CHARACTERIZATION OF THE EXTERNAL ENVELOPE GLYCOPROTEIN OF MAEDI-VISNA VIRUS, AN OVINE LENTIVIRUS

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Declaration

I hereby declare that the composition and experiments of this thesis are my own, unless stated otherwise. No part of this work has been, or is being, submitted for any other degree or qualification.

Signed Date 16.2.96

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ABSTRACT OF THESIS

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Name of Candidate ..... Penelope Jane Carter .....

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Title of Thesis ..... Characterization of the external envelope glycoprotein of Maedi-Visna .....

..... Virus, an ovine lentivirus. .....

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The envelope glycoproteins of Maedi-Visna virus consist of a surface glycoprotein (gp135) which is responsible for the characteristic spikes on the surface of the virion, and a transmembrane protein (gp41) whose function includes linkage to the surface glycoprotein, anchoring it to the virion envelope. The external glycoprotein is required for attachment to the host cell via a receptor molecule present on the surface of the cell. Cells of the macrophage lineage are the main target cells in MVV infection *in vivo*. The host humoral response is targeted to the surface glycoprotein resulting in neutralizing antibody production. The relevance of these antibodies is not understood as virus infection persists despite this active immune response. The external glycoprotein has also been shown to be susceptible to antigenic variation.

Expression of gp135 as three overlapping fragments in the bacterial pGEX system was undertaken with a view of using the recombinant protein as a source of immunogen to raise monoclonal antibodies. These and the three recombinant fragments could be used for epitope mapping. However, these fragments proved to be toxic to bacterial cells resulting in low yields and high levels of contamination. In depth studies were carried out to improve the yield and attempts were made to raise immune polyclonal sera. Characterization of these sera is described.

Recombinant protein studies were extended to express gp135 in the baculovirus expression system. This resulted in a reliable source of recombinant protein that was devoid of contamination and was easily purified. This protein was glycosylated and was recognised by MVV-infected sheep sera. Preliminary studies were carried out to determine its interaction with sheep fibroblasts and hence its use to isolate the host cell receptor.

Attempts were made to raise monoclonal antibodies against gp135 purified from virions by lectin affinity chromatography. The development of a screening assay is described. This approach did not result in the generation of any anti-gp135 monoclonals. The preparation of polyclonal antisera raised against two peptides within the external glycoprotein is reported.

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LIST OF ABBREVIATIONS

Ag	antigen
AIDS	Aquired Immunodeficiency Syndrome
AP	ammonium persulphate
BCIP	bromo-chloro-inodyl phenol toludine salt
BSA	bovine serum albumin
CAEV	caprine arthritis-encephalitis virus
CD	cluster of differentiation
cDNA	DNA complimentary to mRNA
CIP	calf intestinal phosphatase
CNS	central nervous system
ConA	concanavalin A
c.p.e.	cytopathic effect
cpm	counts per minute
CSF	cerberal spinal fluid
CTL	cytotoxic lymphocyte
dH_2O	distilled water
DMEM	Dulbecco's modification of Eagle's medium
DMF	dimethylformamide
DMSO	dimethyl sulphoxide
ds DNA	double stranded DNA
DTT	dithiothreitol
E. coli	*Eschericia coli*
EDTA	ethylenediaminetetra-acetate
EIAV	equine infectious anaemia virus
ELISA	enzyme-linked immunosorbant assay
env	envelope protein
E.R.	endoplastic reticulum
extravadin-AP	extravadin-alkaline phosphatase
FCS	foetal calf serum
FACS	fluorescent activated cell sorter
FITC	flourescein isothiocynate
FIV	feline immunodeficiency virus
GST	glutathione-S-transferase
HBSS	Hank's buffered salt solution
HIV	human immunodeficiency virus

HPEC	high performance electrophoresis chromatography
HPLC	high performance liquid chromatography
HSV	Herpes simplex virus
Ig	immunoglobulin
IL-2	interleukin-2
IPTG	isopropyl-β-D-thiogalactopyranoside
LTR	long terminal repeat
MAb	monoclonal antibody
MHC	major histocompatabilty complex
m.o.i.	multiplicity of infection
MVV	maedi-visna virus
NBT	nitroblue tetrazolium
OLV	ovine lentivirus
orf	open reading frame
PBA	PBS/BSA/azide buffer
PBL	peripheral blood lymphocytes
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
p.f.u.	plaque forming units
PND	principal neutralizing determinant
RNase	ribonuclease
SAP	shrimp alkaline phosphatase
sCD4	soluble CD4
s.d.	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SDW	sterile distilled water
SIV	simian immunodeficiency virus
ss RNA	single stranded RNA
SV40	simian virus-40
$TCID_{50}$	50% tissue culture infectious doses
TEMED	N,N,N',N'-tetramethylethylenediamine
U.V.	ultra-violet
X-gal	5-bromo-4-chloro-3-inodyl-β-D-galactopyranoside
X-gluc	5-bromo-4-chloro-3-inodyl-β-D-glucorinic acid

CHAPTER 1: INTRODUCTION

1.1 Introduction to maedi-visna virus; an ovine lentivirus: isolation, transmission and pathology

The retrovirus family are characterized as RNA viruses that use a viral-encoded reverse transcriptase to replicate via a DNA template. Members of the family include the oncovirinae, the spumavirinae and the lentivirinae. Lentivirus infections are characterized by a period of 'latency' followed by the development of clinical disease which follows a slow, progressive course. Lentiviruses fall into two classes; the immunodeficiency viruses which predominantly infect T-lymphocytes resulting in their depletion, and include the human immunodeficiency viruses; HIV-1 and HIV-2 (Barre-Sinoussi, 1983, Gallo et al., 1984, Levy et al., 1984), simian immunodeficiency virus; SIV (Letvin et al., 1985), and feline immunodeficiency virus; FIV (Pedersen et al., 1987). The second class of lentiviruses infect ungulate animals and are characterized by infection of cells of the monocyte/macrophage lineage resulting in immune-mediated organ specific diseases. These include maedi-visna virus of sheep; MVV (Sigurdsson, 1954), caprine arthritis encephalitis virus; CAEV (Crawford et al., 1980) and equine infectious anaemia virus; EIAV (Valle and Carre, 1904, Cheevers et al., 1985) which has an acute disease progression unique to this lentivirus.

Maedi-visna virus was first isolated from Icelandic sheep in the 1940's (Sigurdsson, 1954, Sigurdsson et al., 1957). The disease was described as an emaciating, respiratory disease and it is from this that the virus derives its name; maedi (dysponea) and visna (wasting) (Gudnadottir and Palsson, 1967). The introduction of this virus to Iceland originated from the importation of a flock of sheep from Germany in 1933, and resulted in a full scale epidemic by 1940 with a subsequent slaughter and devastating depletion of the sheep population. Maedi-visna is now a worldwide problem and is of great economic and veterinary importance. Isolates have been described in the U.S.A., known as ovine progressive pneumonia virus (Marsh, 1923, Kennedy et al., 1968, Cutlip and Laird, 1976), South Africa (Querat et al., 1990), France, the Netherlands where it is known as *zwoegerzietkte* (Ressang et al., 1968, De Boer, 1970) and the U.K. (Dawson et al., 1979, Watt et al., 1990). However, MVV is not found in Australia and New Zealand. More recently, the importance of studying MVV has been linked to its use as a model for HIV-1 infection without the complication of lymphocyte infection, since it shares pathological, virological and epidemiological features.

The major mode of transmission of MVV is horizontal via the respiratory tract. This is probably exacerbated by close housing conditions (Houwers and van der Molen, 1987). A

study by de Boer et al. (1979) indicated that the incidence of transmission is proportional to the length of exposure where 81% of uninfected lambs became infected when housed with a flock of infected ewes for 1 year. The incidence of infection has also been shown to be elevated amongst sheep suffering from sheep pulmonary adenomatosis (SPA) (Dawson et al., 1990), probably as a consequence of increased respiratory fluid caused by SPA (DeMartini et al., 1987). There is no evidence of vertical transmission (de Boer et al., 1979, Sihvonen, 1980), although 2 out of 11 lambs derived by hysterectomy from infected ewes showed progressive pneumonia even though they had been kept in isolation immediately after birth (Cross et al., 1985). This suggested that the lambs had been infected prior to or at birth (Cross et al., 1985). There is no evidence of transmission through the mother's colostrum to the lamb as is seen in CAEV transmission (Ellis et al., 1983). However, the presence of MVV infected cells within colostrum has been reported (Sihvonen, 1980). Minor transmission through faecally contaminated drinking water has been described (Sigurdsson, 1954). MVV is not transmitted sexually as is HIV and other immunodeficiency viruses.

The incubation period of MVV varies from 1 to 4 years. It is thought to be prevalent in 80-85% of the sheep population in Western Europe and U.S.A. (Cutlip et al., 1977), with only 25% developing clinical disease (Crawford et al., 1981). MVV infection is diagnosed by the presence of serum antibodies to the major core antigen (Petursson et al., 1976, Griffin et al., 1978, Oliver et al., 1981) and the envelope glycoprotein (Adams et al., 1985, Adams and Gorham, 1986). Clinical disease is characterized as progressive lesions in one or more organs, including lungs, CNS and joints, leading to severe illness and usually death. Respiratory disease becomes apparent by slight dysponea, loss of condition and listlessness. Dysponea will progress leading to respiratory difficulties, and at post mortem lungs can weigh 2-4 times the normal weight (Marsh, 1923, Sigurdsson, 1954, Cutlip and Laird, 1976, Oliver et al., 1981). Histologically, the alveolar septa are thickened due to the accumulation of plasma cells, mononuclear phagocytes and lymphocytes, which leads to total obliteration of alveoli (Georgsson and Palsson, 1971). There is a presence of lymphoid follicles consisting of lymphocyte aggregates with germinal centres of large lymphoblasts (Lairmore et al., 1986), and smooth muscle hyperplasia (Watt et al., 1992). The histopathology seen in maedi bears some resemblance to lymphoid hyperplasia in children with AIDS, and thus is a useful model for studying HIV infection.

The neurodegenerative aspect of the disease becomes apparent by aberrations in gait, weakness in hind legs and eventually this leads to paralysis. The cause of encephalitis is thought to be due to inflammation within the white matter of the brain and cerebellum which eventually leads to demyelination (Sigurdsson and Palsson, 1958). The primary lesions occur in the glial cells (Sigurdsson and Palsson, 1958), and viral RNA and antigens have been

located in oligodendrocytes and astrocytes (Stowing et al., 1985). The presence of viral antigens in these cells is suggested to influence the inflammatory response which leads to demyelination (Stowing et al., 1985).

Mastitis may be a clinical consequence caused by lymphoid hyperplasia and fibrosis within the mammary gland, with lesions similar to those found in maedi lungs (Cross et al., 1985, Oliver et al., 1981, van der Molen et al., 1985). Non-suppurative arthritis may develop in tarsal and carpal joints (Oliver et al., 1981), but this is a rare event unlike in CAEV infection. Arthritis is caused by swelling and calcification of soft tissue, fibrosis of the joint capsule and synovium, and perivascular lymphocytic infiltration (Oliver et al., 1981).

1.2 Viral tropism

MVV predominantly infects sheep, but infection of goats has also been demonstrated (Banks et al., 1983). There are no reports of infection of any other animal species *in vivo*. The major target cells *in vitro* are fibroblasts cells of sheep and goats. MVV has also been shown to recognise receptors on the surface of cells from a variety of species, including mice, rats and human (Gilden et al., 1981). However, *in vivo* the cells from macrophage/monocyte lineage are the main targets, as demonstrated by the presence of virus in macrophages taken from infected sheep and cocultivated with fibroblasts (Narayan et al., 1982). MVV has been shown to infect and replicate in macrophages *in vitro*, resulting in small quantities of virus produced with non-cytopathic effects (Narayan et al., 1982). The extent of viral gene expression is dependent on the activation state of the cell, such that on increased cellular differentiation virus gene expression is increased (Gendelman et al., 1986). This may be a possible mechanism for viral persistence *in vivo* such that virus may remain latent in undifferentiated cells. In fact, clusters of infected macrophages have been found in the bone marrow of infected sheep, which may act as a virus reservoir since the half-life of circulating monocytes and tissue macrophages is short (Gendelman et al., 1986).

In contrast to HIV infection and other immunodeficiency lentiviruses, infection of T-lymphocytes has not been demonstrated (Gorrell et al., 1992), although one report suggested that cells of lymphocyte morphology in the CNS did express viral core proteins (Georgsson et al., 1989).

Although infection of fibroblasts *in vivo* is not widely observed, a recent report has suggested evidence of virus infection of fibroblasts in the CNS (Zink et al., 1994). Infection of MVV in smooth muscle cells from the ovine aorta has been demonstrated *in vitro* (Leroux et al., 1995). This may reflect of pathogenesis seen in the lungs of infected sheep. Infection of dendritic cells *in vivo* has also been seen (Gorrell et al., 1992).

1.3 Pathogenesis

The cytopathic effects characteristic of MVV infection *in vitro* are not seen *in vivo*, and thus do not explain the pathogenic mechanisms of the virus. Normal host cell defences involve macrophages which are involved in the phagocytosis of virus-infected cells. Macrophages also present viral antigens via MHC classII to helper T cells resulting in the proliferation of lymphocytes whose combined effect eliminates the pathogen. The fact that the major target cell in MVV infection is the macrophage obviously complicates the usual host defence mechanisms. The tropism for macrophages may explain the persistence of the virus in the host, but it is thought that pathogenesis is a consequence of aberrations in the immune system. Evidence for this will be presented below.

Early studies on MVV-induced disease observed that only 1 in 100,000 cells are infected and concluded that the severity of the lesions were not directly due to the existence of virus-infected cells (Petursson et al., 1976). Immunosupression of infected sheep resulted in a decrease in the extent of CNS lesions, suggesting that pathogenesis may be immune-mediated (Nathanson et al., 1976). In contrast to the effect on lesion development, immunosupression did not reduce the amount of recoverable virus indicating the persistence of virus despite an effective immune response (Nathanson et al., 1976). An increase in serum immunoglobulin levels in infected sheep compared to controls was reported (Molitor et al., 1979), although characterization of the increased immunoglobulin was not undertaken. It could be envisaged that elevated levels of immunoglobulin would lead to formation of immune complexes. Since clinical disease is characterised by a local inflammatory response, the clearing of these immune complexes could be seen to influence the pathogenesis of MVV. The envelope glycoprotein is the major target for the immune response *in vivo*, as is discussed in section 1.12. Transgenic sheep have been produced that express the envelope protein *in vivo* (Clements et al., 1994). These sheep expressed envelope proteins in differentiated macrophages and fibroblast cells when cutured *in vitro*. It was not demonstrated whether the envelope protein was expressed *in vivo*, but two out of the three lambs did mount an immune response against envelope protein. These sera were non-neutralizing. The authors comment on the usefulness of this model in looking at the role of envelope in pathogenesis. Infection of these sheep with MVV would provide an opportunity to see if sheep would be protected. Macrophages expressing envelope *in vivo* may desensitize the animal such that on infection, inflammatory responses would be reduced (Clements et al., 1994).

Several studies have been undertaken to characterize the cellular infiltrate in the lungs, which is responsible for the dysponea seen later on in infection. A 1.5-fold increase in

total leukocyte number and a 4-fold increase in lymphocyte numbers in infected lambs compared to normal lambs was reported (Lairmore et al., 1988a). The predominant subset was CD8+ cells (65%) (Lairmore et al., 1988a). This correlates with an increase in absolute numbers of CD8+ lymphocytes found in a clinically infected sheep (Kennedy-Stoskopf et al., 1989). Similar studies in naturally infected sheep showed an increase in cell number but no difference in the percentage of lymphocytes (Cordier et al., 1990). However, there appeared to be an increase in neutrophil numbers and an elevated release of neutrophil chemotactic factor and fibronectin. This could influence the infiltration of inflammatory cells which would correlate with lesion development (Cordier et al., 1990). There was also an increase in MHC classII expression on the surface of macrophages (Cordier et al., 1990), which is consistent with other workers which found that MHC classII was expressed in infected macrophages (Kennedy et al., 1985). A lentivirus-induced interferon was described by Narayan and colleagues who showed that the release of interferon was dependent on the interaction of infected macrophages with T-lymphocytes (Narayan et al., 1985). This data is supported by a similar observation that the spontaneous release of this specific interferon from lymphocytes is increased by the presence of MVV-infected macrophages (Lairmore et al., 1988b). It is postulated that the interaction of infected macrophages with lymphocytes results in the release of the specific interferon that increases the expression of MHC classII on infected macrophages (Kennedy et al., 1985). Since infected cells will also be expressing viral antigens, the increase in MHC classII expression may result in more efficient presentation of viral antigens to helper T cells. This may result in a distortion of the immune response leading to a localised lymphoproliferation and a failure to clear infection (Kennedy et al., 1985).

Virus strains differ in their pathology, such that the Icelandic strains 1514 and K796 are predominantly neurovirulent (Petursson et al., 1976), and virus isolated in the USA appears to be mainly linked to progressive pneumonia (Cutlip and Laird, 1976). Several reports have indicated that the characteristics of virus strains *in vitro* affect the extent of pathogenicity *in vivo*. Several strains isolated form French MVV-infected sheep differed in their replication *in vitro*, that is they were either highly lytic or induced persistent infections. This indicated the existence of genetically distinct strains of virus in the field which may influence the severity of disease (Querat et al., 1984). Another study characterized virus isolates on their ability to lyse fibroblast cells and macrophage cells *in vitro* (Lairmore et al., 1987). Isolates that were able to lytically infect macrophages *in vitro* caused the most severe pathogenic effects (Lairmore et al., 1987). The genetic differences for the existent of pathogenically distinct viruses is not understood.

The literature described so far has suggested that both the interplay of the virus with the immune system and the replication properties *in vitro* may influence the severity of disease. The role of viral antigens, other than stimulating an immune response, have been implicated in pathogenesis. The cysteine-rich domain of MVV tat shares homology with snake venom neurotoxins (Hayman et al., 1993). Tat-derived peptides including this motif were injected into the brains of rats, and were shown to cause lesions (Hayman et al., 1993). It was suggested that tat may provide a source of arginine residues for nitric oxide (NO) synthesis, and the increased levels of NO in the brain results in cell death (Hayman et al. 1993). Transgenic mice carrying the tat gene develop disorders of the lymphoid organs (Vellutini et al., 1994), but no effect in the brains of mice is observed. It is suggested that tat impairs the immune response by stimulating the proliferation of immune cells (Vellutini et al., 1994). Both these studies are preliminary, and the mechanism for tat's role in pathogenesis is not known.

Pathogenesis appears to be mediated by a dysfunction in the regulation of the immune response, but precisely which parts of the immune network are involved is not known.

1.4 Virus structure

MVV, in common with all retroviruses, is characterized morphologically as a crescent shaped, electron dense particle that buds from the plasma membrane. The virus particle comprises a bar-shaped nucleoid, which is characteristic of lentiviruses, surrounded by a lipid coat with protruding spikes, and is distinguishable from type-C retroviruses by the absence of an electron-lucent region between the core and the envelope (Genderblom et al., 1987). The virus particle varies between 80-120nm in size (Gonda et al., 1985).

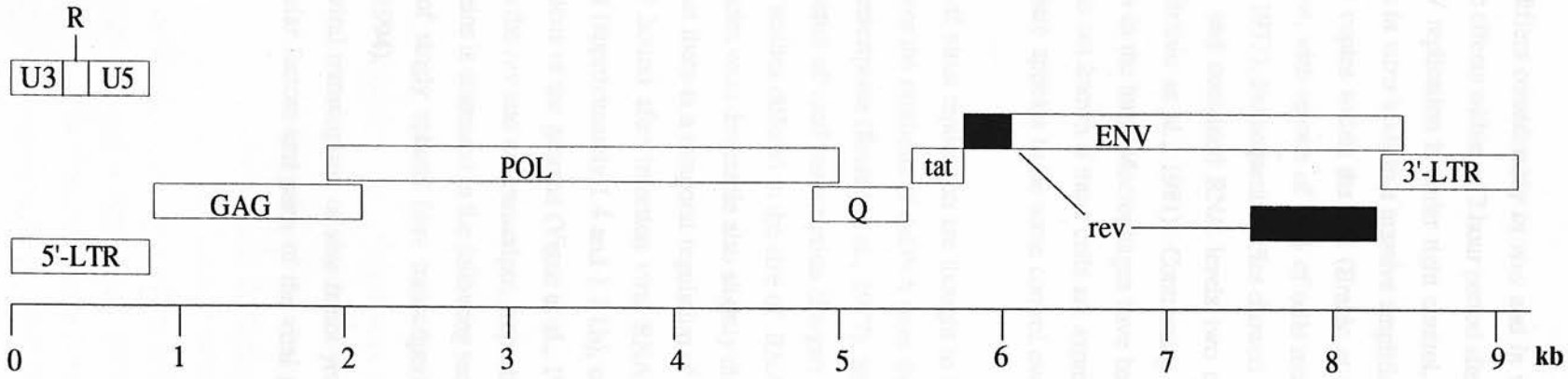
Lentiviruses share a common genomic structure with other retroviruses, that is they possess the *pol*, *gag* and *env* genes (Weiss et al., 1982). In addition, they have a number of additional small open reading frames (orfs) which encode regulatory proteins. The virions contain two copies of a positive-sense single stranded RNA genome (ssRNA) (Brahic et al., 1977), an RNA-dependent DNA polymerase (reverse transcriptase) (Lin and Thormar, 1970), several internal structural proteins and the whole virion is surrounded by the envelope coat containing the major envelope glycoprotein. The virus replicates via a DNA template (Haase and Varmus, 1973), in keeping with all retroviruses. MVV is most closely related to the non-primate lentiviruses by phylogenetic analysis (McClure et al., 1988a, Olmsted et al., 1989), but it is more closely related to HIV than to the onco and spumaviruses (Gonda et al., 1985).

Up to seven different isolates of MVV have been described and sequenced; four of the isolates are derived from the 1514 strain (Sonigo et al., 1985, Braun et al., 1987, Staskus et al., 1991, Andresson et al., 1993), a South African strain (SA-OMVV) (Querat et al., 1990), a British isolate (EV-1) (Sargan et al., 1991) and recently a New York isolate (Campbell et al., 1993). These isolates only show minor differences at the molecular level, and generally share the same genomic organisation. The organisation of the virus genome is shown in figure 1.1. The virus genome is flanked on either side by long terminal repeats (LTRs). These are involved in regulating viral transcription and are divided into three region; U5, R and U3. The target sequences that influence transcription are located in 43 base pair repeats in the U3 region. A number of potential AP-1 binding sites have been located within this region but only a TATA box proximal sequence matches the consensus sequence for the AP-1 site (Hess et al., 1989). The LTR shows strong promoter activity and position independent enhancer activity (Hess et al., 1985). In addition, there is a primer binding site complementary to that for the tRNA $^{lys}_{1,2}$ (Sonigo et al., 1985) and a polypurine tract which acts as an initiation site for positive strand DNA synthesis. There is also a negative response element upstream of the U3 region (Hess et al., 1989).

Figure 1.1: The structure of the MVV genome

The map shows the position and organization of the orfs of MVV. The map gives the approximate nucleotide positions of each orf.

Key: LTR: long terminal repeates comprised of three regions; U3,R and U5.
GAG: encoding internal structural proteins: matrix, capsid and nucleoprotein.
POL: encoding reverse transcriptase, integrase and protease.
Q: vif protein of unknown function.
tat: regulates transcription.
rev: comprised of 2 exons (closed boxes), regulates splicing of mRNA transcripts.
ENV: encoding the envelope glycoproteins.


R
U3
U5
5'-LTR
GAG
POL
Q
tat
ENV
3'-LTR
rev
0
1
2
3
4
5
6
7
8
9
kb

1.5 MVV transcription

The life cycle of MVV differs considerably *in vivo* and *in vitro*. *In vitro* the virus is highly lytic leading to cytopathic effects within a 72 hour period after infection of tissue culture cells. In contrast, *in vivo* MVV replication is under tight control, such that recoverable virus is a rare event. The lytic cycle *in vitro* leads to a massive amplification of virus genome within the infected cell, up to 4000 copies within the cell (Brahic et al., 1977). The number of cells infected *in vivo* is very low, with reports of 18% of cells recovered from the CNS containing viral RNA (Haase et al., 1977). Subsequent studies showed that the level of infected cells *in vivo* is even less, 1-3%, and contained RNA levels two orders of magnitude lower than infected cells *in vitro* (Brahic et al., 1981). Contrasting results have been found when investigating infected cells in the lung. Macrophages have been shown to contain up to 1000 copies of viral RNA, but is not known if these cells are expressing viral antigens (Gendelman et al., 1985). Therefore there appears to be some control over viral transcription *in vivo*, as will be discussed below.

The initial stages of virus replication are thought to be similar both in tissue culture and in the host. This involves the synthesis of dsDNA from the viral ssRNA genome using the virally-encoded reverse transcriptase (Brahic et al., 1977). Several *in vitro* studies have been reported looking at the kinetics of viral transcription (Sargan et al., 1994, Vigne et al., 1987, Davis et al., 1987). These studies differed in the size of RNA transcripts and the time points at which various RNA species were detectable also slightly differed. However, they can all be summarised to conclude that there is a temporal regulation of RNA transcription *in vitro*. At early time points (6 to 24 hours) after infection viral RNA transcripts can be detected as multiply spliced transcripts (approximately 1.4 and 1.7 kb), containing sequences from the 3' end, 5' end and central regions of the genome (Vigne et al., 1987, Davis et al., 1987). These have been shown to encode the *rev* and *tat* transcripts, respectively (Sargan et al., 1994). The functions of these two proteins is discussed in the following section 1.6.1. At later time points, there is also a presence of singly spliced (*env* transcripts) and unspliced (*gag* and *pol* transcripts) (Sargan et al., 1994).

The restriction of viral transcription *in vivo* is not yet understood, but it is probable that a combination of cellular factors and parts of the viral genome, such as the LTR, are involved.

1.6 The proteins of MVV

1.6.1 The regulatory proteins

1.6.1i Rev

Rev is a small regulatory protein of 19kDa in size, that is located predominantly in the cytoplasm of the infected cell (Mazarin et al., 1990), and has been shown to be essential for productive infection (Toohey and Haase, 1994). It is thought to function in a similar manner to that of HIV-1, in that it regulates the splicing of mRNA transcripts and that its relative amount within the cell predicts the type of transcripts present in the infected cell. There is evidence in MVV that early after infection only multiple spliced mRNA transcripts which encode the regulatory proteins of tat and rev are present (as discussed in the above section). There is a shift to incomplete splicing later in infection for the production of *gag* and *env* gene products (Vigne et al., 1987). It is believed that as rev accumulates in the infected cell there is a switch from multiply spliced transcripts to the singly spliced transcripts encoding the structural proteins (Tiley et al., 1990). In contrast to HIV, the first exon of MVV *rev* utilises the first 37 amino acids of the *env* gene (Sonigo et al., 1985) and exon 2 contains the functional domains of rev. Two functional domains within HIV-1 rev have been described: the "RNA binding domain" is present at the N-terminus, contains a highly basic 40 amino acid core and has a nuclear localisation signal; the "activation domain" is located at the C-terminus and contains a leucine rich motif which interacts with cellular proteins (Hope et al., 1990). Rev functions by binding to a rev response element (RRE) which in HIV-1 is located 3' to the *env* gene. The location of the RRE binding domain has been mapped in visna (Tiley et al., 1991), as well as the leucine rich domain. The RRE of visna has been located in a similar place to that of HIV-1 by sequence comparison, and it is predicted to form an RNA stem loop structure, similar to HIV-1 (Tiley et al., 1991).

1.6.1ii Tat

Tat, like rev, is transcribed early in infection and is thought to play a role in the regulation of virus replication. Tat proteins are common to all lentiviruses and all share common protein motifs, but differ in their ability to transactivate their LTR's. HIV tat is derived from 2 exons (Arya et al. 1985) and it strongly activates its LTR by binding to a TAR (tat activated region) element present within the LTR. The TAR element forms an RNA stem loop structure following its transcription to which tat and other transcription factors can bind. In contrast, CAEV and MVV tat are derived from a single exon (Davis and Clements, 1989), weakly transactivate their LTR's and no TAR element exists since mutations within the R element of the LTR have no effect on tat responsiveness (Hess, 1989). Tat is a 10-11kDa protein, sharing

three of the conserved cysteine residues in the cysteine rich domain in HIV tat (Gourdou et al., 1989, Davis and Clements, 1989). Visna tat has distinct domain structures to those of HIV tat and these include an acidic activation domain at the N-terminus, hydrophobic and bulky aromatic residues essential for tat activity and a leucine rich domain, adjacent to the N-terminal activation domain, that acts as a negatively regulating domain (Carruth et al., 1994). Transient expression assays have shown that tat of MVV acts via AP-1 and AP-4 binding sites located in the U3 region of the LTR to increase the rate of transcription (Gdovin and Clements, 1992, Neuvet et al., 1993). Thus tat acts as a positive *trans*-activator of transcription as well as having post transcriptional activities (Gourdou et al., 1989). Post-transcriptional activities of tat have been suggested since deletion of the U3 region does not affect viral transcription but a decrease in viral protein content was observed (Gdovin and Clements, 1992). The importance of tat in the life cycle of ungulate lentivirus infection is not known since it has been shown be is dispensable for viral replication *in vitro* and *in vivo* (Harmache et al., 1995a).

1.6.1iii Vif (Q)

The *vif* gene of MVV encodes at 29kDa protein that is expressed at the later stages of infection (Audoly et al., 1992). It is located within the cytoplasm of infected cells but is not associated with virus particles (Audoly et al., 1992). Deletion mutant studies have shown that vif is essential for the rapid release of virus particles and for efficient virus replication, and that it acts post translationally (Harmache et al., 1995b). These studies revealed the importance of a domain at the C-terminus of vif, which in HIV is membrane associated. This region in HIV-1 vif contains a hydrophobic region with the conserved SLQXL motif which is essential for vif function (Goncalves et al., 1994). This domain is also found in non-viral proteins associated with membranes (Oberste and Gonda, 1992). In HIV, the association of vif with membranes has been correlated with the ability of vif to modulate the gp120 content of newly synthesised virions (Sakai et al., 1993).

1.6.1iv Other regulatory proteins

Sequence comparisons of the SA-OMVV and other MVV isolates identified an additional orf (W) in this South African isolate (Querat et al., 1990). The function of this W gene product is not known.

There are no reports of orfs within MVV related to the other auxiliary proteins of HIV, including *vpr* (Ogawa et al., 1989), *vpu* (Strebel et al., 1987), *tev* (Benko et al., 1990) or *nef* (Ahmad and Venkatesan, 1988).

1.6.2 The non-structural proteins

The *pol* gene encodes for the non-structural proteins which are the reverse transcriptase (p66), the protease (p10) and the integrase (p32). The *pol* gene products are translated directly from the unspliced mRNA and the protease serves to cleave the *gag* (55kDa) and *gag-pol* precursor (150kDa) polypeptides. The *gag* and *pol* genes overlap by approximately 240 nucleotides and the *pol* gene is in a -1 reading frame relative to *gag*. Thus a ribosomal frameshift allows expression of *pol* and this strategy explains the abundance of the *gag* precursor polypeptide in relation to the *pol* precursor (reviewed by Clements and Wong-Staal, 1992). The reverse transcriptase encodes for RNA-dependent polymerase activity (Lin and Thormar, 1970) and RNase H activity that degrades RNA:DNA hybrids during the reverse transcription process. The integrase of MVV shows *in vitro* integrase activities including 3' processing, strand transfer and disintegration (Katzman and Sudol, 1994). Studies with chimeras of HIV-1 and MVV integrase have shown that the N-terminal portion does not contribute to viral DNA specificity (Katzman and Sudol, 1995). To date, there have only been limited reports of integration of visna DNA into the host genome (Vigne et al., 1985) and the function of integrase *in vivo* is not understood.

1.6.3 The structural proteins

The *gag* gene, like the *pol* gene, is synthesized from the unspliced genomic mRNA resulting in a large polypeptide precursor, Pr55gag (Vigne et al., 1982). This polypeptide is cleaved intracellularly to give rise to three internal structural proteins: p30, p16 and p14 (Vigne et al., 1982). Sequence analysis of the MVV genome supported the existence of these proteins (Sonigo et al., 1985). p16 was predicted to lie at the N-terminus of the precursor, followed by the p24 antigen, leaving 79 residues to represent a 8.8 kDa protein. This small protein is postulated to be the nucleocapsid by virtue that it contains a zinc-finger (Cys-X_2-Cys-X_9-Cys) motif for interaction with nucleic acids. This basic motif confers the unusual mobility of these proteins on SDS-PAGE gels, such that they migrate slowly with respect to their actual size. This explains the difference in the predicted size of the gag precursor polypeptide (49.9kDa) (Sonigo et al., 1985) and that observed in infected cells (55kDa) (Vigne et al., 1982). The functions of p16 and p24 are thought to be a membrane associated protein and the major capsid protein, respectively, by analogy to other retroviruses.

Env is translated from a large singly spliced mRNA. The envelope glycoproteins are derived from a 150kDa glycosylated polypeptide to give a mature 135kDa major glycoprotein (Vigne et al., 1982). The *env* gene was identified by the presence of up to 28 N-glycosylation sites

(Sonigo et al., 1985, Sargan et al., 1991). There are also three predicted major hydrophobic regions; the first is thought to represent the signal peptide, the second contains the Arg-Lys-Arg-Lys motif for cleavage between the transmembrane and surface glycoproteins, and the third region probably represents the transmembrane region. No absolute size has been defined to the surface and transmembrane proteins. Gp135 is usually the major glycoprotein seen in cell extracts (Vigne et al., 1982). It is noted that throughout the literature on MVV, there is inconsistency as to the identity of gp135 with some authors referring to it as the surface glycoprotein. The size of the transmembrane protein has not been defined but is thought to be between 38 and 44 kDa (Sonigo et al., 1985). The properties of both of these proteins will be discussed in greater detail below. By analogy to other retroviruses it can be assumed that the precursor protein, gp135, is cleaved, and, on assembly of the virions, the surface and transmembrane proteins become non-covalently linked.

1.7 Viral envelope protein translocation and glycosylation

1.7.1 The signal peptide and glycoprotein translocation; the role of the C-terminus of env

The envelope mRNA is translated on membrane-bound ribosomes and the signal peptide at the N-terminus directs the protein through the secretory pathway. This pathway is common to all cellular glycoproteins which are eventually secreted, and the signal peptide guides the polypeptide to the endoplasmic reticulum (E.R.) via the signal recognition particle (SRP) and the docking protein which is embedded in the membrane of the E.R.. Signal sequences are very varied in length and amino acid composition. The signal or leader sequence is comprised of three regions. The N-terminal region ranges in size, shows the greatest diversity and is generally positive in charge (von Heijne, 1985). A possible role in modulating translocation in bacteria has been proposed, but no such function has been demonstrated with eukaryotic peptides (von Heijne, 1990). The middle hydrophobic region encompasses the signal peptide thought to be responsible for binding to the SRP. However, due to its variation no homologous binding region has been identified, and it may bind non-specifically to the membrane (von Heijne, 1990). The C-terminus can range between 5-7 amino acids and contains the signal peptidase cleavage site. Statistical calculations have predicted that the -3 and -1 residues determine the site of cleavage, and these amino acids are generally uncharged and small (von Heijne, 1983, von Heijne, 1984). Membrane proteins with carboxylic tails longer than 7 amino acids are not cleaved, and will remain membrane-bound with the C-terminus inside the cell. Although the exact mechanism by which the signal sequence translocates the polypeptide through the E.R. is not fully understood, it is proposed that the three regions with their

positive-hydrophobic-polar design may form a loop structure. This allows the middle hydrophobic region to interact with the membrane, leaving the amino-terminus on the cytoplasmic side and the cytoplasmic tail close to the extra-cytoplasmic membrane face where cleavage by the signal peptidase takes place (von Heijne, 1990).

Membrane proteins are classified into four categories; class I, II, III and IV, depending on the number of times they span the membrane, the removal of the signal sequence and the orientation in the membrane. Lentiviruses have class I type glycoproteins, that is their signal sequence is removed, the carboxylic terminus is retained on the cytoplasmic side of the plasma membrane and they only span the membrane once (Hunter and Swanstrom, 1990). In common with retroviruses, lentiviruses have an exceptionally long N-terminus sequence preceding the signal peptide. The function of these additional amino acids is not known. This region in FIV and ungulate lentiviruses is longer than the primate lentiviruses, 149 amino acids in FIV (Stephens et al., 1992) and approximately 80 amino acids for MVV (Sonigo et al., 1985, Sargan et al., 1991). Deletion mutations in this region of FIV has shown that only the initial 47 amino acids at the N-terminus is required for efficient glycoprotein processing (Stephens et al., 1992). Also, the overall charge of this tail does not affect glycoprotein processing, which is a feature of class I membrane proteins.

As the polypeptide is translocated across the rough E.R., the hydrophobic portion of the transmembrane region remains anchored in the membrane and thus acts as a translocation stop signal. The introduction of a stop codon at the N-terminus of this region results in the secretion of the retroviral envelope protein (Perez et al., 1987b, Hallenberger et al., 1993). This translocation anchor confers the bitopic orientation of the retroviral envelope proteins, such that the carboxylic end of the protein is cytoplasmic. The length of this cytoplasmic tail varies amongst retroviruses from 50 amino acids in oncogenic retroviruses to 150 amino acids in HIV-1. The importance of this domain is not fully understood. In the Rous sarcoma virus, deletion of the entire region does not effect biosynthesis, transport and processing of the envelope protein (Perez et al., 1987b). In fact the mutant virus is as infectious as the wild-type, suggesting that the cytoplasmic domain is dispensable to the virus *in vitro* (Perez et al., 1987b). Part of the cytoplasmic tail of the Mason-Pfizer monkey virus is cleaved and involves a viral protease (Brody et al., 1992). This removal of 16-20 amino acids occurs in a late maturation step at post-budding, since this mature form of the transmembrane protein was seen only in infectious virions and not in infected cells. Deletions in the matrix protein affected the cleavage of the cytoplasmic tail of the envelope protein, suggesting some form of interaction between these two proteins (Brody et al., 1992). It is possible that binding of the matrix protein to the cytoplasmic tail brings about a conformational change that allows cleavage by the viral protease (Brody et al., 1992). The cleavage of the cytoplasmic domain of

HIV-1 has not been demonstrated. No cytoplasmic cleavage was seen in the deletion studies carried out by Yu and colleagues (1993), although the vif protein of HIV-1 has been shown to behave as a cysteine protease and cleave envelope proteins (Guy et al., 1991).

Studies linking the cytoplasmic tail of HIV-1 with the signal sequence of the HSV-I glycoprotein D have shown that it is associated with cellular membranes (Haffar et al., 1991). The sequences within this region predict two amphipathic α-helixes, and it is suggested that these two helices form a stable association that spans the lipid bilayer forming a complex analogous to ion channels (Haffar et al., 1991). However, the role in virus assembly and replication is not understood since truncation in this region did not affect replication (Wilk et al., 1992, Earl et al., 1991). Vaccinia viruses were constructed that expressed progressively larger C-terminal deletions, and these were found to have no effect on glycoprotein transport, cleavage or membrane anchorage (Earl et al., 1991). In fact, an enhancement in syncytia formation was observed, suggesting that the cytoplasmic tail may play a role in the conformational state of the extracellular and transmembrane glycoproteins (Earl et al., 1991). More recent data has shown that deletions in the cytoplasmic tail of HIV-1 can effect the infectivity of the virus (Yu et al., 1993). The introduction of stop codons in the cytoplasmic tail resulted in the production of non-infectious virus, suggesting that this domain may function in virus replication as well as virus assembly (Yu et al., 1993), contrasting earlier reports (Wilk et al., 1992).

In summary, the cytoplasmic domain of lentiviruses may be important both in virus assembly and in rendering the virus infectious.

1.7.2 The glycosylation state of lentiviral glycoproteins

Following transport through the E.R., the envelope proteins of lentiviruses are transported through the glycosylation pathway of the host cell (reviewed in Fenouillet et al., 1994). The majority of glycosylation on lentivirus envelope proteins is N-linked and glycosylation is mediated in the lumen of the E.R.. The first step involves the transfer of a dolichol phosphate-linked extended oligosaccharide core to an asparagine residue on the polypeptide backbone. The asparagine is located within the motifs either Asn-X-Thr or Asn-X-Ser, where X can be any amino acid except proline. Dolichol is a long chain of hydrophobic carbon moieties that is able to associate with the lipid bilayer at least three times, resulting in the anchorage of the carbohydrate core in the membrane on the lumen side. Once the oligosaccharide core is linked to the polypeptide, it is rapidly trimmed by the loss of three glucose residues and one mannose residue. The enzymes that catalyse this trimming are α-glucosidases I and II, and mannosidases, receptively. They are inhibited by castanospermine and deoxymannojinimycin, respectively and these provide useful tools when investigating the glycosylation state of viral

proteins. Proteins are then transported to the Golgi apparatus where further trimming occurs. In the *cis*-Golgi, which is the most proximal compartment to the E.R., one mannose residue is trimmed, and a further two mannose residues are trimmed in the *medial*-Golgi. Varying numbers of residues are then added, including N-acetylglucosamine, fucose and sialic acid, and this occurs both in the *medial-* and *trans*-Golgi department catalysed by glycosyl transferases.

In summary, all N-linked glycans are composed of a N-acetylglucosamine$_2$-mannose$_3$ core to which a variety of other residues are added. Depending on the extent of the modification of this core, glycans can be either characterized as the complex-type or when no addition of residues occurs these are called high-mannose glycans.

The envelope glycoproteins of lentiviruses are heavily glycosylated and account for up to 50% of their molecular weight. MVV has up to 29 potential glycosylation sites (Sargan et al., 1991, Sonigo et al., 1985) and HIV-1 has up to 30 sites (Fenouillet et al., 1994). Glycosylation of envelope proteins can be demonstrated by the addition of tunicamycin to infected cells to block glycosylation in the E.R., and this method was used to demonstrate that the envelope protein of MVV was glycosylated (Vigne et al., 1982). The characterization of the glycan residues on HIV-1 gp120 has shown that 13 residues are of the complex-type and 11 are the high-mannose type (Geyer et al., 1988). The glycosylation of the transmembrane protein is not as extensive, with the potential of five sites in HIV-1 gp41 resulting in a much lower molecular weight than the external glycoprotein (Fenouillet et al., 1993). The identity of glycans on MVV have not been characterized although susceptibility to neuraminidase suggests the existence of sialic acid residues (August et al., 1977).

The functional role of glycans in lentiviruses is still not fully understood and the majority of studies have been carried out with HIV-1. Studies on the CD4/gp120 interaction found that deglycosylated gp120 was unable to bind to CD4 on the surface of cells (Matthews et al., 1987). Mutational studies have shown that single point mutations within the glycosylation sites do not affect the processing or function of the envelope glycoprotein, but multiple mutations do inhibit the normal processing of gp160 (Lee et al., 1992). The carbohydrate moieties could be envisaged as a bulk of sugar residues, such that the loss of a single glycosylation site will not disrupt the mass of carbohydrate, and thus its influence on protein conformation. The results from these mutation studies are reinforced by the use of glycosylation inhibitors. The addition of castanospermin which inhibits α-glucosidases in the E.R. resulted in a reduction in syncytium formation (Gruters et al., 1987).

The five putative glycosylation residues of the HIV-1 transmembrane protein, gp41, lie within the ectodomain and are flanked by the immunodominant domain, the putative binding domain between gp120 and gp41, and the gp41 cleavage site (Fenouillet et al., 1993).

It is proposed that in HIV-1, the carbohydrate moieties may play a role in "hiding" the fusion domain prior to membrane fusion. As with gp120 studies, individual mutations within the glycosylation sites of the transmembrane protein had no effect on envelope protein function (Dedera et al., 1992). However, a contrasting study showed that a single mutation in the Asn-642 residue disrupted gp160 processing (Dash et al., 1994). The differences in the results is thought to be a reflection on the experimental protocol used, that is the type of cells used to infect with the mutated proviruses. When three of the five potential glycosylation sites were mutated, Asn-621, Asn-630 and Asn-642, the cleavage of gp160 was slower, and syncytia formation was reduced suggesting a modification in the presentation of the fusion domain (Fenouillet et al., 1993). These mutations also resulted in a non-glycosylated transmembrane protein suggesting that the other two potential glycosylation sites are non-functional (Fenouillet et al., 1993). Subsequent experiments with all three mutations showed that the reduction in syncytia formation and gp160 processing was due to the retention of gp160 in the rough E.R./ *cis*-Golgi compartments (Fenouillet and Jones, 1995). Since cleavage of gp160 occurs in the *trans*-Golgi (see section 1.8.2), the observed reduction in its processing can be explained by its retention, and not by the reduction in glycosylation (Fenouillet and Jones, 1995).

The function of carbohydrate residues on the envelope proteins of MVV is not known, although removal of sialic acid from the envelope glycoprotein of CAEV suggested that these residues may be involved in protection from neutralizing antibodies and host cell proteases, but were dispensable for the infectivity of the virus (Huso et al., 1988).

The existence of O-linked oligosaccharides has recently been demonstrated on HIV-1 gp120 (Bernstein et al., 1994). O-linked glycosylation is less refined than N-linked glycosylation. It is mediated by transfer of N-acetylgalactosamine to a serine or threonine residue, followed by the addition of other sugar residues, such as fucose and sialic acid. Treatment of gp120 with neuraminidase followed by O-glycosidase resulted in a reduction in molecular weight suggesting the presence of O-linked glycosylation, but the function of these residues is not known (Bernstein et al., 1994). A prominence of O-linked oligosaccharides on the surface of the envelope protein of CAEV has been proposed (Huso et al., 1988).

1.8 Viral glycoprotein assembly into virions: oligomerization and proteolytic cleavage

1.8.1 Oligomerization

Prior to transport to the Golgi apparatus, oligomerization of retroviral envelope proteins occurs in the E.R.. The oligomerization of HIV-1 glycoproteins will be discussed as an example of lentivirus oligomerization. Studies with HIV-1 showed that the transmembrane protein gp41, existed as homomeric tetramers (Pinter et al., 1989). These oligomeric complexes were stable under mild reducing conditions and were recognised by antibodies present in patients sera (Pinter et al., 1989). Further investigation of the gp41 protein showed that the ectodomain was required for gp41 oligomerization (Earl et al., 1990). This domain, spanning amino acids 68-129, stabilised gp160 tetramers in a noncovalent fashion (Earl et al., 1990). The ectodomain overlaps with the predicted α-helical structure, which contains a leucine-zipper motif. Leucine zippers within cellular proteins are well characterized as forming dimers with transcription factors, and the proposed role of this motif in the dimerisation of gp160 was investigated. Initial reports suggested that this leucine-repeat motif was not involved in oligomerization, since the substitution of leucine residues with proline to disrupt the α-helix did not destabilise gp160 oligomers (Chen et al., 1993). However, others found that deletions in the N-terminus of the ectodomain resulted in the disruption of gp41 oligomers but not gp160, whereas deletions in the C-terminus did not affect either gp41 or gp160 oligomerization (Poumbourios et al., 1995). Deletion in both these regions resulted in monomeric gp41 and gp160, indicating that both the N-terminus and other regions of gp41 are required to maintain the oligomeric structure of gp160 (Poumbourios et al., 1995). It is suggested that gp41 oligomerization is less stable than that of gp160.

The above studies demonstrated that both gp160 and gp41 are able to form oligomers. Studies with a truncated form of gp160 demonstrated that gp120 is also able to oligomerize in the absence of gp41 (Hallenberger et al., 1993). This is consistent with data that cleavage of gp160 oligomers does not disrupt oligomerization but results in both gp41 and gp120 oligomers (Weiss et al., 1990), and in fact oligomerization is a prerequisite for proteolyitc cleavage (Gabuzda et al., 1992). Oligomerization of the transmembrane protein of CAEV has been demonstrated (McGuire et al., 1992), but the absence of a leucine-zipper motif within this protein means it may use different mechanisms for oligomerization than HIV-1.

In summary, it appears that the glycoproteins of HIV-1 exist as tetramers, and oligomerization involves the leucine-zipper motif located in the ectodomain of gp41, as well as other motifs not yet defined. Disulphide bond formation also occurs in the E.R., and thus

correct folding, oligomerization and subsequent glycosylation are all required for the cleavage of glycoproteins and assembly of virions.

1.8.2 Proteolytic cleavage of envelope precursors

The envelope precursors of lentiviruses must be cleaved for virus infectivity (McCune et al., 1988). The proteolyitc cleavage of HIV-1 gp160 results in the gp120 surface protein and the gp41 transmembrane protein (Willey et al., 1988). Mutations in the tryptic-cleavage site of gp160 resulted in a reduction in virus infectivity and syncytia formation (McCune et al., 1988). This tryptic-like cleavage site, Arg-Gln-Lys-Arg, is conserved in all lentiviruses, including MVV (Sonigo et al., 1985). Studies looking at post-translational processing of the envelope proteins of FIV suggested the existence of two precursors (Verschoor et al., 1993). A 150kDa protein is initially seen in FIV-infected cells and this is replaced by a 130kDa protein, which becomes cleaved into gp100 and gp35 (Verschoor et al., 1993). It is believed that the 150kDa precursor is cleaved to the 130kDa protein by removal of the signal peptide. Two precursor species are also seen with MVV, a 150kDa species followed by a 135kDa protein (Vigne et al., 1982). Nucleotide analysis of MVV predicts that the 135kDa protein is a precursor and is cleaved to a gp90 and gp46 protein, although this has not been experimentally proven (Sonigo et al., 1985). The existence of two precursor species has not been described for HIV and it is suggested that the removal of the signal peptide of HIV-1 occurs co-translationally, presumably as the polypeptide is being translocated through the lipid bilayer of the E.R.. The difference between these lentiviruses may be a consequence of the difference in the length of N-terminal tail preceding the signal peptide (see 1.7.1).

The site within the cell where proteolytic cleavage occurs is debatable. In HIV-1, it is understood that cleavage occurs after it has been translocated from the E.R. and thus is dependent on the correct folding and oligomerization of the precursor, gp160 (Dewar et al., 1989). The use of transport inhibitors suggested that cleavage of HIV-1 gp160 occurred in the *cis/medial*-Golgi compartments (Dewar et al., 1989). A recent study with HIV-1 contradicts this data since they demonstrated using low temperature incubations and membrane traffic inhibitors that cleavage occurred post *trans*-Golgi (Kantanen et al., 1995).

Furin has been suggested as the protease responsible for cleaving the HIV-1 gp160 precursor, since co-expression of furin and gp160 in vaccinia viruses was required for gp160 processing (Hallenberger et al., 1992). Inhibitors of furin prevented cleavage to HIV-1 gp120 and gp41 (Hallenberger et al., 1992). Coexpression studies with baculovirus-derived HIV-1 glycoproteins and baculovirus-derived furin showed that this enzyme is also capable of cleaving the V3 loop of gp120 (Morikawa et al., 1993). The role of V3 loop cleavage is not fully understood since the exposure of the cleavable domain in virion-associated gp120 is not

known (Morikawa et al., 1993).The role of furin *in vivo* is debatable since cleavage has been shown to occur in cells not containing furin (Ohnishi et al., 1994).

1.8.3 Virus assembly

The assembly of MVV has not been widely studied. Following accumulation of the virus envelope at the plasma membrane, virus assembly occurs. It is the gag proteins that are responsible for virus assembly and budding, and only a brief overview will be presented below in reference to HIV assembly.

The gag precursor assembles to an immature core structure which is then cleaved by the virus protease, encoded by the *pol* gene, to the matrix (MA)(p16), capsid (CA)(p30) and nucleocapsid (NC)(p14) proteins (reviewed by Wills and Craven, 1991). It is the matrix protein that is thought to play a major role in virus assembly, since deletions in this protein disrupts the formation of virus particles (Wills and Craven, 1991). Immuno electron microscopy of HIV-1 particles reveals a spherical, sheet-like appearance locating the matrix protein underneath the lipid envelope of the virus (Gelderblom et al., 1987). The *gag* precursor of HIV, in common with other retroviruses (Schultz and Oroszlan, 1983), is myristoylated at the amino terminal, which after cleavage results in the matrix protein retaining this fatty acid tail. The function of this moiety is thought to be important for quaternary structure and involvement in protein-protein or protein-lipid interactions. Substitution of the N-terminal glycine residue, to which myristic acid is attached, with an alanine residue resulted in the loss of myristic acid on the gag precursor and disrupted virus assembly and formation of infectious particles (Bryant and Ratner, 1990). The normal processing of the gag precursor was impaired as was association with the lipid envelope, indicating the importance of myristoylation in membrane targeting (Bryant and Ratner, 1993).

Due to the proximity of the matrix protein to the lipid envelope, an association with viral glycoproteins is postulated, presumably with the the C-terminus of gp41 (see section 1.7.1). Studies involving deletions in the matrix protein of HIV-1 have shown that the N-terminus of the matrix protein is required for a stable association of the envelope protein within virions (Dorfman et al., 1994, Brody et al., 1992). There is an uncertainty as to which portion of the envelope glycoprotein the matrix protein is associated. There are reports that the cytoplasmic tail of gp41 is essential for virus budding (Dubay et al., 1992), whereas other data has suggested that it is dispensable for virus assembly (Earl et al., 1991). It may be possible that the matrix protein can form an association with the hydrophobic, transmembrane portion of gp41. Deletions within other regions apart from that involved with myristoylation have identified other domains in the matrix protein that are required for virus assembly (Spearman et al., 1994). However, complete removal of the matrix protein, except for the

residues involved in myristoylation and a few residues at the C-terminus, did not affect virus assembly, but these studies did suggest an association of the matrix protein with envelope proteins (Wang et al., 1993). The differences between the data produced by Wang et al. (1993) and Spearman et al., (1994) probably lies in the experimental design such that the effect of changing a few amino acid residues will have a more subtle effect on protein function *in vitro* than complete deletion of the matrix protein.

By using recombinant vaccinia vectors, virus assembly can take place in the absence of envelope proteins (Shioda and Shibuta, 1990). The presence of the *pol* gene is required for the complete processing of the *gag* precursor, and this processing was shown to occur post-assembly (Shioda and Shibuta, 1990). HIV has an additional regulatory protein, vpu, not found in other retroviruses, that is expressed at the later stages of virus formation. It has been shown to influence the gag precursor prior to its processing into the individual gag proteins (Gottlinger et al., 1993). HIV-1 vpu was also shown to affect the processing of gag precursors from related viruses, such as MVV, that do not encode a vpu-like protein. Since the matrix protein of MVV is not myristoylated (Towler et al., 1987), the function of myristic acid in virus assembly remains ambiguous. It is proposed that vpu may serve as a substitute of a host cell factor in HIV-1 infection (Gottlinger et al., 1993).

Preliminary studies looking at the budding of HIV from polarized epithelial cells, indicated that it is the envelope protein that governs the site of virus budding (Owens et al., 1991). In the presence of envelope protein, budding occurred at the basolateral surface of epithelial cells, whereas in its absence budding occurred both at the apical and basolateral surface (Owens et al., 1991). The significance of this data *in vivo* may be through virus entry into the host via the mucosal membrane, such that virus particles would then bud directly into the blood stream for transport through the body.

1.9 Viral receptors

The entry of viruses into their host cells is mediated by their attachment to the surface of the target cell. This is accomplished via a virus attachment protein (VAP) which forms the coat of enveloped viruses or the capsid of non-enveloped viruses. The binding of the virus attachment protein to its receptor brings the virus into close proximity to the cell surface providing the opportunity for virus-cell fusion and infection. The virus receptor is defined as a host surface component that participates in virus binding and facilitates virus infection. The receptor also determines the host range and the tissue tropism of the virus. For example, Epstein-Barr virus infects B-lymphocytes and its receptor, C3d receptor CR2, is present on the surface of these

cells (Fingeroth et al., 1984). However, in the case of the poliovirus which replicates only in the gut and motor neurone cells, the tropism is not fully controlled by the distribution of its receptor. In fact, the mRNA of its putative receptor, the lymphocyte homing receptor CD44 (Shepley and Racaniello, 1994), can be found in cells not infected by the virus. It is possible that a post binding event is required to confer the host cell specificity and virus attachment (Mendelsohn et al., 1989).

Recent identification of virus receptors has been facilitated by the use of recombinant DNA technology and monoclonal antibodies. A panel of monoclonal antibodies against a variety of cell surface markers can be used to initially screen for inhibition of infection, and then the gene of the putative receptor can be transfected into a cell not normally expressing this molecule and assessed for its ability to allow virus infection. This approach was used for the identification of the receptor for HIV-1 as CD4 (Klatzmann et al., 1984, Dalgleish et al., 1984, Maddon et al., 1986). Other methods are available for identifying a receptor molecule. The virus overlay protein blot assay (VOPBA) is of some use (Boyle et al., 1987), but only when receptor activity is expressed as a single polypeptide and interaction is not destroyed by denaturation of the protein. Carbohydrate receptors can be identified by separating glycolipids by thin layer chromatography and probing with virus, in a similar manner as a VOPBA. Monoclonal antibodies to different carbohydrate moieties can be used for inhibition of infection studies. One consideration when commencing these studies is that viruses from the same families do not necessarily use the same type of receptors.

Virus receptors may be simple carbohydrate molecules or lipids, or more complicated proteins that may function as recognition molecules on cells. Influenza viruses and paramyxoviruses utilise sialic acid residues (Gottaschalk, 1958), and are thought to bind by hydrogen bonding and van der Walls forces (Weiss et al., 1988). Phosphatidylserine and phosphphatidylinositol are recognised by vesicular stomatitis virus (Mastromarino et al., 1987). The more complex polypeptide receptors include members of the immunoglobulin superfamily, such as CD4, the receptor for HIV (Dalgleish et al., 1984, Klatzmann et al., 1984), the intercellular adhesion molecule 1 (ICAM-1) for the rhinovirus (Greve et al., 1989, Staunton et al., 1989) and aminopeptidase N (CD13) for human and porcine coronaviruses (Yeager et al., 1992, Delmas et al., 1992). Other receptor protein molecules include hormone receptors, such as the epidermal growth factor receptor for vaccinia virus (Eppstein et al., 1985), and neurotransmitters, including the acetylcholine receptor for the rabies virus (Lentz et al., 1982).

The CD4 receptor for HIV has been the most widely studied of the lentiviral receptors. Initial observations that the $CD4^+$ subset of T lymphocytes were the main target cells of HIV infection lead to the confirmation of its identity (McDougal et al., 1985/1986). Monoclonal

antibodies to CD4 blocked virus-induced syncytia formation (Klatzmann et al., 1984), gp120 coprecipitated with CD4 (McDougal et al., 1986) and CD4 expressed in human $CD4^-$ T-cells conferred the cells susceptible to HIV infection (Maddon et al., 1986).

CD4 is a 58kDa transmembrane glycoprotein belonging to the immunoglobulin gene superfamily. The cytoplasmic portion is divided into four domain loop structures, D1 to D4 (Maddon et al., 1985). CD4 is predominantly expressed on the surface of T lymphocytes and is also present on monocytes/macrophages, B cells and certain cells of the central nervous system. The D1 domain has been shown to contain the gp120 binding domain (Richardson et al., 1988) and this has been further mapped to the complimentarity-determining region 2 (CDR2) loop (Landau et al., 1988, Arthos et al., 1989). Mutational studies revealed that residues 42-49, within the CDR2 loop, were required for gp120 binding (Petersen and Seed, 1988). The CDR3 region has also been implicated in HIV entry, although it lies on the opposite face from the CDR2 loop. Initial studies with synthetic peptides indicated that it was involved in the fusion process (Berger et al., 1991). However, further work revealed that this interaction was not specific since these peptides could also disrupt the fusion activity of the human T-cell leukaemia virus I (Repke et al., 1992). Thus the role of CDR3 in HIV binding is ambiguous at present. There is some evidence that a flexible hinge situated between domains 2 and 3 may influence HIV entry since monoclonals to this region are able to block entry but do not effect virus binding (Brady et al, 1993, Hearly et al., 1990).

The principal domain in gp120 for CD4 binding has been mapped to the C-terminus by mutational studies (Pollard et al., 1991, Pollard et al., 1992). A combination of monoclonal antibody mapping and site-directed mutagenesis has finely defined 3 major regions for CD4 binding. These are residues 256-262 in the C2 domain, residues 368-389 in the C3 domain and residues 421-457 in the C4 domain (Cordinnier et al., 1989, Lasky et al., 1987). Antibody epitope mapping has also shown that C4 and C3 domains only are likely to be exposed on the surface of gp120, and thus these will directly interact with CD4 (Olshevsky et al., 1990, Moore et al., 1994). Mutagenesis of single amino acids within these domains has indicated the importance of these residues in retaining the structure of gp120 required for CD4 interaction (Cordonnier et al., 1989).

It has been observed that although expression of CD4 on mouse cells allows virus binding, this is not sufficient for viral entry (Maddon et al., 1986). In fact, studies with vaccinia-derived CD4 and gp120 showed that only human cells expressing these proteins could support fusion (Ashorn et al., 1990). Therefore, the existence of accessory molecules to CD4 have been implicated in virus entry (Stefano et al., 1993). The leukocyte functional antigen 1 (LFA1) has been associated with HIV entry by the virtue that monoclonal antibodies to this molecule inhibit virus fusion (Hildreth and Orentas, 1989). Presumably interaction of LFA1 on the surface of T-cells with HIV-1 infected cells will bring CD4 and gp120 into close

proximity for interaction. The intracellular adhesion molecule 3 (ICAM3) has recently been implicated in HIV entry since monoclonal antibodies to ICAM3 inhibited syncytia formation and virus entry (Sommerfelt and Asjo, 1995). ICAM3 is present on the surface of lymphoid cells, monocyte/macrophage cells and dendritic cells, and thus shares the host range of HIV infection *in vivo*. There is evidence that Fc and complement receptors may enhance uptake of opsonised virus (Homsy et al., 1989) such that anti-gp120 antibodies bound to infected cells interact with Fc receptors and increase the efficiency of virus entry. There is some controversy as to whether this enhancement is dependent or independent on CD4 (McKeating et al., 1990, Takeda et al., 1990). A cellular protease has been proposed as an accessory molecule to CD4 via cleavage of gp120 (Morikawa et al., 1993). The V3 loop within gp120 contains a conserved motif which is a substrate to the dipeptidyl peptidase IV activity of the T cell activation antigen, CD26 (Callebaut et al., 1993). Coexpression of CD4 and CD26 allows virus entry and this is inhibited by monoclonal antibodies to this enzyme (Callebaut et al., 1993). These results have been met with some scepticism, and other workers have been unable to demonstrate a role for CD26 in HIV infection (Cohen, 1993, Wang et al., 1995).

HIV infection has been demonstrated in $CD4^-$ cells, particularly those of the central nervous system (Harouse et al., 1989). The receptor was identified as galactosyl ceramide since monoclonal antibodies to this molecule inhibited virus entry and gp120 binds specifically to galactosyl ceramide and no other glycolipid (Harouse et al., 1991, Bhat et al., 1991). A combination of monoclonal mapping and thin layer chromatography to separate glycolipids indicated that the V3 loop or sites within close proximity are involved in specific interaction with galactosyl ceramide (Cook et al., 1994). Analysis of a $CD4^-$/Gal-Ser^+ cell line with chimeric viruses, derived form a positive or negative infection phenotype, indicated that the V3, V4 and V5 loops are all involved in binding of HIV gp120 to galactosyl ceramide (Harouse et al., 1995). HIV also infects cells of the placenta in a CD4-independent manner. Probing a placental cDNA library, identified a membrane-associated mannose-binding lectin to which gp120 bound (Curtis et al., 1992).

The existence of two receptors for one virus is not unique to HIV. Herpes simplex virus glycoprotein C binds to heparin sulphate, while the glycoprotein D binds to an unidentified protein (Spear, 1993). The fibre protein of adenovirus type 2 binds to an unknown receptor and the penton base protein binds to integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (Wickham et al., 1993). The activity of the two receptors may be cofunctional such that these receptors will modulate each other or act complementary. Alternatively, the receptors may act sequentially. This may be explained by the relative binding affinity of each receptor for the virus. The binding of the virus to the first receptor could be rapid followed by a stronger association with the second receptor leading to virus entry. Thus the initial binding step may be weak enough to allow the

virus to 'browse' over the surface of the cell until it comes into contact with the second receptor required for virus penetration (reviewed by Haywood, 1994).

Secondary binding has been shown to occur only at physiological temperatures, and is usually irreversible committing the virus to penetration. Examples of viruses that show this mechanism include the poliovirus and the herpes simplex virus. The initial binding of poliovirus to the cells is followed by a temperature-sensitive step that is resistant to detergents used to release the virus from cells (Lonberg-Holm, 1989). The binding of herpes simplex virus to cells can be initially reversed with heparin, but this becomes irreversible on secondary binding (Spear, 1993). Secondary binding may be brought about by changes in the conformation of the VAP's or the receptor and thus increase binding affinity. Enveloped viruses usually have their VAP's organised into clusters. The binding of one of the peptides to the receptor may bring about a conformational change that allows stronger binding with the remaining peptides within the multimeric VAP's. The spikes on the surface of HIV represent oligomers of gp120 associated with the transmembrane protein of gp41. The addition of soluble CD4 results in the release of gp120 from the cell only at 37^{o}C, suggesting a strong and stable interaction (Hart et al., 1991). Monoclonal mapping studies have shown that gp120 undergoes a conformational change on binding to CD4 (Sattentau and Moore, 1991). Rearrangements in the oligomeric spikes may influence membrane fusion by exposing the fusion domains and rearranging the spike structure to allow the lipid bilayers to come into close proximity for membrane fusion.

The identity of the receptor for MVV has not been fully characterized. VOPBA experiments revealed that MVV binds to a 30kDa protein present in sheep choroid plexus cell and this binding can be blocked by anti-sheep MHC classII (Dalziel et al., 1991). Antisera to sheep MHC classII was unable to block infection, although soluble MHC classII did block infection. This suggested that MHC classII may only act as a co-receptor (Dalziel et al., 1991). The existence of a secondary receptor is postulated since MVV does not infect all cells expressing MHC classII *in vivo*, such as T lymphocytes. There have also been reports of a 50kDa protein in goat synovium cells to which MVV binds (Crane et al., 1991a). These workers also describe MVV binding to 15 and 30 kDa proteins (Crane et al., 1991a).

In summary, the study of virus receptors is of paramount importance for understanding the initial stages of virus infection and viral tropism. With more refined DNA techniques, the use of transgenic mice expressing a human receptor can be used to study viral pathogenesis, the effects of antivirals and vaccines. The identification of the binding domains within the viral receptor and the VAP's allows the potential for raising antivirals. These can be in the form of

antibodies to these binding sites, anti-idiotype antibodies and ligands for blocking these binding sites.

1.10 Virus Fusion

The entry of enveloped viruses into cells is mediated by attachment to the cellular receptor, as discussed above, followed by virus fusion with the cellular membrane. Some enveloped viruses utilise the receptor-mediated endocytosis pathway. Endocytosis is a common physiological feature of cells and involves the ingestion of variable sized particles into the cell. Receptor-mediated endocytosis allows the selection of specific molecules, such as hormones, nutrients and growth factors. By binding to receptors on the cell surface, viruses will be internalised into coated vesicles. These vesicles loose their coats to form endosomes. The acidic environment of the endosome enables a conformational change in the envelope glycoprotein which allows fusion with the endosomal membrane and release of the virus core into the cytoplasm (reviewed by Marsh, 1984). Uncoating at various acidic pH's depends on the virus. Semliki Forest virus uncoats at a pH close to neutral, whereas Influenza viruses require a more acidic pH of 5 for uncoating. Membrane fusion and release of nucleocapsids is completed before endosomes deliver their contents into lysosymes where enzymes will actively degrade any viral or cellular components. The mechanism of fusion has been most widely studied with influenza (Marsh and Helenius, 1989). It is thought to be mediated by hydrophobic interactions between the fusion domain of the virus and the cellular membrane. The close proximity of the two membranes leads to local dehydration and a change in lipid configuration. This leads to fusion between virus and cellular membranes, and can occur at a single site for the release of the nucleocapsid into the cell (reviewed by Marsh and Helenius, 1989). The fusion peptide of lentiviruses is also hydrophobic and thus the fusion mechanism is likely to follow these events.

Retroviruses, including HIV, fuse to the cell at neutral pH (Stein et al., 1987, McClure et al., 1988b, McClure et al., 1990), and entry occurs either by fusion with the plasma membrane or by a pH-independent endosomal pathway. The fusion mechanism of HIV has been the most thoroughly investigated of all lentiviruses. Various lines of evidence suggest that it is the binding of gp120 to CD4 that activates fusion. The addition of soluble CD4 to infected cells results in the shedding of gp120 (Hart et al., 1991), as discussed in section 1.9. The physiological importance of this event is not fully understood, but it would appear that gp120 shedding results in the exposure of the fusogenic domain. However, shedding has been shown not to be paramount as long as the fusion domain is exposed (Sattentau and Moore, 1991). In

contrast to this, increases in infectivity on binding to soluble CD4 are seen with HIV-2 and SIV, but not with HIV-1 (Allan et al., 1990). HIV-2 can also be induced to infect $CD4^-$ cells by incubation with soluble CD4 (Talbot et al., 1994). The reasons for these differences amongst the primate lentiviruses remain ambiguous, but the reason may lie in the relative stability of the gp120 association with the transmembrane protein *in vitro*.

Cleavage of gp120 prior to fusion has also been proposed as a method for promoting virus entry. The existence of a substrate motif for proteases on the V3 loop has already been discussed (section 1.8.2) (Clements et al., 1991). It is envisaged that following cleavage of the V3 loop, gp120 is displaced to expose the fusion domain (Sattentau and Moore, 1991). Studies have shown that the binding of soluble CD4 to HIV is accompanied by the cleavage of gp120 (Moore et al., 1991). This cleavage can be blocked by monoclonal antibodies to the V3 loop (Moore et al., 1991), and it is proposed that on binding to CD4 a conformational change occurs on gp120 to expose the V3 loop to cleavage. Absence of protease activity in $CD4^+$ cells renders these cells unsusceptible to HIV infection (Werner and Levy, 1993). The interplay between gp120 shedding and the cleavage of gp120 to expose the fusion domain is still not fully understood. It has been suggested that the conformational changes in gp120, gp41 and CD4 are sequential, and gp120 shedding may occur at the later stages of virus fusion or may not occur at the cell surface at all (Moore et al., 1991). The use of soluble CD4 in these studies is also unsatisfactory since it does not represent the true conformational status of this molecule *in vivo*.

Following the exposure of the fusion domain, the events leading to virus fusion are not well understood. The fusion domain present at the amino terminus of HIV gp41 was identified by analogy with that of paramyxoviruses (Gallaher, 1987). Hydrophobic amino acid substitutions to charged amino acids within this domain affects fusion by disrupting its amphipathic α-helical structure (Kowalski et al., 1991). The C1 and C5 domains of gp120 have been associated with gp41 (Helseth et al., 1991), by providing a pocket in which the extracellular loop of gp41 will sit (Schultz et al., 1992). Site-directed mutagenesis within the zipper motif region located in this extracellular loop showed that C-terminus residues were involved in gp120 association (Chen, 1994). Kinetic studies with gp41 have shown that CD4 remains attached during the fusion process, and the CDR-3 region of CD4 has a critical role in virus fusion (Broder and Berger, 1993). In addition, the V3 and V4 regions of CD4 are also critical for virus entry (Poulin et al., 1991), and this data suggests that CD4 attachment is required for virus penetration as well as binding. Association of CD4 with the virus particle within the cell may delay the release of the virus core into the cytoplasm, or retain the nucleocapsid at the plasma membrane halting the release of the virus genome and subsequent viral transcription. Whether this is a protective mechanism of the host cell, or whether this allows

the cell to become physiologically prepared for virus infection is not known. However, there would appear to be a sequence of related events from virus attachment to fusion to nucleocapsid entry. In addition to the fusion peptide at the N-terminus of gp41, other regions have been found to be functionally active. As well as providing the anchorage of gp41 in the membrane, residues within the hydrophobic transmembrane region are important for membrane fusion (Owens et al., 1994). The cytoplasmic domain of gp41 has been shown to play a role in virus penetration and uncoating, which are both post-fusion events (Gabuzda et al., 1992).

The fusion epitope of MVV has been located within the hydrophobic region at the N-terminus of the transmembrane protein, gp46 (Crane et al., 1991b). A peptide encompassing this hydrophobic region was tested for its ability to fuse goat synovial membrane cells. The fusion ability of this peptide and the ability of anti-peptide sera to block fusion suggested that this hydrophobic region contained a fusion epitope. The interaction of the transmembrane glycoprotein with the external glycoprotein, and the conformational changes between these two proteins leading to fusion is not known.

1.11 Functional domains of lentiviral glycoproteins

The regions of HIV-1 envelope glycoprotein involved in receptor binding and virus-cell fusion have been discussed in sections 1.9 and 1.10 above. One of the most widely studied of the epitopes present on gp120 is the V3 loop. It was first characterized for its importance as a neutralizing domain, and its mapping is described in detail in Chapter 4. The V3 loop spans amino acids 308 to 322 on the external envelope protein of HIV-1. As well as its neutralization properties, it has also been implicated in cell tropism and its ability to be cleaved by host proteases has suggested its role in cell fusion (discussed in section 1.10). Synthetic peptides to the V3 loop are able to block virus-cell fusion suggested its role in gp120 binding to CD4 (Rusche et al., 1988). A region of 8 to 15 amino acids within the V3 loop has been mapped as crucial for determining the efficiency with which HIV-1 infects T cells (Nehete et al., 1993).

Different strains of HIV-1 vary in their ability to infect different cell types. Using recombinant viruses with mutations in their envelope region, data has accumulated as to identify the region within gp120 that influences the tropism of a given strain for macrophages (O'Brien et al., 1990, Shioda et al., 1991). A 159 amino acid region encompassing the V1, V2 and V3 loops has been mapped which does not include the CD4 binding domain (Shioda et al., 1991), and

suggests that gp120 alone determines the host cell tropism. Finer mapping of this region has narrowed the macrophage-tropic region to 94 amino acids which include the whole of the V3 loop, and only 2 amino acids from the CD4 binding domain (Westervelt et al., 1991). These authors suggested that the tropism of some strains for macrophages may lie in the specificity of the proteases on the surface of macrophages. By transferring the V3 loop from a macrophage tropic virus to a non-macrophage tropic virus, an alteration in tropism was induced (Cann et al., 1992). This proves that the 94 amino acid region is involved specifying viral tropism. It should be noted that these studies use T cell and macrophage cell lines, which are not true reflection on events *in vivo*. In fact, most of the mutants described in the above studies are able to infect PBMC and primary macrophages.

As was mentioned above, the V3 loop is of particular importance in generating neutralizing antibodies. This is not the only region of HIV-1 envelope that is involved in neutralization. The second conserved region of gp120 has been implicated in inhibition of virus infection (Ho et al., 1988). Antisera were raised to synthetic peptides to this region and shown to neutralize a wide range of HIV-1 strains. This antisera did not block binding of gp120 to CD4, suggesting it is involved in post binding events. The existence of anti-C2 antibodies in the host is a rare event (Ho et al., 1988), but the authors suggest the importance in using these peptides to this region by inhibiting virus infection *in vivo*. Other regions to which neutralizing antibodies are raised include the CD4 binding domain (Lasky et al., 1987), the V2 domain (Ho et al., 1991, see chapter 4) and a conserved domain in gp41 (Muster et al., 1993). The carbohydrate moieties have also been implicated in raising neutralizing antibodies (Hansen et al., 1991). The relevance of neutralizing antibodies *in vivo* is not understood. Antibodies isolated from the serum of infected individuals are capable of neutralizing laboratory strains, but usually are ineffective in neutralizing primary, autologous virus (Weiss et al., 1986). The reason why this occurs is not understood, but has important implications when interpreting results obtained *in vitro* systems to the situation in the host. It is also important to take into consideration the conformation of a given epitope in the native protein when interpreting results obtained using synthetic peptides.

No functional epitopes in MVV have been described. Due to the limited strains of virus available, no sequence comparisons can be made to identify variable and conserved regions, and thus use these as a basis for epitope mapping as has been done for HIV-1. The following two sections will review the knowledge of envelope protein interaction with the immune system and the generation of antigenic variants in the face of neutralizing antibodies.

1.12 Interaction of MVV with the host immune system and the role of envelope

Following infection of the host with MVV, there is an active humoral response. Serum antibody is detected within the first six months after infection and steadily rises over a period of two years (Gudnadottir and Palsson, 1966). These antibodies are able to neutralize virus infection *in vitro*, but despite this humoral response virus infection persists *in vivo* (Gudnadottir and Palsson, 1966). A second longitudinal study involving intracranial injections of sheep with MVV confirmed the presence of neutralizing antibodies 4 weeks post infection, followed by an increase over the next three months when a steady state level was reached (Griffin et al., 1978). Neutralizing antibody first appeared in the serum, but was also present in the cerebral spinal fluid at later stages of infection (Griffin et al., 1978). The characterization of the neutralizing antibodies was further studied to assess their affect on virus entry *in vitro* (Kennedy-Stoskopf and Narayan, 1986). Using a variety of virus binding temperatures, it was shown that the rate of binding of virus to the surface of permissive fibroblast cells was faster than the rate of neutralization. In contrast, transformed macrophage cells showed an increase in virus binding in the presence of neutralizing antibodies and that infectivity was blocked at the RNA transcription level. It is noted, however, that the neutralizing sera used in these assays is hyperimmune, and *in vivo* the antibodies titres would be even lower affinity, supported by the fact that development of neutralizing antibody in the host is slow and titres are low. It was proposed that the ability of virus to bind to the target cells in the presence of an active humoral response may be the mechanism by which the virus is able to persist (Kennedy-Stoskopf and Narayan, 1986). This is supported by the fact that neutralizing ability of sera *in vitro* requires a long incubation period of up to 1 hour at 37^{o}C, which would be inappropriate *in vivo*.

Antibodies against the major core antigen, p25, are the first to appear following infection, followed by the appearance of anti-envelope antibodies and antibodies to other core proteins 4-8 weeks post infection (Kajikawa et al., 1990). This study used two strains of MVV, one which is highly lytic in culture and a second which shows minimal lysis. The appearance of anti-matrix, p14, antibodies appeared sooner with the lytic virus than with the non-lytic strain, presumably since it will be exposed to the immune system sooner in a lytic-type infection. There was no difference between the antibody responses to p24 and envelope antigens between the two strains. This longitudinal study used new-born lambs which will progress to the disease state more rapidly than adults, and thus the appearance of p14 in lytically infected sheep may not be of significance in the field (Kajikawa et al., 1990).

The use of recombinant proteins has allowed the development of more refined assays for analyzing antibody titres. An ELISA assay has been developed for the detection of anti-p25 antibodies using a yeast-derived recombinant protein (Reyburn et al., 1992). The

detection of p25 levels is widely used as a measure of HIV replication during infection, and this assay provides the sensitivity to use in the detection of MVV infection. Expression of a variety of MVV antigens in the pGEX bacterial system has shown that the N-terminus of the transmembrane antigen, gp41, is the immunodominant antibody in infected sheep (Kwang and Cutlip, 1992 a and b). These bacterially-derived recombinant proteins have the potential to be used in a screening assay similar to that described for the p25 antigen above (see also Chapter 5 of this thesis). The immunodominance of the transmembrane protein has been more fully characterized in CAEV (McGuire et al., 1992). Expression of two antigenically distinct strains of the envelope protein of CAEV in the vaccinia system showed that the neutralization epitope is likely to be conserved (Lichtensteiger et al., 1991). The recombinant proteins were able to inhibit heterologous neutralizing antibodies in goat sera, as well as homologous antisera. This suggests that the neutralization epitope is conserved between strains but it is the variation in adjacent amino acids that will alter the conformation and affect the interaction of the epitope with the host immune serum. The location of this neutralization epitope within the envelope protein is not identified in this study. Recent studies using a lambda-expression library have identified four immunodominant epitopes (Bertoni et al., 1994). These epitopes all lie in the external domain of the transmembrane envelope protein. One of these epitopes is conserved in other lentiviruses, such as HIV. Three distinct regions of the external envelope to which sheep mount an immune response have been identified, using yeast-derived recombinant fragments of MVV envelope protein (Carey et al., 1993). To date, the identity of the principal neutralization domain in MVV is not known.

The appearance of anti-envelope antibodies, present in the synovial fluid, in CAEV infection has been correlated to the severity of inflammatory lesions in the joints of infected goats (Knowles et al., 1990). There was no correlation with anti-p25 levels in the blood. This epitope has been mapped to the transmembrane protein (Bertoni et al., 1994). No such association has been described for MVV infection.

The presence of complement-fixing antibodies has also been described in lentiviral infections. In MVV, complement-fixing antibodies are the first to be raised on infection, approximately 1 to 2 months post infection (Gudnadottir and Palsson, 1966). A localised production of complement-fixing antibodies in the central nervous system has also been described, and may explain the clearance of virus in this environment (Petursson et al., 1976). The relevance of these antibodies *in vivo* is disputable since the characterization used a laboratory test which may not be significant *in vivo*. In HIV, complement activation has been implicated as being important for clearing virus by lysing virus infected cells or cell-free virus. The V3 loop of gp120 has been demonstrated as an important region for activating complement-fixing antibodies (Spear et al., 1993).

The presence of enhancing antibodies in lentivirus infections has proved a hindrance when developing potential vaccines. These have been particularly problematic when using recombinant envelope proteins as vaccines followed by challenge with the whole virus (Wang et al., 1994, Sieblink et al., 1995). It is possible that the conformation of the recombinant proteins affect the nature of antibodies raised. However, enhancement has also been observed when using inactivated virus, as with MVV and CAEV vaccine studies (McGuire et al., 1986, Nathanson et al., 1981). The observation that neutralizing antibodies are unable to prevent virus binding and uncoating on the surface of macrophages (Kennedy-Stoskopf and Narayan, 1986) has been investigated further. It was shown that this mechanism may be due to the binding of the Fc portion of the antibody to the Fc receptor on the surface of the macrophage resulting in the internalization of the virus and thus enhancement of infection (Jolly et al., 1989). Antibody-dependent enhancement is defined as the ability of antibody to enhance the infection of the virus in the presence of fresh compliment. This suggests the role of a compliment receptor. In fact, enhancing antibodies can also work with inactivated compliment, but loose their activity by removal of the Fc portion. It has been proposed that the FcRIII is required for enhancement through macrophages in HIV infection (Homsy et al., 1989).

The cell-mediated response to MVV infection is only just beginning to be understood. Early reports described the presence of cell-mediated immunity in peripheral blood leukocytes (Griffin et al., 1978). Following intracerebral infection a response was detectable within one week after virus infection, which peaked at 1 to 2 weeks and then returned to basal levels after 4 to 6 weeks. These stimulated cells were detected in the cerebral spinal fluid, the spleen and the lymph node as well as the blood stream. Lymphoid tissue is the primary region were the immune response is initiated, and by manipulating the cannulation procedure in sheep the early immune event in the lymph node and its ducts have been studied (Blacklaws et al., 1995a). Histological examination of the lymph node after subcutaneous infection of MVV shows an active immune response by the presence of large activated lymphocytes in the T cell rich zones and a large number of plasma cells in the medullary cords (Blacklaws et al., 1995b). Analysis of the efferent lymph duct showed the presence of T lymphocytes which express MHC class II but did not express the IL-2 receptor (Bird et al., 1993). These lymphocytes were further characterized as part of a pool of CTL precursors (Blacklaws et al., 1994). The CTL precursors appeared in the blood stream 3 weeks post infection and remained for up to 3 months (Blacklaws et al., 1994). Similar data has been found with CAEV infection. The subset of CTL precursors in the circulating blood stream were characterized as $CD8^+$ lymphocytes (Lichtensteiger et al., 1993). Studies within the lymph node showed that

the release of CTL precursors was linked to viral replication. This acute immune response occurrs between 10 to 14 days post infection, although viral replication is detectable before 10 days (Blacklaws et al., 1995b). This indicates that this delay in the immune response may allow the virus to establish persistence. Once the immune response is detected the number of infected cells decrease followed by a drop in the number of T cells (Blacklaws et al., 1995b). Thus it appears in the absence of viral antigen T cell proliferation decreases. The viral proteins responsible for inducing a cell-mediated response are not known at present. The cell-mediated response of HIV infection has been more widely studied. This response however is further complicated since the virus infects CD4 lymphocytes, and will not be reported here.

1.13 Antigenic variation of MVV envelope glycoproteins

As was highlighted above, MVV is able to persist in the host despite an active immune response. The ability of the virus to escape immune surveillance has been partly attributed to antigenic variation. Evidence for antigenic variation was gained through longitudinal studies of experimentally infected sheep. Sheep infected with a given strain of MVV all generated neutralizing antibodies within the first six months, but these antibodies were unable to neutralize a different strain (Narayan et al., 1977). After a 22 month period the virus isolates were no longer susceptible to neutralization by the original serum, indicating the existence of mutants. These mutants could be passaged *in vitro* without loss of the change in neutralization properties suggesting they were stable mutants (Narayan et al., 1977). This study was extended over a period of 3.5 years, where virus and serum samples were taken at various time points (Narayan et al., 1978). The viruses isolated after 22 months were poorly recognised by the original neutralization sera, as was seen in the earlier study. By looking at the neutralization patterns over the three years the various virus isolates could be grouped into three types. However, one of the variants was antigenically similar to the original strain suggesting the co-existence of the original strain and the variants. Alternatively the virus may have mutated back to its original form. From this study it was claimed that antigenic mutants were raised after the generation of neutralizing antibodies. The sheep eventually mounted neutralizing antibodies against the variant strain (Narayan et al., 1978). The studies described above have characterized MVV mutants on neutralization properties. This work relies on the fact that the original stock of virus was clonal. Data generated in a subsequent paper puts this claim into dispute (Crane et al., 1988). This experiment involved the hyperimmunization of a sheep with the original 1514 'clonal ' strain of MVV, and this resulted in the generation of antibodies that were able to neutralize both the original virus and a variant, isolated from the

earlier study (Narayan et al., 1978). This data suggests that the original virus was not clonal, and this sheds doubt on the relevance of their earlier work.

Peptide mapping was carried out to ascertain which viral antigen was involved in antigenic variation (Scott et al., 1979). Minor differences were seen in the envelope protein only and not in the capsid proteins. Since these changes were small, it was postulated that the variants had arisen from point mutations rather than recombination. To analyse where on the viral genome mutants were occurring, RNaseT1 oligonucleotide fingerprinting was carried out on viral RNA of the antigenic variants. Small differences were seen in the 3' end of RNA, which corresponds to the envelope region of the genome (Clements et al., 1980). Further studies using a greater number of variants confirmed these results, and demonstrated that these changes were commonly the same point mutation (Clements et al., 1982). These studies also indicated that the variants rose from gradual point mutations in the original strain, and were thus progressive. It should also be noted that these RNA fingerprinting studies were crude and thus results can only be interpreted with caution.

Further evidence for antigenic variation being confined to the envelope region came from studies using a panel of monoclonal antibodies (Stanley et al., 1987). By screening the original parent strain and the subsequent mutant viruses from the earlier study (Narayan et al., 1978), five epitopes were characterized that were implicated in antigenic variation. Three of these epitopes were present on the original virus, and replaced on mutants by other epitopes (Stanley et al., 1987). No similar changes were observed in the core protein. These monoclonal antibodies were unable to neutralize the virus, and the authors claimed the involvement of both neutralizing and non-neutralizing epitopes in antigenic variation. They also claimed that earlier studies using polyclonal serum were unable to detect minor changes in the envelope protein, and thus variation was probably are more frequent event than was reported in other studies (Thormar et al., 1983). They suggest that single point mutations within the envelope gene may have a significant effect on the three-dimensional structure of the envelope, and this will result in the marked change in antigenicity of the protein (Stanley et al., 1987).

Sequence comparisons of different strains of MVV have shown that the majority of variation occurs within the *env* gene (Braun et al., 1987, Querat et al., 1990, Sargan et al., 1991). Sequence comparisons between two strains of the 1514 isolate of MVV showed a hypervariable region within the envelope protein (Braun et al., 1987), but it has not been formally proved whether neutralizing antibodies are directed to this site. The role of neutralizing antibodies in generating mutants is debatable. The fact that they are ineffective in clearing virus from the host would indicate that their role *in vivo* is minor. Sequence comparison of the EV1 strain propagated *in vitro* has shown that variation in the *env* gene can arise in the absence of neutralizing antibodies (Carey and Dalziel, 1994).

Antigenic variation has been described in a variety of viruses, most notably the Influenza viruses. Mutations are seen in the haemaglutinin and neuraminidase proteins (Webster et al., 1982). Of the lentiviruses, it is EIAV that shows the most marked variation. Its disease progression differs from other lentiviruses, in that there are recurrent cycles of viraemia followed by chronic disease episodes. This has been attributed to the constant emergence of antigenic variants, followed by the response by the immune system (Montelaro et al., 1984). The emergence of variation within the genome of lentiviruses may be expected due to the error prone reverse transcriptase (Clements et al., 1988). Several other studies looking at the emergence of variants and their potential role in pathogenesis has suggested that antigenic variants may not be significant (Lutley et al., 1983, Thormar et al., 1983). These longitudinal studies found that antigenic variation was a rare and random event, and that the original virus co-existed with the antigenic variant. They did not observe any correlation with the development of antigenic variants and the severity of disease (Lutley et al., 1983). In summary, it would appear that the role of neutralizing antibodies in generating antigenic variants is debatable.

1.12 Aim of research project

The role of lentiviral envelope glycoproteins in the virus life cycle has been reviewed with particular attention to HIV-1. In contrast, the functional epitopes within the envelope glycoprotein of MVV are not known. It is understood to be a major target for neutralizing antibodies, but the significance of this *in vivo* is not understood. The major epitopes to which the neutralizing response is raised are not known. By analogy with HIV, the envelope glycoprotein of MVV would be expected to interact with the host cell receptor, and thus influence the tropism of the virus. The regions of the envelope which are involved in interaction with its receptor are not known.

To begin to address these questions, expression of the external glycoprotein (referred to as gp135 throughout the thesis) of MVV as a recombinant protein was undertaken. A source of recombinant envelope would be of use both in functional studies and as an immunogen. Studies were undertaken to generate monospecific serum using the bacterial recombinants and lectin-purified envelope from virions as sources of antigen. The use of baculovirus derived envelope to characterize the host cell receptor was also investigated.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

All chemicals were supplied by Sigma Chemical Co., Poole, Dorset, England, or Fisons Scientific Equipment, Loughborough, England, unless stated otherwise. All enzymes used in molecular biology were supplied by Northumbria Biologicals Ltd. (NBL), Cramlington, U.K.. Tissue culture reagents were purchased from GIBCO, Glasgow, U.K. and tissue culture plastic ware was supplied by Nunclon, Denmark. Recipes for frequently used solutions are given in Appendix 2.

2.2 METHODS

2.2.1 Tissue culture

2.2.1i Mammalian cell culture

a] Establishing sheep fibroblast cell lines

Sheep fibroblast cell lines were derived from skin biopsies as described in Rheinwald and Green (1977) and were a kind gift from Dr.B.Blacklaws, Department of Veterinary Pathology, University of Cambridge. Cells were stored in liquid nitrogen at a concentration of $1x10^6$cells/ml in 90%FCS(v/v)/10%DMSO(v/v). Cells were resuscitated by thawing quickly at 37^{o}C and washing out the DMSO in 10%FCS(v/v)/DMEM(v/v). They were grown up as monolayers in a T25 tissue culture flask at 37^{o}C in the presence of 5%CO_2.

Cells were routinely grown in T150 tissue culture flasks with 10%FCS/DMEM. All medium contained penicillin/streptavidin and L-glutamine, unless otherwise stated. When the monolayer was approximately 90% confluent, medium was removed and cells washed twice with versene followed by incubation with 2-3mls trypsin/versene at room temperature until cells were no longer adherent. 10mls of 10%FCS/DMEM was added and cells split 1:3.

b] Visna Virus Strains

All experiments used the EV1 isolate of Maedi-Visna virus (Sargan et al., 1991).

c] Preparation of virus stocks

Skin fibroblasts were grown to 80% confluency in T150 flasks. Medium was removed and virus was added at a m.o.i. of 0.1 in 10ml 2%FCS/DMEM. Cells were incubated with virus at $37^{o}C$ for 60 minutes with gentle agitation every 15 minutes to prevent cells drying out. A further 20mls 2%FCS/DMEM was added and cells left for 6 to 8 days until approximately 50% c.p.e. was apparent. Medium was removed and clarified by centrifugation at 2 500xg for 5 minutes at $18^{o}C$. Supernatant was stored in 1-5ml aliquots at $-70^{o}C$ and viral titration performed as described below.

d] Titration of virus

96 well flat-bottomed tissue culture plates were seeded with $1x10^{4}$cells per well in 100µl 10%FCS/DMEM and left to grow overnight. 10-fold virus dilutions were made in 2%FCS/DMEM, medium removed from the inside 60 wells and virus dilutions added across the plate, 50µl/well, six wells for each dilution. The 10th column was reserved for medium only as a mock infection. Cells were incubated for 2-3 hours after which medium was removed and cells were fed with 50µl 2%FCS/DMEM/well. The plate was fed with an additional 50µl 2%FCS/DMEM/well 4 days later. After another 3 days cells were stained with Giemsa stain as follows.

After removal of medium, cells were fixed in ice-cold acetone:methanol (1:1), 100µl/well, for 5 minutes, removed and air dried for 5 minutes. Cells were then incubated for 30 minutes with 1% potassium dichromate(w/v), 100µl/well. This was removed and cells fixed in acetone:methanol as before. Giemsa stain was freshly diluted in water (1:1), and added to cells, 100µl/well, for 5 minutes. Cells were washed in excess water and destained in water. The plate was air dried and wells examined for presence of syncytia. The 50% tissue culture infectious dose ($TCID_{50}$/ml) was calculated by the method of Reed-Meunch (1937).

e] Virus neutralization assay

96 well plates were seeded with $1x10^{4}$cells/well in 10%FCS/DMEM and grown overnight. Serial dilutions of heat inactivated sera ($56^{o}C$ for 30 minutes to inactivate compliment) were incubated with 500 $TCID_{50}$ visna virus overnight at $4^{o}C$. The next day medium was removed from the inside 60 wells and virus/sera mixtures (50µl/well) were added to the first row and doubling dilutions made across the plate using 2%FCS/DMEM containing appropriate dilution of heat inactivated serum. After a 2 hour incubation, medium was removed and wells were fed with 100µl 2%FCS/DMEM. 7 days later cells were fixed and Giemsa stained as described above. Neutralization was determined by decrease in virus titre, where antibody

titres were defined as greatest dilution of sera which gave 50% reduction in virus titre compared to controls.

2.2.1ii Insect cell culture

a] Establishing insect cell lines SF21 and H5 in monolayers.

The insect cell line SF21, supplied by CLONTECH, is derived from the ovarian tissue of the fall army worm, *Spodoptera frugiperda* (Vaughn et al., 1977). Cells were grown in TC100 medium supplemented with 10%FCS and penicillin/streptomycin. H5 cells are derived from the eggs of the cabbage looper, *Trichoplusia ni*, the natural host of *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV) (Davis et al., 1993). Cells were grown in serum-free medium, SF900.

Insect cells will survive at a pH 6.2-6.4, they grow reasonably well at any temperature from room temperature to 30^{o}C and do not require CO_2. Cells were maintained at 27^{o}C for optimum growth giving a doubling time of 20-24 hours.

Insect cells were resuscitated by thawing at 37^{o}C and adding 5mls of appropriate medium which had been pre-cooled to 4^{o}C. Cells were allowed to attach to T25 flask for 1-2 hours and then medium was replaced with fresh medium. Medium was changed daily on H5 cells until they reached confluence. Cells were passaged when 80-90% confluent by removing medium and adding 5-10mls of fresh medium. Cells were harvested by knocking the flask several times to dislodge the cells and split 1 in 4. Insect cells were maintained in T150 tissue culture flasks.

b] Storage of insect cells in liquid nitrogen

Cells were harvested as described above. Cell viability was assessed using trypan-blue exclusion (dead cells take up the stain). Cells that were at least 90% viable only were used to make stocks. SF21 cells were adjusted to a cell density of 4x10^{6}cells/ml in TC100/FCS medium and chilled to 4^{o}C. Cells were mixed with an equal volume of chilled TC100/FCS containing 20% DMSO(v/v). 1ml aliquots in cryogenic vials were placed at -20^{o}C for 1 hour, followed by overnight storage in -70^{o}C. They were transferred to liquid nitrogen the next day. H5 cells were cryopreserved at a concentration of 3x10^{6}cells/ml in ice cold medium consisting of 45% conditioned medium(v/v) (medium removed from flask of growing cells)/45% fresh medium(v/v)/10% DMSO(v/v). Cells were then placed at -20^{o}C for 1 hour, at -70^{o}C for 24 hours and then transferred to liquid nitrogen.

c] Growing insect cell lines as suspension cultures

Suspension cultures are useful for large scale preparation of recombinant proteins and preparations of virus stocks. Healthy insect cells were seeded into 100ml of appropriate medium at a density of $2x10^5$cells/ml and grown to the required cell density. Cells were grown in a sterile Techne biological spinner (100ml) and cells were stirred gently using a magnetic stirrer (model-IKA-combimag reo, Drehzahl electronic), specifically designed for tissue culture to prevent heating of the plate and thus destruction of cells.

d] Propagation of baculovirus virus stocks

SF21 cells were seeded into a 100ml spinner culture at $1x10^5$cells/ml and grown to a cell density of $4x10^5$cells/ml (approx. 2 days). Cells were infected with baculovirus at a m.o.i. of 0.1 pfu/cell, by addition of calculated volume to spinner culture and grown for a further 4-5 days. Cell suspension was harvested into sterile 50ml tubes (Corning) and centrifuged at 1000xg for 5 minutes at 18^oC to remove cells and debris. Supernatant was transferred to sterile 15ml tubes and/or 1ml screw-top microfuge tubes and stored either at 4^oC for short term storage or at -70^oC for longer term storage. The titre of the virus stock was determined by plaque assay.

e] Baculovirus plaque assay

Plaque assays were used to isolate recombinant viruses and to titre virus. 35mm tissue culture petri dishes were seeded with $1x10^6$cells/1.5ml/dish and grown overnight or seeded with $1.5x10^6$cells/1.5ml/dish and incubated for 1-4 hours to use on the same day. Dilutions of virus were made; 10^{-4}, 10^{-5} and 10^{-6}. 100µl of each dilution was used to infect a single petri dish, 2-3 dishes per dilution. An appropriate dilution of BacPAK6 (Clontech), between 10^{-5} and 10^{-6}, and medium only were used as controls. Petri dishes were incubated for 1 hour at room temperature after which the virus inoculum was removed. The cells were overlaid with 1.5ml of 1% SeaPlaque agarose(w/v)(FMC Bioproducts)/10% FCS/TC100 pre-warmed to 37^oC to prevent agarose setting. The agarose was allowed to set and 1.5ml TC100/FCS/dish containing 450µl X-galactose (X-Gal, 25mg/ml stock in DMF, stored at -20^oC)(Northumbria Biologicals, U.K.) was added. Dishes were incubated at 27^oC for 4-5 days. 1ml of 0.03% neutral red/PBS (0.33%(w/v) neutral red/PBS stock, filter sterilised and stored in dark at room temperature) was added to each dish and incubated at 27^oC for 2-3 hours. Stain was removed and dishes stored upside down overnight in the dark to allow the colour to develop. Neutral red is taken up by healthy cells so that virus infected cells can been seen and counted as clear plaques. Dishes infected with positive control virus, i.e. BacPAK6, will give blue plaques in the presence of X-gal.

Calculation of virus titre:

Plaques were counted from dishes giving a reasonable number of plaques (10-30 plaques). The titre of the virus stock (pfu/ml) was calculated as;

average number of plaques/dish x 10 x (dilution factor)$^{-1}$

2.2.2 Molecular biology

2.2.2i Bacterial culture

a] Bacterial medium and agar plates

Bacteria were routinely grown in Luria Bertoni (LB) broth containing fresh ampicillin (100µg/ml) to select for ampicillin resistant bacteria since all plasmids used in this thesis contained an ampicillin resistance gene. LB/agar plates were prepared by melting 1.5% bacteriological agar(w/v) (Oxoid, U.K.) in L-Broth, cooling to 42^{o}C and adding ampicillin (100µg/ml). For the blue/white colony selection of pCRII™(Invitrogen, USA), agar/amp plates were coated with 25µl X-Gal (40mg/ml stock in DMF) and incubated at 37^{o}C for 1 hour before use.

b] Bacterial strains, growth and storage

Bacterial strains used in this project were competent *E.coli* JM101 (Yannish Perron et al., 1985), DH5α™ (GIBCO, BRL) and INVα-F™ (Invitrogen, USA). Transformed bacteria were streaked onto agar plates and stocks made from single colonies grown up in 10ml L-Broth/ampicillin for 16-18 hours. Bacteria were stored in 20% glycerol(v/v) at -70^{o}C.

c] Transformation of Competent Bacteria

Transformation of INVα-F™ cells was carried out as manufacturer's instructions using β-mercaptoethanol to prepare bacterial cells for uptake of plasmids. The protocol is a modification of the following basic method used for bacterial strains JM101 and DH5-α. Bacterial cells were thawed out on ice and aliquoted into suitable quantities, usually 100µl. Plasmid DNA was added to the cells for a 30 minute incubation on ice followed by heat shock at 42^{o}C for 90 seconds to allow uptake of plasmid. 900µl L-Broth added and cells grown up for 60 minutes to allow for the expression of ampicillin resistance gene. Cells were then plated out onto agar plates. Transformation of bacteria with pCRII used "One-Shot" cells provided in pCRII cloning kit (Invitrogen, USA) and protocol was followed according to the manufacturer's instructions.

2.2.2ii Plasmid cloning

a] Restriction enzyme digestion

DNA was digested for the purposes of checking DNA or for sub cloning fragments into plasmids. DNA was digested in a maximum volume of 25µl using approximately 1 unit of enzyme/µg of enzyme site. Restriction enzymes were supplied by Pharmacia, U.K. and Northumbria Biologicals, U.K.. Digestions were carried out for a minimum of 1 hour at 37^{o}C in the manufacturer's buffer and then analysed by gel electrophoresis using uncut samples as controls.

b] Preparation of linearised plasmid for cloning

Prior to ligation, it was necessary to remove 5' terminal phosphate groups from the digested plasmid to prevent self ligation. A Shrimp Alkaline Phosphatase (SAP) (United States Biochemical, USA) was used as an alternative to more commonly used Calf Intestinal Phosphatase (CIP) since it is irreversibly deactivated at 65^{o}C and is active in most restriction enzyme buffers allowing it to be added directly to restriction digest mixture. 0.1 unit of SAP was added per 1pmol DNA termini (2.5µg of 3kb plasmid) for 1 hour at 37^{o}C followed by a 15 minute deactivation at 65^{o}C. To ensure the removal of protein contamination the DNA sample was phenol:chloroform extracted, run on a 1% agarose gel and DNA purified as described in section 2.2.2iii.

c] Ligations

100ng of dephosphorylated plasmid was routinely ligated to an equal or excess molar ratio of insert which had been cut with the same restriction enzymes. Ligation reactions were carried out in 10µl volumes using 5 units of T4 DNA ligase and the manufacturer's ligation buffer. Reactions were incubated for 16-18 hours at 12^{o}C and appropriate controls were included to ensure the efficiency of T4 ligase, SAP and transformation.

2.2.2iii DNA preparation, purification and analysis

a] Preparation of plasmid DNA

DNA preparations were carried out using the Qiagen purification method (Qiagen Inc., USA) following the manufacturer's protocol. Minipreparations were made using Qiagen-Spin columns (Qiagen Inc., USA) starting with 5ml bacterial culture grown from a single colony for 16-18 hours. Maxipreparations were done using Qiagen tip-500 using 500ml culture of a low copy number plasmid or 150ml culture of a high copy number plasmid. Essentially

bacterial cells are lysed and DNA is denatured by alkali for a limited time. Chromosomal DNA and protein are precipitated using potassium acetate, pH5.5 leaving the smaller plasmid DNA in solution. After centrifugation the supernatant is put through the Qiagen column. Qiagen columns are made from an anion exchange resin that allows binding of DNA which can then be eluted by increasing the salt concentration and pH. Following elution from Qiagen tip-500 it was necessary to precipitate the plasmid DNA using isopropanol to prevent coprecipitation of salt. Plasmid DNA was resuspended in TE, pH8.0 and stored at -20^{o}C.

b] Phenol:chloroform extraction

This was carried out to remove contaminating protein from DNA samples. DNA samples were extracted using phenol:chloroform:isoamylalcohol (25:24:1) twice followed by a single chloroform extraction. DNA was then ethanol precipitated on dry ice for a minimum of 15 minutes using 2 volumes of ethanol and 1/10th volume 0.3M sodium acetate, pH5.5. DNA was pelleted 9 000xg for 10 minutes at 4^{o}C, washed with 70% ethanol, air dried and resuspended in TE, pH8.0.

c] DNA gel electrophoresis

DNA samples were analysed on a 1% agarose(w/v)/TAE gel containing 1μg/ml of ethidium bromide. DNA samples were prepared before loading by mixing them with DNA loading buffer in a ratio of 1:2 loading buffer to sample. Gels were run at a constant voltage of 85-100mV for 60-90 minutes and visualised by UV illumination of ethidium bromide. EcoRI/HindIII λDNA digests markers (Northumbria Biologicals Ltd., U.K.) were run alongside the samples.

d] DNA extraction from agarose

DNA was run on a 1% low melting point agarose (SIGMA)(w/v) gel made up in TAE buffer containing 1μg/ml of ethidium bromide. TAE was used preferentially to TBE buffer since agarose extraction by GenecleanII (Stratech Sci.) is more efficient using TAE buffer. The agarose gel was run at 4^{o}C at a constant voltage of 40mV to prevent melting of the agarose. DNA was visualised by UV illumination and the required fragment cut out of the gel. DNA was extracted and purified following the manufacturer's instructions for the Geneclean II kit (Stratech Sci.) which uses a silica matrix for binding and purifying DNA.

e] Determination of DNA concentration

For small amounts of DNA (pg-ng) estimates were taken by visualising the intensity of fluorescence under UV illumination compared to DNA markers of known concentration. Larger quantities of DNA were measured by reading the absorbance at wavelength 260nm on

a spectrophotometer. An $O.D._{260}$ of 1.0 is equivalent to 50µg/ml double stranded DNA or 40µg/ml of single stranded DNA. Purity of the DNA was measured by calculating the ratio $O.D._{260/280}$ where a ratio of 1.8-2.0 represents a sample clean from protein/phenol contamination (Sambrook et al., 1989).

2.2.2iv Analysis of DNA sequences by blotting and hybridisation

a] Southern blotting by alkali method

Digested DNA samples were run on a 0.8% agarose(w/v)/TBE gel containing 1µg/ml of ethidium bromide for 16-18 hours at a constant voltage of 40mV. The gel was examined under UV light to visualise DNA and photographed alongside a ruler to determine position of DNA fragments. The gel was cut to size removing areas of gel that did not contain DNA and transferred onto an uncharged nylon membrane using a modification to the method first described by Southern (1975). The DNA was denatured by shaking the gel in 0.25M HCl for 30 minutes, washed in distilled water and shaken for a further 20 minutes in 0.25M NaOH to neutralize the DNA. Transfer was carried out onto a nylon membrane (Hybond-N, Amersham) using 0.25M NaOH/1.5M NaCl as the transfer solution. The membrane was prewetted in distilled water for 5 minutes prior to assembly of the transfer pyramid set up using the Whatmann 3mm filter paper wick method described by Sambrook et al. (1989). The transfer was carried out for a minimum of 2 hours after which the filter was washed in 2xSSC and air dried. The DNA was immobilised by baking for 2 hours in a 80°C vacuum oven between two sheets of Whatmann 3mm paper. The membrane was stored at 4°C until required for hybridisation.

b] Slot blotting

Slot blotting was performed using undigested samples of DNA. Nitrocellulose strips (Hybond-C, Amersham) were cut to the size of the slot blot manifold and soaked in dH_2O. The manifold was assembled with the nitrocellulose paper and attached to a vacuum pump. 100-200µl of the DNA sample was added to each slot and allowed to be sucked through the apparatus. The blot was then soaked as follows: 5-10 minutes in 0.5M NaOH, 5-10 minutes in 1M Tris-HCl, pH7.5 and 5-10 minutes in 2xSSC. The blot was placed on a piece of 3mm Whatmann paper that had been soaked in 2xSSC and baked as described above. The blot was air dried and stored at 4°C until ready for hybridisation.

c] Colony Hybridisation

Single colonies were transferred to agar/amp plates and allowed to grow for 16 hours at 37°C, after which they were transferred to nylon filters (Hybond-N Nylon, 0.45u, 82mm diameter,

Amersham UK). The filters were processed by a modification of the method first described by Buluwela et al. (1989). The filters were placed on 3mm Whatmann paper that was pre-soaked in 2xSSC/5% SDS(w/v) for 2 minutes and then microwaved at full power for 2.5 minutes in a 650W Matsui microwave oven. The filters were then rehydrated briefly in 2xSSC/0.1% SDS(w/v) and then prehybridised in aqueous prehybridisation buffer for up to 6 hours at 65^{o}C, followed by hybridisation for 16 hours at 65^{o}C with ^{32}P-labelled probe. The filters were washed as described below and exposed to film (Kodak X-omat S). Colonies that were recognised by the probe were picked and grown up into 10mls L-Broth/amp and analysed as required.

d] Preparation of ^{32}P-labelled probes

DNA fragments were purified from an agarose gel using the Geneclean II kit and labelled as follows. 30ng of DNA fragment was mixed with 6µl of oligonucleotide labelling buffer and made up to 28µl with distilled water. The mixture was boiled for 3 minutes to denature the DNA and cooled slowly to room temperature preventing the DNA from reannealing. 10µCi dCTP-α^{32}P (Amersham, U.K., specific activity 400Ci/mmol) and 1µl Klenow DNA polymerase was added and the reaction carried out for 1-2 hours at 37^{o}C. The reaction was stopped with 170µl of 10xTE, pH8.0. Efficiency of labelling was calculated by removing unincorporated nucleotide by acid precipitation with ice-cold 5% trichloracetic acid(v/v) on glass fibre discs (Whatmann) (Sambrook et al., 1989). Discs were dried and counted on a Beta counter (model 1450 Microbeta PLUS-Liquid scintillation counter, Wallac) in 1ml of 'Optiscint' Hisafe (LKB Scintillation products, Pharmacia Wallac, U.K.). The specific activity was calculated and incorporation was usually between 10-15%, that is a specific activity of 4x10^{7}cpm/µg DNA. Immediately prior to hybridisation the labelled probe was denatured by boiling for 5 minutes and quenching on ice.

e] Hybridisation of DNA blots

Blots were rehydrated in 6xSSC prior to hybridisation which was carried out in glass tubes in a hybridisation oven. Blots were prehybridised in aqueous prehybridisation solution (APH, 1mlAPH/cm^{2} membrane) for 1-4 hours at 68^{o}C. Labelled probe was added to fresh prewarmed APH and blots hybridised at 68^{o}C for 16-18 hours. Blots were washed twice in a low stringency wash, 2xSSC/0.1%(w/v) SDS at 68^{o}C, 30 minutes per wash followed by a further two 30 minute washes in high stringency wash, 0.2xSSC/0.1%(w/v) SDS, at 68^{o}C. Blots were dried between two sheets of Whatmann 3mm paper and placed under film (Kodak-Biomax MR) with an intensifying screen for 3-10 days at -70^{o}C until bands were visible.

2.2.2v DNA sequencing

Sequencing was performed using dideoxy chain termination that was a modification of the basic method first described by Sanger (1977). Sequencing was carried out manually using α-^{35}S-dATP (Amersham: Specific activity 400Ci/mmol) or on an automated sequencer (DNA sequencer model 4000L, Li-COR) using IRD41 labelled primers. Fig.2.1A lists the primers used. Primers 193L and 415H were used in manual sequencing, and primers 124 and 125 were used in automated sequencing.

a] Preparation of DNA prior to sequencing

Mini preparations of DNA were carried out using Qiagen-Spin columns. DNA prepared by this method gave sufficiently pure DNA to use in sequencing reactions and yields were in the range of 3-5µg/ml bacterial culture. Prior to performing sequencing reactions using IR-Dye labelled primers it was necessary to denature the DNA. Approximately 5-10pmol DNA was made up to 18µl with distilled water and 2µl of freshly prepared denaturing buffer (2M NaOH/2mM EDTA) was added. After a 5 minute incubation at 18^{o}C the sample was neutralized with 3µl of 3M sodium acetate, pH5.0 and 7µl of distilled water. DNA was precipitated with 75µl 100% ethanol for 15 minutes at -70^{o}C, pelleted at 13 000xg for 15 minutes at 4^{o}C in a microcentrifuge, washed with 70% ethanol and air dried. Sequencing reactions were immediately carried out.

b] Sequencing with automated sequencer

i) Sequencing reactions

The sequenase V2.0 T7 DNA polymerase sequencing kit (USB, USA) was used to carry out sequencing reactions and labelling was performed using the protocol supplied by the automated sequencer model 4000 using IR-dye labelled primers 124 and 125.

ii) Sequencing gel electrophoresis

Gel electrophoresis was performed according to the protocol in the manufacturer's handbook. Long Ranger™ (AT Biochem, Malvern, USA) acrylamide was used to make up the gels in TBE buffer.

c] Sequencing by manual methods

i) Sequencing reactions

Thermal cycle DNA sequencing was performed using the Circumvent™ Thermal Cycle DNA sequencing kit (New England Biolabs., USA). It was not necessary to denature the DNA

prior to carrying out sequencing reactions. Primers used are listed in Fig.2.1A and reactions were carried out using α-^{35}S dATP incorporation. Cycling conditions were 20 cycles of 20 seconds at 95^{o}C, 20 seconds at 55^{o}C and 20 seconds at 72^{o}C using a Techne Programmable Dri Block PHC-1 model.

ii) Sequencing gel electrophoresis.

Electrophoresis was carried out using a Bio-Rad Sequi-Gen apparatus. Gels were made up with 6% acrylamide(w/v)(acrylamide:N,N'-methylbisacrylamide, ratio 19:1, Biorad) containing 8M urea (Sigma, molecular biology grade) in 0.5xTBE buffer. Plates were sealed at the base with 60ml acrylamide, 200µl TEMED and 400µl 25%AP(w/v) (Biorad). 120mls of acrylamide was degassed and filtered through a 0.45µm filter and 100µl TEMED and 400µl of 25% AP(w/v) added prior to pouring the gel. Gels were run in 0.5xTBE.

Prior to loading the gel, samples were heated to 75^{o}C for 2 minutes and loaded into wells that had been prewashed to remove traces of urea. Gels were prerun for 60-90 minutes until they reached a temperature of 45^{o}-50^{o}C. Electrophoresis was carried out at 60 watts for 3-6 hours, dried down onto Whatmann 3mm paper at 80^{o}C under vacuum (Biorad model 583 gel drier) and put down under film (Kodak X-OMAT R film) for 16-18 hours at -70^{o}C.

2.2.2vi Polymerase chain reaction

a] Primers and Template DNA

Primers used in this thesis are shown in Fig.2.1B. Various considerations were taken when designing primers. Primers were made between 18-34 nucleotides long, were at least 50% GC rich and contained no palindromic sequences to prevent formation of hairpin structures. It was usual to end primers with a G or C residue at the 3' end. The primers were synthesised by Oswell DNA Service (Department of Chemistry, Kings Buildings, West Mains Road, Edinburgh, EH9 3JJ). Primer concentrations were approximately 0.05mmolar.

Unless stated otherwise, the template DNA was taken from a clone containing the whole *env* sequence of the EV1 strain, the original cloning of which was carried out by Dr.R.G.Dalziel (personal communication).

Figure 2.1: Primers

A. Sequencing Primers

Primers shown are annotated with the sequence to which they bind and the nucleotide position in that sequence is also indicated.

Nucleotide positions for the MVV EV1 primers are from Sargan et al., 1991.

B. PCR Primers

Primers 1 and 2 were used to generate the envA fragment of gp135 (see Chapter 3).

Primers 4975 and 4976 were used to generate the external portion of the envelope gene of MVV (see Chapter 5).

Primers 271P and 272P were used as control primers for amplifying the whole env gene of MVV.

The appropriate restriction enzyme site are underlined for each primer.

The terminal codon of the MVV genome is shown in bold typing and the corresponding amino acid for which it codes is given.

The nucleotide position of the terminal base of the MVV genome is also stated.

Sequence positions are taken from Sargan et al., 1991.

A. Sequencing primers

Primer 193L (reverse)

(5')- CTTGCACTCCAATACGG -(3')

MVV EV1 nt 7135 nt 7119

Primer 415H (reverse)

(5')- CCCACTGTTTATATTATGTCT -(3')

MVV EV1 nt 7421 nt 7401

Primer 124 (forward)

(5')- ATGGATATCTGCAGAA -(3')

pCRII™

Primer 125 (reverse)

(5')- CTCGGATCCACTAGTAAC -(3')

pCRII™

B. PCR Primers

Primer 1

(5')- TCCCGG^GATCCTTTGTTTAGG**ATG**GCAAGCAC -(3')

BamHI

nt5984/Met 1

Primer 2

(5')- ACACCGG^GATCCA**GGA**CTCTTCCCTCGTCCTG -(3')

BamHI

nt6922/Pro 312

Primer 4975

(5')- TCCCG^GATCC**AGT**AAGGGTCAATGTCAAGC -(3')

BamHI

nt6306/Ser 110

Primer 4976

(5')- ACACCCGTG^AATTCT*TTA***CAC**CAACCCTATGCC -(3')

EcoRI *stop*

nt8000/Val 669

Primer 271P

(5')- GGGGCG^CTAGCTTTAGG**ATG**GCAAGCAC -(3')

NheI

nt 5996/Met 1

Primer 272P

(5')- GGGGCG^CTAGCTCAGTCATTTCTCT**CA**ATCATGCG -(3')

NheI

nt 8945/End

b] PCR conditions

40ng of DNA template and 25-50pmoles of each primer were used per reaction. Reactions were made up as follows: 100µl of reaction buffer (50mM KCl/10mM Tris-HCl, pH8.8, 1.5mM $MgCl_2$, 3mM dithiothreitol (DTT)) (Ohara et al., 1989), 0.17µg/ml of bovine serum albumin, 100µM of each dNTP (Pharmacia, UK) and 1.7 units of *Thermus aquatus* (Taq) DNA polymerase (GIBCO, 5units/µl). Reactions were made up in 0.5ml microcentrifuge tubes under sterile conditions and wearing gloves to prevent contamination. Before running the reactions, samples were overlaid with 100µl mineral oil to prevent evaporation. PCR cycles were carried out using a Techne Programmable Dri Block PHC-1. Thirty cycles were run: denaturation at 95°C for 0.6 minutes: annealing at 55°C for 2 minutes, extension at 72°C for 2.5 minutes, final extension for 7.5 minutes.

Pilot reactions were carried out using varying concentrations of DMSO, typically 0-10% (v/v). DMSO is a denaturant that reduces secondary structure in the DNA template and is thought to increase the efficiency of PCR (Pomp and Medrano, 1991, Roffs at al., 1992).

10% of the sample was analysed on a gel and the remaining 90% was cloned into pCRII™ plasmid using the TA cloning kit (Invitrogen, USA) following the manufacturer's instructions.

2.2.3 Protein analysis

2.2.3i Quantitation

To determine the amount of protein in a sample the BIO-RAD protein assay was used (Bio-Rad, Germany). The assay utilises the shift in the absorbance maximum of acidic Coomassie Brilliant Blue G-250 from 465nm to 595nm in the presence of protein. Protein concentration was determined following the manufacturer's instructions using BSA made up in PBS(w/v) as a standard. When proteins were not in a form suitable for using in this assay, such as when bound onto glutathione beads, protein was concentration was estimated on a stained SDS-PAGE gel.

2.2.3ii One-Dimensional Gel electrophoresis of proteins

Proteins were separated by gel electrophoresis following the method described by Laemmli (1970) using the Bio-Rad Mini Protean II slab gel apparatus with 0.75mm spacers. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) used either a single concentration or a

gradient of separating gel, using a stock solution of 30% acrylamide(w/v)/0.8% bisacrylamide(w/v) and a final concentration of 0.38M Tris-HCl, pH8.7/0.1% SDS(w/v). Acrylamide was polymerised using 0.05% AP(w/v) and 0.05% TEMED(v/v). The stacking gel contained 3.48%acrylamide(w/v)/0.093%bisacrylamide(w/v)/0.145MTrisHCl,pH6.8/ 0.12% SDS(w/v) and was polymerised with 0.05% AP(w/v) and 0.1% TEMED(v/v). Before loading, samples were mixed in a ratio 1:1 with 2x SDS-reducing buffer and boiled for 3 minutes to denature the proteins. Electrophoresis was carried out in running buffer (0.05M Tris-HCl/0.1% SDS(w/v)/0.37M glycine)for 40-50 minutes at a constant voltage of 200V. Protein standard molecular weight markers were always run alongside the samples (Broad-Range 200-6 kDa, Bio-Rad, Germany).

2.2.3iii Protein Detection

a] Coomassie Brilliant Blue staining

Gels were routinely fixed and stained in 0.25% Coomassie Brilliant blue R250(w/v) in 40% methanol(v/v)/15% acetic acid(v/v) for 30 minutes followed by several destain washes in 20% methanol(v/v)/5% acetic acid(v/v).

b] Silver Staining

This was used when amounts of protein in samples were too low to be detected by Coomassie blue staining, typically 2 to 5 ng/protein band, using the protocol first described by Oekley et al. (1980). Gels were prefixed in 50% methanol(v/v)/10% acetic acid(v/v) for 30 minutes followed by 20 minutes in 5% methanol(v/v)/7% acetic acid(v/v). Gels were then fixed in 10% glutaraldehyde(v/v) for 20 minutes and washed in distilled water extensively over 16 hours. Proteins were detected by incubating gels in 0.1% silver nitrate(w/v) for 15 minutes. Bands were developed using a 0.28M Na_2CO_3 solution containing 0.05% formaldehyde(v/v). Once the desired level of staining had been reached the reaction was stopped using solid citric acid.

A permanent record of the gels was taken by photographing the gel using a commercial gel documentation apparatus (The Imager). Gels were also dried down for 1 hour under vacuum at 80^{o}C on a Bio-Rad model 583 Gel dryer.

c] Immunoblotting

Proteins were separated by SDS-PAGE and then transferred onto nitrocellulose membranes (Hybond-C, 45µ, Amersham, U.K.) using an Ancos semi dry electroblotter A (Harlow and Lane, 1988). Gel, nitrocellulose and paper were pre-soaked in transfer buffer (25mM Tris-HCl/20% methanol(v/v)). The gel was placed on top of the nitrocellulose and these were

sandwiched between 6 sheets of 3mm Whatmann paper and electrophoretically transferred at a constant current of 120mA. Following transfer the protein markers were cut off and stained in 5% Amido-Black(w/v) in 50% methanol(v/v)/5% acetic acid(v/v)and destained in 50% methanol(v/v)/5% acetic acid(v/v).

The nitrocellulose containing proteins of interest was blocked in 5% "Blotto" (5% milk powder (Marvel)(w/v)/0.2% Tween20(v/v)/PBS) for 1 hour at 18^{o}C. Primary antibodies were diluted appropriately in 1% "Blotto" (1% milk powder(w/v)/0.1% Tween20(v/v)/PBS) and membranes were incubated for 16 hours at 4^{o}C. Membranes were washed 5 times in 0.1% Tween20(v/v)/PBS and incubated for 1 hour at 18^{o}C in the presence of alkaline-phosphatase-conjugated secondary antibody diluted in 1% "Blotto" followed by a washing step as before. In some experiments an additional step was added to increase the intensity of staining. This involved using a biotinylated secondary antibody followed by alkaline-phosphatase linked to extravidin. Blots were developed by washing twice in 0.1M Tris-HCl, pH9.5 and using the NBT western blot development solution. Once bands were visible and of suitable intensity compared to background development was stopped by washing the blots in distilled water. Blots were air dried and kept in the dark.

2.2.3iv Immunodetection

a] Enzyme-Linked Immunosorbent Assay (ELISA)

Immulon II or IV (Dynatech, USA) 96-well plates were used for ELISAs. Plates were coated with antigen diluted in PBS (100µl/well) and incubated either for 1 hour at 18^{o}C or overnight at 4^{o}C. Plates were washed five times in 0.1% Tween20(v/v)/PBS with 1 minute soak between each wash. This was followed by a blocking step using 2% BSA(w/v)/PBS with 200µl/well for 30 minutes at 18^{o}C. Antibody was diluted in the blocking buffer and added to plates for up to 1 hour at 18^{o}C, 100µl/well. Plates were washed as before and alkaline phosphatase-conjugated secondary antibody added, 100µl/well, diluted in blocking buffer and incubated for 1 hour at 18^{o}C in the dark. Plates were washed and developed in phosphatase substrate for up to 2 hours at 37^{o}C and read on an ELISA counter (Titertek Multiscan) with a 405nm filter.

b] Immunoflourescence

i) Preparation of cell monolayers

Sterile 13mm round, glass cover slips were placed into wells of a 24-well tissue culture plate (Nunclon) and $1x10^5$cells/well were added, in a total volume of 0.5ml/well. Cells were incubated at 37°C for 16 hours to allow cells to adhere to plastic. Fixing was carried out with ice-cold 0.15M NaCl/ 80% acetone(v/v) for 10 minutes. The coverslips were dried and stored in fresh wells at -20°C.

ii) Immunoflourescent staining

Cells were thawed out at 18°C for a minimum of 45 minutes and blocked in 250µl of 10%normal species serum(v/v)/0.01%Tween80(v/v)/SPBS for 30 minutes at 18°C (normal species serum is that in which the conjugated antibody is raised). Blocking solution was removed and cells incubated for 1 hour at 18°C in 250µl of antiserum, diluted in 2%normal species serum(v/v)/0.01%Tween80(v/v)/SPBS. Cells were washed five times in 0.01%Tween80(v/v)/PBS and incubated in 250µl of FITC-labelled anti-species antibody, diluted as above for 1hour at 18°C in the dark. Cells were washed three times and mounted in 2-3µl of Citiflour. Slides were stored in the dark at 4°C and examined by a Leitz Ortholux II microscope.

c] FACS staining

For single colour immunoflourescent staining, fibroblast cells were resuspended to a concentration of $2x10^6$cells/ml in PBA (PBS/0.5%BSA(w/v)/0.04%azide(w/v)). 10^6cells were taken as a single aliquot and 50µl of primary antibody, diluted appropriately in PBA, was added for a 30 minute incubation at 4°C. Cells were washed three times in PBA and 50µl of FITC-labelled secondary antibody added with the same incubation conditions. Samples were kept in the dark during this incubation. Cells were again washed and reuspended in 250µl of PBA (with or without 250µl of 1% paraformaldehyde).

Fluorescent analysis was carried out using a Bacton Dickinson FACScan cell analyzer. The fluorescent profile is presented as a histogram of fluorescent intensity versus relative cell number.

2.2.3v Preparation and purification of MVV proteins

a] Preparation of MVV-infected cell lysates

Sheep skin fibroblasts were grown to 90% confluency in T150 tissue culture flasks and infected with a low m.o.i. of MVV, between 0.1-0.5 m.o.i./cell, in 10mls of 2%FCS/DMEM for 1 hour. A further 20mls of 2%FCS/DMEM was added. When cells showed extensive c.p.e. sterile glass balls were added to dislodge infected cells. Cell debris was spun down at 10 000xg for 16 hours at $4^{o}C$ in a Beckman JA-20 centrifuge. The pellet was resuspended in a minimum volume of sterile PBS and stored at $-70^{o}C$. Viral antigens were titrated out and analysed by western blot using MVV-infected sheep serum. Mock preparations of antigen were made by the same method adding only 2%FCS/DMEM to the cell monolayers.

b] Purification of gp135 using a lectin affinity column

Sheep fibroblasts were grown up in 8L tissue culture bottles (Corning) in 10%FCS/DMEM containing 25mM Hepes until cells were 80% confluent. Cells were infected as above with a low m.o.i. of MVV and cells monitored for c.p.e.. Medium was removed and clarified at 200xg for 10 minutes at $16^{o}C$. The virus was then pelleted in an ultracentrifuge at 120 000xg for 3 hours at $4^{o}C$. The pellet was resuspended in 2mls of ice-cold resuspending buffer (0.1M NaCl, 1mM EDTA, 20mM Tris-HCl, pH7.4) and this was then disrupted in an equal volume of 2xdisruption buffer (20mM Tris-HCl, pH7.4, 0.15M NaCl, 1mM $CaCl_2$, 1mM $MnCl_2$, 1% Chaps(w/v), 0.1%TritonX-100(v/v), 60mM DTT). The disrupted pellet was kept on ice at all times. The sample was circulated through a lentil lectin-Sepharose 4B (Pharmacia) column for 3 hours at $4^{o}C$, which had been set up and equilibrated according to the manufacturer's instructions. The sample was collected and column washed in 30 column volumes of wash buffer (0.1% Chaps(w/v),0.15M NaCl, 0.02M Tris-HCl, pH7.4, 1mM $MnCl_2$, 1mM $CaCl_2$). Glycoproteins were eluted using 0.2M methyl-α-D-mannopyranoside in wash buffer. 200µl samples were collected and analysed by western blot. The aliquots containing purified protein were pooled and stored at $-70^{o}C$.

2.2.3vi Acetone precipitation

To concentrate protein samples, 5 volumes of ice-cold acetone was added and incubated at $-20^{o}C$ for 16 hours. Precipitated protein was then pelleted by centrifugation at 13 000xg for 10 minutes at $4^{o}C$. The pellet was resuspended in the required buffer.

2.2.3vii Preparation of peptides for immunisation

Peptides were synthesised commercially. Peptides were supplied in a lyophilised state that had been desalted but were not HPLC pure. Peptides were reconstituted in 0.01M HCl (5mg peptide/ml HCl) and extracted in 40mls of ether. The aqueous layer was removed, excess ether was allowed to evaporate in a fume hood and peptides stored at -20^{o}C. 100µl of 0.1M phosphate buffer, pH7.0 was added per ml of peptide immediately before crosslinking.

Prior to immunisation it was necessary to link the peptide to a carrier molecule. Ovalbumin was used as a carrier protein and peptides linked via N-succinyl-3-(2-pyridyldithio) proprionate (SPDP) which provides the thiol groups for crosslinking with cysteine residues at the amino terminal of the peptide. 10mg ovalbumin was dissolved in 1ml PBS. 173µl of 40mM SPDP (dissolved in ethanol), equivalent to 30 molar excess of ovalbumin, was added dropwise to the ovalbumin and incubated at 23^{o}C for 30 minutes. Unlinked ovalbumin was separated from linked by passing through a PD-10 column (Pharmacia) according to the manufacturer's instructions. The linked protein was eluted first followed by the unlinked ovalbumin. To ensure that 2-thiopyridial groups had been introduced onto the amino groups in ovalbumin, 0.01M DTT was added to an aliquot of linked ovalbumin and the release of pyridine-2-thione was measured on a spectrophotometer at an absorbance of 343nm and compared to a non-reduced and an unlinked sample. Linked ovalbumin was stored at -70^{o}C until required.

To link the peptide to its carrier molecule, 30 molar excess of peptide was added dropwise to 2 mg of linked ovalbumin and incubated at 23^{o}C for 1-2 hours. The linking reaction was monitored by measuring the absorbance at 343nm every 30 minutes to measure the release of pyridine-2-thione. When the absorbance readings were constant it was assumed that all 2-pyridial disulphide sites had been reduced by substitution of pyridine-2-thiol with a peptide molecule. A summary of reactions is shown in Fig. 2.2.

2.2.4 Preparation of polyclonal and monoclonal antibodies

2.2.4i Animals

Rabbits used in these studies were either from our own breeding colony or were from a Dutch colony, female only, supplied in the Department of Medical Microbiology, University of Edinburgh. All mice used were 6-8 week old Balb/C females.

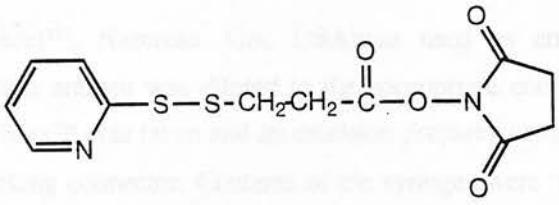
Figure 2.2: Coupling of the carrier molecule to peptides via crosslinking with N-succinyl 3-(2-pyridylthio) proprionate(SPDP)

i) Structure of SPDP

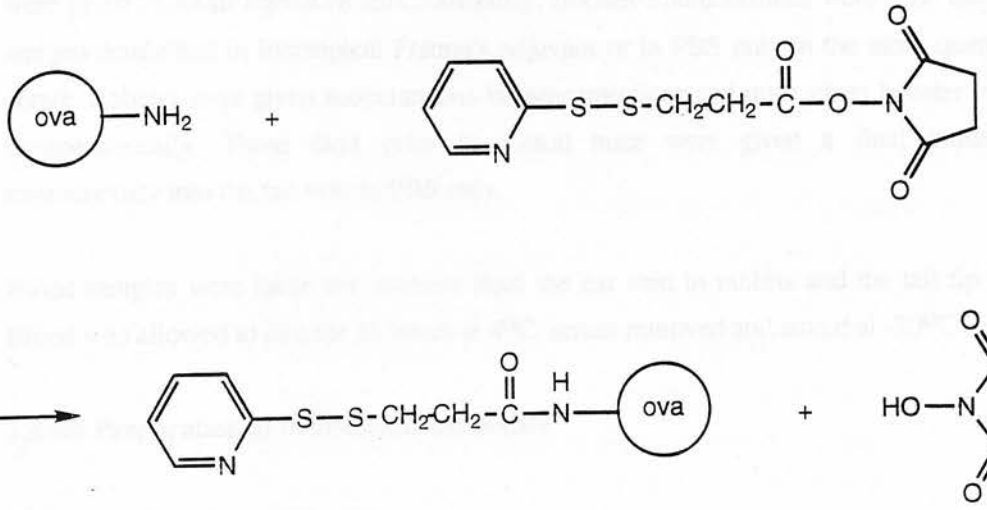
ii) Introduction of 2-pyridial disulphide structures into ovalbumin (ova)

iii) Conjugation of peptide (pep) to ovalbumin (ova) and release of pyridine-2-thione

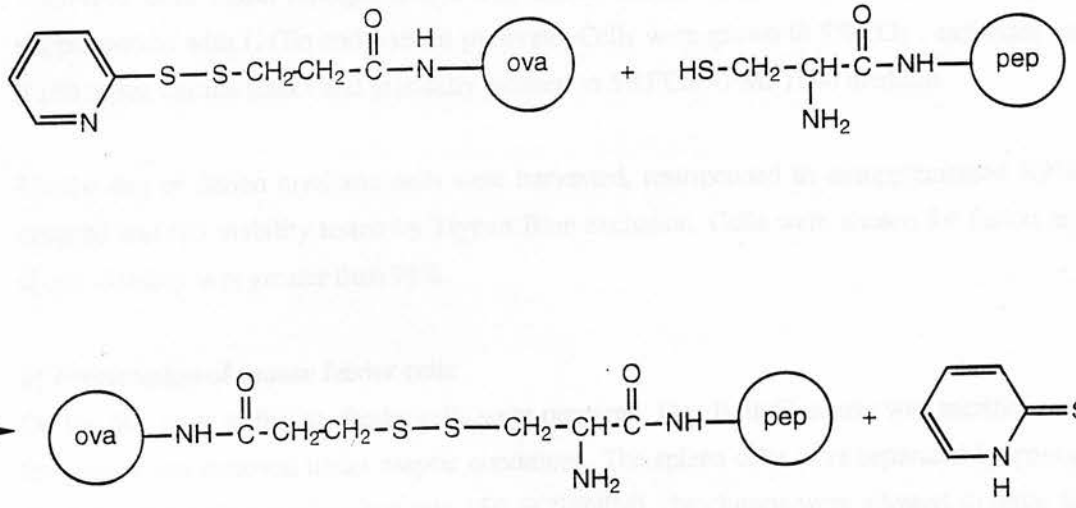
(i)



(ii)



(iii)



2.2.4ii Immunisations

Titre-Max™(Vaxcel™, Narcross, GA, USA)was used as an adjuvant for all primary immunisations. The antigen was diluted to the appropriate concentration in PBS. An equal volume of Titre-Max™ was taken and an emulsion prepared using 2x2ml syringes linked by a double ended locking connector. Contents of the syringes were transferred from 1 syringe to the other for 5 minutes, starting with transfer of antigen to the adjuvant, until an emulsion had formed. Rabbits were given 2x0.5ml injections subcutaneously in their back limbs and mice were given 2x200µl injections subcutaneously. Booster immunisations were done using either antigen emulsified in Incomplete Freund's adjuvant or in PBS only in the same quantities as above. Rabbits were given subcutaneous booster injections and mice given booster injections intraperitoneally. Three days prior to fusion mice were given a final immunisation intravenously into the tail vein in PBS only.

Blood samples were taken for analysis from the ear vein in rabbits and the tail tip in mice. Blood was allowed to clot for 16 hours at 4^{o}C, serum removed and stored at -20^{o}C.

2.2.4iii Preparation of monoclonal antibodies

a] Preparation of myeloma cells

SP2/0 murine myeloma cells were used for fusion. 1 week prior to fusion, myeloma cells were recovered from liquid nitrogen into a T25 tissue culture flask in 15%FCS/RPMI medium supplemented with L-Gln and sodium pyruvate. Cells were grown in 5%CO_2 , expanded into T150 tissue culture flasks and gradually weaned to 5%FCS/RPMI 1640 medium.

On the day of fusion myeloma cells were harvested, resuspended in unsupplemented RPMI, counted and cell viability tested by Trypan-Blue exclusion. Cells were chosen for fusion only if cell viability was greater than 95%.

b] Preparation of mouse feeder cells

On the day prior to fusion, feeder cells were prepared. One Balb/C mouse was sacrificed and the spleen was removed under aseptic conditions. The spleen cells were separated by passing the spleen through a sterile sieve into 15%FCS/RPMI, the clumps were allowed to settle and cell suspension was poured into a fresh universal. Cells were spun down at 200xg for 5 minutes at 16^{o}C and cells resuspended into 7mls 15%FCS/RPMI. Cells were counted in white blood cell counting mix and irradiated.

Cells were adjusted to $5x10^5$cells/ml and plated out onto 96 well plates, 100µl/well, and incubated overnight at 37°C in a CO_2 incubator.

c] Fusion of myeloma cells with immune spleen cells

The fusion protocol used is an adaptation of that described by Galfre et al. (1977).One immunised mouse was sacrificed and spleen removed as above. Cells were extracted into unsupplemented RPMI, washed and resuspended in 10mls RPMI. 0.5mls of cells were removed at this stage and fully supplemented medium was added to these cells which would act as a background control. Spleen cells and SP2 cells were mixed ratios of 3:1 to 8:1 and pelleted at 200xg for 5 minutes. As much medium as possible was removed to leave the pellet dry. 1ml of prewarmed 50% PEG1500(w/v) (Boeringher Mannheim) (at 37°C) was added slowly to the pellet, 50µl at a time over a 5 minute period whilst keeping the pellet warm in a beaker of water at 37°C. The cell suspension was allowed to sit for 3 minutes at 37°C and PEG was then diluted out slowly by adding 1ml RPMI over 5 minutes, followed by 2mls over 2 minutes, followed by 20mls over 4 minutes. Fused cells were spun down gently at 200xg for 5 minutes and carefully resuspended in 20mls of 15%FCS/RPMI. Cells were plated out onto the inner 60 wells of preprepared 96-well plates, 50µl/well. An extra row was plated out with the unfused spleen cells that had been retained at the beginning of the fusion experiment. 24 hours later cells were fed with 15%FCS/RPMI containing 4%hypoxanthine/aminopterin/thymidine (HAT), 50µl/well.

d] Monitoring hybridoma cell lines

10 days post fusion all the wells were examined for clones and scored appropriately. Cells were fed with 15%FCS/RPMI/1%HAT(v/v). When the wells were 50-80% confluent 100µl supernatant was removed and screened. Positive wells were then grown up into 24-well plates by dispersing cells with a Pasteur pipette and transferring to a single well in a 24-well plate that had been preincubated with 15%FCS/RPMI/1%HAT(v/v) medium. Backup wells were grown and duplicate stocks of cells from individual wells were frozen down in 1ml 10%DMSO(v/v)/90%FCS(v/v) and stored in liquid nitrogen.

e] Cloning hybridoma cell lines by agar cloning

When all the original wells containing hybridomas had been tested, up to 4 clones were selected for cloning at one time. Cells were maintained in 24 wells and weaned off HAT medium into HT medium. Agar plates were prepared with 0.5% bacto agar(w/v)(Difco)/20% FCS(v/v)/1% HT(v/v)/RPMI medium supplemented with L-Gln and sodium pyruvate. 90mm dishes were used with 8mls of agar medium/dish, agar was allowed to set and incubated in the CO_2 incubator for 2 hours. Cell dilutions were made in 1ml of 20%FCS/RPMI as 1/5, 1/25,

1/50 and 1/125 dilutions and an equal volume of agar medium was added. The cell preparation was added to the agar plates and maintained in CO_2 incubator for 1-2 weeks. When clones were large enough to be seen by a naked eye they were removed with a Pasteur pipette and transferred to a well in a 24-well plate that had been preincubated with 20%FCS/RPMI. Two rounds of agar cloning was done to obtain a single hybridoma cell line.

2.2.4iv IgG purification of polyclonal serum

This was carried out using a protein G sepharose 4 FF column (Pharmacia, USA). 5mls of serum was clarified at 10 000xg for 10 minutes and passed through a 0.2µm filter. The sample was then diluted 1:1 with binding buffer (supplied by the manufacturer) and IgG purified following the manufacturer's instructions.

2.2.5 Preparation and purification of recombinant proteins

2.2.5i Expression and purification of bacterial glutathione-S-transferase fusion proteins

a] Analyzing transformations by "miniscale" protein preparation

Analysis of transformed bacteria containing cloned pGEX plasmid was carried out as follows. Bacteria were streaked out onto agar/ampicillin plates and colonies grown up over 16 hours at 37^{o}C. Several colonies were picked and grown in 2mls L-Broth/amp for 3-4 hours until the medium was turbid. The culture was induced with 0.1mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1-2 hours. Cells were harvested by centrifugation at 13 000xg for 5 seconds and the pellet was resuspended in 300µl of ice-cold PBS. 10µl was retained for analysis. Cells were lysed with a 2mm probe sonicator (Ultrasonic generator, Dawe instruments Ltd.) for 10 seconds with sufficient power to lyse the cells but prevent frothing. Cell debris was removed by centrifugation at 13 000xg for 5 minutes and supernatant removed. 50µl of 50% glutathione-agarose bead(v/v)/PBS slurry was added to the supernatant and mixed for 2 or more minutes at 18^{o}C. The beads were washed twice in PBS and resuspended in 50µl 2xSDS-reducing buffer, boiled for three minutes and analysed by SDS-PAGE. Bacteria transformed with the native pGEX plasmid was always included as a positive control.

b] Preparation of recombinant fusion protein on a medium/large scale

Medium preparations were made from 100ml cultures and large scale preparations from 1L cultures. Precultures were set up as 10ml or 100ml for medium and large scale preparations, respectively by inoculating the appropriately sized culture with a single colony pick and growing up the cultures for 16 hours. Cultures were diluted 1:10, grown to the required cell density as determined spectrophoretically by absorbance at 600nm and induced with 0.1mM IPTG for a predetermined time. Cells were harvested by centrifugation at 300xg for 5 minutes or for 10 minutes at 4640xg for 10 minutes at $4^{o}C$ in a JA-20 centrifuge for a medium or large scale preparation, respectively. Cell pellets were resuspended in 1/100th of the volume of the original culture in ice-cold PBS and lysed using an ultrasonic probe described in the above section. Medium scale preparations were given 3x1minute pulses with 1 minute on ice in between, and large scale preparations were given 3x3 minute pulses with 3 minute intervals on ice. 1% TritonX-100(v/v)/PBS was added and cells were pelleted by centrifugation at 300xg for 5 minutes at $4^{o}C$. The supernatant was removed and incubated for 30 minutes at $18^{o}C$ with 100µl or 1ml of 50% glutathione-agarose slurry for medium or large scale, respectively. Beads were washed 3 times in ice-cold PBS and resuspended in PBS, in the same volume as beads that were added. An aliquot was analysed by SDS-PAGE.

c] Elution of fusion protein from glutathione beads

Beads were pelleted gently in a bench centrifuge for 10 seconds and PBS removed. An equal volume of 10mM reduced glutathione/50mM Tris-HCl, pH8.0 was added and mixed for 2 minutes at $18^{o}C$. Beads were collected as before, supernatant was retained and elution repeated twice more. Eluted samples were stored at $-70^{o}C$ and analysed by SDS-PAGE .

2.2.5ii Expression and purification of a recombinant protein in the baculovirus system

Expression of a recombinant protein in the Baculovirus system was carried out using the CLONTECH BacPAK™ Baculovirus expression system. Described below are the methods used for production of a recombinant virus.

a] Cotransfection of baculovirus expression vector and baculoviral DNA

SF21 cells were seeded into 35mm dishes at a $1.5x10^{6}$cells/1.5ml/dish for 1-4 hours or $1x10^{6}$cells/1.5ml/dish with overnight incubation. Medium was removed, cells washed with unsupplemented TC100 and a further 2ml TC100 medium added for 30 minutes. It was necessary to exclude FCS since a component of serum inhibits lipofectin mediated transfection. Lipofectin-DNA complexes were made up by mixing the appropriate amounts of plasmid DNA and viral DNA made up to a total volume of 50µl with SDW. 50µl Lipofectin

was added to each sample and Lipofectin-DNA complexes were allowed to form over a 15 minute incubation at room temperature. Lipofectin is a complex of positively charged lipids that binds to nucleic acids allowing uptake by fusion with the plasma membrane. Preparations were made up in sterile polystyrene tubes (Nunclon) since lipofectin sticks to polypropylene. pBacPAK8-GUS was used as a positive control and its plasmid map is shown in appendix 1. The plasmid contains an *E.coli* β-glucorinidase gene down stream of the polyhedron promoter. Recombination with BacPAK6 viral DNA will result in a recombinant virus that expresses β-glucoronidase. This can be detected by a blue colour in the presence of 5-bromo-4cloro-3-indolyl-β-D-glucorinic acid (X-Gluc, 25mg/ml stock in DMSO, CLONTECH).

After preparation of lipofectin-DNA complexes, medium was removed from dishes and 1.5ml of fresh serum-free medium was added to each dish. Lipofectin-DNA complexes were added to each dish, dropwise and gently swirled. After a 5 hour incubation medium was removed and 1.5ml of supplemented medium was added containing 12μl/ml X-gluc. 72 hours post transfection medium containing recombinant viruses was harvested and stored at $4^{o}C$.

b] Isolation of recombinant viruses

To isolate a single clone of recombinant virus from the supernatant of the cotransfection experiment, a virus plaque assay was performed. 10-fold serial dilutions of the cotransfection supernatant were made; 10^{-1}, 10^{-2} and 10^{-3}. A plaque assay was set up using 2-3 dishes per dilution. Control dishes were included using an appropriate dilution of BacPak6™ as a positive control and 10%FCS/TC100 medium as a negative control. Plaque assays were stained with 0.33% neutral-red and X-gal. Plaques were circled and examined under a light microscope to ensure that the plaques contained virus-infected cells, i.e. unstained. Using a pasteur pipette the plaque was gently sucked up including the agar overlay, added to 0.5ml 10%FCS/TC100 medium in a sterile microcentrifuge tube, and allowed to diffuse for 16 hours at $4^{o}C$.

To prepare a primary stock of recombinant virus, 100μl of the "plaque pick" was used to infect a 35mm dish seeded with $5x10^{5}$SF21 cells. 3-4 days post infection the medium was removed and this was stored at $-70^{o}C$ as the primary stock of recombinant virus.

c] Preparation of baculovirus DNA

Virus DNA was extracted from infected cells for the purpose of analysing recombinant viruses by southern blot hybridisation. An infection was set up in 35mm petri dishes as described above and cells were harvested 3-4 days post infection. 1ml PBS was added to the infected-cell monolayer and cells scraped off into a 1.5ml eppendorf tube. Cells were pelleted

at 200xg for 1 minute at 16°C. The supernatant was removed and cells washed again in 0.5ml PBS, repelleted and resuspended in 250µl TE buffer. To the cells 250µl lysis buffer (50mM Tris-HCl, pH8.0, 10mM EDTA, 5% β-mercaptoethanol(v/v), 0.4% SDS(w/v)), 12.5µl 10mg/ml proteinase-K and 2µl 10mg/ml RNase was added and incubated for 30 minutes at 37°C. Protein contaminants were removed by extracting with phenol:chloroform (50:50) three times, the sample was mixed for 5 minutes by inversion and microcentrifuged for 3 minutes to separate the phases. It was important not to vortex the sample since Baculovirus DNA is large and susceptible to shearing. DNA was precipitated with 1/10th volume of 3M sodium acetate and 2 volumes of ethanol. The pellet was soaked in 50µl TE buffer for 16 hours at 4°C since baculovirus DNA solubilises slowly. The DNA was gently resuspended and stored at -20°C.

d] Purification of recombinant protein

Table 2.1 indicates the seeding densities and volume of reagents required for the preparation and purification of recombinant protein.

Two methods were used to purify recombinant protein which was expressed as a protein fused to glutathione-S-transferase.

Method 1: Cell-associated recombinant protein

Protein was extracted from infected cells grown in 35mm dishes. 1ml PBS was added to the cell monolayer and cells scraped off. Cells were pelleted and resuspended in 100µl PBS/0.1% TritonX-100(v/v) and centrifuged at 9 000xg for 10 minutes at 4°C. The supernatant was removed and 50µl of 50% glutathione-agarose beads were added for 30 minutes at 4°C. Beads were washed twice in PBS and resuspended in an equal volume of PBS.

Method 2: Secreted recombinant protein

2-3 days post infection the medium was collected from infected cells and clarified by centrifugation at 200xg for 5 minutes at 18°C. The supernatant containing recombinant protein was dialysed against 1-2 litres PBS for 16 hours at 4°C. The supernatant was removed and an appropriate volume of 50% glutathione-agarose(v/v)/PBS bead slurry was added and mixed for 30 minutes at 4°C. The beads were washed twice in ice-cold PBS and resuspended in same volume as beads.

To elute recombinant protein from the beads, the required volume of elution buffer (50mM Tris-HCl, pH8.0/10mM reduced glutathione) was added and sample mixed for 2 minutes at 4°C. The elution was repeated and samples stored at -70°C.

Table 2.1: Guidelines for the preparation and purification of protein from recombinant baculovirus-infected cells

dish/flask	no. of cells 2 hrs	seeded 16 hrs	virus inoculum	vol. of medium	vol. of beads	vol. of el. buffer
35mm dish	$1.5x10^6$	$1.0x10^6$	0.1-0.5ml	1.5-2.0ml	50µl	n/d
25cm²flask	$2.0x10^6$	$1.5x10^6$	0.4-1.0ml	3.0-5.0ml	100µl	200µl
150cm²flask	$12.0x10^6$	$8.0x10^6$	2.0-6.0ml	20-30ml	600µl	1ml
spinner	$1x10^6$/ml	$2x10^5$/ml (48hrs)	<10ml	100ml	2ml	4ml

CHAPTER 3: THE EXPRESSION AND CHARACTERIZATION OF GP135 EXPRESSED IN pGEX BACTERIAL SYSTEM

3.1 INTRODUCTION

Little is understood as to the identity of functional epitopes on the envelope glycoproteins of MVV, such as their role in receptor binding and induction of host neutralizing antibodies. To carry out studies to delineate functional epitopes within the external glycoprotein, gp135, of MVV, attempts were made at expressing it as a recombinant protein. This would provide a reliable source of gp135 that could be used as an immunogen and also in functional studies. Previous attempts had been made to express gp135 as three overlapping fragments in the yeast Ty-system (Carey et al., 1993). Problems encountered in this system included low yields due to toxicity of gp135 fragments and high contamination.

An alternative approach was taken to express these fragments in a simpler bacterial system and the pGEX expression system was chosen (Smith and Johnson, 1988). Bacteria are the ideal system of choice when expressing recombinant proteins. They are easy to manipulate and can be grown in large cultures in the laboratory, up to 5 litres, without high costs. The main disadvantage of using a prokaryotic expression system is that proteins are not glycosylated. However, this was not a foreseen problem since work with gp120, the external glycoprotein of HIV-1, has shown that sera from infected patients are able to recognise recombinant protein expressed in bacteria and these proteins can be used to raise neutralizing antisera (Putney et al., 1986, Crowl et al., 1985, Lasky et al., 1986).

The pGEX system allows foreign polypeptides to be expressed as recombinant proteins fused to glutathione-S-transferase, GST. GST is a 27kDa cytoplasmic protein, belonging to a family of isoenzymes that detoxify a variety of xenobiotics by catalyzing the interaction of reduced glutathione with these molecules. It was originally isolated by Smith et al. (1986) from the parasitic helminth, *Schistosoma japonicum* (Sj26) and was subsequently expressed as a recombinant protein in bacteria (Smith et al., 1988). This led to its manipulation as a fusion partner for production of foreign polypeptides in bacteria (Smith and Johnson, 1988). Proteins produced are usually soluble and can be purified under non-denaturing conditions by affinity absorption onto glutathione-agarose beads, thus allowing recombinant protein to retain functional activity and antigenicity (Smith and Johnson, 1988). The main advantage of the GST carrier is that it is relatively small compared to other carrier species, for example β-galactosidase, and thus is not likely to interfere with the immunogenicity of the foreign

polypeptide. It also lacks immunological complications as seen when protein A is used as a fusion partner which can interact with a wide variety of antibodies. The GST moiety is under the control of a strong *tac* promoter. The *tac* promoter is derived form the lacUV5 promoter and the trp promoter, which are combined to give a hybrid promoter that is repressed by the *lac* repressor (Amann et al., 1983). By inducing transformed bacterial cells with IPTG, the lac repressor protein is lifted resulting in expression of fusion protein. Bacterial cells are then lysed and fusion proteins are purified. Recombinant proteins can be recovered from the beads by elution with free reduced glutathione at neutral pH.

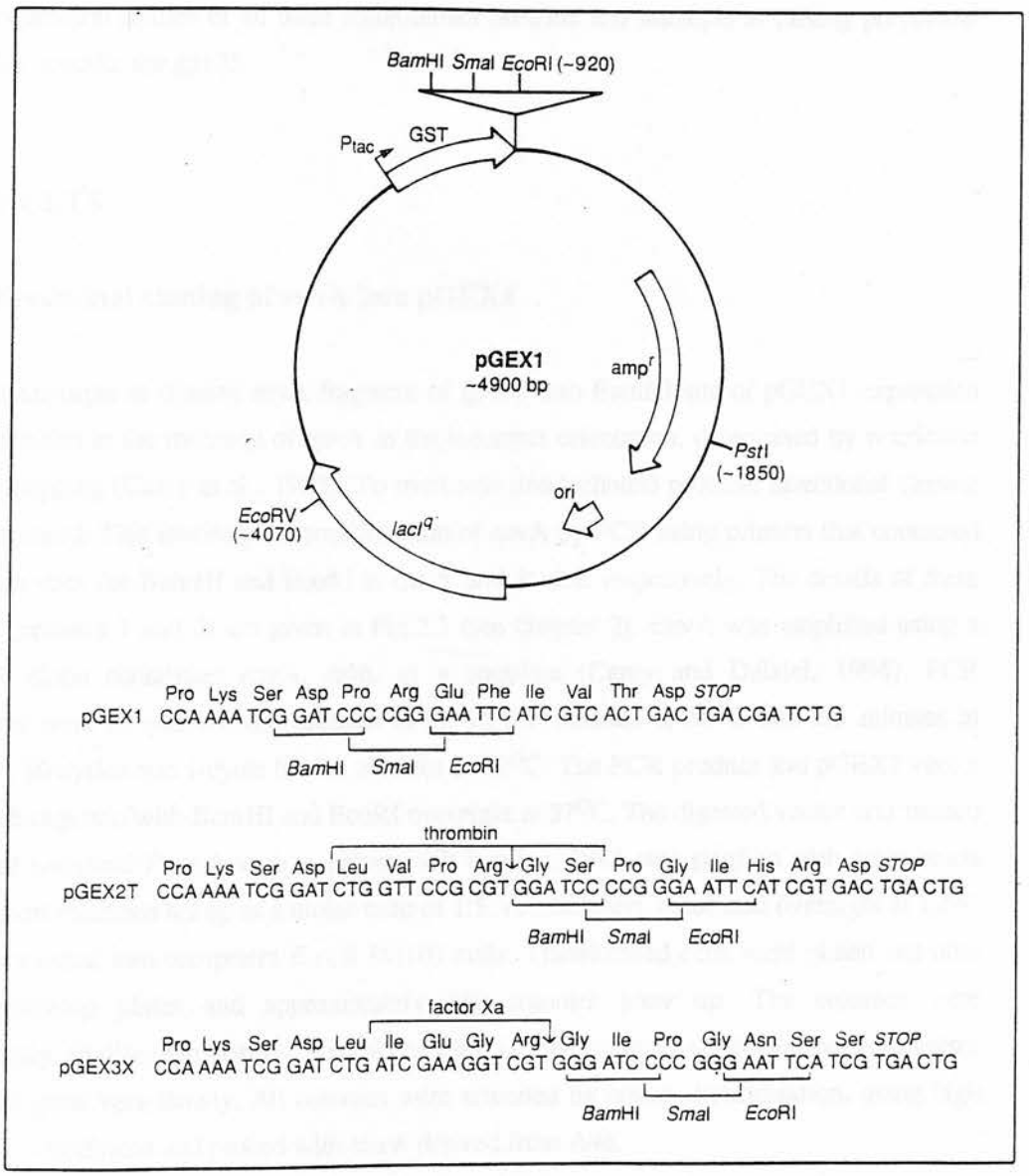
Cloning is performed by ligating the DNA fragment encoding the polypeptide of interest into one of three vectors available and transforming into competent *E. coli* cells. The expression vectors contain an open reading frame with GST followed by a multiple cloning site containing BamHI, SmaI and EcoRI restriction sites and a termination codon set in all three frames (see Fig.3.1). Vectors pGEX2T and pGEX3X also have a thrombin and blood coagulation factor X_a cleavage site, respectively, for the removal of the GST carrier if required. This bacterial expression system has been highly successful for the production of many recombinant proteins. However difficulties can be encountered with foreign proteins >50kDa and with proteins containing large hydrophobic or highly charged regions.

Recombinant proteins can be used in a variety of studies. These include the use of proteins for detecting antibodies from immune sera either for diagnostic means or for analysing neutralizing antibody response (Frosch et al., 1991, Iwayama et al., 1991, Frorath et al., 1991). Since this system allows for the production of large amounts of protein of interest, it is very useful for production of antibodies. Animals can be immunised by a variety of means, for example with eluted protein, with protein immobilised on glutathione-agarose beads or with crushed gel extracts containing the protein of interest (Oettinger et al., 1992). Recombinant proteins have also been used for assessing cellular immune responses in the host as well as detecting a humoral response (Tetzlaff et al., 1992). This system is applicable to construction of cDNA expression libraries which can be adapted to epitope map a panel of monoclonal antibodies (Rizzo and Gray, 1992). Proteins expressed in the pGEX system retain functional activity, demonstrated by large scale expression of a glycoprotein hormone, leukaemia inhibitory factor (LIF) and its use to maintain embryonic stem cells in culture. It should be noted that to retain biological activity it was necessary to cleave LIF from GST via a thrombin cleavage site (Gearing et al., 1989).

Figure 3.1: The structure of pGEX plasmid vector (adapted from Current Protocols, 1990)

The map shows the location of the GST cassette in relation to the multiple cloning site, comprising of BamHI, SmaI and EcoRI restriction enzyme sites. The reading frames of all three pGEX fusion vectors are shown, including protease cleavage sites and the termination codon.

amp^r represents the β-lactamase gene responsible for ampicillin resistance.
ori is the origin of replication.
$lacI^q$ represents the lac repressor gene whose product bind to the fusion protein promoter, P_{tac}. This repression is lifted in the presence of IPTG.


BamHI SmaI EcoRI (~920)
Ptac
GST
pGEX1
~4900 bp
amp^r
PstI
(~1850)
ori
EcoRV
(~4070)
lacI^q
Pro Lys Ser Asp Pro Arg Glu Phe Ile Val Thr Asp STOP
pGEX1 CCA AAA TCG GAT CCC CGG GAA TTC ATC GTC ACT GAC TGA CGA TCT G
BamHI SmaI EcoRI
thrombin
Pro Lys Ser Asp Leu Val Pro Arg↓Gly Ser Pro Gly Ile His Arg Asp STOP
pGEX2T CCA AAA TCG GAT CTG GTT CCG CGT GGA TCC CCG GGA ATT CAT CGT GAC TGA CTG
BamHI SmaI EcoRI
factor Xa
Pro Lys Ser Asp Leu Ile Glu Gly Arg↓Gly Ile Pro Gly Asn Ser Ser STOP
pGEX3X CCA AAA TCG GAT CTG ATC GAA GGT CGT GGG ATC CCC GGG AAT TCA TCG TGA CTG
BamHI SmaI EcoRI

The pGEX system was employed to express the three overlapping fragments of gp135 (map shown in Fig.3.2). The 50-60 amino acid overlap between fragments was included in an attempt to ensure that any putative domain structure was not lost. Fragments envB and envC have been successfully cloned into and expressed in pGEX2T (Carey, 1992). These proteins were shown to be toxic to bacterial cell growth and initial immunisations were performed in an attempt to raise anti-gp135 polyclonal sera. This chapter describes cloning and expression of envA, expression studies of all three recombinant proteins and attempts at raising polyclonal antibodies specific for gp135.

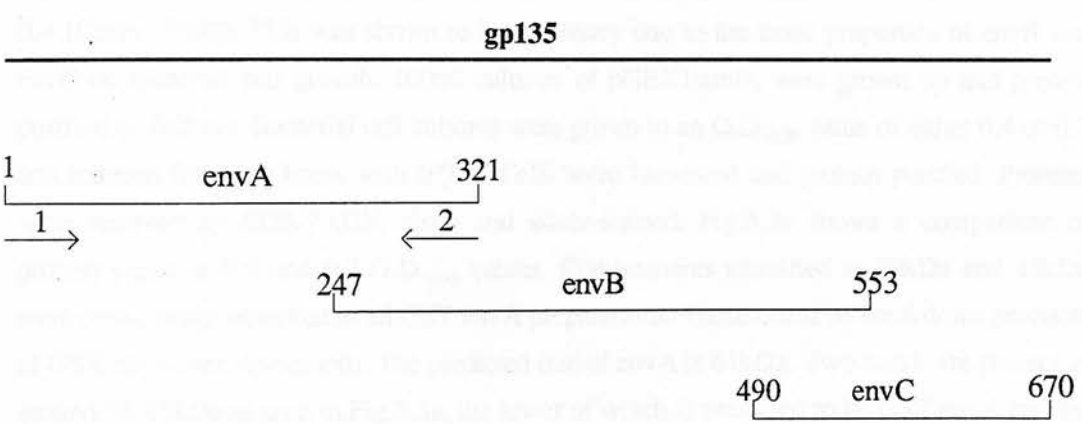
3.2 RESULTS

3.2.1 Directional cloning of envA into pGEX1

Previous attempts at cloning envA fragment of gp135 into BamHI site of pGEX1 expression vector resulted in the insertion of envA in the incorrect orientation, determined by restriction enzyme mapping (Carey et al., 1992). To overcome this technical problem directional cloning was performed. This involved the amplification of envA by PCR using primers that contained restriction sites for BamHI and EcoRI in the 5' and 3' ends respectively. The details of these primers (primers 1 and 2) are given in Fig.2.1 (see chapter 2). EnvA was amplified using a pTZ19R clone containing envA, A46, as a template (Carey and Dalziel, 1994). PCR conditions were as follows: 0.6 minutes at 96^{o}C, 0.2 minutes at 55^{o}C and 2.5 minutes at 72^{o}C for 30 cycles and 1 cycle for 7.5 minutes at 72^{o}C. The PCR product and pGEX1 vector were both digested with BamHI and EcoRI overnight at 37^{o}C. The digested vector was treated with Calf Intestinal Phosphatase to prevent self ligation. DNA was purified with glass beads and ligation reactions set up as a molar ratio of 1:5, vector:insert, incubated overnight at 12^{o}C and transformed into competent *E.coli* JM101 cells. Transformed cells were plated out onto three agar/amp plates and approximately 180 colonies grew up. The colonies were significantly smaller than positive control colonies, i.e. self-ligated, non-phosphorylated vector only, and grew very slowly. All colonies were screened by colony hybridisation, using high stringency conditions and probed with envA derived from A46.

Figure 3.2: Regions of gp135 expressed in pGEX

The diagram illustrates the three overlapping regions of gp135. The number above the lines shows the amino acid position of fragments. Numbering is as in Sargan et al. (1991). Arrows and numbers below the lines refer to primers (for details, see Figure 2.1) used to amplify the envA fragment.


gp135
1
envA
321
1
2
247
envB
553
490
envC
670

Screening resulted in the identification of 12 positive clones and these were selected for analysis. Plasmid DNA was prepared by standard miniprep procedure and DNA cut with BglI. BglI cuts pGEX1 at positions 2009 and 4652, and envA at position 565. EnvA cloned into pGEX1 at BamHI and EcoRI site in the correct orientation would give fragment sizes 1.4, 1.7 and 2.6 kb when cut with BglI. The 12 colonies analysed gave these predicted sizes when cut with BglI, demonstrating that envA had been successfully cloned into pGEX1 in the correct orientation. Double stranded sequencing of the clones was performed to confirm the restriction enzyme result.

3.2.2 Analysis of envA protein expression and the effect on bacterial cell growth

One of the clones that had been isolated above was selected for protein expression analysis. Previous results from studies with envB and envC showed that yields of protein were significantly improved by inducing cell cultures at a higher $O.D._{600}$ value than the standard 0.4 (Carey, 1992). This was shown to be necessary due to the toxic properties of envB and envC on bacterial cell growth. 100ml cultures of pGEX1:envA were grown up and protein purified as follows. Bacterial cell cultures were grown to an $O.D._{600}$ value of either 0.4 or 0.7 and induced for three hours with IPTG. Cells were harvested and protein purified. Proteins were resolved by SDS-PAGE, fixed and silver-stained. Fig.3.3a shows a comparison of protein yields at 0.4 and 0.7 $O.D._{600}$ values. Contaminants identified at 30kDa and 43kDa were consistently observed in all GST:envA preparations. These could be breakdown products of GST:envA (see discussion). The predicted size of envA is 61kDa. Two bands are present at around 55-65kDa as seen in Fig.3.3a, the lower of which is predicted to be GST:envA and the higher is a contaminant of unknown identity. It should be noted that this higher molecular weight contaminant was occasionally observed in preparations of GST:envB and GST:envC. There did not appear to be a significant increase in yield of GST:envA from an $O.D._{600}$ value of 0.4 to 0.7.

To analyse the effect of GST:envA production on bacterial cell growth, transformed JM101 cells containing pGEX1:envA were grown and $O.D._{600}$ values measured every hour. Cells were induced with IPTG after 3 hours growth and $O.D._{600}$ continued to be monitored up to 5 hours after induction. Growth characteristics were compared to JM101 cells transformed with pGEX1 only. Fig.3.3b shows these results graphically. As with envB and envC, production of GST:envA appears to inhibit cell growth and hence is also toxic.

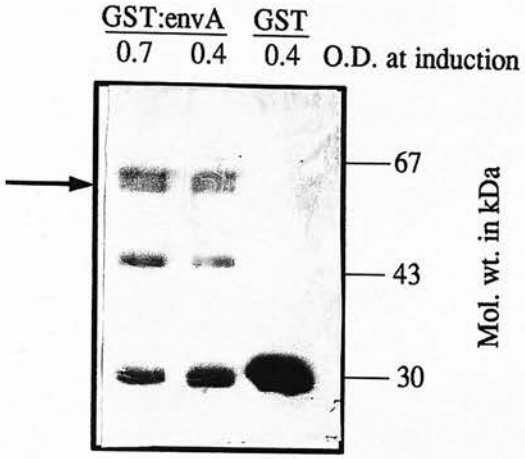
Figure 3.3: An analysis of protein production from JM101 cells transformed with pGEX1:envA and its effect on bacterial cell growth

GST:envA purified from cell cultures induced at $O.D._{600}$ values 0.4 and 0.7 were run on a 12% SDS-PAGE gel and proteins were detected by silver-staining, shown in figure a. The location of GST:envA is highlighted. pGEX1 as the positive control is shown, indicating the position of native GST. Protein markers are shown.

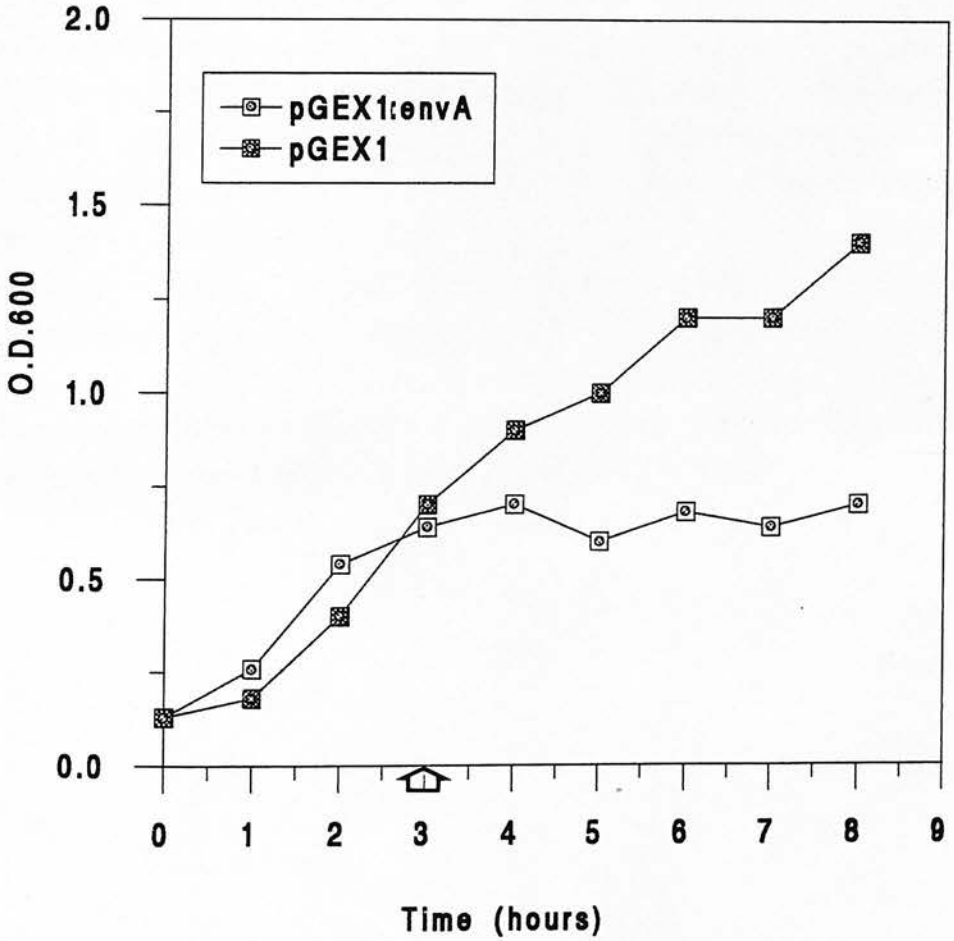
Figure b is a graph to show the effect of GST:envA on bacterial cell growth. pGEX control is shown.

⏢ = time point at which cell cultures were induced with IPTG.

A


GST:envA
GST
0.7
0.4
0.4
O.D. at induction
67
43
30
Mol. wt. in kDa

B


pGEX1:envA
pGEX1
O.D.600
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
7
8
9
Time (hours)

3.2.3 Expression studies on three overlapping fragments of gp135, GST:envA, GST:envB and GST:envC

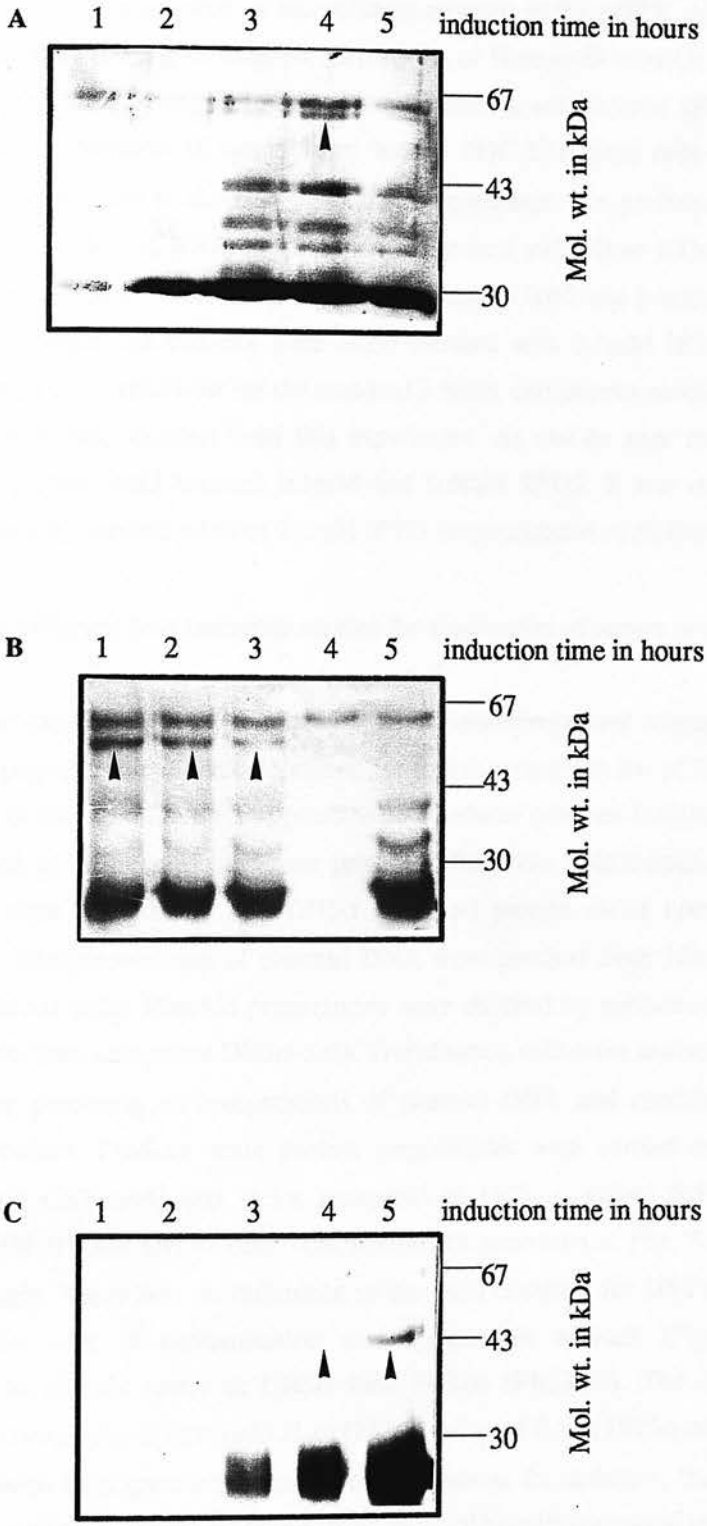
Data presented so far has highlighted the problem in expressing gp135 in a bacterial system by the fact that the three protein fragments appear to be toxic to bacterial cells. This leads to low levels of expression accompanied by protein contamination. To use these proteins for the purpose of raising antisera it was necessary to obtain better and cleaner yields. The following sections deal with attempts at increasing recombinant protein yields.

3.2.3i The effect of varying induction time on protein yield

A standard protocol had been developed in which cell cultures were induced for three hours prior to protein purification (Carey, 1992). To analyse the effect of altering the duration of induction on the yield of all three fusion proteins the following experimental procedure was carried out. A 10ml preculture was set up and grown for 6-8 hours. This culture was transferred to 100ml L-Broth/amp and grown up for a further 16 hours, and then added to 500ml L-Broth/amp in a 2-litre conical flask to allow for maximum aeration. When the $O.D._{600}$ value reached approximately 0.8 the culture was induced with 0.1mM IPTG. At hourly intervals for 5 hours 100ml culture was removed, cells harvested and stored at -70^{o}C. Protein purification was carried out on all 5 samples simultaneously by the usual procedure. Samples were analysed by SDS-PAGE, fixed and silver stained. The results are presented in Fig. 3.4a, b and c for GST:envA, GST:envB and GST:envC, respectively. For GST:envA the yield appeared to be maximum at 3-4 hours post induction. GST:envB shows maximum production at 1 hour induction, with a definite decrease on increasing induction time. It is noted that at 4 hours very little protein was recovered. At 5 hours very little fusion protein by comparison to contaminants, is detectable by silver stain analysis. It is also noted that there is an increase in contamination on increasing induction time. GST:envC showed an opposite effect to GST:envB where maximum yield was at 5 hours. This however was accompanied by an increase in contamination similar to that observed with GST:envB.

Figure 3.4: The effect of varying induction time on the yield of fusion proteins GST:envA, GST:envB and GST:envC

Purified recombinant proteins were separated on a 12% SDS-PAGE gel and detected by silver staining. Figures a, b and c represent GST:envA, GST:envB and GST:envC, respectively, and the position of each fusion protein is marked. Induction time is given in hours. Size of protein markers are indicated.


A
1 2 3 4 5
induction time in hours
67
43
30
Mol. wt. in kDa
B
1 2 3 4 5
induction time in hours
67
43
30
Mol. wt. in kDa
C
1 2 3 4 5
induction time in hours
67
43
30
Mol. wt. in kDa

3.2.3ii The effect of IPTG concentration on protein yield

Published data on the expression of recombinant proteins in the pGEX system uses a variety of concentrations of IPTG. For example, expression of Human 68-kDa(U1) ribonucleoprotein antigen was performed by inducing bacterial cell cultures with 0.5mM IPTG (Frorath et al., 1991), whereas expression of recombinant human PDC-E1α used only 0.01mM IPTG to induce cultures (Iwayana et al., 1991). An initial experiment was performed with GST:envB to see the effect of varying IPTG concentration on protein yield. Four 100ml L-Broth cultures were grown up, 2 cultures induced at an $O.D._{600}$ value of 0.65 and 2 cultures induced at 0.9. For each O.D. value, the cultures were either induced with 0.1mM IPTG or with 0.5mM IPTG. Induction was carried out for the standard 3 hours and protein purified as usual. Figure 3.5 shows the results obtained from this experiment. As can be seen there is no dramatic difference in protein yield between 0.1mM and 0.5mM IPTG. It was decided therefore to maintain a working concentration of 0.1mM IPTG for production of all three fusion proteins.

3.2.3iii Using different host bacterial strains for expression of fusion proteins

Data from the expression of Human 68kDa (U1) ribonucleoprotein suggested that choice of cellular host played a role in yields obtained for fusion proteins in the pGEX system (Frorath et al., 1991). In the literature there appears to be a balance between JM101 and DH5α strains of *E.coli* used to express recombinant proteins. Therefore pGEX:envA, pGEX:envB and pGEX:envC were transformed into DH5α cells and protein yields compared to those in JM101 cells. Minipreparations of plasmid DNA were purified from 10ml cultures of each plasmid in JM101 cells. Plasmid preparations were checked by restriction enzyme digestion and transformed into competent DH5α cells. Transformed cells were analysed for the presence of plasmid by preparing minipreparations of plasmid DNA and checking with restriction enzymes as before. Medium scale protein preparations were carried out for GST:envA, GST:envB and GST:envC and yields compared at $O.D._{600}$ values 0.5 and 0.9 between transformed JM101 and DH5α cells. The results are presented in Fig. 3.6 as silver stained SDS-PAGE gels. There was no difference in the yield obtained for GST:envA in either cell strain and the level of contamination was comparable in each (Fig.3.6a). GST:envB production was slightly better in DH5α than JM101 (Fig.3.6b). The result obtained for GST:envC showed a maximum yield at an $O.D._{600}$ value of 0.5 in DH5α cells (Fig.3.6c). This was accompanied by a greater increase in contamination. In summary, there was no marked improvement in yield obtained for fusion proteins in DHα cells compared to JM101 cells.

Figure 3.5: A comparison of yields obtained from cultures of pGEX2T:envB with different concentrations of IPTG

Protein samples were separated on a 12% SDS-PAGE gel and silver stained to show the effect of inducing pGEX2T:envB cultures with either 0.1mM or 0.5mM IPTG. Protein markers and position of GST:envB are indicated. pGEX control is shown as a positive control for purification.

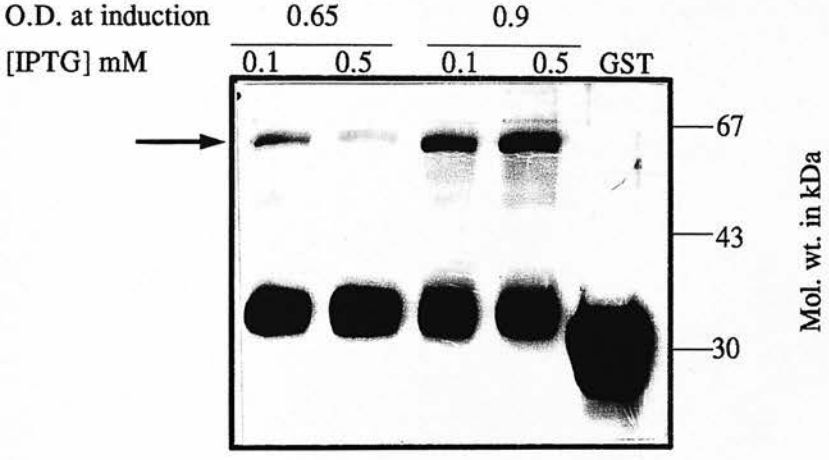

O.D. at induction
0.65
0.9
[IPTG] mM
0.1
0.5
0.1
0.5
GST
67
43
30
Mol. wt. in kDa

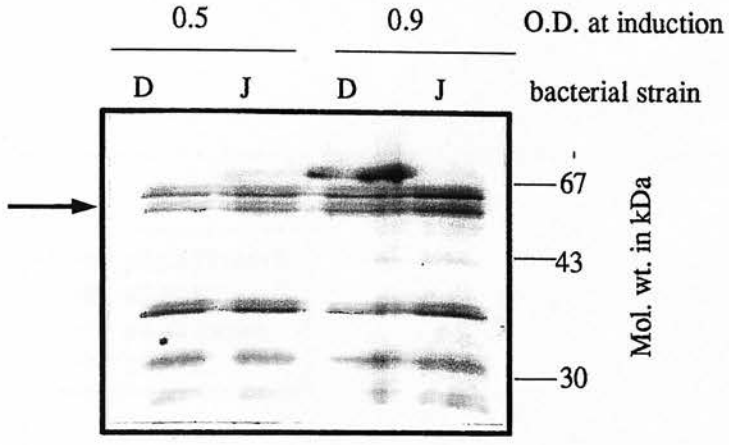
Figure 3.6: A comparison of protein yields in two different strains of *E. coli* transformed with pGEX1:envA, pGEX2T:envB and pGEX2T:envC

Figures a, b and c show the effect of producing GST:envA, GST:envB and GST:envC, respectively in either JM101 (J) and DH5α (D) strains of *E. coli* and are presented as silver-stained 12% SDS-PAGE gels. Protein markers and fusion proteins are indicated.

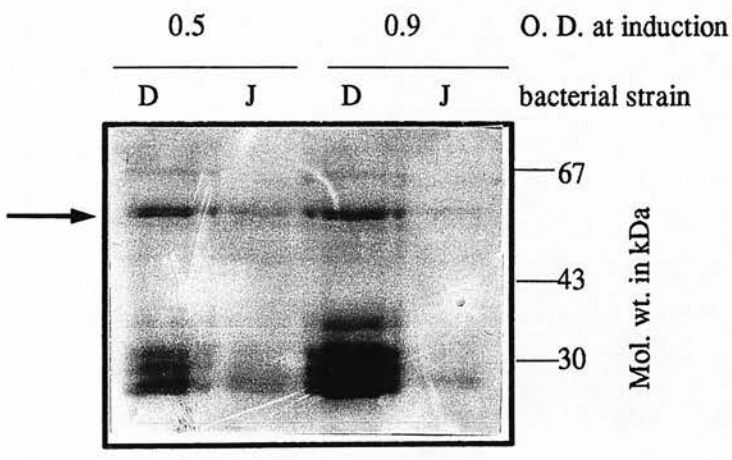
Figure d represents a growth curve to show effect of all 3 fusion proteins on bacterial cell growth in DH5α strain of *E. coli* and compared to pGEX control.

⌂ = time point at which cell cultures were induced with IPTG

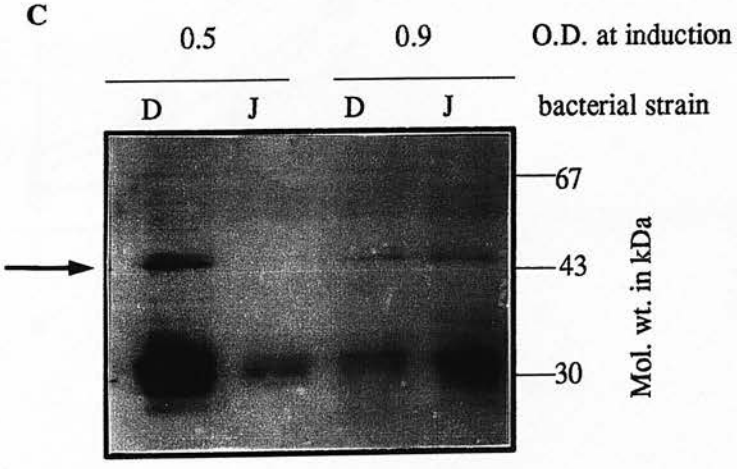
A


0.5
0.9
O.D. at induction
D
J
D
J
bacterial strain
67
43
30
Mol. wt. in kDa

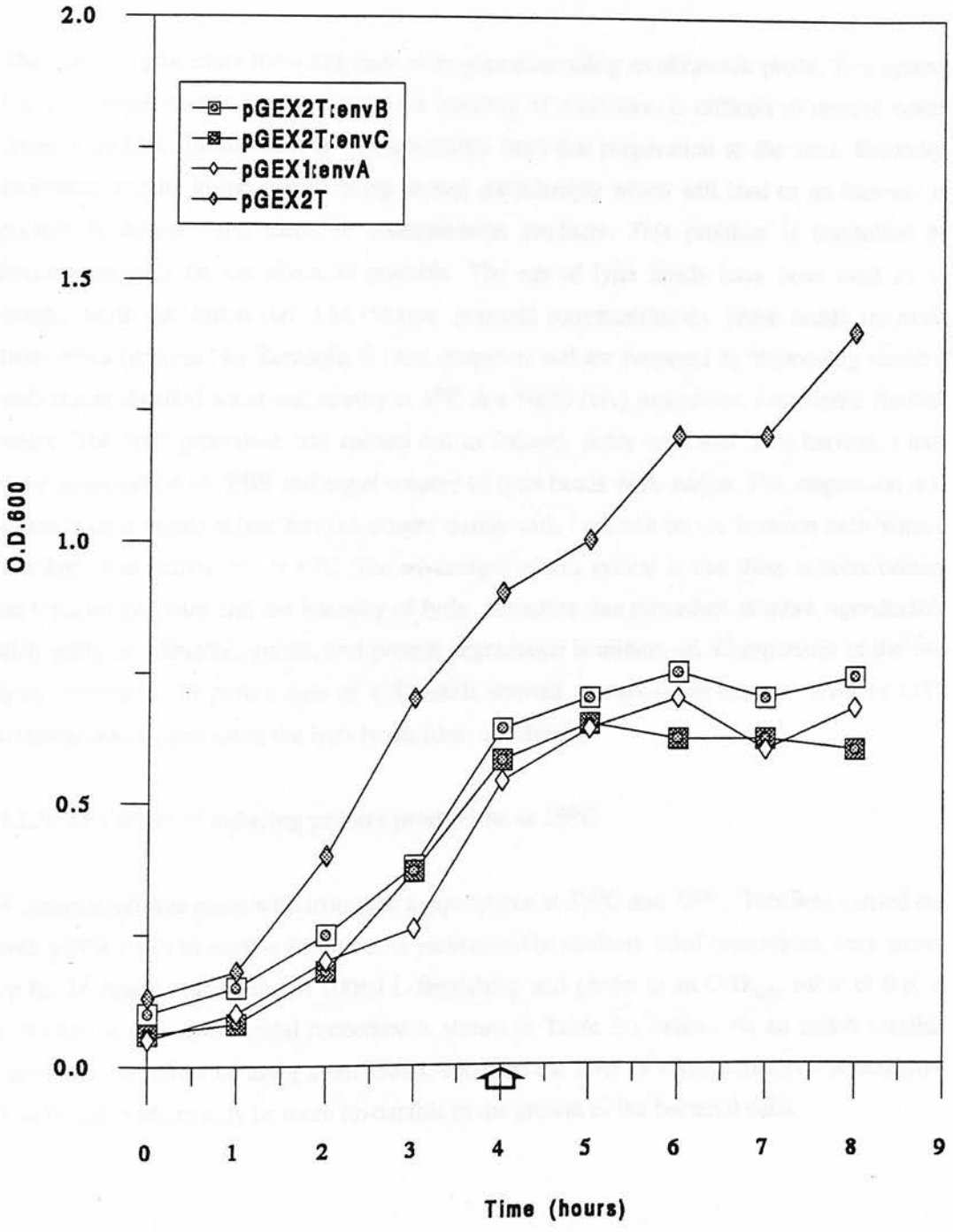
B


0.5
0.9
O. D. at induction
D
J
D
J
bacterial strain
67
43
30
Mol. wt. in kDa

C


0.5
0.9
O.D. at induction
D
J
D
J
bacterial strain
67
43
30
Mol. wt. in kDa

D


pGEX2T:envB
pGEX2T:envC
pGEX1:envA
pGEX2T
O.D.600
2.0
1.5
1.0
0.5
0.0
0
1
2
3
4
5
6
7
8
9
Time (hours)

To analyse if fusion proteins were toxic to DH5α cell growth, $O.D._{600}$ values were monitored at hourly intervals. Cultures were induced with 0.1mM IPTG after 2 hours, and bacterial growth continually monitored. Growth curves showed that these proteins were also toxic in DH5α cells (Fig. 3.6d).

3.2.3iv An alternative lysis procedure for purification of fusion proteins

The standard procedure for lysing cells is by sonication using an ultrasonic probe. This system has two major disadvantages; firstly, the intensity of sonication is difficult to control which raises a problem in maintaining reproducibility from one preparation to the next. Secondly, sonication results in the sample being heated considerably which will lead to an increase in protein breakdown and therefore contamination products. This problem is controlled by keeping samples on ice whenever possible. The use of lysis beads have been used as an alternative to sonication (Dr. J.M. Sharpe, personal communication). These beads are made from silica (supplied by Zerconia, 0.1mm diameter) and are prepared by thoroughly washing with sterile distilled water and storing at 4°C in a 50/50 (v/v) suspension with sterile distilled water. The lysis procedure was carried out as follows. After cells had been harvested they were resuspended in PBS and equal volume of lysis beads were added. The suspension was agitated on a vortex-mixer for 3x3 minute bursts with 1 minute on ice between each vortex. The lysis was carried out at 4°C. The advantages of this system is that there is more control on lysis temperature and the intensity of lysis. Therefore this procedure is more reproducible than using an ultrasonic probe, and protein degradation is minimised. Comparison of the two lysis procedures in purification of GST:envB showed a marked decrease in level of GST contamination when using the lysis beads (data not shown).

3.2.3v The effect of inducing protein production at 25°C

A comparison was made with induction temperatures at 37°C and 25°C. This was carried out with pGEX:envB to see if a difference in yield could be attained. 10ml precultures were grown up for 16 hours, transferred to 100ml L-Broth/amp and grown to an $O.D._{600}$ value of 0.6. A summary of the experimental procedure is shown in Table 3.1 below. As an added variable (sample5), the effect of using a buffered L-broth on the level of contamination was assessed. A buffered medium may be more favourable to the growth of the bacterial cells.

Table 3.1: Growth conditions of bacteria for varying induction temperatures

Sample	Temperature	$O.D._{600}$ at induction
1	37°C	0.9
2*	25°C	0.9
3	25°C	0.6
4	37°C	0.6

Note: * Sample 2; prior to induction cells were harvested and resuspended in 200mls of medium and induced as normal.

Cultures were induced for three hours, cells were harvested, lysed with silica beads as described in 3.2.3iv and purified by usual method. Samples were run on a 12% SDS-PAGE gel, fixed and silver stained. Fig. 3.7 shows the results from this experiment. There was a definite decrease in the level of contamination at 25°C compared to 37°C although the amount of GST:envB remained the same. There was not a comparable difference between the two cultures grown at 25°C indicating that the approach of growing cells to a higher density in order to produce more protein was not necessary at this lower temperature. It is possible that by inducing cultures at a lower temperature there will be a lower activity of host cell proteases which will result in a lower amount of degradation products. No difference in contamination was observed using a buffered L-Broth.

In summary, studies were undertaken in an attempt to improve the yield of fusion proteins. As is discussed above, various parameters were introduced that did improve the yield of fusion protein. This improvement however was not dramatic and was accompanied by increases in contamination. Yields ranged between 50-100µg/litre of culture. This yield is at the bottom end of the scale for anticipated yields of fusion protein, the usual being 1-3mg/litre. Since protein preparations were not pure it was not possible to determine protein concentration by conventional means, and estimations were made from silver-stained SDS-PAGE gels comparing to known concentrations of a standard protein such as Bovine Serum Albumin.

Figure 3.7: The effect on yield of fusion proteins by inducing cultures at different temperatures

100ml cultures of pGEX2T:envB were set up as described in the text. Recombinant protein was purified, resolved on a 12% SDS-PAGE gel and detected by silver staining. The position of GST:envB is indicated.

Lane 1, induced at $O.D._{600}$ =0.9 @ 37°C.

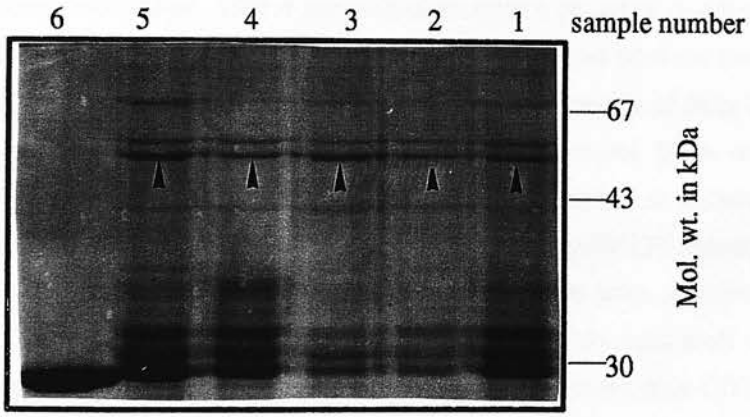
Lane 2, cultures grown to $O.D._{600}$= 0.6 @ 37°C, then to $O.D._{600}$=0.9@ 25°C. Cells resuspended and induced @ 25°C.

Lane 3, grown to $O.D._{600}$= 0.6 @ 37°C, transferred and induced @ 25°C.

Lane 4, grown to $O.D._{600}$=0.6 and induced @ 37°C.

Lane 5, same conditions as 1, but using a buffered L-broth.

Lane 6, GST control purified from pGEX2T cultures.


6
5
4
3
2
1
sample number
67
43
30
Mol. wt. in kDa

3.2.4 Raising immune sera against fusion proteins, GST:envB and GST:envC

GST:envB and GST:envC were used as a source of antigen for raising antisera against gp135. Initially polyclonal sera was made in rabbits with a view of extending immunisations to mice for the production of monoclonal antibodies. GST:envA was not used as an antigen for immunisation due to the consistent presence of contamination.

Large scale preparations were performed for pGEX:envB and pGEX:envC, using modifications described in section 3.2.3, notably a revised induction time and lysis conditions. Rabbits 222 and 217 were given initial subcutaneous injections with approximately 50µg of purified GST:envB and GST:envC, respectively, bound to agarose beads. Proteins were emulsified in Titre-Max™ and rabbits given 0.5ml injections in each hind limb. Pre-bleeds were taken from each animal before immunisation. Rabbits were boosted every 3-4 weeks with the same amount of fusion protein bound to agarose beads suspended in PBS. Rabbits were bled at each immunisation and sera were tested by western blotting to assess the presence of fusion protein antibodies. After 4 immunisations over a period of 4 months, animals were sacrificed and bled out. Sera was tested by western blot against all three fusion proteins, and compared to normal rabbit sera. Fig. 3.8a, b and c shows the results of these blots. Both R222 and R217 sera showed reactivity against all three fusion proteins. There was also an active immune response against contamination products in both rabbits as indicated on the blots. R217 and R222 sera recognised GST purified from control pGEX2T transformed cells (data not shown). The presence of GST antibodies would explain cross reactivity of R217 with GST:envA since anti-envC antibodies would not be expected to cross react with envA. In an attempt to minimise cross reactivity, R222 sera was preabsorbed onto GST immobilised on glutathione-agarose beads overnight at 4^{o}C, beads collected and sera analysed on a western blot. The depleted sera did not cross react with GST but there was a decrease in the intensity of reaction to GST:envB (data not shown). This finding suggests that immunised sera contained a predominance of GST antibodies and envB and envC antibodies were of very low titre. Preabsorbance of R217 antisera onto immobilised GST was not attempted. R217 and R222 did not recognise a specific GST:envC band by comparison to NRS.

In addition, antisera specific for GST was made following the same protocols above. A rabbit, R223, was immunised with GST purified from bacterial cells transformed with pGEX2T only. Interestingly, antisera from R223 recognised the predicted fusion proteins and also contaminant proteins, as well as GST (data not shown).

Western blotting was carried out to see if GST:envB and GST:envC antibodies specifically recognised MVV-derived gp135. Crude cell lysates from MVV infected fibroblasts were separated on a gradient gel and blotted with R217 and R222. These sera failed to recognise gp135 by comparison to MVV-infected sheep sera, 848A (data not shown). Sera were also tested by immunofluorescence on MVV-infected sheep fibroblast cells and compared to uninfected cells (data not shown). Immunofluorescence was unable to show specific binding of antisera, R222 and R217 to infected cell, compared with normal rabbit sera and anti-GST sera from rabbit 223.

In summary, attempts at raising polyclonal antisera with fusion proteins was not successful. Problems encountered included immunogenicity of GST in rabbits which hindered production of anti-gp135 antibodies. The high level of contaminants also resulted in an unclean preparation of polyclonal antibodies. Evidence highlighted above suggested the titre of fusion protein antibodies were low and this may be due to low protein yields obtained from purification of fusion proteins. It is unclear from the above studies whether antibodies to gp135 were raised with the recombinant fragments.

3.2.5 The ability of MVV-infected sheep sera to recognise recombinant env fragments

To determine whether gp135 antibodies in MVV infected sheep sera could recognise GST:envA, GST:envB and GST:envC, western blots were carried out using sera from experimentally infected sheep, 848A and 754N (a kind gift of Dr.B.Blacklaws, personal communication) and compared to normal sheep sera. Results of these blots are shown in Fig.3.9. Immune and normal sheep sera both recognised fusion proteins and contaminants. This result is a reflection on the non-specific reactivity that sheep sera show on western blots. No specific binding of MVV-infected sheep sera to the three fusion proteins was demonstrable.

Figure 3.8: The screening of rabbit polyclonal sera, R222 and R217, against fusion proteins GST:envA, GST:envB and GST:envC

Fusion proteins, GST:envA, GST:envB and GST:envC, were purified and resolved on a 5-15% gradient SDS-PAGE gel, transferred to nitrocellulose and probed. Sera were blotted against each fusion protein GST:envA, GST:envB and GST:envC at a 1/100 dilution and compared to normal rabbit serum. Figures A, B and C represents GST:envA, GST:envB and GST:envC respectively. The identity of each fusion protein is highlighted.

Antibodies were detected by using biotinylated anti-rabbit followed by extravidin-alkaline phosphatase.

Lane a, R222
Lane b, R217
Lane c, normal rabbit serum

Figure 3.9: The screening of MVV-immune sheep sera against fusion proteins, GST:envA, GST:envB and GST:envC

Figures A, B and C show blots of 848A and 754N visna-immune sheep sera against fusion proteins GST:envA, GST:envB and GST:envC, compared to normal sheep sera. Proteins were resolved as described in figure 3.8. Sheep sera were diluted 1/20 and detected with biotinylated anti-sheep and extravidin-AP. The identity of each putative fusion protein is highlighted with an arrow.

Lane d, 754N
Lane e, normal sheep serum
Lane f, 848A

Fig. 3.8

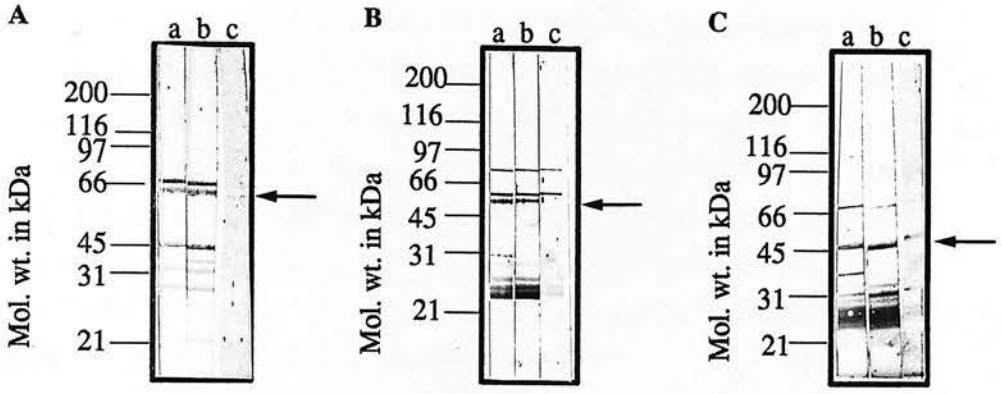
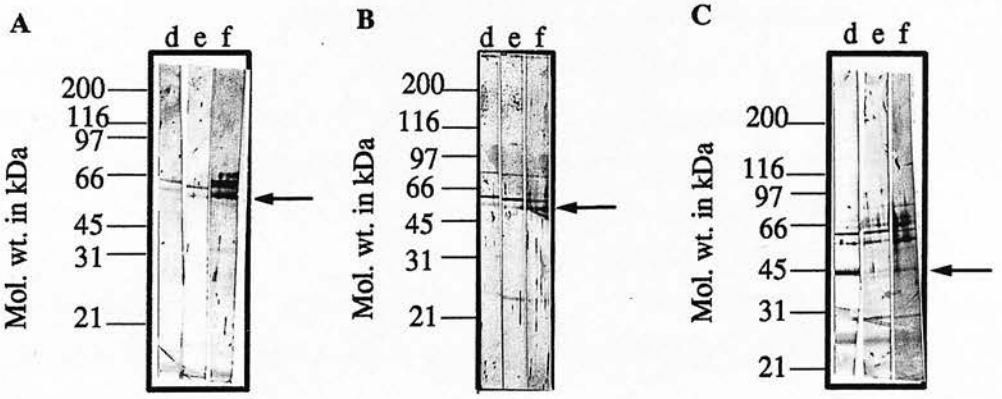

A
a b c
200
116
97
66
45
31
21
Mol. wt. in kDa
B
a b c
200
116
97
66
45
31
21
Mol. wt. in kDa
C
a b c
200
116
97
66
45
31
21
Mol. wt. in kDa

Fig. 3.9


A
d e f
200
116
97
66
45
31
21
Mol. wt. in kDa
B
d e f
200
116
97
66
45
31
21
Mol. wt. in kDa
C
d e f
200
116
97
66
45
31
21
Mol. wt. in kDa

3.3 DISCUSSION

The decision to express gp135 into three overlapping fragments was a historical one in that earlier expression studies of gp135 in yeast required protein fragments less than 40 kDa to ensure reasonable expression of recombinant proteins. For this reason it was decided to attempt to express gp135 as three fragments in the bacterial pGEX system.

Initial cloning of envA into pGEX2T proved to be unsuccessful suggesting that envA was the least suitable of the three fragments for expression (Carey, 1992). The abnormal properties of envA was evident by the morphology of the transformed bacterial colonies. Characterization of protein production of GST:envA demonstrated that envA indeed had toxic effects on bacterial cell growth. The presence of contaminants at 43kDa, 30 kDa and 65 kDa are of concern in the fact that they are produced in equal amounts to GST:envA. This is in contrast to GST:envB and GST:envC where the degree of contamination in GST:envB and GST:envC preparations varied from experiment to experiment. Reasons why envA is especially toxic and hence the problems in expressing it may be related to its structure. EnvA lies at the amino terminus of gp135 and incorporates the putative signal peptide which lies between amino acids 78-100. This means that envA contains a significant degree of hydrophobicity and the presence of a large number of hydrophobic groups is thought to hinder successful expression of foreign polypeptides (Sisk et al., 1992). In addition it should be noted that earlier studies expressing envA in the yeast Ty-system resulted in abnormal Ty particles. EnvA appeared to be glycosylated suggesting that the signal peptide was transporting envA through the glycosylation pathway of yeast cells. It is possible that this may be occurring in the bacterial cells. Bacterial cells have their own protein transport system for targeting proteins to either the plasma membrane, the periplasmic space or the outer membrane which shares various features with the eukaryotic system. Thus bacteria may be manipulating the signal peptide encoded in envA and directing it to one of the compartments mentioned above. This may result in the subsequent cleavage of GST:envA by a bacterial signal peptidase, and hence explain the consisitent presence of contaminants in preparations of GST:envA. The ability for *E.coli* to recognise foreign signal peptides and transport foreign proteins to the periplasmic space has been reported (Gray et al., 1985).

Initial expression studies with all three fusion proteins had highlighted the potential problem in obtaining recombinant protein, in that these proteins were detrimental to the growth of bacteria, and protein was only detectable when bacterial cultures were induced at a higher cell density. Two approaches were taken to improve expression, firstly by attempting to increase the overall yield of protein and secondly to decrease the level of contamination.

By analysing the effect of increasing induction time on the yield of fusion protein an insight into the kinetics of protein production of all three fusion proteins was obtained. GST:envB production appeared to decrease on increasing induction time and this was accompanied by an increase in contamination products. It is possible that a longer induction time allowed host cell proteases to breakdown fusion protein products, presuming that the rate of GST:envB production was constant over time. Alternatively, at later time points the rate of degradation may be greater than the rate of synthesis. With GST:envC production the opposite effect was observed by the fact that increasing induction time increased the overall yield of fusion protein but this was also accompanied by accumulation of breakdown products. Whilst there still seemed to be an effect on protein breakdown by host cell proteases the fact that fusion protein also increased suggests that the expression of GST:envC may be slow and hence a longer induction time is required for protein production. The production of GST:envA was also accompanied by an increase in breakdown products on increasing induction time. Reasons why there should be a difference between the production of all three fusion proteins is not fully understood other than the actual nature of each polypeptide could be affecting expression and this is discussed in further detail below.

An alternative approach to reduce the level of contamination was attempted. By inducing protein production at a lower temperature it was expected that this would have a positive effect on the build up of breakdown products by the fact that host cell proteases will be less active at 25^{o}C than 37^{o}C. The initial results with GST:envB support this hypothesis by the observed reduction in contamination. The assumption was made that protein expression would be slower at a lower temperature, and therefore a three hour induction period was employed instead of one hour. An investigation was also undertaken to see if increasing cell density and resuspending in double the original volume would increase the yield of fusion protein. As was shown in the results this did not result in an increase in protein yield.

The pGEX system uses direct expression of recombinant protein into the cytoplasm to produce high levels of foreign proteins which can be released by cell lysis and purified. This approach is usually rewarding provided that the level of expression is high enough to overcome proteolytic degradation by *E.coli* proteases (Marston, 1986, Pluckthun, 1991). One disadvantage to bacterial expression systems is that overexpression of foreign protein can lead to its accumulation in inclusion bodies which must then be disrupted to release recombinant protein (Harris et al., 1983). It has been reported that by expressing foreign polypeptides fused to bacterial proteins, such as β-galactosidase, degradation is reduced and protein synthesis is higher than by direct expression of recombinant protein (Shine et al., 1980). However, accumulation of inclusion bodies was also observed in this system (Shine et al.,

1980). Although the fusion partner in the pGEX system is not a bacterial protein, fusion protein is generally soluble (Current Protocols, 1990) which is consistent with the absence of inclusion bodies in cell extracts of pGEX1:envA, pGEX2T:envB and pGEX2T:envC (data not shown). Thus the low yields are not due to the accumulation of fusion protein in inclusion bodies. It would appear that the toxic properties hindered overexpression of the recombinant fragments of gp135, such that they were subject to degradation by host cell proteases.

Important factors that influence the level of protein produced include mRNA stability, poor translational or transcriptional efficiency due to the presence of rare codons (Robinson et al., 1984), protein stability and host toxicity due to the overexpression of foreign polypeptides (Reznicoff and Gold, 1986, Brosius, 1984). Areas of hydrophobicity (>15 amino acids) also give toxic properties to recombinant proteins and these can be represented as signal peptides and transmembrane regions in viral glycoproteins (Steinberg et al., 1986). The overexpression of glycoproteins in bacteria is problematic usually by the fact that these proteins are toxic to *E.coli* and inhibit growth resulting in low yields (Amam et al., 1984, Steinberg et al., 1986). A study with envelope glycoproteins of HIV-1 and HTLV-1 showed that by excising hydrophobic domains greater than 15 amino acids long, expression was increased from no protein up to 15% of total cell protein (Sisk et al., 1992). Since envA contains a large section of hydrophobicity (approximately 22 amino acids long) its toxic properties may be attributed to this phenomenon. This applies also to a lesser extent to envB and envC which each contain small regions of hydrophobicity.

There are reports that a large number of GST-fusion proteins are toxic to *E.coli* and the presence of the 'leaky' tac promoter transcriptional control system means that it is unsuitable with potentially toxic proteins. A novel vector has been constructed which incorporates the T7 polymerase promoter enabling tight control over expression. The GST moiety is situated at both N- and C-termini. This is to assist in folding of the foreign polypeptide where GST at the N-terminus may be incompatible to correct folding of the foreign protein (Sharrocks, 1994). It is possible that this vector, pETGEXCT, would alleviate toxic problems encountered with envA, envB and envC. In addition, this work highlights the potential problem in the recombinant protein being folded correctly. If envA, envB and envC are not folding correctly due to the presence of the GST moiety at the N-terminus, then this may explain their observed toxicity in bacteria.

An alternative approach was adopted by a group working on the envelope glycoprotein of HIV-1, gp120. They employed an expression vector carrying the signal peptide of the major outer membrane protein of *E.coli* and inserted gp120 into this vector. Gp120 was expected to

be transported from the reducing environment of the cytoplasm to the more oxidising environment of the periplasmic space allowing formation of disulphide bonds (Morikawa et al., 1990a). This approach generally results in lower levels of recombinant protein compared to direct cytoplasmic expression but proteins are more likely to be biologically active. The three fragments of MVV gp135 will each contain disulphide bridging motifs in the presence of cysteine residues. It may be that by expressing in the cytoplasm disulphide bridging is prohibited resulting in the incorrect folding of GST:envA, GST:envB and GST:envC, and hence confer toxicity to the host cell.

The requirement of specific polyclonal and monoclonal antibodies against different regions of gp135 is important to gain knowledge of the relationship between structure and function. Polyclonal sera could be used to identify the cellular receptor by immunoprecipitating receptor/gp135 complexes. Potential neutralization properties of these sera may provide a crude form of mapping which could be investigated further in finer detail by peptide mapping. The generation of immune sera from recombinant proteins is an approach that has been adopted to study a wide range of viral glycoproteins, including those of lentiviruses such as HIV-1 (Putney et al., 1986) and FIV (Ronde et al., 1994). Initial studies with HIV-1 showed that by raising immune sera to recombinant gp120, antibodies could be raised that recognised native gp120 and this was then used to inhibit neutralization *in vitro*. This was suggested to have direct implications for use both as a vaccine and in understanding the biological function of gp120 (Lasky et al., 1986). Production of recombinant FIV surface glycoprotein was undertaken by expressing it as overlapping fragments in bacterial cells. These recombinant proteins were used successfully to immunise rabbits to produce hyperimmune serum that contained antibodies to FIV envelope protein. This serum was used to identify a neutralizing epitope (Ronde et al., 1994). Thus, the gp135-derived fusion proteins would be expected to be suitable for generation of immune sera. However attempts at raising polyclonal antibodies against GST:envB and GST:envC were not successful. The sera appeared to have a low titre as determined by western blotting and this was probably a reflection on relatively low yield of fusion protein compared to contaminants. In fact, a predominance of anti-GST antibodies were present within the sera. Neither of these sera recognised gp135 derived from EV1-infected cells, indicating that immunizations with these fusion proteins had not resulted in raising antibodies specific to the gp135 fragments.

The fusion proteins could have potential use to map immunodominant epitopes using MVV-infected sheep sera. This approach has been widely used in characterising immunodominant epitopes in HIV-1 and FIV (Kowalski et al., 1987, Samuel et al., 1988, Crowl et al., 1985, Putney et al., 1986, Ronde et al., 1994). There have been limited reports using recombinant

fragments of gp135 to assess the antibody profiles in MVV-infected sheep. Reports by Kwang and Cutlip (1992a and b) suggested that the N-terminus of gp40, the transmembrane protein, was immunodominant compared to the external glycoprotein. and core proteins. They further characterised a potential neutralizing epitope in the N-terminus of the external glycoprotein (Kwang et al., 1995). Bacterially-expressed fragments of the surface and transmembrane glycoproteins were able to induce neutralizing antibodies, suggesting that glycosylation may not be necessary for mounting a humoral response against these proteins (Kwang et al., 1995). In CAEV, the immunodominant epitope has been localised within the transmembrane region (Bertoni et al., 1994), and expression of the hydrophilic portion in bacteria has resulted in its possible use in a diagnostic ELISA (Rosati et al., 1995). Epitope mapping of the external glycoprotein of MVV has been carried out with yeast-derived recombinant protein fragments and three distinct epitopes within sheep sera have been located (Carey et al., 1993). Initial studies with MVV-infected sheep sera suggested that the preparations of recombinant protein were not clean enough to use in epitope mapping studies. Non-specific reactivity of MVV-sheep sera to contaminants was also observed by Kwang et al. (1992a and b).

3.4 SUMMARY

This chapter describes the expression and characterisation of recombinant gp135 fragments in the bacterial pGEX system. These polypeptides proved to be toxic to the host cells as assessed by inhibition of cell growth and resulted in poor yields. Slight improvements in yield were achieved by altering the induction time, and modifying the lysis procedure. However, attempts using the recombinant proteins for raising hyperimmune sera were not successful. This was presumed to be attributed to the low levels of fusion protein compared to contaminants. The potential use of the recombinant proteins in epitope mapping was limited by the non-specific reactivity of sheep sera to contaminants within the protein preparations.

The toxic properties of the recombinant proteins is suggested to be due to hydrophobic regions within the fragments and possible aberrant protein folding in the cytoplasm. More successful expression may be achieved by removing hydrophobic regions, and using alternative bacterial expression systems, such as the pETGEXCT system (Sharrocks, 1994).

CHAPTER 4: GENERATION OF POLYCLONAL AND MONOCLONAL ANTIBODIES AGAINST THE EXTERNAL ENVELOPE GLYCOPROTEIN OF MAEDI-VISNA VIRUS

4.1 INTRODUCTION

This chapter describes attempts to raise specific antibodies to the external envelope glycoprotein, gp135, of MVV for use in characterizing functional epitopes such as receptor binding domains and domains to which neutralizing antibodies are raised.

Epitope mapping of gp135 has not been widely studied. Limited data has identified two separate epitopes for neutralization and fusion on the envelope glycoprotein of the 1514 strain of MVV (Crane et al., 1988). These epitopes were proposed by showing that antiserum specific for the outer envelope protein from one strain of MVV was unable to neutralize an antigenic variant, LV-V1. Inhibition of fusion, however, was observed for both strains by this serum suggesting two separate epitopes. Fusion was monitored by a "fusion from without" assay, which should be interpreted with caution since it does not represent a true physiological occurrence. The assay relies on overloading the virus such that fusion occurs without the need for virus infection. A hydrophobic peptide located within the transmembrane envelope protein has been shown to have fusion activity (Crane et al., 1991b).

There are various reports that identify the existence of epitopes within the external envelope glycoprotein that may be influenced by the host's immune system, and therefore subjected to antigenic variation. Stanley et al. (1987) reported a panel of monoclonal antibodies raised against lectin-purified envelope glycoprotein, which were used to demonstrate topographical differences between antigenic variants of the 1514 strain of MVV. None of these antibodies were neutralizing. Further characterization of this panel of monoclonals has not been published. Another study using yeast-derived overlapping fragments of gp135 identified three distinct epitopes to which sheep infected with MVV mounted an immune response (Carey et al., 1993). Sequence comparison between two antigenically distinct strains of 1514 has identified a hypervariable site within the envelope gene which is comparable in position to the V3 loop in HIV (Braun et al., 1987) but no function has yet been attributed to this site. In summary, the data published so far is very limiting as to the identification of functional epitopes within the envelope glycoprotein of MVV.

In contrast to MVV, the characterization of the envelope glycoproteins of HIV-1 has been widely studied, and epitope function has been mapped to the single amino acid level. Sequence comparison between different isolates of HIV-1 has identified five hypervariable domains, V1 to V5 with five conserved domains, C1 to C5 interspersed between them (Modrow et al., 1987). The following studies are described as an example of the use of MAb for delineating functional epitopes on viral glycoproteins. The existence of a neutralizing epitope on the external glycoprotein, gp120, was first described by Putney et al. (1986). The generation of monoclonal antibodies against either lectin-purified gp120 (Kinney-Thomas et al., 1988), immunoaffinity-purified gp120 (Matsushita et al., 1988), recombinant forms of gp120 (Nakamura et al., 1993) or synthetic peptides (Durda et al., 1988), and assessing their ability to neutralize virus infection has allowed the identification of epitopes within gp120. Monoclonal antibodies raised to lectin purified envelope resulted in the generation of four MAb that recognised a central portion of gp120, and these were able to neutralize the virus (Kinney-Thomas et al., 1988). A fifth MAb directed against gp120 mapped to a conserved region at the carboxyl terminus and this was non-neutralizing. The neutralizing antibodies were only effective against certain isolates of HIV-1 indicating that this epitope lies in a variable domain (Kinney-Thomas et al., 1988). This work was supported by findings of Matsushita et al. (1988), who isolated a MAb that was neutralizing. Epitope mapping using various recombinant protein fragments, cyanogen-bromide fragments of native gp120 and overlapping synthetic peptides mapped this neutralizing domain to a 24 amino acid peptide located within a hypervariable site (amino acids 296-331) known to contain neutralizing epitopes (Matsushita et al., 1988), which had been earlier identified by Putney et al. (1986). The MAbs described by Kinney-Thomas et al. (1988) were further studied using overlapping peptides to finely map the principal neutralizing domain (PND) in order to understand the criteria for a high neutralizing antibody. MAbs that bound to the tip of the loop showed the greatest neutralization activity, whereas those with lower neutralization potential mapped to the N-terminal of the loop (Lanjedijk et al., 1992). Also, antibodies that bound to the same epitope on the loop differed in neutralization activities which was linked to their affinity to the native gp160.

The production of neutralizing MAbs has not been restricted to the PND. Production of MAbs specific for the C4 domain showed that polymorphisms at a specific residue within the C4 domain prevented the neutralization activity of these antibodies on various HIV-1 isolates (Nakamura et al., 1993). This suggested that the ability for some strains to escape neutralization lies in the C4 domain as well as the hypervariable V3 loop. Neutralizing monoclonal antibodies against gp41, the transmembrane glycoprotein of HIV-1, have also been described (Dalgleish et al., 1988). These MAb were raised against a synthetic peptide of

gp41 and were shown to be specific for neutralization of HIV-1 and not HIV-2, and the epitope for binding was exposed before virus binding to the cell surface.

Characterization of the CD4-binding domain of gp120 has also been elucidated with the help of monoclonal antibodies. Dowbenko et al. (1988) describe the generation of a panel of MAb directed against a recombinant form of gp120. Those which blocked CD4 binding were selected for further analysis and epitope mapping carried out using proteolytic fragment of gp120 and a random gp120 expression library produced in the lamda gt11 expression system. These MAb were mapped to a conserved region of gp120, residues 403-457, at the C-terminus. Other MAbs were described that did not block CD4 interaction, but mutant analysis within these domains disrupted gp120 binding to CD4 suggesting they were involved in maintaining the structural confirmation of gp120 required for interaction with its receptor (Dowebenko et al., 1988). A similar approach was taken using immunoaffinity-purified gp120. Monoclonal antibodies that were able to block CD4 interaction were characterised and the epitope mapped to the C3 region (Sun et al., 1989). One major difference in these experiments was that these antibodies were able to neutralize virus infection. This was defined to differences in antibody affinity and avidity for various MAb. Patient sera did not recognise this epitope outlining a possible mechanism by which HIV is able to escape immunosurveilance, by the inability of the host to raise neutralizing antibodies to this site.

Other domains of gp120 have also been characterised with monoclonal antibodies. As has been described above, the V3 loop is the principal neutralizing determinant. The role of V2 as a target for neutralizing antibodies has been described (Ho et al., 1991). A panel of monoclonal antibodies were raised that were dependent on the V2 domain for binding to gp120, as assessed by mutational analysis of the V2 domain. These MAbs were neutralizing but were strain specific. However, no neutralizing antibodies in patient sera against V1 and V2 peptides were found so that the importance of these sites in natural infection is not clear. The use of peptides may not be a true representative of the native confirmation of V2 in gp120 and is therefore of limited value. A subsequent study using competition experiments between human sera and monoclonal antibodies found that antibodies to the V2 loop were present in patient's sera (Moore et al., 1993a). To date no structural analysis of gp120 has been possible, due to the inability to crystallise this protein. The use of MAbs and site-directed mutagenisis has allowed topological characterization of gp120, for example, the demonstration that V3 and C4 regions are in close proximity (Moore et al., 1993b). Subsequent analysis showed that most conserved domains were not present on the surface of a recombinant monomeric form of gp120, and those that were exposed were at the N- or C-termini. These exposed conserved regions were no longer accessible to MAbs in the oligomeric form of gp120 (Moore et al., 1994), presumably due to interaction with other gp120 molecules or gp41. It appears that the conserved regions are located within the core of gp120, which is consistent with their

hydrophobic nature and that very few monoclonal antibodies have been raised to them. The major domains exposed on the surface of the oligomeric protein are V2 and V3 which are both sites for neutralizing antibodies (Moore et al., 1994).

The studies described above are just a few examples of data published for HIV-1 gp120 epitope mapping, but they highlight the importance and usefulness of monoclonal antibodies in understanding protein function. At the commencement of this body of work, it was proposed to generate a recombinant form of gp135 that could be used as a reliable source of antigen to use for generating antibodies. So far attempts at generating such a recombinant protein have been unsuccessful (Chapter 3 and Carey, 1992). This chapter describes attempts at generating monoclonal antibodies to a lectin-purified form of gp135. Since no control monoclonal to gp135 was available within the department at the time of these experiments, various approaches to a suitable screening assay are reported. The generation of two anti-gp135 peptide polyclonal rabbit sera and their characterization is described.

4.2 RESULTS

4.2.1 The generation of monoclonal antibodies to the envelope glycoprotein of MVV

4.2.1i Purification of envelope glycoprotein

For a standard preparation of antigen, sheep fibroblasts were grown to 80% confluency in 8L tissue culture bottles and infected with approximately 0.05 $TCID_{50}$ of EV1/cell in 30mls of 2% FCS/DMEM. By four days post infection extensive c.p.e. was apparent and infected cells were knocked off the plastic surface using sterile glass beads. After clarification, the supernatant was stored at -70^{o}C, and an aliquot was tested on a standard virus titration assay to confirm the presence of virus. The purification of gp135 was carried out using a *Lens culinaris* Sepharose 4B column (Pharmacia). The virus was pelleted, disrupted and loaded on the column (3.5ml), following the manufacturer's instructions. After extensive washing of the column, the glycoprotein was eluted in 3-6mls of elution buffer. 200µl aliquots were collected and analysed by western blotting, probing with EV1 infected sheep serum, 848A, and normal sheep serum as a negative control. It was usual for the glycoprotein to be eluted between the sixth and fifteenth aliquots (data not shown). To ensure that all glycoprotein present in the supernatant had been purified on the column, the 'flow through' was re-circulated on the

column and elutant analysed as before. This revealed that all viral glycoproteins had been purified in the first round of affinity chromatography. Analysis of purified protein showed a single major protein band of approximately Mr 135 000 which was specifically recognised by sheep 848A. To verify that the purified protein was virus-derived, a comparison was made to crude EV1 infected cell lysate. Samples were analysed by western blotting using sheep sera 848A and normal sheep sera (Fig.4.1). A band of comparable size to the purified protein was identified in the crude cell lysates not seen in negative controls. A lectin purification was carried out with supernatant from uninfected sheep fibroblasts and no protein band comparable in size to gp135 was identified (data not shown). To estimate the protein concentration, aliquots containing purified glycoprotein were pooled, run on a SDS-PAGE gel and silver-stained. By comparing to known concentration of bovine serum albumin, the concentration of gp135 was estimated at 7μg/ml. Silver-stain analysis showed there was minimal contamination and that gp135 was the predominant protein (data not shown).

4.2.1ii Development of a screening assay for detection of anti-envelope antibodies

Since the yield of purified protein from the lectin purification was very low, this antigen was reserved for the immunizations carried out below. Therefore, an alternative source of antigen was required for the purposes of screening monoclonals and two approaches are described. One shortcoming in developing a screening assay was the absence of an antiserum specific for gp135, and thus MVV infected sheep sera was used as a positive control.

a] Concanavalin A ELISA

This method was adapted from a protocol outlined by Robinson et al. (1990) developed for screening HIV-1 infected patients.

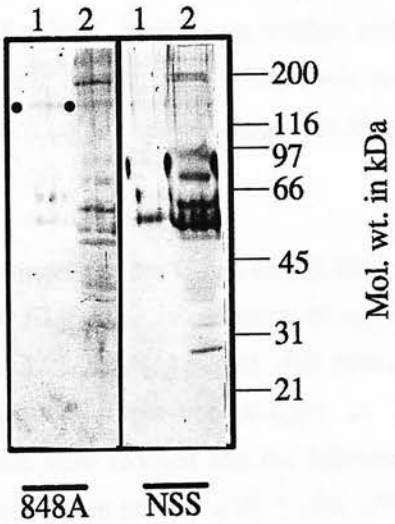
1. Preparation of serum free virus stock

Sheep fibroblasts were grown in roller tissue culture bottles to 80% confluency and infected with 0.05 $TCID_{50}$ of EV1/cell in 30mls of 2%FCS/DMEM. Twenty-four hours later, the medium was replaced with supplemented DMEM containing 1% Nutridoma (Boeringher Mannheim). Three to four days later, extensive c.p.e. was apparent and cells were knocked off the plastic surface with sterile glass beads. The supernatant was clarified and virus disrupted by the addition of 10%(v/v)TritonX-100/PBS, to give a final concentration of 1%TritonX-100. This was incubated on ice for 30 minutes and supernatant was stored in 5ml aliquots at -70°C.

Figure 4.1: Lectin-affinity purification of gp135 from MVV

Proteins were resolved on a 5-20% gradient SDS-PAGE gel and transferred to nitrocellulose. Blots were probed with MVV-infected sheep sera, 848A (1/20) and normal sheep sera, NSS (1/20). Proteins were detected with biotinylated anti-sheep IgG and extravidin-AP.

lane 1, lectin-purified gp135.
lane 2, EV1-infected cell crude lysate.


1 2 1 2
200
116
97
66
45
31
21
Mol. wt. in kDa
848A
NSS

2. ConA envELISA

The basic protocol for the ConA envELISA is as follows. Immulon II 96-well ELISA plates (Dynatech) were coated with 200µg/ml of ConA diluted in 0.1M Na_2CO_3, pH9.0, 100µl/well, for 16 hours at 4°C. The wells were washed 4 times with PBS containing 0.1%(v/v)TritonX-100 (PBS-Tx) and 100µl of a predetermined dilution of serum-free virus stock was added to each well. Virus negative controls were from mock infected cell supernatant, treated in the same way as virus stock. A positive control for the ELISA was immunoaffinity-purified sheep MHC classII, used at 8µg/ml (a kind gift of Dr.B.Dutia, Department of Veterinary Pathology, Edinburgh). Samples were diluted in Hank's buffer (HBSS) and incubated for 1 hour at 37°C. Wells were washed as before and blocked in HBSS/10% FCS, 200µl/well, for 1 hour at 37°C. To test antibody binding to immobilised viral glycoproteins, dilutions of heat-inactivated sheep sera were made in blocking buffer and added at 100µl/well for 1 hour at 37°C. The MHC classII positive control wells were incubated with a combination of two anti-sheep MHC classII monoclonal antibodies (positive control) (α-DR and α-DQ), or a Campylobacter specific monoclonal (negative control), 100µl/well. The wells were washed as before and incubated for 1 hour at 37°C with a anti-species biotinylated antibody, diluted 1/1000 in blocking buffer, 100µl/well. Wells were washed and incubated in extravidin-AP, diluted 1/1000, 100µl/well, for 1 hour at 37°C. The wells were washed and incubated in developing solution at 37°C until colour had developed. ELISAs were read at optical density of 405nm.

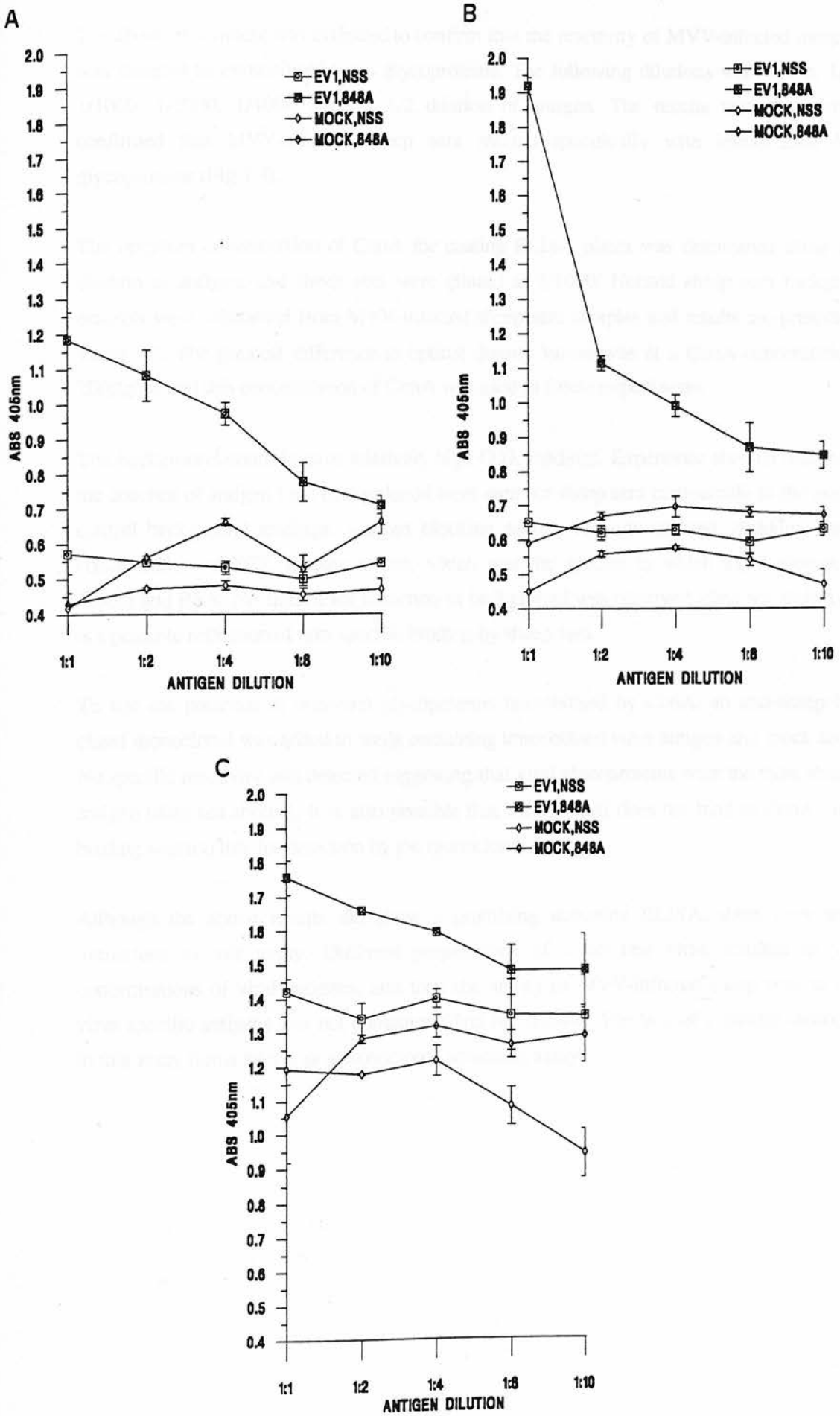
3. Development of optimum conditions for ConA envELISA

To optimise the conditions for ELISA, serial dilutions of serum-free antigen were added to ConA-coated ELISA plates as 1, 1/2, 1/4, 1/8 and 1/10 dilutions in duplicate wells. Plates were set up with mock-infected serum-free antigen as background controls. After immobilisation of antigen, wells were blocked and the following dilutions of MVV-infected sheep sera, 848A, and a negative sheep serum; 1/10, 1/100, 1/500 and 1/1000 were added to the wells to each dilution of antigen. The results are shown in Fig.4.2, for the three serum dilutions; 1/100, 1/500 and 1/1000. Results from the serum dilution 1/10 are not shown since readings were too high to be measured when plates were read at a given time point. Interpretation of the data indicate that at serum dilution 1/500 and 1/1000, MVV-infected sheep sera was reacting specifically with immobilised viral glycoproteins and that the strongest reactivity was seen at dilution 1 and 1/2 of antigen. In subsequent experiments, serum-free virus antigen was usually diluted 1/2.

Figure 4.2: ELISA reactivity of MVV-infected sheep sera against varying dilutions of ConA-immobilised MVV glycoproteins

Details of the ELISA protocol are given in the text. Graphs a, b and c represent dilution of sheep serum at 1/1000, 1/500 and 1/100, respectively. Standard deviations are shown.

Key: EV1, viral antigen
Mock, mock antigen
848A, MVV-infected sheep sera
NSS, normal sheep sera


A
B
C
EV1,NSS
EV1,848A
MOCK,NSS
MOCK,848A
ABS 405nm
ANTIGEN DILUTION
1:1
1:2
1:4
1:8
1:10
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4

The above experiment was extended to confirm that the reactivity of MVV-infected sheep sera was directed to immobilised virus glycoproteins. The following dilutions were made; 1/500, 1/1000, 1/2000, 1/4000 using a 1/2 dilution of antigen. The results from this titration confirmed that MVV-infected sheep sera reacted specifically with immobilised MVV glycoproteins (Fig.4.3).

The optimum concentration of ConA for coating ELISA plates was determined using a 1/2 dilution of antigen, and sheep sera were diluted at 1/1000. Normal sheep sera background controls were subtracted from MVV-infected sheep sera samples and results are presented in Table 4.1. The greatest difference in optical density values was at a ConA concentration of 200μg/ml and this concentration of ConA was used in future experiments.

The background controls gave relatively high O.D. readings. Experience showed that even in the absence of antigen high backgrounds were seen for sheep sera comparable to the negative control background readings. Various blocking agents were investigated, including varying concentration of FCS, donkey serum, which was the species in which the conjugate was raised, and BSA. No significant reduction in background was observed (data not shown), and is a possible reflection of non-specific binding by sheep sera.

To test the presence of non-viral glycoproteins immobilised by ConA, an anti-sheep MHC classI monoclonal was added to wells containing immobilised virus antigen and mock antigen. No specific reactivity was detected suggesting that viral glycoproteins were the most abundant antigen (data not shown). It is also possible that MHC classI does not bind to ConA, or that binding was too low for detection by the monoclonal used.

Although the above results did show a promising screening ELISA, there were several limitations to this assay. Different preparations of serum-free virus resulted in varied concentrations of viral antigens, and thus the ability of MVV-infected sheep sera to detect virus specific antigens was not consistent (data not shown). The lack of a control monoclonal in this assay limits its use as a monoclonal screening assay.

Figure 4.3: Titration of MVV-infected sheep sera reactivity to ConA immobilised MVV glycoproteins

Graph shows the results from the ELISA, details of which are given in the text. Standard deviations are shown.

Key: EV1, viral antigen
Mock, mock antigen
848A, MVV-infected sheep sera
NSS, normal sheep sera

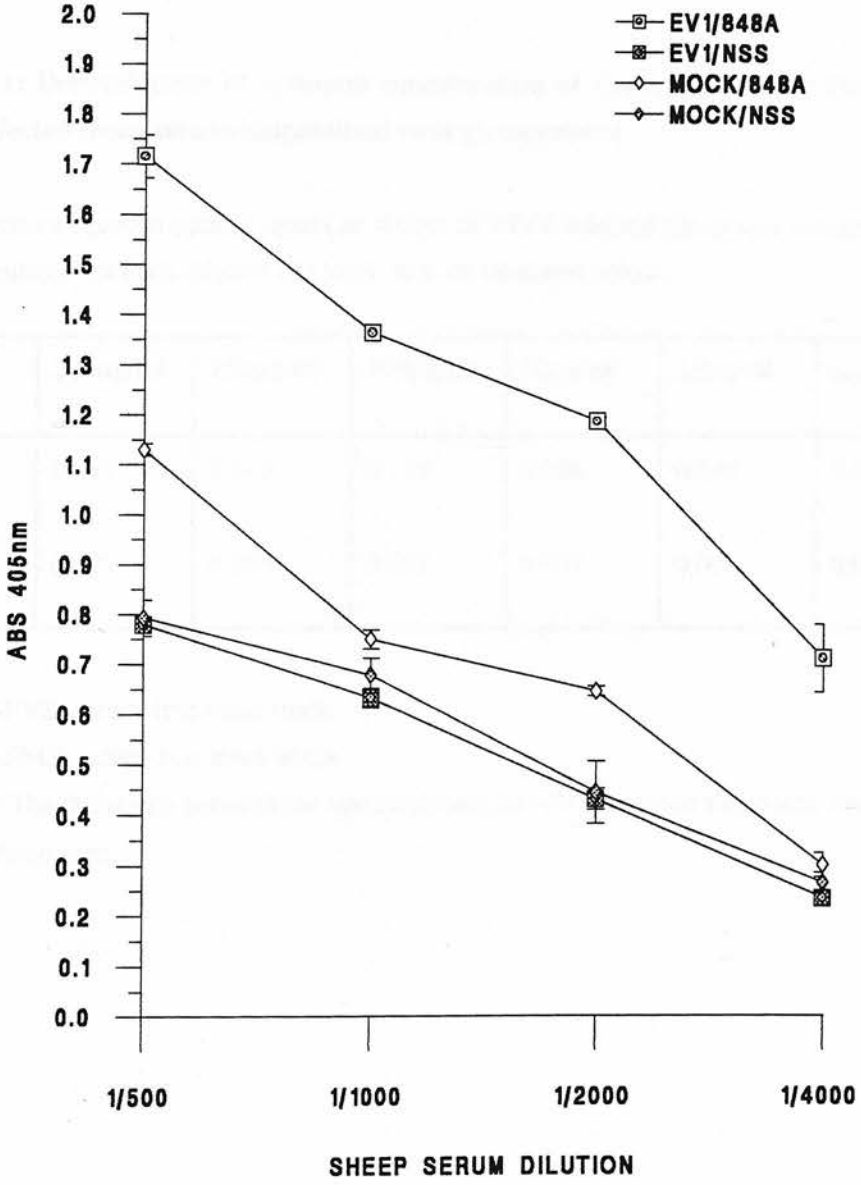

EV1/848A
EV1/NSS
MOCK/848A
MOCK/NSS
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABS 405nm
1/500
1/1000
1/2000
1/4000
SHEEP SERUM DILUTION

Table 4.1: Determination of optimum concentration of ConA for specific reactivity of MVV-infected sheep sera to immobilised viral glycoproteins

The difference between optical values at 405nm of MVV-infected sheep sera and normal sera were calculated for each dilution of ConA, and are tabulated below.

[ConA]	200μg/ml	150μg/ml	100μg/ml	50μg/ml	25μg/ml	none
SFVS	0.995*	0.645	0.749	0.508	0.645	0.081
SFMS	0.127	0.083	0.058	0.007	0.02	0.01

Note: SFVS, serum-free virus stock
SFMS, serum-free mock stock
* The difference between the optical density of MVV-infected sheep sera and normal sheep sera.

b] Fixed-cell envELISA

96-well plates were seeded with 10^4cells/well in the inside 60 wells and incubated for a further 16 hours at 37°C. The cells were infected with 1 $TCID_{50}$/cell, 0.5 $TCID_{50}$/cell or mock-infected in triplicate across the plate. After a 2 hour incubation, virus was removed fresh medium was added. 72 hours later, the medium was removed and cells were fixed in ice-cold acetone:methanol (1:1) for 5 minutes. Well were allowed to dry and stored at -20°C.

Prior to performing an ELISA, plates were thawed for 45 minutes at 18°C. Wells were blocked with 2%BSA(w/v)/PBS for 30 minutes at 18°C (200µl/well). ELISA was then carried out using the standard protocol. To test for the possibility of using this ELISA as a screening assay for *env* monoclonals, the following monoclonal antibodies were tested; anti-p24 (Reyburn et al., 1992), anti-ovine LFA3, anti-ovine LFA1 and anti-ovine MHC classI. Anti-p24 was used to test for the presence of viral antigens, anti-LFA3 and anti-MHC classI to test for surface antigens and anti-LFA1 as a negative control. The results of testing the fixed cell assay are given in Table 4.2. Anti-LFA3 and anti-p24 gave positive readings compared to the negative control, anti-LFA1. Anti-MHC classI gave lower readings, and it is known to give weak staining of fixed tissue by FACS (Dr.B.Blacklaws, personal communication). Readings with anti-LFA3 and anti-MHC classI were higher in uninfected cells than infected and is probably a reflection of cell density in those wells. The opposite is seen for anti-p24 where readings in uninfected cells was comparable to anti-LFA1, indicating a specific reactivity of anti-p24 for viral capsid protein.

Since both positive and negative control monoclonals were available for this assay and that antigen preparation via fixing infected cells was reproducible, this assay was adopted for screening anti-envelope monoclonals. The inside 60 wells of a 96-well plate were routinely infected with an m.o.i. of 1, that is 1 $TCID_{50}$/cell, for 30 wells, and the remaining 30 wells were mock infected. Cells were fixed and plates were stored at -20°C.

Table 4.2: Reactivity of a panel of monoclonals on a fixed-cell assay

m.o.i. of EV1	1	0.5	0
anti-LFA1 (-ve)	0.348+/- 0.014*	0.321+/- 0.018	0.408+/- 0.037
anti-LFA3 (+ve)	0.821+/- 0.1	0.789+/- 0.048	1.059+/- 0.129
anti-MHC classI (+ve)	0.492+/- 0.08	0.428+/- 0.049	0.781+/- 0.06
anti-p24 (+ve)	0.917+/- 0.129	0.795+/- 0.049	0.418+/- 0.057

Note: * optical density values (405nm) +/- s.d.

4.2.1iii Preparation of anti-env monoclonal antibodies

a] Immunizations

Three 7 week old female BALB/c mice were injected at two sites subcutaneously with approximately 1μg of lectin-purified gp135 emulsified in Titer-Max™, to give a final volume of 400μl. Each mouse was given two intraperitoneal boosts of 1μg of antigen in PBS (in the same volume as the initial injections) with an interval of 4 weeks between each boost. Three weeks later, tail bleeds were taken from each mouse and titres were tested on the fixed-cell ELISA assay (Fig 4.4). The titre was measured as the 50% point between the maximum and minimum O.D. values on the titration curve. Mouse 1 gave the highest titre and was selected for fusion. It was noted that there was an immune response to cellular-derived proteins, and although no significant difference was seen for an immune response against viral antigens, it was assumed that since the serum was polyclonal then anti-gp135 antibodies would be present. The two remaining mice were continually boosted as before in an attempt to increase antibody titres. Boosts were given every 10-14 days, and after two more boosts mouse 2 was selected for fusion. Immunizations of mouse 3 was continued over a further two months and a second tail bleed was taken. The titre of this serum, determined on the fixed-cell assay, was estimated at 1/1800 and was much higher than in the original test bleed (Fig.4.5).

b] Preparation of hybridomas and screening

Fusions with the spleen cells of mouse 1 and 2 were unsuccessful, and therefore results from the third fusion only will be reported. Three days prior to fusion, mouse 3 was given an intravenous boost of 1μg of gp135 in PBS (in a volume of 100μl). Spleen cells were isolated, washed and fused with HAT sensitive myeloma cells, SP2 (Schulman et al., 1978), at a fusion ratio of 6:1 of spleen cells to myeloma cells. Ten days post fusion, the wells containing hybridomas were scored and fed with 15%FCS/RPMI supplemented with HAT. When the wells reached 80% confluency, 200μl of supernatant was removed and tested on the fixed-cell assay. If the clone tested positive it was transferred to a single well in a 24-well plate. A total of 350 wells were seeded with fused hybridomas and only 66 of these contained clones. After primary screening, 5 of the 66 clones were positive, that is 7% (see Table 4.3). A positive clone was termed as that which gave an O.D. value greater than the value of the negative control reading plus double its standard deviation.

Figure 4.4: Titration curves for mouse 1, 2 and 3 sera after inoculation with lectin-purified gp135

Mice were immunised as detailed in the text. Tail bleeds were taken and tested on a fixed-cell ELISA by comparison to normal mouse sera. Standard deviations are shown. Graphs a, b and c represent titration curves of mouse 1, mouse 2 and mouse 3, respectively.

Sera titres were estimated as : mouse 1; 1/700
mouse 2; 1/500
mouse 3; 1/450

Key: mock, uninfected fibroblast cells
EV1, MVV-infected fibroblast cells
m1/m2/m3, mouse 1, 2 and 3, respectively
nms, normal mouse serum

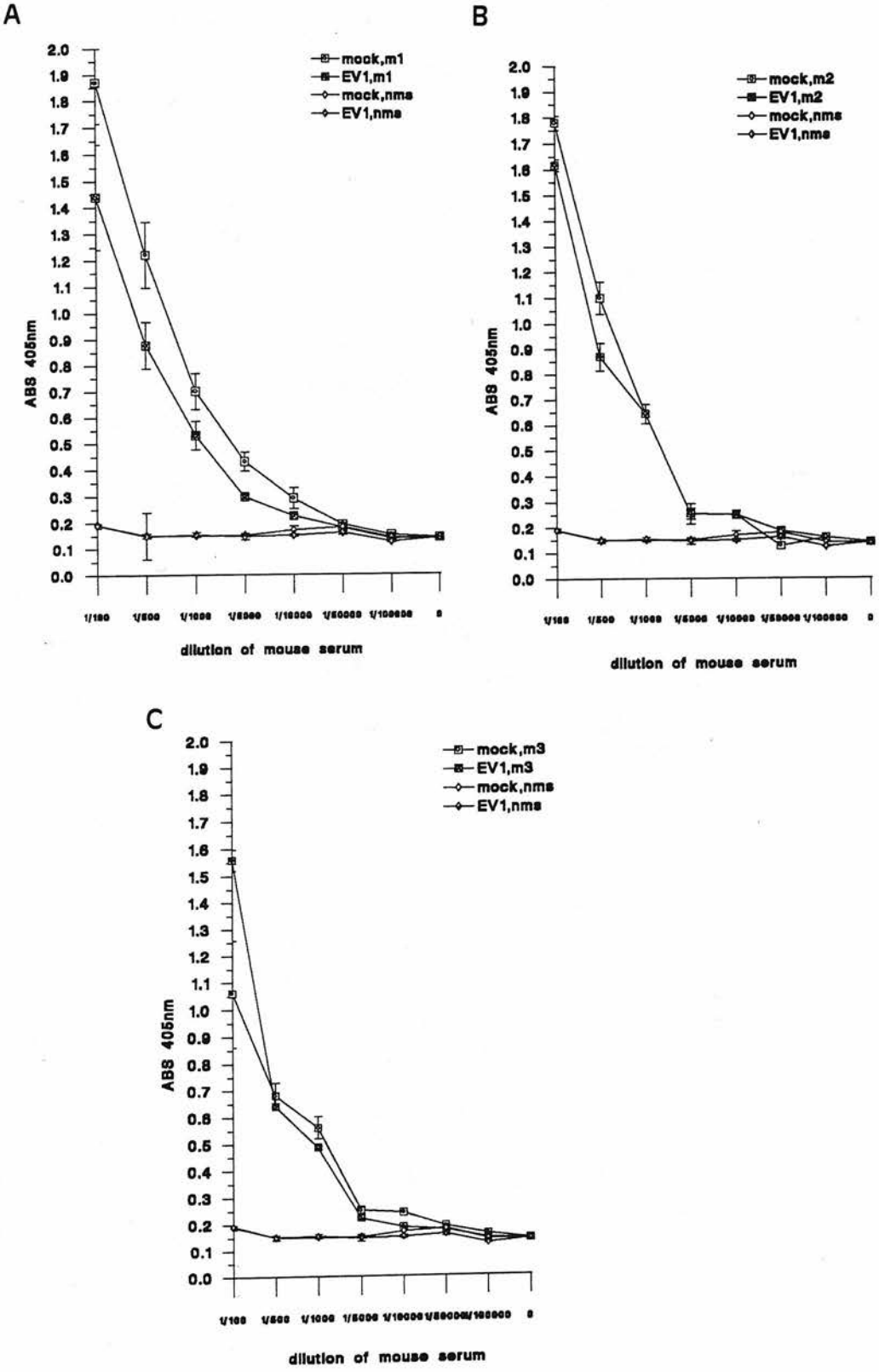

A
mock,m1
EV1,m1
mock,nms
EV1,nms
ABS 405nm
dilution of mouse serum
B
mock,m2
EV1,m2
mock,nms
EV1,nms
ABS 405nm
dilution of mouse serum
C
mock,m3
EV1,m3
mock,nms
EV1,nms
ABS 405nm
dilution of mouse serum

Figure 4.5: Titration of mouse 3 sera on a fixed-cell ELISA after a prolonged immunisation schedule

Mouse 3 was boosted with gp135 as detailed in the text. A second tail bleed was taken and tested on a fixed-cell ELISA, by comparison to normal mouse sera. Standard deviations are shown.

The titre of mouse 3 sera was estimated as 1/1800.

Key: mock, uninfected fibroblast cells
EV1, MVV-infected fibroblast cells
m3, mouse 3
nms, normal mouse serum

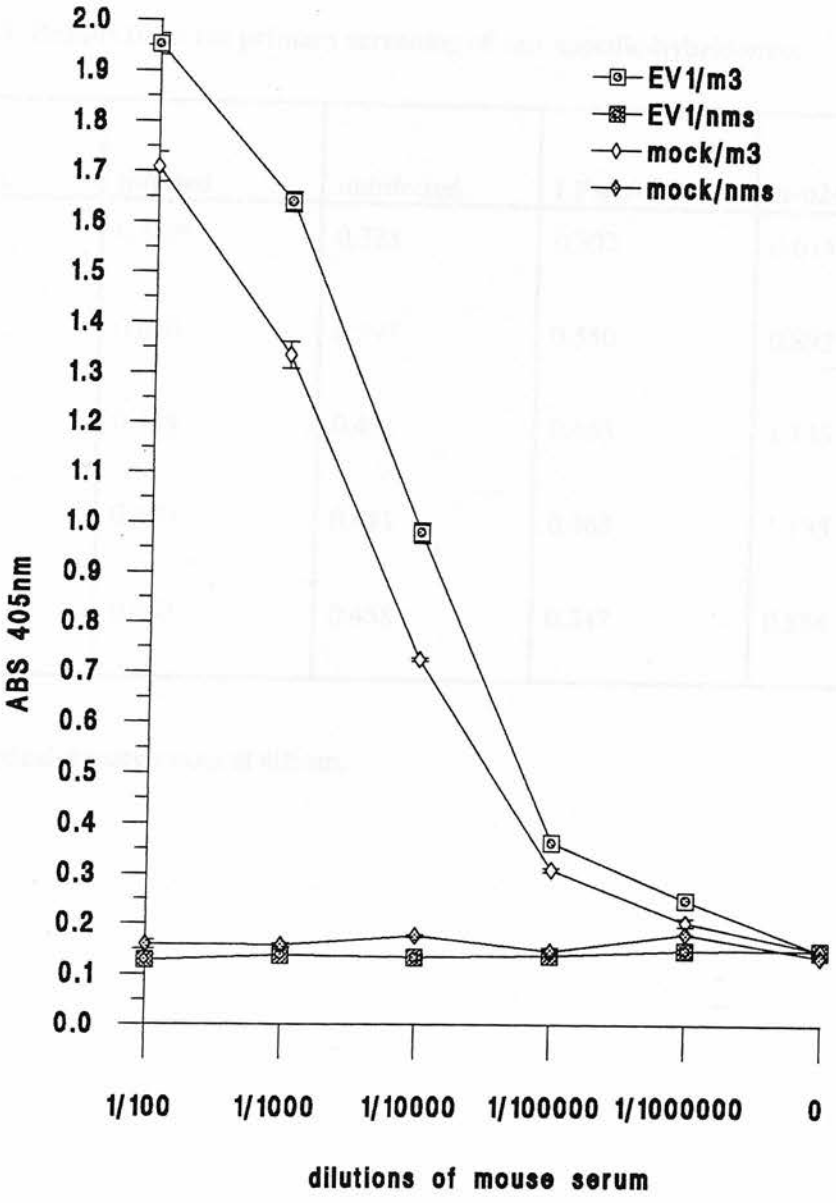

EV1/m3
EV1/nms
mock/m3
mock/nms
ABS 405nm
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1/100
1/1000
1/10000
1/100000
1/1000000
0
dilutions of mouse serum

Table 4.3: Results from the primary screening of env specific-hybridomas

Clone no.	infected	uninfected	LFA1(-ve)	α-p24(+ve)
5F6	0.349*	0.338	0.302	0.614
6G4	0.660	0.597	0.550	0.892
2C7	0.459	0.411	0.463	1.135
2C8	0.459	0.411	0.463	1.135
3F8	0.463	0.458	0.347	0.884

Note: * optical density values at 405nm.

These positive clones were grown in duplicate wells of a 24-well plate and frozen down in liquid nitrogen. Clones 5F6, 2C8, 6G4 and 3F8 were selected for secondary screening by soft-agar cloning. Twenty four colonies were picked for each clone from the agar plates and grown up in 24-well plates. When the wells were confluent the supernatants were tested on the fixed-cell assay. The positive hybridomas isolated from this cloning are listed in Table 4.4 in which their O.D. readings are compared to the positive and negative controls. All these clones were expanded into duplicate wells and stocks frozen down into liquid nitrogen.

Clones 3F8 C3, 5F6 A1, 5F6 C6 and 6G4 D5 were selected for a second round of agar cloning since these clones gave the greatest difference in O.D. values between infected and uninfected cells. Twenty four clones were picked from the agar plates and grown up as before. When tested on the fixed-cell ELISA only 25% of the clones screened positive (data not shown). Since at least 90% of clones would be expected to be positive in the third round of cloning, it was decided to retest the original clones 5F6, 6G4, 2C7, 2C8 and 3F8. These clones were resuscitated and grown up into a single well of a 24-well plate. Supernatants were tested in triplicate on a fixed-cell ELISA and gave negative O.D. readings. The supernatants were also tested by western blotting with MVV-infected cell lysates, using an EV1-infected sheep immune serum and a anti-p24 monoclonal as positive controls. None of the test supernatants bound to virus glycoprotein (data not shown).

Table 4.4: Results of secondary screening by agar cloning

clone	infected	uninfected	LFA1(-ve)	α-p24(+ve)
3F8 A3	0.418*	0.370	0.385	0.667
3F8 C2	0.526	0.356	0.462	0.617
3F8 C3#	0.559	0.306	0.322	0.908
3F8 C4	0.354	0.282	0.322	0.908
3F8 D2	0.442	0.342	0.322	0.908
3F8 D3	0.673	0.562	0.510	1.325
3F8 D5	0.563	0.38	0.372	0.908
2C8 C4	0.450	0.406	0.462	0.617
5F6 A1#	0.673	0.445	0.510	1.325
5F6 C6#	0.706	0.338	0.510	1.325
5F6 D2	0.443	0.359	0.462	0.617
6G4 C6	0.508	0.365	0.372	0.908
6G4 D5#	0.508	0.365	0.672	1.325
6G4 B3	0.615	0.534	0.778	1.358
6G4 C3	0.731	0.650	0.778	1.358

Note: # indicates clones selected for further cloning, as described in text.

* optical density values at 405nm.

4.2.2 Preparation of rabbit anti-serum specific for gp135-derived peptides

4.2.2i Immunizations and determination of antibody titre

Two peptides derived from the external envelope glycoprotein of MVV were synthesized and obtained commercially. Peptide 1 included amino acids 190-209 and peptide 2 included amino acids 419-438. Both are located in predicted hydrophilic sites within α-helical structures and an additional cysteine was added to the carboxyl end of the peptide1, for the purposes of crosslinking (Fig.4.6). Peptides were crosslinked to ovalbumin, the carrier protein, using SPDP as a linker molecule. Two female Dutch rabbits were immunized with either one of the peptides. They were primed with approximately 0.8mg of crosslinked peptide emulsified in Titer-Max™ by giving two injections subcutaneously. The rabbits were boosted with 300μg of antigen in incomplete Freund's adjuvant 10 days later, followed by a second boost 14 days later. Both rabbits were bled prior to commencement of the immunizations and 7 days after the third immunization. Antibody titres were tested by ELISA following standard protocols. Peptides were dissolved in 0.01M HCl, ether extracted and neutralized with phosphate buffer. ImmulonIV plates (Dynatech) were coated with peptide 1, 2 or PBS, 100μl/well at a concentration of 10μg/ml in PBS. Peptides were allowed to adhere to the plastic for 16 hours at 4^{o}C. After blocking, ten fold dilutions of serum were added to the plate, including pre-bleeds. The ELISA was developed with anti-rabbit alkaline phosphatase with the usual developing solution. The titres were determined graphically as described in section 4.2.1iii (Fig. 4.7). Titres were estimated at 1/2000 for rabbit P1 and 1/17000 for rabbit P2. The rabbits were continually bled once a month over a period of 4 months. Titres of each bleed were compared on the ELISA described above, and no significant drop in titre was observed (data not shown). After 6 months since the initial immunisation, it was decided to bleed out the rabbits. They were given a boost of 500μg of peptide and 10 days later they were bled out. Serum was stored at -20^{o}C.

Figure 4.6: The location and amino acid sequence of peptide 1 and peptide 2 on the external glycoprotein of MVV

Amino acid numbering is as stated in Sargan et al. (1991). The location of the three hydrophobic sites and the major hydrophilic region are those predicted in Sargan et al. (1991), and the arg-lys-arg-lys motif is the predicted cleavage site between the surface and transmembrane protein.

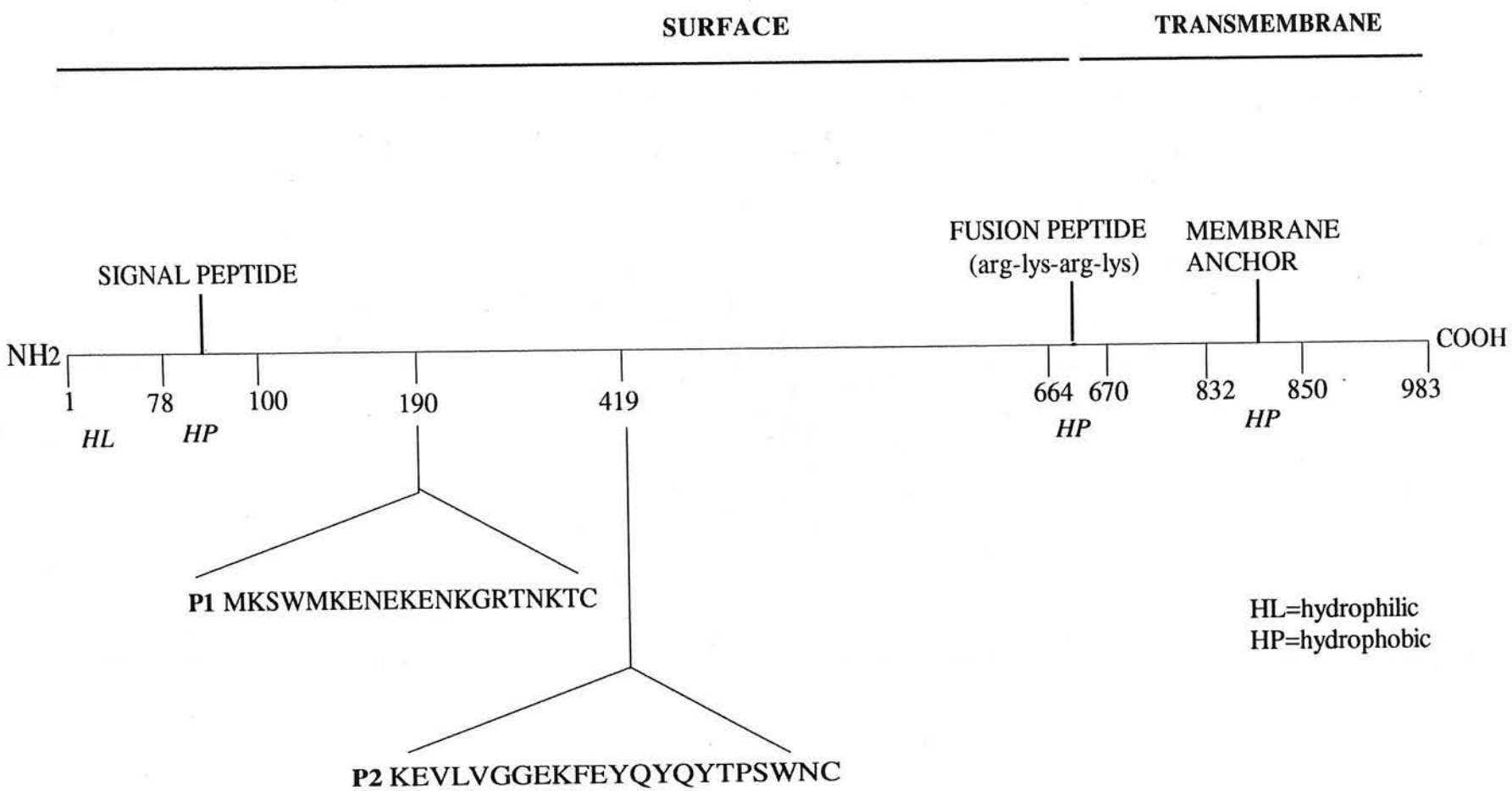
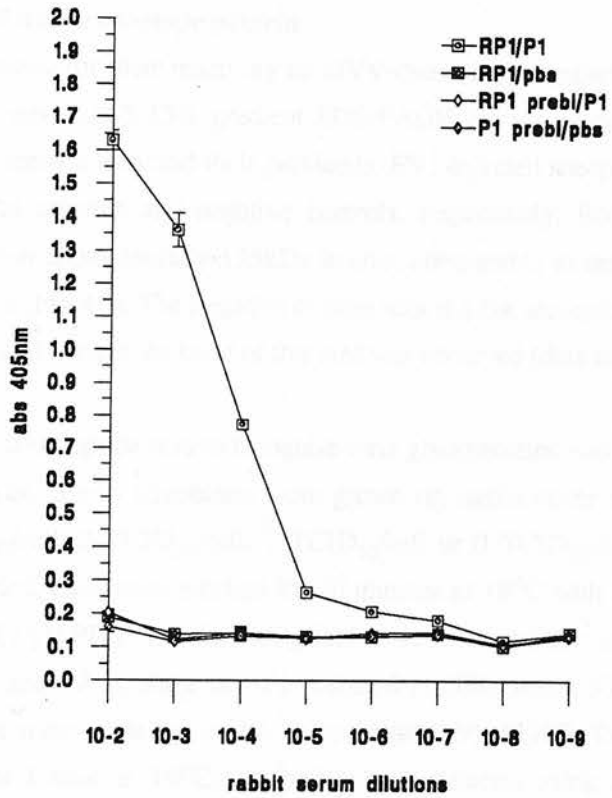

SURFACE
TRANSMEMBRANE
SIGNAL PEPTIDE
FUSION PEPTIDE
(arg-lys-arg-lys)
MEMBRANE
ANCHOR
NH2
COOH
1
78
100
190
419
664
670
832
850
983
HL
HP
HP
HP
P1 MKSWMKENEKENKGRTNKTC
P2 KEVLVGGEKFEYQYQYTPSWNC
HL=hydrophilic
HP=hydrophobic

Figure 4.7: Determination of rabbit sera titre following immunisation with gp135 peptides on ELISA

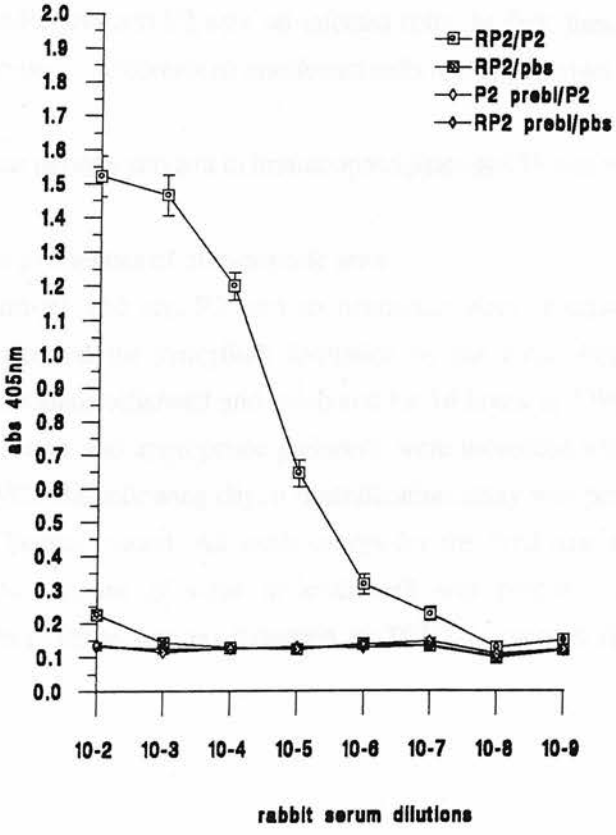
The conditions for the ELISA are given in the text. Graphs a and b represent titration curves for rabbits immunised with peptide 1 and 2, respectively. Standard deviations are shown.

Key: RP1, rabbit peptide1 sera
RP1 prebl, rabbit peptide1 prebleed sera
RP2, rabbit peptide2 sera
RP2 prebl, rabbit peptide2 prebleed sera
PBS, phosphate buffered saline
P1, peptide1
P2, peptide2

A


RP1/P1
RP1/pbs
RP1 prebl/P1
P1 prebl/pbs
abs 405nm
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
10-2
10-3
10-4
10-5
10-6
10-7
10-8
10-9
rabbit serum dilutions

B


RP2/P2
RP2/pbs
P2 prebl/P2
RP2 prebl/pbs
abs 405nm
2.0
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
10-2
10-3
10-4
10-5
10-6
10-7
10-8
10-9
rabbit serum dilutions

4.2.2ii Characterization of anti-gp135 peptide sera

a] Recognition of native envelope protein

Both sera were tested for their reactivity to MVV-derived glycoproteins. EV1-infected crude cell lysates were run on a 5-15% gradient SDS-PAGE gel for western blotting and probed with anti-P1 and anti-P2 sera and their prebleeds. EV1-infected sheep sera and normal sheep sera were used as positive and negative controls, respectively. Both anti-P1 and anti-P2 recognised a protein approximately 135kDa in size, comparable to that recognised by MVV-infected sheep sera (Fig.4.8). The negative control sera did not recognise a protein of this size. In mock-infected cell lysates, no band of this size was observed (data not shown).

The ability of the anti-peptide sera to recognise viral glycoproteins was further investigated by immunoflourescence. Sheep fibroblasts were grown on sterile cover slips and infected with EV1 at 4 $TCID_{50}$/cell, 2 $TCID_{50}$/cell, 1 $TCID_{50}$/cell or 0 $TCID_{50}$/cell. After 3 days, cells were fixed and dried. Cells were blocked for 30 minutes at 18^{o}C with 10% sheep serum(v/v)/ 0.01% Tween 80(v/v)/ PBS. The following rabbit sera were added: anti-P1 (1/100), anti-P1 prebleed (1/100), anti-P2 (1/200), anti-P2 prebleed (1/200), anti-GST (1/100) and anti-p24 (1/500). Dilutions were made up in 2% sheep serum(v/v)/ 0.01% Tween80(v/v)/ PBS and cells incubated for 1 hour at 18^{o}C. Antibodies were detected using anti-rabbit biotinylated antibody (1/250) and extravidin-FITC (1/1000). Coverslips were mounted and examined for fluorescence. The staining pattern in the p24 positive control infected cells indicated that immunoflourescent staining had worked (data not shown). By comparison, no positive staining was seen for anti-P1 and anti-P2 sera on infected cells. In fact, these samples gave staining patterns similar to negative control or uninfected cells (data not shown).

The ability of these peptide antisera to immunoprecipitate gp135 was not investigated.

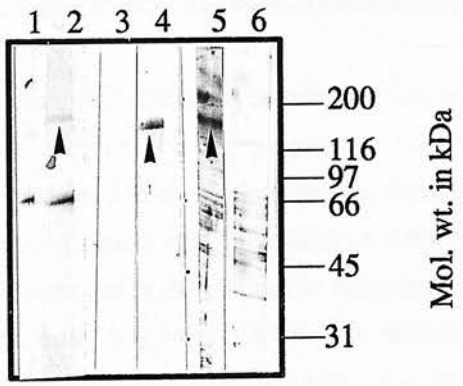
b] Neutralization properties of anti-peptide sera

The ability of anti-P1 and anti-P2 sera to neutralize virus infection was investigated by monitoring the effect on the syncytium formation by the virus. Four 96 well plates were seeded with 10^{4}fibroblast cells/well and incubated for 16 hours at 37^{o}C. Ten fold dilutions of each peptide antiserum and appropriate prebleeds were incubated with 500 $TCID_{50}$ of EV1 for 16 hours at 4^{o}C. The following day, a neutralization assay was performed and seven days later cells were Giemsa stained. All wells except for the final row showed extensive c.p.e. indicating that the amount of virus in each well was probably too high to expect a neutralization effect. There was no reduction in $TCID_{50}$ observed for cells incubated with peptide serum.

Figure 4.8: The ability for anti-peptide sera to recognise the envelope glycoproteins of MVV

Peptide1 and peptide2 sera were tested against EV1-infected crude cell lysates by western blotting as detailed in the text. Sera were diluted 1/100.

lane 1, peptide1 antisera (prebleed).
lane 2, peptide1 immune antisera.
lane 3, peptide2 antisera (prebleed).
lane 4, peptide2 immune antisera.
lane 5, EV1-infected sheep sera, 848A.
lane 6, normal sheep sera.


1 2 3 4 5 6
200
116
97
66
45
31
Mol. wt. in kDa

The neutralization assay was repeated with a lower $TCID_{50}$ start point and using an IgG purified fraction of anti-peptide sera. Doubling dilutions of anti-P1 and anti-P2 were set up starting with 2.4mg/ml of IgG, and 200 $TCID_{50}$ of EV1 was added to each sample for 16 hours at $4^{o}C$. The following day, a neutralization assay was performed with doubling dilutions of virus across the plate. Seven days later, cells were stained with Giemsa and inspected for a reduction in $TCID_{50}$. A control plate was set up with normal rabbit IgG. No inhibition of infection was observed for anti-P1 and anti-P2 sera (data not shown).

4.3 DISCUSSION

This chapter has described attempts at generating antibodies against the external envelope glycoprotein of MVV.

4.3.1 Attempts at generating monoclonal antibodies

Due to the disappointing results on recombinant expression of gp135, an alternative source of immunogen was required for the purpose of raising antisera. A simplified protocol was devised for the purification of envelope protein from virions, adapted from Pyper et al. (1984). Lectin-affinity purification was successful in isolating the envelope glycoprotein of MVV, although yields were not high. The use of lectin-purified gp135 as an immunogen for raising monoclonal antibodies has been described by Stanley et al. (1987). This work resulted in a panel of monoclonal antibodies raised against the envelope glycoprotein of the 1514 strain of MVV, and these were used to localise antigenic variation within the glycoprotein envelope. The absolute identity of lectin-purified gp135 is not clear. In the above publications it is referred as the envelope glycoprotein, and it is not characterised further as representing the external glycoprotein. Crane et al. (1988) describe lectin-purified gp135 as the outer membrane glycoprotein within which two separate epitopes for neutralization and fusion are characterized. During the purification protocol, especially the ultracentrifugation step, the shedding of the external glycoprotein would be expected resulting in the predominance of the precursor glycoprotein in the purified virions. Thus, it would seem probable that the 135kDa protein purified by lectin-chromatography is the uncleaved precursor envelope. The identity and size of the glycoproteins of MVV are discussed further in Chapter 6. It is also noted that the transmembrane glycoprotein of MVV, gp40, was not purified by lectin affinity purification as analysed by western blotting. This failure in purification may be due to its

interaction with the plasma membrane, and possibly stronger detergents would be required to release the transmembrane glycoprotein from the membrane. Alternatively the levels of the transmembrane protein in the purified virions and its subsequent lectin purification may have been too low to detect by western blotting.

The successful development of a screening assay was limited by the absence of an anti-envelope glycoprotein. The first approach using ConA to immobilise viral glycoproteins shared principles similar to that used for the purification of the immunogen. That is, the ability of carbohydrate moieties, present on the surface of glycoprotein, to bind to lectins. The method used for the purification of MVV glycoproteins was less abrasive for the ConA assay than the purification of virions, and thus the preservation of the outer envelope glycoprotein would be expected. One shortcoming was that the concentration of viral glycoproteins in the supernatant of EV1-infected fibroblasts may be too low for adequate binding to ConA. The results for development of the ELISA suggested that EV1-infected sheep sera did recognise immobilised viral glycoproteins (Fig. 4.2 and 4.3). There were several features of this assay that limited its use as a screening assay. The use of sheep sera in development was adequate to demonstrate the specific binding of viral glycoproteins. However, the high backgrounds due to non-specific reactivity of normal sheep sera suggested that sheep sera would not be suitable controls when screening for monoclonals. The non-specific reactivities of sheep sera was suggested, in part, to be due to reactivity to ConA. Despite attempts using various blocking agents, high backgrounds could not be reduced. Non-specific reactivity of sheep sera is seen in western blotting procedures also, which supports the problem in using sheep sera as controls in an ELISA. As was reported in the results, specific reactivity to viral antigens was not observed with a panel of 4 additional EV1-infected sheep sera. There appeared to be a degree of variation between different preparations of viral antigens. This could be due to the state of fibroblasts at the time of infection which will reflect the titre of virus produced. Thirdly, the time required to carry out this assay was longer than for the average ELISA which is not ideal when screening monoclonal antibodies, since a short, concise assay is more favourable. It was reported that MHC classI contaminants were not detectable. However, as was reported for the fixed cell assay, this MAb shows only weak binding to sheep fibroblasts. Therefore the possibility of non-viral glycoprotein contaminants cannot be ruled out, and these may have contributed to the non-specific reactivities of the sheep sera.

The second approach proved to be more suitable for the purposes of screening monoclonals. Firstly, an anti-MVV monoclonal and an anti-sheep fibroblast surface molecule were available. Both these monoclonals gave positive O.D. readings on the ELISA. This suggests that the protocol used allowed the detection of viral antigens as well as preserving the presence

that the protocol used allowed the detection of viral antigens as well as preserving the presence of surface molecules. This is an important result since a control is necessary for the detection of viral glycoproteins which are expected to be present at the surface of the infected cell. This ELISA was more rapid than the ConA ELISA and the plates could be preprepared and stored at -20^{o}C until required. Since during all hybridoma screenings the anti-p24 monoclonal gave positive readings, adequate viral antigens appeared to be present on the fixed-cell plates. The presence of capsid protein is a common detection system for virus infection, suggesting that it is this antigen which is produced to detectable levels during infection (Reyburn et al., 1992). It was not possible to measure whether envelope glycoproteins would be present in detectable levels, due to the absence of anti-envelope antibodies at the time of the monoclonal studies.

Inoculations of three mice were performed. After three immunisations the sera was tested on the fixed-cell ELISA. The titres obtained were at the lower end of the range acceptable for performing fusions. Mice 1 and 2 did not result in successful fusions. In an attempt to increase the antibody titre, mouse 3 was given additional boosts over a period of two months. Retesting of the sera indicated that the titre had increased to a level more acceptable for performing fusions. The usual titre for performing fusions is between 1/1000 to 1/100 000. Although the titration graphs indicated that an immune response had also been mounted against cellular contaminating proteins, subsequent screening of monoclonal antibodies would allow differentiation between these and anti-*env* monoclonals.

Isolation of spleen cells resulted in $9x10^{7}$ cells, which is comparable to the expected result of 10^{8} cells. It is usually expected following fusion that most wells would contain hybridomas, and of those between 10 and 50% will secrete antigen-specific antibody. The results from the fusion of mouse 3 spleen cells to SP2 myeloma cells was disappointing in that only 66 of 300 wells contained hybridomas. This is probably a reflection on the technical expertise required for efficient fusions. However, approximately 7% of these wells screened positive for anti-*env* antibody. The O.D. readings for all these wells was low compared to the positive control. It is noted that even negative hybridomas tested did not give readings comparable to positive controls, thus suggesting that not even anti-cell antibodies were being secreted by the hybridomas, whose presence was predicted on the serum titration curves. Subsequent cloning did not result in the isolation of a single clone secreting anti-*env* antibodies.

Several factors may explain the failure of the fusion. The titre of antibody in the serum may have been too low, such that the proportion of B cells in the spleen secreting the antibodies of interest were low. This may be a reflection on the amount of antigen used to prime the mice. It is usual to prime with 10-50μg of antigen (Hurn and Chantler, 1980, Vaitukaitis, 1981), which was not possible with the low yields obtained during lectin

purification. The use of the prolonged immunisation protocol for mouse 3 would expect to increase the titre, although the affinity of these antibodies would be low. Apart from the small amount of antigen used to prime the mice, the preparation of an adequate emulsion with adjuvant may affect the induction of an effective immune response. An emulsion allows the slow release of antigen into the blood stream which is favourable when attempting to enhance an immune response against a weak antigen. Since the immunization protocol only used adjuvant in the primary inoculation, stimulation of the immune response may not have been adequate on subsequent boosts.

Hybridomas are generally unstable cell lines with a tendency to loose chromosomes on cell division. However, over two rounds of cloning the rate of chromosome loss is slow. In the case of this work, the supernatants gave low readings on the first round of screening, suggesting that the failure of the fusion was not due to chromosome loss during cloning of the hybridomas.

The preparation of monoclonal antibodies is expensive and time consuming. In view of these factors and the problems in developing a suitable screening assay, attempts at raising monoclonal antibodies were halted.

4.3.2 Preparation of anti-peptide polyclonal antibodies.

To date, no antibodies specific for the external envelope glycoprotein of MVV were available within the department. Two peptides derived from the external glycoprotein of MVV were used as immunogens. Peptides can be used to characterize a gene of unknown protein product. One of the shortfalls of using peptides is that they represent continuous epitopes which may not be present on the native protein. However, denatured proteins and peptides can be effectively used to generate antibodies against the native protein (Lerner, 1982). It could be envisaged that the ability of an antibody specific for a continuous epitope to bind to the native antigen is achieved by the mobility of that epitope within the native molecule (Westhof et al., 1984). In a similar way, an antibody may exhibit enough flexibility at the hinge region to fit the antigenic site (Oi et al., 1984). The use of the two peptides derived from gp135 resulted in a polyclonal serum that recognised native gp135 by western blotting. These antisera were not able to recognise antigen by immunoflourescence. In western blotting, proteins are denatured before separation by SDS-PAGE, whereas immunoflourescence was set up to detect non-denatured protein within infected cells. Thus these two peptides may not be present as epitopes in the native protein, although they are both hydrophilic and thus would be expected to lie on the outside of the protein. This is particularly applicable to P1 which lies at amino acid 190 in

gp135, that is 90 amino acids from the N-terminus of the mature protein where the signal sequence lies within the first 100 amino acids (see Chapter 5). The fact that these peptides do not suggest to represent epitopes within the native protein is supported by the fact that the antisera was non-neutralizing, and the peptides did not bind specifically to the surface of sheep cells (see Chapter 5). These peptide antisera have use as a control for western blotting and they could possibly used as a control in a monoclonal screening assay, depending on the nature of the antigen used to coat the ELISA plate.

4.4 SUMMARY

This chapter has described attempts at raising a panel of monoclonal antibodies against lectin purified gp135. The development of two potential screening assays is described and their shortfalls for detecting anti-envelope monoclonals discussed. Reasons why monoclonal antibody production was not successful may be due to immunization strategy and amount of antigen used. The production of polyclonal sera against two peptides derived from the external glycoprotein of MVV is reported. These sera were able to recognise denatured envelope proteins, but did not appear to be represented as epitopes on the native protein.

CHAPTER 5: EXPRESSION AND FUNCTIONAL STUDIES OF THE EXTERNAL GLYCOPROTEIN OF MVV, GP135, IN THE BACULOVIRUS EXPRESSION SYSTEM

5.1 INTRODUCTION

Previous attempts to express the external glycoprotein of Maedi-Visna Virus as a recombinant protein have been frustrated by the toxic properties of this protein which has resulted in low yields accompanied by contaminating products (Carey, 1992, and Chapter 3 of this thesis). The two expression systems employed in this laboratory to date are the yeast Ty system and the bacterial pGEX system. Both systems are simple to manipulate and have the potential to produce large quantities of recombinant protein on a large scale. However, these systems lack the more sophisticated post-translational modifications, such as the glycosylation pathway, that are a feature of the higher eukaryotic systems. Expressing a protein in a eukaryotic system requires the introduction of the gene of interest into mammalian cells via a vector. The vaccinia expression system is the most widely used for production of recombinant protein by constructing recombinant vaccinia virus which can infect mammalian cell lines to express the protein of interest. Preliminary experiments in our laboratory have been unsuccessful in raising a recombinant vaccinia virus, although expression of the surface protein of CAEV, which is the most closely related lentivirus to MVV, in this system has been published (Lichtensteiger et al., 1991). Other mammalian expression systems are more useful to confirm the identity of a certain gene and its function, and involve generating a stable cell line that expresses the protein of interest. An expression system which is becoming more popular is the baculovirus expression system and recent advances in understanding the molecular biology of these insect-specific viruses, especially the very late gene promoters, has lead to refinements in production of recombinant viruses.

Baculoviruses are unique to invertebrates and their infection can lead to complete obliteration of insect populations. It is this for which they first gained their importance as a potential for pest control. Baculoviruses have large, double-stranded, covalently-closed circular DNA genomes that range from 80-200kbp in size. The DNA is associated with a 6.5kDa, highly basic nucleoprotein (Tweeten et al., 1980) and this complex is surrounded by a rod-shaped nucleocapsid containing a 39kDa protein (Theim and Miller, 1989). The nucleocapsids are further packaged into a lipid envelope to form a virus particle which may be assembled into polyhedra depending on the stage of the virus life cycle. Polyhedrin is a 29kDa protein that accumulates in large quantities at the late stages of the virus life cycle (reviewed by

Rohrmann, 1986). Baculoviruses are classified into subgroups depending on the packaging characteristics of the polyhedra. Subgroup A, known as the nuclear polyhedra viruses (NPV) are the type that have been most widely studied, in particular for their potential to control insect pests and manipulation for the production of recombinant protein. This subgroup is characterized by the presence of several virus particles occluded in a single polyhedron, and each virus particle may contain several nucleocapsids. The virus that has been most widely used for expression purposes is *Autographica californica* multiple nuclear polyhedrosis virus (AcMNPV).

Baculoviruses have two life cycles (reviewed by Webb and Summers, 1990): the extracellular virus is the lytic form and is predominant in the initial stages of infection where it is responsible for spread of virus from cell to cell. The polyhedra-associated virus is important in the later stages of the virus life cycle where it is vital for survival outside the insect and therefore for transmission to other hosts. *In vivo*, polyhedra are ingested by the insect host and the polyhedrin protein dissolves in the gut to allow release of the virus particles which then fuse with the plasma membrane of the host cells. The nucleocapsid is important for the entry of the DNA into the host's nucleus where viral replication commences. Nucleocapsids form in the nucleus where they bud from the nuclear membrane. This membrane coat is lost in the cytoplasm and the virus gains a new envelope coat when it buds from the plasma membrane and also aquires a virus encoded glycoprotein, gp67 (Whitford et al., 1989), which is responsible for attachment of the virus to other cells in the host. At later stages in the life cycle the production of extracellular viruses is replaced by the production of virus particles in the nucleus which acquire a lipid coat that is made *de novo* in the nucleus and lacks gp67. These particles accumulate into polyhedra which eventually results in the swelling and destruction of the insect and release of polyhedra into the external environment.

The replication cycle *in vitro* is essentially similar to *in vivo* with the production of polyhedrin in cells at the later time points of infection. The most widely used cell lines that support infection *in vitro* are derived from the Fall army worm, *Spodoptera frugiperda* (Sf) (Vaughn et al, 1977). However, propagation of AcMNPV *in vitro* can lead to incorporation of host cell DNA and transposable elements within its genome so precautions must be undertaken when passaging recombinant viruses *in vitro*. The virus genome is susceptible to mutations but this will rarely affect the production of recombinant protein (Bissard and Rohrmann, 1990). With regard to the use of baculoviruses as expression systems the polyhedrin protein and p10, which forms a crystalline matrix in the polyhedra, are of most importance. These proteins are under the influence of very efficient promoters and can account for up to 50% of total cell protein mass. These proteins are non-essential for the production of progeny virus allowing

them to be manipulated for expression purposes. Baculoviruses are an ideal expression system for the following reasons:

1. The merits of the polyhedrin and P10 have already been highlighted and enable a foreign gene to be expressed in large quantities.
2. The expression of these very late genes occurs after the maturation of the virus particle such that any potentially toxic foreign protein will not disrupt virus replication.
3. Baculovirus genomes are very large and variable in size which allows the insertion of relatively large genes without affecting normal replication.
4. Since baculoviruses infect eukaryotic cells, proteins will be post-translationally modified, although glycosylation pathways are less sophisticated.
5. They are safe to work with since they only infect invertebrates and can be worked up to large scale (500-1000ml preparations) which is not practical with mammalian cell lines.

However, since protein production requires infection of virus in insect cells leading to eventual cell death it is necessary to use fresh cells for every round of protein synthesis.

In summary, the baculovirus expression system involves manipulation of the polyhedrin gene, where it is replaced with the gene of interest resulting in production of large quantities of recombinant protein at the later stages of virus replication. The first proteins to be expressed in this system were β-interferon (Smith et al., 1983) and β-galactosidase (Pennock et al., 1984). Since then a wide variety of proteins have been expressed including viral glycoproteins, for example the hemagglutinin glycoprotein of Influenza virus (Kuroda et al., 1989, Kuroda et al., 1990), the human parainfluenza virus type 3 glycoproteins (Lehman et al., 1993), the membrane fusion and hemagglutinin proteins of the measles virus (Vialard et al., 1990), the envelope fusion glycoprotein gp85 of Epstein-Barr virus (Pulford et al., 1994), the equine herpesvirus 1 glycoprotein D (Love et al., 1993), retrovirus glycoproteins of the avian leukaemia virus (Noteborn et al., 1990) and of the human T cell leukaemia virus type I (Arp et al., 1993). Lentivirus glycoproteins have also been expressed in baculovirus of which HIV-1 has been most widely studied (Hu et al., 1987, Rusche et al., 1987, Murphy et al., 1990, Morikawa et al., 1990b, Culp et al., 1991, Murphy et al., 1993, Wang et al., 1995), the expression of the envelope surface glycoprotein of EIAV (Wang et al., 1994) and of Maedi-Visna virus envelope glycoprotein (Kwang et al., 1995 {published after initiation of this work}) has also been reported.

Attempts at expressing the external glycoprotein of MVV have been hindered by the toxic properties of this protein in the systems chosen so far. The baculovirus system was employed as preference to a mammalian expression system since it generally produces much higher

yields at a significantly lower cost. The production of a glycosylated protein by the baculovirus system is an added benefit since its biological properties would be expected to be similar to that of the native protein. The first consideration taken when expressing a protein in the baculovirus system is choice of transfer vector . Since the genome of baculoviruses is large (128kbp), it is not possible to insert a gene of interest directly into the genome by restriction enzyme manipulation. The original production of a transfer vector for introducing the foreign gene into baculovirus DNA involved the insertion of viral sequences that spanned the polyhedrin gene into bacterial plasmids, followed by removal of the polyhedrin gene by restriction enzyme digestion but leaving the promoter intact. A unique restriction enzyme site was inserted downstream of the polyhedrin promoter to allow insertion of the gene of interest (Smith et al., 1983). The production of recombinant viruses is based on cotransfection of viral DNA with the transfer vector. This leads to recombination between the sequences flanking the foreign gene within the transfer vector and homologous sequences in the viral DNA resulting in the replacement of the polyhedrin gene in viral DNA with that of the foreign gene. The recombinant viruses are then isolated by plaque assay and screened for a non-occlusion, polyhedrin negative phenotype.

There are a wide variety of transfer vectors containing the polyhedrin promoter available (Lucklow and Summers, 1988a and b), including the pAcYM1 type which contain the complete 5' non-coding leader sequence of the polyhedrin gene which is essential for expression of high levels of recombinant proteins (Matsuura et al., 1987), the pAcCL29 series which have a M13 intergenic region necessary for production of single stranded DNA which is useful if modifications to the foreign gene need to be made after the initial insertion (Livingstone and Jones 1989), and the pAcDZ1 type (Zuidema et al., 1990) which have a *lacZ* gene inserted upstream of the polyhedrin promoter such that recombinant viruses can be selected for foreign genes and production of β-galactosidase. Other transfer vectors available use the p10 promoter or allow insertion of two genes of interest (Weyer et al., 1990).

Several considerations were necessary when choosing a suitable vector for the production of external envelope glycoprotein. Yields of glycoproteins in expression systems are generally lower than non-glycosylated proteins. It has been observed in the baculovirus system that maximum yield is achieved earlier in virus infection, rather than later in infection when the activity of the polyhedrin promoter is at its maximum (Murphy et al., 1990). The reasons for this are unclear but the problem may lie in the host cells being unable to recognise processing signals of the foreign protein, such as signal peptides required for efficient translocation through the cell's glycosylation machinery. In fact, there has been some success in increasing yields of recombinant proteins by replacing the signal sequence of the foreign protein with an insect-derived signal peptide, such as the honeybee melittin signal peptide (Tessier et al.,

1991). This approach has been taken further with the construct of baculovirus transfer vectors containing the signal sequence of the membrane glycoprotein of baculovirus, gp67 (Whitford et al., 1989) inserted immediately downstream of the polyhedrin promoter (Murphy et al., 1993, Wang et al., 1995). This approach showed that yields of the external glycoprotein of HIV-1, gp120, was increased by 6- to 20-fold (Murphy et al., 1993). Since expression work in the bacterial pGEX system suggested that the N-terminus of gp135 was especially difficult to express (see Chapter 3), the *env* gene of MVV was manipulated to remove the putative signal sequence and to place *env* under the influence of the baculovirus gp67 signal peptide. The second consideration taken on choosing a transfer vector was the method of purification that would be used. Novel vectors, pAcG1, pAcG2T and pAcG3X, have been described where the coding sequence of glutathione-S-transferase, GST, derived from the pGEX expression system has been introduced into the baculovirus transfer vectors downstream of the polyhedrin promoter (Davies et al., 1993). This allows the gene of interest to be cloned downstream of the GST tag and be produced as a fusion protein which can then be purified by substrate affinity, essentially similar to the bacterial pGEX system. Wang and colleagues have produced a vector, pAcSG2T, which combines both of these attributes, that is the baculovirus-derived signal sequence and the GST tag (Wang et al., 1995). This results in the recombinant protein being secreted into the culture medium, and protein can then be purified by affinity absorption onto glutathione-agarose beads. The fusion protein can also be cleaved with thrombin to remove the GST tag. The map of this vector is given in Fig. 5.1 showing the position of the signal sequence, the GST tag and the multiple cloning site for insertion of the gene of interest.

The original screening of recombinant viruses for a polyhedra negative phenotype required experience and practice to visualise cells that did not contain inclusion particles. Vectors were modified so that recombination resulted in the introduction of β-galactosidase as well as the foreign gene (Pennock et al. 1984) into the baculovirus genome. This allowed recombinant viruses to be screened for a blue phenotype in the presence of X-gal, but the recombinant protein would always be contaminated by the presence of β-galactosidase. However the underlying problem in screening for recombinant viruses was the relative inefficiency of recombination. A novel method was developed by Kitts et al. (1990) which was based on the observation that linear DNA molecules in yeast and mammalian cells are highly recombinogenic (Orr-Weaver et al., 1983, Bollag et al., 1989). Although linear DNA is regarded as non-infectious (Kelly and Wang, 1981), it was proposed that linear DNA would be able to recombine with homologous sequences present in the transfer vector resulting in the recircularisation of the baculovirus genome to produce infectious recombinant viruses. A unique restriction enzyme site (Bsu361) was introduced into AcMNPV DNA to allow it to be linearised. Cotransfection of linear AcMNPV DNA and vector lead to a higher proportion of

progeny being recombinant with a reduction in the background of non-recombinant viruses due to lower infectivity of the linear DNA. The source of wild-type viruses was thought to be derived from recircularisation of linear DNA in the absence of recombination, or incomplete linearisation of the genomic DNA prior to transfection (Kitts et al., 1990). To eliminate this background, the baculovirus DNA was further manipulated by inserting an additional Bsu361 restriction enzyme site within an essential gene, ORF1629. Thus digestion of viral DNA lead to the loss of part of this essential gene that could only be replenished by recombination with the transfer vector which contains an intact copy of ORF1629, and this ensures that the gene of interest is inserted into the viral genome in the correct orientation. Results from cotransfection showed that between 85-99% progeny viruses contained the gene of interest (Kitts and Possee, 1993). A further modification was made to the baculovirus genome whereby the polyhedrin gene was replaced by the *lacZ* gene so that recombinant viruses could be screened for the loss of β-galactosidase phenotype, a simpler phenotype to screen with compared with the more difficult polyhedra phenotype (Kitts and Possee, 1993). This virus, BacPAK6, has been patented, it can be purchased from Clontech and was used as the source of baculoviral DNA in this work.

This chapter describes the expression of the external envelope glycoprotein of Maedi-Visna virus in the baculovirus system. The vector, pAcSG2T was chosen as the transfer vector and was a kind gift from Dr.I.Jones, NERC, Oxford. A recombinant virus is successfully generated and protein production and purification is described. The characterization of the recombinant protein by its ability to be recognised by MVV infected sheep and attempts at its uses in preliminary functional studies are also presented. The BacPAK™ baculovirus expression system was purchased from Clontech to generate recombinant viruses. Additional technical assistance was referenced from King and Possee (1992).

Figure 5.1: The structure of the pAcSG2T transfer vector (adapted from Wang et al., 1995)

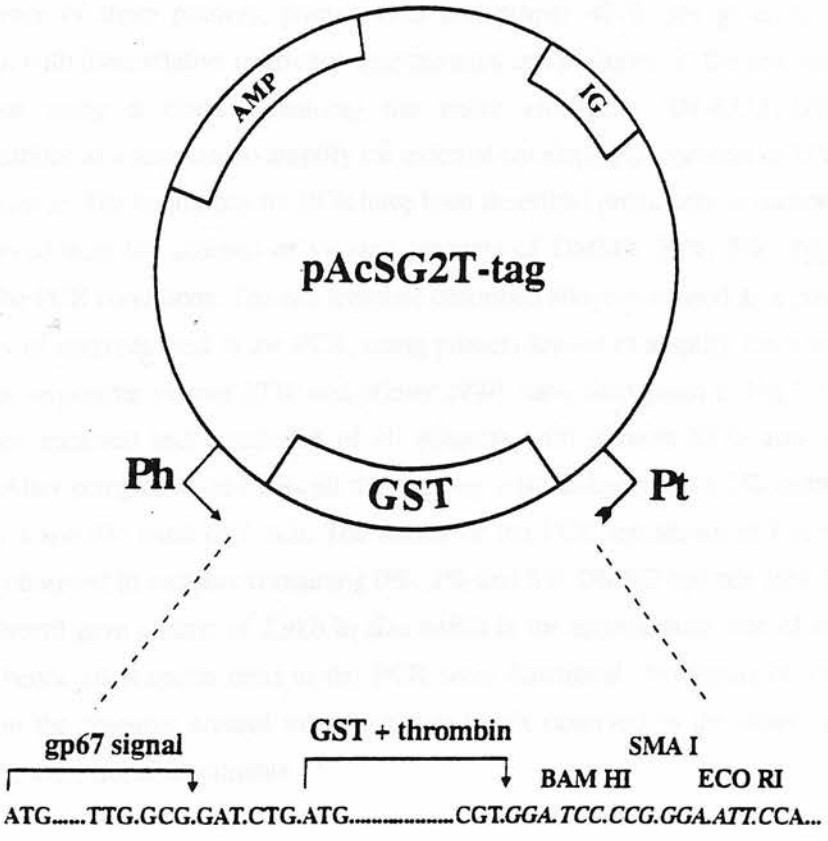
AMP is the gene for β-lactamase giving ampicillin resistance to the plasmid.

IG is the M13 intergenic region.

Ph and Pt are the polyhedrin promoter and terminator, respectively.

The vector backbone is derived from pAcCL29 (Livingstone and Jones, 1989).

The DNA sequences at the 5' and 3' ends of the GST cassette are shown, indicating the position of the multiple cloning site and the thrombin cleavage site. The GST cassette was subcloned from the Sj26 gene, encoding glutathione-S-transferase, and was in phase with the signal peptide sequence from the baculovirus major surface glycoprotein, gp67.


AMP
IG
pAcSG2T-tag
Ph
GST
Pt
gp67 signal
GST + thrombin
SMA I
BAM HI
ECO RI
ATG.....TTG.GCG.GAT.CTG.ATG.....CGT.GGA.TCC.CCG.GGA.ATT.CCA...

5.2 RESULTS

5.2.1 Isolation of *env* fragment by PCR

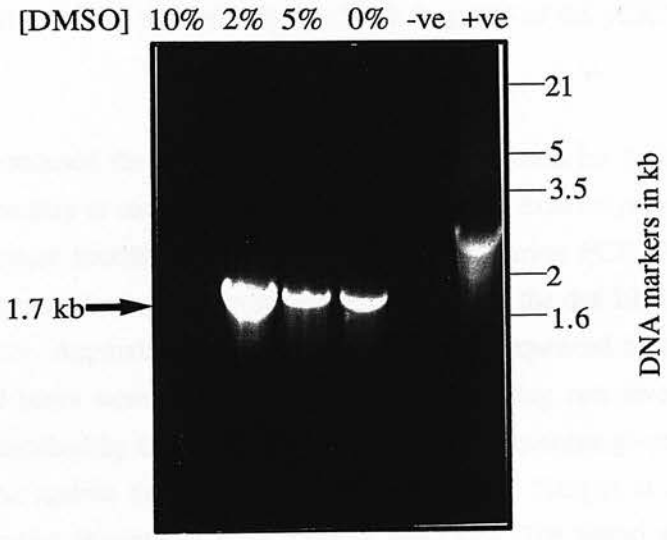
The putative signal sequence of the envelope glycoprotein of MVV is thought to be the first major hydrophobic region from the N-terminus of *env* (Sargan et al., 1991). It is approximately 20 amino acids long and is preceded by a 80 amino acid hydrophilic sequence. PCR primers were designed to amplify the external glycoprotein minus its signal sequence. The sequence of these primers, primer 4975 and primer 4976, are given in Fig.2.1 (see Chapter 2), with their relative restriction enzyme sites and positions on the *env* gene. PCR was carried out using a clone containing the entire *env* gene (Dr.R.G.Dalziel, personal communication) as a template to amplify the external envelope glycoprotein of MVV minus its signal sequence. The conditions for PCR have been described previously in section 2.2.2vi and were followed with the addition of varying amounts of DMSO; 10%, 5%, 2% and 0%, to optimise the PCR conditions. The *env* template described above was used as a positive control for validity of reagents used in the PCR, using primers known to amplify the whole *env* gene. The primer sequences, primer 271P and primer 272P, have been given in Fig.2.1. A negative control was included and comprised of all reagents with primers 4975 and 4976 but no template. After completion of PCR, all the samples were analysed on a 1% agarose/TAE gel to identify a specific band of 1.7kb. The results of the PCR are shown in Fig 5.2. A 1.7kb band was observed in samples containing 0%, 2% and 5% DMSO but not 10% DMSO. The positive control gave a band of 2.9kb in size which is the approximate size of the entire *env* gene and hence all reagents used in the PCR were functional. No bands of any size were observed in the negative control indicating that bands observed in the other samples were specific and were not contaminants.

Since the sample that contained 2% DMSO gave the most efficient PCR, determined by the relative amount of DNA amplified, this was chosen as the optimum conditions. The PCR was repeated to confirm the PCR and 1/10th samples analysed this time on a 1% agarose gel. The concentration was estimated as 4ng/µl by comparing band intensities with those of known amounts of DNA markers.

Figure 5.2: Generation of *env* coding sequence minus the signal peptide sequence

Varying concentrations of DMSO were added to experimental PCR as shown. The negative control was set up as experimental samples, without DMSO and template. The positive control comprised of DNA template with two control primers known to amplify the whole *env* gene. The PCR products were analysed on a 1% agarose/TAE gel.

The 1.7kb fragment is indicated which represents the *env* fragment encoding the external envelope glycoprotein minus its signal sequence.


[DMSO] 10% 2% 5% 0% -ve +ve
1.7 kb
21
5
3.5
2
1.6
DNA markers in kb

The PCR product was then cloned into pCRII™ using the TA cloning kit (Invitrogen, USA) following the manufacturer's instructions. Ligations were carried out with equimolar amounts of vector and insert, and 1µl of the ligation mix was transformed into 'one-shot' INVα-F cells. 1.5% agar plates, precoated with X-gal, were coated with either 25µl or 100µl of transformed cells and colonies allowed to grow for 16 hours at 37°C. The colonies were screened for blue/white phenotype where white colonies represented transformed bacteria containing cloned plasmids. Eight white colonies were picked and one blue colony was picked as a negative control. Colonies were grown up in 5mls of L-Broth/amp for 16 hours and plasmid DNA purified using Qiagen-spin columns. Prior to plasmid DNA purification, 100µl of the cultures were retained and stored in 20% glycerol at -70°C. To determine which of the colonies contained the correct sized PCR fragment, DNA samples were cut with EcoRI and analysed on a 1% agarose/TAE gel. Three out of eight colonies contained a 1.7kb fragment, whilst the other samples contained various sized fragments which were probably contaminants from the PCR. The blue colony selected as a control showed only the 3.9kb fragment of the pCRII™ plasmid when cut with EcoRI.

One of the three colonies that contained the correctly sized fragment was used for further experiments. However, it was necessary to confirm that this fragment was the external portion of *env* and to ensure that no major mutations or deletions had arisen during PCR. The majority of the fragment was sequenced using an automated sequencer with the dye labelled primers described in section 2.2.2v. Approximately 1.3-1.4kb of *env* was sequenced by this method. The remaining 300-400 bases were sequenced by ^{35}S-labelling using two reverse primers (193L and 415H) first described by Carey and Dalziel (1994) and sequences given in Fig 2.1. Sequences were checked against the published sequence of MVV (Sargan et al., 1991) and did not contain any major deletions or other types of mutations. The amino acid sequence was read and compared to that of the published polypeptide sequence (Sargan et al., 1991). The amino acid changes, highlighted in fig.5.3, are consistent with those observed by Carey and Dalziel (1994). Therefore it was acceptable to continue with this clone for further expression work.

Figure 5.3: Amino acid sequence comparison between the published EV1 sequence and the 1.7kb *env* fragment generated by PCR

The predicted amino acid sequence of the *env* clone was compared to that of the published *env* sequence (Sargan et al., 1991). Variable amino acids in the clone are highlighted by astericks. Potential N-glycosylation sites are underlined. Amino acid numbering of the published EV1 sequence is taken from Sargan et al., 1991.

```
        0                                                       49
env     ESKGQCQAEE  VIALIDDPGG  FQRVRQUETV  PVTCVTRNFT  QWGCQPEGAY
EV1     ESKGQCQAEE  LIALIDDPGG  FQRVRQUETV  PVTCVTRNLT  QWGCQPEGAY
        104         *                       *           153

        50                                                      99
env     PDPEIEYRNI  SKEILEQVYC  RDWPWNTYHW  PLWQLENMKS  WMKENEKENK
EV1     PDPEIEYRNI  SKEILEQVYC  RDRPWNTYHW  PLWQLENMKS  WMKENEKENK
        154                     *                       203

        100                                                     149
env     GRTNKTKEDI  DDLLAGRIRG  RFCVPYPYAL  LKCEEWCWYP  TDINEETGHA
EV1     GRTNKTKEDI  DDLLAGRIRG  RFCVPSPYAL  LKCEEWCWYP  TDVNEETGHA
        204                     *                   *   253

        150                                                     199
env     QKIKINCTKA  KAVSCTEKMP  LAGVQRVYWQ  KEDEESMKFM  NIEACTESKL
EV1     QKIKINCTKA  KAVSYTEKMP  LAGVQRVYWE  KEDEETMKFM  NIEACTESKL
        254         *               *       *           303

        200                                                     249
env     RCAQDEKSPG  GCVQGYPIPV  GTEIIPESMK  HLRGKKSPYG  GIKDKNGELK
EV1     RCAQDEKSPG  GCVQGYPIPS  RTEIIPESMK  HLRGKKSPYG  GIKDKNGELK
        304             *   *               353

        250                                                     299
env     LPLTVRVWVU  RMANLSGWVN  GTPPYWSARM  NGSTGINGTR  WYGIGSLHHL
EV1     LPLTVRVWVU  RMANLSGWVN  GTPPYWSARM  NGSTGINGTR  WYGIGSLHHL
        354                                             403

        300                                                     349
env     GFNISSMPNQ  GICNFTKEVL  VGGEKFEYQY  TPSWNCSKNW  TGHPVWHUFR
EV1     GFNISSMPNQ  GICNFTKEVL  VGGEKFEYQY  TPSWNCSKNW  TGHPVWHUFR
        404                                             453
```

```
          350                                                    399
env       YLDMTEHMTS    YCVQRLLRHN    ITVGNGTITG    NCSTTNWDGC    NCSRSGNHLY
EV1       YLDMTEHMTS    YCVQRPLRHN    ITVGNGTITG    NCSTTNWDGC    NCSRSGNHLY
          454                  *                                  503

          400                                                    449
env       NSTTGGLLVL    ICRQSRTIRG    -IMGTNTNWTT   MWEIKNCSSC    ENSTLDRIGN
EV1       NSTTGGLLVL    ICRQSRTITA    IIMGTNTNWTT   MWEIKNCSSC    ENSTLDRIGN
          504                       **   *                       554

          450                                                    499
env       GALGTVKNVN    CRLPHKNESR    KWTCQARRGR--  DRTDSLYIAG    RDFWGRVKAH
EV1       GTLGTIQNIN    CSLPHRNETN    TWTCAARAASRG  NKRDSLYIAG    RDFWGRVKAH
          555  ** *      *   *  **    *   *  *****   ***           606

          500                                                    549
env       YSCESNLGGV    DGMMHQQILL    QKYQIIRVRA    YTGEVDMPQS    YLEKNRRNAF
EV1       YSCESNLGGV    DGMMHQQILL    QKYQIIRVRA    YTGEVDMPQS    YLEKMRRNAF
          607                                                    656

          550  562
env       KKERKKRGIG   LV
EV1       KKERKKRGIG   LV
          657  669
```

5.2.2 Cloning the gene encoding gp135 into the transfer vector, pAcSG2T

A description of pAcSG2T is given in the introduction and it was chosen for the generation of a recombinant baculovirus expressing gp135. The transfer vector contains a multiple cloning site with BamHI, EcoRI and SmaI restriction enzyme sites to facilitate the cloning of foreign DNA. The required portion of *env* was generated by PCR incorporating BamHI and EcoRI restriction sites at the 5' and 3' ends, respectively, to enable directional cloning of *env*.

Initial attempts at inserting *env* into pAcSG2T employed the directional cloning approach. Typically 5μg of pCRII™:env and 8μg of pAcSG2T were both cut with BamHI and EcoRI simultaneously using one-Phor-all™ reaction buffer (Pharmacia, U.K.), following the manufacturer's instructions. Restriction enzyme digests were carried out for 16 hours at 37°C sometimes followed by the addition of an extra aliquot of enzymes for a further 2 hours. Controls were included as two separate digestions of pAcSG2T cut either with BamHI or EcoRI. To prevent religation of the vector pAcSG2T, 1μl of Calf Intestinal Phosphatase (CIP) was added and incubated at 37°C for 1 hour followed by deactivation at 65°C for 15 minutes. The sample was phenol:chloroform extracted twice and DNA was precipitated and resuspended in 10μl TE, pH8.0. An aliquot of cut vector was retained prior to dephosphorylation to control for the efficiency of T4 DNA ligase. All digested samples were analysed on a 1% agarose gel and the correctly sized bands were cut out, extracted from the agarose and resuspended in 10μl TE, pH8.0. 2μl of each sample was run on a 1% agarose/TAE gel to estimate the amount of DNA in the sample. Ligations were set up as previously described in a total volume of 10μl with *env* and pAcSG2T set up in ratios of 4:1, 8:1 and 12:1. Dephosphorylated and phosphorylated, linearised pAcSG2T were included as controls to assess the efficiency of dephosphorylation and activity of the T4 DNA ligase, respectively. 5μl of each ligation reaction was transformed into INVα-F cells and colonies grown on agar plates for 16 hours at 37°C. This approach was repeated 3-4 times without successful ligation between *env* and pAcSG2T, although controls indicated that both CIP and T4 DNA ligase were working.

A second approach was taken whereby cloning was attempted with a single restriction enzyme. The orientation of *env* in pCRII™ was investigated by cutting with BamHI and the rational of this approach is illustrated in Fig.5.4. Option 1 would result in *env* being retained in the vector whereas option 2 would allow *env* to be cut out of the vector and hence cloned into pAcSG2T. Option 2 would result in the additional removal of a small region of the

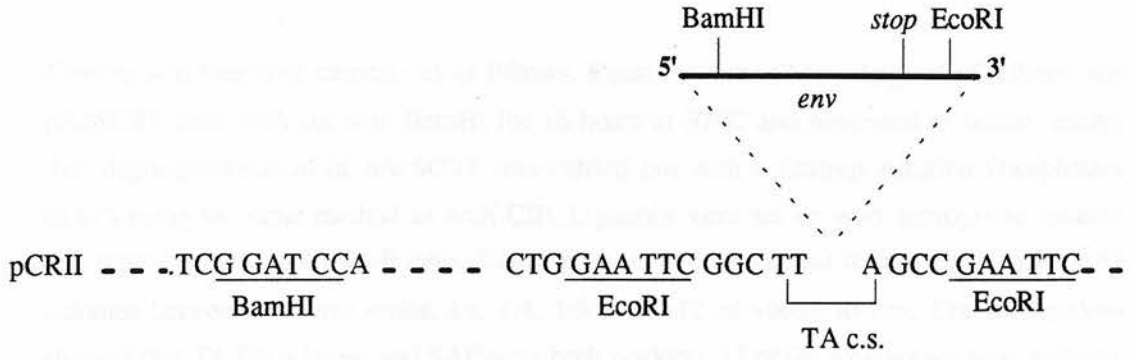
Figure 5.4: Schematic representation of the orientation of *env* in pCRII™

Option1 and option2 show the two possible orientations of *env* in pCRII™ and subsequent position of restriction enzyme sites in *env*.

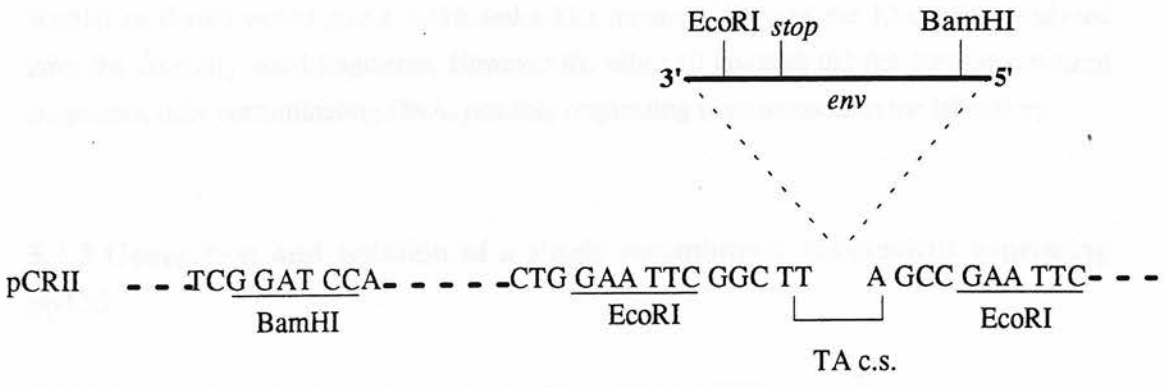
The relevant restriction enzyme sites in pCRII™ vector are underlined and the T.A. cloning site (T.A.c.s.) is indicated.

An explanation of the relevance of option1 and 2 is described in the text.

Option 1



Option 2



pCRII™ vector with *env* but this would not affect the reading frame of gp135 since a stop codon was inserted at the 3' end of *env* by PCR. Cutting with EcoRI was not an option since it would be impossible to determine the orientation of *env* in pCRII™. Subsequent digestion of pCRII™:env with BamHI gave a 3.9kb fragment of pCRII™ and a 1.7kb fragment of *env* indicating that *env* had been cloned in the 3'-5' orientation (Option2).

Cloning was therefore carried out as follows. Equal amounts (2.5μg-4μg) of pCRII:env and pAcSG2T were both cut with BamHI for 16 hours at 37°C and processed as before, except that dephosphorylation of pAcSG2T was carried out with a Shrimp Alkaline Phosphatase (SAP) using the same method as with CIP. Ligations were set up with appropriate controls and transformed into INVα-F cells. Ligations were successful and resulted in a total of 47 colonies between all three ratios, i.e. 1:4, 1:8 and 1:12 of vector to *env*. Control ligations showed that T4 DNA ligase and SAP were both working. 12 of the 47 colonies were selected, 4 from each ratio and grown in 5mls of L-Broth/amp for 16 hours at 37°C. Prior to plasmid purification with Qiagen-spin columns, 100μl of culture was removed and stored in 20%glycerol at -70°C. Plasmid DNA was analysed by restriction enzyme digestion to determine both the presence of *env* and its orientation in pAcSG2T. A correct orientation would give fragments 1.7kb and 8kb when cut with BamHI but only a single cut with EcoRI to give a 10kb fragment. If *env* was in the opposite orientation, 3' to 5', cutting with either BamHI or EcoRI would give a 1.7kb and a 8kb fragment. Two of the 12 colonies analysed gave the correctly sized fragments. However the other 10 colonies did not contain *env*-sized fragments, only contaminating DNA, possibly originating from aerosols in the laboratory.

5.2.3 Generation and isolation of a single recombinant baculovirus expressing gp135

5.2.3i Cotransfection of transfer vector and baculovirus DNA

Insect cells, Sf21, were cotransfected with pAcSG2T:env and baculovirus DNA using Lipofectin™ to introduce DNA into the cells. The viral DNA, BacPAK6, was obtained from Clontech and is supplied as a Bsu361 digested, linear viral DNA. Transfection mixtures included 500ng of transfer vector and 100ng of viral BacPAK6 DNA (Bsu361 digest) made up to 50μl with SDW. As a positive control, the plasmid pBacPAK8-GUS, supplied by Clontech, was cotransfected with viral DNA. This vector has the β-glucuronidase gene located downstream from the polyhedrin promoter and recombination with baculovirus DNA will result in a recombinant virus expressing β-glucuronidase, the presence of which can be

detected by the addition of X-gluc (5-bromo-4-chloro-3-inodyl-β-D-glucurinic acid) to the medium giving a characteristic blue colour. A plasmid map for pBacPAK8-GUS is given in Appendix 1. A negative control was set up with pAcSG2T:env only, to test for contamination in the reagents. To each sample an equal volume of Lipofectin was added and transfection carried out as detailed in section 2.2.5ii. After a 5 hour transfection, medium was replaced with fresh medium. The positive control transfection contained 300μg/ml of X-gluc in the medium. 72 hours after transfection the medium was removed and stored at 4°C as the primary source of recombinant virus. The negative control remained clear and the positive control gave a blue colour in the presence of X-gluc suggesting successful cotransfection and recombination.

5.2.3ii Isolation of a single clone of recombinant virus

To isolate a single clone of recombinant virus it was necessary to perform one round of plaque assay purification. Three ten-fold dilutions were made; 10^{-1}, 10^{-2} and 10^{-3}, of the cotransfection supernatant which contained progeny viruses. A plaque assay was set up with these dilutions, as well as a mock infection and a positive control assay using the BacPAK6 virus, diluted to 10^{-5}. The plaque assays were stained with neutral red in the presence of X-gal. The positive control assay showed numerous plaques that were all stained blue. No blue plaques were observed in the experimental plaques and no plaques were observed in the negative control. A control plaque assay was set up with the cotransfection supernatant derived from the pBacPAK8-GUS transfection to determine the yield and success of generation of recombinant virus expressing GUS. All plaques stained blue in the presence of X-gluc proving a successful and efficient recombination and plaque number increased ten-fold on each dilution indicating a good plaque assay technique. From the experimental assays, 19 plaques were picked, each added to 1ml FCS/TC100 medium and recombinant viruses allowed to diffuse out of the agar for 16 hours at 4°C.

5.2.3iii Screening single recombinant viruses by slot blot hybridisation

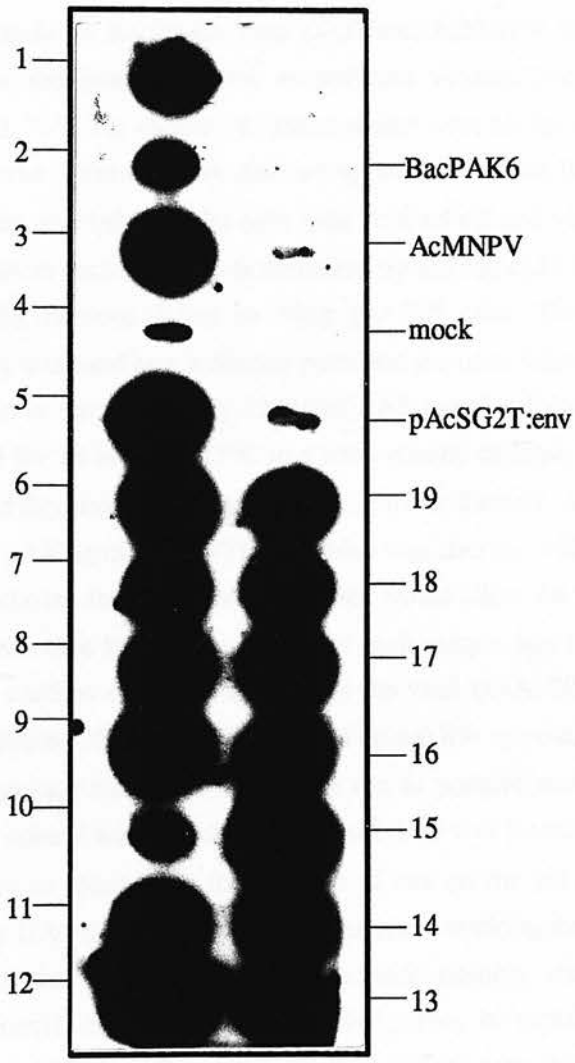
The 19 "plaque picks" isolated above were analysed by slot blot hybridisation for the presence of *env* specific sequences. 35mm petri dishes were seeded with $5x10^5$ cells in 1.5ml of FCS/TC100 and incubated for approximately 2 hours to allow cells to attach to the plastic surface. The medium was removed and each dish was infected with 100μl of one of the "plaque picks". As controls, 100μl of FCS/TC100 only, 100μl of a 10^{-4} dilution of BacPAK6 (parental) virus and 100μl of a 10^{-4} dilution of AcMNPV (wild-type) virus were used to infect separate dishes. After a 1 hour incubation, a further 2mls of medium was added to each dish.

Two to three days post infection the dishes were examined under a light microscope to assess the level of infection. At 72 hours post infection all experimental dishes showed signs of virus infection, that is cells were grainy and misshapen. There were no signs of polyhedra within cells infected with recombinant viruses, a morphology also seen with the parental BacPAK6 virus-infected cells. It was noted that cells from the experimental plates were not as numerous as those in the parental infection. Polyhedra were clearly visible in the AcMNPV infected cells allowing clear differences to be seen between wild-type and BacPAK6 originated viruses. Mock infected cells appeared healthy and showed no signs of virus infection.

The supernatant was removed from each dish and stored at -70^{o}C as the primary stock of that particular virus isolate. 1ml of PBS was added to the infected cell monolayer and cells scraped off into 1.5ml microcentrifuge tubes. DNA was extracted and purified from the cells as described in section 2.2.5ii. Following ethanol precipitation, the viral DNA was incubated in 50µl of TE, pH8.0 for 16 hours at 4^{o}C since baculoviral DNA solubilises slowly due to its large size. The DNA was gently resuspended to avoid shearing. A slot blot was prepared using all 19 experimental samples of DNA, the control DNA and 5pg of pAcSG2T:env as a positive control. Each slot was washed with 200µl of SDW to ensure all viral DNA had passed through onto the filter. The slot blot was probed with ^{32}P-labelled *env*, prepared from pCRII™:env which had been digested with EcoRI and the *env* fragment purified. The filter was exposed to film and allowed to develop for 72 hours at -70^{o}C. The results from the slot blot are shown in Fig. 5.5. A positive band is seen for pAcSG2T:env, although the intensity is weak compared to the experimental samples, due to overloading of cell-extracted DNA. All recombinant viruses gave a positive signal, except 4, compared to negative control backgrounds, BacPAK6, AcMNPV and mock.

Figure 5.5: Slot blot hybridisation of 19 recombinant *env* baculoviruses

Recombinant baculovirus DNA was extracted, purified and probed for *env* sequences by slot blot hybridisation. Samples 1 to 19 represent the recombinant viruses. AcMNPV, BacPAK6 and mock are negative controls and pAcSG2T:env is the positive control.


1
2
3
4
5
6
7
8
9
10
11
12
BacPAK6
AcMNPV
mock
pAcSG2T:env
19
18
17
16
15
14
13

5.2.4 Evaluation of recombinant viruses

5.2.4i Analysis of recombinant virus genomes by Southern hybridisation

Analysis of the "plaque picks" by slot blot hybridisation identified 18 recombinant virus by comparison to the background controls of BacPAK6 viral DNA and AcMNPV DNA. To confirm the presence of *env* within the genomes of the recombinant viruses, 7 of the 18 samples were selected; samples 1, 3, 7, 9, 12, 14 and 16. 35mm dishes were set up as before and infected with 100µl of each virus. Controls were also set up as before with BacPAK6 virus, AcMNPV and mock. 72 hours post infection the cells were scraped off and viral DNA extracted. The concentration of DNA in each sample was measured by reading O.D. values at 260nm and yields ranged typically between 2.5µg to 10µg per 10^6 cells. The sample containing the least amount of DNA was used as a reference point and the other samples were diluted to give the same concentration (approximately 125ng/µl). All samples including the controls were digested with BamHI for 16 hours at 37°C in a total volume of 25µl, followed by the addition of fresh enzyme and digestion was continued for a further 2 hours. A positive control was included: approximately 120ng of pAcSG2T:env which was also cut with BamHI in a volume of 10µl. BamHI was chosen for the digests since this would allow the whole of *env* to be cut out of the viral genome. One fifth of the volume of each sample was run on an analytical 1% agarose/TAE gel to confirm complete digestion of the viral DNA. 20µl of the samples, adjusted to equal concentrations (500ng/20µl), were run on a 0.8% agarose/TBE gel for 16 hours at 18°C. pAcSG2T:env and BacPAK6 DNA were run as positive and negative controls, respectively. The positive control was diluted to 6pg/µl and 10µl was loaded onto the gel as well as an undiluted aliquot to ensure that the position of *env* on the gel could be recorded. The gel was visualised by U.V. illumination and photographed, marking the position of *env* with a ruler. Analysis of the DNA by ethidium bromide staining showed the characteristic band pattern for BamHI-digested AcMNPV DNA. This is explained and illustrated in Fig.5.6a, where the position of *env* is also indicated in respect of the fragments of AcMNPV DNA. DNA was denatured, neutralized and transferred to a nylon membrane. The blot was probed with ^{32}P-labelled *env* prepared as described in section 5.2.3iii. The blot was exposed to film for 3-5 days at -70°C and the autoradiograph developed. The results are shown in Fig.5.6b. All 7 recombinant viruses showed a band of equal size to *env* in the positive control (pAcSG2T:env), that is 1.7kb, and this band was not observed in the negative control, BacPAK6 DNA. A non-specific band, approximately 2.3kb in size, was occasionally observed in all virus derived samples, wild-type or recombinant. An additional band is seen in sample 16 at approximately 2.6kb, and this is explained fully in the discussion. The intensity

of the 1.7kb band in each sample is not equal, despite attempts to load equal amounts of DNA.

5.2.4ii Analysis of protein expression by western blotting

Recombinant protein was expected to be secreted into the medium. Therefore the supernatants from the above experiment were retained for analysis of the protein content. Each sample was dialysed against PBS for 16 hours at 4°C in 1.5ml microcentrifuge tubes sealed with dialysis tubing. This was necessary since the pH of insect medium is between 6.1-6.4, and a pH of 7.2 is required for maximum absorption onto glutathione beads. 50µl of 50% glutathione-agarose bead slurry was added to each sample and mixed for 30 minutes at 4°C. Control samples of AcMNPV, BacPAK6 and mock infected cells were treated in the same way. The beads were washed twice in ice-cold PBS and resuspended in 50µl of PBS. An equal volume of 2x reducing buffer was added to each sample, boiled and loaded onto a 5-15% gradient gel. Proteins were separated by SDS-PAGE using normal conditions and transferred onto a nitrocellulose membrane. After blocking, the blots were probed either with rabbit anti-GST serum or normal rabbit serum, each diluted 1/100. The production of the anti-GST serum is described in Chapter 3. Protein analysis was performed by western blotting since this method is easy to set up and has sufficient sensitivity for detecting small quantities of protein, compared to coomassie-blue staining and radiolabelling. The results from these blots are shown in Fig. 5.7a and b. A single, specific band was observed in all 7 recombinant virus samples which was absent in the negative controls. The estimated molecular weight of this band is 97-100kDa and will be referred to as GST:SUenv. To confirm the identity of GST:SUenv, the protein was run down a second gel and probed with the following antisera: rabbit anti-P1 serum, rabbit anti-P2 serum, including their pre-bleeds (descriptions of these sera are given in Chapter 4), and an anti-Mouse Herpes Virus serum which contains anti-GST antibodies (this was a kind gift from Dr.J.Stewart, Department of Veterinary Pathology, Edinburgh). All three immune sera recognised GST:SUenv, as shown in Fig. 5.7c, and no comparable band was identified by the negative control sera. This confirmed that the band observed was GST:SUenv.

The titres for each recombinant virus stock was calculated by performing a plaque assay.

Figure 5.6:Southern hybridisation of DNA from baculovirus-infected cells

a] Ethidium bromide stained 1% agarose gel of BamHI-digested DNA

Lane 1, recombinant baculovirus; BacPAK:env12
Lane 2, parental virus; BacPAK6
Lane 3, wild-type virus; AcMNPV
Lane 4, mock infected Sf21 cells
Lane 5, transfer vector containing *env*; pAcSG2T:env

The BamHI-digested fragments of baculovirus DNA are indicated (N.B. fragments F and G are not visible) and their sizes are given below (adapted from King and Possee, 1992)

	Kb
A	87
B	23
C	9
D	4.5
E	3.2
F	0.9
G	0.4
mol wt. AcMNPV DNA	128

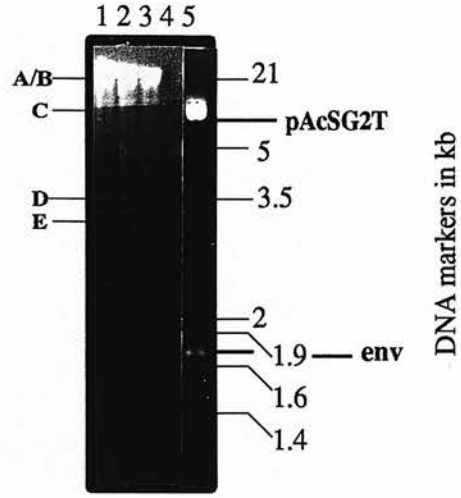
b] Hybridisation results of screening recombinant virus DNA

DNA purified from seven different recombinant viruses were digested with BamHI and probed with a ^{32}P-labelled *env* fragment derived from pCRII:env.

Lane 1,7,9,12,14,16, recombinant baculoviruses; BacPAK:env (refer to Fig.5.5)
Parental virus, BacPAK6 (negative control) and pAcSG2T:env (positive control) are labelled.

The band corresponding to the *env* fragment is indicated.

A


1 2 3 4 5
A/B
C
D
E
21
pAcSG2T
5
3.5
2
1.9
env
1.6
1.4
DNA markers in kb

B

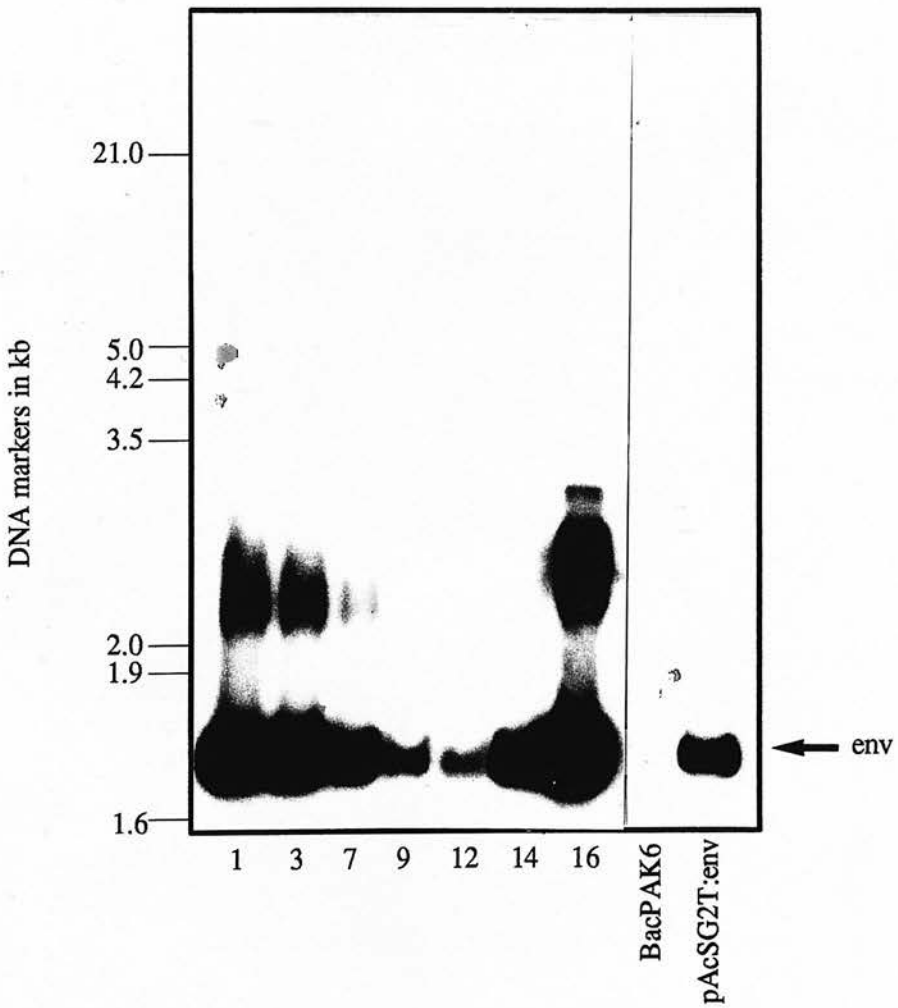

DNA markers in kb
21.0
5.0
4.2
3.5
2.0
1.9
1.6
env
1
3
7
9
12
14
16
BacPAK6
pAcSG2T:env

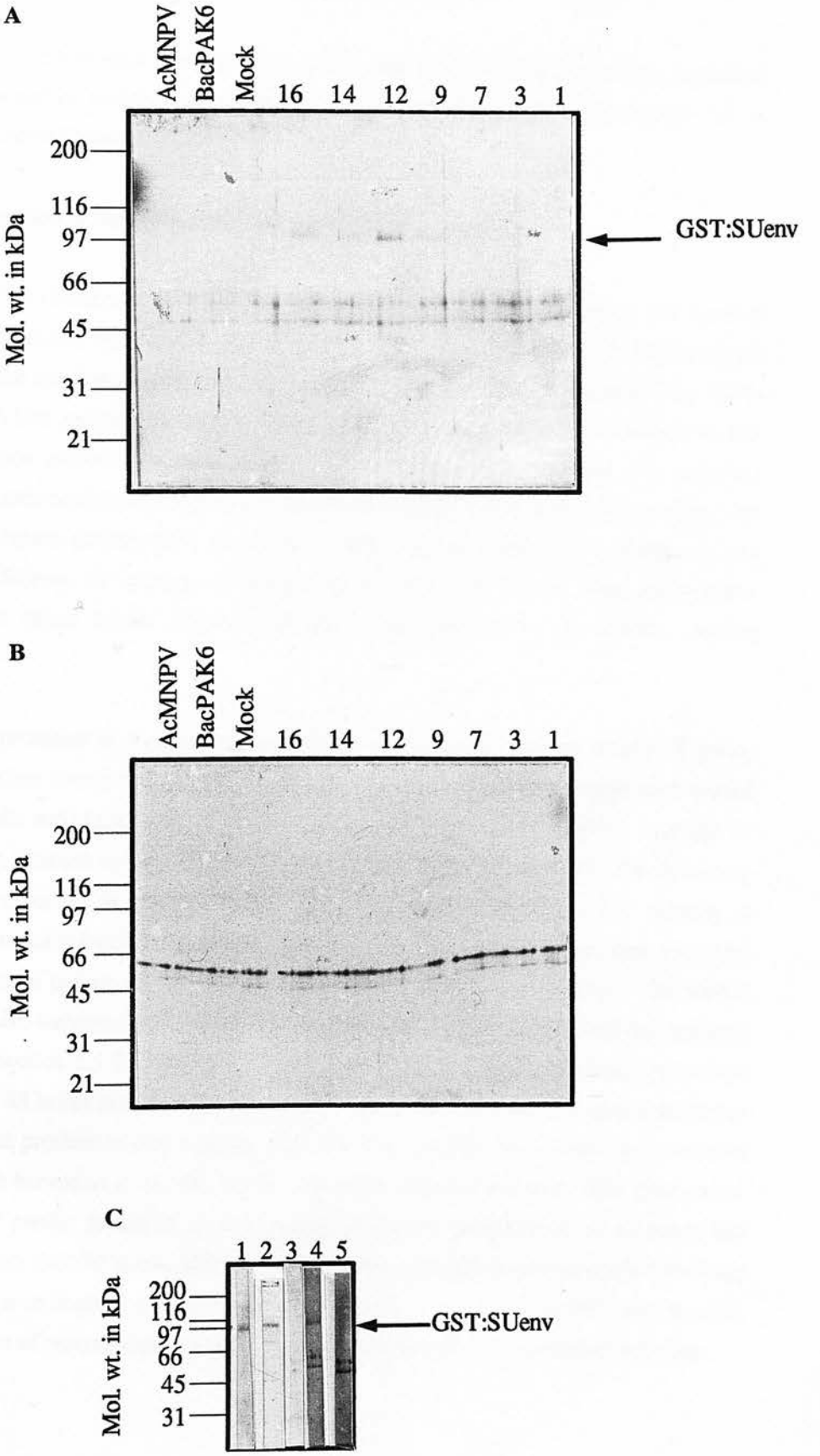
Figure 5.7: Characterization of recombinant protein, GST:SUenv, secreted by baculovirus-infected cells

a and b] Seven recombinant viruses were selected and used to infect insect Sf21 cells. Supernatants were harvested and analysed on 5-15% SDS-PAGE gels, followed by transfer to nitrocellulose for western blotting. Blots were probed with a rabbit antiserum specific for GST (1/100 dilution) (A) or normal rabbit serum (1/100)(B).

Lane 1,3,7,9,12,14 and 16, Recombinant viruses; BacPAK:env
Parental (BacPAK6), wild-type (AcMNPV), uninfected Sf21 cells (mock) are labelled.

c] The identification of GST:SUenv was confirmed using two rabbit antisera specific for a gp135-derived peptide (1/100) (P1 and P2) and a rabbit anti-MHV IgG containing anti-GST antibodies. The prebleed of P1 and P2 (pre-P1 and pre-P2), diluted 1/100, was used as a negative control.

lane 1, anti-GST
lane 2, anti-P1
lane 3, anti-P1, prebleed
lane 4, anti-P2
lane 5, anti-P2, prebleed


A
AcMNPV
BacPAK6
Mock
16
14
12
9
7
3
1
200
116
97
66
45
31
21
Mol. wt. in kDa
GST:SUenv
B
AcMNPV
BacPAK6
Mock
16
14
12
9
7
3
1
200
116
97
66
45
31
21
Mol. wt. in kDa
C
1 2 3 4 5
200
116
97
66
45
31
Mol. wt. in kDa
GST:SUenv

5.2.5 Characterization of recombinant protein production

Sample 12 gave the strongest band by western blot analysis indicating that it was expressing the greatest amount of recombinant protein. It was selected for further analysis and will be referred to as BacPAK:env12.

5.2.5i Determination of the optimum time point for virus production

To quantify the production of GST:SUenv over time, it was necessary to verify that western blotting was a suitable method for quantifying the production of protein. Protein samples obtained from the previous experiment were pooled, titred and proteins separated by SDS-PAGE. Western blot analysis showed that quantitative differences could be visualised by this method (data not shown). Western blotting was chosen as the method for detecting recombinant protein because of its relative ease to perform and it was sufficiently sensitive for detecting recombinant protein (data not shown). Since normal rabbit serum controls did not recognise GST:SUenv, all subsequent figures do not show the results from the negative control. Normal rabbit serum controls (negative) were included in all western blotting experiments.

The polyhedrin promoter in most expression studies is maximum between 18 and 72 hours, and therefore a time course was set up as follows. Ten 35mm petri dishes were each seeded with $1.5x10^5$ cells and these were allowed to attach over a period of 2 hours. 7 of the 10 dishes were each infected at a m.o.i. of 10 p.f.u. of BacPAK:env12 per cell. The remaining three dishes were set up as controls with a 10^{-4} dilution of BacPAK6, a 10^{-4} dilution of AcMNPV and a mock infection. The supernatant from the experimental dishes were harvested at the following time points: 18, 24, 30, 42, 48, 66 and 72 hours post infection. The control infections were also harvested at 72 hours. The recombinant protein was purified and analysed as described in section 2.5.2ii. Fig. 5.8a shows that there is a gradual increase in protein production up to 48 hours post infection. After this time point there did not appear to be any increase in protein production and a steady state was reached. The time course was extended and samples were harvested at 24, 48, 72, 96 and 120 hours post infection. This time course showed a similar profile as before in that protein production increased up to 48 hours and remained maximum until 72 hours, after which there was a decline in protein production (data not shown). This is in contrast to recent work published by Kwang et al. (1995) who reported that the production of recombinant envelope is still maximum after 72 hours post infection.

The kinetics of protein production was investigated further. To understand if protein production was maximum at 48 hours followed by a steady state being reached between protein production and protein degradation, the following experiment was devised. A 35mm dish was seeded with $1.5x10^5$cells as usual and infected with BacPAK:env12 at an m.o.i. of 10 p.f.u. per cell. Every 24 hours, the supernatant was removed and replaced with fresh medium. GST:SUenv was purified from the supernatant as before and mixed in an equal volume of 2x reducing buffer and then analysed by western blotting with anti-GST serum. Fig.5.8b shows the result of this "milking" experiment. Protein production was shown to be maximum at 48 hours after which protein production was no longer detectable suggesting that recombinant protein remains stable in the supernatant for up to 72 hours as was observed in the extended time course experiment.

A comparison was made between cell-associated recombinant protein and secreted protein to extend the above kinetic studies. Ten 35mm dishes were each seeded with $1.5x10^6$cells in 1.5ml of FCS/TC100 medium as before. 7 of the dishes were each infected with $1.5x10^7$ p.f.u. of BacPAK:env12, and the remaining three were set up as controls described previously. The supernatants were harvested and purified every 24 hours as before. The cells were scraped off from the dishes into 1ml of PBS, pelleted, resuspended into 100µl of PBS/0.1%(v/v) TritonX-100 and repelleted. The supernatant was removed and 50µl of glutathione-agarose slurry was added and mixed for 30 minutes at 4^oC. Cell-associated protein and secreted protein were compared and analysed by western blotting using anti-GST serum. The results are shown in Fig.5.8c. The principal observation made was that there appears to be a greater yield of protein from the supernatant than from that extracted from the cells. At 24 and 48 hours the size of recombinant protein seen in cells is approximately 5-10kDa larger than the secreted protein. However at 72 hours, protein purified from cells shows the presence of the same sized protein as the secreted form as well as the larger protein. The significance of these different sized proteins is discussed later. The presence of other contaminating products such as those between 20-35kDa may be a reflection on the state of the insect cells on infection. If cells are past their log-phase the presence of proteases in the medium secreted by dying cells may lead to degradation of recombinant protein.

Figure 5.8: Analysis of the kinetics of GST:SUenv expression

a] A time course for recombinant protein production

Insect sf cells were infected with recombinant baculovirus and protein harvested at: 18, 24, 30, 42, 48, 66 and 72 hours post infection. Protein was purified, analysed on 5-15% SDS-PAGE gels and transferred to nitrocellulose for western blotting. Blots were probed with rabbit anti-GST serum (1/100). Control infections are shown.

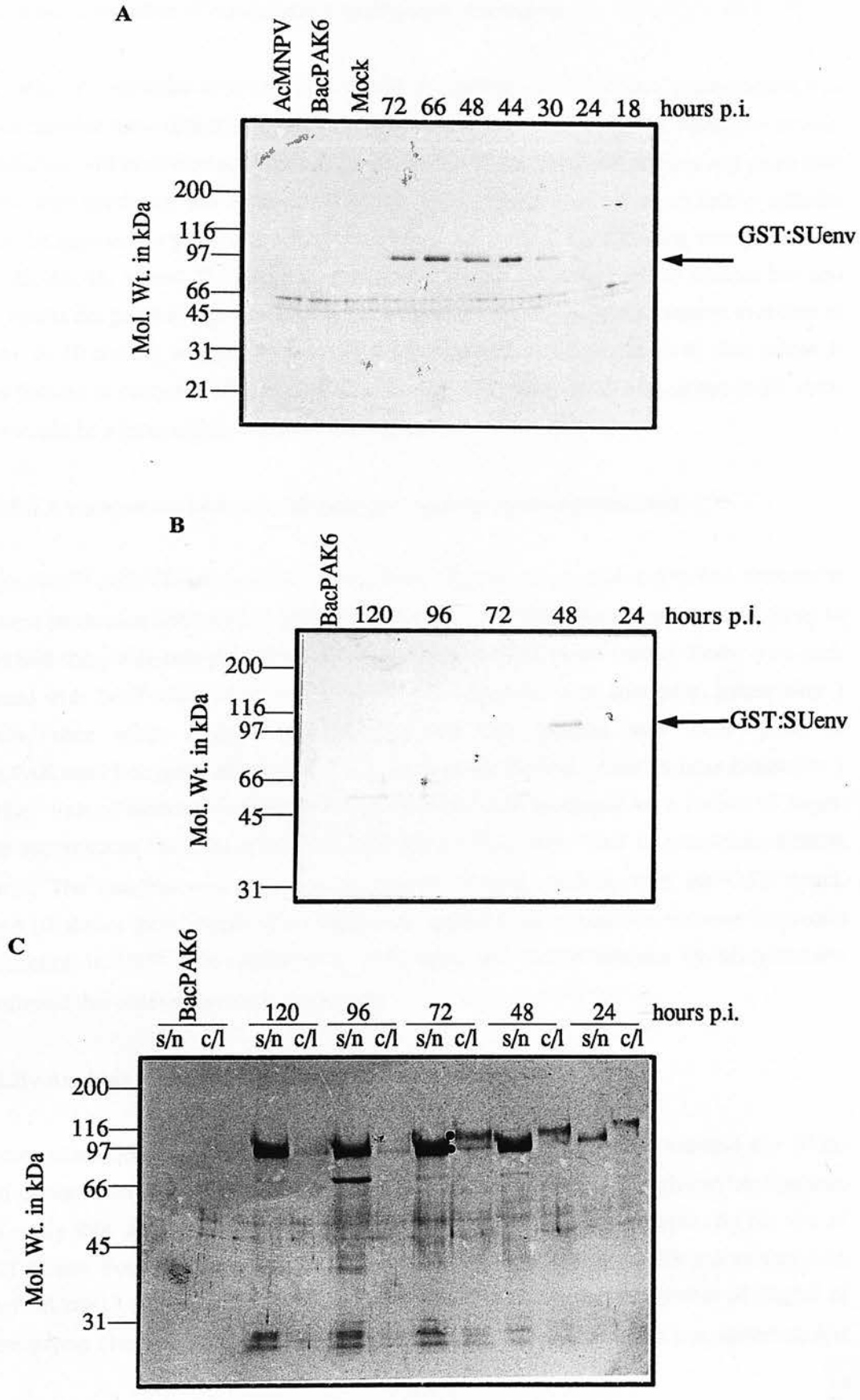
b] Determination of the maximum time point for GST:SUenv production.

Supernatant was collected every 24 hours from insect sf cells infected with recombinant baculovirus and fresh medium was added to the cells. Proteins were purified and analysed as in [a].

c] Characterization of GST:SUenv release from infected cells

A time course was set up and protein was purified from the infected cells as well as the supernatant. Proteins were analysed as in [a].

c/l, cell-associated GST:SUenv
s/n, secreted GST:SUenv


A
AcMNPV
BacPAK6
Mock
72 66 48 44 30 24 18 hours p.i.
200
116
97
66
45
31
21
Mol. Wt. in kDa
GST:SUenv
B
BacPAK6
120 96 72 48 24 hours p.i.
200
116
97
66
45
31
Mol. Wt. in kDa
GST:SUenv
C
BacPAK6
120 96 72 48 24 hours p.i.
s/n c/l s/n c/l s/n c/l s/n c/l s/n c/l s/n c/l
200
116
97
66
45
31
Mol. Wt. in kDa

5.2.5ii Determination of the optimum multiplicity of infection

To assess the optimum m.o.i. required for the production of GST:SUenv, a comparison was made between three different m.o.i.; 5, 10 and 20 p.f.u. per cell. Since the kinetics of protein production will be altered according to the proportion of insect cells infected at any given time point, the experiment was followed over time. 35mm dishes were set up as before, infected with the appropriate p.f.u. of BacPAK:env12 and harvested at the following time points; 18, 24, 30, 42, 48, 60 and 72 hours post infection. Analysis was carried out by western blot and the results are given in Fig. 5.9. The protein production showed as similar pattern over time at m.o.i. of 10 and 20, whereas an m.o.i. of 5 gave lower levels of protein at all time points. It was decided to carry out all subsequent experiment at an m.o.i of 10 as opposed to 20, since this would be a more efficient use of virus stocks.

5.2.5iii A comparison between different cell types for protein production

High Five™ cells (Invitrogen, USA) have been reported to produce a five-fold increase in protein production compared to Sf21 cells (Davis et al., 1993). An experiment was set up to compare the yields between these two cell types. Four T25 tissue culture flasks were each seeded with $2x10^6$cells in 5ml of appropriate medium. Cells were allowed to adhere over 3 hours, after which medium was removed and cells infected with $2x10^7$ p.f.u. of BacPAK:env12 or mock infected in 1ml of appropriate medium. After an hour incubation a further 4mls of medium was added to each flask and cells incubated for a further 48 hours. The supernatants were harvested, dialysed and purified with 100µl of glutathione-agarose slurry. The samples were analysed by western blotting, probing with anti-GST serum. Fig.5.10 shows these results. There does not appear to be a dramatic increase in protein production in H5™ cells compared to Sf21 cells, and further analysis by silver-staining confirmed this observation (data not shown).

5.2.5iv Analysis of the glycosylation state of GST:SUenv

Recombinant GST:SUenv was identified as a 97-100kDa protein. The estimated size of the non-glycosylated protein is 90kDa, which suggests that GST:SUenv is a glycosylated protein. To verify this, an experiment was set up to asses the effect of tunicamycin on the size of GST:SUenv. Four T25 tissue culture flasks were set up as in section 5.2.5iii and infected with BacPAK:env12 at a m.o.i. of 10 or mock infected, in the presence or absence of 1µg/ml of tunicamycin (1mg/ml stock in DMF, stored at -20^{o}C). The supernatant was harvested and

purified as normal 48 hours post infection. Recombinant protein was analysed by western blot, probing with anti-GST serum or an anti-gp135 peptide serum. The results are shown in Fig. 5.11. In the presence of tunicamycin, the size of GST:SUenv is reduced to approximately 85-90kDa, suggesting that GST:SUenv is indeed glycosylated. An accurate prediction of the size of each protein species is difficult to deduce on a mini protein gel, but was deemed sufficient to demonstrated that GST:SUenv is a glycosylated protein. Both sera recognised the same sized protein confirming that it was GST:SUenv which had not been glycosylated in the presence of tunicamycin.

5.2.6 Further purification of GST:SUenv

To further purify GST:SUenv by elution from glutathione beads or removal of the GST moiety, large scale preparation of recombinant protein was routinely carried as follows. 100ml spinner cultures were set up with $2x10^5$cells/ml and grown for approximately 48 hours until the cell density reached $1x10^6$cells/ml. Either H5 or Sf21 cells were used depending on the cell viability at the time of preparation. The culture was infected with $1x10^9$ p.f.u. of BacPAK:env12, that is a m.o.i. of 10 p.f.u. per cell. 48 hours post infection the infected cell culture was collected and the supernatant clarified. Following dialysis against PBS, the supernatant was mixed with 2mls of glutathione-agarose slurry for 30 minutes at 4^oC.

5.2.6i Elution of GST:SUenv from glutathione beads

Attempts at eluting GST:SUenv from the beads were initially unsuccessful. Elution was carried out at room temperature using 10mM reduced glutathione/50mM Tris-HCl, pH8.0, mixing the beads with elution buffer for 2 minutes for every elution. This protocol was not successful, despite increasing both the concentration of reduced glutathione to 20mM and elution time to 30 minutes. An alternative protocol has been reported by Davies et al. (1993), in which elution was carried out at 4^oC. Elution was therefore attempted at this temperature using 10mM reduced glutathione and a two minute incubation period and proved to be successful with the majority of protein eluting in the first elution (data not shown). The yield of eluted GST:SUenv was measured by the Bio-Rad protein assay. Typically yields ranged between 0.5-0.8mg protein per 1 litre of infected cells, that is per $1x10^9$ infected cells.

Figure 5.9 Determination of the optimum m.o.i. for protein expression

Time courses were carried out at three different m.o.i.; 20, 10 and 5 p.f.u. per cell (a, b and c, respectively in figure) and protein harvested from the supernatant at the following time points: 18, 24, 30, 42, 48, 66 and 72 hours post infection. Recombinant protein was purified, analysed on 5-15% SDS-PAGE gels and transferred to nitrocellulose for western blotting. Blots were probed with rabbit anti-GST serum (1/100). Control infections were included as shown.

Figure 5.10 A comparison of GST:SUenv expression in two insect cell types

High five or sf cells were infected with recombinant virus at an m.o.i. of 10 p.f.u. per cell and secreted protein was harvested and purified. Proteins were analysed as above, and uninfected cells were included as controls.

lane 1, H5 cells, uninfected.
lane 2, H5 cells infected with BacPAK:env12.
lane 3, Sf21 cells, uninfected.
lane 4, Sf21 cells infected with BacPAK:env12.

Figure 5.11 Effect of tunicamycin on GST:SUenv expression

Insect cells were infected with recombinant viruses at an m.o.i. of 10 p.f.u. per cell, with or without tunicamycin. Uninfected cells were treated in the same way. Proteins were purified and analysed as above, and probed with rabbit anti-GST serum (1/100) (GST) or a rabbit serum specific for a gp135-derived peptide (1/100) (P2).

T+, tunicamycin treated cells
T-, untreated cells
V, cells infected with BacPAK:env12
M, uninfected cells

Fig. 5.9

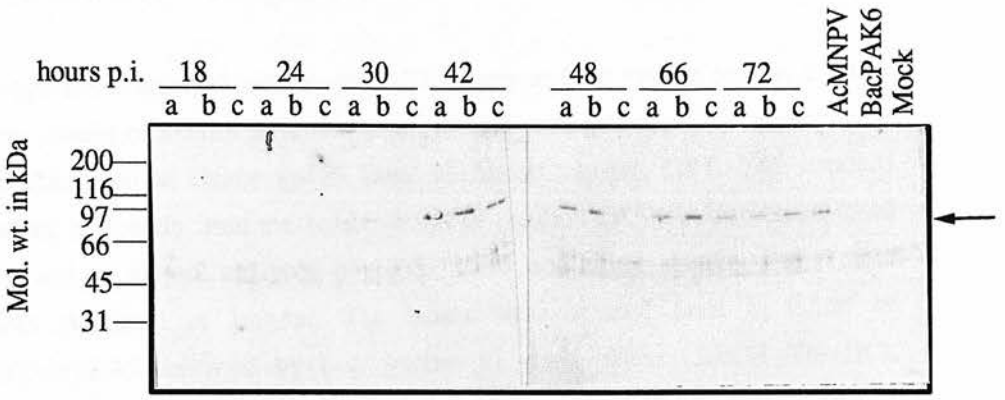


Fig. 5.10

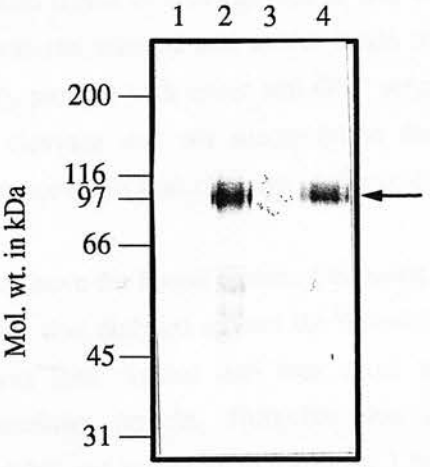
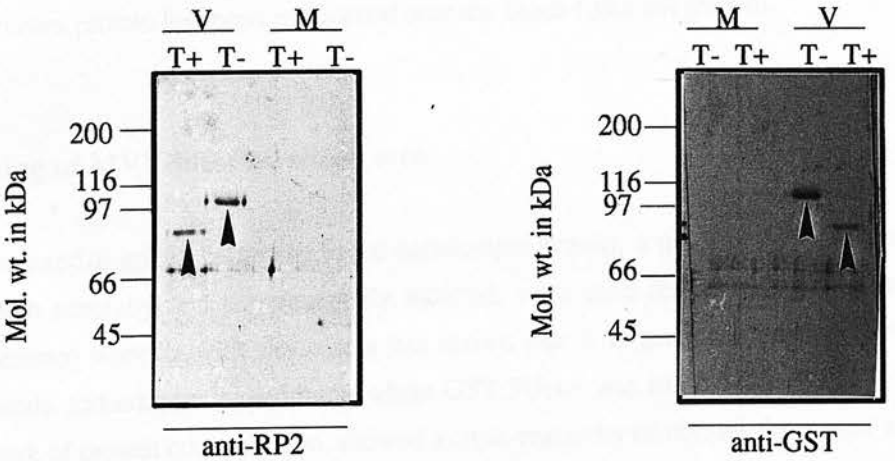


Fig. 5.11



5.2.6ii Cleaving gp135 from the GST moiety

A thrombin cleavage site is situated between the GST moiety and the foreign protein to allow purification of the protein of interest as a non-fused recombinant protein. Two experimental approaches were attempted to cleave gp135 from its fusion partner, GST. The standard protocol for cleaving is directly from the beads. 0.5ml of purified GST:SUenv/agarose-bead slurry was taken and an aliquot analysed by SDS-PAGE and silver staining and protein concentration was estimated at 1mg/ml. The beads were washed once in 0.5ml of 0.1%TritonX-100(v/v)/PBS followed by two washes in wash buffer (50mM Tris-HCl, pH7.5/150mM NaCl) and finally resuspended in 0.5ml of thrombin cleavage buffer (2.5mM $CaCl_2$ in wash buffer). 2.5µg of thrombin was added and incubated for 1 hour at 25°C. The beads were eluted four times with 0.5ml of cleavage buffer and each sample analysed by comparison to a sample that was not cleaved and to the beads remaining after cleavage. Western blotting was performed, probing with either anti-GST serum or anti gp135-peptide serum. Analysis revealed that cleavage was not successful in that GST:SUenv was still detectable on the glutathione-agarose beads post cleavage (data not shown).

A second approach was taken to cleave the fusion protein. Following a large scale preparation and elution, the resulting product was dialysed against the thrombin cleavage buffer for 16 hours at 4°C. The sample was then divided into four equal amounts each containing approximately 50µg of recombinant protein. Thrombin was added in the following proportions; 0%, 0.5%, 1% and 2% and incubated at 25°C for 1 hour. To remove GST and leave the cleaved product in solution, 100µl of glutathione-agarose slurry was added to each sample and mixed for 30 minutes at 4°C. The supernatant was retained and beads were resuspended in a volume equal to the supernatant. Samples were compared by western blot analysis as above. Non of the supernatants contained any cleavage products and it appeared that all recombinant protein had been reabsorbed onto the beads (data not shown).

5.2.7 Screening of MVV-infected sheep sera

To further characterize gp135 expressed in the baculovirus system, a panel of MVV-infected sheep sera, both naturally and experimentally infected, were used for screening purposes. Previous experience working with sheep sera has shown that it is generally "sticky" giving high backgrounds. Indeed, pilot experiments where GST:SUenv was loaded onto SDS-PAGE gels, irrespective of protein concentration, showed a cross-reactivity of normal sheep sera with GST:SUenv. In order to successfully screen sheep sera, it was necessary to titrate out the

antigen to establish the amount of antigen required for specific detection of anti-gp135 antibodies in sheep. Antigen was loaded as three different amounts; 160ng, 80ng and 40ng/lane and probed with four normal sheep sera, one MVV-infected sheep sera and rabbit anti-GST serum. These blots confirmed earlier observations that normal sheep sera does cross-react with antigen but this was reduced to undetectable levels at 40ng/lane (data not shown). The ability of anti-MVV infected sheep sera to recognise GST:SUenv was not proportional to the amount of antigen loaded as determined by the intensity of the specific band for GST:SUenv. Subsequent screening with a panel of sheep sera was carried out as shown in Fig. 5.12. Sheep sera used in this screening where from naturally infected sheep (Dr.N.Watt, personal communication) or from experimentally infected sheep sera (Dr.B.Blacklaws and Dr.P.Bird, personal communication). All sera contained anti-*env* antibodies as determined by western blot analysis with MVV-infected crude cell lysates (data not shown). The results of the screening showed that MVV-infected sheep sera were able to recognise GST:SUenv but not GST (derived from bacteria containing pGEX2T plasmid). Normal sheep sera did not recognise GST:SUenv nor GST. Rabbit anti-gp135 peptide serum (P2) and its prebleed were used as controls (data not shown).

5.2.8 Functional studies with GST:SUenv

Preliminary experiments were carried out to determine the ability of GST:SUenv, expressed in the baculovirus system, to bind specifically to sheep fibroblasts.

5.2.8i Detection of GST:SUenv binding to sheep fibroblasts by immunoflourescence

Twenty four well plates were seeded with $1x10^5$fibroblast cells/well with each well containing a sterile glass coverslip, and incubated for 16 hours in a $5\%CO_2/37^oC$ incubator. The following dilutions of GST:SUenv and GST were made up in 2%FCS/DMEM: 8μg/ml, 4μg/ml and 2μg/ml, and 0.5ml of each dilution was added to a single well in duplicate. As controls, a mock incubation was carried out with medium only. Cells were incubated with antigen for 2 hours in a $5\%CO_2/37^oC$ incubator. Cells were washed three times with unsupplemented DMEM and either rabbit anti-GST serum or normal rabbit serum (diluted 1/100 in 2%FCS/DMEM) was added to the cells. After an hour incubation using the same conditions as previously the wells were washed and incubated in anti-rabbit IgG-FITC (diluted 1/250) for a further hour. After washing, the cells were fixed with ice-cold methanol for 5 minutes, air-dried and mounted onto glass slides in 2-3μl of Citiflour. Cells were examined using a fluorescent microscope to analysis the intensity of fluorescent staining. As a positive

Figure 5.12: Reactivity of MVV-infected sheep sera with GST:SUenv

GST:SUenv was purified from a large scale (100ml) infection. Protein concentration was calculated and analysed on a 5-15% SDS-PAGE gel at a concentration of 400ng/gel. As a control, bacterial-derived GST was also analysed at the same concentrations. Proteins were transferred to nitrocellulose for western blotting and strips were probed with the following panel of sheep sera, using pre-bleeds or normal sheep sera (sheep 05,151,G16) as background controls. Sera were diluted 1/20. Protein markers are not shown to allow comparisons between GST and GST:SUenv to be made. The position of GST:SUenv is indicated by an arrow.

sheep number	type of infection	date of pre-bleed	date of final bleed	reactivity on immunoblot
186	natural	UN	UN	+
187	natural	UN	UN	+
848A	experimental	UN	UN	+
1231T	experimental	29:10:92	25:5:94	+
1299T	experimental	5:12:91	17:10:94	+
1244T	experimental	29:10:92	25:5:94	+
1550	experimental	13:12:88	6:2:91	+
1540	experimental	13:12:88	2:4:92	+
1091R	experimental	20:1:92	30:6:93	+
1071R	experimental	20:1:92	30:6:93	+
1078R	experimental	20:1:92	30:6:93	+
05	-	-	-	-
151	-	-	-	-
G16	-	-	-	-

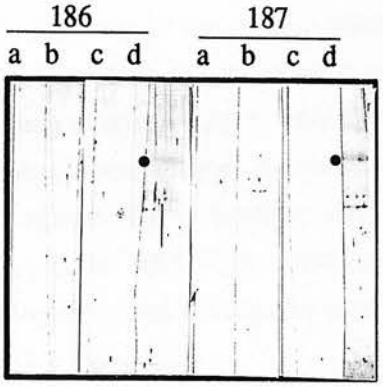
note: UN; unknown

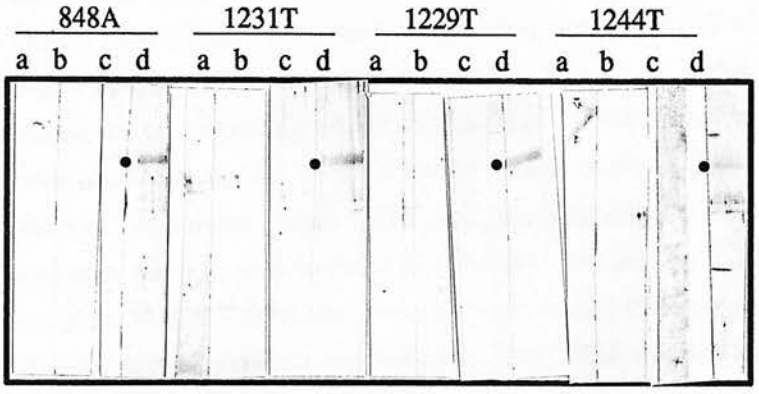
lane a= GST + normal sheep serum

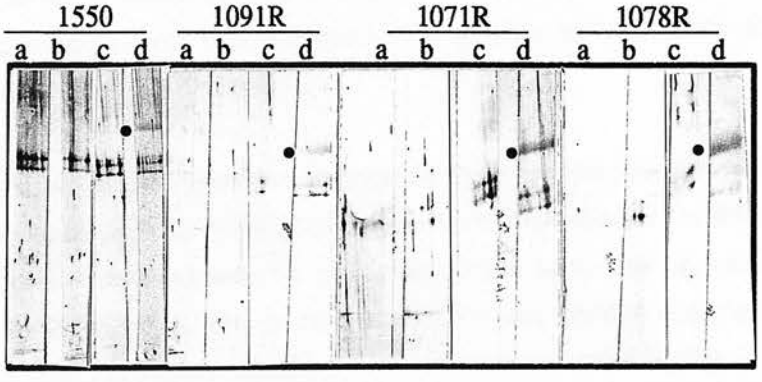
lane b= GST:SUenv + normal sheep serum

lane c= GST + immune sheep serum

lane d= GST:SUenv + immune sheep serum


186
187
a b c d
a b c d


848A
1231T
1229T
1244T
a b c d
a b c d
a b c d
a b c d


1550
1091R
1071R
1078R
a b c d
a b c d
a b c d
a b c d

control, MVV-infected sheep cells that had been prefixed with ice-cold acetone were probed with a rabbit anti-p25 (MVV core protein) serum, diluted 1/1000 (Dr.B.Blacklaws, personal communication). Examination of the positive control indicated that staining procedures were successful, although it is noted that this is not a true control for the experimental procedure. Since the experimental protocol required cells to be fixed post antibody binding, p25 would not be detected since it is not expressed on the surface of MVV-infected sheep fibroblasts. No rabbit sera raised against sheep fibroblast surface molecules were available. No specific fluorescence was seen for GST:SUenv binding to fibroblasts, only background staining comparable to GST controls and the negative controls (data not shown).

5.2.8ii Detection of GST:SUenv binding to sheep fibroblasts by FACS analysis

Immunoflourescence is a less sensitive approach compared to other immunodetection methods available. One of these is flow cytometry. A protocol was set up where sheep fibroblasts were incubated with antigen and this was than detected by FACS analysis. Prior to antigen incubation it was necessary to concentrate the eluted samples of GST:SUenv and this was achieved by acetone precipitation to give an approximate concentration of 150µg/ml. Sheep fibroblasts were grown in T150 tissue culture flasks until they reached 80-90% confluencey. The monolayers were then washed twice in 5mM EDTA/PBS, followed by a 30-45 minute incubation with 5mls of 5mM EDTA/PBS until the cells had detached from the surface of the flask. Cells were counted and adjusted to $2x10^6$cells/ml. Two 200µl aliquots of cells were taken and incubated with 20µg of either GST (derived from pGEX2T) or GST:SUenv in a total volume of 0.5ml. The incubation was carried out for 2 hours at 4^oC. Half of each samples was fixed in 1% paraformaldehyde as well as three 100µl aliquots of untreated cells (Fig.5.13A). All samples were then incubated with primary and secondary antibodies as outlined in Table 5.1.

As discussed in the immunoflourescence experiment there was no absolute positive control available. To test for the validity of FACS staining a monoclonal specific for sheep LFA3 was chosen. All samples were fixed with 1% paraformaldehyde at the end of staining and then analysed on a FACS machine. The positive control showed positive staining (Fig. 5.13a) compared to the autoflourescence observed with the negative controls (Fig. 5.13b and c). There appeared to be a small amount of positive staining for the experimental samples but there was no difference between GST:SUenv and GST (Fig. 5.13d and e). It was noted however that the staining was stronger with cells that had been fixed after incubation with the antigen, by comparison between Fig 5.13A and 5.13B, particularly Fig. d.

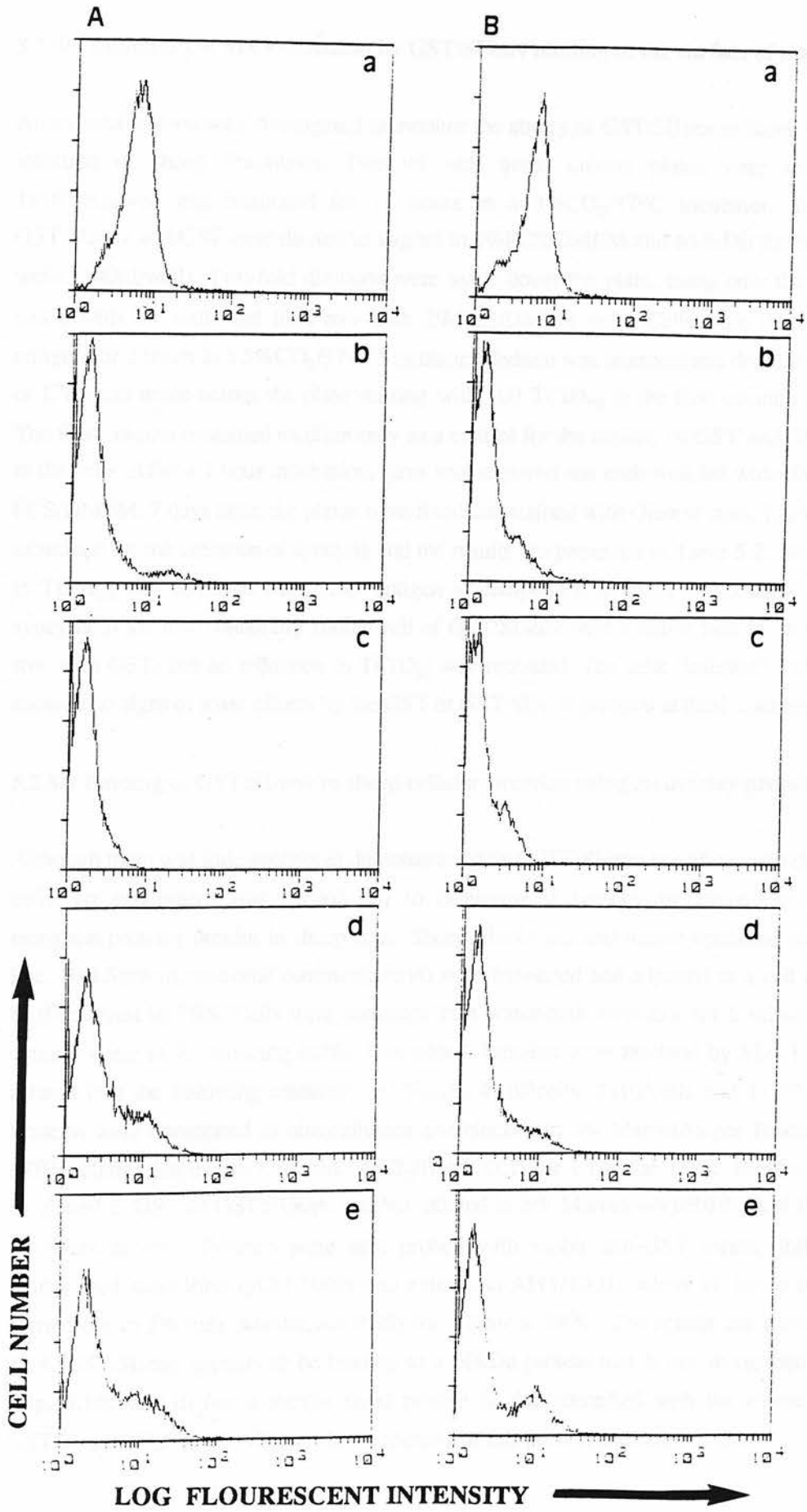
Table 5.1: Staining conditions for FACS analysis experiment

Fixed post Ag binding	Unfixed	Primary antibody (1/100)	Secondary antibody (1/250)
cells + GST:SUenv	cells + GST:SUenv	R-anti-GST	anti-R IgG-FITC
cells + GST	cells + GST	R-anti-GST	anti-RIgG-FITC
cells	cells	R-anti-GST	anti-R IgG-FITC
cells	cells	-	anti-R IgG-FITC
cells	cells	M-anti-LFA3	anti-M IgG-FITC

N.B. R= rabbit, M= mouse

Figure 5.13: Binding of GST:SUenv to the surface of sheep fibroblasts

Frequency histograms showing binding of GST:SUenv (d) to the surface of sheep cells with comparison to GST binding (e). Figures A and B represent different experimental protocols described in the text. Figures a are positive control staining with mouse anti-LFA3. Negative controls are shown in figures b and c, and represent autofluorescence from reagents (see Table 5.1 for details).


A
B
a
b
c
d
e
CELL NUMBER
LOG FLOURESCENT INTENSITY

5.2.8iii Inhibition of MVV-infection by GST:SUenv binding to the surface of sheep cells

An indirect method was investigated to monitor the ability of GST:SUenv to block EV1 virus infection of sheep fibroblasts. Two 96 well tissue culture plates were seeded with $1x10^4$cells/well and incubated for 16 hours in a 5%CO_2/37^oC incubator. Samples of GST:SUenv and GST were diluted to 1μg/ml in 2%FCS/DMEM and added to the first row of wells (100μl/well). Two-fold dilutions were made down the plate, using only the inside 60 wells, with the sixth and final row with 2%FCS/DMEM only. Cells were incubated with antigen for 2 hours in a 5%CO_2/37^oC incubator. Medium was removed and doubling dilutions of EV1 was made across the plate starting with 100 $TCID_{50}$ in the first column, 50μl/well. The final column contained medium only as a control for the toxicity of GST and GST:SUenv to the cells. After a 1 hour incubation, virus was removed and each well fed with 100μl of 2% FCS/DMEM. 7 days later, the plates were fixed and stained with Giemsa stain. Each well was examined for the presence of syncytia and the results are presented in Table 5.2. No reduction in $TCID_{50}$ was observed with either antigen in comparison to mock incubations. However, syncytia in the row containing 100ng/well of GST:SUenv were smaller than in its equivalent row with GST, but no reduction in $TCID_{50}$ was recorded. The cells "infected" with medium showed no signs of toxic effects by the GST or GST:SUenv proteins at these concentrations.

5.2.8iv Binding of GST:SUenv to sheep cellular proteins using an overlay protein blot

Although there was little success in demonstrating that GST:SUenv specifically binds to sheep cells, an experiment was carried out to determine if baculovirus-derived gp135 could recognise proteins present in sheep cells. Sheep fibroblasts and mouse epithelial cells (C127 line, Dr.J.Stewart, personal communication) were harvested and adjusted to a cell density of $8x10^6$cells/ml in PBS. Cells were sonicated in a water-bath sonicator for 5 minutes and an equal volume of 2x reducing buffer was added. Proteins were resolved by SDS-PAGE and titrated into the following amounts: $6x10^4$cells, $4x10^4$cells, $2x10^4$cells and $1x10^4$cells/lane. Proteins were transferred to nitrocellulose and blocked in 5% Marvel/Hepes Binding Buffer (HBB: 20mM Hepes, pH7.5/5mM $MgCl_2$/1mM KCl) for 1 hour at 18^oC. Blots were either incubated in GST or GST:SUenv, 4μg/ml, diluted in 5% Marvel(w/v)/HBB/5mM EDTA for 16 hours at 4^oC. Proteins were then probed with rabbit anti-GST serum, followed by biotinylated anti-rabbit IgG(1/1000) and extravidin-AP(1/1000), where all incubations were carried out in 5% milk powder,w/v/PBS) for 1 hour at 18^oC. The results are shown in Fig. 5.14. GST:SUenv appears to be binding to a 30kDa protein that is not recognised by GST (Fig. 5.14c and d) but a similar sized protein is also identified with the mouse cells by GST:SUenv (Fig. 5.14b). The probable identity of this protein is discussed later.

Table 5.2 Inhibition of MVV infection of sheep fibroblasts by GST:SUenv

$TCID_{50}$		100	50	25	12	6	3	1.5	0.8	0.4	0
100ng/well	GST	**	**	**	*	*	*	*	*	*	#
	GST:SUenv	*	*	*	*	*	*	*	#	#	#
50ng/well	GST	**	**	**	*	*	*	*	*	*	#
	GST:SUenv	**	**	**	*	*	*	*	*	*	#
25ng/well	GST	**	**	**	*	*	*	*	*	#	#
	GST:SUenv	**	**	**	*	*	*	*	*	*	#
12ng/well	GST	**	**	**	*	*	*	*	*	*	#
	GST:SUenv	**	**	**	*	*	*	*	*	*	#
6ng/well	GST	**	**	**	*	*	*	*	*	#	#
	GST:SUenv	**	**	**	*	*	*	*	*	#	#
0ng/well	GST	**	**	**	*	*	*	*	*	#	#
	GST:SUenv	**	**	**	*	*	*	*	*	#	#

Key: **: c.p.e. extensive

*: small syncytia

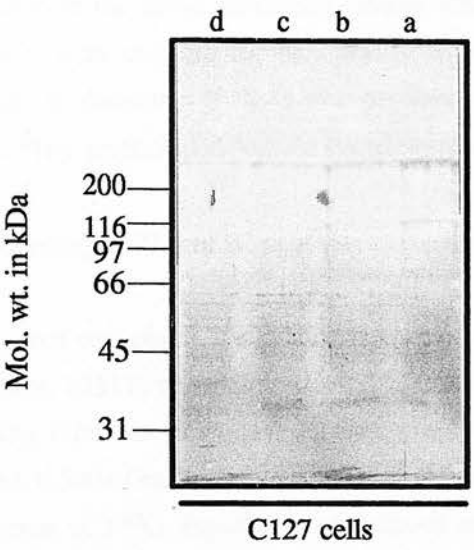
#: no syncytia

Figure 5.14: Identification of polypeptides which bind to baculovirus-derived gp135

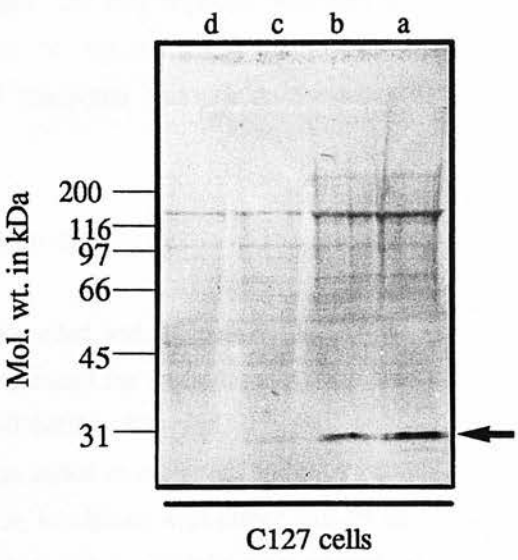
Cell lysates of mouse epithelial cells (C127) and sheep fibroblasts (1231T) were resolved by SDS-PAGE and transferred to nitrocellulose. The filters were incubated with either GST or GST:SUenv and probed with anti-GST serum (1/100).

lane a, $6x10^4$ cells
lane b, $4x10^4$ cells
lane c, $2x10^4$ cells
lane d, $1x10^4$ cells

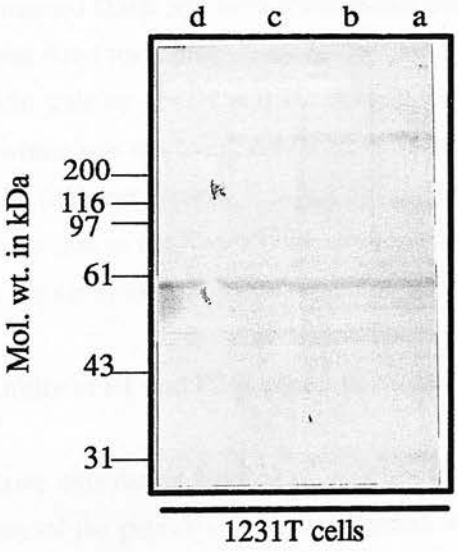
a] GST



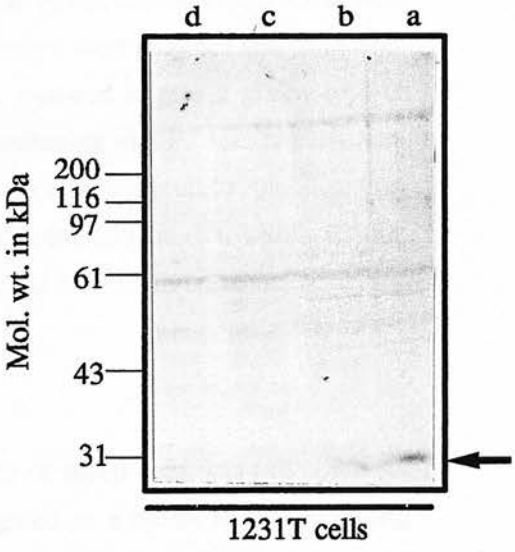
b] GST:env



c] GST



d] GST:env



5.2.9 Functional studies with P1 and P2 peptides

In addition to the above functional studies with GST:SUenv, the two peptides described in Chapter 4 were assessed for their ability to bind to sheep fibroblasts. These studies were necessary to determine if these two peptides represented functional epitopes on the native protein. They are included here for completeness.

5.2.9i Binding of P1 and P2 peptides to the surface of sheep cells

Twenty four well plates, containing sterile coverslips, were seeded with 10^5cells/well of sheep fibroblasts, 1231T, or mouse epithelial cells, C127, and incubated for 16 hours at 37°C. The following dilutions of P1 and P2 were made in 2%FCS/DMEM: 50µg/ml, 25µg/ml and 12µg/ml. 0.5ml of each peptide dilution or medium only was added to each well and incubated for 2 hours at 37°C. Peptides were removed and cells were incubated with either anti-P1 or anti-P2 serum for cells containing P1 or P2, respectively. Mock incubated cells were treated in the same way and control wells were incubated with appropriate prebleeds. Serum dilutions made in 2%FCS/DMEM at 1/100. After a 1 hour incubation at 37°C, cells were washed with unsupplemented DME and further incubated with anti-rabbit-FITC, diluted 1/250. Cells were washed and fixed for 5 minutes in ice-cold methanol. Coverslips were mounted and examined. No specific staining for P1 and P2 binding was seen. P2 appeared to give a grainy type of staining which was not associated with cell morphology, indicating that P2 was binding non-specifically (data not shown). It is noted that P2 was generally more difficult to solubilize than P1, possibly due to the hydrophobic amino acids, valine (V) and leucine (L), within P2 (see Fig. 4.6). This may explain its non-specific binding observed in immunoflourescence.

5.2.9ii Ability of P1 and P2 peptides to block infection

In the above experiment binding of peptides to the surface of sheep cells was not observed. The ability of the peptides to block infection was investigated as a means of demonstrating binding of peptide to sheep cells. 96 well plates were seeded with 10^4cells/well, either with sheep fibroblasts (1231T) or mouse epithelial cells (C127). The following day dilutions of peptides 1 and 2 were made up in 2%FCS/DME as 500, 250, 50, 25, 5 and 2.5µg/ml and 100µl of each dilution was added to each well. A mock incubation was made up with 2%FCS/DMEM only. Peptides were incubated with the cells for 1 hour at 37°C, removed and doubling dilutions of EV1 made across the plate starting with 500 $TCID_{50}$. After a 2 hour incubation, the virus was removed and cells were fed with fresh medium. Seven days later cells were Giemsa stained and examined. The C127 controls showed that they were not

permissible to virus infection. At higher concentrations, greater than 50μg/ml, the peptides were toxic to cells. At lower concentrations of virus, the number of syncytia were fewer with P1-incubated cells than mock incubated (data not shown). This experiment was repeated using the following dilutions of peptide: 50, 40, 30, 20, 10 and 0μg/ml with 100 $TCID_{50}$ of EV1 as the starting point for virus. No reduction in $TCID_{50}$ in the presence of either peptide was observed (data not shown), suggesting that the peptides did not represent functional epitopes on the native envelope protein of MVV.

5.3 DISCUSSION

This chapter has reported the production of a recombinant baculovirus expressing the external envelope glycoprotein, gp135 of MVV. One of the main advantages in using the baculovirus expression system is the ability for recombinant proteins to become glycosylated. This requires the presence of a signal sequence to allow the protein to be transported through the glycosylation machinery of the insect cells. Preliminary data reported by Rafnar et al. (1994), highlighted the toxic properties of the signal peptide of gp135, in that production of recombinant protein in the baculovirus system was only possible when the signal peptide was replaced, in this case with that of mouse-γ-interferon. Work in the HIV field has reported that increase in yield can be achieved by using the signal peptide of gp67, the membrane glycoprotein of AcMNPV (Murphy et al., 1993). Presumably, this signal sequence is more readily recognised and efficiently transported through the translocation machinery of insect cells, and has the additional benefit of producing a secreted protein which can be more easily purified. Our own experience with expressing gp135 as a recombinant protein has shown that the N-terminus of gp135 is especially difficult to express (see chapter 3), and this is most probably due to the presence of a large hydrophobic domain, approximately 80 amino acids from the N-terminus, which is thought to represent the signal peptide. Toxic properties of the signal sequence of other lentiviruses has also been described, such as the expression of HIV-1 gp120 in CHO cells (Lasky et al., 1986). The absolute identity of the signal peptide of gp135 has not been conclusively demonstrated. The presence of the hydrophilic tail preceding the signal peptide is a characteristic of retroviruses (Perez et al. 1987a) and is especially long in FIV, the feline lentivirus (Stephens et al., 1992). The function of the leader sequence prior to the signal peptide of lentiviruses is believed to be non-functional so it was presumed not necessary for the successful production of recombinant MVV envelope protein. It should be noted that this region of the envelope gene incorporates the first exon of rev (Sargan et al.,

1991). The signal peptide was thus identified as the first hydrophobic region from the N-terminus of gp135 and primers were designed to generate a fragment lacking the first 100 amino acids of gp135.

To facilitate the cloning of *env* into pAcSG2T, two primers were designed, primer 4975 and primer 4976, that contained different restriction sites in order to generate a fragment with a BamHI site at the 5' end and EcoRI site at the 3' end. A stop codon was introduced in the 3' end of the *env* fragment since this is absent in the transfer vector. PCR was successful in generating gp135 without the signal peptide as determined by double-stranded sequencing, which showed that no major mutations had arisen from the PCR.

5.3.1 Generation of a recombinant baculovirus

Having generated and confirmed the sequence of the required fragment of *env*, it was necessary to introduce *env* into the baculovirus transfer vector, pAcSG2T. Since pAcSG2T possesses a multiple cloning site, attempts were made to introduce *env* by directional cloning. This proved to be unsuccessful although controls included in restriction enzyme digests indicated that both BamHI and EcoRI were working. It was not possible to determine experimentally if the vector was indeed cut by both enzymes, and if this was not the case it may explain for the failure in cloning *env* into the transfer vector. Reasons why the enzymes may be inefficient at cutting the vector may be explained by the proximity of the two restriction sites in the transfer vector such that both enzymes are unable to cut their respective site. However cloning was possible using the single BamHI restriction enzyme site. Due to the relatively large size of the transfer vector, 8kb, in comparison to *env*, 1.7kb, it was necessary to force *env* into the vector by increasing its proportion relative to that of the vector. As was reported, only two out of the 12 colonies picked contained a 1.7kb fragment of *env* and that *env* was inserted in the correct orientation.

The use of linear viral DNA for the generation of recombinant viruses is described in the introduction. Although most of the viruses in the cotransfection supernatant will be recombinant, this "soup" is not ideal for reproducible results when expressing and purifying recombinant protein. It was therefore necessary to employ a single round of plaque assay to isolate single recombinant viruses and this would also confirm the efficiency of the cotransfection. It should be noted that the use of linearised baculovirus DNA greatly reduces the work required to isolate a single recombinant virus, since original protocols required several rounds of plaque assay purification or limited-dilution assays, before a reliable source of recombinant virus was purified. Plaque assay purification of the cotransfection supernatant

confirmed that all viruses were recombinant by the absence of blue colour in the plaques in the presence of X-Gal.

Following the isolation of single recombinant viruses, all 19 viruses were screened for the presence of *env* by slot-blot as it was felt necessary to confirm recombination between viral DNA and pAcSG2T:env before selecting a smaller number of viruses for characterization. The results from this additional screening step again confirmed the efficiency of recombination to generate recombinant viruses.

It was noted in the results that the number of cells in the experimental dishes were markedly lower than in the control dishes. This observation has also been described for HIV-1 gp120 expression, where a faster decrease in cell viability upon infection with recombinant viruses was noticed (Murphy et al., 1993). This was attributed to the potential toxicity of the signal peptide. Since the signal peptide was not included in this experiment, it could be proposed that other hydrophobic sequences in the envelope are affecting cell viability. These hydrophobic regions were predicted to cause toxic properties when expressed in the bacterial pGEX system (see Chapter 3).

Detailed analysis of seven recombinant viruses was carried out by Southern blot hybridisation to confirm the presence of *env*-specific sequences within the viral genome. The use of Bsu361 digested BacPAK6 Viral DNA ensures that the gene of interest is inserted in the correct position and orientation in the viral genome, as described in the introduction. The gene of interest will be inserted within the 0.8-0.9kb F fragment (not visible in Fig 5.6a), which spans the polyhedrin locus In Fig. 5.6b, sample 16 contained an extra band approximately 2.6kb, which possibly represents incomplete digestion of *env* sequences from the viral genome.

Insect cells infected with all seven recombinant baculoviruses were shown to secrete a recombinant protein that was recognised by GST-specific polyclonal serum. The size of this protein was estimated at approximately 97-100kDa, which gives SUenv a predicted size of 71-74kDa. The expected size of the surface glycoprotein without its signal sequence, in the absence of glycosylation, is 63kDa, suggesting that SUenv has been glycosylated. This is discussed more fully in section 5.3.4. The reported size for the external glycoprotein of MVV varies from 70kDa to 135kDa. Therefore, SUenv produced in the baculovirus system is smaller than the native form. It is usual to observe a smaller recombinant protein expressed in the baculovirus system compared to the native protein due to an immature glycosylation pathway in insect cells. This is well documented and was observed with HIV-1 gp120 expression (Wang et al., 1995, Morikawa et al., 1990b). The identity of the recombinant

protein was confirmed since it was recognised by anti-gp135 peptide antibodies and by another source of anti-GST antibodies.

A recent report has expressed the external glycoprotein of the ovine lentivirus, that is MVV, in the baculovirus system (Kwang et al., 1995). They describe the native external glycoprotein as gp70 and report a 66kDa protein that was recognised by OLV naturally infected sheep sera. Interestingly, they describe a recombinant protein that is transported to the cell surface as demonstrated by immunoflourescence. As far as can be determined, the signal sequence of the external glycoprotein was not included. They do comment that the expressed envelope is glycosylated although no experiment was carried out. Since no signal sequence was incorporated, glycosylation of the recombinant protein would not be expected. Therefore, the 66kDa protein observed is probably comparable to the expected size of the non-glycosylated envelope (63kDa) described in this chapter.

5.3.2 Kinetics of recombinant protein production

In the life cycle of baculoviruses, the polyhedrin promoter is usually active between 18 and 72 hours post infection, and production of polyhedrin protein shows a rapid accumulation between 24 and 30 hours followed by a gradual increase up to 72 hours. The polyhedrin protein is very stable in the cell and will accumulate into characteristic polyhedra crystals. Replacement of the polyhedrin gene with that of the foreign protein does not necessarily mean that the recombinant protein will be stable, and it is more usual to see a maximum time point for production after which protein production is unable to match the degradative processes. The production of GST:SUenv was maximum at 48 hours and thus there is a lag in protein expression compared to polyhedrin production. The kinetic studies suggested that after 48 hours protein production declined considerably, since it was not usual to see accumulation of breakdown products in the supernatant. The varying degrees of contaminating products observed from one preparation to the next may be a reflection on the cell viability at that time.

Work on the expression of gp120 by Morikawa et al. (1990b) observed that the kinetics of protein production differed for secreted and cell-associated protein. A study was undertaken to compare protein production in the cells and that secreted. Protein production in the cells was maximum between 24 and 48 hours, whereas secreted was maximum at 48 to 72 hours. It was noted that examination of cellular-associated proteins identified two species of GST:SUenv, and that the production of larger of the two decreased with time. The smaller protein was identical in size to that of the secreted form. This size difference was presumed to be due to a difference in carbohydrate content, although an experiment to confirm this was not performed. This would require the addition of tunicamycin to the cells with the expected disappearance of both protein species being replaced by a protein of size reported in section

5.2.5iv. Loss of carbohydrate moieties at the later stages of infection was observed by Murphy et al. (1990) and is discussed below.

The optimum m.o.i. was also determined. It is usual in production of recombinant proteins in the baculovirus system to infect at a high m.o.i. to ensure that all cells are infected synchronously which will allow for reproducible results when purifying recombinant protein.

5.3.3 Further characterization of protein production

High five™ (H5) cells are a relatively new cell line. They are derived from the eggs of the native host of AcMNPV, the cabbage looper, *Trichoplusia ni*. They are larger in size than the conventional Sf cells and have a more efficient post-translational modification machinery, and thus give much higher yields of recombinant protein (Davis et al., 1993). These cells are reported to produce 20-fold more protein than Sf21 cells on a cell-to-cell basis (Davis et al., 1992). The cells can also be grown in suspension like Sf21 cells, and this is favourable for protein production since H5 cells are susceptible to contact inhibition. However, a comparison between these two cells for production of GST:SUenv has shown that there was no dramatic increase in the yield of protein in High five™ cells. If the expression of recombinant envelope in the baculovirus system is limited by the toxicity of the protein, then protein production may not be influenced by different cell types. Choice of host cell was governed by cell viability when routinely expressing recombinant protein.

The ability to grow insect cells in suspension lends itself to large scale production. However, suspensions of more than 500ml result in slower growth rates presumably due to a limiting supply of oxygen to the cells (Maiorella et al., 1988). Therefore only 100ml spinner cultures were used to ensure cells were growing in log phase at the point of infection. Another drawback to suspension cultures is ensuring synchronous infection of all the insect cells. This was monitored by checking that the majority of cells showed signs of virus infection prior to harvesting. An alternative method is available instead for infecting spinner cultures but is more cumbersome. This involves the harvesting of cells and allowing viruses to absorb onto the cells. However synchronous infection is still not guaranteed. It is essential that a high-titre virus stocks are used to infect spinner cultures to ensure that the cell density of the culture is not dramatically reduced to the effect that virus-cell interaction is inefficient. The routine yields reported in the results were consistent with those reported for expression of HIV-1 gp120 (Wang et al., 1995).

The affinity purification system using glutathione-agarose beads is a reliable, straightforward system, and elution from the beads resulted in a reliable source of recombinant gp135 in solution. Attempts to remove the fusion partner, GST, from gp135 were not successful. No positive control was available to ensure that thrombin was working, but the expected size, 71-84kDa, of cleaved product was not seen indicating that cleavage had not occurred. Cleaving fusion proteins with thrombin is generally difficult (Dr.J.Stewart, personal communication, Dr.I.Jones, personal communication), requiring the need for various conditions such as detergents for cleavage. Time considerations meant that determination of cleavage conditions to remove GST could not be pursued, and the sequencing of the cleavage site to ensure that it had not been disrupted during cloning was not undertaken.

5.3.4 The glycosylation state of GST:SUenv

As has been discussed previously one of the main advantages of the baculovirus expression system is the ability to produce large quantities of glycosylated recombinant protein. Glycosylation is initiated in the endoplastic reticulum by the addition of an oligosaccharide to the asparagine residues on the protein and this initiation of N-glycosylation is a feature in both mammalian and insect cells. Mammalian cells extensively trim the core oligosaccharide, followed by the addition of sugars such as frucose, glucosamine-galactose and sialic acid to give complex branched oligosaccharides. The glycosylation pathway of insect cells is less refined, such that insect-derived glycoproteins are smaller in size to their mammalian counterparts (Noteborn et al., 1990). The different pathways involved in glycosylation processes in insect cells are not understood. Recently studies on the glycosylation status of the membrane glycoprotein, gp67, of AcMNPV has been undertaken, and it was found that galactose and sialic acid residues were not present on this protein (Jarvis and Finn, 1995). However, studies with plasminogen expression has described sialic acid residues on this recombinant protein when expressed in baculoviruses (Davidson et al., 1990) and the presence of N-acetyl glucosaminyltransferases in insect cells has been demonstrated (Altmann et al., 1993). In depth studies as to the glycosylation state of insect-derived HA of the influenza virus has characterized the carbohydrate content to consist of 50% N-Acetylgalactosamine (GlcNAc) residues and the remaining a variable number of mannose residues (Kuroda et al., 1990). These studies were carried out with a combination of oligosaccharide-modifying enzymes and chromatographic techniques to analyse the type of carbohydrate on the surface of HA.

A variety of techniques can be employed to confirm the presence of oligosaccharides on recombinant protein. The choice of method chosen to investigate the glycosylation state of GST:SUenv was the use of tunicamycin. This is an active irreversible inhibitor of N-

glycosylation in the E.R., where it acts as an analogue to UDP-N-acetylglucosamine preventing the addition of N-acetylglucosamine to dolichol phosphate, the first step in the formation of the core oligosaccharide. Twenty-one of the twenty-three putative glycosylation sites within *env* had been retained (see Fig.5.3) by comparison to the published sequence of EV1 (Sargan et al., 1991). The expected size of the non-glycosylated GST:SUenv is 89kDa; GST is 26kDa and the predicted size of the representative polypeptide portion of gp135 is 63kDa. A protein of approximately this size was identified in the presence of tunicamycin as reported in section 5.2.5iv proving that GST:SUenv is indeed glycosylated in the baculovirus system.

Other methods for detecting the presence of carbohydrate on recombinant proteins include labelling with ^{3}H-mannose, since mannose residues represent a large proportion of carbohydrate on insect-derived glycoproteins. This technique is only feasible when large quantities of recombinant protein are produced and can be distinguished from cellular proteins. Baculovirus-expressed proteins are also sensitive to endoglycosidase H and F and N-glycanase which remove immature high mannose-type oligosaccharides (Noteborn et al., 1990). The use of enzymes is less appealing since conditions will play an important role in the complete removal of the carbohydrate residues which could result in a variety of protein species emerging following digestion.

The kinetics of glycosylation has been investigated fully in the expression of HIV-1 gp120 by the baculovirus system (Murphy et al., 1990). An observation was made on analysing the expression of recombinant protein in that the extent of glycosylation of the recombinant protein was dependent on the time point post infection. At earlier time points or at low levels of expression, glycosylation was efficient. However, at 72 hours post infection, when protein production was maximum the majority of recombinant protein produced was non-glycosylated and insoluble, suggesting that host cell processes had shut down and components required for glycosylation were limiting. Alternatively, the baculovirus itself may affect the glycosylation machinery of the host cell at later time points in infection (Murphy et al., 1990). A 5-10kDa larger protein identified in BacPAK6:env12 infected cells at earlier time points of virus infection, an observation similar to Murphy et al. (1990). The reduction in size of GST:SUenv at later time suggests a loss in carbohydrate. Thus it would seem that the extent of glycosylation was less on the secreted form of SU:env than the cell-associated. However, the yield was better, a reflection on the efficiency of the baculovirus signal sequence. An experiment to see if cell-associated GST:SUenv became insoluble at later time points was not performed. It is noted that Murphy et al (1990) did not incorporate a signal sequence in to the transfer vector. This may reflect the reduction in yields of glycosylated protein at later time points in infection. The fact that the system used in this chapter allowed the secretion of

recombinant protein, may explain why at later time points in infection recombinant protein was still glycosylated, a reflection of subsequent work by Murphy et al (1993). Murphy et al. (1990) comment on the type of vector used may influence the expression of glycosylated proteins. They used the pVL941 transfer vector, whereas pAcSG2T was derived form the pAcCL29 vector. However, they also used the pVL941 vector for expression of secreted gp120 and protein was secreted efficiently at later time points in infection (Murphy et al., 1993). It would suggest that it is the use of a signal sequence allows for more efficient protein production and glycosylation, rather than the transfer vector used. It is noted that the size of the cell-associated protein, 107kDa, is closer in size to that expected of the native protein fused to GST, 116kDa (90kDa plus 26kDa) (see Chapter 6). Whether the secreted form of GST:SUenv is the more mature protein is not known. It would be interesting to know if the extent of glycosylation of the recombinant protein influences its interaction with the host cell receptor.

5.3.5 Potential use of GST:SUenv as an immunogen

Production of glycoproteins in the baculovirus system usually results in the retention of their antigenicity, giving rise to a high titre of antibody. Antibodies are not always neutralising and this may be a reflection on the glycosylation state of the recombinant protein which indirectly effects its conformation and potential exposure of neutralising epitopes (Noteborn et al., 1990, Arp et al., 1993, Bristow et al., 1994). Their use as therapeutic proteins and possible subunit vaccines is debatable, since it has been shown that glycoproteins terminating in mannose, galactose and GlcNAc are more rapidly cleared in the bloodstream (Stahl, 1990). Immunisation with a baculovirus-expressed envelope protein of HTLV was ineffective in inducing neutralising antibodies, and a combination of antigens was required to raise neutralising antibodies as a potential for subunit vaccines (Arp et al., 1993). To determine if the antigenicity of gp135 had been retained by expression in the baculovirus system, the reactivity of MVV-infected sheep sera was assessed by western blotting. As was reported, all infected sheep sera tested were able to recognise GST:SUenv specifically. For controls of naturally infected sheep it was necessary to use non-infected sheep sera since pre-bleed of these sheep were not available. The lower contaminating bands observed between 40-66kDa were present in the negative controls also, and were probably due to non-specific reactivity of sheep sera in immunoblotting. The authenticity of the baculovirus-derived gp135 is an important result, since this allows for the potential of this protein to be used in an ELISA screening assay. This approach has been undertaken with the envelope glycoprotein of HIV-1, which was used in a solid-phase ELISA and was successful in screening 48 positive sera (Hu et al., 1987). Western blot screening involved using sheep experimentally infected with the

EV1 strain of MVV only. Sera from sheep infected with other strains of MVV were not available at the time of screening. However, the ability of naturally-infected sheep to recognise recombinant envelope, suggests that conserved epitopes between different strains of MVV had been retained, since infection in the field will involve a variety of antigenic distinct strains. Development of a screening assay for MVV was not attempted at this stage. Due to time considerations no attempts were made to use GST:SUenv as an immunogen, although the adequate yield and absence of contamination products would suggest that preparations of these reagents would be straightforward.

5.3.6 The use of GST:SUenv in functional studies

The use of baculovirus-derived viral glycoproteins in functional studies has been widely demonstrated with HIV. It is well documented that CD4 is the principal receptor for HIV. Morikawa et al. (1990b) expressed gp120 and CD4 in the baculovirus system and demonstrated that they interacted *in vitro*. A similar approach was taken to examine the ability of another putative receptor for HIV, CD26, to bind to baculovirus-derived gp120. Although binding to CD4 was demonstrable in a variety of assays, this was not reciprocated with CD26 (Wang et al., 1995). The role of carbohydrate in gp120 binding to CD4 was undertaken, and found that removal of carbohydrate from gp120 reduced binding to CD4 (Murphy et al., 1990). It appeared that the high mannose N-linked carbohydrate influenced binding but whether this was due to specific interaction of the carbohydrate with CD4 or that the carbohydrate allows the recombinant protein to fold properly is not well understood.

Preliminary studies undertaken to examine the functional activity of GST:SUenv were inconclusive. Initial studies were carried out using immunofluorescence. The protocol chosen was adapted from that published by Kozak et al. (1995), whereby cells were fixed following staining in an attempt to reduce the disruption of protein-protein interactions. The alternative method of fixing cells prior to protein binding may result in the disruption of epitopes important in receptor binding. Although no specific binding was observed, these experiments were preliminary and adaptations to the protocol are necessary, such as increasing the amount of antigen and fixing cells post-antigen binding.

FACS analysis was used since it is more sensitive than immunoflourescence. Again cells were fixed post antigen binding in an attempt to preserve receptor epitopes that bind to gp135, and this fixing gave a slightly better staining pattern than not fixing presumably by preserving the interaction of surface proteins with antigen. There was high background staining from GST so conditions are needed to be altered to identify specific binding of GST:SUenv to the surface of sheep fibroblasts.

Since binding of recombinant gp135 to the surface of sheep fibroblasts was not shown by the two above methods, an indirect approach was attempted. This involved binding GST:SUenv to the cell surface prior to infection and monitoring of virus-induced c.p.e. visually. If baculovirus-derived gp135 is able to bind to the putative receptor of MVV, it should reduce the c.p.e. of the virus. A reduction in c.p.e. at a concentration of 1μg/ml suggests that GST:SUenv is able to bind to sheep celluar proteins, although gp135 was not shown to neutralise virus infection. Neither GST nor GST:SUenv were toxic to the sheep cells as determined by cell viability after mock infection. In summary, these three experiments were crude in approach but time considerations prevented them from being further developed. The functional studies with the two peptides suggested that they did not represent functional epitopes within the native envelope.

The binding ability of GST:SUenv was supported by the results from the overlay protein assays. The 30kDa protein identified corresponded in size to that seen in sheep choroid plexus cells, thought to represent the putative receptor for MVV (Crane et al., 1991a, Dalziel et al., 1991). The observation that GST:SUenv bound to a 30kDa protein on mouse epithelial cells was also seen in studies by Crane et al. (1991a) who found that MVV did bind at low levels (see fig.5.14). Mouse cells are not permissible to MVV infection as determined by a virus titration assay (data not shown), but have been shown to support virus fusion in "fusion from without" assays (Gilden et al., 1981). Far-western blotting provides only preliminary data as to the identity of the virus receptor. Further experiments would be required to further characterize the identity of the receptor and this is discussed more fully in chapter 6.

5.4 SUMMARY

This chapter describes the construction of a recombinant baculovirus, which expresses a secreted, glycosylated form of the external envelope glycoprotein of MVV. This protein is made in greater amounts than previous experience with other expression systems and is devoid of major contamination. It can be easily purified due to a GST tag and has the potential to be cleaved from its fusion partner, although attempts to do this were not successful. It is recognised by MVV-infected sheep sera and thus could be used to develop a diagnostic assay. It is also of use in identifying the putative host cell receptor, and the presence of the GST tag would allow detection of protein-protein interactions without interfering with important epitope binding sites. Initial attempts to investigate specific binding to sheep cells were inconclusive.

CHAPTER 6: CONCLUSIONS AND FUTURE WORK

This thesis has presented efforts at expressing the external glycoprotein of MVV as a recombinant protein in the bacterial pGEX system and the baculovirus expression system. Attempts at raising monospecific antisera against this protein are also reported. The results have already been discussed in detail at the end of each chapter, and a general discussion will be presented here as to the significance of this work and the use of the generated reagents in future studies.

6.1 Expression of the external envelope glycoprotein of MVV as a recombinant protein

The generation of a recombinant form of the external glycoprotein of MVV has proved to be problematic. The choice was made initially to express the envelope protein as three overlapping fragments. This approach stemmed from earlier expression work in the Ty-VLP expression system (Carey et al., 1993). Expression in fragments would allow the recombinant proteins to be used in epitope mapping, as well as the generation of monospecific sera against three different regions of gp135. The pGEX bacterial system was chosen to express these fragments, due to its relative ease of use with usually high yields. However, all three fragments were found to be toxic to bacterial growth and yields were too poor to use either as immunogens, or in mapping immune responses in sheep.

It was suggested that the regions of hydrophobicity were potentially responsible for the toxicity of gp135 in the bacterial expression system, as seen in other expression work (Sisk et al., 1992). The region that appeared to be especially problematic was the putative signal sequence. This hydrophobic region may have been the cause of unsuccessful cloning, such that directional cloning was required to insert envA into the pGEX1 plasmid. Rev, whose first exon is present in the N-terminal portion of env is also toxic when expressed in the bacterial pGEX system (M.Fotheringham, personal communication). Reports on the expression of the envelope glycoproteins of ungulate viruses in bacterial systems are limited. The N-terminal portion of the transmembrane, and the C- and N-terminal portions of the external glycoproteins of MVV have been expressed in the pGEX system (Kwang and Cutlip, 1992a and b). They also do not include the signal sequence in the N-terminal portion of the external envelope glycoprotein, but do not comment on this. The authors do not state why they chose only to express certain portions of the envelope proteins, but it may be that they also found

these envelope proteins to be toxic. Thus, yields may be improved in the bacterial system by cutting out major hydrophobic regions from the external envelope protein. The pET expression system would probably be a better choice as it incorporates the tightly controlled T7 promoter. This may be more productive since there are reports that the *tac* promoter is 'leaky' (Sharrocks, 1994), which is undesirable when attempting to express toxic proteins.

The toxicity of the recombinant proteins expressed in the pGEX system may also be attributed to the inability to form disulphide bridges when retained in the cytoplasm and thus folding would be abnormal. Incorrect folding may disrupt protein production by some unknown mechanism. Hence, bacterial expression may not be the best choice in the future even with modifications in the hydrophobic regions.

The approach was taken to attempt expression in a eukaryotic system. A eukaryotic system would allow the recombinant protein to be folded correctly, thus reducing the potential risks of toxicity. Since the recombinant protein would be glycosylated in a eukaryotic system, it may be more likely to adopt its native conformational form. From experience in the bacterial system, and the Ty-VLP, it was decided to remove any large regions of hydrophobicity, namely the putative signal sequence. The baculovirus system was chosen, since preliminary efforts in our laboratory to express the envelope glycoprotein in the vaccinia system were unsuccessful. The baculovirus system allows recombinant protein to be folded correctly and glycosylated. In view of the desirability to produce a recombinant protein as close to its native form as possible, the envelope protein was expressed as a whole protein and not as three fragments. The size problem that was of consideration when expressing in the Ty-VLP system, was not an issue in the baculovirus system. The baculovirus transfer vector chosen incorporated a baculovirus-specific signal sequence which is expected to be recognised by the insect cells, and thus transport through the glycosylation machinery will be efficient.

This thesis reported the successful production of recombinant gp135 in the baculovirus system. However, it was noted that there was a reduction in cell viability on infection with recombinant baculovirus, compared to the parental baculovirus. Since recombinant protein yield was at the lower end of the scale expected for the baculovirus expression system (1-500μg/litre), it is possible the gp135 was toxic to the insect cells as well as bacteria. This toxicity may be attributed to the various short hydrophobic regions outside the signal sequence region (Sargan et al., 1991). However, compared to the results obtained in the pGEX system, the expression was considerably improved. The recombinant protein was devoid of major contamination, provided the insect cells were growing at log phase and were at least 95% viable.

6.2 Using baculovirus-derived envelope for raising anti-env antibodies

The result that baculovirus-derived gp135 was recognised by MVV-infected sheep sera was an important one (see Chapter 5). As well as its potential use in a diagnostic assay, it has the potential to be used as an immunogen. The existence of neutralizing epitopes in the recombinant protein could be investigated by incubation of immune sheep sera with the baculovirus-derived envelope to assess the ability to inhibit neutralization. If neutralization epitopes are retained in the baculovirus-derived envelope, it has the potential to be used as an immunogen for producing a panel of neutralizing monoclonal antibodies. The baculovirus-derived envelope would be a suitable immunogen for immunization, because it is produced in adequate amounts and it is free of major contamination. These two criteria proved to be the limiting factors in attempts to generate polyclonal sera against the pGEX-recombinant proteins and in the attempts to generate monoclonal antibodies. Since baculovirus-derived envelope is produced as a soluble protein it could also be used as the source of antigen in the screening assay. The anti-peptide sera potentially provide a positive control for the screening ELISA. However, it is not known whether the peptide sera would be able to recognise GST:SUenv on an ELISA.

If the baculovirus-derived envelope protein is unsuitable for generating neutralizing antibodies, alternative approaches are available. GST:SUenv could be used to raise monospecific polyclonal sera, and this then used to immunopurify envelope glycoproteins from MVV-infected cell extracts. This would then provide an alternative source of native envelope for monoclonal production to be used in a similar manner as the lectin-purified envelope protein. Due to time considerations, the suitability of the fixed-cell ELISA, described in Chapter 4, to detect viral envelope proteins with the anti-peptide sera was not tested.

Although the pGEX-recombinant proteins proved unsuitable in generating immune sera, they may be of use when mapping functional epitopes with MAb. Thus it would be possible to distinguish at least five different domains from the three overlapping fragments, and the properties of the MAb would allow a crude map of functional epitopes on the external envelope glycoprotein.

6.3 Using baculovirus-derived envelope to characterize the host cell receptor

Preliminary studies were undertaken to assess the use of baculovirus-derived envelope as a source of protein for identifying the host cell receptor. One of the considerations when using the fusion moiety, GST, tagged to the envelope glycoprotein is its potential to interrupt the interaction of the envelope with its putative receptor. Studies involving HIV-1 gp120/CD4 interaction indicated that GST did not disrupt binding (Wang et al., 1995). In fact, the GST tag could be seen as a useful marker for detecting immune complexes between the envelope and its receptor, since an antisera to GST is available (see Chapter 3). This was used as a method of detection both in binding assays to sheep fibroblasts, and in the protein overlay assay. Another consideration when using baculovirus-derived recombinant protein is the immature glycosylation state of the protein. However, the binding of HIV-1 gp120 to CD4 has not been impaired by the use of baculovirus-derived gp120 (Murphy et al., 1990, Wang et al., 1995). Once the identity of the receptor has been evaluated then the role of carbohydrate in binding could be investigated, since it possible that carbohydrate may play a non-specific role in determining the structure of the external envelope protein (Murphy et al., 1990). The use of tunicamycin in producing a non-glycosylated recombinant protein was reported in Chapter 5.

The data from the protein overlay assay suggested that baculovirus-derived envelope recognised proteins present in sheep fibroblasts, and the size was consistent with that seen in earlier work (Dalziel et al., 1991, Crane et al., 1991a). Although this assay was straightforward to set up there are several drawbacks. Conformational epitopes that are required for virus binding would be destroyed by resolving cellular proteins on SDS-PAGE. To further characterize the size of the host cell receptor, co-immunoprecipitation studies would be necessary. This would require radiolabelling sheep fibroblast cells, followed by incubation with recombinant envelope. Receptor/envelope complexes would be pulled out with GST antiserum bound to protein A sepharose beads and resolved by SDS-PAGE. Extensions to these studies would include the ability of baculovirus-derived envelope to interact with sheep MHC classII, which has been implicated as a putative receptor for MVV (Dalziel et al., 1991). Co-immunoprecipitation studies would also be carried out with radiolabelled macrophage cells, since these cells are the major target for MVV infection *in vivo* (Narayan et al., 1982). Macrophages could also be used in the binding studies described in Chapter 5, section 5.2.8ii, to show that baculovirus-derived envelope recognises MVV receptors on the surface of these cells.

6.4 The size of the external envelope glycoprotein of MVV

Throughout the literature on both MVV and CAEV there are no consistent reports as to the size of the external and the transmembrane proteins. Genetic analysis of the MVV genome predicts a 114kDa polypeptide backbone as the envelope precursor (Sonigo et al., 1985, Sargan et al., 1991). This 114kDa protein was interpreted as the 150kDa envelope precursor described by Vigne et al. (1982), which would then be processed to the more mature form of gp135, a predicted 105kDa polypeptide (Sonigo et al., 1988). This mature protein is presumed to have lost the signal sequence, which is proposed to lie within the first 100 amino acids at the N-terminus of the envelope protein (Sonigo et al., 1988). The presence of a protease cleavage site at amino acid residue 657, approximately, would result in a 64kDa polypeptide and a 38kDa polypeptide, representing the external and transmembrane proteins, respectively (Sonigo et al., 1988). A gp70 protein has been described which could represent the external envelope protein (Bruns and Frenzel, 1979).

Protein studies are even more ambiguous as to the identity of the external glycoprotein of the ungulate lentiviruses. As has been mentioned, pulse-labelling studies have shown that a 150kDa precursor of the envelope protein is replaced by a 135kDa protein (Vigne et al., 1985). By increasing the [^{35}S]methionine labelling time from 40 minutes to 24 hours, two polypeptides of Mr 85,000 and 50,000 were observed. Glycosylation studies revealed that the 150kDa and 135kDa proteins were glycosylated, but they did not report whether the 85kDa and 50kDa proteins were glycoproteins (Vigne et al., 1985). Most studies that are directed at the function of the envelope glycoprotein use a monospecific guinea-pig antiserum raised against a lectin-purified glycoprotein (see Chapter 4, Crane et al., 1988). This lectin-purified gp135 was used to raise a panel of monoclonal antibodies, although it is not specified whether this is the entire envelope glycoprotein or the external glycoprotein (Stanley et al., 1987). However, studies in identifying the neutralization and fusion epitopes on the envelope protein using this monospecific antisera, describe gp135 as the external glycoprotein (Crane et al., 1988). Work involving the expression of the entire envelope glycoprotein in transgenic sheep characterize the existence of both the external protein, gp135, and the transmembrane, gp50, in the transgenic sheep cells (Clements et al., 1994). The antisera used to carry out these immunoprecipitation studies used the guinea-pig antisera raised against the lectin-purified gp135, that is the external glycoprotein. This clearly shows the inconsistency in identifying the glycoproteins of MVV.

A similar pattern is seen with CAEV. Gp135 was identified as the major glycoprotein of CAEV, and used in immunodiffusion serology (Adams and Gorham, 1986), although they do not speculate whether this is the entire envelope or just the surface glycoprotein. By analogy with MVV, gp135 is commonly described as the surface protein in papers on CAEV

biology (Knowles et al., 1990). Antibody responses to the surface glycoprotein were thought to be correlated with the severity of arthritis, but more recent studies have mapped this to an immunodominant epitope in the transmembrane glycoprotein, gp38 (Bertoni et al., 1994). This suggests that gp135 could be the uncleaved form of the envelope protein, as described by Huso et al. (1988).

In summary, the size of the external envelope glycoprotein is reported to range from 70kDa to 135kDa. Throughout the thesis the external glycoprotein has been referred to as gp135. Howver, results from the baculovirus expression data suggest that this may not be the true size of the external glycoprotein of MVV.

Experience with MVV-infected sheep antisera in our laboratory has lead to observations of a 90kDa protein as well as the 135kDa protein in MVV-infected cell lysates. This 90kDa protein is predicted as the external envelope glycoprotein. The presence of the gp90 protein is not consistent, and greatly depends on the state of the cell cultures at the time of infection and at harvest. It should be noted that gp90 was not purified from the lectin-affinity column described in Chapter 4. This may be explained by the methodology of the purification. Since the virus was collected by ultra-centrifugation, it is likely that the spikes on the surface of the virion will be knocked off, and thus gp90 is lost. This explanation may also be relevant in standard harvesting of MVV-infected cells.

The expression of the external glycoprotein of MVV in the baculovirus system reported in this thesis, described a secreted protein of 70-75kDa, and a cell-associated of approximately 80kDa (see Chapter 5). Since baculovirus produced recombinant proteins are generally smaller than their native form (Morikawa et al., 1990b), the baculovirus-derived protein could be seen as a true representative of gp90. This suggests that gp135 is more likely to represent the uncleaved precursor, with gp90 representing the external. This would predict a 45kDa protein as the transmembrane glycoprotein.

By raising a monospecific antisera against the baculovirus-derived external glycoprotein, the true identity and size could be studied. Immunoaffinity columns could be set up as described above to pull out both the envelope precursor and the external envelope protein form MVV-infected cell lysates. A combination of pulse-chase labelling followed by immunoprecipitation would confirm earlier studies by Vigne et al (1982), and reveal the true identity of the glycoproteins of MVV.

6.5 Conclusions

This thesis has presented studies on the expression of the external glycoprotein of MVV as a recombinant protein. Work in this field has been hampered by the toxicity of the envelope protein as was seen both in the bacterial system and the Ty-VLP expression system. The toxic properties of the envelope fragments was thought to be due to areas of hydrophobicity within the protein. Subsequent expression in the baculovirus system showed that recombinant protein could be produced in adequate amounts with little contamination. Thus, it lends itself to be used in a number of studies, including as a source of antigen and characterization of the host cell receptor. A number of monospecific sera have been raised, including the two anti-peptide and the anti-GST serum, which will be useful in future studies with the baculovirus-derived envelope.

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