

Studies On The Extracellular Envelope
Glycoprotein Of
Maedi-Visna Virus

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This thesis is submitted as part of the course requirements for the degree of Doctor of Philosophy at the University of Edinburgh.

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I would like to thank everyone, both inside and outside the Department of Veterinary Pathology from whom I have received sanity and support during this PhD course.

Declaration

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

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October 1992

UNIVERSITY OF EDINBURGH

ABSTRACT OF THESIS (Regulation 3.5.10)

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Degree PhD Date 29/10/92
Title of Thesis Studies on the extracellular envelope glycoprotein of
..... maedi-visna virus
No. of words in the main text of Thesis 47,142

The extracellular envelope glycoprotein (gp135) of maedi-visna virus interacts with cellular receptor molecules and is the major target of neutralising antisera in vivo. Antigenic drift of gp135 may have an important role in viral persistence.

In order to begin to investigate the roles of different regions of gp135, yeast and bacterial expression systems were used to generate recombinant protein and gp135 was expressed as 3 overlapping fragments. There have been no published reports of expression of recombinant gp135 proteins.

By using the proteins to screen sera from infected sheep it was shown that sheep vary in the regions of gp135 to which they mount an antibody response detectable in this system. At least 3 epitopes on gp135 are recognised by sera from infected sheep.

The recombinant proteins were used to investigate interactions of gp135 with cellular molecules, and as immunogens to raise gp135-specific sera. Possible future experiments using these reagents are suggested.

gp135 fragments derived from different viral stocks of the British isolate of maedi-visna virus were sequenced, to obtain a preliminary estimate of the extent of the variability of the gene. The data suggested the presence of both relatively conserved and variable regions in gp135.

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List Of Abbreviations

BCIP	bromo-chloro-indoyl phenol toluidine salt
BIV	bovine immunodeficiency virus
BSA	bovine serum albumin
CAEV	caprine arthritis-encephalitis virus
CIP	calf intestinal phosphatase
CNS	central nervous system
CPE	cytopathic effects
CSF	cerebrospinal fluid
DMEM	Dulbecco's Modification of Eagle's Medium
DMSO	dimethyl sulphoxide
dsDNA	double stranded deoxyribonucleic acid
DTT	dithiothreitol
<u>E.coli</u>	<u>Eschericia coli</u>
EDTA	ethylenediaminetetra-acetate
EIAV	equine infectious anaemia virus
ELISA	enzyme-linked immunosorbent assay
FITC	fluorescein isothiocyanate
FIV	feline immunodeficiency virus
HIV	human immunodeficiency virus
HSV	herpes simplex virus
IL-2	interleukin-2
IPTG	isopropyl-beta-D-thiogalactoside
kb	kilobase
kDa	kilodalton
LTR	long terminal repeat
MVV	maedi visna virus
NBT	nitroblue tetrazolium
OPD	O-phenylenediamine
orf	open reading frame
PBL	peripheral blood leukocyte
PCR	polymerase chain reaction

PND	principal neutralising determinant
<u>S.cerevisiae</u>	<u>Saccharomyces cerevisiae</u>
sCD4	soluble CD4
SDS	sodium dodecylsulphate
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis
SDW	sterile distilled water
SPA	sheep pulmonary adenomatosis
SPBS	sterile phosphate buffered saline
ssRNA	single stranded ribonucleic acid
SV40	simian virus 40
VAP	viral attachment protein
VLP	virus-like particle
VSV	vesicular stomatitis virus
X-gal	5-bromo-4-chloro-3-indoyl-beta-D- galactoside

1. Introduction

1.1 Brief Historical Background To Maedi-Visna Virus

Maedi-visna virus (MVV) was initially isolated and characterised as a consequence of an epidemic of a progressive pneumonia ("maedi"), accompanied in some cases by progressive paralysis ("visna") which arose in Icelandic sheep flocks between 1939 and 1952. The disease had not previously been recognised. 150,000 animals were lost due to the disease and a further 650,000 destroyed in a successful attempt at disease control (Sigurdsson, 1954a). Transmission of the disease to healthy animals via selected tissue preparations from affected sheep was demonstrated (Sigurdsson, 1954a : Sigurdsson *et al.*, 1957) and in 1967 a single virus was shown to be the causative agent of both sets of symptoms (Gudnadottir & Palsson, 1967).

The outbreak of maedi-visna disease on Iceland has been traced (Sigurdsson, 1954a) to the importation in 1933 of twenty sheep of the Karakul breed from Germany. From the epidemiology of the subsequent outbreak it would appear that at least two of the imported animals were infected with MVV, which had never been seen to cause any disease in Germany. The reasons for its dramatic spread in Iceland probably included the more intensive conditions under which sheep tended to be housed, and its introduction into a sheep population which had been effectively isolated from mainland Europe for hundreds of years.

MVV and the disease which it causes has now been recognised in numerous countries, including the Netherlands where it is known as zweegerziekte (De Boer, 1970), the United Kingdom (Watt *et al.*, 1990), South Africa (Querat *et al.*, 1990), the United States of America, where it is sometimes known as ovine progressive pneumonia virus (Cutlip & Laird, 1976), Greece (Seimenis

et al., 1985) and Belgium (Biront and Deluyker, 1985). In none of these cases has it had the same economic impact as in the Icelandic outbreak although it can be of importance in flocks with a high incidence of MVV infection.

MVV was used (Sigurdsson, 1954b) as a model for a new form of virally-induced disease which Sigurdsson defined as "slow", distinguishing this from a chronic disease such as tuberculosis. Currently MVV has become a focus of research interest because of the increasing level of detection in commercial flocks, and due to its possible use as an animal model for certain aspects of infection by human immunodeficiency virus (HIV).

1.2 Classification Of MVV

MVV is the prototypic member of the Lentivirinae subfamily of the Retroviridae family which includes HIV-1 and HIV-2, feline immunodeficiency virus (FIV) (Pedersen et al., 1987), simian immunodeficiency virus (SIV) (Letvin et al., 1985), caprine arthritis-encephalitis virus (CAEV) (Crawford et al., 1980), bovine immunodeficiency virus (BIV) (Gonda et al., 1987) and equine infectious anaemia virus (EIAV) (Dreguss & Lombard, 1954). With the exception of EIAV in which disease episodes tend to be cyclical the diseases caused by the lentiviruses are characterised by their relatively slow onset and progressively debilitating course.

In common with the other members of the subfamily, MVV possesses a positive-sense single-stranded RNA genome (ssRNA) (Brahic et al., 1977), an RNA-dependent DNA polymerase (reverse transcriptase) (Lin & Thormar, 1970) and replicates via a DNA intermediate (Haase & Varmus, 1973). The lentiviruses are distinguished from the other retroviruses by their more complex genome structure, which in addition to the gag, pol and env genes contains a number of small open reading frames (orfs) which code for regulatory proteins. This results in a complex

transcription pattern (Vigne et al., 1990).

Phylogenetic comparisons of the lentiviruses differ in fine detail, partially dependent on the gene and viral strain studied. However there is reasonable consensus that MVV is more closely related to the non-primate lentiviruses than to the primate species (Olmsted et al., 1986 : McClure et al., 1988), but the relationship between HIV and MVV is closer than that of HIV with various oncogenic retroviruses (Gonda et al., 1985), thereby acting as a further justification for the inclusion of these viruses in the same subfamily.

1.3 The MVV Genome

Six MVV isolates have been sequenced; these represent four variants of the 1514 Icelandic strain (Sonigo et al., 1985; Braun et al., 1987; Staskus et al., 1991), South African-Ovine Maedi Visna Virus (SA-OMVV) (Querant et al., 1990) and the British isolate EV1 (Sargan et al., 1991). The genome structures derived are broadly similar, albeit with some relatively minor differences. Figure 1.1 is a schematic representation of the MVV genome organisation.

The genome size varies from 9202 bases for 1514 to 9256 nucleotides for SA-OMVV. The long terminal repeat (LTR), divided into the U3, R and U5 regions, contains a primer binding site complementary to that for tRNA^{Lys}_{1,2} and a polypurine tract which acts as the initiation site for positive strand DNA synthesis. A well-defined TATA box begins at nucleotide 438 and CCAAT consensus sequences begin at bases 352 and 364 (Hess et al., 1985). The LTR contains target sequences which influence levels of transcription of the genome (section 1.5) many of which appear to be located within the 43base-pair tandem repeats found in the LTRs of all isolates studied. However, while SA-OMVV and 1514 contain two copies of the 43 basepair repeat the majority of the EV1 genomes analysed appeared

Figure 1.1

The MVV genome

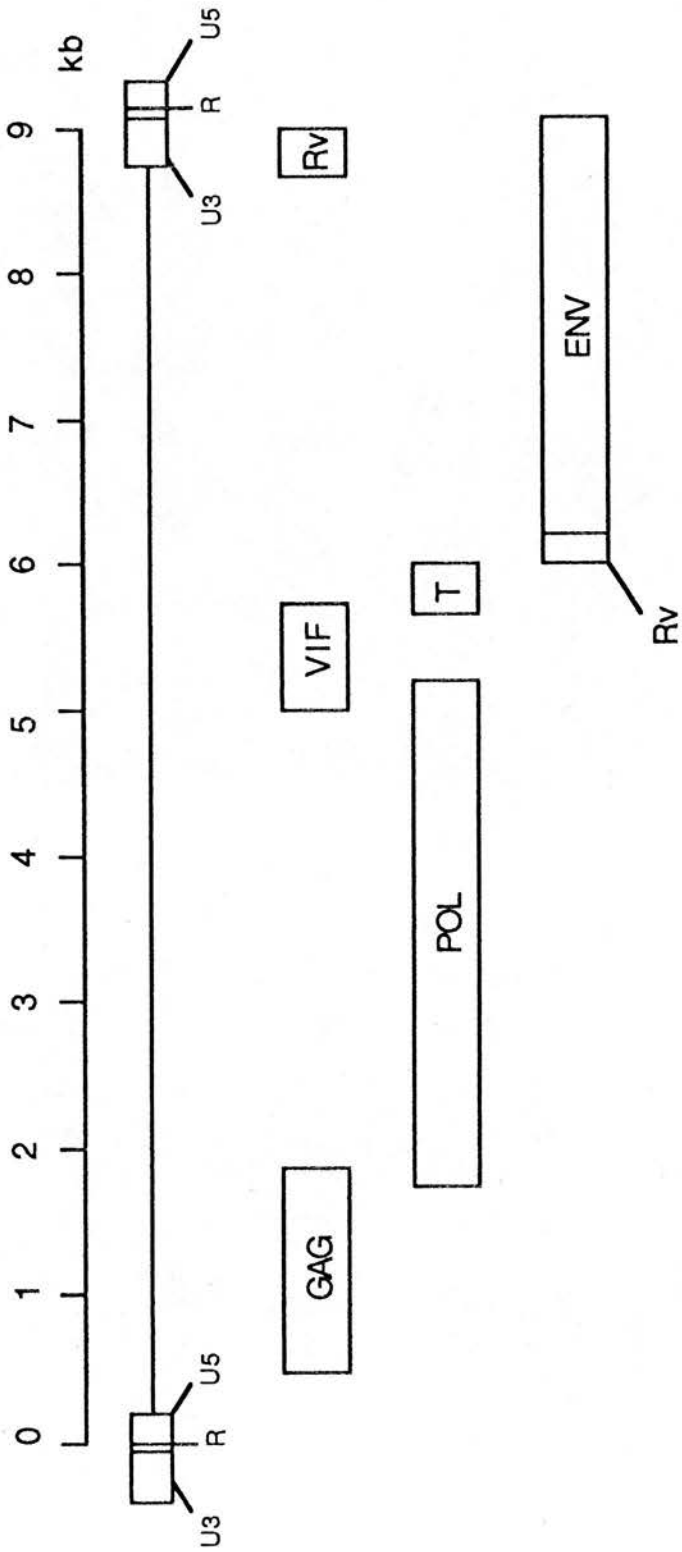
The major open reading frames of MVV are shown. Approximate nucleotide positions are indicated by the scale bar.

Key

T = tat

Rv = rev

Derived from Sargan et al., 1991.



to contain only one such copy. The functional significance of this is unknown.

The viral gag gene encodes the structural nucleoproteins (section 1.4).

By analogy with other retroviruses the pol gene encodes the reverse transcriptase, an endonuclease/integrase and also contains the core sequence of a viral protease, although this activity has not been directly demonstrated for MVV.

The most 3' structural gene is the env gene which codes for the external and transmembrane glycoproteins (section 1.15). All isolates encode an Arg-Lys-Lys-Arg which represents the cleavage site between the outer and transmembrane glycoproteins. A predicted RNA secondary structure within env functions as a cis-acting target sequence for the Rev protein (Tiley et al., 1990).

Of the proteins encoded by the small orfs Tat is encoded by a single exon, unlike the situation in HIV where transcription from two exons is required (Arya et al., 1985). Rev is coded for by two exons, at the 5' and 3' ends of the env gene, the 5' region being in the same reading frame as env. An open reading frame with sequence homology for HIV vif (Strebel et al., 1987) is also present.

There is an additional orf, termed W, in SA-OMVV (Querast et al., 1990). There have been no reports of a functional protein from this orf, and it does not appear to display extensive sequence homology with any other lentiviral orf.

There have been no reports of MVV orfs analogous to the HIV-1 genes vpr (Ogawa et al., 1989), vpu (Strebel et al., 1988), tev (Benko et al., 1990) or nef (Ahmad & Venkatesan, 1988) although an element homologous to a functionally required HIV-1 Nef sequence has been identified in EV1 (Sargan et al., 1991).

1.4 Proteins Of MVV

Initial studies of the proteins synthesised from the MVV genome were hampered by problems in excluding cellular proteins from the assays, and difficulties in the determination of molecular weights, particularly for the glycoproteins. Estimates for the number of polypeptides in the virion ranged from eleven to fourteen (Mountcastle et al., 1972), fifteen (Haase & Baringer, 1974) to twenty five (Lin, 1978). With the advent of monospecific anti-MVV sera, followed by molecular cloning advances, much of this confusion was resolved.

The major precursor of the gag-encoded proteins is a non-glycosylated molecule of 55kDa (Pr55^{gag}) (Vigne et al., 1982) which is cleaved to form the mature proteins p30 (core protein) (occasionally referred to as p25), p16 (matrix protein) and p14 (nucleoprotein). Pulse-chase experiments indicated that the processing and cleavage occurred intracellularly. These are the major internal structural proteins of MVV.

A 150Kda precursor molecule of the gag-encoded products is also detectable in infected cells (Vigne et al., 1982). By analogy with other retroviruses this is believed to be both a Gag and a Pol precursor; this polyprotein is generated by a ribosomal frame-shifting event. The MVV reverse transcriptase is Mg²⁺ dependent but has similar pH, substrate and temperature requirements to the Mn²⁺-reverse transcriptases of the type-C retroviruses (Lin and Thormar, 1970). Production of the predicted integrase and possible protease proteins has not been directly demonstrated, although reports of integration of the MVV genome (Vigne et al., 1985), and the cleavage of the Gag-Pol precursor molecules suggests that both these functions may be present.

Only the env-encoded proteins (section 1.15) of MVV have been shown to be glycosylated. Pulse-chase experiments (Vigne et al., 1982) demonstrated that the Env

glycoprotein is produced as a glycosylated 150kDa precursor, which is subsequently processed to a 135kDa form. The nature of this processing is unclear. Glycosylation inhibitors indicated that the non-glycosylated precursor to the 150kDa molecule had a molecular weight not exceeding 105kDa, and theoretical considerations suggested that the maximum size of the non-glycosylated form of the Env molecule would be 95kDa (Vigne et al., 1982). Env is variously referred to as gp135 or gp105.

The 1982 paper of Vigne et al. does not specifically state whether gp135 represents the entire env-encoded gene product, or the extracellular region only. In addition to the high molecular weight glycoprotein glycosylated proteins with estimated molecular weights of 46kDa and 51kDa have been observed (Haase & Baringer, 1974) in viral preparations. By analogy with other retroviruses, and the fact that most analyses have been conducted using reducing conditions, where the outer and transmembrane glycoproteins would be expected to dissociate it is likely that gp135 represents the extracellular region of Env and the transmembrane region is the gp46/gp51 glycoprotein. For consistency gp135 will refer to the outer membrane glycoprotein, gp46 to the transmembrane glycoprotein, and Env will be used when it is unclear from the literature which region of the env-gene product is under discussion.

The rev gene encodes a 19kDa polypeptide (Mazarin et al., 1990) necessary for the cytoplasmic expression of the incompletely spliced env mRNA (Tiley et al., 1990). HIV-1 Rev contains a region near the N-terminus of forty amino acids, with a highly basic core, mutations of which affect binding of Rev to the Rev-responsive element (RRE) (Hope et al., 1990) and a ten amino acid leucine-rich region towards the C-terminus of the protein, mutations in which have no effect on RRE binding, but which produce a negative viral phenotype (Hope et al., 1990). These major

functional domains are moderately conserved in MVV (Tiley et al., 1991).

The tat gene encodes a protein of 10kDa (Davis & Clements, 1989) or 11kDa (Gourdou et al., 1989) characterised by the presence of a cysteine-rich domain. Tat acts as a positive transactivator of viral transcription; it has been shown (Gourdou et al., 1989) to increase steady-state levels of LTR-directed mRNAs.

The vif gene encodes a 29kDa protein which is detectable late in the lytic cycle in vitro (Audoly et al., 1992). Vif is cytosolic in location and appears to be absent from cell-free virions (Audoly et al., 1992).

1.5 The MVV Life Cycle

In common with the other lentiviruses MVV characteristically displays very different patterns of transcription and replication in vitro and in vivo. In permissive cells in vitro MVV causes extensive cytopathic effect (CPE) with formation of multinucleated giant cells and the release of free infectious virus into the culture medium. This lytic cycle is usually complete in three days (for strain 1514). In vivo however the viral life cycle is interrupted and this has important consequences for viral persistence.

The lytic cycle has been the more thoroughly investigated and will be described first; the in vivo infection will be described in terms of its variation from the lytic process.

1.5.1 In Vitro

Binding of MVV to a cellular receptor (section 1.12) is followed by viral fusion, entry and uncoating. Much work remains to be performed on these early stages in infection. The first stage in viral transcription and replication is reverse transcription of the viral ssRNA to form double-stranded DNA (dsDNA). The basic pathway is thought to be similar to the reverse transcription of most

retroviruses (review see Dahlberg, 1988), and initiated by binding of $\text{tRNA}^{\text{lys}}_{1,2}$ to the primer binding site of the LTR (Sonigo *et al.*, 1985).

By analysis of the low molecular weight DNA fraction from MVV-infected sheep choroid plexus cultures it was determined (Harris *et al.*, 1981) that the major extrachromosomal form of MVV was a linear molecule, comprising a full-length negative strand and a positive strand that contained a gap of 300-500 base pairs just 3' of the centre of the linear molecule. It has been suggested (Sargan *et al.*, 1991) that the presence of a polypurine tract in this region may act as an alternative second strand primer site. While some authors have reported that this gapped linear duplex DNA is by far the predominant form in the infected cell (Blum *et al.*, 1985) other workers (Vigne *et al.*, 1985) have suggested that it forms no more than 20% of the viral DNA. The structure has not been conclusively demonstrated in other lentiviruses and its significance is not clear.

It has been claimed (Harris *et al.*, 1984) that there was no detectable integration of MVV DNA into host chromosomal DNA during a lytic infection. Closed circular DNA is believed to be the topologically preferred form for integration (Panganiban & Temin, 1984) and only 0.1% of the molecules in MVV-infected choroid plexus fibroblasts are in this form (Harris *et al.*, 1981). However, integration has been reported (Vigne *et al.*, 1985) when infecting ovine foetal cornea cells rather than choroid plexus fibroblasts. Integration may therefore be more likely to occur in cells with a higher divisive capacity, or influenced by other cellular factors, but there is no evidence to suggest that it is essential for the infective process in vivo.

dsDNA serves as the template for transcription of viral genes, and for the full-length genomic RNA which will be packaged into virions. MVV has a complex

temporally regulated pattern of transcription. Eight hours after infection of ovine foetal trachea cells with virus strain 1514 no viral RNA was detected (by Northern blotting), presumably as a consequence of reverse transcription of the input RNA (Vigne et al., 1987). At twenty four hours post-infection a low level of mRNA species with apparent sizes of 1.2 to 1.6kb (subsequently redefined as 1.4 and 1.7kb (Gourdou et al., 1989)) were detectable and at seventy two hours post-infection these were accompanied by species of 9.4kb, 3.7kb, 4.3 and 4.8kb. This suggested temporal regulation of expression, with the small mRNAs expressed first, followed by the larger species. A similar pattern has been reported using the same virus strain to infect choroid plexus cells (Sargan & Bennet, 1989).

These results contrast with those obtained by Davis et al., (1987), using the same virus strain and cell type as Sargan & Bennet (1989). Although Davis found a complex transcriptional pattern it differed in a number of details from the work outlined above. At six hours post-infection 9.4, 5.0, 4.3 and 1.8kb mRNA species were detectable. By twelve hours post-infection a 1.5kb mRNA was also detectable, at levels almost equivalent to the 1.8kb molecule, and both continued to increase up to at least twenty-four hours.

All three studies suggested that the two smallest mRNA species were multiply spliced, containing sequences from the 5' end of the genome, the central region between the pol and env genes and terminal 3' sequences. The 3.7 to 4.8kb mRNA species were singly spliced with sequences from the 5' end of the viral genome being spliced to acceptor sites 3' of the pol gene, and the 9.4kb molecule was unspliced. The full-length message is believed to code for the Gag-Pol precursor molecule, and the intermediate molecules for Env, and also the sequences homologous to Vif.

Although they may differ in detail, some of which may be due to experimental variations, all these studies indicate there is complex temporal regulation of expression of the different viral mRNAs, and it is believed that the small, multiply spliced mRNAs play a critical role in this temporal regulation.

The fully spliced transcripts are believed to encode the tat (Gourdou et al., 1989) and rev messages (Tiley et al., 1990). Co-transfection into COS cells of a rev and a LTR-beta globin construct showed that the rev construct trans-activated the viral LTR sequences, either directly or indirectly (Mazarin et al., 1988). Experiments with vectors encoding rev and env demonstrated that cytoplasmic expression of the singly spliced env mRNA required expression in trans of the rev gene product (Tiley et al., 1990) and demonstrated a RRE 3' to the transmembrane protein cleavage site. The mechanism by which Rev increases expression of the singly spliced mRNAs is unclear, and has been referred to as "stabilisation" of the mRNA (Vigne et al., 1990).

In MVV infection therefore Rev appears to be at least dual-functional; it can increase expression of mRNAs from the LTR, and acts to allow the expression of singly spliced env mRNAs in the cytoplasm, the appearance of which is a critical event in progression of a lytic infection in vitro. There is some dispute regarding the cellular localisation of Rev protein in the cell. Mazarin reports a predominantly cytoplasmic location (Mazarin et al., 1990) in MVV-infected ovine cells and a similar pattern has been reported in COS cells transfected with a rev construct (Vigne et al., 1990). However, for a similar construct also transfected into COS cells a nuclear, and specifically nucleoli-based pattern of expression similar to that observed with HIV has been reported (Tiley et al., 1990). There is no clear explanation of this discrepancy.

Co-transfection experiments using Tat-expressing constructs and MVV LTR-beta globin gene constructs showed a dose-dependent steady-state increase in the levels of beta globin mRNA with Tat (Gourdou et al., 1989). The extent of the increase depended on the cell type transfected, suggesting possible interaction(s) with cellular factors. Unlike HIV (Feng & Holland, 1988) no RNA hairpin structure which acts as a target sequence for Tat has been identified in the MVV LTR. It is not known if the nuclear post-transcriptional activity observed with HIV Tat (Braddock et al., 1989) is present in MVV

Translation of the mRNAs, and subsequent processing of the proteins is dependent on cellular enzymes and organelles.

Lytic infection leads to massive amplification of viral genomes with reports of cells containing in excess of 4000 copies of viral RNA (Brahic et al., 1977).

At the end of the lytic cycle the viral genomic RNA and structural and polymerase proteins are packaged. Early work suggested polyploidy of the packaged RNA (Beemon et al., 1976; Vigne et al., 1977) and by analogy with other retroviruses MVV is now described as diploid. Electron microscopic studies (Thormar, 1961 : Coward et al., 1970) of MVV-infected cell cultures in vitro showed MVV virions to have a size range of 65-110mu with a centrally located osmiophilic core, separated from a single outer membrane by a layer of low electron density. The particles form by budding either from the surface of infected cells or into macrophage vacuoles (Georgsson et al., 1990)

1.5.2 In Vivo

While the basic pattern of reverse transcription and gene expression is believed to occur by similar mechanisms in vitro and in vivo the latter situation is subject to much greater constraint. Virus cannot usually be recovered from tissue homogenates from infected animals

but if these are explanted, or if the tissues are maintained in culture with a permissive cell type then infectious virus can be detected. This is consistent with a block on viral replication in vivo which is relieved under tissue culture conditions.

In choroid plexus tissue of experimentally infected lambs proviral DNA was detected in 18% of the cells examined by in situ hybridisation, at levels of 100-200 copies per cell. However the p30 protein was only detected by immunofluorescence in 0.1% of these viral DNA-containing cells (Haase et al., 1977). Similar subsequent experiments (Brahic et al., 1981) found that only 1% to 3% of choroid plexus cells contained viral DNA, at 60-70 copies per cell, and viral RNA was present at levels two orders of magnitude lower than that seen in permissive cells in vitro. This suggested that the restricted expression of MVV in vivo was at least partially due to a transcriptional block.

In contrast, it has also been observed that 15% of alveolar macrophages of experimentally infected lambs contained greater than 1000 copies of viral RNA, but that only approximately 1% of the cells expressing viral RNA produced virus (Gendelman et al., 1985). The authors suggested that this indicated a post-transcriptional block on viral replication. However, the experiments did not involve immunological staining of the same cells which had been assayed by the in situ hybridisation technique. It was not therefore demonstrated that the macrophages which were expressing very high levels of viral RNA were not also expressing viral proteins, since the range of viral RNA expression was from 50-1000 copies per cell.

Restricted replication in vivo has also been demonstrated in monocytes taken from different anatomical locations. Restricted viral RNA expression has been reported in monocytes obtained from the lateral ventricles of experimentally infected sheep (Peluso et al., 1985) and

restricted viral RNA levels have been demonstrated in monocyte/macrophage precursor cells in the bone marrow (Gendelman et al., 1985). Viral genomes in precursor cells may act as a "reservoir" of viral infection in vivo, thereby contributing to viral persistence in the host animal.

The molecular events controlling this restricted replication and gene expression in vivo and release from these restrictions in vitro remain to be fully elucidated. Data suggests that viral gene expression increases during the maturation of monocytes to macrophages (Gendelman et al., 1986) and sequences in the viral LTR may be involved in regulation of transcription in vitro in different cell types.

The 43bp tandem repeats found in all MVV LTRs can act as enhancer elements for a heterologous viral promoter (Hess et al., 1985) and basal transcription levels in sheep choroid plexus cells is highly dependent on sequences within these repeats (Hess et al., 1989). Each repeat contains two copies of a site related to the AP-1 binding site and the LTR also contains a single AP-4 binding site (Gabuzda et al., 1989). In order to investigate the role of cellular factors on activation of the viral LTR mice transgenic for a LTR-CAT (chloramphenicol acetyltransferase) construct were created and LTR-driven CAT expression assessed in various tissues (Small et al., 1989). Expression in resident peritoneal macrophages was low but these levels increased if the macrophages were activated using thioglycolate. This artificial situation may reflect the increase in viral activity observed in the maturation of monocytes to macrophages (Gendelman et al., 1986). Perhaps unexpectedly CAT expression was also observed in splenic lymphocytes (Small et al., 1989) but this was not up-regulated during cellular activation, suggesting an absence in lymphocytes of cellular factors required to

activate the LTR. The AP-1 sites probably mediate the serum responsiveness of the LTR (Hess et al., 1989) and the AP-1 site most proximal to the TATA box is probably the most crucial in regulating induction of gene expression from the LTR (Gabuzda et al., 1989).

Although much remains to be discovered about the restricted life-cycle of MVV in vivo it is probable that interaction of cellular factors with the viral genome, and perhaps particularly the LTR, may play a critical role both in limiting viral replication under the majority of in vivo conditions, and in relieving this block on the viral life-cycle in tissue culture or when a latently infected cell is activated.

1.6 Clinical Consequences Of MVV Infection

The extraordinarily long incubation period for MVV, noted during the initial outbreak as one to three years or more (Sigurdsson, 1954a) is followed by the onset of disease symptoms with little subsequent regression and a generally increasingly pathological course. Various organ systems are affected and histopathologically the lesions are characterised by their inflammatory nature.

The most commonly reported symptom is dyspnoea, which becomes particularly obvious if the affected animal is exercised. On post-mortem examination the lungs may be two to four times heavier than normal with decreased elasticity and a degree of fibrosis (Sigurdsson, 1954a). Histologically the alveolar septa are thickened, due to an accumulation of plasma cells, mononuclear phagocytes and lymphocytes (Georgsson & Palsson, 1971). There is an accompanying formation of lymphoid follicles with active germinal centres (Lairmore et al., 1986) and the regional lymph nodes are enlarged. Interest in the condition has been stimulated by its resemblance to the lymphoid interstitial pneumonia observed in paediatric AIDS cases.

In animals with neurological infection gait is

affected, with paresis of the hindquarters progressing until paraplegia develops. The main histological lesion is demyelination and destruction of white matter in the brain, cerebellum and spinal cord (Sigurdsson et al., 1957). The lesions tend to be initiated periventricularly and are inflammatory in nature. Primary lesions appear to be focussed on the glia with little neuronal destruction (Sigurdsson & Palsson, 1958), and infection of oligodendrocytes and astrocytes by MVV has been confirmed in vivo (Stowring et al., 1985).

The mammary gland has also been shown to be affected by MVV, resulting in a chronic indurative mastitis (van der Molen et al., 1985) again characterised by marked lymphoid hyperplasia and slight to moderate fibrosis.

Arthritis has been observed in MVV-infected sheep, involving swelling and calcification of soft tissue, fibrosis of the joint capsule and synovium and perivascular lymphocytic infiltration (Oliver et al., 1981). This is similar to the arthritis observed in CAEV-infected goats (Crawford et al., 1980).

In certain flocks MVV is found in the same animal as Jaagsiekte retrovirus, the causative agent of sheep pulmonary adenomatosis (Payne et al., 1986). There have been tentative suggestions of in vivo synergism between the two retroviruses (DeMartini et al., 1987), based on accelerated development of lesions when both viruses are present. Readily quantifiable data to support this has not been presented.

In contrast to the oncoviruses there is no clear evidence for any oncogenic action by the Lentivirinae members. Although two early reports (Takemoto & Stone, 1971 : Macintyre et al., 1972) suggested a causative role for MVV in transformation of murine and human cells respectively, these studies were marred by the use of cell lines which showed a high tendency to "spontaneous"

transformation. There are no confirmed reports of a truly oncogenic effect of MVV in ovine cells.

1.7 Pathogenesis

One of the outstanding unsolved problems in lentiviral diseases is the mechanism of pathogenesis. MVV persists in the infected host despite a humoral and cell mediated immune response (section 1.10) In vivo the number of detectable productively infected target cells is low; figures of 1 in 100,000 circulating leukocytes have been quoted for MVV (assayed by virus isolation) (Petursson et al., 1976). While this figure may show some variation within different cell populations in different target tissues it never reaches levels high enough to account directly for the extensive lesions which develop. There have been no reports in vivo of the directly virally mediated formation of syncytia which are observed in vitro.

The predominantly inflammatory nature of the lesions in MVV-induced disease suggested that the disease mechanism could be immunopathological in nature. Support for this hypothesis was obtained from experiments (Nathanson et al., 1976) showing that immunosuppression of sheep led to a major decrease in early central nervous system lesions, but no demonstrable decrease in viral replication.

There have been various reports of alterations in immunological parameters in MVV-infected sheep, although none are as dramatic as the depletion of CD4-positive lymphocytes observed in HIV infection (Gottlieb et al., 1981). Elevated serum immunoglobulin concentrations have been reported in MVV-infected sheep (Molitor et al., 1979) although the technique used was crude (zinc sulphate turbidity) and did not distinguish between MVV-specific and non-MVV-specific antibodies. If levels of MVV-specific immunoglobulin were consistently elevated this

could operate as an immunopathological mechanism, through the formation of immune complexes and aberrant clearing of these. There have however been no direct reports of such a mechanism in MVV-induced disease.

Elevated numbers of cells (not identified) in the CSF of experimentally infected animals have been reported (Petursson et al., 1976); this was generally most marked in the first three months post-infection although most animals continued to show an elevated level thereafter.

It has been reported (Lairmore et al., 1988a) that bronchoalveolar lavage samples from experimentally-infected lambs show a 1.5 fold increase of total leukocytes, and a 4 fold increase of lymphocytes, over the values found for uninfected lambs. 65% of these lymphocytes were CD8-positive, compared with 25% for the control animals. An increase in the absolute numbers of CD8-positive lymphocytes in the circulation of a single clinically affected sheep has also been reported (Kennedy-Stoskopf et al., 1989).

Bronchoalveolar lavage of lungs from naturally infected sheep (diagnosed histopathologically) demonstrated a doubling of total cell number but no significant difference in the percentage of these cells which were lymphocytes (Cordier et al., 1990). No information was available on the clinical status of these sheep (which were obtained from a commercial abattoir) prior to slaughter. There was a significant increase (13% against 5% for normal animals) in the percentage of neutrophils in the lavage. The same paper also demonstrated elevated spontaneous release of a neutrophil chemotactic factor and fibronectin by macrophages obtained from the infected lungs. This activity may play a role in the recruitment of inflammatory cells to the lesion, and in the fibrosis observed in affected lungs.

15.5% of alveolar macrophages from bronchoalveolar lavage material from uninfected sheep expressed major

histocompatibility (MHC) class II antigens but this level was increased to 50.1% in the infected sheep (Cordier et al., 1990). Other workers have also reported increased MHC class II levels on macrophages from infected animals (Kennedy et al., 1985) and have postulated that this is a consequence of induction of an interferon after interaction between lymphocytes and lentivirus-infected macrophages (Narayan et al., 1985). Spontaneous release of interferon by pulmonary leukocytes (Lairmore et al., 1988b) taken from lambs with severe lymphoid interstitial pneumonia, but not from uninfected or asymptomatic controls has been reported. It is not clear if the interferon detected in these sets of experiments belongs to the previously characterised classes of interferons or is a novel molecule.

Much of the data described above for macrophages suggests a possible "over-activation" of the cells, with increased release of inflammatory mediators, which in turn would attract inflammatory cells to a lesion. It is possible that continual low level virus production in vivo may act as a constant stimulus for this immunological activity.

Interleukin-2 (IL-2) activity can be detected in supernatants from lymph node-derived mononuclear cell cultures, stimulated in vitro with concanavalin A. It has been reported (Ellis & DeMartini, 1985) that decreased levels of IL-2 were released when the cultured cells were derived from symptomatic naturally-infected sheep, but not when they were obtained from asymptomatic or uninfected animals. In contrast to the macrophage data this suggests a decrease in immune responsiveness in clinically affected animals.

A sequence homology over twelve amino acids (eight of which are conserved) has been identified between the human and sheep 21.5K myelin basic protein and the visna pol gene product (Carnegie & Weise, 1987). It has been

suggested that this could form the basis for an autoimmune reaction in the demyelination observed in central nervous system lesions. There has been no direct confirmation of this mechanism.

Peptides derived from the gp41 and vif regions of the MVV genome have been reported (Ruegg et al., 1990) to inhibit IL-2 or T-cell antigen receptor driven proliferative responses in vitro. The significance of this is unclear, particularly as immunosuppression has not been identified as a major factor in MVV-induced disease.

Pathogenesis therefore appears to involve disruption of the functioning of the immune system, with possible inappropriate over-activation of effector cells having a role in the observed lesions. While individual pieces of data indicate regulatory dysfunction there is as yet no coherent model for the means by which this dysfunction arises (eg as a direct or indirect consequence of macrophage infection) or precisely which components of the immunological network are involved.

1.8 Phenotypic Variants Of MVV

Various strains of MVV differ in their pathogenicity in vitro and in vivo. Both highly lytic and persistent (in vitro) strains were isolated from naturally infected sheep (Querat et al., 1984), although only one type of isolate could be recovered from an individual animal. Intratracheal injection of either low passage (less than ten passes) or high passage virus into lambs, with sacrifice at various time points up to twenty eight weeks, resulted in the development of histological and clinical lesions only in those animals infected with low passage virus (Lairmore et al., 1986). However, since the high passage isolate had not been derived from the low passage isolate the authors' conclusion that in vitro passage alters viral characteristics is not necessarily valid. Two strains which caused a highly lytic infection of goat

synovial membrane cells in vitro differed in their cytopathic effect on alveolar macrophages in culture. One isolate caused rapid and severe fusion, and this isolate was also highly pathogenic in vivo. The other strain showed little cytopathic effect on alveolar macrophages and no pathogenic effects were observed in vivo (Lairmore et al., 1987). While this study demonstrated a correlation between in vitro and in vivo pathogenicity the attempts to link this with the extent of previous tissue culture passage were again inappropriate.

MVV strains have therefore been shown to vary in their phenotypic characteristics. There is as yet no clear data relating this to any particular MVV genes.

1.9 Transmission

The major route of transmission of MVV is horizontal spread via the respiratory route, and is probably exacerbated by close housing practices. In flocks in which the length of exposure of lambs to infected ewes was varied there was a rising incidence of transmission with increasing periods of contact, rising to 81% of lambs showing evidence of MVV infection when the flock was maintained intact for one year (De Boer et al., 1979). Horizontal transmission between adult sheep has also been demonstrated for flocks maintained under standard management conditions (Houwers & van der Molen, 1987). Concurrent MVV and sheep pulmonary adenomatosis (SPA) may lead to increased transmission of MVV infection (Dawson et al., 1990), possibly as a consequence of the increase in volume of respiratory exudates caused by the SPA.

There is little clear evidence for vertical transmission of MVV. Fifty lambs snatched from their infected dams at birth (De Boer et al., 1979) all remained uninfected (only 80% of the dams may have been infected) and no direct evidence for transplacental transmission could be demonstrated in the experiments of Sihvonon

(1980). However, in experiments with hysterectomy-derived lambs from infected dams two out of eleven lambs from infected ewes showed histological lesions compatible with MVV infection (Cross et al., 1975). It is possible that the virus was transmitted during the surgical manipulation and it is generally accepted that vertical transmission is at best minor. However, as foetuses can be experimentally infected in utero (Narayan et al., 1974) by direct injection of the virus into the foetus the lack of natural vertical transmission may be due to the cell-associated nature of the virus in the blood rather than a lack of foetal susceptibility

Unlike CAEV, in which it has been clearly demonstrated that maternal colostrum is a major infectious route (Ellis et al., 1983), such a mechanism has not been unequivocally demonstrated for MVV, despite the presence in ovine milk of cells from which virus can be intermittently isolated (Sihvonen, 1980).

Transmission of virus has been reported from faecally-contaminated drinking water (Sigurdsson, 1954a). The importance of this as an infectious route is possibly minor.

In contrast to HIV sexual transmission of MVV has not been reported. There have also been no reports of an invertebrate vector, such as that implicated in transmission of EIAV (referenced in Narayan & Clements, 1989).

1.10 Host Immune Responses To MVV

Sequential studies using experimentally infected sheep have demonstrated the generation of complement-fixing and neutralising antibodies to MVV (Gudnadottir & Kristindottir, 1967), the complement-fixing antibodies arising one to two months post-infection and the neutralising antibodies approximately six months after infection. These results were extended to show early

generation of MVV-specific antibodies in experimentally infected sheep which were detectable using an indirect immunofluorescence technique (De Boer, 1970). This response rose more quickly than the complement-fixing response, which again preceded the neutralising reactivity. The minor discrepancies between the two reports in terms of the absolute time-scales of response may reflect differences in technique.

Variations in titre of complement-fixing antibodies between different sheep have been reported (Petursson et al., 1976), varying from 1/32 to 1/1024 and similar variations were found for titres of neutralising antibodies. The same report also suggested local production of complement-fixing antibodies in the central nervous system. Free MVV can be detected in the CSF in the early stages of infection after intra-cerebral inoculation; this local production of MVV-specific immunoglobulin may be implicated in the subsequent disappearance of free virus from the CSF (Petursson et al., 1976).

With the exception of one report (Houwers & van der Molen, 1987) in which two sheep in a naturally infected flock became seropositive (as determined by an ELISA) and then seronegative again the papers referred to above also show persistence of the MVV-specific antibody responses.

Although sheep develop antibodies which are described as neutralising this is often a reflection of an in vitro assay technique involving prolonged prior incubation of MVV with serum and may not be an accurate representation of the in vivo activity of these immunoglobulins. In vitro MVV binds to target cells within the first five minutes of incubation, and virtually maximal attachment occurs within the first fifteen minutes. Incubation of MVV with neutralising antiserum for fifteen minutes results in no detectable fall in virus infectivity. A

possible consequence of this in vivo is that the rate of virus binding to cells may be faster than the rate of neutralisation (Kennedy-Stoskopf & Narayan, 1986).

Neutralisation in vitro has been shown to involve immunoglobulin of the IgG1 class and to be complement-independent (Kennedy-Stoskopf & Narayan, 1986). This contrasts with CAEV where in goats generation of neutralising antibodies is virtually non-existent, and the neutralising action of sera from immunised rabbits is complement-dependent (Klevjer-Anderson & McGuire, 1982).

Neutralising antibodies prevent binding of MVV to the surface of choroid plexus cells but act via a different mechanism in simian virus 40 (SV40)-transformed macrophages. Immune sera enhances binding and entry of MVV in these cells, possibly via an interaction with Fc receptors, but no viral reverse transcription occurs, indicating a post-entry block on the viral life-cycle (Kennedy-Stoskopf & Narayan, 1986). The antibody-dependent enhancement of infection observed for HIV-1 at sub-neutralising levels of antiserum (Takeda et al., 1988) has not been reported for MVV.

The viral proteins against which the humoral response is mounted were determined in a longitudinal study of experimentally and naturally MVV-infected lambs. By immunoblotting it was shown that the first antibody to be generated was anti-p30 followed by anti-gp135, anti-p16 and anti-p14 (Kajikawa et al., 1990), with some minor variation between sheep. Anti-Env immunoglobulins are likely to be the major neutralising antibodies as the Env glycoprotein is external on the virion. Antibodies to the 11kDa Rev protein have subsequently been demonstrated in experimentally-infected sheep (Vigne et al., 1990). However, the functional significance in vivo of the reactivity to each of these viral proteins is unclear.

Much less data is available on the cell-mediated response to MVV infection. An early cell-mediated immune

response to MVV has been demonstrated within one week of experimental intracerebral inoculation (Griffin et al., 1978). This declines to control levels four to six weeks post-inoculation and is typical of the cell-mediated response to an acute infection. However, a proliferative response to virions and recombinant p30 protein, involving CD4-positive and CD8-positive cells, has been consistently demonstrated one to four years post-infection (Reyburn et al., in press).

The available evidence therefore suggests that MVV persists in an infected animal despite an active immune response to the virus. The immune response appears to be incapable of clearing the virus in the early stages of infection, thereby permitting establishment of infection in the host tissues, and virus continues to be produced subsequently despite the continuing specific immune response.

1.11 Tropism Of MVV

1.11.1 Species Tropism

MVV has been shown to infect sheep and also goats (Banks et al., 1983). It has been suggested (Houwens et al., 1989) that different sheep breeds may differ in their susceptibility to MVV but there are difficulties in distinguishing breed variations from inbred familial effects.

In vitro MVV has been reported to infect cells from other species (Gilden et al., 1981), including those of murine, bovine, guinea pig, dog, rat and human origin. There have been no reports of in vivo infection by MVV of any species other than sheep and goats.

1.11.2 Cellular Tropism

Although in vitro MVV replicates to high levels in ovine fibroblasts this is not the major target cell population in vivo. Macrophages were shown to be infectable in vitro and macrophages from infected animals

produced virus detectable by a co-cultivation assay with fibroblasts (Narayan et al., 1982). Cells of the monocyte/macrophage lineage are the major target cell of MVV in vivo. The more highly activated the cell the more susceptible it is to infection, and transcription and translation from the viral genome are dependent on the maturational/activational status of the cell (Gendelman et al., 1986). Clusters of infected macrophage precursors have been found in the bone marrow, which may act as a viral reservoir (Gendelman et al., 1985); this is essential as blood monocytes and tissue macrophages are relatively short-lived. There is selectivity in the macrophage populations which are infected by MVV, for example, there have been no reports of infection of liver Kupffer cells or histiocytes. Restricted cellular tropism in vivo may be related to cellular factors implicated in viral regulation (section 1.5)

There are no convincing reports of MVV infection of lymphocytes. In the description of viral antigens detected in the central nervous system (CNS) of experimentally-infected sheep the identification of lymphocytes which were expressing viral core antigen was based on morphological grounds only (Georgsson et al., 1989). This work also did not exclude the possibility that the detected cells were actually B lymphocytes which had bound MVV antigen via their immunoglobulin molecules. A report of MVV infection of T-helper cells, accompanied by a loss of MVV-specific activity (Jolly et al., 1989) has not been supported by any published data. Infection of dendritic cells in vivo has recently been suggested (Gorrell et al., 1992).

1.12 The Cellular Receptor For MVV

Initial reports of susceptibility to MVV infection in vitro in cells from a wide range of species (Gilden et al., 1981) suggested that the viral receptor could be a

fairly ubiquitous molecule. Subsequent work has not confirmed this. MHC class II antigens have been reported to bind MVV and soluble class II molecules were reported to block infection of permissive choroid plexus cells (Dalziel *et al.*, 1991). MHC class II molecules may therefore be involved under certain circumstances in interaction of the virus with a cellular receptor, although expression of MHC class II on ovine cells is not in itself sufficient to render these cells susceptible to MVV infection. For example, B lymphocytes and activated T lymphocytes express MHC class II molecules but do not appear to be infected by MVV. Antibodies to an unidentified 50kDa protein of sheep choroid plexus cells block binding of MVV to these cells in culture (Crane *et al.*, 1991a). This molecule may represent another component of the cellular receptor for MVV.

1.13 Viral Attachment Proteins and Cellular Receptors For Viruses

The first stage in viral infection of a cell is attachment of the virus via a viral attachment protein (VAP) to a cell surface molecule. These VAPs form either the envelope or the capsid of enveloped and non-enveloped viruses respectively. Major advances have been made in recent years in the identification of viral receptors, and in analysing the stages in viral entry subsequent to viral attachment.

A wide range of cellular surface molecules have been shown to act as viral receptors. These range from relatively wide-spread molecules such as sialic acid residues for influenza virus (Paulson *et al.*, 1979), negatively charged phospholipids for vesicular stomatitis virus (VSV) (Schlegel *et al.*, 1983) and heparan sulphate proteoglycans for herpes simplex virus (HSV) (WuDunn & Spear, 1987) to molecules with more restricted cellular distributions. These include various members of the

immunoglobulin gene superfamily, amongst them CD4 for HIV-1 and HIV-2 (Dalglish et al., 1984; Klatzman et al., 1984), ICAM-1 for major group rhinoviruses (Greve et al., 1989; Staunton et al., 1989), class I MHC molecules for SV40 (Atwood & Norkin, 1989; Breau et al., 1992), a member of the carcinoembryonic antigen family for mouse hepatitis virus (Dveksler et al., 1991) and a molecule of unknown physiological function for poliovirus (Mendelsohn et al., 1989). Other cellular molecules which have been identified as viral receptors include the complement receptor CR2 for Epstein-Barr virus (Fingeroth et al., 1984), a cellular basic amino acid transporter for murine leukaemia virus (Kim et al., 1991; Wang et al., 1991) and aminopeptidase N for certain human and porcine coronavirus strains (Delmas et al., 1992; Yeager et al., 1992).

However, it is becoming clear that while in many instances cellular receptors are necessary for viral infection they may not be in themselves sufficient to render a cell susceptible. For example transfection experiments resulting in expression of CD4 on normally CD4-negative human cells renders these cells susceptible to infection by HIV. Murine cells expressing human CD4 remain non-permissive for infection, but virus can attach to these cells and they will support viral replication if the genome is introduced directly into the cell (Maddon et al., 1986). This suggests a post-receptor block on viral infection in murine cells, possibly at the stage of membrane fusion or uncoating of the virus, which may be dependent on accessory molecules present only on primate cells.

Transcripts for the mRNA which encodes the poliovirus receptor (Mendelsohn et al., 1989) are found in a wide range of cell types in vivo but viral replication is restricted to the oropharyngeal and intestinal mucosa, the Peyer's patches and motor neurons of the CNS. If expression of mRNA correlates with expression of the

receptor molecule on the cell surface this would also suggest that the presence of a cellular receptor is not in itself sufficient to ensure viral replication.

A virus may also be able to infect cells through a molecule other than the primary characterised receptor. HIV-1 for example has been shown to infect CD4-negative cells (in an antibody-independent manner) (Harouse *et al.*, 1989). Certain viruses eg West Nile flavivirus, can subvert the host humoral response by binding to specific immunoglobulin and so infecting macrophages, presumably via their Fc receptors (Peiris & Porterfield, 1979).

VAPs are found in multiple copies on the virion and therefore attachment may potentially involve high affinity interactions or a large number of low affinity reactions, which together generate a high avidity interaction.

Entry of viruses into the host cell can be classified as pH-independent or pH-dependent. Viruses which are internalised by endocytosis, where entry relies upon fusion of the viral and endosomal membranes, are classed as pH-dependent. For example, the influenza glycoprotein precursor HA0 is cleaved by cellular proteases to form HA1 (N-terminal, external glycoprotein) and HA2 (C-terminal transmembrane) which remain associated via a disulphide bridge and form homotrimers on the virion surface (Gething *et al.*, 1986). Upon acid-activation in the endosome there is a configurational change in the homotrimer which exposes a previously hidden hydrophobic domain in the HA2 subunits (Doms & Helenius, 1986) which is believed to mediate fusion of the viral and endosomal membranes. This pH-dependent change is crucial for the establishment of the viral infection of the cell.

The separation of functions between different viral structures during viral attachment and entry is a common feature of viruses from many different families. The Env glycoprotein of HIV contains separate epitopes for receptor binding and fusion, predominantly localised to

the external and cytoplasmic regions (section 1.14). Unlike influenza virus HIV generally fuses with the plasma membrane (Stein et al., 1987), and the reaction is pH-independent.

There is an increasing amount of data to show that binding of a VAP to a cellular receptor results in changes in either the VAP itself, or other viral constituents which influence subsequent stages of the viral life cycle. Binding of poliovirus to cells for example results in the loss of the VP4 viral protein. It is possible that occupation of the receptor binding site disrupts the viral capsid leading to exposure of the N-terminus of VP1, with a likely effect on viral attachment and release of viral RNA into the cell (Hogle, 1988). HSV glycoproteins have been shown to interact in a complex cascade during cellular attachment and entry (Fuller & Lee, 1992).

Interaction of VAPs with cellular receptors is therefore multifactorial and should not be viewed as an isolated event in the viral life cycle. The interaction cannot be considered in isolation as simply a targeting mechanism, as it triggers a range of reactions critically important for the outcome of the viral infection.

1.14 The Envelope Glycoprotein Of HIV

The envelope glycoprotein of HIV is the most thoroughly characterised of all retroviral envelope proteins and it is possible only to discuss some of its major features in this introduction.

The env gene of HIV-1 encodes a precursor molecule of 856 amino acids (gp160) which is processed to produce an external N-terminal glycoprotein of 480 amino acids (gp120) and a transmembrane C-terminal glycoprotein of 345 amino acids (gp41) (Muesing et al., 1985). Minor differences in amino acid number and protein size exist between different isolates (Ratner et al., 1985; Sanchez-Pescador et al., 1985; Wain-Hobson et al., 1985). In HIV-

2 the processed forms are termed gp125 and gp36.

A hydrophobic signal peptide is cleaved from the precursor protein during envelope maturation (Allan et al., 1985). After cleavage of gp160 to gp120 and gp41 the two glycoproteins may remain non-covalently associated, or the gp120 may be shed. Both C- and N-terminal regions of gp120 are involved in its interaction with gp41 (Helseth et al., 1981; Ivey-Hoyle et al., 1991). Mature gp120 is heavily glycosylated, containing twenty-four (Muesing et al., 1985; Ratner et al., 1985) to twenty-six (Sanchez-Pescador et al., 1985) potential N-linked glycosylation sites. Gp120 molecules form oligomers on the surface of virions; however it is not clear if these are trimers (Weiss et al., 1990) or tetramers (Schawaller et al., 1989).

Comparisons of HIV-1 isolates have revealed high sequence diversity in the env gene, particularly in the extracellular region (Starcich et al., 1986). Compared with alterations in the gag and pol genes the env gene changes were not only greater in number but involved a disproportionately higher level of insertions, deletions and non-silent single base changes. Nucleotide changes were not randomly distributed throughout the gp120 coding region; after cleavage of the signal peptide five hypervariable regions (V1 to V5, labelling from the 5' end of the gene) were identified, interspersed with well-conserved sequences (figure 1.2). These regions of high sequence diversity generally coincided with regions of predicted high antigenicity (Starcich et al., 1986).

The eighteen cysteine codons in the gp120 coding sequence were conserved between the five isolates studied (Starcich et al., 1986), indicating a maintained "macrostructure" for HIV-1 gp120, which may be a consequence of functional constraints.

Although infection of cells via Fc receptors has been demonstrated in vitro in a CD4-independent manner

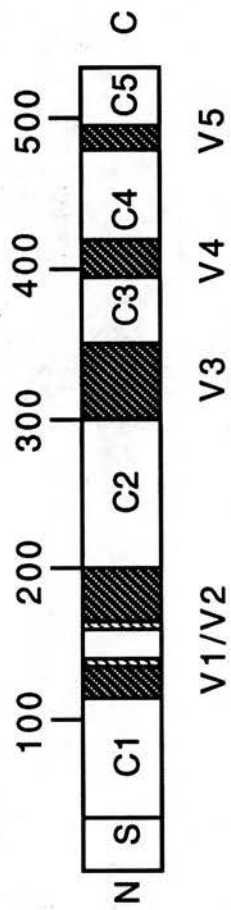
Figure 1.2
HIV gp120

The gp120 glycoprotein of HIV-1 is shown diagrammatically. Regions of high conservation between isolates are unshaded. Regions of high variability between isolates are shaded. Approximate amino acid positions are shown above the diagram.

Key

S = signal sequence

Adapted from Olshevsky et al., 1990.



(McKeating et al, 1990) the binding of gp120 to the CD4 antigen is a major determining event in viral infection under most physiological conditions (Dalgleish et al., 1984; McDougal et al., 1986). The reaction is high affinity with a dissociation constant of at least 4×10^{-9} M (Lasky et al., 1987). Gp120 and the natural physiological ligand of CD4, the MHC class II molecule, bind to different epitopes of CD4 (Lamarre et al., 1989; Fleury et al., 1991). Studies using soluble CD4 antigen (sCD4) (Sattentau & Moore, 1991) have demonstrated that binding of sCD4 to gp120 results in conformational changes in both gp120 and gp41. These changes may be necessary stages in the exposure of a predicted fusion epitope in gp41 such as the highly conserved leucine zipper motif reported to be critical for HIV membrane fusion and viral entry (Dubay et al., 1992). Binding of sCD4 to gp120 can also result in dissociation of the non-covalently linked gp120 and gp41 (Sattentau & Moore, 1991). High level Env glycoprotein expression on the surface of infected cells may result in binding to CD4 antigens on uninfected cells, thereby initiating cell fusion and leading to syncytium formation and cell death (Lifson et al., 1986; Sodroski et al., 1986).

Various regions of gp120 have been identified which are involved, directly or indirectly, in binding to CD4. Deletion mutants and anti-gp120 monoclonal antibodies demonstrated a critical role for amino acids within the protein region 397-439 (Lasky et al., 1987). Three non-continuous regions of the C-terminal half of gp120 were also implicated in gp120/CD4 binding (Kowalski et al., 1987), and a 25kDa fragment of the C-terminal portion of gp120 can bind CD4 (Nygren et al., 1988). However, residues in the N-terminal region of gp120 have also been shown to have a critical role in binding of gp120 to CD4 (Syu et al., 1990). The interaction of gp120 with CD4 therefore appears to involve a number of sequence and

conformational determinants within gp120. This is supported by data reported using a human anti-gp120 monoclonal antibody which inhibits binding to CD4, but which does not map to any of the previously defined binding epitopes (Ho et al., 1991).

Although a region within the second conserved domain of gp120 has been identified which is important in viral infectivity (Ho et al., 1988; Willey et al., 1988) the epitope is probably not exposed under physiological conditions, therefore recent research has been focussed on the V3 domain of gp120 as this may be a possible target for neutralising antisera. A monoclonal antibody reactive with an epitope located between amino acids 303 and 323 neutralised viral infectivity but had no effect on binding of gp120 to CD4 (Linsley et al., 1988). The same epitope, putatively consisting of a loop between cysteine residues at 296 and 331 ("the V3 loop") was shown to elicit type-specific neutralising antibodies in chimpanzees (Goudsmit et al., 1988) and goats (Palker et al., 1988). A peptide spanning amino acids 307 to 330 can completely block fusion inhibition by antisera reactive with the same HIV gp120 type (Rusche et al., 1988). This principal neutralising determinant (PND) in the V3 loop of different isolates consists of a generally conserved Gly-Pro-Gly-Arg sequence at the tip of the loop, flanked by variable amino acids (Javaherian et al., 1989) which may account for the type-specificity of the neutralising antisera.

Different HIV isolates vary in their preferred cellular tropisms, particularly as to whether they replicate more efficiently in monocytes, peripheral blood leukocytes or T cell lines. A number of studies have mapped these tropisms to regions of gp120 which include the PND (Liu et al., 1990; O'Brien et al., 1990; Shioda et al., 1991; Westervelt et al., 1991) and two studies have implicated residues within the PND itself (Hwang et al., 1991; Takeuchi et al., 1991). Direct evidence on the

function of the PND in vivo is limited but it is not believed to interact with CD4. Therefore the neutralising ability of sera directed against the PND must be a consequence of a post-receptor binding event. Mutations in the V3 loop have no effect on Env glycoprotein synthesis, processing or transport (unless one of the conserved cysteine residues is mutated), nor do they affect binding to CD4, but they have been reported to abolish envelope-induced cell fusion (Freed et al., 1991). In vitro various proteinases have been shown to cleave gp120 within the PND (Clements et al., 1991). Cleavage is increased if the gp120 is bound to sCD4 but blocked by binding of neutralising sera. The authors have suggested that cleavage by specific cellular or endosomal proteinases may be a significant event subsequent to binding of gp120 to CD4. Peptides containing the conserved Gly-Pro-Gly-Arg sequence inhibit a membrane-bound serine esterase purified from a human CD4-positive lymphocyte clone (Kido et al., 1990) and a requirement for cleavage by various cellular proteinases could act as a mechanism by which variations in the PND affect cellular tropism. It is probable, however that other regions of the env gene also play a role in cellular tropism as a CD4-binding region of gp120 and the N-terminal portion of gp41 affect the adaptation to growth of certain isolates in tissue culture cell lines (Fujita et al., 1992) in the absence of any variations in the V3 loop.

The g1 envelope glycoprotein has been implicated in cellular tropism of pseudorabies virus (Card et al., 1992), indicating that this phenomenon is not restricted to the lentiviruses. Conversely, among the lentiviruses themselves it may not be the only determinant of cellular tropism as it has been demonstrated in vitro that tropism of EIAV is env-independent (Perry et al., 1992)

1.15 Structure And Function of MVV Envelope Glycoprotein

Due in part to the lack of easily available well-characterised gp135, and the only very recent advances in determining the cellular molecules with which it interacts, information on the structure of gp135 is limited and there has been little progress in assigning functional roles to different regions of the molecule.

The env genes sequenced from various MVV isolates (section 1.3), display both sequence diversity (section 1.15) but also conservation of certain features (figure 1.3). The first hydrophobic region, which is presumed to act as a signal sequence, does not occur at the N-terminus of the protein but between amino acids 78 to 100 (Sonigo et al., 1985) (the actual positions vary slightly between isolates). This may be related to the utilisation of the first forty eight amino acids to encode both Env and Rev. A similar internal location of the hydrophobic signal sequence has also been reported for FIV (Stephens et al., 1992). It is not clear if cleavage of this signal sequence and the amino acids preceding it occurs in MVV, as is found in HIV (Allan et al., 1985) and FIV (Stephens et al., 1992). By analogy with HIV the precursor protein is believed to be cleaved into external and transmembrane components (gp135 and gp46); there is no data on the regions involved in maintaining this presumably non-covalent interaction post-cleavage.

Bromelain digestion experiments indicated that the envelope glycoprotein formed "spikes" on the surface of the virion (Mountcastle et al., 1972). No data is available on any oligomeric organisation of gp135.

The external part of the Env protein encodes twenty three N-linked glycosylation sites (Querat et al., 1989). Sialic acid residues have been demonstrated on the surface of MVV particles, presumably as terminal groups on carbohydrates (August et al., 1977). However, the levels do not appear to be as high as in CAEV, and no clear

Figure 1.3

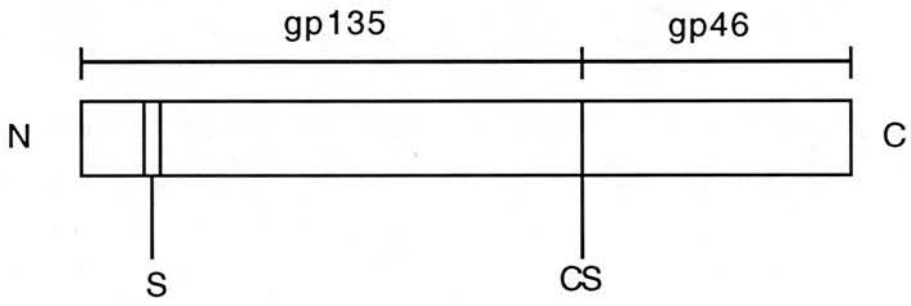
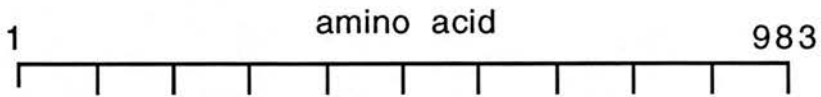
MVV envelope glycoprotein

The envelope glycoprotein of MVV is shown diagrammatically. The full-length glycoprotein is cleaved to form the extracellular region (gp135) and the transmembrane region (gp46). Approximate amino acid positions are shown above the diagram.

Key

S = signal sequence

CS = cleavage site



functional role has been assigned to them. This is in contrast to CAEV where the residues are believed to have an important role in protection of the virus from neutralising antibodies, probably through steric hindrance (Huso et al., 1988).

Gp135 has been identified as an antigenic target during immune responses to MVV infection (section 1.10). Immunisation of guinea pigs with lectin-purified gp135 generated neutralising antisera, whereas antisera raised against p30 had no neutralising effect (Scott et al., 1979). These experiments indicated that gp135 was probably the major target for neutralisation of virus infectivity by specific antisera.

There is little information available on the transmembrane region of Env. In CAEV antibody titres to the transmembrane glycoprotein are high (McGuire et al., 1992) but such a response has not been reported in MVV. A possible fusigenic epitope has been identified in MVV gp46 (Crane et al., 1991b) (section 1.17).

1.16 Antigenic Drift And gp135

Antigenic drift and consequent evasion of host humoral responses has been reported in a number of disease systems including EIAV (Kono et al., 1973), human influenza A virus (Laver & Webster, 1968) and rabies virus (Wiktor & Koprowski, 1980). HIV shows sequence diversity both in isolates from one individual, and between different individuals (Hahn et al., 1986; Saag et al., 1988). While it is not disputed that antigenic variants do arise during MVV infection there is controversy over the frequency of such variants, their in vivo significance, particularly for long-term persistence of the virus in the presence of an active immune response, and the role of neutralising antibody in stimulating the generation of these variants.

1.16.1 Evidence For Antigenic Drift In MVV Infection

Much of the data suggesting a role for antigenic drift in MVV infection and persistence has been derived from longitudinal studies of experimentally infected sheep.

Four lambs were infected intracerebrally with plaque-purified MVV strain 1514, blood samples taken thereafter at regular intervals and virus isolations made from the peripheral blood leukocytes (PBLs) of these animals (Narayan et al., 1977). All sheep raised neutralising antisera to the inoculating viral strain within six months, but the sera all failed to neutralise another strain of MVV, D1-2, indicating strain-specificity in the neutralisation response.

Four strains of virus isolated from leukocytes of the four sheep at twenty two months post-infection were not neutralised by sheep sera, whether collected before or after virus isolation. This indicated the generation of virus mutants which were not neutralised by the host's antibodies. In vitro passage indicated stability of this feature ie virus isolates which were neutralised by a particular sheep serum were still neutralisable by the same serum after a variable number of passages through tissue culture (Narayan et al., 1977).

This work was extended, partly to address the issue of whether the mutant virus replaced the infecting strain, or if the two co-existed. Sheep no. 1 from the experiment described above was studied intensively. The original neutralisation-resistant variant isolated was designated LV₁₋₁ and six further isolates were obtained over the following eighteen months (LV₁₋₂ to LV₁₋₇) (Narayan et al., 1978). All isolates including the parental infecting strain 1514 were tested in neutralisation assays with serum samples collected at different time points post-infection. On the basis of their neutralisation patterns the viruses were placed into three antigenic groups - one isolate had the same neutralisation profile as 1514, while

the other six could be placed into one of two groups. The isolation of a relatively late strain with the same neutralisation profile as the infecting strain suggested that parental and mutant virus strains could co-exist in the same host. From their time-course data on this and other experimentally infected sheep the authors concluded that neutralisation variants did not arise until after the development of antibodies capable of neutralising the parental strain.

However, a sheep infected experimentally with plaque-purified isolate LV₁₋₁ produced antisera which neutralised this infecting viral strain, but also neutralised 1514, and at a higher dilution (Narayan *et al.*, 1978). The authors suggested that this represented some type of "one-way" cross-reactivity between the viral strains but it is not clear that an isolate with similar properties to 1514 has not simply been generated during the LV₁₋₁ infection. It is also important to recognise that there is a slightly arbitrary aspect to the particular strain which is isolated at a given time point if more than one strain is present in the circulating PBL population, particularly if different isolates vary in the efficiency with which they can replicate in tissue culture.

It was claimed (Scott *et al.*, 1979) that these results were reproduced using guinea-pig serum raised to lectin-purified 1514 gp135, but the paper does not actually show strain-specific neutralisation by this serum.

Sera from infected sheep show a broadening of the neutralising range with time (Narayan *et al.*, 1981). An interesting aspect of the development of neutralising sera is differential reactivity to "precursor" (ie viral stocks from which an isolate was derived) and "future" (ie a viral isolate which is obtained subsequently after in vivo passage of a stock) viral mutants. The genealogy of certain viral strains, through in vitro and in vivo

passage is known. Longitudinal studies of experimentally infected sheep show that the early sera of sheep neutralised not only the input virus, but progenitors of the input virus. However, the early sera did not neutralise subsequent variants (Narayan et al., 1981). For example, sheep inoculated with strain 1514 developed almost as much antibody to the progenitor strain K1010 but little to LV₁₋₁; sheep inoculated with strain LV₁₋₁ developed neutralising antisera to 1514. No clear explanation of this phenomenon has been presented and the authors claimed that their initial viral inocula were clonal. However, subsequent data (Staskus et al., 1991, section 1.16.2) suggests this may not be a valid assumption.

Other authors have questioned the frequency and importance of antigenic drift in MVV infection. Six sheep were inoculated intracerebrally with either the K796 or K485 strain of MVV (both early progenitor strains of 1514) and viral isolations made from PBLs at various time points (Thormar et al., 1983). Five of the sheep raised neutralising serum antibodies against the inoculating virus within four months of infection but a wide range of variation in the isolation of different viruses was observed. In one animal all isolates were identical to the infecting strain while in each of the other sheep a variant arose from one to three years post-infection. In no case was replacement of the initial virus clearly demonstrated, and the evidence generally indicated co-existence in vivo of different viruses. The authors claimed that the results did not suggest a smooth progression in the formation of viral isolates, as postulated previously (Narayan et al., 1978) and suggested that in each animal only one distinguishable variant was generated. They concluded that in vivo generation of neutralising variants was a rare event.

In another study seventy six virus isolates were made

over seven years from twenty sheep inoculated intracerebrally with 1514 (Lutley et al., 1983). In six of the sheep isolates not neutralisable by early sera (which did neutralise the inoculating virus) were obtained; in five of these sheep it was obvious that these did not replace the inoculating strain. In the sixth sheep the last six isolates were all different from the inoculating isolate, although it is not clear if they are identical to each other. In contrast to earlier results (Narayan et al., 1981) there was no consistent neutralisation of a progenitor viral strain. The authors concluded that only 16% of the isolates were antigenic variants, and in only one sheep was there reasonable evidence of antigenic drift, where this was defined as the consistent isolation of variant viruses (Lutley et al., 1983).

Part of the controversy surrounding antigenic drift in MVV is one of terminology. For instance, Lutley et al (1983) claim that the figure of 16% quoted for variant virus isolates is low, and indicates a relatively minor role for antigenic drift in viral persistence whereas Stanley et al (1987) interpret the same data as indicting a high frequency of viral variants. Given some of the technical problems associated with the work which suggested a significant role for antigenic shift (section 1.16.2) it is probably reasonable to assume that the importance of the process was initially over-stated. Certainly there is not the massive generation of variants observed in EIAV infection, which can arise at intervals of fourteen to sixteen days (Hussain et al., 1987) and where a distinct virus population persists in each febrile episode (Payne et al., 1987a).

1.16.2 The Role Of Antibody In Generation Of MVV Variants

Maintenance of neutralisation identity in vitro indicated that variations seen in vivo might not simply reflect random sequence changes (Narayan et al., 1977).

Viruses derived after five passages in vitro of plaque-purified virus, in the presence of neutralising antisera, showed significant differences from the parental strain (Narayan et al., 1977). Experiments comparing the mutants which arose in tissue culture following treatment with early or late sera (in which the neutralisation range was broadened) demonstrated that variants arose only rarely from the "late" cultures, and these were antigenically very distinct (Narayan et al., 1981). This was taken to imply that there is a range of "possible" variants, and that selective pressure is applied by the specificities of the neutralising sera.

This is almost certainly too simplistic a proposition. It suggests an ordered progression in the appearance of variants, the existence of which has been disputed (Lutley et al., 1983). The persistence of the initial inoculating strain implies that virus isolates are maintained by the host in the presence of an active immune response, possibly in part due to the slow kinetics of neutralisation (section 1.10). Given that the antibody response is not effective in curtailing the spread of virus it is difficult to envisage how it could operate as an effective selective pressure.

The discovery that a viral stock derived from plaque-purified virus, and subjected to six in vitro passages, differed in nucleotide sequence and neutralisation behaviour from the parental stock also suggested that emergence of variants can be antibody-independent (Staskus et al., 1991) and that plaque-purification may not be a successful procedure for isolating clonal populations of MVV. Doubt can also be cast on the clonal identity of the plaque-purified 1514 by the results obtained in a subsequent paper (Crane et al., 1988). A sheep hyperimmunised with a concentrated plaque-purified 1514 preparation raised antisera which neutralised both 1514 and the variant isolate LV₁₋₁. This may suggest either

that the virus used as an immunogen contained variants or that serum which neutralises 1514 can also neutralise a "future" mutant of the parental stock, in contrast to the results originally obtained with these isolates. Crane et al. (1988) do not discuss this discrepancy, indeed in their discussion they repeat the assertion that neutralisation of MVV is strain-specific despite the fact that their data indicates otherwise. This result casts some doubt upon the conclusions of the earlier work which drew heavily upon postulated differences between 1514 and LV₁₋₁.

1.16.3 The Nature Of Gp135 Variants

In the first formal demonstration that gp135 was a target for neutralising antibodies (Scott et al., 1979) it was demonstrated that purified gp135 glycoproteins from neutralisation variants differed in their chymotryptic peptide maps. No such differences were observed in the chymotryptic digestion of core polypeptides. It was suggested that the minor nature of the changes in the maps implied that variants arose as a consequence of mis-sense mutations, rather than deletions, insertions or recombination events.

In similar studies RNA was isolated from parental and variant strains (Clements et al., 1980) and used in RNase T1 fingerprinting studies. The observed changes were clustered in the 3' region of the genome, consistent with a location in the env gene. A further extension of this work indicated that similar genetic changes occurred during antigenic variation in two animals (Clements et al., 1982).

Monoclonal antibodies have been raised against the gp135 of strain 1514 (Stanley et al., 1987) and checked for reactivity against the envelope glycoprotein of a number of neutralisation variants (Narayan et al., 1978). Fifteen of these monoclonal antibodies were used in epitope mapping studies; they defined five distinct

partially overlapping epitopes, at least one of which was conformational. The monoclonal antibodies all distinguished the parental 1514 virus from the subsequent isolates and distinguished two variant viral subgroups but these did not correspond to the relationships found in the neutralisation assays. The authors claimed that the antigenic variations did not take the form of simple deletions or additions of sites; rather it was suggested that in the variants epitopes were altered in terms of their "exposure" on the molecule; the variations in binding of the monoclonal antibodies possibly reflected alterations in the conformation of the gp135, rather than changes in linear amino acid sequence. This conclusion was reached by assaying the monoclonal antibodies in enzyme-linked immunosorbent assays against the different viral isolates and analysing relative differences in binding (all the monoclonals assayed reacted to a greater or lesser extent with each isolate). It is not clear that such an assay is really suitable for drawing such conclusions. As none of the monoclonal antibodies neutralised the virus it is possible that antigenic drift involves variation in both neutralising and non-neutralising viral epitopes. Variation in non-neutralising epitopes in particular is unlikely to be driven by pressure from neutralising antibodies but in EIAV it has been suggested that some antigenic variation may be due to immune recognition and destruction of certain virus-infected cells, rather than antisera which neutralises infectious virus (Carpenter *et al.*, 1987). It is not clear if such a mechanism operates in MVV infection. The lack of any observed changes in the antigenic structure of the major core protein, also using monoclonal antibodies, indicates that alterations in the env gene may be more readily tolerated than in the other structural genes.

The sequence analyses of different isolates of MVV

have yielded further data on variation in the env gene. (There is some debate as to the actual identities of the viruses sequenced by Sonigo et al., 1985 and Braun et al., 1987. Unless otherwise stated the nomenclature used in Staskus et al., 1991 will be employed.) The 1514-based isolates of Sonigo and Braun (Sonigo et al., 1985; Braun et al., 1987) showed 0.3% sequence diversity over the entire genome and the distribution of mutations was reported to be random except for one cluster of four changes in the env gene (Braun et al., 1987) between nucleotides 7864 to 7890. The likelihood of the cluster arising by chance was calculated at 1 in 2000.

The env gene product of SA-OMVV exhibited 20% mismatched predicted amino acids when compared with 1514 (Querats et al., 1990), higher than the levels found for gag and pol. All but one cysteine and two potential N-linked glycosylation sites were conserved between the isolates (Querats et al., 1990). The variable region previously reported by Braun (Braun et al., 1987) was also detected, as was an N-terminal region of gp135 (coincident with rev) and possibly the regions 7718 to 7753 and 7913 to 7979. Comparison with the EV1 isolate also demonstrated the presence of conserved and variable regions across the env gene (Sargan et al., 1991).

There has as yet been no convincing demonstration that a particular variation in sequence leads to a quantifiable alteration in neutralisation. The data does indicate that the env gene can contain a disproportionately high degree of variation, compared with most of the rest of the viral genome. This can in turn result in a level of amino acid sequence and antigenic variation which is not necessarily reflected in changes in neutralisation of the virus. Whether these changes influence other aspects of viral infection is not clear; it was claimed that sequential isolates varying in their neutralisation patterns replicated as efficiently and as

virulently in vitro as the parental stock, but this does not necessarily preclude the presence of a subtle in vivo effect.

1.17 Functionally Significant Regions Of MVV gp135

Very little has been achieved in delineating structure-functional relationships in MVV gp135. Nothing is known of the regions of gp135 required for interaction with the MVV cellular receptor. Studies using "fusion from without" as an assay system have indicated that immune sera contain two populations of antibodies, one of which neutralises infectivity while the other prevents fusion (Crane et al., 1988). The authors suggested the presence of two epitopes on Env, one responsible for fusion, and one for neutralisation. While the suggestion that two regions of Env are involved in initial binding to a receptor and subsequent fusion of virus with the cell is probably reasonable the authors' contention that viral infection may result from viral entry which is independent of this "fusion" region is not. For fusion from without to occur the virus must be added to cellular cultures at a high multiplicity of infection. Antibodies which inhibit fusion from without could do so by blocking enough sites on the viral glycoprotein to prevent this critical level of virus and cell membrane interaction from occurring, while leaving enough of the "fusion" sites on the molecule exposed to permit normal fusion of the virus with the cellular membrane after receptor binding. The authors also claimed that guinea pig serum raised against purified gp135 blocked virus-induced cell fusion and that therefore both the neutralisation and binding epitopes must be present on gp135. However, as preparation of their immunogen involved lectin-based affinity chromatography of disrupted viral particles the possibility that the antigen preparation also contained antibodies reactive against gp46 cannot be excluded.

A twenty four amino acid peptide from the N-terminal region of gp46 has been shown to induce fusion of goat synovial membrane cells (Crane et al., 1991b). Antisera raised to this peptide blocked fusion from without, but failed to neutralise virus infection. This region may therefore be a candidate for a fusion domain in MVV Env.

Studies of cellular tropisms are not advanced enough to demonstrate any correlation of env variants with particular cellular targets.

It is unclear if antibodies to gp135 are protective, or if they may have a deleterious immunopathological effect. In CAEV the titres of anti-gp135 in the synovia correlated directly with disease severity (Knowles et al., 1990); in studies of lambs experimentally infected with MVV strains of different in vitro replication characteristics the mean titre to gp135 was three times higher in lambs which developed lymphoid interstitial pneumonia (Kajikawa et al., 1990). Although this could suggest a pathological role for anti-gp135 antibodies it might also simply indicate that viruses which replicate to a higher titre in vivo generate more profound lesions, and coincidentally also stimulate a higher level of anti-gp135 antibodies by the presence of a greater antigenic dose. In neither case was a clear cause-and-effect relationship demonstrable and it is also possible that any immunopathological effect of the immune response to gp135 would depend on a balance of different antibody reactivities eg neutralising against non-neutralising.

1.18 Summary

The interaction of virus and host in MVV infection is complex and highly regulated. The Env glycoprotein is a major determinant in the establishment of cellular infection and a highly immunogenic target for the host immune response. Antibodies directed against Env may be involved in pathogenesis and viral persistence. However

previous studies have been hampered by the lack of availability of well-defined well-characterised reagents with which to dissect the structural and functional relationships affecting the glycoprotein.

1.19 Aims Of The Research Project

The gp135 glycoprotein of MVV almost certainly plays a major role in the viral life-cycle by acting as the viral attachment protein. By analogy with other enveloped viruses it is probable that gp135 is involved in post-receptor binding events and it may play a role in the fine details of cellular tropism. It is also the major target for neutralising antisera.

However, virtually no information is available on the regions of gp135 which are involved in these processes. In order to begin to investigate this problem the env gene region encoding gp135 was inserted into expression vectors in the form of 3 overlapping fragments. The expressed recombinant proteins were used to determine if MVV-infected sheep differ in their antibody responses to various regions of gp135. The recombinant proteins were also used in an attempt to localise function to different regions of gp135 eg by inhibiting infection, binding to the surface of susceptible cells, or binding to putative receptor molecules. The recombinant proteins were also used as immunogens in an attempt to raise antisera which could also be used in localising function to regions of gp135, especially by attempting to generate neutralising antisera. Such work has not been previously rigourously attempted because of the lack of suitable recombinant proteins.

An analysis of the sequence diversity of the gp135 region of the env gene was also undertaken in order to determine the extent of variation arising in MVV after culture in vitro.

2. Methods

2.1 Viral Strains

The EV1 British isolate of MVV (Sargan et al, 1991) was used in all studies. Viral stocks described as "low passage" had been passaged a maximum of five times in vitro after the initial isolation, and stocks described as "high passage" had been passaged in vitro at least twelve times.

2.2 Cell Lines

Sheep choroid plexus cells (WSCP cells) were a kind gift of M. Dawson, Central Veterinary Laboratory, Weybridge, UK. 848 skin cells and YT40 skin cells were obtained from sheep 848 and YT40 by the biopsy method of Rheinwald & Green (1977) and were a kind gift of Dr B. Blacklaws, Department of Veterinary Pathology, University of Edinburgh.

2.3 Tissue Culture

Unless otherwise stated cells were maintained in fully supplemented 8% DMEM, at 37°C/5% CO₂ as adherent monolayers in plastic tissue culture flasks (Corning, UK). Once confluence was attained cells were removed from the flask by washing twice in versene, followed by incubation with trypsin-versene at 18°C until the cells were no longer adherent. The trypsin-versene overlaying the cells was immediately diluted with an excess of medium and approximately 25% of the cells re-seeded.

2.4 Propagation Of Virus

The medium was removed from T175 tissue culture flasks containing 80% confluent WSCP cells and the monolayer washed twice with 10ml aliquots of fully supplemented 2% DMEM. Virus diluted in the same medium was added at an MOI of 0.1 in a 5ml volume, and the flask

incubated for 1 hour under the usual tissue culture conditions. 30ml of fully supplemented 2% DMEM were added to the flask, and the cells were maintained under the usual conditions until syncytia formation became clearly apparent. The medium was removed and pooled with that from other flasks if appropriate. Cellular debris was removed by centrifugation at 2500 x g and the supernatant was stored in aliquots at -70°C.

2.5 Titration Of Virus

WSCP or skin cells were seeded into each well of a flat-bottomed 96-well plate (Nunc, Denmark) at a density of 1×10^4 cells/well in 100ul of fully supplemented 8% DMEM. The plates were maintained under the usual conditions until the monolayer was estimated to be 80% confluent. The medium was removed and the cells washed in 100ul of the same medium. Virus stock was serially diluted in fully supplemented 2% DMEM and 50ul of virus added to the appropriate wells, all dilutions being tested at least in triplicate. Mock-infected controls, incubated with fresh fully supplemented 2% DMEM or medium removed from growing cells, were always included. The plates were incubated with the virus for 60 minutes at 37°C/5% CO₂ after which the virus was removed, and the monolayer washed and overlaid with 100ul of fresh fully supplemented 2% DMEM. The plates were incubated under the normal conditions and stained with Giemsa's stain (section 2.19) to detect CPE. The 50% tissue culture infectious dose (TCID₅₀) was determined using the method of Reed & Muench (1938).

2.6 Polymerase Chain Reaction

2.6.1 Template DNA

Hirt DNA (Hirt, 1967) was isolated from 10^7 WSCP cells infected with various isolates of visna virus, and harvested when the cell monolayer was displaying



significant syncytia formation. Hirt's technique was followed by phenol-chloroform extraction, chloroform extraction and ethanol precipitation. The DNA was resuspended in 100ul of sterile distilled water (SDW) and serial 10-fold dilutions used to determine the optimal template concentration for each PCR reaction.

Where purified plasmid DNA was used as the PCR template the plasmid preparation was diluted in SDW to a DNA concentration of 5ng/ul.

2.6.2 Primers

Figure 2.1 shows the primers used to generate the fragments of the gp135 region of the env gene. The primers were synthesised by Oswel DNA Service (Department of Chemistry, Kings Buildings, West Mains Road, Edinburgh, EH9 3JJ). Final primer concentrations ranged from 0.38uM to 0.88uM.

2.6.3 PCR Conditions

Reactions were carried out in 100ul of 50mM KCl/10mM Tris-HCl (pH8.8)/1.5mM MgCl₂/3mM dithiothreitol (Ohara, 1989) with 0.17mg/ml bovine serum albumin (BSA), 100uM of each dNTP (Pharmacia,UK) and 1.7 units Taq DNA polymerase (Boehringer Mannheim) overlaid with 100ul of mineral oil. Thirty cycles of PCR were performed using a Techne Programmable Dri-Block PHC-1: denaturation at 95°C, 0.6 minutes; annealing at 55°C, 2 minutes; extension at 72°C, 2.5 minutes; final extension, 7.5 minutes.

Upon completion of the polymerase chain reaction 10% of the DNA sample was analysed by gel electrophoresis and the remaining 90% was phenol-chloroform extracted, chloroform extracted and resuspended in 10ul of SDW for restriction digestion.

2.7 Restriction Digestion Of DNA Samples

DNA samples for analysis or for insertion into plasmids were digested in a total volume of 20ul using a minimum of 1 unit of enzyme per ug of enzyme site.

Figure 2.1

PCR primers for generation of MVV gp135 fragments

The primers shown were used as 3 pairs - (1 + 4), (2 + 5) and (3 + 6) to generate 3 overlapping fragments of the MVV gp135 gene.

The BamHI restriction site is underlined in each primer. The terminal codon of the relevant env gene region is shown in bold type for each primer.

The terminal MVV EV1 env gene region base is italicised in each primer and the position of the terminal base in the MVV EV1 sequence is shown.

The terminal MVV EV1 env gene region amino acid is shown for each primer.

Sequence positions are from Sargan et al., 1991.

1.

5' TCCCGGGATCCTTTGTTTAGG**ATGG**CAAGCAC 3'

nt 5994, Met 1

2.

5' TCCCGGGATCC**GAG**GAAACAGGACATGCAC 3'

nt 6735, Glu 248

3.

5' TCCCGGGATCC**GGAT**GTAATTGCTCAAGGTCAGG 3'

nt 7464, Gly 491

4.

5' ACACCCGGGATCC**AGG**ACTCTTCTCGTCCTG 3"

nt 6929, Pro 312

5.

5' ACACCCGGGATCC**CCCT**ATTCTGTCTAACGTGC 3'

nt 7652, Gly 553

6.

5' ACACCCGGGATCCT**CACCA**ACCCTATGCCCCCTC 3'

nt 8000, Val 669

Enzymes were obtained from Pharmacia UK or Northumbria Biologicals Ltd UK and digestion was carried out in the manufacturer's buffer. Reactions were incubated at 37°C in a Techne Dri-Block DB-3 for greater than one hour. Digested samples were analysed by electrophoresis.

When digesting a plasmid in order to insert a DNA fragment 5µg were digested as described above, but in a final volume of 50µl. After digestion incubation was continued for a further 30 minutes in the presence of 1 unit of calf intestinal phosphatase (CIP) (Northumbria Biologicals Ltd). A further 1 unit of CIP was added and incubation continued for 5 minutes. The sample was phenol-chloroform extracted twice, followed by chloroform extraction and ethanol precipitation. The DNA was resuspended in 10µl of SDW and 10% of this analysed electrophoretically.

2.8 Ligation Of Insert DNA Into Plasmids

Concentrations of digested fragments and plasmids were selected such that the final amount of plasmid was 100-200ng and the fragment which was to be inserted was present at approximately molar equivalence and at three-fold molar excess. Ligations were performed in a final volume of 20µl for 16 hours at 18°C, using Amersham T4 DNA ligase at 35 new units (1 new unit is the amount of the enzyme which joins more than 90% of 6µg of lambda DNA cleaved with HindIII in 20µl for 30 minutes at 16°C) per reaction, Amersham T4 RNA ligase at 0.1 units per reaction, and the manufacturer's buffer.

2.9 Transformation Of Bacteria And Yeast With Recombinant Plasmids

2.9.1 Generating Competent Bacteria

The JM83 and JM101 strains of E.coli (Pharmacia) were rendered competent using the method of Cooke (H.Cooke, Western General Hospital, Edinburgh, personal

communication). A pre-culture from a single colony was grown in L-broth for 16 hours at 37°C with shaking. 400ul of this pre-culture were inoculated into 40ml of Psi broth and incubated under the same conditions until the optical density at 550nm (OD₅₅₀) of the culture was 0.3. 10ml of this culture were added to 200ml of Psi broth and incubated until an OD₅₅₀ of 0.48 was reached. The cells were cooled briefly on ice and centrifuged at 3840 x g for 5 minutes at 4°C. The cells were resuspended in 66ml of ice-cold transformation buffer 1 (Tfb1) and kept on ice for 10 to 15 minutes. The cells were centrifuged as above and resuspended in 8ml of transformation buffer 2 (Tfb2) and kept on ice for 20 minutes, after which 200ul aliquots were transferred to 1.5ml Eppendorf tubes and frozen immediately on dry ice. The competent cells were stored at -70°C until required.

2.9.2 Transformation Of Bacteria

Competent bacteria were thawed on ice, incubated with the ligated DNA for 30 minutes and then heat-shocked at 42°C for 90 seconds. After brief cooling on ice, 800ul of L-broth were added and the cultures incubated at 37°C for 60 minutes. Typically the competent bacteria were transformed with 10ul i.e. 50 to 100ng of the ligated material.

Bacteria transformed with pGEX-based plasmids were spread onto L-broth/1.5% agar plates (w/v) (Bacteriological Agar, Oxoid) containing ampicillin at 75ug/ml (L-broth/1.5% agar/amp) and incubated for 16 hours at 37°C. Single colonies were picked into 10ml L-broth containing ampicillin at 75ug/ml (L-broth/amp) and analysed (section 2.12.1), or picked onto duplicate L-broth/1.5% agar/amp plates for screening by colony hybridisation (section 2.14.1).

Bacteria transformed with pTZ-based plasmids were spread onto L-broth/1.5% agar/amp plates that had been overlaid with L-broth/0.75% agar/amp containing 200ug/ml

X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactoside, Northumbria Biologicals Ltd) and 0.7mM IPTG (isopropyl-beta-D-thiogalactoside, Northumbria Biologicals Ltd). The plates were incubated for 16 hours at 37°C. Single white colonies were picked into 10ml L-broth/amp and analysed (section 2.12.1), or picked onto duplicate L-broth/1.5% agar/amp plates for screening by colony hybridisation (section 2.14.1).

2.9.3 Transforming Yeast

The BJ2168 strain of Saccharomyces cerevisiae (auxotrophic for leucine biosynthesis) was used and transformations were performed using the spheroplasting method of Hinnen et al. 1978, with the following minor modifications. The yeast cells were washed once in 1M sorbitol before incubation with 2% glucosylase (v/v) (DuPont Co., USA) for 2.5 hours after which the resulting spheroplasts were washed once with 1M sorbitol and once with 1M sorbitol/10mM Tris-HCl pH7.5/10mM CaCl₂. The spheroplasts were resuspended in 1ml of this solution and divided into 100ul aliquots for transformation with either a pOGS recombinant plasmid alone, or the recombinant plasmid and the pUGS 41S plasmid together. The spheroplasts and DNA were incubated for 15 minutes at 18°C after which 1ml of 44% polyethylene glycol (PEG) 3400 was added and incubation continued for a further 10 minutes. The spheroplasts were sedimented by centrifugation at 2500 x g for 10 seconds, resuspended in 1ml of the sorbitol/Tris-HCl/CaCl₂ solution and 0.25ml aliquots added to 20ml of molten regeneration agar. To select for double-transformants, that is containing both the pOGS and pUGS-derived plasmids, the spheroplasts were regenerated in agar containing 0.002% L-tryptophan (w/v) (Sigma). Each 20ml aliquot of regeneration agar containing the transformed spheroplasts was poured into individual sterile 90mm petri dishes. The plates were incubated at

30°C until single colonies were clearly visible (3-6 days).

2.10 Sub-Cloning

For sequencing purposes sub-clones were made from the original clones in pTZ19R. The env gene fragments were excised using two restriction enzymes that cleaved at sites in the multiple cloning site of pTZ19R on either side of the inserted sequence. The fragment was then purified by electrophoresis and the GeneClean II kit (section 2.12.3) and ligated into pTZ18R cut with the appropriate enzymes. Restriction enzymes were also used to cleave a portion of the inserted gene fragment out of the pTZ19R vector using the described protocol. The plasmid plus partial insert that remained was purified by gel electrophoresis, extracted using the GeneClean II kit, and re-ligated to itself to yield a circular plasmid that contained only a portion of the gene fragment of interest.

Where pOGS-based plasmids contained the fragment of interest inserted in the 3' to 5' orientation, but no recombinant plasmid containing the insert in the 5' to 3' orientation had been generated, the 3' to 5' recombinant plasmid was digested with BamHI to excise the fragment. After phenol-chloroform extraction, chloroform extraction and ethanol precipitation the mixture of fragment and linearised plasmid were re-ligated as previously described.

In all cases transformations were performed as previously described.

2.11 Stocks Of Recombinant Transformants

Single colonies of E.coli JM83 and JM101 recombinant bacterial clones were inoculated into 10ml L-broth/amp and incubated for 16 hours at 37°C with shaking, after which aliquots were mixed with equal volumes of sterile 40% glycerol (v/v), transferred to -20°C for 2 hours and the

clones then stored at -70°C . Cultures of BJ2168 recombinant yeast clones were grown in Sc-glc containing 0.002% L-tryptophan (w/v) (Sigma Chemical Company) (Sc-glc/Trp) until the OD_{600} was 1.0. Glycerol stocks were made in the same manner as for bacterial clones.

2.12 Purification Of DNA

2.12.1 Small Scale Purification Of Plasmid DNA

Single colonies were picked from L-broth/amp plates into 10ml of L-broth/amp using a sterile loop, and incubated for 16 hours at 37°C with shaking. Plasmid DNA was extracted using a modification of the alkaline-lysis technique (Birnboim and Doly, 1979). 1.5ml of the culture were centrifuged at $9000 \times g$ for 30 seconds and the bacterial pellet resuspended in 100ul of 25mM Tris-HCl pH8.0/10mM EDTA pH8.0/50mM sucrose containing 5mg/ml of lysozyme and incubated on ice for 10 minutes. 200ul of 0.2M NaOH/1% SDS (w/v) were added and incubation continued on ice for 10 minutes. 150ul of 3M sodium-acetate solution pH4.8 were added and incubation on ice continued for 10 minutes, after which the chromosomal DNA, protein-SDS complexes and high molecular weight RNA molecules were pelleted by centrifugation for 10 minutes at $9000 \times g$. 400ul of supernatant were transferred to a fresh microfuge tube and 1ml of absolute ethanol added. After 30 minutes at -20°C the plasmid DNA and remaining RNA were pelleted as above, and the pellet resuspended in 100ul of 0.1M sodium-acetate solution pH6.0. 200ul of ethanol were added and after a further 10 minutes at -20°C the RNA and DNA were pelleted, resuspended in 100ul of SDW containing DNAase-free RNAase at 10ug/ml and incubated for 30 minutes at 37°C . After phenol-chloroform extraction, chloroform extraction and ethanol precipitation the DNA was resuspended in 20ul of SDW and 4ul were analysed by restriction enzyme digestion followed by gel electrophoresis.

2.12.2 Large-Scale Purification Of Plasmid DNA

A 10ml L-broth/amp pre-culture of recombinant E.coli JM83 or JM101 clones was incubated for 8 hours at 37°C with shaking. The 10ml culture was added to 500ml L-broth/amp and incubation continued for 16 hours. The plasmid DNA was then harvested using pz523 columns (5 Prime-3 Prime, Inc), following the manufacturer's protocol. This was followed by a further round of phenol-chloroform extraction, chloroform extraction and ethanol precipitation. The DNA was resuspended in SDW, typically in a volume of 100 to 200ul and the OD₂₆₀ and OD₂₈₀ values and ratio for a diluted sample of the plasmid determined. The conversion factor of 1 OD₂₆₀ = 50ug/ml DNA was used. Plasmid identity was confirmed by restriction digestion analysis.

2.12.3 Gel Purification Of DNA Fragments

Following restriction digestion and separation of fragments by gel electrophoresis, clean scalpel blades were used to excise the relevant bands from the gel. DNA was extracted from the gel slices using the GeneClean II kit (Stratatech Scientific, UK) and resuspended in 10ul of SDW. 10% of this solution was analysed electrophoretically to allow estimation of DNA concentration.

2.13 DNA Sequencing

All sequencing was conducted using techniques based on the dideoxynucleotide chain termination method (Sanger et al., 1977) using the Sequenase Version 2.0 kit (United States Biochemical Corporation) with the supplied Sequenase Version 2.0 T7 DNA polymerase.

All regions studied were sequenced at least twice. Sequence data was analysed using the Genetics Computer Group Sequence Analysis Software Package Versions 6.0 and 7.0 (Devereux et al., 1984).

Figure 2.2 lists the oligonucleotide primers used in

Figure 2.2

Sequencing primers

The sequence to which each primer binds is shown below the primer name. The terminal nucleotide positions in each primer are shown.

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

M13 Reverse Sequencing Primer

pTZ19R/18R 5' CAGGAAACAGCTATGAC 3'
 nt 267 nt 251

Ty Sequencing Primer

pOGS40/42 5' CAGGAGAAATCCGAGTG 3'
 nt 2587 nt 2603

Primer 191L

MVV EV1 5' TAATGCATAGGGAGATGG 3'
 nt 6692 nt 6675

Primer 193L

MVV EV1 5' CTTGCACTCCAATACGG 3'
 nt 7135 nt 7119

Primer 415H

MVV EV1 5' CCCACTGTTTATATTATGTCT 3'
 nt 7421 nt 7401

sequencing.

2.13.1 Single-Stranded DNA Sequencing

Single-stranded DNA from pTZ-based plasmids transformed into E.coli JM101 cells was generated by following the Pharmacia protocol with a few minor amendments. 10ml of 2 x YT medium containing 150ug/ml ampicillin (2 x YT/amp) were inoculated with transformed JM101 cells and incubated at 37°C with shaking until an OD₆₆₀ of 0.5 to 0.8 was reached. 400ul of this culture were inoculated with M13K07 phage at a multiplicity of infection (MOI) of 10. Incubation was continued for a further hour after which 10ml of 2 x YT/amp, with kanamycin at 70ug/ml were added, and the culture incubated as above for 16 hours. After pelleting out the bacterial cells by centrifugation at 2500 x g for 10 minutes, 2.5ml of 20% PEG 3400/3.5M NaCl were added to the supernatant and the sample incubated on ice for 30 minutes. Precipitated phage was pelleted by centrifugation at 11,000 x g for 30 minutes at 4°C. The supernatant was discarded and the phage resuspended in 500ul STE. After 2 phenol-chloroform extractions, chloroform extraction and ethanol precipitation the ssDNA was resuspended in 30ul of SDW. 7ul were used for each sequencing reaction. Sequencing was performed according to the manufacturer's protocol, each reaction being labelled with 7uCi of ³⁵S-dATP (specific activity of 400Ci/mmol). (For electrophoresis see section 2.15.2.)

2.13.2 Double-Stranded DNA Sequencing

Very pure DNA is required for satisfactory double-stranded DNA sequencing. Material obtained by pz523 column purification (section 2.12.2) of plasmid DNA was suitable. The sequencing reagents from the Sequenase Version 2.0 kit were used.

0.5-1.0 pmoles of DNA were mixed with 0.5 pmoles of primer, and reaction buffer and SDW added to a final volume of 10ul. The samples were boiled for 5 minutes and

placed on ice. The following reagents were then added; DTT to 6.25mM, DMSO to 5% (v/v), 1ul of diluted labelling mix, 7uCi ^{35}S -dATP (specific activity of 400Ci/mmol) and 2ul of diluted Sequenase Version 2.0 enzyme. The reactions were incubated for 2 minutes at 18°C and subsequent stages were performed as for single-stranded DNA sequencing. (For electrophoresis see section 2.15.2.)

2.14 DNA Analysis By Filter Hybridisation

2.14.1 Colony Hybridisation

Colonies were picked from the original plates using a sterile loop onto a pair of duplicate L-broth/1.5% agar/amp plates which were incubated for 16 hours at 37°C. The colonies were then transferred to nylon filters and processed by the method of Buluwela *et al.* (1989). Briefly, dry nylon filters (Hybond-N Nylon, 0.45u, 82mm diameter, Amersham UK) were positioned on the plates for 30 seconds and peeled off to remove the colonies. The filters were placed face upwards on Whatman No. 3 paper pre-soaked in 2 x SSC/5% SDS(w/v) for 2 minutes and then microwaved for 2.5 minutes at full power (650W Matsui microwave oven). After brief pre-wetting in 2 x SSC/0/1% SDS(w/v) the filters were incubated in hybridisation buffer for a minimum of 6 hours at 65°C, followed by hybridisation with a ^{32}P -dCTP-labelled probe in a minimal volume of hybridisation buffer for 16 hours at 65°C. Filters were washed 3 times at 18°C with 2xSSC/0.1%SDS(w,v) and twice at 65°C with 0.1xSSC/0.1%SDS(w/v). Filters were exposed for 16 hours against Kodak X-omat S film at -70°C and developed. Colonies reacting with the labelled probe were picked into 10ml L-broth/amp and treated as for small-scale plasmid preparations (section 2.12.1).

2.14.2 Labelling Probes Using Random Oligonucleotide-Primed Synthesis

PCR fragments to be used as probes were generated as

outlined and resuspended at an approximate concentration of 50ng/ul. Radio-isotopic labelling was performed using a modification of the technique of Feinberg and Vogelstein (1983). Briefly, 1ul of PCR fragment was mixed with 20ul of SDW, boiled for 3 minutes and cooled rapidly on ice. 6ul of 5x oligo-labelling buffer, 5 units of Klenow DNA polymerase I (Northumbria Biologicals Ltd UK) and 20uCi of ^{32}P -dCTP (specific activity of 400Ci/mmol) were added. Labelling was carried out for 2 to 4 hours at 37°C and the reaction stopped by the addition of 170ul of 10xTE. The amount of radiolabel incorporated was assayed by precipitating 1ul of the reaction mixture on glass fibre discs in 5% trichloroacetic acid (w/v). Discs were placed in 2ml of Optiphase Safe scintillant (LKB), activity measured in an LKB Rackbeta Liquid Scintillation Counter and specific activity calculated. With probes of specific activity of approximately 1×10^8 cpm/ug 1.25×10^6 cpm were used in each hybridisation reaction. The labelled probe was boiled for 3 minutes and then cooled rapidly on ice before use.

2.15 Gel Electrophoresis

2.15.1 DNA Analysis Gels

DNA samples were analysed on 1% agarose (w/v)/TAE gels containing 1ug/ml of ethidium bromide. Before loading the samples were mixed with half their volume of DNA sample buffer. Electrophoresis was carried out at a constant voltage of 6.25V/cm² and EcoRI/HindIII-digested lambda DNA molecular weight markers (Northumbria Biologicals Ltd) were included on every gel.

2.15.2 DNA Sequencing Gels

All sequencing reactions were analysed on 6% acrylamide (w/v) gels (acrylamide:N,N'-methylenebisacrylamide ratio of 19:1) containing 8M urea (all molecular biology grade reagents) in 0.5 x TBE buffer using a Bio-Rad Sequi-Gen apparatus. Electrophoresis was

conducted at a maximum voltage of $1212\text{V}/\text{cm}^2$ from 2 to $7\frac{1}{2}$ hours. Gels were dried onto Whatman 3MM paper at 80°C for 2 hours under vacuum on a Bio-Rad Model 583 Gel Dryer and autoradiography was carried out using Kodak X-omat S film. Exposure was typically from 24 to 48 hours at 18°C for single-stranded DNA sequencing, and 7 to 10 days for double-stranded DNA sequencing.

2.15.3 SDS-PAGE Protein Gels

Polypeptides were analysed using a modification of the dissociating discontinuous sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) method of Laemmli (1970). SDS-PAGE was performed using the Bio-Rad Mini Protean II slab gel apparatus, with 0.75mm spacers. Either single concentration or gradient separating gels were used, using a 30% acrylamide (w/v)/0.8% bisacrylamide (w/v) stock solution and a final concentration of 0.38M Tris-HCl pH8.7/0.1% SDS. The stacking gel contained 3.48% acrylamide (w/v)/0.093% (w/v) bisacrylamide/0.145M Tris-HCl pH6.8/0.12% SDS (w/v). Samples were mixed with an equal volume of SDS-PAGE loading buffer, boiled for 3 minutes and gels electrophoresed in running buffer containing 0.05M Tris-HCl/0.1% SDS (w/v)/0.37M glycine. Electrophoresis was performed for 90 to 120 minutes at a constant voltage of $220\text{V}/\text{cm}^2$ and Pharmacia Electrophoresis Calibration Kit markers (molecular weight range 14,400-94,000KDa) were electrophoresed on every gel.

After completion of electrophoresis gels were either stained to localise protein or the polypeptides were electrophoretically transferred onto nitrocellulose membranes for immunodetection (section 2.16).

2.15.3.1 Coomassie Staining Of Protein Gels

Routine staining was performed using 0.1% Coomassie Blue R250 (w/v) (Sigma, UK) in 40% methanol (v/v)/15% acetic acid (v/v) for 30 minutes followed by extensive destaining in 20% methanol (v/v)/5% acetic acid (v/v).

2.15.3.2 Silver Staining Of Protein Gels

Gels were fixed by a modification of the method of Oakley et al., (1980). The gel was pre-fixed in 50% methanol (v/v)/10% acetic acid (v/v) for 30 minutes and then transferred to 5% methanol (v/v)/7% acetic acid (v/v) for 30 minutes. The gel was fixed for 20 minutes in a 10% glutaraldehyde solution (w/v) and washed in double-deionised water for 16 hours. It was then incubated at 18°C for 15 minutes with shaking in a 0.1% (w/v) solution of silver nitrate. Gels were developed in a 0.28M solution of Na₂CO₃ containing 0.05% (v/v) formaldehyde until bands were clearly visible but before background staining became too pronounced. The staining reaction was halted by the addition of 1 to 2g of citric acid to the solution. Staining was fixed by soaking the gels for 60 seconds in a 10% solution (v/v) of Ilford Hypam Fixer.

After staining gels were dried for 1 hour under vacuum at 80°C on a Bio-Rad Model 583 Gel Dryer.

2.16 Western Blotting

Polypeptides separated by SDS-PAGE were electrophoretically transferred onto nitrocellulose membranes (Hybond-C, 0.45u, Amersham, UK) using a modification of the original technique of Towbin et al., (1979). Transfer was performed in 25mM Tris-HCl/20% methanol using an Ancos Semi-Dry Electroblotter A, operated at a constant current of 0.44mA/cm². After transfer the region of the nitrocellulose membrane containing the molecular weight markers was removed and the markers stained in 5% Amido Black (w/v) in 50% (v/v) methanol for 30 minutes, and destained for several hours in 50% methanol (v/v)/5% acetic acid (v/v).

Vacant sites on the nitrocellulose were blocked by incubation at 18°C for at least 1 hour with 5% milk powder (Sainsbury's) (w/v)/0.5% Tween 20 (v/v) (Sigma Chemical Company UK)/sterile phosphate buffered saline (SPBS).

Primary antibody was diluted in 5% milk powder/SPBS and the blot was incubated with the antibody for 16 hours at 4°C. After 5 washes in the same diluent the blot was incubated for 1 hour at 18°C with an alkaline-phosphatase-conjugated second antibody, again diluted in 5% milk powder/ SPBS. The blot was washed twice in the same diluent and 3 times in 0.1M Tris-HCl pH9.5. The blot was developed in NBT western blot development solution and the reaction was stopped before background staining became too pronounced by washing the blot in deionised water.

2.16.1 Preparation Of MVV Antigens From Infected Cells For Western Blotting

Infection of a T175 flask of WSCP or skin cells was carried out as for section 2.4 and the cells incubated until significant synvitya formation was apparent. The medium was removed and the cells dislodged from the flask using sterile glass beads in 500ul of SPBS. An equal volume of SDS-PAGE sample buffer was added and the samples boiled for 3 minutes. Viral proteins were separated by SDS-PAGE and analysed by western blotting.

2.17 Production And Purification Of Recombinant Proteins

2.17.1 Yeast (Ty-VLP) Expression System

2.17.1.1 Small-Scale Purification Of p1-Fusion Proteins

Single colonies from the agar plates were streaked out onto Sc-glc-agar plates containing L-tryptophan at 0.002% (w/v) and incubated at 30°C for 3-5 days until large single colonies were visible. A single colony was picked into 50ml of Sc-glc/Trp and the culture incubated with shaking at 30°C until it had reached an OD₆₀₀ of 0.5-1.0. The cells were centrifuged at 1500 x g for 5 minutes and resuspended in 50ml of Sc-glc-gal containing 0.002% (w/v) tryptophan (Sc-glc-gal/Trp) and incubated as above. Optimal induction time was determined by time-course experiments. 15ml of the culture were centrifuged at 1500 x g for 15 minutes and the cells resuspended in

1ml of TEN. 1g of acid-washed and baked 40 mesh glass beads (BDH,UK) was added, the cells vortexed for 1 minute and then placed on ice for 1 minute. Cell breakage was repeated three times. Cell lysates were mixed with an equal volume of SDS-PAGE reducing gel buffer, boiled for 3 minutes and analysed on SDS-PAGE gels. For Coomassie-stained gels 5ul of sample were loaded, and for Western blot analysis 1-2ul of sample were loaded.

2.17.1.2 Large-Scale Purification Of p1-Fusion Proteins

100ml of Sc-glc/Trp were inoculated with 2ml of a glycerol stock of the appropriate yeast clone and incubated with shaking at 30°C until the culture had reached an OD₆₀₀ of 1. The 100ml culture was added to 500ml Sc-glc/Trp and incubation continued until the same cell density had again been reached. The cells were then split between 8 500ml aliquots of Sc-glc-gal/Trp and incubation continued, the optimal induction time being determined by previous time-course experiments. The cells were harvested by centrifugation at 3480 x g for 20 minutes at 4°C and resuspended in 20ml of SDW per litre of original culture volume. The cells were spun at 1500 x g and washed with SDW. This was repeated twice and followed by one wash in TEN.

7.5ml of packed washed cells were processed using a method based on that of Mellor et al.,1985b. Briefly, the cells were broken open in 4ml of TEN containing protease inhibitors (see Materials) to which 5g of acid-washed and baked 40 mesh glass beads had been added. Cell breakage was achieved by vortexing the cells for 3 one minute intervals, interspersed with one minute intervals on ice. The sample was spun at 1480 x g for 5 minutes at 4°C and the supernatant stored on ice. The process was repeated until greater than 80% breakage was apparent microscopically. The pooled supernatant fractions were centrifuged at 13300 x g for 20 minutes at 4°C to pellet contaminating cellular debris and the supernatant was

layered onto a 2ml 60% sucrose cushion (in TEN with protease inhibitors) and centrifuged at 160,000 x g for 1 hour at 4°C. The layer of protein above the cushion, and the cushion itself, were dialysed overnight at 4°C against TEN and then layered onto a pre-formed 15-45% sucrose gradient, underlayered with a 60% sucrose cushion. After centrifugation at 100,000 x g at 4°C for 3 hours the gradient was fractionated into 2ml samples and the fractions analysed by SDS-PAGE and Western blotting.

2.17.2 Bacterial (pGEX) Expression System

2.17.2.1 Small-Scale Purification Of GST-Fusion Proteins

The protocol of Smith and Johnson (1988) was followed. Briefly, 2ml of L-broth/amp were inoculated with 100ul of a glycerol stock of the pGEX-based clones and incubated at 37°C with shaking until the culture was visibly turbid (typically 4 to 6 hours). IPTG was added to 0.1mM and incubation continued, typically for 2 to 3 hours. The cells were pelleted by centrifugation at 9,000 x g for 30 seconds and the pellet resuspended in 300ul of ice-cold SPBS. A Dawe 2mm probe sonicator was used to lyse the cells in 10 seconds with minimal frothing and cellular debris was removed by centrifugation at 9,000 x g for 5 minutes. 50ul of a pre-swollen 50% slurry of glutathione-agarose beads (Sigma Chemical Company) were added to the supernatant and the samples mixed gently at 18°C for 60 minutes. The beads were resuspended in 1.5ml of SPBS and centrifuged at 9,000 x g for 10 seconds; the supernatant was discarded and the washing procedure repeated twice. The beads were resuspended in 50ul of SDS-PAGE sample buffer and analysed by SDS-PAGE.

2.17.2.2 Large-Scale Purification Of GST-Fusion Proteins

100ml of L-broth/amp were inoculated with 100ul of a glycerol stock of the pGEX-based clones and incubated at 37°C for 16 hours with shaking. This pre-culture was diluted 1:10 with L-broth/amp and incubation continued under the same conditions until an OD₆₀₀ value of 0.6 was

reached. IPTG was added to 0.1mM and incubation continued; the optimal point of induction, and the period of induction were determined experimentally. Once induction was complete the cultures were centrifuged at 4640 x g for 10 minutes at 4°C and the cells resuspended in 15ml of ice-cold SPBS. A Dawe 2mm probe sonicator was used to disrupt the cells in 3 one minute intervals of sonication, with minimal frothing, interspersed with 2 minute intervals on ice. Triton X-100 (Sigma Chemical Co., UK) was added to a final concentration of 1% (v/v) and the samples centrifuged at 10,300 x g for 5 minutes at 4°C to remove cellular debris. 1ml of a pre-swollen 50% slurry of glutathione-agarose beads was added to the supernatant and the samples mixed gently at 18°C for 60 minutes. The beads were resuspended in 50ml of SPBS and centrifuged at 500 x g for 10 seconds; the supernatant was discarded and the washing procedure repeated twice. The beads were resuspended in 1.5ml of SPBS and stored at 4°C until the fusion protein was eluted.

2.17.2.3 Elution Of Glutathione S-Transferase-Fusion Proteins

The supernatant was removed from the glutathione-agarose beads and the beads resuspended in the same volume of 50mM Tris-HCl pH8.0/10mM reduced glutathione (Sigma Chemical Co., UK). The samples were incubated for 2 minutes at 18°C, centrifuged at 500 x g for 10 seconds and the supernatant removed. Elution was repeated twice and the eluted protein stored in aliquots at -20°C.

2.18. Electron Microscopy

Yeast cultures were prepared for electron microscopy by a modification of the protocol of Byers & Goetsch (1975). Briefly, the cells from a 25ml culture were pelleted at 2500 x g for 5 minutes at 4°C and resuspended in 5ml of cacodylate/glutaraldehyde buffer. The cells were incubated for 30 minutes at room temperature and

pelleted as above. After resuspension in 10ml of cacodylate/glutaraldehyde buffer the samples were incubated for 16 hours at 4°C with rotation. The cells were pelleted as above and washed twice in 10ml of pre-treatment buffer. After the second wash the cells were resuspended in 5ml of phosphate/citrate buffer containing 250ul of glusulase and incubated for 60 minutes at 30°C. The spheroplasts were then washed in two changes of 10ml of phosphate/citrate buffer and resuspended finally in 5ml of the same buffer. The samples were post-fixed for 60 minutes at 0°C in 2% w/v osmium tetroxide in cacodylate buffer, rinsed with SDW and incubated for 60 minutes at 20°C with 2% aqueous uranyl acetate. After dehydration through a graded alcohol series and embedding in araldite resin 60nm-thick sections were cut and examined on a Phillips EM400 transmission electron microscope.

2.19 Giemsa Staining Of Cell Monolayers

Medium was removed from the wells of a 96 well plate and replaced with 100ul of ice-cold acetone:methanol (1:1) to fix the cells. After 10 minutes this was replaced with 100ul of 1% potassium dichromate (w/v) solution for 30 minutes. This was replaced with 100ul of acetone:methanol for 10 minutes, and the cells allowed to air-dry for 5 to 10 minutes. 100ul of Giemsa's stain (Gurr's Improved R66, BDH,UK), freshly diluted 1:1 with tap water, were added to each well for 5 minutes after which the stain was removed and replaced by tap water for 60 seconds. Monolayers were examined microscopically for cytopathic effects.

2.20. Immunofluorescent Staining Of Cells

2.20.1 Cell Monolayers

Sterile 10mm coverslips were placed singly in each well of a 24-well plate (Nunclon). 1×10^5 cells were added to each well, in 500ul of fully supplemented 8% DMEM and the plates were incubated at 37°C/5% CO₂ until the

desired level of cell confluency was attained. The medium was removed and the monolayers washed once with 500ul of SPBS, and fixed with 500ul ice-cold 0.15M NaCl/80% acetone for 10 minutes. The coverslips were removed, dried with a hair-dryer and stored dry in fresh plates at -20°C.

2.20.2 Cytospins

Efferent lymph (obtained by lymphatic cannulation of the pre-femoral lymph node of a sheep, a kind gift of Dr J Hopkins, Department Of Veterinary Pathology, University Of Edinburgh) was diluted with SPBS until a cell concentration of 2.5×10^5 cells/ml was reached. Slides coated with 1.25×10^5 cells/cytospin were made using the Shandon Cytospin 2, operated at 500rpm for 5 minutes. After air-drying the cytospins were fixed and stored as in section 2.20.1.

2.20.3 Immunofluorescent Staining

Fixed monolayers and cytospins were thawed at 18°C and blocked for 30 minutes at 18°C with shaking in 250ul of 10% normal species serum/0.01% Tween 80 (v/v)(Sigma Chemical Company, UK)/SPBS. The normal species serum used was from the same species as that in which the conjugated antiserum was raised. After removal of the blocking solution the samples were incubated for 60 minutes at 18°C with shaking in 250ul of the primary antibody, diluted in 2% normal species serum/0.01% Tween 80/SPBS. The samples were washed five times in 0.01% Tween 80/SPBS and incubated with an anti-species antibody, conjugated to fluorescein isothiocyanate (FITC) (Scottish Antibody Production Unit), under identical conditions to the primary antibody incubation. The samples were washed three times in 0.01% Tween 80/SPBS, mounted in 2-3ul of Citifluor (Citifluor Ltd., UK) and stored in the dark at 4°C until examined using a Leitz Ortholux II microscope.

2.21. Enzyme-Linked Immunosorbent Assay (ELISA)

Antigen was diluted in 0.1M NaHCO₃ pH9.5 to an

appropriate concentration and 75ul were used to coat the wells of an ELISA plate (Dynatech UK) for 16 hours at 4°C. The antigen was discarded and the wells blocked with 100ul of 2% BSA (w/v)/SPBS for 45 minutes at 18°C. After washing three times in 0.1% Tween 20 (v/v)/SPBS, 75ul of first antibody, diluted in 2% BSA/SPBS, were added and the plate incubated for 30 minutes at 18°C. The washing was repeated and 75ul of the appropriate anti-species-peroxidase-linked conjugate diluted in 2% BSA/SPBS were added to the wells. After 30 minutes at 18°C the wells were washed as above. 75ul of OPD substrate solution were added to each well and the chromigenic reaction allowed to proceed at 18°C. The reactions were stopped in all wells by the addition of 50ul of 2M H₂SO₄. Absorption at 492nm was determined using a Titertek Multiskan.

Affinity-purified soluble ovine MHC class II antigen, prepared by the method of Dalziel et al. (1991) was a kind gift of Dr B.M. Dutia, Department Of Veterinary Pathology, University Of Edinburgh.

2.22 Glycosidase Treatment Of Proteins

2ug of protein were adjusted to 0.75% SDS in a total volume of 21ul and boiled for 3 minutes. The samples were adjusted to 0.075% SDS/10mM EDTA/10mM Triton X-100 and incubated at 37°C for 16 hours with 0.4U of N-glycosidase F (Boehringer Mannheim Ltd.) or 0.1U of endoglycosidase F (Boehringer Mannheim Ltd.). After digestion the samples were precipitated with 5 volumes of acetone at -20°C for 16 hours and analysed by SDS-PAGE.

2.23. Immunisation Protocols

For the first immunisation antigen was diluted with SPBS, and an equal final volume of Complete Freund's Adjuvant (Sigma Chemical Co., UK) added. Samples were emulsified using a Janhe & Kunkel Ika-Werk Ultra-Turrex and stored at 4°C for 16 hours. Rabbits and mice (strain

Balb/c) were immunised sub-cutaneously with 4ug of antigen in 1ml (at two sites), and 1.5ug in 0.4ml (at two sites) respectively. Subsequent immunisations were performed under the same conditions but Complete Freund's Adjuvant was replaced with Incomplete Freund's Adjuvant (Sigma Chemical Co. UK).

Plain blood samples for analysis were obtained from the ear vein (rabbits) or tail tip (mice). Samples were allowed to clot at 4°C for 16 hours; the serum was removed and stored at -20°C.

3. Analysis Of Sequence Variation In The gp135 Gene Of MVV Strain EV1

3.1 Introduction

In each lentivirus which has been extensively studied significant degrees of variation between isolates have been discovered in the env gene. In MVV this was originally suggested by the isolation of neutralisation variants after in vivo passage of the virus (section 1.16). There is still some debate concerning the frequency with which such variants develop in vivo and the role of neutralising antibodies in generating and maintaining these variants. However it is becoming apparent with the publication of the genome sequence of different strains of MVV that there is significant inter-strain variation in the env gene.

The greatest amount of detail on variation in a lentiviral env gene has been accumulated for HIV. Although an extensive discussion of the data for HIV is outside the scope of this chapter the major salient points will be discussed, particularly where they may have relevance to the interpretation of sequence variations in MVV.

An analysis of HIV variants isolated from geographically distinct individuals demonstrated greater levels of variation between the env genes of different isolates than the gag or pol genes (Starcich et al., 1986). The maximum nucleotide disparity between two gag genes was 5.9%, and 4.4% for pol genes. In contrast the maximum disparity between the gp120 regions of the env genes was 14.0%.

Differences were also apparent in the type of mutations present in different regions of the genome. In the gag and pol genes nucleotide sequence changes were almost exclusively individual point mutations. In env however clustered point mutations, deletions, insertions

and duplications were common (Starcich et al., 1986). The mutations in gag and pol were also less likely to result in amino acid changes than the mutations in env. This suggested a greater "tolerance" of alterations to amino acid sequence in gp120 than in the Gag and Pol proteins. It also indicated a possible bias towards non-silent nucleotide changes in env which may be a result of interaction with host immune responses.

In individuals infected with HIV there is extensive diversity of virus in vivo. While it is obviously impossible under all except very rare circumstances to monitor deviations from a clonal infecting isolate the data suggests evolution of viral quasi-species in vivo. Restriction mapping experiments on sequential isolates from infected individuals found between 9 and 17 related but distinguishable genotypes during viral isolation (Saag et al., 1988) and by analogy with the data of Starcich et al. (1986) the majority of the variation would be expected to be found in the env gene. The insertion of different env sequences, isolated from a single individual, into a "background" viral strain demonstrated that the env gene affected the growth capacity of the virus in different cell types in vitro (Fisher et al., 1988) suggesting that viruses may evolve in an individual host with different cellular tropisms.

Mutations are not spread randomly throughout the env gene (section 1.14) of HIV but are clustered into variable regions (V1 to V5), interspersed with relatively well-conserved domains. The best characterised of these variable regions is the V3 loop, which contains the PND. Although high sequence variability is apparent between the V3 loops from different HIV isolates it has been suggested that certain structural features are maintained between these variants (LaRosa et al., 1990). The virus may be under selective pressure for the kind of amino acid substitution which is consistent with continued function,

such that secondary structure may be maintained even in the presence of primary sequence variation.

Studies on the V3 loop in sequential isolates from children infected by a single plasma source have indicated that the variants which arise during the course of an infection may be host-dependent, as the consensus sequences derived from viral isolations differed for each individual (Wolfs et al., 1990). Fewer changes were observed in the isolates from children in whom progression to AIDS occurred most rapidly, suggesting that generation of variants may be influenced by the immune system (Wolfs et al., 1990).

Extensive variation in the V4 and V5 regions, both between isolates from a single individual and between isolates from different individuals infected from a single source, has also been reported (Simmonds et al., 1990). This work confirmed that a number of env variants co-exist in an infected individual.

Variation in the env gene of HIV has therefore been extensively demonstrated in vivo and is believed to be significant in viral persistence. The initial source of variation is the intrinsically high error rate of reverse transcriptase, due to its lack of a "proof-reading" capacity, but the disparity in the accumulation of mutations, both between genes and within the env gene indicates selection pressures must be operating upon the virus. It has been estimated for HIV that the rate of viable genetic change is 10^{-2} to 10^{-3} substitutions per site per year for env, but is tenfold less for gag (Hahn et al., 1986). Constraints also appear to limit the evolution of variant sequences within an individual, as variation of isolates within an individual is less than variation between individuals (Hahn et al., 1986; Saag et al., 1988).

Variation in the env gene has also been reported for EIAV (Payne et al., 1987b). This may be particularly

significant as in EIAV it is possible to relate specific disease episodes to the emergence of antigenic variants (Montelaro et al., 1984). In sequential isolates from experimentally-infected ponies non-randomly distributed mutations were present in the env gene, particularly in the region encoding the extracellular gp90 (Payne et al., 1987b). Within the gp90 region one hypervariable and two constant regions could be identified by comparison of isolates. Approximately 40% of the amino acid substitutions in gp90 of EIAV resulted in alterations in asparagine residues, and therefore had the potential to affect markedly the glycosylation pattern of the processed glycoprotein (Payne et al., 1987b). This may act in vivo as a further means of protection from neutralising antisera, by masking neutralisation epitopes on the viral envelope.

Relatively little data at the DNA level is available on the variants of MVV which exist in vivo. Section 1.16 summarised the data on sequential isolation of neutralisation variants which arise in vivo. There is a limited amount of data on variants of the 1514 strain of virus, derived from in vivo passage of virus, and sequence comparisons are also available for MVV isolates which have been obtained from geographically distinct variants of the virus. A pattern broadly similar to that seen in HIV is beginning to emerge.

Restriction hybridisation experiments demonstrated that homology between MVV and CAEV was predominantly localised to the gag and pol regions of the genome (Pyper et al., 1984), with homology in env restricted to the 3' region. Similar hybridisation results were obtained with SA-OMVV, in which it was found that homology with the 1514 strain of MVV was greatest in the LTRs and the gag and pol genes (Querat et al., 1987), supporting the hypothesis of relative variability of the env gene.

SA-OMVV was sequenced and compared with MVV strain

1514 (Querat et al., 1990). As anticipated from the hybridisation data (Querat et al., 1987) much higher diversity was observed for env than gag or pol (20% amino acid mismatch, compared with 8.5% and 13% respectively). The only orf in which greater sequence diversity was detected was the rev gene, part of which is coincident with the env orf. Sequence alterations were also heterogeneously distributed within env, which may be analogous to the distribution in HIV env.

A similar pattern was found for the British isolate of MVV, EV1 (Sargan et al., 1991). Comparison of the env sequences of EV1 and the previously sequenced isolates confirmed that the env gene contained both relatively conserved and variable regions. It has not yet proved possible to relate these to functional determinants of the molecule, such as receptor binding domains.

The same pattern may not however be immediately apparent for sequential isolates from an initial viral stock. An analysis of MVV strain 1514 and sequential isolate LV1-1 revealed an almost random distribution of mutations throughout the genome, except for one cluster of mutations between nucleotides 7864 to 7890 which lies towards the putative C-terminus of gp135 (Braun et al., 1987).

MVV strain 1514 was also used as the initial virus stock for experiments in which infectious clones were sequenced and compared (Staskus et al., 1991). Plaque-purified 1514 was passaged in vitro and a progeny stock, 7/86 was obtained which differed from 1514 in its neutralisation properties, suggesting that variations in env may arise in the absence of selective antibody. Cells were infected in vitro and the infectious clones obtained from the Hirt DNA fraction. The two infectious clones differed from the prototype 1514 sequence (Sonigo et al., 1985) by 0.978% and 0.793% but these changes were not evenly spread throughout the genome. The env genes

contained 53.33% and 64.38% of the changes respectively (Staskus *et al.*, 1991). This supported the proposition that even in the absence of selective pressure from a host immune response the env gene is the region of the genome which is most able to sustain mutations.

In order to begin to investigate the variability of MVV strain EV1 a number of in vitro isolates were analysed by DNA sequencing. Hirt DNA was used as the template for PCR generation of env gene fragments. DNA sequencing of these cloned fragments was performed in order to estimate the type of variation observed in vitro for this strain, and to investigate if any major differences were apparent in the gene after long-term passage in tissue culture.

3.1 Cloning Of gp135 Variants

In order to obtain variants of gp135 the PCR was performed using Hirt DNA isolated from a variety of viral sources. The PCR primers shown in chapter 2 (figure 2.1) were used to generate the overlapping fragments of env which encode the protein regions shown in figure 3.1. The fragments generated were inserted into the vector pTZ19R for cloning and sequencing.

For each env region (envA, envB and envC) two clones were sequenced after PCR generation from the Hirt DNA preparation used in the sequencing of the EV1 British isolate of MVV (Sargan *et al.*, 1991) (a kind gift of D.J.Roy, Department Of Veterinary Pathology, University Of Edinburgh). These are referred to in figure 3.2 as "original clones".

One of each of these original clones for each env region was subsequently used as a template in a PCR. The resulting PCR fragment was inserted into the pOGS expression vectors for generation of a recombinant fusion protein (see chapter 4), and was also cloned into plasmid pTZ19R for sequence analysis. These pTZ19R-based clones are referred to in figure 3.2 as "duplicate clones".

Figure 3.1

The regions of MVV strain EV1 gp135 encoded by the products of the PCR using selected primers

PCR primers 1 to 6 (chapter 2, figure 2.1) were used to generate DNA fragments which spanned the coding regions shown. The terminal amino acids of each encoded region are shown (position number is derived from Sargan et al., 1991).

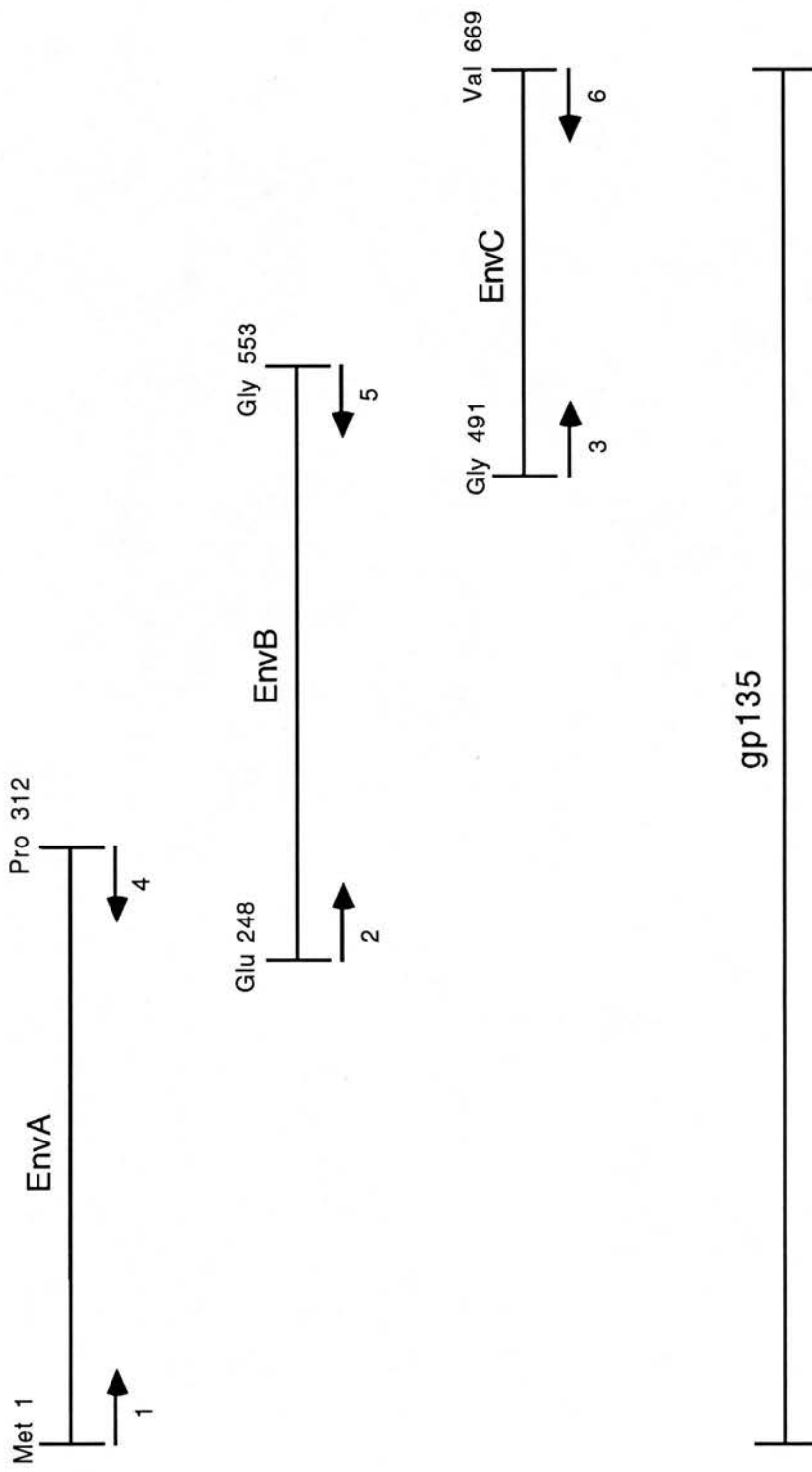


Figure 3.2

MVV strain EV1 gp135 variant clones

Hirt DNA was used as the template for PCR with env-specific primers, and the resulting fragments cloned as in the text. The clone number is that which was assigned to the original clone for each fragment in pTZ19R.

Key

Original	Template = low passage Hirt DNA as used by Sargan <u>et al.</u> , 1991.
Duplicate	Template = purified plasmid DNA from clones A46, BX10 or C45 as appropriate.
Low Passage	Template = Hirt DNA from infection with low passage EV1
High Passage	Template = Hirt DNA from infection with high passage EV1

	<u>ORIGINAL</u>	<u>DUPLICATE</u>	<u>LOW PASSAGE</u>	<u>HIGH PASSAGE</u>
<u>envA</u>	A46 AX11	ADP1	DA3	VA1
<u>envB</u>	BX10 BX11	BDP1	DB2	VB6
<u>envC</u>	C45 CX7	CDP1	DC1	VC1

Hirt DNA isolated from viral stocks of EV1 which had been passaged minimally in vitro (section 2.1) was used as a PCR template. The resulting clones are referred to in figure 3.2 as "low passage clones".

Hirt DNA from viral stocks of EV1 which had been relatively extensively passaged in vitro (section 2.1) was also used as a PCR template. The resulting clones are referred to in figure 3.2 as "high passage clones".

In each case the fragments generated by PCR were digested with BamHI, separated by gel electrophoresis and purified using the Gene-Clean kit. Purified plasmid pTZ19R was digested with BamHI and treated with CIP. The purified digested PCR fragments and the digested plasmid were ligated and the resulting DNA used to transform E.coli strain JM101. Positive clones were initially screened by ampicillin resistance and blue-white selection.

For the original template Hirt DNA approximately 80 white colonies each resulted from the ligation of envA and envC into pTZ19R. 12 of each were selected and screened by small-scale purification of their plasmid DNA followed by digestion with BamHI and analysis by gel electrophoresis. 3 of the clones from the pTZ19R:envA ligation contained a single insert of the expected molecular weight and 2 of these were chosen arbitrarily for sequencing (clones A46 and AX11). 7 of the clones from the pTZ19R:envC ligation contained a single insert of the expected molecular weight and 2 of these were chosen arbitrarily for sequencing (clones C45 and CX7). 5 white colonies resulted from the ligation of envB into pTZ19R. Each clone was screened by analysis of purified plasmid DNA and after BamHI digestion and gel electrophoresis 3 were found to contain a single insert of the expected molecular weight and 2 of these were chosen arbitrarily for sequencing (clones BX10 and BX11).

A similar procedure was followed where clones A46,

BX10 and C45 were used as the PCR template to generate "duplicate" sequences. 4 white colonies from each transformation were analysed, and in each case the plasmid contained a single insert of the expected molecular weight in 3 of the clones. One of each was chosen arbitrarily for sequencing (clones ADP1, BDP1 and CDP1).

For the "low passage" template Hirt DNA 22 white colonies resulted from the ligation of envA into pTZ19R and 32 white colonies from the ligation of envC. Duplicate plates were made from these white colonies and screened by colony hybridisation using the appropriate env region probe. All the colonies hybridised to the env-specific probes and 3 were selected for screening by restriction digestion and gel electrophoresis of plasmid DNA. After digestion with BamHI each of the selected colonies was found to contain a single insert of the expected molecular weight. One of each was chosen arbitrarily for sequencing (clones DA3 and DC1). 2 white colonies resulted from the ligation of envB into pTZ19R and the plasmid DNA was analysed by restriction digestion and gel electrophoresis. After digestion with BamHI one clone was found to contain a single insert of the expected molecular weight and this clone was sequenced (clone DB2).

For the "high passage" template Hirt DNA approximately 75 white colonies resulted from the ligation of either envA or envC into pTZ19R. These were screened as in the paragraph above. All the colonies were positive by colony hybridisation and 3 were further screened by restriction digestion and gel electrophoresis of the plasmid DNA. After digestion with BamHI each clone was found to contain a single insert of the expected molecular weight and one of each was chosen arbitrarily for sequencing (clones VA1 and VC1). 7 white colonies resulted from the ligation of envB into pTZ19R. These were screened by restriction digestion and gel electrophoresis of the plasmid DNA. After digestion with

BamHI it was found that 2 clones contained a single insert of the expected molecular weight and one was chosen arbitrarily for sequencing (clone VB6).

3.3 Sequencing Of gp135 Variant Clones

The various gp135 clones were analysed by single-stranded DNA sequencing using the dideoxynucleotide chain termination technique, and each nucleotide was sequenced at least twice. The sequencing primers used are shown in chapter 2 (figure 2.2) and the basic strategies will be outlined below.

The strategies for sequencing each clone are summarised in figure 3.3. Each env fragment was initially inserted in the 3' to 5' orientation (with respect to the primer binding site) in pTZ19R and all clones were therefore partially sequenced in pTZ19R using reverse sequencing primer.

In order to reverse the orientation of the env gene sequences with respect to the primer binding site the fragments were excised from pTZ19R and ligated into pTZ18R. The two plasmids differ only in the orientation of the multiple cloning site within the plasmid. The envA and envB fragments were excised from purified plasmid DNA with the restriction enzymes EcoRI and PstI. These each cut the plasmid at one site only, one on either side of the inserted DNA, and there are no EcoRI or PstI site within the envA or envB fragments. After excision the fragment was purified in the normal manner, ligated into pTZ18R which had been digested with the same pair of enzymes and used to transform E.coli JM101. Colonies were selected and screened in the same way as for the initial transformations and one positive clone for each fragment was arbitrarily chosen for sequencing (cloning data not shown).

The envC fragment was excised from pTZ19R with EcoRI and HindIII. These each cut the plasmid singly on either

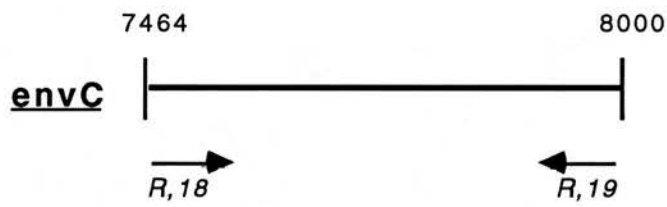
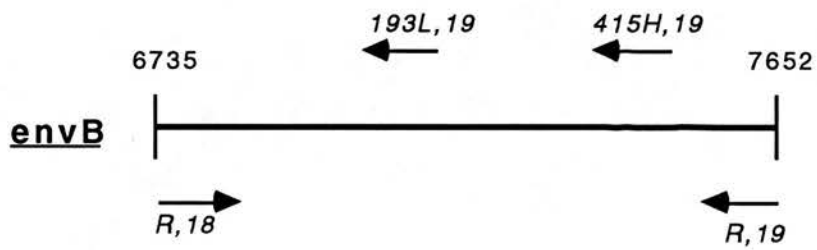
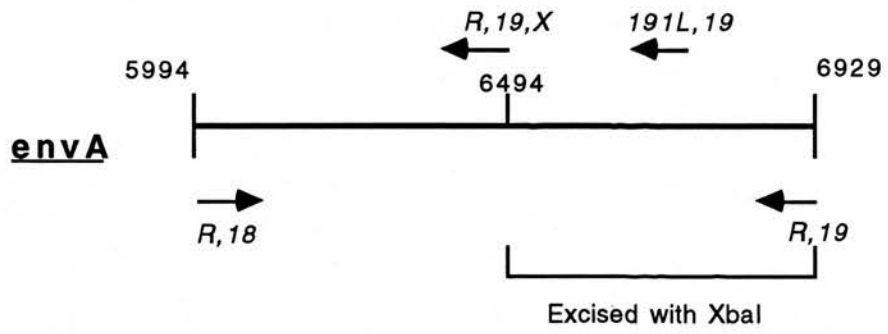
Figure 3.3

Sequencing strategies for MVV strain EV1 gp135 variant clones

Single-stranded DNA was used for dideoxynucleotide chain termination sequencing. Sequencing was in the direction shown by the arrows and the terminal nucleotides for each fragment are indicated (numbering as in Sargan et al., 1991).

Key

R	reverse sequencing primer
191L	sequencing primer 191L
193L	sequencing primer 193L
415H	sequencing primer 415H
19	fragment sequenced in pTZ19R
18	fragment sequenced in pTZ18R
19,X	fragment sequenced in pTZ19R, after part of the <u>envA</u> fragment had been excised with XbaI



side of the inserted fragment, but there are no EcoRI or HindIII sites within envC. The fragment was ligated into pTZ18R which had been digested with the same pair of enzymes and cloning and screening performed as for envA and envB.

The "reversed" clones were then partially sequenced using reverse sequencing primer.

The envA sequence contains a unique XbaI site at nucleotide 6494, and pTZ19R contains a unique XbaI site in the multiple cloning site. Digestion of the pTZ19R:envA plasmid DNA with XbaI resulted in excision of the envA fragment between nucleotides 6494 and 6929 (all positions refer to the nucleotide numbers in the published MVV EV1 sequence - Sargan *et al.*, 1991). The digested plasmid DNA was re-ligated to itself, resulting in a plasmid pTZ19R:envAX where envAX contained the envA fragment 5994 to 6929.

Internal sequencing primers were used in pTZ19R:envA (primer 191L) and pTZ19R:envB (primers 193L and 415H).

The sequences generated for each clone were assembled manually and subsequently analysed using versions 6.0 and 7.0 of the Genetics Computer Group Sequence Analysis Software Package (Devereux *et al.*, 1984).

The nucleotide sequence of each cloned fragment is shown in figure 3.4a-c, along with the published EV1 sequence (Sargan *et al.*, 1991). The same name, for example A46 is used to denote both the clone and the env gene fragment itself. Positions at which the nucleotide is variable between 2 or more clones are marked by asterisks.

The predicted amino acid sequence for each clone was derived from the nucleotide sequence and the results are shown in figure 3.5a-c. Amino acids which vary between 2 or more clones are marked by asterisks. The single letter amino acid code is used.

The percentage identity between each pair of clones

Figure 3.4a

The nucleotide sequences of the MVV strain EV1 gp135 envA variants

The nucleotide sequence of each envA variant is shown, including the published EV1 sequence (EV1, from Sargan et al., 1991).

Nucleotide positions variable between clones are marked by asterisks.

The numbering below each line is derived from Sargan et al., 1991.

The numbering above each line shows the nucleotide position within the fragment itself, beginning with 0 in each fragment.

	0				49
VA1	ATGGCAAGCA	CAAAGAGCAA	GCCAAGCAGA	GCAACCTGGG	CAGACATGGA
DA3	ATGGCAAGCA	CAAAGAGCAA	GCCAAGCAGA	GCAACCTGGG	CAGACATGGA
AX11	ATGGCAAGCA	CAAAGAGCAA	GCCAAGCAGA	GCAACCTGGG	CAGACATGGA
ADP1	ATGGCAAGCA	CAAAGAGCAA	GCCAAGCAGA	GCAACCTGGG	CAGACATGGA
A46	ATGGCAAGCA	CAAAGAGCAA	GCCAAGCAGA	GCAACCTGGG	CAGACATGGA
EV1	ATGGCAAGCA	CAAAGAGCAA	GCCAAGCAGA	GTAACCTGGA	CAGACATGGA
	5994			* *	
					99
VA1	GCCACCGCAA	AAGGAAAAAT	GGGGTCAAGT	AGTACAAGAG	TTGGTGACAG
DA3	GCCACCGCAA	AAGGAAAAAT	GGGGTCAAGT	AGTACAAGAG	TTGGTGACAA
AX11	GCCACCGCAA	AAGGAAAAAT	GGGGTCAAGT	AGTACAAGAG	TTGGTGACAA
ADP1	GCCACCGCAA	GAGGAAAAAT	GGGGTCAAGT	AGTACAAGAG	TTGGTGACAA
A46	GCCACCGCAA	CAGGAAAAAT	GGGGTCAAGT	AGTACAAGAG	TTGGTGACAA
EV1	GCCACCGCAA	AAGGAAAAAT	GGGGTCAAGT	AGTACAAGAG	TTGGTGACAA
	6044	*			*
					149
VA1	GACAACAAAA	CGAAGAGAGG	CAGGGCTTGG	TAACAGGTAA	GAGAAAGCCA
DA3	GACAACAAAA	CGAAGAGAGG	CAGGGCTTGG	TAACAGGTAA	GAGAAAGCCA
AX11	GACAACAAAA	CGAAGAGAGG	CAGGGCTTGG	TAACAGGTAA	GAGAAAGCCA
ADP1	GACAACAAAA	CGAAGAGAGG	CAGGGCTTGG	TAACAGGTAA	GAGAAAGCCA
A46	GACAACAAAA	CGAAGAGAGG	CAGGGCTTGG	TAACAGGTAA	GAGAAAGCCA
EV1	GACAACAAAA	CGAAGAAAGG	CAGGGATTGG	TAACAGGTAA	GAGAAAGCCA
	6094	*	*		
					199
VA1	TGGGTAAGTA	TTGAGATCCT	GGGAACAAAT	CATGATCAGG	AAAAAGAAAA
DA3	TGGGTAAGTA	TTGAGATCCT	GGGAACAAAG	CATGATCAGG	AAAAAGAAAA
AX11	TGGGTAAGTA	TTGAGATCCT	GGGAACAAAG	CATGATCAGG	AAAAAGAAAA
ADP1	TGGGTAAGTA	TTGAGATCCT	GGGAACAAAT	CATGATCAGG	AAAAAGAAAA
A46	TGGGTAAGTA	TTGAGATCCT	GGGAACAAAG	CATGATCAGG	AAAAAGAAAA
EV1	TGGGTAAGTA	TTGAGATCCT	GGGAACAAAG	CATGATCAGG	AAAAAGAAAA
	6144		*		
					249
VA1	GGTAAATGTC	TGGGAACCAT	GTGAAAAATG	GTTTGGCCAA	TTAGTATGGG
DA3	GGTAAATGTC	TGGGAACCAT	GTGAAAAATG	GTTTGGCCAA	TTAGTATGGG
AX11	GGTAAATGTC	TGGGAACCAT	GTGAAAAATG	GTTTGGCCAA	TTATTATGGG
ADP1	GGTAAATGTC	TGGGAACCAT	GTGAAAAATG	GTTTGGCCAA	TTAGTATGGG
A46	GGTAAATGTC	TGGGAACCAT	GTGAAAAATG	GTTTGGCCAA	TTAGTATGGG
EV1	GGTAAATGTC	TGGGAACCAT	GTGAAAAATG	GTTTGGCCAA	TTAGTATGGG
	6194			**	*
					299
VA1	TAACATATGG	ATACTACAGA	CTGGTGTAT	GGGGATGTCT	CATATGGGAA
DA3	TAACATATGG	ATACTACAGA	CTGGTGTAT	GGGGATGTCT	CATATGGGAA
AX11	TAACACTATG	GTACTACAGA	CTGGTGTAT	GGGGATGTCT	CATATGGGAA
ADP1	TAACACTATG	GATGCTACAG	CTGGTGTAT	GGGGATGTCT	CATATGGGAA
A46	TAACACTATG	GATGCTACAG	CTGGTGTAT	GGGGATGTCT	CATATGGGAA
EV1	TAACATCTAG	GTACATCAGA	CTGGTGTAT	GGGGATGTCT	CATATGGGAA
	6244	****	*****		
					349
VA1	ATGCAAAAAG	AAAATAAGGG	TCAATGTCAA	GCAGAAGAAG	TAATAGCGTT
DA3	ATGCAAAAAG	AAAGTAAGGG	TCAATGTCAA	GCAGAAGAAG	TAATAGCATT
AX11	ATGCAAAAAG	AAAGTAAGGG	TCAATGTCAA	GCAGAAGAAG	TAATAGCATT
ADP1	ATGCAAAAAG	AAAGTAAGGG	TCAATGTCAA	GCAGAAGAAG	TAATAGCATT
A46	ATGCAAAAAG	AAAGTAAGGG	TCAATGTCAA	GCAGAAGAAG	TAATAGCATT
EV1	ATGCAAAAAG	AAAGTAAGGG	TCAATGTCAA	GCAGAAGAAT	TAATAGCGTT
	6294	*		*	*
					399
VA1	GATAGATGAC	CCTGGAGGAT	TCCAGAGAGT	AAGACAAGTA	GAGACAGTGC
DA3	GATAGATGAC	CCTGGAGGAT	TCCAGAGAGT	AAGACAAGTA	GAGACAGTGC
AX11	AATAGATGAC	CCTGGAGGAT	TCCAGAGAGT	AAGACAAGTA	GAGACAGTGC
ADP1	GATAGATGAC	CCTGGAGGAT	TCCAGAGAGT	AAGACAAGTA	GAGACAGTGC
A46	GATAGATGAC	CCTGGAGGAT	TCCAGAGAGT	AAGACAAGTA	GAGACAGTGC
EV1	GATAGATGAC	CCTGGAGGAT	TCCAGAGAGT	AAGACAAGTA	GAGACAGTGC
	6344	*			

Figure 3.4a contd.

449

VA1	CAGTGACCTG	TGTAACAAGA	AATTTTACTC	AATGGGGATG	TCAGCCAGAG
DA3	CAGTGACCTG	TGTAACAAGA	AATCTTACTC	AATGGGGATG	TCAGCCAGAG
AX11	CAGTGACCTG	TGTAACAAGA	AATTTTACTC	AATGGGGATG	TCAGCCAGAG
ADP1	CAGTGACCTG	TGTAACAAGA	AATTTTACTC	AATGGGGATG	TCAGCCAGAG
A46	CAGTGACCTG	TGTAACAAGA	AATTTTACTC	AATGGGGATG	TCAGCCAGAG
EV1	CAGTGACCTG	TGTAACAAGA	AATCTTACTC	AATGGGGATG	TCAGCCAGAG
6394			*		

499

VA1	GGAGCATATC	CAGATCCTGA	GATAGAATAT	AGAAATATAT	CAAAAGAAAT
DA3	GGAGCATATC	CAGATCCTGA	GATAGAATAT	AGAAATATAT	CAAAAGAAAT
AX11	GGAGCATATC	CAGATCCTGA	GATAGAATAT	AGAAATATAT	CAAAAGAAAT
ADP1	GGAGCATATC	CAGATCCTGA	GATAGAATAT	AGAAATATAT	CAAAAGAAAT
A46	GGAGCATATC	CAGATCCTGA	GATAGAATAT	AGAAATATAT	CAAAAGAAAT
EV1	GGAGCATATC	CAGATCCTGA	GATAGAATAT	AGAAATATAT	CAAAAGAAAT
6444					

549

VA1	TCTAGAACAA	GTGGATGGAC	GAGACTGGCC	ATGGAATACA	TATCACTGGC
DA3	TCTAGAACAA	GTGGATGGAC	GAGACAGGCC	ATGGAATACA	TATCACTGGC
AX11	TCTAGAACAA	GTGTATGGAC	GAGACTGGCC	ATGGAATACA	TATCACTGGC
ADP1	TCTAGAACAA	GTGTATGGAC	GAGACAGGCC	ATGGAATACA	TATCACTGGC
A46	TCTAGAACAA	GTGTATGGAC	GAGACAGGCC	ATGGAATACA	TATCACTGGC
EV1	TCTAGAACAA	GTGTATGGAC	GAGACAGGCC	ATGGAATACA	TATCACTGGC
6494		*	*		

599

VA1	CTTTATGGCA	GCTGGAAAAC	ATGAAAAGCT	GGATGAAAGA	AAATGAAAAA
DA3	CTTTATGGCA	GCTGGAAAAC	ATGAAAAGCT	GGATGAAAGA	AAATGAAAAA
AX11	CTTTATGGCA	GCTGGAAAAC	ATGAAAAGCT	GGATGAAAGA	AAATGAAAAA
ADP1	CTTTATGGCA	GCTGGAAAAC	ATGAAAAGCT	GGATGAAAGA	AAATGAAAAA
A46	CTTTATGGCA	GCTGGAAAAC	ATGAAAAGCT	GGATGAAAGA	AAATGAAAAA
EV1	CTTTATGGCA	GCTGGAAAAC	ATGAAAAGCT	GGATGAAAGA	AAATGAAAAA
6544					

649

VA1	GAGAATAAGG	GCAGAACAAA	TAAAACAAAG	GAAGATATAG	ATGATCTTTT
DA3	GAGAATAAGG	GCAGAACAAA	TAAAACAAAG	GAAGATATAG	ATGATCTTTT
AX11	GAGAATAAGG	GCAGAACAAA	TAAAACAAAG	GAAGATATAG	ATGATCTTTT
ADP1	GAGAATAAGG	GCAGAACAAA	TAAAACAAAG	GAAGATATAG	ATGATCTTTT
A46	GAGAATAAGG	GCAGAACAAA	TAAAACAAAG	GAAGATATAG	ATGATCTTTT
EV1	GAAAATAAGG	GCAGAACAAA	TAAAACAAAG	GAAGATATAG	ATGATCTTTT
6594	*				

699

VA1	AGCAGGGAGA	ATAAGGGGAA	GATTTTGTGT	TCCATATCCC	TATGCATTAT
DA3	AGCAGGGAGA	ATAAGGGGAA	GATTTTGTGT	TCCATATCCC	TATGCATTAT
AX11	AGCAGGGAGA	ATAAGGGGAA	GATTTTGTGT	TCCATATCCC	TATGCATTAT
ADP1	AGCAGGGAGA	ATAAGGGGAA	GATTTTGTGT	TCCATATCCC	TATGCATTAT
A46	AGCAGGGAGA	ATAAGGGGAA	GATTTTGTGT	TCCATATCCC	TATGCATTAT
EV1	AGCAGGGAGA	ATAAGGGGAA	GATTTTGTGT	TCCATCTCCC	TATGCATTAT
6644				*	

749

VA1	TAAAATGTGA	GGAATGGTGT	TGGTACCCCA	CTGACATTAA	TGAGGAAACA
DA3	TAAAATGTGA	GGAATGGTGT	TGGTACCCTA	CTGACATTAA	TGAGGAAACA
AX11	TAAAATGTGA	GGAATGGTGT	TGGTACCCCA	CTGACATTAA	TGAGGAAACA
ADP1	TAAAATGTGA	GGAATGGTGT	TGGTACCCCA	CTGACATTAA	TGAGGAAACA
A46	TAAAATGTGA	GGAATGGTGT	TGGTACCCCA	CTGACATTAA	TGAGGAAACA
EV1	TAAAATGTGA	GGAATGGTGT	TGGTACCCCA	CTGACGTTAA	TGAGGAAACA
6694			*	*	

799

VA1	GGACATGCAC	AAAAGATAAA	GATAAACTGC	ACAAAAGCAA	AAGCTGTCTC
DA3	GGACATGCAC	AAAAGATAAA	GATAAACTGC	ACAAAAGCAA	AAGCTGTCTC
AX11	GGACATGCAC	AAAAGATAAA	GATAAACTGC	ACAAAAGCAA	AAGCTGTCTC
ADP1	GGACATGCAC	AAAAGATAAA	GATAAACTGC	ACAAAAGCAA	AAGCTGTCTC
A46	GGACATGCAC	AAAAGATAAA	GATAAACTGC	ACAAAAGCAA	AAGCTGTCTC
EV1	GGACATGCAC	AAAAGATAAA	GATAAACTGC	ACAAAAGCAA	AAGCTGTCTC
6744					

Figure 3.4a contd.

849

VA1 TTGCACAGAA AAGATGCCCT TAGCAGGAGT GCAAAGGGTA TATTGGGAAA
DA3 TTGCACAGAA AAGATGCCCT TAGCAGGAGT GCAAAGGGTA TATTGGGAAA
AX11 TTGCACAGAA AAGATGCCCT TAGCAGGAGT GCAAAGGGTA TATTGGGAAA
ADP1 TTGCACAGAA AAGATGCCCT TAGCAGGAGT GCAAAGGGTA TATTGGGAAA
A46 TTGCACAGAA AAGATGCCCT TAGCAGGAGT GCAAAGGGTA TATTGGGAAA
EV1 TTACACAGAA AAGATGCCCT TAGCAGGAGT GCAAAGGGTA TATTGGGAAA
6794 *

899

VA1 AGGAAGATGA GGAGAGTATG AAATTCATGA ATATAGAAGC TTGTACTGAG
DA3 AGGAAGATGA GGAGAGTATG AAATTCATGA ATATAGAAGC TTGTACTGAG
AX11 AGGAAGATGA GGAGAGTATG AAATTCATGA ATATAGAAGC TTGTACTGAG
ADP1 AGGAAGATGA GGAGAGTATG AAATTCATGA ATATAGAAGC TTGTACTGAG
A46 AGGAAGATGA GGAGAGTATG AAATTCATGA ATATAGAAGC TTGTACTGAG
EV1 AGGAAGATGA GGAGACGATG AAATTCATGA ATATAGAAGC TTGCACTGAG
6844 **

936

VA1 TCAAAGTTAA GATGTGCACA GGACGAGAAG AGTCCT
DA3 TCAAAGTTAA GATGTGCACA GGACGAGAAG AGTCCT
AX11 TCAAAGTTAA GATGTGCACA GGACGAGAAG AGTCCT
ADP1 TCAAAGTTAA GATGTGCACA GGACGAGAAG AGTCCT
A46 TCAAAGTTAA GATGTGCACA GGACGAGAAG AGTCCT
EV1 TCAAAGTTAA GATGTGCACA GGACGAGAAG AGTCCT
6894 6929

Figure 3.4b

The nucleotide sequence of the MVV strain EV1 gp135 envB variants

The nucleotide sequence of each envB variant is shown, including the published EV1 sequence (EV1, from Sargan et al., 1991).

Nucleotide positions variable between clones are marked by asterisks.

The numbering below each line is derived from Sargan et al., 1991.

The numbering above each line shows the nucleotide position within the fragment itself, beginning with 0 in each fragment.

0 49

VB6 GAGGAAACAG GACATGCACA AAAGATAAAG ATAAACTGCA CAAAAGCAAA
DB2 GAGGAAACAG GACATGCACA AAAGATAAAG ATAAACTGCA CAAAAGCAAA
BX11 GAGGAAACAG GACATGCACA AAAGATAAAG ATAAACTGCA CAAAAGCAAA
BDP1 GAGGAAACAG GACATGCACA AAAGATAAAG ATAAACTGCA CAAAAGCAAA
BX10 GAGGAAACAG GACATGCACA AAAGATAAAG ATAAACTGCA CAAAAGCAAA
EV1 GAGGAAACAG GACATGCACA AAAGATAAAG ATAAACTGCA CAAAAGCAAA
6735

99

VB6 AGCTGTCTCT TGCACAGAAA AGATGCCCTT AGCAGGAGTA CAAAGGGTAT
DB2 AGCTGTCTCT TGCACAGAAA AGATGCCCTT AGCAGGAGTG CAAAGGGTAT
BX11 AGCTGTCTCT TGCACAGAAA AGATGCCCTT AGCAGGAGTG CAAAGGGTAT
BDP1 AGCTGTCTCT TGCACAGAAA AGATGCCCTT AGCAGGAGTG CAAAGGGTAT
BX10 AGCTGTCTCT TGCACAGAAA AGATGCCCTT AGCAGGAGTG CAAAGGGTAT
EV1 AGCTGTCTCT TACACAGAAA AGATGCCCTT AGCAGGAGTG CAAAGGGTAT
6785 * *

149

VB6 ATTGGGAAAA GGAAGATGAG GAGAGTATGA AATTCATGAA CATAAAAGCT
DB2 ATTGGGAAAGA GGAAGATGAG GAGAGTATGA AATTCATGAA CATAGAAGCT
BX11 ATTGGGAAAA GGAAGATGAG GAGAGTATGA AATTCATGAA TATAGAAGCT
BDP1 ATTGGGAAAA GGAAGATGAG GAGAGTATGA AATTCATGAA TATAGAAGCT
BX10 ATTGGGAAAA GGAAGATGAG GAGAGTATGA AATTCATGAA TATAGAAGCT
EV1 ATTGGGAAAA GGAAGATGAG GAGACGATGA AATTCATGAA TATAGAAGCT
6835 * ** * *

199

VB6 TGTAATGCGT CACAGTTGAG ATGTGAGAAA GAAGATAAGA GTCCTGGAGG
DB2 TGTAATGCGT CAAAGTTAAG ATGTGCACAG GACGAGAAGA GTCCTGGGGG
BX11 TGTAATGAGT CAAAGTTAAG ATGTGCACAG GACGAGAAGA GTCCTGGGGG
BDP1 TGTAATGAGT CAAAGTTAAG ATGTGCACAG GACGAGAAGA GTCCTGGGGG
BX10 TGTAATGAGT CAAAGTTAAG ATGTGCACAG GACGAGAAGA GTCCTGGGGG
EV1 TGCACTGAGT CAAAGTTAAG ATGTGCACAG GACGAGAAGA GTCCTGGGGG
6885 * * * * * ** * *

249

VB6 ATGTGTGCAA GGGTATCCGA TCCCCAGTAG GACTGAAATA ATACCTGAAA
DB2 ATGTGTGCAG GGATATCCGA TCCCAGTAGG GACTGAAATA ATACCTGAAA
BX11 ATGTGTGCAG GGATATCCGA TCCCAGTAGG GACTGAAATA ATACCTGAAA
BXP1 ATGTGTGCAG GGATATCCGA TCCCAGTAGG GACTGAAATA ATACCTGAAA
Bx10 ATGTGTGCAG GGATATCCGA TCCCAGTAGG GACTGAAATA ATACCTGAAA
EV1 ATGTGTGCAG GGATATCCGA TCCCCAGTAG GACTGAAATA ATACCTGAAA
6935 * *****

299

VB6 GTATGAAGCA TTTGAGAGGA AAGAAAAGCC CATATGGAGG AATAAAAGAT
DB2 GTATGAAGCA TTTGAGAGGA AAGAAAAGCC CATATGGAGG AATAAAAGAT
BX11 GTATGAAGCA TTTGAGAGGA AAGAAAAGCC CATATGGAGG AATAAAAGAT
BDP1 GTATGAAGCA TTTGAGAGGA AAGAAAAGCC CATATGGAGG AATAAAAGAT
BX10 GTATGAAGCA TTTGAGAGGA AAGAAAAGCC CATATGGAGG AATAAAAGAT
EV1 GTATGAAGCA TTTGAGAGGA AAGAAAAGCC CATATGGAGG AATAAAAGAT
6985

349

VB6 AAGAATGGAG AATTAAAACT GCCTCTTACA GTAAGAGTCT GGGTAAGAAT
DB2 AAGAATGGAG AATTAAAACT GCCTCTTACA GTAAGAGTCT GGGTAAGAAT
BX11 AAGAATGGAG AATTAAAACT GCCTCTTACA GTAAGAGTCT GGGTAAGAAT
BDP1 AAGAATGGAG AATTAAAACT GCCTCTTACA GTAAGAGTCT GGGTAAGAAT
BX10 AAGAATGGAG AATTAAAACT GCCTCTTACA GTAAGAGTCT GGGTAAGAAT
EV1 AAGAATGGAG AATTAAAACT GCCTCTTACA GTAAGAGTCT GGGTAAGAAT
7035

399

VB6 GGCGAATTTA TCAGGTTGGG TGAATGGAAC TCCCCCGTAT TGGAGTGCAA
DB2 GGCGAATTTA TCAGGTTGGG TGAATGGAAC TCCCCCGTAT TGGAGTGCAA
BX11 GGCGAATTTA TCAGGTTGGG TGAATGGAAC TCCCCCGTAT TGGAGTGCAA
BDP1 GGCGAATTTA TCAGGTTGGG TGAATGGAAC TCCCCCGTAT TGGAGTGCAA
BX10 GGCGAATTTA TCAGGTTGGG TGAATGGAAC TCCCCCGTAT TGGAGTGCAA
EV1 GGCGAATTTA TCAGGTTGGG TGAATGGAAC TCCCCCGTAT TGGAGTGCAA
7085

Figure 3.4b contd.

449

VB6	GAATGAATGG	AAGCACAGGA	ATAAATGGGA	CCAGATGGTA	TGGAATAGGA
DB2	GAATGAATGG	AAGCACAGGA	ATAAATGGGA	CCAGATGGTA	TGGAATAGGA
BX11	GAATGAATGG	AAGCACAGGA	ATAAATGGGA	CCAGATGGTA	TGGAATAGGA
BDP1	GAATGAATGG	AAGCACAGGA	ATAAATGGGA	CCAGATGGTA	TGGAATAGGA
BX10	GAATGAATGG	AAGCACAGGA	ATAAATGGGA	CCAGATGGTA	TGGAATAGGA
EV1	GAATGAATGG	AAGCACAGGA	ATAAATGGGA	CCAGATGGTA	TGGAATAGGA

499

VB6	TCACTGCACC	ATTTAGGGTT	CAACATTAGT	AGTAACCCTG	AGCAGGGGAT
DB2	TCACTGCACC	ATTTAGGGTT	CAACATTAGT	AGTAACCCTG	AGCAGGGGAT
BX11	TCACTGCACC	ATTTAGGGTT	CAACATTAGT	AGTAACCCTG	AGCAGGGGAT
BDP1	TCACTGCACC	ATTTAGGGTT	CAACATTAGT	AGTAACCCTG	AGCAGGGGAT
BX10	TCACTGCACC	ATTTAGGGTT	CAACATTAGT	AGTAACCCTG	AGCAGGGGAT
EV1	TCACTGCACC	ATTTAGGGTT	CAACATTAGT	AGTAACCCTG	AGCAGGGGAT

549

VB6	TTGCAACTTC	ACAAAAGAAG	TTTTGGTAGG	AGGAGAAAAA	TTCGAGTATC
DB2	TTGCAACTTC	ACAAAAGAAG	TTTTGGTAGG	AGGAGAAAAA	TTCGAGTATC
BX11	TTGCAACTTC	ACAAAAGAAG	TTTTGGTAGG	AGGAGAAAAA	TTCGAGTATC
BDP1	TTGCAACTTC	ACAAAAGAAG	TTTTGGTAGG	AGGAGAAAAA	TTCGAGTATC
BX10	TTGCAACTTC	ACAAAAGAAG	TTTTGGTAGG	AGGAGAAAAA	TTCGAGTATC
EV1	TTGCAACTTC	ACAAAAGAAG	TTTTGGTAGG	AGGAGAAAAA	TTCGAGTATC

599

VB6	AGTATACACC	CTCATGGAAT	TGCTCGAAAA	ATTGGACAGG	ACATCCAGTA
DB2	AGTATACACC	CTCATGGAAT	TGCTCGAAAA	ATTGGACAGG	ACATCCAGTA
BX11	AGTATACACC	CTCATGGAAT	TGCTCGAAAA	ATTGGACAGG	ACATCCAGTA
BDP1	AGTATACACC	CTCATGGAAT	TGCTCGAAAA	ATTGGACAGG	ACATCCAGTA
BX10	AGTATACACC	CTCATGGAAT	TGCTCGAAAA	ATTGGACAGG	ACATCCAGTA
EV1	AGTATACACC	CTCATGGAAT	TGCTCGAAAA	ATTGGACAGG	ACATCCAGTA

649

VB6	TGGCATGTAT	TTAGATACCT	AGATATGACA	GAACATATGA	CAAGCTATTG
DB2	TGGCATGTAT	TTAGATACCT	AGATATGACA	GAACATATGA	CAAGCTATTG
BX11	TGGCATGTAT	TTAGATACCT	AGATATGACA	GAACATATGA	CAAGCTATTG
BDP1	TGGCATGTAT	TTAGATACCT	AGATATGACA	GAACATATGA	CAAGCTATTG
BX10	TGGCATGTAT	TTAGATACCT	AGATATGACA	GAACATATGA	CAAGCTATTG
EV1	TGGCATGTAT	TTAGATACCT	AGATATGACA	GAACATATGA	CAAGCTATTG

699

VB6	TGTACAGAGG	CCCTTAAGAC	ATAATATAAC	AGTGGGAAAT	GGCACCATAA
DB2	TGTACAGAGG	CCCTTAAGAC	ATAATATAAC	AGTGGGAAAT	GGCACCATAA
BX11	TGTACAGAGG	CCCTTAAGAC	ATAATATAAC	AGTGGGAAAT	GGCACCATAA
BDP1	TGTACAGAGG	CCCTTAAGAC	ATAATATAAC	AGTGGGAAAT	GGCACCATAA
BX10	TGTACAGAGG	CCCTTAAGAC	ATAATATAAC	AGTGGGAAAT	GGCACCATAA
EV1	TGTACAGAGG	CCCTTAAGAC	ATAATATAAC	AGTGGGAAAT	GGCACCATAA

749

VB6	CAGGAAATTG	TAGCACAACA	AATTGGGATG	GATGTAATTG	CTCAAGGTCA
DB2	CAGGAAATTG	TAGCACAACA	AATTGGGATG	GATGTAATTG	CTCAAGGTCA
BX11	CAGGAAATTG	TAGCACAACA	AATTGGGATG	GATGTAATTG	CTCAAGGTCA
BDP1	CAGGAAATTG	TAGCACAACA	AATTGGGATG	GATGTAATTG	CTCAAGGTCA
BX10	CAGGAAATTG	TAGCACAACA	AATTGGGATG	GATGTAATTG	CTCAAGGTCA
EV1	CAGGAAATTG	TAGCACAACA	AATTGGGATG	GATGTAATTG	CTCAAGGTCA

799

VB6	GGAAATCATT	TATATAACAG	CACCACAGGA	GGATTACTAG	TAATTATATG
DB2	GGAAATCATT	TATATAACAG	CACCACAGGA	GGATTACTAG	TAATTATATG
BX11	GGAAATCATT	TATATAACAG	CACCACAGGA	GGATTACTAG	TAATTATATG
BDP1	GGAAATCATT	TATATAACAG	CACCACAGGA	GGATTACTAG	TAATTATATG
BX10	GGAAATCATT	TATATAACAG	CACCACAGGA	GGATTACTAG	TAATTATATG
EV1	GGAAATCATT	TATATAACAG	CACCACAGGA	GGATTACTAG	TAATTATATG

Figure 3.4b contd.

849

VB6 TAGACAAAGC AGAACAATTA GAG GAAT AATGGGAACT AACACAAATT
DB2 TAGACAAAGC AGAACAATTA GAG GAAT AATGGGAACT AACACAAATT
BX11 TAGACAAAGC AGAACAATTA GAG AAAT AATGGGAACT AACACAAATT
BDP1 TAGACAAAGC AGAACAATTA GAG GAAT AATGGGAACT AACACAAATT
BX10 TAGACAAAGC AGAACAATTA GAG GAAT AATGGGAACT AACACAAATT
EV1 TAGACAAAGC AGAACAATTA CAGCAATAAT AATGGGAACT AACACAAATT
7535 * ****

899

VB6 GGACCACCAT GTGGGAAATA TATAAGAATT GCTCAAGCTG TGAGAACAGC
DB2 GGACCACCAT GTGGGAAATA TATAAGAATT GCTCAAGCTG TGAGAACAGC
BX11 GGACCACCAT GTGGGAAATA TATAAGAATT GCTCAAGCTG TGAGAACAGC
BDP1 GGACCACCAT GTGGGAAATA TATAAGAATT GCTCAAGCTG TGAGAACAGC
BX11 GGACCACCAT GTGGGAAATA TATAAGAATT GCTCAAGCTG TGAGAACAGC
EV1 GGACCACCAT GTGGGAAATA TATAAGAATT GCTCAAGCTG TGAGAACAGC
7585

917

VB6 ACGTTAGACA GAATAGGG
DB2 ACGTTAGACA GAATAGGG
BX11 ACGTTAGACA GAATAGGG
BDP1 ACGTTAGACA GAATAGGG
BX10 ACGTTAGACA GAATAGGG
EV1 ACGTTAGACA GAATAGGG
7635 7652

Figure 3.4c

The nucleotide sequence of the MVV strain EV1 gp135 envC variants

The nucleotide sequence of each envC variant is shown, including the published EV1 sequence (EV1, from Sargan et al., 1991).

Nucleotide positions variable between clones are marked by asterisks.

The numbering below each line is derived from Sargan et al., 1991.

The numbering above each line shows the nucleotide position within each fragment itself, beginning with 0 in each fragment.

0 49

VC1 GGATGTAATT GCTCAAGGTC AGGAAATCAT TTATATAACA GCACCACAGG
DC1 GGATGTAATT GCTCAAGGTC AGGAAATCAT TTATATAACA GCACCACAGG
CX7 GGATGTAATT GCTCAAGGTC AGGAAATCAT TTATATAACA GCACCACAGG
CDP1 GGATGTAATT GCTCAAGGTC AGGAAATCAT TTATATAACA GCACCACAGG
C45 GGATGTAATT GCTCAAGGTC AGGAAATCAT TTATATAACA GCACCACAGG
EV1 GGATGTAATT GCTCAAGGTC AGGAAATCAT TTATATAACA GCACCACAGG
7464

99

VC1 AGGATTACTA GTAATTATAT GTAGACAAAG CAGAACAATT AGGG GAA
DC1 AGGATTACTA GTAATTATAT GTAGACAAAG CAGAACAATT AGAG GAA
CX7 AGGATTACTA GTAATTATAT GCAGACAAAG CAGAACAATT AGAG GAA
CDP1 AGGATTACTA GTAATTATAT GTAGACAAAG CAGAACAATT AGAG GAA
C45 AGGATTACTA GTAATTATAT GTAGACAAAG CAGAACAATT AGAG GAA
EV1 AGGATTACTA GTAATTATAT GTAGACAAAG CAGAACAATT ACAGCAATAA
7514 ** ****

149

VC1 TAATGGGAAC TAACACAAAT TGGACCACCA TGTGGGAAAT ATATAAGAAT
DC1 TAATGGGAAC TAACACAAAT TGGACCACCA TGTGGGAAAT ATATAAGAAT
CX7 TAATGGGAAC TAACACAAAT TGGACCACCA TGTGGGAAAT ATATAAGAAT
CDP1 TAATGGGAAC TAACACAAAT TGGACCACCA TGTGGGAAAT ATATAAGAAT
C45 TAATGGGAAC TAACACAAAT TGGACCACCA TGTGGGAAAT ATATAAGAAT
EV1 TAATGGGAAC TAACACAAAT TGGACCACCA TGTGGGAAAT ATATAAGAAT
7564

199

VC1 TGCTCAAGCT GTGAGAACAG CACGTTAGAC AGAATAGGGA ATGGTGCCTT
DC1 TGCTCAAGCT GTGAGAACAG CACGTTAGAC AGAATAGGGA ATGGTGCCTT
CX7 TGCTCAAGCT GTGAGAACAG CACGTTAGAC AGAATAGGGA ATGGTGCCTT
CDP1 TGCTCAAGCT GTGAGAACAG CACGTTAGAC AGAATAGGGA ATGGTGCCTT
C45 TGCTCAAGCT GTGAGAACAG CACGTTAGAC AGAATAGGGA ATGGTGCCTT
EV1 TGCTCAAGCT GTGAGAACAG CACGTTAGAC AGAATAGGGA ATGGCACCCTT
7614 **

249

VC1 AGGAACTGTA AAAAACGTCA ACTGTAGGTT ACCACACAAA AATGAAAGCC
DC1 AGGAACTGTA CAAAACGTCA ACTGTAGGTT ACCACACAAA AATGAAAGCC
CX7 AGGAACTGTA CAAAACGTCA ACTGTAGGTT ACCACACAAA AATGAAAGCC
CDP1 AGGAACTGTA AAAAACGTCA ACTGTAGGTT ACCACACAAA AATGAAAGCC
C45 AGGAACTGTA AAAAACGTCA ACTGTAGGTT ACCACACAAA AATGAAAGCC
EV1 AGGAACTATA CAGAACATCA ACTGTAGCCT ACCTCACAGA AATGAAACAA
7664 * * * * ** * * ***

299

VC1 GGAAGTGGAC ATGTCAGGCT CG CA GGGGAAGAGA CAAAAGAGAT
DC1 GGAAGTGGAC ATGTCAGGCT CG CA GGGGAAGAGA CAGGACAGAT
CX7 GGAAGTGGAC ATGTCAGGCT CG CA GGGGAAGAGA CAGGACAGAT
CDP1 GGAAGTGGAC ATGTCAGGCT CG CA GGGGAAGAGA CAGGACAGAT
C45 GGAAGTGGAC ATGTCAGGCT CG CA GGGGAAGAGA CAGGACAGAT
EV1 ATACTTGGAC CTGTGCAGCA AGAGCAGCAA GTGCAGGAAA CAAAAGAGAT
7714 ** ** * *** * * ***** ** * * ** *

349

VC1 TCCTTATACA TAGCAGGTAG AGACTTCTGG GGCAGAGTAA AAGCACATTA
DC1 TCCTTATACA TAGCAGGTAG AGACTTCTGG GGCAGAGTAA AAGCACATTA
CX7 TCCTTATACA TAGCAGGTAG AGACTTCTGG GGCAGAGTAA AAGCACATTA
CDP1 TCCTTATACA TAGCAGGTAG AGACTTCTGG GGCAGAGTAA AAGCACATTA
C45 TCCTTATACA TAGCAGGTAG AGACTTCTGG GGCAGAGTAA AAGCACATTA
EV1 TCCTTATACA TAGCAGGTAG AGACTTCTGG GGCAGAGTAA AAGCACATTA
7764

399

VC1 TAGCTGTGAG AGTAACTTAG GTGGATTAGA TGGAAATGATG CATCAACAGA
DC1 TAGCTGTGAG AGTAACTTAG GTGGATTAGA TGGAAATGATG CATCAACAGA
CX7 TAGCTGTGAG AGTAACTTAG GTGGATTAGA TGGAAATGATG CATCAACAGA
CDP1 TAGCTGTGAG AGTAACTTAG GTGGATTAGA TGGAAATGATG CATCAACAGA
C45 TAGCTGTGAG AGTAACTTAG GTGGATTAGA TGGAAATGATG CATCAACAGA
EV1 TAGCTGTGAG AGTAACTTAG GTGGATTAGA TGGAAATGATG CATCAACAGA
7814 *

Figure 3.4c contd

449

VC1 TATTGCTGCA AAAATATCAA ATAATAAGAG TAAGAGCTTA CACATATGGT
DC1 TATTGCTGCA AAAATATCAA ATAATAAGAG TAAGAGCTTA CACATATGGT
CX7 TATTGCTGCA AAAATATCAA ATAATAAGAG TAAGAGCTTA CACATATGGT
CDP1 TATTGCTGCA AAAATATCAA ATAATAAGAG TAAGAGCTTA CACATATGGT
C45 TATTGCTGCA AAAATATCAA ATAATAAGAG TAAGAGCTTA CACATATGGT
EV1 TATTGCTGCA AAAATATCAA ATAATAAGAG TAAGAGCTTA CACATATGGA
7864 *

499

VC1 GTGGTGGATA TGCCTCAATC CTATCTGGAG AAAAATAGAA GAAATGCTTT
DC1 GTGGTGGATA TGCCTCAATC CTATCTGGAG AAAAATAGAA GAAATGCTTT
CX7 GTGGTGGATA TGCCTCAATC CTATCTGGAG AAAAATAGAA GAAATGCTTT
CDP1 GTGGTGGATA TGCCTCAATC CTATCTGGAG AAAAATAGAA GAAATGCTTT
C45 GTGGTGGATA TGCCTCAATC CTATCTGGAG AAAAATAGAA GAAATGCTTT
EV1 GAGGTGGATA TGCCTCAATC CTATCTGGAG AAAAATAGAA GAAATGCTTT
7914 *

537

VC1 TAAGAAAGAA AGAAAGAAGA GGGGCATAGG GTTGGTG
DC1 TAAGAAAGAA AGAAAGAAGA GGGGCATAGG GTTGGTG
CX7 TAGGAAAGAA AGAAAGAAGA GGGGCATAGG GTTGGTG
CDP1 TAAGAAAGAA AGAAAGAAGA GGGGCATAGG GTTGGTG
C45 TAAGAAAGAA AGAAAGAAGA GGGGCATAGG GTTGGTG
EV1 TAAGAAAGAA AGAAAGAAGA GGGGCATAGG GTTGGTG
7964 8000

Figure 3.5a

**The predicted amino acid sequence of the MVV strain EV1
gp135 envA variants**

The predicted amino acid sequence of each envA variant is shown, including the published EV1 sequence (EV1, from Sargan et al., 1991).

Amino acids variable between clones are marked by asterisks.

Potential N-linked glycosylation sites are underlined.

The numbering below each line is derived from Sargan et al., 1991.

The numbering above each line shows the amino acid position within each fragment itself, beginning with 0 in each fragment.

0 49
 VA1 MASTKSKPSR ATWADMEPPQ KEKWGQVVQE LVTGQQNEER QGLVTGKRKP
 DA3 MASTKSKPSR ATWADMEPPQ KEKWGQVVQE LVTRQQNEER QGLVTGKRKP
 AX11 MASTKSKPSR ATWADMEPPQ KEKWGQVVQE LVTRQQNEER QGLVTGKRKP
 ADP1 MASTKSKPSR ATWADMEPPQ EEKWGQVVQE LVTRQQNEER QGLVTGKRKP
 A46 MASTKSKPSR ATWADMEPPQ QEKWGQVVQE LVTRQQNEER QGLVTGKRKP
 EV1 MASTKSKPSR VTWTDMEPPQ KEKWGQVVQE LVTRQQNEER QGLVTGKRKP
 1 * *

99
 VA1 WVSIEILGTN HDQEKEKVVN WEPCEKWFQ LWWVTYGYR LVLWGCLIWE
 DA3 WVSIEILGTK HDQEKEKVVN WEPCEKWFQ LWWVTYGYR LVLWGCLIWE
 AX11 WVSIEILGTK HDQEKRKVVN WEPCEKCVGQ LLWVTLWYR LVLWGCLIWE
 ADP1 WVSIEILGTN HDQEKEKVVN WEPCEKWFQ LWWVTLWMLQ LVLWGCLIWE
 A46 WVSIEILGTK HDQEKEKVVN WEPCEKWFQ LWWVTLWMLQ LVLWGCLIWE
 EV1 WVSIEILGTR HDQEKEKVVN WEPCEKWFQ LWWVTSRYIR LVLWGCLIWE
 51 * *****

149
 VA1 MQKESKGQCQ AEEVIALIDD PGGFQVRQV ETVPVTCVTR NFTQWGCQPE
 DA3 MQKESKGQCQ AEEVIALIDD PGGFQVRQV ETVPVTCVTR NLTQWGCQPE
 AX11 MQKESKGQCQ AEEVIALIDD PGGFQVRQV ETVPVTCVTR NFTQWGCQPE
 ADP1 MQKESKGQCQ AEEVIALIDD PGGFQVRQV ETVPVTCVTR NFTQWGCQPE
 A46 MQKESKGQCQ AEEVIALIDD PGGFQVRQV ETVPVTCVTR NFTQWGCQPE
 EV1 MQKESKGQCQ AEELIALIDD PGGFQVRQV ETVPVTCVTR NLTQWGCQPE
 101 * *

199
 VA1 GAYDPPEIEY RNISKEILEQ VDGRDPWNT YHWPLWQLEN MKSWMKENEK
 DA3 GAYDPPEIEY RNISKEILEQ VDGRDRPNT YHWPLWQLEN MKSWMKENEK
 AX11 GAYDPPEIEY RNISKEILEQ VYGRDPWNT YHWPLWQLEN MKSWMKENEK
 ADP1 GAYDPPEIEY RNISKEILEQ VYGRDRPNT YHWPLWQLEN MKSWMKENEK
 A46 GAYDPPEIEY RNISKEILEQ VYGRDRPNT YHWPLWQLEN MKSWMKENEK
 EV1 GAYDPPEIEY RNISKEILEQ VYGRDRPNT YHWPLWQLEN MKSWMKENEK
 151 * *

249
 VA1 ENKGRTNKTK EDIDLLAGR IRGRFCVPYP YALLKCEEWC WYPTDINEET
 DA3 ENKGRTNKTK EDIDLLAGR IRGRFCVPYP YALLKCEEWC WYPTDINEET
 AX11 ENKGRTNKTK EDIDLLAGR IRGRFCVPYP YALLKCEEWC WYPTDINEET
 ADP1 ENKGRTNKTK EDIDLLAGR IRGRFCVPYP YALLKCEEWC WYPTDINEET
 A46 ENKGRTNKTK EDIDLLAGR IRGRFCVPYP YALLKCEEWC WYPTDINEET
 EV1 ENKGRTNKTK EDIDLLAGR IRGRFCVPS YALLKCEEWC WYPTDVNEET
 201 * *

299
 VA1 GHAQKIKINC TKAKAVSCTE KMPLAGVQRV YWEKEDEESM KFMNIEACTE
 DA3 GHAQKIKINC TKAKAVSCTE KMPLAGVQRV YWEKEDEESM KFMNIEACTE
 AX11 GHAQKIKINC TKAKAVSCTE KMPLAGVQRV YWEKEDEESM KFMNIEACTE
 ADP1 GHAQKIKINC TKAKAVSCTE KMPLAGVQRV YWEKEDEESM KFMNIEACTE
 A46 GHAQKIKINC TKAKAVSCTE KMPLAGVQRV YWEKEDEESM KFMNIEACTE
 EV1 GHAQKIKINC TKAKAVSYTE KMPLAGVQRV YWEKEDEETM KFMNIEACTE
 251 * *

VA1 SKLRCAQDEK SP
 DA3 SKLRCAQDEK SP
 AX11 SKLRCAQDEK SP
 ADP1 SKLRCAQDEK SP
 A46 SKLRCAQDEK SP
 EV1 SKLRCAQDEK SP
 301 312

Figure 3.5b

**The predicted amino acid sequence of the MVV strain EV1
gp135 envB variants**

The predicted amino acid sequence of each envB variant is shown, including the published EV1 sequence (EV1, from Sargan et al., 1991).

Amino acids variable between clones are marked by asterisks.

Potential N-linked glycosylation sites are underlined.

The numbering below each line is derived from Sargan et al., 1991.

The numbering above each line shows the amino acid position within each fragment itself, beginning with 0 in each fragment.

0 49

VB6	EETGHAQKIK	INCTKAKAVS	CTEKMPLAGV	QRVYWEKEDE	ESMKFMNIKA
DB2	EETGHAQKIK	INCTKAKAVS	CTEKMPLAGV	QRVYWEDEDE	ESMKFMNIEA
BX11	EETGHAQKIK	INCTKAKAVS	CTEKMPLAGV	QRVYWEKEDE	ESMKFMNIEA
BDP1	EETGHAQKIK	INCTKAKAVS	CTEKMPLAGV	QRVYWEKEDE	ESMKFMNIEA
BX10	EETGHAQKIK	INCTKAKAVS	CTEKMPLAGV	QRVYWEKEDE	ESMKFMNIEA
EV1	EETGHAQKIK	INCTKAKAVS	YTEKMPLAGV	QRVYWEKEDE	ETMKFMNIEA

248

*

*

99

VB6	CNASQLRCEK	EDKSPGGCVQ	GYPIPSRTEI	IPESMKHLRG	KKSPYGGIKD
DB2	CTESKLRCAQ	DEKSPGGCVQ	GYPIPVGTEI	IPESMKHLRG	KKSPYGGIKD
BX11	CTESKLRCAQ	DEKSPGGCVQ	GYPIPVGTEI	IPESMKHLRG	KKSPYGGIKD
BDP1	CTESKLRCAQ	DEKSPGGCVQ	GYPIPVGTEI	IPESMKHLRG	KKSPYGGIKD
BX10	CTESKLRCAQ	DEKSPGGCVQ	GYPIPVGTEI	IPESMKHLRG	KKSPYGGIKD
EV1	CTESKLRCAQ	DEKSPGGCVQ	GYPIPSRTEI	IPESMKHLRG	KKSPYGGIKD

298

** * ** **

**

149

VB6	KNGELKLPLT	VRVWVRMANL	SGWVNGTPPY	WSARMNGSTG	INGTRWYGIG
DB2	KNGELKLPLT	VRVWVRMANL	SGWVNGTPPY	WSARMNGSTG	INGTRWYGIG
BX11	KNGELKLPLT	VRVWVRMANL	SGWVNGTPPY	WSARMNGSTG	INGTRWYGIG
BDP1	KNGELKLPLT	VRVWVRMANL	SGWVNGTPPY	WSARMNGSTG	INGTRWYGIG
BX10	KNGELKLPLT	VRVWVRMANL	SGWVNGTPPY	WSARMNGSTG	INGTRWYGIG
EV1	KNGELKLPLT	VRVWVRMANL	SGWVNGTPPY	WSARMNGSTG	INGTRWYGIG

348

199

VB6	SLHHLGFNIS	SNPEQGICNF	TKEVLVGGEK	FEYQYTPSWN	CSKNWTGHPV
DB2	SLHHLGFNIS	SNPEQGICNF	TKEVLVGGEK	FEYQYTPSWN	CSKNWTGHPV
BX11	SLHHLGFNIS	SNPEQGICNF	TKEVLVGGEK	FEYQYTPSWN	CSKNWTGHPV
BDP1	SLHHLGFNIS	SNPEQGICNF	TKEVLVGGEK	FEYQYTPSWN	CSKNWTGHPV
BX10	SLHHLGFNIS	SNPEQGICNF	TKEVLVGGEK	FEYQYTPSWN	CSKNWTGHPV
EV1	SLHHLGFNIS	SNPEQGICNF	TKEVLVGGEK	FEYQYTPSWN	CSKNWTGHPV

398

249

VB6	WHVFRYLDMT	EHMTSYCVQR	PLRHNITVGN	GTITGNCSTT	NWDGCNCSRS
DB2	WHVFRYLDMT	EHMTSYCVQR	PLRHNITVGN	GTITGNCSTT	NWDGCNCSRS
BX11	WHVFRYLDMT	EHMTSYCVQR	PLRHNITVGN	GTITGNCSTT	NWDGCNCSRS
BDP1	WHVFRHLDMT	EHMTSYCVQR	SLRHNITVGN	GTITGNCSTT	NWDGCNCSRS
BX10	WHVFRYLDMT	EHMTSYCVQR	SLRHNITVGN	GTITGNCSTT	NWDGCNCSRS
EV1	WHVFRYLDMT	EHMTSYCVQR	PLRHNITVGN	GTITGNCSTT	NWDGCNCSRS

448

*

*

299

VB6	GNHLYNSTTG	GLLVIICRQS	RTIRG	IMGT	NTNWTTMWEI	YKNCSSCENS
DB2	GNHLYNSTTG	GLLVIICRQS	RTIRG	IMGT	NTNWTTMWEI	YKNCSSCENS
BX11	GNHLYNSTTG	GLLVIICRQS	RTIRE	IMGT	NTNWTTMWEI	YKNCSSCENS
BDP1	GNHLYNSTTG	GLLVIICRQS	RTIRG	IMGT	NTNWTTMWEI	YKNCSSCENS
BX10	GNHLYNSTTG	GLLVIICRQS	RTIRG	IMGT	NTNWTTMWEI	YKNCSSCENS
EV1	GNHLYNSTTG	GLLVIICRQS	RTITAIIMGT	NTNWTTMWEI	YKNCSSCENS	

498

VB6	TLDRIG
DB2	TLDRIG
BX11	TLDRIG
BDP1	TLDRIG
BX10	TLDRIG
EV1	TLDRIG

553

Figure 3.5c

**The predicted amino acid sequence of the MVV strain EV1
gp135 envC variants**

The predicted amino acid sequence of each envC variant is shown, including the published EV1 sequence (EV1, from Sargan et al., 1991).

Amino acids variable between clones are marked by asterisks.

Potential N-linked glycosylation sites are underlined.

The numbering below each line is derived from Sargan et al., 1991.

The numbering above each line shows the amino acid position within each fragment itself, beginning with 0 in each fragment.

```

0
VC1 GCNCSRSGNH LYNSTTGGLL VIICRQSRTI RG IMGTNTN WTTMWEIYKN
DC1 GCNCSRSGNH LYNSTTGGLL VIICRQSRTI RG IMGTNTN WTTMWEIYKN
CX7 GCNCSRSGNH LYNSTTGGLL VIICRQSRTI RG IMGTNTN WTTMWEIYKN
CDP1 GCNCSRSGNH LYNSTTGGLL VIICRQSRTI RG IMGTNTN WTTMWEIYKN
C45 GCNCSRSGNH LYNSTTGGLL VIICRQSRTI RG IMGTNTN WTTMWEIYKN
EV1 GCNCSRSGNH LYNSTTGGLL VIICRQSRTI TAIIMGTNTN WTTMWEIYKN
491 ***

99
VC1 CSSCENSTLD RIGNGALGTV KNVNCRPHK NESRKWTCQA RRGR DKRD
DC1 CSSCENSTLD RIGNGALGTV QNVNCRPHK NESRKWTCQA RRGR DRTD
CX7 CSSCENSTLD RIGNGALGTV QNVNCRPHK NESRKWTCQA RRGR DRTD
CDP1 CSSCENSTLD RIGNGALGTV KNVNCRPHK NESRKWTCQA RRGR DRTD
C45 CSSCENSTLD RIGNGALGTV KNVNCRPHK NESRKWTCQA RRGR DRTD
EV1 CSSCENSTLD RIGNGTLGTI QNINCSLPHR NETNTWTCAA RAASRGNKRD
541 * * * * * *** * *****

149
VC1 SLYIAGRDFW GRVKAHYSCE SNLGGLDGMM HQQILLQKYQ IIRVRAYTYG
DC1 SLYIAGRDFW GRVKAHYSCE SNLGGLDGMM HQQILLQKYQ IIRVRAYTYG
CX7 SLYIAGRDFW GRVKAHYSCE SNLGGLDGMM HQQILLQKYQ IIRVRAYTYG
CDP1 SLYIAGRDFW GRVKAHYSCE SNLGGLDGMM HQQILLQKYQ IIRVRAYTYG
C45 SLYIAGRDFW GRVKAHYSCE SNLGGLDGMM HQQILLQKYQ IIRVRAYTYG
EV1 SLYIAGRDFW GRVKAHYSCE SNLGGVDGMM HQQILLQKYQ IIRVRAYTYG
591 *

178
VC1 VVDMPQSYLE KNRRNAFKKE RKKRGIGLV
DC1 VVDMPQSYLE KNRRNAFKKE RKKRGIGLV
CX7 VVDMPQSYLE KNRRNAFKKE RKKRGIGLV
CDP1 VVDMPQSYLE KNRRNAFKKE RKKRGIGLV
C45 VVDMPQSYLE KNRRNAFKKE RKKRGIGLV
EV1 EVDMPQSYLE KNRRNAFKKE RKKRGIGLV
641 * 669

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within a particular group ie envA, envB and envC was calculated using the Bestfit local homology algorithm (Smith & Waterman, 1981). The percentage identity which is calculated excludes variations due to deletions. The results of this calculation are shown in figure 3.6 (identity at the nucleotide level) and figure 3.7 (identity at the amino acid level). The nucleotide data in figure 3.6 demonstrates that the envA fragments all differ more from the published EV1 sequence than from each other. A similar but more pronounced pattern is apparent for envC. For envB however the fragments are all more similar to each other and to EV1 than they are to VB6. Similar relationships can be observed at the amino acid level (figure 3.7).

Predicted secondary motifs, including hydrophilicity, beta sheets and N-linked glycosylation sites are shown in figure 3.8a-c for the first clone in each group to be generated and analysed, that is A46, BX10 and C45. There was no major difference in the plots for the variants (data not shown). These were prepared using the Peptidestructure program on GCG version 7.0.

3.4 Discussion

In this chapter 15 clones were generated from PCR amplification of viral Hirt DNA and sequenced to obtain information on the general variability of the gp135 region of MVV strain EV1.

In the published sequence of strain EV1 (Sargan et al., 1991) almost the entire gp135 region was cloned and sequenced following the same basic protocol as in this chapter; the env gene regions were obtained by PCR from viral DNA. Although there are drawbacks to this method it is appropriate that the same strategy was used in these comparisons.

In order to interpret the data it is necessary to obtain an estimate of the fidelity of the initial PCR

Figure 3.6

**Sequence identity at the nucleotide level between the
MVV strain EV1 gp135 variants**

The percentage identity for each sequence pair was calculated by the Bestfit local homology algorithm of Smith & Waterman (1981).

The published EV1 sequence is included (Sargan et al., 1991)

% IDENTITY

<u>envA</u>	<u>EV1</u>	<u>A46</u>	<u>ADP1</u>	<u>AX11</u>	<u>DA3</u>	<u>VA1</u>
<u>A46</u>	97.094		99.785	99.03	99.461	99.138
<u>ADP1</u>	96.986			98.922	99.353	99.246
<u>AX11</u>	97.094				98.385	98.278
<u>DA3</u>	97.847					99.246
<u>VA1</u>	97.524					

<u>envB</u>	<u>EV1</u>	<u>BX10</u>	<u>BDP1</u>	<u>BX11</u>	<u>DB2</u>	<u>VB6</u>
<u>BX10</u>	98.689		99.891	99.672	99.672	97.596
<u>BDP1</u>	98.579			99.563	99.563	97.486
<u>BX11</u>	98.689				99.563	97.486
<u>DB2</u>	98.579					97.705
<u>VB6</u>	97.596					

<u>envC</u>	<u>EV1</u>	<u>C45</u>	<u>CDP1</u>	<u>CX7</u>	<u>DC1</u>	<u>VC1</u>
<u>C45</u>	93.182		100	99.244	99.811	99.244
<u>CDP1</u>	93.182			99.244	99.811	99.244
<u>CX7</u>	92.803				99.433	98.488
<u>DC1</u>	93.371					99.055
<u>VC1</u>	93.561					

Figure 3.7

**Sequence identity at the amino acid level between the
MVV strain EV1 gp135 variants**

The percentage identity for each predicted amino acid sequence pair was calculated by the Bestfit local homology algorithm of Smith & Waterman (1981).

The published EV1 sequence is included (Sargan et al., 1991)

% IDENTITY

<u>EnvA</u>	<u>EV1</u>	<u>A46</u>	<u>ADP1</u>	<u>AX11</u>	<u>DA3</u>	<u>VA1</u>
<u>A46</u>	97.411		99.676	97.735	97.735	96.764
<u>ADP1</u>	97.087			97.411	97.411	97.087
<u>AX11</u>	97.087				98.058	97.087
<u>DA3</u>	97.411					99.029
<u>VA1</u>	96.44					

<u>EnvB</u>	<u>EV1</u>	<u>BX10</u>	<u>BDP1</u>	<u>BX11</u>	<u>DB2</u>	<u>VB6</u>
<u>BX10</u>	98.361		99.672	99.672	99.344	97.049
<u>BDP1</u>	98.033			99.344	99.016	96.721
<u>BX11</u>	98.361				99.672	97.377
<u>DB2</u>	98.361					97.049
<u>VB6</u>	97.377					

<u>EnvC</u>	<u>EV1</u>	<u>C45</u>	<u>CDP1</u>	<u>CX7</u>	<u>DC1</u>	<u>VC1</u>
<u>C45</u>	93.182		100	99.432	99.432	99.432
<u>CDP1</u>	93.182			99.432	99.432	99.432
<u>CX7</u>	93.75				100	98.864
<u>DC1</u>	93.75					98.864
<u>VC1</u>	93.75					

Figure 3.8a

Predicted structural motifs for EnvA

The predicted structural motifs for envA variant A46 were obtained using the Peptidestructure program of GCG version 7.0.

PLOTSTRUCTURE of: a46.p2s October 19, 1992 16: 46

PEPTIDESTRUCTURE of: a46.pep Ck: 2503, 1 to: 312

EXTRACTPEPTIDE of frames: A from: a46.map

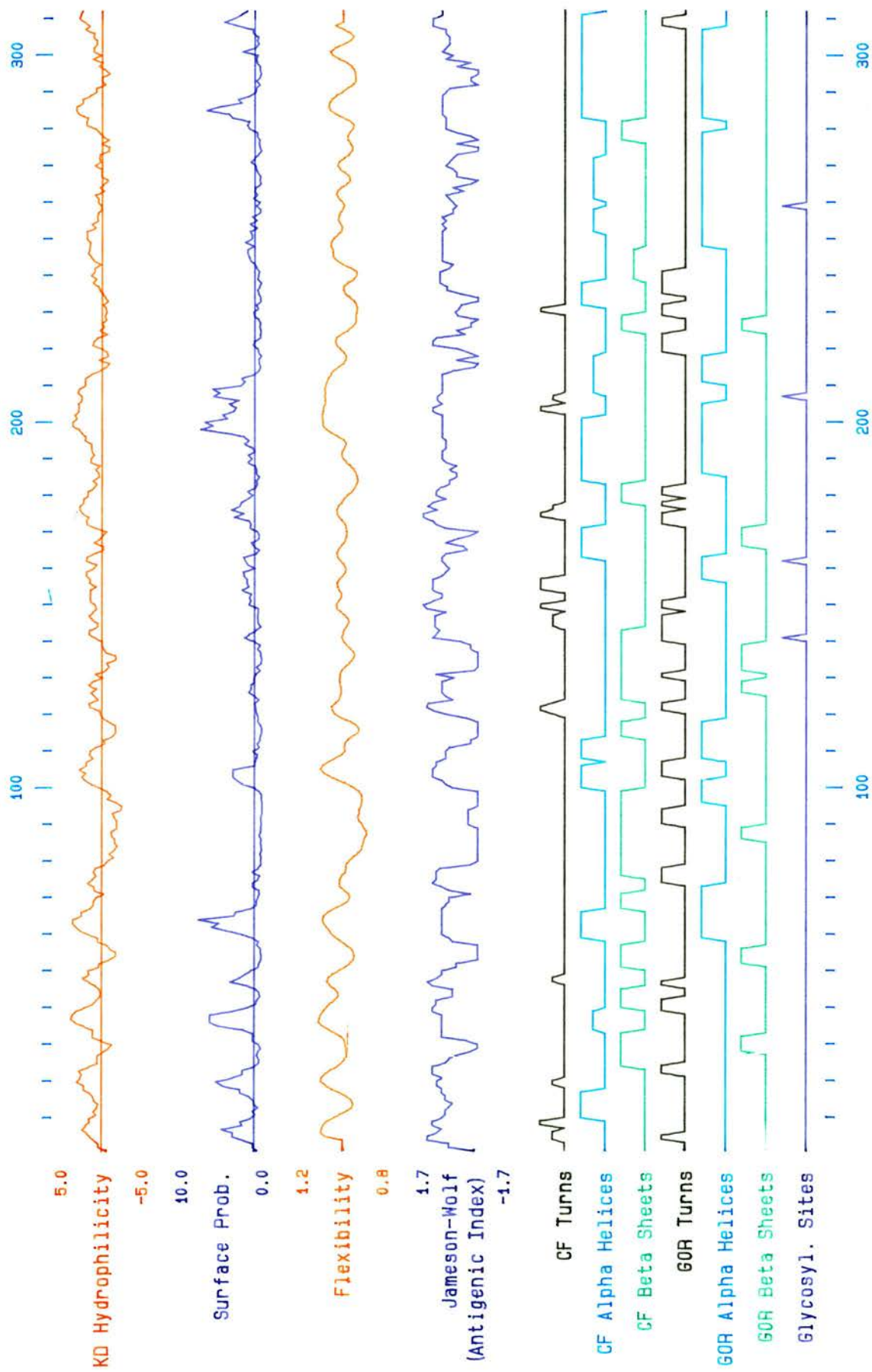


Figure 3.8b

Predicted structural motifs for EnvB

The predicted structural motifs for envB variant BX10 were obtained using the Peptidestructure program of GCG version 7.0.

PLOTSTRUCTURE of: bx10.p2s October 25, 1992 11:20

PEPTIDESTRUCTURE of: bx10.pep Ck: 5923, 1 to: 305

EXTRACTPEPTIDE of frames: A from: bx10.map

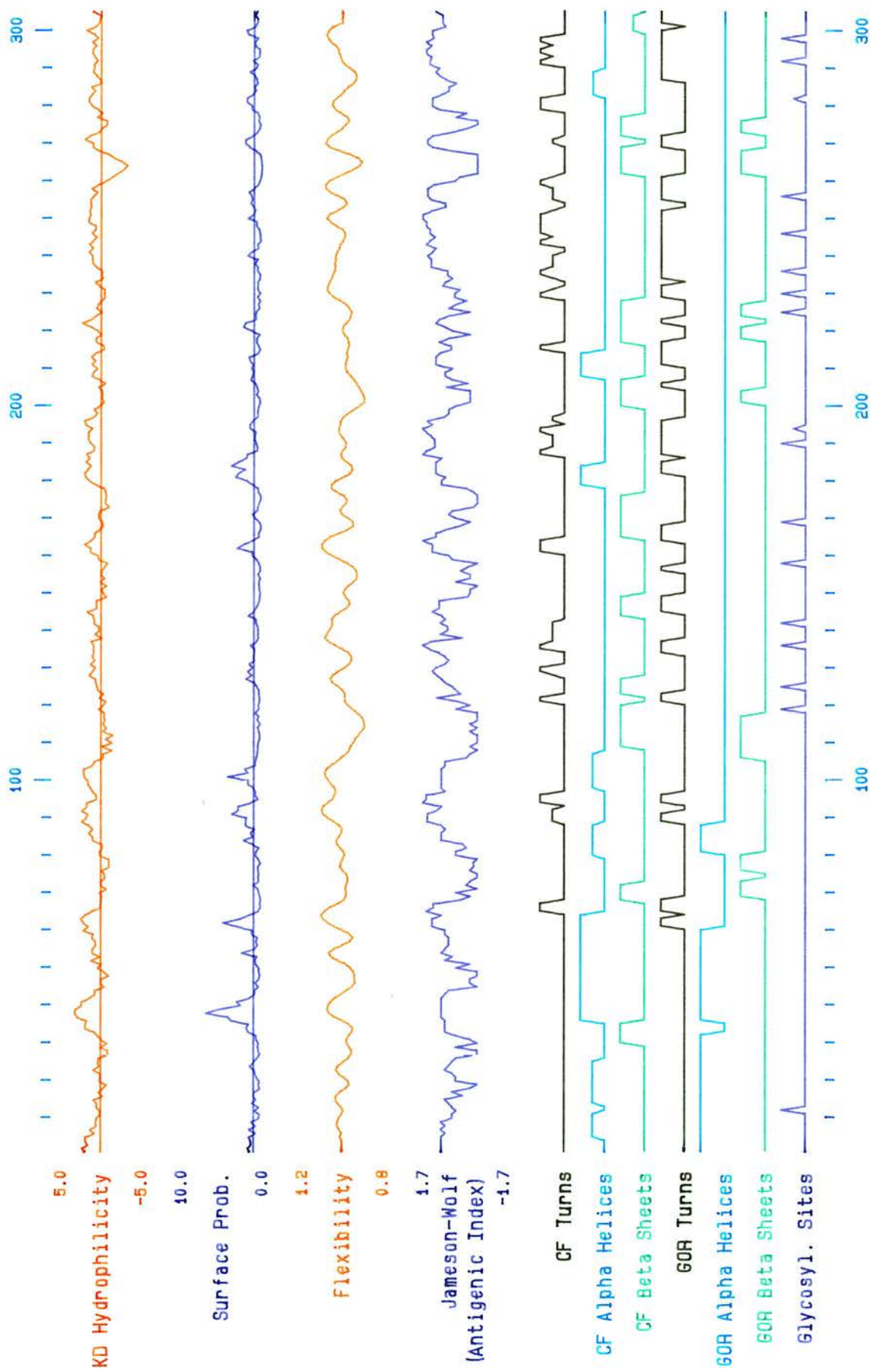


Figure 3.8c

Predicted structural motifs for EnvC

The predicted structural motifs for envC variant C45 were obtained using the Peptidestructure program of GCG version 7.0.

step. DNA from the clones A46, BX10 and C45 was used as template for the PCR under identical conditions to those used for generating the initial clones. By comparing the sequence of the "template" fragment, and the fragment cloned after the subsequent PCR it is possible to estimate the fidelity of the PCR.

As shown in figure 3.4a-c A46 and ADP1 differ at 2 out of 936 bases. BX10 and BDP1 differ at 1 out of 917 bases and C45 and CDP1 are identical, over 537 bases. In total therefore the error rate of the PCR in these experiments is $3/2390$, which is equivalent to approximately 1 in 800, or 0.126%. Any variations between cloned fragments of 0.126% or less may therefore simply reflect inaccuracies in the initial PCR step. This error rate is higher than that previously reported for the PCR of 1 in 1500 (Perrin & Gilliland, 1990) to approximately 1 in 3000 (Meyerhans *et al.*, 1989) and may be a consequence of the template structure or the precise PCR conditions used. The disparity demonstrates the importance of determining the error rate for the system in use, rather than relying upon published data from other models.

In the following discussion of the differences observed for the fragments obtained following amplification of various viral DNA templates, the data for the "duplicate" clones, that is ADP1, BDP1 and CDP1 will generally be excluded as these fragments do not reflect variations in the viral templates.

There are no single base deletions or insertions in any of the fragments and a consistent reading frame is maintained throughout. None of the fragments sequenced contain premature stop codons.

There are no deletions or insertions in any of the envA fragments. A 3 base pair deletion is found in every envB fragment, when compared with the EV1 sequence (EV1 positions 7558-7560). This occurs in the region of envB which overlaps the region contained in the envC fragments,

and the same deletion is also found in all the envC fragments sequenced. This deletion results in the loss of an isoleucine residue (EV1 position 523) in the encoded protein.

A six base pair deletion is present in all the envC fragments when compared with the EV1 sequence (EV1 positions 7736-7741). This deletion results in the loss of an arginine residue and a glycine residue in the encoded protein (EV1 positions 585 and 586). In HIV small deletions are commonly observed between the env genes of different isolates, but are uncommon in the other structural genes (Starcich *et al.*, 1986)

The codons for the 24 cysteine residues in the gp135 region of the env gene of EV1 have been retained in every fragment sequenced. In every envA fragment an additional cysteine residue is encoded at EV1 amino acid position 268. This lies within the region which overlaps with envB and the same mutation is found in all the envB fragments.

The 4 potential N-linked glycosylation sites in the envA region of EV1 have been maintained in all the envA fragments sequenced. There are 17 potential N-linked sites in envB of EV1, and these have been conserved in all the envB fragments sequenced. Fragment VB6 contains an additional site at residue 299 of the EV1 sequence.

There are 8 potential N-linked glycosylation sites in the envC region of EV1. 2 of these have been lost in all the envC fragments sequenced (residues 554 and 564 of the EV1 sequence). At EV1 residue 573 a threonine to serine amino acid change results in the maintenance of a potential glycosylation site, despite the change in amino acid sequence.

This data therefore suggests that in the env fragments analysed in this work changes have been relatively minimal, and generally would be expected to have little effect on the potential for disulphide bonding or N-linked glycosylation. They are unlikely to have a

major effect on the overall three-dimensional structure of the protein. This is consistent with the high degree of conservation of cysteine residues and glycosylation sites previously observed between different MVV isolates (Querast et al., 1990).

In many of the sequences in figure 3.4 it can be shown that at many of the residues identified as variable between the different fragments the variability is limited (the "duplicate" fragments are excluded from this analysis). In envA 3 different nucleotides are found between the 5 sequences (that is, including EV1) at only 3 positions. In 27 positions 4 of the 5 fragments all encode the same base, and in 8 positions 2 bases are used, more than once each between the 5 fragments. A similar pattern is present for envB where at only 1 position are 3 different bases used by the 5 fragments. In 20 positions 4 of the 5 fragments all use the same base, and in 7 positions 2 bases are used, more than once each between the 5 fragments. The most extreme example of this pattern of nucleotide usage is provided by envC where in 32 positions only 1 fragment out of 5 is variable. This is predominantly the EV1 sequence which varies, leading to the nucleotide homology pattern seen in figure 3.6 where the fragments cloned in this chapter all resemble each other more than they resemble EV1.

The distribution of nucleotide changes in the env gene fragments does not appear to be entirely random. In envA there is a cluster of 14 nucleotide changes between residues 6249 to 6263. The remainder of the nucleotide changes occur singly, or at most as a pair and appear to be relatively randomly distributed. The envB fragments also show clustering of certain nucleotide changes, there are 5 nucleotide changes for example between residues 6959 to 6963, and there also appears to be non-random distribution of other changes. Over the 50 residues 6885 to 6934 for example there are 12 nucleotide changes, but

over the 380 residues from 6965 to 7344 no changes were detected. In envC 31 nucleotide changes (excluding deletions) were found between residues 7658 to 7763 (106 residues), while the remaining few changes were relatively randomly distributed.

In envA and envB approximately 40% to 50% of the nucleotide changes which were not tightly clustered result in amino acid alterations. In envC the majority of nucleotide changes are relatively clustered and too few unclustered changes are present to determine reliably the percentage alteration in amino acid sequence. Clustering of point mutations has been reported in the env genes of HIV isolates, but not in the other structural genes (Starcich *et al.*, 1986).

In the comparison of the sequential MVV isolates 1514 and LV1-1 (Braun *et al.*, 1987) it was reported that transitions outnumbered transversions by nearly 6 to 1. In the data reported in this chapter such a pattern has not been found. For positions which use 1 of 2 nucleotides it can be seen that in envA there are 16 transitions and 18 transversions, in envB there are 15 and 12 respectively and in envC there are 14 transitions and 23 transversions. There therefore appears to be no bias amongst the EV1 variants sequenced towards transitions, and in envC it is possible that the opposite may be occurring.

At least 50% of the predicted amino acid substitutions result in no alteration in charge. In the envA encoded sequences only 4 out of 17 substitutions show a change between positively charged and uncharged amino acids, and in one case there is a substitution of a negatively charged residue for an uncharged residue. In envB encoded sequences 7 out of 14 residues remain uncharged, 4 vary between uncharged and positively charged and 3 between uncharged and negatively charged. In envC encoded sequences 10 out of 20 substitutions remain

uncharged, 8 result in a change between uncharged and positively charged residues and 2 vary between negatively charged and uncharged. This pattern of conservative substitutions suggests constraints on the amino acid substitutions tolerated by gp135.

The major sites of amino acid variability of each fragment when compared with EV1 are shown in figure 3.9a-e. These were generated using the Compare program on GCG version 7.0, with windows and stringencies of 10 in all cases. The plots for A46, DA3 and VA1 were identical, as were the plots for BX10, BX11 and DB2, and all the EnvC plots. Representative examples are presented. The identified regions can be compared with the predicted secondary structure graphs (figure 3.8a-c) to determine if they lie within predicted regions of high antigenicity, and if they are likely to adopt a surface configuration.

The major site of variation in the EnvA isolates lies between amino acids 80 to 90 (figure 3.9a,b). This is not predicted to be highly immunogenic nor to be on the surface of the protein.

The major consistent site of amino acid variation in the EnvB isolates lies between amino acids 518 to 528 (of the EV1 sequence) (figure 3.9c,d). This region is not predicted to be highly immunogenic nor to be on the surface of the protein. The additional site of high diversity identified in VB6 (figure 3.9d) lies between amino acids 298 to 308 (of the EV1 sequence) in a region of predicted high antigenicity.

The major sites of amino acid variation in the EnvC isolates (figure 3.9e) lie between amino acids 521 to 531 and between amino acids 571 to 591. The first is the same as the major consistent site of variation in EnvB. The second is a predominantly surface structure which is predicted to be antigenic.

All the major sites of variability between the different EV1 isolates identified in this chapter coincide

Figure 3.9a

Major regions of variability within gp135 of MVV strain EV1

The predicted amino acid sequence of A46 (x-axis) was compared with the published EV1 sequence (Sargan et al., 1991) (y-axis) using the Compare program of GCG version 7.0. Sequences were compared over a 10 amino acid window, at a stringency of 10, and regions of extensive divergence are shown by gaps in the plotted line.

DOTPLOT of: a46.pnt Density: 356.82 October 25, 1992 11:39

COMPARE Window: 10 Stringency: 10.0 Points: 297

Viprov.Pep ck: 3,946, 1 to 312

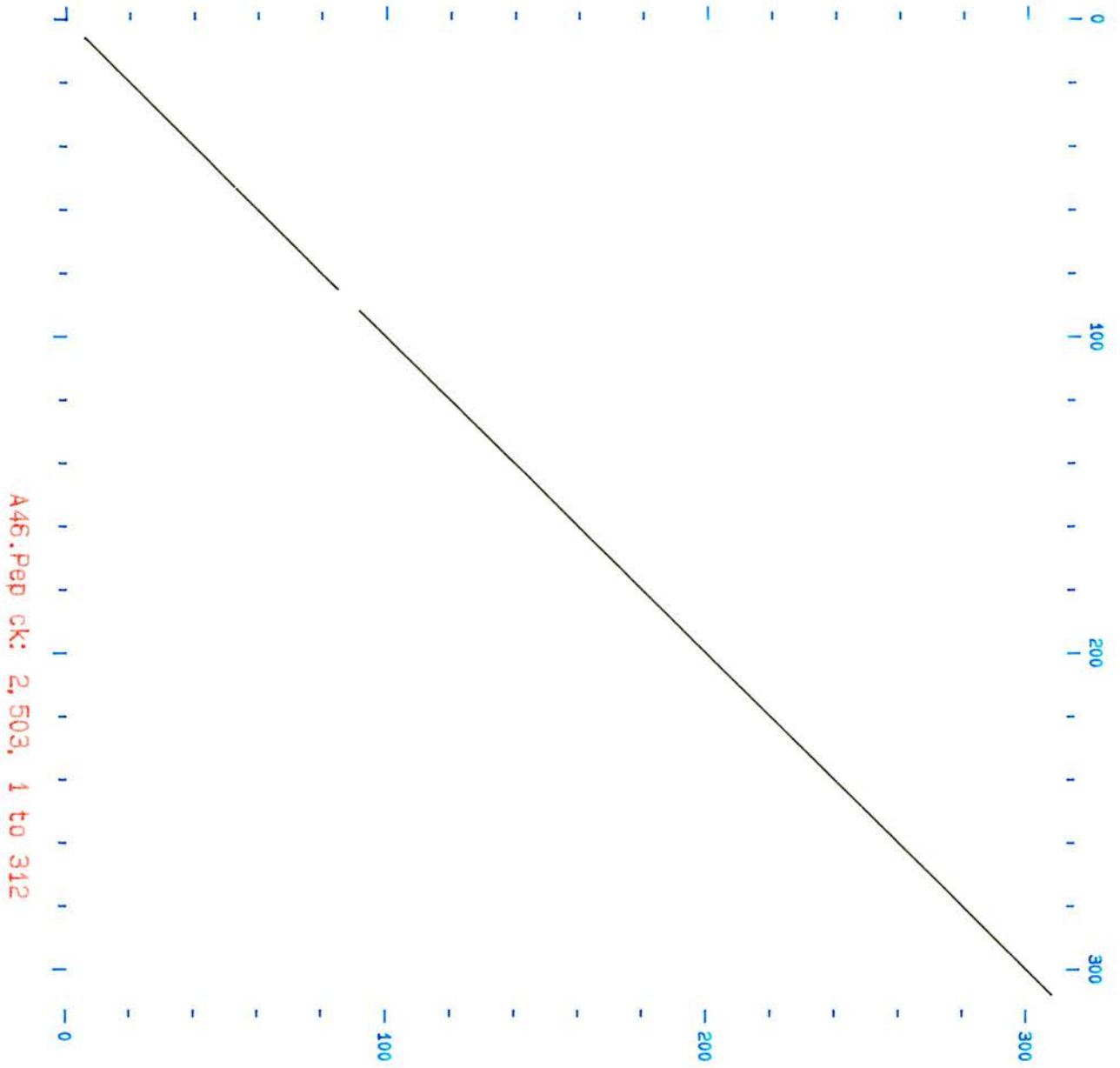


Figure 3.9b

Major regions of variability within gp135 of MVV strain EV1

The predicted amino acid sequence of AX11 (x-axis) was compared with the published EV1 sequence (Sargan et al., 1991) (y-axis) using the Compare program of GCG version 7.0. Sequences were compared over a 10 amino acid window, at a stringency of 10, and regions of extensive divergence are shown by gaps in the plotted line.

DOTPLOT of: ax11.pnt Density: 356.82 October 25, 1992 11:40

COMPARE Window: 10 Stringency: 10.0 Points: 302

Viprov.Pep ck: 3,946, 1 to 312

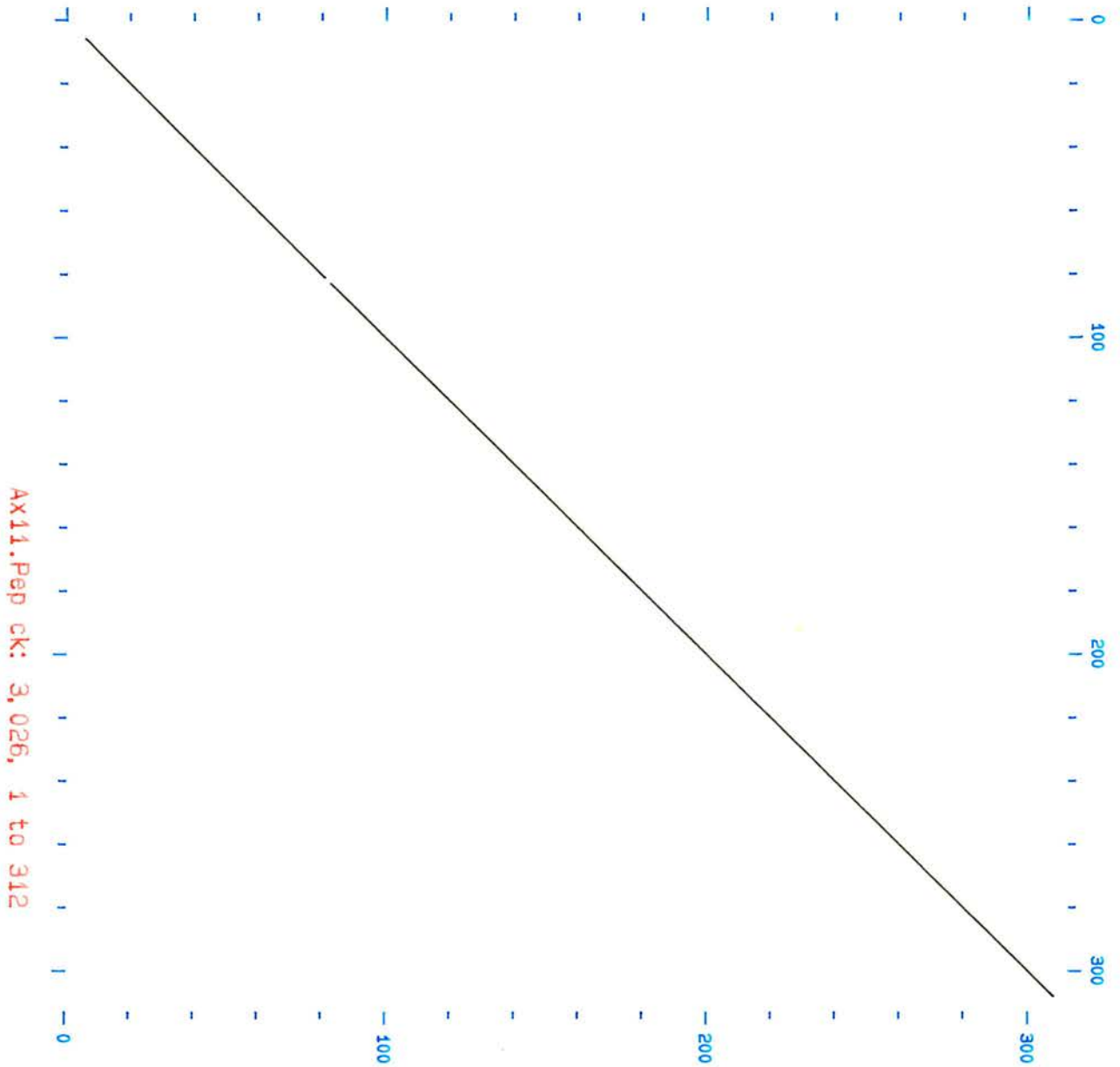


Figure 3.9c

Major regions of variability within gp135 of MVV strain EV1

The predicted amino acid sequence of BX10 (x-axis) was compared with the published EV1 sequence (Sargan et al., 1991) (y-axis) using the Compare program of GCG version 7.0. Sequences were compared over a 10 amino acid window, at a stringency of 10, and regions of extensive divergence are shown by gaps in the plotted line.

POTPLOT of: bx10.pnt Density: 350.00 October 25, 1992 11:41

COMPARE Window: 10 Stringency: 10.0 Points: 292

V1prov.Pep ck: 7,298, 1 to 306

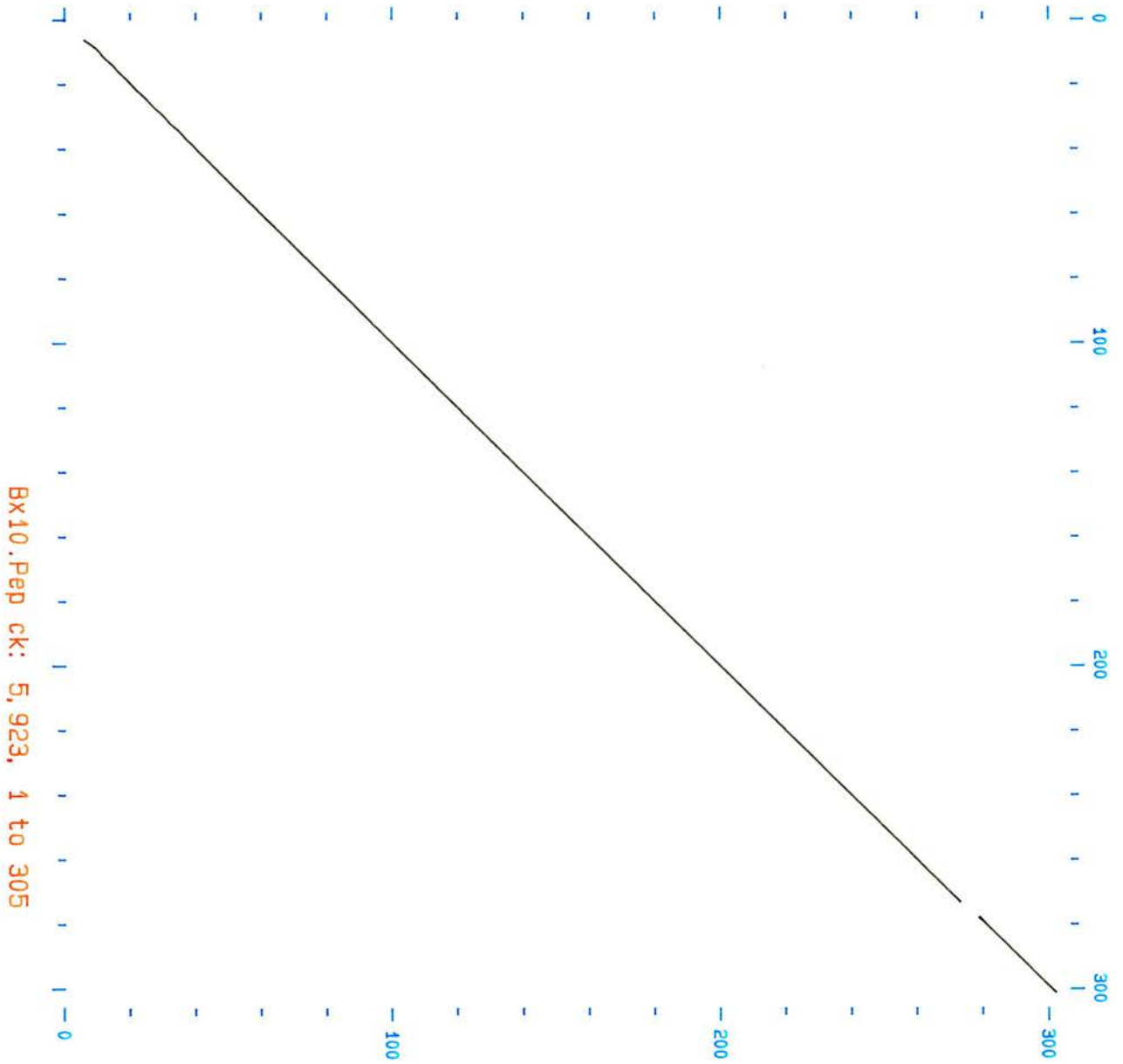


Figure 3.9d

Major regions of variability within gp135 of MVV strain EV1

The predicted amino acid sequence of VB6 (x-axis) was compared with the published EV1 sequence (Sargan et al., 1991) (y-axis) using the Compare program of GCG version 7.0. Sequences were compared over a 10 amino acid window, at a stringency of 10, and regions of extensive divergence are shown by gaps in the plotted line.

POTPLOT of: vb6.pnt Density: 350.00 October 25, 1992 11:42

COMPARE Window: 10 Stringency: 10.0 Points: 289

V1prov.Pep ck: 7,298, 1 to 306

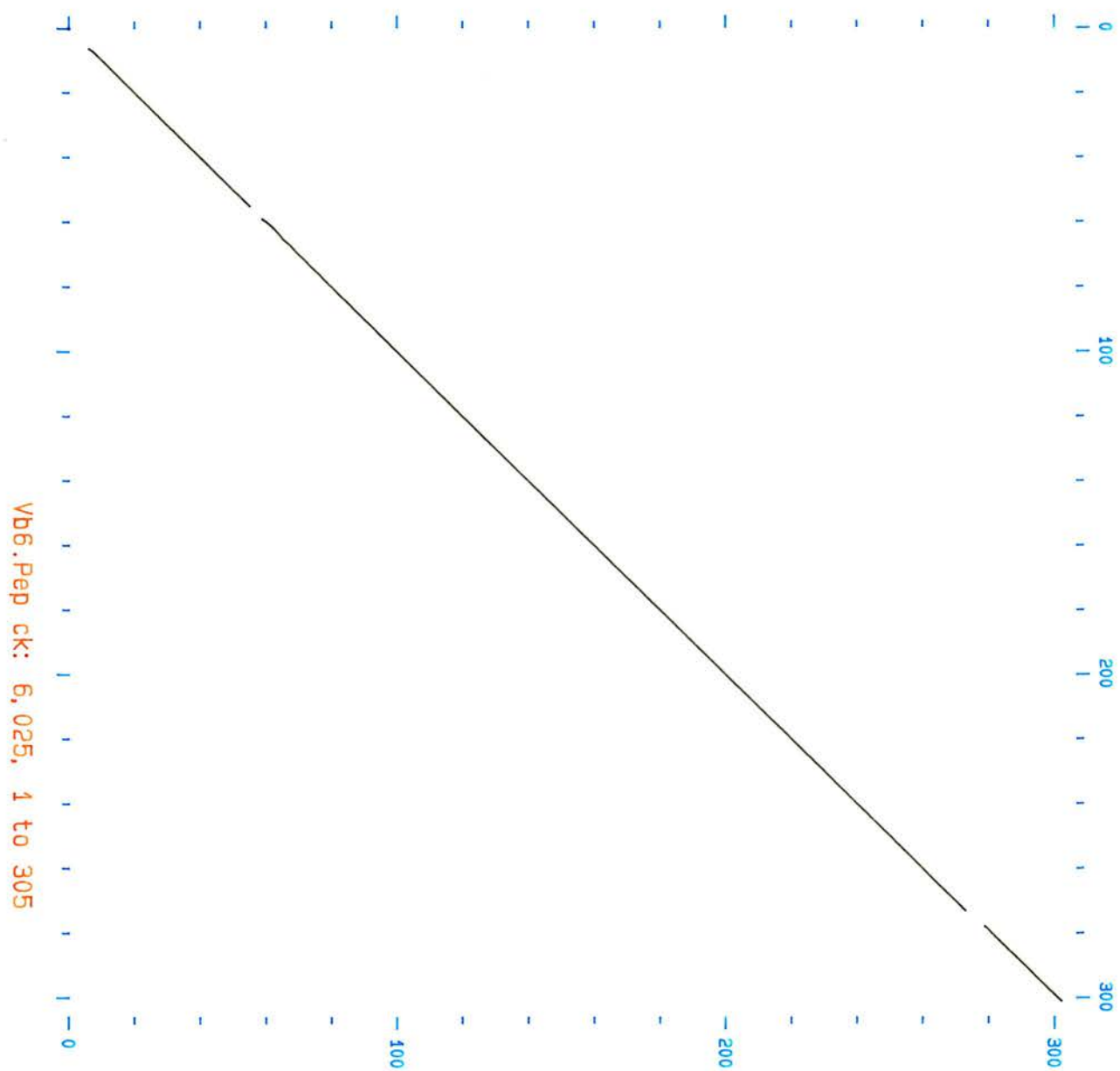


Figure 3.9e

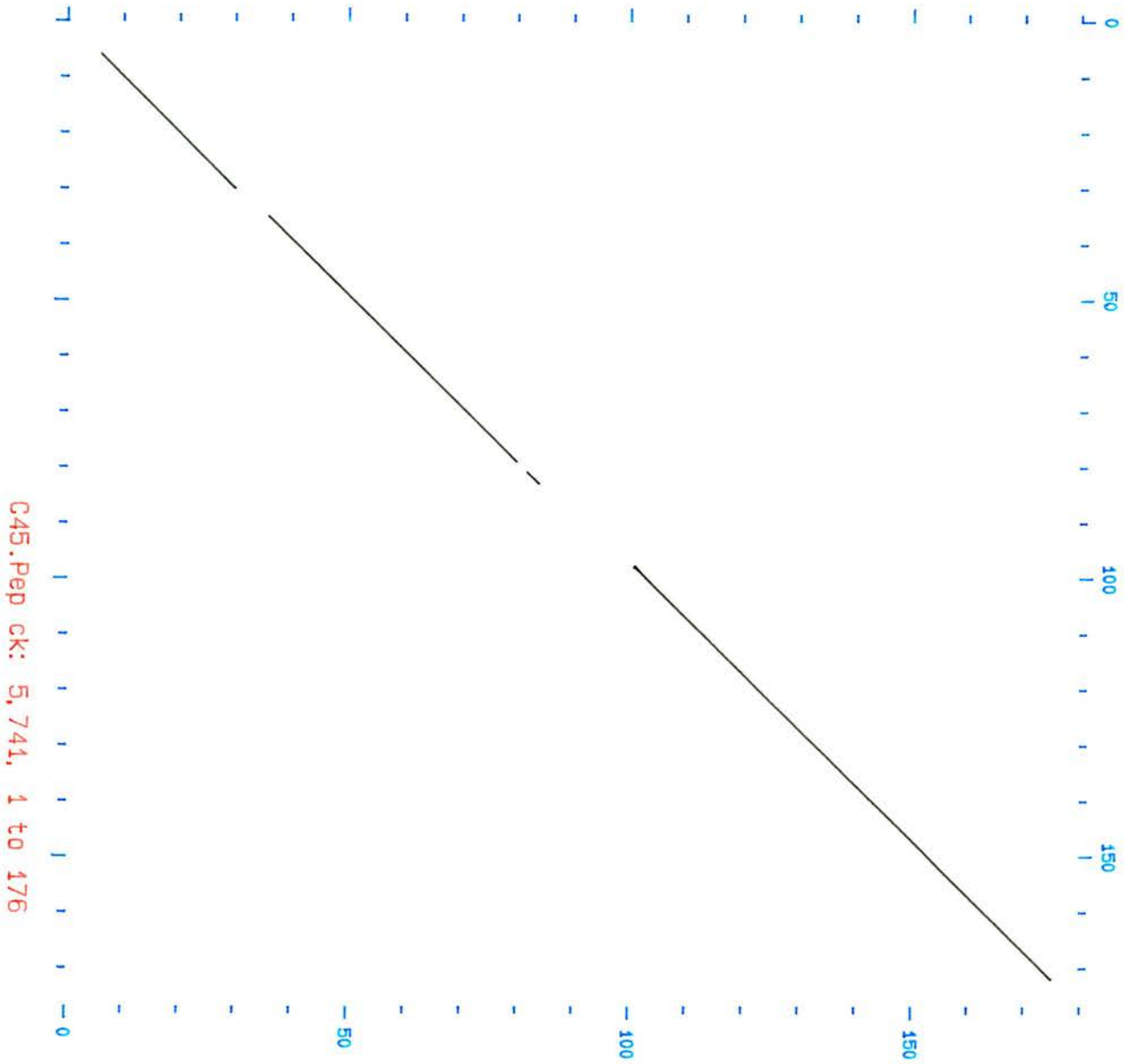
Major regions of variability within gp135 of MVV strain EV1

The predicted amino acid sequence of C45 (x-axis) was compared with the published EV1 sequence (Sargan et al., 1991) (y-axis) using the Compare program of GCG version 7.0. Sequences were compared over a 10 amino acid window, at a stringency of 10, and regions of extensive divergence are shown by gaps in the plotted line.

DOTPLOT of: c45.pnt Density: 205.68 October 25, 1992 11:43

COMPARE Window: 10 Stringency: 10.0 Points: 148

V1prov.Pep ck: 5,354, 1 to 179



with the regions of variability identified between strains by Sargan et al. (1991). An additional variable region at approximately amino acid 400 (Sargan et al., 1991) was not detected in this work. The second major site of variation in the EnvC isolates (amino acids 571-591) was also identified as highly variable by Querat et al. (1990).

This work therefore confirms and extends the previous published data, as it shows that the majority of the regions of gp135 which are variable between geographically isolated strains of MVV are also variable between isolates from a single (but non-clonal) strain. It is not possible to determine if the changes observed have arisen in an antibody-dependent or independent manner. Selection of antigenic variants may have occurred in vivo prior to the isolation of the EV1 viral strain. It appears possible that there may be conserved and variable domains of MVV gp135, analogous to those reported for HIV gp120 (Starcich et al., 1986) although these may not be restricted to regions of high predicted antigenicity.

The majority of the sequenced fragments usually show more than 98% to 99% sequence identity to each other, which may suggest that there is relatively little variation between the env genes of different isolates of the MVV EV1 strain. However it is possible that this under-represents the range of env variants which exist under in vitro conditions. If the initial binding of primers to template in the PCR is a random event then it is likely that the more copies of a particular template that are present in a reaction, the more likely it is that the primers will amplify this template preferentially. However, by chance it is possible that occasionally it will be a template present at a lower frequency which is amplified and cloned (the differences between for example A46 and AX11, which are cloned products of the same PCR, ligation and transformation show that more than one species is amplified by the methodology of these

experiments).

It is possible that the envC sequence published for EV1 (Sargan et al., 1991) represents one of these lower frequency templates, as all the fragments generated and cloned in these results for envC markedly resemble each other more than they resemble EV1.

A similar situation may also have occurred for VB6, the envB fragment which shows the most variability from the other envB regions studied. This is particularly marked when the VB6 envB region which overlaps with the VA1 envA region is analysed. VB6 and VA1 were generated from the same Hirt DNA template. The 3 nucleotide changes (from the published EV1 sequence) found in VA1 in this region are also found in VB6. However VB6 also contains an additional 14 nucleotide changes from the EV1 sequence which are not found in VA1. Minor differences in the "overlaps" between many of the clones generated from one template are present, suggesting a multiplicity of possible env sequences in vitro.

Although certain of the fragments were described as generated from Hirt DNA from in vitro low passage and high passage EV1 it is not possible to determine that any of the nucleotide variations observed are actually a consequence of in vitro culture. The amino acid similarity data may suggest that the observed changes do not necessarily represent a consequence of extensive in vitro passage. A46 and AX11 for example show a percentage similarity of 97.735% whereas DA3 and VA1 show a percentage similarity of 99.029%. This suggests there may be as much variability within a viral stock as there is between stocks. To investigate thoroughly the effect of repeated in vitro passage would require the generation of a replication-competent full-length molecular clone of EV1 which is not currently available.

It cannot be shown that the env sequences analysed in this chapter actually represent "viable" forms of the env

gene which are utilised by the virus. It is possible that they represent aberrant forms, generated as a consequence of the error rate of the viral reverse transcriptase. As there are no stop codons in any of the sequences, and the overall structure has probably been maintained in each encoded molecule it is likely that if the gp135 encoded from these genes was non-functional this would be a consequence of relatively subtle destruction or distortion of important regions. To investigate this fully a replication-competent molecular clone would be required, which could be altered to the env sequences analysed in this chapter either by site-specific mutagenesis, or by the construction of chimeric molecules.

Although all sequences analysed in this chapter were initially compared with the published EV1 sequence of Sargan et al., (1991) this is an arbitrary decision, as the EV1 sequence was also derived from PCR from Hirt DNA. With the exception of the replication-competent molecular clones which were variants of 1514 (Staskus et al., 1991) no full-length functional clones of MVV have been sequenced. There are therefore currently still difficulties in determining if changes observed in vitro relate to phenotypic alterations observed in vivo, or if relevant sequences are actually being detected. This is a problem which has been encountered in much of the work in the lentiviral field.

It has been shown for example that for HIV tat there are major differences between the quasispecies identified in vitro and in vivo (Meyerhans et al., 1989) and that viral isolation by in vitro culture can bias the detection of certain molecular species over others. To minimise such effects other workers have used direct PCR amplification and sequencing of patient-derived HIV sequences (Balfe et al., 1990) but in this case it is difficult to ensure that all the relevant sequences have been amplified, and to determine if changes observed over

time represent true evolution of a new sequence or outgrowth of a previously sequestered variant.

The sequencing of more variants of the EV1 strain of MVV than had previously been reported will allow selection of certain regions for further investigation. These include the identification of highly conserved regions, and regions where there is relatively poor homology between variants. By comparing these regions with the predicted structure of the final protein it may be possible to select protein sequences which may be predicted to have important structural roles or may be an appropriate site for targeting immune responses. This will be discussed further in chapter 7.

3.5 Summary

This chapter reports the comparison of env gene sequences (restricted to the gp135 region) from MVV strain EV1. Homology between different isolates for a selected region of gp135 ranged from 93.182% to 99.811% and regions of high and low variability were distinguished. This data provides basic background information on the in vitro variability of EV1 env which was not previously available, and which may be used for more closely targeted analysis of env gene variants in the future.

4. The Generation And Characterisation Of Recombinant MVV gp135 Fragments - The Yeast Ty-VLP System

4.1 Introduction

Investigations of the structure and function of the env gene product of MVV have been hampered by the paucity of high-quality reagents of known provenance. Previous work with purified MVV gp135 has relied upon lectin-based affinity chromatography of viral preparations (Stanley et al., 1987) but large-scale culture of MVV-infected cells is necessary to obtain useful yields. Not only is this an expensive procedure it is also unsatisfactory in terms of reproducibility, for even if the initial infecting virus stock is clonal viral replication will almost certainly generate variants. This is due in part to the relatively high error rate of retroviral reverse transcriptases, and it has been shown that the env gene in particular is prone to accumulation of mutations, some of which may affect antigenic epitopes, neutralisation epitopes and various structural features of the glycoprotein (sections 1.15 and 1.16). Therefore in order to obtain high yields of envelope glycoprotein of known amino acid sequence it was decided to produce recombinant protein.

The data presented in this chapter describes the generation of fragments spanning the entire gp135 glycoprotein and is the first report of expression of recombinant MVV gp135 polypeptides.

The yeast Ty-VLP expression system was initially selected as the method for producing the recombinant protein as it has previously been reported to produce high yields of recombinant proteins in an easily purifiable, immunologically relevant form.

The Ty-VLP expression system is a derivative of the Ty1 retrotransposon element of yeast. Most Ty elements are approximately 5.9kb in size and contain 5' and 3' LTR-related sequences and two orfs, TYA and TYB. The TYA gene

is an analogue of retroviral gag genes and the TYB gene is an analogue of the retroviral pol genes, encoding a reverse transcriptase, an integrase and a protease. The TYA and TYB genes are expressed as a fusion protein via a frame-shifting event which is probably analogous to the gag/pol frame-shifting observed in retroviruses (Mellor et al., 1985b). The proteins encoded by these orfs can self-assemble into virus-like particles (VLPs) containing reverse transcriptase activity and Ty RNA (Mellor et al., 1985b) but which lack any equivalent of the retroviral envelope protein. The majority of the structural core proteins of the Ty-VLPs are generated by proteolytic cleavage of the primary protein product of the TYA orf, p1 (Adams et al., 1987a).

If over-expressed a truncated form of the p1 protein has been shown to self-assemble into VLPs containing approximately 300 p1 units in the absence of other Ty-encoded proteins (Adams et al., 1987a) and expression vectors have been developed which permit the production of fusion proteins expressed by read-through from the TYA gene into the inserted gene sequence. Hybrid VLPs are formed by the fusion proteins and their particulate nature allows them to be separated from other yeast proteins.

Proteins which have been expressed in this system include alpha₂-interferon (Mellor et al., 1985a), fragments of HIV-1 gp120 (Adams et al., 1987b; Griffiths et al., 1991), HIV-1 p24 (Gilmour et al., 1989), MVV p30 (Reyburn et al., 1992a), HIV-1 Nef (Gilmour et al., 1990), fragments of HIV-1 gp41 and HIV-2 gp36 (Gilmour et al., 1990) and HIV-1 Tat (Braddock et al., 1989). The fusion proteins are immunogenic (Adams et al., 1987b; Griffiths et al., 1991) and the Tat protein and alpha₂-interferon expressed in this system were shown to be functionally active.

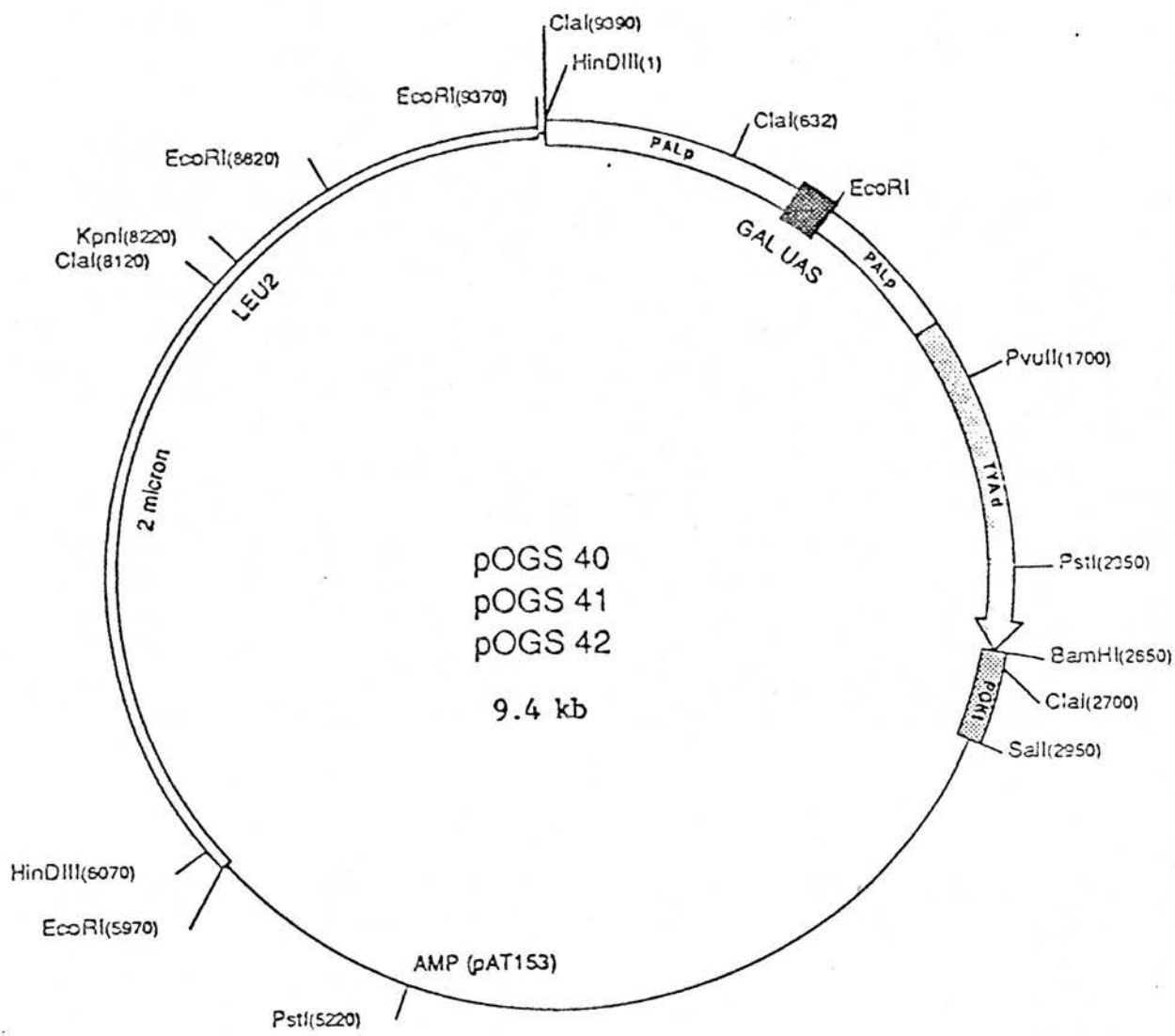
The maximum size of polypeptide which has been fused to the p1 gene without significantly disrupting formation

Figure 4.1

The pOGS expression vectors

The 3 pOGS expression vectors are shown. The major restriction enzyme sites are marked and the diagram also shows the hybrid promoter element and the TYAd gene which encodes the truncated p1 protein.

The cloning site of each vector is shown below the diagram, and the BamHI restriction site is underlined.



pOGS40 : p1---AAA GCC GGA TCC AAA TAA---

pOGS41 : p1---AAA GCC GGA TCA AGG ATC CGA TCC GGG AAA TAA

POGS42 : P1---AAA GCC GGA TCG GAT CCG ATC CGG GAA ATA A

of the Ty-VLP is 43kDa (Burns et al., 1992). As this is significantly smaller than the predicted pre-glycosylation size for gp135 of approximately 77.4kDa (derived from Sargan et al., 1991) the polymerase chain reaction was used to generate overlapping fragments of the gp135 coding region. These gene fragments were predicted to encode proteins of molecular weights of 37.1kDa (EnvA), 34.2kDa (EnvB) and 20.1kDa (EnvC) all of which therefore lie within the size range of fusion proteins which have been successfully expressed in the Ty-VLP system. In order to preserve possible domain structures which could be functionally significant in native gp135, overlaps of 63 or 64 amino acids were introduced between the three fragments (chapter 3, figure 3.1).

Expression vectors with either constitutive or inducible promoters are available for the Ty-VLP expression system. It was not known if the expressed gp135 fusion proteins would be toxic in the transformed cell and therefore it was considered appropriate to use a vector in which fusion protein expression was inducible rather than constitutive. The pOGS family of vectors (Gilmour et al., 1989), (figure 4.1) are high copy number shuttle vectors, that is they can replicate in both E.coli and S.cerevisiae. They are derivatives of the pMA5620 vector (Adams et al., 1987b) in which the constitutive phosphoglycerate kinase (PGK) promoter has been replaced by a hybrid PGK-GAL promoter which is galactose-inducible. In all three pOGS vectors the TYA gene has been truncated at the 3' end to encode the first 381 amino acids of p1 (predicted molecular weight 42kDa). The members of the pOGS family differ only in the reading frame of the BamHI cloning site at codon 381. Stop codons are present in all three reading frames after the BamHI site and the plasmids contain an ampicillin resistance gene, allowing selection in E.coli.

The BJ 2168 strain of S.cerevisiae is phenotypically

leu⁻, ura⁻ and trp⁻ and therefore unable to grow on minimal media in the absence of these amino acids. The strain can be transformed to leucine independence by any pOGS-based plasmid due to the LEU2 gene in the vector. It is also partially protease deficient, a feature which is important in protecting fusion proteins from host cell proteases.

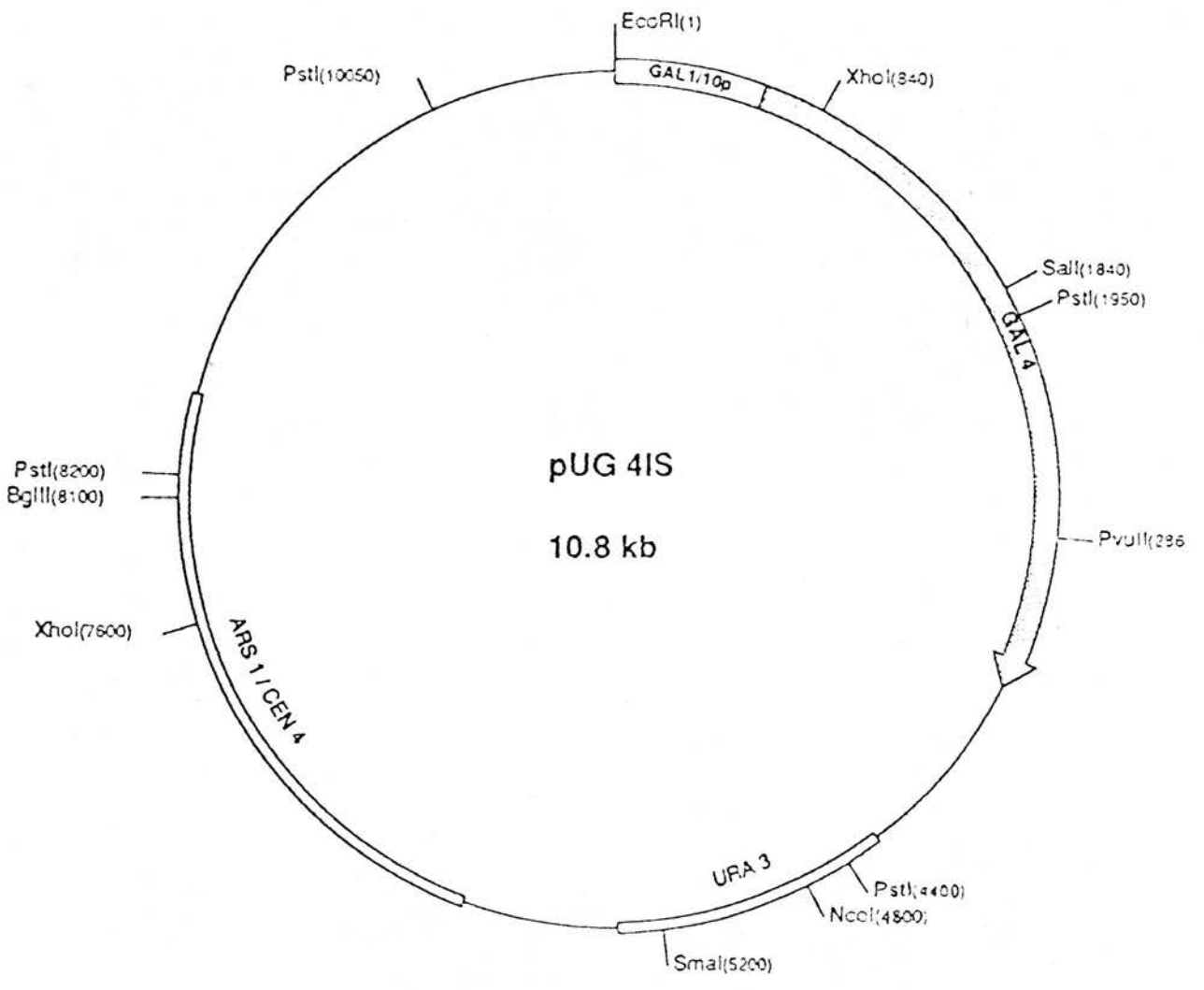
Although the strain contains a mutation in the GAL2 galactose permease gene some galactose can enter the cell by a constitutive pathway, and therefore induce fusion protein expression from the PGK-GAL promoter. Expression from the GAL promoter in yeast is critically dependent on the relative levels of the GAL4 and GAL80 proteins. GAL4 activates expression from the promoter by binding to a specific DNA site and GAL80 antagonises the action of GAL4, either by interacting with the GAL4, the GAL4 binding site on the DNA or both (reviewed in Schultz et al., 1987). As GAL4 levels are often the rate-limiting factor in galactose induction helper plasmids have been developed in which the GAL4 gene has been inserted under the control of the GAL1 or GAL10 promoter (Laughton et al., 1984; Schultz et al., 1987). These preserve the inducibility of the galactose promoter, while increasing the level of expression from it by overcoming the rate-limitation of low GAL4 levels. The low copy number helper plasmid pUG 41S (figure 4.2) can be used with the pOGS plasmids to co-transform S.cerevisiae and so increase the efficiency of induction of the fusion protein. The URA 3 gene of pUG 41S renders the host cell uracil independent allowing selection of double transformants by their uracil and leucine prototrophy.

The env fragments initially generated by the polymerase chain reaction from MVV-infected cell DNA (chapter 3) were inserted into the pOGS expression vectors. After initial selection of positive clones in the JM83 strain of E.coli the expression constructs were

Figure 4.2

The pUG 41S helper plasmid

The pUG 41S helper plasmid which incorporates the GAL4 gene under the control of the GAL1/GAL10 promoter is shown. The major restriction enzyme sites are marked.



co-transformed with pUG 41S into S.cerevisiae and the fusion proteins were expressed and characterised. S.cerevisiae was also co-transformed with pUG 41S and a native pOGS plasmid to obtain a control transformant which expressed the truncated p1 protein alone. The effects of expression of the env gene sequences on the growth of the transformed S.cerevisiae were monitored and compared with the expression of the various fusion proteins at different intervals post-induction. Transformed S.cerevisiae were examined by electron microscopy to investigate any intracellular structural variations in the hybrid-VLPs formed by the different constructs and the fusion proteins were also analysed to determine the extent of glycosylation. Sera from MVV-infected sheep were tested against the fusion proteins to confirm that antigenically relevant polypeptides had been expressed and the fusion proteins were also used as immunogens in rabbits and mice.

4.2 Generation Of Recombinant pOGS/Env Plasmids

The pTZ-env clones A46, BX10 and C45 (see chapter 3) were selected as the env sequences to be expressed in the Ty-VLP system. To maintain the correct reading frame the envA fragment was inserted at the BamHI site of pOGS42 and the envB and envC fragments were separately inserted into the BamHI site of pOGS40.

Initially the fragments were excised from the pTZ plasmids by digestion with BamHI and gel-purified. Insertion of these excised fragments into the appropriate pOGS vector which had been digested with BamHI and treated with CIP was attempted. The ligated DNA was then transformed into competent E.coli JM83. However several attempts using this approach were unsuccessful.

245 colonies were screened for the envA fragment, of which 18 hybridised to an envA-specific probe by colony screening. Gel analysis of the DNA from minipreps showed that the fragment was inserted in pTZ, not pOGS42.

386 colonies were screened for the envB fragment, of which 19 hybridised to the envB-specific probe by colony screening. These envB fragments were also found to be inserted into pTZ.

187 colonies were screened for the envC fragment, of which 6 hybridised to the envC-specific probe by colony screening. The fragments were all found to be inserted in pTZ.

Various stages in the preparation of the env fragments were varied in attempts to exclude the contaminating pTZ DNA. Excised DNA was subjected to two rounds of gel purification, with an additional intervening digestion step with BamHI. The DNA was also digested with PvuI; pTZ19R contains PvuI sites at positions 42 and 1855 but there are no PvuI sites in the env gene fragments. Therefore this procedure should digest within the plasmid sequences lessening the chances of contamination by intact plasmid sequences, while leaving the env sequences intact. However, none of these procedures resulted in the isolation of pOGS plasmids containing the desired fragments.

In order to overcome this problem A46, BX10 and C45 plasmid DNA was used as the template for the polymerase chain reaction, using the original primers. The resulting DNA was digested with BamHI and ligated into the appropriate pOGS plasmid (ligation into pTZ to check the fidelity of the PCR was also performed - chapter 3. The sequences which were therefore used in the ligations into the pOGS vectors are represented by ADP1, BDP1 and CDP1) and E.coli JM83 were transformed.

50 colonies were screened for the envA/pOGS42 ligation by colony screening and 12 hybridised to the envA-specific probe. Gel analysis of plasmid DNA demonstrated that all the positive colonies contained a pOGS plasmid from which a single insert of the appropriate size could be excised with BamHI. The DNA was also

digested with HindIII; pOGS42 contains HindIII sites at positions 1 and 6070 and envA has one HindIII site at position 896. Insertion of the envA coding sequence in the 5' to 3' orientation at the BamHI site at position 2650 would be predicted to yield fragments of 3320, 3460 and 3542 base pairs after digestion with HindIII. Insertion in the opposite orientation would be predicted to yield fragments of 2690, 3320 and 4316 base pairs. By gel analysis nine clones were found to have a HindIII digestion pattern consistent with 5' to 3' insertion and 3 clones to have a pattern consistent with the opposite orientation. Double-stranded DNA sequencing of one of the clones (Ap6) confirmed that the envA fragment had been inserted into the vector in the correct orientation and the correct reading frame. Ap6 was therefore selected as the pOGS42/envA clone to be used to transform S.cerevisiae.

383 colonies were screened after ligation of a PCR envB product into pOGS40, of which 4 hybridised to the envB-specific probe. Restriction digestion of three of these samples resulted in excision of a band of the correct size for envB with BamHI but the plasmid DNA was neither pOGS40 nor pTZ18R/19R (the DNA in these samples was not characterised any further). The restriction pattern of the remaining clone, Bp11 was consistent with a fragment the size of envB ligated into pOGS40. The envB fragment contains a HindIII site at position 145 (the HindIII sites in pOGS40 and pOGS42 are in the same positions) and the digestion pattern with this enzyme was consistent with the bands of predicted sizes 3320, 3422 and 3565 for an envB fragment inserted 3' to 5' in the BamHI site. Plasmid DNA was extracted from the clone, digested with BamHI, re-ligated and transformed into E.coli JM83. 184 colonies were screened and 3 reacted with the B-specific probe. Restriction digestion analysis demonstrated that 2 of the clones contained the fragment

in the 3' to 5' orientation while a third, Bp11R/3 had a HindIII restriction pattern consistent with the band sizes of 2795, 3320 and 4192 predicted for an insertion in the 5' to 3' orientation. Bp11R/3 was therefore selected as the pOGS40/envB clone to be used to transform S.cerevisiae.

10 colonies were analysed by restriction digestion analysis after ligation of an envC PCR fragment into pOGS40. 8 of the clones were pOGS40 with no inserted DNA but BamHI digestion of the DNA from the remaining 2 clones excised a fragment of the expected size for envC. No diagnostic restriction digestion was available to determine the orientation of the C fragment in pOGS40 and therefore double stranded sequencing was performed. This demonstrated that one of the clones, Cp3 contained the envC fragment in the 5' to 3' orientation. Cp3 was therefore selected as the pOGS40/envC clone to be used to transform S.cerevisiae.

4.3 Transformation Of S.cerevisiae With pOGS/Env Plasmids

Purified DNA from pUG 41S and the pOGS/env clones were used to transform S.cerevisiae. A range of DNA concentrations were tested and approximately 3.0ug of the pOGS-based plasmids were co-transformed with 1.0ug of pUG 41S. Double transformants were initially selected on Sc-Glc/Trp plates and then re-streaked onto fresh selection plates. Double transformations (leu^+ , ura^+) are rare events (approximately 1 in 10^8) and 2 transformants were initially obtained with Ap6, 6 with Bp11R/3 and 1 with Cp3. For A and B each transformant was screened for fusion protein expression, using a small-scale culture, and no obvious difference was apparent between the transformants (data not shown). Therefore one of each was chosen arbitrarily and the S.cerevisiae double transformants were designated A2.2, B2.2 and C3.1.

4 double-transformants containing pUG 41S and pOGS40

were also generated. There was no obvious difference in p1 protein expression between these transformants and one was chosen arbitrarily as a control and designated pp8.1.

4.4 Protein Expression From The *S.cerevisiae* pOGS/Env Transformants

Figure 4.3 shows the p1:Env fusion proteins detected by western blotting with a polyclonal rabbit anti-VLP serum (a kind gift of M. Fotheringham, Department Of Veterinary Pathology, University Of Edinburgh) after induction of the cultures with galactose. It should be noted that the bands shown were obtained after large-scale purification of the VLPs, and the loading has been varied for each track. They do not reflect the relative yields of each protein. The p1 protein migrates with an apparent molecular weight of approximately 55kDa-60kDa in the gel system used, higher than its predicted molecular weight (42kDa). This is consistent with previous data (Adams et al., 1987b). p1:EnvA migrates at approximately 94-98kDa, p1:EnvB at approximately 85kDa and p1:EnvC at approximately 76kDa. A number of other bands are visible on the western blots of the p1 and p1:Env fusion protein preparations. These are contaminants of the purification procedure which are reactive with the polyclonal anti-VLP serum.

No obvious bands were visible for the fusion proteins from small-scale protein purifications on Coomassie-stained gels but the p1 band in clone pp8.1 could be distinguished (data not shown). This suggested that the p1:Env fusion proteins might be low-yielding.

Two-litre cultures of the transformants were induced and large-scale protein purification performed. Approximately 20ml of packed cells were obtained from each culture and these were processed in 7.5ml batches. Figure 4.4 shows western blots and Coomassie-stained gels for the large-scale purification of protein from pp8.1.

Figure 4.3

Western blot of p1/p1:Env fusion proteins

Sucrose gradient purified proteins were separated on a 10% SDS-PAGE gel and blotted onto nitrocellulose. The blot was developed with rabbit anti-VLP serum @ 1 in 1500 and anti-rabbit alkaline phosphatase @ 1 in 1000. The p1:Env bands are indicated by dots. The p1 doublet is indicated by 2 dots.

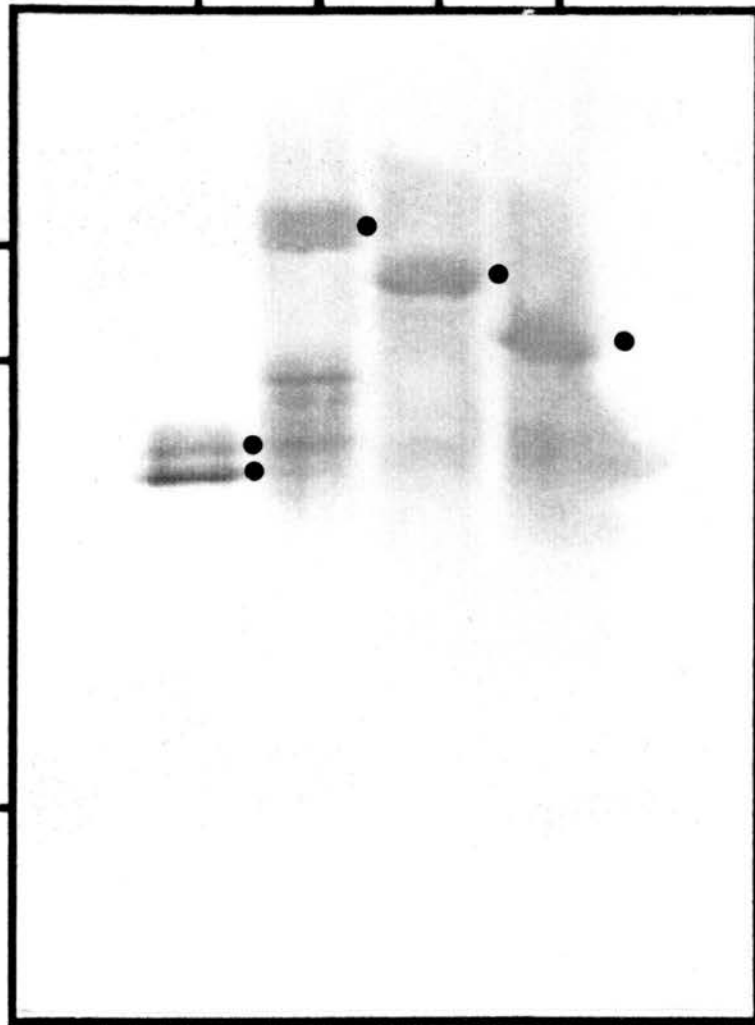
Mol. Wt. / kDa

43

67

94

p1
p1:EnvA
p1:EnvB
p1:EnvC



The western blots showed that the p1 material was concentrated in fractions 8 to 13 (where fraction 1 is the top of the gradient and fraction 19 is the bottom). Similar results were apparent on the Coomassie-stained gel, with a visible p1 band most apparent in fractions 8 to 11. The band consistently ran as a doublet with few contaminating bands present. 5ul from each 2ml fraction were analysed. A BioRad protein dye-binding assay was used (manufacturer's protocol) to estimate the protein concentration of the purified p1 preparations, and the yield of p1 protein was thereby estimated as 2.5mg/L (data not shown).

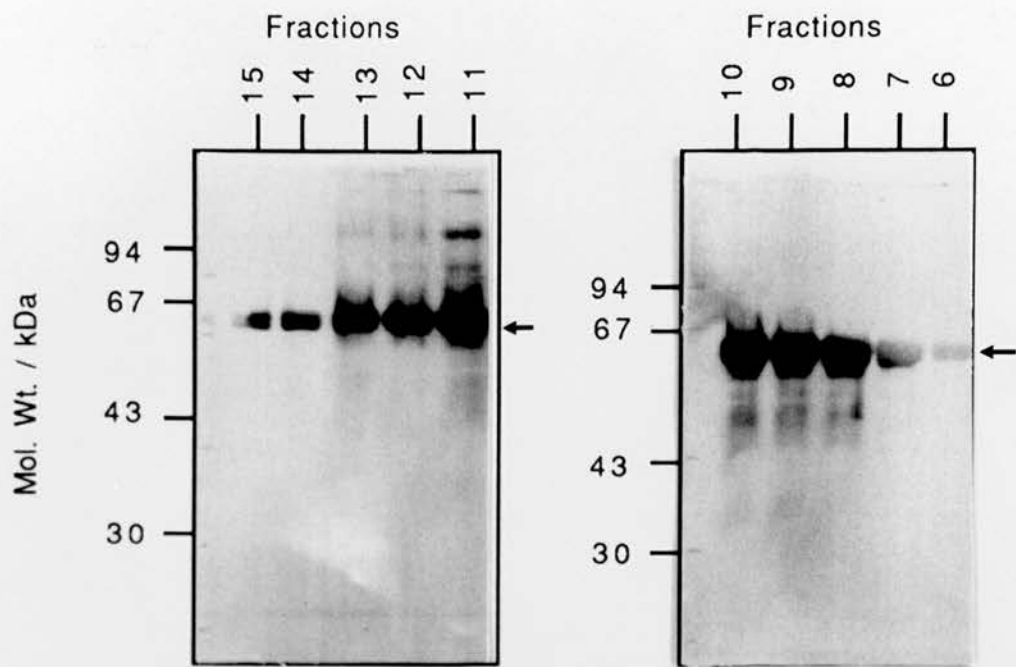
Figure 4.5 shows the western blot for the fractions obtained after sucrose gradient purification of the p1:Env fusion proteins. The p1:EnvA fusion protein is concentrated in fractions 18 and 19, p1:EnvB in fractions 17 and 18 and p1:EnvC in fractions 14 to 17. Other bands are also visible, suggesting the presence of contaminating proteins in the fractions which react with the anti-VLP serum.

Figure 4.6 shows the silver-stained gel for the peak fractions of each of the large-scale purifications. The p1 band from pp8.1 is clearly visible and a band is also visible for p1:EnvA. However despite overloading the gel, and allowing it to become overdeveloped, no p1:EnvB or p1:EnvC bands were visible. It should also be noted that only 1ul was loaded of the protein from pp8.1, but 5ul were loaded for each p1:Env construct. It is therefore apparent that yields of the fusion proteins were much lower than the native p1 protein. Contaminating proteins were also present to a much greater extent in the fusion protein preparations; if equal volumes of the peak fractions from the p1 and p1:Env fusion protein preparations were separated by SDS-PAGE the level of contaminating proteins in the p1 preparation was much lower than for the fusion proteins, while the p1 band

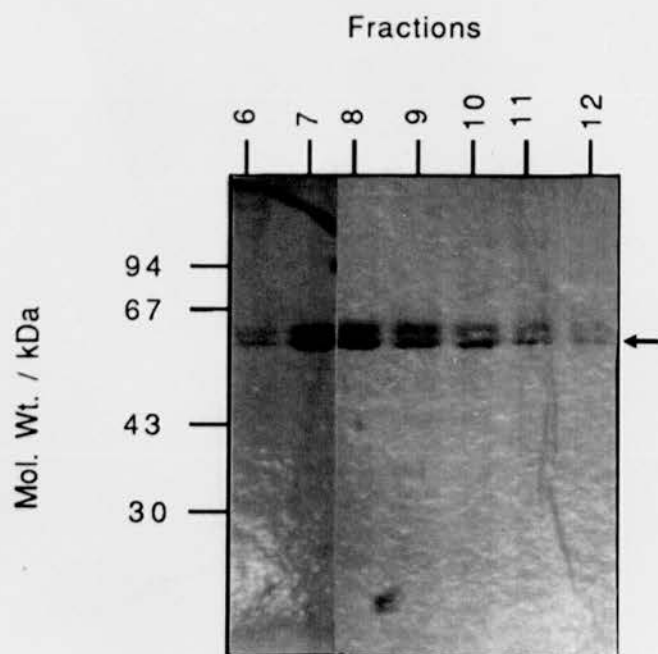
Figure 4.4

Western blot and Coomassie-stained gel of p1

Proteins from the peak fractions of sucrose gradient purified p1 were separated on a 10% SDS-PAGE gel and either Coomassie-stained (b), or blotted onto nitrocellulose (a) and developed with rabbit anti-VLP serum @ 1 in 1500 and anti-rabbit alkaline phosphatase @ 1 in 1000. The p1 doublet is indicated by dots. Sucrose gradient fraction numbers are shown above the tracks.



a



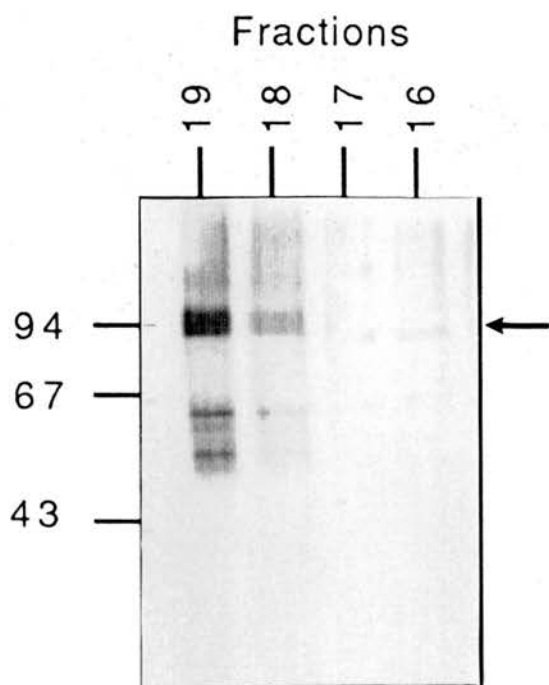
b

Figure 4.5

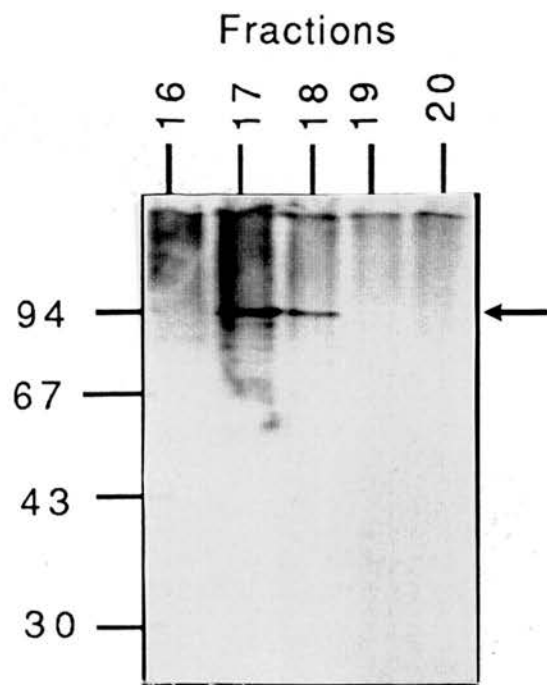
Western blot of p1:Env fusion proteins

Proteins from the peak fractions of sucrose gradient purified p1:Env were separated on a 10% SDS-PAGE gel, blotted onto nitrocellulose and developed with rabbit anti-VLP serum @ 1 in 1500 and anti-rabbit alkaline phosphatase @ 1 in 1000. The fusion protein bands are indicated by arrows. Sucrose gradient fraction numbers are shown above the tracks.

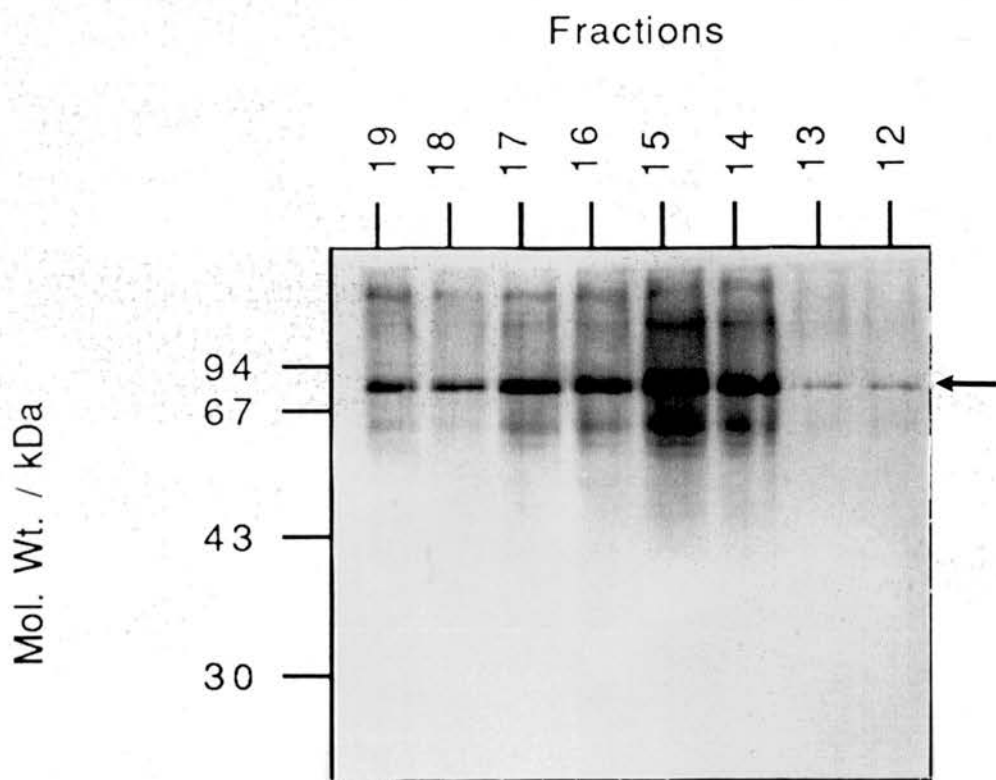
Mol. Wt. / kDa



p1:EnvA



p1:EnvB



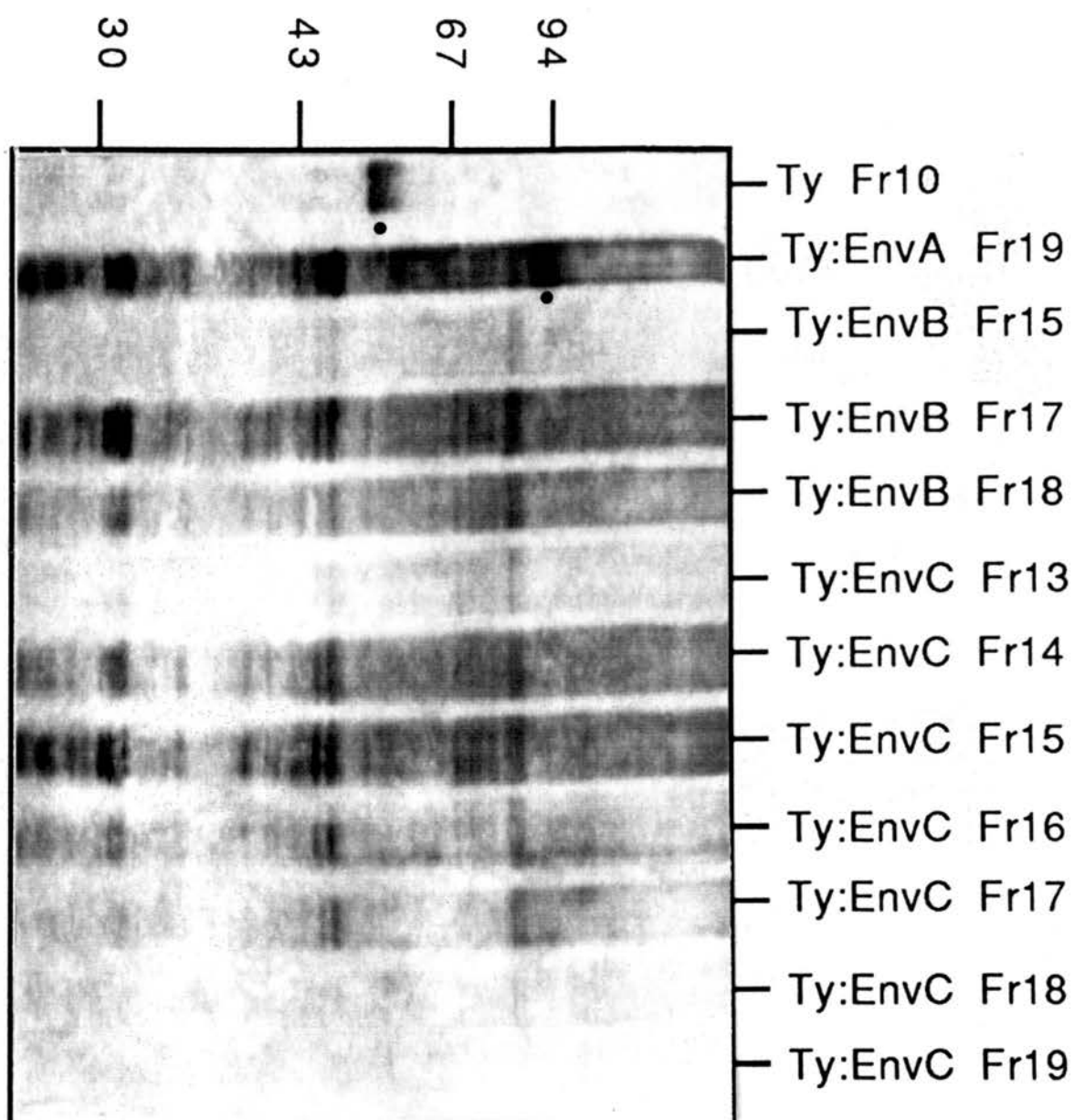
p1:EnvC

Figure 4.6

SDS-PAGE gel of p1/p1:Env fusion proteins

Proteins from the peak fractions of sucrose gradient purified p1/p1:Env were separated on a 10% SDS-PAGE gel and developed by silver staining. The p1 and p1:EnvA bands are indicated by dots. Sucrose gradient fraction numbers are shown above the tracks.

Mol. Wt. / kDa



itself was greatly overloaded (data not shown). As it was not possible to achieve high purification of the fusion proteins no attempt was made to estimate yield by protein assay systems.

4.5 Growth Characteristics Of *S.cerevisiae* pOGS/Env Transformants

The growth characteristics of induced and uninduced *S.cerevisiae* transformants were performed to determine if the low yields of the fusion proteins were a consequence of a toxic effect on the yeast cells. Transformants were grown in Sc-glc/Trp until an OD₆₀₀ of 0.1-0.3 was reached, the cells pelleted and resuspended in fresh pre-warmed Sc-glc/Trp or Sc-glc-gal/Trp and the incubation continued. OD₆₀₀ values were recorded at six-hourly intervals for the first twenty four hours, and finally at forty eight hours post-induction. Small-scale protein preparations were made at 12, 24 and 48 hours post-induction.

Figures 4.7 to 4.10 show the growth profiles for the four transformants, based on the mean of two experiments. Although slight differences are apparent between the induced and uninduced cultures at 12 or 18 hours post-induction these may not be particularly significant, given the large standard deviations of the samples. (The large standard deviations in the early stages of the B2.2 growth curves reflect the different OD₆₀₀ values at which induction was initiated in two separate experiments). By 24 hours post-induction there was no significant difference in the OD₆₀₀ values for the induced and uninduced cultures.

Figure 4.11 shows a comparison of the results for the four induced cultures. No significant differences in the growth characteristics were observed when comparing the Env-expressing transformants with pp8.1 which expresses only p1. Phase contrast microscopy of the cultures revealed no obvious differences in the morphology of the

Figure 4.7

Growth profile of A2.2

An A2.2 transformed yeast culture was induced @ $t=0$, and the OD_{600} of the culture monitored, and compared with the values for an uninduced culture. The standard deviation of each reading (between 2 separate experiments) is shown.

Growth Profile For A2.2

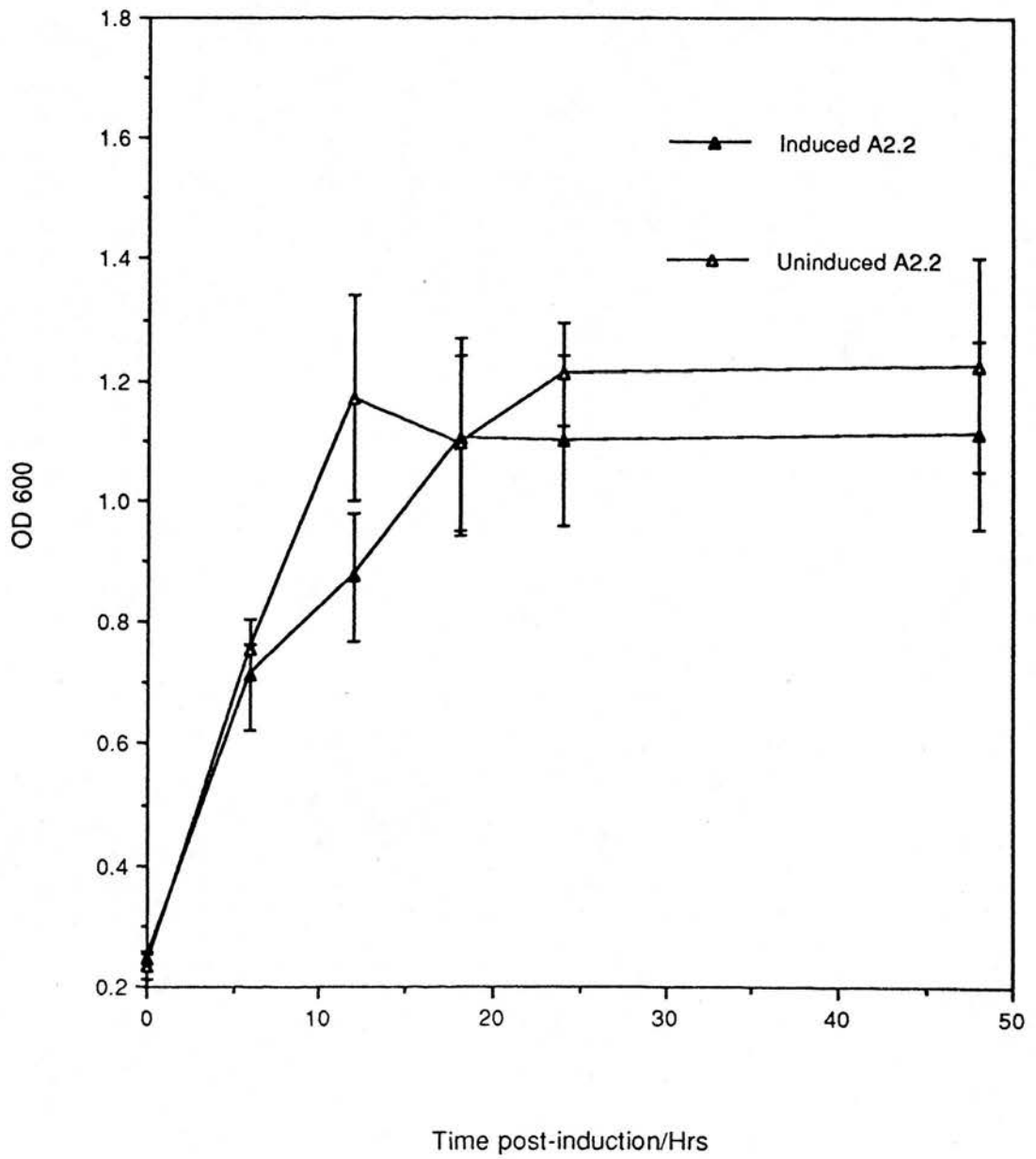


Figure 4.8

Growth profile of B2.2

A B2.2 transformed yeast culture was induced @ $t=0$, and the OD_{600} of the culture monitored, and compared with the values for an uninduced culture. The standard deviation of each reading (between 2 separate experiments) is shown.

Growth Profile For B2.2

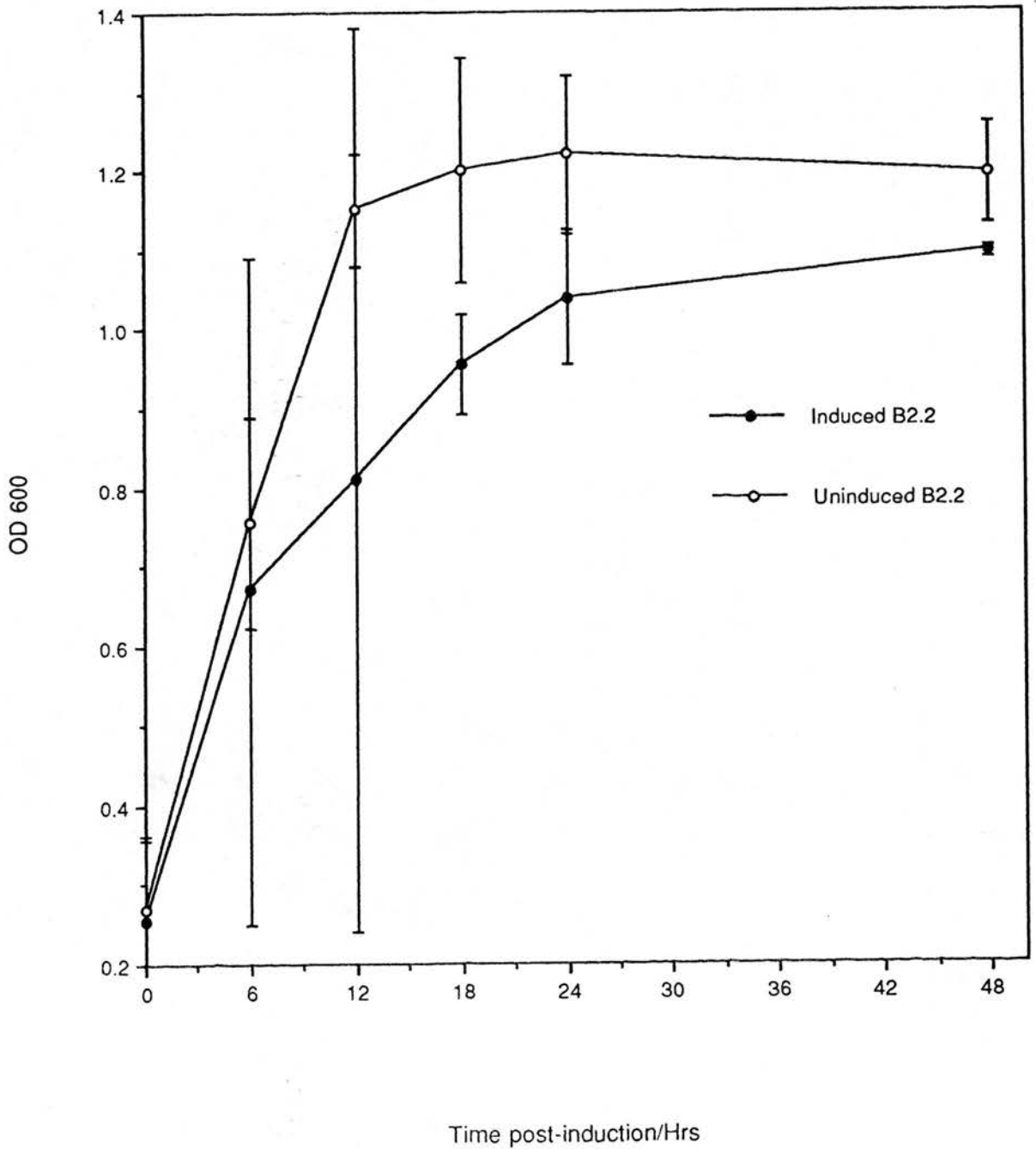


Figure 4.9

Growth profile of C3.1

A C3.1 transformed yeast culture was induced @ $t=0$, and the OD_{600} of the culture monitored, and compared with the values for an uninduced culture. The standard deviation of each reading (between 2 separate experiments) is shown.

Growth Profile For C3.1

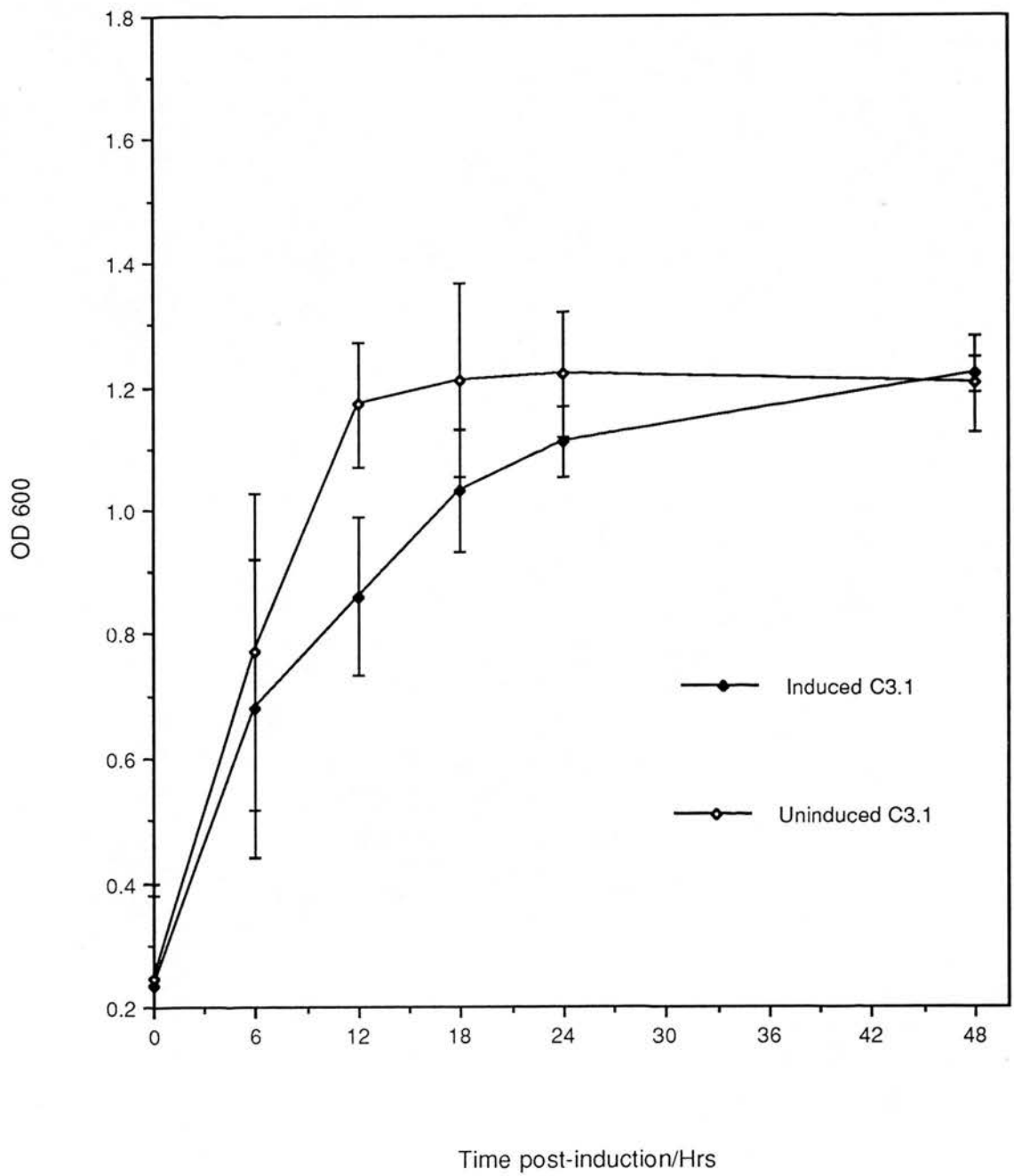


Figure 4.10

Growth profile of pp8.1

A pp8.1 transformed yeast culture was induced @ $t=0$, and the OD_{600} of the culture monitored, and compared with the values for an uninduced culture. The standard deviation of each reading (between 2 separate experiments) is shown.

Growth Profile For pp8.1

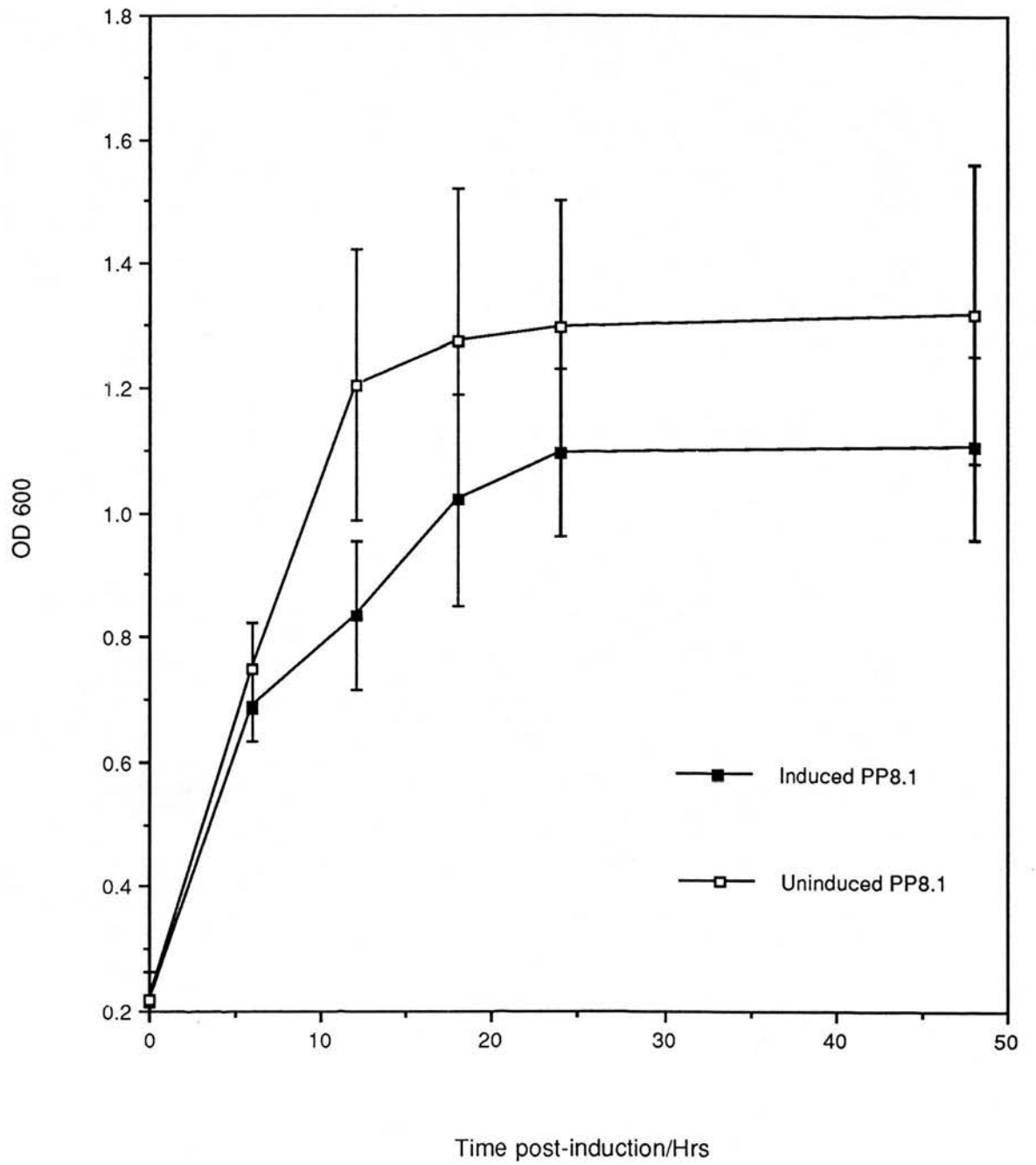
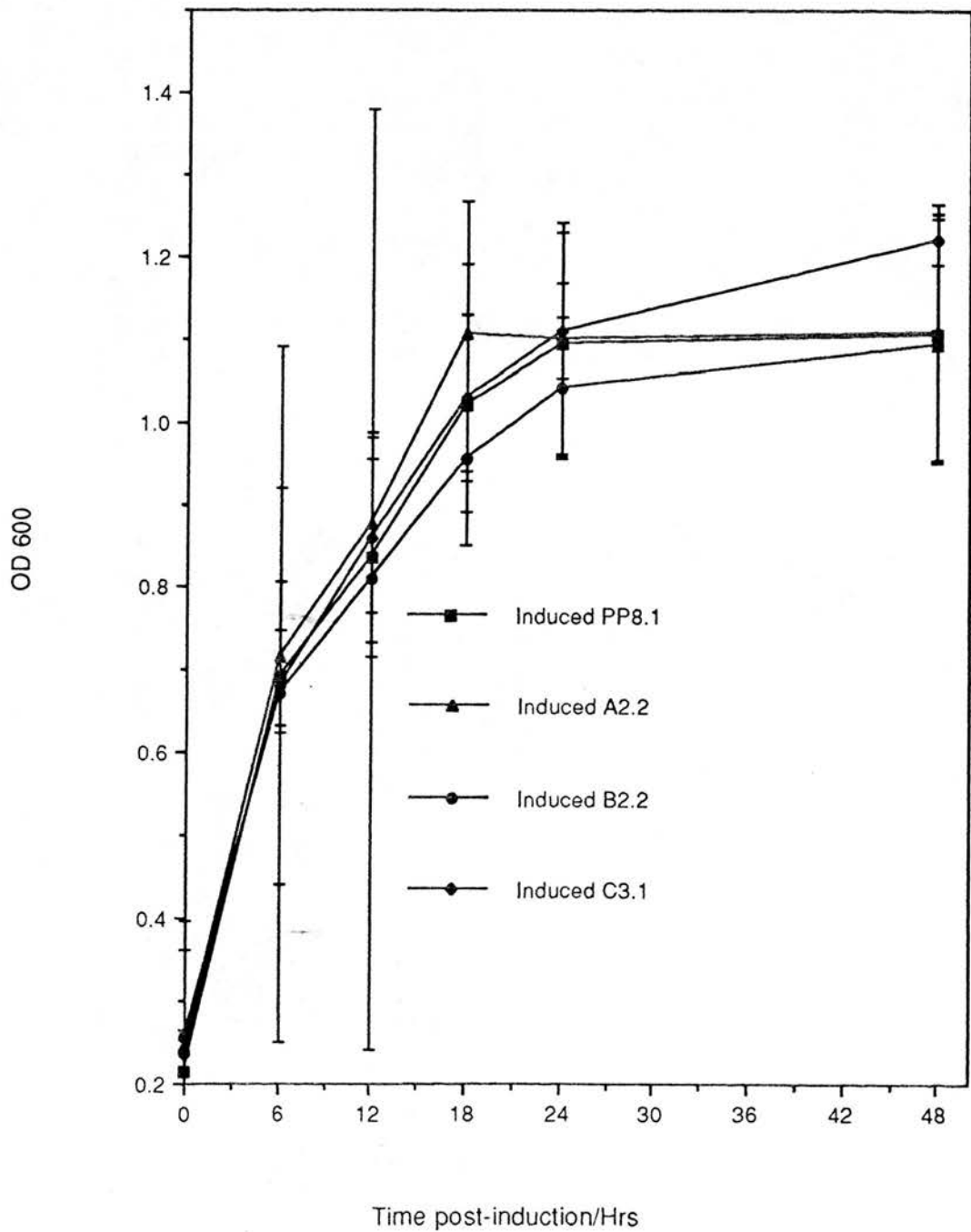


Figure 4.11

Growth profiles of induced yeast transformants

All cultures were induced @ $t = 0$, and the OD_{600} monitored. The standard deviation of each reading (between 2 separate experiments) is shown.

Growth Profiles Of Induced Clones



various induced and uninduced cultures (data not shown).

Recombinant protein expression was monitored by western blotting with the rabbit anti-VLP serum. Figure 4.12d shows that expression of p1 from clone pp8.1 rose over the time-course of the experiment, although expression was apparent from 12 hours post-induction (the first time-point at which protein expression was analysed). For expression of the p1:EnvA fusion protein from clone A2.2 the pattern was very different (figure 4.12a) with very little recombinant protein detectable at 12 or 48 hours post-induction, but appreciable detection at 24 hours post-induction. Figure 4.12b shows that low level expression of p1:EnvB fusion protein was detectable at 12 hours post-induction, had risen markedly by 24 hours post-induction, and possibly had increased slightly further by 48 hours post-induction. Figure 4.12c shows that production of the p1:EnvC fusion protein from clone C3.1 was very poor, as determined by western blotting. Fusion protein could be detected at 12 and twenty-four hours post-induction, but not after 48 hours. The four transformants therefore although showing similar growth characteristics differed markedly in the pattern of recombinant protein production.

4.6 Electron Microscopy Of *S.cerevisiae* Expressing p1:Env Fusion Proteins

In order to investigate the intracellular morphology of the hybrid and non-hybrid VLPs cultures of the 4 transformants were induced for 24 hours and the cells analysed by transmission electron microscopy. Western blotting with the anti-VLP serum was used to confirm that the transformants were expressing the proteins (data not shown). Control uninduced cultures of the same transformants were also analysed by western blotting and transmission electron microscopy.

In figure 4.13d VLPs are visible in the cytoplasm of

Figure 4.12

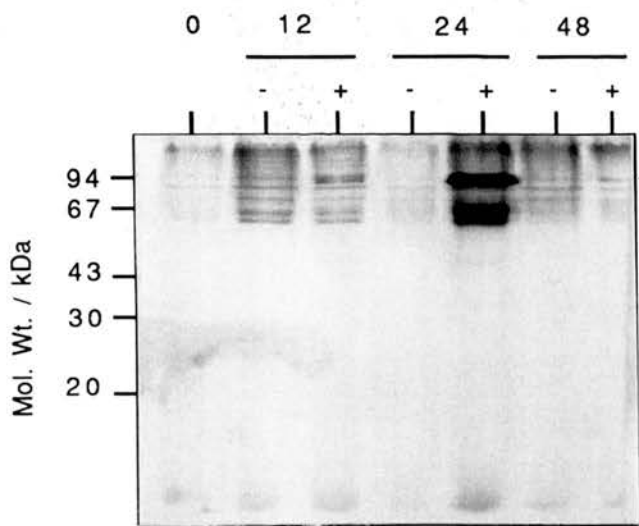
Variation in expression of p1/p1:Env proteins with induction time

The proteins from induced and uninduced transformed yeast cultures were separated on a 10% SDS-PAGE gel, blotted onto nitrocellulose and developed with rabbit anti-VLP serum @ 1 in 1500 and anti-rabbit alkaline phosphatase @ 1 in 1000. The p1/p1:Env bands are indicated by arrows. The time intervals post-induction are shown above each blot.

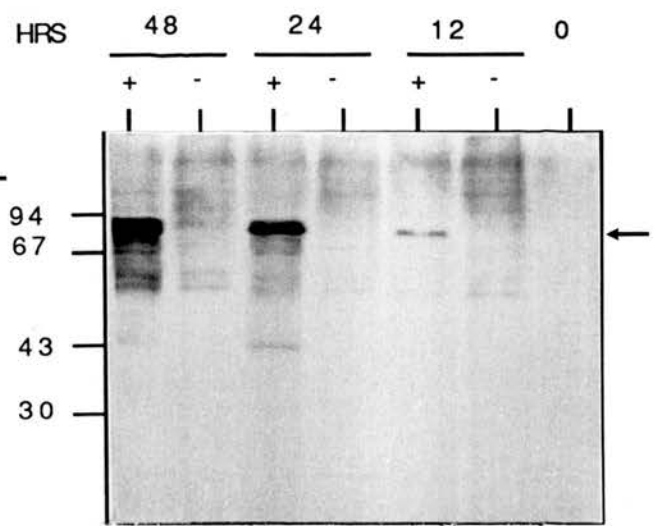
Key

a = transformant A2.2
b = transformant B2.2
c = transformant C3.1
d = transformant pp8.1

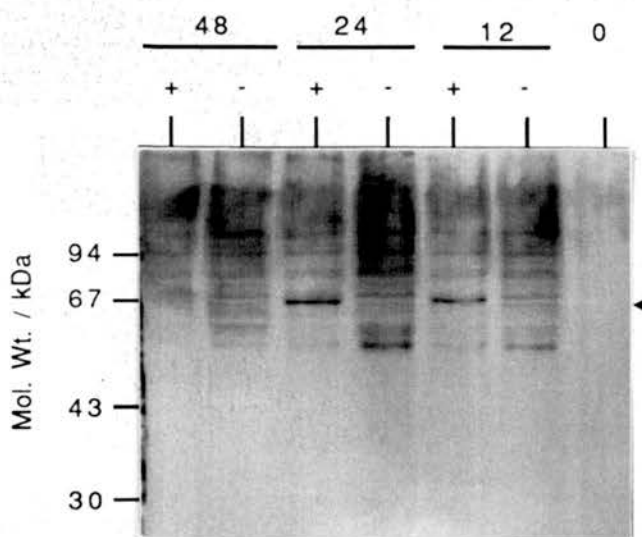
+ = induced culture
- = uninduced culture



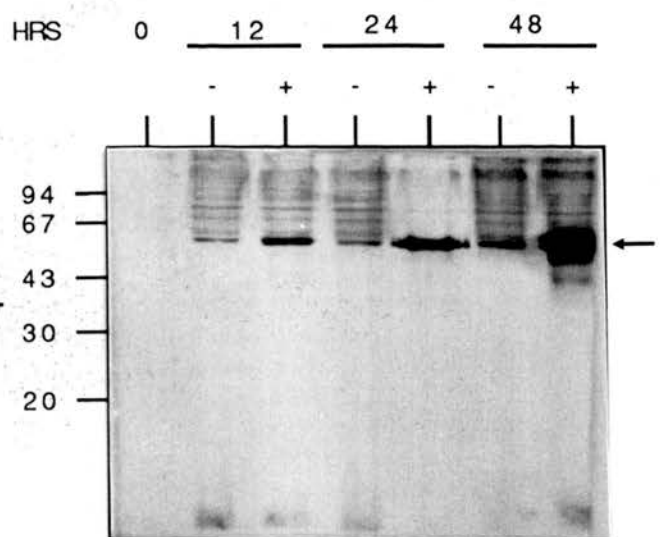
a



b



c



d

Figure 4.13

Transmission electron micrographs of induced p1/p1:Env transformants

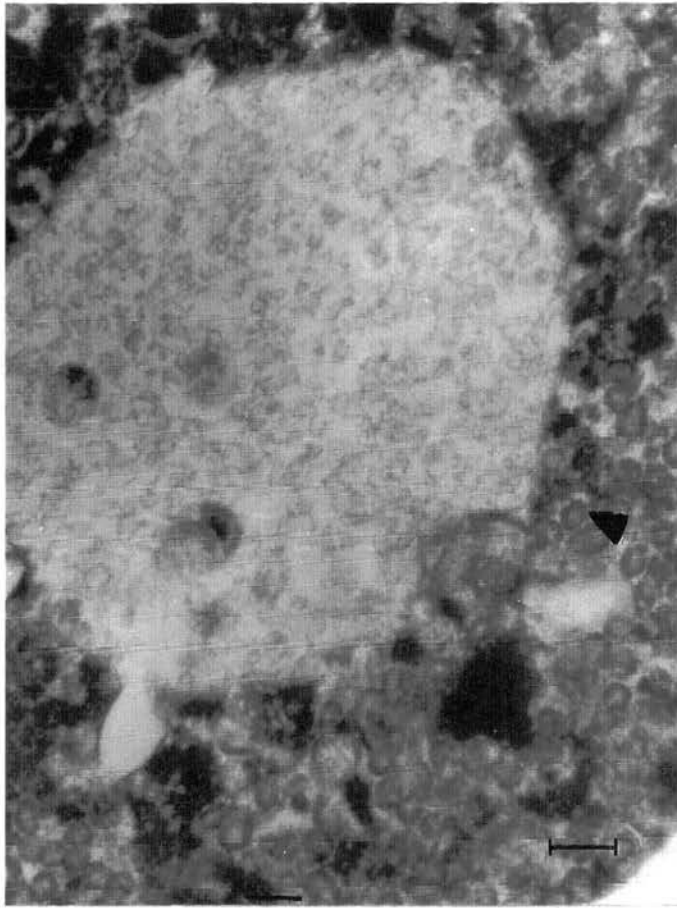
Transformed yeast were grown under inducing conditions for 24 hours and sections of fixed cells prepared for transmission electron microscopy. VLPs are indicated by solid arrows.

Key

- a = transformant A2.2
- b = transformant B2.2
- c = transformant C3.1
- d = transformant pp8.1

Scale bar = 200nm

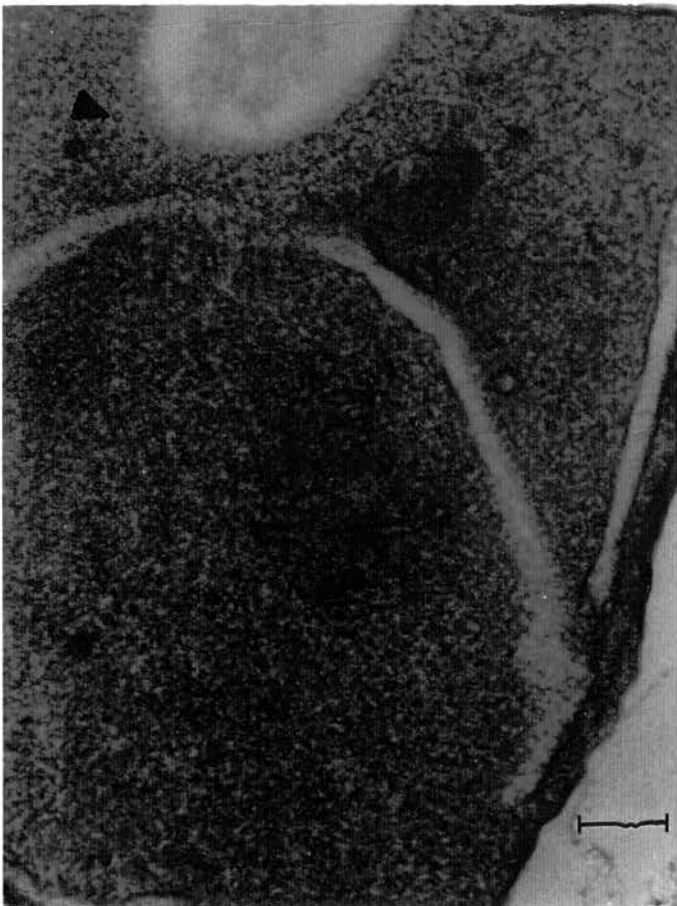
a



b



c



d



induced pp8.1 cells as approximately circular structures with an electron-dense outer region, approximately 15 to 35nm in diameter.

Figure 4.13a demonstrates that the VLPs formed after induction of the p1:EnvA protein were much larger than the control VLPs (approximately 70 to 150nm diameter) and very heterogeneous in morphology. The particles were cytoplasmic and appeared to be circumscribed by two layers of electron-dense material with an intervening layer of electron-light material.

Figures 4.13b and 4.13c show the VLPs present in induced cultures of B2.2 and C3.1 respectively. They were similar in size, cellular location and morphology to the control VLPs formed by clone pp8.1 but were present at much lower levels in the cytoplasm.

No VLPs were found in uninduced cells and there were no obvious major changes in cellular morphology between the various cultures (data not shown).

4.7 Glycosylation Of p1:Env Fusion Proteins

In order to determine if the fusion proteins were glycosylated samples from the peak fractions of sucrose gradient purification were incubated in the presence or absence of N-glycosidase F or endoglycosidase F. N-glycosidase F hydrolyses almost all N-linked carbohydrate residues at the aspartylglycosylamine linkage. Endoglycosidase F hydrolyses the diacetylchitobiose linkage of N-linked high-mannose oligosaccharides, hybrid and to some extent biantennary complex carbohydrates.

Figure 4.14 is a representative example of the results obtained after treatment of p1 and p1:Env fusion proteins with N-glycosidase F. The experiment was repeated four times, using either Triton-X 100 or octylglucoside as a detergent, and similar results were obtained on each occasion.

There was no change observed in the mobilities of the

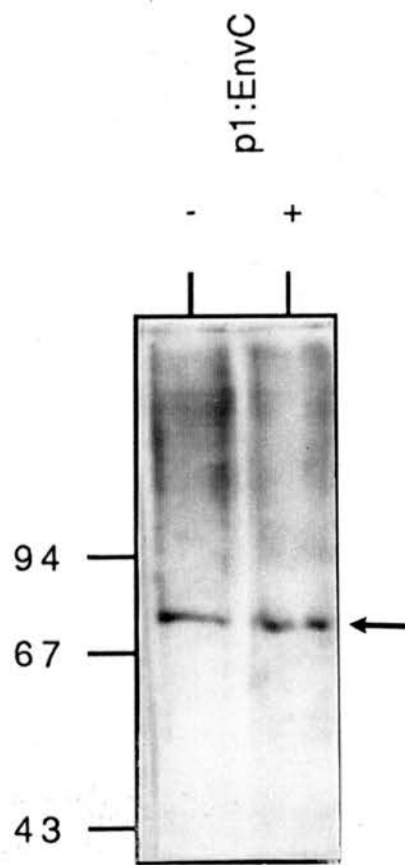
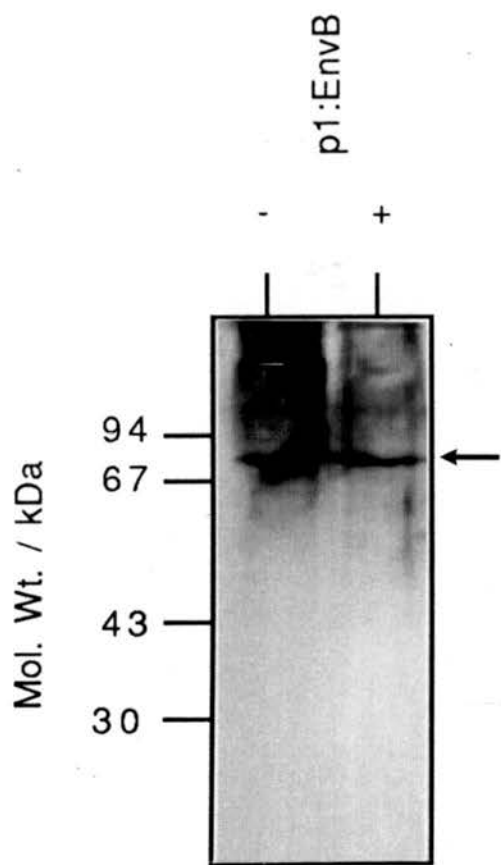
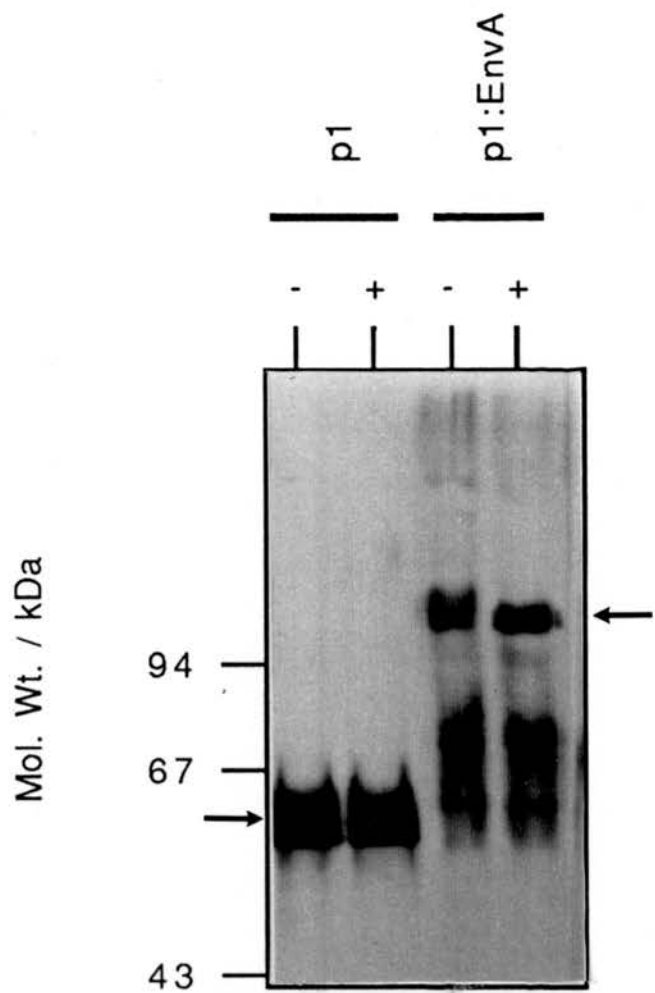
Figure 4.14

Glycosylation of p1/p1:Env fusion proteins

Proteins from the peak sucrose gradient fractions of p1/p1:Env were treated with N-glycosidase F, separated on a 10% SDS-PAGE gel and blotted onto nitrocellulose. Blots were developed with rabbit anti-VLP serum @ 1 in 1500 and anti-rabbit alkaline phosphatase @ 1 in 1000. The p1/p1:Env protein bands are indicated by arrows.

Key

- + = N-glycosidase F treatment
- = no N-glycosidase F treatment



p1, p1:EnvB and p1:EnvC bands after N-glycosidase F treatment but the p1:EnvA band was altered, with the band becoming "sharper" and less diffuse in appearance, although with no detectable alteration in the position of the "leading edge" of the band. This suggests N-linked glycosylation of the p1:EnvA fusion protein.

No alteration in the mobility of p1 alone, or any of the p1:Env fusion proteins was observed when the samples were treated with endoglycosidase F (data not shown).

4.8 Reactivity Of Sera From MVV-Infected Sheep With The p1:Env Fusion Proteins

In order to confirm that the fusion proteins contained MVV gp135 sequences, and to determine if MVV-infected sheep differed in their responses to gp135 western blots of the fusion proteins were analysed using sera from infected sheep.

In order to standardise the amount of fusion protein on each blot peak fractions were diluted to varying degrees in TEN, separated by SDS-PAGE and blotted onto nitrocellulose. The rabbit anti-VLP serum was used to assess the comparative amounts of the p1:fusion proteins present. Loading of the native p1 protein and the fusion proteins was adjusted so that native p1 was present in slight excess compared with the p1:Env fusion proteins. The same blots were then used to screen sera from MVV-infected sheep.

Sheep 754N and 848A had been experimentally infected with the MVV EV1 strain approximately 22 months before the serum samples were obtained, at which point the sheep were asymptomatic. Sheep 2, 68, 74 and G027 had been naturally infected with MVV for an unknown period. The serum samples were obtained at post-mortem and all 4 sheep displayed classical maedi lesions. All sera were used at a dilution of 1 in 20, at which they detected gp135 in western blots of in vitro MVV-infected cell lysates (data

not shown). Pooled normal sheep serum (Scottish Antibody Production Unit) was used as a negative control.

Figure 4.15 demonstrates that sera from infected sheep differed in the extent to which they reacted with the fusion proteins. Sera from sheep G027, 74 and 754N reacted with all three fusion proteins, with 754N the most strongly reactive. Sera from sheep 848A reacted only against the p1:EnvC fusion protein - no reactivity was detected against p1:EnvA or p1:EnvB. Sera from sheep 2 reacted only with the p1:EnvB and p1:EnvC fusion proteins - no reactivity was detected against p1:EnvA. None of these sera reacted against p1 alone. Sera from sheep 68 showed very faint reactivity against p1 alone and also against all three fusion proteins. In view of the relative amounts of p1 on the blots, and the intensities of the bands detected with the sera from sheep 68 it was considered that this sample was genuinely reactive against the Env regions of the fusion proteins. Normal sheep serum reacted very weakly with p1 alone but did not react with any of the fusion proteins.

4.9 Immunisation Of Rabbits And Mice With p1:Env Fusion Proteins

1 rabbit and 3 mice were immunised with each p1:Env fusion protein using the protocol described in section 2.23. Two weeks after the third immunisation the sera were screened by western blotting for reactivity with a whole cell lysate of MVV-infected cells. The presence of Env protein on the blots was confirmed using sera from the MVV-infected sheep 848A, 754N and G027 (data not shown). No anti-Env activity was observed for any mouse serum diluted 1 in 100, or for rabbit sera diluted between 1 in 10 and 1 in 150.

All the sera from the immunised animals were reactive with p1 and figure 4.16 shows representative examples of this reactivity.

Figure 4.15

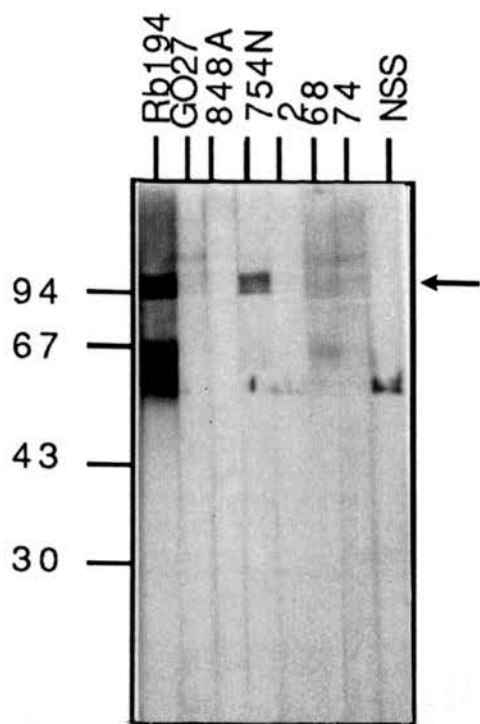
Reactivity of MVV-infected sheep sera with p1/p1:Env fusion proteins

Proteins from the peak sucrose gradient fractions of p1/p1:Env were separated on a 10% SDS-PAGE gel and blotted onto nitrocellulose. The blots were developed with either rabbit anti-VLP serum (Rb194) @ 1 in 1500 or various sheep sera @ 1 in 20, followed by anti-species alkaline phosphatase @ 1 in 1000. The p1/p1:Env protein bands are indicated by arrows.

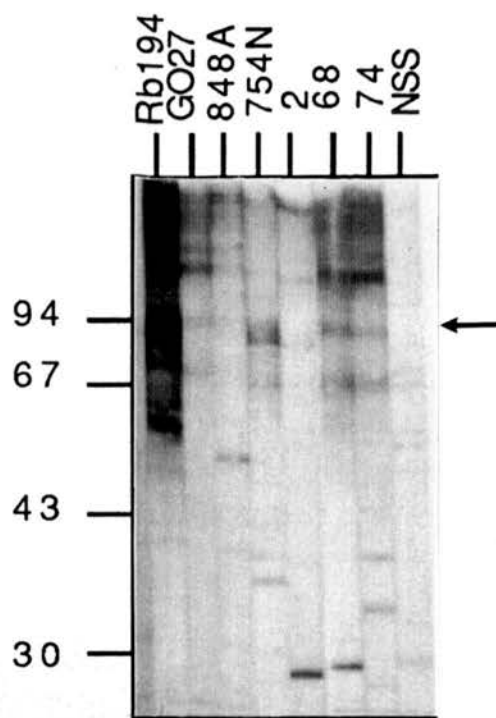
Key

- a = p1:EnvA
- b = p1:EnvB
- c = p1:EnvC
- d = p1

Mol. Wt. / kDa

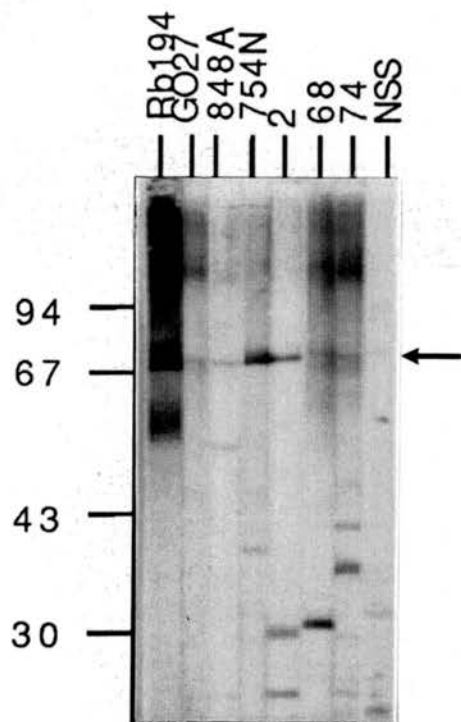


a

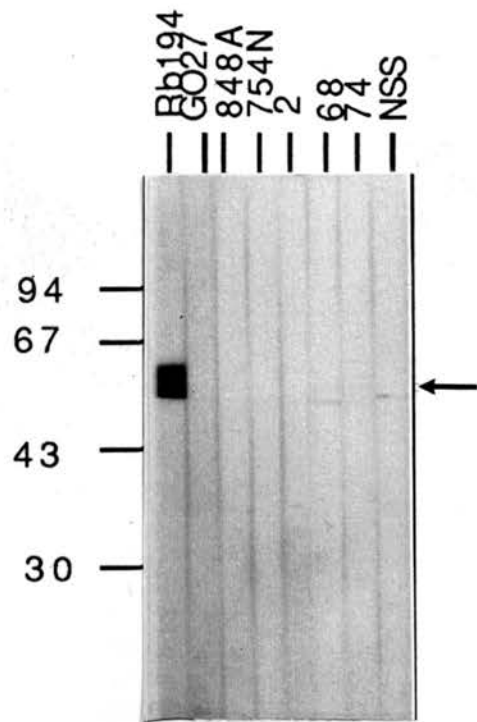


b

Mol. Wt. / kDa



c



d

Figure 4.16

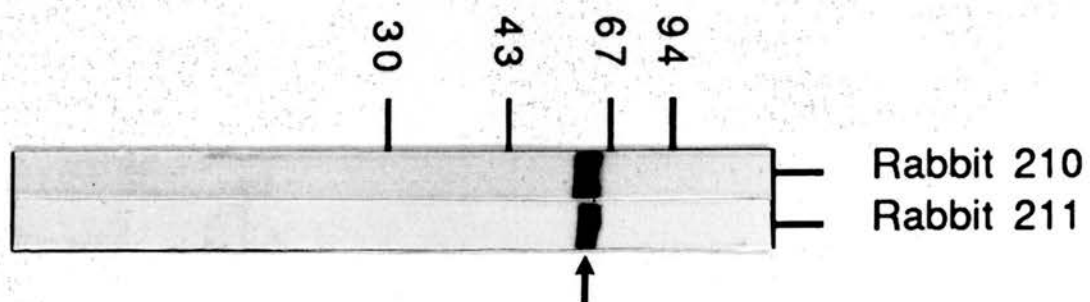
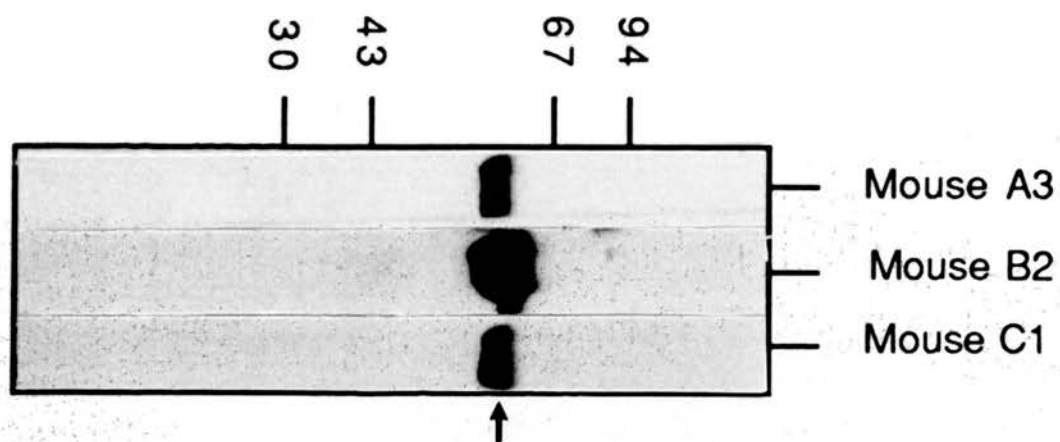
Reactivity of p1:Env-immunised rabbit and mouse sera with p1

Proteins from the peak fractions of sucrose gradient purified p1 were separated on a 10% SDS-PAGE gel and blotted onto nitrocellulose. The blots were developed with sera from mice or rabbits immunised with p1:Env proteins, and anti-species alkaline phosphatase @ 1 in 1000. Arrows indicate the p1 protein band(s).

Key

- Mouse A3 - immunised with p1:EnvA
- Mouse B2 - immunised with p1:EnvB
- Mouse C3 - immunised with p1:EnvC
- Rabbit 210 - immunised with p1:EnvB
- Rabbit 211 - immunised with p1:EnvC

Mol. Wt. / kDa



4.10 Discussion

This chapter has reported the generation of recombinant proteins which span the entire gp135 molecule, and the characterisation of these proteins.

The four transformants generated, pp8.1, A2.2, B2.2 and C3.1 differed only in the coding sequence which was inserted 3' to the p1 gene and therefore the differences observed in the expression and characteristics of the fusion proteins must be a consequence of the Env sequences.

It is not clear why so many difficulties were encountered in sub-cloning the env gene fragments from the pTZ-based clones into pOGS vectors. Part of the difficulty was probably due to the significantly larger size of the shuttle vector (9.4kb cf. 2.9kb for pTZ) as smaller plasmids are known to transform at a higher efficiency than larger plasmids (Hanahan, 1983). It was also suspected that low levels of undigested pTZ plasmids containing the env sequences co-migrated with the excised fragment on a gel, probably by a process of physical entrapment. In order to circumvent this problem procedures such as double digestion and double rounds of gel purification were employed, but these proved to be unsuccessful, possibly because yields of the desired fragment were very low after these multiple steps. Experiments with the bacterial expression system pGEX (chapter 5) demonstrated that expression of the env gene fragments in E.coli resulted in a significant reduction in cell growth. If a basal level of expression from the PGK-PAL promoter occurred after transformation of E.coli the resulting toxicity of the expressed fusion proteins could also result in a reduced effectiveness of transformation by pOGS-based plasmids

One of the most obvious characteristics of the transformants which express the p1:Env fusion proteins was the low expression of the fusion protein compared to that

of p1 alone. On large-scale preparations it became apparent that this was accompanied by extensive contamination of the fractions which contained the fusion proteins with other yeast proteins. This was not merely a technical problem (eg with cell breakage or sucrose gradient formation) as much cleaner preparations were obtained when purifying p1 alone by the same technique. It was noticeable that after sucrose gradient centrifugation the fractions which contained the highest levels of the fusion proteins also contained a large amount of aggregated material, readily visible in the centrifuge tubes. The extensive degree of contamination in these preparations may suggest that the expression of the fusion proteins can lead to the formation of aggregates, either with or between other cellular proteins during the purification stages.

The low level of expression of the fusion proteins was not a consequence of any directly toxic effect upon the host cells. Although induction in all four transformants led to an initial slowing down of cell division this is not an unusual phenomenon, and occurred for both the fusion proteins and p1 alone. Coomassie stained gels from crude lysates of all 4 transformants, harvested at different times post-induction, did not demonstrate any major difference in the level of expression of cellular proteins during induction (data not shown). Although this was only a very crude technique it indicated that expression of the fusion proteins did not result in any massive overall decline in host cell protein synthesis.

The results for the time-course of fusion protein expression suggested differences in the stability of the various fusion proteins. The increasing expression of the p1 protein alone throughout the entire period of the experiment indicated that the protein was stable within the cell, and that expression of the protein could

continue to be induced even in the presence of high intracellular concentrations of p1. The p1:EnvB fusion protein appeared to be the most stably expressed of the Env fusion proteins as it could still be detected at 48 hours post-induction, albeit at levels much lower than for p1. The p1:EnvA and p1:EnvC fusion proteins were detectable at 24 hours post-induction but not at 48 hours, the most likely interpretation being that the proteins had been degraded within the cell and this degradation had not been followed by a significant further degree of fusion protein expression. The alternative possibility of extracellular export appears to be unlikely as there are no reports of a mechanism by which VLPs are transported from a cell. It is not clear how this loss of fusion protein arises, or why it should only become apparent more than 24 hours post-induction. It may be that there is a time-lag in the production of cellular protease activity in the BJ 2168 cells (which are protease-defective) such that initially production of the fusion protein outpaces degradation but that with increasing time there is a relative fall in fusion protein expression such that the degradative process outweighs the synthesis of the p1:Env species. The EnvA and EnvC sequences may also have an inhibitory effect on cellular protein synthesis (either of all cellular proteins or just the fusion proteins) which was not detectable by Coomassie staining of SDS-PAGE gels. However the process must be influenced by regions in EnvA and EnvC (and presumably not in the parts of the molecules which overlap with EnvB) as the same pattern of expression was not apparent for p1 or p1:EnvB.

Electron microscopy revealed that while the VLPs formed by p1, p1:EnvB and p1:EnvC were broadly similar in size, shape and morphology they differed dramatically from those formed by p1:EnvA. The presence of the EnvA protein sequence therefore must exert a major effect during the formation of the VLPs. This is unlikely to be a simple

reflection of the size of the EnvA fragment in the fusion protein as its predicted molecular weight is only slightly greater than that of EnvB and the relative sizes of the monomers of the EnvA and EnvB fusion proteins have been shown by SDS-PAGE and western blotting to be similar. It is therefore likely that a structural feature of the EnvA amino acid sequence leads to this altered assembly of VLPs in the cell.

Treatment of the fusion proteins with N-glycosidase F indicated that only the p1:EnvA fusion protein was glycosylated. N-glycosidase F cleaves the N-glycan linkage of glycoproteins between the carbohydrate chain and the asparagine residue to which it is attached and the results with the p1:EnvA fusion protein indicated that a limited amount of N-linked glycosylation had occurred. The shift in mobility of the band observed after treatment suggested that the p1:EnvA fusion protein produced in this system may be relatively heterogeneous in the degree of glycosylation as the position of the leading edge of the band did not alter after N-glycosidase F treatment. If all molecules of the fusion protein had been deglycosylated to the same extent it would have been expected that the entire band on the western blot would have increased in mobility.

Analysis of the DNA sequence data for the Env clones (see chapter 3) shows that EnvA contains 4 potential N-linked glycosylation sites, EnvB 17 and EnvC 6 so the difference in glycosylation is unlikely to be a consequence of the number of such sites. It is possible that the putative signal sequence in the EnvA fragment (section 1.15) may act to target the fusion protein to the lumen of the endoplasmic reticulum, the cellular site for glycosylation. Signal sequences are normally found at the extreme N-terminus of proteins and it would therefore be unusual for an internal signal sequence to be operational. However, in native MVV gp135 the putative signal sequence

is not N-terminal. If this internal signal sequence is functional during the normal replicative cycle of MVV (and the extracellular expression of gp135 in infected cell cultures suggests that it is) then it is conceivable that it could continue to function as a signal sequence when positioned internally in a fusion protein. However, further experiments using deletion mutants or chimeric proteins would be required to confirm this hypothesis.

Glycosylated recombinant proteins have been produced previously using S.cerevisiae expression systems. These include glycoproteins E1 and E2 of Sindbis virus (Wen & Schlesinger, 1986), the VSV glycoprotein (Wen & Schlesinger, 1986) and the influenza virus haemagglutinin (Jabbar et al., 1985). The recombinant proteins were glycosylated in a manner analogous to that of the native proteins and this was dependent on the presence in the coding regions of sequences which control these events eg when the sequence coding for the signal sequence of the influenza haemagglutinin gene was deleted the recombinant product was no longer glycosylated (Jabbar et al., 1985). However in all of these cases the recombinant proteins were not expressed as fusion proteins.

The retrotransposon element from which the p1 coding sequence is derived is an entirely intracellular molecule and the Ty-VLPs are believed to remain endogenous (Mellor et al., 1986). As the p1 protein therefore does not traverse cellular membranes, and is almost certainly not glycosylated it is unlikely that under normal circumstances polypeptides fused C-terminal to p1 would enter such pathways either.

The results demonstrated that the p1:EnvA fusion protein was glycosylated but they did not indicate which region of the fusion protein had been modified. If the presence of the EnvA sequence altered the pathways into which the fusion protein was transported it is conceivable that the glycosylation could have occurred on the p1

region of the protein (which contains 6 potential N-linked glycosylation sites), the EnvA region or both. It is also possible that the glycosylation detected for the p1:EnvA fusion protein does not accurately mimic that found in native gp135. Endoglycosidase F treatment can remove all the N-linked carbohydrate residues on HIV gp120 (Putney et al., 1986) and FIV envelope glycoprotein (Stephens et al., 1991). It is possible that native MVV gp135 is glycosylated in a similar manner to the glycoproteins of HIV and FIV. If this is the case (no direct evidence is currently available) the lack of digestion of p1:EnvA by endoglycosidase F observed in the experiments reported in this chapter may indicate that the glycosylation of the fusion protein in the Ty-VLP system does not accurately reflect that seen in native MVV gp135.

Although there is no direct evidence it is possible that the presence of carbohydrate residues on the p1:EnvA fusion protein may contribute to the difference in the structure of the VLPs observed by electron microscopy.

Despite the lack of normal glycosylation of the Env polypeptides all three Env regions were recognised by sera from sheep naturally or experimentally infected with MVV, demonstrating that immunologically relevant regions were present in the recombinant proteins and were conserved between MVV strains. Two sheep (G027 and 754N) reacted against all three recombinant proteins. At the very minimum therefore these sera recognised epitopes present in the regions where the N and C terminal portions of EnvB overlapped EnvA and EnvC respectively. Sera from sheep 848A only reacted with the EnvC fusion protein and sera from sheep 2 failed to react with EnvA. It would appear therefore that MVV-infected sheep can raise antibodies against a minimum of three epitopes spread throughout gp135, that is one epitope in EnvC recognised by 848A, one epitope probably in EnvB recognised by all sera except 848A and one epitope probably in EnvA recognised by all

sera except 2 and 848A. This is the minimum number of epitopes predicted by the data, but it may be an underestimate.

The fusion proteins were therefore shown to contain regions of immunological relevance as assessed by their ability to react with antibodies from MVV-infected sheep. As both experimentally and naturally infected sheep had raised antibodies which were reactive with the fusion proteins it is likely that the fusion proteins contained epitopes which are conserved between different MVV isolates, and which therefore may be functionally significant in viral activity, in binding to a cellular receptor for example or in post-receptor binding events.

Attempts to use these fusion proteins to raise antisera which reacted with Env glycoprotein from MVV-infected cells were unsuccessful. Since antisera from infected sheep reacted with the recombinant protein it would seem unlikely that the failure to detect anti-Env specific antibodies after immunisation of rabbits and mice with the fusion proteins was due to differences in the Env sequences of the "natural" gp135 and the fusion proteins. It is also unlikely that the failure is due to the predominantly unglycosylated nature of the Env fragments as antisera can be raised to unglycosylated HIV gp120 which react by western blotting with gp120 purified from infected cell lysates, and which can neutralise viral infectivity (Putney et al., 1986).

The immunised animals all raised a readily detectable anti-p1 response, which indicated that sufficient antigen had been administered to elicit a response. EnvA and EnvB are similar in molecular weight to p1, and EnvC is just under half its size. All three would therefore be expected to be present in sufficient quantities to act as immunogens. However, p1 appears to be highly immunogenic and it is possible that the Env sequences were intrinsically less so and that therefore more antigen

would be required to elicit an Env-specific response.

It is possible that the response to p1 detected in the immunised animals represented the outcome of a secondary immune response to a previously encountered antigen, rather than a primary response. Section 5.5 reports the detection of an anti-p1 response in a rabbit which had been immunised with a GST:EnvC fusion protein preparation, but which had not been immunised with p1 or a p1 fusion protein. This suggests that laboratory animals may encounter p1 proteins naturally, possibly as a result of exposure to yeast. Although at the time of initial immunisation with the p1:Env fusion proteins no anti-p1 response was detectable as shown by using pre-immunisation sera from these animals, this does not exclude the possibility of previous exposure. Consequently immunisation with a p1:Env fusion protein may stimulate a secondary immune response to the p1 region of the fusion protein, and the response elicited to the remainder of the fusion protein may be low.

Hybrid VLPs have been used successfully as immunogens (Adams et al., 1987b; Gilmour et al., 1989; Griffiths et al., 1991) but there are two possible reasons why they may not have been effective in the experiments reported in this results chapter. Higher doses of antigen may be required to promote a fusion protein-specific response than were available here. For example, in raising antisera to the HIV-1 gp120 V3 loop region in this system rabbits were immunised five times with 500ug of VLPs, estimated to contain 50ug of the HIV-specific polypeptide (Griffiths et al., 1991). The low yields of the fusion proteins in the experiments reported here precluded using antigen at these concentration levels. Many fusion proteins in the Ty-VLP system are now produced with a factor Xa cleavage site between the p1 and inserted protein, allowing the foreign protein to be purified away from the p1 (Gilmour et al., 1989) and this purified

protein can be used to boost the non-p1 response of the immunised animal. It has been discovered that this may be essential for eliciting a strong antibody response to the non-p1 component of the fusion protein (Reyburn et al., 1992 (MVV Gag)) (I.Green (ovine tumour necrosis factor alpha) Department Of Veterinary Pathology, University Of Edinburgh, personal communication). As the fusion proteins generated in the experiments reported in this chapter did not contain a factor Xa cleavage site this option could not be followed. However, given the difficulties encountered in purifying the hybrid VLPs it is likely that factor Xa cleavage would not have been a viable option as the level of contaminating proteins was so high that digestion with factor Xa would almost certainly have led to considerable cleavage of these cellular proteins, rendering purification of the cleaved Env polypeptide impossible.

4.11 Summary

The yeast Ty-VLP expression system was used to generate fusion proteins of p1 with fragments of the MVV env gene and the proteins were characterised. The Env sequences markedly influenced the expression of the fusion proteins and the assembly of the VLPs, but had no obviously toxic effect on the cells. Although glycosylation was shown to be either limited or negligible the Env fragments were reactive with sera from MVV-infected sheep and indicated that recognised antigenic epitopes are present in at least three sites on gp135. Attempts to use the recombinant proteins as immunogens were unsuccessful.

5. The Generation And Characterisation Of Recombinant MVV gp135 Fragments - The Bacterial pGEX System

5.1 Introduction

Chapter 4 described the production of recombinant MVV gp135 fragments in the yeast Ty-VLP expression system. Although expression of p1:Env fusion proteins was achieved certain difficulties were encountered which could limit the applications of these recombinant proteins, including low yield, unsatisfactorily high levels of contaminating yeast proteins and no detectable antibody response to the gp135 region of the fusion protein following immunisation of mice and rabbits. In an attempt to overcome some of these difficulties it was decided to express the env gene fragments in an alternative system.

The E.coli pGEX expression system was chosen as it possesses a number of features advantageous when generating recombinant proteins for subsequent use as immunogens. The fusion proteins produced in this system are generally soluble in aqueous solution, they are purified easily from lysed cells under non-denaturing conditions by an affinity reaction and have been used successfully as antigens (Smith & Johnson, 1988). Although fusion proteins expressed in E.coli are not usually glycosylated this was not expected to present a major problem in terms of the antibody response. Unglycosylated fragments of HIV gp120 have been used successfully as immunogens for the production of neutralising antisera (Putney et al., 1986).

The pGEX plasmids used to generate the fusion proteins are shown in figure 5.1. The coding sequences for the recombinant protein are inserted at the 3' end of the glutathione S-transferase (GST) gene of Schistosoma japonicum (Smith et al., 1988) under the control of the strong tac promoter (Smith & Johnson, 1988). The promoter is repressed in the absence of the inducing agent (IPTG)

Figure 5.1

The pGEX1 Expression Vector

The pGEX1 expression vector is shown in the diagram, with the cloning site of each vector reproduced underneath. Single restriction enzyme sites on the plasmid are also marked. The protein cleavage sites for thrombin and factor Xa in the sequences for pGEX2T and pGEX3X respectively are shown.

by the product of the lacI^q allele on the plasmid. Transformants are selected by ampicillin resistance.

Each pGEX vector contains a BamHI site at the 3' end of the gst gene and the reading frame at this cloning site is different in each vector. pGEX2T and pGEX3X also encode a thrombin cleavage site and a factor Xa cleavage site respectively at the insertion site of the foreign sequence (figure 5.1). These allow proteolytic cleavage of the fusion protein into the carrier protein and the expressed protein of interest.

Native GST protein is not sequestered into inclusion bodies after production in transformed E.coli. Following ultrasonic disruption of the cells the protein can be purified by affinity binding to glutathione cross-linked to agarose beads. Bound protein can be eluted in an excess of reduced glutathione.

Proteins which have been successfully expressed in the pGEX system include leukaemia inhibitory factor (Gearing et al., 1989), human pyruvate dehydrogenase E1alpha (Iwayama et al., 1991), a species-specific tegument protein of Echinococcus multilocularis larvae (Frosch et al., 1991), part of the 77kDa merozoite protein of Babesia bovis (Tetzlaff et al., 1992), and the gag proteins of HIV-1, HIV-2 and SIV (Mills et al., 1992). The fusion proteins have been successfully used as antigens (Frosch et al., 1991; Mills et al., 1992; Tetzlaff et al., 1992) and the antisera raised all reacted with the relevant native proteins. The B.bovis merozoite antigen has also been shown to stimulate proliferation of T lymphocytes from B.bovis-infected cattle (Tetzlaff et al., 1992). Therefore proteins expressed in the pGEX system have been shown to resemble the native proteins in the immune responses which they stimulate.

The GST expressed from the pGEX plasmids has a molecular weight of 27.5kDa and fusion proteins with overall molecular weights ranging from 32kDa to 84kDa have

been expressed (Smith & Johnson, 1988). The major technical problem encountered with the expression system is low yields of proteins which contain highly hydrophobic regions. These appear to lead to the formation of insoluble fusion proteins and sequestration into intracellular inclusion bodies.

The env gene fragments were excised from the pOGS-based plasmids into which they had been cloned (chapter 4), ligated into the appropriate pGEX plasmid and transformed into E.coli JM83. Clones which contained an insert of the correct molecular size were further screened by diagnostic restriction digestion and analysed for recombinant protein expression. The effect of expression of the fusion protein on the growth of E.coli was monitored and the expression of the fusion protein compared with production of native GST. Serum from an MVV-infected sheep was used to confirm that antigenically relevant MVV gp135 fragments had been expressed and the fusion proteins were used as immunogens in rabbits and mice.

5.2 Generation Of Recombinant pGEX/Env Plasmids

The pOGS-based clones were chosen in preference to the pTZ-based clones as a source of the env gene fragments in order to minimise cloning difficulties caused by contamination with plasmids during the purification of the desired fragment (section 4.2). As the pOGS plasmids are larger than pGEX (9.4kb and 4.9kb respectively) the probability of contamination by pOGS plasmids during the cloning procedure would be reduced.

The env fragments were excised from the recombinant pOGS-based plasmids with BamHI, gel-purified and ligated into the appropriate pGEX vector which had been digested with BamHI and treated with CIP. In order to maintain the env sequences in the correct reading frames envA was inserted into the BamHI site of pGEX1 and envB and envC were separately inserted into the BamHI site of pGEX2T.

The recombinant DNA was then transformed into competent E.coli JM83.

484 colonies were screened for the envA fragment, of which 13 hybridised to an envA-specific probe by colony hybridisation. The DNA from all 13 clones was screened by restriction digestion and gel analysis. All 13 contained a single insert of the expected molecular weight which could be excised with BamHI. Digestion with BglI was used to determine orientation of the insert. pGEX1 contains BglI sites at nucleotides 2009 and 4652 and envA contains a BglI site at nucleotide 565. Insertion of the envA fragment into the BamHI site at nucleotide 914 of PGEX1 in the 5' to 3' orientation results in predicted BglI fragments of 1476, 1759 and 2643 base pairs. Insertion of the fragment in the opposite orientation results in a predicted restriction pattern of 1575, 1660 and 2643 base pairs after digestion with BglI. All the clones analysed by BglI restriction digestion analysis had a restriction pattern consistent with insertion of the envA fragment in the 3' to 5' orientation. DNA from one clone was digested with BamHI and then re-ligated in an attempt to generate recombinant molecules containing the envA fragment in the 5' to 3' orientation. The resulting DNA was used to transform E.coli JM83 and of the 97 colonies screened 2 hybridised to an envA-specific probe by colony screening. Both clones contained a single insert of the expected molecular weight which could be excised with BamHI, but in each case restriction digestion analysis with BglI demonstrated that the envA fragment was inserted in the 3' to 5' orientation.

Therefore from a total of six separate ligations and transformations no clone was obtained containing envA inserted in the 5' to 3' orientation in pGEX1. Possible reasons for this inability to obtain transformants which contain a suitable recombinant pGEX1/envA plasmid are considered in section 5.6.

32 colonies were screened for the envB fragment, of

which 18 hybridised to an envB-specific probe by colony screening. The DNA from 4 clones was screened by restriction digestion and gel analysis. All 4 contained a single insert of the expected molecular weight which could be excised with BamHI. Digestion with EcoRV was used to determine orientation of the insert. pGEX2T contains an EcoRV site at nucleotide 4070 and envB contains an EcoRV site at nucleotide 214. This results in predicted sizes of 1979 and 3868 base pairs for an insert in the 5' to 3' orientation and 2466 and 3381 base pairs for an insert in the opposite orientation after EcoRV digestion. 2 of the clones had a restriction pattern with EcoRV consistent with 3' to 5' orientation, one clone cut very poorly with EcoRV and the digestion pattern of the remaining clone, gB52, was consistent with insertion in the 5' to 3' orientation. gB52 was therefore selected as the clone for expression of the GST:EnvB fusion protein.

60 colonies were screened for the envC fragment of which 34 hybridised to an envC-specific probe by colony screening. The DNA from 4 clones was screened by restriction digestion and gel analysis. All 4 contained a single insert of the expected molecular weight which could be excised with BamHI. Digestion with HincII was used to determine orientation of the insert. pGEX2T contains HincII sites at nucleotides 184, 1585 and 4137 and envC contains a HincII site at nucleotide 216. This results in predicted sizes of 966, 972, 984 and 2552 nucleotides for an insert in the 5' to 3' orientation, and 887, 972, 1063 and 2552 nucleotides for an insert in the opposite orientation after HincII digestion. 3 of the clones had a HincII digestion pattern consistent with an insert in the 3' to 5' orientation while the pattern for one clone, gC53, was consistent with insertion in the 5' to 3' orientation. gC53 was therefore selected as the clone for expression of the GST:EnvC fusion protein.

5.3 Protein Expression From The pGEX/Env Clones in E.coli

Transformed cultures were induced under identical conditions (section 2.17.2.2), the cells harvested and the fusion proteins purified. Purified material from each culture was separated by SDS-PAGE and the gel silver stained. The results are shown in figure 5.2. There was an obvious difference in the relative yields of GST, GST:EnvB and GST:EnvC. The highest yields were obtained for native GST and the lowest for GST:EnvC. Native GST migrated with an apparent molecular weight consistent with the predicted molecular weight of 27.5kDa. The GST:EnvB fusion protein migrated with an apparent molecular weight of 59kDa and the GST:EnvC fusion protein migrated with an apparent molecular weight of 45.5kDa. These were broadly consistent with the predicted molecular weights of 61.7kDa and 47.6kDa for the GST:EnvB and GST:EnvC fusion proteins respectively.

Contaminating bands were also visible in silver stained gels. These varied slightly between preparations, probably due to minor differences in steps in the purification procedure such as lysis, affinity binding or washing of the glutathione-agarose beads. A cluster of bands migrating at approximately 30kDa were routinely observed and are shown in the GST:EnvB track in figure 5.2. These possibly represent endogenous E.coli GST proteins (eGST). In some preparations bands with apparent molecular weights of 61kDa to 72kDa were also visible (data not shown). Similar contaminating bands have also been observed in GST:fusion protein preparations by other authors (Tetzlaff et al., 1992).

It was not possible to estimate the protein concentrations of the GST:Env fusion proteins by a dye binding assay when the proteins were bound to the glutathione-agarose beads, as poor results are obtained in such assays when the proteins are in a particulate form. Although an assay would have been feasible using eluted

Figure 5.2

Purified native GST and GST:Env fusion proteins

Purified native GST and GST:Env fusion proteins were separated on a 10% SDS-PAGE gel and detected by silver staining. The native GST and GST:Env fusion protein bands are indicated by arrows.

Mol. Wt. / kDa

67

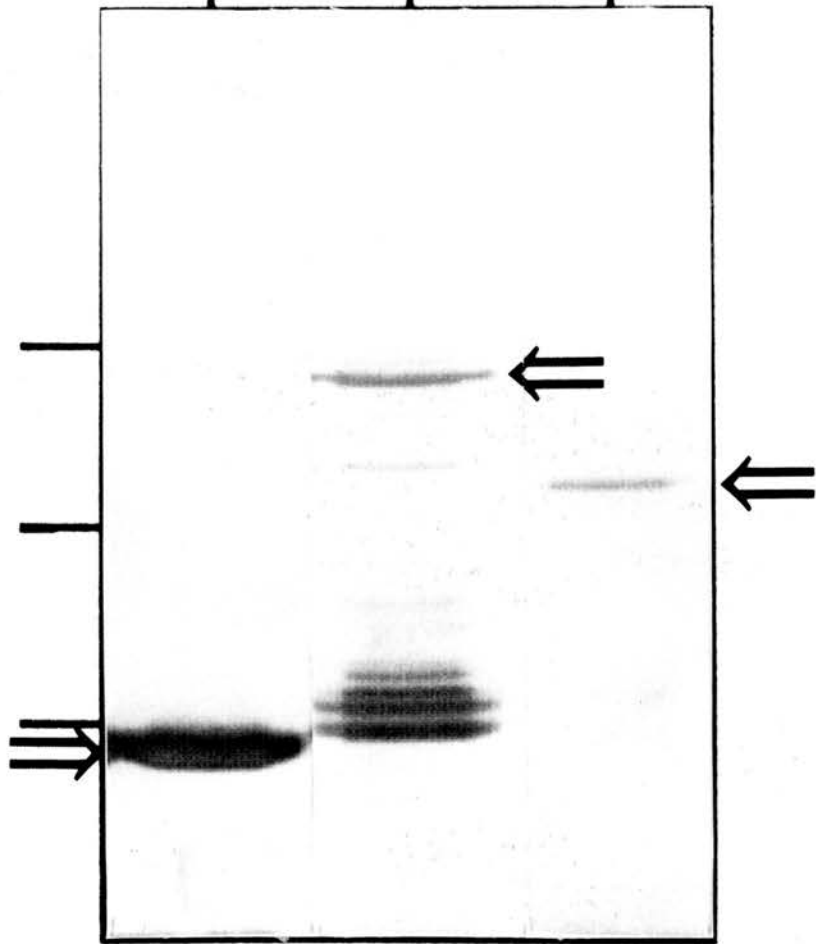
43

30

GST

GST:EnvB

GST:EnvC



protein it would have been necessary to introduce various estimations into the final calculation of fusion protein yield, including the proportion of the protein either uneluted or lost during elution, and the proportion of the total protein represented by the fusion protein itself. As such estimates could introduce large errors into the final values obtained the approximate yields were simply estimated from the silver stained gels, and were in the range of ug/L for the GST:Env fusion proteins and mg/L for GST.

Western blotting with serum from sheep 754N was used to confirm that the fusion proteins contained gp135-specific sequences. Sheep 754N was experimentally infected with MVV strain EV1 and sera from this sheep reacts with gp135 in MVV-infected cell lysates, and with the p1:Env fusion proteins (section 4.8). The reactivity of this serum with the GST:Env fusion proteins is shown in figure 5.3. Although the relevant bands are difficult to see clearly on the photograph of the blots (due to the strong cross-reacting bands migrating with apparent molecular weights similar to those of the fusion proteins) specific reactivity of serum 754N with the fusion proteins was demonstrated. The GST:EnvB band migrates in the expected position, just below a more heavily staining band which is also detected by NSS. The GST:EnvC band also migrates in the expected position, just below a band detected by serum 754N in the GST preparation. There was no detectable reactivity of serum 754N with the native GST protein despite overloading of this protein relative to the fusion proteins, as determined by silver staining of a gel (data not shown). A number of contaminating bands were also detected by both the 754N sera and NSS. These probably represent contaminating bacterial proteins to which the sheep have raised antibody responses during normal exposure to E.coli.

The fusion proteins could be successfully eluted from

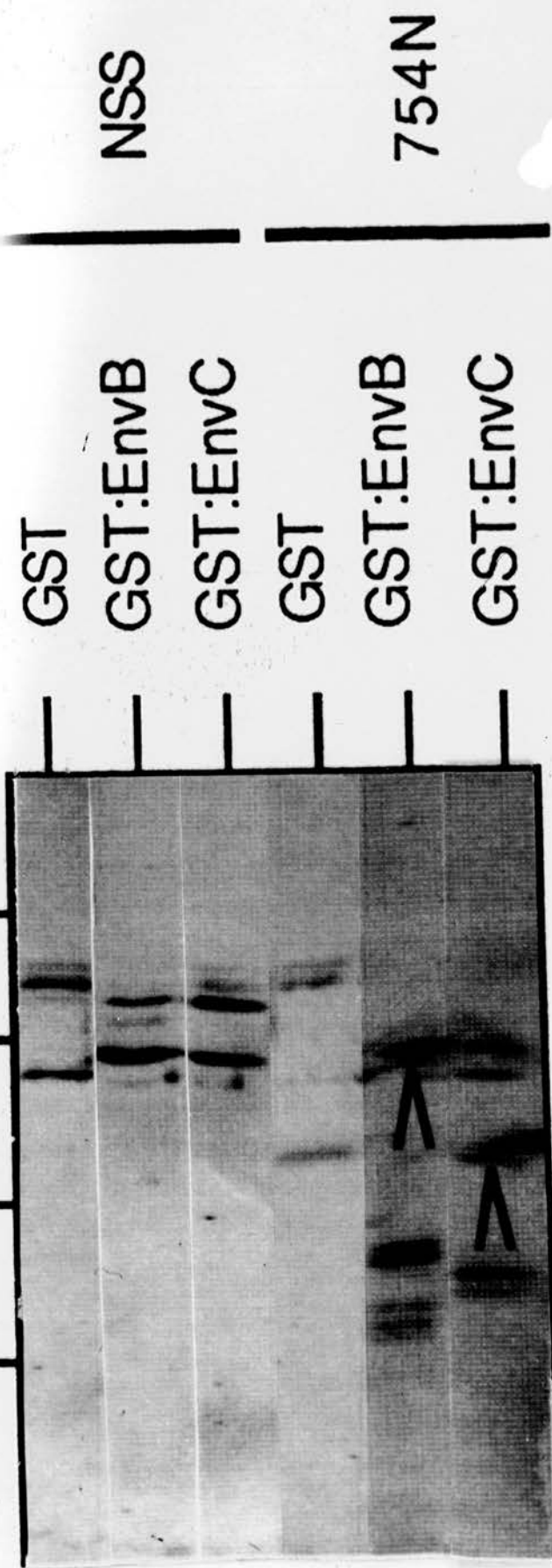
Figure 5.3

Reactivity of MVV-infected sheep serum with GST:Env fusion proteins

Purified GST and GST:Env fusion proteins were separated on a 10% SDS-PAGE gel and blotted onto nitrocellulose. Strips were developed with normal sheep serum (NSS) or MVV-infected sheep serum (754N), @ 1 in 20 and anti-sheep alkaline phosphatase @ 1 in 1000. The GST:Env fusion protein specific bands are indicated by arrows.

Mol. Wt. / kDa

94
67
43
30



the glutathione-agarose beads in the presence of an excess of reduced glutathione. The relative levels of contaminating proteins fell with sequential elution steps leading to increased purity of the eluted protein, albeit with a concomitant decrease in overall fusion protein concentration (data not shown).

It is apparent that the yields of the fusion proteins were considerably lower than the yields of native GST. This appears to reflect a relative decrease in the production of the fusion proteins when compared with the native GST, rather than an indirect effect such as reduced affinity of the GST region of the fusion protein for the glutathione-agarose in the presence of Env fragments. This is demonstrated in figure 5.4 in which crude extracts of the various clones, obtained by pelleting cells from induced cultures and resuspending these in PBS were analysed by SDS-PAGE. The non-recombinant GST band is clearly visible in a Coomassie-stained gel, but a band corresponding in position to GST:EnvB is only barely visible, and no discrete GST:EnvC band can be distinguished. This suggested that expression of the fusion proteins from the recombinant plasmids was less efficient than expression of GST from the non-recombinant pGEX plasmid alone.

5.4 Growth Characteristics Of E.coli Clones Transformed With pGEX/Env

In order to determine if the low yields obtained for the fusion proteins were a consequence of a toxic effect of the constructs on the growth of E.coli a time-course of cell growth after fusion protein induction was performed. Triplicate cultures were induced when the optical densities at 600nm (OD₆₀₀) of the cultures were between 0.35 and 0.5, and growth of the cultures was monitored at 30 minute intervals for 3 hours. At the end of the time-course a protein sample was analysed by SDS-PAGE and

Figure 5.4

Crude extracts from E.coli expressing native GST or GST:Env fusion proteins

Crude extracts were made from cells expressing native GST or GST:Env fusion proteins, separated on a 10% SDS-PAGE gel and detected by Coomassie blue staining. Native GST and GST:EnvB fusion protein bands are indicated by arrows. The expected position of GST:EnvC is indicated by a bracket.

Mol. Wt. / kDa

30

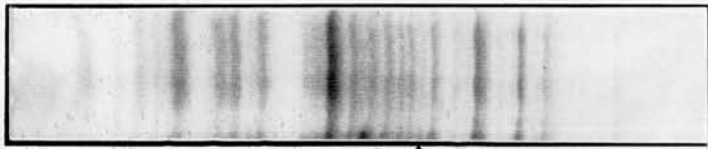
43

67

94



GST



GST:EnvB



GST:EnvC

silver staining to confirm that expression of the fusion proteins had been induced (data not shown).

Figure 5.5 summarises the growth characteristics of the different cultures. The clone transformed with the non-recombinant pGEX plasmid continued to grow vigorously after induction of GST but growth of clones gB52 and gC53 was dramatically reduced following protein induction. This reduction in growth was apparent within one hour post-induction and suggests that expression of the fusion proteins has a toxic effect upon cell growth.

In an attempt to improve the yields of the recombinant proteins cultures were induced at different cell densities. The cultures were either induced at an OD₆₀₀ of 0.45 or of 0.6 to 0.7. After induction the cultures were incubated for a further 3 hours, the cells harvested and the fusion proteins purified. The purified proteins from equivalent volumes of each culture were analysed by SDS-PAGE. Figure 5.6 shows the result of varying the cell density at which expression of the fusion protein was induced. Expression of native GST from the pGEX2T plasmid fell when the culture was induced at the higher OD₆₀₀ reading but the opposite effect was apparent for the GST:EnvB and GST:EnvC fusion proteins. Therefore by varying the point in the growth curve at which fusion protein expression is induced in these clones the yields (per volume) of the recombinant proteins have been significantly improved.

5.5 Immunisation Of Rabbits And Mice With GST:Env Fusion Proteins

In an attempt to raise anti-gp135 specific antisera rabbits and mice were immunised with the fusion proteins. Initially mice were immunised at 2 sites subcutaneously with approximately 150ng of eluted fusion protein in Complete Freund's Adjuvant. After 4 weeks the mice were boosted with a similar amount of eluted fusion protein in

Figure 5.5

Time course of cell growth after induction of E.coli clones expressing native GST or GST:Env fusion proteins

OD600 values were monitored for cultures of E.coli clones pGEX2T (ie transformed with the plasmid pGEX2T), gB52 or gC53. The induction point is indicated by an arrow.

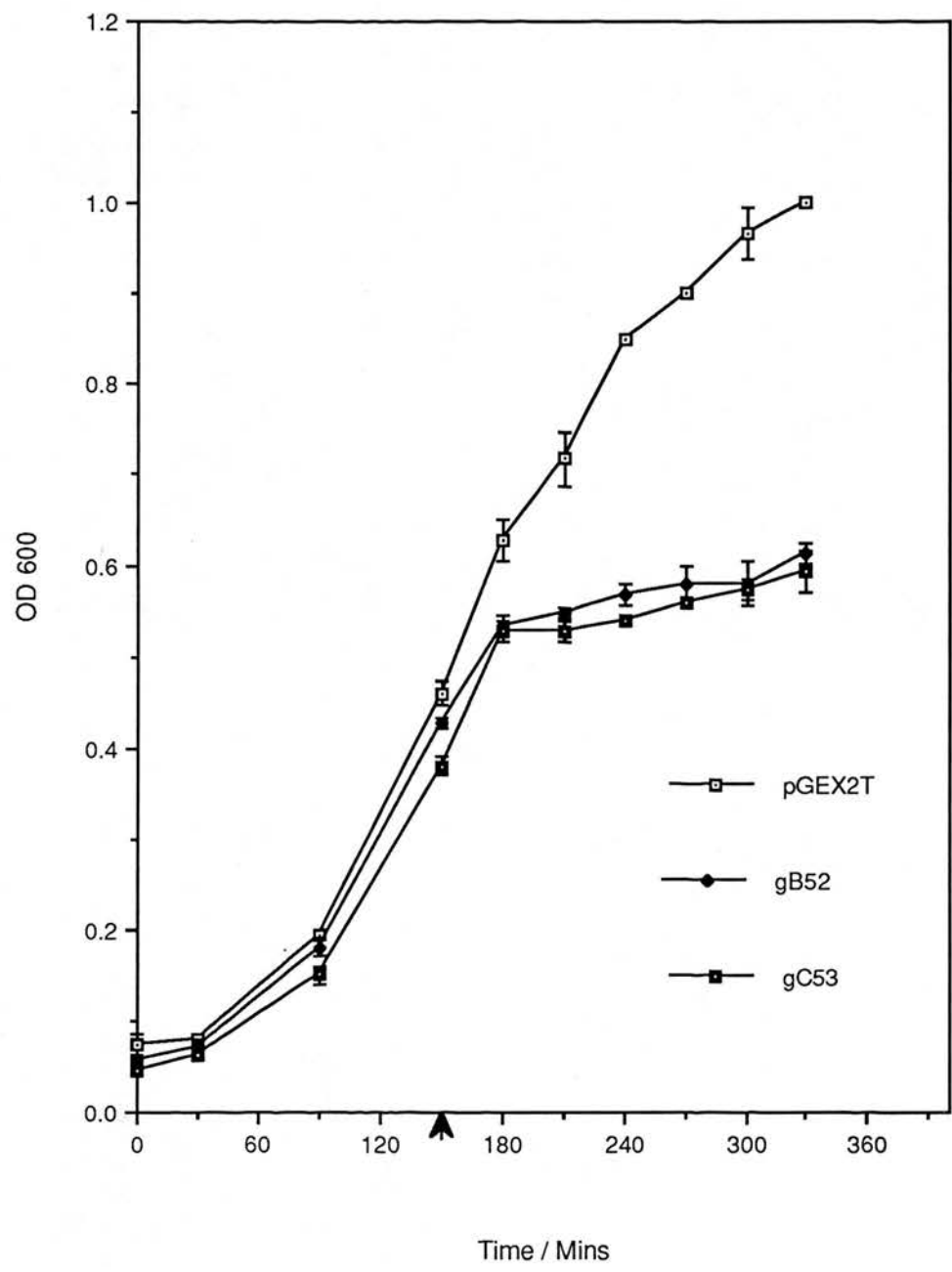
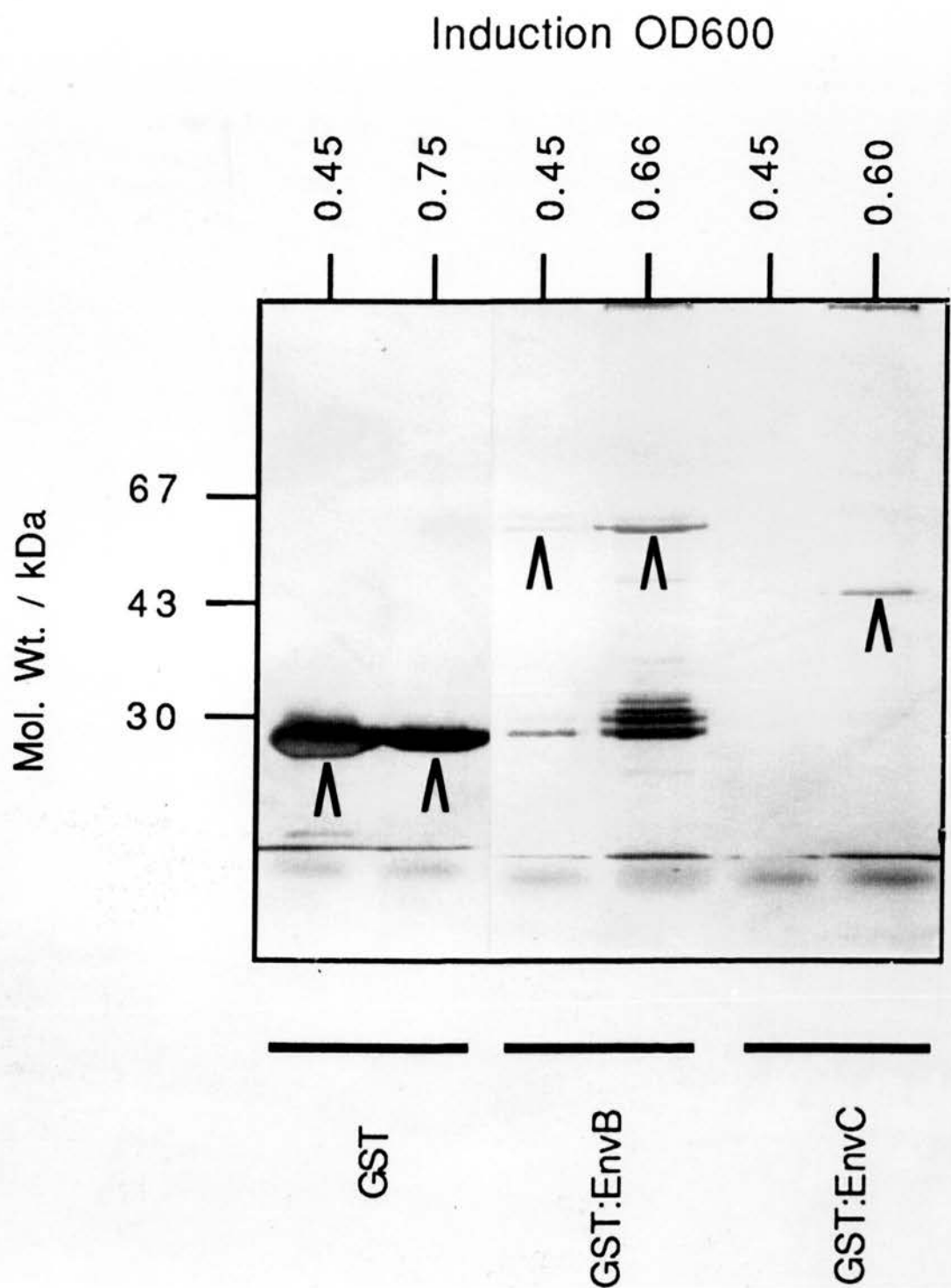


Figure 5.6

The effect of cell density on the induction of native GST and GST:Env fusion proteins

Cultures of E.coli clones pGEX2T (ie transformed with the plasmid pGEX2T), gB52 and gC53 were induced at the OD600 values shown at the top of the figure. Purified native GST and GST:Env fusion proteins were separated on a 10% SDS-PAGE gel and detected by silver staining. The native GST and GST:Env fusion protein bands are indicated by arrows.



Incomplete Freund's Adjuvant, and this was repeated once more for GST:EnvB. Rabbits were immunised at 2 sites subcutaneously with 450ng of eluted fusion protein in Complete Freund's Adjuvant - immunisations B1 and C1 (I_{B1}, I_{C1}), and this was repeated once for the GST:EnvC fusion protein (I_{C2}) and twice for the GST:EnvB fusion protein (I_{B2}, I_{B3}), with the Complete Freund's Adjuvant replaced by Incomplete Freund's Adjuvant. 1ug of the appropriate uneluted fusion protein (that is where the fusion protein was still attached to the glutathione-agarose beads) in Incomplete Freund's Adjuvant was then used to boost the immune response in each rabbit (I_{B4}, I_{C3}).

Only a limited number of tail bleeds can be obtained from a mouse and therefore the rabbits alone were screened initially for a gp135-specific response. When a strongly positive anti-gp135 response was obtained from the rabbits screening of the mouse sera would follow. The fusion proteins had not been cleaved into GST and Env regions therefore to distinguish anti-EnvB/EnvC responses from anti-GST responses the sera were screened against the yeast-derived p1:Env proteins. The sera were also screened against native GST to determine whether a response had been elicited to the "carrier" protein. The reactivity of the sera against protein blots of MVV-infected cell lysates was also analysed.

Figure 5.7a shows that the sera from the immunised rabbits at a dilution of 1 in 100 reacts with a band on a western blot of an eluted GST preparation which had a molecular weight consistent with that of GST. Sera from rabbit 216 which was immunised with the GST:EnvC antigen also recognised a band of unknown identity with an apparent molecular weight of approximately 68kDa.

Figure 5.7b-d shows the results obtained after western blotting of p1, p1:EnvB and p1:EnvC with sera from the immunised rabbits 11 days after I_{B4} and I_{C3} . The

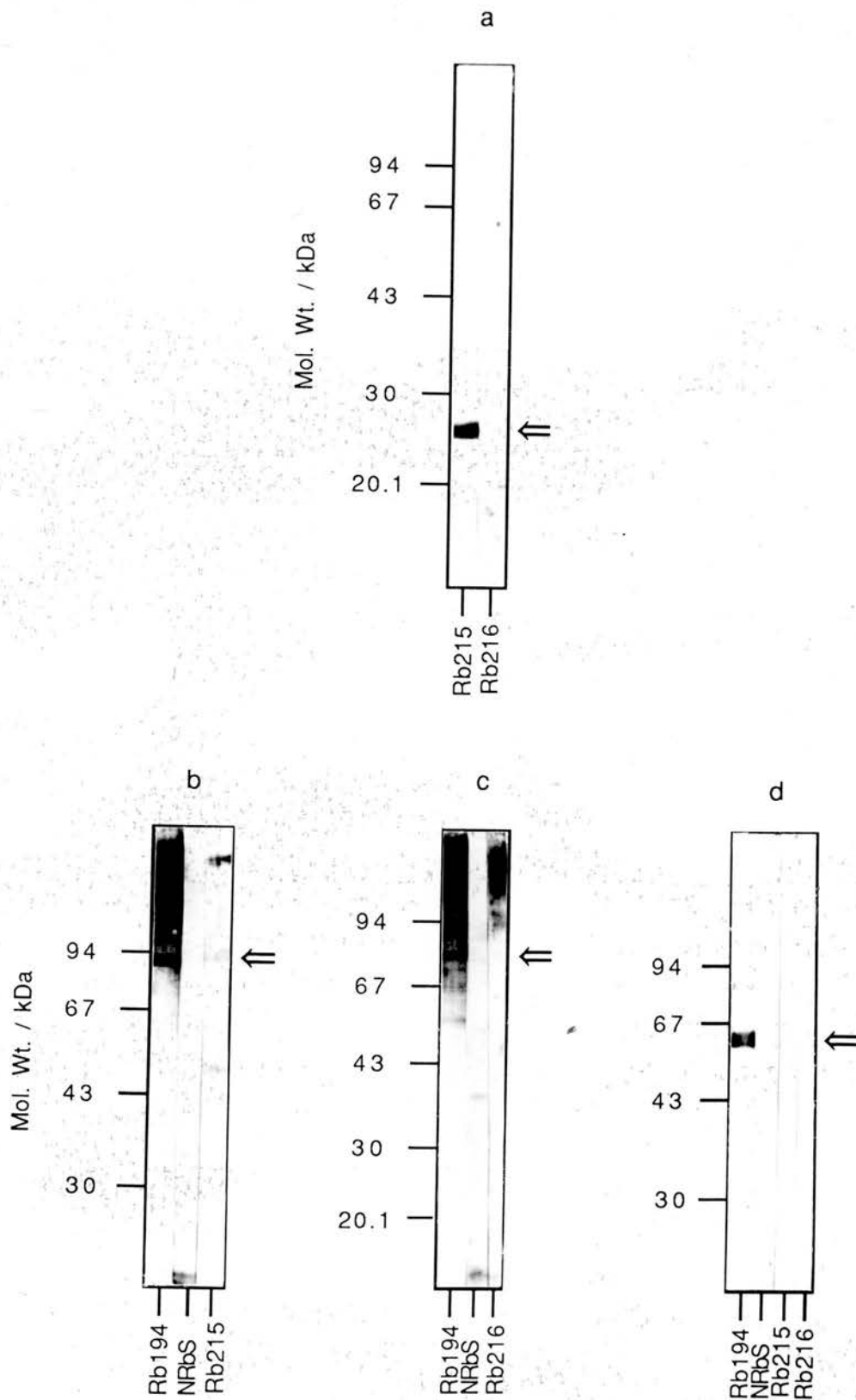
Figure 5.7

Reactivity of sera from rabbits immunised with GST:EnvB and GST:EnvC fusion proteins

Purified proteins were separated on a 10% SDS-PAGE gel and blotted onto nitrocellulose. Strips were developed with normal rabbit serum (NRbS), rabbit serum 215 (Rb215) (immunised with GST:EnvB) or rabbit serum 216 (Rb216) (immunised with GST:EnvC), all @ 1 in 100, or rabbit anti-VLP serum (Rb194) @ 1 in 1500, followed by anti-rabbit alkaline phosphatase @ 1 in 1000. The relevant bands are indicated by arrows.

Key

- a blot of purified native GST
- b blot of purified p1:EnvB
- c blot of purified p1:EnvC
- d blot of purified p1



results with rabbit serum 194 (the polyclonal rabbit anti-VLP serum) show that p1 was loaded in excess on the blots in comparison to p1:EnvB and p1:EnvC. Normal rabbit serum did not react with any of the antigen preparations. Serum from rabbit 215 which was immunised with the GST:EnvB preparation reacted with p1:EnvB but showed no detectable reactivity with p1 alone. Rabbit 215 therefore had raised antibodies specific to the EnvB region of the fusion protein. No reactivity was detected with the gp135 on a western blot from an MVV-infected cell lysate (data not shown).

When the serum from rabbit 216 (which was immunised with the GST:EnvC fusion protein) was screened 14 days after I_{C2} it was found to be strongly reactive with p1, suggesting possible exposure of the rabbit to yeast proteins. The same serum was also faintly reactive with p1:EnvC but given the cross-reactivity observed with p1 alone this could not be considered as gp135-specific (data not shown). When serum from the same rabbit was screened 11 days after I_{C3} the anti-p1 response had decreased, and no band is visible on the p1 blot in figure 5.7d. No reactivity was apparent with p1:EnvC (figure 5.7c) or with the gp135 on an MVV-infected cell lysate (data not shown). It was concluded that rabbit 216 had not mounted a significant humoral immune response to the EnvC portion of the fusion protein. At the time of this negative screening rabbit 216 had a respiratory infection and had developed a large solid mass under its jaw. It was therefore decided that euthanasia was the most appropriate action for this animal.

Rabbit 215 was again boosted with uneluted GST:EnvB and all the mice were boosted with the appropriate uneluted fusion protein. Immunisation of a new rabbit with GST:EnvC was also initiated. The results of these immunisations were not available at the time of writing of this thesis.

5.6 Discussion

This chapter describes the successful generation of clones which express the envB and envC regions of the MVV env gene as fusion proteins with GST. Expression of the fusion protein from these clones has been analysed and conditions determined which improve the yield of these proteins. The proteins have also been employed as immunogens to generate Env-specific antibodies.

Although GST:EnvB and GST:EnvC fusion proteins were successfully expressed the yields of these were low compared with the native GST protein. The poor yields appeared to be predominantly a consequence of low expression of the fusion proteins, rather than a purification problem such as sequestration of the fusion protein into inclusion bodies or steric hindrance of binding of the GST moiety of the fusion protein to glutathione-agarose. The barely detectable levels of the fusion proteins in crude cell lysates indicated that the induced cells only expressed the fusion proteins to a limited extent and the growth curves for induced cultures suggested that expression of the fusion proteins had a toxic effect upon E.coli growth. This is in contrast to the results obtained with S.cerevisiae expressing p1:Env fusion proteins, in which yields of the fusion proteins were low, but this was not a consequence of any detectable toxic effect of the fusion proteins upon cell growth. This implies that the toxicity of the env-encoded regions of the fusion proteins is very dependent on the cell type in which the protein is expressed.

It may be possible to increase the yields of the fusion proteins from clones gB52 and gC53 by altering certain conditions. The data presented in this chapter clearly shows that the yields can be improved by inducing the cultures at a relatively high cell density. As the fusion proteins appear to exert a toxic effect upon cell growth it is advantageous to induce expression of the

fusion proteins when the number of cells present in the culture is high, as this will lead to the greatest production of fusion protein in the absence of effective cell division. It is interesting that the opposite trend was observed for the native pGEX plasmid, suggesting that for proteins which are not toxic the greatest yields can be obtained by inducing the cultures at a relatively low cell density, and allowing the cells to grow and divide while expressing protein.

It may be possible to increase the yields of the fusion proteins by transforming the recombinant plasmids into a different strain of E.coli. In comparative studies using pGEX-based plasmids expressing the human 68kDa (U1) ribonucleoprotein antigen (Frorath et al., 1991) or rat interleukin-6 (Frorath et al., 1992) the highest expression of the fusion proteins in each case was observed in E.coli strain LE392. Although E.coli strain JM83 was not used in these studies this may be a means by which expression of the GST:EnvB and GST:EnvC fusion proteins could be improved. The authors presented no data on the magnitude of the differences in yield between the strains, or the mechanism for the variation.

The yields of the fusion proteins may also be affected by the concentration of IPTG used to induce the cultures. In contrast to the 0.1mM concentrations used to induce the fusion proteins reported in this chapter other workers have used higher levels of the inducing agent, ranging from 0.5mM (Frorath et al., 1991; Frorath et al., 1992) to 10mM (Frosch et al., 1991; Iwayama et al., 1991). The fusion protein yields may also be influenced by the length of time for which the cultures are induced. Due to lack of time it was not possible to investigate these aspect of the fusion protein expression.

The reactivity observed for sheep serum 754N with the GST:EnvB and GST:EnvC fusion proteins by western blotting confirmed that these fusion proteins contained MVV

epitopes against which infected sheep raise antibodies. Although it would be possible to repeat the studies of sera from infected sheep which were detailed in section 4.8 the GST:Env fusion proteins are probably less suited to this work than the p1:Env fusion proteins. This is because there is currently no anti-GST antisera available and therefore it is difficult to confirm that different blots all contain sufficient and equivalent amounts of the GST/GST:Env fusion proteins. Although this can be checked by silver staining this is not as satisfactory as being able to assay the amount on the actual blot which is to be tested, as is possible when the anti-VLP antisera is used with the p1:Env fusion proteins. An indirect check by silver staining has the disadvantages that it relies on precision when loading the relevant samples on separate gels (or different parts of the same gel) and it may be difficult to predict from a silver stained gel if sufficient antigen has been loaded to be detectable by western blotting.

Immunisation of a rabbit with GST:EnvB resulted in the production of anti-EnvB antibodies, as shown by their ability to react with p1:EnvB on a western blot. The time-scale of production was long, and the response did not appear to be strong. This was probably a consequence of the relatively low doses of the fusion protein used to immunise the rabbit. In other experiments it has been reported that rabbits have been immunised with 100ug of fusion protein, of which approximately 30% was the non-GST moiety (Oettinger et al., 1992) in order to raise specific antisera. This is approximately fifty times greater than the estimated amount of purified EnvB with which rabbit 215 was immunised in the results reported in this chapter. No reactivity was observed against gp135 on a western blot of an MVV-infected cell lysate. This was possibly due to a combination of the low titre of the rabbit serum and the relatively low levels of this antigen on the blot.

Alternatively the rabbit may only have raised detectable antibodies against a very limited number of epitopes at this point in its immune response. There is a possibility that the epitopes could be masked by carbohydrate residues in the cell-derived MVV gp135, or that they may be epitopes which vary between MVV isolates. DNA sequencing of envB has shown areas of high amino acid variability between different isolates (chapter 3). It is likely that with further immunisations the response to the EnvB region of the fusion protein will increase and under such circumstances it would be expected that reactivity with MVV gp135 from infected cells would be observed.

The failure to raise rabbit antisera specific to EnvC may also be a consequence of the low doses of the immunogen, especially as EnvC forms a smaller percentage of the total mass of the fusion protein than EnvB (42% and 55% respectively). However the results obtained with GST:EnvB suggest that it will be possible to elicit an antibody response to the fusion protein, especially if uneluted fusion protein is used as the immunogen. This has been shown to be an efficient mechanism for provoking an antibody response (Oettinger et al., 1992) in this system. This may be because by using the uneluted material decreases in final yield due to non-elution of some of the fusion protein, dilution of the sample during elution, and losses during any subsequent concentrating procedures (eg acetone precipitation of the protein) are minimised. Additionally the fusion protein bound to the glutathione-agarose beads may act as a polymeric antigen. Polymeric antigens are generally more immunogenic than monomeric antigens, and therefore may stimulate a stronger antibody response.

Sera from both rabbit 215 and rabbit 216 reacted with a band of the expected molecular weight for native GST by western blotting. However, this does not necessarily indicate that these animals had been immunised with

sufficient of the GST:Env fusion proteins to stimulate an immune response to the GST portion of the fusion protein. The fusion protein preparations which were used to immunise the animals usually contained contaminating proteins with molecular weights of approximately 30kDa, which were believed to represent the endogenous GST protein (eGST) produced by E.coli. It is possible that the anti-GST response observed in the immunised rabbits was originally stimulated by eGST protein, but that there was enough conservation of epitopes between this and the native GST encoded by the pGEX plasmid to allow cross-reactivity of the antisera on a western blot.

Although no data was available on the response of the mice to immunisation with the GST:Env fusion proteins whilst writing this thesis it is likely that if a response can be stimulated in rabbits it will also be possible to use the fusion proteins as effective immunogens in mice. If subsequent tail bleeds from the immunised mice reveal an anti-Env specific response of good titre the spleens from these mice will be used for the production of hybridomas to generate monoclonal antibodies. In order to maximise the number of clones which produce anti-Env antibodies the mice will be boosted intravenously 3 to 4 days prior to sacrifice. The GST:EnvB and GST:EnvC fusion proteins each contain a thrombin cleavage site and if successful cleavage can be achieved (this has not been attempted in the work presented in this thesis) the cleaved material will be used for this final immunisation. If sufficient quantities of cleaved material cannot be obtained the mice will be immunised with eluted uncleaved fusion protein.

The data presented in this chapter suggests that the pGEX system will be suitable for the expression and purification of proteins which can be used to stimulate a response to MVV gp135. Such antisera will be used in a variety of functional and mapping studies (see chapter 7).

For this purpose the pGEX expression system has a number of advantages over the yeast Ty-VLP expression system. Although the yields of the GST:EnvB and GST:EnvC fusion proteins are relatively low (ie ug/L rather than the mg/L observed for the native GST) they are at least comparable with the yields obtained in the Ty-VLP expression system for the same gp135 regions. However the GST-based fusion proteins are of much higher purity than the p1-based fusion proteins and can be produced more rapidly and using simpler and more reliable techniques.

The most obvious problem encountered in the work reported in this chapter was the inability to generate a pGEX1:envA-specific clone. Of 484 colonies screened 13 contained a recombinant pGEX1 plasmid in which the envA fragment had been inserted in the 3' to 5' orientation, and attempts to reverse the orientation by excising the fragment and religating the molecules were unsuccessful. Calculating from a Poisson distribution (see appendix 2) the observed difference between 13 clones in one orientation, and no clones in the opposite orientation is significant at the 0.1% level ($d=3.6$). This suggests that in the cloning system used in these experiments there was selection against clones which contained the envA fragment in the 5' to 3' orientation.

Such a result may suggest that the GST:EnvA fusion protein is toxic to the E.coli JM83 cells into which the plasmid was transformed. Although expression of the p1:EnvA fusion protein did not affect growth of S.cerevisiae (section 4.5) the data presented in figure 5.5 of this chapter demonstrates that toxicity of the env-encoded sequences in one expression system cannot necessarily be used to predict toxicity in another system. Expression of the p1:EnvB and p1:EnvC fusion proteins did not have any significant effect on the growth of transformed S.cerevisiae but expression of the GST:EnvB and GST:EnvC fusion proteins markedly affected the growth

of E.coli JM83. Therefore it cannot be concluded that the lack of an obviously toxic effect of p1:EnvA on the growth of S.cerevisiae would necessarily imply a non-toxic effect of expression of GST:EnvA on the growth of E.coli JM83 transformants.

It may therefore be postulated that the inability to generate any clones transformed with a recombinant pGEX plasmid containing the envA sequence in the 5' to 3' orientation may be a consequence of extreme toxicity of the recombinant protein, such that cells which contain such a plasmid fail to grow on the selection plates. In order for this to be the mechanism leading to the failure to isolate the required clones it is necessary to suggest that there is a degree of constitutive expression of the fusion protein. It is therefore important to consider why E.coli clones containing plasmids into which the envA sequence has been inserted in the 5' to 3' orientation with respect to a promoter have been successfully generated in other experiments.

In chapter 3 envA sequences derived from various MVV preparations were inserted into the pTZ plasmids and used to transform E.coli JM101. The pTZ vectors are not used as a fusion protein expression system but they do have the potential to express low levels of a fusion protein. The foreign DNA is inserted into the beta-galactosidase gene which is inducible by IPTG and it is possible that foreign DNA inserted in the correct reading frame could encode low levels of protein. Although in all cases the envA sequences were found to be in a 3' to 5' orientation with respect to the promoter when initially ligated using the BamHI site, the orientation could be reversed by using a unidirectional cloning strategy. This indicates that although there may be an initial bias towards plasmids with a 3' to 5' orientation of the inserted fragment this can be overcome fairly readily.

In chapter 4 the same envA sequence used in the pGEX

experiments was successfully inserted in the 5' to 3' orientation into a pOGS plasmid, and used to transform E.coli JM83.

These results indicate that the failure to obtain a pGEX-based plasmid in which the envA fragment was inserted in the correct orientation may be the result of the combination of the pGEX plasmid and the bacterial host. Successful cloning of the envA fragment 5' to 3' in the pOGS expression vector into E.coli JM83 may suggest that the sequences encoded by envA are not toxic to E.coli when present as fusion proteins with p1. Alternatively there may be no expression of the p1:fusion protein in E.coli if the yeast promoter on the pOGS plasmid fails to function in the bacterial host.

The successful insertion of envA sequences into pTZ plasmids, and transformation of E.coli JM101 could suggest that the JM101 strain tolerates the envA-encoded sequences better than the JM83 strain of E.coli. However, when inserting the envA sequence into pTZ plasmids no effort was made to ensure that the coding sequence was inserted in the correct reading frame. Therefore the clones isolated which contained the envA sequence in the 5' to 3' orientation may simply reflect the insertion of the sequence in a reading frame in which it does not encode the EnvA protein, and hence it does not have a toxic effect on cell growth.

If the failure to isolate an appropriate pGEX-based envA clone of E.coli is due to extreme toxicity of the fusion protein (or theoretically the mRNA encoding the protein) this must imply some degree of constitutive expression of the fusion protein sequences from the plasmid. This is perhaps surprising, as the product of the lacIq allele of the plasmid should function as a tight repressor of expression from the tac promoter of the plasmid. Constitutive expression from the promoter would indicate some breakdown in this control. In experiments

in which the entire MVV EV1 env gene was inserted into the pRc/RSV plasmid and then transformed into E.coli JM83 and JM101 it was found that in 80 clones which contained the env gene the orientation of the gene was 3' to 5' in all cases (R.G.Dalziel, Department Of Veterinary Pathology, University Of Edinburgh, personal communication) suggesting selection of clones which could not encode the Env protein. This occurred even though no constitutive expression was anticipated from this vector. As the full-length env gene also encoded the gp46 region of Env it cannot be inferred from this data that the envA region was responsible for the failure to obtain any clones which contained the env gene in the 5' to 3' orientation. It is also not possible to determine from the data in this chapter which region of envA was responsible for the observed failure to obtain any clones in which the gene fragment was inserted 5' to 3'. The region which encodes the first 40 amino acids of Rev is not a likely candidate, as Rev expression has been shown to have little toxic effect in this system (M. Fotheringham, Department Of Veterinary Pathology, University Of Edinburgh, personal communication). It is also unlikely that the region of EnvA which overlaps EnvB ie amino acids 247 to 311 is responsible for the presumed toxicity, as envB was successfully cloned in the 5' to 3' orientation into pGEX2T. The hydrophobic signal sequence contained in EnvA is a possible candidate, particularly as strongly hydrophobic regions adversely affect the success of fusion protein expression in the pGEX system but to address this problem more fully it would be necessary to generate truncated envA fragments and attempt to clone these into the same expression system.

It may be possible to increase the chances of obtaining a clone which can encode GST:EnvA by altering the manner in which the recombinant plasmid is generated. As shown in figure 5.1 pGEX1 contains a BamHI site and an

EcoRI site at the cloning site at the 3' end of the gst gene. There are no BamHI or EcoRI sites in the envA sequence. By redesigning the 3' PCR primer for envA, such that the BamHI site is replaced by an EcoRI site and then generating a PCR product in the same way that was used to obtain env fragments for insertion into the pOGS vectors (section 4.2) an envA fragment could be created with a BamHI site at its 5' end and an EcoRI site at its 3' end. If the pGEX1 was similarly digested prior to ligation with envA this would ensure that the envA fragment could only be ligated into the plasmid in the 5' to 3' orientation. Although the number of clones containing such a recombinant plasmid would be expected to be very low given the inferred toxicity of such a construct, screening for such clones should be greatly simplified by the reduced numbers of transformants obtained after a ligation and transformation and therefore the likelihood of isolating an appropriate clone would be improved.

5.7 Summary

The bacterial pGEX system was used to generate fusion proteins of GST with the EnvB and EnvC regions of MVV gp135. The expression of these fusion proteins was shown to be toxic to the host cells but fusion proteins could be produced which were of at least comparable yield to those expressed in the Ty-VLP system, and of greatly improved purity. The GST:EnvB fusion protein was an effective immunogen in the production of a polyclonal anti-EnvB response and by increasing the doses of the GST:EnvC fusion protein it is anticipated that this will also prove to be an effective immunogen. Attempts to obtain a clone which expressed a GST:EnvA fusion protein were unsuccessful, but alterations in the cloning strategy may overcome this problem.

6. Functional Studies Of MVV gp135 Using Recombinant gp135 Fragments

6.1 Introduction

There is currently little information available on the role of different regions of gp135 in the life cycle of MVV. It is not known for example which regions of gp135 bind to the cellular receptor(s), whether sequences within gp135 are important in cell-cell fusion and if neutralising antisera act by blocking gp135-receptor interactions, blocking fusion or perhaps by some other mechanism.

The lentivirus for which there is the most substantial amount of data on the relationships between the structure of the external envelope glycoprotein and its functions is HIV. Some of this data was briefly discussed in section 1.14 and the most apparent conclusion to be drawn is that many different regions of gp120 interact functionally within this molecule. Although a detailed discussion of the HIV gp120 literature cannot be attempted in this chapter a brief description of some of the most salient features will be presented, as this well-characterised molecule may indicate possibly fruitful lines of investigation for MVV gp135.

A major role of HIV gp120 is interaction with CD4, the primary cellular receptor for HIV (McDougal et al., 1986). Antibodies to the amino acid region 397-439 of HIV-1 gp120 block binding to CD4 (Lasky et al., 1987) and this may represent the protein-protein interaction site of the two molecules. However alterations to amino acids 363 and 437 (Kowalski et al., 1987) and 257, 368 and 370 (Olshevsky et al., 1990), and deletion of the C-terminal 44 amino acids of gp120 (Linsley et al., 1988) also influence gp120/CD4 binding. A role for amino acids between residues 42 to 129 of HIV gp120 has also been identified (Syu et al., 1990). Therefore the interaction

of gp120 with its major ligand appears to be dependent on a number of residues spread throughout the glycoprotein, probably as a consequence of the three dimensional configuration of the molecule.

This multiple-site interplay of residues in the function of gp120 is not restricted to its interaction with CD4. Regions critical for the interaction of gp120 with gp41 have been identified in one study in the N-terminal 31 amino acids of gp120 (Ivey-Hoyle et al., 1991) and other reports have shown an involvement of amino acids 36 to 45 and amino acids 491-501 (Helseth et al., 1991).

Virus neutralisation can be achieved by antisera directed against epitopes which are not involved in the interaction with CD4, in particular the PND which lies within the V3 loop formed by the cysteine residues at positions 296 and 331 (Goudsmit et al., 1988).

Mutations within different regions of gp120 have been shown to "compensate" for one another in conferring infectivity on a HIV clone. It was reported that a non-infectious clone which contained a mutation at residue 267 of gp120 was rendered infectious by a subsequent spontaneous mutation at residue 128 (Willey et al., 1988). Following in vitro mutagenesis of this second residue (resulting initially in a non-infectious clone) a further infectious clone was subsequently isolated with a mutation at residue 308, which lies within the PND (Willey et al., 1989). This suggested the interaction of at least three regions in a critical early event in HIV infection.

In order to begin to investigate the potential roles of different regions of MVV gp135 the recombinant proteins described in chapters 4 and 5 were generated and characterised. Initially it had been anticipated that antisera raised against these recombinant proteins would be used to investigate the roles of different regions of gp135. Due to the technical difficulties described previously suitable antisera were not available to

undertake this work at the time of writing. However the recombinant proteins themselves were employed in attempts to begin to relate function to different regions of gp135. Although work with HIV gp120 has shown that residues throughout the protein may interact functionally during viral infection of a cell, useful data has also been obtained using fragments of the glycoprotein. For example, proteolytically cleaved gp120 has been used successfully to investigate regions of gp120 which bind to CD4 (Nygren et al., 1988). Truncated versions of gp120 generated in a vaccinia virus expression system have also been used for similar purposes (Linsley et al., 1988).

In an attempt to investigate the interaction of MVV gp135 with cellular molecules the recombinant Env fusion proteins generated in the yeast Ty-VLP expression system (chapter 4) were used in a variety of tests. The recombinant p1:Env fusion proteins were used in preference to the GST:Env fusion proteins (chapter 5) because no pGEX1:envA clone had been successfully generated at the time of writing, and because the availability of an anti-p1 antiserum formed the basis of the detection system in certain of the experiments. These studies involved attempting to block infection in vitro with the recombinant proteins, detection of interactions with cellular molecules by binding the fusion proteins to intact cells and cell lysates, and analysing possible interactions with MHC class II molecules, which have been identified as a component of a cellular receptor for MVV (Dalziel et al., 1991).

6.2 The Interaction Of The p1:Env Fusion Proteins With Fixed Skin Cell Culture Monolayers

In order to investigate if any of the p1:Env fusion proteins interact with a cellular molecule experiments were performed in which the fusion proteins were added to fixed cell monolayers. The aim of these experiments was

to determine if specific binding of one or more of the p1:Env fusion proteins could be detected as this would indicate that a possible binding site for a cellular molecule was present within the particular region of gp135.

The cell monolayer was fixed using ice-cold acetone in PBS as cells fixed by this method maintain the antigenicity of surface markers. The fusion proteins were added to the cells under non-denaturing conditions to increase the probability of physiologically relevant interactions between the various cellular and non-cellular molecules.

The fixed 848 skin cell monolayers were incubated at 4°C for 16 hours with a 1 in 100 dilution of the peak fractions from sucrose gradient purification of the p1 or p1:Env fusion proteins. Using a standard dilution of the peak fractions resulted in a large relative excess of p1 protein in the p1 control compared with the p1:Env fusion proteins. This was demonstrated by SDS-PAGE and silver staining (data not shown). The monolayers were developed with either polyclonal rabbit serum 194 (anti-VLP) or normal rabbit serum (NRbS) and a donkey anti-rabbit FITC conjugate. After washing the monolayers were examined by fluorescence microscopy.

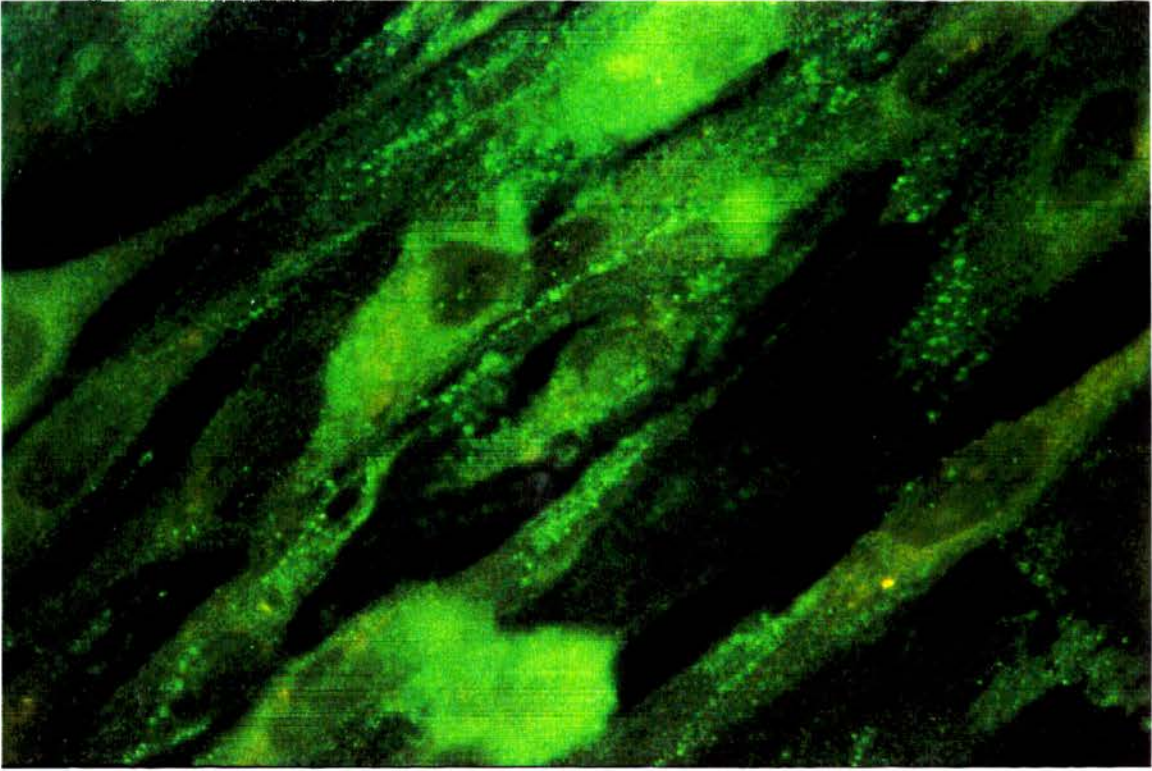
As a control for the fixation and washing procedures and the anti-rabbit FITC conjugate, cytopins of ovine efferent lymphocytes were stained with NRbS or rabbit anti-MHC class II serum. Approximately 15-20% of the efferent lymphocytes were stained by the anti-MHC class II serum, as expected.

There was little non-specific reactivity of the anti-VLP serum (figure 6.1a) or NRbS (figure 6.1b) with the 848 skin cells alone. There was also very little reactivity of NRbS with skin cells pre-incubated with p1:EnvA (Figure 6.2). Similar low reactivity of the NRbS was observed when the cells were pre-incubated with the p1, p1:EnvB or

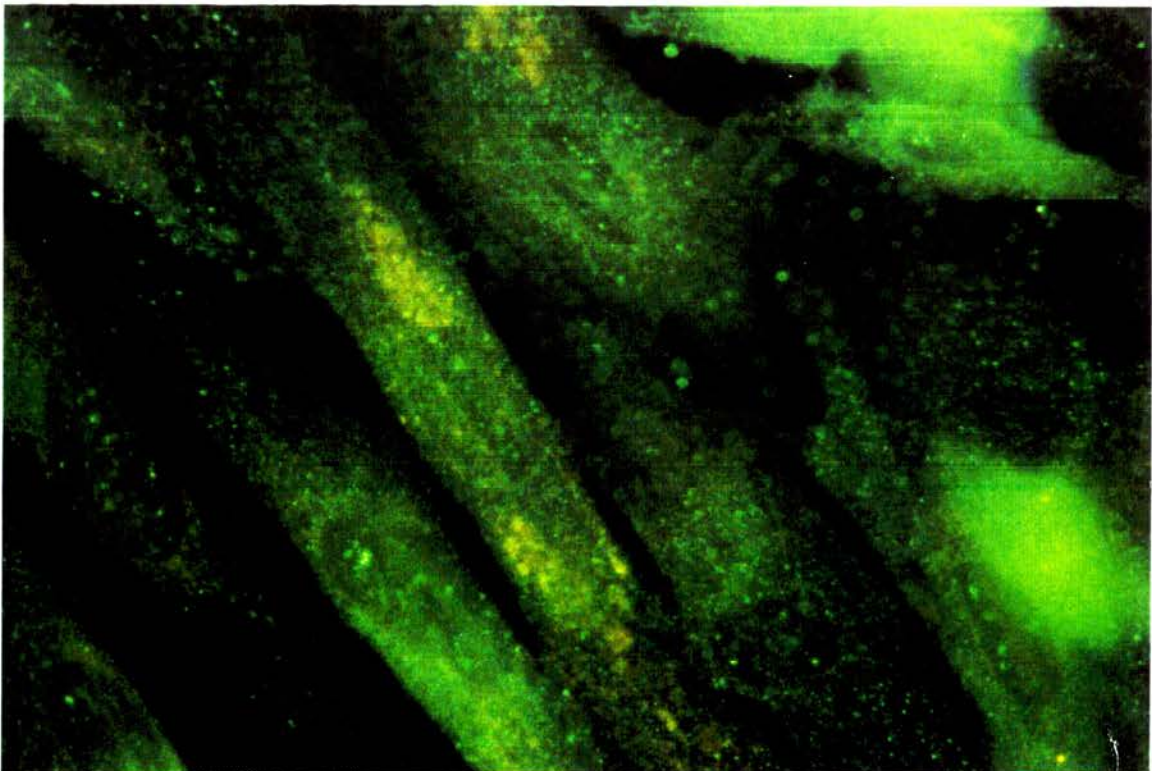
Figure 6.1

Immunofluorescence of 848 skin cells incubated with normal rabbit serum or rabbit anti-VLP serum

Acetone fixed 848 skin cell monolayers were incubated with rabbit anti-VLP serum (a) or normal rabbit serum, @ 1 in 250. Staining was developed using an anti-rabbit FITC conjugate @ 1 in 200.



a

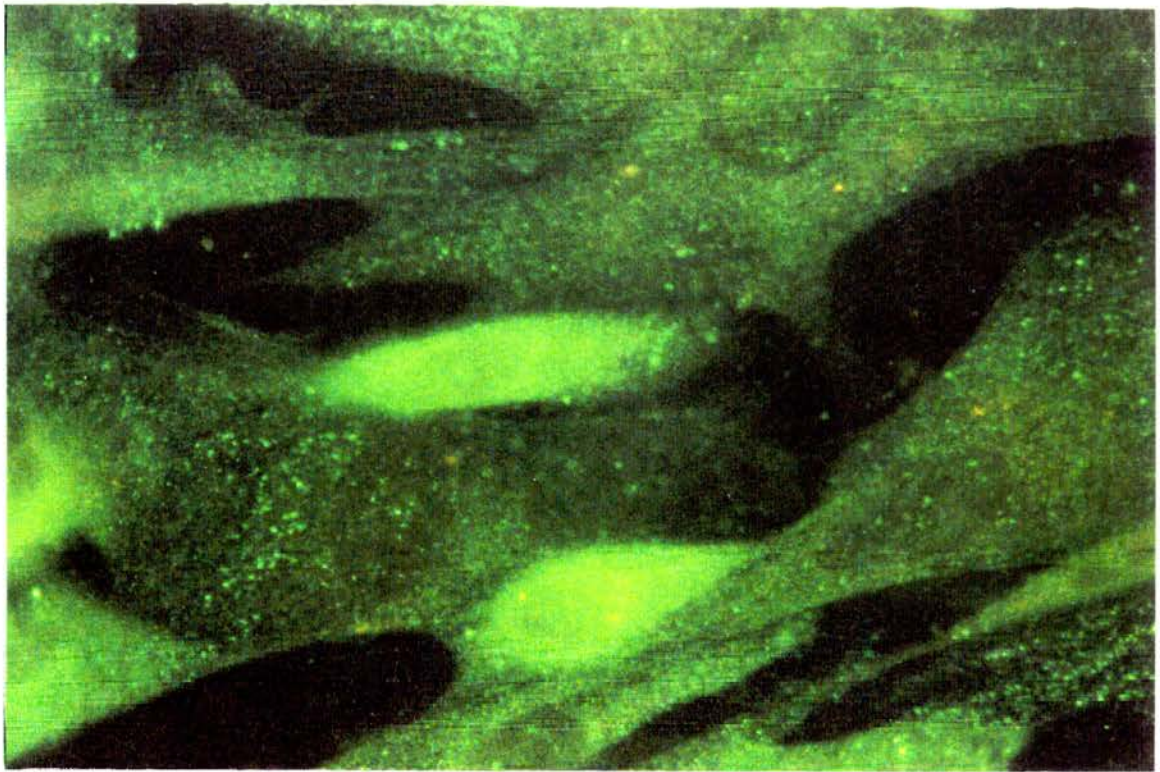


b

Figure 6.2

Immunofluorescence of 848 skin cells incubated with p1:EnvA and developed with normal rabbit serum

Acetone fixed 848 skin cell monolayers were incubated with sucrose gradient purified p1:EnvA. Staining was developed using normal rabbit serum @ 1 in 250 and anti-rabbit FITC conjugate @ 1 in 200.



p1:EnvC preparations (data not shown).

However, as shown in figure 6.3a-d a very different pattern of staining is apparent when cells pre-incubated with p1 or the p1:Env preparations were stained using rabbit serum 194. A very high level of particulate staining was observed. This was so intense that great difficulties were encountered in obtaining photographs in which the cellular morphology could be clearly seen. This is a consequence of an automatic exposure meter on the camera used to record these results. For monolayers such as the NRbS controls a long exposure is set and consequently the cells are visible on these prints. Where high levels of staining are present a short exposure is selected. Unfortunately this results in only the intense staining becoming visible, and is too short for the cells themselves to be distinguishable on the photographs. Examination of the same fields by light microscopy demonstrated that the general cellular appearance had not been altered by the addition of the p1 or p1:Env fusion proteins (data not shown).

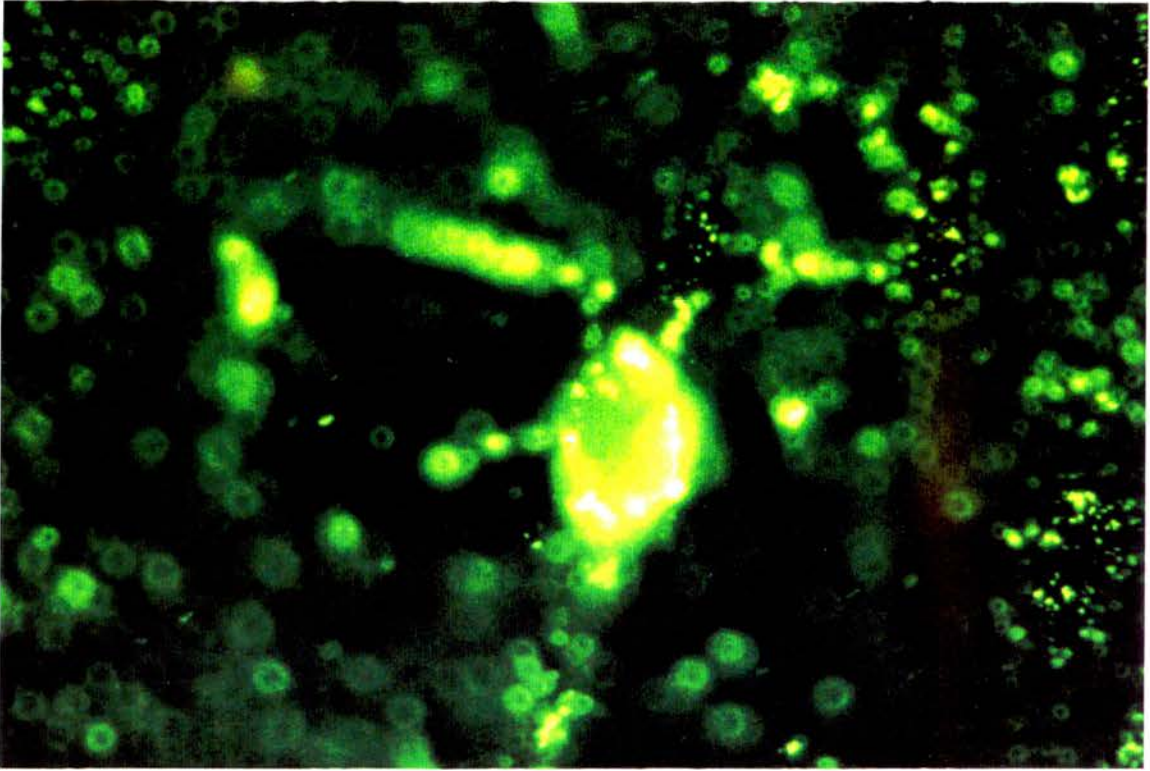
The high level of particulate staining was noticeable for p1 and all the p1:Env fusion proteins, but was especially marked for p1 and p1:EnvA. The data suggests the presence of one or more species in the gradient purified fractions which can interact with both the fixed cells and the anti-VLP serum. As this staining is most marked for p1 and p1:EnvA it suggests these preparations contain the highest levels of the appropriate molecule(s).

As all the preparations including p1 alone resulted in high level staining of the monolayers it was not possible to determine if there was a specific interaction of the gp135 region of any of the fusion proteins with these cells.

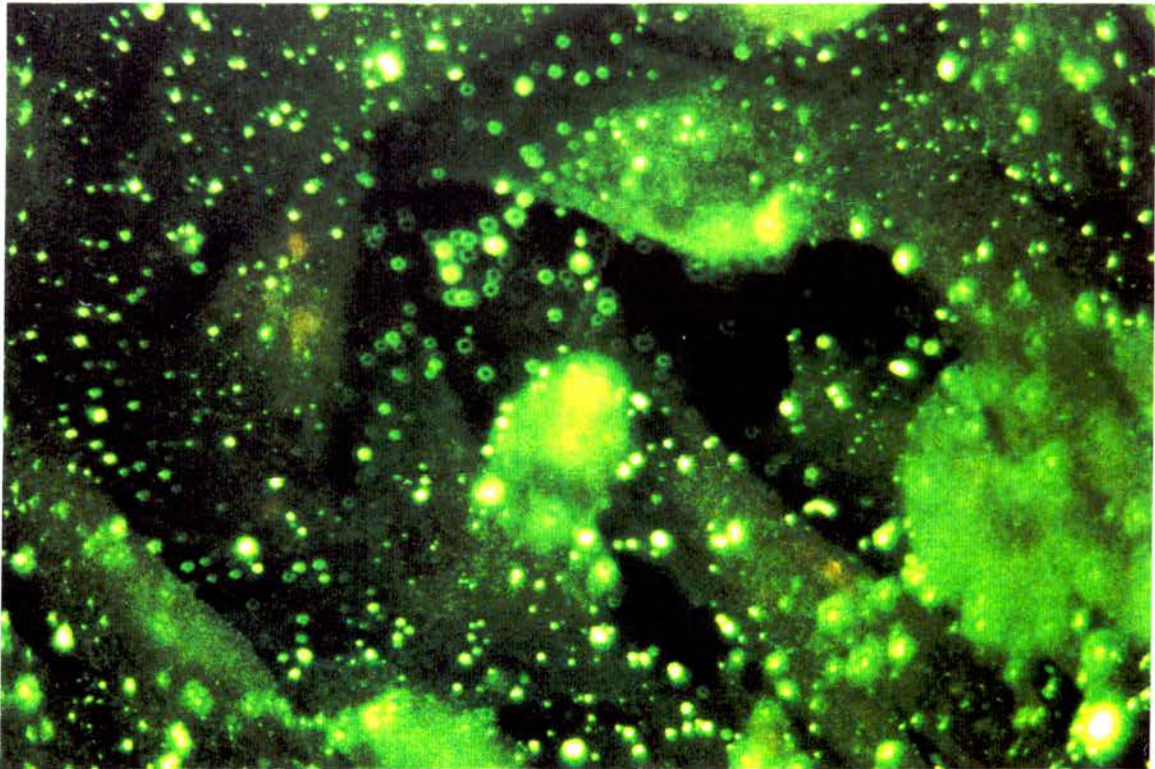
Figure 6.3 (a,b)

Immunofluorescence of 848 skin cells incubated with p1/p1:Env and developed with anti-VLP serum

Acetone fixed 848 skin cell monolayers were incubated with sucrose gradient purified p1:EnvA (a) or p1:EnvB (b). Staining was developed using rabbit anti-VLP serum @ 1 in 250 and anti-rabbit FITC conjugate @ 1 in 200.



a

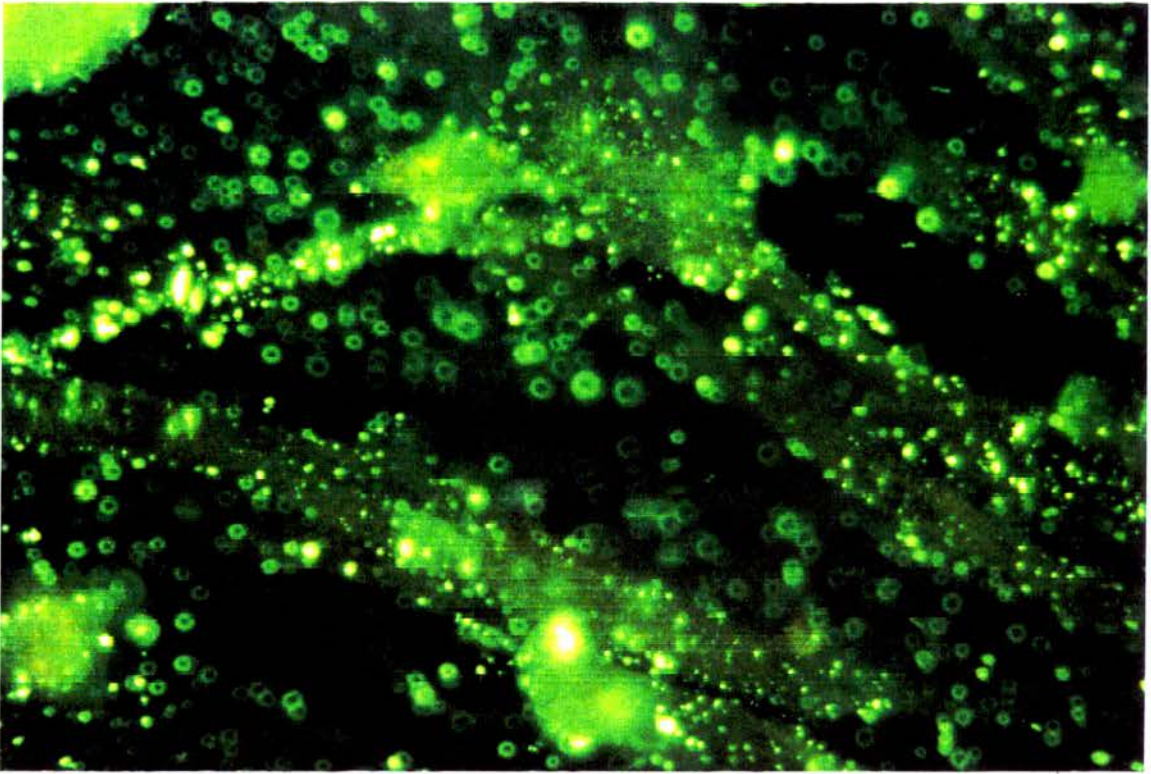


b

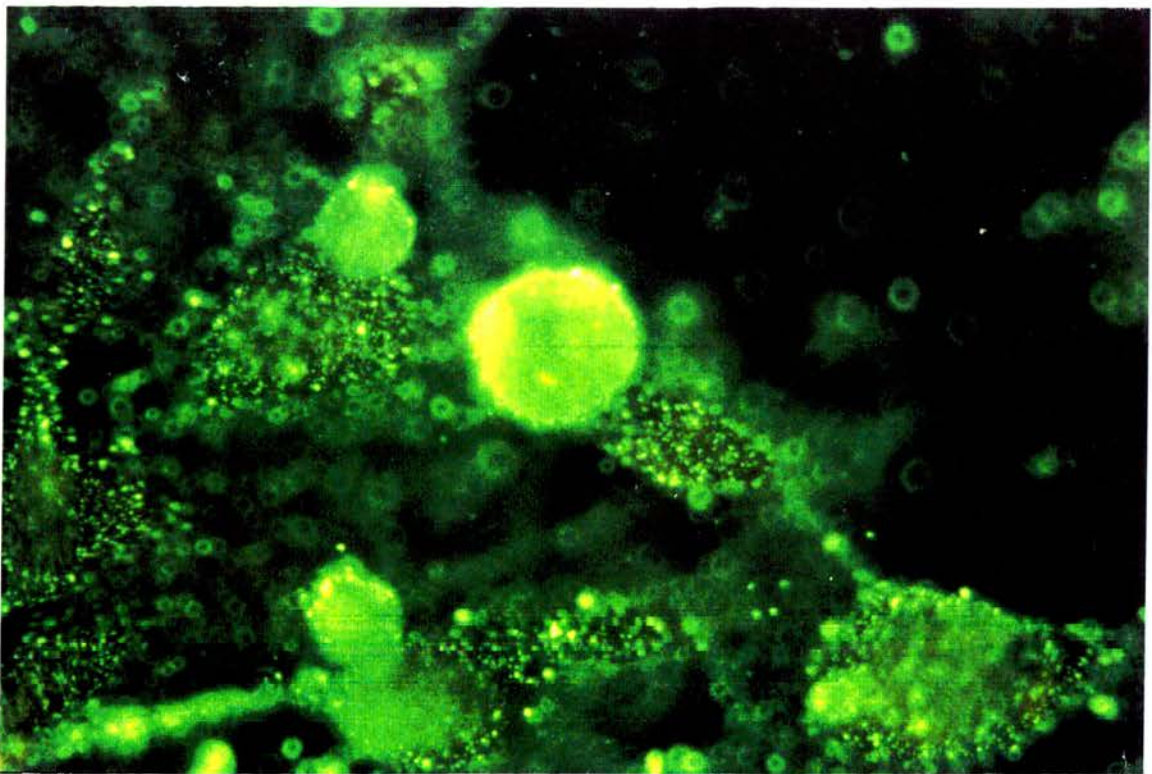
Figure 6.3 (c,d)

Immunofluorescence of 848 skin cells incubated with p1/p1:Env and developed with anti-VLP serum

Acetone fixed 848 skin cell monolayers were incubated with sucrose gradient purified p1:EnvC (c) or p1 (d). Staining was developed using rabbit anti-VLP serum @ 1 in 250 and anti-rabbit FITC conjugate @ 1 in 200.



c



d

6.3 The Interaction Of The p1:Env Fusion Proteins With Skin Cell Lysates

In the report suggesting an involvement of the MHC class II molecule as a component of the cellular receptor for MVV (Dalziel et al., 1991) an interaction was initially demonstrated in vitro between MVV and cellular proteins by a virus overlay protein blot assay (VOPBA) (Boyle et al., 1987). In this system a protein blot of a lysate from a susceptible cell line was incubated with MVV. The complex of cellular components and virus was then localised using an anti-MVV polyclonal antiserum. This putative cell-virus interaction was therefore maintained even where the cellular component was present in a denaturing system.

The p1:Env fusion proteins were used in a similar system to determine if any of the recombinant gp135 sub-regions could be shown to interact with cellular molecules in the same way as observed with intact virus in the VOPBA. This potentially could act as a means of identifying regions of gp135 which interact with cellular molecules.

848 skin cells were lysed when just sub-confluent and the cellular proteins were separated by SDS-PAGE and blotted onto a nitrocellulose membrane. Membrane strips were incubated at 4°C for 18 hours with the peak fractions from sucrose gradient purified p1 or p1:Env preparations. p1 was added to the strips at a dilution of 1 in 50, and the p1:Env fusion proteins were added at 1 in 10. At these dilutions the p1 protein was present in excess in the p1 control (data not shown). The strips were developed with rabbit anti-VLP serum followed by an anti-rabbit alkaline phosphatase conjugate. As a control strips from a blot of p1 alone were developed in the same system, and the p1 band was observed as expected (data not shown).

Figure 6.4a shows the results of this experiment. A

Figure 6.4

Western blot of an 848 cell lysate, incubated with p1/p1:Env fusion proteins

Proteins from an 848 skin cell lysate were separated on a 5-15% SDS-PAGE gel and blotted onto nitrocellulose. Strips were incubated with p1 or p1:Env fusion protein preparations and developed using rabbit anti-VLP serum @ 1 in 1500, and anti-rabbit alkaline phosphatase @ 1 in 1000. The relevant bands are indicated by arrows. Molecular weight standards (kDa) are shown at the left hand side of the figure.

Panel a - incubation with purified preparations.

Panel b - incubation with crude or purified preparations.

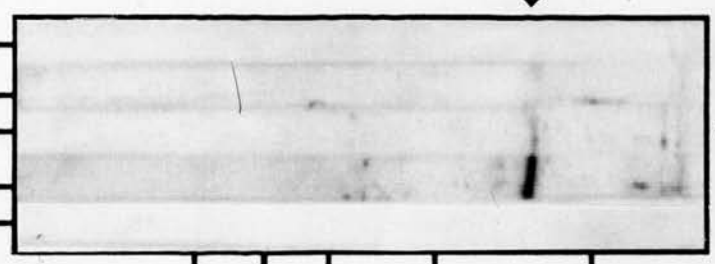
Key

- (P) sucrose gradient purified preparations
- (C) crude preparations (no sucrose gradient purification)
- diluent only

(P)

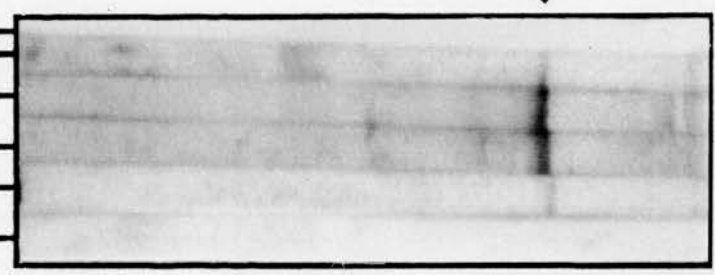
p1
p1:EnvA
p1:EnvB
p1:EnvC

94
67
43
30
20.1



a

p1(P)
p1:EnvA(P)
p1(C)1/10
p1(C)1/20
p1(C)1/50



b

band is visible with an apparent molecular weight of 24kDa when the cell lysates are incubated with each of the p1:Env preparations, but not after incubation with sucrose gradient purified p1 alone. This suggested either that each of the regions of gp135 represented by the fusion proteins could bind to a cellular molecule, or that a contaminant present in the p1:Env preparations but not in the purified p1 alone could bind to a molecule in the 848 cell lysate.

In order to investigate the possibility of contaminants binding the experiment was repeated using a crude preparation of p1 protein. The cells from an induced culture of transformant pp8.1 were harvested and processed as normal up to the stage of centrifuging the cell lysate onto a 60% sucrose cushion. This preparation was stored at -20°C , without any subsequent sucrose gradient purification. Silver staining of an SDS-PAGE gel demonstrated extensive contamination of the sample with non-p1 yeast proteins (data not shown).

In figure 6.4b it can be seen that pre-incubation of the strips with the crude p1 preparation results in detection of a band of the same molecular weight as that observed when the strips are pre-incubated with p1:EnvA. This demonstrates that the band observed in figure 6.4a is the result of an interaction with a yeast-derived contaminant. The results from these experiments therefore do not demonstrate a detectable reactivity of the Env regions of the fusion proteins with cellular molecules present on the protein blots of 848 cell lysates.

6.4 Interaction Of The p1:Env Fusion Proteins With MHC Class II Molecules

From the original description of an interaction between MHC class II and MVV (Dalziel *et al.*, 1991) it can be inferred that MVV can interact with class II when this is denatured (as in the VOPBA) or when the three

dimensional structure is relatively undisturbed (as in the class II antigen-binding ELISA). The p1:Env fusion proteins were used in both types of assay to determine if they displayed any detectable specific interaction with MHC class II.

Blots were made of immunopurified ovine MHC class II antigen and the empty sites blocked. p1 or p1:Env fusion protein preparations were added to the blots as described in section 6.3, and developed in the same way. The blots were also incubated with polyclonal rabbit anti-MHC class II antiserum, to confirm that the blots were positive for class II.

MHC class II was detectable on the blots using the polyclonal anti-MHC class II antiserum. The rabbit anti-VLP serum was reactive with a blot of purified p1. No bands were detectable with the anti-VLP serum after incubation of the MHC class II blots with the p1 or p1:Env preparations (data not shown). Therefore no significant binding of the recombinant gp135 proteins to MHC class II was detectable in this assay system.

In order to determine if an interaction could be observed between the p1:Env fusion proteins and non-denatured MHC class II molecules two variations of an ELISA were performed. In the first, plates were coated with immunopurified MHC class II and the p1 or p1:Env preparations were added. The wells were then incubated with polyclonal rabbit anti-VLP serum, followed by a horseradish peroxidase-linked anti-rabbit antibody and developed.

In the second type of experiment plates were coated with p1 or the p1:Env fusion proteins and immunopurified MHC class II was added. This was followed by a polyclonal rabbit anti-MHC class II serum, and the rest of the development of the ELISA was continued as above.

The results for the first type of ELISA are shown in figure 6.5. It is not possible to determine accurately

Figure 6.5

ELISA to detect binding of p1:Env fusion proteins to MHC class II

Wells were coated with MHC class II and the p1/p1:Env fusion proteins added. Binding was detected using rabbit anti-VLP serum (Rb194) @ 1 in 1500 and anti-rabbit horseradish peroxidase @ 1 in 1000.

The stages up to the addition of the horseradish peroxidase conjugate can be summarised as follows:

<u>Step 1</u>	<u>Step 2</u>	<u>Step 3</u>	<u>Step 4</u>	
cl II	block	-	anti-cl II	=Rb164
p1/Env	block	-	anti-VLP	=Rb194
cl II	block	p1/Env	anti-VLP	=x
-	block	p1/Env	anti-VLP	=y

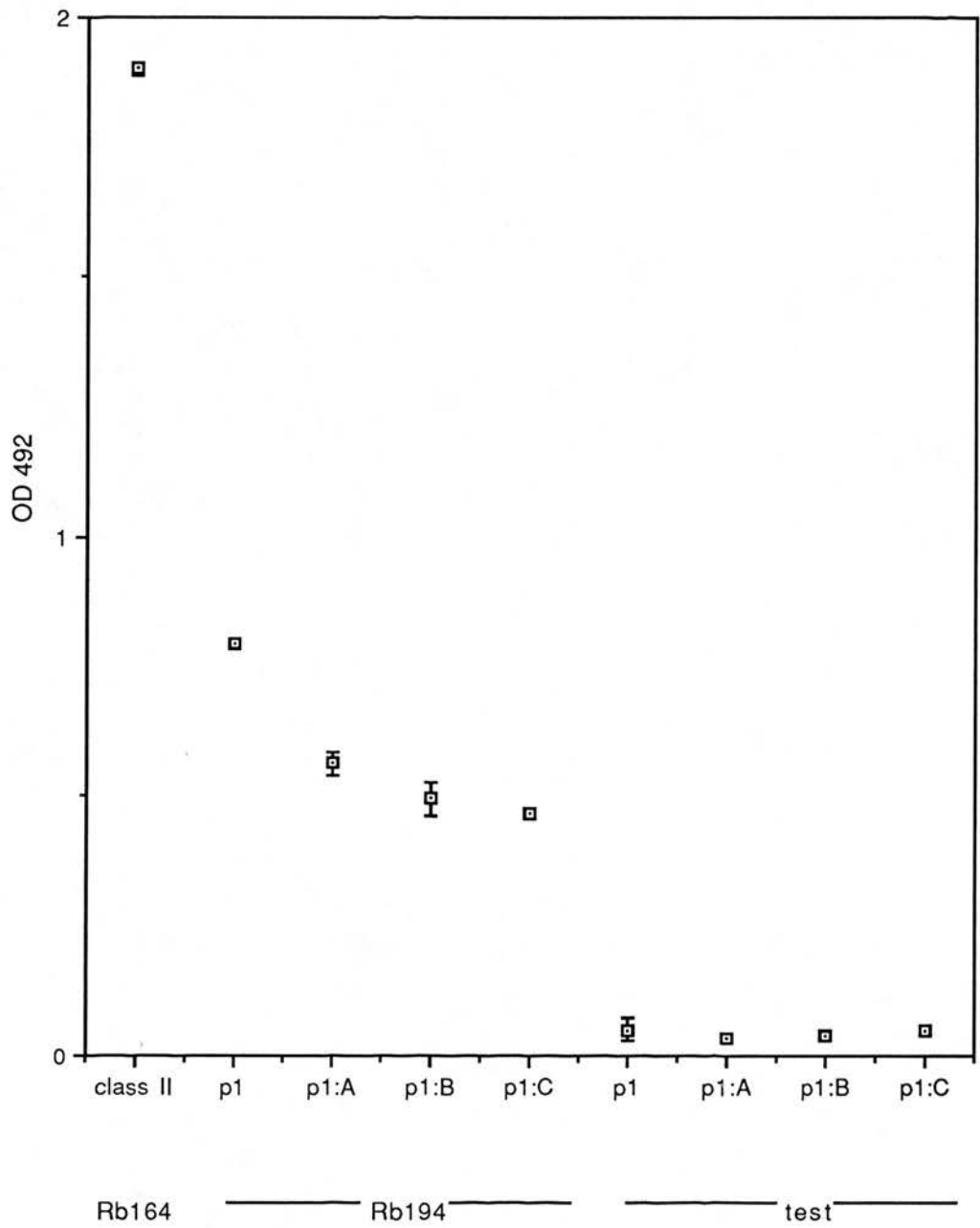
cl II = class II

p1/Env = p1 or p1/Env proteins

(x-y) = "test"

Key

p1:A p1:EnvA
p1:B p1:EnvB
p1:C p1:EnvC



the amounts of MHC class II in the immunopurified preparations, due to the presence of triethanolamine in the solution. From silver stained SDS-PAGE gels the concentration was estimated as 5-10ug/ml and approximately 30ng were used to coat the wells. Control wells coated with MHC class II and developed with MHC class II serum showed that the wells had been successfully coated (figure 6.5). Wells were also coated with the p1 or p1:Env preparations to confirm that the amount of these proteins added to the coated wells was sufficient to be detectable. Figure 6.5 shows this to have been the case. The dilutions of the sucrose gradient purified samples used were: p1 - 1 in 5,000, p1:EnvA - 1 in 20,000, p1:EnvB - 1 in 2,500 and p1:EnvC - 1 in 2,500. It had previously been determined using the same rabbit anti-VLP detection system that coating the plates with these dilutions of the p1 and p1:Env preparations resulted in final absorbance readings which lay within the linear range of ELISA values in a dilution series of each protein (data not shown).

The p1 and p1:Env fusion proteins were added to wells which had been coated with MHC class II. The preparations were also added to wells which had not been coated with MHC class II but had been blocked in the same way as these wells. The values shown in figure 6.5 represent the difference of the two sets of readings for each sample. Four replicates were recorded for binding to the MHC class II coated wells, and two for binding to the control wells. The standard deviations for the binding to the coated wells are shown.

The OD₄₉₂ readings are low for the p1 and the p1:Env fusion proteins, and there is no significant difference between the values for any of the preparations. Therefore there was no detectable binding of the recombinant gp135 proteins to MHC class II in this assay system.

In the second approach to this problem the plates were coated with the p1 or p1:Env preparations. Much

higher concentrations of the sucrose gradient purified fractions were used ie p1 - 1 in 1000, p1:EnvA - 1 in 200, p1:EnvB - 1 in 50 and p1:EnvC - 1 in 50. This was to ensure that the wells were saturated. The OD₄₉₂ readings after development with the rabbit anti-VLP serum are shown in figure 6.6. Wells were also coated with approximately 30ng of MHC class II and developed using the rabbit anti-MHC class II antiserum, to confirm that detectable amounts of MHC class II were added in the test wells. MHC class II was added to p1/p1:Env-coated wells and the ELISA was developed with the rabbit anti-MHC class II serum. As a control the same serum was also used in wells which had been coated with the p1 or p1:Env preparations, but to which MHC class II had not been added. The data shown for the test samples in figure 6.6 represents the difference of these two values. Six replicates were made of the test samples and two of each control. The standard deviations of the test samples are shown.

The results displayed in figure 6.6 indicate that there is no significant difference between the results obtained in the test samples for p1, p1:EnvA and p1:EnvB. There is a slight difference in the results for p1 and p1:EnvC but this is unlikely to be significant. The controls with anti-VLP serum demonstrate approximately 25% higher reactivity with p1:EnvC over p1 alone, suggesting that at the dilutions used the p1:EnvC preparation is present in relative excess compared with p1, for this detection system. If the test result for p1 was raised by 25% to compensate for this relative inequality there would be no significant difference between the results for the samples.

The experiments outlined in this section therefore do not indicate any detectable binding of the recombinant gp135 fragments to immunopurified MHC class II molecules, and it is consequently impossible to speculate which

Figure 6.6

ELISA to detect binding of MHC class II to p1:Env fusion proteins

Wells were coated with p1/p1:Env fusion proteins and MHC class II antigens added. Binding was detected using rabbit anti-MHC class II serum (Rb164) @ 1 in 1000, and anti-rabbit horseradish peroxidase @ 1 in 1000.

The stages up to the addition of the horseradish peroxidase conjugate can be summarised as follows:

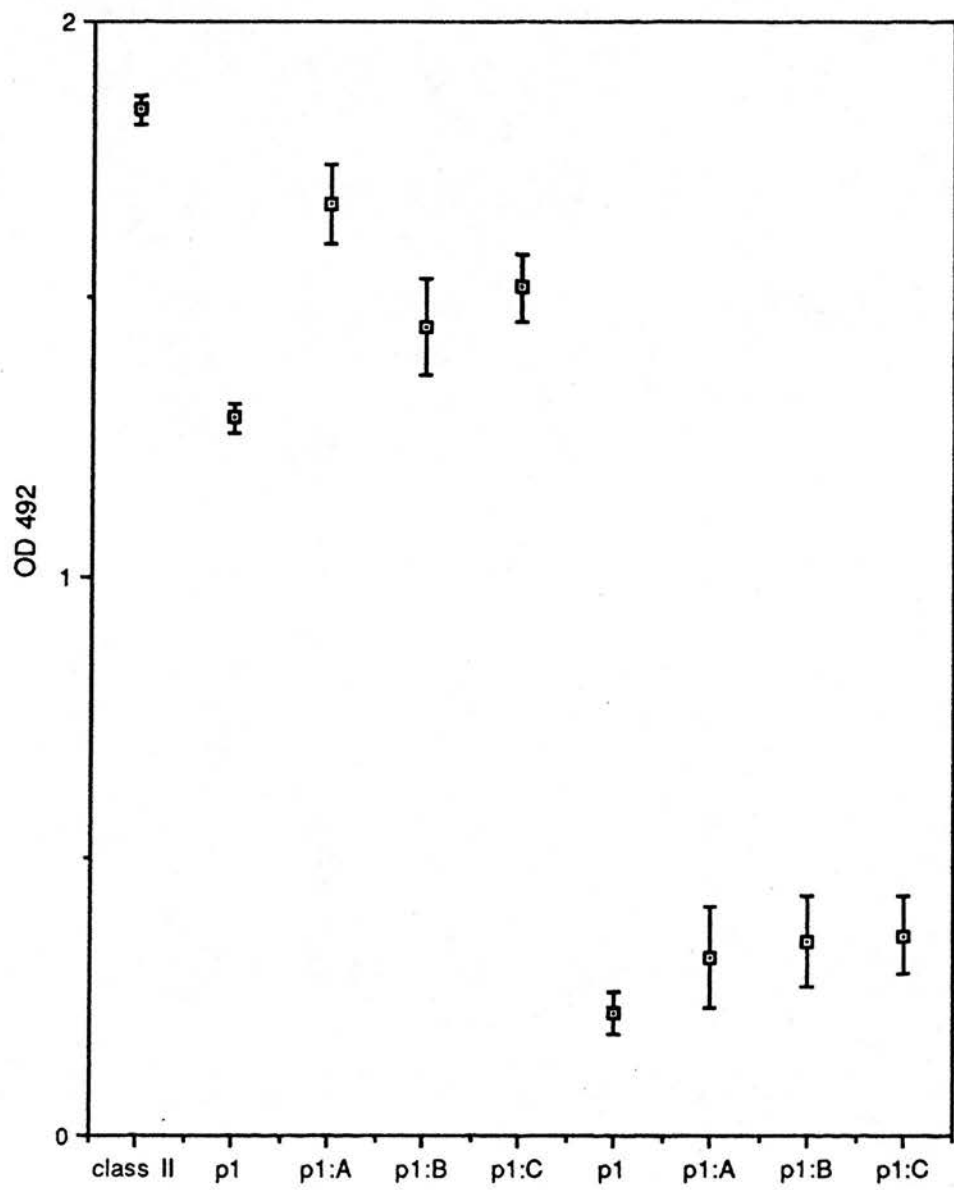
<u>Step 1</u>	<u>Step2</u>	<u>Step3</u>	<u>Step4</u>	
cl II	block	-	anti-cl II	=Rb164
p1/Env	block	-	anti-VLP	=Rb194
p1/Env	block	cl II	anti-cl II	=x
p1/Env	block	-	anti-cl II	=y

cl II = class II

(x-y) = test = (class II + Rb164)-(Rb164)

Key

p1:A p1:EnvA
p1:B p1:EnvB
p1:C p1:EnvC



Rb164 ——— Rb194 ——— - (class II+Rb164)-(Rb164) -

regions, if any, of MVV gp135 interact with this cellular molecule in the infective process.

6.5 Attempts To Block MVV Infection Of Skin Cell Cultures With The p1:Env Fusion Proteins

Under normal circumstances the envelope glycoprotein is the molecule which first mediates the interaction of MVV and a susceptible cell. In HIV it has been shown that preincubation of cells with free gp120 inhibits the fusion of CD4-positive cells with HIV-infected cells (Putney et al., 1986; Matthews et al., 1987) by interacting with the cellular CD4 and thereby preventing attachment by the cell-associated gp120.

Experiments were performed in which susceptible skin cells were preincubated with the p1:Env fusion proteins prior to infection with MVV. The skin cell cultures were subsequently analysed to determine if the preincubation had led to any decline in virally-induced cpe in vitro. 848 skin cells were grown in 96 well plates until just sub-confluent. The medium was removed and the cells washed in fully supplemented 2% DMEM. The peak p1 or p1:Env sucrose gradient purified fractions were diluted 1 in 100 in the same medium. At this dilution the level of yeast cell contaminants in each of the p1:Env preparations is approximately equivalent, and the level of p1 protein in the control is in excess. The cells were incubated for 1 hour at 37°C with 50ul of the diluted fractions. MVV strain EV1 was added in a further volume of 50ul of the same medium. A range of virus concentrations was used ie 6 TCID₅₀, 3 TCID₅₀ and 0.6 TCID₅₀ per well. The plates were incubated under the same conditions for a further hour, the medium was then removed and the cells washed in 100ul of fully supplemented 2% DMEM. 100ul of this medium, containing a 1 in 100 dilution of the appropriate p1 or p1:Env preparations were added to each well. The plates were incubated for 7 days

under standard conditions. At the end of this period the monolayers were stained with Giemsa and examined microscopically. Quadruple replicates were performed for each test. Control wells, which were treated either with MVV alone, cell-conditioned medium or p1/p1:Env proteins alone were also included.

Significant cpe, in the form of large quantities of multi-nucleated syncytia was apparent for all monolayers incubated with MVV, at each of the viral doses used. There was no apparent difference between the cultures incubated with virus in the presence or absence of p1 or the p1:Env preparations. No syncytia were observed in the wells incubated with conditioned medium. There was no apparent toxicity of p1 or the p1:Env fusion proteins at the concentrations used in this experiment.

The experiment was repeated under similar conditions but with YT40 skin cells in place of 848 cells, as the 848 cells available at the time of this repeated experiment were growing very poorly. In an attempt to increase the probability of detecting any inhibitory effect of the fusion proteins on infection some of the assay conditions were altered. MVV strain EV1 was used at 1.5 TCID₅₀, 0.6 TCID₅₀ and 0.3 TCID₅₀ per well. The p1 preparation was used at a dilution of 1 in 100 and all the p1:Env preparations were used at a dilution of 1 in 10.

After incubation and staining it was observed that the monolayers were no longer confluent but consisted of individual cells. This was independent of the different constituents added to the wells. Possibly as a consequence of this disruption of the monolayers no virally-induced cpe was detectable in any of the wells. Therefore no conclusions could be drawn about possible effects of the fusion proteins upon the outcome of viral infection in these cells. However, in all wells to which the p1:EnvC fusion protein had been added there was complete destruction of the monolayer, with only 3 to 4

cells remaining per field. This destruction was observed in both the presence and absence of MVV and indicates that the p1:EnvC preparation is toxic to these cells when added at a high concentration.

The data presented in this section therefore do not show any inhibitory effect of the p1:Env fusion proteins on MVV infection in vitro. In order to confirm this further experiments using a wide range of dilutions of both the virus and the fusion proteins would be required. This was not possible in the time available for these experiments.

6.6 Discussion

In an attempt to correlate function of MVV gp135 with different regions of the molecule the p1:Env fusion proteins expressed in the yeast Ty-VLP expression system were used in a variety of assays. These failed to demonstrate detectable interactions between the fusion proteins and cellular molecules and therefore it has not proved possible to determine which regions of gp135 are for example involved in receptor binding and post-binding entry events. Some of these aspects of the MVV life-cycle may be addressed more successfully when antisera raised against the GST:Env fusion proteins become available. However, for the future design of experimental protocols it is important to consider possible reasons for the lack of detectable activity observed in the assays reported in this chapter. These fall into two major classes: technical problems with the expression and purification of the fusion proteins, and problems which may arise from using fragments of gp135 rather than the whole molecule.

One of the major problems in attempting this work was the combination of poor yields and low purity of the p1:Env fusion proteins. This was exacerbated by the use of a polyclonal anti-VLP serum, which cross-reacted with yeast cell contaminants, as a detection method in many of

the experiments. At the time when the experiments were performed no characterised anti-p1 monoclonal antibody was available. Although controlled for as much as possible in the experiments this may have created conditions unfavourable for detecting any interactions which were relatively low-affinity and/or low frequency.

In section 6.2 it was reported that no specific interaction could be observed for the p1:Env fusion proteins with fixed cell monolayers. There was a high level of non-specific reactivity which was also apparent after incubation of the monolayers with the p1 protein alone. The intensity of this staining was greatest for p1 and p1:EnvA, and was less intense for p1:EnvB and p1:EnvC. The p1 preparation contained few detectable contaminating proteins, and those that were present were at much lower concentrations than in the p1:Env fusion protein preparations. In the p1:Env fusion protein preparations the contaminating proteins were present at approximately equivalent concentrations in all three samples, but levels of the fusion protein itself were greatest in the p1:EnvA preparation. As the intensity of staining observed was greatest with the p1 and p1:EnvA fusion protein it is possible that the molecule which was binding to the fixed cells was p1. In the absence of a p1-specific monoclonal antibody it is not possible to confirm this directly and there is no available data on the cellular molecule which could mediate such an interaction with p1. It is possible that p1 is binding to a cellular molecule which under normal physiological conditions interacts with an unrelated protein. p1 is believed to be permanently intracellular in yeast and there is no known reason why ovine skin cells would interact specifically with such a yeast protein.

Ovine efferent lymph cells were fixed and stained successfully with a polyclonal anti-ovine MHC class II serum, suggesting that the protocol used was effective in

the detection of at least some cellular molecules. However it was not possible to determine directly that the cellular receptor for MVV on the skin cells had been maintained in an intact form. No suitable detection system was available to confirm this.

No specific interaction was demonstrable for the p1:Env fusion proteins and a lysate of MVV-susceptible cells (section 6.3). Where the lysate was incubated with the p1:Env fusion proteins, or a crude preparation of p1 alone a band was detected, but this was not observed following incubation with purified p1. This indicated that a non-p1 yeast component was capable of interacting with a cellular molecule in this assay system. This does not necessarily contradict the result obtained using fixed cell monolayers from the same cell line, in which it appeared likely that the cells were interacting with the p1 component of the fusion proteins. The differences between the two sets of results are possibly a consequence of the non-reduced and reduced nature of the cell preparations in the two systems. The results with the cell lysate demonstrate the importance in these experiments of controlling for both the p1 and non-p1 components of the fusion protein preparations.

In section 6.4 it was reported that there was no detectable interaction between MHC class II molecules and the gp135 region of the fusion proteins. Technical problems with the assay system may have contributed to this failure to observe a specific interaction. In the first ELISA-based experiment reported in this section the four p1/p1:Env fusion preparations were used at different dilutions. These had been previously selected to yield comparable final readings, within the linear range on a dilution series when developed with the anti-VLP antiserum. In order to achieve this the purified p1 and p1:EnvA were used at dilutions of 1 in 5,000 and 1 in 20,000 respectively. However the results in chapter 4

demonstrated that yields of p1 alone were much higher than p1:EnvA, and therefore to obtain comparable ELISA readings it would have been expected that p1 should be diluted to a much greater extent than p1:EnvA. The disparity observed in these experiments highlights the fact that a similar ELISA reading based on reactivity with the anti-VLP serum does not directly correlate with absolute levels of p1. In addition ELISA values were affected by the amounts of contaminating proteins present which are also detectable by the anti-VLP serum. However, despite this it might have been anticipated that if there had been a specific interaction of the Env regions of the fusion proteins with MHC class II this might have been preferentially detected, as coating the plates with MHC class II molecules would have lent specificity to the molecules which would bind to the wells. At the concentrations used in this experiment no such interaction was observed.

No specific interaction was detectable when the plates were coated with the p1 or p1:Env fusion proteins, and then incubated with MHC class II. Much higher concentrations of the fusion proteins were used than in the previous experiment, although this could also result in higher levels of the contaminating proteins which would compete for binding sites on the plates. Again it must be noted that similar ELISA readings for the p1 and p1:Env fusion proteins with the anti-VLP serum do not necessarily imply that equivalent amounts of p1 and p1:Env had coated each well.

From these ELISA results it remains theoretically possible that MHC class II and the Env regions of the fusion proteins do interact but this has been undetected. This could be due to the combination of the low yields and poor purity of the fusion protein preparations, and the polyspecific reactivity of the anti-VLP antiserum which makes precise quantitation of the relative levels of the p1:Env fusion proteins difficult.

No interaction of the p1:Env fusion proteins with MHC class II was observed when the fusion protein preparations were incubated with MHC class II immobilised by blotting onto nitrocellulose after SDS-PAGE. This was despite using the p1:Env preparations at higher concentrations than in the ELISA-based experiments.

In none of the techniques used therefore was a specific interaction between MHC class II and the Env regions of the fusion proteins demonstrated.

In the experiments in section 6.5 the fusion proteins were used in an attempt to block infection of susceptible skin cell lines by MVV. In the first set of experiments no inhibition of infection was detectable. It is possible that the fusion proteins may have been causing some inhibition of infection but that the assay system used (checking for virally-induced syncytia) was too insensitive to detect this decrease. An alternative detection system, based on a p30 assay or monitoring reverse transcriptase levels (possibly at earlier time points post-infection) might yield more definitive results. However, even at the lowest viral dose used (0.6 TCID₅₀) no inhibitory effect of the fusion proteins on virally induced cpe was apparent.

It is possible that the levels of fusion protein added to the cells were inadequate to block viral infection. In HIV concentrations of gp120 as low as 2-4nM have been shown to block the fusion process (Matthews et al., 1987). It is not possible to determine accurately the amount of fusion protein in the p1:Env preparations for the reasons described in chapter 4. However, from a silver stained gel of p1:EnvA it can be estimated that 5ul of the peak sucrose gradient purified fraction contained approximately 50ng (from band intensity). Using a molecular weight of 97kDa for the p1:EnvA fusion protein it is possible to estimate that a concentration of p1:EnvA of approximately 1nM was used in these experiments.

Although this figure is very much an approximation it may indicate that the amount of fusion protein added to the cells was perhaps insufficient to achieve inhibition, had this been possible with these constructs. This does assume however that HIV gp120 can be used as a model for MVV gp135.

In an attempt to check that there was no detectable inhibition of infection by the fusion proteins the amounts added to the cells were increased by an order of magnitude. In this experiment it was difficult to detect MVV-induced cpe in any of the wells, and therefore no conclusions could be drawn regarding the ability of the fusion proteins to block infection. It was observed that at the concentration used p1:EnvC caused almost total destruction of the cell monolayer. As the p1:Env fusion proteins appeared from silver staining of SDS-PAGE gels to contain approximately equivalent levels of contaminating yeast proteins it is probable that this toxicity was due to the p1:EnvC protein itself. There is no direct evidence for the region of p1:EnvC which causes this cellular destruction. EnvC contains highly hydrophobic regions (chapter 3) which may be possible candidates if they lead to non-specific interaction with, and disruption of the cellular membrane. This may be particularly relevant to the highly hydrophobic C-terminal of EnvC, as it would be anticipated that this would be external on the VLPs. It would be necessary to generate deletion mutants or chimeric versions of the fusion protein to confirm this.

It is clear that much of the work reported in this chapter could have been more easily standardised and controlled with recombinant gp135 fragments which were produced with higher yields or at least improved purity (this will be discussed further in chapter 8). The use of a more readily quantifiable assay system for some of the work would also improve the confidence with which these

negative results are interpreted. There was no indication with any of the variety of techniques used that the fusion proteins interacted with any cellular molecules. If it is assumed that this lack of interaction is genuine, and not just a consequence of the low levels of the fusion proteins for example it is important to consider the possible reasons for this.

It has been assumed throughout this work that the Env regions of the fusion proteins are accessible in the VLP preparations. In the formation of hybrid VLPs the p1 molecules are expected to assemble such that the non-p1 fragment of the fusion protein is external on the VLP (Adams et al., 1988). However there was no direct confirmation of this for the fusion proteins used in these experiments. If the particles had mis-assembled such that all the gp135 fragments were completely sequestered within the VLP it would be impossible for them to interact with any cellular molecules, and therefore negative results would be obtained in all the assays attempted in the experiments reported in this chapter.

For expression in the Ty-VLP system large overlapping fragments of gp135 were chosen. This was to attempt to maintain, at least partially, the three-dimensional integrity of the intact protein. There is however no published data on the secondary, tertiary or quaternary structure of MVV gp135. There are 24 cysteine residues in MVV EV1 gp135 (Sargan et al., 1991) and therefore there is the potential for the formation of extensive secondary and tertiary structure. It is possible that even the use of relatively large fragments of gp135 as fusion proteins has resulted in the loss of structural motifs critical for interaction with the cellular receptor(s).

It is possible that the lack of any observed interaction between the fusion proteins and cellular molecules is due to the relative lack of glycosylation of the fusion proteins, compared with the glycosylation of

native gp135. As described in chapter 4 only the p1:EnvA fusion protein may be glycosylated, and it is possible that the glycosylation of this fragment does not mimic glycosylation of the native protein.

The role of glycosylation in the function of lentiviral envelope proteins has been most extensively studied for HIV gp120 but much of the data is contradictory. It has been reported that the removal of a single glycosylation site at amino acid 400 of HIV-2 gp105 leads to a 50-fold decrease in the efficiency of binding to CD4 (Morikawa et al., 1991). However it is not clear that the decreased affinity for CD4 was a direct consequence of the loss of carbohydrate at the residue. It has been shown that the loss of infectivity observed when a potential glycosylation site of HIV-1 gp120 was mutated was actually due to alteration in the secondary/tertiary structure of the protein, not the loss of carbohydrate (Willey et al., 1988).

It has been reported that unglycosylated recombinant full-length and truncated gp120, and deglycosylated native gp120 lose the ability to bind to CD4, and to block fusion (Putney et al., 1986; Matthews et al., 1987; Morikawa et al., 1990). It was therefore suggested that glycosylation of gp120 was essential for receptor binding. However similar experiments with recombinant gp120 and gp120 on intact virions has shown a diminution in binding to CD4 and infection, but not an abolition of the interaction (Fenouillet et al., 1990). The variation observed by different workers may reflect differences in technique, whether in the deglycosylation process or the subsequent functional assays. The presence of carbohydrate on gp120 appears therefore to affect the interaction of the molecule with its cellular receptor but the degree of importance of this in the viral life cycle is currently unclear. A major role of the extensive glycosylation of gp120 may be protection in vivo from

potentially neutralising antisera (Davis et al., 1990).

It is therefore possible that the inability to detect any interaction of the MVV gp135 fragments with cellular molecules reported in this chapter is a consequence of the apparent lack of appropriate glycosylation of the fusion proteins. Carbohydrate residues may be required for effective interaction of the virus and its receptor(s). In order to address this problem it would be necessary to express the gp135 fragments in an expression system in which normal glycosylation could take place.

It is also possible that the lack of interaction observed with MHC class II molecules may indicate that it is not gp135 that interacts with this molecule. The data of Dalziel et al. (1991) does not conclusively demonstrate that the postulated interaction with MHC class II is dependent on MVV gp135. If another viral protein for example gp46 is involved then it would not be expected that an interaction would be observed with the fusion proteins used in these experiments. However, this cannot explain the lack of interaction with skin cell lysates, which are expected to contain the cellular receptor which interacts with gp135.

6.7 Summary

This chapter reports attempts to use the p1:Env fusion proteins to identify regions of gp135 which are functionally involved in interactions with cellular molecules. It was not possible to demonstrate any specific binding of these fusion proteins in the variety of assays used in these investigations. This may have been due to the rather unsatisfactory yields and purity of the fusion proteins, or it may have been a consequence of structural features of gp135 itself which had been lost in generating these fragments.

7. Conclusions

Each results chapter in this thesis has contained an extensive discussion of the data presented, and these will not be repeated substantially in this chapter. This final discussion will attempt to summarise the major findings of this study on MVV gp135, and to indicate new areas of research which have been suggested by the work reported here.

There have been no previous reports of the generation of MVV gp135 recombinant proteins. In the work reported here the entire gp135 protein was expressed in the form of 3 overlapping fragments in the yeast Ty-VLP expression system, and the C-terminal 60% of the protein has also been expressed in the bacterial pGEX expression system. Parameters affecting the success of recombinant fusion protein production in these systems have been analysed, and the env gene sequences inserted into the expression vectors have been shown to exert a major effect on fusion protein expression. As poor yields were encountered in both expression systems it is possible that sequences within MVV gp135 tend to have a generally deleterious effect upon fusion protein production. This could not realistically have been predicted in advance. It may be possible to improve the yields of recombinant MVV gp135 by cloning smaller fragments of the gene into expression vectors. This could also allow the identification of those features which appear to lead to poor yields of the proteins.

Although some of the work reported in this thesis was hampered by the poor yields and low purity of the fusion proteins certain interesting observations were made. Perhaps unexpectedly the p1:EnvA fusion protein generated in the yeast Ty-VLP expression system was shown to be glycosylated. There have been no previous reports of glycosylation in this expression system, and it is possible (although not actually demonstrated) that the

glycosylation of the fusion protein affected assembly of the VLPs. By cloning smaller fragments of the envA region of gp135 into the same expression system it may be possible to determine which part of the EnvA protein leads to this glycosylation. Any candidate region could also be used to generate a chimeric molecule, for example with one of the other gp135 regions, to determine if this leads to glycosylation of a previously unglycosylated fusion protein.

By using the recombinant proteins it was demonstrated that MVV-infected sheep differ in the regions of gp135 to which they have mounted a detectable antibody response, and this observation may lead to interesting subsequent lines of investigation. A preliminary retrospective longitudinal study is about to be undertaken in the Department Of Veterinary Pathology (Dr. R.G. Dalziel, personal communication) to determine if the response of individual sheep to different regions of gp135 varies during the course of infection. It will also be interesting to investigate if there is any correlation of disease progression with the anti-gp135 responses. It may also be possible to link the anti-gp135 response to the neutralisation capability of sheep sera in vitro and so begin to identify regions of gp135 involved in the infective process. Prior to the generation of the recombinant proteins this work would not have been feasible.

Although none of the p1:Env fusion proteins were found to interact detectably with cellular molecules, or to inhibit infection in vitro the recombinant proteins may still be useful novel reagents for functional studies of MVV gp135. If an E.coli clone which expressed GST:EnvA can be successfully generated it would be appropriate to repeat the experiments reported for the p1:Env fusion proteins in chapter 6, but using the GST:Env fusion proteins. This would require an anti-GST antiserum (or

preferably a monoclonal antibody) and immunisation of rabbits and mice has begun at the time of writing.

The GST:Env proteins will be useful reagents for these functional studies, as they can be produced with much higher purity than the p1:Env fusion proteins. As there are fewer contaminants it may be possible to use much higher absolute levels of the fusion proteins themselves in these experiments, which may improve the likelihood of detecting interactions with cellular molecules. It will be interesting to determine if eluted GST:EnvC shows the same toxicity for skin cell cultures as p1:EnvC or if this is dependent on assembly into the VLPs.

Both the p1:Env and GST:Env fusion proteins could be used in inhibition of neutralisation assays. In these assays neutralising sera are preincubated with the fusion proteins, before incubation with virus. If any of the fusion proteins inhibit viral neutralisation this may indicate regions of gp135 which are targets in vivo for neutralising antisera, and which may be important in the initial stages of viral infection of cells. This would be best attempted using a sensitive detection system, for example monitoring reverse transcriptase levels, or p30 antigen detection.

The data reported for GST:EnvB suggested that rabbits immunised with this protein were beginning to mount an immune response to the gp135 region. If the GST:Env fusion proteins prove to be effective immunogens a wide range of experiments will become possible, which may begin to identify regions of gp135 involved in interactions with cells.

Both polyclonal antisera and monoclonal antibodies could be screened to determine if they are able to inhibit viral infection in vitro. This will allow identification of regions of gp135 which are involved in viral infection, information which does not currently exist for MVV. Monoclonal antibodies may be particularly informative for

this purpose. The monoclonal antibodies can be mapped crudely to different regions of gp135 rapidly and simply. For example, a monoclonal antibody raised initially against EnvA, but which also cross-reacted with EnvB could be predicted to recognise an epitope in the overlap between the 2 proteins. Similar mapping will be possible using the overlap between EnvB and EnvC. The N-termini of the Rev and gp135 proteins are coincident, and the availability of recombinant Rev protein in the Department Of Veterinary Pathology will allow further mapping of anti-EnvA monoclonal antibodies.

If any of the antisera are found to be neutralising it will be of interest to investigate at which point they interrupt the infection process. It is likely that antibodies to different regions of gp135 may affect different events for example initial binding, or post-binding entry events.

There still remains considerable interest in the identity of the cellular receptor(s) for MVV. Antisera raised against the recombinant proteins may be useful reagents for analysing this interaction. Immunoprecipitation of infected cells using the antisera (pooled polyclonal antisera would be the most appropriate initial choice) could be used to precipitate viral gp135 cross-linked with cellular molecules with which it interacts. These molecules could be analysed by SDS-PAGE.

The data reported in chapter 3 demonstrated that within the EV1 strain of MVV there appear to be conserved and variable regions of amino acids. The majority of these coincide with conserved and variable regions identified previously in the comparison of different isolates of MVV. The inherent variability of MVV, which is characteristic of the lentiviruses may lead to difficulties of interpretation in some experiments. For example in longitudinal studies of the response to MVV strain EV1 the risk is always present that an infected

animal fails to respond to a particular gp135 region because it has never encountered the antigenic sequence of the recombinant protein. However, although much of the work could be simplified if an infectious clone was available the data presented in chapter 3 does suggest possible further avenues of investigation.

Any neutralising antisera raised against the recombinant gp135 fragments could be tested against different viral strains eg Icelandic variant 1514, and against sequential isolates of EV1, or British field isolates of MVV.

It was demonstrated in chapter 3 that there are variations in the amino acid sequences between certain of the gp135 variants. These variants could all be expressed individually, possibly using an in vitro translation system and tested with sera from infected sheep. As the regions of amino acid diversity are already known for these fragments it would be possible to predict the regions which resulted in any change in reactivity of sheep sera with a particular gp135 variant.

The same approach could also be used to map the monoclonal antibodies obtained using the GST:Env fusion proteins.

The data in chapter 3 indicated regions of gp135 which are variable between viral isolates. It will be interesting to expand this work to investigate the extent of this variation in vivo, in both experimentally and naturally infected sheep (again an infectious clone would be useful for this study). From the data in chapter 3 it is possible to select PCR primers which would generate fragments which span regions of high predicted variability. Rather than introduce selection of viral variants which grow well under tissue culture conditions it may be more appropriate to perform the PCR without an intervening in vitro passage. The generated fragments could be sequenced directly, and they could also be

inserted into expression systems for further analysis with monoclonal antibodies. As controls the same protocol could be used to generate fragments of predicted high conservation.

Summary

Reagents have been generated which allow potential investigation of the interaction of MVV gp135 with the host animal, both at the level of infection of individual virus-susceptible cells, and the host immune response to this protein. There have been no published reports of the generation of recombinant MVV gp135 proteins previously, and these are reagents which may be critical in investigating the structural-functional relationships of this protein. The level of variability of this protein in vitro has been investigated, and this data will allow the selection of regions of gp135 for further study with more confidence than was previously possible.

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Appendix 1

Materials

All reagents supplied by BDH (Analar grade) or Sigma Chemical Company, unless otherwise stated.

General Solutions

STE

Tris HCl pH7.4	0.01M
NaCl	0.01M
EDTA	0.001M

20xSSC

NaCl	3M
Tri-sodium citrate	0.3M

1xTAE

Tris-acetate	0.04M
EDTA	0.001M

0.5xTBE

Tris-borate	0.045M
EDTA	0.001M

10xTE

Tris-HCl pH8	0.1M
EDTA	0.01M

1xTEN

Tris pH7.4	0.01M
EDTA	0.002M
NaCl	0.14M

SDS-PAGE sample buffer

Tris-HCl pH6.8	0.125M
Glycerol	20% (v/v)
SDS	2%
2-Mercaptoethanol	0.5%
Bromophenol blue	0.025%

Phosphate Buffered Saline

NaCl	0.137M
KCl	0.0027M
Na ₂ HPO ₄	0.008M
KH ₂ PO ₄	0.0015M
Adjust to pH 7.2	

Molecular Biology Reagents

Hybridisation Buffer

Milk powder	0.75%
Sodium phosphate buffer pH7	0.02M
SSC	2.82x
EDTA	0.001M
SDS	0.1%
Yeast tRNA	0.1mg/ml

Tfb1

Sodium acetate	0.035M
CaCl ₂	0.01M
RbCl	0.1M
MnCl ₂	0.068M
Glycerol	15% (w/v)
pH5.9	

Tfb2

MOPS	0.01M
(3-[N-Morpholino] propanosulphonic acid)	
RbCl	0.01M
CaCl ₂	0.075M
Glycerol	15% (w/v)
pH6.8	

5x Oligo-Labeling Buffer

Tris-HCl pH8	0.27M
MgCl ₂	0.027M
2-mercaptoethanol	0.37%
dATP	1.1 x 10 ⁻⁴ M
dGTP	1.1 x 10 ⁻⁴ M
dTTP	1.1 x 10 ⁻⁴ M
HEPES pH6.6	1.1M
(N-2-hydroxyethylpiperazine-N'-3-propanesulphonic acid)	
Hexadeoxyribonucleotides	1.2 x 10 ⁻³ M

DNA Sample Buffer

Sucrose	40% (w/v)
Bromophenol blue	0.25% (w/v)
Xylene cyanol FF	0.25% (w/v)

Phenol-chloroform

Phenol:chloroform	1:1
Hydroxyquiniline	0.05%w/v
Equilibrated to pH7.4 with 1 x TE	

Bacterial Culture Reagents And Media

Psi broth

Tryptone	2%
Yeast extract	0.5%
NaCl	0.01M
MgSO ₄	0.02M
KCl	0.005M

L-broth

Tryptone	1%
Yeast extract	0.5%
NaCl	0.17M
pH7.0	

2 x YT Medium

Tryptone	1.6%
Yeast extract	0.8%
NaCl	0.085M
pH7.0	

Yeast Culture Reagents

Sc-glc

Yeast N ₂ -base (no amino acids)	0.67%
D-glucose	1%

Sc-glc-agar

Yeast N ₂ -base (no amino acids)	0.67%
D-glucose	1%
Agar	2%

Sc-glc-gal

Yeast N ₂ -base	0.67%
D-glucose	0.3%
D-galactose	1%

Regeneration Agar

Sorbitol	1M
Yeast N ₂ -base (no amino acids)	0.67%
Glucose	1%
Agar	3%

Protease Inhibitors (In TEN)

Aprotonin	1.25ug/ml
Antipain	1.25ug/ml
Chymostatin	1.25ug/ml
Leupeptin	1.25ug/ml
Pepstatin	1.25ug/ml
Phenylmethylsulfonyl fluoride	50ug/ml

Tissue Culture Reagents

Fully Supplemented 8% Dulbecco's Modification Of Eagle's
Medium (8% DMEM)

DMEM	1x
(with non-essential amino acids)	
Foetal calf serum	8%
NaHCO ₃	0.036M
L-glutamine	0.002M
Penicillin/streptomycin	100U/ml

Fully Supplemented 2% DMEM

DMEM	1x
(with non-essential amino acids)	
Foetal calf serum	2%
NaHCO ₃	0.036M
Fungizone	2ug/ml
Gentamycin sulphate	0.2ug/ml

Versene

EDTA	5 x 10 ⁻⁴ M
Phenol red	1.3 x 10 ⁻³ %w/v

Trypsin-versene

Trypsin	0.05%w/v
EDTA	5 x 10 ⁻⁴ M
Phenol red	1.3 x 10 ⁻³ %w/v

Electron Microscopy Reagents

Cacodylate/Glutaraldehyde Buffer

Cacodylate pH6.8	0.1M
Glutaraldehyde	3%v/v
CaCl ₂	0.005M

Pre-Treatment Buffer

2-mercaptoethanol	0.1M
EDTA	0.02M
Tris-HCl pH8.1	0.2M

Phosphate/Citrate Buffer pH5.8

Citric acid	0.04M
Na ₂ HPO ₄	0.06M

Cacodylate Buffer

Cacodylate pH6.8	0.1M
CaCl ₂	0.005M

Western Blot Solutions

NBT Western Blot Development Solution

Tris-HCl pH9.5	0.1M
MgCl ₂	3.6 x 10 ⁻⁵ M
NBT (Nitroblue Tetrazolium)	0.18mg/ml
BCIP (Bromo-chloro-indoyl phenol toluidine salt)	0.09mg/ml

ELISA Solutions

OPD Substrate Solution

OPD (o-Phenylenediamine)	0.0012%w/v
Na ₂ HPO ₄	0.05M
Citric acid	0.023M
H ₂ O ₂	4.8 x 10 ⁻⁵ %v/v

Appendix 2

Statistical Formula

$$d = \bar{x}_1 - \bar{x}_2$$
$$\sqrt{\frac{\bar{x}_1}{n_1} + \frac{\bar{x}_2}{n_2}}$$

P	0.05	0.02	0.01	0.002	0.001
d	1.960	2.326	2.576	3.090	3.291