Insect Expression Systems: Improving intracellular and regulated heterologous gene expression

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Abstract

Expression systems (ES) in a wide variety of biological systems are used to provide foreign protein. Protein production in mammalian cells is a labour-intensive and expensive process and insect cells have been used as cheaper alternatives. Insect ES are based on two types of vector; viral- and plasmid-based. The baculovirus ES (BES) offers high-level transient production of intracellular proteins. Yields of secreted and membrane-targeted proteins have until recently been relatively low, however, the development of a novel vector, lacking chitinase, has overcome these limitations. Plasmid-based vectors can be used for transient and stable expression in insect cells but most utilise the relatively weak *Autographa californica* multiple nucleopolyhedrovirus (*Ac*MNPV) immediate early-1 (*ie*-1) promoter, producing low-levels of intracellular recombinant protein. More recent vectors have used stronger promoters, however, compared with mammalian stable ES these are limited in use and most notable is the lack of an efficient inducible ES in insect cells. The work in this thesis describes methods, with which intracellular levels of foreign protein may be increased, while also developing the grounding work for the development of a fully functional insect tetracycline regulatory system.

To improve stable and regulated expression of heterologous genes in insect cells, work was undertaken to compare and characterise the transcriptional activity of a range of promoters successfully used in insect and mammalian systems. Of the promoters tested, *Orgyia pseudotsugata* MNPV (*Op*) *ie*-2 and *Bombyx mori* actin 3 (*BmA*3+E) were found to be transcriptionally stronger than *Ac*MNPV *ie*-1. *Drosophila melanogaster* metallothionein and actin 5.1 did not produce any detectable activity, and the Cytomegalovirus (CMV) *ie* promoter, although active, was weaker than *Ac*MNPV *ie*-1. Attempts to develop a new inducible ES for use in stable cell lines was based on modifying a mammalian tetracycline-inducible ES. The original *CMVie* promoters were replaced by the *Bm A*3+E and *Op ie*-2 promoters that had been shown to be optimal in insect cells. The *Bm A*3+E promoter was successfully used to express the tetracycline transcriptional activator protein. Studies also demonstrated that the constitutive transcriptional activity of *Op ie*-2, used to drive expression of a reporter gene, was successfully suppressed in normal medium. However, in the presence of the inducer, doxycycline, transcriptional activity of *Op ie*-2 was not activated. Work to elucidate why *Op ie*-2 remain repressed in the presence of the inducer, indicated that other, uncharacterised vector sequences may have interfered with the activation process.

To determine whether stable insect cell lines could be used as an effective alternative to the BES for producing large quantities of intracellular foreign proteins, protein production from stable cell lines, using the *Bm A*3+E promoter, were compared to the BES using a range of reporter proteins. It was concluded that with vectors currently available, stable cell lines would not normally provide an effective alternative to the BES. However, a stable insect cell line expressing *Discosoma* red was used to develop methods for scaling-up continuous cultivation of cells in an open fermenter system. This stable cell line was successfully maintained without contamination or total loss of cell viability for 4 weeks. Improving intracellular expression of foreign genes using the BES, initially focused on investigating whether a baculovirus vector (BV) lacking chitinase could produce larger yields than normal BV. It was concluded that recombinant BV lacking chitinase could be used to improve intracellular levels of foreign protein, but demonstrated the importance of optimising production conditions for each recombinant protein (e.g., vector and cell line). Intracellular levels of foreign protein were further improved from the BES using different culturing methodologies (fermentation and shaker flask) and concluded that fermenters produced optimal conditions for intracellular protein production, probably due to maintaining a constant level of dissolved oxygen concentration during virus infection.

Presentations and Publications

Much of the work described in this thesis has been presented in the following:

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List of abbreviations

α	alpha
A_{600}	spectrophotometric absorbance at 600
ABP	auxin binding protein
Ac	*Autographa californica*
*Ac*5.1	actin 5.1
Amp^r	ampicillin resistance gene
APS	ammonium persulphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
AFU	arbitrary fluorescence units
APRT	adenyl phosphoribosyl transferase
β	beta
bA	basal actin promoter
BAC	bacterial artificial chromosome
BHK	Baby hamster kidney
BES	baculovirus expression system
Bm	*Bombyx mori*
*Bm*A3+E	*Bombyx mori* cytoplasmic actin 3 plus enhancers
bp	base pair
Bsd	blasticidin
BSA	bovine serum albumin
C	contaminant
CAP	catabolic activator protein
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
chiA	chitinase
CHO	Chinese hamster ovary
CIP	calf intestinal alkaline phosphatase
cm	centimetre
CMV	*Cytomegalovirus*
CMVie	*Cytomegalovirus* immediate early gene
CO_2	carbon dioxide
CsCl	Caesium chloride
$CuSO_4$	cupric sulphate
CV	column volumes
Δ	delta
DCIP	2,6 dichlorophenylindolphenol
DE	delayed early
DES	*Drosophila* expression system
dH_2O	distilled water
DHFR	dihydrofolate reductase
DHODH	dihydroorotate dehydrogenase
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
dO_2	dissolved oxygen concentration
Dox	Doxycycline
*Ds*Red	*Discosoma* red
DTT	dithiothreitol
Dm	*Drosophila melanogaster*

ϵ	millimolar extinction coefficient
EC_{50}	effective concentration
E.coli	*Escherichia coli*
Ed.	edited
EDTA	ethylene-diamine-tetra acetic acid
EGFP	enhanced green fluorescent protein
EF	elongation factor
ER	endoplasmic reticulum
ERAD	ER-associated degradation
ERE	ecdysone-responsive elements
et al.	*et alia* (Latin) – and others
EtBr	ethidium bromide
FA	formaldehyde-agarose
FBS	foetal bovine serum
FCS	foetal calf serum
g	gram
GA	Golgi apparatus
GFP	green fluorescent protein
gp	glycoprotein
GS	glutamine synthetase
Gus	β-glucuronidase
$G418^r$	geneticin resistance gene
h	hours
Hi5	high five
His	histidine
HIV	Human immunodeficiency virus
hpi	hours post infection
hpt	hours post transfection
hr	homologous region
hsp70	heat shock protein 70
HSV	Herpes simplex virus
HTS	high-throughput screening
HygB	hygromycin B
ie	immediate early
IPTG	isopropyl β-D-thiogalactopyranoside
Kan^r	kanamycin resistance gene
kb	kilobase pairs (1000bp)
kDa	kilo Dalton
KDEL	lysine, aspartic acid, glutamic acid, leucine
L	litres
Lac	lactose
lacZ	β-galactosidase
Ld	*Lymantria dispar*
LGT	low gelling temperature
LTR	long terminal repeat
luc	firefly luciferase
M	molar
Mb	*Mamestra brassicae*
µCi	micro Curie
MCS	multiple cloning site
MI	mock infected
µg	microgram
MgCl	magnesium chloride

µl	microlitre
µm	micrometer
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mmol	millimole
MNPV	multiple nucleopolyhedrovirus
moi	multiplicity of infection
MOPS	3-[N-morpholino]propanesulfonic acid
mRNA	messenger ribonucleic acid
MT	metallothionein
MT	mock transfected
MW	molecular weight
N	asparagine
NaCl	sodium chloride
NaOH	sodium hydroxide
NEB	New England Biolabs
Neo^r	neomycin resistance gene
Ng	nanogram
NP	nuclear protein
NPV	nucleopolyhedrovirus
nm	nanometer
nmol	nanomole
nt	nucleotide
OD	optical density
Op	*Orgyia preudotsugata*
ORF	open reading frame
p	probability
PA	plasminogen activator
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PBST	phosphate buffered saline Tween 20
PCR	polymerase chain reaction
pfu	plaque forming units
p.i	post-infection
polh	polyhedrin
poly-A	poly-adenylation
psi	pounds per square inch
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RT-PCR	reverse-transcription polymerase chain reaction
rTetR	tetracycline repressor protein
rtTA	reverse tetracycline-controlled transactivator
S	Schneider
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulphate
Sf	*Spodoptera frugiperda*
STR	stirred tank bioreactor
SV40	Simian virus 40
T-test	value for student's t test

T1 / 2	transcription termination sequences
T	tumour
TAE	Tris-acetate-EDTA buffer
TAF's	transcription activation factors
TBP	transcription binding protein
Tc	tetracycline
TE	Tis-HCl-EDTA
tet	tetracycline-resistance
Tet	Tetracycline inducible
tetO	tetracycline operator sequences
TetR	tetracycline repressor protein
TF	transcription factor
TEMED	N-N-N'-N'tetramethylethylenediamine
Tet-On	tetracycline On expression system
Tet-Off	tetracycline Off expression system
TLC	thin layer chromatography
T.ni	*Trichoplusia ni*
TOPO	topoisomerase
t-PA	tissue plasminogen activator
TRE	tetracycline response element
TRES	tetracycline-regulated gene expression system
Tris	Tris(hydroxymethy)-aminoethane
Tris-HCl	Tris(hydroxymethy)-aminoethane hydrogen chloride
tTA	tetracycline-controlled transactivator
U	units
UC	uncut
UK	United Kingdom
UPE	upstream promoter elemnets
USA	United States of America
UV	ultra violet
v-cath	viral cathepsin
VP16	viral protein 16
vs.	versus
v / v	volume per volume
wt	weight
w / v	weight per volume
XG-PRT	xanthine-guanine phosphoribosyl transferase
Zeo	Zeocin
°C	degree centigrade
%	per cent
<	less than
>	more than
=	equal to
-	negative
+	positive
£	pounds sterling
5'	5 prime
3'	3 prime
20E	20-hydroxy-ecdysone

CHAPTER 1: INTRODUCTION

1.0 Introduction

Protein production is a core practice for both academic and industrial-based research. Expression systems provide the basis for the development of therapeutics, vaccines, and antibodies, and for this they are studied in great detail. The production of heterologous proteins also provides material for studies in x-ray crystallography, functional genomics, verification of protein identity, structure, function, and localisation. However, this is just the tip of the iceberg concerning the importance of protein roles. Obtaining large quantities of endogenous proteins from their natural hosts (whole animals / organisms) falls short of today's requirements for research and commercialisation. This has prompted the development of alternative protein production systems, which mimic those of natural hosts and assimilate the complex post-translational modification processes of higher eukaryotic cells (Pfeifer, 1998).

Expression systems are based upon the insertion of a foreign gene, under the control of a strong trans-activated promoter (for example, the promoter may have originated from a virus or mammalian cell line), which is transcriptionally trans-activated in a heterologous host organism / cell line to allow over-expression of a recombinant gene. This in turn produces varying amounts of heterologous protein, which can be attributed to promoter activity (Pfeifer, 1998; Huynh and Zieler, 1999), choice of cell line used to host expression (Farrell *et al.*, 1998; Keith *et al.*; 1999) and a range of other factors.

In the early stages of heterologous protein production, the choice of expression system was limited. The production of recombinant proteins requiring authentic higher eukaryotic glycosylation, phosphorylation, methylation, and acetylation were manufactured using surface adherent or suspension rodent cell lines (Chinese Hamster Ovary cells [CHO]; Geisse *et al.*, 1996), whilst heterologous proteins that necessitate less complex glycosylation could be expressed in yeast (*Sacharomyces cerevisiae*). A third host utilised for the expression of recombinant genes includes *Escherichia coli*. This system has been well documented for producing high-levels of foreign protein (Nolan and Shatzman, 1998). However, problems associated with this system limits the types of heterologous protein expressed, due to the hosts inability to correctly fold and glycosylate proteins. The periplasm of *E.coli* allows for the formation of disulfide bonds, whereas the cytoplasm is a fully reductive environment preventing disulfide bond formation and hence proper protein folding to occur (Lorenzo and Fernandes, 2000). Therefore, only the production of small,

simple proteins, unmodified post-translationally, or at least active and non-toxic without post-translational modifications that were apparent in its native state, could be produced using this systems.

Extensive research and development to generate alternative expression systems has led to the choice becoming more diverse and complicated; heterologous expression systems currently encompass five kingdoms of organism: bacteria (Jacobs *et al.*, 1985; Dietrich *et al.*, 1992; French *et al.*, 1995), fungi (Beggs *et al.*, 1980; Cregg *et al.*, 1993), mammalian (Hentschel, 1991; Barr, 1995; Geisse and Kocher, 1999), insect and plant (Miller, 1996; Pfeifer, 1998; Geisse and Kocher, 1999; Huynh and Zieler, 1999), as well as viruses targeted to their host (Yates *et al.*, 1985; Blissard and Rohrmann, 1990; Adams and McClintock, 1991; King and Possee, 1992, Xiang *et al.*, 1994; Schlesinger and Dubensky, 1999).

From a commercial value, bacterial, mammalian and viral expression systems are most favoured for the production of recombinant therapeutic proteins. However, no one expression system is sufficient for the production of all recombinant proteins (Geisse *et al.*, 1996). Furthermore, the development of alternative protein production systems within insect cell lines has become extremely attractive to industries and researchers due to the number of advantages they hold over mammalian and bacterial producer organisms. Two approaches have been made with regards to recombinant protein production in insect cells, both of which are studied in this thesis. First is the reputable baculovirus expression system (BES) and second is the more recently developed stable insect cell expression system.

The choice of a suitable expression system depends largely on the biochemical and biological properties of the protein of interest, as well as on the nature of the experiment and the amount of recombinant protein required. Importantly, the choice of expression system now involves consideration of the problem not only from the point of view of the molecular biologist, but also from those of specialist niches, such as microbial and cell physiologists, fermentation specialist, downstream process engineer, and the clinician (Hodgson, 1993).

1.1 Mammalian versus insect: Hosts for the expression of heterologous proteins

Authentic processing of proteins from higher organisms often requires eukaryotic cell lines in order to retain full biological activity. Mammalian cell lines have in the past, been the main choice of heterologous gene expression systems. However, a rival competitor has emerged, which makes use of insect cell lines as an alternative for the production of recombinant proteins.

Mammalian expression systems use a plethora of different cell lines, the most common of which include CHO, established in 1957 by Puck and is by far the most popular (König *et al.*, 1989; Cockett *et al.*, 1990; Page and Sydenham, 1991), human embryonic kidney (HEK)-293 (Berg *et al.*, 1993; Dovey *et al.*, 1993), baby hamster kidney (BHK; Wirth *et al.*, 1988), mouse L-cells, and myeloma cell lines like J558L (Traunecker *et al.*, 1991; Lane *et al.*, 1993) and *Sp2/0* (Gillies *et al.*, 1989; Lo *et al.*, 1992). However, the type of expression and protein required will affect the choice of cell line use. For instance, for transient expression, the most common cells used are the HEK-293, COS (Gluzman, 1981), and BHK; whereas stable gene expression utilises, CHO, myeloma cell lines and Sp2/0 (Gillies *et al.*, 1989; Lo *et al.*, 1992).

The advantages of using mammalian cell lines and expression vectors for the production of recombinant proteins include:

- Constitutive and inducible recombinant gene expression;
- The capacity to produce post-translationally modified and appropriately folded heterologous proteins;
- Long-term continuous culture;
- The ability to characterise the impact of specific mutations on cell metabolism;
- The capability to alter cellular phenotype as a function of stable transgene expression.

The key disadvantages of mammalian cells as hosts for recombinant protein production include; slow growth, relatively poor yields, and expensive to maintain. Areas of expense include:

- Stacked multilayer systems utilized for growth of cells for large-scale recombinant protein production (Wurm and Bernard, 1999);
- Cells are cultivated at 37°C / 5% (v/v) CO_2 in a humidified atmosphere and are sub-cultured twice a week (Geisse and Kocher, 1999);
- Complex medias and fetal bovine serum (FBS) requirements that may have to be met (i.e. tetracycline free);
- Regulatory issues concerning the used of FBS;
- Screening cell lines for adventitious agents / latent viruses.

The considerations of all these factors mean researchers working within academia and industry welcome alternate expression systems. Studies into the development of alternative expression systems, which will compete with the mammalian expression systems, include the insect cell expression systems.

Insect cell lines have provided researches with an alternative tool for the production of recombinant proteins. The insect cell lines most commonly used are summarised in **Table 1.1.** The advantages of recombinant protein production in insect cells include those described for mammalian cells, in addition to those described below:

- Lower growth temperature 28°C;
- Does not require CO_2 for growth;
- Fast growth;
- Adaptability to large-scale fermentation;
- The capability to produce high-levels of recombinant protein;
- Less complex growth conditions;
- Often null expression background.

The main limitations of insect cell lines as hosts for heterologous gene expression include a number of those outlined for mammalian cells, plus those listed below:

- Possess limited *N*-glycosylation capability for the production of complex-proteins;
- Poor methods of detection for adventitious agents (mycoplasma);
- Regulatory issues concerning the used of FBS;
- Screening cell lines for adventitious agents / latent viruses.

Table 1.1: Summary of eight insect cell lines used as hosts to produce recombinant proteins[1].

Cell line	Species
*Bm*5	*Bombyx mori*
*Bm*N4	*Bombyx mori*
High five (*T.ni* Hi5)	*Trichoplusia ni*
IPLB-*Ld*FB	*Lymantria dispar*
IZD-*Mb*-0503	*Mamestra brassicae*
Schneider's line 2 (*Dm* S2)	*Drosophila melanogaster*
Sf-21	*Spodoptera frugiperda*
Sf-9	*Spodoptera frugiperda*

[1]Keith *et al* (1999)

Eukaryotic protein processing capabilities in insect cell lines is one of the most appealing features of recombinant protein production using insect expression systems as an alternative to the mammalian systems. A plethora of eukaryotic gene products have been successfully expressed with authentic functional activity, using both the baculovirus and stable insect expression systems (Guarino and Summers, 1987; O'Reilly and Miller, 1989; Jarvis *et al.*, 1990; King and Possee, 1992; Lu *et al.*, 1997; Ikonomou *et al.*, 2003). Accordingly, insect expression systems are widely considered to be excellent tools for recombinant glycoproteins. However, this perception is inconsistent with the fact that there are fundamental differences in the glycoprotein processing pathway in insects and higher eukaryotes (Stollar *et al.*, 1976; Hsieh and Robbins, 1984; Marz *et al.*, 1995; Jarvis and Finn, 1996; Hollister and Jarvis, 2001; Hollister *et al.*, 2002), in particular the *N*-glycosylation pathway.

The *N*-glycosylation pathway in higher eukaryotes has been well characterised (Hunt and Dayhoff, 1970; Kornfield and Kornfield, 1985; Dorner *et al.*, 1987). This pathway starts with the translocation of a nascent polypeptide across the membrane of the rough endoplasmic reticulum (RER) and transfer of a pre-assembled oligosaccharide from a lipid carrier to an asparagine residue within a specific recognition sequence (Hunt and Dayhoff, 1970). Glucosidases within the RER remove terminal glucose residues to create a side chain with a distinguishing structure known as high mannose, in which all of the outer-chain sugars are mannose residues. A quantity of *N*-linked oligosaccharides remain in this relatively unprocessed form, while others are further processed by other glycosidases and glycosyltransferases in the RER and Golgi apparatus (GA), which can remove specific mannose residues and append *N*-acetylglucosamine, galactose, and sialic acid residues (Kornfield and Kornfield, 1985; Paulson and Colley, 1989; Moremen *et al.*, 1994).

Studies on *N*-glycan structures produced by mosquito cells provided the earliest view of the insect *N*-glycosylation pathway (Stollar *et al.*, 1976; Hsieh and Robbins, 1984). These studies investigated potential differences in the structure and function of Sindbis viris glycoproteins produced during its replication in mammalian cells, compared to insect cells. The result indicated that there were striking differences, as the structures of the *N*-glycans on the viral glycoproteins produced in insect cells were less complex than those produced in mammalian cells.

These observations suggested that the insect cell *N*-glycosylation processing pathway is truncated relative to the mammalian pathway. The insect *N*-glycan processing pathway appeared to include all the enzymes involved in *N*-glycan trimming (e.g. α1,2-glucosidase I, α1,3-glucosidase II, α-mannosidase I [RER / GA], α-mannosidase II), but few of the enzymes involved in *N*-glycan elongation in mammalian cells (e.g. *N*-acetylglucosaminyltransferase I and II, *N*-acetylglucosaminidase and fucosyltransferases).

Contrary to the evidence described above, structural analysis of the recombinant human plasminogen provided the first direct evidence that the baculovirus-insect cell system might be able to produce complex, terminally sialylated *N*-glycans (Davidson *et al.*, 1990, Davidson and Castellino, 1991). However, studies by Jarvis and Finn. (1995) confirmed significant *N*-glycan structural differences between *Autographa californica* multicapsid nuclear polyhedrosis virus (*Ac*MNPV) major structural glycoprotein ~ gp64, produced in insect cells (*Sf*9, *T.ni* Hi5, and *Estigmene acrea*) and mammalian cells (COS-1). gp64 produced in insect cells lacked detectable galactose and sialic acid; whereas, at least one *N*-linked oligosaccharide side chain on gp64 was converted to a complex form containing galactose and sialic acid in mammalian cells. Therefore, indicating that gp64 produced in insect cells lack complex side chains like those produced by mammalian cells. It was concluded from these data that *Ac*MNPV-infected insect cells cannot process *N*-linked carbohydrates on most glycoproteins to complex forms, unless the glycoprotein is an unusually good substrate for the late processing reactions.

Although post-translational processes in insect cells are more similar to mammalian cells than to bacterial and yeast, the limited *N*-linked carbohydrate processing abilities of insect cells (Jarvis and Finn, 1996) may affect protein function. Such as protein half-life within blood, and uptake / clearance through the liver. Therefore, correct post-translational

modifications of proteins produced for the application of therapeutics are very critical (Jarvis and Finn, 1996).

The problem of foreign protein post-translational processing may also be influences by viral and plasmid based vectors. However, improper secretory processing can be especially problematic within viral-based expression systems such as the baculovirus-insect expression system. At several days post-infection with recombinant baculovirus, the host cell's post-translational processing machinery becomes dramatically compromised, producing some protein that may be incorrectly modified and not representing native structure or function (Ailor and Betenbaugh, 1999). An innovative approach to overcome these limitations in the baculovirus-insect cell system was to engineer the secretory pathway of the insect host cells by supplementing secretory processing proteins absent or limited in supply in the host insect cell intracellular environment. A number of studies were carried out, by which co-expression of chaperones (Hsu *et al.*, 1994), peptidases (Ailor *et al.*, 1999), foldases (Lenhard and Reilander, 1997), and glycosylating enzymes (Wagner, *et al.*, 1996; Hollister *et al.*, 1998) have proven effective in enhancing secretion, processing, and glycosylation of several heterologous proteins expressed in insect cells.

However, more recently the establishment of genetically transformed lepidopteran insect cell lines with constitutively expressible mammalian genes, has yielded transgenic insect cell lines with normal growth properties that can support baculovirus infection, have new *N*-glycan processing enzyme activities, and produce humanised heterologous glycoproteins (Hollister *et al.*, 1998; Breitbach and Jarvis, 2001; Hollister and Jarvis, 2001; Hollister *et al.*, 2002). These cells require an extracellular sialoglycoprotein for *de novo* glycoprotein sialylation, which provides the first evidence that these cells have an interesting sialic acid salvaging pathway (Nakata *et al.*, 2000; Lawrence *et al.*, 2001).

The advantages of insect cells over mammalian cells as hosts for expressing properly processed eukaryotic proteins that do not require complex *N*-glycosylation, has already received much attention by industry and academia. Subsequently, new developments in insect cell technology described in this Section further strengthen the establishment of insect cells as alternate hosts for heterologous protein production.

1.2 Classification of gene expression and expression systems

Proteins are produced in a number of different expression systems; however, these systems are classified into three main classes of gene expression, which include transient, inducible, and stable. It is extremely important that novice researchers understand the main differences between the types of expression available, consequently they must choose the protocol that is most suitable for their needs, as this could effect recombinant protein quantities, and authenticity (Hodgson, 1993).

1.2.1 Classes of gene expression

1.2.1.1 Transient constitutive gene expression

The first category of gene expression is referred to as "transient" expression. Transient expression can be subdivided into two forms of expression, constitutive and inducible. Inducible expression will be discussed in the following Section 1.2.1.2. Constitutive expression refers to the continuous transcriptional activity of a promoter constantly transcribing a gene. Transient expression of a heterologous gene occurs when the production of a foreign protein is temporary. Two types of expression vector operate using transient expression; plasmid-based / viral-based vectors (Sections 1.3-1.3.3). The temporary nature of this process occurs when a foreign gene is present within the nucleus of the host, utilising host cell's DNA replicative enzymes in order to make copies of itself, which in turn are transcribed to produce protein. The gene is not integrated into the host genome, but remains extra-chromosomal element (episomal) so will be lost / degraded within a short time (plasmid-based vectors; 48-72 hours [h]). Viral expression system are categorised as transient, due to the completion of viral replication causing death to the host, hence the termination of protein production (72-96 h).

1.2.1.2 Transient inducible gene expression

The second form of gene expression, "inducible" expression, involves the manipulation of inducible elements from prokaryotic or eukaryotic cells into expression vectors containing foreign genes. These vectors now produce conditional expression, which is extremely advantageous to overcoming the problem of expressing cytotoxic gene products. One example of an inducible expression system is the tetracycline-On / tetracycline-Off

expression system (Tet-On / Tet-Off, as described in Section 4.1; Gossen and Bujard, 1992; Saez *et al.*, 1997).

1.2.1.3 Stable gene expression

The third type of gene expression is, "stable" expression. Stable expression can also be constitutive or inducible (plasmid-based vectors). The term stable expression comes from the stable integration of a heterologous gene into the host's genome, under chronic selection (plasmid contains a selectable marker). This is an added advantage because the foreign gene is mistaken as part of the host cells make-up and escapes degradation. Therefore, the foreign gene is continuously translated to produce heterologous protein (McCarroll and King, 1997). When cells undergoing mitosis contain stably integrated foreign genes, the genetic information is passed to the daughter cells, therefore, producing a continuous cell line that produces exogenous protein.

If the promoter / enhancer complex can modulate transcription of the recombinant gene, the expression can be modified, such that it can be enhanced or decreased by environmental factors and components that are added to the growth medium. Components that can modulate transcription of the foreign gene include; hormones (Ponta *et al.*, 1985; Israel and Kaufman, 1989; Ko *et al.*, 1989), metal ions (Hu and Davidson, 1990), antibiotics and environmental factors including heat shock (Schweinfest *et al.*, 1988).

A selectable marker employed within the expression vector facilitates enrichment of cells that contain the heterologous gene of interest. A typical plasmid vector used to enrich successfully transfected cells will carry a gene essential for the survival of a given cell line that is either defective in the gene or void of the gene altogether. Classic selectable markers such as the dihydrofolate reductase (DHFR), adenyl phosphoribosyl transferase (APRT), and glutamine synthetase (GS) genes can only be used in cell deficient in DHFR, APRT or GS, respectively (Ausubel, et al., 1997). Alternatively, genes that confer resistance to cytotoxic drugs are quite effective in situations when the cell line is not defective in one of the endogenous genes mentioned earlier. The most common selection marker is the aminoglycoside phosphotransferase gene that confers resistance to antibiotics such as kanamycin (Kan^r), neomycin (Neo^r) and geneticin ($G418^r$). Genes encoding either for hygromycin B phosphotransferase (HygB), xanthine-guanine

phosphoribosyl transferase (XG-PRT), Zeocin™ (Zeo), or blasticidin (Bsd) have also been effectively used, and to date this is the most commonly used selection method (Fehse *et al.*, 1998). Because each cell line is different in its sensitivity to these compounds, the optimal drug concentration must be empirically determined for each cell type. The selection typically requires long-term (1 to 5 weeks) *in vitro* cultivation in the presence of a drug (Colosimo *et al.*, 2000).

1.2.2 Transient versus stable gene expression

Both transient and stable expression of heterologous genes have merits and must be carefully considered before expressing the gene of choice. The key features of most transient expression systems are:

- Simplicity, in particular in the construction of expression vectors;
- Extremely short time-frame for the generation of product or if it is important to have experimental results within a short time-frame (days);
- Antibiotic selective pressure not required;
- Intrinsic genetic stability and consistency due to extremely short time-frame between generation of vector and product recovered;
- Applicability to a wide range of host cell lines;
- Suitability to multiple processing, allowing study of many genes or mutants at the same time.

However, disadvantages related to this type of expression include:

- Narrow time-frame for producing recombinant protein;
- Difficult to scale-up transient plasmid-based expression;
- Protein production is generated as a single batch;
- Labour-intensive and expensive process (producing large volumes of high-titre virus inoculum, and producing multiple batches of recombinant protein).

Transient expression studies are predicated on a burst of gene expression between 12 and 72 h after transfection. This burst of gene expression is followed by a rapid deterioration in expression of the transgene because of cell death or loss of the expression plasmid (Section 1.2.1.1; Colosimo *et al.*, 2000). The optimal time to assay expression

needs to be determined empirically and will depend on the cell type, the cell doubling time and the characteristics of the vector regulatory elements. Transient expression systems can be evaluated in terms of the protein products synthesised in the transfected cells, such as the activity of a reporter gene that is not endogenously expressed in the cell type utilised. The most common reporter gene systems include bacterial chloramphenicol acetyltransferase (*cat*), β-galactosidase (*lac*Z), firefly luciferase (*luc*), β-glucuronidase (*gus*) and more recently green fluorescent protein (*gfp*; Alam and Cook, 1990), and *Discosoma* red (*Ds*Red; Brooke and Glick, 2002).

Transient expression is a particularly valuable method for studying initial gene products, gene function / characterisation via mutational analyses, and expression regulatory mechanisms (Carson *et al.*, 1991). However, transient expression from viral-based expression systems, which utilise promoters with high rates of transcriptional activity (as described in Section 1.3.2) tend to generate significantly larger quantities of recombinant protein than compared to plasmid-based vectors. For this reason transient expression is effectively used for producing large quantities of foreign protein (King and Possee, 1992; Blasey *et al.*, 1997; Lundstrom, 1997; Possee, 1997; Vlak *et al.*, 1998; Wurn and Bernard, 1999; Jayakumar *et al.*, 2004). The key disadvantage of transient expression for this procedure is that recombinant protein is produced as a single batch, therefore, to generate additional protein the experimental procedure must be repeated, which is costly and labour-intensive.

Stable expression prevails this problem, as plasmid-based vectors integrate into the host cell genome, therefore, avoiding degradation by host cell mechanisms. Subsequently, transformed cell lines are established with either constitutive or inducible transgene expression. A number of advantages acknowledged for transient expression also apply to stable expression, in addition to those listed below:

- Development of transformed cell lines producing continuous foreign protein (inducibly or constitutively);
- Simplicity, in particular in the construction of expression vectors;
- Applicability to a wide range of host cell lines;
- Transformed cell line is immortal;
- Established cell lines require low maintenance;
- Adaptability to large-scale fermentation;

- Flexibility to vary the quantities of recombinant protein required.

In contrast, the disadvantages of stable expression include:

- Selective pressure required;
- Initial development of stable cell lines is labour-intensive;
- Expression of intracellular recombinant proteins is relatively low, due to transcriptionally weak promoters (plasmid-based insect expression systems);
- Transformed cell lines must be routinely screened for adventitious agents.

Generating stable expression is a time-consuming process, in particular establishing clonal cell lines and identifying the highest protein producers. However, this method is widely used for producing large quantities of recombinant protein and is perceived as more desirable than the labour-intensive transient vial expression system (Fussenegger *et al.*, 1999; Colosimo *et al.*, 2000; Zahn-Zabal *et al.*, 2001).

Transient and stable expression are both important. However, transient expression will continue to dominate studies on elements that regulate gene expression or when it is important to have experimental results within a short time frame. Stable expression is not effective for these preliminary studies, as production of a stable cell line is costly and time consuming.

1.3 Eukaryotic vector systems

Mammalian and insect cell expression systems can be divided into two broad categories: viral vectors and plasmid vectors. Viral vectors are essentially modified viruses into which heterologous genes are cloned. Viral expression systems delivered to mammalian and insect cell lines for production of recombinant proteins are summarised in **Table 1.2.**

Table 1.2: Summary of mammalian and insect viral based expression systems.

Mammalian viral based systems	Insect viral based systems
Adeno-X expression system Adeno-X Tet-off expression system MSCV Retroviral expression system Pantropic Retroviral expression system Sindbis expression system	Baculovirus expression system

Plasmid vectors are more complex than viral vectors as their functional components may include prokaryotic, eukaryotic and viral sequences. The prokaryotic elements facilitate bacterial propagation and maintenance of the vector, while eukaryotic and viral elements comprise transcriptional elements and sequences coding for selectable markers. Both types of vectors are generally classified with respect to their viral backbone or the sequence elements that regulate the expression of the gene to be studied or utilised.

Promoters and enhancer sequences, whether viral or plasmid based, control the regulation of gene expression. The most common promoters / enhancers are of viral origin. However, bacteriophage, nuclear, cytoplasmic, cellular-tissue type-specific and inducible promoters can also be used (Gossen and Bujard, 1992; Lu *et al.*, 1997; Colosimo *et al.*, 2000).

1.3.1 Viral-based vectors

Insect cell lines as alternatives to mammalian cell lines have stimulated extensive gene expression research and (Friesen and Miller, 1986; Weyer *et al.*, 1990; Weyer and Possee, 1991; Bishop and Possee, 1990; King and Possee, 1992; Possee, 1997; Grabherr *et al.*, 2001) have produced a vial-based delivery and expression system, which is known as the BES. A greater number of mammalian viral-based expression systems exist than insect ones, as indicated in **Table 1.2.** However, my emphasis in this thesis will focus on the BES.

1.3.2 The baculovirus expression system

Baculovirus expression vectors provide a versatile and dependable expression system. Baculoviruses comprise many isolates; however, *Ac*MNPV is most widely used within academia and industry (Possee, 1997; Schwartz *et al.*, 1997; Stewart and Champoux, 1999; Jayakumar *et al.*, 2004). Frequent use of the baculovirus expression vectors arises from the relative ease and speed with which a heterologous protein can be expressed on a laboratory scale or industrially scaled-up in bioreactors with a high chance of obtaining a biologically active protein.

Baculoviruses are large double-stranded DNA viruses, comprising covalently closed genomes of 80-133-kb, which are pathogenic to invertebrates. Baculovirus are species

specific; however, studies have reported that replication in non-targeted organisms (i.e. mammalian cell lines) has been revealed (Miltenburger, 1979).

Within baculovirus-infected cells, viral proteins are expressed in a cascade fashion; three main transcriptional phases are recognised, early, late, and very late (Guarino and Summer, 1986; Carson *et al.*, 1988; Kovacs *et al.*, 1991; Mikhailov, 2003). Upon viral infection host RNA polymerase II transcribes the viral early genes (Blissard and Rohrmann, 1989; Blissard *et al.*, 1992; Lu and Carstens, 1992; Dai *et al.*, 2004). Early gene expression may be subdivided into immediate early and delayed early (Guarino and Summer, 1986; Carson *et al.*, 1988). The expression levels obtained in each successive phase are higher than that of the preceding phase **Figure1.1**. Viral replication is temporally regulated to produce two different types of infectious virion; polyhedra-derived virions, which persist outside the insect host and initiate host viral infection, and extracellular virus / budded virus, which spreads from cell to cell. The latter of these infectious virion forms the basic of the BES, establishing the mean by which recombinant viruses are propagated (**Figure 1.2**).

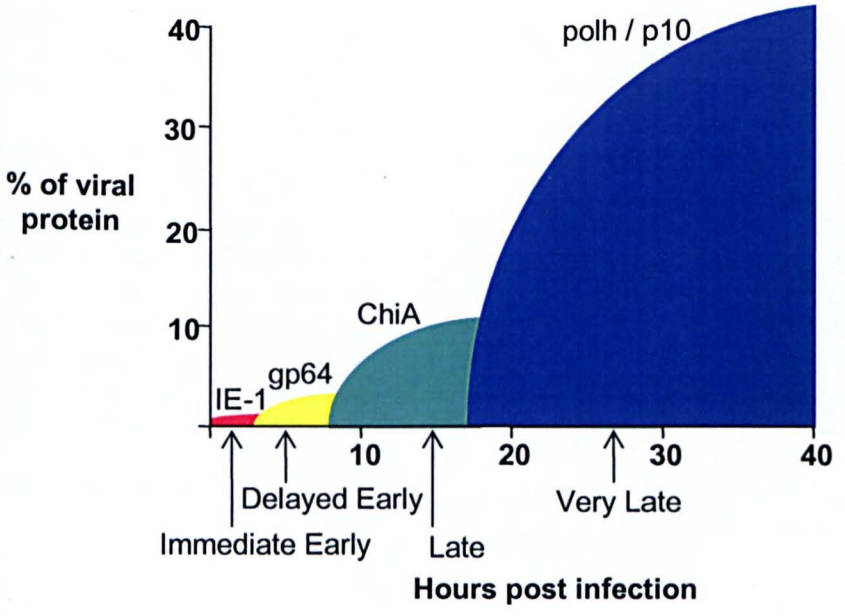
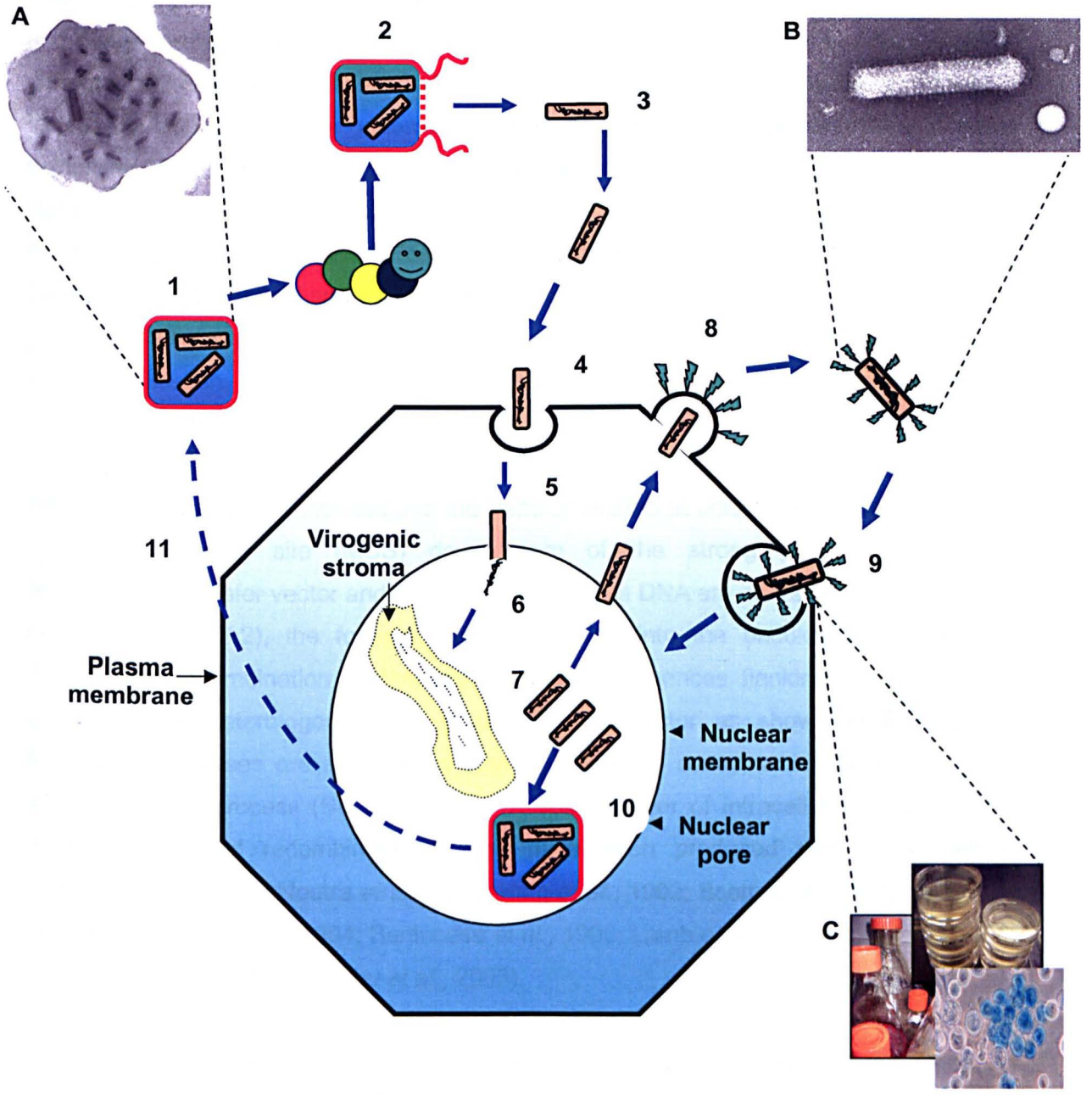


Figure 1.1 Temporal gene expression during baculovirus infection. Baculovirus genes are divided into three classes based on their temporal expression, consisting of early, late and very late genes. The early gene class may be further subdivided into immediate early and delayed early genes. Examples of genes identified in these phases are illustrated (immediate early 1 [IE-1]; glycoprotein 64 [gp64]; chitinase [ChiA]; polyhedrin [polh] and p10).

Figure 1.2: The replication cycle of *Ac*MNPV.

1. Virus occlusion bodies (polyhedra; **A**) are ingested by a non-infected susceptible larvae.
2. The polyhedrin matrix of the ingested occluded virus is dissolved in the alkaline conditions of the midgut lumen.
3. Infectious virus particles are released (polyhedra-derived virions).
4. Midgut cells are infected with polyhedra-derived virions by crossing the peritrophic membrane via fusing with membranes of the microvilli of the cell.
5. Nucleocapsids move to the nucleus where they interact end-on with the nuclear pore.
6. Upon entering the nucleus, viral DNA is released from the nucleocapsid.
7. Viral DNA is transcribed, replicated, and packaged into nucleocapsids in association with the virogenic stroma.
8. During the late stage of infection, nucleocapsids leave the nucleus and migrate to the plasma membrane where they bud from the membrane to produce budded virus (**B**). This form of virus has been effectively exploited to form the basis of the baculovirus expression system (BES). Budded virus is used to amplify stocks of recombinant baculovirus in insect cell culture (**C**), while also acting as a efficient transfer method of recombinant viral DNA into insect cells for the production of recombinant protein.
9. The budded virus is then able to cause secondary infection of neighbouring cells.
10. During the very late phase of infection, nucleocapsids are enveloped within the nucleus. Intra-nuclear virions are then embedded in a polyhedrin (polh) matrix to form occlusion bodies (**A**). The *polh* gene is non-essential for virus replication in insect cell culture, and the *polh* gene promoter has been characterised with extremely high transcriptional activity. For these reasons, the *polh* gene coding-region can be substituted for a foreign gene inserted, which is subsequently under the control of the polh promoter, therefore produce large quantities of recombinant protein.
11. Polyhedra (**A**) are released in to the environment (or cell culture) upon cell lysis and host cell death.


A
B
C
1
2
3
4
5
6
7
8
9
10
11
Virogenic stroma
Plasma membrane
Nuclear membrane
Nuclear pore

The BES is a highly efficient method for producing recombinant proteins. The success of this system has promoted its extensive use by biotechnology investigators (King *et al.*, 1994; Schwartz *et al.*, 1997; Stewart and Champoux, 1999; Jayakumar *et al.*, 2004). The very late baculovirus genes encode structural proteins under the control of promoters with very high transcriptional activity, and have been determined non-essential for *in vitro* replication. Subsequent removal of these gene-coding regions enables the replacement and over-expression of a heterologous gene of choice. Due to the size of the *Ac*MNPV genome (133-kb) and the absence of unique restriction sites, it is impossible to introduce foreign DNA directly into the genome. However, the development of baculovirus transfer vectors surmount this problem. The transfer vectors consist of a prokaryotic origin of replication for high plasmid copy number, and the promoter / flanking sequence of the polyhedrin (*polh*), or *p*10 gene (Possee, 1986; Possee and Howard, 1987; Luckow and Summers, 1988; Bishop and Possee, 1990; Weyer and Possee, 1991). However, it is possible to use any vial promoter / flanking gene sequence determined non-essential for *in vitro* replication, although the rate of promoter transcriptional activity may be less than those characterised for *polh* and *p*10.

Heterologous genes are inserted into the transfer vectors at unique restriction sites within a multiple cloning site (MCS) downstream of the strong promoter sequence. Recombinant transfer vector and infectious baculovirus DNA are co-transfected into insect cells (Section 2.3.2), the foreign gene is inserted into the baculovirus genome by homologous recombination involving the identical sequences flanking the native *polh* gene and the heterologous gene in the transfer vector as shown in **Figure 1.3.** Recombinant viruses are selected by rounds of plaque assay, which is a skilled and labour-intensive process (Section 2.3.3). A vast number of intracellular, secreted and membrane-targeted recombinant proteins have been produced using this method (Atkinson *et al.*, 1992; Neutra *et al.*, 1992; King *et al.*, 1992; Scott *et al.*, 1992; Nguyen *et al.*, 1993; van Lier *et al.*, 1994; Benincasa *et al.*, 1996; Lamb *et al.*, Wu and Chiang, 1996; Schwartz *et al.*, 1997; Ikonomou *et al.*, 2003).

The identification of recombinant baculoviruses previously was determined using an inverted light microscope to identify polyhedrin negative viruses. This was both a time consuming and very difficult technique to carry out. Therefore, to simplify the identification of recombinant baculovirus, the *polh* gene of *Ac*MNPV was replaced with a *lac*Z gene, to derive *Ac*MNPV.*lac*Z (Possee and Howard, 1987). Insect cells were co-transfected with a recombinant transfer vector and *Ac*MNPV.*lac*Z DNA and recombinant

baculoviruses were selected against parental virus (present as blue plaques) as a colourless plaque following application of 2% X-gal, a substrate for *lac*Z, **Figure 1.3.**

An improvement of the BES was developed in which non-infectious, linearised *Ac*MNPV.*lac*Z (*Ac*BacPAK6) was co-transfected with a recombinant transfer vector (Kitts *et al.*, 1990; Kitts and Possee, 1993). Homologous recombination must occur between the *Ac*MNPV and transfer vectors DNA, in order to produce a viable virus. Following the introduction of three *Bsu*361 sites within the sequence flanking the *polh* expression locus, a recombination frequency approaching 100% may be attained. This is because restriction of the viral DNA using *Bsu*361 removes an essential fragment of the virus genome (open reading frame *1629*; ORF*1629*), rendering it non-viable. Rescue with a recombinant transfer vector, restores the integrity of the essential gene enabling the production of a recombinant viable virus, **Figure 1.3.**

The BES has been well documented for producing large quantities of intracellular recombinant protein, and is usually favoured over insect plasmid-based expression. However, yields of secreted foreign proteins produced by the BES are usually low (Jarvis *et al.*, 1990; Thomas, 1997; Farrell *et al.*, 1998; Thomas *et al.*, 1998; Saville *et al.*, 2002).

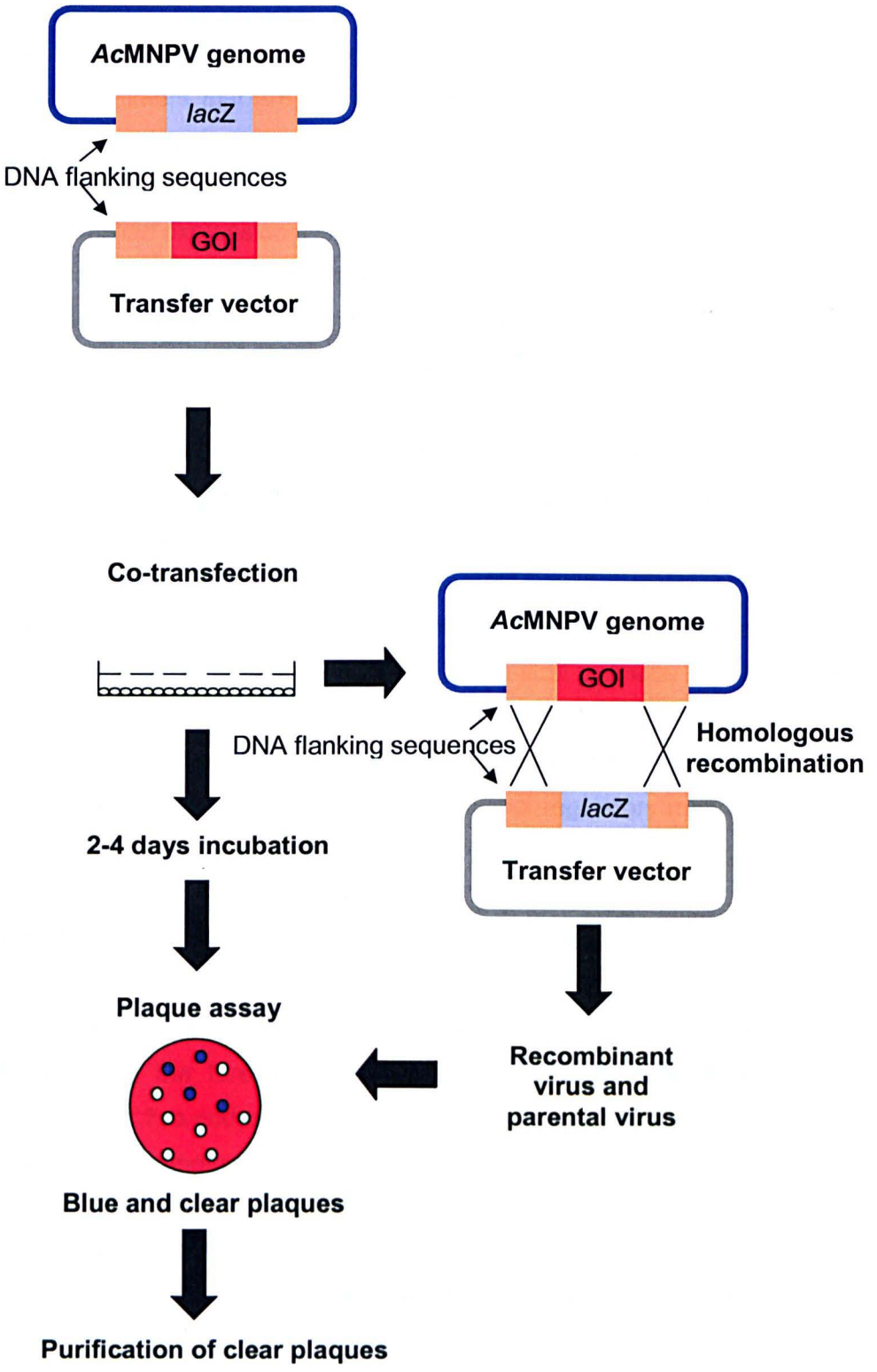
Further developments to the *Ac*BacPAK6 virus, was the removal of the chitinase (*chi*A) gene from the genome (Hawtin *et al.*, 1997), which subsequently increased the yields of secretory proteins (McCarroll, 1997; Possee *et al.*, 1999). Studies revealed that the *chi*A gene encoded an endoplasmic recticulum (ER) retrieval sequence (KDEL; Thomas *et al.*, 1998), which retained chitinase within the ER throughout virus replication (Thomas *et al.*, 1998). It was hypothesised that chitinase was blocking further processing of proteins in the secretory pathway, therefore, reducing the yield of secreted recombinant proteins and localised plasma-membrane proteins. The removal of the *chi*A gene from the *Ac*MNPV genome produced the *Ac*BacPAK6.Δ*chi*A virus (Hawtin *et al.*, 1997). *Ac*BacPAK6.Δ*chi*A was used to construct recombinant viruses, which lead to the production of membrane secreted proteins. The results indicated that the ER was no longer blocked by chitinase and levels of recombinant membrane secreted proteins were increased (McCarroll, 1997; Possee *et al.*, 1999).

The BES has also been used to develop multiple expression vectors for simultaneous expression of two or more heterologous genes, by the co-infection of two or more recombinant viruses (St Angelo *et al.*, 1987; Belyaev and Roy, 1993). A number of

proteins have been co-expressed using this method, including: Cowpea mosaic virus capsid proteins (Shanks and Lomonossoff, 2000), human cytochrome P4501A1 and human NADPH-cytochrome P450 reductase (Schwarz *et al.*, 2001), $GABA_A$ subunits and G-protein coupled 5-HT receptors (Obosi *et al.*, 1996).

Use of the transient BES does, however, gives rise to several significant problems. These include termination of recombinant protein expression with the end of virus infection cycle, complex downstream purification due to the release of intracellular proteins by the virus-lysed host cells, and low secreted protein yield, suggesting the host cell secretory pathway is compromised towards the end of infection (Jarvis and Summers, 1989; McCarroll and King, 1997; Thomas *et al.*, 1998; Ailor and Betenbaugh, 1999; Keith et al., 1999). However, as mentioned above an innovative approach to overcome the latter problem of the BES was to engineer *Ac*MNPV genome.

Figure 1.3: Schematic representation of the production of recombinant baculovirus. The transfer vector contains a polyhedrin promoter and transcription termination sequence flanked by *Ac*MNPV sequence (orange). The coding sequence for the gene of interest (GOI) within the transfer vector (red) is inserted in place of the deleted polyhedrin sequence under control of the polyhedrin promoter. Insect cells are co-transfected with the recombinant transfer vector and non-infectious, linearised *Ac*MNPV.*lacZ*. Homologous recombination replaces the *lacZ* gene (light blue) with the GOI-coding region and progeny viruses are screened by plaque assay. Recombinant viruses are identified by the production of colourless, *lacZ* negative plaques (modified from King and Possee, 1992).


AcMNPV genome
lacZ
DNA flanking sequences
GOI
Transfer vector
Co-transfection
AcMNPV genome
GOI
DNA flanking sequences
Homologous recombination
lacZ
Transfer vector
2-4 days incubation
Plaque assay
Recombinant virus and parental virus
Blue and clear plaques
Purification of clear plaques

1.3.3 Plasmid-based vectors

Plasmid-based expression vectors for mammalian and insect cell lines often utilise immediate early viral promoters that are transcriptionally active in the absence of other viral promoters (Section 1.5-1.5.4).

A vast number of mammalian expression systems have been developed to date; however, one viral promoter dominates the mammalian expression vectors, due to its strong transcriptional properties within a range of mammalian cell lines (Colosimo *et al.*, 2000). The immediate early promoter from the cytomegalovirus (CMV) has been used in over 40 mammalian expression vectors. Expression systems include the Tet-On / Tet-Off (described in Chapter 4, Section 4.1), retroviral, bicistronic, and epitope tagged expression systems. Additional promoters include the *Drosophila hsp*70 and elongation factor (EF)-1 (non-viral), retroviral (5'LTR), and a simian virus 40 promoter (SV40).

More recently non-viral promoters have received strong endorsement, as they appear to be as strong or stronger than some viral promoters. A non-viral promoter that has been exploited commercially is the *Drosophila hsp*70 promoter. The *hsp*70 promoter is used within a number of mammalian expression systems, one of which being the ecdysone-inducible expression system that is based on a unique insect regulatory mechanism (No *et al.*, 1996).

The ecdysone-inducible mammalian expression system, is based on the constitutive expression of a human retenoid X receptor and a modified ecdysone receptor from *Drosophila*. These receptor subunits interact to form a functional ecdysone receptor. A second plasmid containing the foreign gene downstream of the ecdysone-response promoter, which consists of five ecdysone-response element juxtaposed upstream of the modified *Drosophila hsp*70 promoter. When both vectors are present in mammalian cells, the expressed functional ecdysone receptor bind the ecdysone-response promoter in the presence of the induce (ponasterone A or muristerone A) and activates promoter transcriptional activity, thus transcribing the heterologous gene (No *et al.*, 1996; Saez *et al.*, 1997).

Even though the promoters used to transcribe heterologous genes in mammalian plasmid-based expression vectors are characterised with strong transcriptional activity.

The disadvantages of using these vectors for foreign protein production, relates to the drawbacks associated with mammalian cells as hosts for expression (Section 1.1).

The first insect plasmid-based expression vectors comprised early baculovirus promoters (Jarvis and Summers, 1989; Jarvis *et al.*, 1990). The main focal point of researchers, for developing insect plasmid-based expression vectors, was to use the immediate early promoter of the *ie*-1 gene. Those used at the time were *ie*-1 genes identified and characterised in *Ac*MNPV and *Orgyia preudotsugata* multicapsid nucleopolyhedrovirus (*Op*MNPV; Guarino Summers, 1986a; Carson et al., 1988; Nissen and Friesen, 1989; Theilmann and Stewart, 1991).

Immediate early genes are trans-activated under the control of host cell's RNA polymerase II soon after being transfected, and therefore make good candidates for plasmid-based expression. The most common *ie*-1 promoters used within plasmid vectors to drive heterologous gene expression are taken from, *Ac*MNPV (Guarino Summers, 1986a; Jarvis and Summers, 1989; Jarvis *et al.*, 1990), *Bombyx mori* (*Bm*) multicapsid nucleopolyhedrovirus (Lu *et al.*, 1997; Farrell et al., 1997), and *Op*MNPV (Theilmann and Stewart, 1991; Slack and Blissard, 1997). These vectors provide moderate transcriptional rates in most lepidopteran and dipteran cell lines (Pfeifer *et al.*, 1997). A number of other insect promoters used include the actin 3, *Drosophila* heat-shock protein 70 (*hsp*70), metallothionein (*MT*), and copia, which function in lepidopteran cell lines albeit at much lower levels (Pfeifer *et al.*, 1997; Hegedus *et al.*, 1998). In *Spodoptera* cells, it has been shown that the *Ac*MNPV *ie*-1 promoter produces 10-fold higher levels of gene activity than the *hsp*70 promoter (McLachlin and Miller, 1997).

Plasmid-based vectors that utilise the *ie*-1 promoter of *Ac*MNPV have been a useful tool for transient and stable expression studies (Jarvis *et al.*, 1990; Henderson *et al.*, 1995; Jarvis *et al.*, 1996; Murges *et al.*, 1997). However, use of the *Ac*MNPV *ie*-1 promoter for stably expressing large quantities of intracellular recombinant protein is usually inadequate for industrial application, for example, structural crystallography, and drug compound library screening. On the contrary, transformed insect cell lines constitutively expressing secreted recombinant proteins from *Ac*MNPV *ie*-1, have successfully yielded quantities equal to or greater than those of the BES (Jarvis *et al.*, 1990).

It has been possible to elevate *ie*-1 promoter activity with the use of enhancer sequences (Section 1.5.2). The use of baculovirus homologous repeat (*hr*) sequences in *cis* to the

*Ac*MNPV *ie*-1 promoter provides up to two-fold enhancement of transcription over the *ie*-1 promoter alone (Guarino and Summers, 1986a, b; Pfeifer, 1998). The effects of *hr* sequences do not appear to be limited to viral promoters as the *Bm hr*3 sequence can function as an enhancer in *cis* to the *Bm* cytoplasmic actin 3 gene (*BmA*3; Farrell *et al.*, 1997; Lu *et al.*, 1997).

Additional factors that have been shown to enhance heterologous gene transcription in insect cells, is the presence of a 70-180bp intron. The 70-180bp intron has been shown to increase levels of transcription by fivefold over a non-intron-containing gene in *Drosophila melanogaster* (Dunkler *et al.*, 1997). Hypotheses as to why introns boost transcription levels range from *cis*-acting elements to the presence of spliceosomes stabilising the mRNA (Pfeifer, 1998).

Since the development of the first insect plasmid-based expression vectors, the range of promoters available has expanded, offering different types of expression and a broader choice of insect cell lines for hosting protein production. However, these choices are still limited compared with the plethora of mammalian vector available. Examples of the insect plasmid-based expression systems available to date include; *Drosophila* expression system (DES®), Insectselect™, and Express-insect™ expression systems (Invitrogen™). These expression systems permit the choice of either transient / stable, and inducible / constitutive expression (Section 1.2.1.1-1.2.1.3). Stable expression subsequently overcomes the major disadvantage of the BES, which is the cytolytic properties of infectious baculovirus that narrow the window for optimal foreign protein production.

The DES is sub-divided into two types of expression, constitutive and inducible. The constitutive expression vectors exploit the *Drosophila actin* 5C promoter, while the inducible vectors utilise the *Drosophila MT* promoter. The inducible *MT* promoter is dependent on metal ions for full activity (Otto *et al.*, 1987; Bunch *et al.*, 1988). This expression system has been used in tissue culture systems and functions efficiently in dipteran cell lines (Kovach *et al.*, 1992). The actin 5C gene is transcriptionally complex, containing two promoters from which a variety of stage-specific transcripts are generated (Bond and Davidson, 1986; Bond and Davidson, 1988). The actin 5C distal promoter appears to be active in early embryos and in all *Drosophila* tissue (Burn *et al.*, 1989) and is often referred to as the strongest constitutive promoter in dipteran (Chung and Keller,

1990; Huynh and Zieler, 1999). The DES® combines the advantages of high-level insect expression systems, with the non-lytic stable mammalian expression systems. The DES® uses the well-characterised *Drosophila* Schneider S2 cells and simple expression vectors to allow stable or transient expression of recombinant proteins. Transient expression can be observed as early as two days post-transfection. Stable polyclonal cell lines can be established in 4 weeks with antibiotic selection (HygB).

InsectSelect™ is system designed for continuous, non-lytic insect expression, which utilises the constitutive *Op ie*-2 promoter (Theilmann and Stewart, 1992a), which is functional in lepidopteran and dipteran cell lines (Hegedus *et al.*, 1998). Stable expression using the InsectSelect™ expression system can be achieved in three steps: clone a gene of interest into an InsectSelect™ vector, transfect insect cells, and select stable clones (selection based on Zeo^r). This system has been successfully used for the production of secreted monoclonal antibodies directed against the type 2 adenovirus penton based protein in *Sf*9 cells (Li *et al.*, 2001).

ExpressInsect™ is a new non-lytic constitutive expression system. Work described by Lu *et al.* (1997), demonstrated that two baculovirus-specific genetic elements, the *hr*3 and *ie*-1 gene product (IE-1) of *Bm*NPV, are capable of enhancing co-operatively the activity of the *BmA*3 promoter by three orders of magnitude in a virus-free environment. These finding lead to the development of the ExpressInsect™ expression system.

The DES®, InsectSelect™, and ExpressInsect™ systems are very successful tools. However, the need for new and improved insect expression systems, to increase the diversity of expressed recombinant proteins, and to produce competitive intracellular protein yields to that of the BES, means that there is a great potential for future research, as during the last five to ten years, development has been progressively slow.

Of the insect plasmid-based expression systems described above only one offers the choice of regulated expression (DES®, *MT* promoter; *Dm* S2 cells). Therefore, it would be beneficial to expand the choice of insect cell line used to host regulated expression (e.g lepidopteran), while increasing the diversity of regulated expression mechanisms available.

1.4 Industrial applications: Scale-up of heterologous gene expression

Mammalian and insect cell culture are two commercially important technologies for the production of heterologous proteins. Although neither are routinely carried out at large scale, considerable basic information necessary for scale-up of these systems has been developed recently. The emphasis from evaluation of different bioreactor configurations has shifted of towards understanding what controls protein production in these two systems.

1.4.1 Classification of eukaryotic cell growth requirements

Mammalian cells of interest to biotechnology are classified either by their requirement for anchorage-dependent growth (CHO, BHK, hepatocytes) or by their ability to grow in suspension (CHO, BHK, hybridoma, and mouse connective tissue [3T3]). Whereas, insect cell lines characterised as excellent hosts for heterologous gene expression can be grown using either suspension or adherent cultivation methodologies (*Sf*9, *Sf*21, *T.ni* Hi5, and *Dm* S2). Therefore, the choice of cultivation can be reflected by the cell type used, which undoubtedly places restricts on mammalian cells.

1.4.2 Large-scale cultivation of eukaryotic cells

The stirred tank bioreactor (STR) is one of the simplest and most widely used of all fermenter design for culturing mammalian cell suspension cultures. The STR has been developed commercially in large-scale mammalian cell culture processing up to a volume of at least 10,000 litres (Feder and Tolbert, 1985; Bliem and Katinger, 1988). For laboratory use, there are also numerous bench-top STRs (1-5 litres) available commercially (Applikon, and New Brunswick). The bench-top models are generally made of glass with a stainless steel head-plate, whereas the larger fermenters are made entirely of stainless steel. The head-plate of the STR consists of a range ports and pipes, which allows for electrodes to be inserted (pH, temperature and dissolved oxygen probes) and tubing to be attached for air, oxygen, and media input or sampling (refer to Chapter 5; **Figure 5.16**). This design has been used extensively in all microbial fermentation and more recently insect cell fermentation (Agathos *et al.*, 1990; Caron *et al.*, 1990; Kuna and Papoutsakis, 1990; van Lier *et al.*, 1994; Hu and Bentley, 1999). Insect cells can also be maintained in suspension culture using less-complex methods, for instance shaker (*Sf*9,

T.ni Hi5) and spinner (*Sf*21) flasks. These system can be used to cultivate insect cells from 10ml to 5 litres. An alternate type of bioreactor developed for eukaryotic cell suspension cultures includes the airlift (Wood and Thompson, 1987) and wave fermenters (Singh, 1999).

Anchorage-dependent mammalian cell culture has also been successfully scaled-up using ceramic (Lydersen, 1987), hollow-fibre (Tyo *et al.*, 1988), packed-bed (Whiteside and Spier, 1981), and fluidised-bed (Runstadler and Cernek, 1988) bioreactors.

1.4.3 Optimising growth of eukaryotic cell culture and foreign protein production

The successful scale-up of eukaryotic cell lines expressing large quantities of recombinant protein using the systems described previously, is attributed to the vast number of studies that have determined factors that limit these processes. These factors include; pH, temperature, dissolved oxygen concentrations, and defined nutrient (glucose) and amino acid (glutamine) consumption (Maiorella *et al.*, 1988; Scott *et al.*, 1992; Lin *et al.*, 1992; Borys *et al.*, 1993; Racher *et al.*, 1993; Raghunand and Dale, 1999; Hu and Bentley, 1999). In particular, oxygen demand of insect cells is higher than that of mammalian cells (Tramper *et al.*, 1986; Maiorella *et al.*, 1988), and oxygen uptake increases after infection with virus (Streett and Hink, 1978; Maiorella *et al.*, 1988; Hu and Bentley, 1999). Therefore, supplementing culture with the required factors can facilitate optimal cell growth and conditions for heterologous gene expression.

1.4.4 Large-scale transient expression in eukaryotic cell for recombinant protein production

Stable expression technologies, based on chromosome-integrated plasmid sequences, have delivered large quantities of recombinant proteins from eukaryotic cells (Hill *et al.*, 2001; Pfeifer *et al.*, 2001; Shin and Cha, 2002). The development of these technologies requires considerable investment in time, human resources and bioreactor equipment. Due to high demand, the need for faster and cheaper approaches for the production of recombinant proteins is evident. The consideration of transient systems for the production of milligram to gram quantities of recombinant protein are rather new. With better technologies for reliable growth of eukaryotic cells and with better nucleic acid transfer

systems, the opportunities to explore scale-up of transient expression in eukaryotic cells has become feasible.

A promising development in transient expression, is the use of elements that provide extended maintenance of plasmid-based vectors in cells. A plasmid-based vector has been developed, which contains two expression cassettes, the first contains the foreign gene of interest and the second, the large T-antigen from SV40 (Geisse *et al.*, 1997). The large T-antigen promotes episomal plasmid replication, which has subsequently improved recombinant protein production 3- to 5-fold compare to the same vector minus the large T-antigen (Geisse *et al.*, 1997). However, a disadvantage of "naked DNA" systems is the requirement for large quantities of highly purified plasmid DNA.

Viral systems have become easier to use because the methods to create recombinant virus vectors have been optimised. The alphavirus and baculovirus expression systems have provided the most efficient mammalian and insect expression for scale-up (Liljeström and Garoff, 1991; King *et al.*, 1992; Berglund *et al.*, 1993; Nguyen *et al.*, 1993; Paul *et al.*, 1993; Liljeström, 1994; Blasey *et al.*, 1997; Lundstrom, 1997; Vlak *et al.*, 1998; Yamanaka, 2004). However, a disadvantage of the transient viral systems is the large quantity of inoculum requirement to infect scaled-up eukaryotic cell cultures.

Transformed eukaryotic cell lines expressing high-levels of recombinant protein are perceived as more advantageous over the systems described above, due to the relative ease with which cultures can be scaled-up without enduring further expenses.

1.5 Transcriptional activation in eukaryotic cells

Eukaryotic cells must control their patterns of gene expression in response to physiological signals, generated outside the cell, which are transduced to the nucleus through a series of complex biochemical interactions. These transduced signals can either amplify or attenuate temporally or spatially regulated gene expression by altering the interactions of transcription factors with their cognate targets (De La Brousse and Mcknight, 1993; Beckett, 2001; Johnson *et al.*, 2001; Warren, 2002). It is now generally accepted that gene expression is predominantly regulated at the level of transcription (reviewed by Latchman, 1995; Naar *et al.*, 2001), and the majority of data derived from baculovirus-infected insect cells has shown that this is true. When studying expression systems for

the purpose of high-level protein production, it is essential to understand factors governing gene regulation. Factors involved in the regulation of eukaryotic gene transcription fall into two categories: promoter / enhancer elements (DNA sequences) and activator elements (protein).

1.5.1 Eukaryotic gene promoters

The region immediately upstream (25-30 nucleotides) of transcription initiation has been the centre of significant study. Evaluating different genes and deleting / mutating sequences with significant homology to identified putative regulatory sequences. Many gene promoters are found to contain an AT-rich consensus sequence 'TATA$^{A}/_{T}A^{A}/_{T}$', which was determined as the TATA box. The TATA box is situated approximately 30 nucleotides upstream from the transcriptional start site (Sassone-Corsi and Borrelli, 1986). This sequence appears to be essential in determining accurate positioning of the transcriptional start site. However, not all genes contain the TATA box, in some cases a GC or CCAAT box is found in place of the TATA box, **Table 1.3** (Mitchell and Tjian, 1989; Gill, 2001; Butler and Kadonaga, 2002; Woychik and Hampsey, 2002).

Promoters are defined as the region of the gene bracketed by the TATA box motif and the site of transcriptional initiation (the catabolic gene activator protein [CAP] site). However, the presence of *cis* / *trans*-activiating gene-specific sequences may result in a dramatic increase of expression over those demonstrated by the TATA box alone. The effect of the *Bm*NPV *hr*3 consensus and IE-1 activator element on the *BmA*3 promoter, and heavy metal binding element in *MT* genes (Glanville *et al.*, 1981; Carter *et al.*, 1984; Farrell *et al.*, 1997; Lu *et al.*, 1997; Chen *et al.*, 2004) provide excellent examples of this mechanism (enhancer mechanisms are described in Section 1.5.3). Where present, these sequences have been shown to have an essential role in the up-regulation of transcription (Latchman, 1990, 1995; Farrell *et al.*, 1997; Lu *et al.*, 1997; Farrell *et al.*, 1999; Chen *et al.*, 2004).

Table 1.3: Consensus sequences found in the vicinity of eukaryotic RNA polymerase II start points[1].

Element	Consensus sequence	General transcriptional factor
BRE	$^{G}/_{C}{}^{G}/_{C}{}^{G}/_{A}$CGCC	TFIIB
TATA	TATA$^{A}/_{T}$A$^{A}/_{T}$	TBP
INR	$^{C}/_{T}{}^{C}/_{T}$AN$^{T}/_{A}{}^{C}/_{T}{}^{C}/_{T}$	TFIID
DPE	$^{A}/_{G}$G$^{A}/_{T}$CGTG	TFIID

[1]The name given to each consensus sequence (first column) and the general transcription factor (TF) that recognises it are indicated (last column). N indicated any nucleotide, and two nucleotides separated by a slash indicate an equal probability of either nucleotide at the indicated position (Alberts *et al.*, 2002).

Baculovirus genes have been shown to contain promoter elements that are important to transcription. Transcription by eukaryotic RNA polymerase II is most often initiated by recognition of a TATA box motif. Although not always present, the TATA box motif is the most commonly conserved sequence element associated with RNA polymerase II-transcribed promoters. Several baculovirus early genes, including *ie*-1, *ie*-2 (*ie*-N), *gp*64, *pe*-38 and *pe*-39 have been shown to have a consensus TATA box motif and tetranucleotide "CAGT" motif at / near the transcription start site (Blissard and Rohrmann, 1989; Blissard *et al.*, 1992; Lu and Carstens, 1992). This conserved start site sequence is similar to the consensus (ATCA$^{G}/_{T}$ T$^{C}/_{T}$) originally described at the mRNA start site of *Drosophila* heat shock protein gene and found a few nucleotides downstream of the mRNA start site in a variety of other insect genes (Hunltmark *et al.*, 1986). In *p*143, however, transcription is initiated at an unconventional 'CGTGC' mRNA start site consensus.

Baculovirus late genes (*p*10 and *polh*) appear to be transcribed from a consensus late promoter element "ATAAG" or "GTAAG," which are also the transcriptional start sites, and is the minimal sequence requirement for gene expression (Weyer and Possee, 1989). It has been shown that the 5' non-coding leader sequence of the *polh* gene is very important in the regulation of gene expression. The seven nucleotides before the ATG are particularly important for maximum promoter activity. Repeating the eight nucleotides spanning the 'TAAG' motif with synthetic oligonucleotides results in a 2000-fold reduction of expression. Sequences upstream of the mRNA start site appear to be relatively

unimportant. Similar results have been obtained with *p*10, *vlf*-1 and *lef*-6 (Weyer and Possee, 1989, Yang and Miller, 1998; Yang and Miller, 1999; Lin and Blissard, 2002).

1.5.2 Enhancers

The process of transcriptional control in eukaryotic cells is undoubtedly complex, and the mechanism of which is not fully understood. Two types of regulatory sequences have been described that control transcription initiation by RNA polymerase II: promoters and enhancers (Serfling *et al.*, 1985; Maniatis *et al.*, 1987; Müller *et al.*, 1988; Blackwood and Kadonaga, 1998; Butler and Kadonaga, 2002; Woychik and Hampsey, 2002). Enhancers are sequences typically composed of a variety of short sequence motifs (*cis*-elements). Together with the promoter, enhancers can have profound effects on the level of gene expression (Atchison, 1988; Butler and Kadonaga, 2002; Chen *et al.*, 2004). The definition of an enhancer is based on three criteria: first, an enhancer is capable of augmenting the level of foreign gene expression directed by the promoter in a position- and orientation-independent manner. Second, enhancers are unable to stimulate the expression of a promoter in *trans*. However, work carried out by Müller and Schaffner, (1990) suggest transcriptional enhances can act in *trans*. Third, increased transgene expression from the enhanced promoter is due to significant increases in transcription rates (Lu *et al.*, 1997).

Enhancers were originally identified in the DNA tumor virus simian virus 40 (SV40; Picard, 1985; Sassone-Corsi *et al.*, 1984; Sassone-Corsi and Borrelli, 1986) as *cis*-acting DNA elements able to dramatically increase transcription from RNA polymerase II-transcribed promoters independent of orientation and distance. Studies revealed that when a 200bp-long DNA fragment of the SV40 was linked to a reporter gene, transcription of the reporter gene increased more than 100-fold over. Activation was found to occur over large distances of more than 3000bp independent of the enhancer position or orientation. Enhancer elements have since been found in many other viruses (Serfling *et al.*, 1985; Voss *et al.*, 1986). However, the initial observations that the SV40 enhancer element augmented transcription from certain heterologous promoters (β-globin), became apparent that the magnitude of the stimulation also depended on the promoter examined. Studies carried out on the original SV40-β-globin chimera proved to have been a fortuitous choice of construct in terms of revealing an enhancer effect. Subsequent work has shown that as far as independence of distance was concerned, the SV40 enhancer had less dramatic

effects on other promoters, with both the SV40 early and chicken conalbumin promoters exhibiting a biphasic decrease in activity with increased distance between enhancer and promoter (Wasylyk *et al.*, 1984).

Nucleotide sequences exhibiting enhancer function have been identified in baculoviruses. *Ac*MNPV consists of primarily unique sequences with the potential capacity to encode more than 100 proteins. In addition, eight homologous regions (*hr*s) of 30- to 800-bp in length were identified as potential enhancers of transcription (Guarino *et al.*, 1986; Guarino and Summers, 1986; Rodems and Friesen, 1995; Viswanathan *et al.*, 2003; Chen *et al.*, 2004). The unique distribution of the largest *Ac*MNPV *hrs* (*hr*1 to *hr*5) and the identification of similar DNA elements in the genome of other baculoviruses (Ayers *et al.*, 1994; Majima *et al.*, 1993; Pearson and Rohrmann, 1995; Acharya and Gopinathan, 2001) suggest that the *hrs* perform an essential function (s) during the multiphase replication cycle of these invertebrate viruses. The *Ac*MNPV *hrs* also function as enhancer elements for early viral genes by stimulating transcription from transfected plasmids (Guarino *et al.*, 1986; Guarino and Summers, 1986; Carson *et al.*, 1991; Chen *et al.*, 2004). Viral enhancers differ from other eukaryotic enhancers in that they are interspersed throughout the viral genome. *hrs* were shown to have an average A / T content of 68%, significantly higher than the 58% content for the rest of the *Ac*MNPV genome. Similar observations have been made for other baculoviruses, such as *Op*MNPV (Theilmann and Stewart, 1992a: Theilmann and Stewart, 1992b), *Trichoplusia ni* granulosis virus (Hashimoto *et al.*, 1996) and *Choristone*ura *fuminferana* MNPV (Arif and Doerfler, 1984; Kuzio and Faulkner, 1984; Qiu *et al.*, 1996).

The 485-bp *hr*5 enhancer located immediately downstream from the *Ac*MNPV apoptotic suppresser *p*35, stimulates expression of viral genes in a promoter-specific manner. It consists of six 26- to 30-bp imperfect palindromes each bisected by an *Eco*RI site. Studies have indicated that the central 28-bp repeat (28-*mer*; TTTACGAGTAGAATTCTACTTGTAACGC) represents the smallest functional unit of *hr*5 capable of position- and orientation-independent transcriptional activation (Rodems and Friesen, 1993). Consistent with a modular organisation of *hr*5, repetition of the palindrome increases enhancement. It has subsequently shown that *hr*5 stimulates early gene expression in the *Ac*MNPV genome but is not required for virus replication. It has also been suggested that the *hr* region may be utilised by the virus as origins of DNA replication (Kool *et al.*, 1993). The highly conserved imperfect palindrome of 28-*mer* has been identified in all *hr*s (Rodems and Friesen, 1995; Olson *et al.*, 2003).

Activity of the *hr* enhances is stimulated by the *Ac*MNPV trans-regulator gene *ie*-1. The *ie*-1 gene product (IE-1) is a 67-kDa multifunctional protein that trans-regulates the expression of transfected genes under control of viral and heterologous promoters by *hr*-dependent and *hr*-independent mechanisms. Studies revealed that transcription of *ie*-1 is not affected by *cis*-activation of the *hr*5 enhancer, whereas expression of the delayed early gene *39K* was increased up to 1000-fold in the presence of the *hr*5 enhancer (Guarino *et al.*, 1986; Guarino and Dong, 1994). A 1-bp mismatch was found to occur in the same location in *hr*1 to *hr*4, *hr*5 contained a 2-bp mismatch in the same region. *hr*1 to *hr*5 have varying effects on *39K* expression. Out of all the *hr* enhancer *hr*5 exhibits greater *cis*-activation of *39K* and *hr3* the lowest. However, studies carried out by Lu *et al.* (1997) first reported that when linked to gene constructs expressing reporter proteins under control of the *BmA*3 gene promoter, a DNA fragment containing the *hr3* of *Bm*NPV enhances significantly the *in vitro* expression of reporter proteins. Secondly it was show that linkage of the *hr*3 enhancer to the *ac BmA*3 promoter, combined with the presence of the *Bm*NPV IE-1 protein in transfected cells resulted in an augmentation in the level of *BmA*3 promoter strength by three orders of magnitude relatively to that of the basic *BmA*3 gene promoter. *hr*1 has been shown to be important in regulating *Ac*MNPV *ie*-2, while *Ac*MNPV IE-1 down-regulates *ie*-2, both in the presence and absence of *hr*1 (Carson *et al.*, 1991).

1.5.3 The enhancer mechanism

Numerous models have been proposed to explain the mechanism of gene activation by remote enhancers and distal promoter sequences. Some of these models (the 'sequence-specific gyrase' model) are now considered unlikely (Plon and Wang, 1986). Also, it is unlikely that the main action of enhancers is the formation of a specific 'chromatin structure', since enhancers can stimulate transcription *in vitro* in the adsence of chromatin, albeit with a more pronounced distance-dependent attenuation than *in vivo* (Müller *et al.*, 1988; Carey *et al.*, 1990; Blackwood and Kadonaga, 1998). Although considerable progress has been made in understanding the structure and function of transcription factors that bind to promoters and enhancers, there is still little known about the way these factors interact with the general transcription apparatus.

Two principle models have been the most often considered for this process: looping and scanning (Müller *et al.*, 1988; Blackwood and Kadonaga, 1998). In the looping model, initiation of transcription is stimulated by the interaction of enhancer / upstream promoter

elements with proximal promoter elements through proteins bound to the DNA. The DNA between remote sequences and proximal promoter elements is thereby looped out (Picard and Schaffner, 1985; Ptashne, 1986). The main problem for communication over large distances is low concentration of the DNA regions (such as an enhancer and a promoter) in the vicinity of each other (Rippe *et al.*, 1995). Measurements of local concentrations of linear DNA ends in the vicinity of each other suggests that it is relatively high only when the distance between DNA ends is 100- 900-bp (Shore *et al.*, 1981). When distance between the DNA ends is increased to 3-kb, their local concentration is decreased by an order of magnitude (Shore *et al.*, 1981; Liu *et al.*, 2001). Thus, it is remarkable that enhancer and promoter regions positioned far away from each other can communicate efficiently.

Several models have been proposed to explain the mechanism of enhancer action over a large distance. One class of models suggests that initial communication of an enhancer with a promoter leads to the formation of a stable DNA-protein complex in the vicinity of the promoter. This stable complex may facilitate subsequent rounds of transcription serving as a "memory" of initial enhancer-promoter interaction (Blackwood and Kadonaga, 1998). Alternatively, the average distance between promoter and enhancer could be considerably decreased if the intervening DNA is super-coiled or bent (Shore *et al.*, 1981, Liu *et al.*, 2001).

Work to determine the mode of enhancer promoter interaction over large distances in prokaryotes, favours the looping model. Work by Liu *et al.* (2001) has demonstrated that short distances of 100- 900-bp between an enhancer and promoter does not necessitate DNA super-coiling to result in enhancement of promoter activity. However, when the enhancer was placed 2.3- or 2.5-kb upstream of the promoter, DNA super-coiling was required to elect equivalent up-regulation of promoter activity, as observed for short distances. There are several possible explanations for the effect of DNA super-coiling on the rate of enhancer-promoter communication. Local concentration of DNA sites separated by 2.5-kb is quite low ($\approx 10^{-8}$ M; Shore *et al.*, 1981) and could be dramatically increased by DNA super-coiling. Metropolis-Monte Carlo simulations of equilibrium DNA conformation suggested that DNA super-coiling increases the probability of juxtaposition of two sites (enhancer and promoter) spaced by 3-kb by about two orders of magnitude as compared with relaxed DNA (Vologodskii and Cozzarelli, 1996). Understanding the mechanism of action of prokaryotic enhancers could subsequently be important for better understanding of the mechanism of eukaryotic enhancer action.

The scanning (or entry site) model proposed by Moreau *et al.* (1981), suggests that enhancers / upstream promoter elements are recognised by RNA polymerase II / or transcription factors, which binds and then slides in either direction along the DNA, until it reaches the proximal promoter elements where it facilitates the formation of a transcriptional pre-initiation complex.

The mechanism of enhancer action has previously been proposed as a *cis*-activation regulatory element. However, a number of studies have demonstrated that enhancer sequences can also stimulate transcription in *trans* (Müller *et al.*, 1988; Morris *et al.*, 1999; Chen *et al.*, 2002).

1.5.4 Mechanism of transcriptional activation

Eukaryotic RNA polymerase II is a large multi-subunit enzyme comprising at least 12 distinct subunits and possessing a molecular mass in excess of 500 kiladaltons (Woychik, 1998). Over the last 25 years, biochemical studies aimed at reconstituting promoter-dependent transcription by RNA polymerase II with pure proteins and elucidating its mechanism have bought to light the remarkable complexity of the initiation stage of eukaryotic mRNA synthesis. Previous studies (Fire *et al.*, 1984; Brown *et al.*, 1985; Reinberg and Roeder, 1987; Ptashne, 1988; Zhang *et al.*, 1990; Bates and Maxwell, 1993; Buratowski, 1994; Stargell and Struhl, 1996; Beckett, 2001; Warren, 2002) revealed that transcription initiation by RNA polymerase II is an elaborate multi-step process that requires as a minimum five general transcription initiation factors: TFIIB, TFIIE, TFIIF (Reinberg and Roeder, 1987), TFIID (Lin, Y-S *et al.*, 1991) or TBP (Carthew *et al.*, 1985), TFIIH (Xiao *et al.*, 1994) and an ATP cofactor. Assembly of the above transcription factors on a core promoter causes the formation of the pre-initiation complex, **Figure 1.4** (Fire *et al.*, 1984; Bates and Maxwell, 1993; Buratowski, 1994; Stargell and Struhl, 1996; Dvir *et al.*, 2001; Naar *et al.*, 2001; Warren, 2002).

The basic transcriptional factors essential for transcription by RNA polymerase II is more complex than that of RNA polymerase I or III. The first step in the assembly of the pre-initiation complex is binding of TFIID (TBP and TAFs) sequence-specifically to the TATA element (Stringer *et al.*, 1990; Takada *et al.*, 1992; Dvir *et al.*, 2001; Woychik and Hampsey, 2002). This results in protection of the –35 to –19 upstream region of the transcription start site, **Figure 1.4**, which subsequently establishes a nucleoprotein

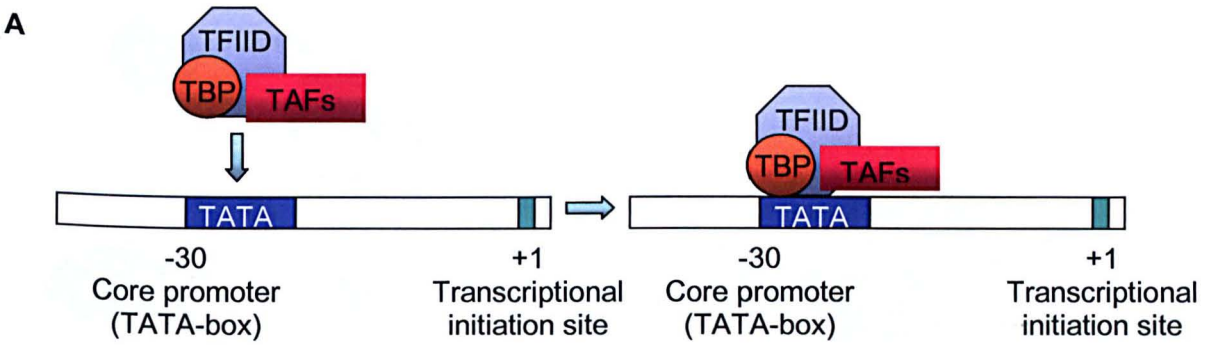
recognition site for RNA polymerase II on the DNA. This is the earliest step in the formation of the stable transcriptional complex (Baldick *et al.*, 1994; Woychik and Hampsey, 2002) and is facilitated by TFIIA. TFIIA binds to the promoter-TFIID complex (Buratowski *et al.*, 1989), TFIIA is not essential for basal transcription but it does function to stabilise the pre-initiation complex under more physiological conditions (Lee *et al.*, 1992). Several negative regulators of transcription have been discovered (Auble and Hahn, 1993; Gill, 2001; Wei *et al.*, 2003). Some of these inhibitors act by displacing TFIID from the core promoter, while others block interactions between TFIID and the other basal factors. TFIIA appears to counteract these repressors of transcription, presumably through its direct interaction with TBP. This antagonism may represent a mechanism for regulation of the pre-initiation complex.

The second step of pre-initiation complex assembly is the recruitment of TFIIB to the TFIID-TFIIA-TATA-element complex (Lin and Green, 1991; Kimura and Ishihama, 2000). TFIIB functions as an adaptor to promote selective binding of RNA polymerase II to TFIID at the promoter by binding specifically to both polymerase and the TBP subunit of TFIID to from the Dbpol II complex (Johnson and Mcknight, 1989, Misra *et al.*, 1995; Dvir *et al.*, 2001). The recruitment of RNA polymerase II, brings with it TFIIF. TFIIF performs dual roles in assembly of the pre-initiation complex by binding to and strongly stabilising the Dbpol II intermediate and by recruiting TFIIE into the complex. TFIIF also has an enzymatic activity needed to unwind the DNA helix. TFIIE incorporation into the pre-initiation complex appears necessary for subsequent recruitment of TFIIH. TFIIE is a DNA-dependent ATPase and is probably needed for generating the energy for transcription (Bunick *et al.*, 1982; Sawadogo and Roeder, 1984). Evidence that TFIIE makes contact with promoter DNA both upstream and downstream of the transcriptional start site suggests that this initiation factor accenuate DNA bending, and promotes the complete wrapping of promoter DNA around the pre-initiation complex. Leaving DNA upstream and downstream of the transcriptional start site juxtaposed (Robert *et al.*, 1996; Robert *et al.*, 1998; Johnson *et al.*, 2001).

TFIIH is one of the last additions to the pre-initiation complex. TFIIH is a multi-subunit complex associated with several biochemical activities. TFIIH possesses the serine / threonine protein kinase activity that is capable of phosphorylating the carboxyl-termini-domain of RNA polymerase II (Buratowski, 1994; Kimura and Ishihama, 2000; Butler and Kadonaga, 2002). TFIID cannot bind this heavily phosphorylated region and releases the RNA polymerase enabling transcription of DNA. During transcription, TFIIA and TFIID

(Takada *et al.*, 1992) remain bound to the promoter region, allowing repeated rounds of transcription, (Mastsui *et al.*, 1980; Samuels *et al.*, 1982; Kadonaga, 1990; Wampler *et al.*, 1990; Latchman, 1995; Gill, 2001; Johnson *et al.*, 2001; Butler and Kadonaga, 2002) **Figure 1.4.** Even though the formation of the transcriptional complex is undoubtedly important, it has been demonstrated that DNA binding *per se* does not initiate transcription (Keegan *et al.*, 1986; Gill, 2001; Johnson *et al.*, 2001; Butler and Kadonaga, 2002; Woychik and Hampsey, 2002).

Figure 1.4: The formation of the active eukaryotic pre-initiation complex. The diagrams represent the complexes formed on the TATA box by the transcription factors and RNA polymerase II. **A:** The TFIID complex binds to the TATA box through its TBP subunit. **B:** TFIIA binds promoter-TFIID complex and stabilizes TFIID. **C:** TFIIB is recruited to the complex on the TATA box. **D:** The recruitment of RNA polymerase II, brings with it TFIIF. RNA polymerase II is positioned by TFIIB, and its carboxy-terminal domain (CTD) is bound by TFIID. **E:** TFIIE and TFIIH bind to the complex. **F:** The CTD is phosphorylated by TFIIH and is released by TFIID. The RNA polymerase II is now competent to transcribe mRNA from the gene.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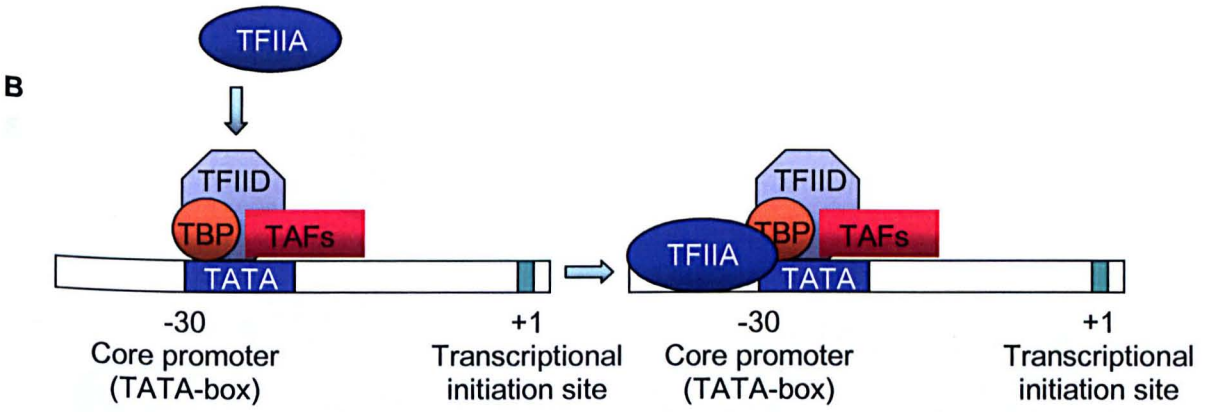
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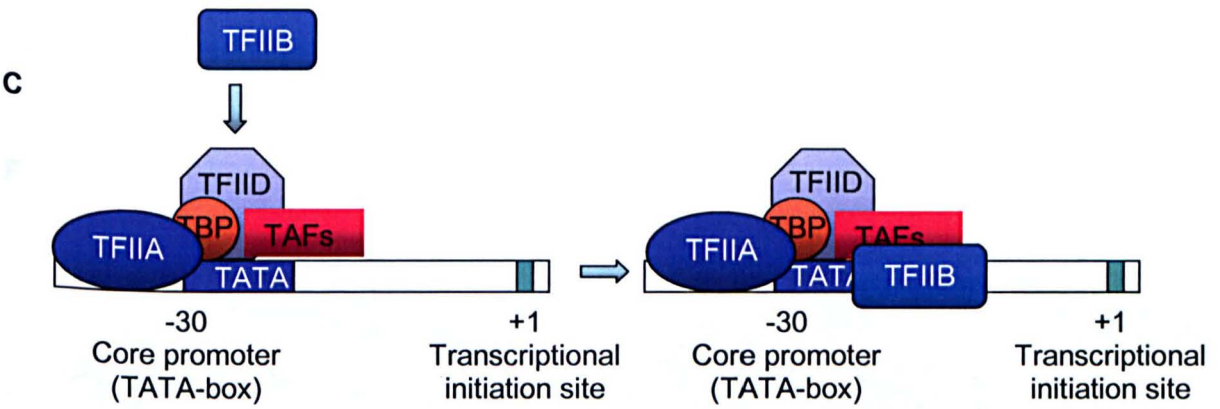
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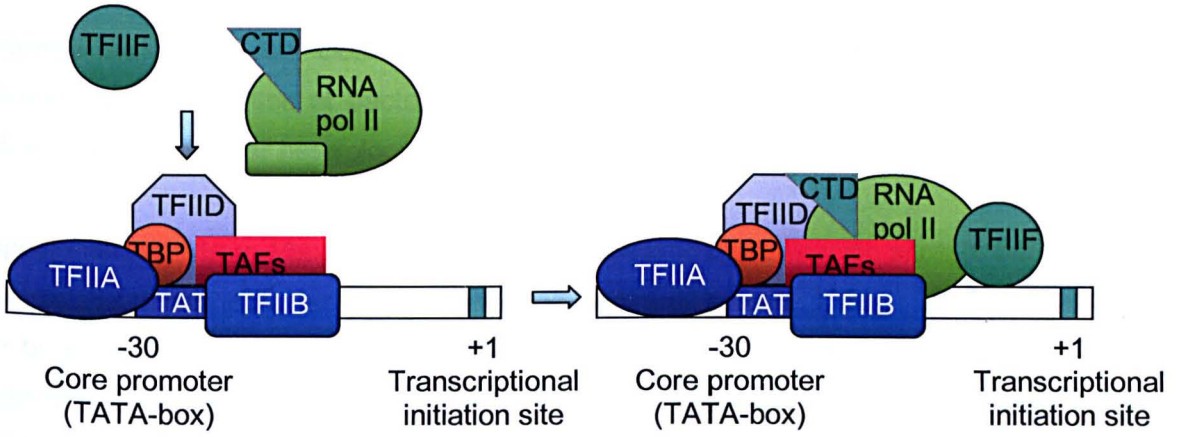
D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Core promoter
(TATA-box)
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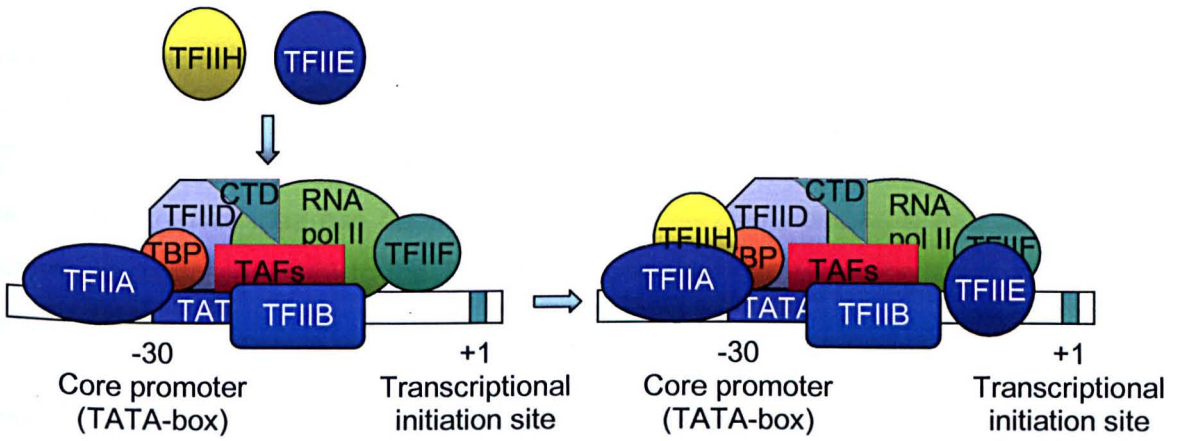
TFIIH
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TFIIB
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Transcriptional
initiation site
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TFIID
RNA pol II
TFIIH
BP
TAFs
TFIIA
TFIIE
TFIIB
-30
Core promoter
(TATA-box)
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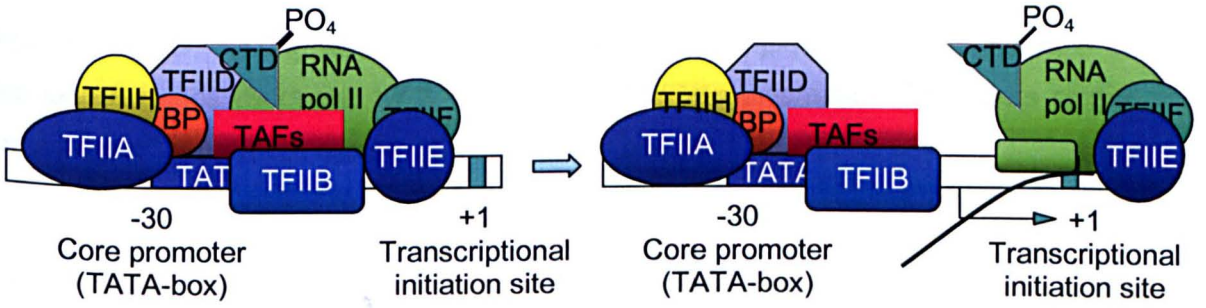
initiation site

F


PO4
CTD
TFIIH
TFIID
RNA pol II
BP
TAFs
TFIIA
TFIIE
TAT
TFIIB
-30
Core promoter
(TATA-box)
+1
Transcriptional
initiation site
PO4
CTD
TFIIH
TFIID
RNA pol II
BP
TAFs
TFIIA
TFIIE
TFIIB
-30
Core promoter
(TATA-box)
+1
Transcriptional
initiation site

1.6 TOPO® cloning

Gateway™ is a universal cloning technology that takes advantage of the site-specific recombination properties of bacteriophage lamda (Landy, 1989) to proved a rapid and efficient way to transfer heterologous genes of interest into multiple vector systems

Topoisomerase I from *Vaccinia* virus binds to duplex DNA at specific sites (CCCTT) and cleaves the phosphodiester backbone in one strand (Shuman, 1991). The energy from the broken phosphodiester backbone is converted, by the formation of a covalent bond between the 3' phosphate of the cleaved strand and a tyrosyl residue (Tyr 274) of topoisomerase I. The phospho-tyrosyl bond between the DNA and enzyme can subsequently be attacked by the 5' hydroxyl of the original cleaved strand, reversing the reaction and releasing topoisomerase (Shuman, 1994). TOPO® cloning exploits this reaction to efficiently clone polymerase chain reaction (PCR) products.

Directional joining of double-stranded DNA using TOPO®-charged oligonucleotides occurs by adding a 3' single-stranded end (overhang) to the incoming DNA (Cheng and Shuman, 2000). This single-stranded overhang is identical to the 5' end of the TOPO®-charged DNA fragment. This idea has been modified (Invitrogen™) by adding a four nucleotide overhang sequence (GTGG) to the TOPO®-charged DNA and adapting it to a plasmid-based vector format.

In this system, PCR products are directionally cloned by adding four nucleotides to the forward primer (CACC). The overhang in the plasmid-based vector (GTGG) invades the 5' end of the PCR product, anneals to CACC, and stabilises the PCR product in the correct orientation. Inserts can be cloned in the correct orientation with efficiencies equal to or greater than 90%.

Directional TOPO® cloning was used in this thesis for the rapid and efficient insertion of heterologous genes (Sections 1.7.2, 1.7.4 and 1.7.5) into plasmid-based vectors of the ExpressInsect™ expression system (Chapters 3, 4, and 5).

1.7 Heterologous genes used in this thesis

1.7.1 Reporter genes

Reporter genes are those, which are introduced into cells, which allow the detection or visualisation of modified gene expression levels, signal transduction rates and cell metabolism activities. The detection of reporter-proteins is generally both quick and easy to assay for and give quantitative and qualitative results. Reporter genes used in this thesis are all intracellular and include: chloramphenicol acetyl transferase (CAT), green fluorescent protein / enhanced green fluorescent protein (GFP / EGFP), *Discosoma* red (*Ds*Red), and dihydroorotate dehydrogenase (DHODH).

1.7.2 Chloramphenicol acetyl transferase

CAT is a commonly used reporter gene when accurate quantitative results are required, such as promoter comparative assays (Roelvink *et al.*, 1992). For this reason *cat* will be used in this project to compare the activity of different promoters. Assaying for CAT is undertaken in the presents of [^{14}C] chloramphenicol, CAT catalyses the conversion of chloramphenicol to the inactive mono- or diacetylated forms, which can the be separated by thin layer chromatography (Section 2.8.1). Percentage conversion is calculated by use of the software program Image-Quant 5.1.

1.7.3 Green fluorescent protein / enhanced green fluorescent protein

The cloning of the gene for green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* and its subsequent expression in heterologous systems has established GFP as a unique genetic reporter system for use in a variety of organisms (Chalfie *et al.*, 1994; Kain *et al.*, 1995; Tsien, 1998). Unlike other reporters, GFP fluorescence emerges in the absence of substrates or cofactors and allows for non-invasive monitoring in living and in paraformaldehyde-fixed cells. Enhancement of wild-type GFP by human codon optimisation (Yang *et al.*, 1996) and fluorophore mutation (EGFP) resulted in higher expression levels and brighter fluorescence (Yang *et al.*, 1996; Zhang *et al.*, 1996).

1.7.4 *Discosoma* red fluorescent protein

Recently a novel red fluorescent protein DsRed, was isolated from reef coral (*Discosoma* sp.). DsRed retains the same advantages as described for GFP and EGFP as a reporter gene (Section 1.7.3). DsRed fluorescence is excited optimally at 558 nm, but can also be excited by a standard 488 nm laser (Hawley *et al.*, 2001). DsRed has a high quantum yield and is photo-stable (Baird *et al.*, 2000). These characteristics make DsRed an ideal candidate for fluorescence imaging. However, problems with DsRed have been characterised recently, such that they form tetrameric forms (Rodrigues *et al.*, 2001; Sacchetti *et al.*, 2002). These tetrameric forms have a tendency to self-associate to form higher-order aggregates when over-expressed to very high-levels and on occasion these have been shown to be toxic to cells (Baird *et al.*, 2000; Goss *et al.*, 2000; Heikal *et al.*, 2000; Jakobs *et al.*, 2000). Despite this possible disadvantage, use of DsRed as a reporter gene is widespread and generally its advantages outweigh the disadvantages.

1.7.5 Dihydroorotate dehydrogenase (*Candida albicans*)

Dihydroorotate dehydrogenases (DHODH) have been identified as flavoproteins. Flavoproteins are enzymes or proteins that have a flavin nucleotide as a coenzyme or prosthetic group (Bader *et al.*, 1998). Eukaryotic DHODH are a protein component located in the inner mitochondrial membrane and possess a quinone as its electron acceptor (Dietz *et al.*, 2000; Carrey *et al.*, 2002). DHODH catalyses the fourth of six consecutive enzymatic reactions in the *de novo* biosynthesis of the pyrimidine, UMP. It allows for the oxidation of dihydroorotate, which is not a true pyrimidine, to orotate (Löffler *et al.*, 1997). The reducing equivalents resulting from dihydroorotate may be utilised to push ATP synthesis through oxidative phosphorylation (Carrey *et al.*, 2002). DHODH was used as a reporter gene in this thesis, as it has industrial importance and relevance to Pfizer.

1.8 Aims of the project

The overall aim of this thesis was to improve intracellular expression and regulation of heterologous genes in insect cells (lepidopteran). This work was subdivided into more specific aims, which are described below:

- To characterise α-amanitin-sensitive RNA polymerase promoters with stronger transcriptional activity than the *Ac*MNPV *ie*-1 promoter.

The use of *Ac*MNPV *ie*-1 for the stable production of intracellular recombinant proteins in insect cells has been well documented as producing low yields (Guarino and Summers, 1987; Jarvis *et al.*, 1990; Joyce *et al.*, 1993). Therefore, comparative expression analyses of the *Ac*MNPV *ie*-1 promoter to a number of promoters demonstrated to be useful in other eukaryotic expression systems (Section 1.3.3) was undertaken. These data would subsequently aid the development of further work described in this thesis.

- To develop a new plasmid-based inducible insect cell expression system.

Mechanisms that will permit tight regulation of inducible gene expression in insect cell lines are very limited (Sections 1.3.3, 4.1), compared to those developed for mammalian cells. Therefore, work was undertaken with the aim of developing one of the mammalian inducible expression systems to lepidopteran cells. The tetracycline inducible system was selected as a good candidate to develop to insect cell lines (Chapter 4). The tetracycline inducible expression system offers a number of advantages for adaptation to insect cells, these include; extremely tight on / off regulation, no pleiotropic effects, and expression is induced by a well-characterised inducer (tetracycline / doxycycline).

- To investigate ways of improve transient expression of intracellular recombinant proteins, using a novel baculovirus expression vector and determining optimal scale-up cultivation methodologies.

The removal of chitinase from *Ac*BacPAK6, to derive *Ac*BacPAK6.Δ*chi*A, has demonstrated improved yields of secreted and membrane targeted proteins (Section 1.3.2). Experiments to determine if recombinants derived from *Ac*BacPAK6.Δ*chi*A could augment intracellular protein production over those observed from *Ac*BacPAK6 were undertaken. In addition, different culturing methodologies (fermenter and shaker) were used to scale-up (1-litre) intracellular protein production using the recombinant viruses derived from *Ac*BacPAK6.Δ*chi*A and *Ac*BacPAK6 (Chapter 5).

- To examine ways to improve stable expression of intracellular recombinant proteins, using promoters characterised with stronger transcriptional activity than *Ac*MNPV *ie*-1.

The data derived from the promoter comparison study (Chapter 3) conducted at the beginning of this thesis, was used to identify the promoter with the strongest transcriptional activity in lepidopteran cells. This promoter was subsequently used to stably express intracellular heterologous genes (Sections 1.7.4 and 1.7.5) to address whether stable insect cell lines could be used as an alternative to the BES for the production of intracellular proteins (Chapter 5).

- To develop methods for continuous cultivation of transformed insect cell lines.

Transformed insect cells generated in this thesis were also used for the development of a protocol that would maintain continuous cultivation of scaled-up stable insect cell lines in an open fermenter system (Chapter 5).

CHAPTER 2: MATERIALS AND METHODS

2.1 General information

This chapter gives a summary of materials and methods used throughout this thesis.

2.1.1 Chemicals and reagents

General chemicals, solvents and reagents were purchased from Sigma Chemicals Company Ltd., UK, BDH Chemicals Ltd., UK, Invitrogen™, Life Technologies, Gibco BRL, USA., and BD Biosciences, unless otherwise stated. All solutions were made as weight per volume (w / v) unless otherwise indicated. Solutions were sterilised, when necessary, either by autoclaving (15 pounds per square inch (psi) for 15 mins or 10psi for 20 mins) or by filter sterilisation.

2.1.2 Radioisotopes

The following radioisotope was obtained from Amersham Pharmacia Biotech, UK Ltd. and used as appropriate:

1-Deoxy[dichloroacetyl-1-^{14}C] chloramphenicol (0.025μCi)

2.1.3 Enzymes

Restriction endonucleases were purchased from Promega or New England Biolabs (NEB). Specific modifying enzymes were obtained from the following:

Proof*sprinter*™ DNA polymerase:	Hybaid-AGS
Gateway™ LR Clonase™ Enzyme mix:	Invitrogen™
Shrimp Alkaline Phosphatase (SAP)	Boehringer Mannheim Ltd.
T4 DNA ligase	Promega / NEB
T4 polynucleotide kinase	Promega / NEB
Klenow DNA polymerase	Promega / NEB
Omniscript reverse transcriptase	QIAGEN

All enzymes were accompanied with the appropriate buffers and reagents.

2.1.4 Oligonucleotides

All oligonucleotides were custom synthesized by Invitrogen™.

2.1.5 Bacterial strains

The following modified *Escherichia coli* K12 strains were used for prokaryotic cloning and plasmid propagation:

MAX Efficiency® DH5α™	F⁻ϕ80*lac*ZΔ(*lac*ZYA-*arg*F)U169*deo*R*rec*A1 *end*A1*hsd*R17(r_k^-,m_k^-)*pho*A*sup*E44*thi*-1*gyr*A96*rel*A1*ton*A (confers resistance to phage T1) (Invitrogen™)
DB3.1™	F⁻ *gyr*A462*end*A-delta(*sr*l-*rec*A) *mcr*B *mrr hsd*S20 (r_{B-},m_{B-}) *sup*E44 *ara*-14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Sm^r) *xyl*5 lambda⁻ *leu mtl*1 (Invitrogen™)
TOP10™	F⁻ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L(Str^R) *end*A1 *nup*G (Invitrogen™)

2.1.6 Glossary of mutations

ϕ80	cell carries F80 prophage.
ara-leu 7697/*ara*D139	blocks arabinose catabolism and leucine biosynthesis.
ara-14	blocks arabinose catabolism.
*arg*F	ornithine carbamoyltransferase mutation blocks the ability to use arginine.

Δ*lac*X74	entire deletion of *lac* operon and varying amounts of flanking DNA.
*deo*R	regulatory mutant for constitutive deoxyribose synthesis to allow uptake of large plasmids.
*end*A1	DNA specific endonuclease. Mutation shown to increase quantity of plasmid yields.
F^-	lacks the F' episome.
galK / *galK*2	galactokinase mutation blocks catabolism of galactose.
galU	glucose-1-phosphate uridylyltransferase mutation blocks the ability to use galactose.
gyrA	mutation affects DNA gyrase, subunit A, conferring resistance to nalidixic acid.
*gyrA*96	DNA gyrase mutant produced resistance to nalidixic acid.
*hsd*RMS	mutation in the methylation system that *E. coli* use to recognise foreign DNA. There are two parts to this system, methylation and restriction.
*hsd*R17(r_k^-,m_k^-)	restriction minus (via *Eco*K1 restriction system mutant, so no restriction at unmethylated *Eco*K1 sites), modification minus (no methylation at *Eco*K1 sites.

*hsd*S20(r_{B-}, m_{B-})	mutation in the site recognition gene for strain B restriction endonuclease system. This makes the strain deficient for both restriction and modification of DNA.
*lac*Y1	blocks use of lactose via β-D-galactosidase mutant.
*lac*Z	mutation yields colourless (vs. blue) colonies in the presence of X-gal.
*lac*ZΔM15	a partial deletion of the NH_2-terminal region of β-D-galactosidase, permits alpha-complementation with certain vectors that encode this region (pUC, M13) thus producing blue / white colour selection.
leu	mutation in the *leu* operon.
*mcr*A	blocks restriction of DNA methylated at G(m)CGC.
*mcr*B	blocks restriction of DNA methylated at AG(mC)T.
mrr	blocks adenine methylation; prevents cleavage of C(m)AG and G(mA)C.
*mcr*BC	*Gme*C or *Ame*C *Hha* II, *Pst* I methylases methylate these sequence Gm6AC and Cm6AG respectively.
*mtl*1	blocks catabolism of mannitol.
*nup*G	a mutation for the transport of nucleosides.
*pho*A	encodes the periplasmic enzyme alkaline phosphatase.

*pro*A2	requires proline for growth on minimal media.
*rec*A / *rec*A1	involved in DNA repair and recombination. Mutation reduces homologous recombination of vector with host DNA giving more stable inserts.
*rel*A	RNA is synthesized in the absence of protein synthesis (relaxed phenotype).
*rps*L20(Sm^R) / *rps*L(Str^R)	confers resistance to streptomycin due to a mutation in S12 of 30S ribosome.
*sup*E44	carries a tRNA suppressor gene for amber or ochre termination codons.
thi-1	requires thiamine for growth on minimal media.
*ton*A	confers resistance to the lytic bacteriophage *T*1, *T*5 and *Phi* 80, via a mutation in an outer membrane protein.
U169	this refers to a deletion in this part of the *E. coli* genome, including the *lac* Z, Y, and A genes.
*xyl*5	blocks catabolism of xylose.

2.1.7 Non-viral insect expression systems

Constitutive / Inducible *Drosophila* Expression System (*version F*), Invitrogen™ Life Technologies, Gibco BRL, USA.

Constitutive	pAc5.1/V5-His A, B, C
Inducible	pMT/V5-His A, B, C

EXPRESS INSECT™ vector set, Invitrogen™, Life Technologies, Gibco BRL, USA:

Constitutive	pXINsect-DEST38
Constitutive	pXINsect-DEST39
Constitutive	pBmA:neo

Insect Select™ vector set, Invitrogen™, Life Technologies, Gibco BRL, USA:

Constitutive	pIZT/V5-His
Constitutive	pIZT/V5-His/CAT

2.1.8 Non-viral mammalian expression system

Tet-on™ Gene Expression System, BD Biosciences Clontech:

Constitutive	pTet-on
Inducible	pTRE2

Living colours expression vectors, BD Biosciences Clontech:

Constitutive	pEGFP-N1
Constitutive	pDsRED-N1

2.1.9 Insect virus transfer vectors

BacPAK™ transfer vectors, BD Biosciences Clontech:

pBacPAK8

pBacPAK9

2.1.10 Insect viruses

Viruses used for the construction of recombinant viruses are:

BacPAK6™	R.D. Possee, CEH, Oxford
BacPAK6 (*chiA*$^-$)	L.A. King, Oxford Brookes University
BacPAK6.*DsRed*	A. Patmanidi, Oxford Brookes University
BacPAK6.*chiA*$^-$.*DsRed*	C. Pritchard, Oxford Brookes University

2.1.11 Insect cell lines

Insect cell lines:

Spodoptera frugiperda (*Sf*21)	R.D. Possee, CEH, Oxford.
Spodoptera frugiperda (*Sf*9)	Invitrogen™, Life Technologies, Gibco BRL, USA.
Drosophila melanogaster (*Dm* S2)	Invitrogen™, Life Technologies, Gibco BRL, USA.
Trichoplusia ni (*T.ni* Hi5)	JRH Biosciences.

2.1.12 Insect cell culture medium

TC100	Invitrogen™,LifeTechnologies, Gibco BRL, USA.
Sf900II serum-free	Invitrogen™, Life Technologies, Gibco BRL, USA.
Ex-cell 405 serum-free	JRH Biosciences.
Foetal Calf Serum (FCS)	Invitrogen™/ Harlan-Seralab / Globepharm.
Schneider's (DES®Expression Medium with L-glutamine containing 10% heat-inactivated FCS)	Invitrogen™, Life Technologies, Gibco BRL, USA.

2.2 Standard cell culture procedures

2.2.1 Insect cell culture

*Spodoptera frugiperda (Sf*21) cells were maintained at a low passage in spinner cultures (35 rpm) and in disposable 75cm^3 T-flasks for seed stocks. Cells were maintained at a low-density stock (0.2x10^6 cell / ml) and growth to high-density stocks (2x10^6 cell / ml) before passaging. In both cases the cells were maintained in TC100 medium supplemented with 10% foetal calf serum (FCS).

Spodoptera frugiperda (*Sf*9) cells, and *Trichoplusia ni* (*T.ni* Hi5) cells were maintained at a low passage in shaker culture and in disposable 75cm^3 T-flasks for seed stocks. Cells were maintained at a low-density stock (0.35x10^6 cell / ml) and growth to high-density stocks (6-8x10^6 cell / ml) before passaging. In both cases the cells were maintained in serum-free medium (*Sf*9 cells in Sf900II and *T.ni* Hi5 cells in Ex-cell 405).

Drosophila melanogaster Schneider S2 (*Dm*) cells, were maintained at a low passage in complete *Drosophila* expression system® (DES®) medium, containing L-glutamine and 10% heat-inactivated FCS within disposable 75cm^3 T-flasks. Cells were maintained at a low-density stock (1x10^6 cell / ml) and growth to high-density stocks (6-20x10^6 cell / ml) before passaging.

*Sf*21, *Sf*9, and *T.ni* Hi5 cell lines were incubated at 28°C whilst *Dm* cells were incubated at 24°C. *Sf*9 and *T.ni* Hi5 shaker cultures were grown on Denley orbital shakers (135 rpm). *Sf*21 spinner cultures were grown on biological stirrers (Techne MCS-104L). Prior to use, cell numbers were determined using a Neubauer heamocytometer. Cell viability was determined by trypan blue staining (Section 2.2.6).

2.2.2 Long term storage of cells

All cell lines were frozen slowly in liquid nitrogen in the appropriate growth medium containing 10% DMSO (FCS was added if required), for long-term storage. Cells were stored in 1ml aliquots in sterile cryovials at a cell density of 1-3x10^7 cell / ml. Recovery of cells involved the rapid thawing of the aliquot at 37°C. In the case of monolayer-forming cells, cells were transferred initially into disposable 25cm^3 T-flasks, 4ml of fresh medium was added, and the cells were allowed to settle. The medium was decanted and fresh medium added. The cells were expanded as required. In the case of suspension cultures, cells were removed from storage medium by gentle centrifugation (1400 *xg*, 10mins Bechman J6B) and resuspended into 10ml fresh medium and incubated at 28°C on an orbital shaker or biological stirrers.

2.2.3 Seeding insect cells

Cells were seeded in either 35mm tissue culture dishes. Seeding densities used for 35mm dishes were 1x10^6 for *Sf*9, *Sf*21 and *T.ni* Hi5, or 3x10^6 for *Dm*. *Sf*21, *Sf*9, and *T.ni*

Hi5 cells were incubated at 28°C, *Dm* cells at 24°C, for 2-3 h to allow the cells to adhere prior to use.

2.2.4 Transfer of heterologous DNA into insect cells

Transfer of plasmid DNA into *Sf*9 or *T.ni* Hi5 cells for transient or stable protein production was carried out using Lipofectin™ (Invitrogen™, Life Technologies, Gibco BRL, USA). For transfection into 35mm tissue culture dishes, 1ml total aliquots containing appropriate growth medium, Lipofectin™ and plasmid DNA was added to the cells. Lipofectin™ was diluted 1 in 100 with growth medium (5µl:495µl) to give a volume of 500µl. Plasmid DNA was used at varying concentrations (1-10µg per transfection), which was added to growth medium to give a volume of 500µl. The DNA / growth medium was added to the Lipofectin™ / growth medium and gently vortexed to mix. The 1ml transfection mixtures were incubated at room temperature for 40 mins to form DNA / lipid complexes. The transfection mixtures were applied to $1x10^6$ *Sf*9 or *T.ni* Hi5 cells previously seeded into 35mm tissue culture dishes (section 2.2.3). Dishes of cells were incubated at 28°C in a moist box for 5 h, a further 1ml of appropriate growth medium was then added.

Transfer of plasmid DNA into *Dm* cells for transient or stable protein production was carried out by calcium phosphate transfection. *Dm* cells were seeded (section 2.2.3) into 35mm tissue culture dishes. In a microcentrifuge tube, the following components were mixed together to form solution A: 2M $CaCl_2$ (36µl), plasmid DNA (19µg), tissue culture sterile water (to a final volume of 300µl). In a second microcentrifuge tube the following was added to form solution B: 300µl 2X HEPES-Buffer Saline (HBS) (50mM HEPES, 1.5mM Na_2HPO_4, 280mM NaCl, pH 7.1). Solution A was slowly added to solution B in a drop wise fashion with continuous gentle vortexing to mix the solutions together. Continuous mixing ensured production of a fine white precipitate necessary for efficient transfection. The resulting solution was incubated at room temperature for 40 mins. The solution was swirled to mix and added drop wise to the seeded *Dm* cells. The transfections were incubated for 16 h at 24°C. The calcium phosphate solution was removed and the cells washed twice with fresh DES complete medium. The cells were washed by re-suspending in DES complete medium and pelleted (100 *xg*, 5 mins, microfuge). The medium was decanted and fresh medium was added, cells were re-plated into the same 35mm tissue culture dishes. Dishes were incubated at 24°C to allow protein production.

2.2.5 Production of stable insect cell lines

Cells were seeded (Section 2.2.3) and transfected with DNA as described in Section 2.2.4, transfections were incubated at 28°C for 48 h. Medium was removed and replaced with complete growth medium containing 700μg / ml for *T.ni* Hi5 cells or 400μg / ml for *Sf*9 cells. Selective pressure was reduced to 300μg / ml for *T.ni* Hi5 cells and 200μg / ml for *Sf*9 cells following 7-21 days incubation at 28°C. Medium was changed at weekly intervals until clones of cells were visible by eye. Individual clones were picked using a blue Gilson tip and seeded into 24 well plates. Once monolayers were confluent, cells were screened for the presence of the heterologous gene using PCR (Section 2.5.5).

2.2.6 Trypan blue staining

Cell samples were removed from the stock culture and an equal volume of 0.2% w / v trypan blue added. The cells were examined immediately using phase-contrast microscopy. Non-viable (stained) and viable (non-stained) cells were counted.

2.3 Standard Baculovirus Procedures

2.3.1 Virus infection of insect cells

Cells were seeded (Section 2.2.3) and incubated at 28°C for 1-2 h to allow cells to adhere. Medium was completely removed from the cell monolayer, 100μl of virus inoculum was added and incubated at room temperature for 1 h, with gentle agitation. Virus inoculum was removed and replaced with 2ml of fresh medium.

2.3.2 Production of recombinant baculoviruses

*Sf*21 cells were seeded into 35mm tissue culture dishes (Section 2.2.3) and allowed to adhere. Co-transfection mixtures were set-up as described in section 2.2.4. Linear viral DNA was added to the plasmid DNA / TC100 minus FCS to give a volume of 500μl. Cell monolayers were co-transfected with 100ng linearised BacPAK6 or BacPAK6.*chiA*$^-$ and 200ng of the appropriate recombinant baculovirus transfer vector, or 200ng virus and 1μg of transfer vector. Following a 48-h incubation at 28°C, co-transfection mixes were

removed and subjected to plaque assay (section 2.3.3). Putative recombinant baculoviruses were plaque-picked for further analysis (section 2.3.4).

2.3.3 Titration of viruses by plaque assay

Virus stock titres were assessed by plaque assay, as described by King and Possee (1992). *Sf*21 cells were seeded at $1.5x10^6$ cells per 35mm tissue culture dish in 2ml of TC100 medium supplement with 10% FCS and allowed to adhere. Virus inoculum was initially diluted (1:10) and subsequently used for serial (log) dilutions in TC100 medium supplemented with 10% FCS. Medium was removed from the cells; 100µl of the appropriate virus/medium dilution was added to the cells, infections were incubated at room temperature for 45 mins, allowing the virus to enter the cells. Virus inoculum was removed completely, 2ml of 2% low gelling temperature (LGT) agarose overlay mixed with an equal volume of TC100 medium supplemented with 10% FCS was applied to the cell monolayer and incubated at room temperature for 15 mins to set. A 1ml liquid overlay of TC100 medium supplemented with 10% FCS was added to each dish. Dishes were incubated at 28°C for 3-4 days.

Plaques were visualized by staining with Neutral Red (0.5% w/v stock solution, Sigma) for a minimum of 2 h at 28°C. In the case of viruses expressing the *lac*Z gene, 12.5µl of 2% X-gal (5-bromo-4-chloro-3-indoyl-β-galactoside) was added to the 1ml liquid overlay, dishes were incubated at 28°C for a minimum of 4 h. For both staining methods, after the incubation period, the dishes were emptied of their liquid content into 1% Virkon, dishes were then inverted and stored in the dark until the plaques had cleared. To determine the virus titre plaques were counted from the plate with best countable number. The average number of plaques counted was multiplied by the dilution factor and by 10 to give plaque-forming units per ml (pfu / ml).

Average number of plaques × dilution factor × 10 = pfu / ml

2.3.4 Amplification of virus stocks

All viruses used for comparative studies were amplified to high titre working stocks, in order to achieve valid date. Virus stocks were amplified from plaque picks.

Screening for recombinant virus against background parental virus in a plaque assay was based on blue, white selection: blue plaques represent parental virus expressing *lac*Z, white plaques (colourless) for recombinant viruses with insertional inactivation of *lac*Z.

From dishes with minimal background, well-isolated white plaques of recombinant virus were picked, using a sterile Pasteur pipette. The plaques were dispersed in 500µl of TC100 medium supplemented with 10% FCS by vortexing to assist release of the virus particles. This virus suspension was used to establish seed stocks (King and Possee, 1992). *Sf*21 cells were seeded in disposable 25cm^3 T-flasks at a density of 1x10^6 per flask. Medium was removed from the cell monolayer, 250µl of plaque-picked virus suspension was applied to the cells, the cells were incubated for 45 mins at room temperature. The inoculum was removed and replenished with 5ml fresh TC100 plus 10% FCS and incubated at 28°C for 4-7 days. At four to seven days post infection, the medium was harvested and cells were removed by centrifugation (2700 *xg*, 4 °C, 10 mins, Beckman J6B). The medium containing the recombinant virus was stored at 4°C. This first inoculum is referred to as the P1 stock.

From a P1 stock it was possible to increase amplification stocks to a P2 and P3 stock. The production of a P2 stock began with a 50ml spinner culture of *Sf*21 cells seeded at 0.5x10^6 cells / ml in TC100 plus 10% FCS. The spinner culture was inoculated at a low multiplicity of infection (moi) of 0.1 pfu / cell. The pfu / cell was determined by plaque assay (Section 2.3.3). The infection was incubated at 28°C for five days. At five days post-infection, cells were removed by centrifugation (1900 *xg*, 4°C, 10 mins Beckman J6B). The medium containing the virus was stored at 4°C. P3 stocks were carried out as described above, however 100-500ml spinner cultures of *Sf*21 cells were inoculated.

2.3.5 Production of high titre recombinant baculovirus inoculum from existing stocks

A shaker culture (50-1000ml) of *Sf*9 cells at a density of 2x10^6 cells / ml was infected with 0.1 multiplicity of infection (moi). At three days post infection, cells were pelleted (1900 *xg*, 4 °C, 15 mins, Beckman J6B) and discarded. The medium containing the virus was stored at 4°C and titrated by plaque assay (Section 2.3.3).

2.3.6.1 Recombinant protein production in insect cell lines

Cells were seeded at a final density of $2x10^6$ / ml (*Sf*9) or $1x10^6$ / ml (*T.ni* Hi5) in shake flasks, unless otherwise stated. Cells initially seeded in sterile shake flasks were as a concentrated volume, virus inoculum was added using a specific moi (2-10 pfu / cell). Infections were incubated at 28°C for 1 h on a Denley orbital shaker (135 rpm). Appropriate medium was added to adjust the cell density to the correct final cell density, and re-incubated at 28°C on a Denley orbital shaker (135 rpm). Infections were harvested at specified times post-infection. Proteins were visualised on SDS-PAGE and detected using Western blot analysis (Sections 2.7.5 / 2.7.6).

2.3.6.2 Recombinant protein production in insect cell lines using fermenters

Culturing of insect cells (*T.ni* Hi5 or *Sf*9) in fermenter vessels (Applikon®) was carried out in a final volume of 1-litre. Cells were seeded initially into sterile Duran bottles with vessel connecters at a density of $0.35x10^6$ cells / ml. Fermenter vessels were assembled and autoclaved empty (121°C, 10min) prior to use with pH and dissolved oxygen concentration (dO_2) probes attached. The vessels were washed with 100ml sterile medium prior to use via a headspace inlet line. The medium was removed through the vessel harvest line and discarded. All connections were made in the presence of 70% ethanol spray in order to maintain a sterile environment. The 1-litre cell culture ($0.35x10^6$ cells / ml) was connected to the harvest line and pumped into the sterile fermenter.

The cells were kept in suspension by stirring the culture with impellers (1x marine at the bottom of the vessel, 1x rushton at the culture surface) at 120 rpm. A supply of compressed air was introduced into the headspace using a filtered headspace inlet at a rate of 500ml / min. The same inlet was used to attach an oxygen supply line to provide oxygen to the headspace at a rate of 500ml / min (when required). A further oxygen supply was connected to the sparger intake, which delivered oxygen at a rate of 300ml / min. This supply was only activated when the dO_2 was lower than could be controlled from the oxygen in the headspace, which was set to maintain 60%. The temperature probe was inserted into the vessel and the exterior heating jacket connected. Using a bio-controller (Applikon® ADI 1030), the temperature was set to regulate cell growth at 27°C. Once 27°C was obtained, the interior cooling-finger was connected.

Cells were grown to the correct density, *Sf*9 2x10^6 cells / ml and *T.ni* Hi5 1x10^6 cells / ml. Cell viability and total counts were taken (section 2.2.6) to monitor cell growth. At the correct cell density, cells were infected with the appropriate recombinant baculovirus at the required moi. The virus was added aseptically via a sterile syringe and needle through a self-sealing septum at the top of the vessel, in the presence of 70% ethanol spray.

The fermentation was monitored using BioXpert software to measure changes in temperature, pH and dO_2 during the fermentation. These data were recorded on a graph throughout the period of fermentation.

To monitor virus infection, 1.5ml samples of the virus-infected cells were taken every 24 h. Viral-proteins were visualised on SDS-PAGE (Section 2.7.5) and recombinant proteins detected using western-blot analysis (Section 2.7.6). Harvesting infections were carried out using the vessel harvest line and collected into 4x 250ml disposable centrifuge tubes (Corning). Samples were centrifuged (1800 *xg*, 4°C, 30min, Beckman GS-6R) to pellet the infected cells. The medium was removed and discarded into 1% Virkon. Pellets were stored at –80°C prior to further analysis.

2.4 Standard Bacterial Manipulations

2.4.1 Culturing and storage of *E. coli* strains and clones

Bacterial cultures were grown in L-broth (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl, 2mM NaOH) or SOC medium (2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose) at 37°C on an orbital shaker rotating at 150 rpm (Certonat® BS-1) unless otherwise stated. The medium was supplemented with antibiotics where appropriate. This was to exert selective pressure on untransformed bacteria and to ensure only transformed bacteria expressing the appropriate antibiotic resistance gene were able to grow.

L-agar was prepared by adding 4.8g technical agar number 3 (Oxoid) to 400ml L-broth and autoclaved. Antibiotic L-agar plates were prepared by adding the appropriate filter-sterilised antibiotic (ampicillin 100μg / ml, kanamycin 50μg / ml) to cooled L-agar prior to plate pouring.

2.4.2 Preparation of competent bacterial cells

A variation to the method described in Sambrook *et al.* (1989) was employed to prepare competent cells for cloning purposes. Starter cultures of DH5α bacterial cells were propagated and then inoculated into 400ml of L-broth at a 1:100 dilution. The cultures were incubated on an orbital shaker (150 rpm) at 37°C for 1-2 h until the A_{600} reached an optical density (OD) of 0.4-0.5. This was determined using a spectrophotometer (Shimadzu). At this stage the cells were incubated on ice in the cold room (4°C) for 30 mins. The cultures were transferred to chilled universal centrifuge bottles and pelleted (1900 *xg*, 4°C, 15 mins, Beckman J6B centrifuge). The cell pellets were washed in reducing volumes of calcium chloride (50mM $CaCl_2$ containing 15% glycerol, 4°C). The final pellet was resuspended in 2ml of $CaCl_2$ and the bacteria aliquoted (50μl) into pre-chilled microfuge tubes and stored at -70°C. The competent cells were then tested for transformation efficiency with 1ng of known plasmid.

2.4.3 Transformation of competent cells

Competent cells were transformed with 1-5μl of ligation reaction or 10ng of circularised plasmid. Transformation procedure was based on either, the method described in Sambrook *et al.* (1989), or provided by the supplier. Microfuge tubes containing 50μl (DH5α, TOP10) or 100μl (DB3.1) of competent cells were mixed gently with ligation mix or circularised plasmid DNA and incubated on ice for 30 mins. The transformation mix was then heat-shocked for 45 seconds (DB3.1), or 30 seconds (DH5α, TOP10) at 42°C. Transformations were placed on ice and appropriate growth medium was added (DH5α, 450μl SOC, TOP10, 250μl SOC, DB3.1, 900μl SOC). Transformations were incubated for 1 h (DH5α, DB3.1) or 30 mins (TOP10) on an orbital shaker (225 rpm, 200 rpm) at 37°C to allow expression of the plasmids antibiotic resistance gene for selection purposes. The culture was then spread onto L-agar plates with the appropriate antibiotic and incubated at 37°C overnight. For long-term storage, the plates were sealed with parafilm and kept at 4°C.

2.5 Standard DNA Manipulation

2.5.1 Restriction endonuclease digestion

For analysis of DNA, up to 1μg of DNA was digested using 5-10 unit (U) of enzyme in the buffer recommended by the enzyme manufacturer. Restriction digests were incubated for 1-3 h at 37°C unless otherwise stated. For preparative digests, the amount of both DNA and enzyme were increased as required. Multiple digests were carried out simultaneously in universal buffer (multi-core) unless otherwise stated. Confirmation that DNA was digested appropriately was achieved by agarose gel electrophoresis (Section 2.7.1).

2.5.2 Filling recessed 3' termini

Recessed 3' ends of double-stranded DNA, generated by restriction enzyme digestion, were repaired to produce blunt ended double-stranded DNA fragment, with the use of large fragment DNA polymerase I (Klenow). Following digestion with the appropriate restriction enzyme, dNTPs (final concentration 0.25mM each) and Klenow fragment (1 unit μg DNA) was added. Samples were incubated at room temperature for 10 mins, reactions were terminated either by heating to 75°C for 10 mins or by extraction with equal volumes of phenol chloroform (1:1), followed by precipitation of the DNA with two volumes of absolute ethanol and 10% 3M sodium acetate.

2.5.3 Dephosphorylation of linear plasmid DNA

Following restriction enzyme digestion of plasmid DNA, shrimp alkaline phosphatase (SAP) was added directly to the reaction mix (>1 unit μg DNA) and then re-incubated at 37°C for 30 mins. Enzymes were inactivated by phenol:chloroform extraction and DNA was concentrated by ethanol precipitation. The dephosphorylated plasmid DNA was resuspended in TE buffer (10mM TrisHCl pH 8.0, 0.1mM EDTA) or distilled water (20-50μl). Recovery of DNA was confirmed by agarose gel electrophoresis (Section 2.7.1).

2.5.4 Ligation of DNA with blunt and cohesive ends

Ligation of DNA fragments to vector DNA was performed using a range of insert to vector concentrations (vector to insert 1:1, 1:2, 1:3, 1:4 and 1:5). Reactions were set up to a final

volume of 20µl, containing 30mM Tris-HCl (pH 7.8), 10mM $MgCl_2$, 10mM DTT and 1mM ATP (ligation buffer), vector and insert of known concentration, sterile water and 2.5 units of T4 DNA ligase per reaction. Ligations were incubated at 16°C for 16-18 h or 22°C for 3 h. A vector only control was included. Ligations were transformed as described in Section 2.4.3.

2.5.5 Amplification of DNA sequences using the polymerase chain reaction (PCR)

Amplification of DNA sequences using the polymerase chain reaction (PCR) was carried out in a Robocycler® gradient 96 thermo-cycler (Stratagene®). Reactions were carried out using template sequences requiring amplification with an initial concentration of 200ng / µl of DNA. Final reaction volumes of 100µl were routinely used containing 1x reaction buffer (50mM KCl, 10mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 1.5nM $MgCl_2$), primers (200ng / µl), dNTP's (final concentration 0.2mM) and 1 unit of Proof*sprinter*™ DNA polymerase. A standard program was followed for routine amplifications. The PCR program is detailed in **Table 2.1.**

Table 2.1: Programme for amplification of DNA using PCR

Temperature block	Temperature °C / duration mins	No. of cycles
1	95 for 6	1
1	95 for 1	30-35
2	n^1 for 1	30-35
3	72 for n^2	30-35
3	72 for 10	1
4	6 for storage	0

n^1 Depends on Tm of primers.
n^2 depends on the size of DNA fragment wished to be amplified.

2.5.6 Reverse transcription polymerase chain reaction (RT-PCR)

Synthesis of cDNA was achieved using the Omniscript reverse transcriptase (QIAGEN) is a recombinant heterodimeric enzyme expressed in *E. coli.* For the reverse transcription of total insect RNA to single stranded DNA, 2µg of RNA was mixed on ice with 2µl 10µM oligo-dT primers (15mers), 2µl 10x buffer RT, 2µl dNTP mix (5mM each dNTP), 1µl RNase inhibitor (10 units / µl), 1µl Omniscript reverse transcriptase (4 units / µl), and RNA

free water to a final reaction volume of 20µl. The reaction was incubated at 37°C for 60 mins.

For analysis of cDNAs by PCR (Section 2.5.5), Omniscript reverse transcriptase was inactivated by incubating the reaction at 93°C for 5 mins followed by rapid cooling on ice. To a fresh, 10µl of the reverse transcriptase reaction was added, and subjected to PCR (final volume 50µl) for further analysis.

2.5.7 Column purification of DNA

2.5.7.1 From PCR reactions

Amplified DNA was purified from PCR reactions using QIAGEN QIAquick® Mini columns and method. Confirmation of a correct PCR product was confirmed by running 1:10 of the PCR reaction on an agarose gel (Section 2.7.1). The remaining PCR reaction was mixed with five volumes of PB buffer (supplied by, QIAGEN) and mixed; the solution was applied to a QIAquick column and pulled through by centrifugation (11300 *xg*, 1 min, microfuge). The DNA binds to the column while unwanted impurities pass through. The columns were washed with 750µl PE buffer (supplied by, QIAGEN). DNA was eluted in 30-50µl of sterile distilled water.

2.5.7.2 From agarose gels

Restriction digested DNA was visualised using agarose gel electrophoresis (Section 2.7.1). Specific DNA bands were excised from the agarose gel in order to obtain cloning-quality DNA. The gel extraction method was based on the QIAquick gel extraction protocol (QIAGEN).

Target DNA bands were excised from the agarose gel using a scalpel and placed into 1.5ml microfuge tubes with three times the volume of QG buffer, supplied by, QIAGEN (for example, 300µl of QG buffer was added to each 100mg of gel / DNA excised) and dissolved. To assist dissolving the gel, the tubes were incubated at 50°C for 10 mins and vortexed every 2 mins. Once the gel had completely dissolved, one volume of isopropanol was added (only if band size was <0.5kb and >5kb) and the mixture was applied onto a QIAqiuck column and centrifuged (11300 *xg*, 1 min, microfuge). For DNA

subsequently used for direct sequencing, *in vitro* transcription or microinjection, the DNA was washed additionally with 500µl of QG buffer prior to an ethanol based wash with 750µl of PE buffer. The DNA fragments were eluted in 30-50µl of distilled water. Purified linear DNA was analysed using agarose gel electrophoresis (Section 2.7.1) to determine its concentration.

2.5.8 DNA sequencing

Sequencing was performed at the DNA sequencing facility, Department of Biochemistry, University of Oxford, using ABI Big Dye terminator chemistry. Reactions used the cycle sequencing method (**Table 2.2**) to generate authentic data. A standard set of cycling conditions was used and is listed in **Table 2.2.**

Table 2.2: Programme used for sequencing of DNA, at the DNA sequencing facility, Department of Biochemistry, Oxford.

BigDye™ terminator cycling conditions	
Pre-denaturation	10 seconds @ 96°C
Followed by:	
Denaturation	30 seconds @ 98°C
Annealing	15 seconds @ 50°C
Extension	4 mins @ 60°C
Number of cycles	25 cycles

2.5.9 Purification of plasmid DNA

2.5.9.1 Small-scale plasmid DNA purification (mini-prep)

Isolated colonies of bacteria transformed with the specified plasmid of choice were inoculated into 5ml of L-broth containing the appropriate antibiotic (starter culture). Cultures were incubated at 37°C for 7-18 h with continuous agitation (150 rpm). From the transformed bacterial culture 1.5ml was transferred to a microfuge tube and pelleted (1100 *xg*, 4°C, 5 mins, microfuge). The medium fraction was discarded into 1% Virkon, whereas the bacterial pellet was resuspended in 100µl of glucose-based solution (50mM glucose, 25mM Tris-HCl, pH 8.0, 10mM EDTA). Bacterial lysis was achieved with the addition of 200µl of cell lysis buffer (200mM NaOH, 1% SDS and 100µg / ml ribonuclease A), which was incubated at room temperature for 5 mins. Cell lysis was terminated by the addition

of 150µl neutralization buffer (5M KAc, 110ml glacial acetic acid, and distilled water, 4°C) and incubation on ice for 10 mins. Cell debris was removed by centrifugation (11300 *xg*, 20°C, 10 mins, microfuge). The supernatant fraction containing plasmid DNA was transferred to a fresh microfuge tube.

Protein traces were removed by phenol / chloroform extraction (1:1 v / v) and the DNA precipitated with two volumes of absolute ethanol and 10% 3M sodium acetate. The ethanol precipitate was incubated at -20°C for 1 h. DNA was pelleted (14100 *xg*, 20°C, 10 mins microfuge) and washed twice with 75% ethanol, all traces of ethanol were removed and the DNA was left to air-dry for 5 mins. The DNA was resuspended in 20µl of sterile distilled water and subsequently used for restriction digestion for identification of positive clones. Initial cultures were stored at 4°C until their further use in the production of large quantities of DNA.

2.5.9.2 Large-scale plasmid DNA purification (pure-prep)

Plasmid DNA was extracted from transformed bacteria using a modification of the method of Sambrook *et al.* (1989). A 1-litre flask containing 400ml L-broth and the appropriate antibiotic were inoculated with 1-3ml of the specific starter culture transformed with the appropriate plasmid. The 400ml culture was propagated for 14-18 h at 37°C with shaking (150 rpm).

The cells were pelleted (2500 *xg*, 4°C, 10 mins, Beckman J-6B centrifuge) and the medium fraction discarded into 1% Virkon, the pellet was resuspended in 7.5ml of sucrose buffer (50mM Tris-HCl, pH 8.0, 25% sucrose). The cell suspension was transferred to two chilled Beckman centrifuge tubes before adding 1.5ml lysozyme (10mg / ml made up fresh in sucrose buffer) and incubated on ice for 10 mins. RNase A (125µl; 10mg / ml) and 3ml Tris / EDTA solution (250mM Tris-HCl, pH 8.0, 250mM EDTA) was added and re-incubated on ice for 5 mins. Triton lysis buffer (12.5ml; 2% v/v Triton X-100, 50mM Tris-HCl, pH 8.0, 10mM EDTA) was added, samples were mixed gently and re-incubated on ice for 10 mins.

Cellular debris was pelleted (26000 *xg*, 4°C, 1 h, Beckman Ti-70 rotor, Beckman ultra-centrifuge). The supernatant fraction containing plasmid DNA was transferred to a fresh 50ml polypropylene tube and SDS was added to 1% (w / v). Protein traces were removed

by phenol / chloroform extraction (1:1 v / v). Equal volumes of phenol / chloroform were added to the supernatant (5ml of each), the solution was mixed vigorously by vortexing then incubated at room temperature for 10 mins. To separate the aqueous phases the solutions were centrifuged (1900 *xg*, 4°C, 20 mins, Beckman J-6B centrifuge). The top clear aqueous phase of the solution was carefully transferred to a fresh polypropylene tube. Two volumes of absolute ethanol and 10% 3M sodium acetate were added and incubated at –20°C for 1 h, to precipitate the DNA. The DNA was pelleted (1800 *xg*, 4°C, 30 mins, Denley BS400 centrifuge), washed in 75% ethanol and re-pelleted. The ethanol was removed and the pellet was briefly air-dried.

The pellet was resuspended in 7.5ml TE buffer to which 8g cesium chloride (CsCl) and 75µl ethidium bromide (10mg / ml) was added. The solution was loaded into a Beckman quick-seal polyallomer tube, filled with liquid paraffin and heat-sealed. Tubes were centrifuged (133042 *xg*, 20°C, 16-20 h, Beckman Ti-70 rotor, Beckman ultra-centrifuge).

The plasmid DNA was harvested and ethidium bromide removed by repeated extractions with an equal volume of butanol. The DNA / CsCl solution was diluted 1:3 by the addition of two volumes of TE and sodium acetate to 300mM, the DNA was ethanol precipitated. DNA was pelleted (1800 *xg*, 4°C, 30 mins, Denley BS400 centrifuge), washed in 75% ethanol and re-pelleted. The DNA was resuspended in 1ml TE buffer and SDS to 1%, followed by phenol / chloroform extraction. The DNA was re-precipitated, pelleted (600 *xg*, 4°C, 5 mins, microfuge) and washed twice in 75% ethanol. The plasmid DNA pellet was air-dried and resuspended in 500µl TE and stored at –20°C. The concentration and purity of the plasmid was determined using a spectrophotometer (Shimadzu) at 260 / 280nm.

2.5.10 Extraction of DNA from virus-infected cells

The method described by King and Possee (1992) was employed to extract DNA from virus-infected cells. Cells were seeded at the required density as described in Section 2.2.3, the medium was removed and discarded into 1% Virkon. Cells were infected using a moi of 10 pfu / cell, and incubated at room temperature for 1 h. The inoculum was removed and discarded into 1% Virkon, and 1.5 ml appropriate medium was carefully added to the cell monolayer. Cells were incubated for 18-26 h at 28°C. Cells were harvested with a Pasteur pipette and transfer to a 1.5ml microfuge tube, and pelleted (600

xg, 10 mins, microfuge). The medium was discarded into 1% Virkon. Pellets were washed once with PBS and resuspended in 250µl TE, 250µl of cell lysis buffer (50mM Tris-HCl, pH 8.0, 5% 2- mercaptoethanol, 0.4% w / v SDS, 10mM EDTA) was added and gently mixed. Proteinase K (12.5µl; 10mg / ml made up fresh in TE and pre-incubated at 37°C for 30 mins prior to use) and 2.5µl RNase A (10mg / ml) was added to the sample, and incubated at 37°C for 30 mins.

The cellular lysate was extracted with an equal volume of phenol / chloroform as described in Section 2.5.9.1 (violent shaking was avoided as this shears the high molecular-weight virus DNA), a second extraction with phenol / chloroform was repeated. The top aqueous phase was transferred to a fresh microfuge tube, and 50µl 3M sodium acetate and two volumes of absolute ethanol were added to precipitate the DNA. The DNA was pelleted (14100 *xg*, 4°C, 10 mins, microfuge) and washed twice with 75% ethanol. All traces of ethanol were removed with a drawn-out Pastuer pipette. The pellet was air-dried and resuspended in 100µl TE by socking overnight at 4°C, then incubating at 37°C for 10 mins, and finally gently resuspended the pellet with a Gilson tip. The DNA was stored at –20°C.

2.5.11 Extraction of total RNA from insect cells

Total cytoplasmic RNA was isolated from $1.5x10^6$ *Sf*9 or *T.ni* Hi5 cells. RNA extraction was carried out using the Rneasy kit from Qiagen. The kit encompassed the selective purification of cytoplasmic RNA, which comprises approximately 85% of the total cellular RNA content.

Cells grown in $25cm^3$ T-flasks were harvested and transferred to 15ml centrifuge tubes. The cells were pelleted (200 *xg*, 10min, Denley BS400 centrifuge), medium was removed and discarded into 1% Virkon. Cell pellets were resuspended into 175µl of cold RLN buffer (50mM Tris-HCl, pH 8.0, 140mM NaCl, 1.5mM $MgCl_2$, 0.5% (v / v) Nonidet P-40) and incubated on ice for 5 mins to allow complete cell lysis to occur. Cell debris was pelleted (11300 *xg*, 4°C, 10 mins, microfuge) and the supernatant transferred to a fresh 1.5ml microfuge tube. RNase traces were removed by addition of 600µl of RLT buffer (supplied by, QIAGEN) to the cytoplasmic extract, which contained guanidine isothiocyanate supplemented with 10µl / ml β-mercaptoethanol. To the homogenate 430µl of ethanol (100%) was added to create the appropriate conditions for selective binding of

RNA onto the silica-gel based membrane of the RNeasy columns. The RNA-containing mix was transferred onto the column and RNA bound to the membrane by centrifugation (6700 *xg*, 4°C, 15 sec, microfuge). The flow-through was discarded before 700µl of RW1 buffer (supplied by, QIAGEN) was added to the column. The column was washed by centrifugation (6700 *xg*, 4°C, 15 sec, microfuge), before two final washes with 500µl RPE buffer (supplied by, QIAGEN). The bound RNA was eluted in DEPC-treated water (0.01% of DECP). The recovered RNA was visualised on a formalin-based gel (Section 2.7.2) and quantified using a spectrophotometer (Shimadzu) at 260/280nm. Purified RNA was stored at –70°C.

2.5.12 Directional TOPO® cloning

Directional TOPO® cloning was performed using two sub-cloning steps. In this system, PCR products were directionally cloned into a TOPO® entry vector by adding four bases to the beginning of the forward primer (CACC). DNA of interest was amplified by PCR (Section 2.5.5). PCR products were visualised on an agarose gel (Section 2.7.1), and purified (Section 2.5.7.1). Purified PCR product (10-100ng) was added to a standard cloning reaction as described in **Table 2.3** to generate a TOPO® entry clone.

Table 2.3: Standard cloning reaction set up to generate a TOPO® entry clone.

Reagent	Volumes added to 1.5ml microfuge tube (µl)
Fresh PCR product	0.5 to 4
Salt solution	1
Sterile water	Add to a final volume of 5
TOPO® entry vector	1

The cloning reaction was mixed gently and incubated for 5 mins at room temperature (22-23°C). From the TOPO® cloning reaction 2µl was transformed using TOP10 chemically competent *E. coli* (Section 2.4.3). Mini-preps were performed to identify positive clones (Section 2.5.9.1). Once positive clones were identified, a pure-prep was performed (Section 2.5.9.2).

Once the TOPO® entry clone had been constructed, it was then possible to generate an expression construct by performing a recombination reaction between the entry clone and

a linear Gateway™ destination vector of choice (pXINsect-DEST38, pXINsect-DEST39). The protocol for producing LR Clonase™ reactions is provided in **Table 2.4.**

Table 2.4: Standard LR Clonase™ reaction to generate a TOPO® expression construct.

Components	Negative control	Sample
LR reaction buffer (5x)	4μl	4μl
Entry clone (100-300ng / reaction)	--	1-11μl
Destination vector (100-300ng / reaction)	1-11μl	1-11μl
TE buffer	To 16μl	To 16μl
LR Clonase™ Enzyme mix	4μl	4μl

The LR Clonase™ reactions were incubated at 25°C for 60 mins. Proteinase K solution (2μl; 2mg / ml) was added to the LR Clonase™ reaction and incubated at 37°C for 10 mins, this terminated the LR Clonase™ reaction. From the LR Clonase™ reaction 1μl was transformed using DH5α chemically competent *E. coli* (Section 2.4.3). Mini-preps were performed to identify positive clones (Section 2.5.9.1). Once positive clones were identified, a pure-prep was performed (Section 2.5.9.2).

2.6 Protein Purification

2.6.1 Purification of 6x-Histidine-tagged recombinant proteins

Cell pellets containing recombinant protein were retrieved from the –80°C freezer (Section 2.3.6.1 / 2.3.6.2) and thawed on ice for 30min. Pellets were resuspended in 10ml cell lysis buffer per gram of cell pellet (50mM Tris-HCl, pH8.0, 10% v / v glycerol, 150mM sodium chloride, protease inhibitor cocktail EDTA-free tables, Sigma (one tablet per 50ml), 0. 1% v / v Triton X-100). A 50μl sample of the lysate was taken for SDS-PAGE analysis (Section 2.7.5). The lysate was passed through a cell disrupter (20 kpsi, 13°C, Constant systems Ltd, Cell disruption equipment B series) collected in a clean beaker and placed on ice. The lysate was centrifuged to pellet cellular debris (36514 *xg*, 4°C, 45min, SW-34 rotor, Pu Pont instruments, Sorvall® RC-5B). The supernatant fraction containing soluble histidine-tagged (His) recombinant protein was transferred to a sterile Duran bottle and placed on ice. The pellet was resuspended in 10ml PBS. Samples (50μl) of the supernatant and pellet were taken for SDS-PAGE analysis (Section 2.7.5). The pellet was decontaminated by autoclaving (121°C, 10min) and discarded into 1% Virkon.

A HiTrap chelating sepharose™ high performance 5ml (Amersham Biosciences) protein purification column was used to bind 6xHis-tagged recombinant proteins. The column was charged with metal ions prior to use. First the column was washed with 15ml de-ionised water using a syringe, followed by charging with 7ml 0.1mM nickel sulphate. A second 15ml wash with de-ionised water was carried out to remove unbound nickel. The charged column was connected to a ÄKTA explorer chromatography system (Amersham Biosciences). The ÄKTA explorer pumps were washed with 10ml binding buffer pH 8.0 (50mM Tris-HCl, pH 8.0, 10% v / v glycerol, 150mM sodium chloride, 5mM β-mercaptoethanol, 20mM imidazole) and 10ml elution buffer pH 8.0 (50mM Tris-HCl, pH 8.0, 10% v / v glycerol, 150mM sodium chloride, 5mM β-mercaptoethanol, 500mM imidazole) at a rate of 5ml / min, before the sample was injected onto the HiTrap chelating sepharose™ 5ml column. The sample was injected into a super-loop (Amersham Biosciences) using a syringe, and connected to the injection-valve of the ÄKTA explorer. Binding buffer from the ÄKTA pumps was injected into the super-loop, causing the sample to be injected into ÄKTA explorer. The sample was then passed through the column, binding proteins that contained histidine repeats. The column was first equilibrated with one column volume (CV) of binding buffer. The sample was then injected into the column at a rate of 2ml / min. The flow through was collected in 10ml fractions using a Frac-900 (Amersham Biosciences). The column was then washed with ten CV of binding buffer, followed by 5 CV of 92% binding buffer and 8% elution buffer. This was to remove weakly bound his-containing proteins and non-specific binding.

Samples (50μl) of each step were taken for SDS-PAGE analysis (Section 2.7.5). The process was paused to attach a Hi-Prep™ G-25 fine desalting-column (Amersham Biosciences) to the bottom of the HiTrap chelating sepharose™ column. It was important at this step to remove any imidazole present in the eluted sample. This was achieved by passing the eluted sample through the desalt column. The desalt-column was washed prior to use with desalt buffer pH 8.0 (50mM Tris-HCl, pH 8.0, 10% v / v glycerol, 50mM sodium chloride) at a rate of 5ml / min for 20mins.

Protein bound to the HiTrap chelating sepharose™ column was eluted with 20 CV of 100% elution buffer. The eluted protein sample was desalted and collected in 2ml fractions using a Frac-900. A computer connected to the ÄKTA explorer chromatography system monitored the specific time of protein elution from the column. This identified, which 2ml fractions of elution buffer contained eluted protein. The fractions were pooled and

analysed using SDS-PAGE / western blot analysis (Sections 2.7.5 / 2.7.6). The remaining sample was stored at –20°C in 2ml aliquots prior to further analysis.

2.7 Electrophoretic techniques

2.7.1 Agarose gel electrophoresis for DNA

Horizontal agarose gels were used routinely for DNA analysis, using apparatus supplied by Stratagene®. Concentrations of agarose (Invitrogen™, LifeTechnologies, Gibco BRL, USA.) between 0.7-2.0% were used, depending on the size of DNA to be analysed. Submerged gel electrophoresis was performed in 1x TAE buffer (2M Trizma base, 50mM EDTA, pH 8.0 using glacial acetic acid, make up to 1 litre with sterile water, makes 50x). Samples were mixed with 5x DNA loading dye (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) prior to electrophoresis. Gels were resolved at 100 volts for 1 h or 20 volts overnight. DNA was visualised by the addition of ethidium bromide (5µl of 10mg / ml) to the gel prior to casting. Stained gels were placed on a Chromato-vue UV transilluminator (UVP, Inc, USA) and photographed using a UVP video camera.

2.7.2 Agarose gel electrophoresis for RNA

Analysis of RNA was carried out in 1.2% formaldehyde-agarose (FA) gels. The gels consisted of 1.2g of agarose (Sigma-Aldrich, UK), 10xFA gel buffer (200mM 3-[N-morpholino]propanesulfonic acid (MOPS), 50mM sodium acetate and 10mM EDTA), and were adjusted to 100ml with RNAse-free water. After dissolving the agarose and cooling to 65°C, 1.8ml of 37°C (12.3M) formaldehyde and 1µl ethidium bromide from a 10mg / ml stock were added. Prior to electrophoresis, the gels were submerged in 1xFA buffer for 30mins. Gels were resolved at 100 volts for 1 h or 20 volts overnight. RNA was visualised using a Chromato-vue UV transilluminator (UVP, Inc, USA) and photographed using a UVP video camera.

2.7.3 Extraction of protein from insect cells for analysis by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Cells were harvested from 35mm tissue culture dishes by removing the medium and adding 500µl cell lysis buffer (10% 1M Tris-HCl, pH 8.0, 0.1% Triton® X-100, 89.9% sterile

water), cells were resuspended into the cell lysis buffer and transferred to a 1.5ml eppendorf tube and vortexed. Cell nucleus and membranes were pelleted (11300 *xg*, 2mins, microfuge). The supernatant fraction containing the intracellular proteins was transferred to fresh 2ml microfuge tubes. The cell pellet was resuspended in 100µl cell lysis buffer. Samples at this stage were either stored at –70°C or 4x SDS-PAGE sample buffer (50% 0.5M Tris-HCl, pH 6.8, 40% glycerol, 8% β-mercaptoethanol, 0.04g Bromophenol Blue, 0.8g SDS) was added, 125µl to the supernatant fraction, and 25µl to the pellet fraction. All samples were incubated at 100°C in a boiling incubator for 5 mins prior to electrophoresis.

2.7.4 Protein estimation using the Bio-Rad protein assay

The protein content of all samples was determined using the Bio-Rad protein assay (Bio-Rad). This method of protein quantification is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm, when binding to protein occurs (Bradford, 1976). Protein estimation was carried out according to the manufactures instructions. Samples optical density (OD) was read (595nm) using a biowave S2100 Diode Array spectrophotometer (WAP Ltd).

2.7.5 SDS-PAGE

Glass plates were assembled according to manufacturers instructions (Invitrogen™, LifeTechnologies, Gibco BRL, USA.). Acrylamide gels at a concentration of 12% w / v were de-gassed and cast (32.6% sterile water, 40% protoGel [30% acrylamide, 0.8% Bis-acrylamide] 25.3% Tris-HCl, pH 8.8, 1% SDS, 1% Ammonium sulfate at 100mg / ml, 0.04% TEMED; all v / v) unless otherwise stated. Water-equilibrated butanol was layered onto the gel, which was then allowed to set. Once set, the butanol was removed and the exposed gel and glass washed three times with sterile water, and all traces of water were removed. A stacker gel was cast on top of the resolving gel (70% sterile water, 16.6% protoGel [30% acrylamide, 0.8% Bis-acrylamide], 12.5% Tris-HCl, pH 6.8, 1% SDS, 1% Ammonium sulfate at 100mg / ml, 0.1% TEMED; all v / v). Previously denatured (Section 2.7.3) samples were loaded into preformed sample wells in the stacker gel. Samples were resolved at 150 volts for 2 h. Gels were either stained with Coomassie brilliant blue (0.25g Coomassie brilliant blue, 45ml methanol, 45ml sterile water, 10ml glacial acetic acid) and destained by washing with destain (45% methanol, 45% sterile water, 10%

glacial acetic acid) as detailed in Sambrook *et al.* (1989), or stained with antibody via the procedure of Western blotting (Section 2.7.6).

2.7.6 Analysis of proteins by Western blot

Immunological detection of proteins on nitrocellulose membrane was employed as a means to visualise the presence of specific polypeptides / proteins. Following SDS-PAGE, proteins were transferred onto nitrocellulose membranes using a wet-blot apparatus (King and Possee, 1992). The nitrocellulose membrane was washed in blocking solution (10% w / v non-fat milk, 0.1% v / v Tween 20 in PBS) for 1 h at room temperature. Primary antibody specific to the recombinant protein was diluted (1 / 500-1 / 10000) in blocking solution and applied to the membrane for 1 h at room temperature. The excess antibody was removed and the membrane washed five times for 5 mins at room temperature in PBST buffer (0.1% v / v Tween 20 in PBS). Secondary anti-antibody (alkaline phosphatase-conjugated antibody) was applied to the membrane and incubated for 1 h at room temperature. The excess secondary anti-antibody was removed and the membrane washed five times for 5 mins at room temperature in PBST buffer. The membrane was developed in an alkaline phosphatase development buffer (20mls alkaline phosphatase buffer, 100mM Tris-HCl, pH 9.5, 5mM $MgCl_2$, 100mM NaCl,132µl nitro blue tetrazolium chloride, Sigma, 66µl 5-bromo-4-chloro-3-indolyl phosphate, Sigma) to detect proteins. The membrane was rinsed in sterile water to terminate the development reaction.

2.8 Enzyme assays

2.8.1 Chloramphenicol acetyltransferase assay

A variation of the method described by Gorman *et al.* (1982) was employed to assay for chloramphenicol acetyltransferase (CAT) production. Cell samples were harvested by resuspending the cells into the medium, and transferred to 2ml microfuge tubes. Cells were pelleted (1700 *xg*, 4°C, 10 mins, microfuge) and washed twice in PBS and re-pelleted. Cell pellets were resuspended in 100µl 250nM Tris-HCl, pH 7.8. Cells were lysed by three freeze-thaw cycles in a dry ice ethanol bath followed by incubation at 37°C until thawed. Samples were subjected to sonication (3 mins) to complete cell lysis. Cell debris was pelleted (1700 *xg*, 4°C, 5 mins, microfuge) and 80µl of the supernatant was

transferred to a fresh 1.5ml microfuge tube containing 20µl sterile water. To each sample 5µl of 1-Deoxy[dichloroacetyl-1-^{14}C] chloramphenicol (0.004625Mbqi) was added and incubated at 37°C for 10 mins. Acetyl coenzyme A (10µl; 4mM) was added to each reaction and incubated at 37°C for 1 h. Samples were briefly centrifuged, 1-Deoxy[dichloroacetyl-1-^{14}C] chloramphenicol was extracted using 1ml of ethyl acetate. Reactions were vortexed for 30 seconds and centrifuged (14100 *xg*, 3 mins, microfuge) to separate the aqueous phases. The upper aqueous phase was transferred to a fresh 1.5ml microfuge tube and dried under heat-vacuum centrifugation for 1 h. Dry samples were resuspended in 20µl ethyl acetate (vortex 30 seconds / sample). Reactions were dotted onto an aluminum backed silica gel thin layer chromatography (TLC) plate (Whatman) in 2µl aliquots, allowing spots to dry between applications until all the reaction mix was absorbed.

The TLC plate was then subjected to ascending chromatography in a tank pre-equilibrated with a 95:5 mixture of chloroform and methanol (final volume 300ml). Plates were air-dried and exposed to a blanked storage-phosphor screen for 1 h. The storage-phosphor screen was scanned using a Typhoon 8600 variable mode imager (Molecular dynamics subdivision of Amersham Pharmacia Biotech) and results quantified by calculating the percentage conversion of 1-Deoxy[dichloroacetyl-1-^{14}C] chloramphenicol to acetylated chloramphenicol using the software program Image-Quant 5.1.

2.8.2 Dihydroorotate dehydrogenase (DHODH) assay

A variation of the method described by Löffle *et al.*, (1997) was used to assay dihydroorotate dehydrogenase (DHODH) functional activity. DHODH produced using the baculovirus expression system in insect cells (Sections 2.3.6.1 / 2.3.6.2) was purified (Section 2.6.1), and total protein of each purified sample quantified (Section 2.7.4).

To a fresh 1.5ml microfuge tube on ice, 36µl 2,6 dichlorophenylindolphenol (1mM 2,6 dichlorophenylindolphenol [DCIP] / 0.1mM Tris-HCl, pH 8.0), 50µl Co-enzyme Q6 (5mM Co-enzyme Q6 / 0.1mM Tris-HCl, pH 8.0), 20µl L-dihydroorotate (1mM L-dihydroorotate / 0.1mM Tris-HCl, pH 8.0) was added. The volume of Tris-HCl (0.1mM Tri-HCl, pH 8.0) varied according to the sample volume that was added. The final volume totalled 1ml.

Samples were immediately read using a Cary 300 scan UV visible spectrophotometer at 595nm over a period of 2mins. The specific activity of DHODH was calculated using the following equations.

$\frac{\Delta A / min}{\epsilon}$ = mM conversion of DCIP / min

Where ΔA = slope of absorbance over linear range (1min).

ϵ = millimolar extinction coefficient of DCIP ($21 mM^{-1} \cdot cm^{-1}$ at 595nm).

To convert mM conversion of DCIP / min to nmoles conversion of DCIP / min the following equation was used:

M x V = nmoles conversion of DCIP / min

Where M = molarity and V = volume (litre)

To convert nmoles conversion of DCIP / min to µmoles conversion of DCIP / min / mg of total protein the following equation was used:

$\frac{\text{nmoles conversion of DCIP / min}}{\text{mg of total protein}}$ = µmoles conversion of DCIP / min / mg

2.9 Fluorimetric assays

2.9.1 DsRed fluorescent assay

One ml cell samples were harvested into 1.5ml microfuge tubes and pelleted (600 *xg*, 10 mins, microfuge). The growth medium was removed and transferred to fresh 1.5ml microfuge tubes. The cell pellets and growth medium was assayed immediately or stored at –80°C pending further analysis.

Cell pellets were washed in 1ml PBS and re-pelleted (600 *xg*, 10 mins, microfuge). PBS was removed and discarded into 1% Virkon. Cell pellets were resuspended into 100µl cell lysis buffer (10% 1M Tris-HCl, pH 8.0, 0.1% Triton® X-100, 89.9% sterile water). The samples were centrifuged (14100 *xg*, 5 mins, microfuge) to pellet cellular debris. The intracellular supernatant fractions (100µl) were transferred to individual wells of a black

96-well, flat bottom microplate and the OD read using a LAMBDA FLUORO-320 microplate fluorescent reader (MWG-Biotech) at 530 / 590nm. The growth medium (100μl) was assayed as described above.

CHAPTER 3: ANALYSIS OF TRANSIENT PROMOTER ACTIVITY IN INSECT CELLS

3.1 Introduction

The ability to produce high-levels of authentic recombinant proteins relies on the successful interaction between an expression system and the host in which it functions. Insect cells have been used as successful hosts for the production of recombinant proteins, since they are generally able to perform the post-translational modifications often necessary for proteins to be functionally active (O'Reilly and Miller, 1989; King and Possee, 1992; Altmann *et al.*, 1999). One of the first insect expression systems to be exploited for the production of high-level recombinant proteins was the baculovirus expression system, in which a foreign gene is placed under the control of the promoter for the non-essential viral polyhedrin (*polh*) gene, (Smith *et al.*, 1983a,b; Pennock *et al.*, 1984). The most significant advantage of this expression system is the strength of the *polh* promoter, compared to others used in eukaryotic expression vectors. Although yields of recombinant protein produced are generally high, the disadvantage is expression can only be sustained for up to 96 hpi due to the lytic nature of the virus infection. Also, the level of expression is not entirely reproducible and depends on the degree of infection of the cells (Broeck *et al.*, 1995).

One way to overcome the limitations of the baculovirus expression system would be to develop a baculovirus vector with only those viral genes required to initiate optimal transcription from the *p10* or *polh* promoters. Unfortunately, the size and complexity of the baculovirus genome makes this approach difficult.

A simpler strategy has been adopted, which takes advantage of the same principles used to develop mammalian plasmid-based expression systems (Wigler *et al.*, 1980; Southern and Berg, 1982; Bebbington *et al.*, 1992). Foreign gene products are expressed under the control of a viral or cellular promoter, which is transcriptionally controlled by an α-amanitin-sensitive RNA polymerase (Fuchs *et al.*, 1983; Glocker *et al.*, 1992). The characterisation of *ie*-1, an immediate early gene of the baculovirus *Ac*MNPV, was found to contain such a promoter (Guarino and Summers, 1986; 1987). Since the *Ac*MNPV *ie*-1 promoter requires only host cell RNA polymerase II to be transcribed, it was initially used for the development of a plasmid-based expression system, which has been shown to work in insect cells (Jarvis and Summers, 1989; Jarvis *et al.*, 1990).

Insect cells are transformed by co-transfecting a mixture of two plasmids, one containing a dominant selectable marker e.g. neomycin-resistant gene, the second a foreign gene, both under the control of the *Ac*MNPV *ie*-1 promoter. Transformed cells were found to be stable, expressed foreign genes continuously, and were able to process complex glycoproteins, such as tissue plasminogen activator (Jarvis *et al.*, 1990).

A plethora of recombinant cell lines have been produced using this method (Johansen *et al.*, 1989a,b; Joyce *et al.*, 1993; Broeck *et al.*, 1995; Henderson *et al.*, 1995; McCarroll and King, 1997; Hegedus *et al.*, 1998). The main advantage of the plasmid-based system over the baculovirus system is its non-lytic nature; therefore, expression of a foreign gene is continuous, allowing long-term expression without the detrimental effects of virus infection. However the disadvantage of this system is the weak transcriptional activity of the *Ac*MNPV *ie*-1 promoter; expression levels are very low and do not compare favourably to other expression systems (Guarino and Summers, 1988; Jarvis *et al.*, 1990; Joyce *et al.*, 1993).

Levels of protein production equal to those observed using the baculovirus expression system have been reported using the *Bm*NPV *ie*-1 promoter in *Drosophila* cells (Broeck *et al.*, 1995). It was also reported that over-expression of the secreted glycoprotein, juvenile hormone esterase, yielded 47-fold more active protein in stable *Bm*5 cells, under the control of the *BmA*3 gene promoter / enhancers, compared to levels achieved using the *p10* promoter of *Ac*MNPV in infected *Sf*21 cells (Farrell *et al.*, 1998).

Technological advances in stable insect expression systems, has been inherently slow over the last 10 years, and only a few new systems have been developed. The discovery of new promoters for mammalian based expression over the last decade has led to the development of numerous plasmid-based expression systems, offering control of expression and enhanced transcriptional activity. The common factor between current plasmid-based expression systems is the large percentage of promoters that constitutively express foreign genes. A range of inducible promoters are available for use in mammalian cell lines, whilst the choice of inducible promoter for use in insect cells is very limited. The only inducible insect expression system available commercially, is the inducible *Drosophila* system (Invitrogen™).

Inducible expression offers the choice to produce cytotoxic proteins, which can be detrimental to the cells viability if expressed under the control of a constitutive promoter.

The *Drosophila melanogaster* (*Dm*) metallothionein (*MT*) promoter (Maroni *et al.*, 1986; Otto *et al.*, 1987; Bunch *et al.*, 1988) has been characterised as an inducible promoter. The *Dm MT* gene promoter has been used to express foreign genes upon induction with copper sulphate (Otto *et al.*, 1987; Hu and Davidson, 1990) and is currently employed in the inducible *Drosophila* system.

In order to further the use of plasmid-based expression systems, it was necessary to attempt to identify promoters that significantly improved foreign gene expression above the existing *Ac*MNPV *ie*-1 promoter in *Sf*9 and *T.ni* Hi5 cell lines. The identification of such promoters would subsequently lead to the attempt to generate a novel inducible plasmid-based expression system for use in *Sf*9 and *T.ni* Hi5 cell lines.

The gene promoter used in this system need not be of baculovirus in origin providing transcription can be initiated by insect cell transcription factors. Eukaryotic gene promoters and viral immediate early promoters, identified as having been shown to induce high levels of expression in other cell lines, were tested to see if they were functional in *Sf*9 and *T.ni* Hi5 cell lines. These included the human cytomegalovirus immediate early (*CMVie*; Chan *et al.*, 1996), *Bm A*3 and enhancers *Bm*NPV *ie*-1 and *hr*3, (Mounier and Prudhomme, 1986; Johnson *et al.*, 1992; Lu *et al.*, 1996, 1997), *Orgyia pseudotsugata* MNPV *ie*-2 (Theilmann and Stewart, 1992; Pfeifer *et al.*, 1997; Hegedus *et al.*, 1998), *Dm MT* (Maroni *et al.*, 1986; Otto *et al.*, 1987; Bunch *et al.*, 1988), and the *Dm actin* 5.1 gene promoter (Bond and Davidson, 1986; Vigoreaux and Tobin, 1987; Bond-Matthews and Davidson, 1988).

This Chapter first describes attempts to identify transcriptional activity of the *CMVie* promoter in *Sf*9 and *T.ni* Hi5 cell lines. Expression of the green fluorescent protein and enhanced green fluorescent protein (GFP / EGFP) were used because of their ease of detection. Secondly, attempts to identify increased levels of expression in *Sf*9 and *T.ni* Hi5 cell lines, by comparing transient expression of CAT from a number of baculovirus and eukaryotic gene promoters would be investigated. The *cat* gene was selected for use as a reporter gene because of the ease of detection of the gene product and the ability to accurately quantify the levels of expression. The identification of transcriptionally strong promoters active in insect cells would enable further progress for the development of a novel stable inducible expression system for use in insect cells, as described in Chapter 4.

3.2 Functional analysis of the human *cytomegalovirus* immediate early (*CMVie*) gene promoter in insect cells.

3.2.1 Human *CMVie* gene promoter

The human *CMVie* promoter was chosen because it has been characterised as a very strong promoter in mammalian cell lines (Furth *et al.*, 1991; Furth *et al.*, 1994) and is transcriptionally active early after infection *in vivo* / *in vitro* suggesting that it is dependent on eukaryotic host cell transcription factors. In this respect it is similar to the *Ac*MNPV *ie*-1 promoter. A plasmid (pEGFP.N1) containing the *CMVie* promoter, with the *egfp* gene in-frame was obtained (BD Biosciences, Clontech) for the purpose of this work. This construct was used in transient experiments to determine the functional activity of the *CMVie* promoter in insect cells. The detection of EGFP from *CMVie* was used to analyse promoter function. A genetic map of pEGFP.N1 is shown in **Figure 3.1**

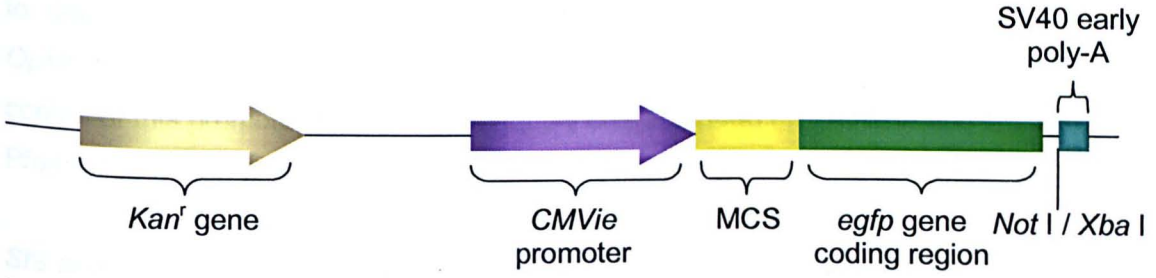


Figure 3.1: Genetic map of pEGFP.N1 (BD Biosciences, Clontech), shows the *CMVie* promoter, a multiple cloning site (MCS), *egfp* gene-coding region, SV40 early poly-adenylation (poly-A), and *kanamycin* (*Kan*^{r}) resistance gene.

3.2.2 *Op*MNPV *ie*-1 gene promoter

Plasmid pIZT/V5-His (Invitrogen™) contains the *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus (*Op*MNPV) *immediate early* (*Op*MNPV *ie*-1 / *ie*-2) promoters. Downstream of the *Op*MNPV *ie*-1 promoter is the coding region of a fused GFP-Zeocin resistance gene. The *Op*MNPV *ie*-1 promoter has been characterised previously (Theilmann and Stewart, 1991; Pfeifer *et al.*, 1997). This construct was used as a positive control in transient experiments to determine functionally active promoters in insect cell lines. pIZT/V5-His provided basal levels of GFP production. A genetic map of pIZT/V5-His is shown in **Figure 3.2**.

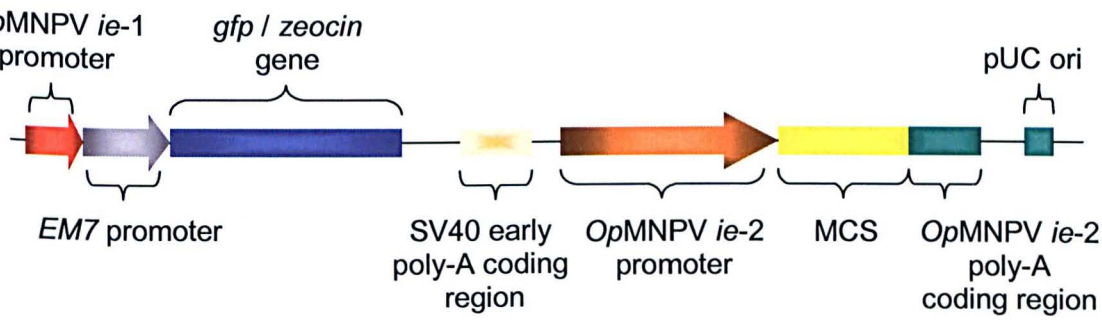


Figure 3.2: Genetic map of pIZT/V5-His (Invitrogen™), shows the pUC origin of replication (pUC ori) and two expression cassettes, the first contains the coding region of the *gfp-zeocin* chimeric gene in-frame with the *Op*MNPV *ie*-1 and *EM7* promoter and SV40 early poly-A coding region; the second, *Op*MNPV *ie*-2 promoter with a downstream MCS, and *Op*MNPV *ie*-2 poly-A coding region.

3.2.3 Analysis of *CMVie* promoter activity in insect cells

Having obtained the expression vectors outlined in Section 3.2.1 / 3.2.2, they were tested to determine their transient activity in *Sf*9 and *T.ni* H5 cells. Expression using the *Op*MNPV *ie*-1 promoter was used as a standard because foreign gene expression under control of this promoter had been previously characterised (Theilmann and Stewart, 1991; Pfeifer *et al.*, 1997).

*Sf*9 and *T.ni* H5 cells were seeded (Section 2.2.3) into 8 well chamber slides, and left to adhere for 1 h. The cell mono-layers were transfected (Section 2.2.4) using 2μg of plasmid DNA (pIZT/V5-His, pEGFP.N1). Transfections were carried out in triplicate. Cells were visualised for the production of EGFP and GFP on a Carl Zeiss Axiovert 510 CLSM microscope, using a standard Argon 488nm laser, and 534nm Helium/Neon laser, after 48 h incubation at 28°C.

Figure 3.3 shows results obtained for promoter activity in *Sf*9 (**panel A**) and *T.ni* Hi5 (**panel B**) cells. Expression of *gfp* from the *Op*MNPV *ie*-1 promoter was shown to be detectable by visualisation of green fluorescence. The fluorescence observed from the level of GFP produced by *Op*MNPV *ie*-1 served as a positive control. The detection of EGFP production from the *CMVie* promoter was also shown, as fluorescence was detected. The levels of EGFP produced by the *CMVie* promoter were visibly much lower than the levels of GFP produced by the *Op*MNPV *ie*-1 promoter. Mock-transfected *Sf*9 cells after 48 h did not show any detectible level of background auto-fluorescence.

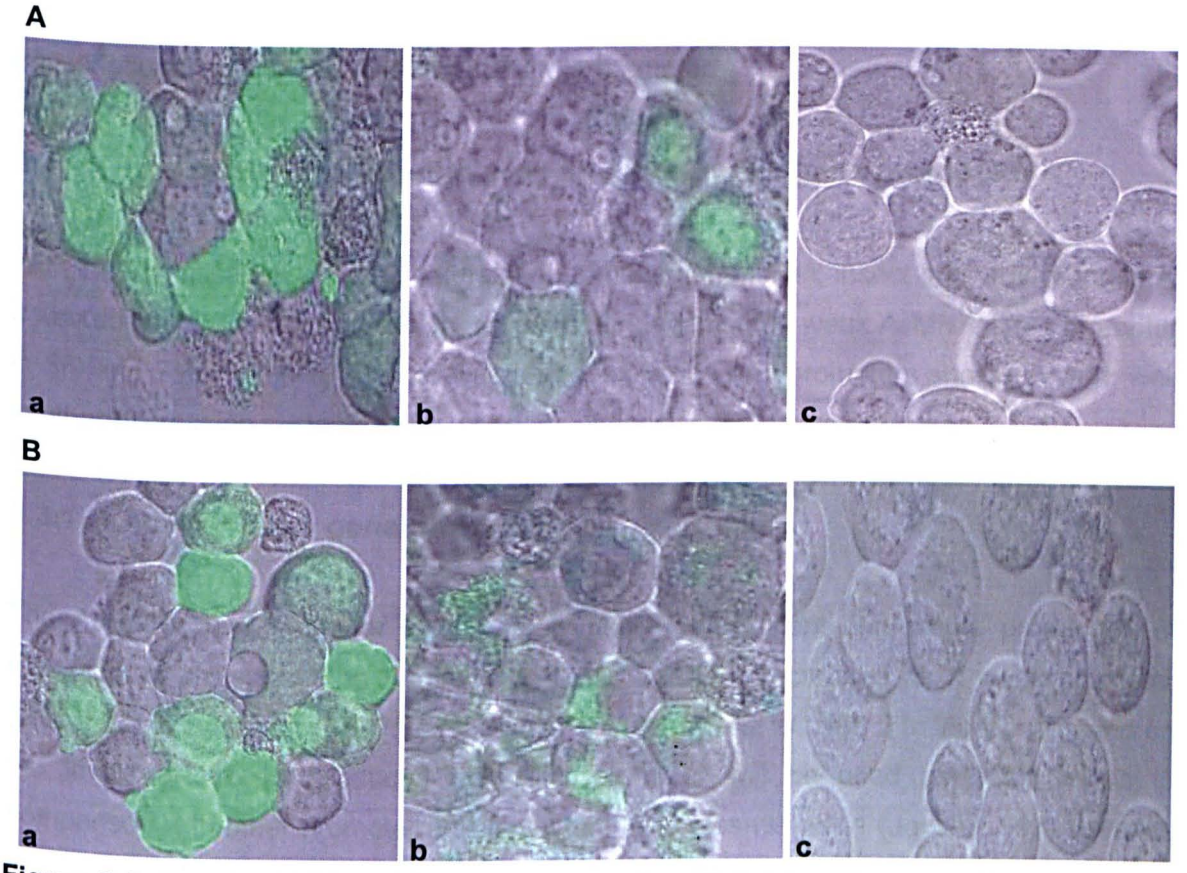


Figure 3.3: Transient GFP / EGPF production after 48 hpt in *Sf*9 (**panel A**) and *T.ni* Hi5 (**panel B**) cells. Transient GFP production shown under control of the *Op*MNPV *ie*-1 promoter (**a**), EGFP under the *CMVie* promoter (**b**) and mock-transfected cells showing no detectable levels of background auto-fluorescence (**c**).

The results show that the *CMVie* promoter is transcriptionally active in *Sf*9 and *T.ni* Hi5 cells. However, the intensity of EGFP produced by the *CMVie* promoter was much lower in comparison to the intensity of GFP produced by the *Op*MNPV *ie*-1 promoter, suggesting that the *CMVie* promoter is a weaker promoter than the *Op*MNPV *ie*-1 promoter. These findings correlate with reports suggesting that the activity of the *CMVie* promoter is weak in insect cells (Walker, 1989; Pfeifer *et al.*, 1997). The activity of the *Op*MNPV *ie*-1 promoter has been characterised as moderate in insect cells (Shotkoski *et al.*, 1996; Pfeifer *et al.*, 1997), whilst *Ac*MNPV *ie*-1 has been determined as weak. The *CMVie* was shown to function in insect cells, however it was not possible to quantify the strength of this promoter, or compare promoter activity directly with *Ac*MNPV *ie*-1. Therefore the *CMVie* promoter was used in the following investigation to determine its transcriptional activity in insect cells. This involved comparing the expression of CAT to the commonly used weak *Ac*MNPV *ie*-1 promoter and a number of other baculovirus and eukaryotic gene

promoters. This investigation was an attempt to identify promoters with significantly higher transcriptional activity than the *Ac*MNPV *ie*-1 promoter.

3.3 Use of alternate baculovirus and eukaryotic gene promoters

With the identification and characterisation of new mammalian, insect and viral promoters, it was decided to test a range of these promoters against the weak *Ac*MNPV *ie*-1 promoter in *Sf*9 and *T.ni* Hi5 cells. Baculovirus and eukaryotic gene promoters shown to work well in other expression systems in the absence of specific factors were chosen.

3.3.1 *Ac*MNPV *ie*-1 gene promoter

The *Ac*MNPV *ie*-1 promoter is commonly used for transient and stable production of recombinant proteins. The *Ac*MNPV *ie*-1 has been characterised as a weak promoter (Guarino and Summers, 1987; Jarvis *et al.*, 1990; Joyce *et al.*, 1993) in insect cells and so the levels of protein produced from this promoter will be used as basal levels in comparison with other promoter activity tested. The plasmid pIE-1 (Joyce, 1993) contains the *Ac*MNPV *ie*-1 promoter, gene-coding region and transcription termination sequence. This construct was used in transient experiments as a negative control, because there were no foreign genes present within it. A restriction enzyme map of pIE-1 is shown in **Figure 3.4A**.

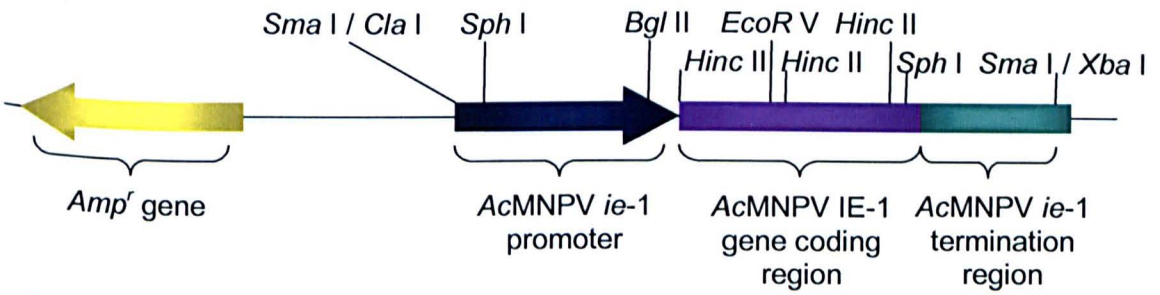


Figure 3.4A: Restriction map of pIE-1 (Joyce, 1993), shows the *Ac*MNPV *ie*-1 promoter, gene-coding region and termination region, and *Amp*^{r} gene.

Plasmid pIEK1.*cat* is similar to pIE-1, except the *Ac*MNPV *ie*-1 coding region was removed and replaced with the *cat* gene downstream of the *Ac*MNPV *ie*-1 promoter (Joyce, 1993). Since the *Ac*MNPV *ie*-1 promoter has been characterised previously (Jarvis and Summers, 1989; Joyce, 1993; Atkinson *et al.*, 1996), pIEK1.*cat* was used in all transient

experiments in order to obtain basal levels of expression. A restriction enzyme map of pIEK1.*cat* is shown in **Figure 3.4B**.

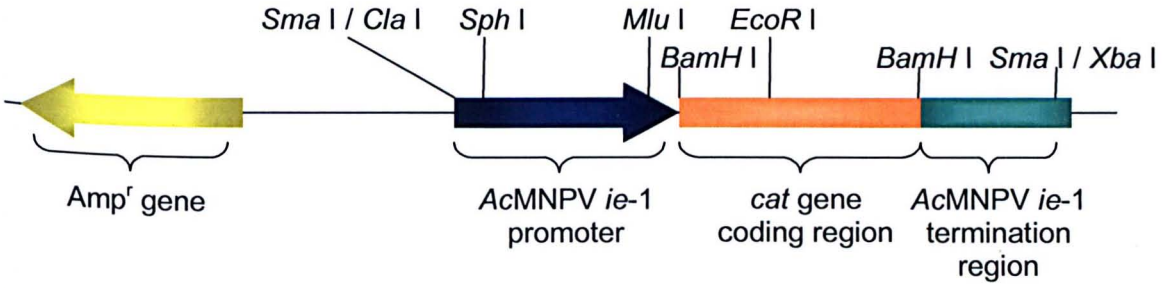


Figure 3.4B: Restriction map of pIEK1.*cat* (Joyce, 1993) is similar to pIE1 described in Figure 3.4A, except the *ie*-1 gene coding region has been replaced with that of *cat*.

3.3.2 Human *CMVie* gene promoter

The human *CMVie* promoter has been shown in Section 3.2.3 to be transcriptionally active in *Sf*9 and *T.ni* Hi5 cells, albeit at relatively low levels. To analyse the strength of the *CMVie* promoter in comparison to the *Ac*MNPV *ie*-1 promoter, the *cat* gene (Joyce, 1993) was inserted into pEGFP.N1. pEGFP.N1 was digested using *Bgl*II and *BamH*I (Section 2.5.1), and treated with shrimp alkaline phosphatase (Section 2.5.3). The *cat* gene from pIEK1.*cat* (Joyce, 1993) was removed using *BamH*I and cloned into pEGFP.N1. Digestion using *EcoR*I and *Not*I identified the correct orientation of the *cat* gene, to derive plasmid pEGFP.N1.*cat* (**Figure 3.5**).

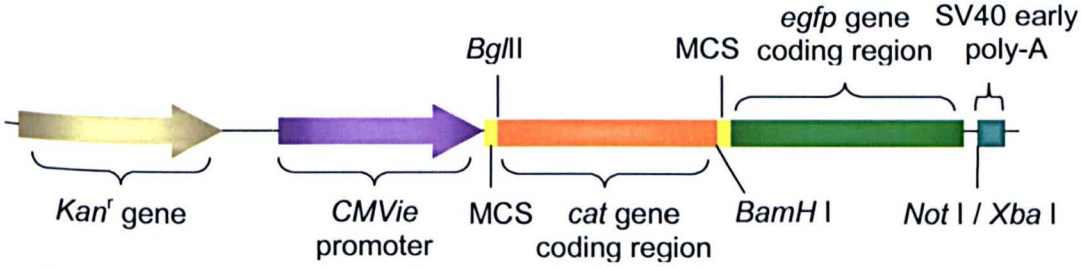


Figure 3.5: Genetic map of pEGFP.N1.*cat*. pEGFP.N1.*cat* is similar to pEGFP.N1 (BD Biosciences, Clontech) described in Figure 3.1, except the *cat* gene has been cloned into the *Bgl*II and *BamH*I cloning sites of the MCS.

3.3.3 *BmA3* gene promoter and *Bm*NPV enhancers

The *Bm A3* gene promoter (Mounier and Prudhomme, 1986; 1991) has been reported to be functionally active in Lepidopteran cell lines (Johnson *et al.*, 1992). Work to develop an *in vitro* expression system that employed the *Bm A3* promoter to drive expression of foreign genes identified two *cis*-acting enhancers (Lu *et al.*, 1996; Farrell *et al.*, 1998; 1999; Keith *et al.*, 1999). The presences of both *cis*-acting enhancers in transfected cells, has been shown to increase level of the *Bm A3* promoter strength by three orders of magnitude, relative to that of the basic *Bm A3* promoter (Lu *et al.*, 1996; Farrell *et al.*, 1998, 1999; Keith *et al.*, 1999). From this evidence, the *Bm A3* promoter and enhancers (*Bm A3*+E) were chosen to analyse in this expression study.

The *cat* gene from pEGFP.N1.*cat* was amplified using PCR (Section 2.5.5), and inserted into the entry vector pENTR.D.TOPO (Invitrogen™) using directional TOPO® cloning (Section 2.5.12). The correct sequence of *cat* was confirmed by sequencing (as describe in Section 2.5.8). A genetic map of pENTR.D.TOPO.*cat* is shown in **Figure 3.6**.

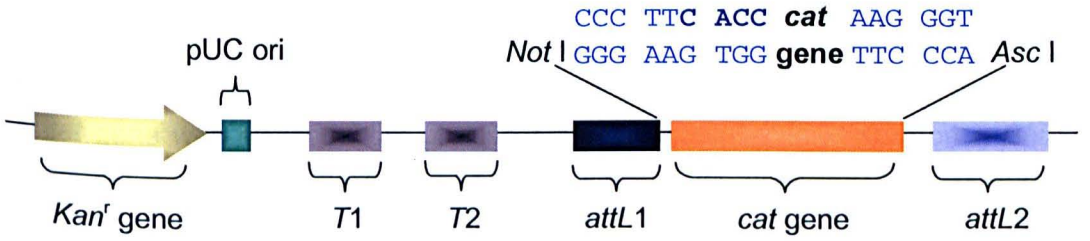


Figure 3.6: Genetic map of pENTR.D.TOPO.*cat*, shows the *Kan*^{r} gene, pUC ori, transcriptional termination sequences (*T1* / *T2*), phage λ recombination sites (*attL1* / *attL2*) with *cat* inserted amidst.

Following the construction of pENTR.D.TOPO.*cat*, this entry vector was used to transfer *cat* into the expression vector (Section 2.5.12) pXINsect.DEST38 (Invitrogen™), downstream of the *Bm A3* promoter. Plasmid pXINsect.DEST38.*cat* was derived. A genetic map of pXINsect.DEST38.*cat* is shown in **Figure 3.7**.

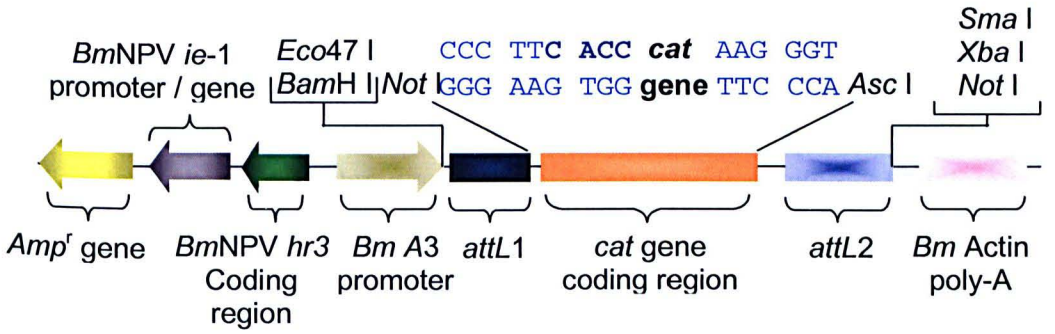


Figure 3.7: Genetic map of pXINsect.DEST38.*cat* shows the *Amp*^{r} gene, *Bm*NPV *ie*-1 promoter / gene, *Bm*NPV *hr*3 coding region, *Bm A*3 promoter with *cat* inserted downstream, *attL*1 / *attL*2 recombination sites, and the *Bm* actin poly-A.

3.3.4 *Op*MNPV *ie*-2 gene promoter

The *Op*MNPV *ie*-2 promoter was chosen because it has been shown to be functionally active in Lepidopteran cell lines (Pfeifer *et al.*, 1997, Hegedus *et al.*, 1998; Invitrogen™). It has also been reported that the *Op*MNPV *ie*-2 promoter provides relatively high levels of constitutive expression, and has been shown to be about 5-10-fold stronger than the *Op*MNPV *ie*-1 promoter (Pfeifer *et al.*, 1997, Hegedus *et al.*, 1998). The plasmid pIZT/V5-His (Invitrogen), described in Section 3.2.2 was used for the purpose of this experiment. The *cat* gene was excised from pXINsect.DEST38.*cat* (Section 3.3.3) on a *Bam*HI and *Not*I fragment and cloned into complementary restriction enzyme sites of pIZT/V5-His, downstream of the *Op*MNPV *ie*-2 promoter. Plasmid pIZT/V5-His.*cat* was subsequently generated. A genetic map of pIZT/V5-His.*cat* is shown in **Figure 3.8**.

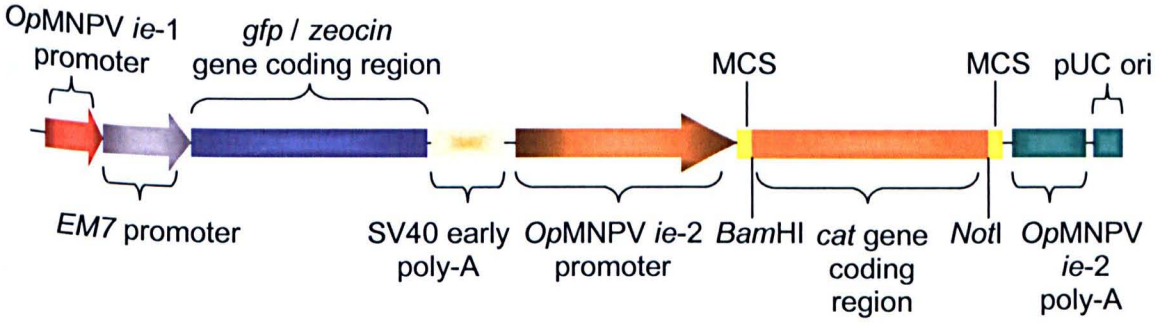


Figure 3.8: Genetic map of pIZT/V5-His.*cat* (Invitrogen™). pIZT/V5-His.*cat* is similar to pIZT/V5-His described in **Figure 3.2**, except that *cat* has been cloned downstream of the *Op*MNPV *ie*-2 promoter using the restriction enzyme sites *Bam*HI and *Not*I present in the MCS.

3.3.5 *Dm Ac*5.1 gene promoter

The *Dm Ac*5.1 promoter has previously been used in transient and stable *Drosophila* cell line work (Chung and Keller, 1990), and was chosen because of its functional activity in *Sf*21 cells (Han *et al.*, 1989; Chung and Keller, 1990). It was of interest to determine if the *Dm Ac*5.1 promoter was functionally active in alternative insect cell lines. The plasmid pAc5.1/V5-His was obtained from Invitrogen™. pAc5.1/V5-His was digested using *Not*I (Section 2.5.1). The *cat* gene was excised from pXINsect.DEST38.*cat* using *Not*I and inserted into pAc5.1/V5-His. Digestion, using *Xba*I and *Xho*I, identified the correct orientation of *cat*, to derive plasmid pAc5.1/V5-His.*cat*. A genetic map of pAc5.1/V5-His.*cat* is shown in **Figure 3.9**.

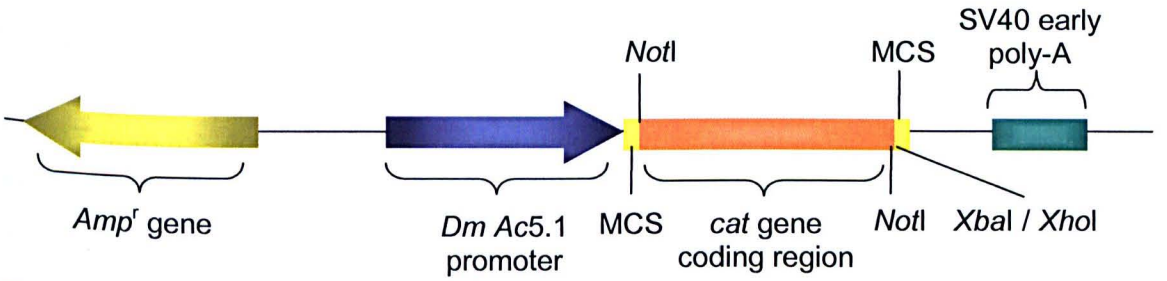


Figure 3.9: Genetic map of pAc5.1/V5-His.*cat*, shows the *Amp*^{r} gene, *Dm Ac*5.1 promoter, *cat* gene inserted downstream of the *Dm Ac*5.1 promoter using the restriction site *Not*I present in the MCS, *Xba*I and *Xho*I restriction sites to identify correct orientation of *cat*, and SV40 poly-A.

3.3.6 *Dm MT* gene promoter

The *Dm MT* promoter was chosen because it is an inducible promoter and shows functional activity in *Drosophila* cells (Bunch *et al.*, 1988). It was of interest to determine if this promoter was functionally active in alternative insect cell lines. The *Dm MT* promoter has been shown to be induced by low levels of heavy metals (Stuart *et al.*, 1984; Bunch *et al.*, 1988; Kovach *et al.*, 1992). Plasmid pMT/V5-His was obtained from Invitrogen™. The *cat* gene from pXINsect.DEST38.*cat* was inserted into the *Not*I cloning site downstream of the promoter. Section 3.3.5 described how the correct orientation of *cat* was identified. A restriction enzyme map of pMT/V5-His.*cat* is shown in **Figure 3.10**.

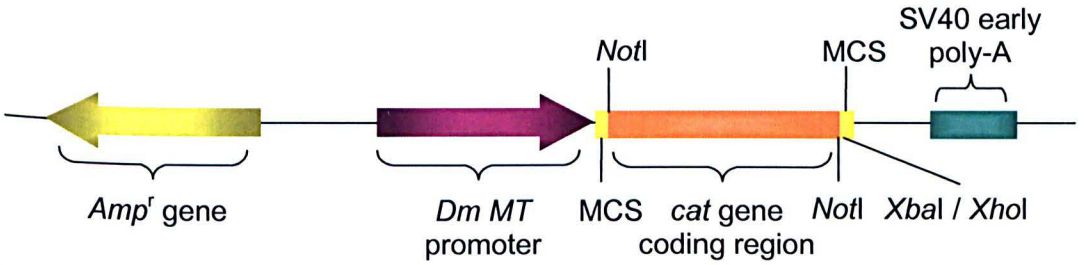


Figure 3.10: Genetic map of pMT/V5-His.*cat*, shows an *Amp*^{r} gene, *Dm MT* promoter, *cat* gene inserted downstream of the promoter using the restriction site *Not*I present in the MCS, *Xba*I and *Xho*I restriction sites to identify correct orientation of *cat*, and SV40 poly-A.

3.4 Analysis of promoter activity in insect cells

Following construction of the expression vectors, as described in Section 3.3, the constructs were tested for their ability to produce CAT in insect cell lines. The expression of CAT from the *Ac*MNPV *ie*-1 promoter was used as the standard because foreign gene expression had been previously characterised using this promoter in insect cells (Guarino and Summers, 1986b; Jarvis *et al.*, 1990; Henderson *et al.*, 1995). As the promoters tested were from a variety of expression systems, their ability to function to produce CAT was tested in two different insect cell lines (*Sf*9 and *T.ni* Hi5 cells).

Cells were seeded as described in Section 2.2.3, and monolayers transfected (Section 2.2.4) using 5μg of each construct (pIE-1, pIEK1.*cat*, pEGFP.N1.*cat*, pXINsect.DEST38.*cat*, pIZT/V5-His.*cat*, pAc5.1/V5-His.*cat*, pMT/V5-His.*cat*). Transfections were carried out in triplicate. After 24 h incubation at 28°C, cells transfected with pMT/V5-His.*cat* were treated with 500μM $CuSO_4$ to induce transcription. Cells were harvested every 24 h, over a period of 120 h, and CAT activity quantified. The assays were carried out in triplicate as described in Section 2.8.1.

Following phosphor-imaging, the percentage conversion from non-modified to the 3-acetylated form of chloramphenicol by each cellular extract was calculated using the software program Image-Quant 5.1 (Section 2.8.1). CAT activity was expressed as a total percentage conversion per 10^6 cells. Since the *Ac*MNPV *ie*-1 promoter has been used previously in stable insect cell lines and is known to give low levels of expression in these circumstances, expression using this promoter in *Sf*9 and *T.ni* Hi5 cells was considered as the basal level, and all data was compared to this (**Figure 3.11A-B**). Mock-transfected

*Sf*9 and *T.ni* Hi5 cells were used as a negative control to determine non-specific background expression levels, and commercially purified CAT (Sigma) was used to determine 100% chloramphenicol conversion (positive control). Statistical analyses were carried out using the unpaired 2 sample T-test at a 95% confidence level. In experiments the level of CAT activity detected was assumed to be directly proportional to the level of CAT produced under control of the promoters used.

In both cell lines tested, CAT activity observed from the negative control, *CMVie*, *Dm* Ac5.1 and *Dm MT* promoters (induced/non-induced) was significantly lower than that observed from using *Ac*MNPV *ie*-1 (**Figure 3.11A-B**; $p<0.05$ in all cases). These gene promoters would not be appropriate for high level continuous or inducible protein production in *Sf*9 or *T.ni* Hi5 cells.

Figure 3.11A shows results obtained from the promoter activity assays in *Sf*9 cells. The production of CAT at 0 hpt from all promoters tested yielded no detectable CAT activity ($p>0.05$ in all cases) above background levels. Expression of *cat* using *Ac*MNPV *ie*-1, produced CAT activity at 24 hpt of 1.4 %. At 48 hpt the level of activity increased to 3.9%, and by 72 hpt levels had augmented to 5.3%. The levels of CAT produced were low, which is characteristic of this weak promoter. At 96 hpt levels of CAT activity decreased by 0.78-fold (4.1%), and by 120 hpt, activity was not observed above background levels. These data, demonstrating CAT activity produced from the *Ac*MNPV *ie*-1 promoter, are used as basal levels in the cell lines tested to which other promoters are compared in this assay.

The production of CAT from *Bm* A3+E resulted in a significant increase compared with *Ac*MNPV *ie*-1 at 24 hpt (CAT activity, 20.2%; $p<0.0001$), which corresponds to a 14-fold increase in expression. At 48 hpt CAT activity from *Bm* A3+E increased to 39.8%, a 10-fold increase compared to *Ac*MNPV *ie*-1 at this time-point ($p<0.0001$). At 72 hpt CAT activity from *Bm* A3+E remained significantly higher (38.1%; $p<0.0001$) than that detected from *Ac*MNPV *ie*-1 at this time-point (7-fold increase). At 96 hpt the levels of CAT activity from *Bm* A3+E were similar (4.5%) to those produced by *Ac*MNPV *ie*-1 ($p=0.607$). No significant CAT activity (0.8%) was detected above background levels at 120 hpt ($p=0.197$; **Figure 3.11A**).

Cells producing CAT under control of *Op*MNPV *ie*-2, showed CAT activity was significantly increased by 24-fold in comparison to those observed from *Ac*MNPV *ie*-1 at 24 hpt (CAT

activity, 34%; p<0.0001). Expression from *Op*MNPV *ie*-2 also results in a 1.7-fold increase in CAT activity (34%) over those observed from *Bm* A3+E (20%) at 24 hpt. At 48 hpt CAT activity from *Op*MNPV *ie*-2 was detected at 28.9%, which corresponds to a 7-fold increase in expression compared to *Ac*MNPV *ie*-1, but a 1.4-fold decrease compared to *Bm* A3+E at this time-point (p<0.0001). The levels of CAT activity at 72-96 hpt from *Op*MNPV *ie*-2 (72hpt, 5.2%; 96hpt, 4.9%) were similar to those observed from *Ac*MNPV *ie*-1, hence not significantly different (p>0.05). However at 72 hpt, *Bm* A3+E produced a 7.6-fold increase in CAT activity compared to *Op*MNPV *ie*-2 at this time-point. At 120 hpt CAT activity was still detectable from *Op*MNPV *ie*-2 and resulted in levels of activity that were significantly higher than both *Ac*MNPV *ie*-1 (p=0.007) and *Bm* A3+E (p=0.002), though overall levels were low (3.2%). It was concluded, that *Op*MNPV *ie*-2 and *Bm* A3+E would be suitable for high level transient or stable expression of foreign genes in *Sf*9 cells.

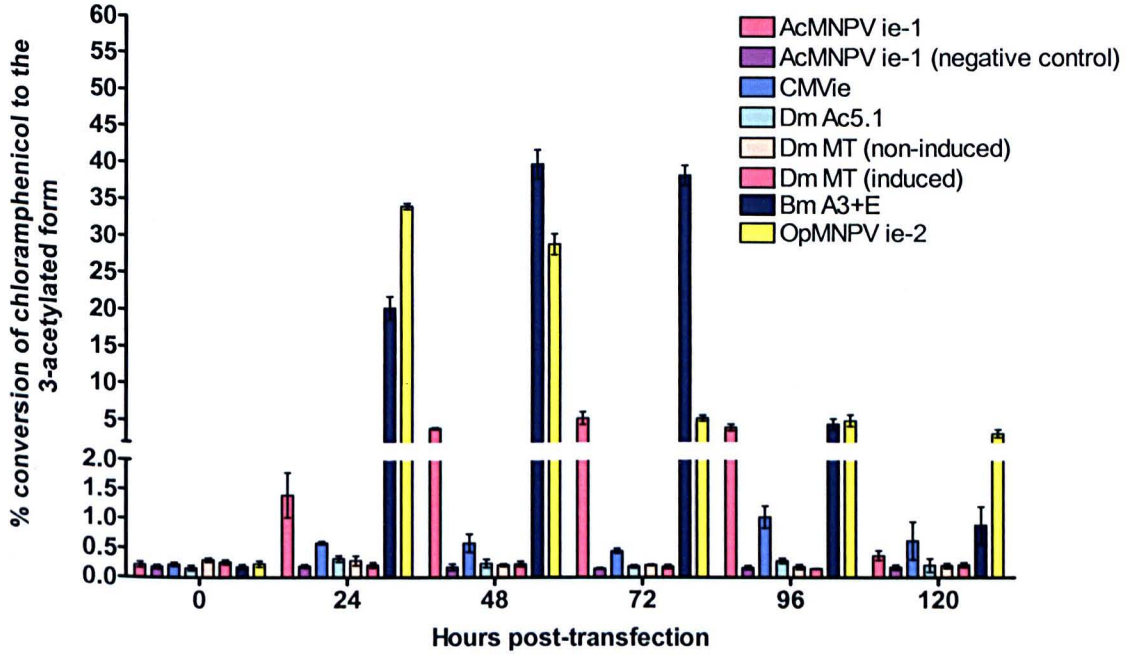


Figure 3.11A: Time course of transient CAT activity in *Sf*9 cells following transfection with plasmids containing different promoters to drive expression of *cat* (relative to CAT activity produced by the *Ac*MNPV *ie*-1 promoter).

Figure 3.11B shows results obtained from the promoter activity assays in *T.ni* Hi5 cells. At 0 hpt no detectable levels of CAT activity above background levels were observed for any of the promoters tested (p>0.05 in all cases). The *Ac*MNPV *ie*1 promoter appears to be only active at 24 hpt, producing CAT activity of 3.1%, which was characteristically low, although an increase compared to levels observed using *Sf*9 cells (**Figure 3.11A**). The

*Op*MNPV *ie*-2 and *Bm* A3+E produced significantly higher levels of CAT activity (*Op*MNPV *ie*-2, 51.3%; *Bm* A3+E, 41.1%) than those obtained from *Ac*MNPV *ie*1 at 24 hpt (*Op*MNPV *ie*-2, 17-fold increase; *Bm* A3+E, 13-fold increase; $p<0.0001$). A comparison of CAT activity at 24 hpt from *Op*MNPV *ie*-2 and *Bm* A3+E, showed *Op*MNPV *ie*-2 resulted in 1.2-fold greater expression. Both promoters were shown to have a greater transcriptional activity at 24 hpt than in *Sf*9 cells (**Figure 3.11A**). At 48 hpt *Op*MNPV *ie*-2 shows detectable CAT activity of 5%, which was significantly higher than the basal levels from *Ac*MNPV *ie*-1 ($p<0.0001$), but no detectable CAT activity was observed above background levels from this promoter at 72-120 hpt ($p>0.05$). The *Bm* A3+E produced no detectable CAT activity above background levels from 48-120 hpt, which correlates with the same expression profile of the *Ac*MNPV *ie*1 promoter ($p>0.05$), suggesting no functional promoter activity. It was concluded that *Op*MNPV *ie*-2 and *Bm* A3+E would be suitable for high level transient and stable expression of foreign genes in *T.ni* Hi5 cells. *T.ni* Hi5 cells are also shown to produce the highest levels of CAT activity compared to levels observed using *Sf*9 cells (**Figure 3.11A**).

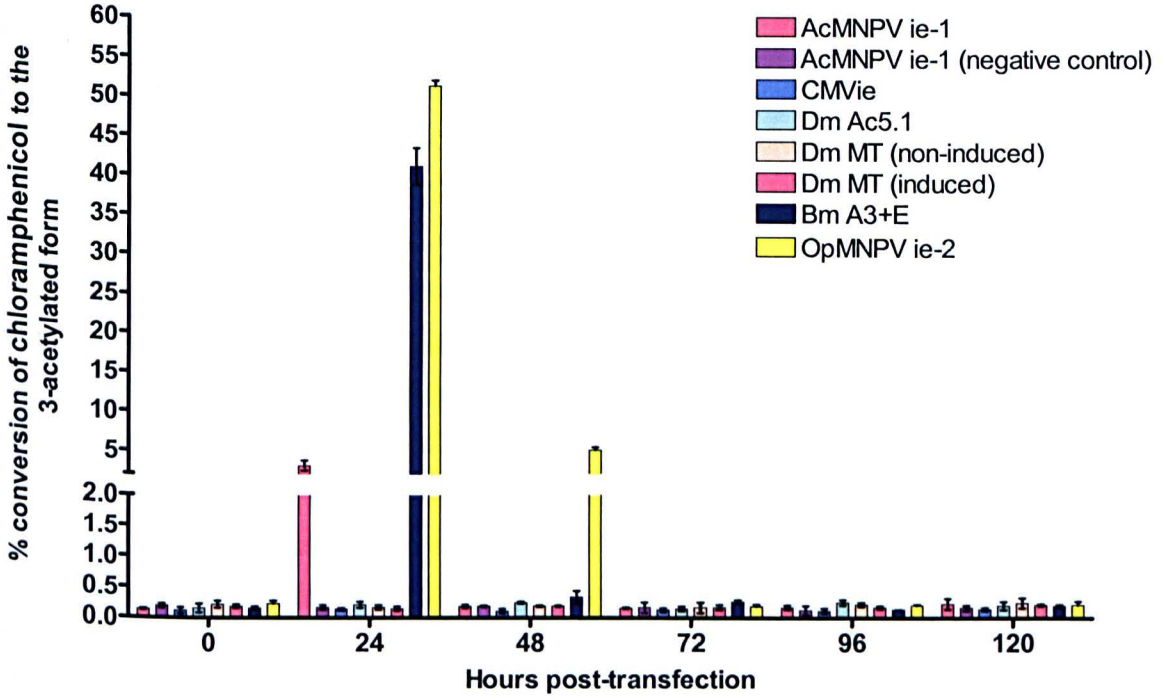


Figure 3.11B: Time course of transient CAT activity in *T.ni* Hi5 cells following transfection with plasmids containing different promoters to drive expression of *cat* (relative to CAT activity produced by the *Ac*MNPV *ie*-1 promoter).

It was therefore shown that, *Op*MNPV *ie*-2 or *Bm A*3+E transfected *T.ni* Hi5 and *Sf*9 cells would appear to be the most useful combination in producing stable cell lines. Both these promoters would also be suitable as potential candidates to drive foreign gene expression in a developmental inducible expression system (described in Chapter 4), for use in *T.ni* Hi5 or *Sf*9 cell lines.

3.5 Discussion

Early studies on the development of non-lytic expression systems for generating continuous insect cell lines have shown that the *Ac*MNPV *ie*-1 promoter could be used to produce heterologous proteins in Lepidopteran (*Sf*9) cells (Jarvis *et al.*, 1990). Levels of foreign protein produced by *Ac*MNPV *ie*-1 were found to be very low and did not compare favourably with other expression systems. Often, demand for high levels of recombinant protein is generally required, such as that for screening large compound libraries for drug targets, consequently the use of *Ac*MNPV *ie*-1 would not be appropriate. The work in this Chapter was carried out to determine whether strong eukaryotic promoters could be used in place of *Ac*MNPV *ie*-1 in stable insect cell lines for the production of recombinant proteins, yielding levels comparable with other expression systems. Those promoters matching the above description would be used in the development of a novel inducible insect expression system, as described in Chapter 4.

Endogenous cellular promoter activation is reliant on host cell transcription factors (TF) to initiate transcription of endogenous / heterologous genes. Heterologous promoters may be used if the transcriptional machinery recognises complementary DNA-protein binding motifs to initiate transcription. Viral immediate early (*ie*) promoters have been shown to be similar in structure to mammalian counterparts (Miller, 1996). It follows that other eukaryotic promoters may be able to function using the same host TF. Studies have shown (Morris and Miller, 1992) that promoter choice strongly influences the range of insect cells that can efficiently support the production of heterologous proteins. To determine if there were host cell differences in the regulation of the promoters tested, constructs were transfected into *Sf*9 and *T.ni* Hi5 cell lines, and assayed for transient CAT activity.

Results in Section 3.4 show that neither *Dm Ac*5.1, nor *Dm MT* (non-induced / induced) promoters were active in the insect cell lines tested. One explanation is likely to be due to

the incompatibility of promoter DNA-protein binding sites with TF endogenous to *Sf*9 and *T.ni* Hi5 cells. If the TF of insect cells used cannot recognise *trans*-activating domains of the promoters to initiate formation of the transcription initiation complex, then promoter activation is inherently silent and gene expression will not occur. This work is believed to be the first testing of the *Dm Ac*5.1 promoter function in *Sf*9 cells. The lack of detectable transcription from this promoter suggests, it would not be suitable for generating stable *Sf*9 or *T.ni* Hi5 cell lines. The lack of transcription from *Dm MT* was disappointing, as previous reports (McCarroll, 1997) have shown this promoter to be active in Lepidopteran cells (*Sf*21, *T.ni*), albeit at levels that are barely detectable when using CAT assay analysis. In a direct comparison with the weak *Ac*MNPV *ie*-1, it has been shown that *Dm MT* activity was not detectable above background levels. Due to the transcriptionally weak nature of *Dm MT* reported in *Sf*21 and *T.ni* cells, attempts to augment activity levels in *Sf*9 and *T.ni* Hi5 cells failed. Given these and previous data, levels of expression as low as those reported would not be useful for developing a stable cell line with the view to developing an expression system.

The strong eukaryotic promoter *CMVie* was found to be functionally active in *T.ni* Hi5 (as described in Section 3.2.3) and *Sf*9 cells (as described in Section 3.2.3 / 3.4), albeit at low levels. An explanation of why CAT activity from the *CMVie* promoter was detected in one cell line, but not the other (Section 3.4), could also explain why CAT activity from the *Dm* promoters was not observed. If the transfection efficiency was low then the number of cells transformed may have been inadequate to detect CAT activity. DNA transfer into cells was liposome-mediated (Lipofectin, Invitrogen™), whereas in some previous studies the calcium phosphate method was utilised. However, it is generally accepted that Lipofectin works more efficiently in Lepidopteran cell lines (Mann and King, 1989). Similar studies have also shown the *CMVie* promoter to be active in *Dm* cells (Bello *et al.*, 1998), although levels of CAT were only just detectable above background. Similar findings were reported in *Sf*9 cells (Section 3.4). These data characterise the *CMVie* as a weak promoter in insect cell lines, as CAT production was significantly lower than levels observed from *Ac*MNPV *ie*-1. The *CMVie* promoter would be an inappropriate choice for further investigation of constitutive or inducible heterologous gene expression in stable insect cell lines.

The *Bm A*3+E and *Op*MNPV *ie*-2 promoters were the most successful in transcribing *cat* in transient studies. Both promoters produced significantly higher levels of CAT in comparison to *Ac*MNPV *ie*-1 in both insect cell lines tested. As the promoters were

identified as being transcriptionally stronger than *Ac*MNPV *ie*-1, a comparison of *Bm* A3+E and *Op*MNPV *ie*-2 was investigated using the data obtained from Section 3.4. Firstly, a comparison of promoter activity was conducted, followed by a comparison to determine if there were host cell differences in the regulation of the two promoters. In *Sf*9 cells the overall expression profile of CAT from *Bm* A3+E produced the most significant result compared with *Op*MNPV *ie*-2 (**Figure 3.11A**). Optimal levels of CAT were produced between 24-72 hpt from *Bm* A3+E and 24-48 hpt from *Op*MNPV *ie*-2 (**Figure 3.11A**). These data show that the *Bm* A3+E was the stronger of the two promoters. However, both promoters were sufficiently stronger than *Ac*MNPV *ie*-1, and are potential candidates for increasing yields of recombinant protein in stable *Sf*9 cells.

Both *Op*MNPV *ie*-2 and *Bm* A3+E promoters were functional in *T.ni* Hi5 cells. The production of CAT was detectable from both these promoters at 24 hpt, but only *Op*MNPV *ie*-2 at 48 hpt (**Figure 3.11B**). At 24 hpt, expression of *cat* from *Op*MNPV *ie*-2 was significantly elevated in comparison to levels observed from *Bm* A3+E, and in fact the level of CAT activity from *Op*MNPV *ie*-2 at this time was significantly higher than all other promoters tested in both cell lines (**Figures 3.11A / 3.11B**). Comparison of optimal *cat* expression from *Bm* A3+E in *Sf*9 / *T.ni* Hi5 cells shows no significant difference apart from the time (hpt) CAT was expression. The difference in expression profiles, observed from the same promoters in different hosts, indicates that there are differences in host cell regulation of heterologous promoters. The characterisation of *Bm* A3+E and *Op*MNPV *ie*-2 promoter activity in *T.ni* Hi5 cells has demonstrated that these promoters have a significantly increased transcriptional activity compared to the weak *Ac*MNPV *ie*-1 and should be classified as strong promoters. These promoters are therefore eligible for generating high level recombinant protein in stable *T.ni* Hi5 cell lines.

These data coincide with findings from earlier studies, that have shown *Op*MNPV *ie*-2 to be functionally active in *Sf*9 (Hegedus *et al.*, 1998; Pfeifer *et al.*, 1997; Theilmann and Stewart, 1991) and *T.ni* Hi5 cells (Invitrogen™), and *Bm* A3+E in *T.ni* Hi5 cells (Keith *et al.*, 1999). The data presented in this thesis characterising the strong transcriptional activity of *Bm* A3+E in *Sf*9 cells, is the first acknowledged study to date of its kind. However, evidence supporting *Bm* A3+E activity in *Sf*21 cells has been shown previously (Keith *et al.*, 1999; Lu *et al.*, 1997).

The difference in CAT production from the cell lines used in Section 3.4, may be explained by a number of hypotheses. Transient expression studies rely on a burst of gene

expression between 12-72 hpt. This burst of gene expression is most often followed by a rapid deterioration in expression of the heterologous gene because of cell death. Due to the episomal nature of expression vectors used for transient expression studies, the vector does not integrate into the cell genome, but remains extra-chromosomal in the nucleus as multiple copies. Loss of the expression vector (Colosimo *et al.*, 2000) from the cell consequently results in decreased expression of the transgene. It is possible that the expression vectors housing the *Bm* A3+E and *Op*MNPV *ie*-2 promoters were degraded by the cell, or cell viability was compromised by over-expression of *cat*. This would explain the rapid decrease in *cat* expression observed from *Bm* A3+E and *Op*MNPV *ie*-2 promoters in *Sf*9 cells (**Figure 3.11A**) at 72-96 hpt, and may account for the loss of CAT activity after 24 hpt from *Bm* A3+E and 48 hpt from *Op*MNPV *ie*-2 in *T.ni* Hi5 cells (**Figure 3.11B**).

The concept of protein degradation may have also contributed to loss of CAT activity in *Sf*9 and *T.ni* Hi5 cells. Similar constructs (pXINSect.DEST38 or pIZT/V5-His) used in Section 3.4, were transfected into *Sf*9 and *T.ni* Hi5 cells, although *cat* was replaced with a red fluorescent reporter gene called *Ds*Red, as described in Chapter 5 (BD Biosciences, Clontech). Both cell lines were shown to produce DsRed from 24 to 120 hpt (data not shown). However, levels of fluorescence were not quantified; fluorescence was clearly visible throughout transfection (data not shown). The expression of *cat* in *T.ni* Hi5 cells may therefore have still been active, but cellular defence mechanisms may have been triggered, directing CAT to lysosomal compartments for degradation. Finally CAT may have been inactivated by some other cellular mechanism rendering the recombinant protein catabolically ineffective, detection via CAT assay would not have accounted for the non-active form of CAT respectively.

Figure 3.12 shows partial sequence alignment of all promoters tested in this Chapter. The promoter may be considered to be central in its role in gene transcription (Latchman, 1995); without promoter activation, gene expression is essentially silent. Studies of promoter regions have identified putative regulatory sequences immediately upstream of the transcribed region. These regulatory sequences have shown some level of homology across many gene classes (Dynan and Tjian, 1985; Berg and von Hippel, 1988; Latchman, 1995). A short sequence primarily composed of A and T nucleotides known as the TATA box (consensus $TATA^{A}/_{T}A^{A}/_{T}$), is found about 25-30bp upstream of the transcriptional start site (+1) of many, but not all genes (Section 1.5.1). Analysis of published sequences for those promoters used in this thesis showed that all contained a TATA box (**Figure 3.12**).

Interestingly, the baculovirus promoters *Ac*MNPV *ie*-1 and *Op*MNPV *ie*-2 contain identical TATA boxes, whilst the two *Dm* promoters retain identical homology to one another, but differ from *Ac*MNPV *ie*-1 and *Op*MNPV *ie*-2. The TATA box sequences of the *Bm A3* and *CMVie* differ from all TATA boxes identified in this study. Notably all promoters tested showed a high level of homology (>71%) to the *Ac*MNPV *ie*-1 TATA box.

The distance of the TATA box in relation to the RNA start site was also found to differ between promoters (**Table 3.1**). A comparison of TATA box homology with *Ac*MNPV *ie*-1, and the distance relationship of the RNA start site showed *Op*MNPV *ie*-2 to be highly comparable (**Figure 3.12 / Table 3.1**). This may explain its promoter activity in the cell lines tested in this thesis. It was disappointing to find the *Dm Ac*5.1 promoter functionally inactive given it has 86% homology with the *Ac*MNPV *ie*-1 TATA box, and a similar distance from the RNA start site.

The activity of *CMVie* was surprising because it resembles the least homology (71%) and a closer RNA start site, also the first nucleotide of the *CMVie* initiator motif is T (**Table 3.1**), whereas *Ac*MNPV *ie*-1 and *Op*MNPV *ie*-2 utilises the nucleotide A (**Table 3.1**), with exception to *Op*MNPV *ie*-2, which can use the nucleotide C as well (**Table 3.1**; Theilmann and Stewart, 1992). It was surprising to see no activity from *Dm MT* considering the similarity it possessed with *CMVie* (**Figure 3.12 / Table 3.1**). This may support a previous argument that the transfection efficiency was very low in this study. Unfortunately, the *Bm A3* RNA start site has not been elucidated, so a comparison could not be made. However, this promoter was characterised as transcriptionally active.

Viral immediate early genes may play a role in host specificity or virulence since the promoter of these genes must be able to utilise host transcriptional machinery. Studies have identified a conserved putative sequence motif (Blissard and Rohrmann, 1989; Pullen and Friesen, 1995a; Theilmann and Stewart, 1992), at the RNA start site of a number of genes from both *Op*MNPV (*gp*64, *ie*-2) and *Ac*MNPV (*gp*67, *ie*-1, 39*K*). This motif is depicted by the sequence CAGT, and is located at the transcriptional start site 21-24 nucleotides downstream of the TATA box. It is noteworthy that many baculovirus genes lack an obvious CAGT motif at their RNA start sites. For these genes, the TATA box may suffice to position the RNA start site, as is the case for the *p*35 gene of *Ac*MNPV (Dickson and Friesen, 1991).

```
AcMNPV ie-1 ATATTGTTAT  CGTGTTCGCC  ATTAGGGCAG  TATAAATTGA  CGTTCATGTT
OpMNPV ie-2 ACACGAGGCG  CCCGTCCCGC  TTATCGCGCC  TATAAATACA  GCCCGCAACG
Bm A3       TGGACGCAAA  TAAATTTGTC  GTTTACTTAG  TATAATGTAT  GGAATGAGAA
CMVie       GGGCGGTAGG  CGTGTACGGT  GGGAGGTCTA  TATAAGCAGA  GCTGGTTTAG
Dm MT       GACCCGTGTG  AAAGCCGCGT  TTCCAAAATG  TATAAAACCG  AGAGCATCTG
Dm Ac5.1    GCTGATAAGG  TTTTAGCGCT  AAGCGGGCTT  TATAAAACGG  GCTGCGGGAC

AcMNPV ie-1 GGATATTGTT  TCAGTTGCAA  GTTGACACTG  GCGGCGACAA  GATCGTGAAC
OpMNPV ie-2 ATCTGGTAAA  CACAGTTGAA  CAGCATCTGT  TCGAA
Bm A3       TGTAGTTTGA  ATTGTTTTTT  TTCTTTTCTT  GCAGACTAAT  TCAAGAGGTG
CMVie       TGAACCGTCA  GATCGCCTGG
Dm MT       GCCAATGTGC  ATCAGTTGTG  GTCAGCAGCA
Dm Ac5.1    CAGTTTTCAT  ATCACTACCG  TTTGAGTTCT  TGTGCTGTGT  GGATACTCCT
```

Figure 3.12: Partial nucleic acid sequence alignment of eukaryotic gene promoter regions. The TATA boxes of the published sequences of *Ac*MNPV *ie*-1 (Guarino and Summers, 1987), *Op*MNPV *ie*-2 (Theilmann and Stewart, 1991) *Bm A*3 (Invitrogen) *CMVie* (BD biosciences, Clontech) *Dm MT* (Maroni *et al.*, 1986) *Dm Ac*5.1 (Chung and Keller, 1990) promoters were lined up and are shown in red. The location of other known transcription motif were also identified from published data: CAGT in blue (Blissard *et al.*,1992; Lu and Carstens, 1992, Pullen and Friesen, 1995a; Pullen and Friesen, 1995b), and transcriptional initiation sites mark by +1 directly above the essential nucleotide(s).

Table 3.1: Nucleotide distance of the upstream TATA box of eukaryotic gene promoters from the transcription initiation site[1].

Promoters	TATA Box distance from initiator element	Nucleotide at position +1 of the transcriptional initiation site
*Ac*MNPV *ie*-1	-32	A
*Op*MNPV *ie*-2	-31 / -30	C / A
*Bm A*3	Unknown	Unknown
CMVie	-27	T
Dm MT	-27	T
*Dm Ac*5.1	-30	A

[1]The first nucleotide of the RNA start site is depicted as +1

Of the promoters studied in this thesis, two contained the CAGT motif surrounding the recognised RNA initiation site (*Ac*MNPV *ie*-1 and *Op*MNPV *ie*-2; **Figure 3.12**) and two (*Dm MT* and *Dm Ac*5.1; **Figure 3.12**) contained the motif close to the vicinity of the recognised initiation point. The *Bm A*3 and *CMVie* promoter do not have a CAGT motif, suggesting the TATA box can work independently of the CAGT motif. Although CAGT is present in both *Dm* promoters, promoter activation was not identified, suggesting that

CAGT does not function to activate these promoters in the cell lines tested. Pullen and Friesen (1995b) showed that the CAGT motif could function to initiate gene expression in the absence of the TATA element. Point mutation or complete deletion of CAGT from the *Ac*MNPV *ie*-1 promoter, revealed a significant decrease in promoter activity. It was concluded that CAGT is sufficient to direct proper transcription from *Ac*MNPV *ie*-1, and the TATA box helps to boost basal transcriptional level.

In all promoters, whether a TATA element is present or not, the innate low activity of the promoter itself can be significantly increased by elements located upstream (Latchman, 1995); these are formally known as upstream promoter elements (UPE). Analyses of published sequences for the identification of known higher eukaryotic UPE in promoters used in this thesis, shows a high degree of variation (**Table 3.2**).

Table 3.2: Identification of upstream promoter elements from known higher eukaryotic promoters in published sequences of *Ac*MNPV *ie*-1[1], *Op*MNPV *ie*-2[2], *Bm A3*[3], *CMVie*[4], *Dm MT*[5] and *Dm Ac*5.1[6].

Promoters	GC Box Consensus GGGCGG	CCAAT Box Consensus CCAAT	GATA Box* Consensus $^{C}/_{T}$TATC$^{A}/_{T}$	CREB Box Consensus TGACGTCA	AP1 Box Consensus TGACGTCA
*Ac*MNPV *ie*-1	NI	GGCATAAT	TTATCA	NI	NI
*Op*MNPV *ie*-2	NI	NI	TTATCT	NI	NI
Bm A3	GGGCGG	NI	TTATCA TTATCT	NI	NI
CMVie	GGGCGG	CCAAT	NI	NI	TGACGTCA
Dm MT	NI	CCAAT	NI	NI	NI
*Dm Ac*5.1	GGGCGG	CCAAT	TTATCT	TGACGTCA	NI

[1]Guarino and Summers, 1987; [2]Theilmann and Stewart, 1991; [3]Invitrogen; [4]BD biosciences, Clontech; [5]Maroni *et al.*, 1986; [6]Chung and Keller, 1990. *insect binding consensus. Consensus not identified (NI).

Examination of *Bm A3*, *CMVie*, and *Dm Ac*5.1 promoters revealed that all contained at least one or more copies of a GC-rich sequence, known as the GC or Sp1-box (Dynan and Tjian, 1983a, b; Courey and Tjian, 1988). The GC-box binds Sp1, an essential TF for the formation of transcription initiation complexes (Pugh and Tjian, 1990; Sune and Garcia-Blanco, 1995). Studies to identify the role of Sp1 in the regulation of the retroviral long terminal repeat (LTR) have demonstrated a strict requirement for Sp1 involvement for both basal transcription and Tat-mediated *trans*-activation of the human immunodeficiency virus type1 LTR (Sune and Garcia-Blanco, 1995). The ability of this protein to activate

transcription has been mapped to two glutamine-rich regions. These regions interact with transcription activation factors (TAFs) that are associated with the transcriptional binding protein in the TFIID complex (**Figure 1.4**). The absence of Sp1 or TAFs from the cell renders the promoter transcriptionally inactive (Sune and Garcia-Blanco, 1995). *Drosophila* cells have been shown to produce endogenous Sp1 (Hoey *et al.*, 1990), but it is not clear whether other insect cells produce Sp1. If not, this may explain why *Dm Ac*5.1 was not active in the cell lines tested here.

Both *Bm A*3 and *CMVie* promoters contain GC-boxes and were active, suggesting a separate transcriptional mechanism may have surpassed Sp1 activation, which employs *cis* or *trans*-activating enhancers to boost transcription above basal levels.

The pentanucleotide sequence CCAAT is commonly found 50 to 100 nucleotides upstream from the RNA start site (Gidoni *et al.*, 1984; Dynan and Tjian, 1985; Johnson and McKnight, 1989). Like the GC-box, the CCAAT-box plays an essential role in the activity of a wide range of promoters. In contrast to the GC-box, the motif binds a number of TFs, some of which are produced in all tissues, whilst others are expressed in a tissue-specific manner (Johnson and McKnight, 1989). Of the promoters used in this study, *CMVie*, *Dm MT* and *Dm Ac*5.1 contain a CCAAT-box. Guarino and Summers (1987) identified a sequence similar to CCAAT in *Ac*MNPV *ie*-1 (GGCATAAT), located 69 to 77 nucleotides upstream of the start site for transcription (**Table 3.2**). Expression from the promoters containing the CCAAT motif in this thesis were all classified as weak or functionally inactive. Since the two promoters (*Bm A*3 and *Op*MNPV *ie*-2), shown to produce the strongest transcriptional activity do not contain this motif, a different mechanism of transcriptional activation may have been employed.

The GATA TF family has been characterised as recognising the hexanucleotide $^{A}/_{T}GATA^{T}/_{C}$ motif (Evans and Felsenfeld, 1989). The GATA-motif has been found to be recognised not only by vertebrate proteins, but also invertebrate and fungal proteins (Evans and Felsenfeld, 1989; Fu and Marzluf, 1990; Skeiky and Iatrou, 1991, Spieth *et al.*, 1991). The best-characterised member of the GATA family is the erythroid-specific GATA-1 factor (Evans *et al.*, 1988). Studies have shown (Krappa *et al.*, 1992) the interaction of an insect GATA-like protein with the *PE*38 promoter of *Ac*MNPV, which has been termed *S. frugiperda* nuclear protein 1 (*Sf*NP-1). The characterisation of an insect GATA-motif (consensus $^{C}/_{T}TATC^{A}/_{T}$) upstream of the TATA element (Krappa *et al.*, 1992) was used to analyse the promoters used in this study. Interestingly all but two (*CMVie* and *Dm MT*)

promoters contain one or more copies of this specific motif (**Table 3.2**). Furthermore, the two strong promoters (*Bm A*3 and *Op*MNPV *ie*-2) are included in these findings. It is possible that insect GATA TF may be involved in the regulation of *Bm A*3, *Op*MNPV *ie*-2 and *Ac*MNPV *ie*-1 transcriptional activation in *Sf*9 cells, although evidence from transient expression studies suggests mutations in GATA sequences do not influence *PE*38 promoter activity in insect cell culture (Krappa *et al.*, 1992).

A common factor identified among the strong *trans*-acting *Bm A*3 and *Op*MNPV *ie*-2 promoters is the inherent presence of *cis* and *trans*-acting enhancer-like elements (Section 1.5.2, 1.5.3). The *CMVie* also contains a strong eukaryotic *cis*-acting enhancer element 89 to 495 nucleotides upstream of the TATA-box, yet activation of this promoter was only just detectable in this study. It is possible the enhancer was able to aid transcriptional activation of *CMVie* to levels of activity observed. Both *Dm* promoters used did not contain identifiable enhancer-like sequences, and may be a contributing factor to why activity from these promoters was not detected in *Sf*9 and *T.ni* Hi5 cells.

Early studies using deletion analysis experiments to characterise the *Op*MNPV *ie*-2 gene and its promoter (Theilmann and Stewart, 1991) show that, of the 656 nucleotides that comprised the proposed promoter region, only 275 nucleotides upstream of the RNA start site were needed to activate full promoter activity in *Sf*9 and *Lymantria dispar* (*LD*652Y) cells. The identification of an 18 base pair (bp) sequence (consensus CTTATCGGNACAGGACGC), contained among the 275-bp previously identified, was almost completely repeated at two other locations in the promoter, and partially repeated at six other locations in the promoter (Theilmann and Stewart, 1991). Deletion of one or more copies of the 18-bp dramatically decreased promoter activity; deletion of all 8 repeat sequences rendered the promoter completely inactive (Theilmann and Stewart, 1991). Comparison of the 275-bp *Op*MNPV *ie*-2 promoter with the same region of *Ac*MNPV *ie*-1 shows very little homology. From previous work by Theilmann and Stewart (1991), the identified sequences in *Op*MNPV *ie*-2 are thought to act as *cis*-acting enhancers. The presence of these sequences in their entirety augment promoter activity above levels observed from *Ac*MNPV *ie*-1 generating a strong promoter capable of high level heterologous gene expression in *Sf*9 and *T.ni* Hi5 cells.

The strong promoter activity observed in this Chapter from *Bm A*3+E complements findings from a number of previous studies (Lu *et al.*, 1996; Farrell *et al.*, 1998; Farrell *et al.*, 1999; Keith *et al.*, 1999). The effect of *trans*-acting enhancers on the *Bm A*3 gene

promoter was first described when the *Bm*NPV *ie*-1 gene was characterised as a co-activator of the *Bm A*3 promoter in transfected insect cells and was shown to increase the level of transcription from this promoter by two orders of magnitude (Lu *et al.*, 1996). Following further work on this system, the *hr* 3 from *Bm*NPV was identified (Lu *et al.*, 1997) and characterised as a second enhancer of the *Bm A*3 promoter. Interestingly, comparisons of *hr* 3 from *Bm*NPV with *hr* 1 to 5 of *Ac*MNPV show identical homology between the highly conserved palindrome consensus TTTACAAGTAGAATTCTACT (Guarino *et al.*, 1986; Lu *et al.*, 1997). The *hr*s of *Ac*MNPV are known to increase expression of viral genes in *Sf* and *T.ni* cells (Rodems and Friesen, 1993); it may be that *hr* 3 from *Bm*NPV would also function to enhance specific promoters in the same cell lines. When *hr* 3 from *Bm*NPV was linked to gene constructs expressing CAT under the control of the *Bm A*3 promoter, *hr* 3 was found to enhance significantly the *in vitro* expression of CAT in *Sf*21 and *Bm*5 cell lines (Lu *et al.*, 1997). Furthermore, when CAT was expressed under the *Bm A*3 promoter in the presence of both *cis* and *trans*-acting enhancers in transfected cells, the results indicated an augmentation in the level of *Bm A*3 promoter strength by three orders of magnitude relative to that of the basic *Bm A*3 promoter (Lu *et al.*, 1996; Farrell *et al.*, 1998; Farrell *et al.*, 1999; Keith *et al.*, 1999). The apparent increased promoter activity from *Bm A*3+E compared to *Ac*MNPV *ie*-1 may be explained by the presence of enhancers; no enhancer-like elements were identified in the *Ac*MNPV *ie*-1 promoter used in this study.

Gene expression is a complex process, which is tightly regulated at the transcriptional level. Although the interaction of multiple proteins is likely to be important in eukaryotic gene expression, the absence or inactivity of a single factor does not necessarily lead to termination of a transcription complex (Johnson and McKnight, 1989). Although the data in **Figure 3.12** and **Tables 3.1 / 3.2** allows interesting comparisons to be made, it is by no means conclusive.

The results presented and discussed in this Chapter give an insight into promoters and DNA elements able to stimulate increased transcription activity that may be used in a stable insect cell expression system. Transient, *in vitro* assays were deemed to be the simplest way of indicating likely results in a stable cell system without generating recombinant cell lines for all promoters tested in this study.

In conclusion, *Sf*9 and *T.ni* Hi5 cells were deemed suitable for generating stable insect cell lines with the use of *Bm A*3+E or *Op*MNPV *ie*-2 promoters, as an alternative to *Ac*MNPV

ie-1, driving expression of a foreign gene of interest. The *Bm A*3+E and *Op*MNPV *ie*-2 were also identified as potential candidates for the development of a novel insect expression system (described in Chapter 4) for use in *Sf*9 and *T.ni* Hi5 cells.

CHAPTER 4: CONSTRUCTION OF A TETRACYCLINE INDUCIBLE EXPRESSION SYSTEM FOR INSECT CELLS

4.1 Introduction

Stringent regulation of gene expression offers a number of advantages for studying gene function or production of recombinant proteins that are either cytotoxic or cytostatic (Yarronton, 1992; Rossi and Blau, 1998). Early attempts to develop inducible expression vectors for mammalian cells relied upon the use of heat shock promoters (Schweinfest *et al.*, 1988), metallothionein promoters with heavy metal ion control (Hu and Davidson, 1990) and steroid regulatory promoters (Israel and Kaufman, 1989; Ko *et al.*, 1989). A number of transcriptional control systems have been described (Yarronton, 1992), in which the expression of heterologous genes by RNA polymerase II promoters is susceptible to outside stimuli. These endogenous systems usually suffer from one, or both, of the following drawbacks. First, inducement treatment (such as heat shock) can invariably lead to the co-ordinate induction of a variety of cellular genes, which evoke pleiotropic effects that render analysis of the resulting phenotype difficult (Lee *et al.*, 1988). Second, most regulated promoters have high basal levels of activity in the non-induced state, which prevents the total shut-off of gene expression. The resulting leaky activity of these promoters generally precludes them from applications such as gene function studies. Even so, when the primary goal is the induction of high level protein synthesis, and negative effects that interfere with the physiology of the cell can be tolerated, such systems may be employed.

In an endeavour to overcome some of the problems of utilising endogenous control elements, attempts have been made to use regulatory elements distant in evolution from eukaryotic cells. In a number of cases, regulatory elements from *E. coli* have been used successfully to regulate foreign gene expression in eukaryotic cells (Brown *et al.*, 1987; Hu and Davidson, 1987; 1990). Several studies have shown that the lactose (*lac*) repressor / operator / inducer system of *E. coli* functions in mammalian cells. Three fundamentally different approaches have been described: (i) prevention of transcription initiation by properly placed *lac* operators at promoter sites (Brown *et al.*, 1987; Hu and Davidson, 1987; Figge *et al.*, 1988; Deuschle *et al.*, 1989; Fuerst *et al.*, 1989); (ii) blockage of transcribing RNA polymerase II during elongation by a *lac* repressor / operator complex (Deuschle *et al.*, 1990), and (iii) activation of a promoter responsive to a fusion between *lac* repressor and the activating domain of virion protein 16 (VP16) of herpes simplex virus (HSV; Lawbow *et al.*, 1990; Baim *et al.*, 1991).

Studies have shown that utility of the *lac* repressor / operator system in mammalian cells is limited since the inducer isopropyl β-D-thiogalactopyranoside (IPTG), despite its rapid uptake and intracellular stability (Wyborski and Short, 1991), acts rather slowly and inefficiently, resulting in only moderate induction.

The tetracycline-resistance (*tet*) operon identified as a set of prokaryotic control elements, has also been characterised as functional in higher eukaryotic cells (Gossen and Bujard, 1992; Gossen *et al.*, 1995), without the interference of physiological processes.

In *E. coli*, the tetracycline repressor protein (TetR) negatively regulates the genes of the *tet* operon on the Tn*10* transposon. The TetR blocks transcription of these genes by binding to the tetracycline operator sequences (*tetO*) in the absence of tetracycline (Tc) (**Figure 4.1A-C**). The TetR and *tetO* provide the basis of the tetracycline-Off (Gossen and Bujard, 1992) and-On (Gossen *et al.*, 1995) inducible systems developed for use in mammalian cell lines, such as CHO and Hela.

The Tetracycline inducible (Tet) system is comprised of two plasmid-based vectors. The first vector (pTet-Off / pTet-On depending on the system of choice) contains the first critical component of the Tet system, which is the regulatory protein based on the TetR. In the Tet-Off system, this protein is a fusion of amino acids 1-207 of the TetR and the C-terminal 127 amino acids of the HSV VP16 activation domain (Triezenberg *et al.*, 1988). The VP16 domain converts the TetR from a transcriptional repressor to a transcriptional activator, the resulting chimeric protein is known as the tetracycline-controlled transactivator (tTA; Gossen and Bujard, 1992). The gene encoding tTA has been subsequently placed under the control of the human cytomegalovirus immediate early (*CMVie*) promoter, to derive the pTet-Off regulator plasmid.

The Tet-On system is very similar to Tet-Off. However, the regulatory protein is based on a reverse TetR (rTetR), which has been created by 4 amino acid changes in the TetR (Hillen and Berens, 1994; Gossen *et al.*, 1995). The rTetR is fused with the VP16 activation domain to generate the reverse tTA (rtTA). The resulting protein, rtTA is encoded by the pTet-On regulator plasmid (**Figure 4.2**). Both pTet-On / Off regulator plasmids also include a neomycin-resistance (*Neo*^{r}) gene to permit selection of stably transfected cell line.

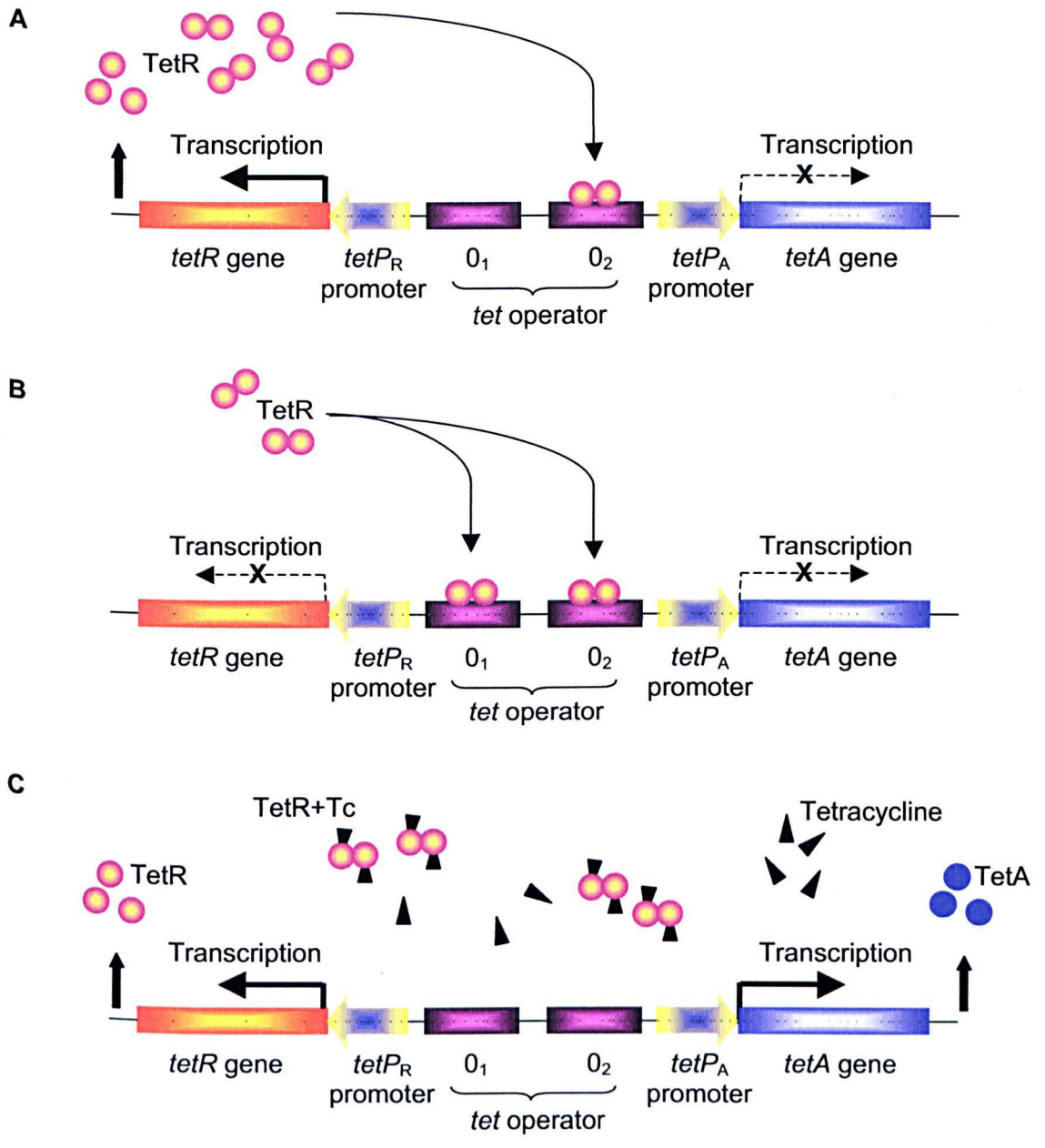


Figure 4.1A-C: Schematic of the mechanism of regulation from the Tn*10*-encoded Tc-resistance operon in *E. coli*. **A / B:** In the absence of tetracycline (Tc), expressed TetR forms a dimer and binds to the two tandem *tet* operators 0_1 and 0_2 located between *tetA* and *tetR*. The affinity of TetR to operator 0_2 is about 2-5 fold higher than it is to operator 0_1. This causes TetR to bind operator 0_2 first, which means the $tetP_R$ promoter retains transcriptional activity and *tetR* is expressed, whilst the $tetP_A$ promoter is repressed and no TetA is produced (**A**). Subsequent binding of both operators renders $tetP_A$ and $tetP_R$ promoters transcriptionally repressed therefore neither *tetA* or *tetR* are expressed (**B**). **C:** In the presence of Tc, Tc binds the TetR-operator complex and triggers a conformational change in TetR so it can no longer bind to the *tet* operator, enforcing rapid dissociation from the DNA. The dissociation of TetR from the *tet* operator, activates $tetP_A$ and $tetP_R$ promoter activity and *tetA* or *tetR* expression.

The second critical component is the response plasmid (pTRE), which expresses a heterologous gene of interest under the control of the tetracycline-response element (*TRE*). The *TRE* consists of seven tandem repeats of a 42-bp sequence containing the *tetO*, and is located just upstream of the of the minimal *CMVie* promoter (Gossen and Bujard, 1992), which lacks the strong enhancer elements normally associated with the *CMVie*. Because these enhancer elements are missing, there is no leaky expression of the heterologous gene from the *TRE* / minimal *CMVie* promoter in the absence of binding tTA or rtTA (**Figure 4.2**).

The ultimate goal in setting up a functional Tet system is to create a double-stable Tet cell line, which contains both the regulatory and response plasmids. When cells containing both pTet-On (or pTet-Off) and pTRE (includes the heterologous gene of interest), the heterologous gene is expressed upon binding of the (r)tTA protein to the *TRE* (**Figure 4.2**). The Tet-Off system operates by tTA binding to the *TRE* in the absence of Tc or doxycycline (Dox), whilst the Tet-On system work by rtTA binding to the *TRE* in the presence of Dox.

The Tet systems have been shown to modulate gene expression 10^5-fold (Gossen and Bujard, 1992) from the repressed state to the activated state. The very tight control of gene expression has earned it increasing popularity since its development (Gatz and Quail, 1988; Gossen and Bujard, 1992) and it has been successfully employed in a number of studies (Damke *et al.*, 1994; Resnitzky *et al.*, 1994; Yin and Schimke, 1995; Gallagher *et al.*, 2003; Martel-Renoir *et al.*, 2003; Modha *et al.*, 2003; Qu *et al.*, 2004). The Tet system has also been shown to function as a tetracycline-regulated transcriptional system with high induction rates in several other eukaryotic systems, including plant cells (Gatz and Quail, 1988; Gatz *et al.*, 1992), transgenic mice / rats (Fishman *et al.*, 1994; Efrat *et al.*, 1995; Ewald *et al.*, 1996; Kistner *et al.*, 1996; Mayford *et al.*, 1996; Harding *et al.*, 1998), *Drosophila* spp. (Bello *et al.*, 1998) and a variety of cells infected with virus (Kim *et al.*, 1995; Hwang *et al.*, 1996).

Studies by Wu *et al.* (2000) have demonstrated the functional activity of the Tet-Off system in insect cells. However, levels of tTA were considerably low and the heterologous gene activity observed was exceptionally weak. These studies concluded that the activity of the *CMVie* promoter in insect cells was very weak (these findings correlate with results obtained in Chapter 3, showing the *CMVie* promoter is transcriptionally weak in insect

cells, **Figures 3.3, 3.11 A / B**), and that promoters with stronger transcriptional activity were required to drive the Tet system in insect cells.

To develop a Tet system for use in *Sf*9 and *T.ni* Hi5 cells, it was necessary to replace the *CMVie* promoters of the Tet system with promoters capable of high-level foreign gene expression in lepidopteran cells. Work described in chapter 3 has characterised the *Bombyx mori cytoplasmic actin* 3 promoter / enhancers (*Bm A*3+E), and the *Orgyia pseudotsugata* MNPV *ie*-2 promoter, as functionally strong in lepidopteran cells. These promoters were identified as potential candidates for replacing the *CMVie* promoters of the Tet-On system.

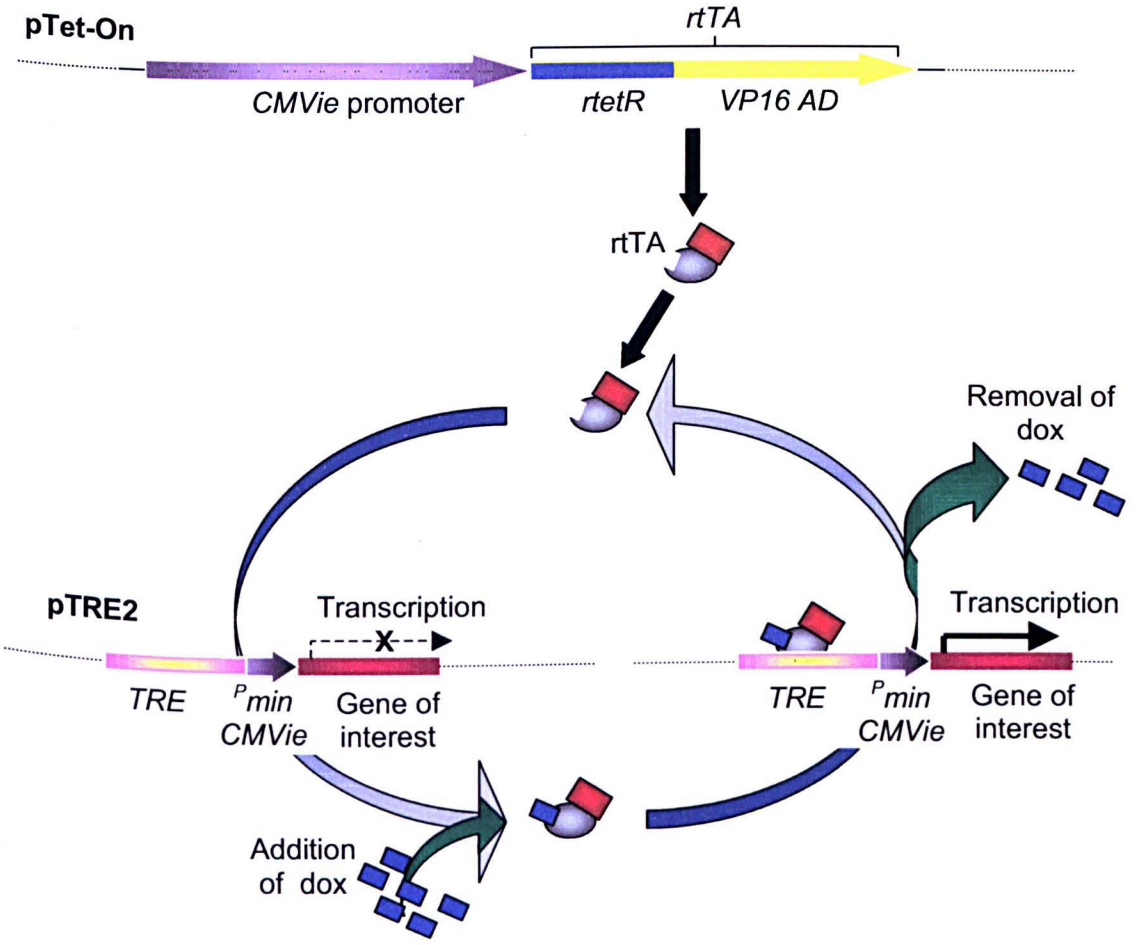


Figure 4.2: Schematic of gene regulation in the Tet-On System. The Tetracycline response element (*TRE*) is located upstream of the minimal *cytomegalovirus immediate early* gene promoter (*PminCMVie*), which is silent in the absence of activation. The reverse tetracycline transcriptional activator (rtTA) is expressed constitutively from pTet-On, which binds the *TRE* (pTRE2) and activates transcription in the presence of doxycycline (dox). When dox is remove the rtTA no longer has the properties to bind *TRE* and transcription is silent.

This chapter describes the construction of two plasmid-based expression vectors, which were used in an attempt to develop an inducible expression system for use in insect cell lines. Elements of the "Express Insect™ system", "InsectSelect™" (Invitrogen™) and "Tet-On™ system" (BD Biosciences Clontech) were used in the construction of this system. The gene *cat* (as described in Sections 1.7.2 and 3.1) was used as a reporter to determine functional promoter activity.

4.2 Construction of the insect Tet regulatory vector

Construction of the insect Tet regulatory plasmid required placing the regulatory gene *rtTA* from pTet-On (**Figure 4.3**) under the control of a strong promoter, functional in lepidopteran cells.

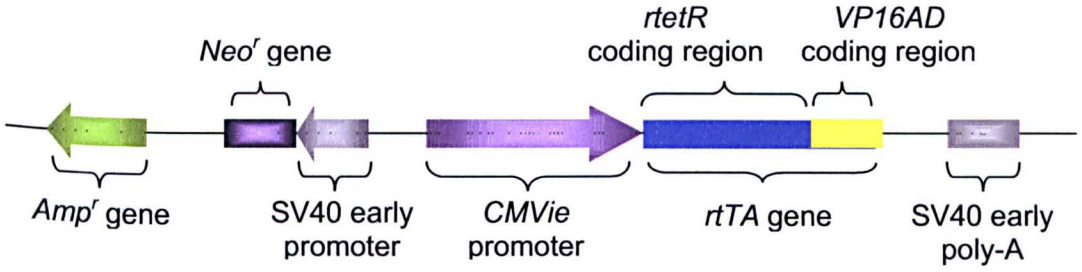


Figure 4.3: Genetic map of pTet.On (BD Biosciences, Clontech) shows the *CMVie* promoter, *rtetR / VP16AD,* fusion, which encodes the *rtTA* gene, SV40 early poly-A coding region, SV40 early promoter, *neomycin* resistance (*Neo*^{r}) gene and *Amp*^{r} gene.

Of the promoters tested in this thesis (Chapter 3) the *Op*MNPV *ie*-2 and *Bm* A3+E promoters were identified as transcriptionally strong in lepidopteran cells. Comparison of expression levels from these promoters revealed not a great deal of difference. However, for technical reasons the *Bm* A3+E promoter was used to transcribe *rtTA*. The main technical problem was the removal of *rtTA* from pTet-On for insertion downstream of the *Op*MNPV *ie*-2 promoter in pIZT/V5-His, as no compatible restriction sites flanked *rtTA*. The vector pXINect.DEST38 containing *Bm* A3+E utilises the efficient Gateway™ TOPO® cloning technology (Section 1.6). This allows for fast and efficient cloning of foreign genes, compared to the conventional ligation dependent method, used to place foreign genes downstream of promoters. Due to the nature of TOPO® cloning, the *rtTA* gene was first inserted into an entry plasmid (pENTR/D-TOPO) to produce an entry clone.

For insertion of *rtTA* into pENTR/D-TOPO, primers were designed to amplify the *rtTA* coding-region from pTet-On. Amplification of *rtTA* using PCR (Section 2.5.5) was carried out. The results shown in **Figure 4.4** clearly show, in lanes 2-5, amplified DNA fragments of a size relative to the *rtTA* gene (1010-bp).

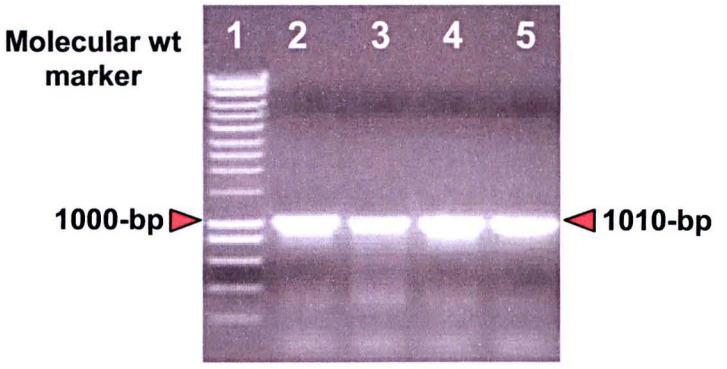


Figure 4.4: Analysis of the amplification of the *rtTA*-coding region from pTet-On, using PCR (Section 2.5.5). Positive amplified *rtTA* coding regions produced DNA fragments banding at 1010-bp (*rtTA*). The above gel shows:

Track 1: Smart Ladder (Eurogentec 1kb)
Track 2-5: Positive amplification of the *rtTA* coding region

The *rtTA* gene was purified (Section 2.5.7.2) and cloned into pENTR/D-TOPO (Section 2.5.12). Positive clones were identified using the restriction enzymes *Xba*I and *Hind*III (Section 2.5.1; data not shown). The correct sequence of *rtTA* was confirmed by sequencing (Section 2.5.8). The resulting entry clone pENTR/D-TOPO.*rtTA* was derived (**Figure 4.5**).

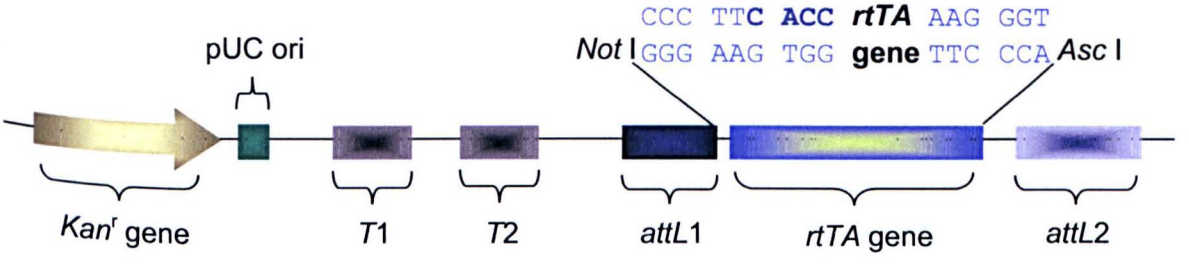


Figure 4.5: Genetic map of pENTR.D.TOPO.*rtTA*, shows the *Kan*^{r} gene, pUC origin of replication, *T1* / *T2* , *attL1* / *attL2* recombination sites with *tTA* inserted.

The *rtTA* gene was subsequently inserted into pXINsect-DEST38 expression vector, using TOPO site-specific recombination between the entry clone (pENTR/D-TOPO.*rtTA*) and the linearised destination vector (pXINsect-DEST38; Section 2.5.12). Positive clones were identified using the restriction enzyme *Xba*I (Section 2.5.1; **Figure 4.6**) and the resulting plasmid was named p*BmA*3+E.*rtTA* (**Figure 4.7**).

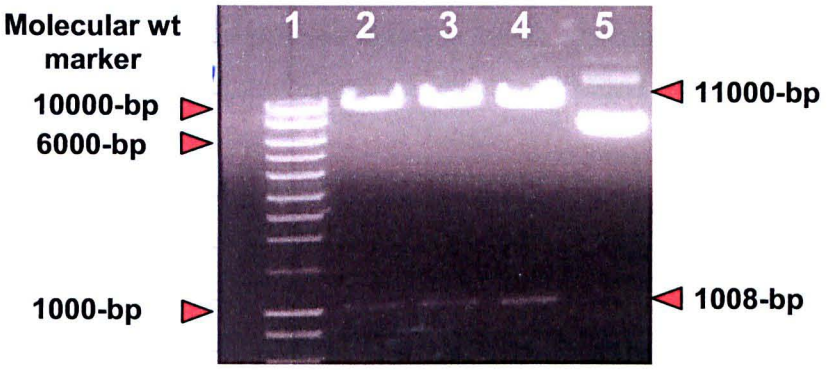


Figure 4.6: Analysis of plasmid DNA, to positively identify the insertion of the *rtTA* gene into pXINsect-DEST38 to derive p*BmA*3+E.*rtTA*.

Plasmid DNA was extracted from the *E. coli* TOP10 bacterial strain by mini-prep purification (Section 2.5.9.1). Plasmid DNA was cut using the restriction enzyme *Xba*I (Section 2.5.1), and run on a 0.7% agarose gel (Section 2.7.1).

Positive clones with the *rtTA* coding region produced two DNA fragments banding at 1008-bp (*rtTA*) and 11000-bp (linear pXINsect-DEST38). The above gel shows:

Track 1:	Smart Ladder (Eurogentec 1kb)
Track 2-4:	p*BmA*3+E.*rtTA* / *Xba*I positive clones
Track 5:	p*BmA*3+E.*rtTA* / uncut

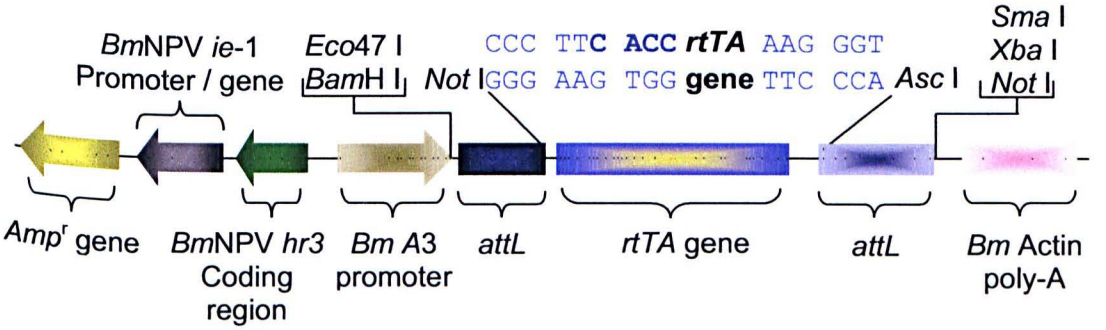


Figure 4.7: Genetic map of p*BmA*3+E.*rtTA*, shows the *Amp*^{r} gene, *Bm*NPV *ie*-1 promoter / gene, *Bm*NPV *hr*3 coding region, *Bm A*3 promoter with *rtTA* inserted downstream, *attL*1 / *attL*2 recombination sites, and the *Bm* actin poly-A coding region.

4.3 Transient expression of the *rtTA* gene in insect cells

Transient expression of *rtTA* from the *BmA*3+E promoter in insect cells was evaluated to determine, firstly, if the production of rtTA was at suitable levels required for the insect *tet* response vector to work, and secondly, to confirm that the levels of rtTA produced were not cytotoxic to the host cells used. This was important as there is evidence to suggest over-expression of rtTA can have detrimental effects on host cells, if levels produced are above the critical threshold of the cell (Bujard, 1999). A comparison of *rtTA* expression from the *CMVie* (in pTet-On) and *BmA*3+E (in p*BmA*3+E.*rtTA*) promoters in insect cells was undertaken to compare level of rtTA produced.

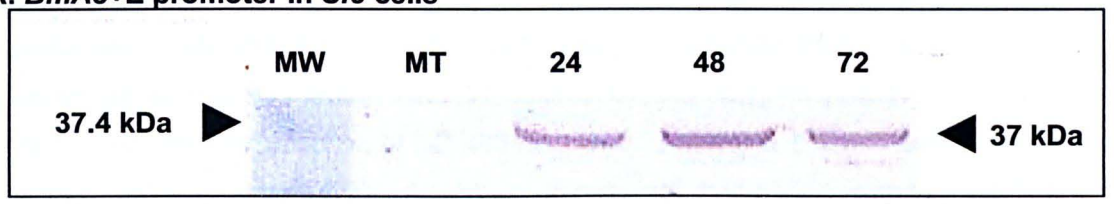
*Sf*9 and *T.ni* Hi5 cells were seeded (Section 2.2.3) into 35mm tissue culture dishes, and left to adhere for 1 h. The cell mono-layers were transfected (Section 2.2.4) using 1μg of plasmid DNA (p*BmA*3+E.*rtTA* or pTet-On). Transfections were carried out in triplicate. Cells were harvested after 24, 48 and 72 h incubation at 28°C. Standard amounts of total protein (25μg; Section 2.7.4) from each sample were subjected to SDS-PAGE and Western blot analysis (Section 2.7.5, 2.7.6).

Figure 4.8 shows results obtained for *rtTA* expression from the *BmA*3+E promoter in *Sf*9 (**A**) and *T.ni* Hi5 (**B**) cells. Qualitative analysis of rtTA production using a polyclonal antibody specific to the *VP16* domain (BD Biosciences) of *rtTA* (molecular weight 37 kDa), showed no detectable levels of rtTA in mock-transfected *Sf*9 or *T.ni* Hi5 cells (**A / B: lane MT**). In both cell lines transfected with p*BmA*3+E.*rtTA*, production of rtTA was observed at 24-72 hpt (**A / B: lanes 24-72**). The levels of rtTA produced from the *BmA*3+E promoter were shown to vary between the cell lines used, without detrimental effects to the cells. The highest level of rtTA produced was observed in *T.ni* Hi5 cells, at 24-48 hpt (visual observation: **B: lanes: 24-48**); at 72 hpt (**B: lane 72**) levels were shown to decrease. Production of rtTA in *Sf*9 cells was shown to peak at 48 hpt, while producing relatively similar levels at 24 and 72 hpt (**A: lanes 24-72**).

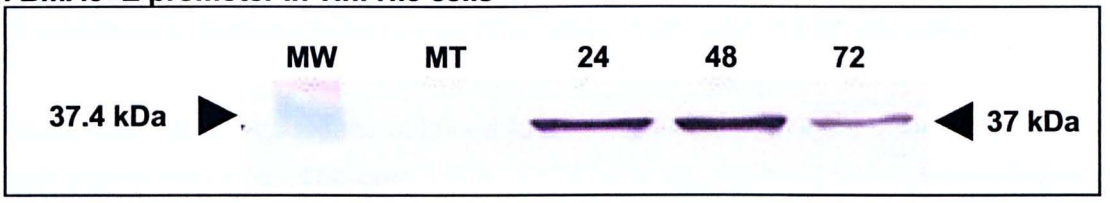
Figure 4.8 also shows results obtained for *rtTA* expression from *CMVie* in *Sf*9 (**C**) and *T.ni* Hi5 (**D**) cells. The production of rtTA was not observed in mock-transfected cells (**C / D: lane MT**). Expression of *rtTA* from the *BmA*3+E promoter at 72 hpt was incorporated as a positive control (**C / D: lane +ve**). In both cell lines transfected with pTet-On, no detectable levels of rtTA were observed at 24-72 hpt (**C / D: lanes 24-72**).

The insect Tet regulatory plasmid (p*BmA*3+E.*rtTA*) was shown to produce detectable levels of rtTA in *Sf*9 and *T.ni* Hi5 cells, without detrimental effects to the cells (cell viability studies Section 4.4, data not shown). However, pTet-On produced no detectable levels of rtTA. The functional working of the insect Tet regulatory plasmid meant this vector could be used to generate stable insect cell lines expressing rtTA, which would be used to test the insect Tet response plasmid (Section 4.5).

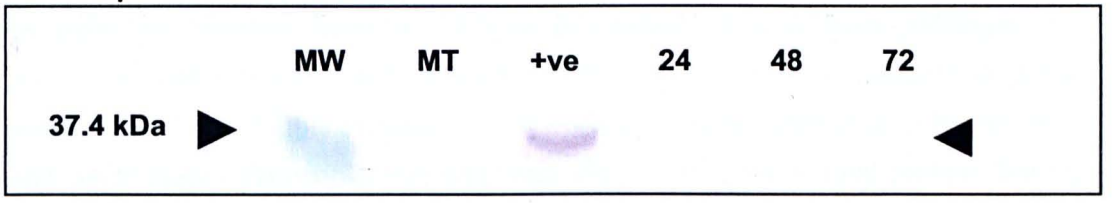
A: *BmA*3+E promoter in *Sf*9 cells



B: *BmA*3+E promoter in *T.ni* Hi5 cells



C: *CMVie* promoter in *Sf*9 cells



D: *CMVie* promoter in *T.ni* Hi5 cells

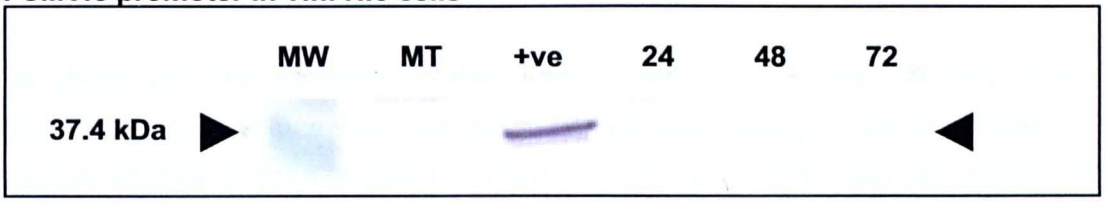


Figure 4.8A-D: Western blot analysis of *Sf*9 and *T.ni* Hi5 cells transfected with 1μg of p*BmA*3+E.*rtTA* (**A / B**) or pTet.On (**C / D**). In all panels, lane MW shows a protein marker (Invitrogen). Lane MT shows intracellular cell extract (25μg / lane) from mock-transfected *Sf*9 and *T.ni* Hi5 cells (negative control). **A / B**: Lanes 24-72 shows intracellular cell extract (25μg / lane) from *Sf*9 (**A**) and *T.ni* Hi5 (**B**) cells transfected with 1μg of p*BmA*3+E.*rtTA*, 24-72 hpt. **C / D**: Lanes +ve shows intracellular cell extract (25μg / lane) from *Sf*9 (**C**) and *T.ni* Hi5 (**D**) cells transfected with 1μg of p*BmA*3+E.*rtTA*, 72 hpt (positive control). Lanes 24-72 show intracellular cell extract (25μg / lane) from *Sf*9 (**C**) and *T.ni* Hi5 (**D**) cells transfected with 1μg of pTet-on, 24-72 hpt. Position of molecular weight (MW) size marker is shown in kDa and the position of rtTA is indicated (37kDa).

4.4 Production of stable insect cell lines expressing rtTA

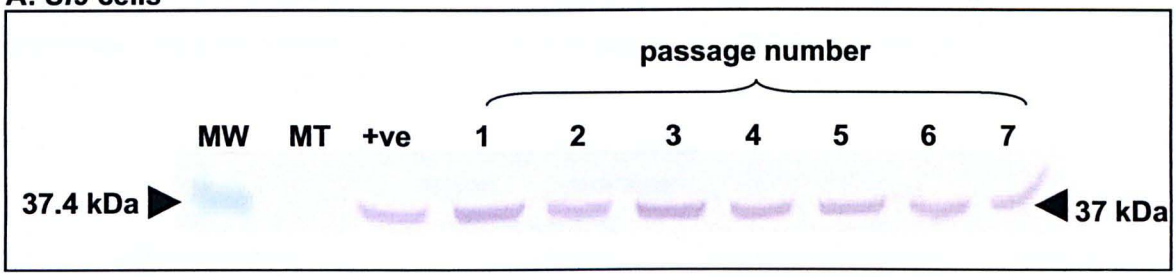
Having constructed and transiently tested the insect Tet regulatory plasmid (Section 4.3), the next step was to produce stable *Sf*9 and *T.ni* Hi5 cells expressing rtTA. These cell lines provided constitutive expression of rtTA, which was required to activate gene expression in the presence of the Tet response plasmid and inducer (Section 4.1).

Stable *Sf*9 and *T.ni* Hi5 cell lines were produced as described in Section 2.2.5. Co-transfection of p*BmA*3+E.*rtTA* (900ng) with a second plasmid (pBmA:neo, 100ng) carrying a dominant selectable marker, *Neo*^{r}, was used for this purpose (Section 3.1; Jarvis *et al.*, 1990). Transient transfection of p*BmA*3+E.*rtTA* into *Sf*9 and *T.ni* Hi5 cells was used as a positive control (Section 4.3). Analyses of transient and stable expression of rtTA was determined by SDS-PAGE / Western blot analysis (Sections 2.7.5 / 2.7.6). To determine cell viabilities at each passage, trypan blue staining (Section 2.2.6) was used.

Figure 4.9A / B shows results obtained for passages 1-7 of *Sf*9 (**A**) and *T.ni* Hi5 (**B**) cells, stably expressing rtTA. The production of rtTA was not observed in mock-transfected cells (**lane MT**). Transient expression of rtTA from the *BmA*3+E promoter at 24 hpt (**lane +ve**) was detected (positive control). Stable production of rtTA from passages 1-7 was observed in both cell lines used (**lanes 1-7**). The levels of rtTA produced from passage 1-7 were shown to be highly consistent. No apparent visible difference in the levels of rtTA could be detected between these cell lines (equal amounts of total protein [25mg] were loaded in each lane).

The stable cell lines generated above were named *Sf*9.rtTA and *T.ni* (Hi5).rtTA. The viability of these cell lines was determined before each passage, and was shown to vary between 88-98% in *Sf*9.rtTA and 86-92% in *T.ni* (Hi5).rtTA cells (data not shown). These stable cell lines will be used to test the functionality of the insect Tet response plasmid as described in Section 4.5.

A: *Sf*9 cells



B: *Tni.* Hi5 cells

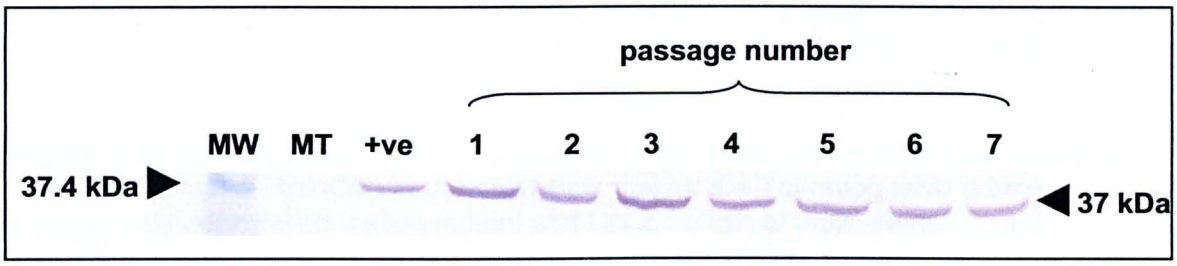


Figure 4.9A / B: Western blot analysis of *Sf*9 (**A**) and *T.ni* Hi5 (**B**) cells stably expressing rtTA. In panels **A / B** lane MW shows a protein marker (Invitrogen). Lane MT shows intracellular cell extract (25μg / lane) from mock-transfected *Sf*9 and *T.ni* Hi5 cells (negative control). Lane +ve shows intracellular cell extract (25μg / lane) from *Sf*9 and *T.ni* Hi5 cells transfected with 1μg of p*BmA*3+E.*rtTA*, 24 hpt (positive control). Lane 1-7 shows intracellular cell extract (25μg / lane) from passages 1-7 of *Sf*9 (**A**) and *T.ni* Hi5 (**B**) cells stably expressing rtTA. Position of molecular weight (MW) size marker is shown in kDa and the position of rtTA is indicated (37kDa).

4.5 Construction of the insect Tet response vector

The original Tet response plasmid (pTRE; **Figure 4.10**), designed for the use in mammalian cell lines, contains the minimal *CMVie* promoter (Section 4.1). Whilst this vector has been shown to function in insect cells, the levels of expressed protein were minimal (Lee *et al.*, 1998; Wu *et al.*, 2000). To overcome this problem it was suggested that by replacing the minimal *CMVie* promoter of with a strong promoter capable of functioning in lepidopteran cells, the inducible system could be adapted for use in insect cells.

The baculovirus *Op*MNPV *ie*-2 promoter was chosen to replace the minimal *CMVie* promoter of pTRE, as it has a strong transcriptional activity with a *cis*-activating enhancer sequence integrated into the short promoter sequence (Chapter 3). The *BmA*3 promoter relies on *cis* and *trans*-activating enhancers external to the promoter, which would have made using this promoter very difficult to engineer into a new vector. Also due to the

dependence of the external enhancers boosting the transcriptional activity of the *BmA3* promoter, regulating transcription from this promoter would be very difficult.

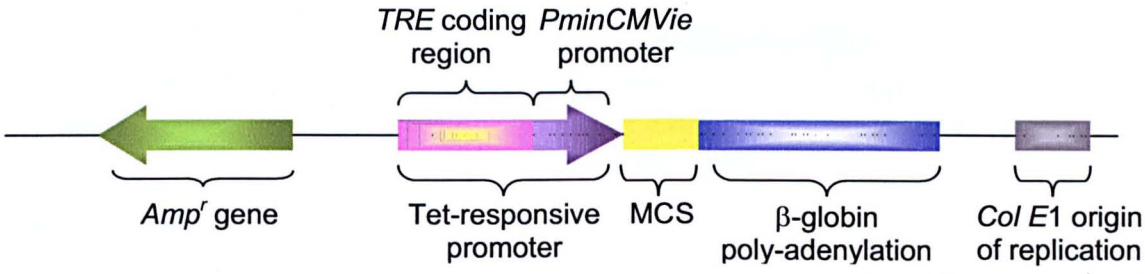


Figure 4.10: Genetic map of pTRE, shows the *Amp*^{r} gene, tetracycline response element (*TRE*) fused to the *PminCMVie* promoter (this makes the Tet-responsive promoter), MCS, β-globin poly-adenylation coding region, and *Col E*1 origin of replication.

The pTRE was used as a backbone plasmid for the development of the insect Tet response plasmid. Modifications to pTRE involved removing the minimal *CMVie* promoter followed by the insertion of *Op*MNPV *ie*-2 immediately downstream of the *TRE* (**Figure 4.11A-C**). The minimal *CMVie* promoter is flanked by the restriction enzyme sites *Asp718*I and these sites were used to excise (Section 2.5.1) the promoter sequence from pTRE (**Figure 4.11A-B**) to derive pTREΔ. The *Op*MNPV *ie*-2 promoter was amplified (Section 2.5.5) from pIZT/V5-His using PCR. The flanking regions of *Op*MNPV *ie*-2 were modified to incorporate *Asp718*I restriction sites for insertion of the promoter into the modified promoter-less pTREΔ vector (**Figure 4.11B**). The *Asp718*I-digested pTREΔ vector was shrimp alkaline phosphatase-treated to prevent self-ligation (Section 2.5.3), and the *Op*MNPV *ie*-2 promoter inserted (Section 2.5.4) to derive pTREΔ.*ie*-2 (**Figure 4.11C**). Digestion using *Hind*III identified the correct orientation of the *Op*MNPV *ie*-2 promoter. Subsequently, the *Op*MNPV *ie*-2 promoter region was sequenced (Section 2.5.8) to confirm sequence integrity.

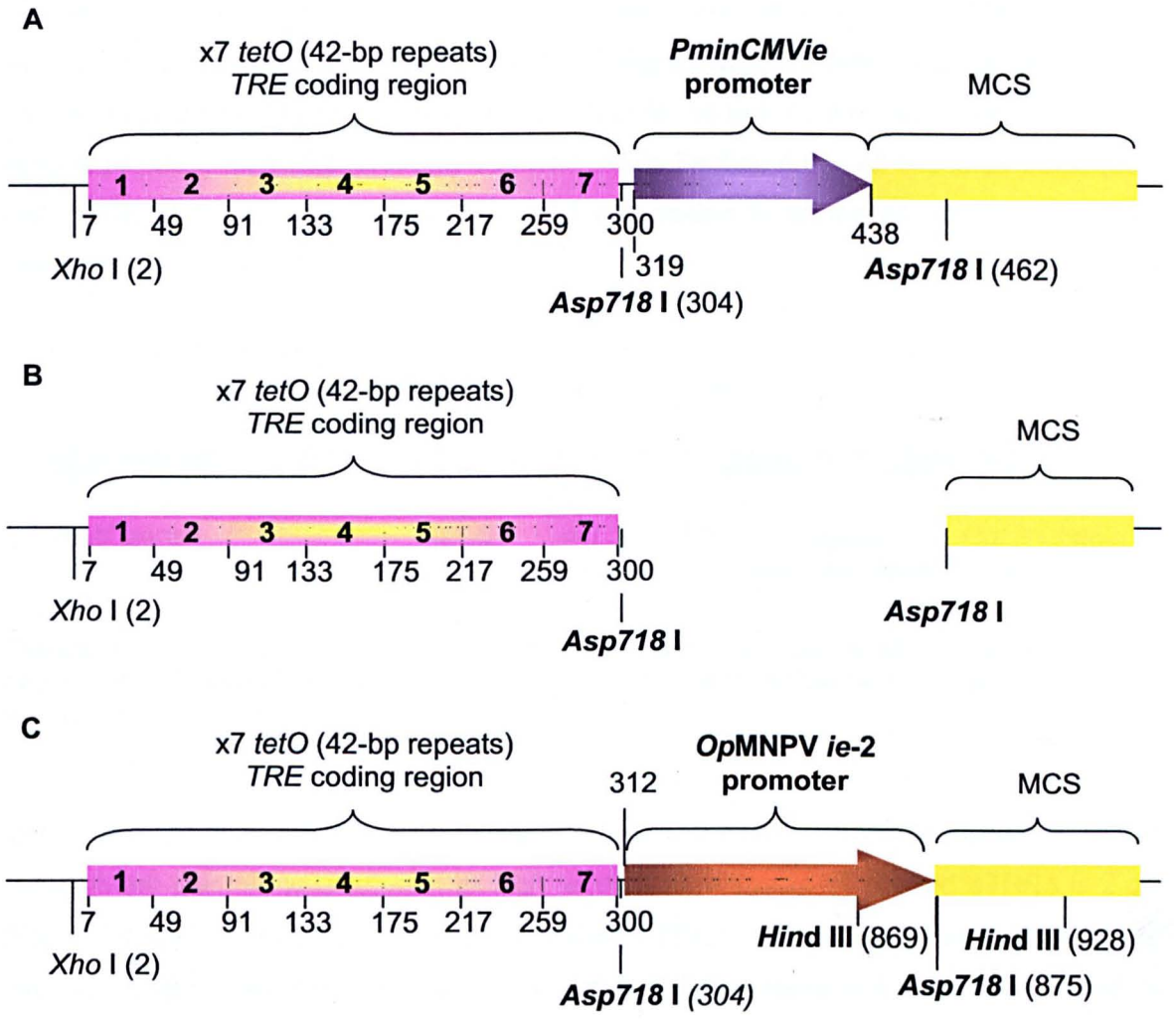


Figure 4.11A: Genetic map of pTRE (Figure 4.10), shows the restriction sites *Asp718*I bisecting the *PminCMVie* promoter, and the tetracycline response element (*TRE*), which is constructed from seven tetracycline operator sequences (x7 *tetO*) 42-bp in length. **B:** The *PminCMVie* promoter was excised from pTRE on an *Asp718*I fragment to generate a linear promoter-less pTREΔ vector, which was used for the insertion of the *Op*MNPV *ie*-2 promoter. **C:** Genetic map of pTREΔ.*ie*-2; pTREΔ.*ie*-2 is similar to pTRE except the *Op*MNPV *ie*-2 promoter has been cloned in place of the *PminCMVie* promoter.

4.6 Problems encountered during the construction of the insect Tet response vector

Having produced the expression vector pTREΔ.*ie*-2, the reporter gene *cat* was chosen to analyse expression from the insect Tet-responsive promoter (*TRE* / *Op*MNPV *ie*-2 promoter). In order to do this *cat* was cloned into pTREΔ.*ie*-2. The *cat* gene was excised from pIZT/V5-His (Section 2.5.1) on a *Bam*HI and *Not*I fragment and cloned into

complementary restriction enzyme sites of pTREΔ.*ie*-2, downstream of the insect Tet-responsive promoter, to derive pTREΔ.*ie*-2.*cat* (**Figure 4.12**). pTREΔ.*ie*-2.*cat* was used to transfect (Section 4.7) normal *Sf*9 and *T.ni* Hi5 cells, as well as the stably transformed cell lines expressing rtTA (*Sf*9.rtTA and *T.ni* (Hi5).rtTA; Section 4.4). All cell lines transfected with pTREΔ.*ie*-2.*cat* were analysed for CAT expression in an induced and non-induced state.

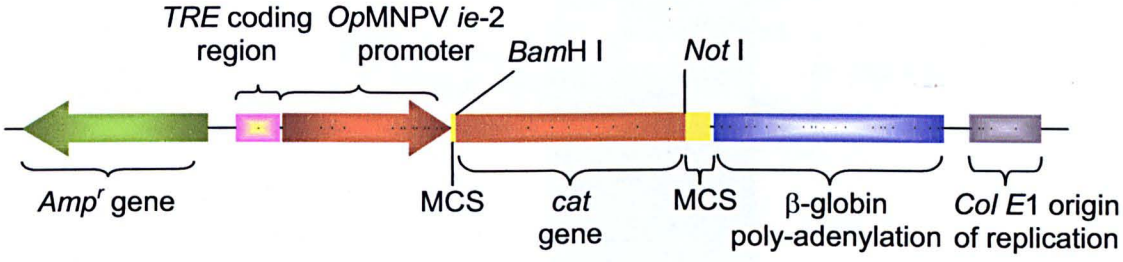


Figure 4.12: Genetic map of pTREΔ.*ie*-2.*cat*, pTREΔ.*ie*-2.*cat* is similar to pTREΔ.*ie*-2 described in Figure 4.11C, except the *cat* gene has been cloned into the *BamH*I and *Not*I restriction sites.

During analyses to confirm the identity of pTREΔ.*ie*-2.*cat*, it became apparent that a contaminant plasmid was present at high levels in the stocks of purified pTREΔ.*ie*-2.*cat* (**Figure 4.13**). Repeated attempts to isolate pTREΔ.*ie*-2.*cat* from the contaminating plasmid failed. Experiments to determine where the contaminant came from failed to produce a definitive answer, although the original stock of pTRE bought from BD Biosciences was suspected. Therefore, the initial pTREΔ.*ie*-2.*cat* ligation stock, which yielded the original pTREΔ.*ie*-2.*cat* was re-transformed (Section 2.4.3), and a new stock of pTREΔ.*ie*-2.*cat*, without the contaminating DNA was eventually derived (**Figure 4.14**), called pTREΔ.*ie*-2.*cat2*. However, a second problem was then identified; enzymatic digest profiles revealed an estimated 210-bp had been deleted from pTREΔ.*ie*-2.*cat2* (**Figure 4.14**). To confirm this plasmid was pTREΔ.*ie*-2.*cat2*, primers were designed to amplify the *Op*MNPV *ie*-2 promoter and *cat* coding-region. The *Op*MNPV *ie*-2 promoter and *cat* coding-region were amplified from both pTREΔ.*ie*-2.*cat* and pTREΔ.*ie*-2.*cat2* using PCR (Section 2.5.5; **Figure 4.15**). The results shown in **Figure 4.15** clearly show in lane 3 an amplified DNA fragment of a size relative to the *Op*MNPV *ie*-2 / *cat* coding-region amplified from pTREΔ.*ie*-2.*cat* (lane2; 1297-bp). Sequencing of these regions were also confirmed as authentic. However, this does not confirm the whereabouts of the deletion in the pTREΔ.*ie*-2.*cat2* vector.

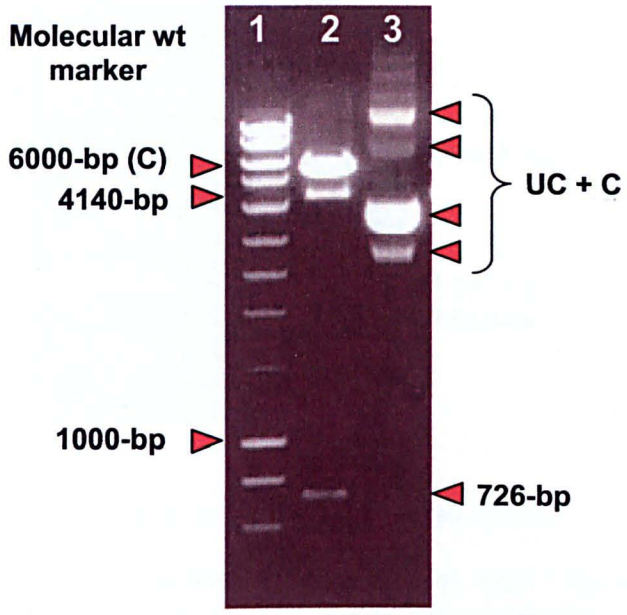


Figure 4.13: Analysis of plasmid DNA, to positively identify pTREΔ.*ie*-2.*cat*.

Plasmid DNA was extracted from the *E. coli* TOP10 bacterial strain by large scale plasmid DNA purification (Section 2.5.9.2). Plasmid DNA was cut using the restriction enzymes *Bam*HI and *Not*I (Section 2.5.1), and run on a 0.7% agarose gel (Section 2.7.1).

Positive identification of pTREΔ.*ie*-2.*cat* produced two DNA fragments banding at 726-bp (*cat*) and 4140-bp (linear pTREΔ.*ie*-2). The above gel shows:

Track 1:	Smart Ladder (Eurogentec 1kb)
Track 2:	pTREΔ.*ie*-2.*cat* / *Bam*HI / *Not*I (4140 / 726-bp) + contaminant (C) DNA (6000-bp)
Track 3:	pTREΔ.*ie*-2.*cat* / uncut (UC) + contaminant DNA (6000-bp)

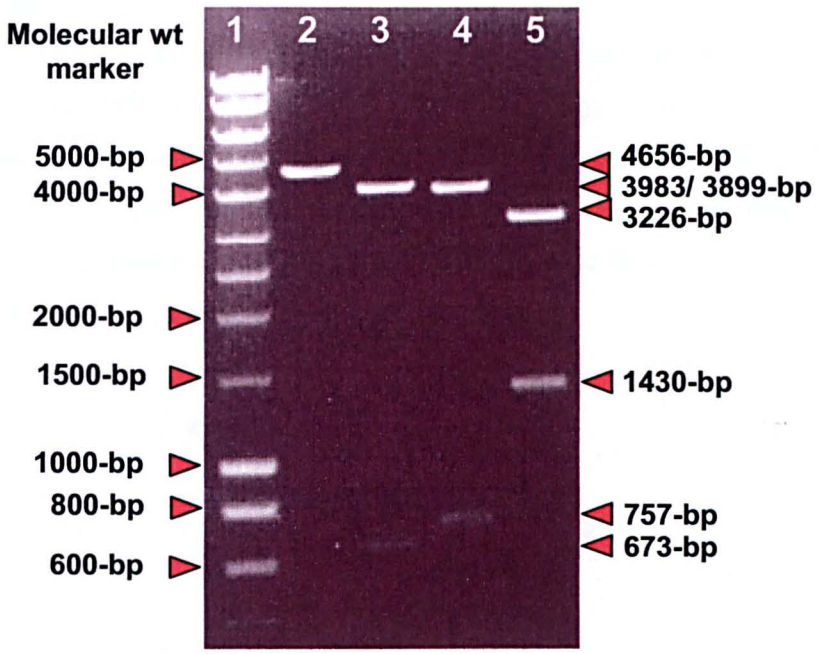


Figure 4.14: Restriction enzyme digest analysis of pTREΔ.*ie*-2.*cat*2.

Plasmid DNA was cut using the restriction enzymes *Bam*HI, *Xho*I / *Bam*HI, *Bam*HI / *Xba*I, and *Xho*I / *Xba*I (Section 2.5.1), and run on a 0.7% agarose gel (Section 2.7.1).

The pTREΔ.*ie*-2.*cat*2 plasmid is 4856-bp (linear), although the restriction profiles shown in tracks 2-5 revealed an estimated 200-bp decrease in size. The above gel shows:

Track 1:	Smart Ladder (Eurogentec 1kb)
Track 2:	pTREΔ.*ie*-2.*cat*2 / *Bam*HI (predicted size 4886-bp, actual size 4656-bp)
Track 3:	pTREΔ.*ie*-2.*cat*2 / *Xho*I/ *Bam*HI (predicted size 3983 / 883-bp, actual size 3983 / 673-bp)
Track 4:	pTREΔ.*ie*-2.*cat*2 / *Bam*HI / *Xba*I (predicted size 4109 / 757-bp, actual size 3899 / 757-bp)
Track 5:	pTREΔ.*ie*-2.*cat*2 / *Xho*I / *Xba*I (predicted size 3226 / 1640-bp, actual size 3226 / 1430-bp)

The next region of the pTREΔ.*ie*-2.*cat*2 vector to be checked for authenticity was the *TRE* coding-region. Primers were designed to amplify the *TRE* / 5' *Op*MNPV *ie*-2 coding-region from pTREΔ.*ie*-2.*cat*2 and pTREΔ.*ie*-2.*cat* vector (positive control). Amplification of *TRE* / 5' *Op*MNPV *ie*-2 coding-region using PCR (Section 2.5.5) was carried out. The results shown in **Figure 4.16** show, in lane 2, the positive amplification of the *TRE* / 5' *Op*MNPV *ie*-2 coding region (611-bp) from the positive control. Whilst, in lane 3, a DNA fragment of 401-bp was amplified from the pTREΔ.*ie*-2.*cat*2 vector, which does not correlate with the size of the *TRE* / 5' *Op*MNPV *ie*-2 coding region, but reveals a difference in size of 210-bp.

As the *Op*MNPV *ie*-2 promoter has been shown previously in this chapter to be present in this vector, it was suggested that the deletion had occurred within the *TRE* coding-region. Subsequently the *TRE* and *Op*MNPV *ie*-2 coding-regions of the pTREΔ.*ie*-2.*cat*2 plasmid were sequenced to confirm sequence integrity (Section 2.5.8). The correct sequence of the *Op*MNPV *ie*-2 coding-region was confirmed, but the sequencing data revealed that five of the seven *tetO* coding-regions had been deleted. The five *tetO* coding-regions deleted were internal repeats 2-6 (**Figure 4.17A / B**).

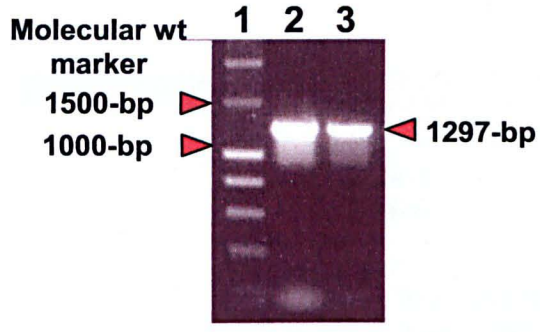


Figure 4.15: Analysis of the amplification of *Op*MNPV *ie*-2 / *cat* coding-region from pTREΔ.*ie*-2.*cat*2, using PCR (Section 2.5.5). A positive control was included, which involved amplifying the *Op*MNPV *ie*-2 / *cat* coding-region from pTREΔ.*ie*-2.*cat*. Positive amplification of *Op*MNPV *ie*-2 / *cat* coding-region produced a DNA fragment banding at 1297-bp. The above gel shows:

Track 1: Smart Ladder (Eurogentec 1kb)
Track 2: Positive amplification of the *Op*MNPV *ie*-2 / *cat* from pTREΔ.*ie*-2.*cat*
Track 3: Positive amplification of the *Op*MNPV *ie*-2 / *cat* from pTREΔ.*ie*-2.*cat*2

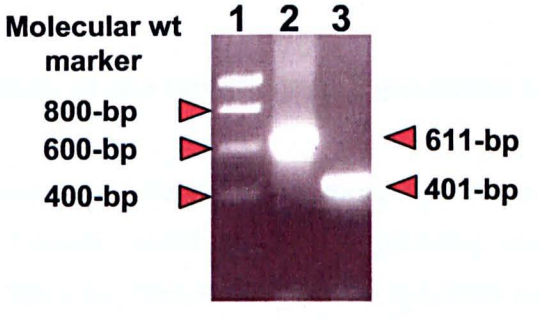


Figure 4.16: Analysis of *TRE* amplification from pTREΔ.*ie*-2.*cat*2, using PCR (Section 2.5.5). A positive control was included, which involved amplifying the *TRE* coding-region from pTREΔ.*ie*-2.*cat*. Positive amplification of *TRE* produced a DNA fragment banding at 611-bp. The above gel shows:

Track 1: Smart Ladder (Eurogentec 1kb)
Track 2: Positive amplification of the *TRE* (611-bp) from pTREΔ.*ie*-2.*cat*
Track 3: Amplification of an unidentified DNA fragment (401-bp) from pTREΔ.*ie*-2.*cat*2

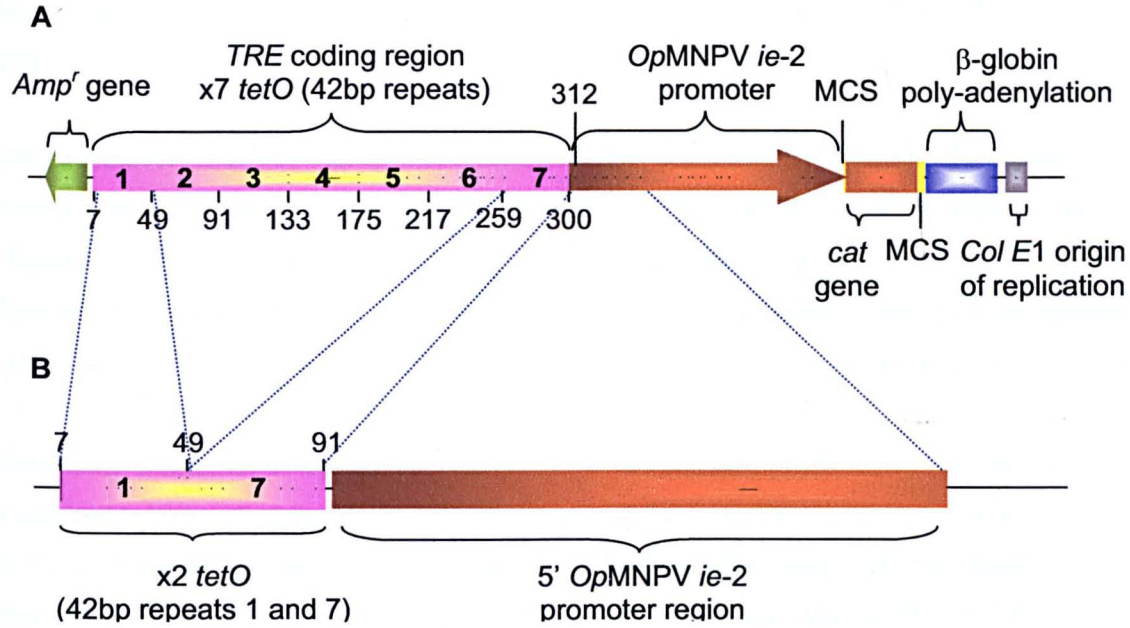


Figure 4.17A / B: Analysis of the *TRE* / 5' *Op*MNPV *ie*-2 coding-region form pTREΔ.*ie*-2.*cat* (**A**; as described in Figure 4.11) and pTREΔ.*ie*-2.*cat*2 (**B**), after identification of a mutation, which caused 210-bp to be deleted from pTREΔ.*ie*-2.*cat*2. Sequencing identified 5 of the 7 *tetO* coding-regions (coding-regions 2-6) had been deleted from pTREΔ.*ie*-2.*cat*2 leaving the remaining 2 *tetO* coding-regions (coding-regions 1 and 7) intact (**B**).

Earlier studies had shown that the Tet system can work effectively with as little as one or two *tetO* coding-regions to suppress / activate promoter activity (Gossen and Bujard, 1992). In light of this evidence the pTREΔ.*ie*-2.*cat*2 vector was established as the insect Tet response plasmid.

4.7 TRE suppression of the *Op*MNPV *ie*-2 promoter in insect cells

The construction of the insect regulatory and response plasmids (Section 4.2, 4.5), and subsequent generation of stable insect cell lines expressing the regulatory protein rtTA (Section 4.4), completed the initial development of the inducible insect expression system. To test the functionality of this system in insect cells it was essential to first confirm that the *TRE* of pTREΔ.*ie*-2.*cat*2 would repress the transcriptional activity of the *Op*MNPV *ie*-2 promoter. In order to test this *Sf*9 and *T.ni* Hi5 cells were transfected with either pTREΔ.*ie*-2.*cat*2 or pIZT/V5-His.*cat*, the latter being a positive control in which *Op*MNPV *ie*-2 expresses *cat* constitutively. In addition, pTREΔ.*ie*-2.*cat*2 and pIZT/V5-His.*cat* (positive control) were transfected into the stable cell lines *Sf*9.rtTA and *T.ni* (Hi5).rtTA to

show that in the absence of any inducer, expression of rtTA does not affect expression of *cat*.

*Sf*9 or *Sf*9.rtTA and *T.ni* Hi5 or *T.ni* (Hi5).rtTA cells were seeded (Section 2.2.3) into 35mm tissue culture dishes, and left to adhere for 1 h. The cell mono-layers were transfected (Section 2.2.4) using 1μg of plasmid DNA (pTREΔ.*ie*-2.*cat*2 or pIZT/V5-His.*cat*). Transfections were carried out in triplicate. Cells were harvested after 24 h incubation at 28°C, and CAT activity assayed. The assays were carried out in triplicate (Section 2.8.1).

Following phosphor-imaging, the percentage conversion from non-modified to the 3-acetylated form of chloramphenicol by each cellular extract was calculated using the software program Image-Quant 5.1 (Section 2.8.1). CAT activity was expressed as a total percentage conversion per 10^6 cells. Since the constitutive activity of the *Op*MNPV *ie*-2 promoter has been characterised previously in *Sf*9 and *T.ni* Hi5 cells (Chapter 3; Section 3.4) expression from this promoter was considered the basal level, and all data was compared to this (**Figure 4.18A-B**). Mock-transfected *Sf*9 and *T.ni* Hi5 cells were used as a negative control to determine non-specific background expression levels, and commercially purified CAT (Sigma) was used to determine 100% chloramphenicol conversion (positive control). Statistical analyses were carried out using the unpaired 2 sample T-test at a 95% confidence level.

In all cell lines tested, no detectable levels of CAT activity above background levels was observed from mock- transfected cells, or cells transfected at 0 h (**Figure 4.18A-B**; $p>0.05$ in all cases).

Figure 4.18A shows results obtained from pTREΔ.*ie*-2.*cat*2 (*TRE* / *Op*MNPV *ie*-2) and pIZT/V5-His.*cat* (*Op*MNPV *ie*-2 without *TRE*, control) promoter activity assays in *Sf*9 and *Sf*9.rtTA cells. Expression of *cat* from pIZT/V5-His.*cat* produced CAT activity levels at 24 hpt of 35% in both *Sf*9 and *Sf*9.rtTA cells. At 24 hpt, very low levels of CAT were observed from pTREΔ.*ie*-2.*cat*2, which were significantly lower (*Sf*9, 1.2%; *Sf*9.rtTA, 1.3%) than those observed from pIZT/V5-His.*cat* (*Sf*9, $p<0.0001$; *Sf*9.rtTA, $p<0.0001$).

Figure 4.18B shows results obtained from pTREΔ.*ie*-2.*cat*2 (*TRE* / *Op*MNPV *ie*-2) and pIZT/V5-His.*cat* (*Op*MNPV *ie*-2 without *TRE*, control) promoter activity assays in *T.ni* Hi5 and *T.ni* (Hi5).rtTA cells. At 24 hpt, the levels of CAT activity detected from pIZT/V5-

His.*cat* was 51% in *T.ni* Hi5 and 50% in *T.ni* (Hi5).rtTA cells. At 24 hpt, expression of *cat* from pTREΔ.*ie*-2.*cat*2 was detected at an activity level of 3.9% in *T.ni* Hi5 and 4.2% in *T.ni* (Hi5).rtTA cells. A significantly reduced level of CAT activity from pTREΔ.*ie*-2.*cat*2 was observed compared with that of pIZT/V5-His.*cat* (*T.ni* Hi5, $p<0.0001$; *T.ni* Hi5.rtTA, $p<0.0001$).

These data show that the transcriptional activity of the *Op*MNPV *ie*-2 promoter was successfully reduced when placed downstream of the *TRE* (pTREΔ.*ie*-2.*cat*2). Therefore, this evidence suggested that the *TRE* was actively suppressing the *Op*MNPV *ie*-2 promoter.

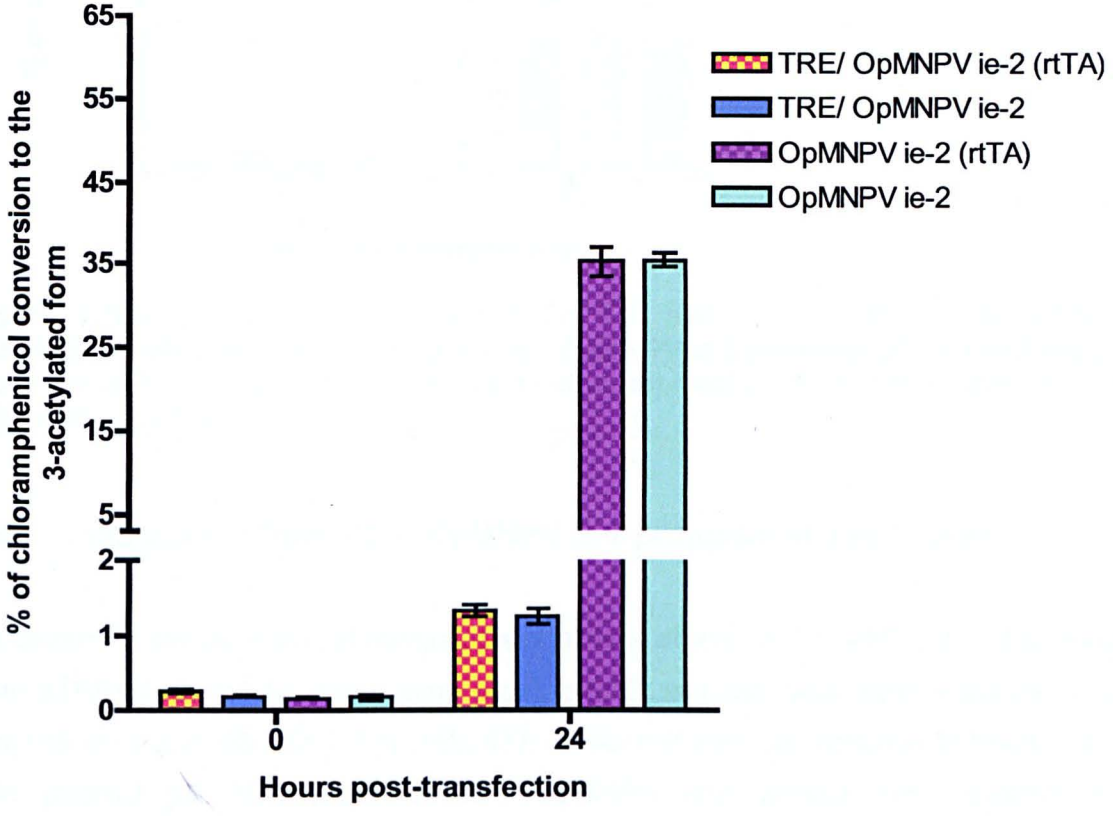


Figure 4.18A: Transient CAT activity in *Sf*9 and *Sf*9.rtTA cells following, transfection with plasmids containing a *TRE* / *Op*MNPV *ie*-2 promoter (pTREΔ.*ie*-2.*cat*2) or *Op*MNPV *ie*-2 promoter (pIZT/V5-His.*cat*) to drive expression of *cat* in the absence of the inducer doxycycline.

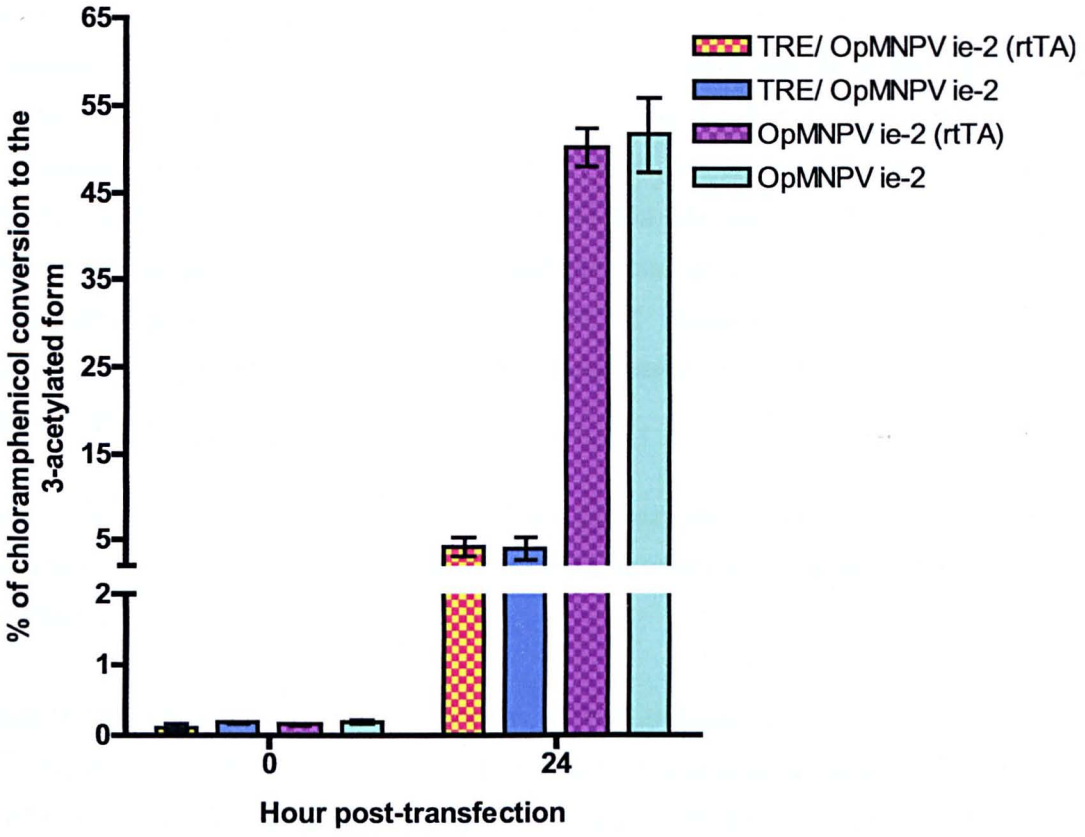


Figure 4.18B: Transient CAT activity in *T.ni* Hi5 and *T.ni* (Hi5).rtTA cells, following transfection with plasmids containing a *TRE* / *Op*MNPV *ie*-2 promoter (pTREΔ.*ie*-2.*cat*2) or *Op*MNPV *ie*-2 promoter (pIZT/V5-His.*cat*) to drive expression of *cat* in the absence of the inducer doxycycline.

4.8 Induction of the TRE / *Op*MNPV *ie*-2 promoter in insect cells

To determine the induction of transcriptional activity of the *TRE* / *Op*MNPV *ie*-2 promoter from pTREΔ.*ie*-2.*cat*2 by doxycycline (dox), pTREΔ.*ie*-2.*cat*2 was transfected into *Sf*9 / *T.ni* Hi5 cells and *Sf*9.rtTA / *T.ni* (Hi5).rtTA stable cell lines (as describe in Section 4.7). The plasmid pIZT/V5-His.*cat* (contains *Op*MNPV *ie*-2 without *TRE*, control) was transfected in parallel, and used as a control to determine if the inducer affected constitutive activity of the non-modified *Op*MNPV *ie*-2 promoter. After 5 hpt, all transfected cell lines were treated with 1μg / ml of dox, to induce promoter activity, and subsequently *cat* expression. Cells were harvested after 24 h incubation at 28°C, and CAT activity assayed for.

Following phosphor-imaging, the percentage conversion from non-modified to the 3-acetylated form of chloramphenicol by each cellular extract was calculated using the

software program Image-Quant 5.1 (Section 2.8.1). CAT activity was expressed as a total percentage conversion per 10^6 cells. Since the constitutive activity of the *Op*MNPV *ie*-2 promoter has been characterised previously in *Sf*9 and *T.ni* Hi5 cells (Chapter 3; Section 3.4) expression from this promoter was considered the basal level, and all data was compared to this (**Figure 4.19A-B**). Mock-transfected *Sf*9 and *T.ni* Hi5 cells were used as a negative control to determine non-specific background expression levels, and commercially purified CAT (Sigma) was used to determine 100% chloramphenicol conversion (positive control). Statistical analyses were carried out using the unpaired 2 sample T-test at a 95% confidence level.

In all cell lines tested, no detectable levels of CAT activity above background levels was observed from mock- transfected cells, or cells transfected at 0 h (**Figure 4.19A-B**; $p>0.05$ in all cases).

Figure 4.19A shows results obtained from pTREΔ*.ie*-2.*cat*2 (*TRE* / *Op*MNPV *ie*-2) and pIZT/V5-His.*cat* (*Op*MNPV *ie*-2 without *TRE*, control) promoter activity assays in *Sf*9 and *Sf*9.rtTA cells, in the presence of dox. Expression of *cat* from pIZT/V5-His.*cat* was observed at 24 hpt, and produced CAT activity levels of 36% in *Sf*9 and 39% in *Sf*9.rtTA cells. A comparison of induced vs. non-induced pIZT/V5-His.*cat* (**Figure 4.18A**) *Op*MNPV *ie*-2 activity showed, that there was no significant difference (*Sf*9, $p=0.745$; *Sf*9.rtTA, $p=0.264$) in the levels of CAT activity detected. Consequently, the presence of the inducer did not interfere with the constitutive activity of this promoter. At 24 hpt, levels of CAT activity detected from pTREΔ*.ie*-2.*cat*2 was 1.3% in *Sf*9 and 1.8% in *Sf*9.rtTA cells. The levels detected were significantly lower than those observed from the pIZT/V5-His.*cat* (*Sf*9, $p<0.0001$; *Sf*9.rtTA, $p<0.0001$). Comparison of CAT activity from the induced pTREΔ*.ie*-2.*cat*2 with the non-induced (**Figure 4.18A**) showed, that there was no significant difference (*Sf*9, $p=0.897$; *Sf*9.rtTA, $p=0.135$).

Figure 4.19B shows results obtained from pTREΔ*.ie*-2.*cat*2 (*TRE* / *Op*MNPV *ie*-2) and pIZT/V5-His.*cat* (*Op*MNPV *ie*-2 without *TRE*, control) promoter activity assays in *T.ni* Hi5 and *T.ni* (Hi5).rtTA cells, in the presence of dox. At 24 hpt, the levels of CAT activity detected from pIZT/V5-His.*cat* was 56% in *T.ni* Hi5 and 52% in *T.ni* (Hi5).rtTA cells. No significant difference (*T.ni* Hi5, $p=0.340$; *T.ni* (Hi5).rtTA, $p=0.609$) was observed between the levels of CAT activity detected from pIZT/V5-His.*cat* non-induced (**Figure 4.18B**) and the induced *Op*MNPV *ie*-2 promoter. The levels of CAT activity detected from the

pTREΔ.*ie*-2.*cat*2 at 24 hpt, were shown to be 3.3% in *T.ni* Hi5 and 3.6% in *T.ni* (Hi5).rtTA cells. The levels detected from pTREΔ.*ie*-2.*cat*2 were significantly lower than those observed from pIZT/V5-His.*cat* (*T.ni* Hi5, $p<0.0001$; *T.ni* (Hi5).rtTA, $p<0.0001$). These data were compared with the non-induced pTREΔ.*ie*-2.*cat*2 results (**Figure 4.18B**), which identified no significant difference (*T.ni* Hi5, $p=0.694$; *T.ni* (Hi5).rtTA, $p=0.736$) in the production of CAT.

It was therefore shown that in the presence of the inducer dox, the transcriptional activity of the *TRE* / *Op*MNPV *ie*-2 promoter from pTREΔ.*ie*-2.*cat*2 could not be restored to the levels observed from *Op*MNPV *ie*-2 of pIZT/V5-His.*cat*. Attempts were made to activate the suppressed *TRE* / *Op*MNPV *ie*-2 promoter, by varying the concentration of dox needed to induce promoter activity. However, no significant effect was observed (data not shown), compared with the data shown in **Figures 4.18 / 19 A, B**. To identify if any other sequence element in pTREΔ.*ie*-2.*cat*2 besides *TRE* was suppressing *Op*MNPV *ie*-2 activity, a rescue plasmid was generated to try and restore the transcriptional activity of *Op*MNPV *ie*-2 in pTREΔ.*ie*-2.*cat*2.

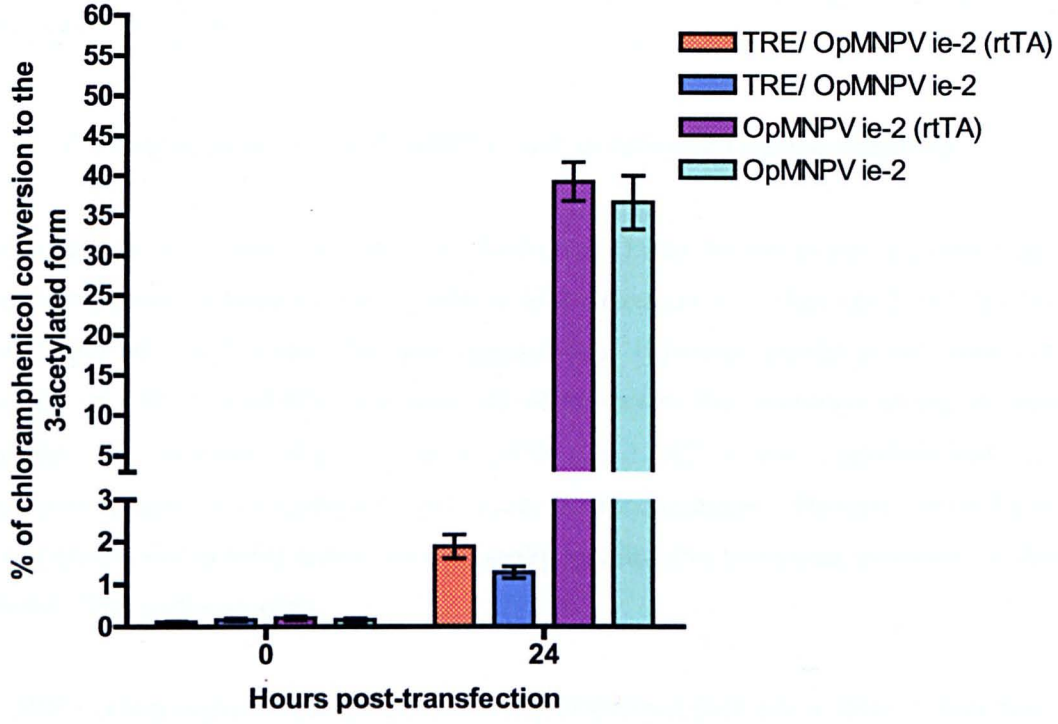


Figure 4.19A: Transient CAT activity in *Sf*9 and *Sf*9.rtTA cells, following transfection with plasmids containing the *TRE* / *Op*MNPV *ie*-2 promoter (pTREΔ.*ie*-2.*cat*2) or *Op*MNPV *ie*-2 promoter (pIZT/V5-His.*cat*) to drive expression of *cat* in the presence of the inducer doxycycline.

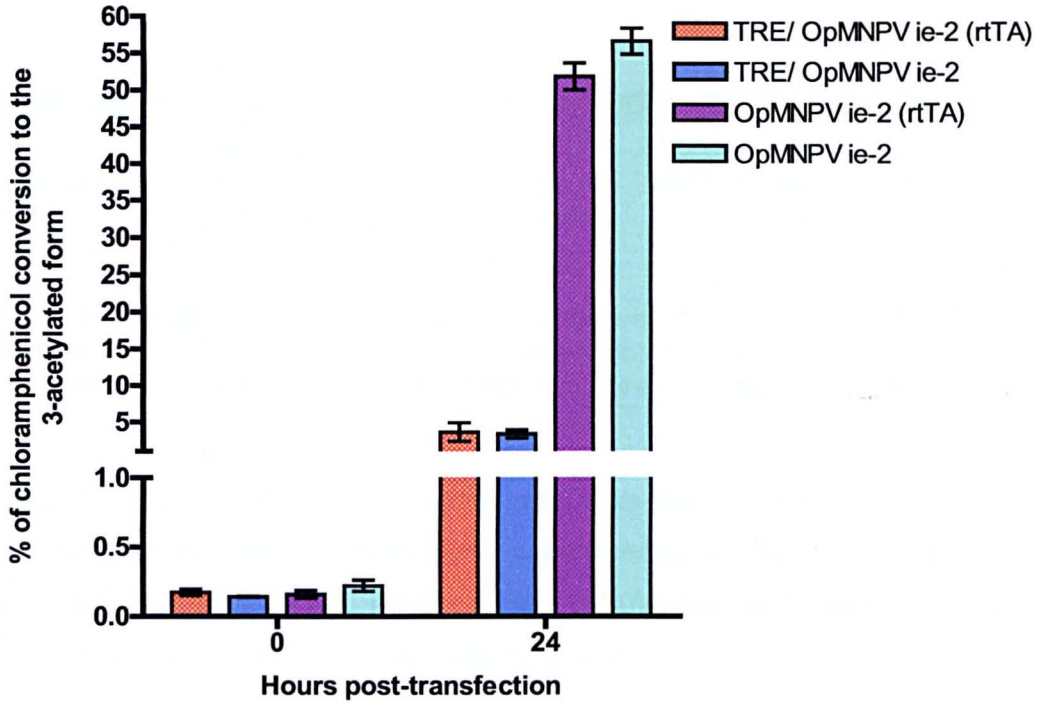


Figure 4.19B: Transient CAT activity in *T.ni* Hi5 and *T.ni* (Hi5).rtTA cells, following transfection with plasmids containing the *TRE* / *Op*MNPV *ie*-2 promoter (pTRE∆.*ie*-2.*cat*2) or *Op*MNPV *ie*-2 promoter (pIZT/*V*5-His.*cat*) to drive expression of *cat* in the presence of the inducer doxycycline.

4.9 Construction of the *Op*MNPV *ie*-2 promoter rescue plasmid

In the absence of the inducer, the *TRE* (Section 4.7) was shown to successfully suppress the transcriptional activity of the *Op*MNPV *ie*-2 promoter in pTRE∆.*ie*-2.*cat*2 by 99% in *Sf*9.rtTA and 98% in *T.ni* Hi5.rtTA cells respectively. However, partial or full transcriptional activation of *TRE* / *Op*MNPV *ie*-2 was not observed in the presence of the inducer, as expected. By removing the *TRE* from pTRE∆.*ie*-2.*cat*2, it was hypothesised that the transcriptional activity of *Op*MNPV *ie*-2 could be reconstituted. Restoration of *Op*MNPV *ie*-2 transcriptional activity would subsequently localise the problems observed in Section 4.7 to the *TRE* coding-region.

The *TRE* coding-region was removed from pTRE∆.*ie*-2.*cat*2 on a *Xho*I / *Sac*I fragment (Section 2.5.1), and a *Xho*I / *Sac*I linker was inserted to religate the linear vector (Section 2.5.4). The resulting plasmid pRescue.*cat*1 was derived (**Figure 4.20**).

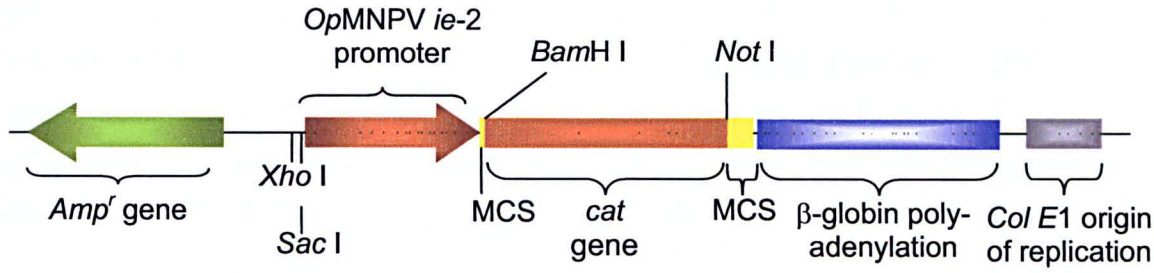


Figure 4.20: Genetic map of pRescue.*cat*1; pRescue.*cat*1 is similar to pTREΔ.*ie*-2.*cat*2 described in Figure 4.12, except the *TRE* coding-region has been removed using the *Xho*I and *Sac*I restriction sites. A *Xho*I / *Sac*I linker was used to relegate this vector.

*Sf*9 / *Sf*9.rtTA and *T.ni* Hi5 / *T.ni* (Hi5).rtTA cells were seeded (Section 2.2.3) into 35mm tissue culture dishes. The cell mono-layers were transfected (Section 2.2.4) using 1μg of plasmid DNA (pTREΔ.*ie*-2.*cat*2, pRescue.*cat*1, pIZT/V5-His.*cat*). Cells were harvested after 24 h incubation at 28°C, and CAT activity quantified.

In all cell lines tested, no detectable levels of CAT activity above background levels were observed from mock-transfected cells, or cells transfected at 0 h (**Figure 4.21A / B**; $p>0.05$ in all cases).

Figure 4.21A shows results obtained from the non-induced pTREΔ.*ie*-2.*cat*2 (*TRE* / *Op*MNPV *ie*-2), pRescue.*cat*1 (*Op*MNPV *ie*-2.Δ*TRE*) and pIZT/V5-His.*cat* (*Op*MNPV *ie*-2, control) promoter activity assays in *Sf*9 and *Sf*9.rtTA cells. Expression of CAT from pIZT/V5-His.*cat* was observed at 24 hpt, producing CAT activity levels of 37% in *Sf*9 and 36% in *Sf*9.rtTA cells. The levels of CAT activity detected from pRescue.*cat*1 (*Sf*9, 1.9%; *Sf*9.rtTA, 1.6%) and pTREΔ.*ie*-2.*cat*2 (*Sf*9, 1.7%; *Sf*9.rtTA, 1.6%) at 24 hpt, were not significantly different ($p>0.05$ in all cases). However, the basal levels produced by pIZT/V5-His.*cat* were significantly higher than those from pRescue.*cat*1 (*Sf*9, $p<0.0001$; *Sf*9.rtTA, $p=0.003$). These data show that no increase in *Op*MNPV *ie*-2 promoter activity was observed with the removal of the *TRE* from pTREΔ.*ie*-2.*cat*2.

Figure 4.21B shows results obtained from the non-induced pTREΔ.*ie*-2.*cat*2 (*TRE* / *Op*MNPV *ie*-2), pRescue.*cat*1 (*Op*MNPV *ie*-2.Δ*TRE*) and pIZT/V5-His.*cat* (*Op*MNPV *ie*-2, control) promoter activity assays in *T.ni* Hi5 and *T.ni* (Hi5).rtTA cells. The levels of CAT activity detected from pIZT/V5-His.*cat* at 24 hpt, were 47% in *T.ni* Hi5 and 46% in *T.ni* (Hi5).rtTA cells. Like the results shown in **Figure 4.21A**, the levels of CAT activity

detected from pRescue.*cat*1 (*T.ni* Hi5, 4.7%; *T.ni* (Hi5).rtTA, 4.6%) and pTREΔ.*ie*-2.*cat*2 (*T.ni* Hi5, 4.1%; *T.ni* (Hi5).rtTA, 4.4%) at 24 hpt, did not differ significantly ($p>0.05$ in all cases). A comparison of CAT activity from pRescue.*cat*1 and pIZT/V5-His.*cat* shows that pIZT/V5-His.*cat* produced significant more CAT activity than that observed from pRescue.*cat*1 (*T.ni* Hi5, $p<0.0001$; *T.ni* (Hi5).rtTA, $p<0.0001$).

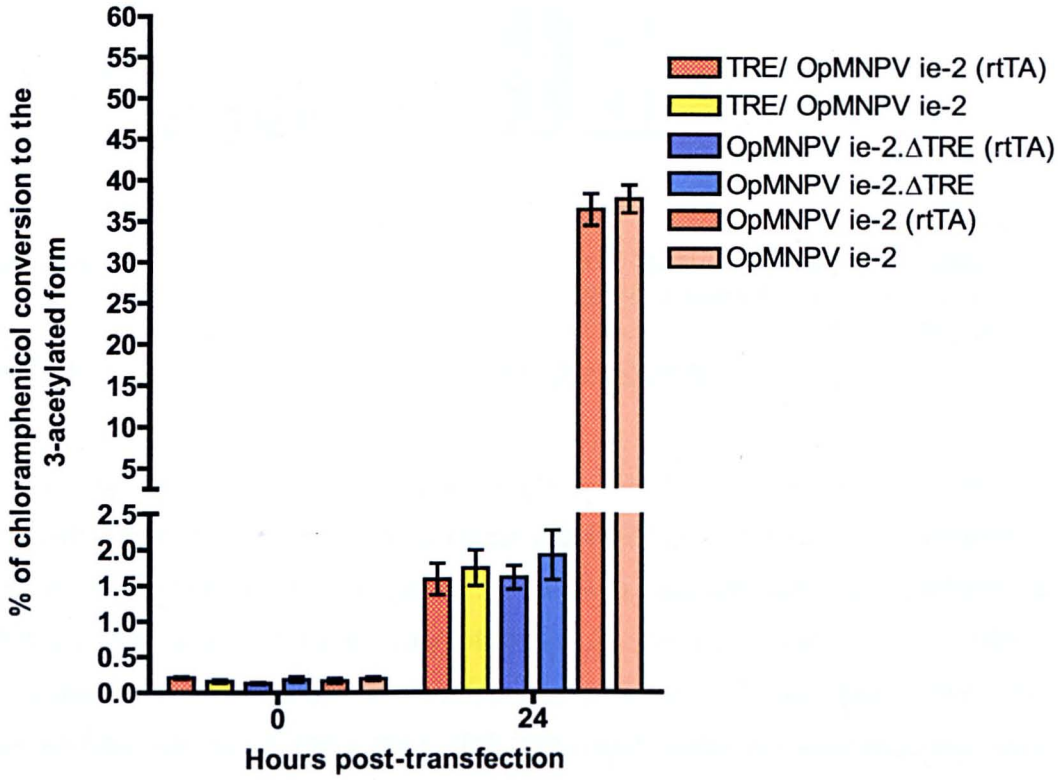


Figure 4.21A: Transient CAT activity in *Sf*9 and *Sf*9.rtTA cells, following transfection with plasmids containing the *TRE* / *Op*MNPV *ie*-2 (pTREΔ.*ie*-2.*cat*2), *Op*MNPV *ie*-2.Δ*TRE* (pRescue.*cat*1) or *Op*MNPV *ie*-2 promoter (pIZT/V5-His.*cat*) to drive expression of *cat* in the absence of the inducer doxycycline.

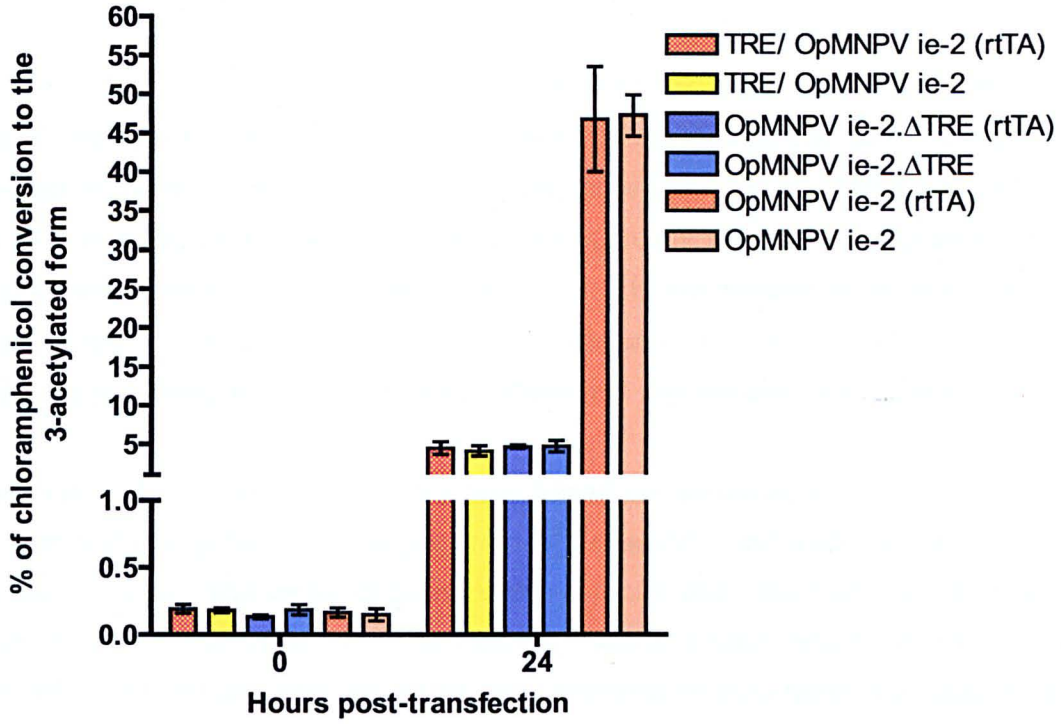


Figure 4.21B: Transient CAT activity in *T.ni* Hi5 and *T.ni* (Hi5).rtTA cells, following transfection with plasmids containing the *TRE* / *Op*MNPV *ie*-2 (pTREΔ.*ie*-2.*cat*2), *Op*MNPV *ie*-2.Δ*TRE* (pRescue.*cat*1) or *Op*MNPV *ie*-2 promoter (pIZT/V5-His.*cat*) to drive expression of *cat* in the absence of the inducer doxycycline.

These results have shown that the removal of the *TRE* from pTREΔ.*ie*-2.*cat*2 was not able to restore basal transcriptional activity of the *Op*MNPV *ie*-2 promoter, as demonstrated by the positive control (pIZT/V5-His.*cat*). Therefore, suppression of *Op*MNPV *ie*-2 in pTREΔ.*ie*-2.*cat*2 was not exclusively attributed to the mechanism of the *TRE*. Two hypotheses were put forward to resolve this problem. Firstly, that pTREΔ.*ie*-2.*cat*2 contained DNA elements, other than *TRE* that might inhibit the transcriptional activity of *Op*MNPV *ie*-2. Secondly, that the published literature used to identify the *Op*MNPV *ie*-2 promoter coding-region from pIZT/V5-His.*cat* may be incorrect, and that a crucial sequence, not identified in the literature, was missing from the promoter sequence amplified. Therefore, as a consequence cause a dramatic reduction in the transcriptional activity of *Op*MNPV *ie*-2. This prompted the construction of a second *Op*MNPV *ie*-2 rescue plasmid.

4.10 Transcriptional restoration of the *Op*MNPV *ie*-2 promoter

To address the problems encountered in Section 4.8 / 4.9, a second *Op*MNPV *ie*-2 rescue plasmid was constructed. This vector would hopefully resolve one of two hypothesises proposed in Section 4.9; were there DNA sequences present in pTREΔ.*ie*-2.*cat*2, other than *TRE* that may inhibit the transcriptional activity of the *Op*MNPV *ie*-2 promoter; or was the amplified *Op*MNPV *ie*-2 promoter from pIZT/V5-His.*cat* missing an essential sequence not elucidated in the literature? However, sequencing of *Op*MNPV *ie*-2 from pTREΔ.*ie*-2.*cat*2 was confirmed with published data (Theilmann and Stewart, 1991; Section 4.5).

Construction of the new rescue vector was based on the excision of the *Op*MNPV *ie*-2 promoter and *cat* gene coding-region from pRescue.*cat*1 and insertion into a selected expression vector. The vector chosen for this purpose was pBacPAK9 (BD Biosciences; **Figure 4.22A**). This vector is a baculovirus transfer vector, which contains the *polh* promoter. The *polh* promoter cannot be *trans*-activated by eukaryotic RNA polymerase II, but only works in the presence of late / very late viral transcription factors. However, to rule out any possible activation of this promoter, which may cause interference with the *Op*MNPV *ie*-2, *polh* was removed by restriction digestion (Section 2.5.1) from pBacPAK9 using *Eco*RV and *Sma*I. These restriction sites once digested generate blunt 5' / 3' ends, which were ligated together (Section 2.5.4) to derive plasmid pBacPAK9Δ*polh* (**Figure 4.22B**).

The pBacPAK9Δ*polh* vector was digested using *Not*I (Section 2.5.1) and purified using column purification (Section 2.5.7.2). The *Op*MNPV *ie*-2 promoter / *cat* gene coding-region of pRescue.*cat*1 was removed using *Not*I and inserted into pBacPAK9Δ*polh*. Digestion using *Bam*HI / *Xho*I and *Eco*RI identified the correct orientation of the *Op*MNPV *ie*-2 promoter / *cat* gene coding-region. The plasmid pRescue.*cat*2 was derived (**Figure 4.23**).

*Sf*9 / *Sf*9.rtTA and *T.ni* Hi5 / *T.ni* (Hi5).rtTA cells were seeded (Section 2.2.3) into 35mm tissue culture dishes, and left to adhere for 1 h. The cell mono-layers were transfected (Section 2.2.4) using 1μg of plasmid DNA (pTREΔ.*ie*-2.*cat*2, pRescue.*cat*1, pRescue.*cat*2, pIZT/V5-His.*cat*). Transfections were carried out in triplicate. Cells were harvested after 24 h incubation at 28°C, and CAT activity assayed for. The assays were carried out in triplicate (Section 2.8.1).

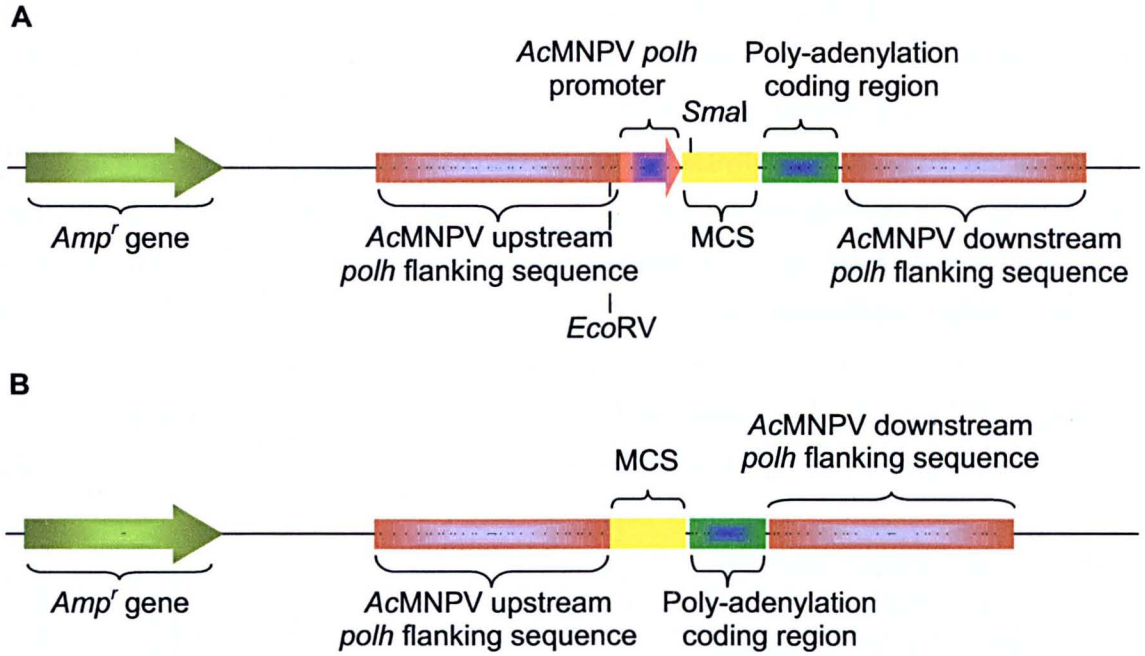


Figure 4.22A: Genetic map of pBacPAK9, shows the *Amp*^{r} gene, *Ac*MNPV up / downstream *polh* flanking sequences, *Ac*MNPV *polh* promoter, MCS, and poly-adenylation coding-region. **B:** Genetic map of pBacPAK9Δ*polh*, which is similar to pBacPAK9 except the *polh* promoter has been removed.

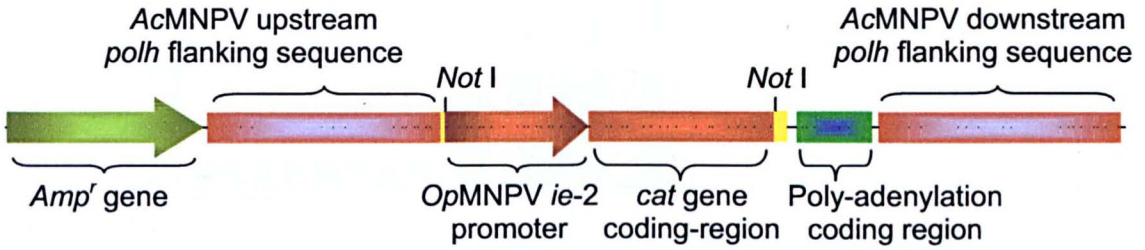


Figure 4.23: Genetic map of pRescue.*cat*2, which is similar to pBacPAK9Δ*polh* (Figure 4.20B), except the *Op*MNPV *ie*-2 promoter and *cat* gene coding-region have been inserted.

In all cell lines tested, no detectable levels of CAT activity above background levels were observed from mock-transfected cells, or cells transfected at 0 h (**Figure 4.21A / B**; $p>0.05$ in all cases).

Figure 4.24A shows results obtained from the non-induced pTREΔ.*ie*-2.*cat*2 (*TRE* / *Op*MNPV *ie*-2), pRescue.*cat*1 (*Op*MNPV *ie*-2.Δ*TRE*), pRescue.*cat*2 (*Op*MNPV *ie*-2 rescue) and pIZT/V5-His.*cat* (*Op*MNPV *ie*-2, control) promoter activity assays in *Sf*9 and

*Sf*9.rtTA cells. The levels of CAT activity detected from pIZT/V5-His.*cat* at 24 hpt (36% in both cell lines used), were determined as the basal transcriptional activity of the *Op*MNPV *ie*-2 promoter. Expression of *cat* from pTREΔ.*ie*-2.*cat*2 and pRescue.*cat*1 at 24 hpt, were similar to that shown in **Figure 4.21A**; although CAT activity was detected, the levels were significantly lower than those observed from pIZT/V5-His.*cat* ($p<0.05$ in all cases). The production of CAT from pRescue.*cat*2 was observed at 24 hpt; the levels of CAT activity detected (*Sf*9, 24%; *Sf*9.rtTA, 26%) were shown to be significantly higher than those observed from pTREΔ.*ie*-2.*cat*2 and pRescue.*cat*1 (*Sf*9, $p=0.003$; *Sf*9.rtTA, $p=0.003$). However, the levels of CAT activity produced from pRescue.*cat*2 were still significantly lower than those observed from pIZT/V5-His.*cat* (*Sf*9, $p=0.010$; *Sf*9.rtTA, $p=0.018$).

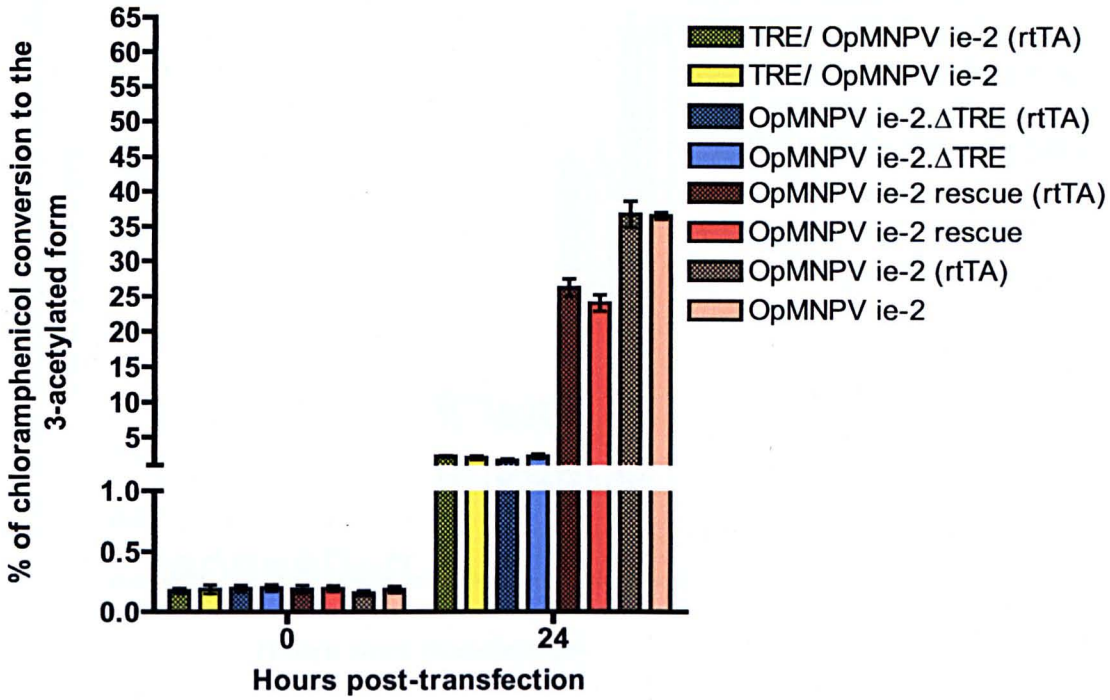


Figure 4.24A: Transient CAT activity in *Sf*9 and *Sf*9.rtTA cells, following transfection with plasmids containing the *TRE* / *Op*MNPV *ie*-2 (pTREΔ.*ie*-2.*cat*2), *Op*MNPV *ie*-2.Δ*TRE* (pRescue.*cat*1), *Op*MNPV *ie*-2 rescue (pRescue.*cat*2) or *Op*MNPV *ie*-2 promoter (pIZT/V5-His.*cat*) to drive expression of *cat* in the absence of the inducer doxycycline.

Figure 4.24B shows results obtained from the non-induced pTREΔ.*ie*-2.*cat*2 (*TRE* / *Op*MNPV *ie*-2), pRescue.*cat*1 (*Op*MNPV *ie*-2.Δ*TRE*), pRescue.*cat*2 (*Op*MNPV *ie*-2 rescue) and pIZT/V5-His.*cat* (*Op*MNPV *ie*-2, control) promoter activity assays in *T.ni* Hi5 and *T.ni* (Hi5).rtTA cells. The levels of CAT activity detected from pIZT/V5-His.*cat* at 24 hpt (56% in both cell lines), were determined as the basal activity of the *Op*MNPV *ie*-2 promoter. The pTREΔ.*ie*-2.*cat*2 and pRescue.*cat*1 produced very low levels of CAT

activity at 24 hpt (as seen previously in **Figure 4.21B**). However, the levels produced did not significantly differ between these promoters tested ($p>0.05$ in all cases); although the levels were significantly lower than those detected from pIZT/V5-His.*cat* ($p<0.05$ in all cases). The pRescue.*cat*2 produced significantly higher levels of CAT activity (*T.ni* Hi5, 37%; *T.ni* (Hi5).rtTA, 39%) than those observed from the pTREΔ.*ie*-2.*cat*2 (*T.ni* Hi5, 4%; *T.ni* (Hi5).rtTA, 5%) and pRescue.*cat*1 (*T.ni* Hi5, 4%; *T.ni* (Hi5).rtTA, 4%; *T.ni* Hi5, p=0.006; *T.ni* (Hi5).rtTA, p=0.004). Conversely, the levels of CAT activity produced from pRescue.*cat*2 were shown to be significantly lower than those observed from pIZT/V5-His.*cat* (*T.ni* Hi5, p=0.020; *T.ni* (Hi5).rtTA, p=0.002).

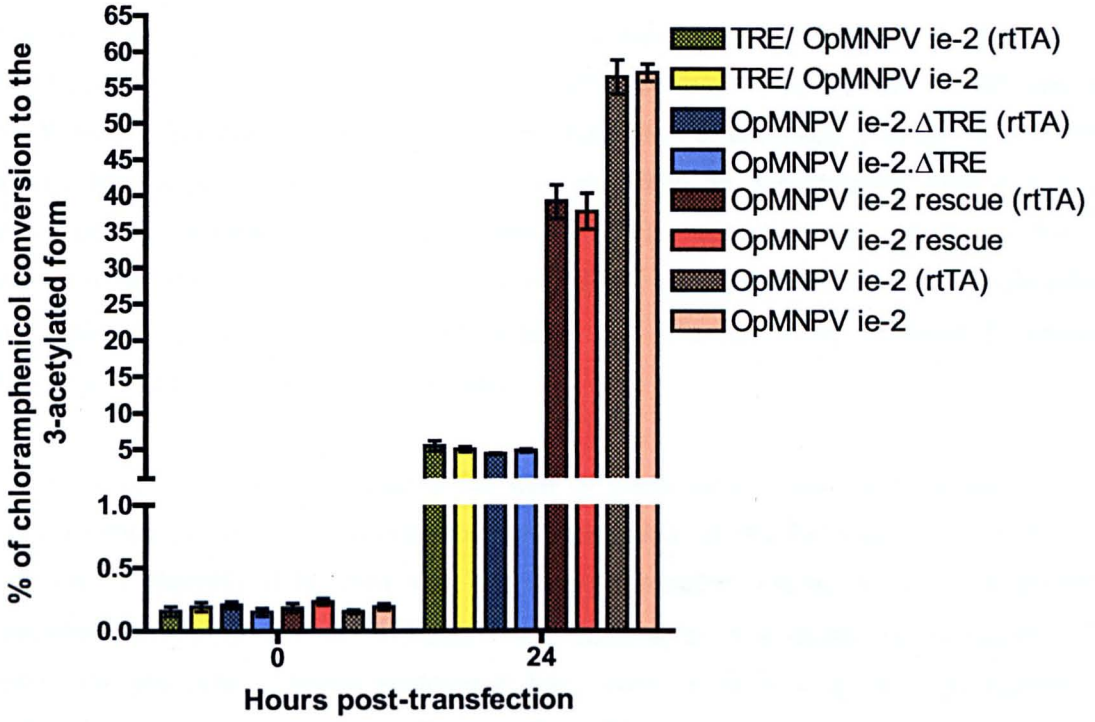


Figure 4.24B: Transient CAT activity in *T.ni* Hi5 and *T.ni* (Hi5).rtTA cells, following transfection with plasmids containing the *TRE* / *Op*MNPV *ie*-2 (pTREΔ.*ie*-2.*cat*2), *Op*MNPV *ie*-2.Δ*TRE* (pRescue.*cat*1), *Op*MNPV *ie*-2 rescue (pRescue.*cat*2) or *Op*MNPV *ie*-2 promoter (pIZT/V5-His.*cat*) to drive expression of *cat* in the absence of the inducer doxycycline.

It was therefore shown that, even though complete transcriptional activity of *Op*MNPV *ie*-2 rescue was not restored (pRescue.*cat*2), compared with the levels observed from pTREΔ.*ie*-2.*cat*2 and pRescue.*cat*1, activity was shown to increase 10.9-fold in *Sf*9; 13.4-fold in *Sf*9.rtTA; 7.6-fold in *T.ni* Hi5; and 8-fold in *T.ni* (Hi5).rtTA cells.

4.11 Discussion

The availability of inducible expression systems for use in insect cells is very limited. Systems designed to direct inducible gene expression in insect cells are currently based on the use of endogenous transcriptional regulatory elements, such as the *Drosophila heat shock protein* 70 (*Hsp*70; Bunch *et al.*, 1988), and *MT* (Maroni *et al.*, 1986) promoters. The *Hsp*70 promoter is heat-induced and has been shown to function in *Drosophila* (Di Nocera and Dawid, 1983; Steller and Pirrotta, 1984), mosquito (Lycett and Crampton, 1993; Shotkoski *et al.*, 1996), and lepidopteran (Helgen and Fallon, 1990; Morris and Miller, 1992) cell lines. However, this promoter is not available commercially. The *MT* promoter (commercially available) depends on metal ions for full activation (Otto *et al.*, 1987; Bunch *et al.*, 1988; Johansen *et al.*, 1989; van der Straten *et al.*, 1989), and has been shown to function in dipteran cell lines (Johansen *et al.*, 1989; Kovach *et al.*, 1992). Due to a high level of basal expression from *Hsp*70 in an non-induced state (Clem and Miller, 1994; Prikhod'ko and Miller, 1996), and the relatively low activity of the *MT* promoter in an induced state (Hegedus *et al.*, 1998), combined with the pleiotropic effects bought about by inducement, these systems lack the specificity required to regulate cytotoxic genes successfully in insect cells.

More advanced schemes developed for use in mammalian cells have sought to avoid these problems by constructing inducible systems, such as the Tet expression system (as described in Section 4.1), that rely on non-mammalian elements, or re-engineered mammalian proteins, that are incapable of responding to endogenous inducers. The fundamental principle of these systems is the existence of an inducer that modifies the activity of a synthetic TF, and subsequently acts through a heterologous promoter to regulate the expression of a target gene. Increased specificity of these systems has been achieved by selecting inducers that do not affect cellular physiology, and by assembling chimeric transactivators with minimal homology to natural TF, which consequently do not interact with endogenous promoters (Gossen and Bujard, 1992; 1995; No *et al.*, 1996).

The work in this chapter was carried out to determine whether the Tet expression system (Tet-On) could be adapted for use in insect cell lines. For this system to be successfully adapted, the *CMVie* promoters of the Tet-On system were first replaced, due to their inherent low activity in insect cells (Wu *et al.*, 2000; this thesis Chapter 3). These promoters were replaced with eukaryotic / viral promoters, which have been characterised

as having strong transcriptional activity in insect cell lines (Theilmann and Stewart, 1991; Lu *et al.*, 1996; Farrell *et al.*, 1998; this thesis Chapter 3). The re-engineering of the Tet-On system was hypothesised to generate a functionally novel inducible insect expression system.

The functionality of the Tet expression system (as describe in Section 4.1) is governed by a number of factors, such as the availability of a constitutively expressed transactivator protein (rtTA), the presence of the *TRE* promoter, and presence / absence of the inducer dox (Gossen and Bujard, 1992; Gossen *et al.*, 1995; Baron *et al.*, 1997). The combination of these factors was perceived as essential for this system to work efficiently in insect cells. Therefore, the first step towards producing this system was developing the insect regulatory vector, which would constitutively produce rtTA to detectable levels in insect cells.

The results described in Section 4.2 show the methodology used to construct the insect Tet regulatory vector. The *BmA*3+E promoter was chosen to express the *rtTA* gene in replace of the *CMVie* promoter. To determine the productivity of rtTA from the *BmA*3+E promoter, compared with that from the *CMVie* promoter, pXINsect.DEST38.*rtTA* and pTet-On were transfected into *Sf*9 and *T.ni* Hi5 cells, and subjected to Western blot analysis for detection of rtTA.

Early studies (Wu *et al.*, 2000) to try and adapt the Tet system for use in insect cells have shown that the strength of promoter used to drive expression of *rtTA* in insect cells is crucial to the functioning of the Tet system. A Tet regulatory vector was developed by Wu *et al.* (2000), which utilised the *Dm* actin promoter to drive expression of *rtTA* in insect cells. However, the levels detected were extremely low, which subsequently caused problems with the inducible expression system they developed. To overcome the problem of low rtTA production the *rtTA* was placed under the control of the late *Ac*MNPV *p*10 promoter. Insect cells (*Sf*21, *Sf*9, *TN*368) were transfected with a Tet response vector (pTRE.Luc) and subsequently infected with the recombinant baculovirus expressing *rtTA*. The result was a transient inducible insect expression system that was shown to function with some varying degree of success. The disadvantage of this system is that it can only be used for transient expression, and cannot be use to generate stable insect cell lines, which is one of the aims of this thesis.

Results in Section 4.3 show that the production of rtTA was successfully detected at 24-72 hpt, from the *BmA*3+E promoter in *Sf*9 and *T.ni* Hi5 cells (**Figure 4.8A**). Optimal production of rtTA was observed between 24-48 hpt, in *T.ni* Hi5 cells. At 72 hpt, the levels of rtTA produced from *BmA*3+E were shown to decrease. The reduction in expression may be explained by the transient nature of plasmid-based expression, as proposed in Chapter 3. Transient expression studies rely on short bursts of gene expression (12-72hr), these bursts of gene expression are most often followed by a rapid deterioration in expression of the heterologous gene because of cell death. A decrease in plasmid copy number may also explain the possible decrease observed in rtTA production. The production of rtTA in *Sf*9 cells was shown to be relatively consistent between 24-72 hpt. However, the levels detected were less than those observed in *T.ni* Hi5 cells. No detectable levels of rtTA were observed from the full length *CMVie* promoter (**Figure 4.8B**), or mock transfected *Sf*9 and *T.ni* Hi5 cells. The lack of detectable rtTA from the *CMVie* promoter was undoubtedly due to the weak transcriptional activity of this promoter in insect cells (Wu *et al.*, 2000; this thesis Chapter 3). However, rtTA may have been produced, but at levels not detectable by Western blot analyses. Subsequently, levels this low could not be used to develop a functional inducible insect expression system.

The insect Tet regulatory vector was shown to function efficiently in both insect cell lines used. The rtTA detected on the Westerns (**Figure 4.8A**) suggests that production in these cell lines was between moderate to high levels. These data prompted the next stage of development, which was to use the insect Tet regulatory vector to generate stable insect cell lines expressing *rtTA* constitutively.

Results in Section 4.4 show the successful generation of two stable insect cell lines expressing *rtTA* (*Sf*9.*rtTA* and *T.ni* (Hi5).*rtTA*). The results in **Figure 4.9A / B** show that the production of rtTA, from passages 1-7, in both cell lines was detectable; rtTA has also been detected from passages 10, 20, 30 and 40, with no significant decrease in expression (data not show). The levels of rtTA detected from passages 1-7 in both cell lines was shown to be consistent, suggesting that the levels of rtTA produced were stable.

The level of rtTA produced is one of the crucial factors required for the successful functioning of the Tet expression system. If the levels produced are low, the *TRE* promoter from the Tet response vector will remain suppressed in the presence of the inducer. However, if the levels produced are above the host cells critical threshold, then rtTA becomes cytotoxic, which subsequently has a detrimental affect to the host cells

(Gossen and Bujard, 1992; Baron *et al.*, 1997; Bujard, 1999). The levels of rtTA produced in *Sf*9.*rtTA* and *T.ni* (Hi5).*rtTA* cell lines was considered to be below the crucial level proposed to induce cytotoxicity, but high enough for detection using Western blot analyses, and subsequent *TRE* promoter activation. The viability of *Sf*9.*rtTA* and *T.ni* (Hi5).*rtTA* cells was determined at each passage, which indicated greater than 85% of cells were viable (data not shown). This suggests that stable production of rtTA from *Bm*A3+E is not toxic to the cell lines used. The successful generation of *Sf*9.*rtTA* and *T.ni* (Hi5).*rtTA* cell lines, provides the first critical components necessary to develop the insect inducible expression system.

Results in Section 4.5 describe the methodology used to construct the insect Tet response plasmid (pTREΔ.*ie*-2.*cat*). The pTREΔ.*ie*-2.*cat* plasmid is the second critical component necessary to complete the development of the insect inducible expression system. The *Op*MNPV *ie*-2 promoter was chosen to replace the minimal *CMVie* promoter of the *TRE* promoter, which is used to control expression of a heterologous gene in mammalian cells. It was hypothesised that the *TRE* upstream of the *Op*MNPV *ie*-2 promoter would mimic the original *TRE* promoter by suppressing / activating the *Op*MNPV *ie*-2 promoter in insect cells, in the absence/ presence of the inducer dox.

The pTREΔ.*ie*-2.*cat* vector was originally constructed with seven *tetO* sequences (each *tetO* is 42-bp), upstream of the *Op*MNPV *ie*-2 promoter. However, a contaminating DNA was isolated with the stock of pTREΔ.*ie*-2.*cat*. Attempts to isolate pTREΔ.*ie*-2.*cat* from the contaminant failed. This lead to the original ligation reaction stock of pTREΔ.*ie*-2.*cat* being transformed in *E. coli* to generate a clean stock of pTREΔ.*ie*-2.*cat*. A contaminant-free stock of pTREΔ.*ie*-2.*cat* was produced and renamed pTREΔ.*ie*-2.*cat*2. However, a second problem was identified; five of the seven *tetO* sequences were identified as missing. The deleted *tetO* sequences were identified as sequences 2-6 (**Figure 4.17A / B**).

An explanation to account for this mutation may be justified by the occurrence of transposition. Transposition is the mechanism used to move transposable elements, such as transposons, from one location to another on the genome; for example, from one chromosomal site to another, from a chromosome to a plasmid, or from a plasmid to a chromosome (Hillen and Berens, 1994). Because transposons occur in plasmids, they can also be transmitted from one cell to another. The Tet system is based on regulatory elements of the Tn*10* transposon-specified tetracycline-resistance operon of *E. coli* (Hillen

and Wissmann, 1989; Gossen and Bujard, 1992). It is possible that when the ligation reaction was transformed in *E. coli*, the five *tetO* sequence (2-6) of pTREΔ*ie*-2.*cat*2 became mobile and integrated into the bacterial genome. Hence, being lost from the plasmid.

Early studies on the development of the Tet-Off system (Gossen and Bujard, 1992) have shown that, one, two, and seven *tetO* sequences can function to suppress / activate the minimal *CMVie* promoter in the presence / absence of Tc. This evidence suggested that, with the mutation identified in pTREΔ*ie*-2.*cat*2, control of the *Op*MNPV *ie*-2 promoter would still be possible. To determine if the *TRE* could suppress / activate the constitutive activity of the *Op*MNPV *ie*-2 promoter, pTREΔ*ie*-2.*cat*2 was transfected into *Sf*9 / *Sf*9.*rtTA* and *T.ni* Hi5 / *T.ni* (Hi5).*rtTA* cell lines, and assayed for transient CAT activity.

Results in Section 4.7 show that, in the absence of dox, the levels of CAT activity produced from the pTREΔ*ie*-2.*cat*2 (*TRE* / *Op*MNPV *ie*-2) was significantly lower than those observed from the pIZT/V5-His.*cat* (*Op*MNPV *ie*-2) in all the cell lines used (*Sf*9 / *Sf*9.*rtTA*, 26.9-fold respectively; *T.ni* Hi5 / *T.ni* (Hi5).*rtTA*, 12.5-fold respectively; **Figures 4.18A / B**). The activity of the *TRE* / *Op*MNPV *ie*-2 promoter compared with those observed from the *Op*MNPV *ie*-2 promoter, was shown to decrease by 99% in *Sf*9 / *Sf*9.*rtTA*, and 98% in *T.ni* Hi5 / *T.ni* (Hi5).*rtTA* cells respectively. These data suggest that the *TRE* was suppressing the *Op*MNPV *ie*-2 promoter; complete suppression was not observed, which may be correlated to the length of the *Op*MNPV *ie*-2 promoter used. The minimal *CMVie* promoter of the Tet systems is 120-bp, compared with its full-length sequence (587-bp). Minimal promoters are used because promoters such as the *CMVie* have upstream *cis*-acting enhancer elements capable of up-regulating the minimal promoter to exceptional levels. These promoters do, however, retain a high transcriptional rate with the removal of the enhancer element (Gossen and Bujard, 1992; Yin *et al.*, 1996). The removal of the strong enhancer element allows the minimal promoter to be tightly regulated by the *TRE*, but still produce high levels of recombinant protein in an induced state.

The full length *Op*MNPV *ie*-2 promoter (548-bp) was used in the development of this system. Studies have shown that the minimal *Op*MNPV *ie*-2 sequence required for basal activity in *Sf*9 cells is 125-bp upstream of the RNA start site (Theilmann and Stewart, 1992). The level of promoter activity observed from the minimal *Op*MNPV *ie*-2 are shown

to be weak compared with those observed from the full length *Op*MNPV *ie*-2 promoter. If the minimal *Op*MNPV *ie*-2 promoter had been used in the development of this system, complete suppression of the *Op*MNPV *ie*-2 promoter may have occurred, but the ability to produce high levels of recombinant protein would inevitably be compromised. However as described in Chapter 3, to retain **full-activity** of the *Op*MNPV *ie*-2 promoter, the minimal sequence required is 275-bp upstream of the RNA start site (Theilmann and Stewart, 1992). By removing the full-length (548-bp) *Op*MNPV *ie*-2 from pTREΔ.*ie*-2.*cat*2 and replacing it with the 275-bp *Op*MNPV *ie*-2 promoter, complete suppression of this promoter may be achieved, without compromising transcription activity when induced.

Analysis of induced transcriptional activity from pTREΔ.*ie*-2.*cat*2 (*TRE* / *Op*MNPV *ie*-2) in the presence of dox was determined to verify the complete functionality of the Tet inducible insect expression system in insect cell lines. Production of CAT from pTREΔ.*ie*-2.*cat*2 in the presence of dox showed (Section 4.8) that no significant increase of CAT activity was observed from the *TRE* / *Op*MNPV *ie*-2 promoter, in all cell lines used, when compared with those observed from *TRE* / *Op*MNPV *ie*-2 in the absence of dox (**Figure 4.18A / B**). The levels of CAT activity detected from pTREΔ.*ie*-2.*cat*2 in the presence of dox, was shown to be significantly lower than those observed from pIZT/V5-His.*cat* (*Op*MNPV *ie*-2), in all cell lines used (**Figure 4.19A / B**). The levels of CAT activity detected from pIZT/V5-His.*cat* in the presence of dox was not shown to differ significantly from those observed in the absence of dox, in all cell lines used (**Figure 4.18 / 19A, B**), highlighting that the concentrations of dox used did not affect the production of CAT from this promoter. Dox concentrations were varied (1ng-10μg/ml) to try and induce pTREΔ.*ie*-2.*cat*2 activity, although with little success. However, concentrations above 5μg/ml were observed to have cytotoxic affects to the host cells (data not shown). Subsequently these concentrations were not used to test for induction of pTREΔ.*ie*-2.*cat*2.

The transient insect Tet inducible expression system, developed by Wu *et al.* (2000), employs the original *TRE* promoter and the reporter gene *luc*, to detect changes in promoter regulation. The drawbacks of this system are, firstly, it uses the minimal *CMVie* promoter to drive foreign gene expression in insect cell lines. The full length *CMVie* promoter has already been characterised as functionally weak in insect cells, thus removal of the strong enhancer may potentially reduce transcriptional activity of this promoter in insect cells further. Secondly, the means by which suppression / activation of the minimal *CMVie* promoter was analysed in insect cells was reliant on the production of luciferase.

The assays used to detect the production of luciferase are highly sensitive, compared with those used in this thesis (CAT), and may explain why Wu *et al.* (2000), were able to detect activation of this promoter.

The transcriptional activity of a plethora of prokaryotic / eukaryotic promoters have been shown to be significantly decreased or silenced by various cellular processes / mechanisms. Studies have shown that it is possible to suppress heterologous prokaryotic / eukaryotic promoters by flanking sequence elements (Hu and Davidson, 1987; Gossen and Bujard, 1992; Schnetz, 1995), such as the *bgl* operon (Schaefler, 1967), *lac* operon (Miller and Reznikoff, 1980), and the Tn*10*-specified tetracycline-resistance operon (as describe in this Chapter; Hillen and Wieemann, 1989). However, the *lac* and Tn*10* operons have been manipulated to re-activate heterologous promoter activity (Hu and Davidson, 1987; Gossen and Bujard, 1992). The inability to induce activity of the *TRE* / *Op*MNPV *ie*-2 promoter from pTREΔ.*ie*-2.*cat*2 may be due to the number of *tetO* sequences present. The two *tetO* sequences upstream of *TRE* / *Op*MNPV *ie*-2 may be sufficient to suppress significant promoter activity as observed, but might not be satisfactory to induce *TRE* / *Op*MNPV *ie*-2 promoter activity. With only two *tetO* sequences controlling the *TRE* / *Op*MNPV *ie*-2 promoter, it is possible that the rtTA binding to these sequences is insufficient to trans-activate the *TRE* / *Op*MNPV *ie*-2 promoter. However, the evidence described previously in this Chapter by Gossen and Bujard (1992) has shown it is possible to suppress and activate the minimal *CMVie* promoter with two *tetO* sequences, although Gossen and Bujard used the heptameric version of the *tetO* sequences because enhanced activation of promoter activity was observed.

Promoter activities of prokaryotic / eukaryotic genes may be modulated by their sequence context. Studies have shown that the deletion of the 5' long terminal repeat (LTR) of the avian leukosis retrovirus activates its 3' LTR promoter (Cullen *et al.*, 1984). Similar transcriptional interference was also shown to occur between duplicated alpha-globin gene constructs (Proudfoot, 1986). This phenomenon was attributed to the mechanism identified as promoter occlusion (Adhya and Gottesman, 1982). The mechanism of promoter occlusion is likely to be caused by an inhibition of transcriptional initiation from a downstream promoter as read-through transcripts from an upstream promoter destabilise initiation complexes within the downstream promoter region (Adhya and Gottesman, 1982; Cullen *et al.*, 1984; Jansen *et al.*, 1993; Meng *et al.*, 1997; Greger *et al.*, 1998). Promoter occlusion has not been found to occur when antisense RNA production transpires from

two promoters that are in opposite directions (Brondyk, 1995), which is the case for *TRE* / *Op*MNPV *ie*-2 and the upstream *EM7* promoter (**Figure 4.12**) in pTREΔ*ie*-2*cat*2. Therefore, the inability to induce *TRE* / *Op*MNPV *ie*-2 could not be explained by the mechanism of promoter occlusion.

A second mechanism discovered, which serves to silence promoter activity is CpG island promoter hypermethylation (Greger *et al.*, 1989). CpG island promoter hypermethylation was identified as a common mechanism of inactivating the tumor suppressor gene $p16^{ink4a}$ in human cancers (Gonzalez-Zulueta *et al.*, 1995; Herman *et al.*, 1995; Merlo *et al.*, 1995). DNA methylation, catalysed by DNA methyltransferase, involves the addition of a methyl group to the carbon-5 position of the cytosine ring in the CpG dinucleotide and results in the generation of methylcytosine (Singal and Ginder, 1999; Baylin and Herman, 2000; Robertson and Wolffe; 2000). Hypermethylation of gene promoters has been shown to result in suppression of promoter activity and gene silencing (Garinis *et al.*, 2002; Kalpana *et al.*, 2002; Kikuchi *et al.*, 2002; Kin and Shibata, 2002). Work to determine the exact mechanism of how promoter methylation leads to gene silencing is still ongoing; there is evidence that promoter hypermethylation is associated with histone de-acetylation, change in chromatin structure and thus transcriptional inhibition (Clark and Melki, 2002; Esteller, 2002; Garinis *et al.*, 2002; Kikuchi *et al.*, 2002; Turker, 2002).

Analysis of the *TRE* / *Op*MNPV *ie*-2 promoter sequence revealed forty eight potential CpG islands and four prospective *Hpa*II-methylase sites (CCGG; **Figure 4.25**). The enzyme, *Hpa*II-methylase is endogenous to bacteria and methylates the central C nucleotide of the sequence CCGG, to protect bacterial DNA against its own *Hpa*II restriction nuclease (Barras and Marinus, 1989; Holliday, 1993). It is possible that the *TRE* / *Op*MNPV *ie*-2 promoter has been hypermethylated, which subsequently resulted in the repression of this promoter, thus explaining why it was not possible to induce transcriptional activity of the *TRE* / *Op*MNPV *ie*-2 promoter.

DNA methyltransferases have been identified and characterised in bacteria and mammalian cell lines, and more recently in insect cells (*Drosophila melanogaster*; Tweedie *et al.*, 1999). It is feasible that the CpG islands and methylation sites identified in *TRE* / *Op*MNPV *ie*-2 may have been hypermethylated when pTREΔ*ie*-2*cat*2 was propagated in *E. coli* (TOP10 *E. coli* contains all genomic methyltransferases;

Invitrogen™), or transfected in *Sf*9 and *T.ni* Hi5 cells if, like *Drosophila melanogaster* cells, they contain endogenous methyltransferases.

Further possible explanations to explain suppression of the activity of *TRE* / *Op*MNPV *ie*-2 in the presence of dox, are the presence of unidentified flanking element sequences in pTREΔ.*ie*-2.*cat*2, that may contribute to *TRE* repression of *Op*MNPV *ie*-2. It may also be suggested that the *Op*MNPV *ie*-2 sequence amplified from pIZT/V5-His.*cat* may be missing a critical transcriptional component, which was not elucidated in the literature. To resolve this problem a rescue vector was constructed, which was proposed to restore the promoter activity of the *Op*MNPV *ie*-2, by removing *TRE* from pTREΔ.*ie*-2.*cat*2. The subsequent plasmid pRescue.*cat*1 was derived.

CTCGAGTTTA CCACTCCCTA TCAGTGATAG AGAAAAGTGA AAGTCGAGTT TACCACTCCC
TATCAGTGAT AGAGAAAAGT GAAAGTCGAG CTCGGTACCG ATCATGATGA TAAACAATGT
ATGGTGCTAA TGTTGCTTCA ACAACAATTC TGTTGAACTG TGTTTTCATG TTTGCCAACA
AGCACCTTTA TACTCGGTGG CCTCCCCACC ACCAACTTTT TTGCACTGCA AAAAAACACG
CTTTTGCACG CGGGCCCATA CATAGTACAA ACTCTACGTT TCGTAGACTA TTTTACATAA
ATAGTCTACA CCGTTGTATA CGCTCCAAAT ACACTACCAC ACATTGAACC TTTTTGCAGT
GCAAAAAAGT ACGTGTCGGC AGTCACGTAG GCCGGCCTTA TCGGGTCGCG TCCTGTCACG
TACGAATCAC ATTATCGGAC CGGACGAGTG TTGTCTTATC GTGACAGGAC GCCAGCTTCC
TGTGTTGCTA ACCGCAGCCG GACGCAACTC CTTATCGGAA CAGGACGCGC CTCCATATCA
GCCGCGCGTT ATCTCATGCG CGTGACCGGA CACGAGGCGC CCGTCCCGCT TATCGCGCCT
ATAAATACAG CCCGCAACGA TCTGGTAAAC A^{+1}CAGTTGAA CAGCATCTGT TCGAA

Figure 4.25: Nucleic acid sequence of the *TRE* / *Op*MNPV *ie*-2 promoter. CpG islands are indicated as purple CG boxes, and *Hpa*II-methylase sites as yellow/ purple / yellow CCGG boxes. The TATA box has been underlined and the RNA start site is indicated by +1.

The results in section 4.9 show that, surprisingly the removal of *TRE* from pTREΔ.*ie*-2.*cat*2 did not restore the constitutive activity of pRescue.*cat*1 (*Op*MNPV *ie*-2.Δ*TRE*) in any of the cell lines used (**Figure 4.21A / B**). In fact, the levels of CAT activity detected from pRescue.*cat*1 were shown not to significantly differ from those observed from pTREΔ.*ie*-2.*cat*2. These data suggest that either the *Op*MNPV *ie*-2.Δ*TRE* promoter was silenced by one of the mechanisms describe above, or the promoter sequence amplified from pIZT/V5-His.*cat* was incorrect. Further investigation of this enigma lead to the

construction of a second rescue plasmid. Attempts to restore the constitutive activity of the *Op*MNPV *ie*-2.Δ*TRE* promoter from pRescue.*cat*1 were made by removing the *Op*MNPV *ie*-2.Δ*TRE* promoter from pRescue.*cat*1 and cloning it into a completely different promoter-less vector; the plasmid pRescue.*cat*2 was derived.

Results in Section 4.10 show that the constitutive activity of the *Op*MNPV *ie*-2 rescued promoter was restored in all cell lines used. The levels of CAT activity produced from pRescue.*cat*2 were shown to be significantly higher than those observed from pTREΔ.*ie*-2.*cat*2 and pRescue.*cat*1. However, compared with the pIZT/V5-His.*cat*, complete restoration of transcriptional activity from *Op*MNPV *ie*-2 of pRescue.*cat*2 was not achieved. Levels of transcriptional activity were, however, restored to 70% of the *Op*MNPV *ie*-2 promoter from pIZT/V5-His.*cat* in *Sf*9 / *Sf*9.*rtTA* cells and 74% in *T.ni* Hi5 *T.ni* (Hi5).*rtTA* cells respectively.

Conclusions drawn from these data suggest that the *TRE* / *Op*MNPV *ie*-2 and *Op*MNPV *ie*-2.Δ*TRE* promoters were not subjected to hypermethylation, as *Op*MNPV *ie*2 rescue was shown to function in all insect cell lines used, albeit in a different vector. The second possible explanation eliminated was the authenticity of the *Op*MNPV *ie*-2 promoter sequence used, as confirmed by the literature. Even though low levels of CAT activity were detected from the *TRE* / *Op*MNPV *ie*-2 and *Op*MNPV *ie*-2.Δ*TRE* promoters, it was hypothesised that a crucial sequence may be missing, which may be used to augment activity of *Op*MNPV *ie*-2 in pIZT/V5-His.*cat*. Finally, the explanation most likely to account for why activity of *TRE* / *Op*MNPV *ie*-2 was not augmented in the presence of dox, and *Op*MNPV *ie*-2.Δ*TRE* constitutive activity was not fully restored, focuses on the presence of other flanking element sequences (other than *TRE*) present in pTREΔ.*ie*-2.*cat*2 capable of causing repression of the *Op*MNPV *ie*-2 promoter. The flanking elements, hypothesised to attenuate the transcriptional activity of *Op*MNPV *ie*-2 in pTREΔ.*ie*-2.*cat*2, have yet to be identified.

The results obtained in this chapter address some of the problems associated with constructing novel plasmid-based expression vectors. The complexity of adapting the Tet-On expression system for use in insect cells was acknowledged in the development of the insect Tet response vector. The strategy used to construct this vector was found to be compromised, due to unforeseen phenomena such as unidentified, repressor-like elements attenuating the activity of the *Op*MNPV *ie*-2 promoter. The conclusion drawn

from these data is that the original Tet response vector cannot be directly re-engineered to incorporate the *Op*MNPV *ie*-2 promoter in place of the minimal *CMVie* to generate a functional insect Tet response vector.

The foundation work for adapting the Tet-On expression system for use in insect cells has been initiated, with the successful development and use of the insect Tet regulatory vector (pXINsect-DEST38.*rtTA*). It is likely that the problems identified with the insect Tet response vector (pTREΔ.*ie*-2.*cat*2) can be overcome with further investigation, to generate a fully functional insect Tet inducible expression system that will offer the researcher a more diverse choice of expression system and cell lines for recombinant protein production.

CHAPTER 5: METHODS OF IMPROVING INTRACELLULAR PROTEIN PRODUCTION USING INSECT EXPRESSION SYSTEMS

5.1 Introduction

Advances in the development of plasmid-based expression systems for the generation of stable insect cell lines has progressed slowly over the last fifteen years, and has been dominated by the transcriptionally weak activity of the *Ac*MNPV *ie*-1 promoter (Jarvis and Summers, 1989; Jarvis *et al.*, 1990). However, a number of new RNA polymerase II transcribed insect and viral promoters (*Bm A*3+E / *Op*MNPV*ie*-2) have been shown to increase foreign gene expression above those observed from *Ac*MNPV *ie*-1 in insect cells (Johnson *et al.*, 1992; Jarvis *et al.*, 1996; Lu *et al.*, 1996; 1997; Hegedus *et al.*, 1998). Subsequently, new plasmid-based expression vectors have been developed using these promoters (*Bm A*3+E and *Op*MNPV *ie*-2). These vectors are currently used for transient and stable foreign gene expression in insect cell lines.

The baculovirus expression system (BES) is widely used as an alternative to stably-transformed insect cell lines for transient expression of heterologous genes, as levels of foreign protein produced are generally high. However, expression levels of secreted and membrane-targeted proteins may be considerably lower than those directed to the cytoplasm or nucleus (King and Possee, 1992). Studies have shown that stably transformed insect cells, continuously expressing secreted recombinant proteins can produce levels comparable to those reported for baculovirus vectors (Farrell *et al.*, 1998; Li *et al.*, 2001), although, stably expressed foreign proteins targeted to intracellular compartments have been shown to be significantly lower than those observed form the BES (Jarvis *et al.*, 1990). In this Chapter attempts were made to compare intracellular foreign protein production from an improved stable insect plasmid-based expression system (Express Insect™ system; *Bm A*3+E), to that of the BES.

The inherent lower levels of secreted recombinant proteins from baculovirus-infected cells has been well documented, and has been attributed to the onset of baculovirus infection compromising both the cell secretory pathway and viability (Thomas *et al.*, 1998; Saville *et al.*, 2002). Baculovirus infected cells have been shown to retain the baculovirus expressed enzyme, chitinase (*chiA*) in the endoplasmic reticulum (ER), which subsequently causes blockage of the secretory pathway (Thomas *et al.*, 1998; Saville *et al.*, 2002), and as a consequence reduces secretion of proteins. Further-more, the cytolytic effect of the baculovirus on insect cells inevitably leads to the release of cell / viral

proteases into the culture medium, which inexorably cause proteolytic degradation of secreted foreign proteins (Li *et al.*, 2001).

Previous work in our laboratory has shown that deletion of *chiA* from the *Ac*MNPV genome improves yields of secreted foreign proteins (urokinase / tissues-plasminogen activator; McCarroll, 1997) from infected insect cells. However, no evidence for improved intracellular foreign protein production has been attained using this virus. It was for this reason, that analyses of intracellular levels of foreign protein produced from the *polh* promoter of *Ac*BacPAK6.Δ*chiA* (designated here as *Ac*.Δ*chiA*; Possee *et al.*, 1999) was compared to those of *Ac*BacPAK6 (designated here as *Ac*; Kitts and Possee, 1993).

Small-scale protein production in insect cells is commonly carried out in T-flasks (cell monolayers) or shaker cultures (suspended culture), and often provides sufficient protein for research purposes. However, industrial scale projects demand large quantities of foreign protein, which cannot often be obtained from T-flasks. To meet these demands, industries have turned to using large shaker flasks (3-litre) or fermenter vessels to scale-up protein production. The disadvantages associated with using fermenter vessels are inevitably the initial setting up costs; also these systems are very time-consuming and labour-intensive, compared to the simplicity and relative inexpensive shaker flasks. However, the advantages of recombinant protein production in fermenters includes the ability to control environmental factors associated with maintaining optimal culturing conditions for maximising protein production. Such factors include dissolved oxygen concentration and pH (Scott *et al.*, 1992; Wong *et al.*, 1994; Hu and Bentley, 1999). These factors can be regulated and monitored throughout fermentation, which is not possible in shake cultures.

The work in this Chapter examines these two different scale-up culturing methodologies (fermenter versus shaker) for recombinant protein production in insect cell lines. Attempts were made to scale-up (1-litre) recombinant protein production from stably transformed insect cells, and from baculovirus-infected (recombinant *Ac* or *Ac*.Δ*chiA*) insect cell cultures. Subsequently the relative yields of recombinant protein produced from fermenter or shaker cultures were determined and compared.

All studies conducted in this Chapter utilised the intracellular fluorescence reporter gene *Discosoma* red (*DsRed*) because of its ease of detection, and intracellular *dihydroorotate*

dehydrogenase (*Candida albicans, DHODH*), due to its industrial importance and relevance to Pfizer, the collaborating institution involved in this project. From these studies a comprehensive analysis of insect expression systems and culturing methodology was anticipated.

5.2 Comparison of heterologous protein production from *Ac* and *Ac.ΔchiA* in insect cell lines

To examine and compare the levels of intracellular recombinant protein produced from the baculoviruses, *Ac* and *Ac.ΔchiA*, *DsRed* and *DHODH* were inserted under control of the transcriptionally strong *polh* promoter, using the methods described in Chapter 1 (Section 1.3.2) and 2 (Sections 2.3.2, 2.3.3 and 2.3.4; King and Possee, 1992). Subsequently the recombinant baculoviruses, *Ac.DsRed*, *Ac.DHODH*, *Ac.ΔchiA.DsRed* and *Ac.ΔchiA.DHODH* were derived.

An important fundamental consideration for the production of foreign protein is the choice of cell type used to host expression. Several studies have shown that this can dramatically affect the levels of expression obtained (Lynn and Hink, 1980; King and Possee, 1992). Another significant elemental deliberation for the production of foreign protein is the moi used to infect cells. Experiment were carried out in this Chapter to determine the most suitable insect cell line and virus moi for the expression of recombinant DsRed and DHODH, using the recombinant baculoviruses described above.

Small (25ml) shaker cultures of *Sf*9 and *T.ni* Hi5 cells were set-up as previously described in Section 2.2.3. Cells were infected (moi 2, 5 and 10) at the appropriate density with *Ac.DsRed*, *Ac.DHODH*, *Ac.ΔchiA.DsRed* or *Ac.ΔchiA.DHODH*. Mock-infected *Sf*9 and *T.ni* Hi5 cells were used as a negative control. Samples (1ml) were harvested at 0, 24, 48, and 72 hpi and the intracellular total cell protein content was estimated using the Bradford BioRad assay (Section 2.7.4). Standard amounts of total protein (25μg) from each sample were run on SDS-PAGE for Western blot analyses (Sections 2.7.5, 2.7.6) to determine the levels of intracellular DsRed and DHODH produced. Separate samples (1ml) were also harvested at 0, 12, 24, 36, 48, 60, 72, 84 and 96 hpi and subjected to fluorometric analyses (as described in Section 2.9.1), quantifying the levels of intracellular fluorescence generated by the production of DsRed. *Sf*9 and *T.ni* Hi5 cells infected with *Ac.DHODH* or

Ac.ΔchiA.DHODH were used to detect background auto-fluorescence (negative controls). As expected, the production of DHODH was not detected from mock-infected *Sf*9 cells.

Figure 5.1 shows results obtained for optimal DHODH expression from *Ac.DHODH* and *Ac.ΔchiA.DHODH* in *T.ni* Hi5 cells. To detect the production of DHODH, six juxtaposed histidine residues were inserted in-frame at the N-terminus of the *DHODH*-coding region. An anti-polyhistidine monoclonal antibody (Sigma) was used to detect these residues, therefore producing qualitative / semi-quantitative analyses of DHODH (molecular weight 43 kDa) production. The production of DHODH was not detected from mock-infected or *Ac.DHODH* / *Ac.ΔchiA.DHODH* infected *T.ni* Hi5 cells at 0 hpi (**lanes MI, 0a, 0b**). However, DHODH was detected at varying time-points in *Ac.DHODH* and *Ac.ΔchiA.DHODH* using an moi of 2, 5 and 10 (selected data shown). However, an moi of 5 was shown to produce optimal DHODH production. Expression of DHODH from *Ac.DHODH* (moi of 5) was detected at 48-72 hpi (**lanes 48a, 72a**), optimal levels were detected at 48 hpi. At 72 hpi the production of DHODH was dramatically decreased, and a second His-tagged protein (estimated molecular weight 28kDa) was detected, which may have been a possible degradation product of DHODH (**lane 72a**). The production of DHODH from *Ac.ΔchiA.DHODH* (moi of 5) was observed at 24-72 hpi (**lanes 24b-72b**). Although DHODH was detected at 24 hpi from *Ac.ΔchiA.DHODH* none was detectable from *Ac.DHODH* at the same time-point (**lane 24a**). The highest levels of DHODH produced from *Ac.ΔchiA.DHODH* were observed at 48-72 hpi (**lane 48b**), and was shown to be greater than those observed from *Ac.DHODH* (48-72 hpi). Interestingly, the very apparent 28kDa product detected from *Ac.DHODH* at 72 hpi was also observed from *Ac.ΔchiA.DHODH* at the same time-point (**lanes 48b, 72b**), but at much lower levels. Unexpectedly, the production of DHODH was not detected from *Ac.DHODH* / *Ac.ΔchiA.DHODH* infected *Sf*9 cells (data not shown) at 0-72 hpi.

Figure 5.2A / B shows results obtained for optimal DsRed expression from *Ac.DsRed* and *Ac.ΔchiA.DsRed* in *Sf*9 (**A**) and *T.ni* Hi5 (**B**) cells. Semi-qualitative analyses of DsRed production using a DsRed polyclonal antibody (BD Biosciences; molecular weight 25 kDa), showed no detectable levels of DsRed in mock-infected or *Ac.DsRed* / *Ac.ΔchiA.DsRed* infected *Sf*9 and *T.ni* Hi5 cells at 0 hpi (**A / B: lane MI, 0a, 0b**). In both cell lines infected with *Ac.DsRed* or *Ac.ΔchiA.DsRed*, production of DsRed was observed from all moi's used at 48-72 hpi (moi 2,5 and 10; selected data shown). However, an moi of 2 was shown to produce the highest levels of DsRed in the insect cells used (**A / B:**

lanes 48-72a, 48-72a). The levels of DsRed produced from *Ac.DsRed* at 48-72 hpi were shown to be higher than those detected from *Ac.ΔchiA.DsRed* in both cell lines. Optimal production of DsRed from both viruses in *Sf*9 and *T.ni* Hi5 cells were observed at 72 hpi. Overall production of DsRed from *Ac.DsRed* and *Ac.ΔchiA.DsRed* in *Sf*9 and *T.ni* Hi5 cells, was shown to be higher in *Sf*9 cells.

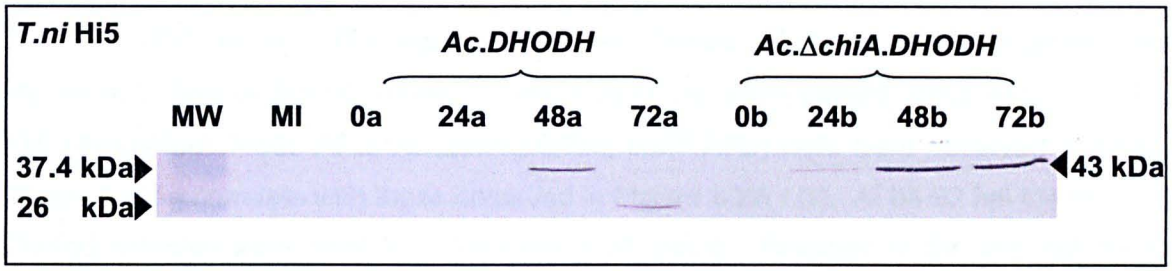


Figure 5.1: Western blot analyses of *Ac.DHODH* and *Ac.ΔchiA.DHODH* infected *T.ni* Hi5 cells expressing DHODH. Lane MW (molecular weight), shows protein markers (Invitrogen). Lane MI (mock-infected), shows intracellular cell extract (25μg / lane) from mock-infected *T.ni* Hi5 cells (negative control). Lane 0-72a shows intracellular cell extract (25μg / lane) from *T.ni* Hi5 cells infected with *Ac.DHODH* at 0-72 hpi (moi of 5). Lane 0-72b shows intracellular cell extract (25μg / lane) from *T.ni* Hi5 cells infected with *Ac.ΔchiA.DHODH* at 0-72 hpi (moi of 5). Position of molecular weight (MW) size marker is shown in kDa and the position of DHODH is indicated (43kDa).

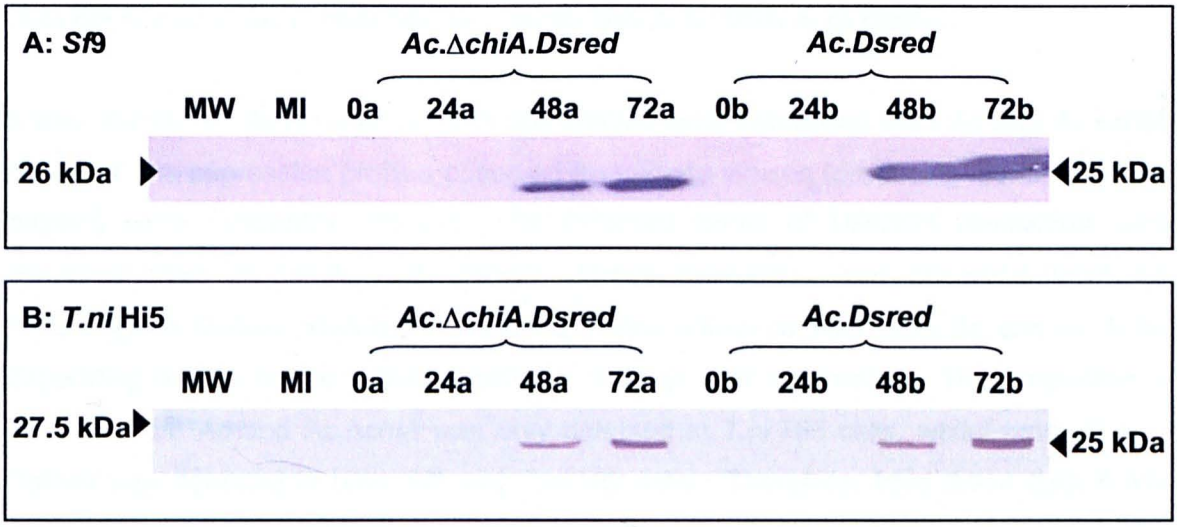
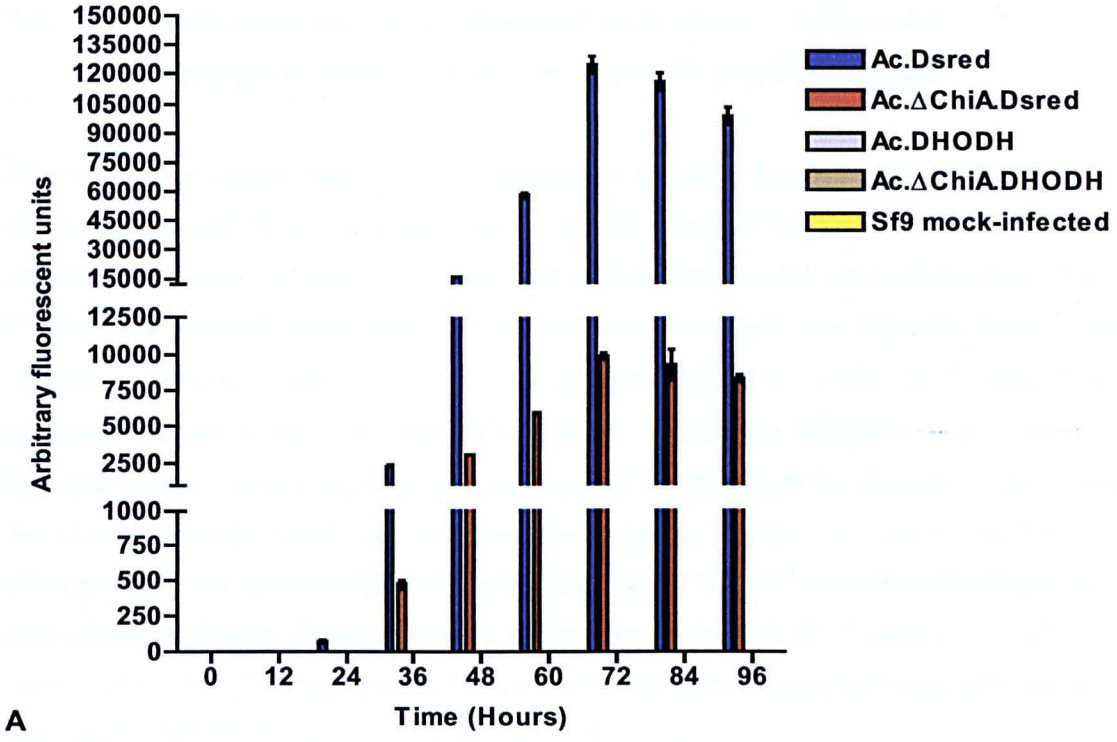


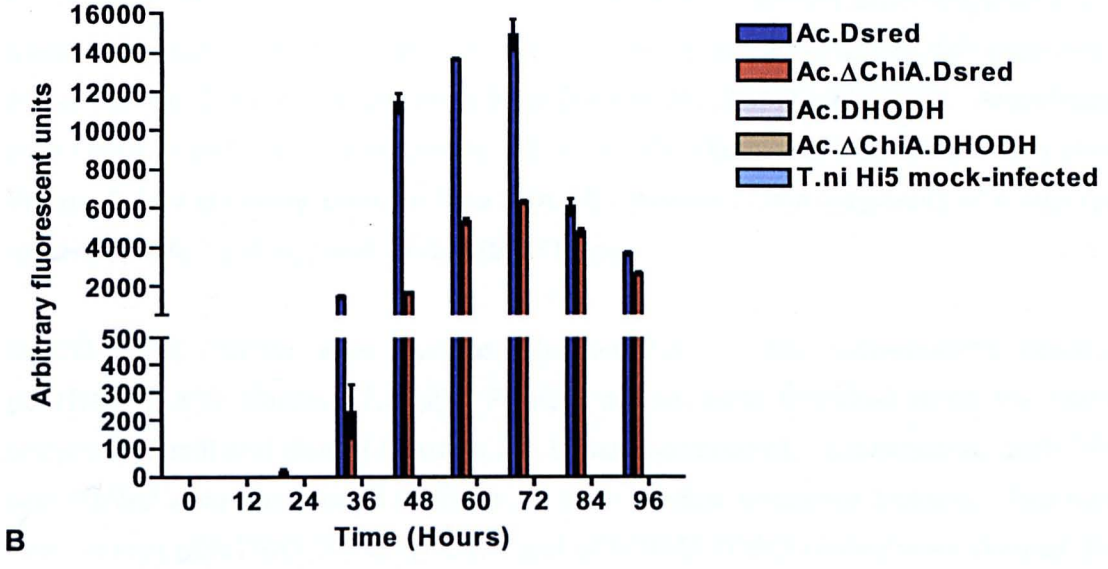
Figure 5.2A / B: Western blot analysis of *Ac.DsRed* and *Ac.ΔchiA.DsRed* infected *Sf*9 (**panel A**) and *T.ni* Hi5 (**panel B**) cells expressing DsRed. Lanes MW (molecular weight), shows protein markers (Invitrogen). Lanes MI (mock-infected), shows intracellular cell extract (25μg / lane) from mock-infected *Sf*9 and *T.ni* Hi5 cells (negative control). Lanes 0-72a shows intracellular cell extract (25μg / lane) from *Sf*9 and *T.ni* Hi5 cells infected with *Ac.DsRed* at 0-72 hpi (moi of 2). Lane 0-72b shows intracellular cell extract (25μg / lane) from *Sf*9 and *T.ni* Hi5 cells infected with *Ac.ΔchiA.DsRed* at 0-72 hpi (moi of 2). Position of molecular weight (MW) size marker is shown in kDa and the position of DsRed is indicated (25kDa).

Figure 5.3 A / B shows results obtained for DsRed production from *Ac.DsRed* and *Ac.ΔchiA.DsRed* (moi 2) in *Sf*9 (**A**) and *T.ni* Hi5 cells (**B**). No background levels of fluorescence were observed from mock-infected, or *Ac.DHODH / Ac.ΔchiA.DHODH* infected *Sf*9 and *T.ni* Hi5 cells. The production of DsRed from *Ac.DsRed* in *Sf*9 (71 arbitrary fluorescence units [AFU]) and *T.ni* Hi5 (16.6 AFU) cells was first observed at 24 hpi, whilst production from *Ac.ΔchiA.DsRed* was initially detected at 36 hpi (*Sf*9, 475 AFU; *T.ni* Hi5, 231 AFU). The highest levels of DsRed produced from *Ac.DsRed* and *Ac.ΔchiA.DsRed* in *Sf*9 (*Ac.DsRed*, 124873 AFU; *Ac.ΔchiA.DsRed*, 9853 AFU) and *T.ni* Hi5 (*Ac.DsRed*, 14930 AFU; *Ac.ΔchiA.DsRed*, 6357 AFU) cells were observed at 72 hpi (these finding correlate with those observed in **Figure 5.2A / B**). At 84-92 hpi the levels of DsRed detected were shown to decrease in all cases. Analyses of the cell viability for each infected culture, showed cell viability was severely compromised by virus infection at 72-96 hpi (data not shown). Therefore, the reduction in the detected levels of DsRed from each infection at 84-96 hpi correlates with cell death observed at these same time points. A comparison of DsRed production from *Ac.DsRed* and *Ac.ΔchiA.DsRed* in *Sf*9 and *T.ni* Hi5 cells, showed *Ac.DsRed* produced significantly higher levels of DsRed than those observed from *Ac.ΔchiA.DsRed* ($p<0.001$ in all cases). The levels of DsRed produced from *Ac.DsRed* and *Ac.ΔchiA.DsRed* were also shown to be significantly higher in *Sf*9 cells compared to those observed in *T.ni* Hi5 cells ($p=0.0001$ in all cases).

It was, therefore, shown that *DHODH* and *DsRed* were expressed from *Ac* and *Ac.ΔchiA*. However, the expression profiles observed from these viruses (producing identical foreign protein) were noticeably different. As optimum levels of DHODH production were observed from *Ac.ΔchiA*, and optimal DsRed production was observed from *Ac.* Heterologous protein production levels were also shown to vary from *Ac* and *Ac.ΔchiA* depending on the choice of insect cell line used to host expression. The production of DHODH from *Ac* and *Ac.ΔchiA* was only detected in *T.ni* Hi5 cells, whilst production of DsRed was detected in both *Sf*9 and *T.ni* Hi5 cells. Therefore, from these data it was concluded that *Ac.ΔchiA* improved the production of specific recombinant proteins, but highlights the need to characterise gene expression from these expression vectors, as well as determine the cell line required for optimal protein production.



A



B

Figure 5.3 A / B: Fluorescence detected for DsRed production in *Ac.DsRed* and *Ac.ΔchiA.DsRed* infected *Sf*9 (**A**) and *T.ni* Hi5 (**B**) cells. Mock-infected and *Ac.DHODH* / *Ac.ΔchiA.DHODH* infected *Sf*9 and *T.ni* Hi5 cells were tested for background fluorescence and used as negative controls. Cell samples (1ml) were harvested at 12 h intervals over a period of 96 h. These sample were subjected to fluorometric analyse to determine the levels of fluorescence produced from DsRed expression.

5.3 Construction of plasmid-based expression vectors for comparison of foreign protein production in stable insect cell lines

The advances made over the last decade to improve foreign protein production from stable insect cell lines, promoted work in this Chapter to compare relative yields of intracellular foreign protein, produced from stable insect cell lines to those from the BES. Of the new plasmid-based expression systems developed, the Express Insect™ system (Invitrogen™), which utilises the *Bm A*3+E promoter (Lu *et al.*, 1996; 1997), was chosen to generate stably transformed insect cell lines expressing DHODH and DsRed. The Express Insect™ system was chosen because of the excellent transcriptional activity of the *Bm A*3+E promoter used to transcribe foreign genes in insect cell lines (as described in Chapter 3). This system also utilises the directional TOPO® cloning technology, for rapid and efficient cloning of heterologous genes (as described in Chapter 1.6). Due to the nature of TOPO® cloning, the *DHODH* and *DsRed* genes were first inserted into an entry plasmid (pENTR/D-TOPO) to produce an entry clone.

For insertion of *DHODH* or *DsRed* into pENTR/D-TOPO, primers were designed to amplify these gene coding-regions. *DHODH* was amplified from pET-21b.*DHODH* (obtained from Pfizer), whilst *DsRed* was amplified from p*DsRed*.N1 (BD Biosciences). Amplification of both *DHODH* and *DsRed* was carried out using PCR (Section 2.5.5). The results shown in **Figure 5.4A / B** clearly show, in lane 2 (**A / B**) amplified DNA fragments of a size relative to *DHODH* (**A**; 1210-bp) and *DsRed* (**B**;677-bp).

DHODH and *DsRed* were purified (Section 2.5.7.2) and subsequently cloned into pENTR/D-TOPO (Section 2.5.12). Positive clones were identified using the restriction enzymes *Hin*dIII and *Bam*HI (Section 2.5.1; data not shown). Subsequently both *DHODH* and *DsRed* were sequenced (Section 2.5.8) to confirm sequence integrity. The resulting entry clones pENTR/D-TOPO.*DHODH* and pENTR/D-TOPO.*DsRed* were derived (**Figure 5.5A / B**).

DHODH and *DsRed* were then inserted into pXINsect-DEST38, using TOPO site-specific recombination (Section 2.5.12). Positive clones were identified using the restriction enzymes *Hin*dIII and *Bam*HI (Section 2.5.1; **Figure 5.6A / B**). The plasmids derived were named p*BmA*3+E.*DHODH* and p*BmA*3+E.*DsRed* (**Figure 5.7A / B**).

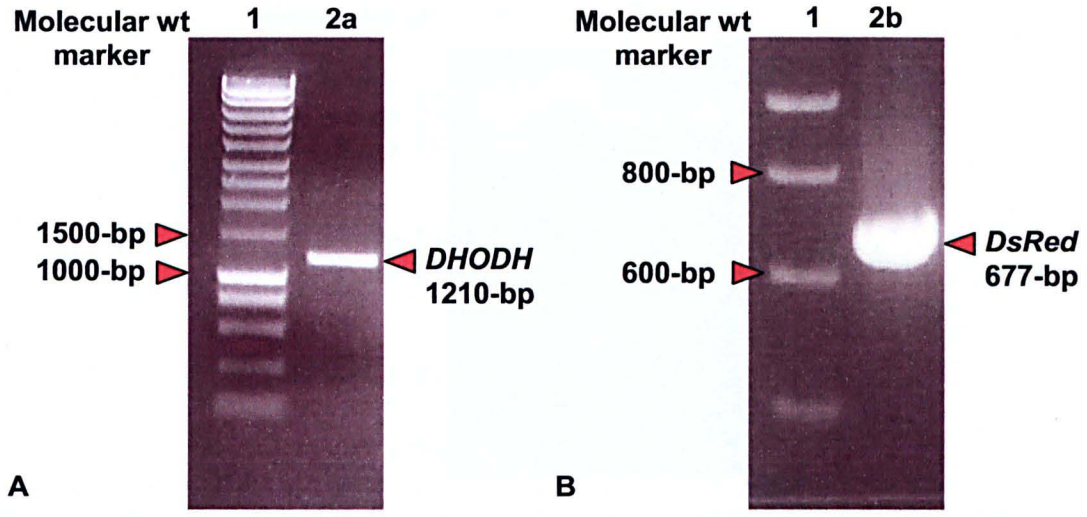


Figure 5.4A / B: Analysis of the amplification of the *DHODH* (**A**) coding region from pET-21b.*DHODH* and *DsRed* (**B**) coding region from p*DsRed*.N1, using PCR (Section 2.5.5). Positive amplified *DHODH* and *DsRed* coding-regions produced DNA fragments banding at 1210-bp (*DHODH*) and 677-bp (*DsRed*). The above gels show:

Track 1: Smart Ladder (Eurogentec 1kb)
Track 2a: Positive amplification of the *DHODH* coding-region
Track 2b: Positive amplification of the *DsRed* coding-region

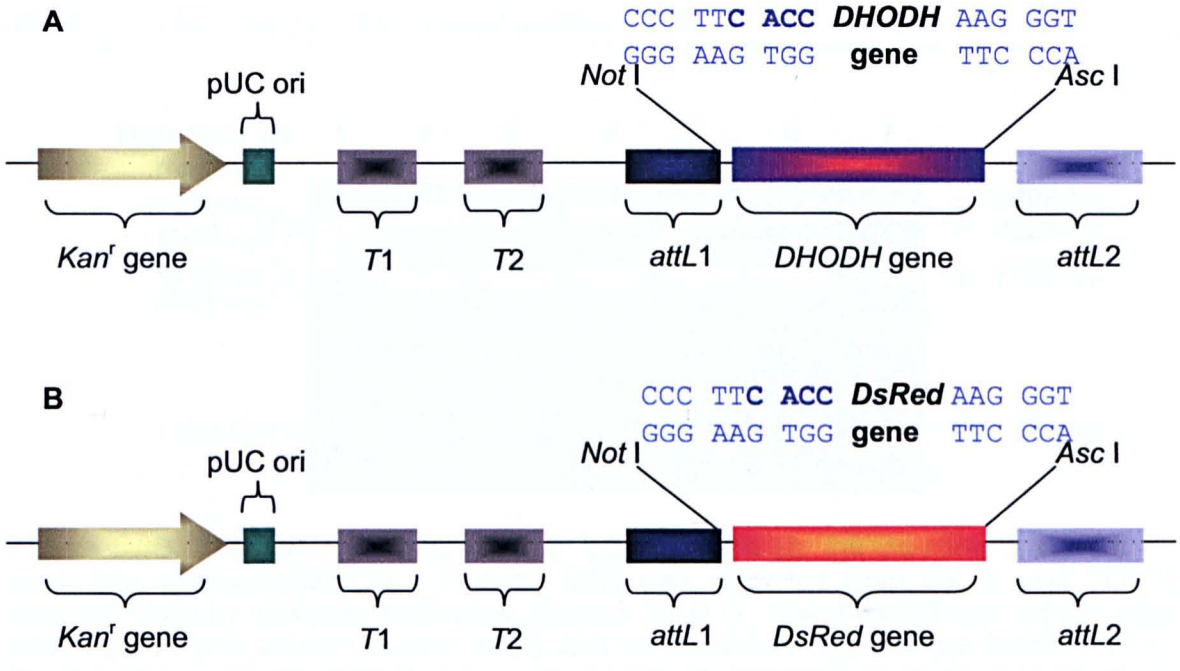


Figure 5.5A / B: Genetic map of pENTR.D.TOPO.*DHODH* (**A**) and pENTR.D.TOPO.*DsRed* (**B**), showing a *Kan*^{r} gene, pUC origin of replication, *T1* / *T2* , *attL1* / *attL2* recombination sites with *DHODH* or *DsRed* inserted amidst.

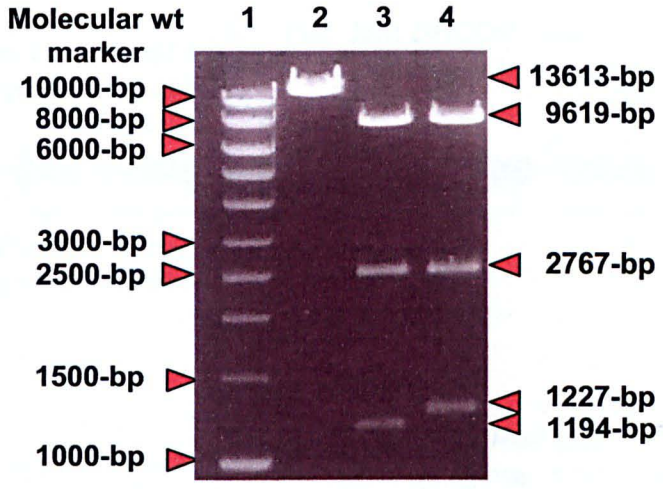


Figure 5.6A: Analysis of plasmid DNA, to positively identify the insertion of the *DHODH* gene into pXINsect-DEST38. Plasmid DNA was extracted from the *E. coli* TOP10 bacterial strain by mini-prep purification (Section 2.5.9.1). Plasmid DNA was cut using the restriction enzymes *Bam*HI and *Hin*dIII (Section 2.5.1), and run on a 0.7% agarose gel (Section 2.7.1). Positive clones with the *DHDOH* coding-region produced DNA fragments banding at 13613-bp (*Bam*HI), 9619-bp, 2767-bp, 1210-bp and 33-bp (*Bam*HI / *Hin*dIII), 9619-bp, 2767-bp and 1227-bp (*Hin*dIII). The above gel shows:

Track 1: Smart Ladder (Eurogentec 1kb)
Track 2: p*BmA*3+E.*DHODH* / *Bam*HI positive clone
Track 3: p*BmA*3+E.*DHODH* / *Bam*HI / *Hin*dIII positive clone (33-bp was to small to detect)
Track 4: p*BmA3*+E.*DHODH* / *Hin*dIII positive clone

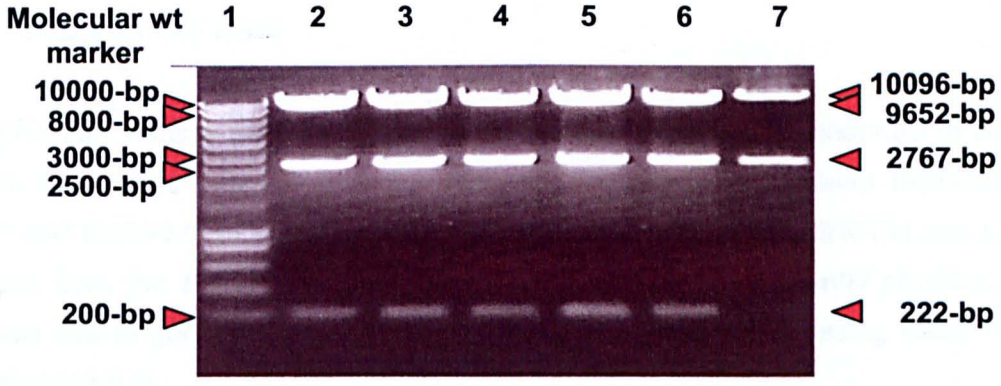


Figure 5.6B: Analysis of plasmid DNA, to positively identify the insertion of the *DsRed* gene into pXINsect-DEST38. Plasmid DNA was extracted from the *E. coli* TOP10 bacterial strain by mini-prep purification (Section 2.5.9.1). Plasmid DNA was cut using the restriction enzyme *Hin*dIII (Section 2.5.1), and run on a 0.7% agarose gel (Section 2.7.1). Positive clones with the *DsRed* coding-region produced DNA fragments banding at 10096-bp, 2767-bp and 222-bp (*Hin*dIII). The above gel shows:

Track 1: Smart Ladder (Eurogentec 1kb)
Track 2-6: pBmA3+E.*DsRed* / *Hin*dIII positive clones
Track 7: pBmA3+E / *Hin*dIII (negative control; 9652 / 2767-bp)

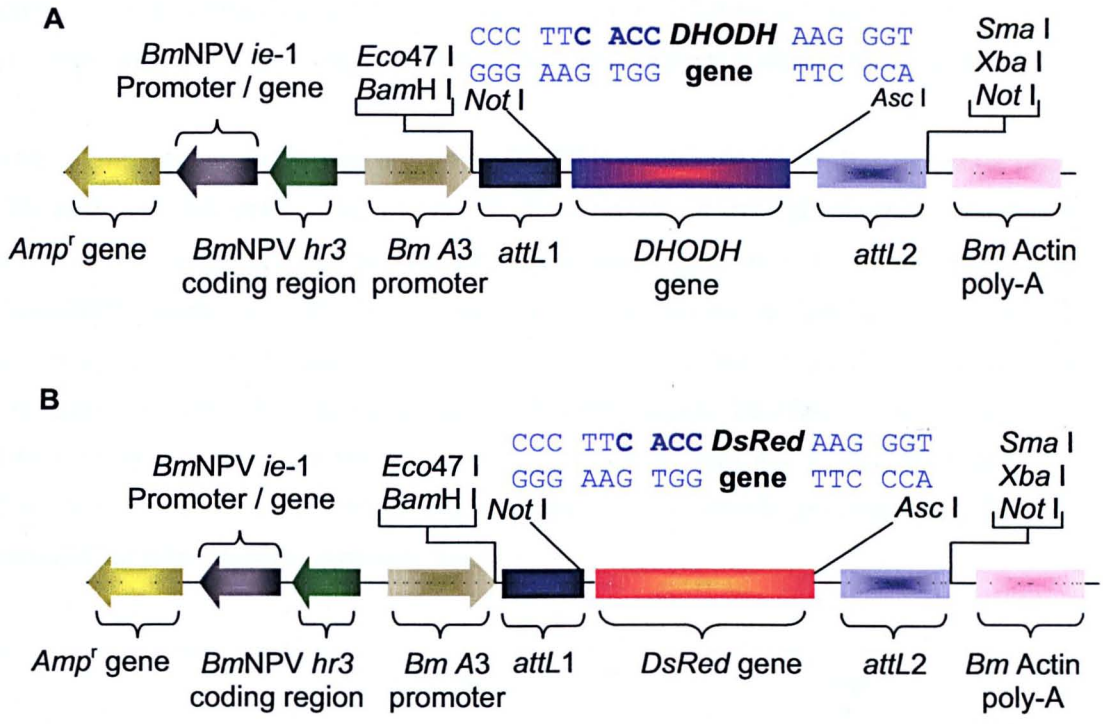


Figure 5.7A / B: Genetic map of p*BmA*3+E.*DHODH* (**A**) and p*BmA*3+E.*DsRed* (**B**), showing a *Amp*^{r} gene, *Bm*NPV *ie*-1 promoter / gene, *Bm*NPV *hr*3 coding region, *Bm A*3 promoter with *DHODH* or *DsRed* inserted downstream, *attL*1 / *attL*2 recombination sites, and the *Bm* actin poly-A coding region.

5.4 Transient plasmid-based expression of *DHODH* and *DsRed* in *Sf*9 and *T.ni* Hi5 cells

The expression vectors p*BmA*3+E.*DHODH* and p*BmA*3+E.*DsRed* generated in Section 5.3 were transfected into *Sf*9 and *T.ni* Hi5 cells to determine transient expression of *DHODH* and *DsRed* from the *BmA*3+E promoter. The detection of DHODH and DsRed production from the *BmA*3+E promoter in these cell lines subsequently provided data, which was use to generate stably transformed insect cell lines expressing these foreign genes (Section 5.5).

*Sf*9 and *T.ni* Hi5 cells were seeded (Section 2.2.3) into 35mm tissue culture dishes, and left to adhere for 1 h. The cell mono-layers were transfected (Section 2.2.4) using 1μg of plasmid DNA (p*BmA*3+E.*DHODH* or p*BmA*3+E.*DsRed*). Cells were harvested after 24, 48 and 72 h incubation at 28°C. Standard amounts of total protein (25μg; Section 2.7.4) from each sample were run on SDS-PAGE and subjected to Western blot analyses (Section 2.7.5, 2.7.6). Cells transfected with p*BmA*3+E.*DsRed* were also visualised for the

production of DsRed using a Carl Zeiss Axiovert 510 Confocal Laser Scanning Microscope (CLSM), with a standard Argon 488nm laser, and 534nm Helium / Neon laser.

The results shown in **Figure 5.8** show DHODH expression from the *BmA*3+E promoter in *Sf*9 and *T.ni* Hi5 cells. Expression of DHODH was detected using an anti-polyhistidine monoclonal antibody (Sigma). Mock-transfected *Sf*9 and *T.ni* Hi5 cells showed no detectable levels of DHODH (**lanes MT**). Expression of *DHODH* from the *BmA*3+E promoter in *T.ni* Hi5 cells were shown to be detected at 24-72 hpt (**lanes 24-72a**), however, no DHODH was detected in *Sf*9 cells (**lanes 24-72b**). The visual detection of DHODH by Western blot analyses was observed as very low signal intensities, indicating that the levels of DHODH produced from the *BmA*3+E promoter in *T.ni* Hi5 cells throughout transfection were very low.

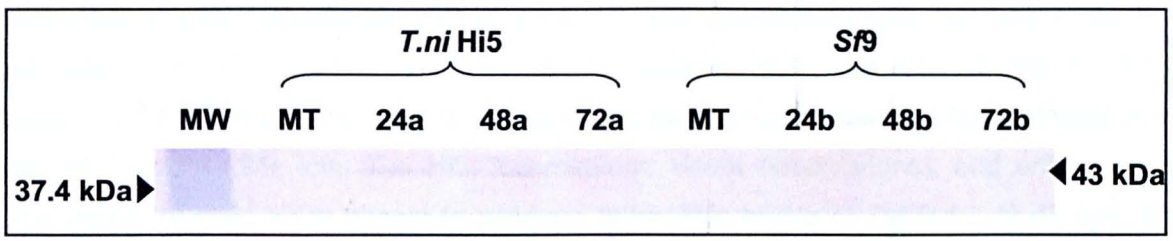


Figure 5.8: Western blot analysis of *T.ni* Hi5 and *Sf*9 cells expressing DHODH. Lane MW (molecular weight), shows protein markers (Invitrogen). Lane MT (mock-transfected), shows intracellular cell extract (25μg / lane) from mock-transfected *T.ni* Hi5 and *Sf*9 cells (negative control). Lane 24-72**a / b** shows intracellular cell extract (25μg / lane) from *T.ni* Hi5 (**a**) and *Sf*9 (**b**) cells transfected with 1μg of p*BmA*3+E.*DHODH* at 24-72 hpt. Position of molecular weight (MW) size marker is shown in kDa and the position of DHODH is indicated (43kDa).

Figure 5.9 shows results obtained for DsRed expression from the *BmA*3+E promoter in transfected *Sf*9 and *T.ni* Hi5 cells. The production of DsRed was detected using a anti-DsRed polyclonal antibody (BD Biosciences; molecular weight 25 kDa), no detectable levels of DsRed were observed from mock-transfected *Sf*9 or *T.ni* Hi5 cells (**lanes MT**). DsRed production was not detected in transfected *Sf*9 cells at 24-72 hpt (**lanes 24-72a**), nor in *T.ni* Hi5 cells at 24 hpt (**lane 24b**). At 48-72 hpt, DsRed was detected in *T.ni* Hi5 cells (**lanes 48-72b**). However, the signal intensity produced by Western blot were very weak, indicating that the DsRed levels detected were very low.

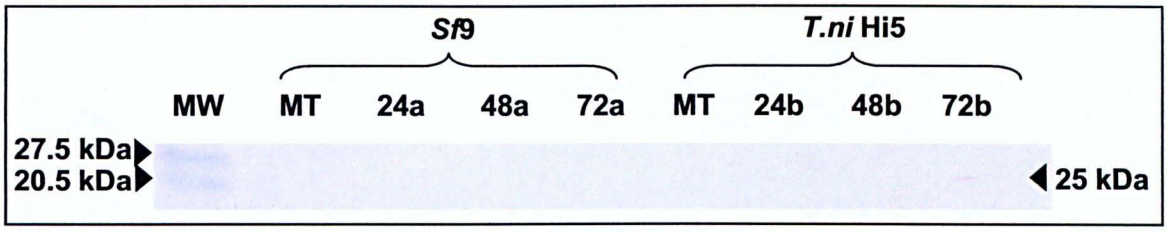


Figure 5.9: Western blot analysis of *Sf*9 and *T.ni* Hi5 cells expressing DsRed. Lane MW (molecular weight), shows protein markers (Invitrogen). Lane MT shows intracellular cell extract (25μg / lane) from mock-transfected *Sf*9 and *T.ni* Hi5 cells (negative control). Lane 24-72**a / b** shows intracellular cell extract (25μg / lane) from *Sf*9 (**a**) and *T.ni* Hi5 (**b**) cells transfected with 1μg of p*BmA*3+E.*DsRed* at 24-72 hpt. Position of molecular weight (MW) size marker is shown in kDa and the position of DsRed is indicated (25kDa).

DsRed expression from the *BmA*3+E promoter in *Sf*9 and *T.ni* Hi5 cells was also monitored by the CLSM (**Figure 5.10A-H**). DsRed-specific fluorescence was not observed in mock-transfected *Sf*9 or *T.ni* Hi5 cells (**panels: A / E**). At 24-72 hpt, the production of DsRed was observed in both *Sf*9 (**panels: B-D**) and *T.ni* Hi5 (**panels: F-H**) cells. At 24 hpt the intensity of fluorescence observed from these cell lines ranged from low to medium (*Sf*9, low; *T.ni* Hi5, low-medium; visual observations), and only a small population of cells were shown to produce detectable levels of DsRed. At 48 hpt, the population of both cell lines expressing DsRed were shown to increase, producing low to high fluorescence levels (*Sf*9, low-medium; *T.ni* Hi5, low-high). At 72 hpt, the population of cells producing DsRed was shown to further increase (visual observation), and fluorescence levels were shown to range from low to high (*Sf*9, low-medium; *T.ni* Hi5, low-high). Overall, fluorescence intensity was higher in *T.ni* Hi5 cell throughout transfection, and a greater population of cells were shown to express detectable levels of DsRed.

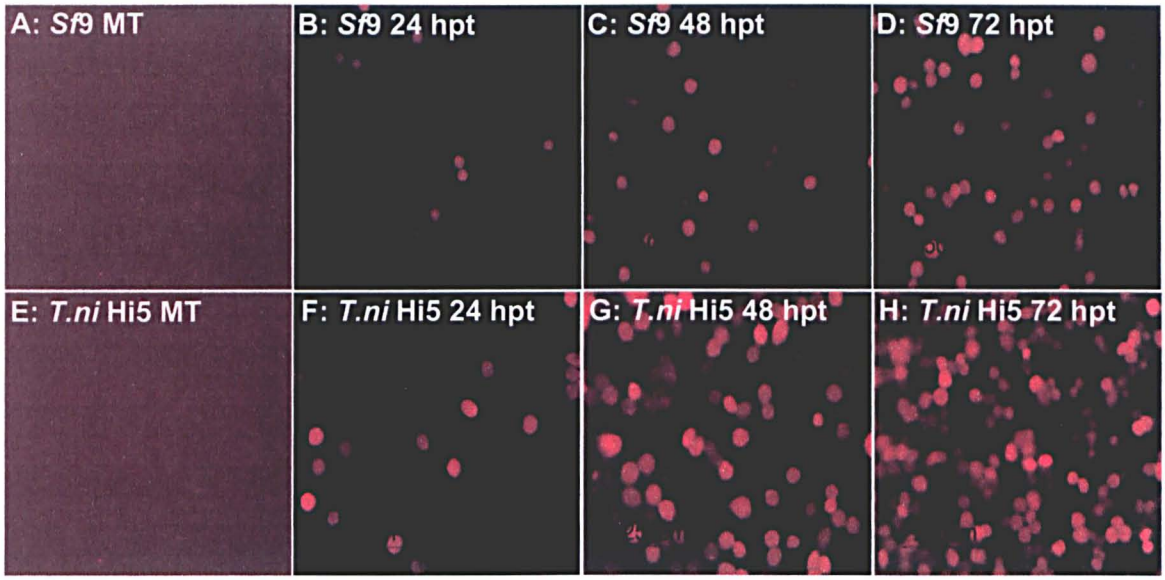


Figure 5.10A-H: Transient DsRed production from the *BmA*3+E promoter 24-72 hpt in *Sf*9 (**panels B-D**) and *T.ni* Hi5 (**panels F-H**) cells. Mock-transfected *Sf*9 (**A**) and *T.ni* Hi5 cells (**E**).

Western blot analyses showed transient expression of DHODH and DsRed from the *BmA*3+E promoter was only detected in *T.ni* Hi5 cells (DHODH, 24-72 hpt; DsRed, 48-72 hpt). However, fluorescence microscopy showed DsRed production was detected in transfected *Sf*9 cells, and also at an earlier time-point for both cell lines (24-72 hpt). Overall, *T.ni* Hi5 cells were shown to produce higher levels of DsRed, compared to those observed in *Sf*9 cells. As the production of DsRed was not observed in *Sf*9 cells using Western blot analyses, but was observed using other means of detection, it may be that DHODH was also produced in *Sf*9 cells. However, the levels produced were to low to detect using Western blot analyses.

5.5 Production of stable insect cell lines expressing DHODH or DsRed

Having constructed and tested p*BmA*3+E.*DHODH* and p*BmA*3+E.*DsRed* in transient expression assays (Section 5.4), the next step was to produce stable insect cell lines expressing these foreign genes. Although the transient results in Section 5.4 show variety, it was decided to make both *Sf*9 and *T.ni* Hi5 cells stably expressing DHODH or DsRed. The generation of these stable insect cell lines will provided the basis for comparing intracellular foreign protein production, against that produced from the BES (*Ac.DHODH* / *Ac.DsRed* and *Ac.*Δ*chiA.DHODH* / *Ac.*Δ*chiA.DsRed*).

Stable *Sf*9 and *T.ni* Hi5 cell lines were produced as described in Section 2.2.5. Cells were co-transfected with p*BmA*3+E.*DHODH* or p*BmA*3+E.*DsRed* (900ng) and a second plasmid (pBmA:neo, 100ng) carrying a dominant selectable marker *neo*^{r} (Section 3.1; Jarvis *et al.*, 1990). Analyses of stable DHODH and DsRed expression was determined by SDS-PAGE (25 mg of protein sample loaded / lane, Section 2.7.4) and Western blot analyses (Sections 2.7.5 / 2.7.6).

Analyses of DsRed production was also determined using quantification of red fluorescence (Section 2.9.1). Shaker cultures containing stably transformed (p*BmA*3+E.*DsRed*) *Sf*9 or *T.ni* Hi5 cells were seeded (20ml at $0.5x10^6$ / ml), and cell samples (1ml) harvested after 0, 12, 24, 36, 48, 60, 72, 84 and 96 h incubation at 28°C, 135rpm. The cells were subsequently analysed using fluorometric analyses (as described in Section 2.9.1). Cell samples were also taken for visualisation of DsRed using a CLSM.

Analyses of DHODH expression from cells of passage numbers 1-18 of transformed *Sf*9 and *T.ni* Hi5 cells by Western blot did not detect production of DHODH (data not shown). To verify *DHODH* had been successfully incorporated into the genomic DNA of *Sf*9 and *T.ni* Hi5 cells, and remained stably integrated, analyses of whole genomic DNA from the transformed cell lines was carried out.

Genomic DNA was extracted from passage 1, 10 and 18 of *Sf*9 and *T.ni* Hi5 cells stably transformed with p*BmA*3+E.*DHODH* (Section 2.5.10). Genomic DNA was also extracted from non-transformed *Sf*9 and *T.ni* Hi5 cells (negative control). To confirm the presence of *DHODH*, primers utilised in Section 5.3 were used to amplify the *DHODH* coding-region from stably transformed *Sf*9 and *T.ni* Hi5 genomic DNA and p*BmA*3+E.*DHODH* (positive control). Amplification of *DHODH* was carried out using PCR (Section 2.5.5). The results shown in **Figure 5.11** clearly show, in lanes 3-5 (stably transformed *Sf*9 cells) and 6-8 (stably transformed *T.ni* Hi5 cells) amplified DNA fragments of a size relative to the *DHODH* coding-region amplified from the positive control (**lane 2; 1210-bp**). Lanes 9 (*Sf*9) and 10 (*T.ni* Hi5) show *DHODH* was not detected from non-transformed *Sf*9 and *T.ni* Hi5 genomic DNA (negative control). These results confirm that *DHODH* was stably integrated into *Sf*9 and *T.ni* Hi5 genomic DNA, but expression of this foreign gene was still elusive.

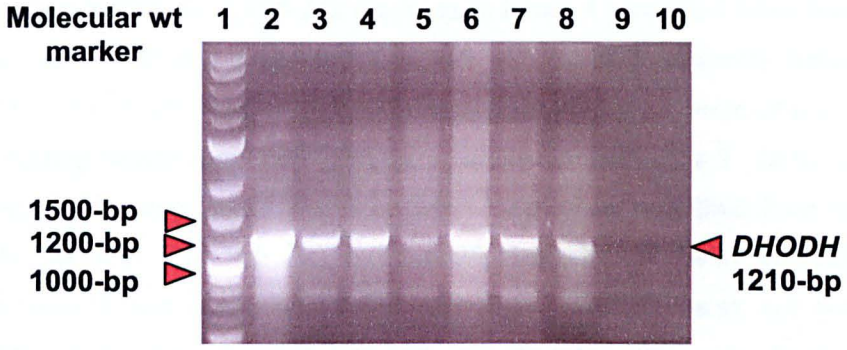


Figure 5.11: Analysis of the amplification of the *DHODH* coding-region from genomic DNA of *Sf*9 and *T.ni* Hi5 cells stably transformed with p*BmA*3+E.*DHODH* (passage 1, 10 and 18), non-transformed *Sf*9 and *T.ni* Hi5 cells and plasmid DNA p*BmA*3+E.*DHODH*, using PCR (Section 2.5.5). Positive amplified *DHODH* coding-region produced a DNA fragment banding at 1210-bp (*DHODH*). The above gels show:

Track 1:	2-Log ladder (New England Biolabs)
Track 2:	Positive amplification of the *DHODH* coding-region (p*BmA*3+E.*DHODH*; positive control)
Track 3-5:	Positive amplification of the *DHODH* coding-region (stably transformed *Sf*9 cells passage 1, 10 and 18)
Track 6-8:	Positive amplification of the *DHODH* coding-region (stably transformed *T.ni* Hi5 cells passage 1, 10 and 18)
Track 9:	Negative control, no amplification of the *DHODH* coding-region (non-transformed *Sf*9 cells)
Track 10:	Negative control, no amplification of the *DHODH* coding-region (non-transformed *T.ni* Hi5 cells)

To confirm DHODH was expressed in stably transformed *Sf*9 and *T.ni* Hi5 cell lines, and had not been integrated into a transcriptionally repressed region of the genome, reverse transcriptase PCR (RT-PCR) was used to detect *DHODH* mRNA transcripts.

Total RNA was extracted (Section 2.5.11) from *Sf*9 and *T.ni* Hi5 cells stably transformed with p*BmA*3+E.*DHODH* (passage 19). Total RNA was also extracted from non-transformed *Sf*9 and *T.ni* Hi5 cells (negative control), and *Sf*9 and *T.ni* Hi5 cells transfected with p*BmA*3+E.*DHODH* 48hpt (positive control). The RNA extracted from the samples specified above was subsequently used to generate cDNA as described in Section 2.5.6. To detect the presence of *DHODH* from the cDNA, cDNA was analysed using PCR. Primer used in previous experiments specific for amplification of *DHODH* were used to amplify the *DHODH* coding-region from cDNA and confirm DHODH was produced in stably transformed *Sf*9 and *T.ni* Hi5 cells.

The results shown in **Figure 5.12A / B** illustrate in lanes 4 amplified DNA fragments from total genomic cDNA of *Sf*9 (**A**) and *T.ni* Hi5 (**B**) cells, transiently transfected with p*BmA*3+E.*DHODH* (48hpt). The amplified DNA shown in lanes 4 were of a size relative to the *DHODH* coding-region amplified from the positive control (**lane 2;** 1210-bp). Lanes 5 and 6 also show the same identically sized DNA fragments, amplified from total genomic cDNA of *Sf*9 (**A**) and *T.ni* Hi5 (**B**) cells stably transformed with p*BmA*3+E.*DHODH* (passage 19; lane 5 and 6 are replicated samples). *DHODH* was not amplified from genomic cDNA of non-transformed *Sf*9 (**A**) and *T.ni* Hi5 (**B**) cells (**lane 3**, negative control). These results confirm that *DHODH* was stably expressed from the *BmA*3+E promoter in transformed *Sf*9 and *T.ni* Hi5 cells.

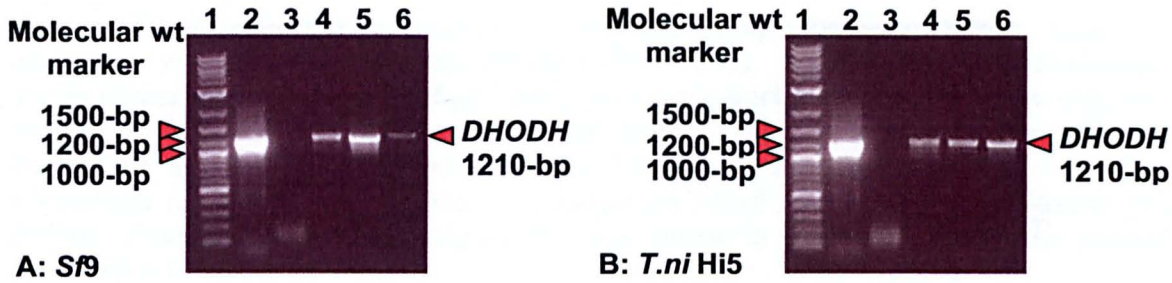


Figure 5.12A / B: Analysis of the amplification of the *DHODH* coding-region from cDNA produced by RT-PCR from total RNA extracted from *Sf*9 and *T.ni* Hi5 cells stably transformed with p*BmA*3+E.*DHODH* (passage 19), p*BmA*3+E.*DHODH* transfected *Sf*9 and *T.ni* Hi5 cells 48 hpt and non-transformed *Sf*9 and *T.ni* Hi5 cells, using PCR (Section 2.5.5). p*BmA*3+E.*DHODH* was used to amplify the *DHODH* coding-region as a positive control. Positive amplified *DHODH* coding-region produced a DNA fragment banding at 1210-bp (*DHODH*). The above gels show:

Track 1:	2-Log ladder (New England Biolabs)
Track 2:	Positive amplification of the *DHODH* coding-region (p*BmA*3+E.*DHODH*; positive control)
Track 3:	Negative control, no amplification of the *DHODH* coding-region (non-transformed *Sf*9 (**A**) *T.ni* Hi5 (**B**) cells)
Track 4:	Positive amplification of the *DHODH* coding-region (p*BmA*3+E.*DHODH*, 48 hpt in *Sf*9 (**A**) *T.ni* Hi5 (**B**) cells)
Track 5-6:	Positive amplification of the *DHODH* coding-region (p*BmA*3+E.*DHODH* stably transformed *Sf*9 (**A**) *T.ni* Hi5 (**B**) cells passage 19)

Expression of DsRed was detected by Western blot analyses in *T.ni* Hi5 cells stably transformed with p*BmA*3+E.*DsRed* (**Figure 5.13**), but not *Sf*9 cells.

The stable expression of DsRed from *T.ni* Hi5 cells, passages 1-8 are shown in **Figure 5.13**. The production of DsRed was not observed in mock-transfected *T.ni* Hi5 cells (**lane MT**). Transient expression of DsRed from the *BmA*3+E promoter in *T.ni* Hi5 cells was

detected at 72 hpt (**lane +ve**; positive control). Stable production of DsRed from passages 1-8 was also observed (**lanes 1-8**). The levels of DsRed produced from passage 1-8 were considered low, as the signal intensity produce by Western blot was very weak. No apparent difference among the levels of DsRed produced between passages were evident from these results.

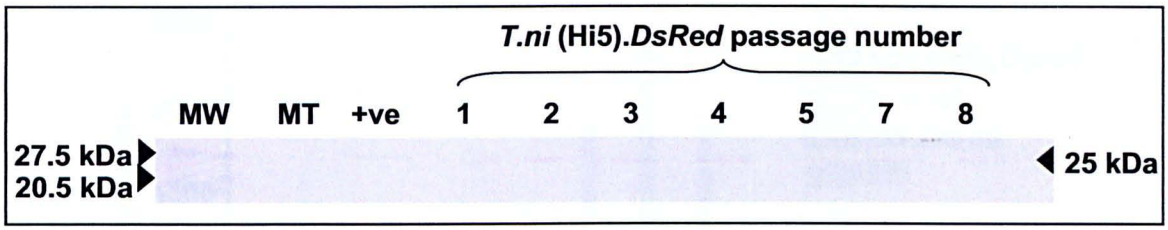


Figure 5.13: Western blot analysis of *T.ni* Hi5 cells stably expressing DsRed. Lane MW (molecular weight), shows protein markers (Invitrogen). Lane MT (mock-transfected), shows intracellular cell extract (25μg / lane) from mock-transfected *T.ni* Hi5 cells (negative control). Lane +ve shows intracellular cell extract (25μg / lane) from *T.ni* Hi5 cells transfected with 1μg of p*BmA*3+E.*DsRed* at 72 hpt (positive control). Lane 1-8 shows intracellular cell extract (25μg / lane) from passages 1-8 of *T.ni* Hi5 cells stably expressing DsRed. Position of molecular weight (MW) size marker is shown in kDa and the position of DsRed is indicated (25kDa).

The stable production of *DsRed* from the *BmA*3+E promoter in transformed *Sf*9 and *T.ni* Hi5 cells was further analysed using fluorometric analyses (**Figure 5.14**) and fluorescence microscopy (**Figure 5.15A-D**). Subsequently these cell lines were named *Sf*9.*DsRed* and *T.ni* (Hi5).*DsRed*.

Figure 5.14 shows results obtained from passage 9 of *Sf*9.*DsRed* and *T.ni* (Hi5).*DsRed*. No detectable levels of background fluorescence were observed from non-transformed *Sf*9 or *T.ni* Hi5 cells (negative control). Fluorescence was detected from both *Sf*9.*DsRed* and *T.ni* (Hi5).*DsRed*, which confirmed production of DsRed in *T.ni* (Hi5).*DsRed* (these findings correlate with those observations in **Figure 5.13**), and also established that DsRed was produced in *Sf*9.*DsRed* albeit at a level that was not possible to detect by Western blot analyses. Even though DsRed was shown to be stably expressed in *Sf*9.*DsRed* (**Figure 5.14**), the levels produced were too low to detect at 0-12 h (0.5-0.75x10^6 cell / ml). However, with increased cell density DsRed was detected from *Sf*9.*DsRed* at 24-96 h, whilst in *T.ni* (Hi5).*DsRed*, the levels of DsRed produced were sufficient to be detected at 0-96 h. Comparison of DsRed production from these cell lines showed that *T.ni* (Hi5).*DsRed* produced significantly higher levels of DsRed than those

observed from *Sf*9.*DsRed* (p=0.0001, in all cases), at all time-points. A decrease in DsRed production was observed from *T.ni* (Hi5).*DsRed* at 96 h, these finding were shown to correlate with *T.ni* (Hi5).*DsRed* cell death, as at 96 h *T.ni* (Hi5).*DsRed* cells enter the decline phase of growth (data not shown; this finding correlates with those observed in Section 5.6, **Figure 5.17 / 5.19**).

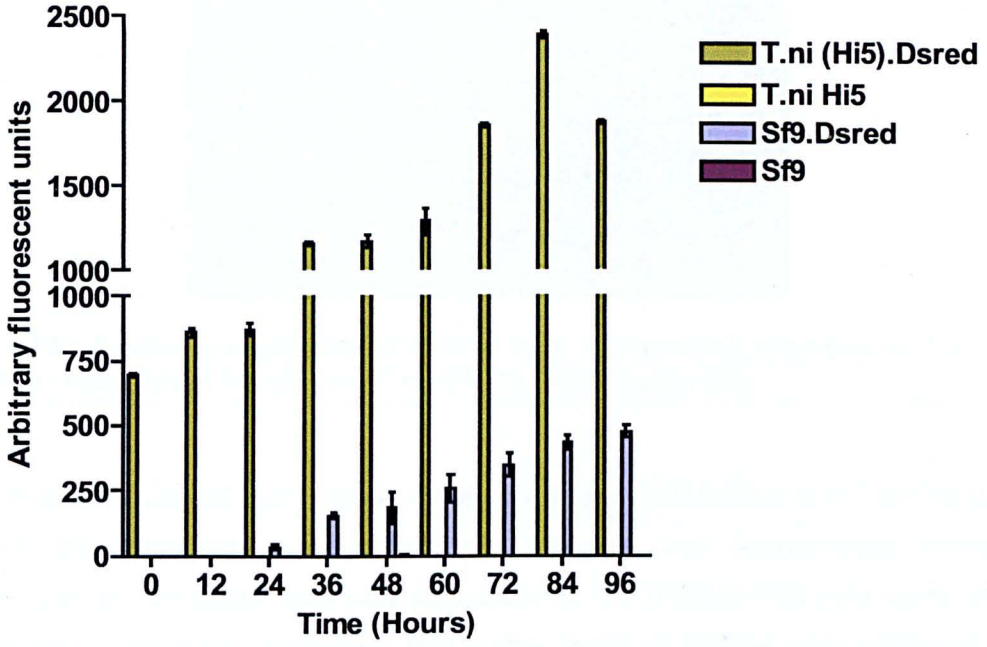


Figure 5.14: Red fluorescence detected from the production of DsRed in *Sf*9.*DsRed* and *T.ni* (Hi5).*DsRed* cell lines. Cell samples (1ml) were harvested at 12 h intervals over a period of 96 h, these sample were subjected to fluorometric analyses to determine the levels of DsRed produced.

The results shown in **Figure 5.15A-D**, show visual evidence of DsRed production in *T.ni* (Hi5).*DsRed* (**B**) and *Sf*9.*DsRed* (**D**) cell lines. Expression of DsRed was not detected in non-transformed *Sf*9 (**C**) or *T.ni* Hi5 (**A**) cells.

These data show that the production of DHODH was difficult to express under control of the *BmA*3+E promoter in stably transformed *Sf*9 and *T.ni* Hi5 cell lines. As expression of DHODH was only detectable by analyses of mRNA transcripts it was concluded that the levels of DHODH produced in these stable insect cell lines was too low for further comparisons to be made between stable cell lines and production using the BES.

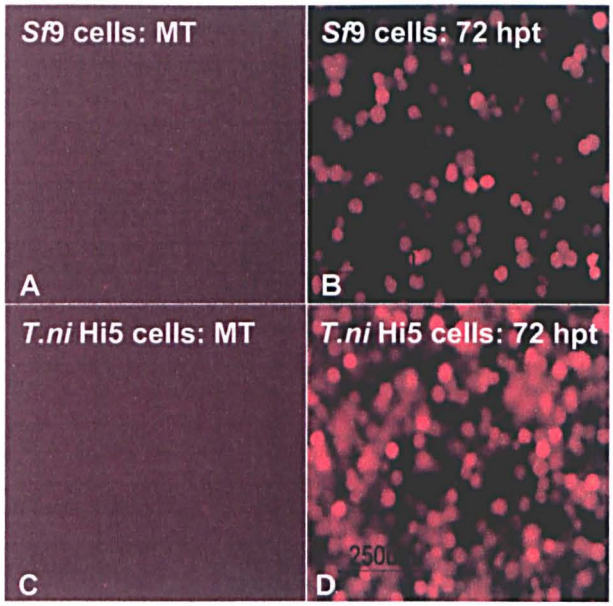


Figure 5.15A-D: Stable production of DsRed from the *BmA*3+E promoter in *T.ni* Hi5 (**B**) and *Sf*9 (**D**) cells. Mock-transfected *T.ni* Hi5 (**A**) and *Sf*9 cells (**C**).

The production of DsRed from the *BmA*3+E promoter in *Sf*9.*DsRed* and *T.ni* (Hi5).*DsRed* cell lines was detected using fluorometric analyses and fluorescence microscopy. However, DsRed production was only detectable in *T.ni* (Hi5).*DsRed* cells using Western blot analyses. Therefore concluding, that higher levels of DsRed were produced in *T.ni* (Hi5).*DsRed* than those observed from *Sf*9.*DsRed* (these results correlate with fluorometric analyses, (**Figure 5.14**). The fluorometric data obtained for maximal DsRed production from *Sf*9.*DsRed* (96 h) and *T.ni* (Hi5).*DsRed* (84 h) cell lines (**Figure 5.14**), was subsequently compared to *Ac.DsRed* (moi 2) in *Sf*9 cells (Section 5.2; **Figure 5.3 A**), which was shown to be the highest expressing DsRed baculovirus. These results showed that optimal DsRed production from *Ac.DsRed* (moi 2) in *Sf*9 cells (72 hpi) was 52.5-fold greater than those detected from *T.ni* (Hi5).*DsRed* cells, and 262.9-fold higher than those observed from *Sf*9.*DsRed* cells. From these data it was concluded that DsRed production from *Sf*9.*DsRed* and *T.ni* (Hi5).*DsRed* cell lines was not comparable to those observed from baculovirus expression.

However, the *T.ni* (Hi5).*DsRed* cell line was subsequently used at Pfizer to generate a protocol for continuous stable insect cell line cultivation in 1-litre fermenters (Section 5.6).

5.6 Scale-up of stable insect cell cultivation using a 1-litre open fermenter system

Attempts were made to establish a protocol for scaling-up stable insect cell cultivation, using a 3-litre open fermenter system. Development of a standardised procedure would provide useful data for future projects involving necessary scale-up of stable insect cell cultures for maximizing foreign protein yield.

An open culturing system allows both the removal and addition of cells and medium to a vessel once set-up and running. The aims of this experiment were to inoculate a 3-litre fermenter with 1-litre of low density stable insect cells and continuously cultivate a viable culture, without contamination or total cell death occurring.

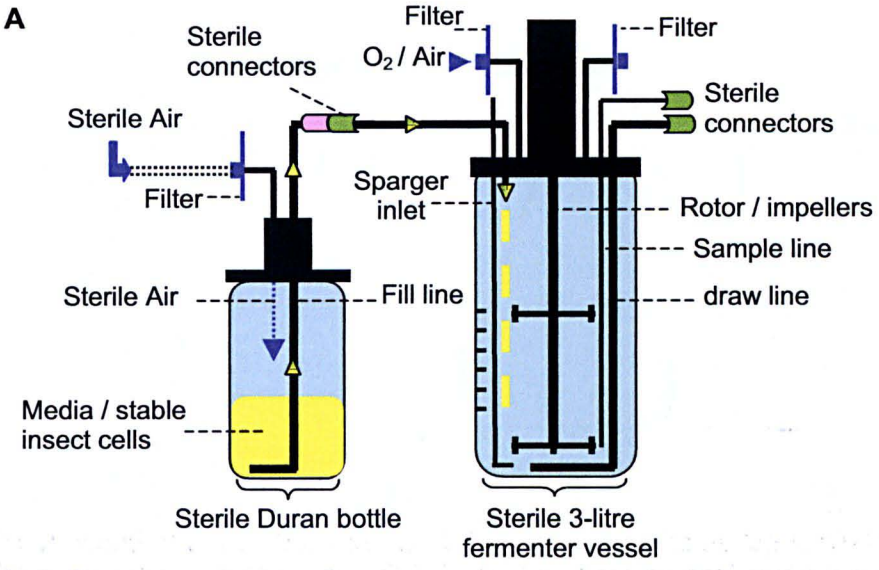
The *T.ni* (Hi5).*DsRed* cell line generated in Section 5.5 was chosen as a model to simulate culture dynamics throughout the culturing process. A technique described as the fill and draw method (**Figure 5.16A-C**), which is similar to the fed-batch method, was used to inoculate cells into the fermenter vessel and passage them during fermentation, enabling continuous culturing to be achieved. The cell culture was analysed for cell growth, viability and stability of foreign gene expression.

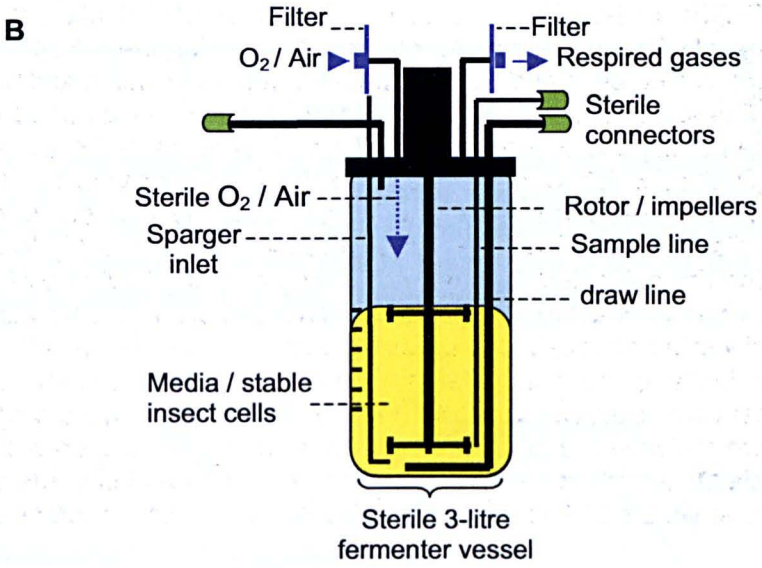
A 1-litre culture of *T.ni* (Hi5).*DsRed* cells was seeded at a density of $0.5x10^6$ cells / ml in a 1.5-litre sterile Duran bottle with vessel connectors, and used to inoculate a 3-litre fermenter vessel as described in Section 2.3.6.2 (**Figure 5.16A**). A 1ml cell sample was drawn-off and re-counted to determine the accuracy of the inoculation process, this count was termed day 1. Cell samples (1ml) were harvested at intervals of 24 h for analyses of cell growth and viability. Cell viability was determined using trypan blue staining (Section 2.2.6). Confirmation of stable DsRed production was monitored using a fluorescence microscope, but no camera facility was available (Pfizer) to record data. However, DsRed production was visualised from all cell samples taken over the period of culturing.

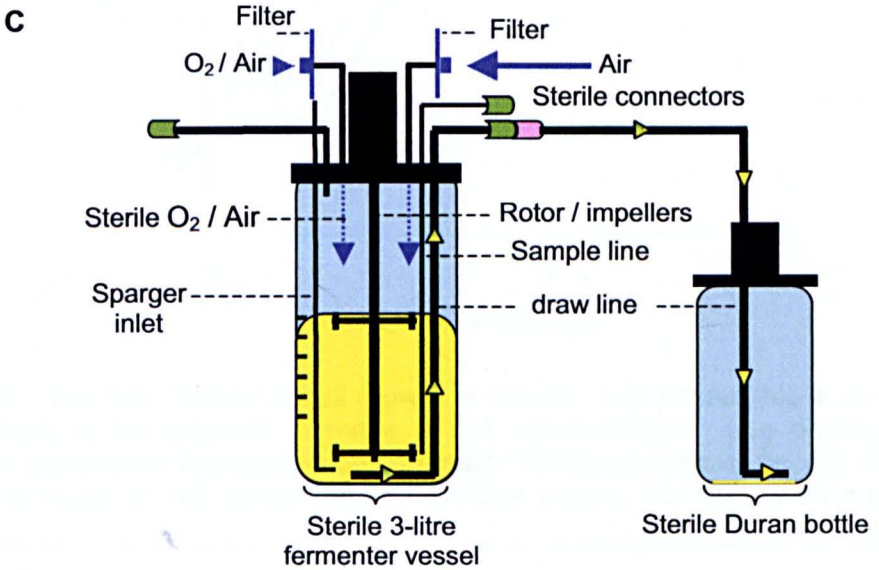
The results shown in **Figure 5.17** show *T.ni* (Hi5).*DsRed* cell growth, from a 1-litre open fermenter system, after two passages. Day 1, the fermenter vessel was inoculated with 1-litre of *T.ni* (Hi5).*DsRed* cells at a density of $0.5x10^6$ cells / ml. Day 2, *T.ni* (Hi5).*DsRed* cell density was shown to increase to $0.8x10^6$ cells / ml. Day 3, the cell density of the culture was shown to have doubled ($1.6x10^6$ cells / ml), compared to those observed on

day 2. Day 4, cell density was shown to have increased to $2.8x10^6$ cells / ml. However on day 5, the cell density was shown to decrease, and was observed at $2.5x10^6$ cells / ml. It was suggested, that cell growth had shifted from log-phase to decline phase (**Figure 5.18**), therefore the cells were passaged to continue growth. The fill and draw technique was used to passage these cells and re-establish a 1-litre *T.ni* (Hi5).*DsRed* cell culture at a density of $0.5x10^6$ cells / ml. Continued monitoring of *T.ni* (Hi5).*DsRed* cell growth from days 5-8, showed a similar pattern of growth to the initial observations made from days 1-4. On day 8 the cell density of the culture was observed at $2.4x10^6$ cells / ml, so a second passage was performed. The *T.ni* (Hi5).*DsRed* cell culture was again re-established as a 1-litre culture at $0.5x10^6$ cells / ml. Further monitoring of cell growth from day 8-11 showed no significant difference ($p>0.05$ in all cases) in cell density compared to those observed from days 1-4 and 5-8.

Figure 5.16A-C: A schematic of the fill and draw technique used to continuously cultivate stable insect cells in a fermenter vessel. **A:** A 3-litre fermenter vessel was used to culture 1-litre of *T.ni* (Hi5).*DsRed* cells. The vessel was assembled and sterilized (Section 2.3.6.2). Filling the vessel with the cell culture, required connecting the sterile Duran bottle pre-filled with *T.ni* (Hi5).*DsRed* cells and Sf900II medium at the correct inoculating density ($0.5x10^6$ cells / ml). The sterile Duran bottles have one inlet, which was used to pass air through a filter into the headspace of the Duran bottle and force the cell culture out of the Duran bottle via the fill line, which was connected to the fermenter vessel. The culture enters the fermenter via the vessel head-unit and inoculates the fermenter. Subsequently the Duran bottle was disconnected from the vessel and the vessel is ready to culture the cells (**B**). **C:** Passaging the cell culture required removing a defined volume of cells (1ml) and medium from the vessel via the sample line. The sample line was also used to sample the culture daily to monitor cell growth and viability. Passaging of cells required a second sterile Duran bottle to be connected to the draw line of the vessel. Air was pumped into the vessel via a filter, which allows release of respired gases during culturing. This caused the culture to leave the vessel under-pressure through the draw line and be collected into the sterile Duran bottle. A defined volume of cells was left in the vessel and fresh medium was added via the fill line (**A**) to dilute the culture for continuous culturing.


A
Filter
Sterile connectors
O_2 / Air
Filter
Sterile connectors
Sterile Air
Filter
Sparger inlet
Rotor / impellers
Sample line
Sterile Air
Fill line
draw line
Media / stable insect cells
Sterile Duran bottle
Sterile 3-litre fermenter vessel


B
Filter
Filter
O_2 / Air
Respired gases
Sterile connectors
Sterile O_2 / Air
Rotor / impellers
Sparger inlet
Sample line
draw line
Media / stable insect cells
Sterile 3-litre fermenter vessel


C
Filter
Filter
O_2 / Air
Air
Sterile connectors
Sterile O_2 / Air
Rotor / impellers
Sample line
Sparger inlet
draw line
Sterile 3-litre fermenter vessel
Sterile Duran bottle

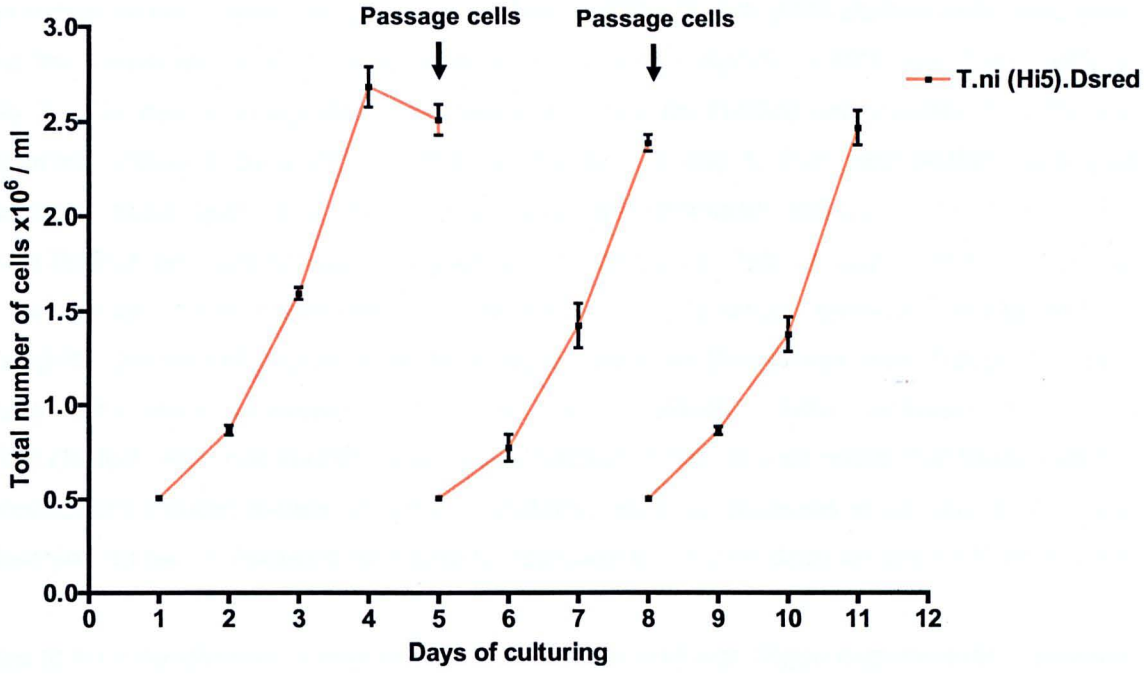


Figure 5.17: Total number of *T.ni* (Hi5).*DsRed* cells / ml, following inoculation of an open fermenter system. A 3-litre open fermenter system was inoculated with 1-litre of *T.ni* (Hi5).*DsRed* cells (day 1). Cell samples (1ml) were taken daily over a period of 11 day, which included passaging the cell culture twice (days 5 and 8), the total number of cells were counted to determine *T.ni* (Hi5).*DsRed* cell growth.

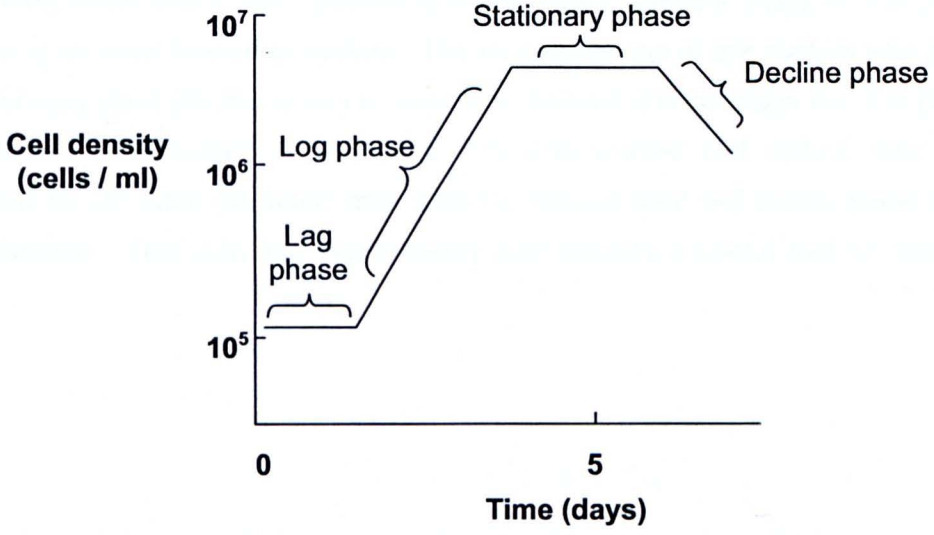


Figure 5.18: The four phases of cell growth in culture. Lag phase; this is an early phase in which there is no apparent increase in cell concentration. Log phase; this phase involves an exponential increase in cell number. Stationary phase, occurs when there is no further increase in cell concentration. Decline phase; this occurs as a result of cell death.

Figure 5.19 shows results obtained for *T.ni* (Hi5).*DsRed* cell viability, from a 1-litre open fermenter system, after two passages. The viability of *T.ni* (Hi5).*DsRed* cells inoculated into the fermenter on day 1 was 90%, which dropped slightly to 89% day 2 and 88% on day 3. On day 4, a significant decrease in *T.ni* (Hi5).*DsRed* cell viability, to 74% was observed, followed by a drop to 51% on day 5. On day 5, *T.ni* (Hi5).*DsRed* cells were therefore passaged, and the cell viability re-determined (53%). On day 6, *T.ni* (Hi5).*DsRed* cell viability had increased to 71% and on to 76% on day 7. However on day 8, the viability of the culture decreased again to 70%. As shown previously in **Figure 5.17**, *T.ni* (Hi5).*DsRed* cell growth shifts from log phase to decline phase after 4 days, therefore these cells were passaged to continue culture growth. After passaging these *T.ni* (Hi5).*DsRed* cells, cell viability was re-established, 71%. It was noted that these cell had establishing a cyclic pattern of growth / viability, again an increase in viability, to 77% was observed on day 9, followed by a steady decrease to 72% on days 10 and 67% on day 11.

Due to time constraints, it was not possible to proceed with these experiments. However, Colin Robinson and Chris Carter carried-out a further four successful passages before terminating these studies. Similar results were obtained to that described above, but it was unfortunate that samples could not be taken 24 h intervals, therefore these results were not included.

These data show that it was possible to continuously cultivate 1-litre of *T.ni* (Hi5).*DsRed* cells using an open fermenter system. The successful use of this system was attributed to the technique used (fill and draw) to inoculate, harvest and passage the *T.ni* (Hi5).*DsRed* cells from the fermenter. The 1-litre *T.ni* (Hi5).*DsRed* cell culture was successful sustained for 27 days (selected data shown), without total cell death, yeast or bacterial contamination. This culturing methodology may provide a useful tool for future work at Pfizer.

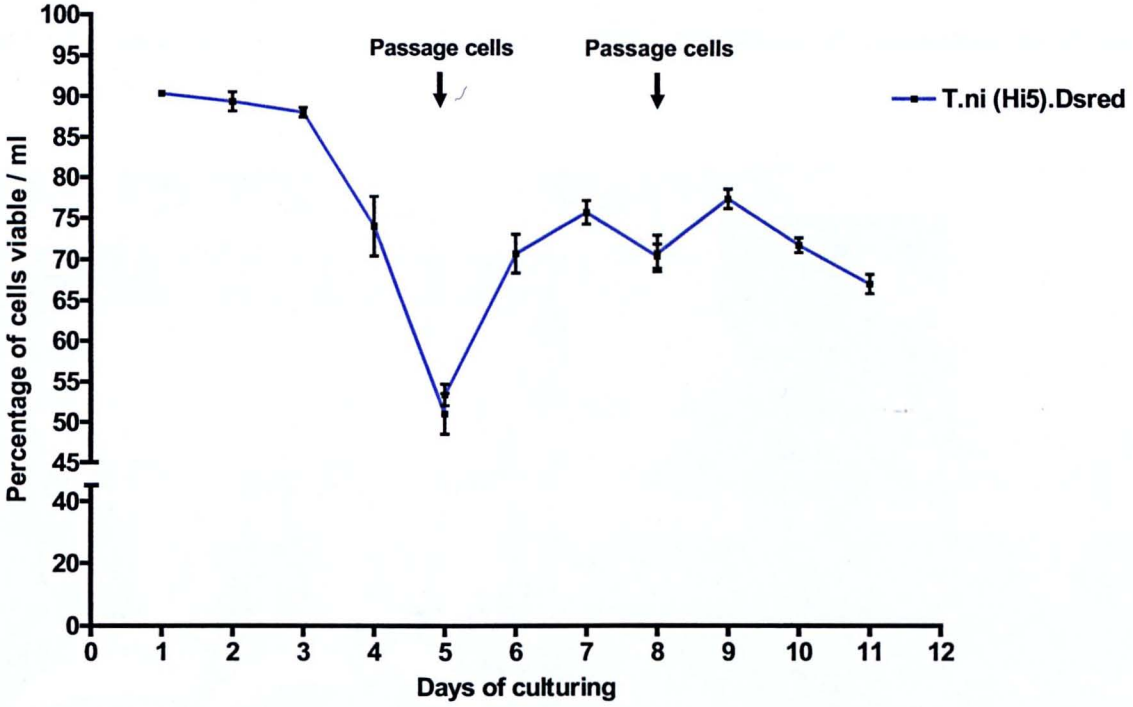


Figure 5.19: Viability of *T.ni* (Hi5).*DsRed* cells, cultured using an open fermenter system. A 3-litre open fermenter system was set-up and inoculated with 1-litre of *T.ni* (Hi5).*DsRed* cells (day 1). Cell samples (1ml) were taken daily over a period of 11 day, which included passaging the *T.ni* (Hi5).*DsRed* cell culture twice (days 5 and 8). Samples were subjected to trypan blue staining to determine *T.ni* (Hi5).*DsRed* cell viability.

5.7 Comparison of recombinant protein production from *Ac* and *Ac.ΔchiA* using different scale-up culturing methodologies

The successful production of high-level recombinant protein (mg / litre) from the BES has been well documented over the years (King *et al.*, 1992; Schwartz *et al.*, 1997; Stewart and Champoux, 1999; Jayakumar *et al.*, 2004). However, to achieve these quantities, protein production often requires scaling-up. Two successful methodologies currently used for scaling-up protein production include; shaker culture and fermentation, as described in Section 5.1 (**Figure 5.20A / B**). These culturing methodologies are extremely different to one another; shaker cultures are simplistic and easy to set-up, and relatively inexpensive (£54, 3-litre disposable flask) to use, compared with the very technical, expensive (£15000, 3-litre complete set-up) and laborious, fermenter cultures. One of the key differences of fermenter and shaker culturing, focuses on the regulation of dissolved oxygen concentration in the culture. The dissolved oxygen concentration can be regulated using fermenter culturing, whilst this is not possible for shaker culturing. In this Section,

attempts were made to determine whether these different scale-up culturing methodologies (shaker vs. fermenter), could affect the yields of baculovirus expressed recombinant protein.

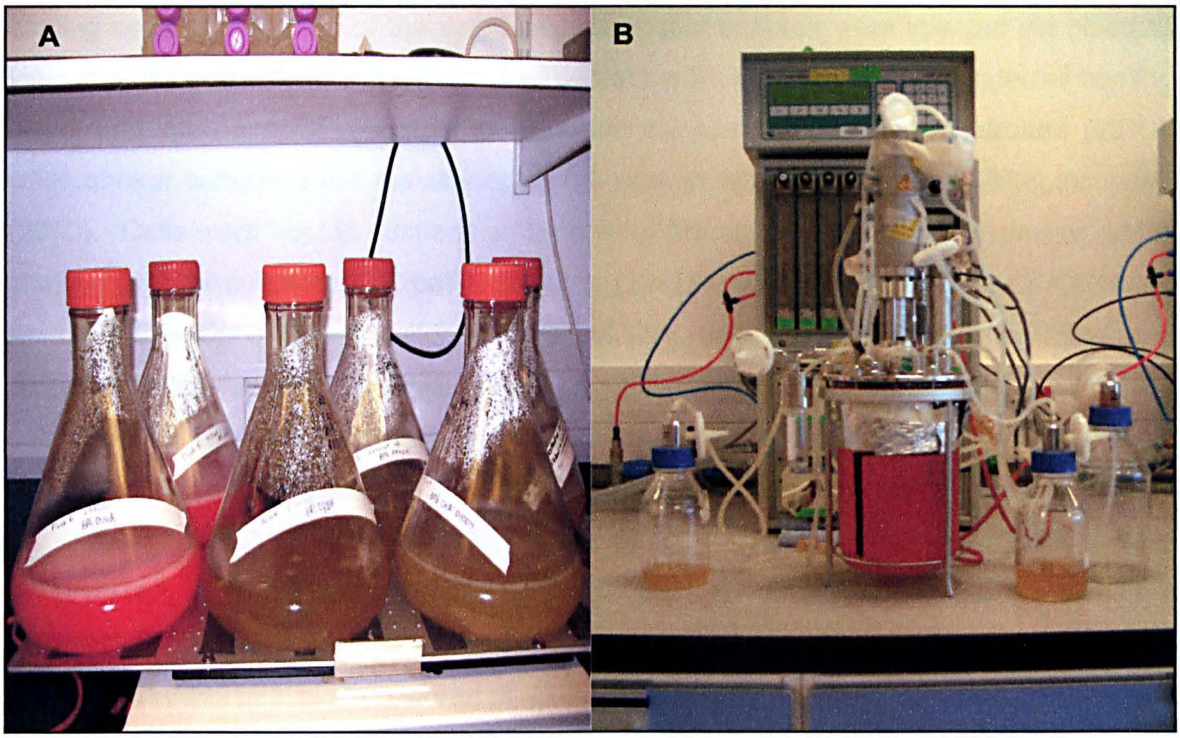


Figure 5.20A / B: Two different culturing methods used to scale-up protein production using the BES. **A:** Shaker culture. **B:** Fermenter culture (Applikon®).

To investigate the affects described above, the recombinant baculoviruses generated in Section 5.2 (*Ac.DsRed*, *Ac.DHODH*, *Ac.ΔchiA.DsRed* and *Ac.ΔchiA.DHODH*) were used to express intracellular foreign protein (DHODH or DsRed) in 1-litre *Sf*9 and *T.ni* Hi5 shaker and fermenter cultures.

One-litre *Sf*9 and *T.ni* Hi5 cell cultures were seeded into 1-litre sterile Duran bottles with vessel connectors, or directly into 3-litre shaker flasks (as described in Sections 2.3.6.1 and 2.3.6.2). Small-scale (25ml) *Sf*9 and *T.ni* Hi5 shaker cultures were also set-up (Section 2.2.3) as mock-infected controls (negative control). The pre-filled 1-litre sterile Duran bottles were subsequently used to inoculate 3-litre fermenter vessels (as described in Section 2.3.6.2).

Cells cultured in fermenter vessels were grown to the required density, then infected with the appropriate recombinant baculovirus at a specified moi (as determined in Section 5.2; *Ac.DsRed* / *Ac.ΔchiA.DsRed*, moi 2, *Ac.DHODH* / *Ac.ΔchiA.DHODH*, moi 5). Fermenter cultures were infected with virus inoculum via a sterile syringe and needle through a self-sealing septum at the top of the vessel, whilst shaker cultures were infected via pipetting virus inoculum directly into the flask. Fermenters were equipped with an external heating jacket and internal cooling system to regulated the correct culturing temperature (28°C), while shaker cultures were maintained at the correct temperature in an orbital incubator (28°C). Cells were kept in suspension by stirring (fermenter cultures; 120 rpm) or orbital shaking (shaker cultures; 135 rpm). Samples (1ml) were harvested from *Ac.DHODH* and *Ac.ΔchiA.DHODH* infected cultures at 24-48 hpi, and 24-72hpi from *Ac.DsRed* and *Ac.ΔchiA.DsRed* infected cultures. The intracellular supernatant of each cell sample was subsequently subjected to protein estimation assays. Standard amounts of total protein (25μg) from each sample were run on SDS-PAGE for Western blot analyses (Sections 2.7.4, 2.7.5, 2.7.6) to determine the levels of DsRed and DHODH produced (visual observation). The remaining sample volume of each infection (*Ac.DHODH* / *Ac.ΔchiA.DHODH*, 48 hpi and Ac.DsRed / *Ac.ΔchiA.DsRed*, 72 hpi) was harvested and pelleted. Dry cell pellets were stored at -80°C to await further analyses.

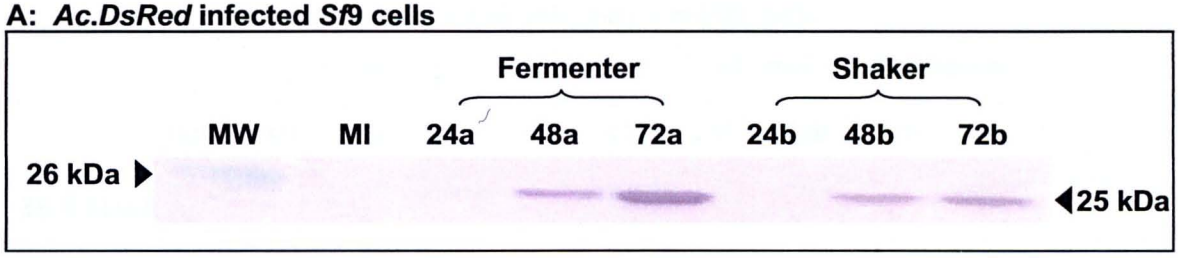
The results shown in **Figure 5.21A-D** show DsRed expression from *Ac.DsRed* and *Ac.ΔchiA.DsRed* in 1-litre *Sf*9 and *T.ni* Hi5 fermenter and shaker cultures. The detection of DsRed was analysed using a DsRed polyclonal antibody (BD Biosciences; molecular weight 25 kDa). No detectable levels of DsRed were observed in mock-infected *Sf*9 or *T.ni* Hi5 cells (**A-D: lane MI**). *Sf*9 and *T.ni* Hi5 fermenter / shake cultures, infected with *Ac.DsRed* or *Ac.ΔchiA.DsRed*, were all shown to produce detectable levels of DsRed at 48-72 hpi (**A-D: lane 48-72 a, b**). However, at 72 hpi, DsRed production was shown to be higher than those observed at 48 hpi. *Sf*9 fermenter cultures infected with *Ac.DsRed* or *Ac.ΔchiA.DsRed*, were also shown to produce higher levels of DsRed than those observed from the shaker cultures (**A / B: lanes 48-72 a, b**). However, this was not the case for *T.ni* Hi5 cells, as the shaker cultures produce higher levels of DsRed than those observed from the fermenter cultures (**C / D: lanes 48-72 a, b**). A comparison of DsRed production from *Ac.DsRed* infected *Sf*9 and *T.ni* Hi5 fermenter / shaker cultures, showed production of DsRed was higher in both *Sf*9 cultures (**A / C: lanes 48-72 a, b**). Similar observations were also shown for the comparison of DsRed production from *Ac.ΔchiA.DsRed* infected *Sf*9 and *T.ni*Hi5 fermenter / shaker cultures (**B / D: lanes 48-72 a, b**). A comparison of

DsRed expression from *Ac.DsRed* and *Ac.ΔchiA.DsRed* in *Sf*9 fermenter / shaker cultures, showed *Ac.DsRed* produced higher levels of DsRed in both cultures, than those detected from *Ac.ΔchiA.DsRed* (**A / B: lanes 48-72 a, b**). Similar DsRed expression profiles were also observed from *Ac.DsRed* and *Ac.ΔchiA.DsRed* in *T.ni* Hi5 fermenter / shaker cultures (**C / D: lanes 48-72 a, b**), however, at much lower levels than those observed in *Sf*9 cultures.

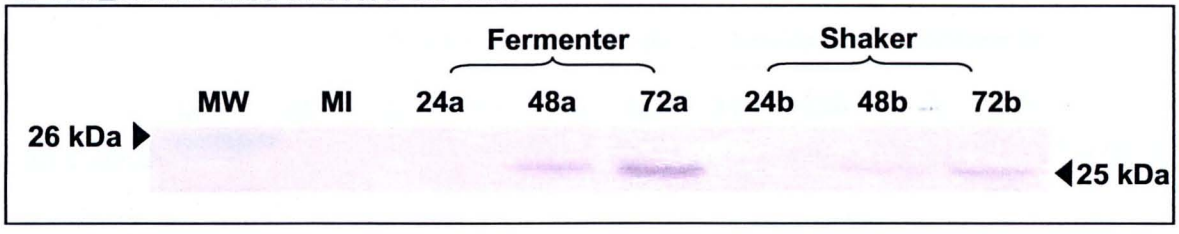
Figure 5.22 A / B shows results obtained for DHODH expression from *Ac.DHODH* and *Ac.ΔchiA.DHODH* in 1-litre *T.ni* Hi5 (**A**) and *Sf*9 (**B**) fermenter / shaker cultures. The production of DHODH was detected using an anti-polyhistidine monoclonal antibody (as described in Section 5.2). No detectable levels of DHODH were observed in mock-infected *T.ni* Hi5 or *Sf*9 cells (negative control; **A / B: lane MI**). Production of DHODH from *Ac.ΔchiA.DHODH* infected *T.ni* Hi5 fermenter / shaker cultures was detected at 24-48 hpi (**A: 24-48a**), whilst from *Ac.DHODH*, DHODH was only detected at 48 hpi (**A: 48b**). Optimal DHODH production from all *T.ni* Hi5 infected cultures was observed at 48 hpi (**A: 48a, b**). Expression of DHODH from *Ac.DHODH* and *Ac.ΔchiA.DHODH* infected *Sf*9 fermenter / shaker cultures, was only detected at 48 hpi (**B: 24-48a, b**). The levels observed were also considerably lower (at all time points), than those observed from infected (*Ac.DHODH* or *Ac.ΔchiA.DHODH*) *T.ni* Hi5 cultures (these findings support previous results described in Section 5.2).

A comparison of DHODH production from *Ac.ΔchiA.DHODH* infected *T.ni* Hi5 cultures at 24-48 hpi, showed the fermenter culture produced higher levels of DHODH than those observed from shaker culture, at the same time points. Similar results were also observed from *Ac.DHODH* infected *T.ni* Hi5 cultures (48 hpi), and *Ac.ΔchiA.DHODH / Ac.DHODH* infected *Sf*9 cultures (48 hpi). Furthermore, a comparison of *Ac.ΔchiA.DHODH* infected *T.ni* Hi5 (at 24-48 hpi) and *Sf*9 (at 48 hpi) fermenter / shaker cultures, showed levels of DHODH produced were higher than those observed from *Ac.DHODH* at the same time points.

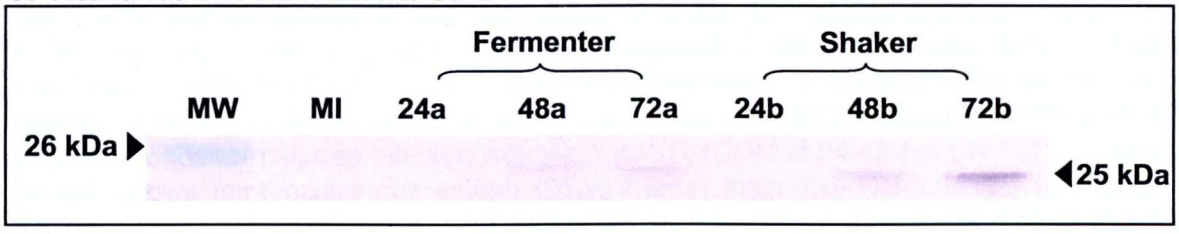
A: *Ac.DsRed* infected *Sf*9 cells



B: *Ac.ΔchiA.DsRed* infected *Sf*9 cells



C: *Ac.DsRed* infected *T.ni* Hi5 cells



D: *Ac.ΔchiA.DsRed* infected *T.ni* Hi5 cells

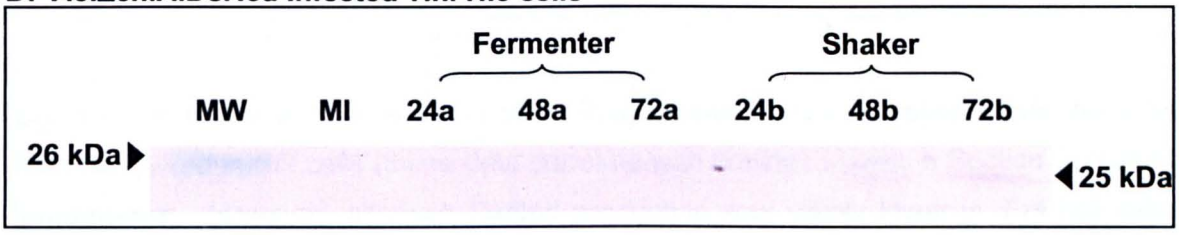
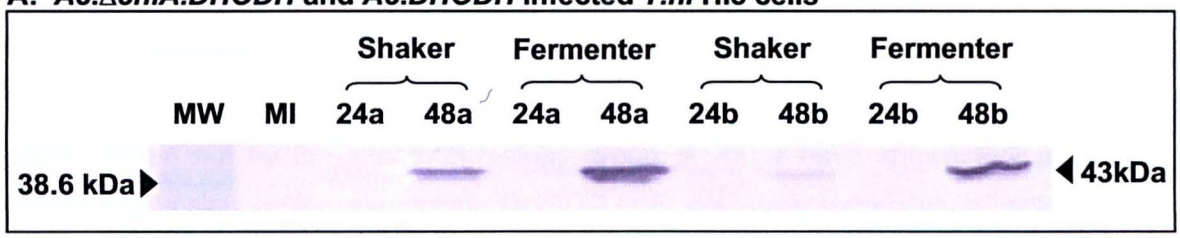


Figure 5.21A-D: Western blot analysis of *Ac.DsRed* (**panel A / C**) and *Ac.ΔchiA.DsRed* (**panel B / D**) expressing DsRed in *Sf*9 (**panel A-B**) and *T.ni* Hi5 (**panel C-D**) fermenter and shaker cultures. Lanes MW (molecular weight), shows protein markers (Invitrogen). Lanes MI (mock-infected), shows intracellular cell extract (25μg / lane) from mock-infected *Sf*9 and *T.ni* Hi5 cells (negative control). Lanes 24-72a shows intracellular cell extract (25μg / lane) from *Sf*9 and *T.ni* Hi5 fermenter cultures infected with *Ac.DsRed* or *Ac.ΔchiA.DsRed* at 24-72 hpi (moi of 2). Lane 24-72b shows intracellular cell extract (25μg / lane) from *Sf*9 and *T.ni* Hi5 shaker cultures infected with *Ac.DsRed* or *Ac.ΔchiA.DsRed* at 24-72 hpi (moi of 2). Position of molecular weight (MW) size marker is shown in kDa and the position of DsRed is indicated (25kDa).

A: *Ac.ΔchiA.DHODH* and *Ac.DHODH* infected *T.ni* Hi5 cells



B: *Ac.ΔchiA.DHODH* and *Ac.DHODH* infected *Sf*9 cells

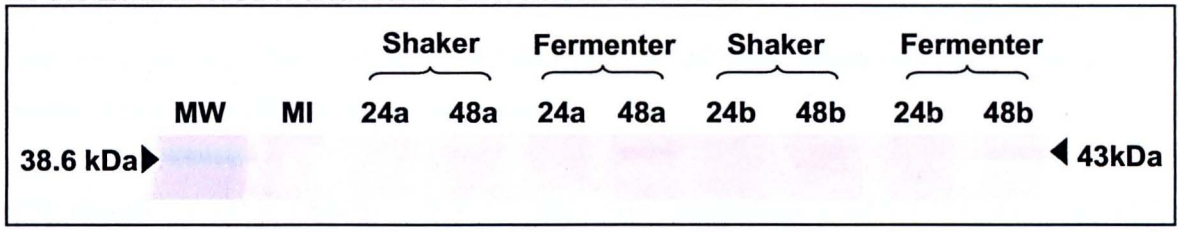


Figure 5.22A / B: Western blot analysis of *Ac.ΔchiA.DHODH* and *Ac.DHODH* expressing DHODH in *T.ni* Hi5 (**panel A**) and *Sf*9 (**panel B**) fermenter / shaker cultures. Lanes MW (molecular weight), shows protein markers (Invitrogen). Lanes MI (mock-infected), shows intracellular cell extract (25μg / lane) from mock-infected *T.ni* Hi5 and *Sf*9 cells (negative control). Lanes 24-48a shows intracellular cell extract (25μg / lane) from *T.ni* Hi5 and *Sf*9 fermenter / shaker cultures infected with *Ac.ΔchiA.DHODH* at 24-48 hpi (moi of 5). Lanes 24-48b shows intracellular cell extract (25μg / lane) from *T.ni* Hi5 and *Sf*9 fermenter / shaker cultures infected with *Ac.DHODH* at 24-48 hpi (moi of 5). Position of molecular weight (MW) size marker is shown in kDa and the position of DHODH is indicated (43kDa).

Conclusions drawn from these data show that optimal DsRed expression was achieved from *Ac.DsRed* in *Sf*9 cells (these data correlate with findings shown in Section 5.2) using fermentation. However, although DsRed production was visibly lower in *T.ni* Hi5 cells, optimal production was also obtained from *Ac.DsRed*, but using shaker culture. Interestingly optimal DHODH production was obtained from *Ac.ΔchiA.DHODH* in *T.ni* Hi5 cells (these data correlate with finding shown in Section 5.2) using fermentation. Similar results were also observed in *Sf*9 cells, although the levels of DHODH detected were extremely low.

These data show only semi-quantitative results, which cannot be used to determine accurately the quantities of foreign protein (DsRed and DHODH) produced. Therefore, in order to quantify the amounts of recombinant protein produced, heterologous protein was purified and analysed using densitometry. DHODH was chosen for this analysis since the sequence of DHODH in the construct used here contained an N-terminal His-tag (described in Section 5.2), which was subsequently used to purify the protein.

5.8 Purification of His-tagged DHODH from *Ac* and *Ac.ΔChiA T.ni* Hi5 cultures

Work carried out by the structural biology group at Pfizer have shown that a plasmid-based expression vector, expressing *DHODH* in *E. coli* using fermentation, yielded 2mg / litre of biologically active DHODH (Robert Moore; personal communication). To determine whether the yields of DHODH produced from fermenter / shaker culturing of *Ac.DHODH* and *Ac.ΔchiA.DHODH* in insect cell lines was higher than those observed from *E. coli*, yields of purified DHODH were quantified.

The results shown in **Figure 5.22 A / B** show that production of DHODH from *Ac.DHODH* and *Ac.ΔChiA.DHODH* in *Sf*9 fermenter / shaker cultures was extremely low compared to those observed from infected *T.ni* Hi5 cultures. For this reason, quantification of DHODH production was determined from infected *T.ni* Hi5 cultures.

The purification of His-tagged DHODH from *Ac.DHODH* and *Ac.ΔchiA.DHODH* infected *T.ni* Hi5 fermenter / shaker cultures (dry cell pellets stored dry at -80°C, Section 5.7) was carried out using HiTrap chelating sepharose™, high performance protein purification columns (Amersham Biosciences), as described in Section 2.6.1. Technical problems prevented successful purification of DHODH from *Ac.DHODH* infected *T.ni* Hi5 shaker culture, but protein was readily purified from both *Ac.ΔchiA.DHODH T.ni* Hi5 fermenter / shaker cultures and from the *Ac.DHODH T.ni* Hi5 fermenter culture (**Figure 5.22 A**).

Purified DHODH was visualised using SDS-PAGE and Western blot analyses (Sections 2.7.5 / 2.7.6; selected data shown). Quantification of DHODH production (mg / litre) was determined by densitometry (Section 2.7.4). The percentage of purified DHODH to total protein was then estimated optically using a Coomasse stained SDS-PAGE (**Figure 5.23**). From these results and observations an estimation of DHODH production from each culture was calculated (mg / litre).

Figure 5.23 shows results obtained for purification of His-tagged DHODH from *Ac.ΔchiA.DHODH* and *Ac.DHODH* in 1-litre *T.ni* Hi5 fermenter and *Ac.ΔchiA.DHODH* 1-litre *T.ni* Hi5 shaker culture. The purification of DHODH from these cultures described above, showed that optimal DHODH production was obtained from *Ac.ΔchiA.DHODH* infected *T.ni* Hi5 fermenter culture, producing an estimated 7.2mg / litre of DHODH (**lane:**

1; 18 mg of total purified protein / litre; 40% DHODH). The *Ac.DHODH* infected *T.ni* Hi5 fermenter culture produced an estimated 2mg / litre of DHODH (**lane: 2**; 4 mg of total purified protein / litre; 50% DHODH). The lowest level of DHODH production was observed from *Ac.ΔchiA.DHODH* infected *T.ni* Hi5 shake culture (**lane: 3**), which was shown to produce approximately 1.1mg / litre of DHODH (7.5 mg of total purified protein / litre; 15% DHODH).

These results correlate with the finding shown and described in **Figure 5.22 A** (Section 5.7), DHODH production from *Ac.DHODH* infected *T.ni* Hi5 shaker culture, which had been lost, might therefore be predicted to be even lower than those observed from the latter culture.

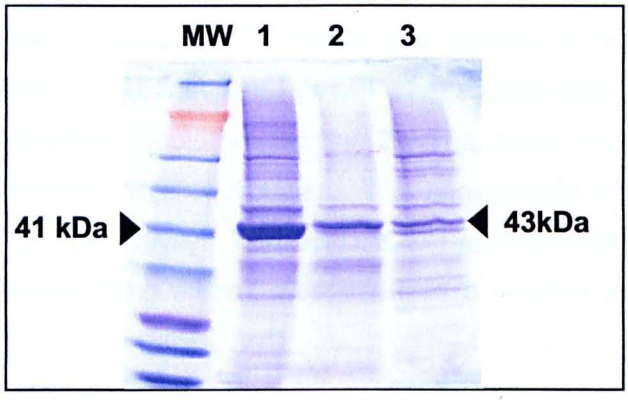


Figure 5.23: Coomasse stained SDS-PAGE analysis of purified His-tagged DHODH from *Ac.ΔchiA.DHODH* and *Ac.DHODH* in 1-litre *T.ni* Hi5 fermenter cultures and *Ac.ΔchiA.DHODH* 1-litre *T.ni* Hi5 shaker culture. Lane MW (molecular weight), shows protein markers (Invitrogen). Lanes 1-3 show total purified cell protein (25μg / lane) from *Ac.ΔchiA.DHODH* (lane 1) and *Ac.DHODH* (lane 2) 1-litre *T.ni* Hi5 fermenter cultures and *Ac.ΔchiA.DHODH* 1-litre *T.ni* Hi5 shaker culture (lane 3). Position of molecular weight (MW) size marker is shown in kDa and the position of DHODH is indicated (43kDa).

These data show that the production of DHODH from *Ac.ΔchiA.DHODH T.ni* Hi5 shaker culture (1.1mg / litre) yielded 0.5-fold less DHODH than those obtained from plasmid-based expression in *E. coli* using fermentation (2mg / litre). However, the yield of DHODH obtained from *Ac.DHODH T.ni* Hi5 fermenter culture (2mg / litre), was comparable to that obtained from *E. coli*. The highest yield of DHODH obtained was observed from *Ac.ΔchiA.DHODH T.ni* Hi5 fermenter culture (7.2mg / litre), which produced 3.6-fold more DHODH than those obtained from *E. coli*.

5.9 Functional analyses of baculovirus expressed DHODH

Having expressed (Section 5.7) and purified (Section 5.8) DHODH from *Ac.DHODH* / *Ac.ΔchiA.DHODH* in *T.ni* Hi5 cells, DHODH was tested to verify its authentic biological activity.

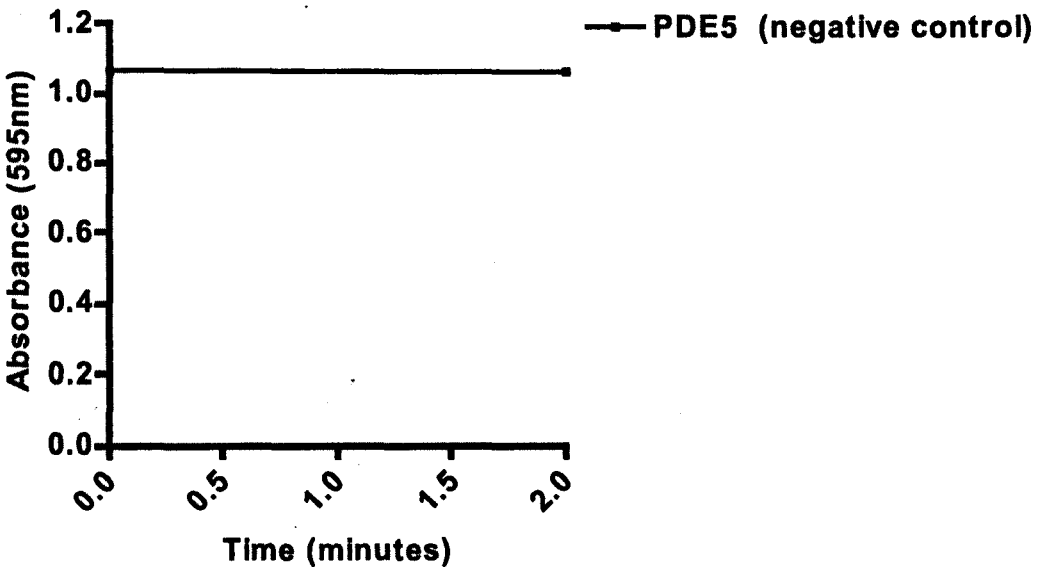
DHODH purified from *Ac.ΔchiA.DHODH* / *Ac.DHODH T.ni* Hi5 fermenter cultures and *Ac.ΔChiA.DHODH T.ni* Hi5 shaker culture (Section 5.8), were subjected to DHODH activity assays to determine the specific activity (μmoles conversion of DCIP / min / mg) of DHODH in each sample (Section 2.8.2). To establish that DHODH was actively converting the substrate 2,6 dichlorophenylindolphenol (DCIP) and not any non-specific proteins co-purified with DHODH, it was important to have a control for non-specific proteins that may have co-purified. To obtain this control, a non His-tagged protein (PDE5) expressed using the BES in *T.ni* Hi5 cell was passed through a HiTrap chelating sepharose™ protein purification column (Section 2.6.1) to purify any non-specific proteins that may be also present in DHODH samples. This sample was named PDE5 and used as a control for non-specific activity. Purified DHODH from plasmid-based expression in *E. coli* using fermentation (2mg / litre), was used as a positive control (obtained from Robert Moore; Pfizer).

Table 5.1 and **Figure 5.24 A-E** show results obtained form DHODH activity assays. Non-specific proteins purified from baculovirus expressed PDE5 *T.ni* Hi5 cell sample, were shown to have no specific activity (**A**; negative control). However, activity was detected from DHODH produced in *E.coli* (**B**; positive control). The specific activity of DHODH from this sample was calculated to be 1.2 μmoles conversion of DCIP / min / mg. DHODH produced from *Ac.ΔchiA.DHODH* (**C** / **E**) and *Ac.DHODH* (**D**) *T.ni* Hi5 fermenter / shaker cultures, was also shown to be active. DHODH purified from *Ac.ΔchiA.DHODH T.ni* Hi5 fermenter produced a specific activity of 0.5 μmoles conversion of DCIP / min / mg, while *Ac.DHODH T.ni* Hi5 fermenter produced approximately 0.1 μmoles conversion of DCIP / min / mg, and *Ac.ΔchiA.DHODH T.ni* Hi5 shaker culture produced 0.02 μmoles conversion of DCIP / min / mg.

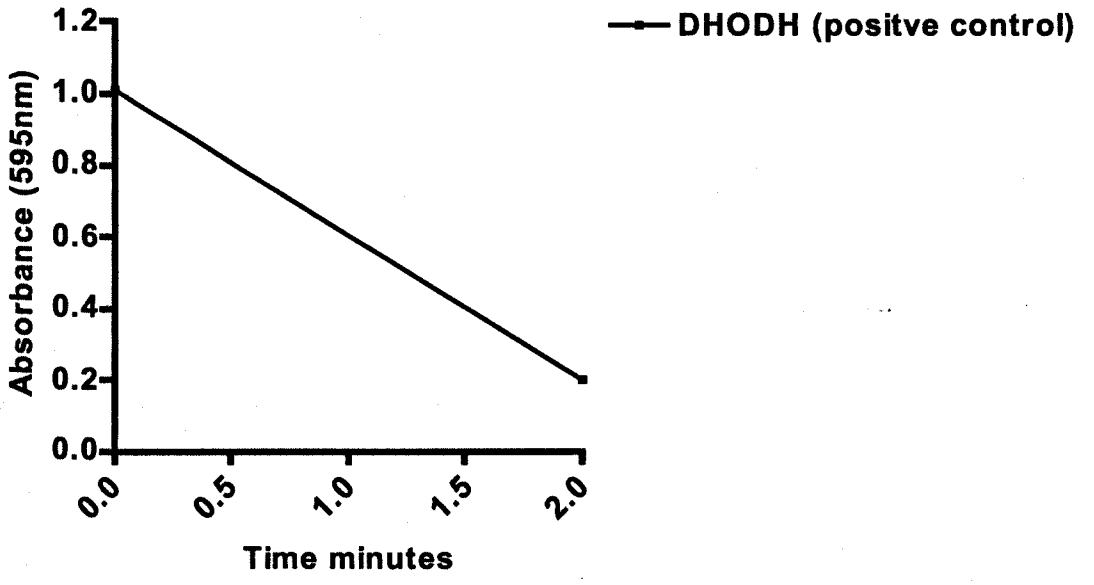
Table 5.1: A summary of DHODH activity assay results shown in **Figure 5.24.**

Sample	ABS at 595nm 0 mins	ABS at 595nm 2 mins	Estimated DHODH specific activity (μmoles conversion of DCIP / min / mg)
PDE5 *T.ni Hi5* (negative control)	1.0666	1.0607	0
DHODH (*E.coli*; positive control)	1.0112	0.2022	1.2
Ac.ΔchiA.DHODH T.ni Hi5 fermenter	1.1047	0.9354	0.5
Ac.DHODH T.ni Hi5 fermenter	1.0800	0.5762	0.1
Ac.ΔchiA.DHODH T.ni Hi5 shaker	1.0950	0.9635	0.02

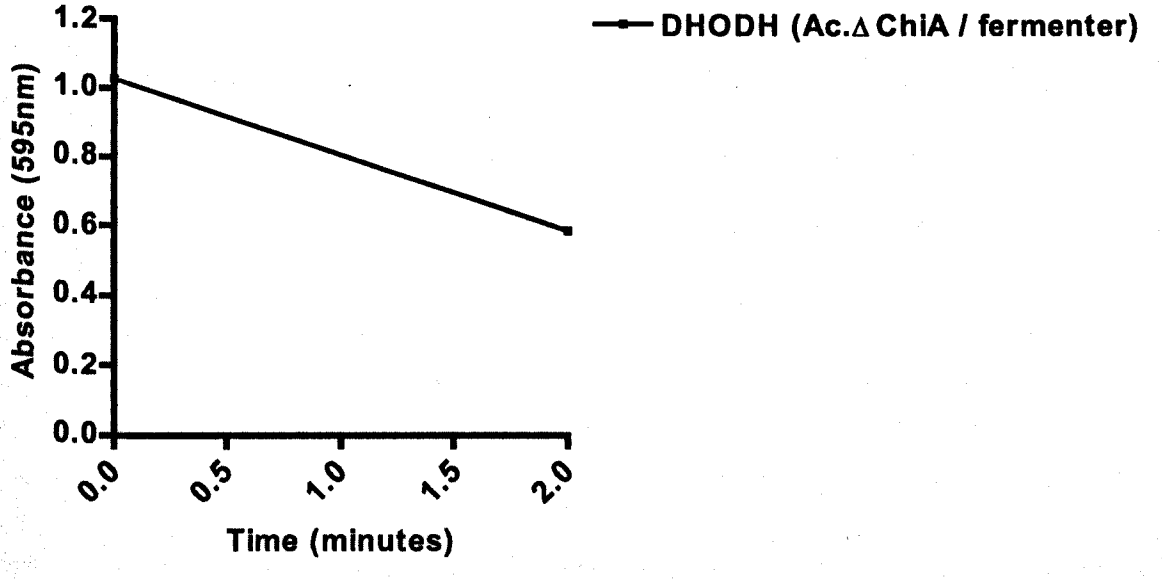
A: Non-specific protein



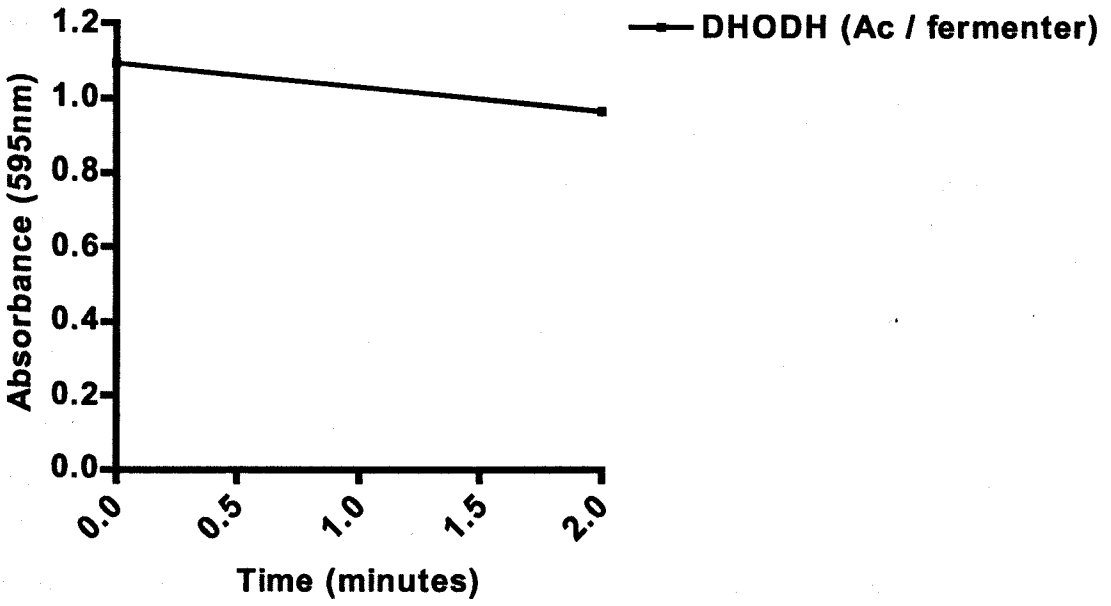
B: DHODH produced from plasmid-based expression in *E.coli* using fermentation



C: DHODH produced from *Ac.ΔChiA.DHODH T.ni* Hi5 fermenter culture



D: DHODH produced from *Ac.DHODH T.ni* Hi5 fermenter culture



E: DHODH produced from *Ac.ΔChiA.DHODH T.ni* Hi5 shaker culture

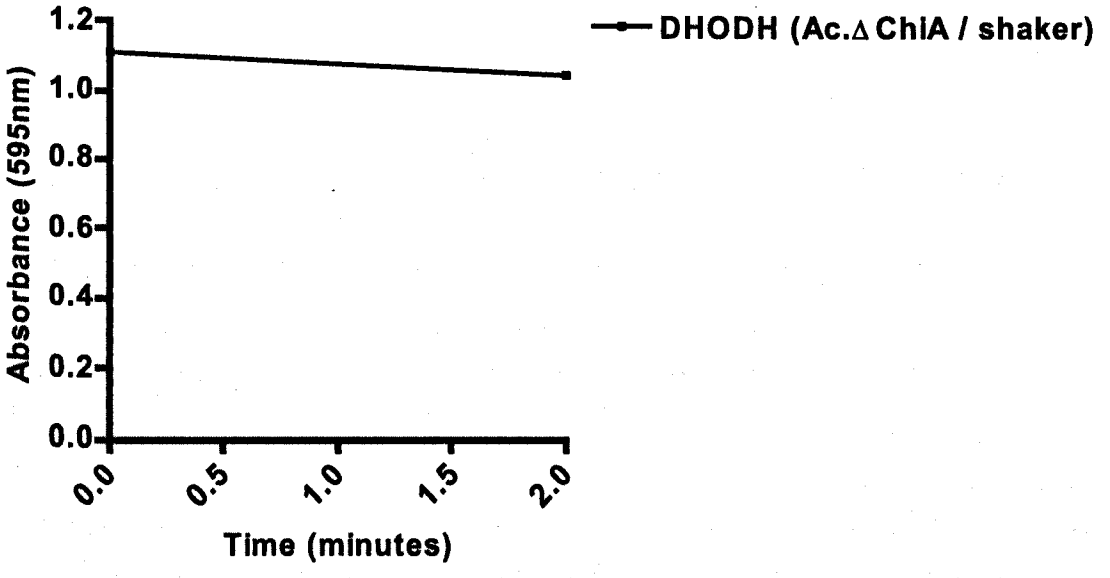


Figure 5.24 A-E: Functional analyses of recombinant DHODH activity. **A:** Non-specific proteins purified from baculovirus expressed PDE5 in *T.ni* Hi5 cells, were used as a negative control to determine possible background activity levels, no activity was observed. **B:** Purified DHODH produced from plasmid-based expression in *E. coli* by means of fermentation, showed DHODH activity was detected (positive control). **C-E:** The production and purification of DHODH from *Ac.ΔchiA.DHODH* (**C**) / *Ac.DHODH* (**D**) *T.ni* Hi5 fermenter cultures and *Ac.ΔchiA.DHODH T.ni* Hi5 shaker culture (**E**), were also shown to be active.

In conclusion these data show that DHODH produced from *Ac.ΔchiA.DHODH* and *Ac.DHODH T.ni* Hi5 fermenter cultures and *Ac.ΔchiA.DHODH T.ni* Hi5 shaker culture was biologically active. As expected, *Ac.ΔchiA.DHODH T.ni* Hi5 fermenter culture was shown to have a higher specific activity (0.5 μmoles conversion of DCIP / min / mg) than those observed from *Ac.DHODH T.ni* Hi5 fermenter culture (0.1 μmoles conversion of DCIP / min / mg) and *Ac.ΔchiA.DHODH T.ni* Hi5 shaker culture (0.02 μmoles conversion of DCIP / min / mg). These results correlate with those shown in Section 5.7 and 5.8, which show DHODH production was optimal from *Ac.ΔchiA.DHODH T.ni* Hi5 fermenter culture. The specific activity of DHODH produced in *E.coli* was shown to be higher than those obtained from baculovirus in *T.ni* Hi5 cells, even though the purified yield of DHODH was lower (*E.coli*; 2mg / litre) than that obtained from *Ac.ΔchiA.DHODH T.ni* Hi5 fermenter culture (7.2mg / litre). This was firstly explained by the *E.coli* purified DHODH sample being passed through an ion-exchange purification column to remove some of the non-specific proteins present in the sample, therefore producing a cleaner sample. Secondly, the sample was concentrated, to increase the amount of DHODH / μl (this work was carried out by Robert Moore at Pfizer) and as a consequence increase the specific activity of DHODH.

5.10 Discussion

To date, many heterologous genes have been expressed successfully using the BES. It has been well documented that recombinant baculoviruses usually express high levels of intracellular foreign proteins (nuclear and cytoplasmic; King and Possee, 1992; Vlak *et al.*, 1998; Stewart and Champoux, 1999; Ikonomou *et al.*, 2003; Jayakumar *et al.*, 2004). However, virus infection severely compromises the insect cells secretory pathway, which inevitably has been shown to effect the expression of recombinant proteins destined for the plasma membrane or secretion (Jarvis and Summers, 1989; Jarvis *et al.*, 1990; King and Possee, 1992; Thomas, 1997; Saville *et al.*, 2002). As described in Section 5.1, work carried out in our laboratory has identified a non-essential viral gene in the *Ac* genome called *chiA* (Hawtin *et al.*, 1995). The expression of chitinase from *Ac* has been shown to accumulate at high levels in the ER late in virus infection (Thomas, 1997; Thomas *et al.*, 1998; Saville *et al.*, 2002), inadvertently blocking the insect cells secretory pathway. The removal of *chiA* from the *Ac* genome (*Ac.ΔchiA*) has subsequently been shown to improve yields of secreted foreign proteins from infected insect cells (urokinase / tissues-plasminogen activator; McCarroll, 1997).

The initial work in this Chapter was carried out to determine whether DHODH and DsRed were expressed from the *polh* promoter of *Ac* and *Ac.ΔchiA* in *Sf*9 and *T.ni* Hi5 cells. Subsequently, the intracellular levels of DHODH and Dsred from *Ac.DHODH / Ac.DsRed* and *Ac.ΔchiA.DHODH / Ac.ΔchiA.DsRed* were quantified and compared. The results shown in **Figure 5.1** and **5.2 A / B** showed that DHODH and DsRed were expressed from both *Ac* and *Ac.ΔchiA*. However, DHODH was only detectable in *T.ni* Hi5 cells, whilst DsRed was observed in both *Sf*9 and *T.ni* Hi5 cells.

This was the first known study of *Ac.ΔchiA* expressing intracellular targeted foreign proteins (DHODH and DsRed). From what is currently known about the localisation of chitinase (retained in the ER, due to the presence of a KDEL-ER retention motif; Thomas *et al.*, 1998) in *Ac* infected insect cells, it was hypothesised that the removal of *chiA* from *Ac* should not significantly increase the levels of expressed DHODH or DsRed in *Sf*9 or *T.ni* Hi5 cells: Noting that DHODH and DsRed are not produced in the ER or processed through the cell secretory pathway.

However, the results shown in **Figure 5.1** indicated that the production of intracellular DHODH from *Ac.ΔchiA.DHODH* was significantly higher than that observed for *Ac.DHODH* in *T.ni* Hi5 cells at all time-points (24-72 hpi). Interestingly, the expression of DHODH from *Ac.ΔchiA.DHODH* was observed at an earlier time-point (24 hpi) than that detected for *Ac.DHODH* (48 hpi). At 72 hpi, the production of DHODH from *Ac.DHODH* was barely detectable but a lower molecular weight protein was observed (estimated 28KDa), which was postulated to be a degraded form of DHODH. This protein was also observed at 72 hpi in *Ac.ΔchiA.DHODH*-infected cells; however, the levels were drastically lower than that detected in *Ac.DHODH*-infected cells, and the levels of DHODH were significantly higher than those observed from *Ac.DHODH*.

Conversely, expression of intracellular DsRed was observed in both *Ac.DsRed*-and *Ac.ΔchiA.DsRed*-infected *Sf*9 / *T.ni* Hi5 cells (**Figure 5.2 A / B, 5.3A / B**). However, of the two recombinant viruses expressing DsRed, *Ac.DsRed* produced higher levels in both insect cell lines used. Fluorometric analyses revealed *Ac.DsRed* produced DsRed earlier in infection (24 hpi) than *Ac.ΔchiA.DsRed* (36 hpi). The choice of insect cell line used to produce DsRed from *Ac.DsRed* and *Ac.ΔchiA.DsRed* also showed a considerable variation in the levels produced, as optimal DsRed production from both recombinant viruses was observed in *Sf*9 cells.

For reasons unclear at this time, it was difficult to address the reasons why the removal of *chiA* from the *Ac* genome increased the production of intracellular DHODH. However, this was not the case for the production of intracellular DsRed, as the removal of *chiA* from the *Ac* genome reduced the levels of DsRed produced. Therefore, it is difficult to generalise which viral expression vector (*Ac* or *Ac.ΔchiA*) would be most suited for optimal intracellular protein production in insect cells. This study does show that *Ac.ΔchiA* can be used to produce improved yields of intracellular foreign protein, although it seems to be dependent on the nature of the protein produced. This work also stresses the importance of optimising foreign protein production from different expression vectors and insect cell lines.

Studies have shown that promoter choice strongly influences the range of insect cells that can effectively support the production of heterologous genes (Morris and Miller, 1992). However, in this study DHODH and Dsred were expressed from identical promoters (*polh* promoter of *Ac* and *Ac.ΔchiA*) in both *Sf*9 and *T.ni* Hi5 cells. Subsequently, optimal DHODH production was observed in *T.ni* Hi5 cells, while optimum DsRed production was detected in *Sf*9 cells. From these data, it was concluded that promoter choice was essential for the production of foreign proteins, but the nature of the foreign protein under control of the *polh* promoter may also determine the range of insect cells used to host optimal expression.

As described above, *Ac.DsRed* was shown to produce higher levels of DsRed than *Ac.ΔchiA.DsRed* in both insect cell lines used. A proposed hypothesis, to explain these results, addresses the involvement of *chiA*, ER-associated degradation (ERAD) pathway and the ubiquitin-mediated proteolytic pathway. Eukaryotic cells have several intracellular proteolytic pathways for degrading misfolded (cytosol / ER proteins), viral, aggregated or denatured proteins (Laney and Hochstrasser, 1999). The ubiquitin-mediated proteolytic pathway is the main mechanism for degrading cytosolic proteins (Kirschner, 1999; Kornitzer and Ciechanover, 2000), and proteins that become misfolded in the course of their synthesis in the ER (Hiller *et al.*, 1996; Lord *et al.*, 2000; Jarosch *et al.*, 2003; Kostova and Wolf, 2003). Proteins that are misfolded in the ER are transported back to the cytosol by the ERAD pathway via the *sec61* translocon (Hiller *et al.*, 1996; Plemper *et al.*, 1998) and subsequently degraded by the ubiquitin-mediated proteolytic pathway. The process of protein degradation occurs due to the chemical modification of a target protein lysine side chain with the addition of ubiquitin molecule, a 76-residue polypeptide.

Subsequently, further ubiquitin molecules are added to the preceding one, resulting in a polyubiquitin chain that is recognised by the 26S proteosome (Ciechanover, 1998). The 26S proteosome then cleaves the protein into numerous small peptide fragments.

DsRed is known to exist in the cytosol of both prokaryotic and eukaryotic cells as monomeric and tetrameric forms (Rodrigues *et al.*, 2001; Sacchetti *et al.*, 2002). However, only the tetrameric form emits red fluorescence (Sacchetti *et al.*, 2002). DsRed tetramers also have a strong tendency to self-associate to form higher-order aggregates, which has subsequently been shown to be toxic in both prokaryotic and eukaryotic cells (Baird *et al.*, 2000; Goss *et al.*, 2000; Heikal *et al.*, 2000; Jakobs *et al.*, 2000).

It was hypothesised that DsRed expression from *Ac.DsRed* and *Ac.ΔchiA.DsRed* formed aggregates in the cytosol of *Sf*9 and *T.ni* Hi5 cells, which were then targeted for ubiquitin-mediated proteolytic degradation. However, it is thought that the production of chitinase from *Ac.DsRed* compromised the functional role of the ER / insect cell secretory pathway, inadvertently causing an increase in misfolded host cell ER proteins. This possible increase in ER misfolded proteins may have increased the activity of the ubiquitin-mediated proteolytic pathway, and as a consequence over-worked the proteosome machinery. The stressed proteosomes may have, therefore, only degraded a small proportion of the aggregated intracellular DsRed present. In contrast, as *chiA* was removed from *Ac.ΔchiA.DsRed* the insect cell secretory pathway was free of chitinase, thus the levels of misfolded host cell ER proteins may have been lower, allowing the ubiquitin-mediated proteolytic pathway to degrade higher levels of aggregated intracellular DsRed.

Attempts to overcome these detrimental affects of the BES (lytic system) has led to the development of stable (non-lytic system), plasmid-based expression systems (Bourouis and Jarry, 1983; Jarvis *et al.*, 1990; Joyce *et al.*, 1993; Jarvis and Finn, 1996; Lu *et al.*, 1996; Farrell *et al.*, 1998, 1999), capable of continuous protein production in insect cell lines (Henderson *et al.*, 1995; McCarroll and King, 1997). A variety of recombinant proteins targeted for secretion (serine protease inhibitor neurosepin, juvenile hormone esterase) or the plasma membrane (maize ABP1, chick nicotinic acetylcholine receptor α-subunit) have been effectively produced using stable insect cell lines (Henderson *et al.*, 1995; Atkinson *et al.*, 1996; Farrell *et al.*, 1998; Hill *et al.*, 2001). In a number of reported cases, the expression of secreted foreign proteins from stable insect cell lines has been

equal to or greater than those observed from the BES (Jarvis *et al.* 1990; Farrell *et al.*, 1998; Jarvis *et al.*, 1996). However, the expression of intracellular foreign proteins in stable insect cell lines (plasmid-based expression systems) are usually very low compared to those detected from the BES.

Work demonstrated by Jarvis *et al.* (1990), showed that stable expression of β-galactosidease from the *Ac*MNPV *ie*-1 promoter in *Sf*9 cells (48 h growth), ranged from 100 to 5000-fold less than that detected from the *polh* promoter of recombinant *Ac*MNPV infected *Sf*9 cells (48 hpi). As previously described in Chapter 3, the *Ac*MNPV *ie*-1 promoter has been characterised as a transcriptionally weak promoter. However, work in Chapter 3 characterised two RNA polymerase II transcribed insect / viral promoters (*Op*MNPV *ie*-2 / *BmA*3+E) with significantly stronger transcriptional activity than *Ac*MNPV *ie*-1 in *Sf*9 and *T.ni* Hi5 cells. Therefore, work in this Chapter was carried out to compare the intracellular levels of expressed DsRed and DHODH from the *BmA*3+E promoter to those produced from the *polh* promoter of *Ac* and *Ac.ΔchiA* in *Sf*9 and *T.ni* Hi5 cells. This comparison addresses whether it is feasible to use stable insect cell lines to produced intracellular foreign protein as an alternative to the BES.

Western blot analyses showed transient DHODH and DsRed expression from the *BmA*3+E promoter was only detected in *T.ni* Hi5 cells (**Figures 5.8 / 5.9**). However, visualisation of red fluorescence using a CLSM microscope, showed DsRed was detectable in both *Sf*9 and *T.ni* Hi5 cells (**Figure 510 A-H**), although the levels of red fluorescence observed were lower in *Sf*9 cells compared to those detected in *T.ni* Hi5 cells. No assays were available for the detection of low level DHODH production, it was however, postulated that DHODH may have been expressed in *Sf*9 cells, but at very low levels. Therefore, the production of stable insect cell lines (*Sf*9 and *T.ni* Hi5 cells) expressing DHODH or DsRed from the *BmA*3+E promoter were generated (Section 5.5).

Stably transformed *Sf*9 and *T.ni* Hi5 cells expressing DHODH were analysed for DHODH production using Western blot analyses, although DHODH was not detected. Possible explanations why DHODH was not detected include: firstly, the *BmA*3+E promoter / *DHODH* coding-region may not be integrated into the genome of transformed *Sf*9 and *T.ni* Hi5 cells, due to a low transfection efficiency (as described in Chapter 3 Section 3.5). Secondly, the *BmA*3+E promoter / *DHODH* coding-region may have been integrated into a silent region of the genome (O'Gorman *et al.*, 1991), inadvertently repressing the *BmA*3+E

promoter of its transcriptional activity. Finally, it is possible that the levels of DHODH produced were extremely low, due to the nature of this protein.

To identify if the *DHODH* coding-region was stably integrated into the genomic DNA of transformed *Sf*9 and *T.ni* Hi5 cells, total genomic DNA was isolated from these cell lines and screened for the *DHODH* coding-region using PCR. The results in **Figure 5.11** show that the *DHODH* coding-region was amplified successfully from total genomic DNAs tested. Therefore, the *DHODH* coding-region was integrated into the genomic DNA of stably transformed *Sf*9 and *T.ni* Hi5 cells.

If the *BmA*3+E promoter / *DHODH* coding-region was integrated into a silent region of these genomes then the *BmA*3+E promoter would not transcribe *DHODH*. Consequently, no DHODH mRNA transcripts would be detected. Analyses of total RNA isolated from these insect cell lines revealed that DHODH was expressed as mRNA transcripts (**Figure 5.12 A / B**). These data conclude that the *BmA*3+E promoter / *DHODH* coding-region was not integrated into a silent region of the *Sf*9 or *T.ni* Hi5 genome, but was expressed from the *BmA*3+E promoter at very low levels. The production of DHODH from the *polh* promoter of *Ac* and *Ac.ΔchiA* in *Sf*9 fermenter / shaker cultures was also shown to be extremely low (Section 5.7), suggesting that *DHODH* was difficult to express under the control of certain promoters and insect cell lines (*BmA*3+E, *Sf*9 / *T.ni* Hi5 cells; *polh* promoter, *Sf*9 cells). Therefore, DHODH was not used to compare expression levels from the *BmA*3+E promoter in stably transformed *Sf*9 and *T.ni* Hi5 cells with levels produced from the *polh* promoter of *Ac* and *Ac.ΔchiA*.

The production of intracellular DsRed from the *BmA*3+E promoter in passage 1-8 of stably transformed *Sf*9 and *T.ni* Hi5 cells (*Sf*9.*DsRed* and *T.ni* (Hi5).*DsRed*) was initially only detected in *T.ni* (Hi5).*DsRed* cells (**Figure 5.13**). The levels were barely detectable; however, the levels observed were consistently low from each passage. Fluorometric analyses detected the production of intracellular DsRed in both *Sf*9.*DsRed* and *T.ni* (Hi5).*DsRed* cell lines. However, the *T.ni* (Hi5).*DsRed* cell line produced significantly higher levels of intracellular DsRed than those observed from *Sf*9.*DsRed* cell ($p=0.0001$ in all cases). Therefore, it was concluded that the transcriptional activity of the *BmA*3+E promoter was stronger in *T.ni* Hi5 cells compared to that in *Sf*9 cells (these findings correlate with those of Farrell *et al.*, 1998).

A comparison of optimal intracellular DsRed production from *Sf*9.*DsRed* (**Figure 5.14**, 94 h growth [estimated $7x10^6$ cells / ml, data not shown]) and *T.ni* (Hi5).*DsRed* (**Figure 5.14**, 84 h growth [$2.8x10^6$ cells / ml, data not shown]) cell lines to those detected from the BES revealed (as described above optimal DsRed expression was detected in *Ac.DsRed* infected *Sf*9 cells at 72 hpi; Section 5.2) that *Ac.DsRed* infected *Sf*9 cells produced significantly higher levels of DsRed than those observed from both stable insect cell lines ($p=0.0001$ in all cases). An estimated intracellular DsRed productivity difference of 262.9-fold greater than *Sf*9.*DsRed* cells and 52.5-fold greater than *T.ni* (Hi5).*DsRed* cells was observed from *Ac.DsRed* infected *Sf*9 cells at 72 hpi.

From these findings it was estimated that to produce an equivalent amount of DsRed from the *T.ni* (Hi5).*DsRed* cell line to that obtained from a 1-litre *Ac.DsRed* infected *Sf*9 culture at 72 hpi (estimated medium requirements 500ml = £17.5), *T.ni* (Hi5).*DsRed* cells would have to be amplified to 52.8 litres (estimated medium requirements 42L = £1470) at a cell density of $2.8x10^6$ cells/ ml. The implications for producing these volumes, described above, increases the scope for experimental error (contamination of culture), and would not be cost-effective due to the cost of medium (£35 / litre) required to cultivate these volumes of cells. Additionally, downstream processing of large volumes may also be problematic, costly and labour-intensive. Therefore, it was concluded that the BES was likely to remain the most effective method for producing large amounts of intracellular foreign protein in insect cell lines, using current promoters studied for cell lines.

However, with reports of stable insect cell lines producing secreted foreign proteins equal to or greater than those observed from the BES (Jarvis *et al.* 1990; Farrell *et al.*, 1998; Jarvis *et al.*, 1996), a method for scaling-up stable insect cell cultivation was investigated using *T.ni* (Hi5).*DsRed* cells as a model for culture dynamics (Section 5.6).

The protocol developed in this Chapter for maintaining large volumes (1-litre) of stable insect cells in an open fermenter system, was successfully piloted using the fill and draw technique (previously described in Section 5.6). *T.ni* (Hi5).*DsRed* cells were seeded in a 3-litre fermenter vessel at a density of $0.5x10^6$ cells / ml (90% viable). Subsequently, the culture was monitored at 24 h intervals for cell growth (**Figure 5.17**) and viability (**Figure 5.19**).

Over a period of 4 days *T.ni* (Hi5).*DsRed* cells were shown to proliferate to a density of $2.8x10^6$ cells / ml (74% viable). However, on day 5, cell density decreased ($2.5x10^6$ cells /

ml), which correlates with the dramatic decrease in cell viability observed (51%). Subsequently, *T.ni* (Hi5).*DsRed* cells were passaged using the fill and draw technique, and re-established at a density of $0.5x10^6$ cells / ml. *T.ni* (Hi5).*DsRed* cell growth from days 5-8 were shown to replicate the data obtained from days 1-4, with no significant difference ($p>0.05$ in all cases), as did the data observed from days 8-11. However, the viability of the *T.ni* (Hi5).*DsRed* cells increase from 51% on day 5, to 76% on day 7, before decreasing in day 8 to 70%. This apparent cyclic pattern of increased viability followed by a decrease was observed throughout the course of culturing (further 5 passages, data only shown for 1 [days 8-11]). From the results shown in **Figure 5.19**, it was proposed that if *T.ni* (Hi5).*DsRed* cells were passaged on day 4, then the cyclic pattern of cell viability observed throughout cultivation may have been slightly higher, inevitably maintaining a healthier culture.

The evident increase in cell viability after passaging was attributed to nutrient metabolic consumption kinetics. Maintaining healthy insect cells in a culture is reliant on growth medium (Grace, 1962) supplying essential nutrients, carbon sources (glucose, fructose and sucrose), organic acids (fumarate, malate, succinate and α-ketoglutarate) and amino acids (L-glutamine). As insect cells proliferate from a low-high cell density numbers, certain components of the growth medium become depleted or limited (e.g. glucose and L-glutamine) and waste metabolic products accumulate (ammonia, uric acid and lactate; Bedard *et al.*, 1993), which shifts insect cell growth from log phase to stationary or decline phase of cell growth (Raghunand and Dale, 1999). At this critical point cells are passaged into fresh growth medium, replenishing essential compounds required for efficient viable growth.

In our laboratory, non-transformed *T.ni* Hi5 cells are routinely passaged at $6\text{-}8x10^6$ cells / ml (5 days growth from $0.5x10^6$ cells / ml). Contrary to this, the highest density of *T.ni* (Hi5).*DsRed* cells obtained in this study was between $2.5\text{-}8x10^6$ cells / ml, at 4-5 days. This, dramatic shift of cell growth may be partly due to maintaining *T.ni* (Hi5).*DsRed* cells in medium (Ex-cell 405, JRH Biosciences) containing the antibiotic G418 (concentration of G418 per ml of medium, 300μg / ml), inadvertently stressing the cells and changing their growth dynamics. Work by Keith *et al.* (1999), has shown that growth dynamics of an established stably transformed *T.ni* Hi5 cell line (expressing granulocyte-macrophage colony-stimulating factor) maintained in growth medium containing a selective antibiotic

(HygB) was also significantly affected. The highest cell density obtained from their culture was $2.8x10^6$ cells / ml after 9 days.

A second contributing factor of altering the growth dynamics of *T.ni* (Hi5).*DsRed* cells may be explained by the stable production of DsRed. As described previously, the expression of DsRed has been shown to be toxic to eukaryotic cells due to the formation of aggregates (Baird *et al.*, 2000; Goss *et al.*, 2000; Heikal *et al.*, 2000; Jakobs *et al.*, 2000). Therefore, it is proposed that in proliferating *T.ni* (Hi5).*DsRed* cell culture producing constitutively low levels of DsRed, as levels increase over time (e.g. 1-5 days) DsRed surpasses the cells critical threshold (not known), consequently having detrimental affect on cell viability.

Further possible conclusions for the substantially long *T.ni* (Hi5).*DsRed* population doubling-time, with respect to non-transformed *T.ni* Hi5 cells include: (i) transformed cells redirected energy from cell proliferation towards recombinant protein production, (ii) the enhancing transcription factor IE-1, produced to increase the transcriptional activity of the *BmA*3 promoter (Section 3.3.3), may also affect cellular metabolism, or other unknown factors may be responsible.

The fill and draw technique applied to maintaining a viable, contaminant free stable insect cell line in an open fermenter system may prove to be a useful tool in the future. This method of cultivating large volumes of stable insect cells can essentially be applied to increasing yields of secreted foreign proteins. By increasing the culture volume of stably transformed insect cells expressing a foreign gene, the amount of recombinant protein produced should also effectively increase. However, from the data obtained for *T.ni* (Hi5).*DsRed* cell growth dynamic, it is postulated that growth dynamics of different stable insect cell lines may vary depending on the foreign protein produced. Therefore, growth curves of individual stable insect cell lines should be determined before using this method of culturing.

The final Section of this Chapter, investigates the process of scaling-up intracellular recombinant protein production (DsRed and DHODH) from the BES (*Ac* and *Ac.ΔchiA*) in insect cells (*Sf*9 and *T.ni* Hi5 cells), using two different culturing methodologies (fermenter verses shaker). A number of reported studies have shown that fermentation and shaker culturing can be successfully applied to scaling-up heterologous protein production from *Ac* in insect cells (King *et al.*, 1992; Neutra *et al.*, 1992; Scott *et al.*, 1992; Nguyen *et al.*,

1993; van Lier *et al.*, 1994; Schwartz *et al.*, 1997; Ikonomou *et al.*, 2003). However, these are the first reported studies comparing scale-up of intracellular foreign protein production (DsRed and DHODH) from *Ac.ΔchiA* in 1-litre *Sf*9 and *T.ni* Hi5 fermenter / shaker cultures.

Comparison of intracellular DsRed production from *Ac.DsRed* and *Ac.ΔchiA.DsRed* in 1-litre *Sf*9 and *T.ni* Hi5 fermenter / shaker cultures, using Western blot analyses revealed that optimal intracellular DsRed expression was produced from *Ac.DsRed* in *Sf*9 cells using fermentation (**Figure 5.21 A**). Production of intracellular DsRed from *Ac.ΔchiA.DsRed* in *Sf*9 fermenter / shaker cultures also showed that fermentation produced higher levels than those observed from the shaker cultures (**Figure 5.21 B**). However, these levels were lower than those detected from *Ac.DsRed.* The production of intracellular DsRed from *Ac.DsRed* and *Ac.ΔchiA.DsRed* in *T.ni* Hi5 fermenter / shaker cultures was extremely low. Interestingly the highest levels observed were also from *Ac.DsRed*, but in shaker cultures (**Figure 5.21 C**). Similar results were also observed from *Ac.ΔchiA.DsRed* producing intracellular DsRed in *T.ni* Hi5 fermenter / shaker culture, but at considerably lower levels (**Figure 5.21 D**).

The levels of intracellular DHODH produced from *Ac.DHODH* and *Ac.ΔchiA.DHODH* in 1-litre *Sf*9 and *T.ni* Hi5 fermenter / shaker cultures were initially semi-quantified using Western blot analyses. However, intracellular DHODH was subsequently purified from the highest producing cultures to accurately determine the amount produced (mg / litre). The overall levels of intracellular DHODH produced from *Ac.DHODH* and *Ac.ΔchiA.DHODH* in *T.ni* Hi5 fermenter / shaker cultures were significantly higher than in *Sf*9 cultures (barely detectable; **Figure 5.22 A / B**). Therefore, intracellular DHODH produced from infected *Sf*9 cultures was not purified. However, infected *Sf*9 fermenter cultures produced higher levels of intracellular DHODH than those observed from shaker cultures. Optimal intracellular DHODH production from both *Ac.DHODH* and *Ac.ΔchiA.DHODH* in *T.ni* Hi5 cells was observed from fermenter cultures. Maximal production of intracellular DHODH was observed from *Ac.ΔchiA.DHODH* in *T.ni* Hi5 cells using fermentation, producing an estimated 7.2mg / litre. The *Ac.DHODH* infected *T.ni* Hi5 fermenter culture produced 2mg / litre, and *Ac.ΔchiA.DHODH* in *T.ni* Hi5 shaker culture was estimated to produce 1.1mg / litre, which was higher than those detected from *Ac.DHODH* in *T.ni* Hi5 shaker culture (mg / litre was not determined due to technical problems). Subsequently, recombinant intracellular DHODH produced using these recombinant viruses was shown to have authentic biological activity.

From these results it was concluded that foreign protein production using fermentation supports optimal production from recombinant baculoviruses in insect cells. Even though the production of intracellular DsRed from *Ac.ΔchiA.DsRed* in *T.ni* Hi5 shaker culture was higher than those observed from the fermenter culture, DsRed production was not optimal. These studies yet again stress the importance of optimising the expression vector and insect cell line used to host expression. The results obtained from small-scale DsRed and DHODH production (Section 5.2; 25ml shaker culture) provided a good indicator of which recombinant virus / insect cell line would provide optimal expression when scaled-up.

A number of proposed explanations have been previously described in this Section, explaining why higher levels of intracellular DsRed may be observed from *Ac.DsRed* compared to *Ac.ΔchiA.DsRed* in small shaker culture. The increased levels of intracellular foreign proteins observed in fermenter culture may be attributed to maintaining a consistent dO_2 concentration (minimum of 60% was used in these studies; Colin Robinson; personal communication) throughout infection. Similar properties may be effective in explaining DHODH product levels.

Studies by Scott *et al.* (1992) has shown the importance of oxygen demand in baculovirus infected insect cell cultures producing heterologous protein. Their results show oxygen consumption of insect cells rapidly increases after baculovirus infection (Streett and Hink, 1978; Maiorella *et al.*, 1988). The high oxygen demand of baculovirus infected insect cells has been attributed to maintaining cellular activities such as oxidative phosphorylation, coordinate synthesis of adenosine triphosphate, viral DNA synthesis and recombinant protein production (Maiorella *et al.*, 1988). Therefore, the effect of maintaining an adequate oxygen supply to the culture can prolong infected insect cell viability and as a consequence increase recombinant protein production (Lindsay and Betenbaugh, 1992; Scott *et al.*, 1992; Malinowski and Daugulis, 1993; Wang *et al.*, 1996; Taticek and Shuler, 1997; Hu and Bentley, 1999).

The major variable distinguishing between fermenter and shaker culturing of infected insect cells is essentially the level of dO_2 concentration maintained in the culture throughout infection. Shaker flasks are simplistic vessels and do not have the means of ensuring the dO_2 concentration of an infected insect culture is maintained. As a consequence, oxygen from the headspace and culture is rapidly depleted, which subsequently affects foreign protein production. However, fermentation offers the

advantage of aerating infected insect cultures by supplying oxygen and air to the headspace of the vessel and culture via gentle sparging. It is also possible to control the dO_2 concentration of a culture maintained in a fermenter, as different cell lines respond to different dO_2 concentrations, e.g. mammalian cell require less O_2 than insect cells (Maiorella *et al.*, 1988).

The flavoenzyme DHODH is a mitochondrial protein that performs the fourth enzymatic step of the *de novo* pyrimidine biosynthesis, the ubiquinone-mediated oxidation of dihydroorotate to orotate (Jone, 1980; Section 1.7.5). This enzyme links the biosynthesis of pyrimidine nucleotides to functional mitochondria and the aerobic metabolism of cells (Löffler *et al.*, 1997). Because it connects two essential pathways of a living cell, this enzyme has received attention from numerous clinical researchers in different fields investigating mitochondrial diseases (Naviaux, 2000).

Therefore, producing high yields of biologically active recombinant DHODH has been an aim at Pfizer, for in-house studies. Their initial attempts using *E.coli* to host expression were successful, producing 2mg / litre of biologically active enzyme. However, a significant draw-backs of using *E.coli* is that many expressed proteins accumulate intracellularly in the form of insoluble, inactive inclusion bodies, from which biologically active recombinant proteins must be recovered using complicated and costly denaturation and refolding processes (Jeong and Lee, 1999; 2001). These processes can inevitably lead to the loss or biological inactivation of recombinant protein. However, an alternative method of producing DHODH was determined, as studies in this thesis show intracellular biologically active DHODH was produced effectively from the BES (*Ac.ΔchiA.DHODH*) in insect cells (*Sf*9 cells). The yields produced were equivalent to or greater than (7.2mg / litre; 3.6-fold increase) those observed from *E.coli* (2mg / litre), and purification of intracellular DHODH from infected insect cells did not require costly denaturation and refolding processes.

Finally the conclusions drawn from work in this Chapter show that *Ac.ΔchiA*, originally shown to improve yields of secreted foreign proteins, can effectively be used to produce high level intracellular recombinant protein. However, the levels produced may be dependent on the nature of the protein expressed. Therefore, *Ac.ΔchiA* may be considered as a novel expression vector, when optimising intracellular heterologous protein production in insect cells. Further investigations for improving heterologous gene

expression in insect cells, addressed different culturing methodologies (fermenter versus shaker) used to scale-up production of recombinant proteins. Fermentation was determined as the pre-eminent method for obtaining optimal yields of foreign protein from scaled-up BES infected insect cells. This method is expensive and can be justified for use in industry, but the balance of cost versus yield will lie at the users discretion. Cheaper alternatives (shaker culture) are available, but levels of foreign protein may be compromised.

Intracellular foreign protein production was also analysed from an improved stable insect expression system to determine if the levels produced were sufficient enough to use as an alterative to the BES, if scaled-up. However, the levels produced from the stable insect cell lines were insufficient for obtaining cost-effective yields of recombinant protein, therefore strengthening the cost effectiveness of the BES for producing large amounts of intracellular foreign protein.

Subsequently, the stable insect cell line *T.ni* (Hi5).*DsRed* was used to develop a protocol for large-scale, long-term cultivation of stable insect cell lines. The success of this work will provide researchers with an alternative tool for scaling-up stable insect cells expressing heterologous protein, with the aim of improving yields (e.g. secreted recombinant proteins).

CHAPTER 6: FINAL DISCUSSION AND FUTURE WORK

The work in this thesis was undertaken to address and improve various problems allied with the production of intracellular recombinant proteins in insect cells, in particular with a focus on the industrial sector. The production of heterologous proteins is an elemental facilitator in drug discovery programmes for the identification of new therapeutics. The cost of producing therapeutics is an expensive process, which is reflected by "on the shelf" sale prices. Therefore, developing methods to reduce the cost of producing therapeutics would subsequently impact their economic value.

One of the key problems pharmaceutical companies are facing is the need to increase compound testing *in vitro*, due to high throughput programs in place producing vast numbers of compound libraries. The cost of producing heterologous proteins for *in vitro* testing using mammalian and viral systems, is both expensive and labour-intensive. Therefore, cheaper alternatives for producing high-levels of recombinant protein, especially those requiring post-translational modifications, are in high demand (e.g. for structural genomics / drug screening *in vitro*).

Work previous to this thesis has demonstrated that insect cell lines (e.g. lepidopteran) can be used successfully for foreign protein production. Insect cells possess the capability to perform most post-translational modifications performed by mammalian cells. Compared to mammalian cells, insect cells also have a faster growth rate and require less complex growth conditions, which inevitably can reduce the cost of foreign protein production. Overall, these properties make insect cells strong candidates as alternative hosts for the production of recombinant proteins.

The most widely used expression system using insect cells is the BES (Section 1.3.2). However, the BES encompasses a number of limitations (lytic system, transient expression) that limits its use for foreign protein production (e.g. producing high-levels of secreted proteins; Jarvis *et al.*, 1990). An alternate expression system, stably transformed insect cell lines, was subsequently developed to overcome the limitation of the BES (Jarvis and Summers, 1989). This system has shown particular promise for the production of secreted foreign proteins (Jarvis *et al.*, 1990, 1996). However, very low levels of intracellular recombinant protein were recorded (Jarvis and Summers, 1989; Jarvis *et al.*, 1990).

Since these studies, an increased number of RNA polymerase II transcribing promoters have been identified and characterised for transient / stable foreign gene expression in

eukaryotic cells. The initial aim of this project was to characterise the transcriptional activity of a number of these promoters (eukaryotic / viral) in lepidopteran cell lines (*Sf*9 / *T.ni* Hi5 cells; Chapter 3). The developed aim led to production of a comprehensive promoter analysis, which identified promoters with strong transcriptional activity that may be used to generate stably transformed *Sf*9 / *T.ni* Hi5 cell lines, or alternatively for the development of new plasmid-based expression cassettes (as described in Chapter 4).

The continuous insect cell expression system developed by Jarvis and Summers (1989) utilises the *Ac*MNPV *ie*-1 promoter to transcribe foreign genes. However, previous studies have characterised this immediate early baculovirus promoter as transcriptionally weak in insect cell lines (Jarvis *et al.*, 1990; Joyce *et al.*, 1993; McCarroll, 1997). Therefore, in terms of high-level intracellular heterologous gene expression, it was determined that the *Ac*MNPV *ie*-1 promoter would be of limited use. However, expression levels from the *Ac*MNPV *ie*-1 promoter were used throughout this study as basal levels for comparison.

Work in this thesis to examine the transcriptional activity of alternative eukaryotic and viral promoters in *Sf*9 / *T.ni* Hi5 cells has focused on gene promoters that have previously demonstrated moderate to high levels of foreign protein production in insect (dipterans / lepidopteran) and other expression systems (mammalian [Sections 3.3, 3.4]). It was anticipated that if these promoters were functional in *Sf*9 or *T.ni* Hi5 cells, high levels of expression would be obtained. In order to rapidly test the chosen promoters to give an indication of how well each functioned, the *cat* gene was placed under control of each promoter (Section 3.3) and the expression cassettes tested in transient assays (Section 3.4).

The *CMVie* promoter has been previously characterised as a strong promoter in mammalian cell lines and therefore functions well in mammalian expression systems (Chan *et al.*, 1996). However, the *CMVie* promoter did not result in significantly improved gene expression over that exhibited by using *Ac*MNPV *ie*-1 in both *Sf*9 or *T.ni* Hi5 cells. Two *Dm* gene promoters *Ac*5.1 (constitutive) and *MT* (inducible), were also tested in *Sf*9 and *T.ni* Hi5 cells, but no detectable levels of CAT activity were observed from these promoters (*Dm MT* non-induced / induced). These results demonstrated that the transcriptional activities of *CMVie*, *Dm Ac*5.1 and *MT* gene promoters were extremely low in the lepidopteran cell lines used, suggesting that these promoters are not amenable to generating stably transformed lepidopteran cell lines for the efficient production of intracellular foreign proteins.

Since the characterisation of insect cell TF (e.g. host RNA polymerase II) transcribing foreign genes from the *Ac*MNPV *ie*-1 promoter (Jarvis and Summers, 1989; Jarvis *et al.*, 1990), a number of other baculovirus gene promoters (*Ac*MNPV *ie*-n; *Op*MNPV *ie*-1; *Op*MNPV *ie*-2; *Bm*NPV *ie*-1) have been shown to function via the same cellular mechanism (Theilmann and Stewart, 1991, 1992a; Vulsteke *et al.*, 1993; Lu *et al.*, 1996, 1997). Studies by other research groups have shown that the *Op*MNPV *ie*-2 gene promoter is transcriptionally active in *Sf*9 and *T.ni* Hi5 cells. A promoter isolated from the *BmA*3 gene, has also been characterised as transcriptionally active in lepidopteran and dipteran cells, but as a weak promoter (Johnson *et al.*, 1992). However, studies by Lu *et al.* (1996, 1997) have shown that the transcriptional activity of *Bm A*3 in lepidopteran cells is enhanced by the presence of *Bm*NPV TF, IE and *hr*3. These finding prompted the testing of both the *Op*MNPV *ie*-2 and *BmA*3+E promoters in this study. The levels of transcriptional activity detected from these promoters were shown to significantly improve gene expression over that demonstrated by *Ac*MNPV *ie*-1 in *Sf*9 and *T.ni* Hi5 cells. Relative to *Ac*MNPV *ie*-1, these results clearly show both *Op*MNPV *ie*-2 and *BmA*3+E as stronger promoters in lepidopteran cells, which subsequently could be used to generate stable *Sf*9 and *T.ni* Hi5 cells for the production of intracellular foreign protein.

For future studies, work to improve foreign gene expression levels from plasmid-based cassettes in insect cell lines may focus on identifying new baculovirus immediate early / delayed early and insect cellular promoters that are transcribed by insect RNA polymerase II, as well as enhancers that may up-regulate new and existing promoters. Subsequent identification of new promoters / enhancers may be characterised for transcriptional activity across a range of insect cell lines to optimise promoter specificity for host RNA polymerase II / TF. In contrast, the late and very late baculovirus gene promoters are known to be particularly strong. Approximately 50% of the total cell protein in an *in vitro* infection is the product of the late and very late promoters (*p*10 and *polh*). Baculovirus gene expression is regulated as a cascade system; each phase depends upon the presence of viral gene products of the proceeding phase (Section 1.3.2). Therefore, identifying all the essential genes involved in the *trans*-activation of the *p10* and *polh* promoters, and discarding the non-essential genes may provide a method for harnessing the strong activity of these promoters in a plasmid-based vector.

Through intense research, Lois K. Miller and collaborators recognised the potential for developing such a vector, and to date have identified nineteen baculovirus open reading frames that support late gene expression within plasmid-based vectors (Li *et al.*, 1993;

Passarelli and Miller, 1993a, b, c; Lu and Miller, 1994; Morris *et al.*, 1994; Passarelli and Miller, 1994; Passarelli *et al.*, 1994; Lu and Miller, 1995). The open reading frames include *ie*-1, *ie*-2, late expression factors 1-12, *p143*, *dnapol*, *p35*, *p47*, *and 39k* (Rapp *et al.*, 1998; Li *et al.*, 1999). Once all known essential genes have been identified and in which order they *trans*-activate one another, to finally *trans*-activate *p10* and *polh* promoters, expression vectors may be designed either on the basis of expressing constitutively or by induction (chemically / physical stimulus).

The second aim of this project was to endeavour to develop a novel stable insect inducible expression system (Chapter 4). The problems associated with existing insect inducible expression systems is the lack of complete promoter suppression. Both the *Dm MT and Hsp* 70 gene promoters have been characterised as inducible promoter, but retain a low to high constitutive activity in a non-induced state (Johansen *et al.*, 1989; Clem and Miller, 1994; Prikhod'ko and Miller, 1996; McCarroll, 1997; Hegedus *et al.*, 1998). Therefore, expression systems utilising these promoters lack the specificity required to regulate cytotoxic genes successfully in insect (dipteran) cells.

Work by Wu *et al.* (2000) devised a method for the stringent regulation of foreign gene expression in insect cells using a modified tetracycline-regulated gene expression system (TRES; Section 4.1, 4.11). However, this system utilises the late *p10* baculovirus promoter to indirectly induce gene expression from the minimal *CMVie* promoter in insect cells (*CMVie* promoter has been characterised in this thesis as transcriptionally weak in insect cells). Due to the lytic-nature of the BES, induction of foreign gene expression can only exist transiently. Therefore, this system may have limited use for the production of cytotoxic genes that may cause detrimental effects to cells hosting expression, as viral infection will inevitably kill the host cell at 72-96 hpi. One could argue the BES is an inducible system itself, therefore, as an alternative it may be simpler to express cytotoxic genes using the BES.

On the other hand, the development of a plasmid-based TRES for generating stable insect cell lines with inducible expression may appear more attractive economically for the production of cytotoxic genes. The main disadvantage of the BES is the transient expression of foreign genes. Even though high-levels of recombinant protein can be obtained, this system is extremely labour-intensive, compared with having an immortal stable insect cell line, which may be induced to transcribe foreign genes. The work by Wu *et al.* (2000) highlights a number of key issues for adapting the TRES for use in insect

cells, which were addressed in the current study. The main issue addressed was the necessity to substitute the *CMVie* promoters from the tetracycline regulatory and response cassettes, with strong promoters functionally active in insect cells.

The promoter comparison study previously described (Chapter 3) characterised two promoters with strong transcriptional activity in lepidopteran cells; the *Op*MNPV *ie*-2 and *BmA*3+E promoters. These promoters were subsequently determined as potential candidates for the development of the stable insect TRES.

The first critical component of this system, the insect tetracycline regulatory plasmid, was developed using the *BmA*3+E promoter. The regulatory gene-coding sequence *rtTA* was amplified from pTet-On (mammalian tetracycline regulatory vector) and inserted downstream of the *BmA*3+E promoter, to derive p*BmA*3+E.rtTA (Section 4.2). Transient expression of rtTA from p*BmA*3+E.rtTA revealed rtTA was successfully transcribed in both *Sf*9 and *T.ni* Hi5 cells (Section 4.3). Subsequent to these results, stably transformed *Sf*9 and *T.ni* Hi5 cells expressing *rtTA* were generated and rtTA production analysed (Section 4.4). The levels of rtTA produced from these cell lines were highly consistent, demonstrating the successful establishment of *Sf*9.*rtTA* and *T.ni* (Hi5).*rtTA* cell lines, which represent the first functional components necessary for developing the stable insect TRES.

The second essential constituent constructed to complete the development of the stable insect TRES was the insect tetracycline response vector. While constructing this vector a number of problems were encountered, which were successfully overcome (as described in Section 4.5, 4.6). The insect tetracycline response vector was derived by modifying the mammalian tetracycline response vector (pTRE), substituting the minimal *CMVie* promoter for the *Op*MNPV *ie*-2 promoter (pTREΔ.*ie*2). It was hypothesised that the *TRE* would serve to regulate the transcriptional activity of the *Op*MNPV *ie*-2 promoter and, therefore, adapt this vector for use in insect cells (e.g. *Sf*9.*rtTA* and *T.ni* (Hi5).*rtTA* cell line). The reporter gene *cat* was placed under control of the *TRE* / *Op*MNPV *ie*-2 promoter and the expression cassette tested in transient assays (pTREΔ.*ie*2.*cat*2; Section 4.7-10).

Repressing the constitutive activity of the *Op*MNPV *ie*-2 promoter using the *TRE* in *Sf*9 / *Sf*9.*rtTA* and *T.ni* Hi5 / *T.ni* (Hi5).*rtTA* cells was successful in the absence of the inducer (dox). Complete suppression was not observed, but the *TRE* was estimated to reduce the

transcriptional activity of *Op*MNPV *ie*-2 by 99% in *Sf*9 / *Sf*9.*rtTA* and 98% in *T.ni* Hi5 / *T.ni* (Hi5).*rtTA* cell lines, compared with the normal constitutive activity of *Op*MNPV *ie*-2 in these cell lines (Section 4.7). However, it was not possible to induce the transcriptional activity of the *TRE* / *Op*MNPV *ie*-2 promoter in the presence of dox using the cell lines described above (Section 4.8). Experimental work carried out to resolve this problem (as discussed in Sections 4.9-10), addressed the strong possibility of additional DNA sequences present in pTREΔ.*ie*2.*cat*2, other than *TRE*, which have the ability to facilitate the repression of *Op*MNPV *ie*-2 transcriptional activity. However, these DNA sequences were not elucidated.

Further avenues of exploration for the development of a functional insect tetracycline response vector involve the removal of *TRE* (x7 *tetO* repeats) from pTRE for insertion upstream / adjacent the *Op*MNPV *ie*-2 promoter in pIZT.V5-His.*cat* (described in Section 3.3.4; Invitrogen™) or pRescue.*cat*2 (constructed to restore *Op*MNPV *ie*-2 activity; Section 4.10). The *Op*MNPV *ie*-2 promoter has been characterised as transcriptionally active in these vectors, here the problem of additional DNA sequences that suppress the activity of *Op*MNPV *ie*-2 (as observed in pTREΔ.*ie*2.*cat*2) is thought not to be an issue. As a result, *TRE* alone should suppress *Op*MNPV *ie*-2 in the absence of dox and importantly, activate the *TRE* / *Op*MNPV *ie*-2 promoter in the presence of dox, to overcome the problems described in Sections 4.9-10.

An alternative method for developing a new stable insect inducible expression system, to that describe in this thesis, may be accomplished by adapting the mammalian TRES (T-REx™; Invitrogen™) developed by Yao *et al.* (1998). The T-REx™ system works on the principle of positioning two juxtaposed *tetO* sequences between the TATA box and the transcriptional start site of the full-length *CMVie* promoter. The *tetO* sequences themselves have no effect on expression (Yao *et al.*, 1998). However, when the tetracycline repressor protein (tetR) is present, it effectively binds the two *tetO* sequences and blocks transcription initiation. The addition of Tc subsequently changes the conformation of the TetR protein, which causes TetR to dissociate from the *tetO* sites and activate transcription from the *CMVie* promoter (Yao *et al.*, 1998; Yao and Eriksson, 1999). Therefore, it may be possible to use the *Op*MNPV *ie*-2 or *BmA*3+E promoters to adapt this system for use in insect cells.

A recent publication by Swevers *et al.* (2004), describes briefly the development of a new inducible plasmid-based expression cassette for use in *Bm*5 cells, which was designed for a cell-based high-throughput screening (HTS) system for detecting ecdysteroid agonists and antagonists in plant extracts and libraries of synthetic compounds. The inducible expression cassette was developed using a basal actin promoter (*BmbA*) derived from the silkmoth actin gene. Seven juxtaposed ecdysone-responsive elements (*ERE*) were subsequently cloned upstream of the *BmbA* promoter, which transformed it into an inducible promoter (Luc Swevers; personal communication). The inducible expression vector derived, p*BmbA/ERE.cat*, was transiently tested in *Bm*5 cells for the induction of the *BmbA* promoter with the addition of the inducer, active moulting hormone 20-hydroxy-ecdysone (20E). Following transfection of p*BmbA/ERE.cat* into *Bm*5 cells, the addition of 20E resulted in a dose-dependent stimulation of *cat* reporter gene activity. Maximal levels of inducibility were ~ 1800-fold; the effective concentration (EC_{50}) of 20E for the *ERE*-dependent reporter was between 75 and 100nM (Swevers *et al.*, 2004).

It may be possible to broaden the applications of the 20E-inducible expression cassette, such as generating stable insect cell lines, from which the production of foreign proteins are regulated for therapeutic use (e.g. cytotoxic genes). A particular merit of this inducible system is its simplicity, which entails the generation of single-stable insect cell lines, compared with the tetracycline-regulated expression system, which requires the generation of double-stable insect cell lines for the production of recombinant protein (Section 4.1). However, this system does have one main disadvantage, which focuses primarily on the promoter used to express foreign genes (*BmbA* promoter). The *BmbA* promoter is functionally active in insect cells (Johnson *et al.*, 1992; Swevers *et al.*, 2004); but the overall transcriptional activity from this promoter compared with that of the *BmA*3+E promoter (characterised as a strong promoter in lepidopteran cells, Section 3.4) is three orders of magnitude lower (Lu *et al.*, 1997). Therefore, in order to utilise the 20E-inducible expression system for the production of foreign proteins in insect cells, it may be necessary to substitute the *BmbA* promoter with either the stronger *BmA*3+E or *Op*MNPV *ie*-2 promoter (*Op*MNPV *ie*-2 has been characterised as possessing comparative transcriptional activity to that of *BmA*3+E lepidopteran cells; Section 3.4). As a result, these promoters may be transformed by the *ERE* into inducible promoters, which retain their stronger transcriptional activity.

The final aim of the work in this thesis was to investigate methods of improving intracellular protein production using insect expression systems. Initial work in Chapter 5

focuses on using the BES to achieve this. It has been well documented that the baculovirus expression vector *Ac* (Section 5.1) can produce high-levels of intracellular recombinant protein (nuclear and cytoplasmic; Vlak *et al.*, 1998; Stewart and Champoux, 1999; Ikonomou *et al.*, 2003; Jayakumar *et al.*, 2004). However, the levels of secreted and membrane targeted foreign proteins produced from *Ac* are usually low (Jarvis and Summers, 1989; Jarvis *et al.*, 1990; Thomas, 1997; Saville *et al.*, 2002). The deletion of a non-essential viral gene (*chi*A) from *Ac* (*Ac.ΔchiA*; Hawtin *et al.*, 1995), which is not required for virus replication in insect cell culture, has subsequently improved the yields of secreted and membrane targeted recombinant proteins produced (Section 5.1; McCarroll, 1997). However, no published data exists to address whether *Ac.ΔchiA* improves the yields of intracellular foreign protein; therefore, a study to address this was carried out.

In order to compare the levels of intracellular protein produced from both *Ac* and *Ac.ΔchiA*, *DHODH* or *DsRed* gene coding-regions were inserted under control of the *polh* promoters. The data presented in this thesis for this comparison, is the first acknowledged study of its kind (Sections 5.2, 5.7).

The infection of *Sf*9 and *T.ni* Hi5 cells with the resulting recombinant viruses (*Ac.DsRed*, *Ac.DHODH*, *Ac.ΔchiA.DsRed* and *Ac.ΔchiA.DHODH*) demonstrated improved levels of intracellular DHODH from *Ac.ΔchiA.DHODH* infected *T.ni* Hi5 cells, compared to levels detected in *Ac.DHODH* infected *T.ni* Hi5 cells. DHODH was not detected from either recombinant virus in *Sf*9 cells. Detection of DHODH from these viruses in *T.ni* Hi5 cells also demonstrated that *Ac.DHODH* produced significantly higher levels of degrading DHODH than *Ac.ΔchiA.DHODH*. Therefore, it may be suggested that the removal of *chiA* from *Ac* indirectly reduced proteolysis of DHODH. On the contrary, the levels of intracellular DsRed in *Ac.DsRed* infected *Sf*9 and *T.ni* Hi5 cells was higher than that detected in *Ac.ΔchiA.DsRed* infected insect cell lines.

These results demonstrate that *Ac.ΔchiA* may be used as a new baculovirus expression vector for improving yields of intracellular protein. However, the use of this vector may be dependent on the nature of the protein produced and the cell line used to host expression. Therefore, these results verify the importance of optimising conditions for the expression of each recombinant gene. The potential for improved foreign protein production, following deletion of viral genes that have been found to be non-essential for virus replication in cell culture, has been confirmed and opens the possibilities for similar studies in the future.

A number of studies by other laboratories have demonstrated improved quality and yields of intracellular / secreted foreign proteins from the BES, by the addition of protease inhibitors to culture medium to prevent proteolysis (Grosch, 1998; Naggie and Bentley, 1998; Hu and Bentley, 1999; Martensen and Justesen, 2001). However, the identification and characterisation of baculovirus cathepsin (*v-cath*), a cysteine protease and a non-essential gene for virus replication in cell culture (Slack *et al.*, 1995), has lead to the development of a baculovirus expression vector (BacVector-3000; Novagen) from which *v-cath*, *chiA*, and five other viral genes have been removed. Heterologous proteins produced using this virus are cited as exhibiting improved stability at 4°C. However, improved recombinant protein secretion confirmed by McCarroll (1997) and intracellular protein production demonstrated in this thesis as a result of the absence of *chiA* has not yet been reported for BacVector-3000.

To meet the needs of industrial requirements for the production of recombinant protein for the post-genomic era, simple, rapid and efficient expression systems are required. Both mammalian and viral expression systems fall short of these objectives. Recombinant protein production from mammalian systems is a labour-intensive process, although these systems are a necessity for producing complex foreign proteins. The downfall of both the mammalian and viral systems is their complexity, which makes them not amenable to high through-put applications. Stable insect cell lines are an attractive alternative, possessing fast growth, simple generation, and adaptability for high throughput applications, which include generating thousands of clones that can be screened to determine cells expressing high-levels of recombinant protein. However, the main disadvantage of using stable insect cell lines (lepidopteran) for the production of intracellular recombinant protein, is the inherently low levels they produce, which subsequently has been contributed to the weak promoters used to express foreign genes (*Ac*MNPV *ie*-1).

The characterisation of insect / viral promoters (*BmA*3+E / *Op*MNPV *ie*-2) with stronger transcriptional activity, than the weak *Ac*MNPV *ie*-1 promoter (Chapter 3), lead to the development of stable insect cell lines (*Sf*9 and *T.ni* Hi5) expressing intracellular foreign genes (intracellular DHODH or DsRed) from the *BmA*3+E promoter (sections 5.5). The levels of recombinant protein produced were determined and subsequently compared to levels from the BES, thereby, determining whether the developments over the last decade, to improve intracellular foreign protein production from stable insect cell lines, are sufficient to use as an alternative to the BES.

Stably transformed *Sf*9 and *T.ni* Hi5 cells expressing intracellular DsRed were initially identified by Western blot and fluorometric analyses (Section 5.5). The production of intracellular DsRed in *T.ni* (Hi5).*DsRed* cells was observed by both methods of analyses. Intracellular DsRed detected by Western blotting produced a very weak signal intensity, indicating that the levels produced were significantly low. However, intracellular DsRed produced in *Sf*9.*DsRed* cells was only detected by fluorometric analyses. The data obtained from fluorometric testing indicated that DsRed expression was significantly higher in *T.ni* (Hi5).*DsRed* cells compared to that observed in *Sf*9.*DsRed* cells ($p=0.0001$, in all cases).

A comparison of intracellular DsRed production from both *Sf*9.*DsRed* and *T.ni* (Hi5).*DsRed* cell lines with that from the BES (optimised conditions; *Ac.DsRed* infected *Sf*9 cells, 72hpi), showed that DsRed expression from *Ac.DsRed* in *Sf*9 cells was 52.5-fold greater than those detected from *T.ni* (Hi5).*DsRed* cells, and 262.9-fold higher than those observed from *Sf*9.*DsRed* cells. From these data it was concluded that DsRed production from *Sf*9.*DsRed* and *T.ni* (Hi5).*DsRed* cell lines was not comparable to those observed from baculovirus expression.

Stable insect cell lines (*Sf*9 and *T.ni* Hi5) expressing intracellular DHODH were also generated and analysed using Western blot analyses. However, DHODH was not detected from either cell line (Section 5.5). Subsequent analyses of total cellular RNA from both cell lines indicated that DHODH mRNA was produced, therefore demonstrating that DHODH was expressed from the *BmA*3+E promoter, but at extremely low levels. The results presented in this thesis indicate that, at present, stably-transformed insect cells expressing intracellular foreign proteins are not a viable alternative to baculovirus expression using the conditions and heterologous genes described.

As previously stated (Sections 5.1, 5.10) yields of secreted recombinant proteins expressed from stable insect cell lines have been reported to be equal to or greater than those produced from the BES. Therefore, to maximise the production of secreted recombinant protein from stable insect cell lines, cultures should be continuously cultivated at scaled-up volumes. Subsequently, a method to successfully scale-up the continuous cultivation of stable insect cell lines was investigated. This work was based on using the *T.ni* (Hi5).*DsRed* cell line (Section 5.5) as a model for culture dynamics (cell growth / viability; Section 5.6). *T.ni* (Hi5).*DsRed* cells were expanded to generate a 1-litre culture, which was inoculated into a 3-litre fermenter vessel at a low cell density ($0.5x10^6$/ml) using

the fill and draw technique described in Section 5.6 (**Figure 5.16A-C**). The *T.ni* (Hi5).*DsRed* cell culture was successfully maintained (requiring a total of six passages) as a viable 1-litre open fermenter culture for four weeks. Continuous intracellular DsRed production was also observed (data not shown). The development of this protocol may provide useful data for future work involving the scale-up of continuous stable insect cell line cultivation. To develop this work, it would be of interest to repeat the work described above using stable insect cell lines constitutively expressing secreted foreign proteins. This process would address the levels of secreted foreign proteins produced using the open fermenter system in comparison to those produced from the BES (fermenter culture), and to what order of magnitude expression can be elevated that being the case.

In order to maintain the importance of baculoviruses as expression vectors, it was essential to investigate means by which baculovirus-mediated foreign gene expression could be improved. Previous studies have demonstrated the importance of optimising conditions for each recombinant gene expressed (Section 5.2; Lynn and Hink, 1980). It has been demonstrated that yields of many recombinant proteins can be significantly increased by maintaining the dO_2 concentration of baculovirus infected insect cultures (Lindsay and Betenbaugh, 1992; Scott *et al.*, 1992; Malinowski and Daugulis, 1993; Wang *et al.*, 1996; Taticek and Shuler, 1997; Hu and Bentley, 1999). Therefore, two different insect cell cultivation methods (shake cultures and fermentation, as described in Sections 5.1, 5.7, 5.10), used to maintain recombinant baculovirus infections, were subsequently investigated to determine their effects on foreign protein productivity (Sections 5.7-5.9). The recombinant baculoviruses used to infect *Sf*9 and *T.ni* Hi5 cells included; *Ac.DsRed*, *Ac.DHODH*, *Ac.ΔchiA.DsRed* and *Ac.ΔchiA.DHODH*.

The expression profiles of intracellular DHODH / DsRed from infected insect fermenter / shaker cultures were shown to correlate with the data described in Section 5.2 (*Ac.ΔchiA.DHODH* infected *T.ni* Hi5 cells produced optimal levels of intracellular DHODH; *Ac.DsRed* infected *Sf*9 cells produced optimal levels of intracellular DsRed). However, the levels of intracellular DHODH / DsRed produced by fermenter and shaker culturing were shown to significantly differ. The highest levels of intracellular DHODH / DsRed produced were detected from infections maintained as fermenter cultures. These infected cultures were sustained at a minimum dO_2 concentration of 60%, as opposed to the infected shaker cultures, which did not possess the ability to control the culture dO_2 concentration. Therefore, the dO_2 concentration of the shaker culture was rapidly depleted, which

inevitably was shown to effect the overall yields of DHODH / DsRed produced. Quantitative analyses of DHODH production (Section 5.8) demonstrated that *Ac.ΔchiA.DHODH* infected *T.ni* Hi5 fermenter culture produced 7.2-fold more DHODH than that produced by *Ac.ΔchiA.DHODH* infected *T.ni* Hi5 shaker culture, and 1.8-fold more DHODH than that generated by *Ac.DHODH* infected *T.ni* Hi5 fermenter culture.

The results of this study correlate with published reports, which demonstrate that the dO_2 concentration of recombinant virus infected insect cell culture is an important factor for maintaining high-level foreign protein production (Scott *et al.*, 1992; Taticek and Shuler, 1997; Hu and Bentley, 1999). Other factors also found to affect baculovirus recombinant protein production include; availability of specific medium constituents ([glucose] Raghunand and Dale, 1999; [glutamine] Hu and Bentley, 1999) and the addition of specific protease inhibitors to infected cultures (Grosch, 1998; Naggie and Bentley, 1998; Hu and Bentley, 1999; Martensen and Justesen, 2001).

It is clear from the data presented in this thesis that the BES is still a very powerful tool for producing intracellular recombinant proteins. The method used to generate the recombinant viruses (BacPAK6) for the work in this thesis is not amenable to high throughput applications due to its complexity, which ideally is what industry aspire to use. However, a new baculovirus expression system based on *Ac.ΔchiA* has been recently developed in our laboratory in collaboration with Professor Robert Possee (Centre for Ecology and Hydrology Oxford), which meets the requirements of industry in the post genomic era. This system (*flash*BAC; Oxford Expression Technologies) is simple to use, generating recombinant viruses in a one step process; insect cells are co-transfected with *flash*BAC DNA and transfer vector, incubate at 28°C for 5 days and harvested. There is no need to isolate recombinants virus from parental virus via skilled and tedious methods such as plaque assays, as this system generates 100% recombinants. Production of high-titre recombinant virus stocks is rapid, reducing the time from 17-20 days using traditional methods (BakPAK6) to 7-10 days using *flash*BAC. *flash*BAC also retains the advantages of *Ac.ΔchiA*, which increases yields of secreted and membrane targeted proteins as well as specific intracellular proteins.

This system is also importantly amenable to high throughput applications. Work in our laboratory has automated the production of recombinant viruses in a 24-well plate and is currently evaluating automated trials to produce recombinants using a 96-well format. It is

possible to produce multiple copies of a single recombinant virus or different recombinants using the automated technology.

The limitation of the BES to produce humanised recombinant proteins is also another disadvantage of this system. However, work by Donald Jarvis and collaborators have recently addressed this problem by developing new lepidopteran insect cell lines, which constitutively express mammalian glycosyltransferase genes. Subsequently this has yielded transgenic insect cell lines with normal growth properties that can support baculovirus infection, have new *N*-glycan processing enzyme activities, and can produce humanised recombinant glycoproteins (Section 1.1).

These new developments in insect expression technology may be used to expand the range of recombinant protein produced, therefore reducing the dependence on mammalian expression systems to express complex foreign proteins; and, in turn, reduce the overall costs of producing recombinant protein.

In conclusion, this thesis has demonstrated advances made over the last decade, to improve stable insect expression systems following the identification and characterisation of novel promoters, has enhanced intracellular recombinant protein production. Secondly, these advancements have aided the groundwork for development of a new stable insect inducible expression system. Finally, the development of stably-transformed insect cell lines, utilising promoters with stronger transcriptional activity than conventional insect expression systems, still have limited potential for producing intracellular foreign proteins at present, when compared to the BES. However, the inherent advantages continuous and inducible insect expression systems possess over transient systems (BES), is expected to drive continued development of these systems; thereby, further elevating yields of intracellular recombinant protein produced. At the present time it is improbable that continuous expression will supplant the efficient use of the BES. A plethora of intracellular recombinant proteins have been expressed successfully using the BES and with developments and improvements in foreign protein production described recently, this system will undoubtedly lead to further popularity for the expression of foreign genes in the post genomic era.

References

Acharya, A. and Gopinathan, K.P. (2001) Identification of an enhancer-like element in the polyhedrin gene upstream region of *Bombyx mori* nucleopolyhedrovirus. *J. Gen. Virol.* **82**:2811-2819.

Adams, J.R. and McClintock, J.T. (1991) Nuclear. Polyhedrosis viruses of insects. *In* "Atlas of Invertebrate Viruses". Ed. Adams, J.R. and Bonami, J.R. CRC Press, Boca Raton, FL:87-204.

Adhya, S. and Gottesman, M. (1982) Promoter occlusion: transcription through a promoter may inhibit its activity. *Cell.* **29(3)**:939-944.

Agathos, S.N., Jeong, Y-H. and Venkat, K. (1990) Growth kinetics of free and immobilized insect cell cultures. *Ann. NY. Acad. Sci.* **589**:372-398.

Ailor, E. and Betenbaugh, M. (1999) Modifying secretion and post-translational processing in insect cells. *Current Opinion in Biotechnology.* **10**:142-145.

Ailor, E., Pathmanathan, J., Jongbloed, J.D.H. and Betenbaugh, M.J. (1999) A bacterial signal peptidase enhances processing of a recombinant single chain antibody fragment in insect cells. *Biochem Biophys Res Comm.* **255**:444-450.

Alam, J. and Cook, J.L. (1990) Reporter genes: application to the study of mammalian gene transcription. *Anal Biochem.* **188(2)**:245-254.

Alberts, B., Johnson, A., Raff, M., Roberts, K. and Walter, P. (2002) How cells read the genome: From DNA to protein. *Molecular Biology of the Cell.* 4th Ed. Garland Publishing, New York, USA.

Altmann, F., Staudacher, E., Wilson, I.B.H. and Marz, L. (1999) Insect cells as hosts for the expression of recombinant glycoproteins. *Glycoconj. J.* **16**:109-123.

Arif, B.Y. and Doerfler, W. (1984) Identification and localisation of sequences in the *Choristoneura fumiferana* MNPV genome. *EMBO. J.* **3**:525-529.

Atchison, M.L. (1988) Enhancers: mechanisms of action and cell specificity. *Ann. Rev. Cell. Biology.* **4**:127-153.

Atkinson, A.E., Henderson, J., Hawes, C.R. and King, L.A. (1996) Efficient membrane targeting of the chick nicotinic acetylcholine receptor α-subunit in stable insect cell lines. *Cytotechnology.* **19**:37-42.

Atkinson, A.E., Bermudez, I. Darlinson, G.M., Barnard, E.A., Earley, F.G.P., Possee, R.D., Beadle, D.J. and King, L.A. (1992) Assembly of functional $GABA_A$ receptor in insect cells using baculovirus expression vectors. *Molecular Neuroscience.* **3(7)**:597-600.

Auble, D.T. and Hahn, S. (1993) An ATP-dependent inhibitor of TBP binding to DNA. *Genes Dev.* **7(5)**:844-856

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl. (1997) *Current Protocols in Molecular Biology.* John Wiley and sons, New York.

Ayers, M.D., Howard, S.C., Kuzio, J. Lopez-Ferber, M. and Possee, R.D. (1994) The complete DNA sequence of *Autographa californica* nuclear polyhedrosis virus. *Virology.* **202**:586-605.

Bader, B., Knecht, W., Fries, M. and Löffler, M. (1998) Expression, purification, and characterization of histidine-tagged rat and human flavoenzyme dihydroorotate dehydrogenase. *Protein Express. Purif.* **13**:414-422.

Baim, S.B., Labow, M.A., Levine, A.J. and Shenk, T. (1991) A chimeric mammalian transactivator based on the *lac* repressor that is regulated by temperature and isopropyl β-D-Thiogalactopyranoside. *Proc. Natl. Acad. Sci. USA.* **88**:5072-5076.

Baird, G.S., Zacharias, D.A. and Tsien, R.Y. (2000) Biochemistry, mutagenesis, and oligomerization of DsRed, a red fluorescent protein from coral. *Proc. Natl. Acad. Sci. USA.* **97**:11984-11989.

Baldick, C .J., Cassetti, M.C., Harris, N. and Moss, B. (1994) Ordered assembly of a functional preinitiation transcription complex containing vaccinia virus early transcription factor and RNA polymerase on a immobilised template. *J. Virol.* **68(4)**:6052-6056.

Baron, U., Gossen, M. and Bujard, H. (1997) Tetracycline-controlled transcription in eukaryotes: novel transactivators with graded transactivation potential. *Nucleic Acids Revs.* **14**:2723-2729.

Barr, P.J. (1995) Mammalian cell technology: secretion on demand. *Bio/technology.* **13**:1158-1159.

Barras, F. and Marinus, M.G. (1989) The great GATC: DNA methylation in *E.coli. Trends Genet.* **5(5)**:139-143.

Bates, A.D. and Maxwell, A. (1993) DNA topology. IRL Press, Oxford University Press. Ed. Rickwood, D.

Baylin, S.B. and Herman, J.G. (2000) DNA hypermethylation in tumorigenesis: epigenetics joins genetics. *Trends. Genet.* **16**:168-174.

Bebbington, C.R., Renner, G., Thompson, S., King, D., Abrams, D. and Yarranton, G.T. (1992) High-level expression of a recombinant antibody from myeloma cells using a glutamine synthetase gene as an amplifiable selectable marker. *Bio/Technology.* **10**:169-175.

Beckett, D. (2001) Regulated assembly of transcription factors and control of transcription initiation. *J. Mol. Biol.* **314(3)**:335-352.

Bedard, C., Tom, R. and Kamen, A. (1993) Growth nutrient consumption, and end-product accumulation in Sf-9 and BTI-EAA insect cell culture: insight into growth limitation and metabolism. *Biotechnol. Prog.* **9(6)**:615-624.

Beggs, J.D., van der Berg, J., van Ooyen, A. and Weissmann, C. (1980) Abnormal expression of chromosomal rabbit β-globin gene in *Saccharomyces cerevisiae*. *Nature.* **283**:835-840.

Bello, B., Resendez-Perez. and Gehring, W.J. (1998) Spatial and temporal targeting of gene expression in *Drosophila* by means of a tetracycline-dependent transactivator system. *Development.* **125**:2193-2202.

Belyaev, A.S. and Roy, P. (1993) Development of baculovirus triple and quadruple expression vectors: co-expression of three or four bluetongue virus proteins and the synthesis of bluetongue virus-like particles in insect cells. *Nucleic Acids Res.* **21(5)**:1219-1223.

Benincasa, D., Silberklang, M., Mark III, G.E. and Ludmerer, S.W. (1996) Rapid, high-level transient expression of papillomavirus-like particles in insect cells. *BioTechniques.* **20**:890-895.

Berg, D.T., McClure, D.B. and Grinnel, B.W. (1993) High-level expression of secreted proteins from cells adapted to serum-free suspension culture. *BioTechniques.* **14**:972-978.

Berg, O.C. and von Hippel, P.H. (1988) Selection of DNA binding sites by regulatory proteins. *TIBS.* **13**:207-211.

Berglund, P., Sjöberg, M., Garoff, H., Atkins, G.J., Sheahan, B.J. and Liljeström, P. (1993) Semliki forest virus expression system: production of conditionally infectious recombinant particles. *Bio/Technology.* **11**:916-920.

Bishop, D.H.L. and Possee, R.D. (1990) Baculovirus expression vectors. *Advances in Gene Technology.* **1**:55-72.

Blackwood, E.M. and Kadonaga, J.T. (1998) Going the distance: a current view of enhancer action. *Science.* **281**:61-63.

Blasey, H.D., Lundström, K., Tate, S. and Bernard, A.R. (1997) Recombinant protein production using the Semliki Forest Virus expression system. *Cytotechnology.* **24**:65-72.

Bliem, R. and Katinger, H. (1988) Scale-up engineering in animal cell technology. *Trends in Biotechnology.* **6**:190.

Blissard, G.W. and Rohrmann, G.F. (1989) Location, sequence, transcriptional mapping, and temporal expression of the gp64 envelope glycoprotein gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. *Virology.* **170**:537-555.

Blissard, G.W. and Rohrmann, G.F. (1990) Baculovirus diversity and molecular biology. *Annu. Rev. Entomol.* **36**:127-155.

Blissard, G.W., Kogan, P.H., Wei, R. and Rohrmann, G.F. (1992) A synthetic early promoter from a baculovirus: roles of the TATA box and conserved start site CAGT sequence in basal levels of transcription. *Virology.* **190**:783-793.

Bond, B.J. and Davidson, N. (1986) The *Drosophila melanogaster* actin5C gene uses two transcription initiation sites and three polyadenylation sites to express multiple mRNA species. *Mol. Cell. Biol.* **6**:2080-2088.

Bond-Matthews, B. and Davidson, N. (1988) Transcription from each of the *Drosophila* act5C leader exons is driven by a separate functional promoter. *Gene.* **62**:289-300.

Borys, M.C. Linzer, D.I.H. and Papoutsakis, E.T. (1993) Culture pH affects expression rate and glycosylation of recombinant mouse placental lactogen by Chinese Hamster Ovary (CHO) cells. *Biotechnology.* **11**:720-724.

Bourouis, M. and Jarry, B. (1983) Vectors containing a prokaryotic dihydrofolate reductase gene transform *Drosophila* cells to methotrexate-resistance. *EMBO. J.* **2**:1099-1104.

Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry.* **72(7)**: 248-254.

Breibach, K. and Jarvis, D.L. (2001) Improved glycosylation of a foreign protein by Tn-5B1-4 cells engineered to express mammalian glycosyl-transferases. *Biotechnol. Bioeng.* **74**:230-239.

Broeck, J.V., Vulsteke, V., Huybrechts, R. and De Loof, A. (1995) characterisation of a cloned locust tyramine receptor cDNA by functional expression in permanently transformed *Drosophila* S2 cells. *Journal of Neurochemistry.* **64(6)**:2387-2395.

Brondyk, W.H. (1995) The pCI-neo mammalian expression vector. *Promega Notes Magazine.* **51**:10.

Brooke, B.J. and Glick, B.S. (2002) Rapidly maturing variants of the *Discosoma* red fluorescent protein (DsRed). *Nature Biotechnolgy.* **20**:83-87.

Brown, M., Figge, J., Hansen, U., Wright, C., Jeang, K.T., Khoury, G., Livingston, D.M. and Roberts, T.M. (1987) *lac* repressor can regulate expression from a hybrid SV40 early promoter containing a *lac* operator in animal cells. *Cell.* **49**:603-612.

Brown, R.S., Sander, C. and Argos, P. (1985) The primary structure of transcription factors TK111A has 12 consecutive repeats. *FEBS.* **186(2)**:271-274.

Bujard, H. (1999) Controlling genes with tetracyclines. *J.Gene. Med.* **1**:372-374.

Bunch, T.A., Grinblatt, Y. and Goldstein, L.S.B. (1988) Characterisation and use of the *Drosophila* metallothionein promoter in cultured *Drosophila melanogaster* cells. *Nucl. Acids Res.* **16**:1043-1061.

Bunick, D., Zandomeni, R., Ackerman, S. and Weinmann, R. (1982) Mechanism of RNA polymerase II--specific initiation of transcription in vitro: ATP requirement and uncapped runoff transcripts.*Cell.* **29(3)**:877-886.

Buratowski, S. (1994) The basics of basal transcription by RNA polymerase II. *Cell.* **77**:1-3.

Buratowski, S., Hahn, S., Guarente, L. and Sharp, P.A. (1989) Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell.* **56(4)**:549-561.

Burn, T.C., Vigoreaux, J.O. and Tobin, S.L. (1989) Alternative 5C actin transcripts are localized in different patterns during *Drosophila* embryogenesis. *Dev. Biol.* **131**:345-355.

Butler, J.E. and Kadonaga, J.T. (2002) The RNA polymerase II core promoter: a key component in the regulation of gene expression. *Genes Dev.* **16**:2583-2592.

Carey, M., Leatherwood, J. and Ptashne, M. (1990) A Potent GAL4 derivative activates transcription at a distance *in vitro*. *Science.* **247**:710-712.

Caron, A.W., Archambault, J. and Massie, B. (1990) High-level recombinant protein production in bioreactors using the baculovirus-insect cell expression system. *Biotechnol Bioeng.* **36**:1133-1140.

Carrey, E.A., Dietz, C., Glubb, D.M., Löffler, M., Lucocq, J.M. and Watson, P.F. (2002) Detection and location of the enzyme of *de novo* pyrimidine biosythesis in mammalian spermatozoa. *Eur. J. Biochem.* **268**:1861-1868.

Carthew, R.W., Chodosh, L.A. and Sharp, P.A. (1985) An RNA polymerase II transcription factor binds to an upstream element in the adenovirus major late promoter. *Cell.* **43**:439-448.

Carson, D.D., Guarino, L.A. and Summer, M.D. (1988) Functional mapping of an *Ac*MNPV immediate early gene which augment expression of the IE-1 trans-activated 39K gene. *Virology.* **162**:444-451.

Carson, D.D., Summers, M.D. and Guarino, L. A. (1991) Molecular analysis of a baculovirus regulatory gene. *Virology.* **182**:279-286.

Carter, A.D., Felber, B.K., Walling, M., Jubier, M., Schmidt, C.J. and Hamer, D.H. (1984) Duplicated heavy metal control sequence of the mouse metallothionein-I gene. *Proc. Natl. Acad. Sci. USA.* **81**:7392-7396.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W. and Prasher, D.C. (1994) Green fluorescent protein as a marker for gene expression. *Science.* **263**:802-805.

Chan, Y-S., Tseng, W-P. and Hayward, G.S. (1996) Two distinct upstream regulatory domains containing multicopy cellular transcription factors binding sites provide basal repression and inducible enhancer characteristics to the immediate-early IES(US3) promoter from human cytomegalovirus. *J. Virol.* **70(8)**:5312-5328.

Chen, J-L., Huisinga, K.L., Viering, M.M., Ou, S.A., Wu, C-T. and Geyer, P.K. (2002) Enhancer action in trans is permitted throughout the *Drosophila* genome. *PNAS.* **99(6)**:3723-3728.

Chen, Y., Yao, B., Zhu, Z., Yi, Y., Lin, X., Zhang, Z. and Shen, G. (2004) A constitutive super-enhancer: homologous region 3 of *Bombyx mori* nucleopolyhedrovirus. *Biochem Biophys. Res. Commun.* **318(4)**:1039-1044.

Cheng, C. and Shuman, S. (2000) Recombinogenic flap ligation pathway for intrinsic repair of topoisomerase IB-induced double-stranded breaks. *Mol. Cell. Biol.* **20**:8059-8068.

Chung, Y.-T. and Keller, E.B. (1990) Positive and negative regulatory elements mediating transcription from the *Drosophila melanogaster* actin 5C distal promoter. *Mol. Cell. Biol.* **10**:6172-6180.

Ciechanover, A. (1998) The ubiquitin-proteasome pathway on protein death and cell life. *EMBO. J.* **17**:7151-7160.

Clark, S.J. and Melki, J. (2002) DNA methylation and gene silencing in cancer: which is the guilty party? *Oncogene.* **21**:5380-5387.

Clem, R.J. and Miller, L.K. (1994) Control of programmed cell death by the baculovirus genes *p35* and *iap. Mol. Cell. Biol.* **14**:5212-5222.

Cockett, M.I., Bebbington, C.R. and Yarranton, G.T. (1990) The use of engineered E1A genes to tranactivate the hCMV-MIE promoter in permanent CHO cell lines. *Nucleic Acid Res.***19**:319-325.

Colosimo, A., Goncz, K.K., Holmes, A.R., Kunzel-mann, K., Novelli, G., Malone, R.W., Bennett, M.J. and Gruenert, D.C. (2000) Transfer and expression of foreign genes in mammalian cells. *Biotechniques.* **29**:314-331.

Courey, A.J. and Tjian, R. (1988) Analysis of Sp1 in vivo reveals multiple transcriptional domains, including a novel glutamine-rich activation motif. *Cell.* **55**:887-898.

Cregg, J.M., Vedvicks, T.S. and Raschke, W.C (1993) Recent advances in the expression of foreign genes in *Pichia pastoris. Bio/technology.* **11**:905-910.

Cullen, B.R., Lomedico, T. and Ju, G. (1984) Transcriptional interference in avin retroviruses-implications for the promoter insertion model of leukemogenesis. *Nature.* **307**:241-245.

Dai, X., Willis, L.G., Huijskens, I., Palli, S.R. and Theilmann, D.A. (2004) The acidic activation domains of the baculovirus transactivators IE1 and IE0 are functional for transcriptional activation in both insect and mammalian cells. *J. Gen. Virol.* **85(3)**:573-582.

Damke. H., Baba, T., Warnock, D.E. and Schmid, S.L. (1994) Induction of mutant dynamin specifically blocks endocytic coated vesicle formation. *J. Cell Biol.* **127(4)**:915-934.

Davidson, D.J. and Castellino, F.J. (1991) Asparagine-linked oligosaccharide processing in lepidopteran insect cells. Temporal dependence of the nature of the oligosaccharides assembled on asparagine-289 of recombinant human plasminogen produced in baculovirus vector infected *Spodoptera frugiperda* (IPLB-SF-21AE) cells. *Biochemistry.* **29**:6167-6174.

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

De La Brousse, F.C. and McKnight, S.L. (1993) Glimpses of allostery in the control of eukaryotic gene expression. *Trends Genet.* **9(5)**:151-154.

Deuschle, U., Hipskind, R.A. and Bujard, H. (1990) RNA polymerase II transcription blocked by *Escherichia coli lac* repressor. *Science.* **248**:480-483.

Deuschle, U., Pepperkok, R., Wang, F.B., Giordano, T.J., McAllister, W.T., Ansorge, W. and Bujard, H. (1989) Regulated expression of foreign genes in mammalian cells under the control of coliphage T3 RNA polymerase and *lac* repressor. *Proc. Natl. Acad. Sci. USA.* **86**:5400-5404.

Di Nocera, P.P. and Dawid, I.B. (1983) Transient expression of genes introduced into cultured cells of *Drosophila. Proc. Natl. Acad. Sci. USA.* **80**:7095-7098.

Dickson, J.A. and Friesen, P.D. (1991) Identification of upstream promoter elements mediating early transcription from the 35,000-molecular-weight protein gene of *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* **65**:4006-4016.

Dietrich, A., Meister, M., Spicher, K., Schultz, G., Camps, M. and Gierschik, P. (1992) Expression, characterization and purification of soluble G-protein by dimers composed of defined subunits in baculovirus-infected insect cells. *FEBS Letters.* **313(3)**:220-224.

Dietz, C., Hinsch, E. and Löffler, M. (2000) Immunocytochemical detection of mitochondrial dihydroorotate dehydrogenase in human spermatozoa. *Int. J. Androl.* **23**:294-299.

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

Dovey, H.F., Suomensaari-Chryler, S., Lieberburg, I., Sinha, S. and Keim, P.S. (1993) Cells with a familial Alzheimer's disease mutation produce authentic b-peptide. *NeuroReport.* **4**:1039-1042.

Dunkler, B.P., Davies, P.L. and Walker, V.K. (1997) Intron boost transgene expression in *Drosophila melanogaster. Mol Gen Genet.* **254**:291-296.

Dvir, A., Conaway, J.W. and Conaway, R.C. (2001) Mechanism of transcription initiation and promoter escape by RNA polymerase II. *Current Opinion in Genetics and Development.* **11**:209-214.

Dynan, W.S. and Tjian, R. (1983a) Isolation of transcription factors that discriminate between two different promoters recognised by RNA polymerase II. *Cell.* **32**:669-680.

Dynan, W.S. and Tjian, R. (1983b) The promoter specific transcription factor Spl binds to upstream sequences in the SV40 early promoter. *Cell.* **35**:79-87.

Dynan, W.S. and Tjian, R. (1985) Control of eukaryotic messenger RNA synthesis by sequence-specific DNA-binding proteins. *Nature.* **316**:774-778.

Efrat, S., Fusco-DeMane, D., Lemberg, H., al Emran, O. and Wang, X. (1995) Conditional transformation of a pancreatic beta-cell line derived from transgenic mice expressing a tetracycline-regulated oncogene. *Proc. Natl. Acad. Sci. USA.* **92**:3576-3580.

Esteller, M. (2002) CpG island hypermethylation and tumor suppressor genes: a booming present, a brighter future. *Oncogene.* **21**:5427-5440.

Evans, T. and Felsenfeld, G. (1989) The erythroid-specific transcription factor Eryf1: a new finger protein. *Cell.* **58**:877-885.

Evans, T., Reitman, M. and Felsenfeld, G. (1988) An erythrocyte-specific DNA-binding factor recognizes a regulatory sequence common to all chicken globin genes. *Proc. Natl. Acad. Sci. USA.* **85**:5979-5980.

Ewald, D., Li, M., Efrat, S, *et al.* (1996) Time-sensitive reversal of hyperplasia in transgenic mice expressing SV40 T antigen. *Science.* **273**:1384-1386.

Farrell, P.J., Behie, L. and Iatrou, K. (1999) Transformed lepidopteran insect cells: new sources of recombinant human tissue plasminogen activator. *Biotechnol. Bioeng.* **64**:426-433.

Farrell, P.J., Johnson, R. and Iatrou, K. (1997) A baculovirus (*Bombyx mori* nuclear polyhedrosis virus) repeats element functions as a powerful constitutive enhancer in transfected insect cells. *J Biol Chem.* **272**:30724-30728.

Farrell, P.J., Lu, M., Prevost, J., Brown, C., Behie, L. and Iatrou, K. (1998) High-level expression of secreted glycoproteins in transformed lepidopteran insect cells using a novel expression vector. *Biotechnology and Bioengineering.* **60(6)**:656-663.

Feder, J. and Tolbert, W.R. (1985) large scale mammalian cell culture. Academic Press, New York.

Fehse, B., Li, Z., Schade, U.M., Uhde, A. and Zander, A.R. (1998) Impact of a new generation of gene transfer markers on gene therapy. *Gene. Ther.* **5**:429-430.

Figge, J., Wright, C., Collins, C.J., Robert, T.M. and Livingston, D.M. (1988) Stringent regulation of stably integrated chloramphenicol acetyl transferase genes by *E.coli lac* repressor in monkey cells. *Cell.* **52**:713-722.

Fire, A., Samuels, M. and Sharp, P.A. (1984) Interactions between RNA polymerase II, factors, and template leading to accurate transcription. *J. Biol. Chem.* **259(4)**:2509-2516.

Fishman, G.I., Kaplan, M.L. and Buttrick, P.M. (1994) Tetracycline-regulated cardiac gene expression *in vivo*. *J. Clin. Invest.* **93**:1864-1868.

French, C.E., Halles, A.M., Ratbone, D.A., Long, M.T., Willey, D.L. and Bruce, N.C. (1995) Biological production of semisynthetic opiates using genetically engineered bacteria. *Bio/technology.* **13**:674-676.

Friesen, P.D. and Miller, L.K. (1986) The regulation of baculovirus gene expression. *Current topics in Microbiology and Immunology.* **131**:31-49.

Fu, Y-H. and Marzluf, G.A. (1990) *nit-2*, the major nitrogen regulatory gene of *Neurospora crassa*, encodes a protein with putative zinc finger DNA-binding domain. *Mol. Cell. Biol.* **10**:1056-1065.

Fuchs, Y.L., Woods, M.S. and Weaver, R.F. (1983) Viral transcription during *Autographa californica* nuclear polyhedrosis virus infection: A novel RNA polymerase induced in infected *Spodoptera frugiperda* cells. *J. Virol.* **48**:641-646.

Fuerst, T.R., Fernandez, M.P. and Moss, B. (1989) Transfer of the inducible *lac* repressor / operator system from *Escherichia coli* to a vaccinia virus expression vector. *Proc. Natl. Acad. Sci. USA.* **86**:2549-2553.

Furth, PA., Hennighausen, L., Baker, C., Beatty, B. and Woychick, R. (1991) The variability in activity of the universally expressed human cytomegalovirus immediate early gene 1 enhancer/promoter in transgenic mice. *Nucleic. Acids. Revs.* **19(22)**:6205-6208.

Furth, PA., St Onge, L., Boger, H., Gruss, P. Gossen, M., Kistner, A., Bujard, H. and Hennighausen, L. (1994) Temporal control of gene expression in transgenic mice by a tetracycline responsive promoter. *Proc. Natl. Acad. Sci. USA.* **91(20)**:9302-9306.

Fussenegger, M., Bailey, J., Hauser, H. and Mueller, P. (1999) Genetic optimization of recombinant glycoprotein production by mammalian cells. *Trends Biotechnol.* **17**:35-42.

Gallagher, A.R., Schonig, K., Brown, N., Bujard, H. and Witzgall, R. (2003) Use of the tetracycline system for inducible protein synthesis in kidney. *J. Am. Soc. Nephrol.* **14(8)**:2042-2051.

Garinis, G.A., Patrinos, G.P., Spanakis, N.E. and Menounos, P.G. (2002) DNA hypermethylation: when tumour suppressor genes go silent. *Hum. Genet.* **111**:115-127.

Gatz, C. and Quail, P.H. (1988) Tn-10-encoded tet repressor can regulate an operator-containing plant promoter. *Proc. Natl. Acad. Sci. USA.* **85**:1394-1397.

Gatz, C., Frohberg, C. and Wendenburg, R. (1992) Stringent repression and homogeneous de-repression by tetracycline of a modified CaMV 35S promoter intact transgenic tobacco plants. *Plants J.* **2**:397-404.

Geisse, S., Gram, H., Kleuser, B. and Kocker, H.P. (1996) Eukaryotic expression systems: A comparison. *Protein Expression and Purification.* **8**:271-282.

Geisse, S. and Kocker, H.P. (1999) Protein expression in mammalian and insect cell systems. *Methods Enzymol.* **306**:19-42.

Geisse, S., Patoux, A., Rohr, F., Glueck, D., Hagen, S., Rinaldi, D. and Gram, H. (1997) EBNA-1/ori P driven expression in HEK293 cells. *Transient gene expression in animal cells.*

Gidoni, D., Dynan, W.S. and Tjian, R. (1984) Multiple specific contacts between a mammalian transcription factor and its cognate promoters. *Nature.* **312**:409-413.

Gill, G. (2001) Regulation of the initiation of eukaryotic transcription. *Essays. Biochem.* **37**:33-43.

Gillies, S.D., Dorai, H., Wesolowski, J., Majeau, G., Young, D., Boyd, J., Gardner, J. and James, K. (1989) Expression of human anti-tetanus toxoid antibody in transfected murine myeloma cells. *BioTechnology.* **7**:799-804.

Glenville, N., Durnam, D.M. and Palmiter, R.D. (1981) Structure of mouse metallothionein-I and its mRNA. *Nature.* **292**:267-269.

Glocker. B., Hoopes, R.R. and Rohrmann, G.F. (1992) *In vitro* transactivation of baculovirus early genes by nuclear extracts from *Autographa californica* nuclear polyhedrosis virus-infected *Spodoptera frugiperda* cells. *J. Virol.* **66(6)**:3476-3484.

Gluzman, Y. (1981) SV40-transformed simian cells support the replication of early SV40 mutants. *Cell.* **23**:175-182.

Gonzalez-Zulueta, M., Bender, C.M., Yang, A.S., Nguyen, T., Beart, R.W., Van Tornout, J.M. and Jones, P.A. (1995) Methylation of the 5'CpG island of the p16/CDKN2 tumor suppressor gene in normal and transformed human tissue correlates with gene silencing. *Cancer Res.* **55(20)**:4531-4535.

Gorman, C.L., Merlino, G.T., Willingham, M.C., Pastan, I., Howard, B.H. (1982) The Rous sarcoma virus long terminal repeat is a strong promoter when introduced into a variety of eukaryotic cells by DNA-mediated transfection. *Proc. Natl. Acad. Sci. USA.* **79(22)**: 6777-6781.

Goss, L.A., Baird, G.S., Hoffman, R.C., Baldridge, K.K. and Tsien, R.Y. (2000) The structure of the chromophore within DsRed, a red fluorescent protein from coral. *PNAS.* **97(22)**:11990-11995.

Gossen, M. and Bujard, H. (1992) Tight control of gene expression in mammalian cells by tetracycline responsive promoter. *Proc. Natl. Acad. Sci. USA.* **89**:5547-5551.

Gossen, M. and Bujard, H. (1995) Efficacy of tetracycline-controlled gene expression is influenced by cells type.*BioTechniques.* **89**:213-215.

Gossen, M., Freundlieb, S., Bender, Muller, G. Hillen, W. and Bujard, H. (1995) Transcriptional activation by tetracycline in mammalian cells. *Science.* **268**:1766-1769.

Grabherr, R., Ernst, W., Oker-Blom, C. and Jones, I. (2001) Development in the use of baculoviruses for the surface display of complex eukaryotic proteins. *Trends Biotechnol.* **19(6)**:231-236.

Grace, T.D. (1962) Establishment of four strains of cells from insect tissues grown *in vitro.* *Nature.* **195**:788-789.

Greger, I.H., Demarchi, F., Giacca, M. and Proudfoot, N.J. (1998) Transcriptional interference perturbs the binding of Sp1 to HIV-1 promoter. *Nucleic. Acids. Revs.* **26(5)**:1294-1300.

Greger, V., Passarge, E., Hopping, W., Messmer, E. and Horsthemke, B. (1989) Epigenetic changes may contribute to the formation and spontaneous regression of retinoblastoma. *Hum. Genet.* **83(2)**:155-158.

Grosch, H-W. (1998) Protection of proteolysis-prone recombinant proteins in baculovirus expression system. *BioTechniques.* **24**:930-934.

Guarino, L.A. and Dong, W. (1994) Functional dissection of the *Autographa californica* nuclear polyhedrosis virus enhancer element *hr5. Virology.* **200**:228-335.

Guarino, L.A., Gonzalez, M.A. and Summes, M.D. (1986) Complete sequence and enhancer function of the homologous DNA regions of *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* **60**:224-229.

Guarino, L.A. and Summers, M.D. (1986a) Functional mapping of a *trans*- activating gene required for expression of a baculovirus delayed early gene. *J. Virol.* **57**:563-571.

Guarino, L.A. and Summers, M.D. (1986b) Interspersed homologous DNA regions of *Autographa californica* nuclear polyhedrosis virus enhances delayed early gene expression. *J. Virol.* **60**:215-223.

Guarino, L.A. and Summers, M.D. (1987) Nucleotide sequence and temporal expression of a baculovirus regulatory gene. *J. Virol.* **61(7)**:2091-2099.

Han, K., Levine, M.S. and Manley, J.L. (1989) Synergistic activation and repression of transcription by *Drosophila* homeobox proteins. *Cell.* **56**:573-583.

Harding, T.C., Geddes, B.J., Murphy, D., Knight, D. and Uney, J.B. (1998) Switching transgene expression in the brain using an adenovirus tetracycline-regulatable system. *Nat. Biotechnol.* **16**: 553-555.

Hashimoto, Y., Hayashi, K., Okuno, Y., Hayakawa, T., Saimoto, A., Granados, R.R. and Matsumoto, T. (1996) Physical mapping and identification of interspersed homologous sequences in *Trichoplusia ni* granulosis virus genome. *J. Gen. Virol.* **77**:555-563.

Hawley, T.S., Telford, W.G., Ramezani, A. and Hawley, R.G. (2001) Four-color flow cytometric detection of retrovirally expressed red, yellow, green, and cyan fluorescent proteins. *BioTechniques.* **30**:1028-1034.

Hawtin, R.E., Arnold, K., Ayres, M.D., Zanotto, P.M.D.A., Howard, S.C., Gooday, G.W., Chappell, L.H., Kitts, P.A., King, L.A. and Possee, R.D. (1995) Identification and preliminary characterization of a chitinase gene in the *Autographa californica* nuclear polyhedrosis virus genome. *Virology.* **212**:673-685.

Hawtin, R.E., Zarkowska, T. Arnold, K., Thomas, C.J., Gooday, G.W., King, L.A., Kuzio, J.A. and Possee, R.D. (1997) Liquefaction of *Autographa californica* nucleopolyhedrovirus-infected insects is dependent on the integrity of virus-encoded chitinase and cathepsin genes. *Virology.* **238**:243-253.

Hegedus, D.D., Pfeifer, T.A., Hendry, J., Theilmann, D.A. and Grigliatti, T.A. (1998) A series of broad host range shuttle vectors for constitutive and inducible expression of heterologous proteins in insect cell lines. *Gene.* **207**:241-249.

Heikal, A.A., Hess, S., Baird, G.S., Tsien, R.Y. and Webb, W.W. (2000) Molecular spectroscopy and dynamics of intrinsically fluorescent proteins: Coral red (dsRed) and yellow (Citrine). *PNAS.* **97(22)**:11996-12001.

Helgen, J.C. and Fallon, A.M. (1990) Polybrene-mediated transfection of cultured lepidopteran cells: induction of a *Drosophila* heat shock promoter. *Vitro Cell. Dev. Biol.* **26**:731-736.

Henderson, J., Atkinson, A.E., Lazarus, C.M., Hawes, C.R., Napier, R.M., Macdonald, H. and King, L.A. (1995) Stable expression of maize auxin-binding protein in insect cell lines. *FEBS Letters.* **371**:293-296.

Hentschel, C.C. (1991) Recent developments in mammalian expression systems. ESACT 10TH Meeting, Ed. Spier, R.E., Griffiths, J.B. and Meigner, B. Butterworth Heinman Ltd. Oxford

Herman, J.G., Merlo, A., Mao, L., Lapidus, R.G., Issa, J.P., Davidson, N.E., Sidransky, D. and Baylin, S.B. (1995) Inactivation of the CDKN2/p16/MTS1 gene is frequently associated with aberrant DNA methylation in all common human cancers. *Cancer Res.* **55**:4525-4530.

Hill, R.M., Brennan, S.O, Birch, N.P. (2001) Expression, purification, and fuctional characterization of the serine protease inhibitor neuroserpin expressed in Drosophila S2 cells. *Protein Expr. Purif.* **22(3)**:406-413.

Hillen, W. and Berens, C. (1994) Mechanisms underlying expression of Tn10-encoded tetracycline resistance. *Annual Rev. Microbiol.* **48**:345-369.

Hillen, W. and Wissmann, A. (1989) TetR-*tetO* interaction. *Protein-Nucleic Acid Interactions. Topics in Molecular and structural Biology.* Ed. Saenger, W. Heinemann, U. **10**:143-162. London: Macmillan Press.

Hiller, M.M., Finger, A., Schweiger, M. and Wolf, D.H. (1996) ER degradation of a misfolded luminal protein by the cytosolic ubiquitin-proteasome pathway. *Science.* **273**:1725-1728.

Hodgson, J. (1993) Expression systems: A user's guide. *Bio/Technology.* **11**:889-893.

Hoey, T., Dynlacht, B.D., Peterson, M.G., Pugh, B.F. and Tjian, R. (1990) Isolation and characterization of the *Drosophila* gene encoding the TATA box binding protein, TFIID. *Cell.* **61(7)**:1179-1186.

Holliday, R. (1993) Epigenetic inheritance based on DNA methylation. *EXS.* **64**:452-468.

Hollister, J.R., Grabenhorst, E., Nimtz, M., Conradt, H.O. and Jarvis, D.L. (2002) Engineering the protein *N*-glycosylation pathway in insect cells for the production of biantennary, complex *N*-glycans. *Biochemistry.* **41**:15093-15104.

Hollister, J.R. and Jarvis, D.L. (2001) Engineering lepidopteran insect cells for sialoglycoprotein production by genetic transformation with mammalian β1-4-galactosyltransferase and alpha 2,6-sialyltransferase genes. *Glycobiology.* **11**:1-9.

Hollister, J.R., Shaper, J.H. and Jarvis, D.L. (1998) Stable expression of mammalian β1-4-galactosyltransferase extends the *N*-glycosylation pathway in insect cells. *Glycobiology.* **8**:473-480.

Hsieh, P. and Robbins, P.W. (1984) Regulation of asparagines-linked oligosaccharide processing. Oligosaccharide processing in *Aedes albopictus* mosquito cells. *J. Bio. Chem.* **259**:2375-2382.

Hsu, T-A., Eiden, J.J., Bourgarel, P., Meo, T. and Betenbaugh. (1994) Effect of co-expressing chaperone BiP on functional antibody production in the baculovirus system. *Protein Expr Purif.* **5**:595-603.

Hwang, J.J., Scuric, Z. and Anderson, W.F. (1996) Novel retroviral vectors transferring a suicide gene and a selectable marker gene with enhanced gene expression by using a tetracycline responsive expression system. *J. Virol.* **70**:8138-8141.

Hu, M.C.T. and Davidson, N. (1987) The inducible *lac* operator-repressor system is functional in mammalian cells. *Cell.* **48**:555-566.

Hu, M.C.T. and Davidson, N. (1990) A combination of derepression of the *lac* operator-repressor system with positive induction by glucocorticoid and metal ions provides a high-level-inducible gene expresion system based on the human metallothionein IIA promoter. *Mol Cell Biol.* **10**:6141-6151.

Hu, Y-C. and Bentley, W.E. (1999) Enhancing yield of infectious bursal disease virus structural proteins in baculovirus expression systems: Focus on Media, protease inhibitors, and dissolved oxygen. *Biotechnol. Prog.* **15**:1064-1071.

Hunltmark, D., Klemenz, R. and Gehring, W. (1986) Translational and transcriptional control elements in the untranslated leader of the heat shoch gene hsp22. *Cell.* **44**:429-438.

Hunt, L.T. and Dayhoff, M.O. (1970) The occurrence in proteins of the tripeptide Asn-X-Ser and Asn-X-Thr and of bound carbohydrate. *Biochem. Biophys. Res. Commun.* **39**:757-765.

Huynh, C. Q. and Zieler, H. (1999) Construction of modular and versatile plasmid vectors for the high-level expression of single or multiple genes in insects and insect cell lines. *Journal of Molecular Biology.* **288**:13-20.

Ikonomou, L., Schneider, Y.J. and Agathos, S.N. (2003) Insect cell culture for industrial production of recombinant proteins. *Appl. Microbiol Biotechnol.* **62(1)**:1-20.

Israel, D.I. and Kaufman, R.J. (1989) Highly inducible expression from vector containing multiple GRES in CHO cells overexpressing the glucocorticoid receptor. *Nucl Acids Res.* **12**:4589-4604.

Jacobs, P., Cravador, A., Loriau, R., Brockly, F., Colau, B., Chuchana, P., Van Elsen, A., Herzog, A. and Bollen, A. (1985) Molecular cloning, sequence, and expression in *Escherichia coli* of human preprourokinase cDNA. *DNA.* **4(2)**:139-146.

Jakobs, S., Subramaniam, V., Schonle, A., Jovin, T.M. and Hell, S.W. (2000) EGFP and DsRed expressing cultures of Escherichia coli imaged by confocal, two-photon and fluorescence lifetime microscopy. *FEBS. Lett.* **479(3)**:131-135.

Jansen, R., Tollervey, D. and Hurt, E.C. (1993) A U3 snoRNP protein with homology to splicing factor PRP4 and Gβ domains is required for ribosomal RNA processing. *EMBO. J.* **12(6)**:2549-2558.

Jarosch, E., Lenk, U. and Sommer, T. (2003) Endoplasmic reticulum-associated protein degradation. *Int. Rev. Cytol.* **223**:39-81.

Jarvis, D.L. (2003) Developing baculovirus-insect cell expression systems of humanized recombinant glycoprotein production. *Virology.* **310**:1-7.

Jarvis, D.L. and Finn, E.E. (1995) Biochemical analysis of the N-glycosylation pathway baculovirus-infected lepidopteran insect cells. *Virology.* **212**:500-511.

Jarvis, D.L. and Finn, E.E. (1996) Modifying the insect cell N-glycosylation pathway with Immediate early baculovirus expression vector. *Nature Biotechnology.* **14**:1288-1292.

Jarvis, D.L. and Summers, M.D. (1989) Glycosylation and secretion of human tissue plasminogen activator in recombinant baculovirus-infected cells. *Molecular and Cellular Biology.* **9 (1)**:214-223.

Jarvis, D.L., Fleming, J.G.W., Kovacs, G.R., Summer, M.D. and Guarino, L.A. (1990) Use of early baculovirus promoters for continuous expression and efficient processing of foreign gene products in stably-transformed lepidopteran insect cells. *Bio/Technology.* **8**:50-55.

Jarvis, D.L., Weinkauf, C. and Guarino, L.A. (1996) Immediate-early baculovirus vectors for foreign gene expression in transformed or infected insect cells. *Protein Expression and Purification.* **8**:191-203.

Jayakumar, A., Cataltepe, S., Kang, S., Frederick, M.J., Mitsudo, K., Henderson, Y., Crawford, S.E., Silerman, G.A. and Clayman, G.L. (2004) Production of serpins using baculovirus expression systems. *Methods.* **32(2)**:177-184.

Jeong, K.J. and Lee, S.Y. (1999) High-level production of human leptin by fed-batch cultivation of recombinant *Escherichia coli* and its purification. *Appl. Environ, Microbiol.* **65(7)**:3027-3032.

Jeong, K.J. and Lee, S.Y. (2001) Secretory production of human granulocyte-colony stimulating factor in *Escherichia coli. Protein Expres. Purif.* **23(2)**:311-318.

Johansen, H., van der Straten, A., Sweet, R., Maroni, G. and Rosenberg, M. (1989a) Regulated expression at high copy number allows production of a growth-inhibitory oncogene product in *Drosophila* Schneider cells. *Genes and Development.* **3**:882-889.

Johansen, H., van der Straten, A. and Rosenberg, M. (1989b) Expression of foreign genes in *Drosophila* cells. European Patent Application Number 88304093.3, publication number 0290 261 A1 1988.

Johnson, K.M., Mitsouras, K. and Carey, M. (2001) Eukaryotic transcription: the core of eukaryotic gene activation. *Curr. Biol.* **11(13)**:510-513.

Johnson, P.F. and McKnight, S.L. (1989) Eukaryotic transcriptional regulatory proteins. *Annu. Rev. Biochem.* **58**:799-839.

Johnson, R., Meldinger, R.G. and Iatrou, K. (1992) A cellular promoter-based expression cassette for generating recombinant baculoviruses directing rapid expression of passenger genes in infected insect cells. *Virology.* **190**:815-823.

Jone, M.E. (1980) Pyrimidine nucleotide biosynthesis in animals: genes, enzymes and regulation of UMP biosynthesis. *Annu. Rev. Biochem.* **49**:253-279.

Joyce, K.A. (1993) Heterologous expression in insect cell lines. *Mphil thesis.* Oxford Brookes University.

Joyce, K.A., Atkinson, A.E., Bermudez, I., Beadle, D.J. and King, L.A. (1993) Synthesis of functional $GABA_A$ receptors in stable insect cell lines. *FEBS Letters.* **335 (1)**:61-64.

Kadonaga, J.T. (1990) Assembly and disassembly of the *Drosophila* RNA polymerase II complex during transcription. *J. Biol. Chem.* **265(5)**:2624-2631.

Kain, S.R., Adams, M., Kondepudi, A., Yang, T.T., Ward, W.W. and Kitts, P. (1995) Green fluorescent protein as a reporter of gene expression and protein localization. *Biotechniques.* **19**:650-655.

Kalpana, G., Majumder, S. and Jacob, S.T. (2002) Analysis of promoter methylation and its role in silencing metallothionein I gene expression in tumor cells. *Methods. Enzymol.* **353**:476-486.

Keegan, L., Gill, G. and Ptashne, M. (1986) Separation of DNA binding from the transcription-activating function of a eukaryotic regulatory protein. *Science.* **230**:699-704.

Keith, M.B., Farrell, P.J., Iatrou, K. and Behle, L.A. (1999) Screening of transformed insect cell lines for recombinant protein production. *Biotechnol. Prog.* **15**:1046-1052.

Kikuchi, T., Toyota, M., Itoh, F., Suzuki, H., Obata, T., Yamamoto, H., Kakluchi, H. *et al.* (2002) Inactivation of p57KIP2 by regional promoter hypermethylation and histone deacetylation in human tumors. *Oncogene.* **21**:2741-2749.

Kim, H.J., Gatz, C., Hillen, W. and Jones, T.R. (1995) Tetrcycline repressor-regulated gene repression in recombinant human cytomegalovirus. *J. Virol.* **69**:2565-2573.

Kim, K-M. and Shibata, D. (2002) Methylation reveals a niche: stem cell succession in human colon crypts. *Onogene.* **21**:5441-5449.

Kimura, M. and Ishihama, A. (2000) Involvement of multiple subunit-subunit contacts in the assembly of RNA polymerase II. *Nucleic Acids Res.* **28(4)**:953-959.

King, G.A., Daugulis, A.J., Faulkner, P. and Goosen, M.F. (1992) Recombinant beta-galactosidase production in serum-free medium by insect cells in a 14-L airlift bioreactor. *Biotechnol. Prog.* **8(6)**:567-571.

King, L.A., Mann, S., Lawrie, A.M. and Possee, R.D. (1994) Baculovirus expression vector systems: production and isolation of recombinant viruses. In: *Cell Biology: a laboratory handbook.* Academic Press, Inc.

King, L.A. and Possee, R.D. (1992) The *Baculovirus expression system: A laboratory manual.* Chapman and Hall.

Kirschner, M. (1999) Intracellular proteolysis. *Trends. Cell. Biol.* **9**:42-45.

Kistner, A., Gossen, M., Zimmermann, F., *et al.* (1996) Doxycycline-mediated quantitative and tissue-specific control of gene expression in transgenic mice. *Proc. Natl. Acad. Sci. USA.* **93**:10933-10938.

Kitts, P.A., Ayres, M.D. and Possee, R.D. (1990) Linearisation of baculovirus DNA enhances the recovery of recombinant virus expression vectors. *Nucleic Acids Res.* **18(19)**:5667-5672.

Kitts, P.A. and Possee, R.D. (1993) A method for producing recombinant baculovirus expression vectors at high frequency. *BioTechniques.* **14(5)**:810-817.

Ko, M.S.H., Sugiyama, N. and Takano, T. (1989) An auto-inducible vector conferring high glucocorticoid inducibility upon stable transformant cells. *Gene.* **89**:383-389.

König, R., Ashwell, G. and Hanover, J.A. (1989) Over-expression and biosynthesis of CD4 on Chinese hamster ovary cells: Coamplification using the multiple drug resistance gene. *Proc. Natl. Acad. Sci. USA.* **86**:9188-9192.

Kool, M.J., Voeten, T.M., Goldbach, R.W., Tramper, J. and Vlak, J.M. (1993) Identification of seven putitive origins of *Autographa californica* multiple nuclear polyhedrosis virus DNA replication. *J. Gen. Virol.* **74**:2661-2668.

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

Kornitzer, D. and Ciechanover, A. (2000) Modes of regulation of ubiquitin-mediated protein degradation. *J. Cell Physiol.* **182**:1-11.

Kostova, Z. and Wolf, D.H. (2003) For whom the bell tolls: protein quality control of the endoplasmic reticulum and the ubiquitin-proteasome connection. *EMBO. J.* **22(10)**:2309-2317.

Kovach, M.J., Carlson, J.O. and Beaty, B.J. (1992) A *Drosophila* metallothionein promoter is inducible in mosquito cells. *Insect Mol. Biol.* **1**:37-43.

Kovacs, G.R., Guarino, L.A. and Summers, M.D. (1991) Novel regulatory properties of the IE-1 and IE-0 transactivator encoded by the baculovirus *Autographa californica* multicapsid polyhedrosis virus. *J. Virol.* **65**:5281-5288.

Krappa, R., Behn-Krappa, A., Jahnel, F., Doerfler, W. and Knebel-Morsdorf, D. (1992) Differential factor binding at the promoter of early baculovirus gene PE38 during viral infection: GATA motif is recognized by an insect protein. *J. Virol.* **66(6)**:3494-3503.

Kuna, K.T. and Papoutsakis, E.T. (1990) Damage mechanisms of suspended animal cells in agitated bioreactors with and without bubble entrainment. *Biotechnol Bioeng.* **36**:476-483.

Kuzio, J. and Faulkner, P. (1984) Regions of repeated DNA in the genome of *Choristoneura fumiferana* NPV. *Virology.* **139**:185-188.

Labow, M.A., Baim, S.B., Shenk, T. and Levine, A.J. (1990) Conversion of the *lac* repressor into an allosterically regulated transcriptional activator for mammalian cells. *Mol. Cell. Biol.* **10**:3343-3356.

Lamb, J.W., Duncan, G.H., Reavy, B., Gildow, F.E., Mayo, M.A. and Hay, R.T. (1996) Assembly of virus-like particles in insect cells infected with a baculovirus containing a modified coat protein gene of potato leafroll luteovirus. *J. Gen. Virol.* **77**:1349-1358.

Landy, A. (1989) Dynamic, structural, and regulatory aspect of lambda site-specific recombination. *Annu. Rev. Biochem.* **58**:913-948.

Lane, P., Brocker, T., Hubele, S., Padovan, E., Lanzavecchia, A. and McConnell, F. (1993) Soluble CD40 ligand can replace the normal T cell-derived CD40 ligand signal to B cells in T-cell dependent activation. *J. Exp. Med.* **177**:1209-1213.

Laney, J.D. and Hochstrasser, M. (1999) Substrate targeting in the ubiquitin system. *Cell.* **97**:427-430.

Latchman, D.S. (1990) Eukaryotic transcription factors. *Biochem. J.* **270**:281-289.

Latchman, D.S. (1995) Eukaryotic transcription factors. 2nd ED. Academic Press INC., San Diego, California.

Lawrence, S.M., Huddleston, K.A., Tomiya, N., Nguyen, N., Lee, Y.C., Vann, W.F., Coleman, T.A. and Betenbaugh, M.J. (2001) Cloning and expression of human sialic acid pathway genes to generate CMP-sialic acids in insect cells. *Glycoconj. J.* **18**:205-213.

Lee, D. K., DeJong, J., Hashimoto, S., Horikoshi, M. and Roeder, R.G. (1992) TFIIA induces conformational changes in TFIID via interactions with the basic repeat. *Mol. Cell. Biol.* **12(11)**:5189-5196.

Lee, J.C., Chen, H.H. and Chao, Y.C. (1998) Persistent baculovirus infection results from deletion of the apoptotic suppression gene *p*35. *J. Virol.* **72**:9157-9165.

Lee, S.W., Tsou, A-P., Chan, H., Thomas, J., Petrie, K., Eugui, E.M. and Allison, A.C. (1988) Glucocorticoids selectivity inhibit the transcription of the interleukin 1 beta gene and decrease the stability of interleukin 1 beta mRNA. *Proc. Natl. Acad. Sci. USA.* **85(4)**:1204-1208.

Lenhard, T. and Reilander, H. (1997) Engineering the folding pathway of insect cells: generation of a stably transformed insect cell line showing improved folding of a recombinant membrane protein. *Biochem Biophys Res Commun.* **238**:823-830.

Li, E., Brown, S.L., Dolman, C.S., Brown, G.B. and Nemerow, G.R. (2001) Production of functional antibodies generated in a nonlytic insect cell expression system. *Protein Expression and Purification.* **21**:121-128.

Li, L., Harwood, S.H. and Rohrmann. (1999) Identification of additional genes that influence baculovirus late gene expression. *Virology.* **255**:9-19.

Li, Y., Passarelli, A.L. and Miller, L.K. (1993) Identification, sequence, and transcriptional mapping of *lef-3*, a baculovirus gene involved in late and very late gene expression. *J. Virol.* **67**:5260-5268.

Liljeström, P. (1994) Alphavirus expression system. *Curr. Opin. Biotechnol.* **5**:495-500.

Liljeström, P. and Garoff, H. (1991) A new generation of animal cell expression vectors based on the Semliki Forest virus system. *Bio/Technology.* **9**:1356-1361.

Lin, A.A., Kimura, R. and Miller, W.M. (1992) Production of tPA in recombinant CHO cells under oxygen-limited conditions. *Biotechnol Bioeng.* **42**:339-350.

Lin, G., and Blissard, G.W., (2002) Analysis of an *Autographa californica* multicapsid nucleopolyhedrovirus *lef-6*-null virus: LEF-6 is not essential for viral replication but appears to accelerate late gene transcription. *J. Virol.* **76(11)**:5503-5514.

Lin, Y-S. and Green, M.R. (1991) Mechanism of action of an acidic transcriptional activator *in vitro*. *Cell.* **64**:971-981.

Lin, Y-S., Ha, I., Maldonado, E., Reinberg, D. and Green, M.R. (1991) Binding of general transcription factors TFIIB to an acidic activating region. *Nature.* **353**:569-571.

Lindsay, D.A. and Betenbaugh, M.J. (1992) Quantification of cell culture factors affecting recombinant protein yields in baculovirus-infected insect cells. *Biotechnol. Bioeng.* **39**:614-618.

Liu, Y., Bondarenko, V., Ninfa, A. and Studitsky, M. (2001) DNA supercoling allows enhancer action over a large distance. *PANS.* **98(26)**:14883-14888.

Lo, K.-M., Lynch, C.A. and Gillies, S.D. (1992) The use of a wild-type dihydrofolate reductase-encoding cDNA as a dominant selectable marker and induction of expression by methotrexate. *Gene.* **121**:365-369.

Löffler, M., Jöckel, J., Schuster, G. and Becker, C. (1997) Dihydrooratate-ubiquinone oxidoreductase links mitochondria in the biosynthesis of pyrimidine nucleotides. *Mol. Cell. Biochem.* **174**:125-129.

Lord, J.M., Davey, J., Frigerio, L. Roberts, L.M. (2000) Endoplasmic reticulum-associated protein degradation. *Semin. Cell. Dev. Biol.* **11(3)**:159-164.

Lorenzo, V. and Fernandez, J.M. (2000) Expression vectors and delivery systems playing alien genes in remote theaters. *Current Opinion in Biotechnology.* **11**:427-428.

Lu, A. and Carstens, E.B. (1992) Nucleotide sequence and transcriptional analysis of the p80 gene of *Autographa californica* nuclear polyhedrosis virus: a homologue of the *Orgyia pseudotsugata* nuclear polyhedrosis virus capsid-associated gene. *Virology.* **190**:201-209.

Lu, A.. and Miller, L.K. (1994) Identification of three late expression factor genes within the 33.8- to and 43.4-map unit region of *Autographa californica* nuclear polyhedrosis virus. *J. Virol.* **68**:6710-6718.

Lu, A.. and Miller, L.K. (1995) The role of eighteen baculovirus late expression factor genes in transcription and DNA replication. *J. Virol.* **69**:975-982.

Lu, M., Farrell, P.J., Johnson, R. and Iatrou, K. (1997) A baculovirus (*Bombyx mori* Nuclear Polyhedrosis Virus) repeat element functions as a powerful constitutive enhancer in transfected insect cells. *The Journal of Biological Chemistry.* **272(49)**:30724-30728.

Lu, M., Johnson, R. and Iatrou, K. (1996) *Trans*-Activation of a house-keeping gene promoter by the IE1 gene product of baculoviruses. *Virology.* **218**:103-113.

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

Lundström, K. (1997) Alphaviruses as expression vectors. *Curr. Opin. Biotechnol.* **8**:578-582.

Lycett, G.J. and Crampton, J.M. (1993) Stable transformation of mosquito cell lines using a hsp70::neo fusion gene. *Gene.* **136**:129-136.

Lydersen, B.K. (1987) Perfusion cell culture system based on ceramic matrices. *Large scale culture technology.* Ed. Lydersen, B.K. 169-185.

Lynn, D.E. and Hink, W.F. (1980) Comparison of nuclear polyhedrosis virus replication in five lepidopteran cell lines. *J. Inv. Pathol.* **35**:234-240.

Maiorella, B., Inlow, D., Shauger, A. and Harano, D. (1988) Large-scale insect cell-culture for recombinant protein production. *Bio/Technology.* **6**:1406-1410.

Majima, K., Kobara, R. and Maede, S. (1993) Divergence and evolution of homologous regions of *Bombyx mori* nuclear polyhedrosis virus. *J. Virol.* **67**:7513-7521.

Malinowski, J.J. and Daugulis, A.J. (1993) Bioreactor design for insect cell cultivation: a review. *Insect cell culture engineering.* Ed. Goosen, M.F.A., Daugulis, A.J., Faulker, P. and Marcel, D. New York. 51-67.

Maniatis, T., Goodbourn, S. and Fischer, J.A. (1987) Regulation of inducible and tissue-specific gene expression. *Science.* **236**:1237-1245.

Mann, S.G. and King, L.A. (1989) Efficient transfection of insect cells with baculovirus DNA using electroporation. *J. Gen. Virol.* **70**:3501-3505.

Maroni, G., Otto, E. and Lastowski-Perry, D. (1986) Molecular and cytogenetic characterization of a metallothionein gene of Drosophila. *Genetics.* **112**:493-504.

Martel-Renoir, D., Trochon-Joseph, V., Galaup, A., Bouquet, C., Griscelli, F., Opolon, P., Opolon, D., Connault, E., Mir, L. and Perricaudet, M. (2003) Co-electrotransfer to skeletal muscle of three plasmids coding for antiangiogenic factors and regulatory factors of the tetracycline-inducible system: tightly regulated expression, inhibition of transplanted tumor growth, and antimetastatic effect. *Mol. Ther.* **8(3)**:425-433.

Martensen, P.M. and Justesen, J. (2001) Specific inhibitors prevent proteolytic degradation of recombinant proteins expressed in High Five™ cells. *BioTechniques.* **30**:782-792.

Marz, L., Altmann, F., Staudacher, E. and Kubelka, V. (1995) Protein glycosylation in insects: Elsevier, Amsterdam, Ed. Montreuil, J., Vliegenthart, J.F.G. and Schachter, H. *Glycoproteins.* **29a**:543-563.

Mastsui, T., Segall, J., Weil, P.A. and Roeder, R.G. (1980) Multiple factors required for accurate initiation of transcription by purified RNA polymerase II. *J. Biol. Chem.* **255(24)**:11992-11996.

Mayford, M., Bach, M.E., Huang, Y.Y., Wang, L., Hawkins, R.D. and Kandel, E.R. (1996) Control of memory formation through regulated expression of a CaMKII transgene. *Science.* **274**:1678-1683.

McCarroll, L. (1997) Characterisation of heterologous gene expression in insect cell lines. *PhD thesis.* Oxford Brookes University.

McCarroll, L. and King, L.A. (1997) Stable insect cell cultures for recombinant protein production. *Current Opinion in Biotechnology.* **8(5)**:590-594.

McLachlin, J.R. and Miller, L. (1997) Stable transformation of insect cells to co-express a rapidly selectable marker gene and an inhibitor of apoptosis. *In Vitro Cell Dev Biol [Anim].* **33**:575-579.

Meng, W., Green, J. and Guest, J.R. (1997) FNR-dependent repression of *ndh* gene expression requires two upstream FNR-binding sites. *Microbiology.* **143**:1521-1532.

Merlo, A., Herman, J.G., Mao, L., Lee, D.J., Gabrielson, E., Burger, P.C., Baylin, S.B. and Sidransky, D. (1995) 5' CpG island methylation is associated with transcriptional silencing of the tumour suppressor p16/CDKN2/MTS1 in human cancers. *Nat. Med.* **1(7)**:686-692.

Mikhailov, V.S. (2003) Replication of the baculovirus genome. *Mol. Biol.* (*Mosk*). **37(2)**: 288-299

Miltenburger, H.G. (1979) The replication of insect pathogenic viruses (nuclear polyhedrosis viruses) in cell culture. 3rd general meeting of ESACT, Oxford 1979. *Develop. Biol. Standard.* **46**:295-300.

Miller, J.H. and Reznikoff, W.A. (1980) The Operon. Cold Springs Harbor, New York. Cold Springs Harbor Laboratory.

Miller. L.K. (1996) Insect viruses in: *Field virology* (3rd ed). Chapter 13. Edited by Fields, B.N., Knipe, D.M., Howley, P.M, *et al.* Lippincott-Raven Publishers, Philadelphia.

Misra, V., Walker, S., Haynes, S. and O'Hare, P. (1995) The bovine herpesvirus A gene *trans*-inducing factors activates transcription by mechanism different from those of its herpes simplex virus type I counterpart VP16. *J. Virol.* **69(9)**:5209-5216.

Mitchell, P.J. and Tjian, R. (1989) Transcriptional regulation in mammalian cells by sequence-specific DNA binding proteins. *Science.* **245**:371-378.

Modha, G., Blanchard, A., Sidorchuk, J., Venditti, M., Shiu, R. and Myal, Y. (2003) Inducible expression of dominant negative insulin-like growth factor I receptor in MCF-1 breast cancer cells. *Endocrine.* **22(3)**:293-303.

Moreau, P., Hen, R., Wasylyk, B., Everett, R., Gaub, M.P. and Chambon, P. (1981) The SV40 72 base repair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. *Nuclaic Acids Res.* **9(22)**:6047-6068.

Moremen, K.W., Trimble, R.G. and Herscovics, A. (1994) Glycosidases of the asparagine-linked oligosaccharide processing pathway. *Glycobiology.* **4**:113-125.

Morris, J.R., Geyer, P.K. and Wu, C-T. (1998) Core promoter elements can regulate transcription on a separate chromosome in *trans. Gene. Develop.* **13**:253-258.

Morris, T.D. and Miller, L.K. (1992) Promoter influence on baculovirus-mediated gene expression in permissive and nonpermissive insect cell-lines. *J. Virol.* **66(12)**:7397-7405.

Morris, T.D., Todd, J.W., Fisher, B. and Miller, L.K. (1994) Identification of *lef-7*; a baculovirus gene affecting late gene expression. *Virology.* **200**:360-369.

Mounier, N. and Prudhomme, J-C. (1986) Isolation of actin genes in *Bombyx mori*: the coding sequence of a cytoplasmic actin gene expressed in the silk gland is interrupted by a single intron in an unusual position. *Biochimie.* **68**:1056-1061.

Mounier, N. and Prudhomme, J-C. (1991) Expression of a cytoplasmic actin gene in relation to the silk production cycle in the silk gland of *Bombyx mori*. *Insect Biochem.* **21**:293-301.

Müller, M.M., Gerster, T. and Schaffner, W. (1988) Enhancer sequence and the requlation of gene transcription. *Eur. J. Biochem.* **176**:485-495.

Müller, H-P. and Schaffner, W. (1990) Transcriptional enhancer can act *in trans*. *TIG.* **6(9)**:300-304.

Murges, D., Kremer, A. and Knebel-Mörsdorf, D. (1997) Baculovirus transactivator IE1 is functional in mammalian cells. *Journal of General Virology.* **78**:1507-1510.

Naar, A.M., Lemon, B.D. and Tjian, R. (2001) Transcriptional coactivator complexes. *Annu. Rev. Biochem.* **70**:475-501.

Naggle, S. and Bentley, W.E. (1998) Appearance of protease activities coincides with p10 and polyhedrin-driven protein production in the baculovirus expression system: Effects on yield. *Biotechnol. Prog.* **14**:227-232.

Nakata, D., Close, B.E., Colley, K.J., Matsuda, T. and Kitajima, K. (2000) Molecular cloning and expression of the mouse *N*-acetylneuraminic acid 9-phoshate synthase which does not have deaminoneuraminic acid (KDN) 9-phoshate synthase activity. *Biochem. Biophys. Rev Commun.* **273**:642-648.

Navlaux, B. (2000) Options and challenges in the treatment of mitochondrial disease. *Genet. Med.* **3**:56.

Neutra, R., Levi, B.Z. and Shoham, Y. (1992) Optimization of protein-production by the baculovirus expression vector system in shake flasks. *Appl. Microbiol. Biotechnol.* **37(1)**:74-78.

Nguyen, B., Jarnagin, K., Williams, S., Chan, H. and Barnett, J. (1993) Fed-batch culture of insect cells: a method to increase the yield of recombinant human nerve growth factor (rhNGF) in the baculovirus expression system. *J. Biotechnol.* **31(2)**:205-217.

Nissen, M.S. and Friesen, P.D. (1989) Molecular analysis of the transcriptional regulatory region of an early baculovirus gene. *J. Virol.* **63(2)**:493-503.

No, D., Yao, T-P. and Evans, R.M. (1996) Ecdysone-inducible expression in mammalian cells and transgenic mice. *Proc. Natl. Acad. Sci. USA.* **93**:3346-3351.

Nolan, G.P. and Shatzman, A.R. (1998) Expression vector and delivery systems. *Current Opinion in Biotechnology.* **9**:447-450.

Obosi, L.A., Schutte, D.G., Europe-Finner, G.N., Beadle, D.J., Hen, R., King, L.A. and Bermudez, I. (1996) Functional characterisation of the *Drosophila* 5-HT_{dro1} and 5-HT_{dro2B} serotonin receptors in insect cells: activation of a G_{as} –like protein by 5-HT_{dro1} but lack of coupling to inhibitory G-proteins by 5-HT_{dro2B}. *FEBS letters.* **318**:233-236.

O'Gorman, S., Fox, D.T. and Wahl, G.M. (1991) Recombinase-mediated gene activation and site-specific integration in mammalian cells.

Olson, V.A., Wetter, J.A., Friesen, P.D. (2003) The highly conserved basic domain I of baculovirus IE1 is required for hr enhancer DNA binding and hr-dependent transactivation. *J. Virol.* **77(10)**:5668-5677.

O'Reilly, D.R. and Miller, L.K. (1989) A baculovirus blocks insect molting by producing ecdysteroid UDP-glucosyl transferase. *Science.* **245**:1110-1112.

Otto, E., Allen, J.M., Young, J.E., Palmiter, R.D. and Maroni, G. (1987) A DNA segment controlling metal-regulated expression of the *Drosophila melanogater* metallothionein gene Mtn. *Mol. Cell. Biol.* **7**:1710-1715.

Page, M.J. and Sydenham, M.A. (1991) High level expression of humanized monoclonal antibody Campath-1H in Chinese Hamster Ovary Cells. *BioTechnology.* **9**:64-68.

Passarelli, A.L. and Miller, L.K. (1993a) Three baculovirus genes involved in late and very late gene expression: *ie-1*, *ie-n*, and *lef-2*. *J. Virol.* **67**:2149-2158.

Passarelli, A.L. and Miller, L.K. (1993b) Identification and characterization of *lef-1*, a baculovirus gene involved in late and very late gene expression. *J. Virol.* **67**:3481-3488.

Passarelli, A.L. and Miller, L.K. (1993c) Identification of genes encoding late expression factors located between 56.0 and 65.4 map units of the *Autographa californica* nuclear polyhedrosis virus genome. *Virology.* **197**:704-714.

Passarelli, A.L. and Miller, L.K. (1994) Identification and transcriptional regulation of the baculovirus *lef-6* gene. *Virology.* **68**:4458-4467.

Passarelli, A.L., Todd, J.W. and Miller, L.K. (1994) A baculovirus gene involved in late gene expression encodes a large polypeptide with a conserved motif of RNA polymerases. *J. Virol.* **68**:4673-4678.

Paul, N.L., Marsh, M., McKeating, J.A., Schulz, T.F., Liljeström, P., Garoff, H. and Weiss, R.A. (1993) Expression of HIV-1envelope glycoproteins by Semliki forest virus vectors. *AIDS. Res. Hum. Retroviruses.* **9**: 963-970.

Paulson, J.C. and Colley, K.J. (1989) Glycosyltransferses-Structure, localization, and control of cell type-specific glycosylation. *J. Bio. Chem.* **264**:17615-17618.

Pearson, M.N. and Rohrmann, G.F. (1995) *Lymantria dispar* nuclear polyhedrosis virus homologous regions: characterization of their ability to function as replication origins. *J. Virol.* **69**:213-221

Pennock, G.D., Shoemaker, C. and Miller, L.K. (1984) Strong and regulated expression of *Escherichia coli* β-galactosidase in insect cells with a baculovirus vector. *Mol. Cell. Biol.* **4**:399-406.

Pfeifer, T.A. (1998) Expression of heterologous proteins in stable insect cell culture. *Current Opinion in Biotechnology.* **9**:518-521.

Pfeifer, T.A., Guarna, M.M., Kwan, E.M., Lesnicki, G. Theilmann, D.A., Grigliatti, T.A. and Kilburn, D.G (2001) Expression analysis of a modified factor X in stably transformed insect cell lines. *Protein Expr. Purif.* **23(2)**:233-241.

Pfeifer, T.A., Hegedus, D.D., Grigliatti, T.A. and Theilmann, D.A. (1997) Baculovirus immediate-early promoter-mediated expression of the zeocin resistance gene for use as a dominant selectable marker in dipteran and lepidopteran insect cell lines. *Gene.* **188**:183-190.

Picard, D. (1985) Viral and cellular transcription enhancers. *Oxford Surv Eukaryotic Genes.* **2**:24-48.

Picard, D. and Schaffner, W. (1985). Cell-type preference of immunoglobulin kappa and lambda gene promoters. *EMBO J.* **4(11)**:2831-2838.

Ptashne, M. (1986) Gene regulation by proteins acting nearby and at a distance. *Nature.* **322**:697-701.

Ptashne, M. (1988) How eukaryotic transcriptional activators work. *Nature.* **335**:683-689.

Plemper, R.K., Egner, R., Kuchler, K. and Wolf, D.H. (1998) Endoplasmic reticulum degradation of a mutated ATP-binding cassette transporter Pdr5 proceeds in a concerted action of Sec61 and the proteasome. *J. Biol. Chem.* **273(49)**:32848-32856.

Plon, S.E. and Wang, J.C. (1986) Transcription of the human beta-globin gene is stimulated by an SV40 enhancer to which it is physically linked but topologically uncoupled. *Cell.* **45(4)**:575-580.

Ponta, H., Kennedy, N., Skroch, P., Hynes, N.E. and Groner, B. (1985) Hormonal response region in the mouse mammary tumor virus long terminal repeat can be dissociated from proviral promoter and has enhancer properties. *Proc. Natl. Acad. Sci. USA.* **82**:1020-1024.

Possee, R.D. (1986) Cell-surface expression of influenza virus haemagglutinin in insect cells using a baculovirus vector. *Virus Res.* **5**:43-59.

Possee, R.D. (1997) Baculoviruses as expression vectors. *Current Opinion in Biotechnology.* **8**:569-572.

Possee, R.D. and Howard, S.C. (1987) Analysis of the polyhedrin gene promoter of the *Autographa californica* nuclear polyhedrosis virus. *Nucleic Acids Res.* **15**:10233-10248.

Possee, R.D., Thomas, C.J. and King, L.A. (1999) The use of baculovirus vectors for the production of membrane proteins in insect cells. *Biochemical Society Transactions.* **27(6)**:928-932.

Prikhod'ko, E.A. and Miller, L.K. (1996) Induction of apoptosis by baculovirus transactivator IE1. *J. Virol.* **70**:7116-7124.

Proudfoot, N.J. (1986) Transcriptional interference and termination between duplicated alpha-globin gene constructs suggests a novel mechanism for gene regulation. *Nature.* **322**:562-565.

Pugh, F.B. and Tjian, R. (1990) Mechanism of transcriptional activation by Sp1: Evidence for coactivators. *Cell.* **61**:1187-1197.

Pullen, S.S. and Friesen, P.D. (1995a) The CAGT motif functions as an initiator element during early transcription of the baculovirus transregulator *ie-1. J. Virol.* **69(6)**:3575-3583.

Pullen, S.S. and Friesen, P.D. (1995b) Early transcription of the *ie-1* transregulator gene of *Autographa californica* nuclear polyhedrosis virus is regulated by DNA sequences within its 5' noncoding leader region. *J. Virol.* **69(1)**:156-165.

Qiu, W., Liu, J. and Carstens, E.B. (1996) Studies of *Choristoneura fumiferana* nuclear polyhedrosis virus gene expression in insect cells. *Virology.* **217**:564-572.

Qu, Z.C., Thottassery, J.V., Van Ginkel, S., Manuvakhova, M., Westbrook, L., Roland-Lazenby, C., Hays, S. and Kern, F.G. (2004) Homogeneity and long-term stability of tetracycline-regulated gene expression with low basal activity by using the rtTA2S-M2 transactivator and insulator-flanked reporter vectors. *Gene* **327**:61-73.

Racher, A.J., Looby, D. and Griffiths, J.B. (1993) Influence of ammonium ion and glucose on mAb production in suspension and fixed bed hybridoma cultures. *J. Biotechnol.* **29**:145-156.

Raghunand, N. and Dale, B.E. (1999) Alteration of glucose consumption kinetics with progression of baculovirus infection in *Spodoptera frugiperda* cells. *Appl. Biotechem. Biotechnol.* **80**:231-242.

Rapp, J.C., Wilson, J.A. and Miller, L.A. (1998) Nineteen baculovirus open reading frames, including LEF-12, support late gene expression. *Journal of Virology.* **72(12)**:10197-10206.

Reinberg, D. and Roeder, R.G. (1987) Factors involved in specific transcription by mammalian RNA polymerase II. *J. Biol. Chem.* **262(7)**:3310-3321.

Resnitzky, D., Gossen, M., Bujard, H. and Reed, S.I. (1994) Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol. Cell. Biol.* **14(3)**:1669-1679.

Rippe, K., Von Hippel, P.H. and Langowski, J. (1995) Action at a distance: DNA-looping and initiation of transcription. *Trends Biochem. Sci.* **20(12)**:500-506.

Robert, F., Douziech, M. Forget, D., Egly, J.M., Greenblatt, J., Burton, Z.F. and Coulombe, B. (1998) Wrapping of promoter DNA around the RNA polymerase II initiation complex induced by TFIIF. *Mol. Cell.* **2**:342-351.

Robert, F., Forget, D., Li, J., Greenblatt, J. and Coulombe, B. (1996) Localization of subunits of transcription factors IIE and IIF immediately upstream of the transcriptional initiation site of the adenovirus major late promoter. *J. Biol. Chem.* **271**:8517-8520.

Robertson, K.D., Wolffe, A.P. (2000) DNA methylation in health and disease. *Nature.* **1**:11-19.

Roelvink, P.W., van Meer, M.M.M., de Kort, C.A.D., Possee, R.D., Hammock, B.D. and Vlak, J.M. (1992) Dissimilar expression of *Autographa californica* multiple nucleocapsid nuclear polyhedrosis virus polyhedrin and p10 genes. *Journal of General Virology.* **73**:1481-1489.

Rodems, S.M. and Friesen, P.D. (1993) The *hr*5 transcriptional enhancer stimulates early expression from the *Autographa californica* nuclear polyhedrosis virus genome but is not required for virus replication. *J. Virol.* **67(10)**:5776-5785.

Rodems, S.M. and Friesen, P.D. (1995) Transcriptional enhancer activity of *hr*5 requires dual-palindrome half sites that mediate binding of a dimeric form of the baculovius transregulator IE1. *J. Virol.* **69(9)**:5368-5375.

Rodrigues, F., van Hemert, M., Yda Steensma, H., Corte-Real, M. and Leao, C. (2001) Red fluorescent protein (DsRed) as a reported in *Saccharomyces cerevisiae. J. Bacteriol.* **183(10)**:3791-3794.

Rossi, F.M.V. and Blau, H.M. (1998) Recent advances in inducible gene expressions. *Curr. Opin. Biotechnol.* **9**:451-456.

Runstadler, P.W., and Cernek, S.R. (1988) Large-scale fluidized bed, immobilized cultivation of animal cells at high density. *Animal cell Biotechnology.* Ed. Spier, R.E. and Griffiths, J.B. Academic Press, London. **3**:305-320.

Sacchetti, A., Subramaniam, V., Jovin, T.M. and Alberti, S. (2002) Oligomerization of DsRed is required for the generation of a functional red fluorescent chromophore. *FEBS. Letters.* **525**:13-19.

Saez, E., No, D., West, A. and Evans, R.M. (1997) Inducible gene expression in mammalian cells and transgenic mice. *Curr. Opin. Biotechnol.* **8**:608-616.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular cloning: a laboratory manual.* Cold Springs Harbour Laboratory Press, New York.

Samuels, M., Fire, A. and Sharps, P.A. (1982) Separation and characterisation of factors mediating accurate transcription by RNA polymerase II. *J. Biol. Chem.* **257(23)**:14419-14427.

Sassone-Corsi, P., Dougherty, J.P., Wasylyk, B. and Chambon, P. (1984) Stimulation of *in vitro* transcription from heterologous promoters by the simian virus 40 enhancer. *Proc. Natl. Acad. Sci. USA.* **81**:308-312.

Sassone-Corsi, P. and Borrelli, E. (1986) Transcriptional regulation by *trans*-acting factors. *TIGS.* 215-219.

Saville, G.P., Thomas, C.J., Possee, R.D. and King. L.A (2002) Partial deletion redistribution of the *Autographa californica* nucleopolyhedrovirus chitinase in virus-infected cells accompanies mutation of the carboxy-terminal KDEL RE-retention motif. *J. Gen. Virol.* **83**:685-694.

Sawadogo, M. and Roeder, R.G. (1984) Energy requirement for specific transcription initiation by the human RNA polymerase II system. *J. Biol. Chem.* **259(8)**:5321-5326.

Schlesinger, S. and Dubensky, T.W. (1999) Alphavirus vectors for gene expression and vaccines. *Current Opinion in Biotechnology.* **10**:434-439.

Schaefler, S. (1967) Inducible system for the utilization of beta-glucosides in *Escherichia coli.* I. Active transport and utilization of beta-glucosides. *J. Bacteriol.* **93(1)**:254-263.

Schnetz, K. (1995) Silencing of *Escherichia coli bgl* promoter by flanking sequence elements. *EMBO. J.* **14(11)**:2545-2550.

Schwartz, D., Kisselev, P., Honeck, H., Cascorbi, I., Schunck, W.H. and Roots, I. (2001) Co-expression of human cytochrome P4501A1 (CYP1A1) variants and human NADPH-cytochrome P450 reductase in the baculovirus/insect cell system. *Xenobiotica.* **31(6)**:345-356.

Schwartz, J.L., Ferrari, E.B., Terracciano, J., Troyanovich, J., Gunnarsson, I., Wright-Minogue, J., Chen, J.W. and Kwong, A.D. (1997) Production of recombinant herpes simplex virus protease in 10-L stirred vessels using a baculovirus-insect cell expression system. *J. Ind. Microbiol. Biotechnol.* **19(2)**:87-91.

Schweinfest, C.W., Jorcyk, C.L., Fujiwara, S. and Papas, T.S. (1988) A heat shock-inducible eukaryotic expression vector. *Gene.* **71**:207-210.

Scott, R.I., Blanchard, J.H. and Ferguson, C.H.R. (1992) Effect of oxygen on recombinant protein production by suspension culture of *Spodoptera frugiperda* (Sf-9) insect cells. *Enzyme Microb. Technol.* **14**:798-804.

Serfling, E., Jasin, M. and Schaffner, W. (1985) Enhancers and eukaryotic gene transcription. *TIG.* **1**:224-230.

Shanks, M. and Lomonossoff, G.P. (2000) Co-expression of the capsid proteins of Cowpea virus in insect cells leads to the formation of virus-like particles. *J. Gen. Virol.* **81(12)**:3093-3097.

Shin, H.S. and Cha, H.J. (2002) Facile and statistical optimization of transfection conditions for secretion of foreign proteins from insect Drosophila S2 cells using green fluorescent protein reporter. *Biotechnol. Prog.* **18(6)**: 1187-1194.

Shore, D., Langowski, J. and Baldwin, R.L. (1981) DNA flexibility studied by covalent closure of short fragments into circles. *Proc. Natl. Acad. Sci. USA.* **78(8)**:4833-4837.

Shotkoski, F., Zhang, H.G, Jackson, M.B. and French-Constant, R.H. (1996) Stable expression of insect GABA receptors in insect cell lines: promoters for efficient expression of *Drosophia* and mosquito *Rdl* GABA receptors in stably transformed mosquito cell lines. *FEBS. Lett.* **380**:257-262.

Shuman, S. (1991) Recombination mediated by Vaccinia virus DNA topoisomerase I in *Escherichia* is sequence specific. *Proc. Natl. Acad. Sci. USA.* **88**:10104-10108.

Shuman, S. (1994) Novel approach to molecular cloning and polynucleotide synthesis using Vaccinia virus DNA topoisomerase. *J. Biol. chem.* **269**:32678-32684.

Singal, R. and Ginder, G.D. (1999) Methylation. *Blood.* **93**:4059-4070.

Singh, V. (1999) Disposable bioreactor for cell culture using wave-induced agitation. *Cytotechnology.* **30**: 149-158.

Skeily, Y.A.W. and Iatrou, K. (1991) Synergistic interactions of silkmoth chorion promoter-binding factors. *Mol. Cell. Biol.* **11**:1954-1964.

Slack, J.M. and Blissard, G.W. (1997) Identification of two independent transcriptional activation domains in the *Autographa californica* multicapsid nuclear polyhedrosis virus IE1 protein. *J. Virol.* **71(12)**:9579-9587.

Slack, J.M., Kuzio, J. and Faulkner, P (1995) Characterization of *v-cath*, and cathepsin L-like proteinase expressed by the baculovirus *Autographa californica* multiple nuclear polyhedrosis virus. *J. Gen. Virol.* **76**:1091-1098.

Smith, G.E., Fraser, M.J. and Summers, M.D. (1983a) Molecular engineering of the *Autographa californica* nuclear polyhedrosis genome: deletion mutation within the polyhedrin gene. *J. Virol.* **46**:584-593.

Smith, G.E., Summers, M.D. and Fraser, M.J. (1983b) Production of human beta interferon in insect cells infected with a baculovirus expression vector. *Mol. Cell. Biol.* **3**:2156-2165.

Southern, P.J. and Berg, J. (1982) Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Gen.* **1**:327-341.

Spieth, J., Shim, J.Y.H., Lea, K., Conrad, R. and Blumenthal, T. (1991) *elt*-1, an embryonically expressed *Caenorhabditis elegans* gene homologous to the GATA transcription factor family. *Mol. Cell. Biol.* **11**:4651-4659.

St Angelo, C., Smith, G.E., Summers, M.D. and Krug, R.M. (1987) Two of the three influenza viral polymerase proteins expressed by using baculovirus vectors form a complex in insect cells. *J. Virol.* **61**:361-365.

Stargell, L.A. and Struhl, K. (1996) Mechanism of transcriptional activation *in vivo*: two steps forward. *TIG.* **12(8)**:311-315.

Steller, H. and Pirrotta, V. (1984) Regulated expression of genes injected into early *Drosophila* embryos. *EMBO. J.* **3**:165-173.

Stewart, L. and Champoux, J.J. (1999) Purification of baculovirus-expressed human DNA topoisomerase I. *Methods Mol. Biol.* **94**:223-234.

Stollar, V., Stollar, B.D., Koo, R., Harrap, K.A. and Schlesinger, R.W. (1976) Sialic acid contents of sindbis virus from vertebrate and mosquito cells. Equivalence of biological and immunological viral properties. *Virology.* **69**:104-115.

Streett, D.A. and Hink, W.F. (1978) Oxygen consumption of Trichoplusia ni (TN-368) insect cell line infected with *Autographa californica* nuclear polyhedrosis virus. *J. Invert. Pathol.* **32**:112-113.

Stringer, K.F., Ingles, C.J. and Greenblatt, J. (1990) Direct and selective binding of an acidic transcriptional activation domain to the TATA-box factor TF11D. *Nature.* **345**:783-786.

Stuart, G.W., Searle, P.F., Chen, H.Y., Brinster, R.L. and Palmiter, R.D. (1984) A 12-base-pair DNA motif that is repeated several times in metallothionein gene promoter confers metal regulation to a heterologous gene. *Proc. Natl. Acad. Sci. USA.* **81**:7318-7322.

Sune, C. and Garcia-Blanco, M.A. (1995) Sp1 transcriptional factor is required for *in vitro* basal and Tat-activated transcription from the human immunodeficiency virus type 1 long terminal repeat. *J. Virol.* **69(10)**:6572-6576.

Swevers, L., Kravariti, L., Ciolfi, S., Xenou-Kokoletsi, M., Ragoussis, N., Smagghe, G., Nakagawa, Y., Mazomenos, B. and Iatrou, K. (2004) A cell-based high-throughput screening system for detecting ecdysteroid agonists and antagonists in plant extracts and libraries of synthetic compounds. *FASEB. J.* **18**:134-136.

Takada, R., Nakatami, Y., Hoffmann, A., Kokubo, T., Hasegawa, S., Roeder, R.G. and Horikoshi, M. (1992) Identification of human TFIID components and direct interaction between a 250-kDA polypeptide and the TATA box-binding protein (TFIIDt). *Proc. Natl. Acad. Sci. USA.* **89**:11809-11813.

Taticek, R.A. Shuler, M.L. (1997) Effect of elevated oxygen and glutamine levels on foreign protein production at high cell densities using the insect cell-baculovirus expression system. *Biotechnol. Bioeng.* **54**:142-152.

Theilmann, D.A. and Stewart, S. (1991) Identification and characterization of the IE-1 gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. *Virology.* **180**:492-508.

Theilmann, D.A. and Stewart, S. (1992a) Molecular analysis of the trans-acting IE-2 gene of *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus. *Virology.* **187**:84-96.

Theilmann, D.A. and Stewart, S. (1992b) Tandemly repeated sequence at 3' end of the IE-2 gene of the baculovirus *Orgyia pseudotsugata* multicapsid nuclear polyhedrosis virus is an enhancer element. *Virology.* **187**:97-102.

Thomas, C.J. (1997) Role of chitinase and cathepsin in baculovirus infection. *PhD thesis.* Oxford Brookes University.

Thomas, C.J., Brown, H.L., Hawes, C.R., Lee, B.Y., Min, M.K., King, L.A. and Possee, R.D. (1998) Localization of a baculovirus-induced chitinase in the insect cell endoplasmic reticulum. *J. Virol.* **72**:10207-10212.

Tramper, J., Williams, J.B., Joustra, D. and Vlak, J.M. (1986) Shear sensitivity of insect cells in suspension. *Enzyme Microb. Technol.* **8**:33-36.

Traunecker, A., Oliveri, F. and Karjalainen, K. (1991) Myeloma based expression system for production of large mammalian proteins. *Tibtech.* **9**:109-113.

Triezenberg., S.J., Kingsbury, R.C. and McKnight, S.L. (1988) Functional dissection of VP16, the *trans*-activator of herpes simplex virus immediate early gene expression. *Genes Devel.* **2**:718-729.

Tsien, R. (1998) The green fluorescent protein. *Annu. Rev. Biochem.* **67**:509-544.

Turker, M.S. (2002) Gene silencing in mammalian cells and the spread of DNA methylation. *Oncogene.* **21**:5388-5393.

Tweedie, S., Ng, H-H., Barlow, A.L., Turner, B.M., Hendrich, B. and Bird, A. (1999) Vestiges of a DNA methylation system in *Drosophila melanogaster*? *Nat. Genet.* **23(4)**:389-390.

Tyo, M.A., Bulbulian, B.J., Menken, B.Z. and Murphy, T.J. (1988) Large-scale mammalian cell culture utilizing Acusyst technology. *Animal cell biotechnology.* Ed. Spier, R.E. and Griffiths, J.B. Academic Press, London. **3**:358-371.

van der Straten, A., Johansen, H., Sweet, R. and Rosenburg, M. (1989) Efficient expression of foreign genes in cultured *Drosophila melanogaster* cells using hygromycin B selection. *Invertebrate Cell System Applications.* Ed. Mitsuhashi, J. 183-195. CRC Press, Boca Raton, FL.

Van-Lier, F.L.J., Van-Duijnhoven, G.C.F., de-Vaan, M.M.J.A.C.M., Vlak, J.M. and Tramper, J. (1994) Continuous β-galactosidase production in insect cells with a p10 gene based baculovirus vector in a two-stage bioreactor system. *Biotechnol. Prog.* **10**:60-64.

Vigoreaux, J.O. and Tobin, S.L. (1987) Stage-specific selection of alternative transcriptional initiation sites from the 5C actin gene of *Drosophila melanogaster. Genes. Dev.* **1**:1161-1171.

Viswanathan, P., Venkaiah, B., Kumar, M.S., Rasheedi, S., Vrati, S., Bashyam, M.D. and Hasnain, S.E. (2003) The homologous region sequence (hr1) of *Autographa californica* multinucleocapsid polyhedrosis virus can enhance transcription from non-baculoviral promoters in mammalian cells. *J. Biol. Chem.* **278(52)**:52564-52571.

Vlak, J.M., Putb, B., Desernob, M.L., Schoonevelda, H. and Smida, H.M. (1998) Expression of a male accessory gland peptide of Leptinotarsa decemlineate in insect cells infected with a recombinant baculovirus. *J. Insect Physiol.* **44(3-4)**:255-262.

Vologodskii, A. and Cozzarelli, N.R. (1996) Effect of supercoiling on the juxtaposition and relative orientation of DNA sites. *Biophy. J.* **70(6)**:2548-2556.

Voss, S.D., Schlokat, U. and Gruss, P. (1986) The role of enhancers in the regulation of cell-type-specific transcriptional control. *TIBS.* **11**:287-289.

Vulsteke ,V., Janssen, I., Vanden Broeck, J., De Loof, A. and Huybrechts, R. (1993) Baculovirus immediate early gene promoter based expression vectors for transient and stable transformation of insect cells. *Insect Mol. Biol.* **2(4)**:195-204.

Wagner, R., Lledke, S., Kretzschmar, E., Geyer, H., Geyer, R. and Klenk, H-D. (1996) Elongation of the *N*-glycans of fowl plague hemagglutinin expressed in *Spodoptera frugiperda* (Sf9) cells by co-expression of human β1,2-N-acetlglucosaminyltransferase I. *Glycobiology.* **6**:165-175.

Walker, V.K (1989) Gene transfer in insect cells. *Advances in cell culture.* **7**. Ed. Maramorosch, K. and Sato, G. Academic Press, New York.

Wampler, S.L., Tyree, C.M. and Kadonaga, J.T. (1990) Fractionation of the general RNA polymerase II transcription factors from *Drosophila* embryos. *J. Biol. Chem.* **265(34)**:21223-21231.

Wang, M-Y., Pulliam, T.R., Valle, M., Vakharia, V.N. and Bentley, W.E. (1996) Kinetic analysis of alkaline protease activity, recombinant protein production and metabolites for infected insects (Sf-9) cells under different DO levels. *J. Biotechnol.* **46**:243-254.

Warren, A.J. (2002) Eukaryotic transcription factors. *Curr. Opin. Biol.* **12(1)**:107-114.

Wasylyk, B., Wasylyk, C. and Chambon, P. (1984) A novel eukaryotic promoter element: the simian virus 40 72 base pair repeat. *Fed. Proc.* **43(2)**:226-34.

Wei, W., Gu, J.X., Zhu, C.Q., Sun, F.Y., Dorjsuren, D., Lin, Y. and Murakami, S. (2003) Interaction with general transcription factor IIF (TFIIF) is required for the suppression of activated transcription by RPB5-mediating protein (RMP). *Cell Res.* **13(2)**:111-120.

Weyer, U., Knight, S. and Possee, R.D. (1990) Analysis of very late gene expression by *Autographa californica* nuclear polyhedrosis virus and the further development of multiple expression vectors. *J. Gen. Virol.* **71**:1525-1534.

Weyer, U. and Possee, R.D. (1989) Analysis of the promoter of the *Autographa californica* nuclear polyhedrosis virus p10 gene. *J. Gen. Virol.* **70**:203-208.

Weyer, U. and Possee, R.D. (1991) A baculovirus dual expression vector derived from the *Autographa californica* nuclear polyhedrosis virus polyhedrin and p10 promoters: co-expression of two influenza virus genes in insect cells. *J. Gen. Virol.* **72**:2967-2974.

Whiteside, J.P. and Spier, R.E. (1981) The scale-up from 0.1 to 100 litre of a unit process system based on 3 mm diameter glass spheres for the production of four strains of FMDV from BHK monolayer cells. *Biotechnol. Bioeng.* **23**:551.

Wigler, M., Perucho, M., Kurtz, D., Dana, S., Pellicer, A., Axel, R. and Silverstein, S. (1980) Transformation of mammalian cells with an amplifiable dominant-acting gene. *Proc. Natl. Acad. Sci. USA.* **77**:3567-3570.

Wirth, M., Bode, J., Zettlmeissl, G. and Hauser, H. (1988) Isolation of overproducing recombinant mammalian cell lines by a fast and simple selection procedure. *Gene.* **73**:419-426.

Wong, T.K., Nielsen, L.K., Greenfield, P.F. and Reid, S. (1994) Relationship between oxygen uptake rate and time of infection of Sf9 insect cells infected with a recombinant baculovirus. *Cytotechnology.***15(1-3)**:157-167.

Wood, L.A. and Thompson, P.W. (1987) Applications of the air lift fermenter. *Appl. Bio. Biotechnol.* **15**:131.

Woychik, N.A. (1998) Fractions to funcions: RNA polymerase II thirty years later. *Cold Spring Harb Symp Quant Biol.* **63**:311-317.

Woychik, N.A. and Hampsey, M. (1998) The polymerase II machinery: structure illuminates function. *Cell.* **108**:453-463.

Wu, S-Y. and Chiang, C-M. (1996) Establishment of stable insect cell lines expressing potentially toxic proteins by tetracycline-regulated and epitope-tagging methods. *BioTechniques.* **21(4)**:718-725.

Wu, T-Y., Liona, L., Chen, S-L., Chen, C-Y. and Chao Y-C. (2000) Expression of highly controllable genes in insect cells using a modified tetracycline-regulated gene expression system. *Journal of biotechnology.* **80**:75-83.

Wurm, F. and Bernard, A. (1999) Large-scale transient expression in mammalian cells for recombinant protein production. *Current Opinion in Biotechnology.* **10**:156-159.

Wyborski, D.L. and Short, J.M. (1991) Analysis of inducers of the E.coli lac repressor system in mammalian cells and whole animal. *Nucleic Acids Revs.* **19(17)**:4647-4653.

Xiang, Z.Q., Spitalnik, S., Tran, M., Wunner, W.H., Cheng, J. and Ertl, H.C.J. (1994) Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. *Virology.* **199**:132-140.

Xiao, H., Pearson, A., Coulombe, B., Truant, R., Zhang, S., Regier, JL., Triezenberg, SJ., Reinberg, D., Flores, O., Ingles, CJ., *et al.* (1994) Binding of basal transcription factor TFIIH to the acidic activation domains of VP16 and p53. *Mol. Cell. Biol.* **14(10)**:7013-7024.

Yamanaka, R. (2004) Alphavirus vectors for cancer therapy. *Int. J. Oncol.* **24(4)**:919-923.

Yang, S. and Miller, L.K. (1998) Expression and mutational analysis of the baculovirus very later factor 1 (*vlf-1*). *Virology.* **245**:99-109.

Yang, S. and Miller, L.K. (1999) Activation of baculovirus very later promoter by interaction with very later factor 1. *J. Virol.* **73(4)**:3404-3409.

Yang, T.T., Cheng, L. and Kain, S.R. (1996) Optimized codon usage and chromophore mutation provides enhanced sensitivity with the green fluorescent protein. *Nucl Acids Res.* **24**:4592-4593.

Yao, F., Svensjo, T., Winkler, T., Lu, M., Eriksson, C. and Eriksson, E. (1998) Tetracycline repressor, tetR, rather than the tetR-mammalian cell transcription factor fusion derivatives, regulates inducible gene expression in mammalian cells. *Hum. Gene. Ther.* **9**:1939-1950.

Yao, F. and Eriksson, E. (1999) A novel tetracycline-inducible viral replication switch. *Hum. Gene. Ther.* **10**:419-427.

Yarranton, G.F. (1992) Inducible vectors for expression in mammalians cells. *Curr. Opin. Biotechnol.* **3**:506-511.

Yates, J.L., Warren, N. and Sugden, B. (1985) Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. *Nature.* **313**:812-815.

Yin, D.X. and Schimke, R.T. (1995) BCL-2 expression delays drug-induced apoptosis but does not increase clonogenic survival after drug treatment in HeLa cells. *Cancer Res.* **55**:4922-4928.

Yin, D.X., Zhu, L. and Schimke, R.T. (1996) Tetracycline-controlled gene expression system achieves high-level and quantitative control of gene expression. *Analytical Biochemistry.* **235**:195-201.

Zahn-Zabal, M., Kobr, M., Girod, P-A., Imhof, M., Chatellard, P., de Jesus, M., Wurm, F. and Mermod, N. (2001) Development of stable cell lines for production or regulated expression using matrix attachment regions. *J. Biotech.* **87**:29-42.

Zhang, G., Gurtu, V. and Kain, S.R. (1996) An enhanced green fluorescent protein allows sensitive detection of gene transfer in mammalian cells. *Biochem. Biophys. Rev. Commun.* **227**:707-711.

Zhang, J., Logan, K., Yu, X-B. and Davis, E.A. (1990) Early events of RNA polymerase II transcription initiation. *Life Sciences.* **46**:729-735.