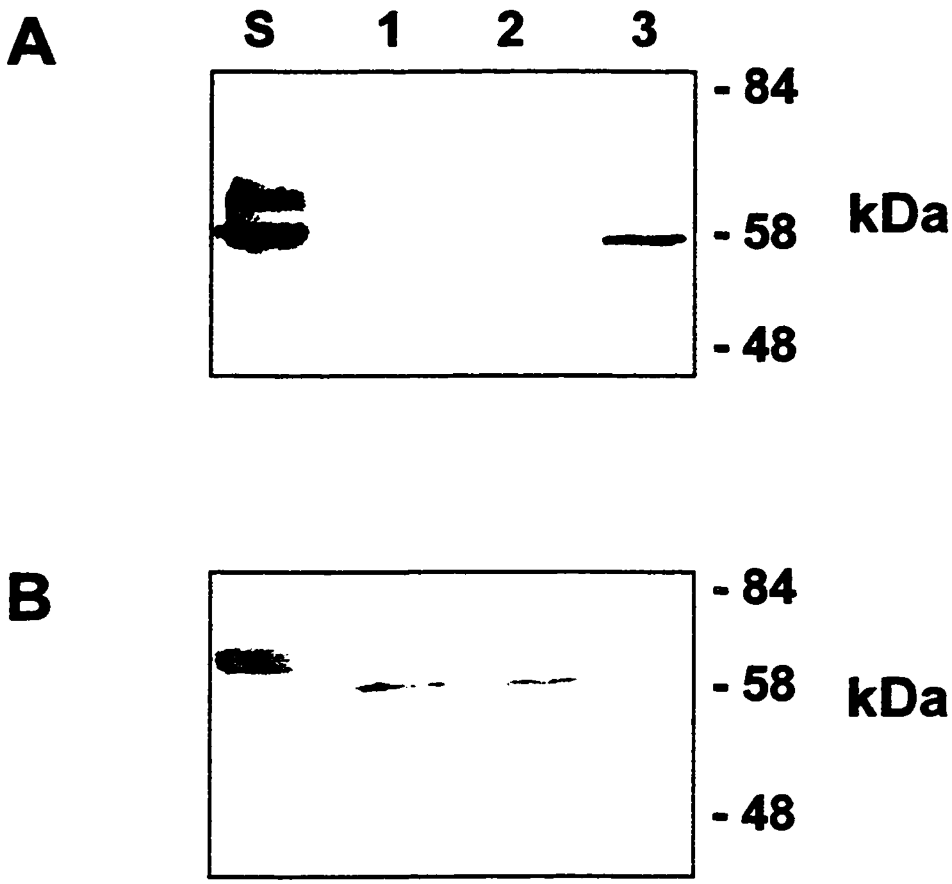


Figure 6.4: Western analyses of expression of a non-glycosylated form of bovine transferrin (ngbTf) by transformed High Five™ cells A) Comparison to the baculovirus expression systems. The lane marked 'S' contains 25 µg bTf standard, Lane 1 contains 25 µL ngbTf from baculovirus infected Sf9 cells, Lane 2 contains 25 µL of day 7 supernatant from a polyclonal population of transformed High Five™ cells, Lane 3 contains 25 µL supernatant of day 7 supernatant from the transformed High Five™ clone ngbTf#14. B) Comparison to the *Pichia pastoris* expression system. The lane marked 'S' contains 25 µg bTf standard, Lane 1 contains 200 µL of supernatant from a day 2 culture of *Pichia pastoris*, Lane 2 contains 20 µL of day 7 supernatant from a polyclonal population of transformed High Five™ cells, Lane 3 contains day 7 conditioned medium from non-transformed High Five™ cells (control).

possesses a trypsin cleavage site in the N-terminal extracellular domain and, upon exposure to trypsin, reveals an new amino-terminal sequence (SLIGRL) that stimulates receptor function and results in a physiological vascular relaxant response (Hollenberg et al., 1996). Furthermore, the trypsin-activating activity can be mimicked in the absence of trypsin merely by exposure to the synthetic peptide SLIGRL (Hollenberg et al., 1996).

Several G-protein coupled receptors have already been expressed in Sf9 insect cells following infection with recombinant baculoviruses including the human thrombin receptor (Chen et al., 1996), rat odorant receptors (Raming et al., 1993) and muscarinic receptors (Hu et al., 1994), and resulted in similar Ca^{2+} signaling mechanisms essentially similar to mammalian cells. However, expression by baculoviruses is transient and a short window of opportunity exists for functional studies following infection, due to decreased viability 72 h post-infection (p.i.), with a maximal response 24 h p.i. (Chen et al., 1996; Hu et al., 1994; King et al., 1993). Furthermore, expression levels obtained for membrane targeted proteins are significantly lower than for those proteins destined for the cytoplasm or nucleus (Jarvis and Summers, 1989). On the other hand, expression of membrane receptors by transformed mammalian cell lines and their functional analysis is often complicated by the presence of endogenous receptors which are absent in insect cell lines. Thus, this collaboration provided an opportunity to evaluate our transformed insect cell technology for functional G-protein coupled receptor expression.

6.4.2 Materials and Methods

A 1,300 *HindIII/NotI* fragment from pCDNA3.rPAR2 (Dr. Hollenberg's laboratory), containing the rPAR-2 open reading frame, was blunt ended with *klenow* enzyme and ligated into the unique *SmaI* site of PIE1/153A to form pIE1/153A.PAR2.

Live cells were immuno-labeled for flourescent activated cell sorting (FACs) as described in Section 2.5.4, except that cells were not fixed or permeabilized. Polyclonal rabbit anti-rat PAR-2 (Dr. Hollenberg's laboratory) and FITC-conjugated goat anti-rabbit IgG (Jackson Immuochemicals) were used as the primary and secondary antibodies respectively. The following FACs settings were used for analysis: FSC = E00 (linear), FL1 = 500 (log), SSC = 350 (linear), threshold = 0.48 (SSC). Individual cell clones were sorted by the University of Calgary core flow cytometry laboratory into 96-well plates in 50% conditioned medium.

For measurement of peptide induced calcium signals, cells were pelleted, and loaded with calcium by resuspending in 1 mL of culture medium containing 25 µg/mL fluo-3 (Molecular Probes Inc.), a flourescent calcium indicator, and 0.25mM sulfinepyrazone peptide, and incubated for 20 min at room temperature. Cells were rinsed twice and cells were resuspended to a viable cell density of 6 x 10^6 cells/mL in either insect cell buffer (50 mM NaCl, 16 mM KCl, 8 mM $MgSO_4$, 8 mM $NaH_2PO_4.H_2O$, 14 mM D-glucose, 170 mM sucrose, 0.25 mM sulfinepyrazone, and 25 mM ACES pH = 6.4) or mammalian cell buffer (150 mM NaCl, 3 mM KCl, 3 mM $CaCl_2$, 10 mM D-glucose, 0.25 mM sulfinepyrazone, and 20 mM HEPES pH = 6.4). Fluorescence measurements reflecting elevations of intracellular calcium were conducted at 24°C using a fluorescence spectrophotometer with an excitation wavelenght of 480 nm and emission wavelenght of 530 nm. The peptide agonist (SLIGRL) or trypsin was added to cuvets containing the suspended cells and changes in flourescence were monitored.

6.4.3 Cloning of High Five™ Cells Expressing PAR-2 using Flow Cytometry

Following transfection of High Five™ cells and selection in 1.0 mg/mL hygromycin B using routine methods, a polyclonal population of transformed cells was obtained and verified to express rPAR-2 by surface immunoflourescent staining (data not shown). Because rPAR-2 is expressed on the surface and some epitopes targeted by the polyclonal antibody are extracellular, it was decided to use immunofluorescent labeling and FACs sorting of live cells to clone high expressors. The top 1% flourescent cells of the polyclonal population were individually sorted into wells of a 96-well culture dish, allowed to grow, and clones re-analyzed by FACs to accurately quantitate the rPAR-2 expression levels. A pooled population of the top 5% immunofluorescent cells was also obtained by FACs sorting for comparison.

One clone Hi5.rPAR-2#41 was selected for functional studies on the bases of high mean immunofluorescence and low standard deviation in fluorescence. The distribution of fluorescent cells in Hi5.rPAR-2#41 and comparison to the polyclonal population, the top 5%

population and background normal High Five™ cells is shown in Figure 6.5 (parts A to D), and clearly shows that Hi5.rPAR-2#41 cells are more immunofluorescent. Furthermore, it was established that the higher the immunofluorescence of individual clones, the higher the functional activity of the rPAR-2 receptor (data not shown). A FACs comparison of the Hi5.rPAR-2#41 cell line with a KNRK cell line stably transformed to express rPar2 reveals that more receptors are expressed by transformed High Five™ cells (Figure 6.6).

6.4.4 Expression of Biologically Active PAR2 and Comparison to KNRK Cells

To test the G-protein coupled receptor activity, calcium signaling assays were used. This assay measures intracellular calcium signals using a fluorescent calcium indicator. Figure 6.7A shows that a significant calcium response occurs when Hi5.rPAR-2#41 are exposed to the PAR-2 peptide agonist SLIGRL. A higher calcium response, however, occurs when transformed KNRK cells are exposed to the peptide agonist (Figure 6.7B).

6.4.5 Conclusions

In this section, it was demonstrated that the G-protein coupled receptor, rPAR-2, could be expressed in High Five™ cells with a higher receptor density per cell than stably transformed KNRK cells. Furthermore, rPAR-2 was found to be functional in High Five™ cells when calcium signaling was observed upon exposure to an agonist of this receptor. However, the magnitude of the transduced calcium signal in High Five™ was smaller than that observed in KNRK cells. This is either due to biological differences in G-protein receptor coupled signal transduction pathways between insect and mammalian cells, or differences in the experimental conditions used for mammalian and insect cells in the calcium signaling assays.

6.5 Comparison of the Expression of a Secreted Form of *Bombyx mori* Chorion Factor 1 from Stably Transformed and Cloned Bm5 and High Five™ Cell Lines

6.5.1 Introduction

In Chapter 5, a module was developed for the secretion of normally intracellular proteins such as cytoplasmic proteins and nuclear factors. The successful secretion of

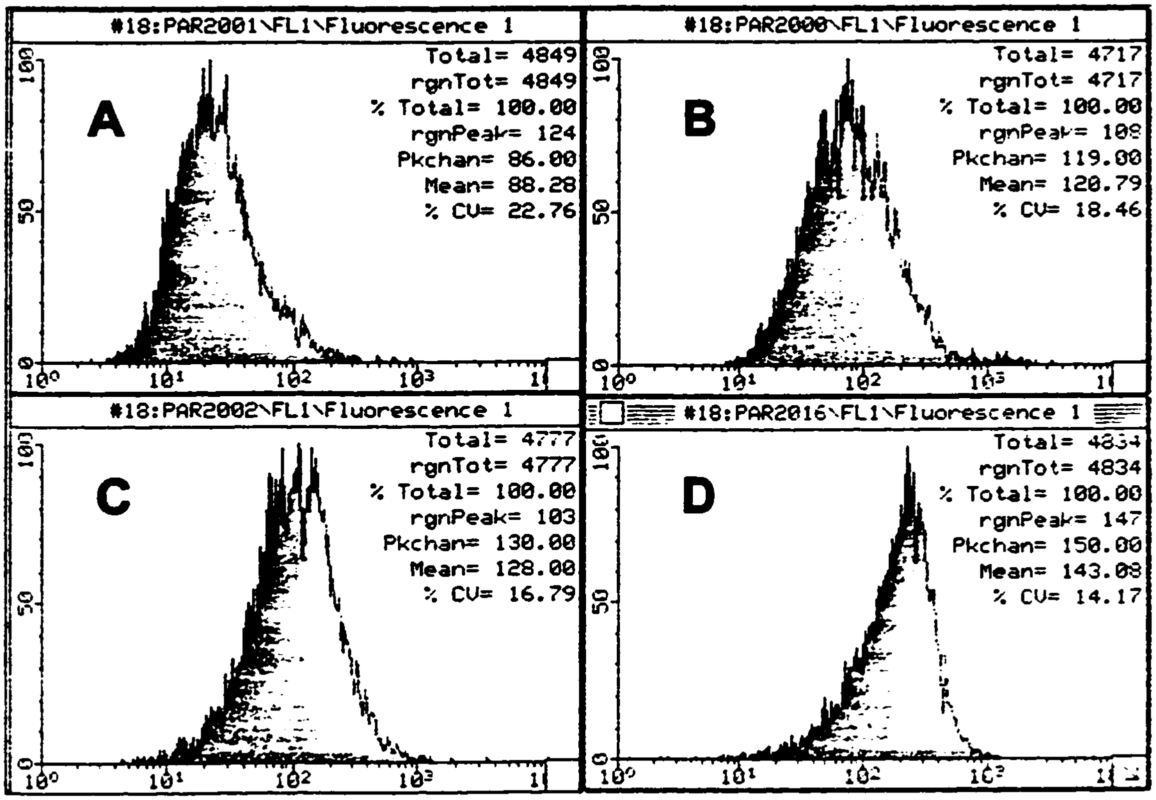


Figure 6.5: FACs analysis of High Five™ cells to detect the expression rPAR-2. Samples were labeled by immunocytochemistry before analysis. The horizontal axis of each frequency histogram is the log of the green fluorescent intensity of each labeled cell, corresponding to the number of rPAR-2 receptors present on the cell surface. A) Control High Five™ cells. B) The polyclonal stable transformed population expressing rPAR-2. C) A pooled population of the top 5% rPAR-2 expressors obtained by fluorescent activated cell sorting of the polyclonal transformed population. D) A cloned cell line, HI5-rPAR-2#41, obtained by fluorescent activated cell sorting of the polyclonal transformed population.

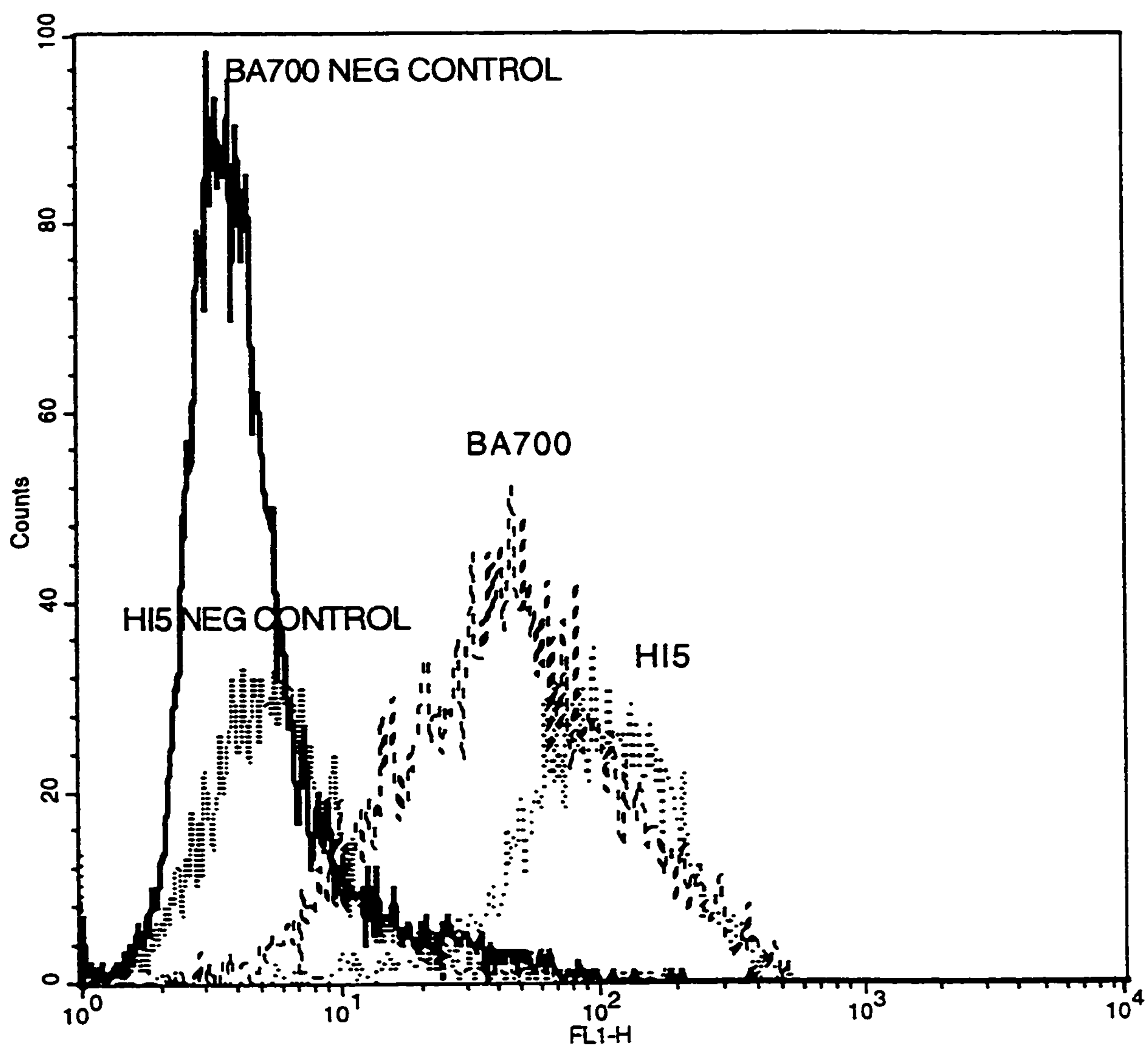


Figure 6.6: Immunofluorescent labeling and FACs analysis of Hi5.rPAR-2#41 (Hi5) and comparison to a transformed KNRK (a mammalian cell line) clone (BA700) over-expressing rPAR-2. The flourescent intensity of the horizontal axis corresponds to the number of receptors per cell. Negative controls of each cell line are only labeled with the secondary antibody.

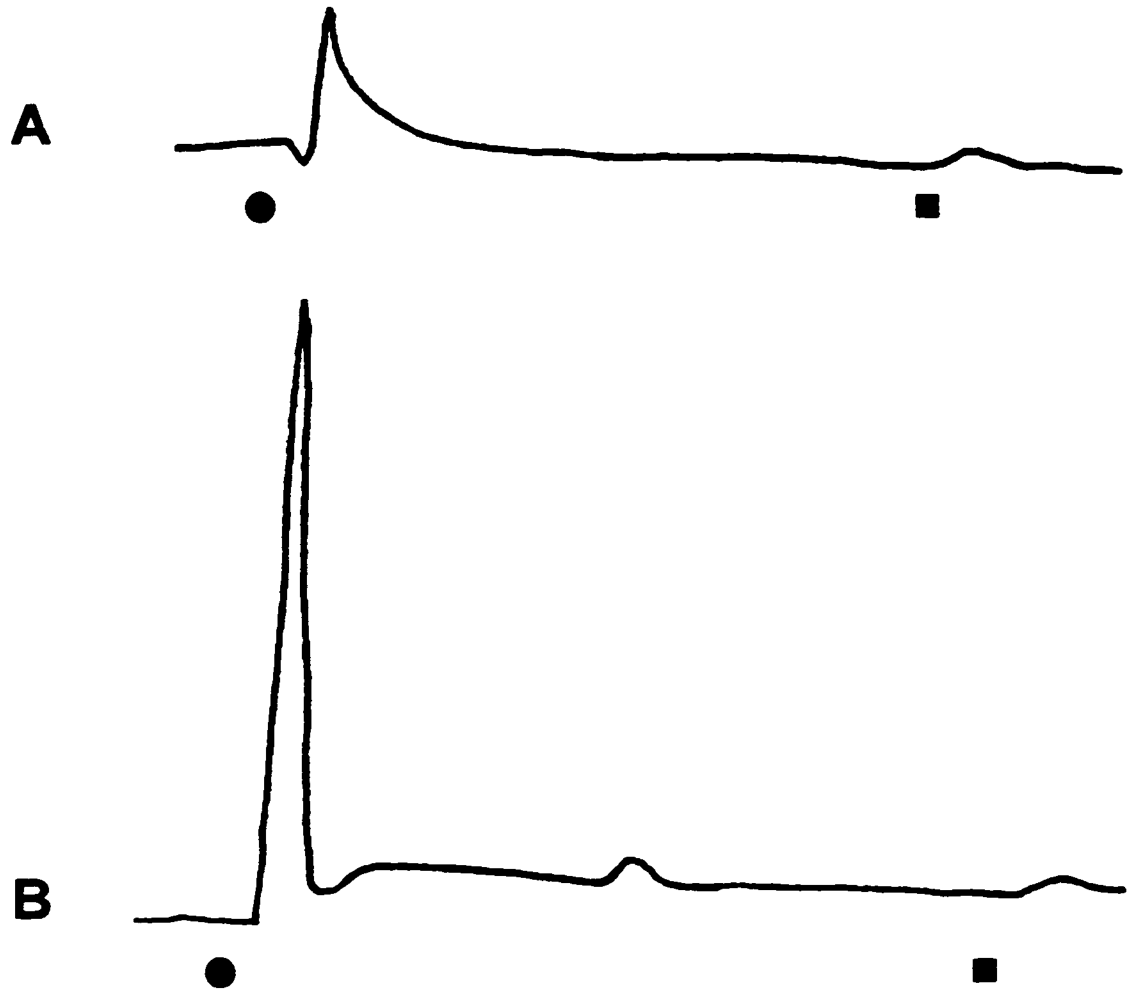


Figure 6.7: Fluorescent spectrophotomer outputs of calcium signaling assays to test the G-protein coupled receptor response of A) Hi5.PAR-2#41, and B) KNRK cells stably transformed to over-express rPAR-2. The amplitude reflects elevations in intracellular calcium with time. Both cell lines respond when exposed to the rPAR-2 peptide agonist, SLIGRL, at the time point denoted by the filled circle. Due to continued binding of SLIGRL to the rPAR-2 activation site, only a small response occurs when cells are exposed to the natural protease activator, trypsin, at the time point denoted by the filled square.

Bombyx mori Chorion Factor 1 (BmCF1) was demonstrated from transfected Bm5 cells. In this section, stably transformed and cloned Bm5 and High Five™ cell lines over-expressing BmCF1 were obtained and characterized.

6.5.3 Materials and Methods

Materials and methods pertaining to the expression of BmCF1 are already described in Section 5.3.

6.5.3 A High Five™ Clone is Superior to a Bm5 Clone in its Ability to Over-Express BmCF1

Following standard protocols for Bm5 and High Five™ transfection with pIE1/153A.jhe.6H.EP.BmCF1 and pBmA.HmB and antibiotic selection, cloned cell lines over-expressing secreted BmCF1 from High Five™ and Bm5 cells were finally obtained after 6 and 11 weeks respectively. The characterization of their growth and the BmCF1 expression level (based on JHE activity assays) in static culture over 14 days is shown in Figure 6.8 and reveals that Bm5 cells grew to 2.0×10^6 viable cells/mL after 10 days and produced 10 µg/mL BmCF1 after 14 days while High Five™ cells grew to only 1.4×10^6 viable cells/mL after 8 days and produced 28 µg/mL BmCF1 after 14 days.

Western blot analysis of day 8 samples of supernatant probed with both anti-BmCF1 and anti-JHE antibodies. More antigenic BmCF1 was produced by High Five™ cells (Figure 6.9A). However two discrete bands of higher molecular mass are present and differ by approximately 10 kDa, suggesting that some degradation of the product had occurred, presumably at the C-terminus. This is suspected because the anti-BmCF1 antibody is monoclonal and could not detect the lower molecular mass degradation products that were identified with the anti-JHE polyclonal antibody (Figure 6.9B). No degredation of JHE was observed in other Western blots presented in this dissertation.

6.5.4 Conclusions

For over-expression of BmCF1 using the secretion module described in Chapter 5, High Five™ cells were transformed and cloned in almost half-the time as Bm5 cells and

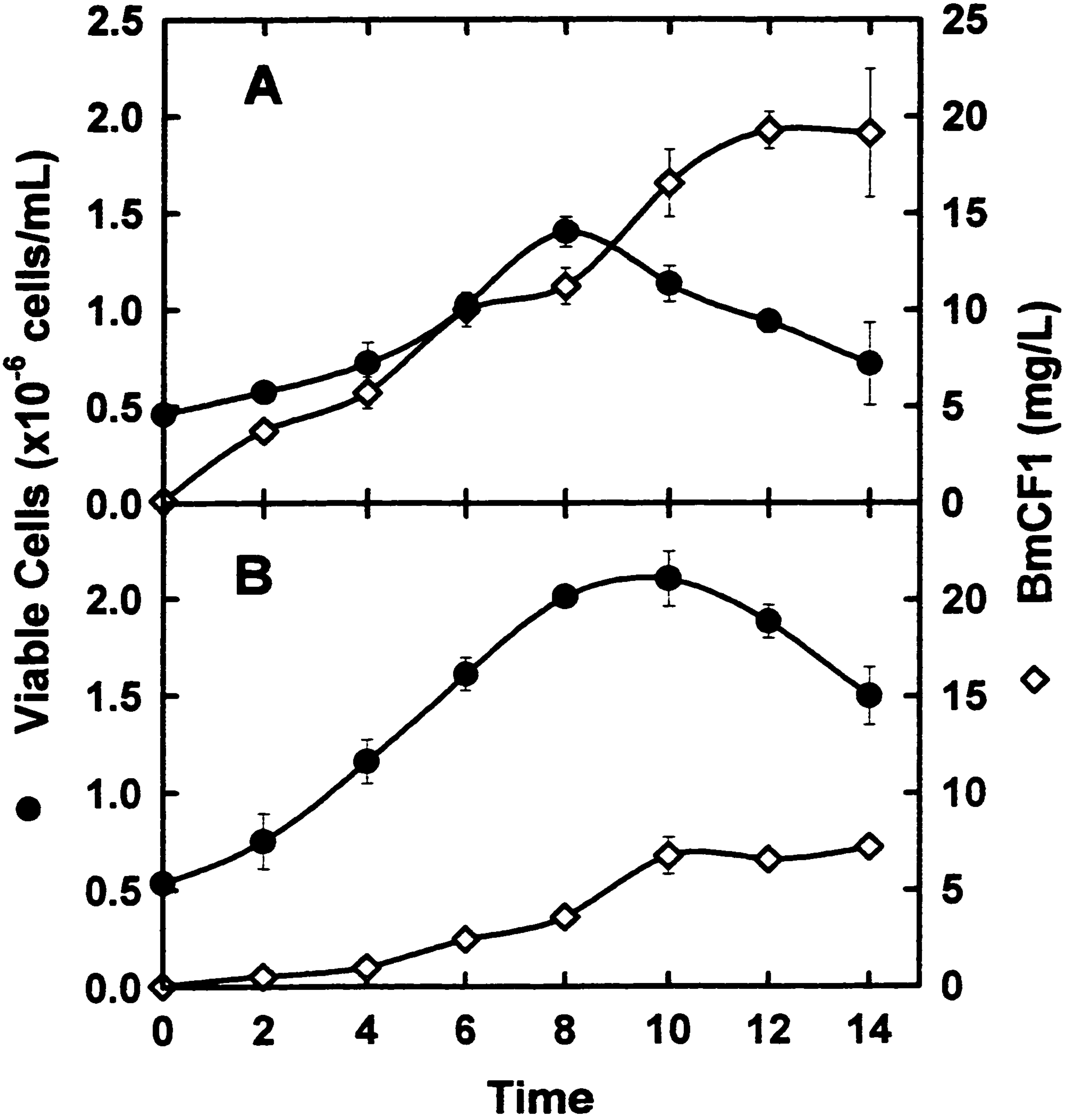


Figure 6.8: Comparison of the cell growth and expression of a secreted form of BmCF1 by both a cloned High Five™ (A) and Bm5 (B) cell line stably transformed with pIE1/153A.jhe.6H.EP.BmCF1. Cells were grown in static culture in 6-well plates. The expression levels were determined by JHE assays.

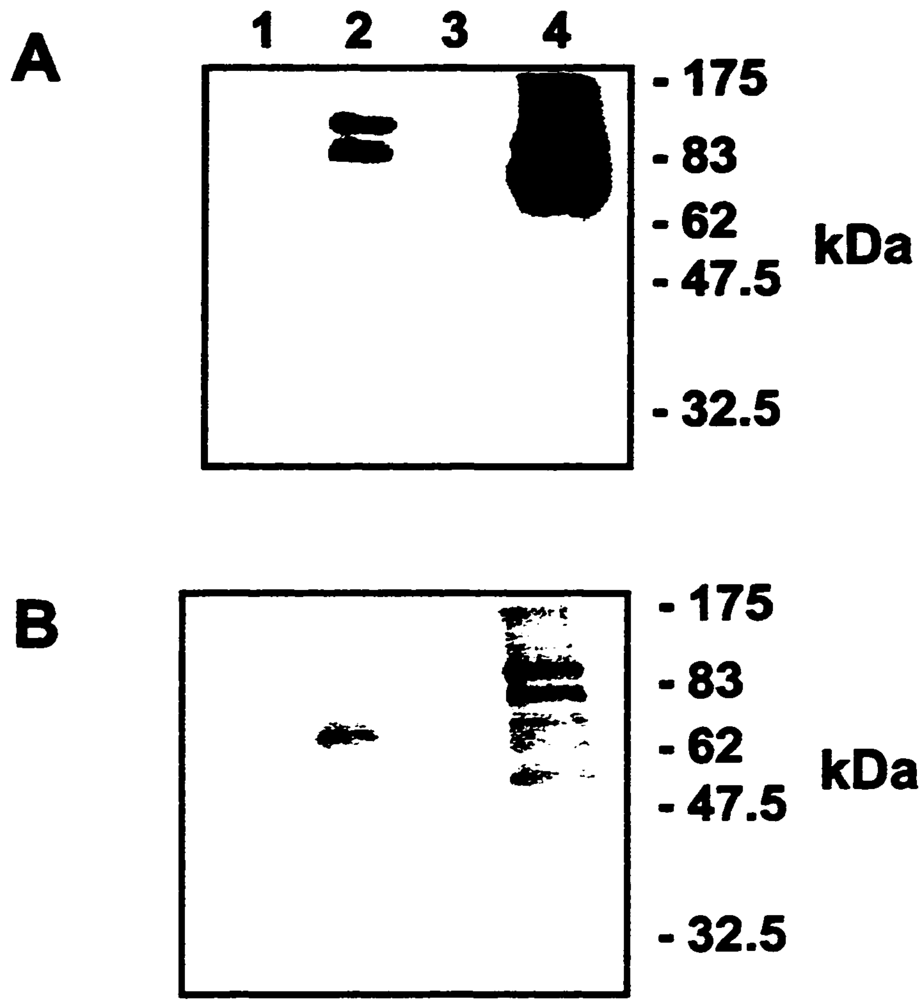


Figure 6.9: Western analysis of 20 µL of day 8 supernatants probed for the JHE-BmCF1 fusion protein produced from transformed Bm5 and High Five™ clones in 6-well plates. The blot in A) is probed for BmCF1 and the blot in B) is probed for JHE. Lane 1 contains Bm5 control conditioned medium, lane 2 contains supernatant from transformed Bm5 cells, lane 3 contains control High Five™ conditioned medium, and lane 4 contains supernatant from transformed High Five™ cells.

yielded 3-fold higher BmCF1 (based on JHE activity assays). However, it appears that significant degradation of BmCF1 occurred in both the insect cell cultures.

CHAPTER 7

Generation of a Baculovirus Artificial Chromosome (BVAC) Using a Stably Transformed Bm5 Rescuing Cell Line

7.0 Summary

In previous chapters, transformed insect cell technology was used for the purpose of over-expressing recombinant proteins. However, this technology can also be used as a research tool in insect biology and for the study of baculoviruses. In this chapter, stably transformed insect cell lines were used to create baculovirus artificial chromosomes (BVACs). BVACs can potentially be used *in vitro* for basic research and large-scale recombinant protein production, or *in vivo* to generate transgenic insects for study and biopesticide industry-related applications. The approach outlined in this chapter has been to inactivate a single baculovirus gene, namely BmNPV *lef-8*, rendering a baculovirus as an infectious, yet harmless, self-replicating extra-chromosomal entity that can carry useful genes of scientific or commercial value into lepidopteran insect cells. Such viral genomes may replicate without harming the host cell, and be shared between daughter cells following mitosis and cell division, thereby acting as an artificial chromosome. Rescuing cell lines were generated to constitutively express *lef-8* in order to generate BVACs and infectious BVAC inocula. It appears, however, that the successful generation of pure BVACs was hampered by recombination events where *lef-8* was regained by the BVACs from the rescuing cell line. Unfortunately, the virulence was then restored to these pseudo wild-type baculoviruses. Research is still in progress to correct this problem.

7.1 Introduction to the Insect Transducing Technology

The technology to generate genetically engineered (transgenic) non-drosophilid insects has many potential applications, many of which are analogous to the uses of transgenic mammals and plants. These include the manipulation of breeding stocks of *Apis mellifera* (Honeybee) or *Bombyx mori* (silkworm), the exploitation of transgenic insects as biological factories for the production of recombinant proteins, and as a basic research tool in the field of insect biology. Other potential applications, that are discussed below, include biological pest control where insects, pathogenic to humans, and agricultural insect pests would be replaced with genetically engineered non-pest strains.

The biological control of human pathogens, particularly mosquitoes, has been proposed (Collins and James, 1996). Mosquitoes are vectors of several protozoan, metazoan, and viral pathogens, such as malaria that alone causes 2 million deaths a year. It has been suggested that transformation of mosquitoes could yield transgenic strains that are resistant to disease transmission. The goal would then be to replace a wild mosquito population with the harmless transgenic strain in areas inhabited by humans to prevent the spread of disease.

The biological control of insect pests is an alternative to the use of chemical insecticides which can have toxic effects on humans following prolonged exposure, can kill other non-pest arthropods indiscriminately and to which insects develop resistance. In agriculture the cost of chemical control of insects approaches $10 billion annually (Estruch et al., 1997). To date, biological pest control of agricultural insect pests has taken several different strategies: first, transgenic crops have been developed to express insect specific toxins from *Bacillus thuringiensis* (Bt). These crops have provided good protection against cotton bollworms and potato beetle larvae (in Estruch et al., 1997), although it now appears that insect pests may adapt quickly to become resistance to Bt toxins (Gould et al., 1997). Second, wild-type baculoviruses can be sprayed on crops as bioinsecticides to target specific populations of insect pests; however, it can take several days or weeks to kill an insect, during which time it continues to feed. Thus genetically engineered baculoviruses were developed to reduce insect feeding. This has been achieved by disrupting a gene (*egt*) to inhibit insect molting (O'Reilly and Miller, 1991), or by inserting insect-specific genes for toxins, hormones, or enzymes into the virus genome uncluding the Bt toxin (Merryweather et al., 1990), scorpion toxin (McCutchen et al., 1991), diuretic hormone (Maeda et al., 1989), and juvenile hormone esterase (Hammock et al., 1990). Although, crop damage is usually reduced, it is not sufficient to make these recombinant viruses commercially viable alternatives to conventional crop protection methods. A third approach has been the programmed release of sterile insects. In one field current study, fruit orchards are being flooded with irradiated sterile *Laspeyresia pomonella* (coddling moths - a fruit pest; Warner, 1997). Wild moths mating with sterile moths will produce no offspring and this is expected to eliminate the wild fertile population over a three year period. Transgenic insects could potentially be used in this third strategy for biological control of insect pests.

Unfortunately the technology to generate non-drosophilid transgenic insects is primitive, yet over 1 million species of insects have been identified. Transgenic mammals have been created either by transfecting germ- or stem-cells with a transfer vector, followed by antibiotic selection and embryo implantation into a surrogate female, or using retroviruses. Unfortunately efficient selection and culturing methods are not developed in insect cells for the former approach, and there are no known retroviruses that function in non-drosophilid insects for the latter. Most research approaches towards the creation of transgenic insects exploit short inverted repeat-type transposable DNA elements (reviewed by O'Brochta and Atkinson, 1996). Transposable elements naturally have the ability to promote recombination reactions that result in the movement of the element from one location in the genome to another. The short inverted repeat-type transposable elements have two functional components, a transcription unit that encodes a transposase protein required for transpositional recombination (to cut and paste DNA) and terminal inverted repeats of approximately 12 to 31 bp that guide the element to other points in the genome. In an attempt to create transgenic insects, vectors to recreate this recombination process and promote the integration and expression of heterologous gene sequences into insect host chromosomes are being developed based on the *P*-element (Brennan et al., 1984), the *Mariner* element (Lidholm et al., 1993), the *Hermes* element (O'Brochta et al., 1996), the *Minos* element (Franz and Savakis, 1991), and the *PiggyBac* element (Cary et al., 1989). Recently, successful transformation of the yellow fever mosquito (*Aedes aegypti*) was accomplished using both the *Mariner* (Coates et al., 1998) and *Hermes* elements (Jasinskiene et al., 1998). Currently, however, the technology for routine transformation of non-drosophilid insects is still not available.

As an alternative to transposable elements for the generation of transgenic insects, baculoviruses may potentially be used to transport heterologous genetic information into a host cell (Iatrou et al., 1995). It is predicted that a baculovirus can function either as a *transducing* vector, which means that the genetic information introduced into the host cell by the virus is maintained as an extrachromosomal element without impairing the host's normal function, or a *transforming* vector, where the genetic information introduced into the host cell by the virus is integrated into the host genome and transmitted into the progeny. Members of the baculovirus family can infect over 600 species of arthropods.

In order for the baculovirus to function as either a transducing or transforming vector without impairing the host's (i.e. the insect or *in vitro* cultured insect cells) normal function, the malevolent effects of the baculovirus must be inactivated. Using the *Bombyx mori* nuclear polyhedrosis virus (BmNPV) as a model baculovirus, the gene expression cascade of a baculovirus is illustrated in Figure 7.1. For simplicity the BmNPV life cycle is divided into two halves: an early phase beginning immediatly after infection of the host cell and terminating with DNA replication, and a late phase of events that occur after DNA replication and results in new viral particles, occluded virus, apoptosis and cell death. By blocking entry into the late phase, viral chromosomes may replicate without harming the host cell, and be shared between daughter cells following mitosis and cell division. The block may be achieved by permanently inactivating specific gene(s) whose protein products are required for progression into the late phase of infection, thus trapping the baculovirus in the early phase of its life cycle. A recombinant baculovirus with these properties would be known as a baculovirus artificial chromosome or BVAC.

Possible genes regulating the transition from the early to late phases that are candidates for inactivation include 18 late expression factors (*lefs*) that have been identified both in a related nuclear polyhedrosis virus *Autographa californica* nuclear polyhedrosis virus (AcNPV; Todd et al., 1995) and BmNPV (Gomi et al., 1997). These *lefs* control the expression of late and very late genes in the second half of the baculovirus life cycle and

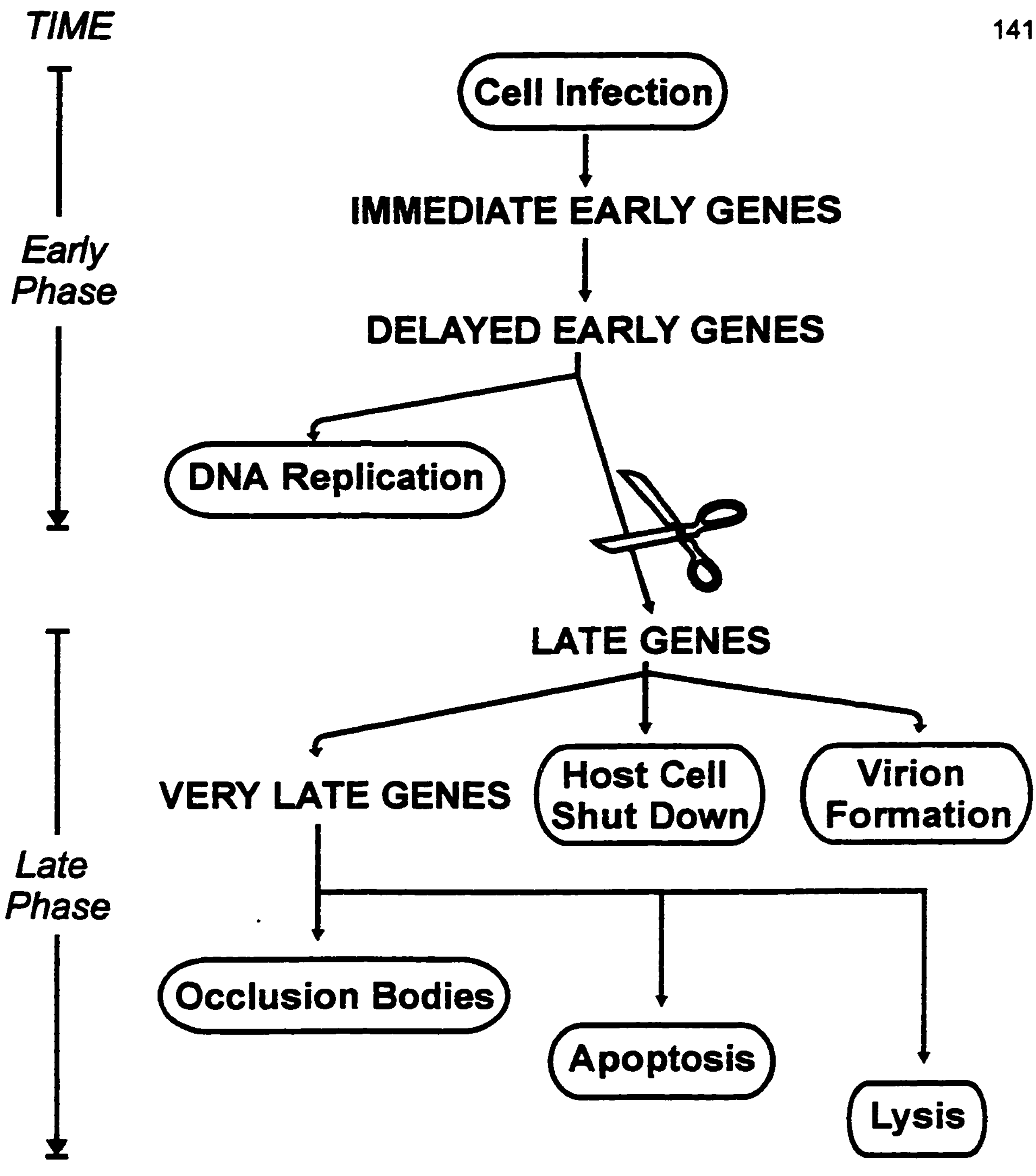


Figure 7.1: Cacscade of baculovirus gene expression events following infection of a host cell. The baculovirus life cycle can be divided into 2 temporal halves: an early phase terminating just after viral DNA replication, and a late (virulent) phase that results in budded virus production, shutting down of normal host cell functions, occlusion body formation, apoptosis, and lysis. By preventing entry into the late phase, the baculovirus is trapped in the early phase of infection.

were mainly identified using a transient expression assay system in which the expression level of a reporter gene under late baculovirus promoter control was measured (Lu and Miller, 1995).

In this chapter, a single *lef* in BmNPV, namely *lef-8*, was identified to regulate the transition from early to the late phase of the BmNPV life cycle. This was facilitated using a temperature-sensitive mutant BmNPV, previously characterized with the phenotype to be defective in budded virus and occlusion body formation, but not in viral synthesis. This baculovirus, reported in a recent publication (Shikata et al., 1998), has contributed to the generation of BVACs in this thesis and was kindly provided by Dr. Y. Hashimoto (Kyoto Institute of Technology, Japan).

7.2 Materials and Methods

7.2.1 Plasmid Constructions

The expression plasmid pIE1/153A.lef-8 (Figure 7.2A) was obtained as follows. PCR amplification using *Pfu* polymerase, wild-type BmNPV DNA as a template, and the mutagenic PCR primers,

5'-CAAAGGATCCATGACGGACGTAC-3' (forward)

5'-CTTTTCTAGAGTTATCAATTTTTCATTATCG-3' (reverse),

yielded a 2.6 kbp PCR product that was digested with *BamHI* and *XbaI* and cloned into the unique *BamHI/XbaI* sites of the pBSK+. To minimize possible mutations created through PCR amplification, ninety-one percent of the PCR product was replaced with an authentic 2.4 kbp *AvaII/EcoRV* fragment from the *ApaI*-C fragment from genomic BmNPV DNA to yield the plasmid plef-8(91%). The 2.6 kbp *BamHI/XbaI* fragment containing the *lef-8* open reading frame was ligated into the unique *BamHI/XbaI* sites of pIE1/153A to yield pIE1/153A.lef-8.

The expression plasmid, pBmA.lef-8 (Figure 7.2B), was generated by isolating the 2.6 kbp *BamHI/XbaI* fragment containing the *lef-8* open reading frame from pIE1/153A.lef-8 and ligating it into the unique *BamHI/XbaI* sites of pBmA.

Five types of transfer vectors were generated to target the *lef-8* region of BmNPV (Figure 7.3A) for homologous recombination. Each transfer vector is capable of expressing

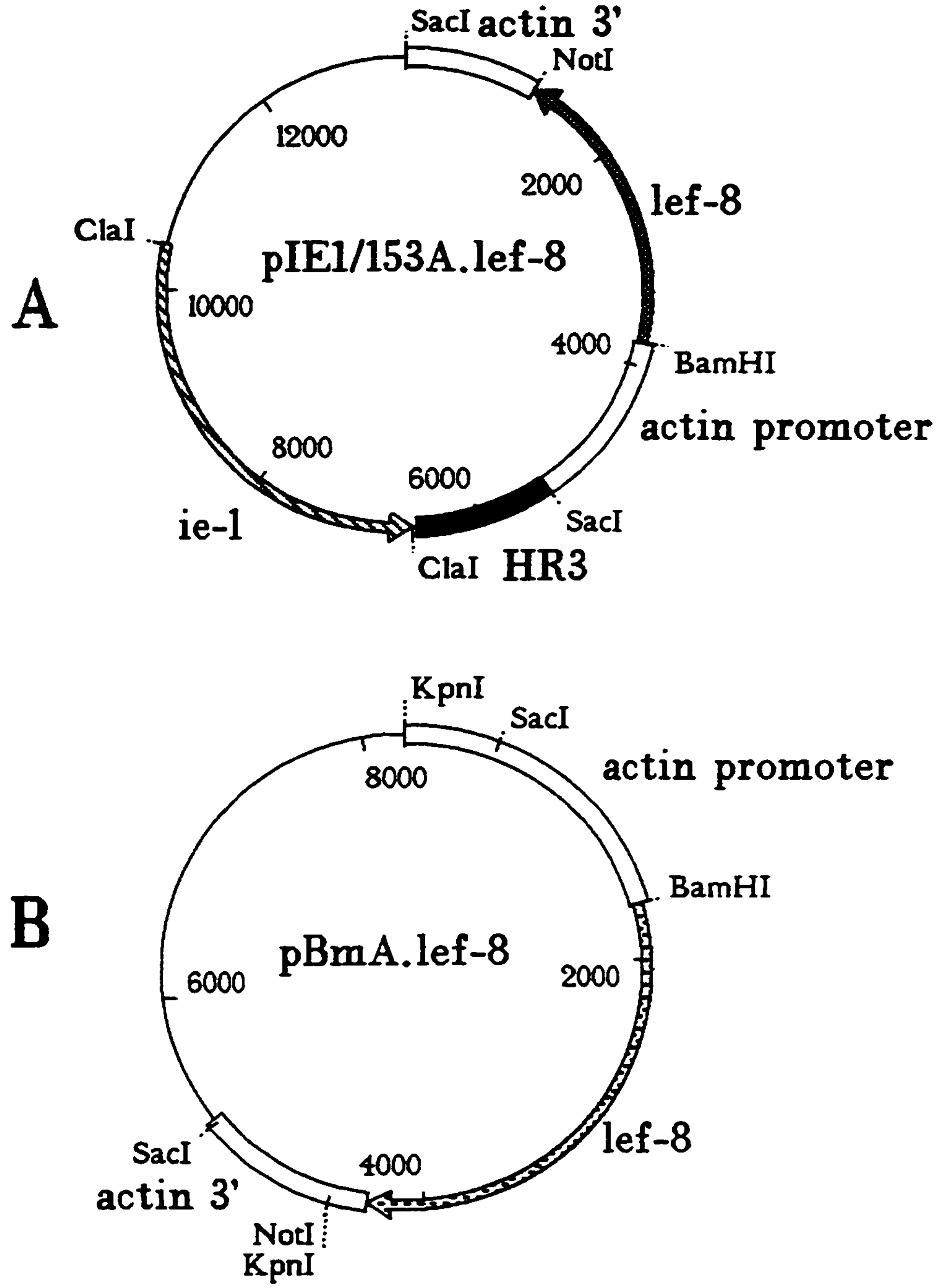


Figure 7.2: Two expression plasmids used to generate stably transformed Bm5 cell lines expressing *lef-8*.

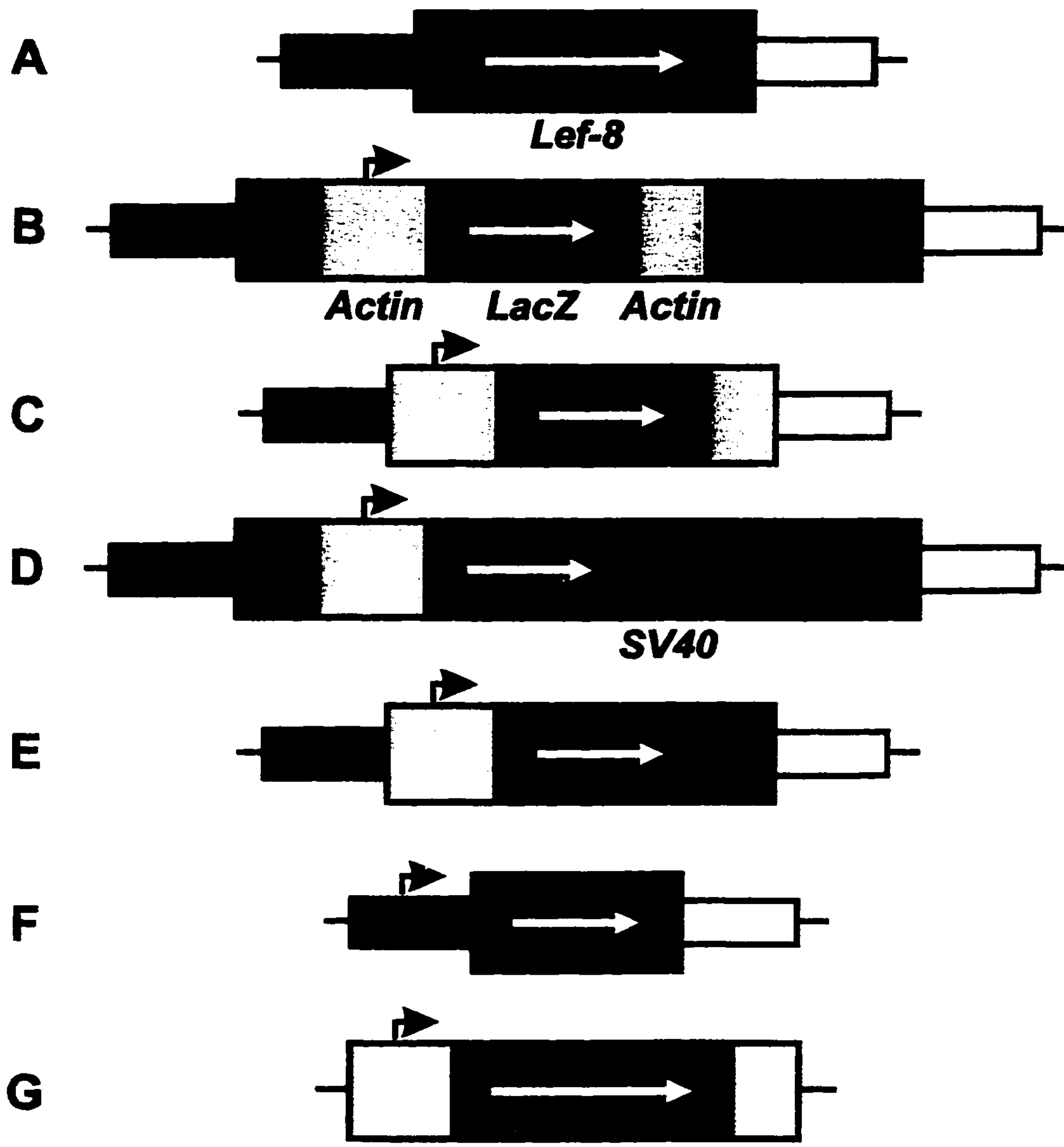


Figure 7.3: Summary of transfer vectors used in attempts to generate *lef-8* deficient/β-galactosidase expressing BVACs by double crossover homologous recombination: A) the targeted *lef-8* region in wild-type BmNPV, B) pTV#1.A.LacZ is a transfer vector used to insert the actin cassette expressing *LacZ* into the *lef-8* gene, C) pTV#2.A.LacZ is a transfer vector that substitutes the *lef-8* gene with the actin cassette expressing *LacZ*, D) pTV#1.A.LacZ is used to insert the actin cassette/SV40 polyadenylation and transcription termination region expressing *LacZ*, E) pTV#1.A.LacZ substitutes the *lef-8* gene with the actin, cassette/SV40 polyadenylation and transcription termination region expressing *LacZ*, F) pTV#2.LacZ expresses *LacZ* from a putative *lef-8* promoter with *lef-8* transcription termination/polyadenylation signals, G) is the region of the expression cassettes shown in 7.2A and B that express *lef-8* from the actin gene promoter.

a reporter gene, such as β-galactosidase (*lacz*), green fluoresence protein (*gfp*), or juvenile hormone esterase (*jhe*).

A) The transfer vector pTV#1.A.lacZ (Figure 7.3B) targets the wild type BmNPV genome for homologous recombination and insertion of β-galactosidase under control of the actin gene promoter at a point located 1,008 bp downstream downstream from the ATG initiation codon of the *lef-8* ORF. This vector was generated as follows. First the O-fragment from *HindIII* digested wild-type BmNPV DNA (Maeda and Majima, 1990), containing some of the *lef-8* open reading frame, was cloned into pBKS+. This plasmid was digested with *SphI*, blunt ended with T4 DNA polymerase, and ligated with a *SacI* linker (Sigma) to generate a *SacI* restriction site approximately 1,010 bp from the ATG initiation codon of the *lef-8* ORF. Next, some unwanted restriction sites existing in the pBKS+ polylinker region of this reulting plasmid (such as *SacI*) were removed by *SacI* and *EcoRV* partial digestions, blunt ending with T4 DNA polymerase and self ligation to yield pTV#1. The 2.4 kbp *SacI* fragment from pBmA containing the actin cassette was cloned into the unique *SacI* site of pTV#1 to yield pTV#1.A. A 3.2 kbp *SpeI/BamHI* fragment containing the *lacZ* open reading frame from pD16.43 (Fire et al., 1990) was previously cloned into the unique *SpeI/BamHI* sites of pBmA to yield pBmA.lacZ. A 3.2 kbp *NotI/BamHI* fragment containg the *lacZ* ORF from pBmA.lacZ was cloned into the unique *NotI/BamHI* site of pTV#1.A to yield pTV#1.A.lacZ.

B) The transfer vector pTV#2.A.lacZ (Figue 7.3C) targets the wild type BmNPV genome for homologous recombination and insertion of β-galactosidase under control of the actin gene promoter by substitution of the complete *lef-8* ORF. This vector was generated as follows. PCR amplification using *Pfu* polymerase, wild-type BmNPV DNA as a template and the mutagenic primers,

5-GAAGGCAGCTGCGGCCCTCACGCGT-3' (forward)

5'-GGAGGAGCTCTTGCACGATTGCAAACATGATAAAACCG-3' (reverse),

yielded a 2.1 kbp fragment flanking the 3' end of the *lef-8* ORF. This was digested with *SacI* and *PvuII* and cloned into pBSK+ that had been digested with *SacI* and partially with *PvuII*. Seventy-seven percent of the PCR product was replaced with a 1.6 kbp *MluI* fragment from the *ApaI*-C fragment of wild type BmNPV (Maeda and Majima, 1990) to yield pLeft(77%).

PCR amplification using *Pfu* polymerase, wild-type BmNPV DNA as a template and the mutagenic primers,

5'-GGGGGGAGCTCGTAAAGCGATTATTGCACACTAATTATGTC-3' (forward)

5'-GAAAGGGTACCGTCGCGGACCATACGTTA-3' (reverse),

yielded a 2.0 kbp fragment flanking the 5' end of the *lef-8* ORF. This was digested with *SacI* and *KpnI* and cloned into the unique *SacI/KpnI* sites of pBSK+. Ninety percent of the PCR product was replaced with a 1.8 kbp *AvaII* fragment from the *ApaI*-C fragment of wild type BmNPV (Maeda and Majima, 1990) to yield pRight(90%). A 2.0 *SacI/KpnI* kbp fragment from pRight(90%) was cloned into pLeft(77%) to yield the plasmid pTV#2. The 2.4 kbp *SacI* fragment from pBmA containing the actin cassette was cloned into the unique *SacI* site of pTV#2 to yield pTV#2.A. A 3.2 kbp *NotI/BamHI* fragment containg the *lacZ* ORF from pBmA.lacZ was cloned into the unique *NotI/BamHI* site of pTV#2.A to yield pTV#2.A.lacZ.

C and D) To correct potential homologous recombination with the packaging cell line by a double crossover event, the region containing the actin polyadenylation and transcription termination signals in both pTV#1.A.lacZ and pTV#2.A.lacZ was replaced an SV40 early genes polyadenylation signal and transcription termination sequence. Mutagenic PCR amplicication using *Tsg* DNA polymerase (Angon), pcDNA-1 template (Invitrogen), and the primers 5'-3' (forward) and 5'-3' (reverse) yielded a 700 bp fragment that was digested with SacI/XhoI and ligated into the 4.6 Kbp fragment of pBmA that had been partially digested with *SacI* and *XhoI* to yield pBmA/SV40. To test the efficiency of this polyadenylation signal, a 1.8 Kbp *NotI* fragment from pIE1/153A.jhe(kk) containing the juvenile hormone esterase (kk) ORF was cloned into the resulting plasmid to yield pBmA/SV40.jhe(kk). The 2.4 kb *SacI* fragment isolated from pBmA/SV40 and containing the actin cassette with SV40 polyadenylation signal and transcription termination sequence was ligated into the unique *SacI* sites of pTV#1 and pTV#2 to yield pTV#1.A/SV40 and pTV#2.A/SV40 respectively. A 3.2 kbp *NotI/BamHI* fragment containg the *lacZ* ORF from pBmA.lacZ was cloned into the unique *NotI/BamHI* sites of both pTV#1.A/SV40 and pTV#2.A/SV40 to yield pTV#1.A/SV40.lacZ (Figure 7.3D) and pTV#2.A/SV40.lacZ (Figure 7.3E).

E) To utilize the putative *lef-8* promoter present in pTV#2, a polylinker was first generated by mutagenic PCR amplification with *Tsg* DNA polymerase, pBSK+ plasmid DNA template, and the following primers

5'-ACCCTCACTAAAGGGAACAAAAGC-3'

5'-CTTTGAGCTCGAGGTCGACGG-3'.

The 130 bp PCR product was digested with *SacI* and cloned into the unique *SacI* site of TV#2 to yield TV#2.mcs1 (with the multiple cloning site in the same orientation to that in pBmA) and TV#2.mcs2 (with the multiple cloning site in reverse orientation to that in pBmA). To test expression of a reporter gene from the putative *lef-8* promoter, a 1.8 kbp *NotI* fragment containing the juvenile hormone esterase (kk) ORF from pIE1/153A.jhe(kk) was cloned into TV#2.mcs1 to yield TV#2.jhe(kk). Also a 3.2 kbp *NotI/BamHI* fragment containg the *lacZ* ORF from pBmA.lacZ was cloned into the unique *NotI/BamHI* sites of pTV#2.mcs1 to yield TV#2.lacZ (Figure 7.3F).

7.2.2 Co-Transfections to Generate Recombinant Baculoviruses

Recombinant baculoviruses were generated by co-transfecting the rescuing cell line with 2 µg of transfer vector and 1 µg of wild-type baculovirus DNA. Seven days following transfection, 100 µL of supernatant containing wild-type and recombinant viruses was used to infect the rescuing cell line and expand the virus population for 3 days. Purification of the recombinant virus from this supernatant followed using limiting dilution.

7.3 Results

7.3.1 Bm5 Cells Infected with a Temperature Sensitive Mutant BmNPV (TS-S1) is Trapped in the Early Phase of Infection at the Non-Permissive Temperature

Bombyx mori Bm5 tissue culture cells were infected with a temperature-sensitive mutant of BmNPV (TS-S1) for 1 h at room temperature at a multiplicity of infection of 5 and maintained at the non-permissive temperature of 33°C for 10 days. Viable cell counts of uninfected and TS-S1 infected Bm5 cells were taken every 2 days. Figure 7.4A shows that TS-S1 infected Bm5 cell grew normally compared to uninfected Bm5 cells.

Bm5 cells were also infected with wild-type BmNPV and TS-S1 at a multiplicity of infection (m.o.i) of 5 for 1 h and maintained at the non-permissive temperature of 33°C for

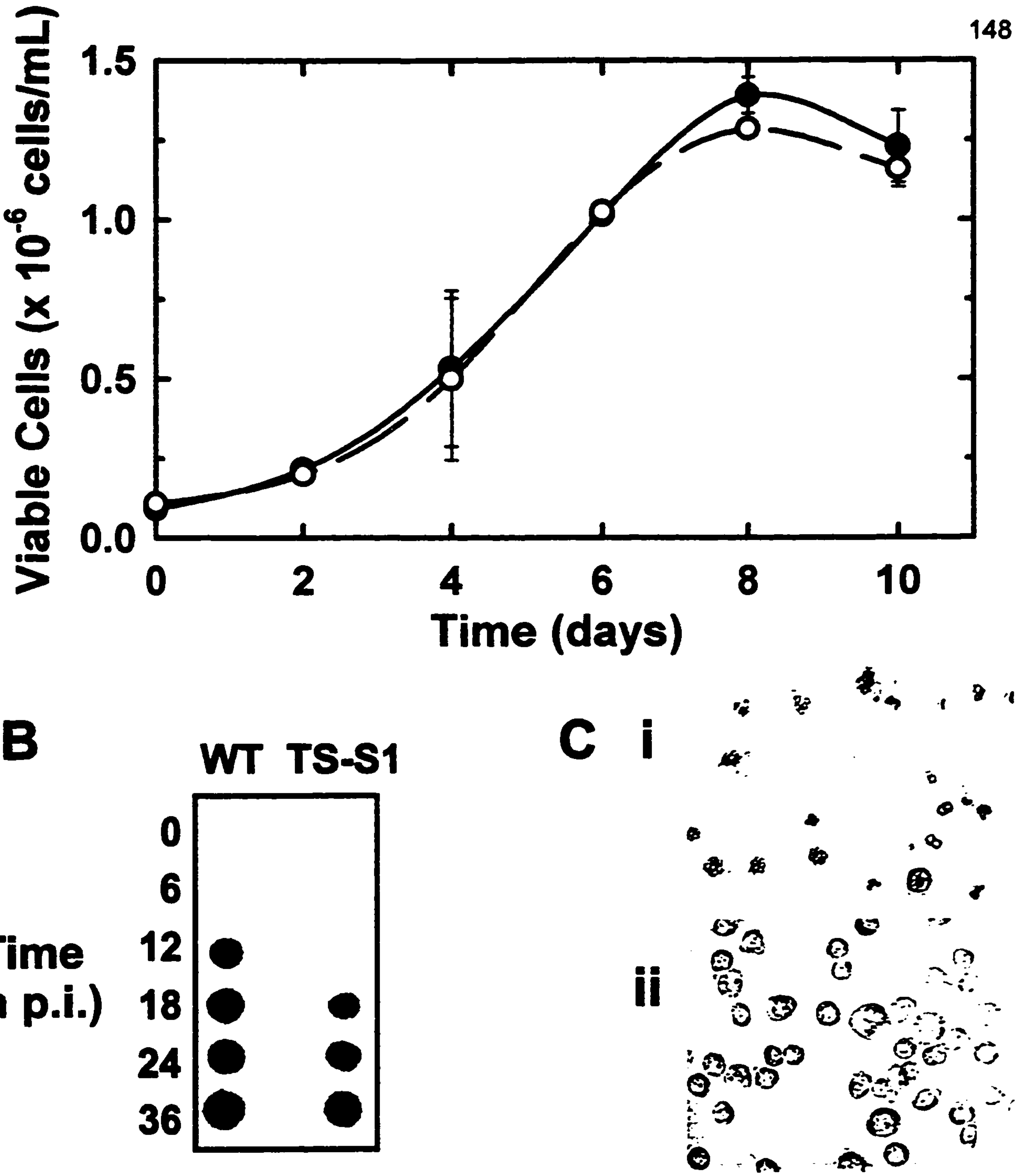


Figure 7.4: Characterization of the TS-S1 temperature sensitive mutant BmNPV at 33 °C. A) No difference in cell growth was observed between TS-S1 infected (dashed line) and normal uninfected (solid line) Bm5 cells. B) The dot blot hybridization to detect BmNPV genomic DNA shows that the genome replication of TS-S1 is indistinguishable from wild-type BmNPV. C) Wild-type BmNPV can complete its infection cycle in Bm5 cells at 33°C, as evidenced by the presence of occlusion bodies (i), while TS-S1 cannot (ii).

3 days. Figure 7.4B is a dot blot hybridization using a ^{32}P-labeled BmNPV DNA probe of cell samples taken up to 36 h post-infection. It shows that the TS-S1 mutant virus can replicate its genome in a manner that is very similar, if not identical, to the replication of wild-type BmNPV at the non-permissive temperature of 33°C. Figure 7.4C (part ii) shows that the temperature sensitive mutant BmNPV is unable to progress into the virulent phase of infection, as evidenced by the absence of occlusion bodies 3 days after infection; in contrast, Bm5 cells infected with a wild type BmNPV contain occlusion bodies 3 days after infection at 33°C (Figure 7.4C, part i). These effects were thought to be due to a mutation in the *lef-8* region in the TS-S1 baculovirus.

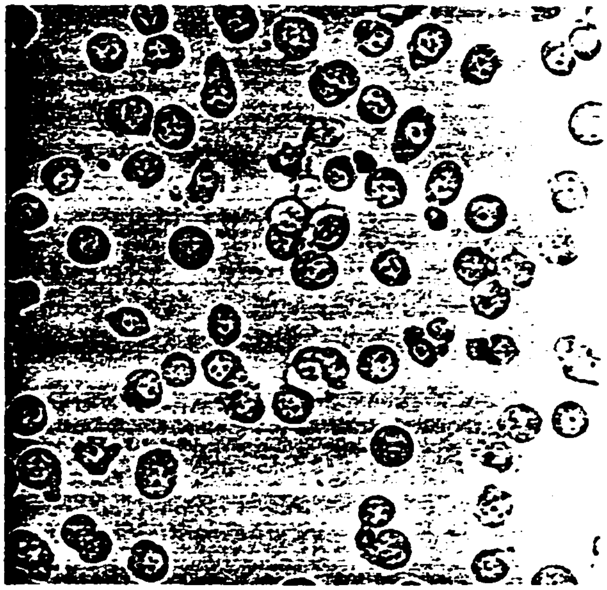
7.3.2 Generation of a Transformed Bm5 Cell Line Capable of Rescuing TS-S1

In order to confirm that a mutation in the *lef-8* gene was responsible for preventing the TS-S1 baculovirus from progressing into the virulent phase of infection, a stably transformed Bm5 cell line was generated to over-express the wild-type *lef-8* gene. Bm5 cells were transformed by co-transfecting them with a 100:1 molar ratio of the plasmids pIE1/153A.lef-8 and pBmA.hmB respectively, followed by antibiotic selection in 0.25 mg/mL hygromycin B. After 5 weeks, a stably transformed polyclonal population was obtained that weakly rescued the TS-S1 BmNPV at the non-permissive temperature of 33°C, as evidenced by the presence of occlusion bodies in approximately 1% of the cells. Several cloned cell lines capable of rescuing TS-S1 at 33°C were obtained by limiting dilution cloning, and one of these cell lines, Bm5.lef-8(371), was selected for further experiments. When infected with TS-S1 for 1 h at room temperature and incubated at 33°C for 3 days, occlusion bodies were present in most of the Bm5.lef-8(371) cells (Figure 7.5B), while normal Bm5 cells infected with TS-S1 for 1 h at room temperature and incubated at 33°C showed no sign of occlusion bodies after 3 days (Figure 7.5A). These results indicate that the Bm5.lef-8(371) cell line can be employed as a packaging cell line for the generation of BVACs.

7.3.3 Genetic Instability of the Packaging Cell Line

To test whether this cell line was genetically stable, the Bm5.lef-8(371) packaging

A



B

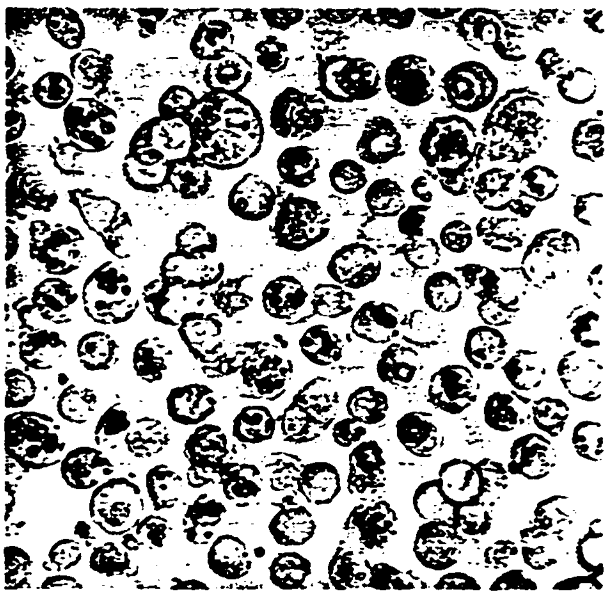


Figure 7.5: Establishment that *lef-8* gene expression is required for rescuing the TS-S1 mutant BmNPV at 33°C. The TS-S1 virus is unable to complete its infection cycle in normal Bm5 cells, as evidenced by the lack of occlusion bodies in A), however expression of *lef-8* by the Bm5.lef-8(371) cell line allows the TS-S1 virus to complete its infection cycle in B).

cell line was passaged weekly for 52 weeks in the presence of 0.25 mg/mL hygromycin B selective pressure and then tested for its ability to rescue the TS-S1 virus at the non-permissive temperature of 33°C. Bm5.lef-8(371) cells that had been preserved in liquid nitrogen since the first passage were used as a control.

Following infection with the TS-S1 virus at a m.o.i. of approximately 5 for 1 h, cells were placed at 33°C for 3 days. The photographs in Figure 7.6A clearly show that cells Bm5.lef-8(371) from passage 52 contained less occlusion bodies than those from passage 1 at 33°C. Northern hybridisation of a ^{32}P-labeled *lef-8* probe to RNA isolated from both normal Bm5 and Bm5.lef-8(371) cells at passage 1 and 52, clearly show that a reduction in *lef-8* mRNA had occured over 52 passages (Figure 7.6B). Also Southern hybridization of a ^{32}P-labeled *lef-8* probe to genomic DNA isolated from normal Bm5 cells, and Bm5.lef-8(371) at passage 1 and 52, indicated that the loss of *lef-8* expression was connected to a loss of the *lef-8* gene from the genome of transformed Bm5.lef-8(371) over 52 passages (Figure 7.6C). In Chapter 4, the transformed Bm5 cell JHE#1724, over-expressing the secreted protein JHE, was shown to exhibit stable expression over a 4 month period. Whether negative selective pressure, such as a growth disadvantage, is inherent to transformed cells overexpressing an intracellular protein remains to be established as the cause for this genetic instability.

7.3.4 Generation Two Recombinant Baculoviruses with Inactivated *lef-8* Genes, Capable of Replication but Incapable of Virulence

The transfer vectors pTV#1.A.lacZ and pTV#2.A.lacZ (Figure 7.3B and C) were used to generate BVACs that were expected to act as harmless, self-replicating extrachromosomal entities passed on from parent to daughter cells following cell division. To facilitate the detection of recombinant baculoviruses, the actin promoter present in each transfer vector directs the expression of the β-galactosidase reporter gene which forms a blue colored precipitate in β-galactosidase staining assays

The desired homologous recombination event with wild-type BmNPV was achieved by co-transfecting Bm5.lef-8(371) cells with each transfer vector DNA and wild-type BmNPV DNA at a mass ratio of 2:1. Seven days following co-transfection, 0.2 mL of supernatant

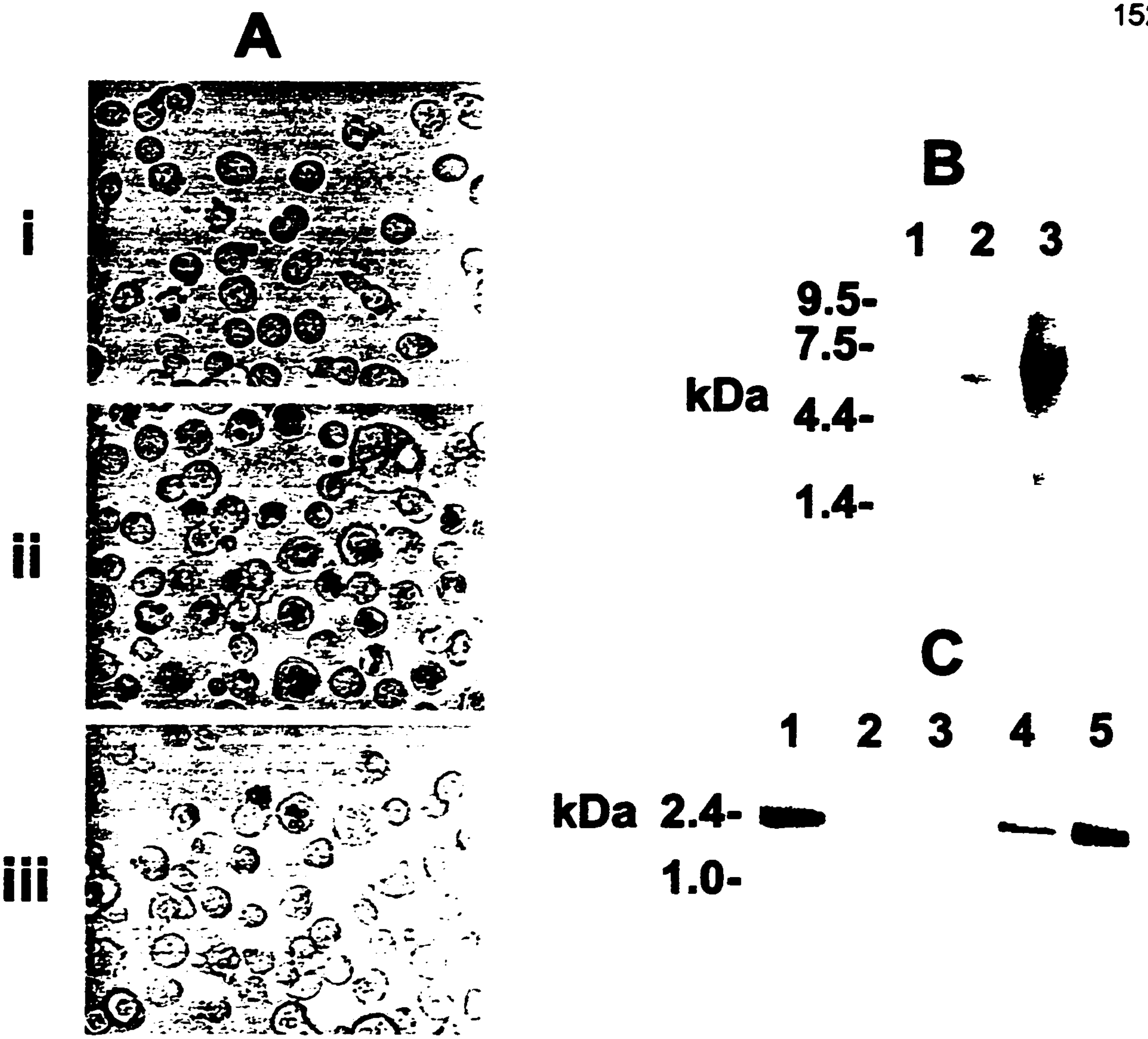


Figure 7.6: Possible genetic instability of the Bm5.lef-8(371) rescuing cell line that expresses *lef-8* over 52 passages. A) Infection of normal Bm5 cells (i), Bm5.lef-8(371) cells at passage 1 (ii), and Bm5.lef-8(371) cells at passage 52 (iii) with TS-S1 mutant BmNPV at 33°C shows that the stably transformed cell line loses its ability to efficiently rescue the TS-S1 after 52 passages. This is due to a reduction in *lef-8* mRNA expression, as illustrated by the Northern blot in B; lanes 1 to 3 contains RNA isolated from normal Bm5 cells, Bm5.lef-8(371) cells at passage 52, and Bm5.lef-8(371) cells at passage 1 respectively. Loss of mRNA expression is due to loss of the *lef-8* gene from the population of Bm5.lef-8(371) cells over 52 passages, as shown by the Southern blot in C). Lanes1 and 2 contains 10 and 0 pg of pIE1/153A.lef-8 plasmid DNA digested with *NotI/BamHI*, and lanes 3 to 5 contain 5 μg of *NotI/BamHI* digested genomic DNA isolated from normal Bm5 cells, Bm5.lef-8(371) cells at passage 52, and Bm5.lef-8(371) cells at passage 1 respectively.

was used to infect Bm5.lef-8(371) cells and increase the virus titre for 2 to 3 days. At this point the cells were treated to detect the β-galactosidase and revealed that approximately 1 in 1000 infected cells harbored the recombinant viruses. Attempts to isolate pure recombinant baculoviruses were made using serial dilution cloning in Bm5.lef-8(371) cells with β-galactosidase staining criteria. If the correct homologous recombination event had occurred, the *lef-8* gene should be inactivated and the recombinant virus would not complete its infection cycle.

Normal Bm5 cells were infected with supernatants containing either wild-type BmNPV or purified recombinant viruses generated using transfer vectors pTV#1.A.lacZ and pTV#2.A.lacZ, incubated for 3 days at 28°C, and stained for the presence of β-galactosidase. The results are shown in Figure 7.7, parts A to C. Bm5 cells infected with wild-type BmNPV show occlusion bodies (Figure 7.7A), indicating that wild-type BmNPV could complete its infection cycle but do not produce β-galactosidase. Bm5 cells infected with supernatant from the recombinant virus generated using transfer vector pTV#1.A.lacZ, do not show occlusion bodies, indicating that this virus did not complete its infection cycle, but stain for β-galactosidase indicating that the virus is present in the cells (Figure 7.7B). Similarly, Bm5 cells infected with supernatant from the recombinant virus generated using transfer vector pTV#2.A.lacZ do not show occlusion bodies, indicating that this virus did not complete its infection cycle, but stain for β-galactosidase indicating that the virus is present in the cells (Figure 7.7C).

7.3.5 Failure to Isolate Pure Recombinant Viruses Due to Homologous Recombination with the Rescuing Cell Line

Unfortunately, further propagation of both types of recombinant viruses in the Bm5.lef-8(371) rescuing cell line lead to a sub-population of pseudo wild-type recombinant baculoviruses that could complete their infection cycle in normal Bm5 cells, despite further attempts to isolate pure viruses using plaque purification. It was predicted that other homologous recombination events could occur with the rescuing cell line to restore the expression of *lef-8*, under control of the actin promoter, from the BmNPV genome. Potential recombination events are shown in Figure 7.8A. To test these possibilities, budded viral

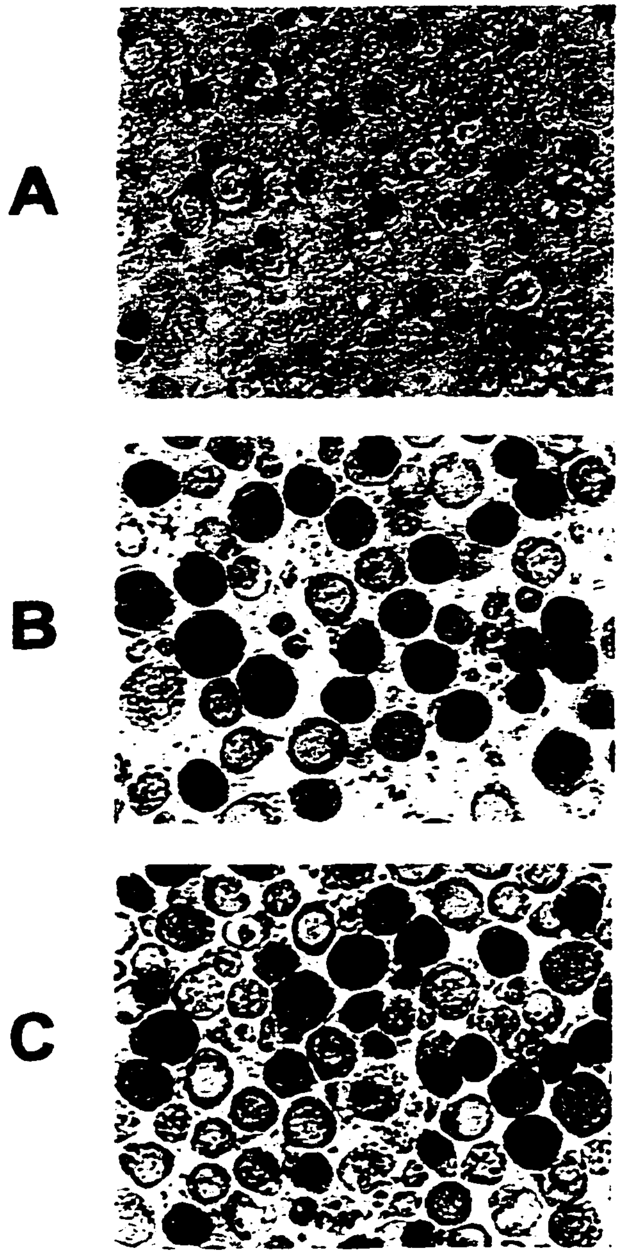


Figure 7.7: Demonstration of the successful creation of first generation BVACs expressing the *LacZ* transgene. Normal Bm5 cell cultures were infected with either wild-type (A) or recombinant baculoviruses generated with the transfer vectors pTV#1.A.LacZ (B) and pTV#2.A.LacZ (C), incubated for 3 days at 28°C, and then stained for the presence β-galactosidase. Occlusion bodies are only present in Bm5 cells infected with the wild-type virus, while blue cells (indicating the presence of β-galactosidase) are only found in the cultures infected with the recombinant baculoviruses.

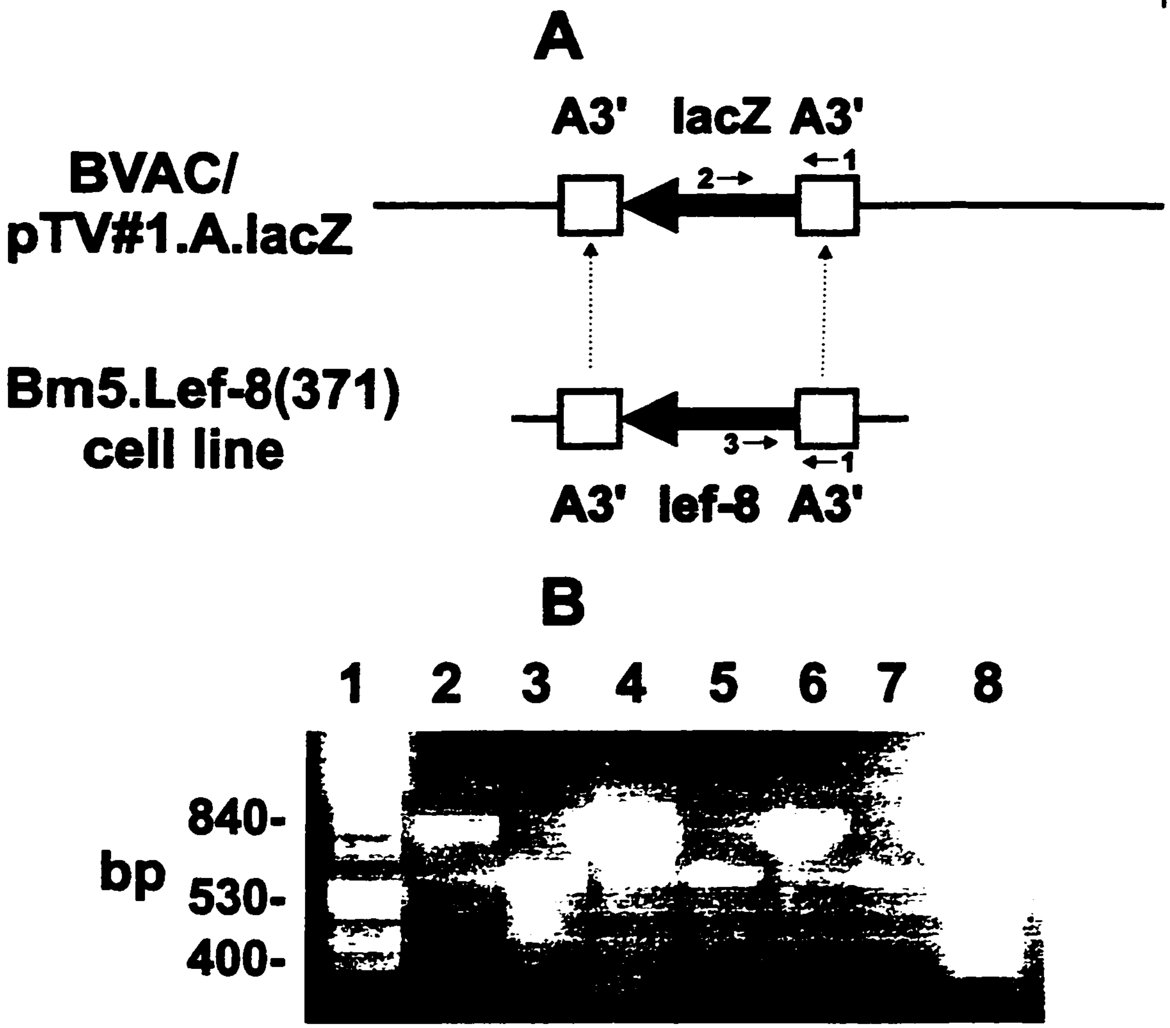


Figure 7.8: Homologous recombination between the packaging cell line and the BVACs leads to a pseudo-wild type baculovirus with the ability to express *lef-8* under control of the actin promoter. One potential homologous recombination event is shown in (A). PCR amplification with either primer pairs 1 and 2, or 1 and 3, would amplify a 750 bp *LacZ* fragment from an actin.*lacZ* template or a 580 bp *lef-8* fragment from an actin.*lef-8* template respectively. B) An ethidium bromide stained agarose gel of electrophoresed PCR amplification reactions; lanes 1 and 8 are molecular weight markers; lanes 2, 4, and 6 are PCR reactions using primers 1 and 2, and control pTV#1.A.lacZ DNA, DNA isolated from the supernatant of Bm5 cells infected with a BVAC generated with pTV#1.A.lacZ, and Bm5 cells infected with a BVAC generated with pTV#2.A.lacZ respectively; lanes 3, 5, and 7 are PCR reactions using primers 1 and 3, and control pIE1/153A.lef-8 DNA, and DNA isolated from the supernatant of Bm5 cells infected with a BVAC generated with pTV#1.A.lacZ, and Bm5 cells infected with a BVAC generated with pTV#2.A.lac respectively.

DNA, isolated from the supernatant of infected Bm5 cells, was used as a template for diagnostic PCR analysis. The ethidium bromide stained analytical gel of diagnostic PCR products in Figure 7.8B reveals that at least two species of recombinant viruses per transfer vector are present in the Bm5 supernatants. One of these species contains *lacZ* and actin promoter, while the other species identified confirms the prediction that a pseudo wild-type virus expressing *lef-8* from the actin promoter was generated, presumably by double homologous crossover with the expression cassette contained in the chromosomes of the rescuing cell line Bm5.lef-8(371).

7.3.6 Failure to Prevent Homologous Recombination by Replacing the Actin Polyadenylation Signal with a SV40 Early Genes Polyadenylation Signal

Whilst recombination by double crossover is a relatively efficient, single crossover events are expected to be less efficient. Therefore the actin polyadenlyation signal and transcription termination (polyA) region in the transfer vectors pTV#1.A.lacZ and pTV#2.A.lacZ was replaced with a SV40 early genes polyA region to yield the second generation transfer vectors pTV#1.A/SV40 and pTV#2.A/SV40. (Figure 7.3, parts D and E), in order to reduce the efficiency of a recombination event with Bm5.lef-8(371) cells to create the pseudo wild-type baculoviruses detected in Section 7.3.4.

The SV40 polyA sequence was verified to be functional and compared to the actin polyA sequence using transient expression assays with JHE as the reporter protein. The relative expression levels of JHE from each construct are shown in Figure 7.9, and indicate that the transfer vectors pTV#1.A/SV40.jhe(kk) and pTV#2.A/SV40.jhe(kk) are functional but over 50% less efficient at expressing JHE with the actin polyA sequence than that used by SV40 early genes.

Co-transfection of the transfer vectors pTV#1.A/SV40.LacZ and *pTV#2*.A/SV40.lacZ with wild-type BmNPV and purification of the recombinant baculoviruses proceeded in an identical manner as that described in Section 7.3.3. As before, pure viruses expressing β-galactosidase but failing to complete their infection cycle in normal Bm5 cells could not be obtained, despite several rounds of dilution and plaque purification. It appeared that the SV40 early genes polyA in each new transfer vector was sufficiently homologous to the

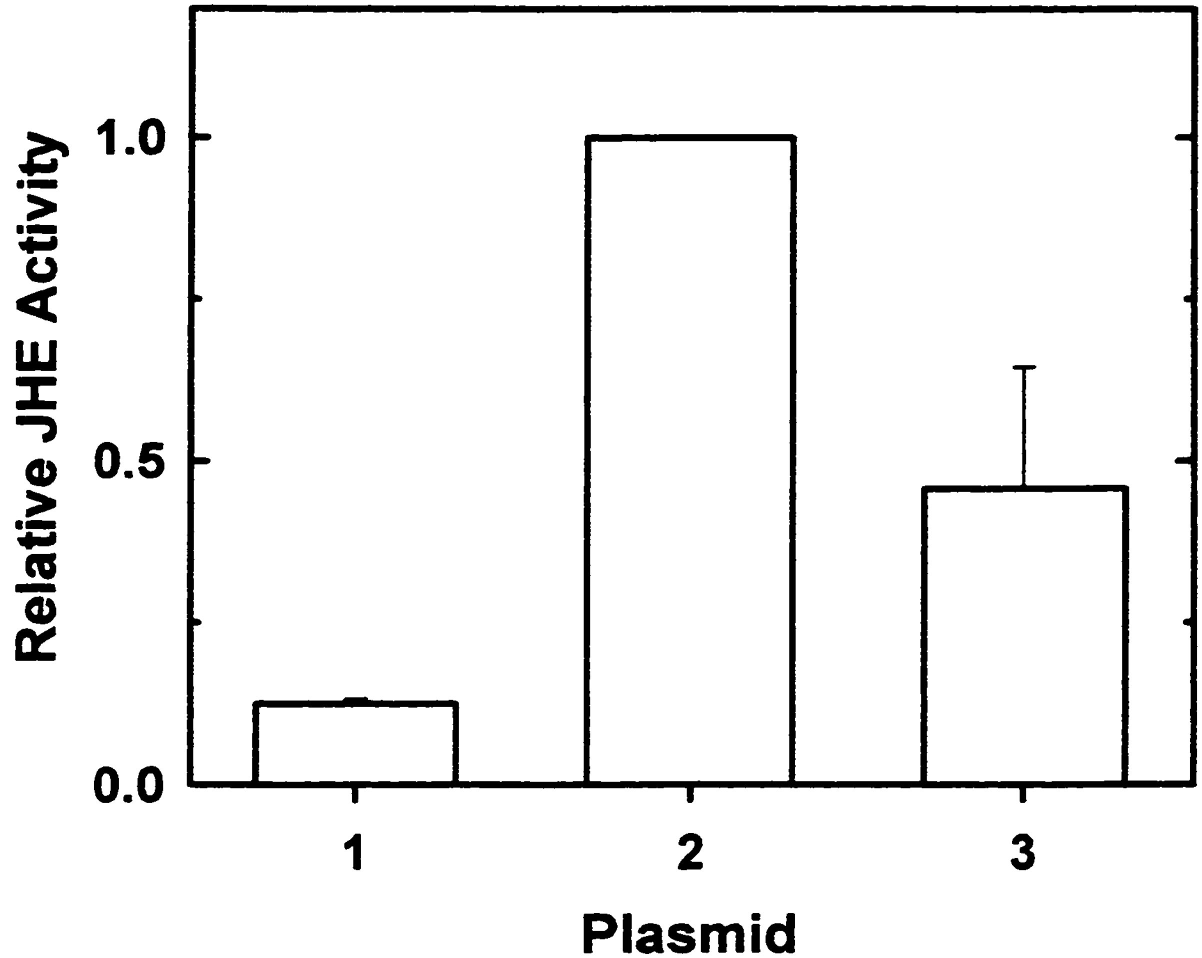


Figure 7.9: Relative JHE activities in the supernatant of Bm5 cells 3 days following transfection with the expression plasmids pBmA (plasmid 1), pBmA.JHE(kk) (plasmid 2), and pBmA/SV40.JHE(kk) (plasmid 3) to test the ability of the SV40 transcription termination and polyadenylation signal to function in gene expression. Although less JHE resulted from using the SV40 3' region than the actin 3' region, this SV40 3' region was used in transfer vectors pTV#1.A/SV40.LacZ and pTV#2.A/SV40.LacZ to reduce homologous recombination events with the packaging cell line.

actin polyA to allow a double homologous crossover recombination event to occur with Bm5.lef8(371) cells to form pseudo wild-type baculoviruses expressing *lef-8* (data not shown). The SV40 early genes polyA is approximately 50% identical to the *Bombyx mori* cytoplasmic actin polyA on a nucleotide level.

7.3.7 Testing of a Third Generation Transfer Vector Incapable of Homologous Recombination with the Actin Cassette in the Rescuing Cell Line

To completely exclude any possible homologous recombination with the actin cassette in the rescuing Bm5.lef-8(371) cell line, it was decided to dispose of the actin cassette and exploit the putative *lef-8* promoter and *lef-8* polyA signals already present in pTV#2 instead. A multiple cloning site was introduced into the *SacI* site of pTV#2 in different orientations to yield pTV#2.mcs1 and pTV#2.mcs2. To determine if there was any promoter activity, the juvenile hormone esterase open reading frame was introduced into pTV#2.mcs1 in two orientations to yield pTV#2.mcs1.jhe(forward) and pTV#2.mcs1.jhe(reverse), and these were used to transfect normal Bm5 cells. Very early BmNPV gene products were anticipated to be required for the *lef-8* promoter to function efficiently, therefore some Bm5 cells were infected with wild-type BmNPV at a multiplicity of infection of approximately 5 viruses/cell for 1 h immediatly prior to transfection. The control plasmid expressing *jhe* under control of the actin promoter was used for comparison. Figure 7.10 shows the relative levels of JHE produced 3 days post-transfection. It shows that the putative *lef-8* promoter can drive foreign gene expression at a basal level in the absence of other BmNPV factors, but expression is enhanced 4-fold in the presence BmNPV factors. Conversely, the level of juvenile hormone esterase expressed from the actin gene promoter is reduced 7-fold in the presence of BmNPV factors. And, the *lef-8* promoter is 2.5-fold more active than the actin promoter in the presence of BmNPV factors, making it suitable to drive foreign gene expression in recombinant viruses, such as a BVAC.

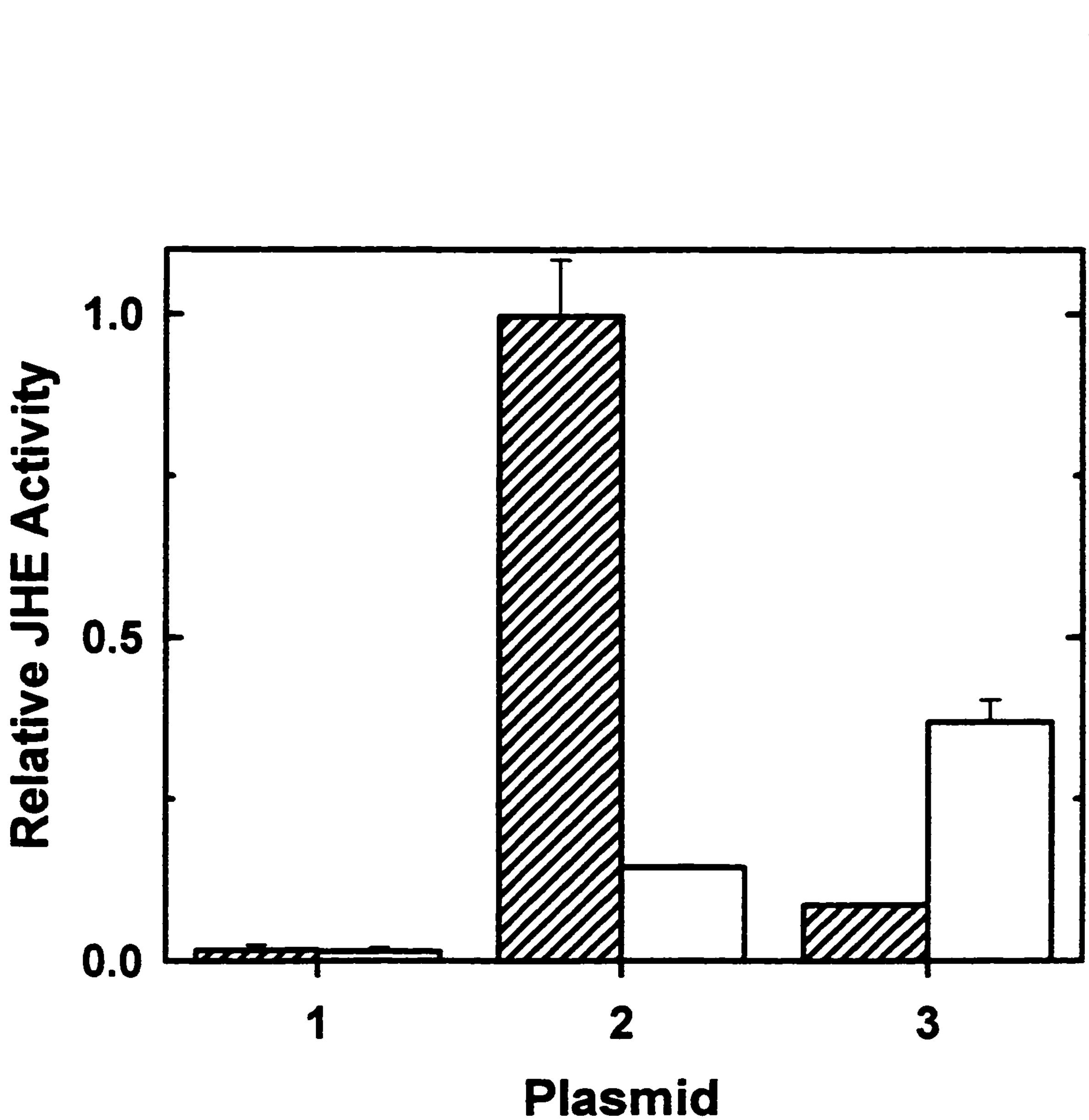


Figure 7.10: Relative JHE activities in the supernatant of Bm5 cells 3 days following transfection with the expression plasmids pTV#2 (plasmid 1), pTV#2.A.jhe(kk), and (plasmid 2), and pTV#2.mcs1,jhe(kk) (plasmid 3) to test the ability the putative *lef-8* promoter to drive foreign gene expression in the absence (shaded bars) or presence (unfilled bars) of wild-type BmNPV infection. In the presence of BmNPV, the *lef-8* promoter seems superior to the actin promoter in driving foreign gene expression, hence suitable for use in the transfer vector pTV#2.mcs1.LacZ.

7.3.8 Failure to Isolate a Pure Recombinant Viruses with the Third Generation Transfer Vector

Co-transfection of the transfer vector pTV#1.mcs1.lacZ with wild-type BmNPV and purification of the recombinant baculoviruses proceeded in an identical manner as that described in Section 7.3.3. As before, pure viruses expressing β-galactosidase but failing to complete their infection cycle in normal Bm5 cells could not be obtained, despite 5 rounds of serial dilution cloning and 2 rounds of plaque purification. This was not expected, as the possibility of homologous recombination with the actin cassette to restore *lef-8* expression from the baculovirus genome was eleminated with pTV#2.mcs1.lacZ. Although unlikely, one further homologous recombination event was predicted to create a pseudo wild type BmNPV. This would involve the acquisition of the complete *lef-8* expression cassette from the rescuing cell line, via an *ie-1*/HR3 and *ie-1*/HR5 double crossover homologous recombination with BmNPV (Figure 7.11A).

To test this possibility, baculovirus DNA was extracted from Bm5 cultures infected the "pure" virus species isolated after 7 rounds of purification, digested with *SpeI* restriction enzyme, and analyzed by Southern hybridization using ^{32}P-labeled *lacZ*, *lef-8*, and *actin* probes. The Southern blot indicates that the desired recombination event that placed the *lacZ* gene into the viral genome had occurred (Figure 7.11B, part i) because the *LacZ* probe hybridized to the predicted 4.3 kbp *SpeI* fragment (the origin of the 5.3 kbp *SpeI* fragment is unknown). However, the recombinant viral species still contained *lef-8*, through either an unknown recombination event or by contamination with wild-type BmNPV (Figure 7.11B, part ii). In wild-type BmNPV, a *lef-8* probe is predicted to hybridize to a 3.2 kbp *SpeI* fragment, and, to a lesser extent, to a 4.5 kbp *SpeI* fragment. Actin was not found to be present in the viral genome (data not shown), and thus this data does not support the proposed *ie-1*/HR3 and *ie-1*/HR5 double crossover homologous recombination hypothesis.

7.3.9 Redesign of the Rescuing Cell to Prevent Recombination

The expression vector, pIE1/153A.lef-8, that was used to generate the Bm5.lef-8(371) rescuing cell line does contain two baculovirus elements, *ie-1* and HR3, that may

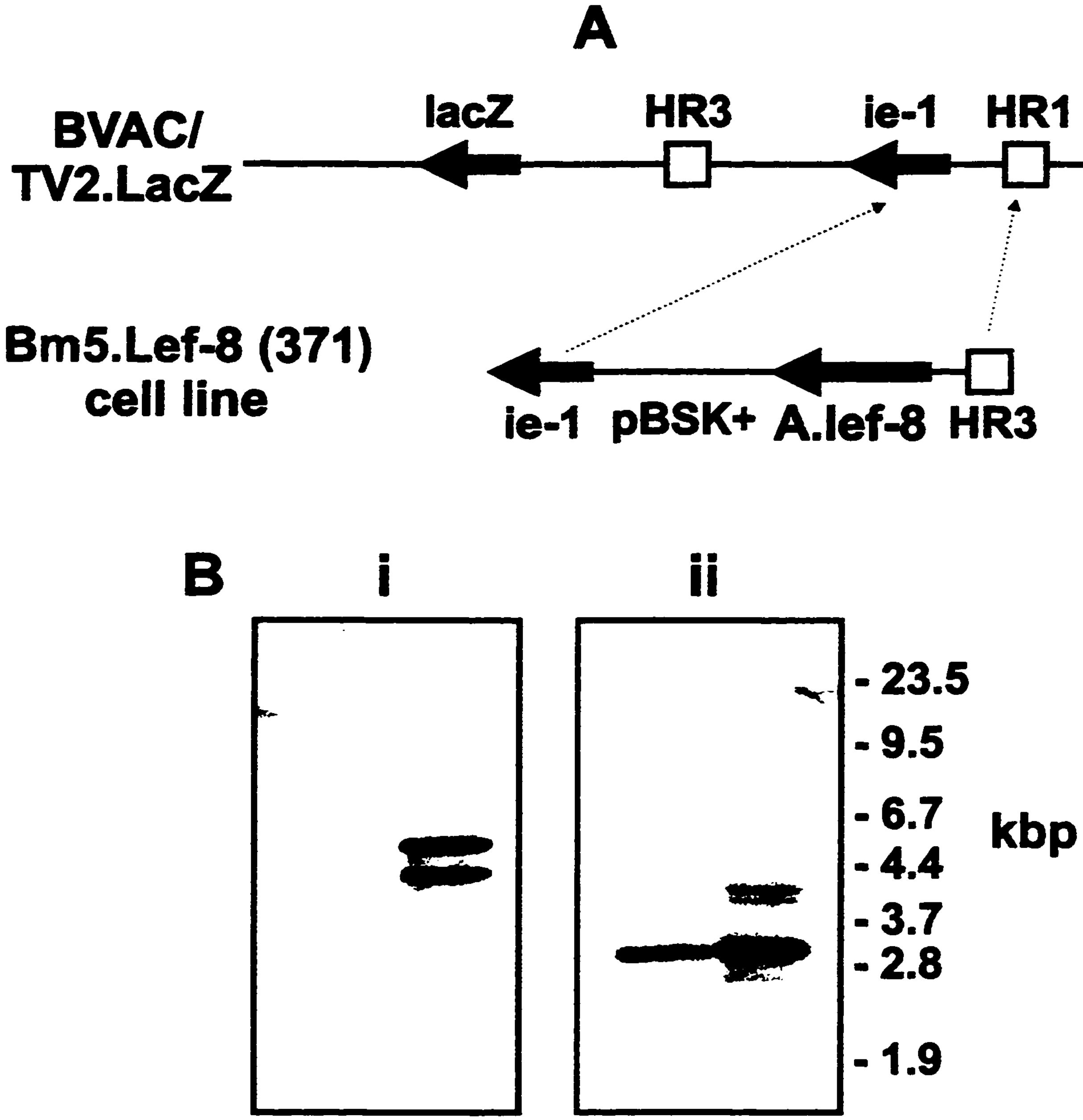


Figure 7.11: A) Predicted recombination events between the rescuing cell line Bm5.Lef-8(371) and the BVAC created with TV#2.LacZ that could restore *lef-8* expression to the BVAC. B) Southern hybridizations of *SpeI* digested DNA isolated from wild-type BmNPV (left lanes) and the BVAC species arising from recombination events (right lanes) using i) the 3.2 kbp *BamHI/NotI* fragment containing the *LacZ* ORF from pBmA.lacZ and ii) the 2.6 kbp *BamHI/XbaI* fragment containing the *lef-8* ORF from pIE1/153A.Lef-8 as gene probes. No hybridization signal was detected with an *actin* probe.

enable a BVAC to acquire the capacity for *lef-8* expression through an unlikely double homologous recombination crossover event. To exclude this event, an alternative expression vector, pBmA.lef-8 (Figure 7.2B), was constructed and contains no homology with the recombinant baculovirus expected to be created using the pTV#2.mcs1 transfer vector. A stably transformed Bm5 cell line expressing *lef-8* from this expression vector is currently being characterized for use as the next generation of rescuing cell line for BVAC generation and propagation.

7.3.10 Demonstration of BVAC Transduction of Silkworms for Generating Transgenic Lepidopteran Insects

Despite problems with recombination to form pseudo wild type BmNPV, the first generation BVACs were tested for their ability transduce *Bombyx mori* larvae and generate a transgenic germline. Fourth instar male larvae and day 5 pupae were injected through the lower abdomen with cell culture supernatant containing BVACs. Actually, the presence of a pseudo wild-type BmNPV in the supernatant was expected to act as a helper virus and rescue enough BVACs to spread infection more efficiently throughout larval tissue, particularly to the gonads. This is shown in the dissected day 7 infected 4th instar larvae that was assayed for expression of the β-galactosidase transgene from the BVAC in Figure 7.12. Some injected pupae were able to metamorphose into adult moths without dying from pseudo wild-type BmNPV infection. Male moths were mated with uninfected females and the larvae that hatched from the resulting eggs were reared and analyzed at the fourth instar for transgenic f1 progeny. Both β-galactosidase staining assays and more sensitive PCR analysis were inconclusive in determining whether transgenic f1 progeny were actually obtained (Swevers and Iatrou, unpublished data).

7.4 Discussion

In this chapter it was established by rescue of the temperature sensitive TS-S1 mutant BmNPV by a transformed Bm5 cell line expressing BmNPV *lef-8*, that the *lef-8* gene product is responsible for progression of the baculovirus into the late (virulent) phase of infection. The study of the BmNPV *lef-8* counterpart in AcNPV was previously reported to

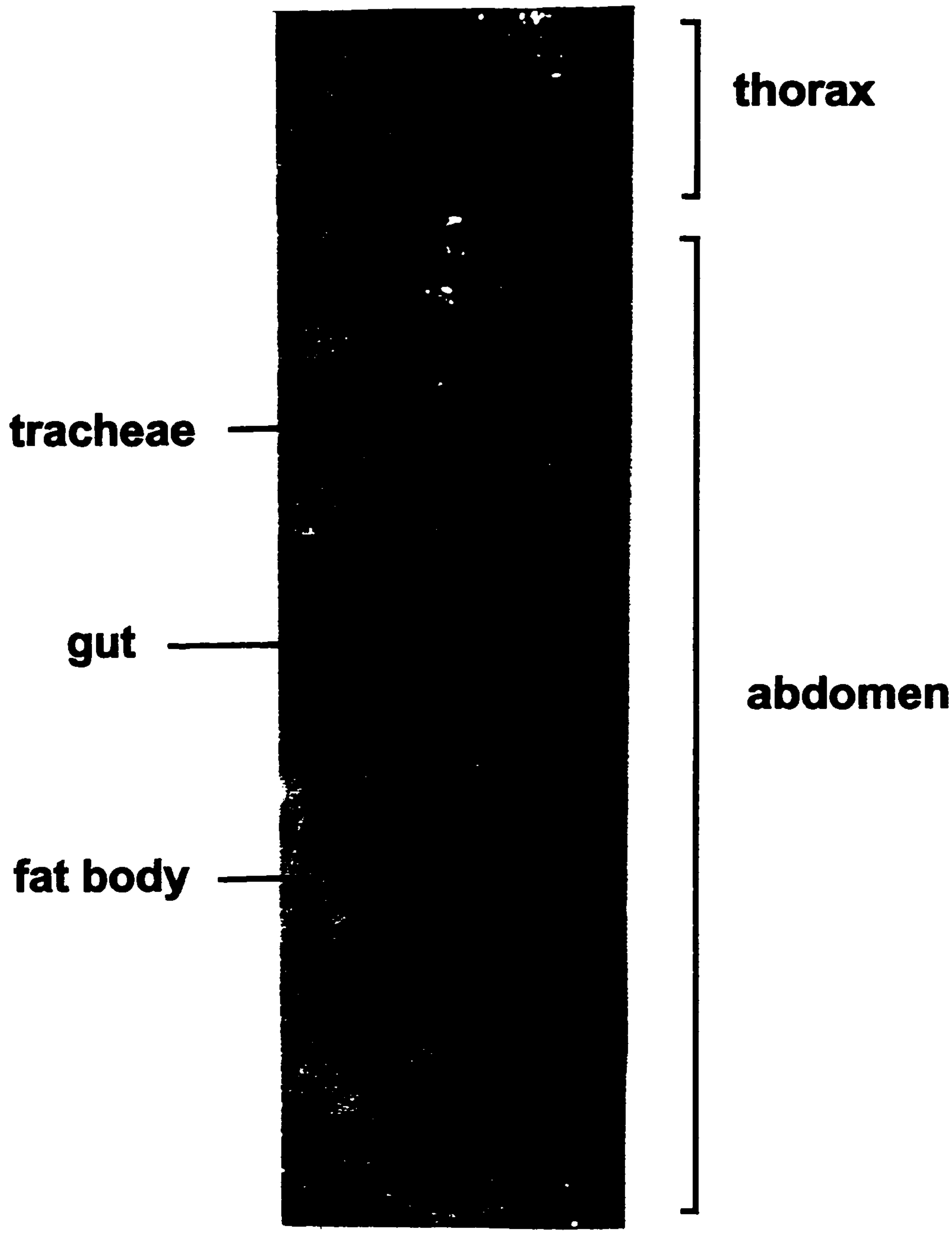


Figure 7.12: β-galactosidase staining assay of a dissected 5[th] instar *Bombyx mori* larvae 7 days after injection with pTV#1.A.LacZ/BVAC containing supernatant. The blue regions confirm that the transgene was expressed in most of the tissues (taken by K. Iatrou).

be essential for efficient transactivation of a reporter gene under late viral promoter control (Passarelli et al., 1994). The predicted protein product of this gene, LEF-8, contains a conserved amino acid motif found at the active site of DNA-directed RNA polymerases from both prokaryotes and eukaryotes (GIKICGIHGQKGV, Passarelli et al., 1994), and thus LEF-8 is likely to be a novel viral RNA polymerase subunit necessary for mRNA transcription from the promoters of late and very late-phase baculovirus genes.

Attempts to inactivate the *lef-8* gene in the BmNPV genome, either by disruption or elimination, were only partially successful for the generation of BVACs. In every case, this was found to be due to recombination events where *lef-8* was restored to the BVACs from the rescuing cell line. Immense selective pressure exists for the occurrence of both homologous and non-homologous recombination events to create pseudo wild-type viruses from BVACs; even if they occurred in only 1 in 10^6 viruses, the virulence obtained by a pseudo wild-type virus would rapidly dominate the non-virulent BVAC population. Therefore research efforts are still underway to at least completely eliminate any chance of *homologous* recombination with a BVAC. This is necessary before any further attempts to generate transgenic insects are made.

CHAPTER 8

Conclusions and Recommendations

9.1 Conclusions

The focus of this thesis has been to develop transformed insect cell protein expression technology with characteristics that are superior to other protein expression systems, particularly the baculovirus expression system.

In the first stage of this project, expression (pIE1/153A) and transformation (pBmA.hmB) vectors were initially constructed, and protocols for transfection and transformation were optimized for use with Bm5 cells. A stably transformed cell line could be generated by initially transfecting 10^6 Bm5 cells with lipofectin reagent and 3 μg DNA at a 100:1 molar ratio of expression plasmid:hygromycin B resistance plasmid, followed by antibiotic selection with 0.25 to 0.5 mg/mL hygromycin B with weekly subculturing for 4 to 6 weeks, and finally isolation of clones by limiting dilution cloning and screening clones for high producers over a further 4 week period. Using this protocol and the insect glycoprotein juvenile hormone esterase (JHE) as a reporter gene, levels of up to 200 mg/L JHE were obtained in stirred suspension culture in serum-containing medium, and 135 mg/L JHE in static culture in serum-free medium. The expression levels for are 30 to 50 fold higher than those obtained from the baculovirus expression system, and are among the highest reported to date for secreted glycoproteins using any expression system.

Although apparently successful for secreted proteins, it was found that expression of intracellular proteins in transformed insect cells would offer no advantages over the baculovirus expression system. In an attempt make this system useful for expression of intracellular proteins, a *Bombyx mori* chorion signal peptide was tested for its ability to secrete intracellular proteins. While this signal peptide functioned efficiently for the secretion of a normally secreted protein such as JHE and human tissue plasminogen activator (t-PA), chimeras of the chorion signal peptide fused to intracellular proteins were not secreted. It was realized that additional signals were required for successful passage through the secretory pathway, and an intracellular protein could only be secreted when a complete secreted protein was fused in-frame to N-terminus of the intracellular protein. Auxiliary vectors for in-frame attachment of JHE to an intracellular protein were constructed and were used to successfully secrete a bacterial cytoplasmic protein, chloramphenicol acetyl-transferase (CAT), and a nuclear factor, *Bombyx mori* chorion factor 1 (BmCF1). To our knowledge, these are the only clear examples of successful secretion of intracellular proteins.

Other proteins were also expressed and characterized in this dissertation to further evaluate this system. These include the secreted glycoproteins human tissue plasminogen activator (t-PA), human granulocyte-macrophage colony stimulating factor (GM-CSF), a soluble isoform of the alpha subunit of the human granulocyte-macrophage colony stimulating factor receptor (solGMRα), and a non-glycosylated form of bovine transferrin (ngbTf). Furthermore, one G-protein coupled membrane receptor [rat protease activated receptor 2 (rPAR-2)], and two ion exchangers [native bovine retinal rod Na^+-Ca^{2+}-K^+ exchanger (bNCKX) and a modified bovine retinal rod Na^+-Ca^{2+}-K^+ exchanger (bNCKXdd)] were also successfully expressed. Whenever possible, direct comparisons of expression levels or biological activity were made with other eukaryotic expression systems including the baculovirus expression system, transformed mammalian cells, and yeast. These comparisons were all found to favor the use of transformed insect cells over other expression systems for recombinant protein expression.

Transformed insect cells are not only potentially useful for the expression of recombinant proteins but also can be employed as a research tool in the field of insect biology. This was demonstrated in attempts to generate baculovirus artificial chromosomes (BVACs) using a packaging cell line (Bm5 cells stably expressing a baculovirus transcription factor). BVACs themselves could be used as a powerful transient expression vector (they have a 100% transfection efficiency and should persist in cell culture), but their most important application would be for the generation of non-drosophilid transgenic insects. Although the goal to create the first transgenic lepidopteran insect was not realized in this dissertation, due to undesirable homologous recombination events between the BVACs, these problems should be eliminated in the near future.

9.2 Recommendations

The major disadvantage with this expression system is the fact that it takes approximately 2 months to generate stably transformed and cloned insect cell lines. There are three obvious approaches that should improve this. The first is to develop protocols to clone cell lines as they are being transformed. This could be achieved by serial diluting cells in antibiotic selective medium directly following transfection, and visually identifying colonies of transformed cells. Four to six weeks could be eliminated using this strategy. Second, the use of a puromycin antibiotic resistance-selection scheme may be advantageous over the use of hygromycin B. Puromycin acteyl-transferase was reported to function in lepidopteran cells and allowed the selection of stably transformed Sf21 cells in the presence of puromycin in less than one week because puromycin acts within hours (McLachlin and Miller, 1997), whereas lepidopteran cells continue to divide for a considerable time in the presence hygromycin B. The third approach to reducing transformation time, that was explored briefly in this thesis, is to use alternative lepidopteran cell lines. A reduction in transformation time is seen with faster growing insect cell lines.

Not only can the use of alternate lepidopteran insect cell lines be used to save time, but may also offer advantages for recombinant protein yield, specific productivity, large-scale growth in bioreactors, and post-translational modifications. For example, Sf21, High Five™, and Ea4 (derived from *Estigmena acrea*) cells have been found to have the useful capacity for some complex N-linked glycosylation (Davis and Wood, 1995; Ogonah et al., 1997). Therefore it is recommended that different insect cell lines be screened for the desirable properties in order to identify an optimal cell line.

There is also potential for improved recombinant protein yields through the use of alternative bioreactor configurations, such as continuous perfusion systems (Piret et al., 1994; Docoslis et al., 1997), and carefully designed insect cell culture media. Fortified growth media have been reported to increase the density of lepidopteran cells to 8×10^6 viable cells/mL in batch suspension cultures (Rhiel et al., 1997). Rather than develop our own media, which is a tedious and expensive undertaking, several commercial serum-free and protein-free media formulations for high cell density insect cell culture are available and should be tested for use with this new expression system.

To improve the properties of the secretion modules described in Chapter 5, other secreted proteins should be explored as alternatives to JHE for their ability to "piggy-back" intracellular proteins into the supernatant. Furthermore, it may be possible to identify the specific peptide signals required for successful passage through the secretory pathway, and generate small synthetic peptide coding for attachment to the intracellular protein's open reading frame. A smaller molecule may be more efficiently secreted.

To facilitate the purification of recombinant proteins using this system, it is recommended that all proteins expressed in the future contain the same affinity tag for purification. This would obviate the need to develop and optimize a protocol for the purification of every individual protein, which is expensive and time consuming, because a common affinity tag would allow the same purification protocol be used for each protein. It is expected that the affinity tag would rarely affect the biological activity of a protein.

Due to time constraints, no attempt to scale-up the production of recombinant proteins from transformed insect cells beyond 100 mL was made in this dissertation. However, normal Bm5 cells have already been well characterized in 1.5 L batch suspension cultures (Zhang et al., 1994), while many other insect cell lines, including High Five™ and Sf21 cells, have proven scalable up to 150 L owing to their use as hosts cells for the baculovirus expression system (Guillaume et al., 1992). Nevertheless, it is recommended that at least one stably transformed insect cell line over-expressing a recombinant protein be grown in a pH and dissolved oxygen controlled stirred-tank bioreactor in the near future, to verify that this system is indeed scalable.

Finally, we anticipate that pure BVACs will soon be isolated. The question remains, however, as to whether a BVAC can be stably maintained as an extra-chromosomal entity to generate transgenic insects. If necessary, steps may be required to encourage integration of the BVAC into the germ cell chromosomes. This could be achieved by equipping the BVAC with either regions targeting the host genome for homologous recombination, or transposabe elements.

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APPENDIX A

pIE1/153A Lepidopteran Expression Vector Sequence
Version 3 compiled by Patrick Farrell on 7/4/98

1..2750 = Actin Cassette (polylinker 1149..1210)
2763..3950 = HR3 Element
3978..7814 = IE-1 gene
7815..10699 = pBSK+ backbone

1 gagctcgtag ctccaccgcg gcggggatct cgacgaccgg tgacactacg caatgacgtt
61 gacaagtcac tcaaatctca gacgggtcaa gacacagacg catcaaagga tgttcatctt
121 gtgtgtaacc tgctcgtatg tattgtaaaa acgcgaacag tcatcttatg cctaagcgta
181 acctgggtac ctctacacag atttctacgg aattagtgag tccgcataaa attaatttgg
241 tgaatctttt gaaaggtaga aagtacagaa ccagtttcct ttgcattttg cgaattttag
301 aatgtttcgt attaagcggt caacgactcg cattccaatt aaagactgca gctgcagtaa
361 acaacggtat gcagctggga gatttgtgct cctagcgtaa ctgtctcaaa cggtgtacga
421 aatcggtaat ttcggtgcac acgacgtact gttagtcggg agcggggaca gcaagtgaaa
481 aacgtcagtt attataccgg tatataaatt tattttatta aaaagttttg gggttagtaa
541 ctttaataaa taaaaatctt tgtcaaaaaa aaaacggcaa caacaacatt ccgttcgttt
601 tacgtaatat gcatattaca ataccttaca acctacatta aatactgaat ttatttacaa
661 gatcacaagc gtaatttgag ccaccctctt gaggttctgc tgtagggcat cataacttaa
721 agtctcaacg cttagaagca cttcctgtgt acaatggagg gaccagactc gtcgtactcc
781 tgtttcgaga tccacatctg ttggaaggta gagagggagg cgaggatcga tccaccgatc
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1141 taagacttct ***cgaggcggcc gctctagaac tagtggatcc cccgggctgc aggaattcga***
1201 tatcaagctt *atcgataccg tctactacca acgcggcaac ttcttcgtcg cacctcttga*
1261 attagtctgc aagaaaagaa aaaaaacaat tcaaactaca ttctcattcc atacattata
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1681 ataaatatgt ttttttttct ttcgcaattt taaaattaaa acttacatag tattattaaa
1741 taagtgacaa gtacgtagat gcgaatgcgc actgttcgag cacaccttag taaatgagaa
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1861 ccagtttgtt tttccaccaa tcgcgggctg actcagtttt tgtcaccata tatggtaacg
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1981 ggcacctcgc ggtcgcttgt aacctctccc cgactcccgc cgaagtttca tagaatttca
2041 ctcgacgtgc tttcactaga ttctgtaccg atgtaaatca taacccagaa aatccggtta
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2701 tcacggcaga aataggcagg gtggtggtac ctacccgcga ggactcacaa gagctccccc
2761 ccaatattag **acaacaaaga tttattttat tcatgccact actcggttcc gtttttcaag**
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2881 aacgttaaag tggacgctga ttgcgaattg gccaaagacc tatcgcacgt tttaaacgcg
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3001 gtaaaaaaag aagccgtgcc cagtcacgtg tacgccaacc tgaacacgca atccaacgac
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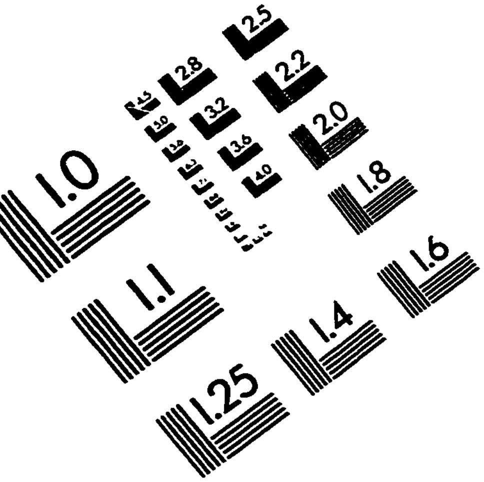
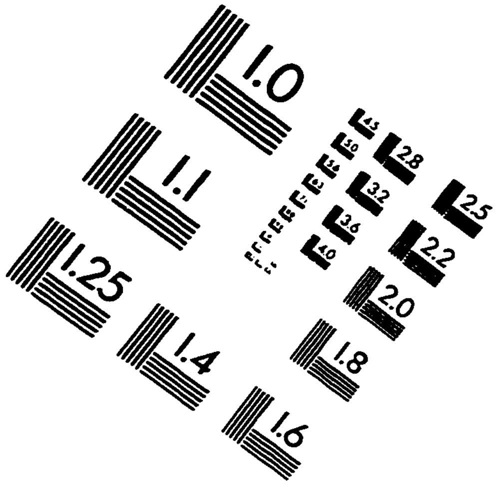
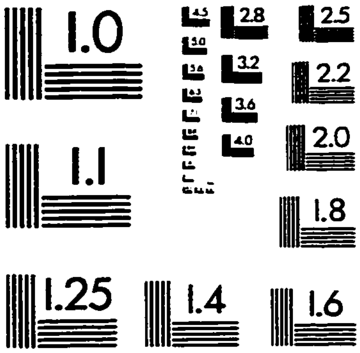
IMAGE EVALUATION TEST TARGET (QA-3)







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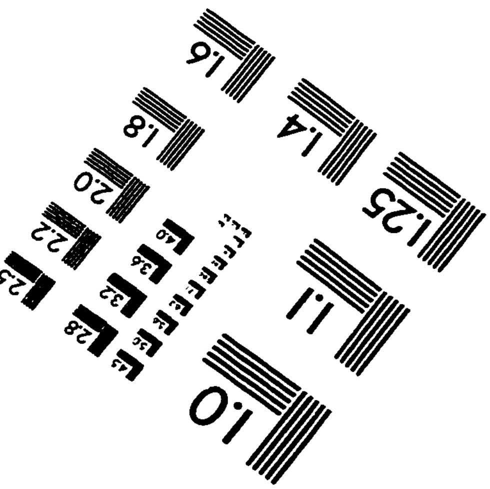

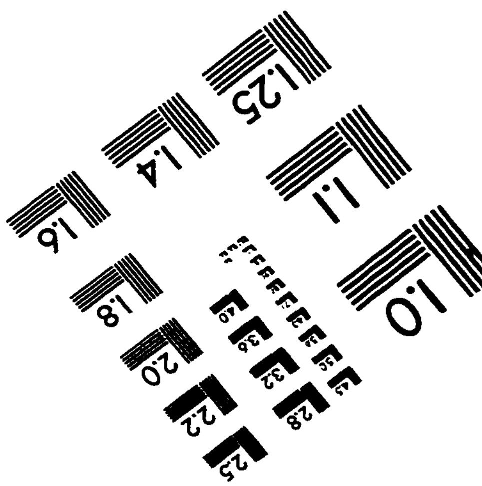

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