MEMBRANE FUSION AND THE TRANSMEMBRANE ENVELOPE GLYCOPROTEIN OF MAEDI VISNA VIRUS

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DECLARATION

I declare that this thesis has been composed by myself and has not been submitted for any other degree. The work described herein is my own except where otherwise indicated and all work of other authors is duly acknowledged.

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ABSTRACT

The transmembrane envelope glycoprotein, gp46 of maedi visna virus (MVV) was proposed to mediate membrane fusion between the virus and cell and between infected and uninfected cells on the basis of analogy with other viruses.

In order to look at the relationship between fusion and gp46, which is difficult to purify in quantity from virus preparations, recombinant gp46 (rgp46) was made. This first required cloning of the part of the MVV (strain EV1) *env* gene which encodes gp46. Sequence comparison with other viruses showed that gp46 is a typical transmembrane fusion protein, with a hydrophobic N-terminal fusion peptide. This putative fusion peptide was highly conserved between different MVV isolates although gp46 as a whole was only about 80% conserved.

rgp46 was expressed as fusion proteins in yeast and in bacteria, but could only be prepared at low yield and purity principally due to its toxicity to host cells. Immunised animals raised antibodies to rgp46 and sera from MVV-infected sheep specifically reacted with rgp46, whereas serum from uninfected sheep did not.

A fusion assay was developed using MVV to determine the effect of serum on MVV-mediated fusion. A significant difference was shown between the activity of sera from MVV-infected and uninfected sheep in the fusion assay: Serum from uninfected sheep generally enhanced fusion at low dilutions of serum (<1:64) and had no effect at higher dilutions, whereas serum from MVV infected animals tended to inhibit fusion at low serum dilutions (<1:16) and enhance at higher dilutions.

The effect of serum in the fusion assay was shown to be principally mediated by IgG and correlated with serum ELISA activity against a rgp46 preparation. This suggested a relationship between the presence of antibodies to gp46 and fusion modulatory activity.

These results may have important implications in consideration of potential vaccines and also in understanding how the virus can continue to replicate and eventually cause disease in the face of an apparently normal specific immune response.

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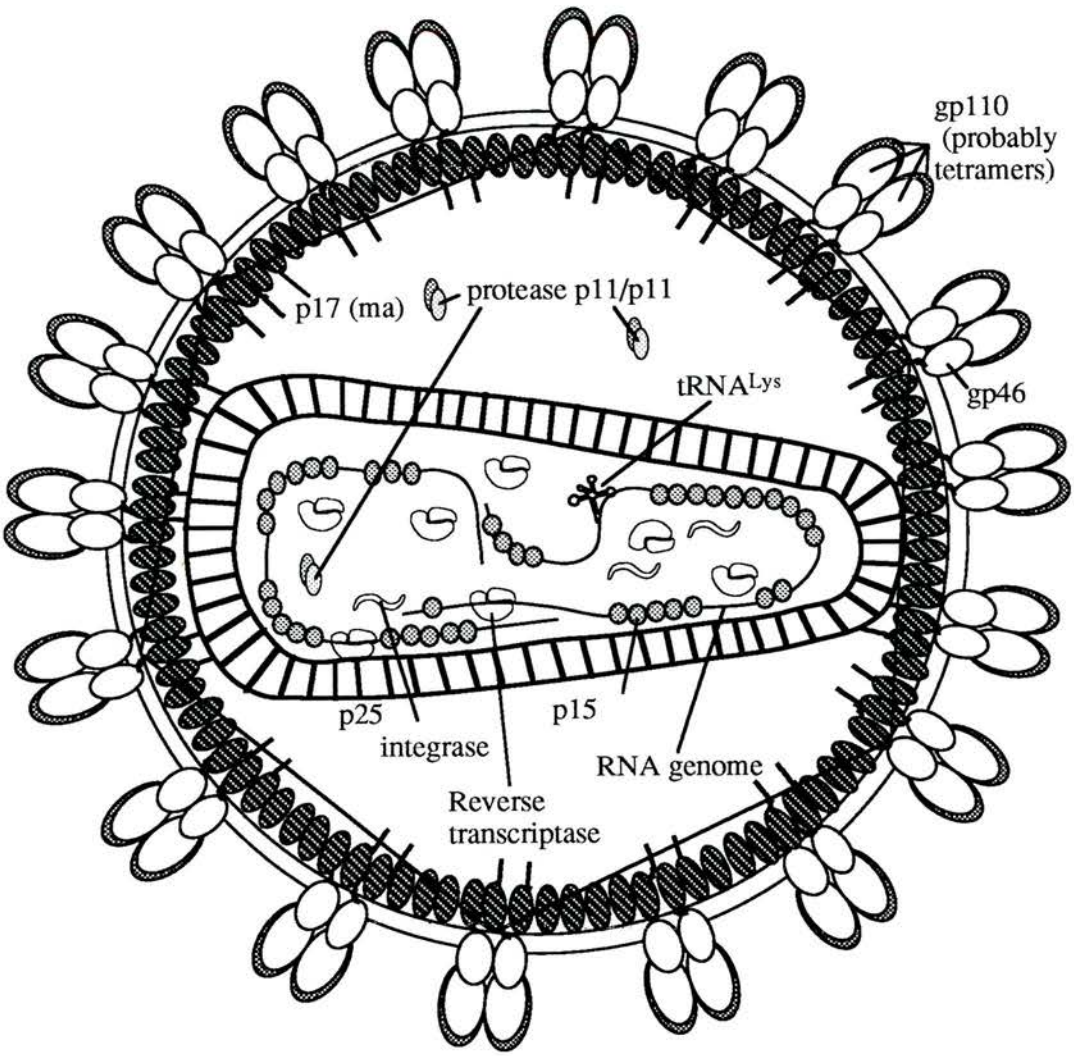
CHAPTER 1: Introduction and literature review

1.1: MAEDI-VISNA VIRUS

Maedi-visna virus, (MVV), (also known as visna virus), is a lentivirus, a member of the same sub-family of non-oncogenic retroviruses that includes the human, simian, bovine, and feline immunodeficiency viruses, (HIV, SIV, BIV, and FIV), caprine arthritis encephalitis virus, (CAEV), and equine infectious anemia virus, (EIAV). (Narayan *et al.*, 1980, Popovic *et al.*, 1984, Letven *et al.*, 1985, Gonda *et al.*, 1987, Pedersen *et al.*, 1987). The lentiviruses are enveloped viruses with a genome consisting of a positive stranded RNA molecule which encodes the three major structural genes 5'-*gag-pol-env* -3' (Sonigo *et al.*, 1985, Wain-Hobson *et al.*, 1985). The *env* gene encodes the envelope glycoptoteins (transmembrane and external), *gag* encodes the viral core proteins and a viral RNA binding protein, and *pol* encodes a complex of enzymes involved in reverse transcription of the viral RNA to proviral DNA and integration of the proviral DNA. In addition, a number of small open reading frames encode regulatory proteins involved in the complex regulation of lentiviral gene expression (Vigne *et al.*, 1990, Cullen 1992). A diagram of the structure of MVV is shown in Figure 1.1.

Maedi-visna virus causes a slow progressive fatal disease of sheep with a prolonged incubation period of months to years. Its name, derived from Icelandic, describes two of the principal clinical manifestations of the disease; maedi - shortness of breath and visna - wasting (Siggurdsson, 1954, Siggurdson and Palsson, 1958). The same virus is responsible for both maedi, the pulmonary form of the disease with dyspnea and loss of lung function, and visna, the neurological form which appears as loss of coordination, encephalopathy and eventually paralysis of the hind limbs. The range of clinical signs, which develop only gradually, make early diagnosis difficult in the absence of serological evidence of infection (Siggurdson and Palsson, 1958, Watt *et al.*, 1992). Loss of condition or wasting may be the first sign of the disease before the more severe pulmonary and/or neurological symptoms become manifest. Inflamed joints (arthritis) or mammary tissue (mastitis) may also be seen in some MVV infected sheep.

Figure 1.1: Stucture of MVV



The same strain of MVV can cause different disease conditions in susceptible breeds or infection can be asymptomatic. It is clear that the genetic background of the host can have a direct effect on the progression and outcome of infection by the virus (Houwers *et al.*, 1989).

Replication and cytopathic effect (CPE) of MVV differs depending on virus strain (Trowbridge *et al.*, 1976) as well as on the source and maturation state of the host cell (Gendelman *et al.*, 1986). Different MVV isolates may also differ in their ability to induce disease. Lairmore *et al.*, (1987), showed that MVV isolates which were cytopathic for alveolar macrophages *in vitro* were pathogenic when inoculated into neonatal lambs. However it should be noted that the strains they used were isolated from MVV-infected animals suffering from lymphocytic interstitial pneumonitis. A correlation was shown between pathogenicity and *in vitro* CPE on macrophages but not on choroid plexus or fibroblast cells.

1.1.2: **Epidemiology**

MVV was first described and isolated in Iceland (Siggurdsson, 1954, Siggurdson and Palsson, 1958). It appears that asymptomatic MVV-infected sheep of Karakul breed which were imported from Germany in 1933 brought the infection to Iceland (Siggurdsson, 1954). Infection of the susceptible indigenous population of Icelandic sheep with MVV resulted in epidemic outbreaks of MVV disease with a mortality of 10-20% per year on many farms. It is likely that Icelandic farming practices whereby large numbers of sheep were housed together at certain times of the year contributed to the rapid spread of the disease. Today the disease has been eradicated from Iceland by culling all infected sheep flocks but MVV infection still occurs in sheep in many other parts of the world (Adams and Gorham, 1986, East *et al.*, 1987). Often the infection does not become apparent and no disease symptoms appear within the normal lifespan of sheep (Cutlip, Jackson and Laird, 1977, de Boer *et al.*, 1979) but in some flocks the disease is a major cause of morbidity and sickness and is therefore of economic importance (Watt *et al.*, 1990, Dawson, 1980).

The virus is spread in colostrum, milk or bronchoalveolar secretions. It therefore passes to sucking lambs and also passes horizontally particularly between sheep housed in close proximity.

1.1.3: ***In vitro* culture of MVV**

In vitro MVV can be grown in monocyte/macrophage cells or fibroblast-like cells derived from a variety of foetal or adult ovine tissue such as choroid plexus, synovial membrane, or skin. MVV induces a cytopathic effect with the formation of syncytia. It replicates to fairly high titre *in vitro* and infectious virus can be recovered from the supernatant. It should be noted, however, that the strains which grow well *in vitro* in fibroblasts are tissue culture adapted strains.

1.1.4: **Replication of MVV *in vivo***

In vivo MVV replicates initially at the site of infection, probably the lung, and subsequently spreads. Infection and replication occurs preferentially in cells of the macrophage/monocyte lineage and dendritic cells causing a non-cytopathic, persistent infection of these cells in the bone marrow, blood, and inflamed tissues (Narayan *et al*, 1982, Gendelman *et al.*, 1985). In contrast to the *in vitro* situation, *in vivo* expression of the viral RNA and proteins is restricted to a minimal rate except under certain conditions, such as activation of an infected monocyte, when replication is stimulated (Narayan *et al.*, 1983, Gendelman *et al.*, 1985, Gendelman *et al.*, 1986). As macrophages are terminally differentiated cells with a relatively short lifespan, (about 3 months for alveolar macrophages, longer for some other tissue macrophages), it is likely that other cells are infected and provide a reservoir for lifelong persistence of the virus. One theory is that proviral DNA becomes integrated into the genome of myeloid stem cells in the bone marrow where infection becomes established early after infection of the sheep (Narayan and Jolly, 1990). Alternatively non-productive infection of non-macrophage cells such as fibroblasts may provide a harbour of persistency escaping immune surveillance by shutting down expression of viral proteins.

Little cell free virus is detectable in infected sheep (Kennedy-Stoskopf *et al.*, 1989). This may be due to the inability of field isolates to grow *in vitro* in the indicator lines used, or it may be that there is very little cell free virus *in vivo* and thus the majority of virus spread would be intracellular. During disease, replication becomes amplified and more virus can be isolated, especially from inflammatory lesions.

1.1.5: Immune responses to lentivirus infection

All arms of the immune system are involved in the response to infection by viruses. In addition to antibody, cell mediated immunity, phagocytes, complement, and interferon all play an important role in immune defence. However viruses exploit weaknesses in the host defences evading, avoiding or subverting the antiviral forces. The lentiviruses have evolved mechanisms of escaping immune destruction by all three of these strategies; despite the formation of both cellular and humoral immune responses, neither arm of the immune system seems to be able to indefinitely control the replication and spread of the virus and eventually most infected individuals develop pathological symptoms. The long clinically latent period during lentivirus infection suggests that for a long time the immune system may be able to control virus infection even though it is unable to clear it. During this time the virus may continue to replicate albeit at a restricted rate and eventually the cumulative adverse effects resulting from persisting virus on the immune system mean that the virus can no longer be controlled. The host immune responses may ultimately cause clinical disease rather than any direct effects of the virus, many of the symptoms being the result of chronic inflammatory effects. Whilst the immune response is sufficient to cause long-term tissue damage it is unable to clear the virus which suggests the immune response to MVV is inappropriate. Nevertheless the clinically latent period gives hope that augmenting the immune response to further shift control in favour of the immune system may enable virus clearance or at least prevention of disease.

1.1.6: **Immune response to MVV infection**

Some MVV infected animals may take more than six months to seroconvert, others may seroconvert and later become seronegative, others may never seroconvert (Houwers and van der Moulen, 1987). As a result of viral persistence in apparently seronegative carriers MVV may reappear in a flock from which seropositives have been culled (Houwers and van der Moulen, 1987). Despite the presence of neutralising antibody the virus may persist and continue to replicate. Some infected animals remain seropositive for life which is indicative of persistence and low level production of virus. Also virus can be isolated from the circulating blood monocytes of infected sheep at all times after infection (Narayan *et al.*, 1982, Gendelman *et al.*, 1986, Gendelman *et al.*, 1985).

MVV specific lymphocyte proliferative responses have been reported in sheep persistently infected with MVV (Reyburn *et al.*, 1992). In most animals the

major lymphocyte subset in PBMCs shown to proliferate in response to viral antigen were CD4+ T-lymphocytes although in some sheep CD8+ were also shown to respond.

1.1.7: **Virus neutralisation**

The binding of antibodies to viruses can result in loss of infectivity or neutralisation. Production of neutralising antibodies is a protective immune response which occurs early in most viral infections. The molecular principles underlying antibody neutralisation are less well understood than the other effector functions of antibodies e.g. opsonisation and complement activation. Neutralisation of viruses can occur by various mechanisms depending on factors such as the nature of the virus, the epitope recognised, the antibody isotype, the cell type/receptor for the virus and the virus:antibody ratio. Neutralising antibodies may act at various stages in the virus life cycle. Mechanisms of neutralisation include aggregation of virions, prevention of binding to the cell receptor and inhibition of events following attachment such as fusion of viral and cellular membranes and secondary uncoating of the virion.

Neutralisation is an *in vitro* phenomenon and cannot necessarily be extrapolated to *in vivo* activity. Neutralisation assays test the ability of a serum or antibody to reduce virus infectivity *in vitro*. The neutralisation titre is normally considered to be the highest dilution of serum/antibody at which the infectivity of the virus is reduced by 50% as measured by CPE, detection of viral antigen or reverse transcriptase assay. A large number of factors affect the result of neutralisation assays. These factors include the viral genotype used, the cell type used to propagate the virus stocks, the target cells used in the assay, the parameters used to measure virus production, and the time course of the assay.

Although neutralising antibodies may be demonstrated by *in vitro* assays, their *in vivo* role in prevention of virus infection or disease progression is not well understood. This is particularly true in cases where disease progression continues despite the presence of neutralising antibody, e.g. HIV or MVV. This is complicated by the fact that most studies have used assays against standard strains rather than testing the ability of the infected individual's serum to neutralise their own virus isolates.

1.1.8: **Neutralisation of MVV**

In studies with experimentally infected sheep it has been reported that there is a delay between the detection of non-protective complement fixing antibodies, (1-2 months p.i.), and the detection of neutralising antibody (~6 months p.i.) (Gudnadottir and Kristindottir, 1967). This time interval is variable even between different sheep experimentally infected with the same inoculate of virus. Other workers report detection of neutralising antibodies 3-4 weeks after experimental infection (Narayan *et al.*, 1977 Griffin *et al.*, 1978) but this may be the result of the route of experimental infection; Griffin *et al.*, (1978), inoculated each sheep intracerebrally with 10^8 pfu of MVV-1514.

Antibody titres are also variable, (1:32 to 1:1024 in studies by Petursson *et al.*, 1976). Often any neutralising antibodies detected are of very low affinity (Gudnadottir, 1990) and are inefficient at neutralisation (Kennedy-Stoskopf and Narayan, 1986). Assay techniques require prolonged incubation of virus with serum/antibody prior to addition to cells. *In vitro* MVV binds to cells within five minutes of incubation and attachment is maximal within fifteen minutes incubation. Incubation of MVV with neutralising antiserum for fifteen minutes does not reduce infectivity (Kennedy-Stoskopf and Narayan, 1986). This suggests that *in vivo* binding of virus to target cells may be more rapid than neutralisation and may be one reason why neutralising antibodies are not protective. The same authors showed that *in vitro* neutralisation involved IgG subclass 1 and was complement independent.

At first MVV neutralising antibodies are directed only against strain restricted envelope epitopes but the strain specificity broadens with time (Narayan *et al.*, 1981).

1.1.9: **ADCC**

Monoclonal antibodies against envelope glycoproteins have been shown to mediate lysis of infected cells (antibody dependent cell mediated cytotoxicity or ADCC) by NK cells, K cells or macrophages. Antibody bound to an infected cell is recognised by Fc gamma receptors on these immune cells and activates them to kill the infected cell. Some antibodies which direct ADCC have been localised to specific epitopes of HIV env, e.g. aa 644-653 and 579-604 of gp41 (Tyler *et al.*,1990), but it is likely that any antibody that recognises viral antigen on an infected cell would be able to direct ADCC provided it is of the right isotype.

ADCC responses may be important in restricting viral replication early in infection (Sawsyer *et al.*, 1990). In the case of HIV antibodies capable of inducing ADCC appear may be less strain specific than neutralising antibodies (Lyerly *et al.*,

1987). The same is likely to be true for MVV.

In sheep IgG2 mediates ADCC (Grant, 1975). Recent work by Bird and Reyburn (Personal communication), suggests there may be a specific deficiency in IgG2 against MVV proteins in the MVV-infected sheep although both subclasses are produced against other antigens by the MVV-infected sheep and immunisation of uninfected animals with recombinant MVV proteins induces both IgG1 and IgG2 antibodies. It is not known yet whether this deficiency occurs very early in infection when it would be particularly disadvantageous to the host.

If there is sufficient antibody coating the cell surface it may activate complement by the alternative pathway and the infected cell may be lysed by complement lysis complexes. This antibody dependent complement mediated cytotoxicity has been demonstrated using sera from MVV-infected sheep against MVV infected cells (Reyburn *et al.*, 1992).

1.1.10: **Enhancement of infection by anti-envelope antibodies**

The best known example of antibody dependent enhancement of infection is by Dengue virus, an enveloped virus, which uses non-neutralising antibody to enhance infection of monocytes/macrophages via binding to the Fc receptor (Halstead, 1988).

HIV infection can be enhanced by antibody mediated via Fc receptors (Takeda *et al.*, 1988) or complement receptors (Robinson *et al.*, 1988) which may also enable infection of cells not expressing the normal HIV receptor CD4 (Homsy *et al.*, 1989). Very low concentrations of antibody (0.05 - 5ng/ml) have been shown to enhance infection with limiting dilutions of virus (Perno *et al.*, 1990). Antibodies against two immunodominant regions of HIV gp41 may be particularly important in complement mediated antibody dependent enhancement (C-ADE) of HIV infection as synthetic peptides to which these monoclonal antibodies bind not only blocked the C-ADE mediated by these human mabs but also blocked C-ADE by polyclonal anti-HIV serum whilst other peptides did not. (Robinson *et al.*, 1990b, Robinson *et al.*, 1991). These two domains are highly conserved between all HIV-1, HIV-2 and SIV envelope proteins sequenced to date.

1.1.11: **Hidden Epitopes**

If functional epitopes are hidden the virus may escape neutralisation and the immune system may instead be wasting effort making antibodies of limited antiviral function. If of higher affinity these antibodies may actually be inhibiting the binding of neutralising antibodies.

The virus most closely related to MVV, CAEV, does not induce detectable

neutralising antibodies in the host, even though antibodies develop to all the CAEV proteins and there is no evidence to suggest impairment of the host immune system (Crawford and Adams, 1981, Klevjer-Anderson and McGuire, 1982). It has been suggested that the epitopes responsible for CAEV neutralisation are masked from the immune system.

1.1.12: **Antigenic variation**

One mechanism by which to escape either the humoral or cell mediated immune response is by antigenic drift or shift. In all lentiviruses antigenic variation occurs because frequent point mutations occur during replication due to the error-prone reverse transcriptase which lacks editing function. The greatest divergence between variants is seen within the *env* gene (Hahn *et al.*, 1985, Clements *et al.*, 1988). Antigenic variation may enable virus to escape immune surveillance by allowing the generation of neutralisation escape mutants. In the presence of strain specific neutralising antibody viral variants (or recombinants) with mutated epitopes which no longer bind the circulating neutralising antibody have a selective advantage.

The higher the frequency a viral variant reaches the higher the probability of its recognition and neutralisation. Consequently variants found at low frequency will have a selective advantage and will increase in frequency until they too are countered by the immmune response. A longitudinal study of HIV variation by Holmes *et al.*, (1992), showed that generally the most frequent sequence that appears then disappears completely probably due to neutralisation. In both EIAV and MVV antigenically distinct *env* variants arise progressively during infection and always precede their associated neutralising antibodies (Scott *et al.*, 1979, Salinovich *et al.*, 1986). In EIAV, as in HIV, neutralising antibodies seem to eradicate the strain of virus against which they have arisen. However, in MVV the original strains of virus do not completely disappear and parental strains can still be detected late in experimental infection long after the emergence of new variants (Lutley *et al.*, 1983).

In MVV, and other lentiviruses, a small number of base changes, deletion insertion, or changes in glycosylation patterns may have a profound effect on the ability of antibodies to neutralise the virus by altering the tertiary structure of the envelope protein thus exposing new epitopes and burying previous ones, rather than creating new antigenic sites (Stanley *et al.*, 1987, Salinovich *et al.*, 1986).

Although EIAV is structurally and morphologically similar to the other lentiviruses, the disease pathogenesis is significantly different. EIAV infection is typified by viral persistence, with recurrent episodes of fever due to the sequential

evolution of new variants escaping neutralisation by the antibodies directed against previous variants which results in an acute hemolytic crisis coinciding with a rapid round of replication by the new viral variant (Issel and Coggins, 1979, Montelaro *et al.*, 1984, Salinovich *et al.*, 1986). Distinct antigenic variants of EIAV are associated with each disease episode. The time taken for new EIAV variants to arise which can escape host immune defences can be as short as 28 days as clinical episodes may appear at 4-8 week intervals (Salinovich *et al.*, 1986). It is likely that emergence of new variants is associated with episodic disease in other lentiviral infections; minor and short-lived upsurges of viremia are seen in some asymptomatic HIV-infected patients (Nowak *et al.*, 1991) and also in SIV-infected Rhesus monkeys (Martin *et al.*, 1990). In the latter case recurrent antigenemia has been shown to be predictive of disease (Martin *et al.*, 1990).

In MVV it has been reported that antigenically distinct virus strains take 1-2 years to appear in the persistently infected animal (Clements *et al.*, 1980, Scott *et al.*, 1979). There is no reported evidence that the emergence of new variants is associated with episodic disease. In sheep persistently infected with MVV mitogen activation of a cannulated lymph node induces a surge of MVV in the afferent lymph, but this appears to be due to activation of immune cells rather than being related to a change in the virus antigenicity (Bird and Blacklaws, Personal communication).

MVV escape mutants can be generated *in vitro* by passaging virus in the presence of monospecific neutralising antibody (Clements *et al.*, 1988). The variants which emerge are closely related to the parental strain differing by only a few changes localised in the 3' end of *gp135* in the *env* gene. Many of the same point mutations were common amongst different variants isolated showing the occurrence of antigenic drift in a progressive manner. Mutations other than in *env* were rare and random, conveying no apparent selective advantage. *In vivo* common changes from parental to variant mutants in different sheep suggests that there is selective pressure on particular changes (or mutational hotspots).

Antigenic variation *in vivo* was only detected in 25% of infected sheep in antigenic cross-reactivity studies by Thormar *et al.*, (1983), and Lutley *et al.*, (1983). This low rate of variation would be insufficient for it to be an important mechanism for persistence. It may be that the methods used for detecting variation give a low estimate of the rate of antigenic variation. Using RNA footprinting, or peptide mapping it has been shown that MVV variants do emerge in infected animals (Scott *et al.*, 1979, Clements *et al.*, 1988). As described above, although MVV antigenic variants do arise the presence of neutralisation antibodies to a virus strain does not

enable it to be completely cleared and the parental strain may still be detected late in infection. (Narayan *et al.*, 1978, Ellis *et al.*, 1987, Clements *et al.*, 1988). One reason for this may be that bone marrow and other cell types which contain integrated provirus may act as a reservoir of the original strain as well as emerging variants (Gendelman *et al.*, 1985). The inability of the immune response to confine the virus to latently infected cells may in part be due to the emergence of variants that are no longer neutralised by antibodies against the parental strain. This gives the new strain time to spread before the host can mount an effective immune response. The restricted replication of MVV in vivo may account for the apparently gradual rate of variation (discussed below) compared to EIAV where a variant escaping immune control replicates very rapidly.

Large numbers of HIV variants exist both between and within patients as determined by nucleic acid sequence analyses. The envelope gene is the most divergent of the HIV genes especially in the hypervariable regions of *env* (Starcich *et al.*, 1986, Hahn *et al.*, 1985). Both convergent and divergent evolution of HIV *env* sequences have been reported (Holmes *et al.*, 1992). HIV variants can be generated experimentally *in vitro* and *in vivo* by selective pressure (McKeating *et al.*, 1988, Nara *et al.*, 1990).

In HIV immunoselective pressure varies over the course of infection as immune dysfunction/deficiency develops during disease progression. Different types of variant strain tend to appear at particular stages in the course of disease. Strains with with slow replication kinetics and low cytopathicity ("slow, low strains") predominate in infected individuals who are clinically healthy (Fenyo *et al.*, 1988, Tersmette *et al.*, 1988, Albert *et al.*, 1989). Progression to the disease state of AIDS is associated with a shift in the predominant strains of virus to "rapid, high strains" (rapid replication kinetics, increased cytopathicity) (Fenyo *et al.*,1988, Tersmette *et al.*, 1988). This suggests that early in infection the "slow, low" variants are better able to replicate in the presence of an active immune system and have a selective advantage over the "rapid, high" variants. As the total number of antigenically distinct viral strains increases over time the total number of CD4+ cells goes down. To start with specific CD4+ cells replicate in response to virus, but then as CD4+ cell numbers decline later in the course of HIV infection the total virus population escapes regulation by the immune system and consequently the faster replicating strains begin to predominate over the earlier slow growing strains.

Nowak *et al.* (1991) go as far as to suggest that viral antigenic diversity is the cause not a consequence of AIDS. They proposed that the immune system is only

able to mount an effective response against HIV quasispecies (populations of closely related genomes) within a range of diversity but that once the population of viral strains exceeds the "diversity threshold" the immune system is no longer able to control viral regulation.

1.1.13: **Antigenic variation and escape from cell mediated immunity**

Antigenic variation can also allow escape from specific T-cell activity. Presentation of viral peptides in the context of MHCII induces activation and proliferation of T-cells expressing a T-cell receptor (TCR) which binds the peptide/MHCII ligand. Antigenic variation resulting in presentation of an altered peptide may alter the affinity of the TCR for the peptide/MHC complex and therefore alter the T-cell response. For example, HIV variants which escape CTL (cytotoxic T-lymphocyte) recognition by generating an epitope which no longer stimulates a CTL response have been reported (Phillips *et al.*, 1991).

A recent paper by Sloan-Lancaster *et al.*, (1993) demonstrated that presentation of an altered peptide by antigen presenting cells to a cytotoxic T-cell clone, which had been selected against an immunogenic peptide, either reduced the affinity such that the TCR did not bind the altered peptide and the T-cell clone did not respond or such that the TCR bound peptide but only at low affinity which induced partial activation and subsequent anergy of the T-cells. These cells consequently became unable to respond to the altered peptide or to the original immunogenic peptide. Whether this *in vitro* phenomenon, namely, induction of clonal anergy occurs *in vivo* has yet to be investigated. If it occurs *in vivo* it is possible that mutant/variable viral peptides presented by APCs actually provide protection by inducing anergy of T-cells responsive to previous virus variants.

1.1.14: **Avoidance of the immune system : Latency**

Some virus infected cells escape elimination by the immune system because synthesis of viral antigen is restricted by tight regulation of viral gene expression, i.e. by virus latency. Virus latency is possible due to the highly regulated expression of lentiviral genes controlled by complex mechanisms involving transcriptional as well as post-transcriptional events (reviewed by Allan, 1992).

At the clinical level, lentiviral latency may last many years, e.g. HIV infection has on average a clinically latent period of 8-12 years during which time the individual remains in good health and generally maintains normal levels of CD4 cells. Similarly MVV infection may have no clear pathological signs until years after infection. However during the clinical period virus replication may actually be continuing albeit at a low level. Apart from possible infection of haematopoietic

stem cells, (Kaczmarski *et al.*, 1992), the lentiviruses infect cells with a relatively short life in comparison to the neurones in which latent herpes virus persists, therefore it may be necessary for replication to be ocurring in at least some of the infected cells at all times for the virus to infect new cells and survive in the host. HIV-1 RNA can be detected in infected individuals at all stages in disease suggesting some virus replication is continuing even during the clinically asymptomatic phase when the majority of infected cells are harbouring latent virus (Harper *et al.*, 1986, Schnittman *et al.*, 1991). The lymph node cells are more likely to be producing infectious virus than cells in the blood (Pantaleo *et al.*, 1993, Embretson *et al.*, 1993).

In the case of MVV, *in situ* hybridisation shows that transcription is markedly restricted *in vivo* compared to productively infected cells in culture (Haase et al, 1977, Geballe *et al.*, 1985, Brahic *et al.*, 1987). There is also post-transcriptional regulation of antigen expression (Gendelman *et al.*, 1985, Stowring *et al.*, 1985). In cell culture the level of virus transcription and production is directly related to the rate of synthesis of proviral DNA (Haase *et al.*, 1982) suggesting that there is no negative control acting upon viral transcription and translation *in vitro*.

1.1.15: **Subversion/destruction of the immune system**

One way to escape immune destruction is to actually destroy components of the immune system. As their names imply, the immunodeficiency viruses have the most severe detrimental effects on the immune system. This may be by direct destruction of immune cells, by causing dysfunction of infected or uninfected cells and/or by the effects of chronic stimulation of the immune system.

HIV infection leads to a myriad of immunologic defects resulting in AIDS. There is depletion of CD4 positive lymphocytes, dysfunction of remaining CD4 cells, abnormal cytokine production, B-cell hyperreactivity, clonal defects of certain Vβ subfamilies, defects in antigen presentation, anergy in CD8+ cells and other immunologic disorders. Some of these abnormalities may be a direct result of CD4 cell infection or be a result of HIV infection of other immune cells in particular cells of the monocyte macrophage lineage.

Altered CD4:CD8 cell ratios and T-cell numbers are often seen in MVV infected animals, although there is no evidence that MVV directly affects CD4+ T-cells. Watt *et al.*, (1992), reported that the number of T-cells was significantly increased in the lungs of MVV infected sheep but the CD4:CD8 ratio was similar to the ratio in uninfected lungs. However in synovial fluid of MVV arthritic joints CD8+ cells were the primary cells and a decreased CD4:CD8 ratio was seen in the

peripheral circulation of diseased animals. In HIV a decreased CD4:CD8 ratio results from destruction of CD4 cells, whereas in MVV infected sheep depressed CD4:CD8 ratios were found due to decreased numbers of CD4 cells and/or increased numbers of CD8 cells (Kennedy-Stoskopf *et al.*, 1989). In MVV-infected lymph nodes significantly increased numbers of CD8 cells with only a slight increase in the number of CD4 cells resulting in a significantly decreased CD4:CD8 ratio have been reported (Watt *et al.*, 1992).

1.1.16: **Chronic inflammation as a response to persistent infection**

Lentivirus infection, like any persistent antigen (e.g. asbestos fragments in the lung), results in the local production of excessive chemotactic factors which recruit leucocytes to areas of inflammation (Koenig *et al.*, 1986, Lairmore *et al.*, 1987). Recruitment of lymphocytes may serve to promote tissue damage and further accelerate the influx of monocyte/macrophages into the lesion. The inflammatory process thereby provides an influx of cells which may either already be carrying virus or which are susceptible to infection. In addition the incoming cells may be responsible for tissue injury and clinical symptoms. For example, in other viral infections cytotoxic T-cells have been shown to clear virus but augment pulmonary disease (Cannon *et al.*, 1988). Successful treatment of HIV-lymphocytic interstitial pneumonia (LIP) patients by steroid therapy (immunosuppressive), (Morris *et al.*, 1987), and the prevention of MVV induced encephalomyelitis in immunosuppressed sheep (Nathanson *et al.*, 1976) supports the critical role of the inflammatory process in lentiviral disease. Increased virus and antigen loads have also been shown to correlate with disease severity both in MVV, (Brodie *et al.*, 1992), and in HIV infection (Schnittman *et al.*, 1990). Antigen challenge caused virus reactivation in MVV infected sheep (B.A. Blacklaws and P.Bird, personal communication) suggesting that the inflammatory or DTH response increases virus production which in turn induces inflammation that, unless controlled, could give a downward spiral in disease progression.

Chronic inflammation triggered by the anti-viral immune response probably leads to the lesions described in MVV which eventually cause manifest disease (Griffin *et al.*, 1978). Post-infection immunisation with whole inactivated virus enhances the severity of visna disease whilst immunosuppression reduces the inflammatory lesions (Nathanson *et al.*, 1985, Georgsson *et al.*, 1976).

Similar inflammatory pathologies to those described for MVV have been reported in the lung , joints, and CNS of patients infected with HIV (Scott *et al.*, 1984, Joshi *et al.*, 1985, Rynes *et al.*, 1987, Sharer, 1992). In HIV, as in MVV,

infected alveolar macrophages are likely to be involved in the induction of immunoproliferative and inflammatory reactions as they are targets for CD8+ cytotoxic T-cells (Plata *et al.*, 1990).

It has been suggested that MVV may predispose the lung to certain secondary infections (Watt *et al.*, 1992, Brodie *et al.*, 1992). Whether this is a particular property of MVV infection or may result from any chronic inflammatory condition is not clear. Note for example that asthma sufferers, in whom the lung suffers chronic inflammation, are predisposed to lung infections (Corrigan and Kay, 1992).

1.1.17: **Summary of Section 1.1**

In summary the lentiviruses have evolved a range of strategies to enable persistence and avoid immune clearance. MVV differs from HIV in that there is no significant destruction of one particular subset of immune cells. Nevertheless the immune system is unable to clear MVV and the virus continues to replicate in the face of active immune responses. Eventually continued persistence of MVV leads to pathological changes probably resulting from tissue damage by the inflammatory response. There appears to be a delicate balance between the immune system and MVV infection/replication. The control of this balance remains to be elucidated.

1.2: RELEVANCE OF IMMUNE RESPONSES WHICH AFFECT FUSION

1.2.1: **Correlation of fusion inhibitory antibodies with disease state in HIV infection**

A humoral immune response to HIV envelope has been shown to affect disease outcome. Symptom-free HIV-positive individuals are more likely to have higher neutralising antibody titres than symptomatic patients (Weber *et al.*, 1987, Robert-Guroff *et al.*, 1987). The presence of antibodies to the putative immunosuppressive region of HIV gp41 has also been linked to the absence of disease symptoms (Klasse *et al.*, 1988).

A study by Brenner *et al.* (1991) suggested that there is a relationship between the presence of HIV syncytium inhibiting antibodies and the clinical outcome in children infected with HIV. 65% (15/23) of a group of children who had detectable antibodies to HIV by western blotting had syncytium inhibitory antibodies. All of the eight children with lymphocytic pneumonitis had syncytium inhibitory antibody titres of 1:40 to 1:320, whereas only two of seven children with

opportunistic infections had syncytium inhibitory activity and these two both had syncytium inhibitory titres of 1:20. Whether syncytium inhibiting antibodies play a role in the pathology of lymphocytic pneumonitis or whether this state is more likely to induce syncytium inhibitory antibodies cannot be determined from this study.

1.2.2: **Vaccines**

An immune response to the envelope glycoproteins appears to be a vital part of the immune response to enveloped viruses. Experience with a number of viruses has shown that eliciting an immune response to the envelope proteins may be an important way to induce immunity to that virus or to decrease the severity of disease and aid virus clearance if infection does occur. For example, vaccinia virus recombinants which express paramyxovirus F protein induce a protective immune response (Merz *et al.*, 1980). Similarly vaccination with a recombinant fowlpox virus expressing the F protein of the paramyxovirus Newcastle disease virus, (NDV), protects against subsequent challenge with NDV (Taylor *et al.*, 1990). Also, a recombinant canarypox virus expressing measles virus F and HA glycoproteins protected dogs against subsequent challenge with canine distemper virus, which is closely related to measles virus (Taylor *et al.*, 1992). Interestingly the host-restricted fowlpox recombinant which could not replicate in the vaccinee induced a similar level of measles virus neutralising antibodies and resistance to CDV infection as the equivalent replication competent vaccinia recombinant.

To be effective vaccines must induce protective immunity so that upon exposure the immune system rapidly recognises and destroys the pathogen. Immunisation must present, in the correct form, those elements of the pathogen which can elicit a protective immune response taking into account that some elements of the pathogen may elicit immune responses which negate those that are beneficial. Obviously it is better for a vaccine not to include such negative elements. Not only are the epitopes included important but also the titre of antibody induced. Antisera against HIV which are neutralising against a moderate viral dose have been shown to enhance infection at low m.o.i.s (Perno *et al.*, 1990). If this phenomenon also occurs *in vivo* vaccination which induces only a low anti-viral titre might have the opposite to the desired effect.

1.2.3: **Potential problems of using inactivated virus vaccines with altered envelope glycoprotein immunogenicity**

The importance of inducing a balanced immune response to viral envelope glycoproteins has been shown by experience with several human vaccines.

Vaccination with formalin inactivated measles virus served to potentiate rather than prevent infection by inducing immunity to the H protein but not the F protein (Fulginiti *et al.*, 1967). Formalin inactivated respiratory syncytial virus also induced an unbalanced immune response which exacerbated disease (Murphy and Walsh, 1988). The problem appeared to be that a high concentration of anti-viral antibodies was raised only to non-protective epitopes. The use of vaccines with such altered immunogenicity primes for an accelerated immune response against non-protective epitopes so upon subsequent infection effective resistance is not provided but immune complexes may form with subsequent complications such as an Arthus reaction in the lung which can contribute to potentiation of disease.

1.2.4: **Immunisation with HIV envelope**

The protection demonstrated by vaccines based on HA and F glycoproteins is one reason why the HIV-1 envelope glycoprotein has been the major target of HIV vaccine development to date.

Many test animals immunised with HIV-1 envelope-derived immunogens elicit a type-specific neutralising response (Matthews *et al.*, 1986). Numerous attempts have been made to raise cross-reactive neutralising antibodies against conserved regions of gp120 using viral envelope or sub-units (e.g. Girard *et al.*, 1991, Berman *et al.*, 1990). Antisera raised against the principal neutralisation determinant (PND) in the gp120 V3 loop can inhibit HIV replication and inhibit syncytium formation *in vitro*, but only in a type-specific manner, even though the 3D structure of the V3 loop and the amino acids at the top of the loop are conserved (Palker *et al.*, 1988, Goudsmit *et al.*, 1988, Javaherian *et al.*, 1990). To date studies have not uncovered a dominant neutralising epitope from a conserved region which suggests that potential broadly neutralising epitopes are inaccessible to antibodies.

A cross-reactive epitope (with reference to different strains of HIV) has been found in gp41; a synthetic peptide representing the region from 735-752 of HIV gp41 is recognised by antisera from HIV positive patients and induces antibodies which neutralise a range of HIV-1 isolates (Kennedy *et al.*, 1986, Chanh *et al.*, 1986, Dalgliesh *et al.*, 1988). When an engineered poliovirus chimaera containing this epitope was used to immunise rabbits antibodies specific for HIV gp41 were elicited, as measured by peptide binding assays and by Western blotting (Evans *et al.*, 1989). The antisera also neutralised a range of HIV-1 isolates and inhibited HIV induced cell-to-cell fusion. As well as demonstrating the possibility of a broadly-reactive vaccine this shows the potential of using poliovirus to present HIV antigens.

1.2.5: **Development of HIV vaccines**

A number of vaccine studies have been conducted in chimps using a variety of immunogens. In the first experiments, from 1985-1989, vaccination did not protect from challenge infection (Reviewed by Girard and Eichberg, 1990). However neutralising antibodies were low or absent and cellular immunity was weak at the time of challenge. Berman and colleagues (1990) succeeded in protecting chimpanzees from infection by improving the properties of the immunogen and immunisation schedule and inducing a good titre of neutralising antibody particularly against the principal neutralising domain (PND) of gp120. A consistent correlation between neutralising antibodies to the PND and protection from challenge infection with a homologous HIV strain has been shown (Emini *et al.*, 1990 and 1992), but have been shown not to protect against a distinct strain of HIV (Fultz *et al.*, 1987). This corresponds to in vitro assays where neutralisation by antibodies to the PND are type-specific.

Protection against heterologous strains of HIV has yet to be acheived. Although neutralising antibodies against the PND are important for protection against homologous strains, other forms of anti-viral immunity, which are less strain-specific, need to be induced. One option would be to identify more epitopes such as the gp41 epitope described above which can neutralise a range of strains and perhaps to include a range of peptides representing different groups of strains to induce a series of antibodies which together might be broadly protective.

1.2.6: **A universal vaccine against enveloped viruses ?**

As there are such striking similarities between the different virus fusion proteins it is very likely that the actual mechanism of fusion when it is finally worked out will be very similar. Although this suggests a possibility of a single vaccine or chemotherapeutic agent to prevent or treat infection by all fusogenic viruses, it also appears that some normal cellular mechanisms of membrane fusion may be very similar to viral mediated fusion and therefore may also be a target for any potential cross-species anti-virals. Therefore there is still a need to further elucidate viral fusion mechanisms and to identify specific viral components which may act as conserved targets and which are accessible to antibodies to prevent infection. It may be that no such targets exist.

1.3: VIRUS MEDIATED MEMBRANE FUSION

Surrounding the virion of enveloped viruses is an envelope or viral membrane which consists of host derived lipid bilayer containing virus encoded envelope proteins. In order for enveloped viruses to infect a cell fusion of the viral and cell membrane must occur thus releasing the capsid into the cytoplasm of the new host cell.

Membrane fusion can also occur late in the infection cycle when viral proteins become expressed in the cell membrane and may mediate fusion of infected cells with neighbouring uninfected cells. Cell-to-cell fusion results in the formation of large multinucleate cells called syncytia. Cell fusion and the formation of multinucleate syncytial cells *in vivo* can result in localised tissue damage and be a major cause of pathology. For example, the major CPE on cells in culture by measles virus is syncytium formation, whilst *in vivo*, a similar mechanism may enable cell-to cell spread of virus which sometimes results in a more serious complication of measles infection, giant cell pneumonia (Mitus *et al.*, 1959). Another example of fusion related cytopathology is by HIV which causes giant cell formation followed by rapid cell death. This may be one way by which susceptible cells are lost during HIV infection (Lifson *et al.*, 1986a, Sodroski *et al.*, 1986, Lifson, 1986b).

1.3.2: ***In vitro*** **virus-induced cell fusion**

When a high m.o.i. of fusogenic virus is added to susceptible cells *in vitro*, fusion from without (FFWO) can occur. FFWO occurs rapidly and is independent of virus replication. This is shown by the fact that it can be mediated by non-infective virus as long as the viral membrane integrity is maintained and also can occur with cells in which the virus is unable to replicate (e.g. MVV can cause FFWO in hamster kidney cells). MVV can cause fusion from without at a multiplicity of 4 virions per cell (Harter and Choppin, 1967).

Fusion from within (FFWI) occurs late in the viral life cycle. It involves fusion of infected cells with neighbouring uninfected cells. FFWI is inhibited by metabolic inhibitors. This shows the requirement for the synthesis of new virus specific macromolecules for FFWI to occur. Expression of the virus receptor on the uninfected cell is also necessary for FFWI to occur.

FFWO is a convenient method for studying membrane fusion in vitro. The degree of cell fusion seen by FFWO *in vitro* is variable and is dependent on a number of factors. The extent of cell fusion and size of polykaryons increases with

virus concentration and with degree of confluence of monolayered cells. Cell susceptibility is also an important factor. It is affected by number, availability and type of virus cell surface receptors, by the lipid composition of the cell membrane and the nature of the cytoskeletal network of the cells as well as by a number of ill-defined factors such as cell passage number, time from subculture to virus exposure and stage in the cell cycle.

1.3.3: **Virus-mediated membrane fusion**

Entry of the virus into the host cell is a multi-step process. Infection is initiated by attachment of the virus to the prospective host cell bringing the opposing membranes into close proximity to allow the next step, membrane fusion, to proceed. Binding of the virus to the target cell is generally via the interaction of a viral attachment protein with a specific molecule (receptor) expressed on the surface of the target cell. Different viruses exploit different cell surface molecules as receptors (Reviewed by Lentz, 1990). For example, influenza HA binds to sialic acid containing receptors on the target membrane, HIV gp120 mediates virus binding to CD4 molecules expressed only on certain immune cells, whilst MVV interacts with MHCII and may use it as a receptor (Dalziel *et al.*, 1991). The specificity of the virus:receptor reaction is in part responsible for restricting the cell types which the virus is able to infect (including species specificity). However, it is clear that secondary receptors or other binding mechanisms may also be used as viruses sometimes infect cells where the usual receptor cannot be detected e.g. HIV infection of CD4 negative glial cells. In addition antibody or complement can mediate binding of virus to cells expressing FcR or complement receptors.

Binding of the virus to the host cell holds the two membranes close together as a prerequisite to membrane fusion but is not sufficient alone to trigger membrane fusion. Viral receptor binding and membrane fusion are mediated by proteins which form spike-like projections from the viral envelope. In some viruses two sub-units derived from a single glycoprotein or two separate proteins which are generally linked non-covalently or by di-sulphide bonds mediate binding and membrane fusion.

Two separate activation steps regulate virus fusion and ensure that assembly and entry functions do not interfere with one another. Typically the functions required for virus entry are not activated before the final stages in virus maturation and release from the cell. For most enveloped viruses a host-cell-mediated limited proteolysis of the viral fusion protein makes it sensitive to the later activation event. In some cases the cleavage generates a fusion protein that is activated upon receptor

binding thereby resulting in fusion directly with the target cell surface (e.g. HIV or MVV). In other cases the acidic conditions of the endosome following receptor mediated endocytosis are required for activation and fusion occurs with the endosomal membrane. This difference can be used to classify viral fusion reactions into two general groups; pH independent and low pH dependent, respectively. Some pH independent viruses such as the herpesviruses have been shown to enter the cell by either route i.e. by fusion with the plasma membrane or via the endosome.

1.3.4: **pH dependent virus fusion**

Virus families which are dependent on an acid pH to enable fusion to occur include orthomyxoviruses, e.g. influenza virus, togaviruses, e.g. Semliki Forest Virus (Lobigs and Garoff, 1990) , and flaviviruses e.g. Dengue virus (Halstead,1988, Summers *et al.*,1989). Semliki Forest Virus was the first virus used to delineate the acidic/endocytic fusion pathway which has proved to be very similar for all these viruses. Virus particles bind to cell surface receptors and are endocytosed in coated pits and coated vesicles (receptor mediated endocytosis). As the endosome becomes acidic to effect its normal function the low pH induces a conformational change in the envelope glycoprotein(s) converting it to its fusogenic form so that virus-host membrane fusion takes place. Fusion with the endosomal membrane releases the nucleocapsid into the cytoplasm.

In the case of Influenza virus the low pH induces an irreversible conformational change in the HA glycoprotein which converts it to its fusogenic form. (This will be described in much greater detail below). Similarly low pH triggers Semliki forest virus to fuse by inducing irreversible conformational changes in the viral spike protein which mediates fusion.

1.3.5: **pH independent virus fusion**

Paramyxoviruses, herpesviruses, and some retroviruses fuse with cells in a pH independent manner. The mechanism by which pH independent fusion reactions occur remains to be elucidated. As with pH dependent fusion it is mediated by specific fusion glycoproteins in the viral membrane and receptor binding precedes a conformational change which then allows fusion to occur. Fusion is thought to be mediated by a very hydrophobic region at the amino-terminal end ofthe F1 sub-unit in the case of paramyxoviruses or of the transmembrane envelope glycoprotein in the retroviruses (Richardson *et al.*, 1980, Richardson and Choppin, 1983, Stein *et al.*, 1987. Retrovirus fusion will be discussed in some detail in section 1.3.9.

1.3.6: **The mechanism of membrane fusion**

Because of the stability of the lipid bilayer membrane structure, fusion of two membranes is an energetically unfavourable event. The first requirement for fusion is to overcome the electrostatic force which repels two membranes and to bring them close together (Rand, 1981). It is thought that destabilisation of the structure of the opposing membranes is the next step in fusion. Changes in molecular organisation to create an area of lipid bilayer free of integral proteins may be necessary. Then there must be mixing of the two membranes and finally reformation of a stable lipid bilayer.

It has been suggested that membrane destabilisation occurs by the calcium induced formation of domains of crystalline acidic phospholipids within the surrounding non-crystalline lipid. (Reviewed by Poste and Pasternak, 1978). The boundaries of crystalline domains on adjacent membranes may represent sites at which fusion occurs (Papahadjopoulos *et al.*, 1974). Fusion susceptibility of a membrane has been shown to be dependent on the phospholipid type, density and orientation. This may be accounted for by the fact that some phospholipids crystallise at concentrations within physiological ranges while others do not.

1.3.7: **Mechanism of virus membrane fusion**

Although the exact mechanism is unknown it appears that viruses make use of envelope glycoproteins to disrupt membrane stability and induce fusion. Attachment of the virus to its receptor is temperature independent, whilst fusion of the virus envelope to the cell membrane is optimum around 37°C and is much slower at reduced temperatures. Although it is generally accepted that fusion is inhibited below 18°C, Stegmann and colleagues, (1990), showed that fusion of influenza virus with liposomes could still occur at 0°C albeit very slowly. This suggests a requirement for fluidity of the membrane lipids as this is inhibited at low temperatures. The lateral movement of intramembranous particles within the membrane also seems to be important. For example, red blood cell ghosts treated with anti-spectrin antibodies to cross-link the protein and prevent it clustering in the membrane are not able to be fused by Sendai virus. The opposite effect is shown by exogenous proteolytic enzymes which release membrane proteins from the contractile cytoskeletal networks and can fuse human erythrocytes in the absence of any virus. However not all chemically induced cell fusion seems to depend on proteolytic breakdown of the membrane skeleton, and there is no evidence that viral membrane fusion relies on a breakdown of the membrane skeleton. Nor is there evidence that a functional membrane skeleton is required for viral fusion to cells

since treatment with Cytochalasin-B, which is a cytoskeletal inhibitor, permits initial fusion of the virus. Cytochalasin-B blocks subsequent cell-to-cell fusion, but this merely confirms the expectation that participation of the membrane associated microfilament system is required to enable the transport/organisation of newly synthesised viral membrane proteins necessary for cell-to-cell fusion. Whilst disturbance of microfilament function suppresses virus-induced cell fusion, disruption of microtubules enhances cell fusion (Yamamoto *et al.*, 1984).

All viral fusion proteins are glycosylated. The glycosylation pattern of the envelope glycoprotein is determined by the host cell and can affect the host range of the progeny virions (Feizi and Larkin, 1990) perhaps by affecting the interaction with carbohydrate-binding proteins on the target cell surface. Glycosylation is also important in defining secondary or tertiary structure of the fusion proteins. For example, it has been shown in mutation studies that, whilst individual glycosylation sites on HIV gp41 are dispensable, if glycosylation of all the sites is prevented the conformation of *env* products is altered and fusion activity is lost (Dedera and Ratner, 1992, Fenouillet *et al.*, 1993). However, enzymatic removal of glycans from the mature HIV envelope glycoproteins, under non-denaturing conditions, diminishes but does not abolish virus binding to its receptor nor infection of susceptible cells (Fenouillet *et al.*, 1989).

1.3.8: **Influenza virus as a model for virus membrane fusion**

Influenza hemagluttinin (HA) is the best characterised of all the viral fusion proteins. Because of this a number of models for the mechanism of viral fusion have been proposed based on studies with influenza virus.

The Influenza HA spike protrudes from the membrane and is responsible for virus binding to the cell as well as for membrane fusion, and is active even in the absence of any other viral proteins (White, 1982a). HA is an integral membrane protein composed of three 84kDa subunits. Each subunit is composed of two polypeptides HA1 and HA2 linked by di-sulphide bonds. The external structure of the spike extends 135Å from the viral membrane with a long highly alpha-helical stem region composed mainly of HA2 supporting a large globular domain composed of HA1 (Wilson *et al.*, 1981, Weiss *et al.*, 1988). Domains in HA1 mediate receptor binding, whilst a very hydrophobic region at the NH_2-terminal end of HA2, called the fusion peptide, mediates fusion. The fusion peptide is highly conserved in HAs from different influenza virus strains and mutations within it alter or abolish fusion activity (Daniels *et al.*, 1985, Gething *et al.*, 1986). At neutral pH the amino-terminal three residues of the fusion peptides are inside the spike at the subunit

interface whilst the next 14 residues wrap around the stem held by a network of hydrogen bonds (Wilson *et al.*, 1981). At pH values needed to trigger fusion an irreversible conformational change occurs exposing the HA2 amino-terminal fusion peptides which are then able to insert into the outer leaflet of an opposing membrane to initiate fusion (Harter *et al.*, 1989, Brunner, 1989).

At acid pH influenza virus can fuse with liposomes which do not contain receptors for HA demonstrating that the receptor binding function of HA1 is not vital for fusion (Stegmann, White and Helenius, 1990, White *et al.*, 1982b). In the absence of sialic acid (or ganglioside) receptors HA2 may be bringing the membranes close together by inserting its N-terminal end (following low pH induced exposure of the hydrophobic fusion peptides) into the target membrane whilst its C-terminal end is anchored in the viral membrane. The role of the spike proteins may be simply to bring the two membranes close enough together to perturb their hydration shells thereby permitting contact between the lipids in the two membranes (Rand, 1981).

The fusogenic state seems to be a transient intermediate in the series of changes induced by low pH. Whilst the first conformational change of HA on exposure to low pH exposes the hydrophobic fusion peptides and allows fusion to occur, the later conformational change(s) appear to inhibit fusion and also make the HA become sensitive to protease digestion (Stegmann, White and Helenius, 1990). It may be that following its role in viral fusion this allows the HA to be degraded in the lysosome to prevent its recycling from the endosome to the cell surface thus evading detection by antibody until after virus replication has occurred.

The primary conformational change occurs very rapidly upon acid exposure and is followed almost instantaneously, even at 0°C, by attachment of the virus to the target membrane most likely by insertion of the exposed fusion peptides into the target bilayer. The next detectable event is fusion between the viral and target membrane which occurs following a lag of less than 1 up to 30 minutes depending on temperature, pH and membrane lipid composition. The lag involves a series of changes in the virus-target membrane complex which are continuous, additive and irreversible (Stegmann, White and Helenius, 1990). The lag does not involve further intra-molecular changes in HA but may reflect reorganisation, (rotational and lateral movements of HA within the plane of the viral membrane of the molecules within the membrane), the net result of which is thought to be aggregation of HA spikes to form a fusion complex. Following the lag phase further changes in the virus-target membrane complex result in merger of the two membranes. Unlike the earlier steps in fusion this final stage is not low pH dependent. The HA complex may be forming

a fusion pore which allows fusion to begin from its centre and then to open outwards. Electrophysiological and electron microscopic studies back up the existence of an HA fusion pore (Spruce *et al.*, 1991, Burger *et al.*, 1988).

As the ectodomains of HA do not dissociate Stegmann *et al.*, (1990), proposed a model in which the HA trimers tilt upon contact with the target membrane to allow at least one of the fusion peptides of each trimer to come into contact and insert into the target membrane. Another model suggests that the trimers remain upright and that the fusion peptides project into the fusion pore acting as a solvating mechanism and promoting lipid mixing (Ellens *et al.*,1990).

Recent work by White and colleagues, (1992), suggests that the transmembrane domain of the fusion protein may have an important role in fusion.

Ultimately fusion releases the contents of the viral envelope into the cytoplasm, and mixes the phospholipids derived from the membrane of the previous host cell with those of the new host cell, whilst the domains previously on the outside of the virus remain within the endosome where they will be degraded.

1.3.9: **Retrovirus fusion**

The retroviruses also enter into cells by pH independent membrane fusion which is mediated by envelope glycoproteins (Stein *et al.*, 1987). The retroviral envelope glycoprotein is produced as a precursor polypeptide which, after processing and glycosylation, is cleaved at a basic site in the C-terminal half of the molecule to form the mature transmembrane subunit, (TM), and surface subunit, (SU) (Reviewed by Hunter and Swanstrom, 1990). In the lentiviruses the SU subunit is non-covalently bound to the TM subunit. The external subunit mediates viral binding to the cell surface receptor which then acts in concert with the TM subunit to mediate fusion of the viral envelope with the cell plasma membrane. As retroviruses bind to a wide range of receptors their SU glycoproteins are correspondingly extremely divergent. However the fusion mechanism is likely to be broadly similar irrespective of cell surface receptor and, although the primary sequences of retroviral TM proteins are poorly conserved, they do share common tertiary structural features (Gallagher *et al.*, 1989).

Retrovirus membrane fusion is a multi-step process involving both the products of *env*. This is confirmed by the range of different mutations of *env* which have been shown to affect the ability of HIV to mediate membrane fusion as judged by syncytium formation *in vitro*. Such mutations can be divided into two groups; mutations that disrupt binding to the cell surface receptor and mutations that affect a post binding fusion event. Similarly neutralising antibodies against the envelope

glycoproteins may act to block infection at either the binding or post-binding fusion event.

As HIV is now the best studied of the retroviruses more is known about the structure and function of the envelope glycoproteins for this virus and this will be described in some detail in the following paragraphs. Studies of HIV may be applicable to the other lentiviruses.

1.3.10: **The HIV envelope glycoproteins - structure and function**

The envelope glycoprotein of HIV is multi-functional having evolved for the function of gaining entry of the virus into the host cell in the face of negative selection by the host immune responses. It is responsible for receptor binding, fusion, immunomodulation, and it has packaging signals to enable incorporation into the virus.

The HIV envelope glycoprotein is synthesised as a 160kDa precursor which, following oligomerisation, is proteolytically processed to form the mature envelope glycoprotein subunits, the transmembrane glycoprotein, gp41, and the external glycoprotein, gp120, which remain non-covalently associated (McCune *et al.*, 1988). Intracellular transport and processing proceeds through the rough endoplasmic reticulum and golgi complex and generates a protein highly glycosylated with asparagine N-linked carbohydrates (Allan *et al.*, 1985). Proteolytic cleavage of gp160 occurs prior to viral assembly and budding (McCune *et al.*, 1988) and has been shown to be vital for fusion but not for transport to the cell surface or for binding to CD4 (Earl *et al.*, 1991, Freed *et al.*, 1989).

The envelope glycoprotein consists of interspersed variable and conserved domains (Starcich *et al.*, 1986, Willey *et al.*, 1986). In the constant domains conservation is greater than 80% whereas in the variable domains it may be as little as 20-30%. Conservation of domains presumably indicates selective pressure to maintain those sequences suggesting that these are functional domains or (partly) that there is a lack of selective pressure for divergence so that conserved sequences may represent non-immunodominant areas. In contrast variable domains suggest areas where there is no requirement to maintain a specific sequence, a non-functional region, and where the appearance of new variants may give that strain of virus a selective advantage (as discussed in Section 1.1.14). The envelope cysteine residues are strongly conserved between strains of HIV-1 and are neccessary for the functional integrity of the envelope (Freed *et al.*, 1991a, Tschachler *et al.*, 1990, Syu *et al.*, 1991).

Both envelope sub-units are required for infection; gp120 binds to the viral

cell surface receptor (Lasky *et al.*, 1987), whilst gp41 mediates the subsequent fusion of the viral membrane with the cell membrane (Kowalski *et al.*, 1987).

Receptor binding - gp120:CD4 interaction

HIV predominantly infects cells that express CD4 since this molecule serves as a receptor for HIV and is therefore critical in determining the cellular tropism of the virus (Dalgleish *et al.*, 1984, McDougal *et al.*, 1986). CD4 is found on some immune system cells including a subset of T-lymphocytes, on monocytes/macrophages, Langerhans cells, dendritic cells and some cells in the brain. A conformational epitope in the carboxyl-terminal region of gp120 forms the binding domain for CD4 (Lasky *et al.*, 1987 , Olshevsky *et al.*, 1990 , Ho *et al.*, 1991) and binding can be blocked by monoclonal antibodies against CD4 (Dalgleish *et al.*, 1984) or by soluble CD4 (Deen *et al.*, 1988).

Human cells which are refractory to HIV infection become infectable if they are made to express CD4 by transfection with a cDNA clone encoding CD4 (Maddon *et al.*, 1986). However, the differences in the ability of different strains to infect different cell lines, and the inability to infect CD4+ (transfected) cells from other species indicates that other proteins, or a hypothetical "second receptor", may be required in addition to CD4 for efficient infection and these may interact differently with different strains of virus (Maddon *et al.*, 1986, Cann *et al.*, 1990, Aoki *et al.*, 1990, Ashorn *et al.*, 1990. A candidate for the secondary factor is a cellular tryptase-like protease which is thought to cleave the gp120 V3 loop between residues 308-331 after binding of gp120 to CD4. (Hattori *et al.*, 1989, Kido *et al.*, 1991). This is supported by evidence that HIV syncytium formation is blocked by tryptase inhibitors. A 44kDa cell surface protein of unidentified function has been implicated as a possible secondory factor (Ebenbichler *et al.*, 1993).

HIV cell tropism is further complicated by the fact that gp120 may also bind to galactocerebroside and galactosyl-sulphatide expressed on the surface of oligodendrocytes and other neural cells (Harouse *et al.*, 1989a and 1989b). These molecules may act as alternative receptors for HIV and allow infection of these CD4 negative cells.

The HIV transmembrane envelope glycoprotein, gp41

The HIV transmembrane envelope glycoprotein, gp41, is known to be important in viral membrane fusion. It consists of a C-terminal region to the cytoplasmic side of the membrane, a membrane spanning region, (the transmembrane domain) which acts as a stop transfer signal and membrane anchor

(Earl *et al.*, 1991), and an N-terminal region to the outside of the membrane, (the ectodomain).

The fusion peptide

The hydrophobic amino terminal end of gp41 (residues 1-28) is vital for fusion. It contains a conserved fusion motif homologous to that of other viral fusion proteins (Gallaher, 1987, Gonzalez-Scarano *et al.*, 1987). The fusion proteins of the paramyxoviruses and orthomyxoviruses include a phenylalanine-x-glycine, (F x G), motif. This motif is found twice in HIV-1 and once in HIV-2 and SIV near the N-terminal end of the TM glycoprotein (Bosch *et al.*, 1989). Mutations within any of the first 29 residues of gp41 affect syncytium formation: generally, mutations which decrease the hydrophobicity of the fusion peptide reduce or abolish syncytium formation whereas exchange of more hydrophobic residues for the wild-type sequence enhances syncytium formation (Bosch *et al.*, 1989, Helseth *et al.*, 1990, Freed *et al.*, 1990).

Cotransfection of wild type and mutant envelope expression vectors showed that a mutation at the second amino acid of gp41 dominantly interferes with syncytium formation and infection by wild-type envelope glycoproteins even in the presence of a great excess of wild type proteins (Freed *et al.*, 1990). This indicates that multiple envelope protein:CD4 interactions are required for syncytium formation and virus infection which is consistent with the fusion pore model as suggested for influenza virus (discussed earlier).

Oligomerisation of envelope glycoproteins

It is thought that the envelope protein forms non-covalently linked homodimers in the endoplasmic reticulum (from sedimentation values) which may later assemble into tetramers (Earl *et al.*, 1990, Pinter *et al.*, 1989, Schawaller *et al.*, 1989). It has been shown that the first 129 aa of the HIV-1 gp41 ectodomain are important for the assembly of env protein oligomers (Earl *et al.*, 1990a) and that after gp160 cleavage the interaction is maintained between gp41 molecules but, from cross-linking studies, there is no evidence that gp120 sub-units interact with each other (Earl *et al.*, 1990b) . Work with other viruses has shown that oligomerisation of the envelope glycoproteins is essential for ER transport (Copeland *et al.*, 1986, Gething *et al.*, 1986, Einfeld and Hunter, 1988). Multimerisation of the envelope glycoproteins may also be important for fusion activity (Schwaller *et al.*, 1989, Serl *et al.*, 1990). Co-expression studies have shown that mixed dimers can form between the envelope glycoproteins of HIV-1 and HIV-2 or SIV (Doms *et al.*, 1990).

This suggests the presence of a conserved structural domain and indeed the gp41 ectodomain is the most conserved region of the envelope protein between HIV-1, HIV-2 and SIV (Doms *et al.*, 1990).

The carboxy-terminal region of the fusion peptide is a "leucine zipper"-like motif (Delwart *et al.*, 1990). Such motifs have been shown to be involved in protein:protein interactions, which suggests that this region may be important for oligomerisation. However it has been shown that mutations within the leucine zipper can affect HIV fusion and infectivity whilst oligomerisation does not appear to be altered (Dubay *et al.*, 1992).

The cytoplasmic domain

Sequences on the C-terminal side of the membrane appear not to be necessary for fusion function but may be important in envelope processsing (Kowalski et al, 1987) and for ensuring incorporation of the envelope glycoproteins into the budding virus (Gebhardt, *et al.*, 1984, Dubay *et al.*, 1991). *In vivo* there must be selective pressure to retain the untruncated cytoplasmic tail, although *in vitro* , truncation of the cytoplasmic domain to 15-20 aa can increase the ability of the HIV-1, HIV-2 and SIV to replicate (Kodama *et al.*, 1989).

gp41:gp120 association

The association of gp120 with gp41 is necessary for infection. Following proteolytic processing of the env precursor gp160, non-covalent interactions maintain multiple contacts between the N-terminal of gp120 and the external portion of gp41 (Kowalski *et al.*, 1987, Willey *et al.*, 1988, McPhee *et al.*, 1989). Computer models suggest that the TM subunit may form a knob-like structure which associates with a socket-like structure in gp120 (Schulz *et al.*, 1992).

Changes in the envelope glycoproteins following receptor binding

Binding of gp120 to the CD4 receptor appears to reduce the affinity of the gp120/gp41 interaction. This may result in dissociation of gp120 and exposure of the gp41 fusogenic domain as the initial stage in virus-cell or cell-to-cell fusion (Moore *et al.*, 1990).

gp120 is shed from infectious virions independently of CD4 binding, predominantly as the virus particle matures and buds from the infected cell (shown by EM) and also as they age (Gelderblom *et al.*, 1985, Gelderblom *et al.*, 1987). The rate and extent of HIV-1 gp120 dissociation before and after CD4 binding is highly variable but is low in most primary isolates of HIV-1 (Kirsh *et al.*, 1990, Moore *et*

al., 1990, Moore *et al.*, 1992). Shedding of gp120 has been shown to correlate with a loss of viral infectivity (McKeating *et al.*, 1991).

Some of the gp120 appears to be resistant to sCD4-induced dissociation, a similar fraction to that which is resistant to shedding with age. These resistant molecules perhaps are required to continue holding the two membranes together whilst the other molecules dissociate to allow the fusion process to occur. This fits in with the case of most C-type retroviruses, (oncoviruses), where a small proportion of their TM and SU subunits are covalently linked by an intramolecular disulphide bond with the remainder being non-covalently associated (reviewed by Schulz *et al.*, 1992).

The gp41 glycoprotein appears to be degraded more rapidly in the cell membrane following gp120 dissociation compared to gp41:gp120 together (Helseth *et al.*, 1990). Thus gp120 dissociation may be advantageous to the virus by reducing viral antigen on the cell surface after infection until replication.

It has been shown that the V3 loop region of gp120, formed by a disulphide bridge between two conserved cysteine residues at amino acids 303 and 338 (Leonard *et al.*, 1990), is also necessary for fusion. Although most of the amino acids within the V3 loop are hypervariable between different strains of HIV, a highly conserved motif is located in the middle of the loop (LaRosa *et al.*, 1990). Whilst the cysteine residues forming the V3 loop are crucial in determining the proper conformation required for processing and CD4 binding, mutations of the conserved amino acids of the V3 loop either block completely or greatly reduce HIV-induced fusion without influencing envelope glycoprotein synthesis, processing, transport or binding to CD4 (Freed *et al.*,1991, Freed and Risser 1991, Helseth *et al.*, 1990, Tschachler *et al.*,1990, Chiou *et al.*,1992). Neutralising antibodies to this region of the V3 loop can block HIV-1 infection and inhibit fusion without preventing gp120 binding to CD4 (Skinner *et al.*, 1988). It is thought that cellular endopeptidases cleave gp120 within this region and that this is a vital step in the fusion process. The V3 domains of all published HIV-1, HIV-2 and SIV sequences contain a trypsin, chymotrypsin or aspartic acid protease cleavage site near the crown of the V3 loop (Clements *et al.*, 1991). Antibodies to this region neutralise and inhibit cleavage (Schulz *et al.*, 1991). Furthermore syncytium formation by HIV infected cells is blocked by protease inhibitors (Hattori *et al.*, 1989).

It may be that dissociation of gp120 from gp41 is partly an *in vitro* artefact and that it may be cleavage of the V3 loop following gp120:CD4 binding which induces the conformational change which allows exposure of the fusion peptide to the opposing membrane.

1.3.11: **Membrane fusion and cytopathicity of HIV**

There is some evidence that HIV interaction with the plasma membrane of CD4+ cells causes alterations in the membrane (Fermin and Garry, 1992). Although HIV penetration was shown to be predominantly via fusion with the plasma membrane EM ultrastructural studies showed that virus attachment appeared to initiate formation of a coated pit (Fermin and Garry, 1992). Viral attachment/penetration was also shown to disrupt the plasma membrane. Effects included partial separation of the bilayers, and formation of pores, perturbations or membrane thickenings. The mechanism by which HIV disrupts the plasma membrane is unclear.

HIV may have similarities with several lytic viruses including myxoviruses and togaviruses which encode proteins that form functional transmembrane ion channels. It has been suggested that the extreme C-terminal end of HIV gp41 resembles the S4 ion-transducing segment of monovalent cation channels (Garry *et al.*, 1991, Miller *et al.*, 1991). Synthetic peptides representing this region of gp41 alter ion fluxes and are cytotoxic in micromolar concentrations (Miller *et al.*, 1991). Thus HIV infection may result in insertion or activation of a transmembrane channel which allows the influx of ions , water and perhaps also other molecules (Miller *et al.*, 1991, Cloyd and Lynn, 1991). Following HIV infection cells may become swollen and lose viability (Garry, 1989). This is sometimes referred to as "ballooning" degeneration and may be responsible for the death of single or syncytial HIV infected cells , perhaps by induction of apoptosis (Garry, 1989) or by disruption of cellular functions due to osmotic effects and an irreversable loss of the ability of the cell to maintain volume homeostasis. Cellular changes associated with apoptosis have been reported to occur in cultures infected with HIV prior to the synthesis of most viral proteins. Sufficient numbers of virions interacting with a cell may cause membrane alterations lethal to the cell. This could account for the ability of UV irradiated virus to kill cells (Rasheed *et al.*, 1986). On the other hand ionic changes induced by infection with a low number of virions could be compensated initially by the cellular ion transport systems but these may be swamped following *de novo* synthesis of viral membrane proteins resulting in ionic changes and finally degeneration or lysis of the infected cell.

Proteins encoded by HIV may also affect the movement of ions by mimicking neurotoxins which interact with transmembrane ion channels (Garry *et al.*, 1991, Werner *et al.*, 1991).

Blocking of syncytium formation by monoclonal antibodies against leucocyte adhesion factor (LFA-1) indicated that this molecule is involved in HIV-1 membrane fusion (Hildreth and Orentas, 1989, Valentin *et al.*, 1990). HIV can infect T cells from a leucocyte adhesion deficiency patient, which are deficient in expression of LFA-1, but these cells do not form syncytia (Pantaleo *et al.*, 1991) suggesting that LFA-1 may be required for cell-to-cell fusion but not for virus to cell fusion. More recently Golding *et al.* (1992) reported that initial cell-to-cell fusion (CD4/envelope mediated) did not require LFA-1, but it was required for syncytia formation in lymphoid cells. It was suggested that LFA-1 may be acting to stabilise aggregates of cells to allow syncytium formation. Aggregates of non-lymphoid adherent cells may be stabilised by other molecules e.g. integrins involved in adherence, and thus may able to form syncytia in the absence of LFA-1.

Syncytium formation by HIV *in vitro* is also dependent on the presence of serum factors (Ushijima *et al.*, 1992). The requirement for serum is not species specific in that the addition of foetal calf, rabbit or human serum to serum-free medium restored fusion by HIV-1 MOLT-4 cells added to uninfected MOLT-4 cells. Fibrinogen, fibronectin, and alphaglobulin were identified as being involved in the promotion of HIV-induced fusion. Both fibrinogen and fibronectin are coagulation factors present at high concentrations in serum (2-4mg/ml fibrinogen and approximately 300μg/ml of fibronectin in human serum) Both these proteins are known to mediate cell attachment (Ruoslahti and Pierschbacher, 1987, Auffray and Navotny, 1986). These two proteins include the tetrapeptide motif R-G-D-S which represents the cell attachment site. The same sequence is present in the MHC antigens and in HIV-1 gp120. The additional factors may be involved in stabilising the interaction between cells to enable syncytium formation but are not required for infection.

1.3.12: **Hypothetical mechanism of HIV membrane fusion**

The mechanism of HIV membrane fusion may be hypothesised drawing together the available information as discussed above;

The viral and cell plasma membrane are brought close together by gp120 binding to CD4 (or some other receptor) which, dependent upon the correct tertiary and quarternary structure of the membrane glycoproteins gp120 and gp41, causes a conformational change in gp120:gp41 exposing the fusion peptide of gp41 which may insert in the opposite membrane linking the two. It may be necessary for the CD4:gp120 complex to move aside in order for the fusion peptide to gain physical access to the opposing membrane. The dissociation of gp41 from gp120 , and/or of

gp41 from core proteins may be necessary to enable this, as lateral mobility of gp41 within the viral membrane may be restricted by the interaction between the C-terminal tail and the core proteins. Translocation of the TM glycoproteins within the membranes may enable the formation of a fusion pore causing disruption of the stable lipid bilayers, mixing, and reformation of lipid bilayers in a different plane, as hypothesised for influenza fusion pores. The formation of coated-pit-like structures at the virus/plasma membrane interaction site may also be important for fusion as coated pits contain proteins involved in cellular fusion pathways which are necessary for endocytosis. It may be that the viral fusion pore components, i.e. the envelope glycoproteins, and the cellular fusion pore components interact in some way to enable destabilisation of the two membranes, mixing and reformation of the lipid bilayer.

A common mechanism involving the fusion protein NSF (N-ethylmaleimide-sensitive fusion protein) may underlie diverse types of fusion events in eukaryotes including in the pathway of endocytosis (Malhotra *et al.*, 1988). NSF appears to assemble with other proteins to form an active multi-subunit "fusion machine". It may be that the viral transmembrane protein is involved in such an assembly which mediates membrane fusion. Perhaps in addition to using host cellular machinery for RNA and protein synthesis the virus is also very efficient at hijacking cell proteins involved in fusion pathways.

The influenza fusion pore appears to be a tight structure which does not allow ions or water through, but it appears that in HIV the structure may allow water and ions through. Alternatively, HIV glycoproteins may alter water and ion balance by acting as calcium channels; Calcium channels are involved in membrane fusion between intracellular vesicles, and the C-terminal end of gp41 is "calcium channel-like". Similar properties have not been identified for influenza glycoproteins.

The disruption of ion transport which occurs late in infection after synthesis of viral proteins may be by a different mechanism altogether, perhaps by expression of envelope proteins interfering with recycling of cellular integral membrane proteins and leading to progressive loss of important cellular functions. For example, it has been suggested that the fibrinogen receptor is down regulated on HIV-infected cells (Ushijima *et al.*, 1992) An alternative reason for this disruption of ion transport may be that envelope proteins might interact with cellular fusion pore proteins on cell surface and disrupt ion exchange.

1.3.13: **Conserved features of virus membrane fusion**

Many of the features of fusion are similar amongst all the fusogenic viruses.

The viral fusion proteins are in a class of integral sub-unit proteins which are characterised by a three-domain structure with a large hydrophilic carbohydrate containing domain on the external surface of the membrane, a small, uncharged hydrophobic domain spanning the membrane and a fairly small hydrophilic domain on the internal side of the membrane. Similar proteins in eukaryotic cells have enzyme or recognition activities e.g MHC antigens.

Proteolytic processing of the envelope glycoprotein precursor gives rise to two proteins which remain associated (non-covalently or by di-sulphide bonding). The heterodimer subunit combines into trimers or tetramers; Vesicular Stomatitis virus protein G which mediates binding and fusion associates into trimers (Crise *et al.*, 1989), Semliki Forest virus E1-E2 heterodimers trimerise (Fuller, 1987), whilst HIV envelope proteins most likely form tetramers (Pinter *et al.*, 1989, Schawaller *et al.*, 1989, Earl *et al.*, 1990). Proteolytic maturation, glycosylation and oligomerisation are vital for fusion but not for processing or transport of the envelope proteins to the cell surface. A conformational change is required to trigger fusion. This is thought necessary to expose the N-terminal hydrophobic region of the glycoprotein, the fusion peptide, to the target membrane. This conformational change may be induced by binding of the associated envelope glycoprotein (HN/HA or surface glycoprotein) to its specific receptor and/or by a drop in pH.

The fusion peptide, a stretch of relatively hydrophobic amino acids within a membrane anchored subunit, has been identified as a key feature of most viral fusion proteins. Fusion peptide sequences are short, 16-26 a.a., and relatively hydrophobic. If modelled as alpha-helices they display one face with a high hydrophobicity index and a back face that has hydrogen bonding potential (White, 1990). Fusion peptides are highly conserved within but not between virus families. There are no major features that distinguish the fusion peptides of acid dependent from neutral pH fusion proteins (White, 1992). Therefore it seems that it is the tertiary structure of the protein which is dependent on the low pH for correct exposure/conformation. The fusion peptide is thought to insert into the opposing membrane destabilising the lipid bilayer to initiate fusion.

Some non-viral proteins which have a role in fusion have many similarities with the viral fusion proteins e.g. PH-30 a sperm surface antigen involved with fusion with the oocyte (Primakoff *et al.*, 1987, Blobel *et al.*, 1990, Blobel *et al.*, 1992). PH-30 is thought to be involved in both binding and fusion. It is a complex of two integral membrane proteins formed by cleavage of larger precursors and has a typical fusion peptide.

CHAPTER 2: Cloning and Sequencing of DNA encoding EV1 *gp46*

2.1: INTRODUCTION

The *env* gene encodes the envelope glycoprotein which is subsequently cleaved into the external surface envelope glycoprotein (gp135) and the transmembrane envelope glycoprotein (gp46). The aim of this work was to clone the whole of the region encoding the transmembrane envelope glycoprotein, (hereafter referred to as *gp46*), of MVV strain EV1. I wished firstly to sequence *gp46* of strain EV1 for comparison with other strains and to try to identify conserved epitopes on gp46 which might be involved in fusion. The cloning was planned bearing in mind that I wished subsequently to express the gene to produce recombinant protein for studies on the immune response of MVV infected animals to gp46.

By analogy with other viruses it is likely that gp46 plays a major role in mediation of membrane fusion by the virus. As discussed in Chapter 1, it is apparent that there is variation in the MVV genome as shown by the appearance of neutralisation escape mutants. Variation in *env* may be important in survival and persistence of the virus in the host. However, functional epitopes are generally conserved.

The reason strain EV1 of MVV was chosen for use in this project was that the other MVV strains were isolated outside the UK and containment requirements pose difficulties for their use to establish experimental infections or to challenge animals in the UK. MVV strain EV1 was originally isolated from the peripheral blood of a sheep displaying symptoms of pneumonia and arthritis. After brief passage in sheep skin fibroblasts, the virus was used to infect choroid plexus (SCP) cells and stocks of virus were aliquoted and stored at -70°C.

Much of EV1 had already been sequenced from cDNA clones prepared from EV1 infected SCP cells when I began this project, but no clone spanning the whole of *gp46* had been found despite extensive screening of the lambda gt10 library which had yielded the other EV1 clones.

The sequence of MVV strain 1514, an Icelandic isolate, had been ascertained but only after extensive *in vitro* passaging. LV1, a strain derived from a sheep experimentally infected with 1514 had also been sequenced (Braun *et al.*, 1987). EV1 is thus the closest to a "field" isolate.

Chapter 2:

METHODS

2.2.1: **Screening of an EV1 lambda- gt10 library for EV1 *env* (*gp46*)**

Attempts were made to obtain a clone of MVV-EV1 *gp46* from a library made from cDNA from EV1 infected WSCP (Sheep choroid plexus cells) ligated into lambda- gt10 (as described in our paper, Sargan *et al.*, 1991). An 1180 base *DdeI* fragment of the MVV strain 1514 genome, positions 7152-8332, was obtained from a genomic clone of MVV-1514, pVisJC8.5, (Molineaux and Clements, 1983) by sequential *SacI/HindIII* and *DdeI* digestions and gel purification and phenol extraction. Full length 1514 insert was used to probe for MVV specific plaques whilst the 1180bp. fragment was used to identify MVV *gp46* specific clones.

2.2.2: **Cloning of MVV EV1 *env* (*gp46*) via Polymerase Chain Reaction (PCR)**

Extrachromosomal DNA purified from EV1 infected cells by the method of Hirt (1987) was used as template for amplification of MVV-specific DNA. Partially redundant primers, 140D and 141D, were used to amplify DNA encoding EV1 gp46.

```
140D-   5'-   GAA TTC TCA ATC ATG GGC ATC TGT TCT C    -3'
                                                  A
                                                  T
141D-   5'-   GAA TTC ATG GGI ATA GGI TTG GTI ATA GT G    -3'
                                                    C
```

The PCR reaction conditions were as follows; 67mM Tris-Cl pH8.8, 16.6mM Ammonium sulphate, 6.7mM Magnesium chloride, 10mM 2-mercaptoethanol, 6.7µM EDTA, 25µM dATP, 25µM dCTP, 25µM dGTP, 25µM dTTP, 0.17µg/µl BSA, and 1 unit Taq polymerase made up to 100µl with sterile distilled water and overlaid with paraffin oil. A Techne Programmable Dri-block (PHC-1) was programmed to provide 35 cycles of 0.6 minutes melting at 95°C, 1 minute annealing at 45°C and 5 minutes extension at 70°C, and a 5 minute final extension at 70°C.

10µl samples of each PCR reaction were analysed by electrophoresis. The gel was Southern blotted onto nitrocellulose and probed with ^{32}P labelled pVisJC8.5 insert (Appendix III).

2.2.3: **Preparation of PCR product for ligation**

gp46 PCR product was phenol extracted and ethanol precipitated. A portion of the product was digested with *EcoRI* in the conditions recommended by the manufacturer and then gel purified by excising a band of the predicted size from an LGT agarose gel following electrophoresis.

The remainder of the *gp46* PCR product was gel purified without restriction. A band of approximately 1kb was excised. The DNA in LGT was made up to a volume of 159µl with SDW, melted by heating to 60°C, then kept liquid at 37°C. 22µl buffer E14 (10mM Tris-Cl(pH7.5), 1mM MgCl2, 7mM B-mercaptoethanol), 37µl 3mM dNTPs and 2µl klenow polymerase (4U/µl) was added and incubated at 37°C for 45 minutes. DNA was extracted and the agarose removed using 60°C phenol/chloroform/isoamyl alcohol followed by ethanol precipitation. The precipitate was redissolved in 10 µl of SDW, heated to 60°C for 2 minutes and cooled to 37°C before adding 5µl of 10 x reaction exchange buffer (0.5M Tris-Cl (pH 7.6), 0.1M $MgCl_2$, 50mM dithiothreitol, 1mM spermidine HCl, 1mM EDTA (pH 8.0)), 5µl 10µM ATP, 3µl T4 polynucleotide kinase and 27µl SDW.

Concentrations were estimated by electrophoresis of sample dilutions on a 1% agarose gel containing 0.5 µg/ml EthBr.

2.2.4: **Ligation of gp46 PCR product into pTZ19R**

Plasmid was prepared for ligation by digestion with *HincII* (as manufacturers instructions) and treatment with Calf Intestinal Phosphatase (CIP). to remove the 5' terminal phosphate and prevent recircularisation of the plasmid. Linear plasmid was gel-purified and recovered by Geneclean.

Ligation reactions were set up at molar ratios of approximately 0:1, 4:1, and 10:1, of insert:vector (klenow/kinase treated *gp46* PCR product : *HincII*/CIP-treated pTZ19R). 200ng of pTZ19R was used for each reaction. Vector, insert and SDW were heated to 60°C for 2 minutes. After cooling briefly on ice, ligase buffer and enzyme were added. The ligation reactions were incubated overnight at 14°C.

Half of each ligation was used to transform competent *E.coli* JM101. Transformants were selected by plating onto LB agar containing Ampicillin (150µg/ml), X-Gal, and IPTG (Appendix I). Colonies were picked and inoculated onto a fresh X-Gal/IPTG/Amp plate and into Universals containing 5ml LB/150µg/ml Amp. and incubated overnight at 37°C. Plasmid mini-preps were prepared as described in Appendix II. Precipitated DNA from 1.5ml of culture was resuspended in 8.5µl TE/50µg/ml RNase A, 1µl E13 buffer (6mM Tris-Cl(pH7.5), 6mM NaCl, 6mM $MgCl_2$, 6mM B-mercaptoethanol, 0.02% w:v BSA), and 0.5µl

KpnI (8U/µl) and incubated at 37°C for 3 hours 45 minutes. 15µl samples were electrophoresed.

New plasmid mini-preps were made from the possible positives, (i.e. those plasmids which were larger than pTZ19R when linearised with *KpnI*), and digested with *HindIII* followed by *XbaI*. The restriction digests were electrophoresed alongside super-coiled pTZ19R, linear pTZ19R and *gp46* PCR product .

2.2.5: **Preparation of single-stranded pTZ-derivatives**

Single-stranded DNA was prepared, (as described in Appendix IV), from four different clones, pCC19.12, pCC19.13, pCC19.15 and pCC19.25, consisting of the gp46 PCR product inserted into pTZ19R .

2.2.6: **Inversion of gp46 insert**

pCC19.25 was digested with *HindIII* and *XbaI*. The resultant 1kb restriction fragment was gel purified and ligated into *HindIII*/*XbaI*-cut, CIP-treated pTZ18R. Transformants containing plasmid derived from pTZ18R with *gp46* insert were selected, (named pCC18.n), and single stranded plasmid DNA was prepared for sequencing (Appendix IV).

2.2.7: **Subcloning from gp46 clone for sequencing**

pCC18.10 and pCC19.15 were digested with *EcoRI* producing two fragments of about 500bp from the gp46 insert. The 500bp material was gel purified and ligated into *EcoRI* cut, CIP-treated pTZ18R. Plasmid minipreps were made from overnight cultures of transformants selected on LB Amp/X-Gal/IPTG agar, and digested with *EcoRI* to characterise the insert. Both single stranded and double stranded DNA was prepared for sequencing of the plasmids containing a 500bp *gp46* insert (Appendix IV).

2.2.8: **Sequencing**

DNA sequencing was performed by the dideoxy chain termination method of Sanger *et al.*, (1977), using a commercial kit (USB Biochemicals). Double stranded as well as single stranded DNA sequencing allowed the sequence of both strands of *gp46* to be determined and multiple runs allowed the elimination of ambiguities.

The University of Wisconsin Genetics Computing Group (UWGCG) package version 6.2 (Devereux *et al.*, 1984) was used for assembly and analysis of the sequence data obtained.

Chapter 2:

RESULTS

2.3.1: **Cloning and sequencing of *gp46***

PCR with primers 140D and 141D using a template of DNA prepared from EV1 infected sheep skin cell fibroblasts amplified a product of approximately 1kb (Figure 2.1). When DNA prepared from uninfected sheep cells was used as PCR template for primers 140D and 141D no product was made. As a positive control primers derived from the sequence of an EV1 *gag* clone were used. These amplified a product of 635 bases. On southern blots of the PCR products ^{32}P labelled insert from pVisJC8.5 (*SstI* fragment, nucleotides 562-9132 of Visna-1514) hybridised with the EV1 *gag* and *gp46* PCR products .

Restriction digestion of the PCR product revealed an *EcoRI* cleavage site approximately in the middle of MVV-EV1-*gp46* which was not present in MVV-1514-*gp46*. This meant that the planned ligation of *EcoRI*-treated PCR product into *EcoRI*-cut pTZ19R would be unsuitable. Therefore the PCR product was treated with Klenow polymerase then T4 polynucleotide kinase to ensure blunt and phosphorylated ends, and was blunt-end ligated into the *HincII* site of pTZ19R. Transformants were selected and plasmid minipreps were made. Six were larger than pTZ19R alone when linearised with *KpnI*. Plasmid DNA from these 6 clones was digested with *HindIII* and *XbaI* to excise any insert by cutting either side of the *HincII* site within the pUC19 multilinker. Four clones, namely CC19.12, CC19.13, CC19.15 and CC19.26, contained plasmids with inserts of the required size.

Single stranded plasmid DNA was prepared using helper phage M13K07. Sequencing of the single stranded plasmids using the reverse sequencing primer showed that each of the four plasmids contained the insert in the same orientation. The sequence obtained corresponded to pTZ19R multilinker, primer 140D then sequence complementary to the 3' end of *gp46* (as previously sequenced by DRS).

In order to enable single stranded sequencing from either end it was neccessary to turn the insert round relative to the M13 packaging signal. This was done by cutting the insert out of pTZ19R using *XbaI* and *HindIII* and religating it into *XbaI*/*HindIII* cut pTZ18R. Again, transformants were selected. Three plasmids, pCC18.7, pCC18.8 and pCC18.9, were found which contained an insert of 1Kb. Single stranded plasmid DNA was prepared and sequenced using the reverse sequencing primer. This produced sequence corresponding to the 5' end of gp46 as shown by comparison with the published sequence of MVV 1514.

Figure 2.1: Results of PCR to amplify EV1 *gp46*

i) PCR products separated on a 1% agarose mini-gel

ii) Southern blot of the same gel probed with pVisJC8.5

A : EV1-gp46 primers with sheep DNA template (negative control)
B : EV1-gag primers with EV1 HIRT DNA template
(positive control)
C -E: EV1-gp46 primers with EV1 HIRT DNA template
(Decreasing amount of primer 141D with constant amount of 140D)
C=1.2μM 140D, 4.5μM 141D
D=1.2μM 140D, 1.35μM 141D
E=1.2μM 140D, 0.45μM 141D

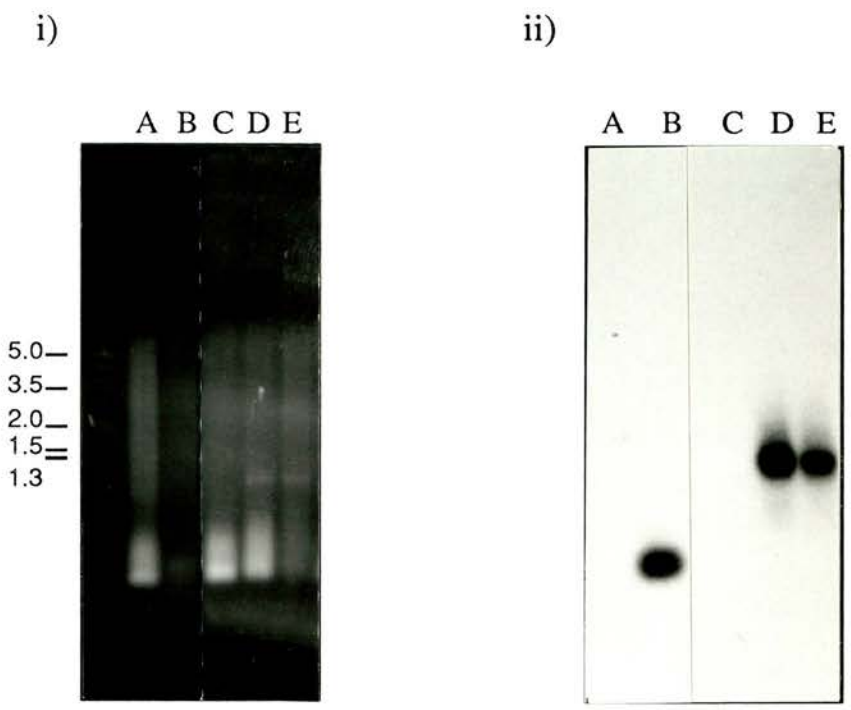
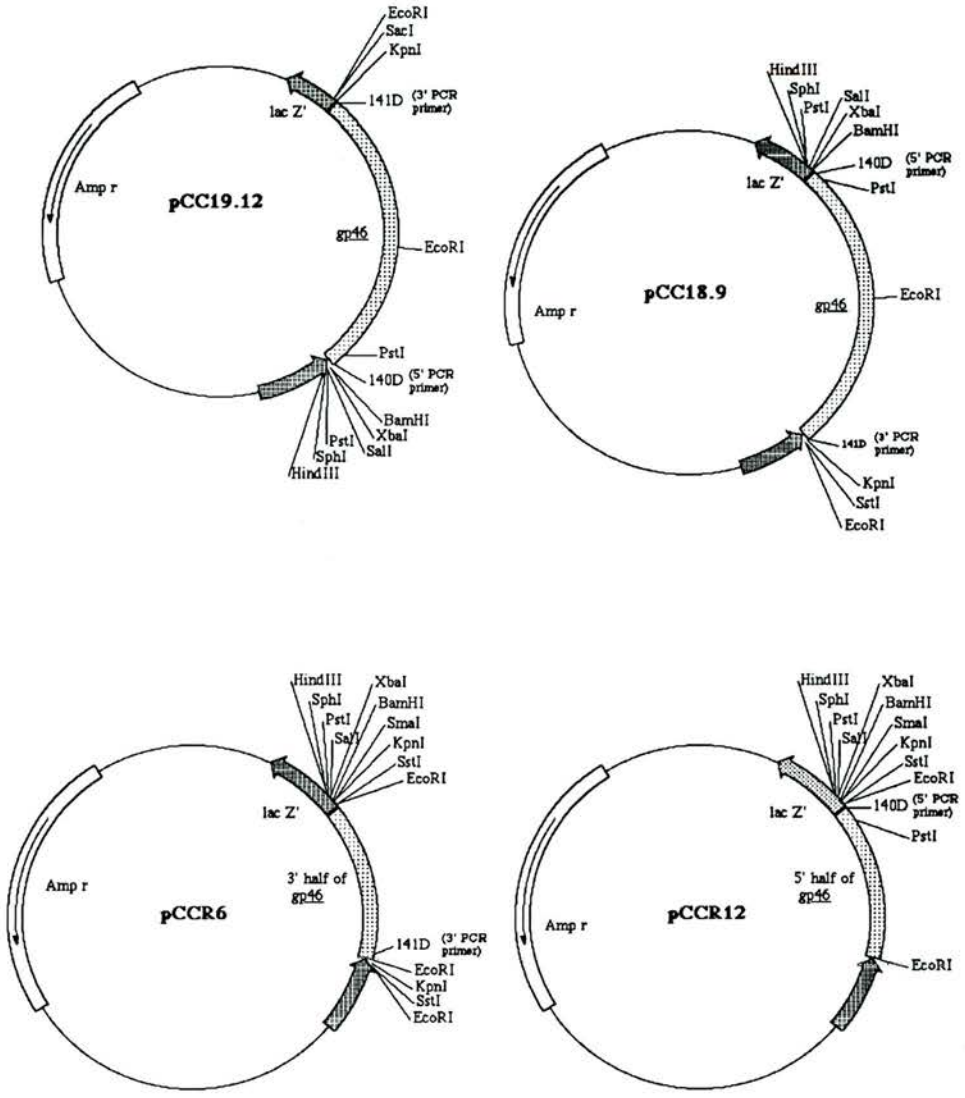


Figure 2.2: Diagrammatic representation of clones encoding EV1 gp46

Diagram of the plasmids constructed to clone and sequence EV1 gp46. Sequencing using the reverse or universal primers (USB) enabled the directionality of the inserts to be determined.



The insert of 1kb was too large for complete sequencing from either end so the central *EcoRI* site was utilised to make plasmids with half length inserts. pCC18.9 was digested with *EcoRI*. The two 500bp restriction fragments, which could not be seperated by electrophoresis, were ligated with *EcoRI*-cut, CIP-treated pTZ19R or pTZ18R. Transformants were selected. Plasmids were prepared and those with inserts of 500bp were used for double stranded sequencing. Using either the reverse or universal sequencing primers, sequence from either end of the insert was obtained.

Restriction maps of some of the plasmids constructed for sequencing of EV1 *gp46* are shown in Figure 2.2. Multiple sequencing reactions were done to enable the sequence of the entire gp46 coding region to be determined (Figure 2.3).

A cDNA clone pV1LTR (IB and DRS) overlapped the *gp46* coding sequence from the *EcoRI* site to the 3' end. The sequence obtained was confirmed by the sequence of pV1LTR therefore I concentrated on sequencing of the 5' half of *gp46*.

The sequences obtained were aligned using the the GCG "Bestfit" program. Where ambiguities occurred between sequences from different reactions, sufficient additional runs were performed to obtain a correct sequence. Finally the sequences were joined up using GCG "Assemble" to complete the sequence of EV1 *gp46* (Figure 2.4).

Although the sequence through the *EcoRI* site in *gp46* was not confirmed, the translation phase was conserved, and sequence alignment with sequence from other strains of MVV backs up the sequence shown in Figure 2.4. Therefore, this can be considered to be a faithful representation of the sequence encoding MVV EV1 gp46.

2.3.2: **Sequence Comparison between gp46 of EV1 and other MVV isolates**

The EV1 *gp46* sequence was compared with the other available MVV sequences, visna-1514 (Molineaux and Clements, 1983) and SAOMVV (Querat et al , 1990), using the GCG "Bestfit" and "Lineup" programs (Figure 2.5). Percentage similarity between them was calculated: EV1 and 1514 were 81.6 % identical, EV1 and SAOMVV were 82.2 % identical, and 1514 and SAOMVV were 81.3 % identical.

Figure 2.3: Diagrammatic representation of sequencing results

The arrows in the diagram represent sequence obtained and show that the sequence for the whole of the region encoding EV1 gp46 was the result of at least three independent sequencing reactions.

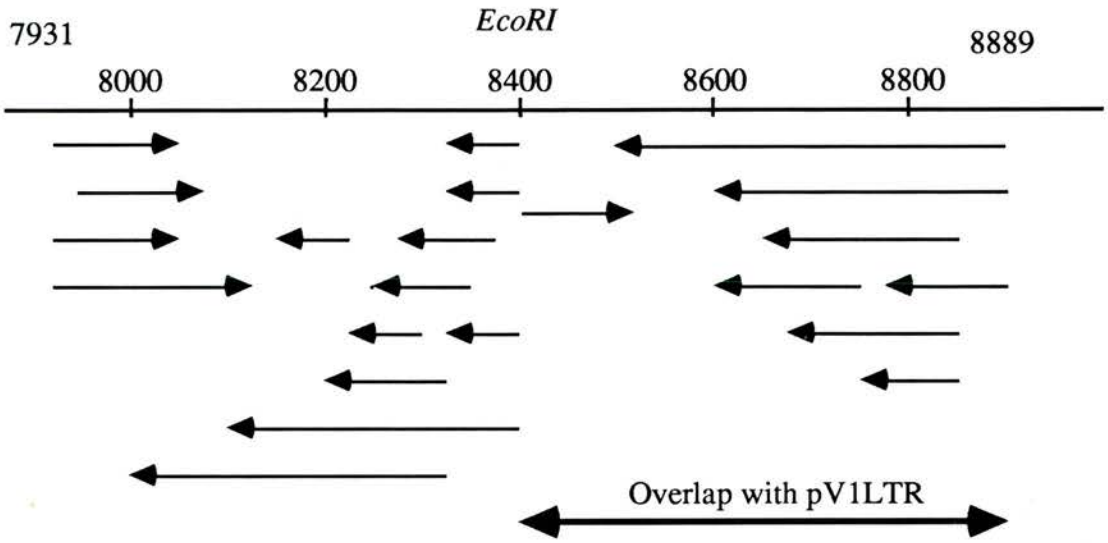


Figure 2.4: Nucleotide sequence and amino acid translation of MVV-EV1 *gp46*

The first four nucleotides, and the initial amino acid, cysteine, are derived from the 5' PCR primer. In the completed sequence of EV1 *env* the protease recognition sequence ArgLysLysArg lies immediately upstream of the first amino acid, glycine, of gp46.

```
    ATGTGGGATAGGCTTGGTGATAGTGCTCGCTATCATGGCAATAATAGCTGCTGCAGGAGC
  1 ---------+---------+---------+---------+---------+---------+ 60
    TACACCCTATCCGAACCACTATCACGAGCGATAGTACCGTTATTATCGACGACGTCCTCG

     CysGlyIleGlyLeuValIleValLeuAlaIleMetAlaIleIleAlaAlaAlaGlyAla  -

    TGGTCTCGGTGTTGCAAATGCCGTGCAGCAGTCCTATACAAGGACGCTTGTCCAGTCTCT
 61 ---------+---------+---------+---------+---------+---------+ 120
    ACCAGAGCCACAACGTTTACGGCACGTCGTCAGGATATGTTCCTGCGAACAGGTCAGAGA

     GlyLeuGlyValAlaAsnAlaValGlnGlnSerTyrThrArgThrLeuValGlnSerLeu  -

    TGCTAACGCAACTGCTGCTCAGCAGAATGTGTTAGAAGCAACTTATGCCATGGTGCAGCA
121 ---------+---------+---------+---------+---------+---------+ 180
    ACGATTGCGTTGACGACGAGTCGTCTTACACAATCTTCGTTGAATACGGTACCACGTCGT

     AlaAsnAlaThrAlaAlaGlnGlnAsnValLeuGluAlaThrTyrAlaMetValGlnHis  -

    TGTAGCCAAAGGAGTAAGAATATTAGAGGCAAGAGTTGCCCGAGTGGAAGTCCTGGTAGA
181 ---------+---------+---------+---------+---------+---------+ 240
    ACATCGGTTTCCTCATTCTTATAATCTCCGTTCTCAACGGGCTCACCTTCAGGACCATCT

     ValAlaLysGlyValArgIleLeuGluAlaArgValAlaArgValGluValLeuValAsp  -

    TAGGATGATGGTATATCAGGAATTAGATTGTTGGCATTATCAGCACTATTGTGTAACCTC
241 ---------+---------+---------+---------+---------+---------+ 300
    ATCCTACTACCATATAGTCCTTAATCTAACAACCGTAATAGTCGTGATAACACATTGGAG

    ArgMetMetValTyrGlnGluLeuAspCysTrpHisTyrGlnHisTyrCysValThrSer  -

    AACAAAAAGTGAAGTAGCAAAGTATGTGAATTGGACAAGATACAGGGATAATTGTACATG
301 ---------+---------+---------+---------+---------+---------+ 360
    TTGTTTTTCACTTCATCGTTTCATACACTTAACCTGTTCTATGTCCCTATTAACATGTAC

     ThrLysSerGluValAlaLysTyrValAsnTrpThrArgTyrArgAspAsnCysThrTrp  -

    GCAGCAATGGGAAGAAGAGATAGAACAGCATGAAGCGAATTTAAGTCAGTTGCTTAGGGA
361 ---------+---------+---------+---------+---------+---------+ 420
    CGTCGTTACCCTTCTTCTCTATCTTGTCGTACTTCGCTTAAATTCAGTCAACGAATCCCT

     GlnGlnTrpGluGluGluIleGluGlnHisGluAlaAsnLeuSerGlnLeuLeuArgGlu  -

    AGCTGCATTGCAAGTACACATAGCTCAAAGGGATGCACAAAGAATTCCAGATGTATGGAC
421 ---------+---------+---------+---------+---------+---------+ 480
    TCGACGTAACGTTCATGTGTATCGAGTTTCCCTACGTGTTTCTTAAGGTCTACATACCTG

     AlaAlaLeuGlnValHisIleAlaGlnArgAspAlaGlnArgIleProAspValTrpThr  -
```

```
    AGCGTTGCAAGGAGCATTTGATTGGGCAGGATGGTTCGCATGGTTGAAATATATACCCTG
481 ---------+---------+---------+---------+---------+---------+ 540
    TCGCAACGTTCCTCGTAAACTAACCCGTCCTACCAAGCGTACCAACTTTATATATGGGAC

     AlaLeuGlnGlyAlaPheAspTrpAlaGlyTrpPheAlaTrpLeuLysTyrIleProTrp  -

    GATTGTGATGGGAATCATAGGACTAATCTGTTTTAGAATAATAATGTGTGTGGTGTCACT
541 ---------+---------+---------+---------+---------+---------+ 600
    CTAACACTACCCTTAGTATCCTGATTAGACAAAATCTTATTATTACACACACCACAGTGA

     IleValMetGlyIleIleGlyLeuIleCysPheArgIleIleMetCysValValSerLeu  -

    GTGCCTACAGGCCTACAAGCAGATTCAACAGATCAGATATACACAGGTAACAGTGGTGAT
601 ---------+---------+---------+---------+---------+---------+ 660
    CACGGATGTCCGGATGTTCGTCTAAGTTGTCTAGTCTATATGTGTCCATTGTCACCACTA

     CysLeuGlnAlaTyrLysGlnIleGlnGlnIleArgTyrThrGlnValThrValValIle  -

    AGAAGAACCAGTGGACCTCGAGGAAAAACCAGGAGAAGAAAAGGATGGTTTAAATGGCTT
661 ---------+---------+---------+---------+---------+---------+ 720
    TCTTCTTGGTCACCTGGAGCTCCTTTTTGGTCCTCTTCTTTTCCTACCAAATTTACCGAA

     GluGluProValAspLeuGluGluLysProGlyGluGluLysAspGlyLeuAsnGlyPhe  -

    CGAAGACTTAAGGCAAGAGAAAAGAACATCCCGGCGCACTTTTACCCAGATATGGAGAGC
721 ---------+---------+---------+---------+---------+---------+ 780
    GCTTCTGAATTCCGTTCTCTTTTCTTGTAGGGCCGCGTGAAAATGGGTCTATACCTCTCG

     GluAspLeuArgGlnGluLysArgThrSerArgArgThrPheThrGlnIleTrpArgAla  -

    AACGTGGCAGGCCTGGAAAAACTCACCTTGGAAGAAAAGTTGGAGGAGAAGCCTATATAT
781 ---------+---------+---------+---------+---------+---------+ 840
    TTGCACCGTCCGGACCTTTTTGAGTGGAACCTTCTTTTCAACCTCCTCTTCGGATATATA

     ThrTrpGlnAlaTrpLysAsnSerProTrpLysLysSerTrpArgArgSerLeuTyrMet  -

    GAGTCTACTACCTCTACTGGCGGTACATCAGTGGCTGGAAGAGACTGGATGGATTGGCGG
841 ---------+---------+---------+---------+---------+---------+ 900
    CTCAGATGATGGAGATGACCGCCATGTAGTCACCGACCTTCTCTGACCTACCTAACCGCC

     SerLeuLeuProLeuLeuAlaValHisGlnTrpLeuGluGluThrGlyTrpIleGlyGly  -

    GAATCAGCTCAAAAATAAAAAAGAAAGGGTGGACTGTCAGGGCAGAGAACAGATGCCCAT
901 ---------+---------+---------+---------+---------+---------+ 960
    CTTAGTCGAGTTTTTATTTTTTCTTTCCCACCTGACAGTCCCGTCTCTTGTCTACGGGTA

     AsnGlnLeuLysAsnLysLysGluArgValAspCysGlnGlyArgGluGlnMetProMet  -

    GATTGAGAATGACTATGTAGAAATGAAGTAG
961 ---------+---------+---------+- 991
    CTAACTCTTACTGATACATCTTTACTTCATC

     IleGluAsnAspTyrValGluMetLysEnd  -
```

The EV1 DNA sequence was translated and the predicted amino acid sequence was compared with other MVV isolates. EV1 and 1514 were 79.5 % identical (88% similarity), EV1 and SAOMVV were 79.9 % identical , and 1514 and SAOMVV were 79.8 % identical. The similarity between these isolates suggests that each may be equally diverged from a common ancestor. Comparison of the sequences using the GCG "Lineup" program, allowed conserved regions to be visualised (Figure 2.6).

Translation of the EV1 sequence revealed five potential N-linked glycosylation sites in gp46 (as identified by the motif N X S/T) all occuring in the region thought to lie to the external side of the membrane. These occur at 42-44, 110-112, 117-119, and 133-135 and are conserved in all three MVV isolates except that 1514 has one extra potential site at 167-169. The conservation of sequence at the four sites listed suggests that glycosylation at these sites is necessary for the function of gp46. Alterations in glycosylation may alter the structure of the glycoprotein and may alter its antigenicity.

There are seven conserved cysteines. These are found at residues 90, 97, 118, 190, 196, 201, and 312. Conservation of these residues may be important in maintaining the required secondary structure of the glycoprotein. Conversely additional cysteines at non-conserved sites may alter the protein structure and therefore alter the antigenicity of the protein.

The amino-terminal end of gp46, which is predicted to be involved in mediating cell fusion, is well conserved with only two amino acid changes within the first 50 bases. Indeed in the first 106 amino acids there are only 8 differences between these three strains and each is a conservative change in that the charge and polarity of the amino acid stays the same. Within the nucleotide sequence encoding these amino acids there are 54 differences between the three sequences. The majority of these changes are at the third base of the codon and do not alter the amino acid sequence which suggests that there is positive selection for conservation of the amino acid sequence.

The sequence 115-131, (DNCTWQQWEEEIEQHE), known as the Kennedy motif, and the sequence 87-100, (ELDCWHYQHYCVTS), the immunodominant region, are the same for all three isolates.

The sequence 177-195, predicted to be trans-membrane in the native protein, is similar for all three strains. Where there are changes they are semi-conservative in that all the changes are from one hydrophobic amino acid to another. The exception is at aa 183 which is cysteine in SAOMVV and glycine in 1514 and EV1. Both cysteine and glycine are uncharged polar amino acids so this is also a semi-

Figure 2.5: Comparison of MVV *gp46* nucleic acid sequences .

The EV1 *gp46* sequence was aligned with two other MVV *gp46* sequences using the GCG "Lineup" program. A capital letter is shown in the consensus sequence where all three bases match, a small letter is shown where two of the three strains have that base, an x is shown where the bases are different for each MVV strain.

```
          1                                                   50
Consensus xxxxGGGATA GGcTTGGTxA TaGTGCTxGC tATCATGGCa ATAATaGCTG
   Saomvv ....GGGATA GGATTGGTCA TAGTGCTTGC TATCATGGCT ATAATAGCTG
     1514     GGGATA GGCTTGGTTA TTGTGCTAGC CATCATGGCA ATAATCGCTG
      EV1 ATGTGGGATA GGCTTGGTGA TAGTGCTCGC TATCATGGCA ATAATAGCTG
          51                                                  100
Consensus CTGCAGGAGC TGGtCTCGGt GTtGCaAAtG CCGTGCAGCA aTCCTAtACc
   Saomvv CTGCAGGAGC TGGACTCGGC GTTGCTAATG CCGTGCAGCA ATCCTACACC
     1514 CTGCAGGAGC TGGTCTCGGT GTCGCAAACG CCGTGCAGCA ATCCTATACC
      EV1 CTGCAGGAGC TGGTCTCGGT GTTGCAAATG CCGTGCAGCA GTCCTATACA
          101                                                 150
Consensus AGGACGgcTG TcCAGTCTCT TGCTAACGCA ACTGCTGCtC AGCAGaAtGT
   Saomvv AGGACGGCTG TTCAGTCTCT TGCTAACGCA ACTGCTGCTC AGCAGAATGT
     1514 AGGACGGCTG TCCAGTCTCT TGCTAACGCA ACTGCTGCCC AGCAGGAAGT
      EV1 AGGACGCTTG TCCAGTCTCT TGCTAACGCA ACTGCTGCTC AGCAGAATGT
          151                                                 200
Consensus GTTaGAAGCA aCtTATGCCA TGGTxCAGCA TgTaGCcAAA GGagTAAGAA
   Saomvv GTTAGAAGCA ACTTATGCCA TGGTTCAGCA TGTGGCAAAA GGGGTAAGAA
     1514 GTTGGAAGCA TCGTATGCCA TGGTACAGCA TATAGCCAAA GGAATAAGAA
      EV1 GTTAGAAGCA ACTTATGCCA TGGTGCAGCA TGTAGCCAAA GGAGTAAGAA
          201                                                 250
Consensus TatTAGAGGC AaGAGTxGCt cGaGTgGAAG cccTgGTaGA TaGaATGATG
   Saomvv TATTAGAGGC ACGAGTAGCT CGAGTAGAAG CTATAGTCGA TAGAATGATG
     1514 TCCTAGAGGC AAGAGTGGCT AGGGTGGAAG CCCTGGTAGA TTGAATGATG
      EV1 TATTAGAGGC AAGAGTTGCC CGAGTGGAAG TCCTGGTAGA TAGGATGATG
          251                                                 300
Consensus gTaTAtCAxG AATTgGATTG tTGGCATTAT CAaCACTAtT GTGTAACCTC
   Saomvv CTCTATCATG AATTGGATTG TTGGCATTAT CAACACTATT GTGTAACCTC
     1514 GTATACCAAG AATTGGATTG CTGGCATTAT CAACACTACT GTGTAACCTC
      EV1 GTATATCAGG AATTAGATTG TTGGCATTAT CAGCACTATT GTGTAACCTC
```

```
          301                                                 350
Consensus aACaAgAAgt GAAGTAGCaa AxTATGTaAA tTGGACAAGA TatAagGATA
   Saomvv TACTAGAACA GAAGTAGCAC AATATGTAAA TTGGACAAGA TATAAGGATA
     1514 AACAAGAAGT GAAGTAGCCA ATTATGTAAA CTGGACAAGA TTTAAAGATA
      EV1 AACAAAAAGT GAAGTAGCAA AGTATGTGAA TTGGACAAGA TACAGGGATA
          351                                                 400
Consensus AtTGtACaTG GCAgCAgTGG GAaGAAGAgA TAGAaCAaCA cGAgGcaAAt
   Saomvv ATTGCACATG GCAGCAGTGG GAAGAAGAGA TAGAGCAACA CGAGGCAAAT
     1514 ACTGTACCTG GCAACAGTGG GAGGAAGAAA TAGAACAACA CGAGGGAAAC
      EV1 ATTGTACATG GCAGCAATGG GAAGAAGAGA TAGAACAGCA TGAAGCGAAT
          401                                                 450
Consensus TTaAGtCtaT TgCTcAgaGA AGCaGCaTTa CAAGTACAcA TAGCtCAAAG
   Saomvv TTAAGCCTAT TGCTCAAAGA AGCAGCGTTA CAAGTACAAA TAGCACAAAG
     1514 TTGAGTCTAT TACTCAGAGA AGCAGCATTA CAAGTACACA TAGCTCAAAG
      EV1 TTAAGTCAGT TGCTTAGGGA AGCTGCATTG CAAGTACACA TAGCTCAAAG
          451                                                 500
Consensus aGATGCacaa AGAATxCCaG ATGtxTGGAa aGCatTaCAA GaAGCATTTg
   Saomvv AGATGCACAA AGAATACCAG ATGTCTGGAA GGCAATACAA GAAGCATTTG
     1514 AGATGCTAGG AGAATCCCAG ATGCTTGGAA AGCAATACAA GAAGCATTTA
      EV1 GGATGCACAA AGAATTCCAG ATGTATGGAC AGCGTTGCAA GGAGCATTTG
          501                                                 550
Consensus AcTGGtCagg aTGGTTCtCx TGGcTcAAAT ATATACCCTG GATtgTxaTg
   Saomvv ACTGGTCAGG ATGGTTCTCT TGGCTCAAAT ATATACCCTG GATAGTAGTA
     1514 ACTGGTCCTC TTGGTTCTCG TGGCTCAAAT ATATACCCTG GATTATCATG
      EV1 ATTGGGCAGG ATGGTTCGCA TGGTTGAAAT ATATACCCTG GATTGTGATG
           551                                                 600
Consensus gGaATxgTAG GAxTAATxTG TTTTAGAaTA cTAATGTGTG TGaTatCAaT
   Saomvv TGCATAGTAG GAGTAATATG TTTTAGACTA CTAATGTGTG TGATAACAAT
     1514 GGAATTGTAG GATTAATGTG TTTTAGAATA CTAATGTGTG TGATATCAAT
      EV1 GGAATCATAG GACTAATCTG TTTTAGAATA ATAATGTGTG TGGTGTCACT
          601                                                 650
Consensus GTGttTaCAG GCcTACAaGC AagTtcagcA GATCaGATAT ACACaGGTAA
   Saomvv GTGTTTACAG GCCTACAGGC AAGTTCGGGA GATCCGATAT ACACGGGTAA
     1514 GTGTTTGCAG GCTTACAAGC AAGTAAAGCA GATCAGATAT ACACAGGTAA
      EV1 GTGCCTACAG GCCTACAAGC AGATTCAACA GATCAGATAT ACACAGGTAA
```

```
          651                                                   700
Consensus CAgTgGTGAT AGAAGcACCx GTGGAccTgG AGGAAAAACa AagAgaAgAa
   Saomvv CACTAGTGAT AGAAGCACCC GTGGACCTGG AGGAAAAACA AAGAGAAGAA
     1514 CAGTGGTGAT AGAAGCACCG  GTGGAATTGG AGGAAAAACA AAAAAGAAAC
      EV1 CAGTGGTGAT AGAAGAACCA GTGGACCTCG AGGAAAAACC AGGAGAAGAA
          701                                                   750
Consensus agGGATGGTt caAaTGGCTx cGaAaaCTTA gaGCAcGAGA aAAGAACATC
   Saomvv AGGGATGGTT CCAGTGGCTC AGAAAACTTA GAGCACGAGA AAAGAACATC
     1514 GGGGATGGTA CAAATGGCTG CGCAAGCTTA GAGCACGAGA GAAGAACATC
      EV1 AAGGATGGTT TAAATGGCTT CGAAGACTTA AGGCAAGAGA AAAGAACATC
          751                                                   800
Consensus CCxtCGCAgT TTTAtCCAGA TATGGAGaGC AACxtgGcaG GCxTGGAAAA
   Saomvv CCCTCGCAGT TTTATCCAGA TATGGAGGGC AACTGTGCAG GCTTGGAAAA
     1514 CCATCGCAGT TTTATCCAGA TATGGAGAGC AACATGGTGG GCATGGAAAA
      EV1 CCGGCGCACT TTTACCCAGA TATGGAGAGC AACGTGGCAG GCCTGGAAAA
          801                                                   850
Consensus cCTCACCtTG GaggaAxagx TGGAgGAxAA tcCtaTATAT gActCTaCTa
   Saomvv CCTCACCCTG GGGGAAGGGA TGGAAGAAAA TCCTATATAT GACTCTACTG
     1514 CCTCACCTTG GAGACACAAC TGGAGGACAA TGCCCTATAT AACCCTGCTA
      EV1 ACTCACCTTG GAAGAAAAGT TGGAGGAGAA GCCTATATAT GAGTCTACTA
          851                                                   900
Consensus CCxcTAcTgg cgxTacaxca aTGGaTGGAA GAgAcTGGAT GGAaTGGaGa
   Saomvv CCGCTACTAA CACTGCAAAT ATGGATGGAA GAAACTGGAT GGAATGGAGA
     1514 CCCATATTGG TGATATGGCA ATGGATGGAA GAGAATGGAT GGAATGGAGA
      EV1 CCTCTACTGG CGGTACATCA GTGGCTGGAA GAGACTGGAT GGATTGGCGG
          901                                                   950
Consensus gAAtcaGcxC AAaAAgAAAA AAGAAAGGGT GGACTGTCAG GaCAGAGAAc
   Saomvv TAAGAGGTGC AAGAAGAAAA AAGAAAGGGT GGACTGTCAG GACAGAGAAA
     1514 GAATCAGCAC AAAAAGAAAA AAGAAAGGGT GGACTGTCAG GACAGAGAAC
      EV1 GAATCAGCTC AAAAATAAAA AAGAAAGGGT GGACTGTCAG GGCAGAGAAC
          951                                             991
Consensus axATGCCtac caTxGAgAAT GAcTATGTAG AgxTgaagtA g
   Saomvv GCATGCCTGC CATAGAGAAT GAGTATGTAG AGCTGAGCTA G
     1514 AAATGCCTAC CCTGGAAAAT GACTATGTAG AGTTATAGGA A
      EV1 AGATGCCCAT GATTGAGAAT GACTATGTAG AAATGAAGTA G
```

Figure 2.6: Comparison of MVV gp46 amino acid sequences.

The GCG "Lineup" program was used to align the gp46 amino acid sequence of MVV EV1 with those of strains 1514 and SAOMVV. A capital letter is shown in the consensus sequence where all three amino acids match, a small letter is shown where two of the three strains have that amino acid, an x is shown where the amino acid is different for each MVV strain.

```
          0                                                  49
Consensus .GIGLVIVLA IMAIIAAAGA GLGVANAVQQ SYTRTaVQSL ANATAAQQnV
   Saomvv .GIGLVIVLA IMAIIAAAGA GLGVANAVQQ SYTRTAVQSL ANATAAQQNV
     1514 .GIGLVIVLA IMAIIAAAGA GLGVANAVQQ SYTRTAVQSL ANATAAQQEV
      EV1 .GIGLVIVLA IMAIIAAAGA GLGVANAVQQ SYTRTLVQSL ANATAAQQNV

         50                                                  99
Consensus LEAtYAMVQH vAKGvRILEA RVARVEalVD rMMvYqELDC WHYQHYCVTS
   Saomvv LEATYAMVQH VAKGVRILEA RVARVEAIVD RMMLYHELDC WHYQHYCVTS
     1514 LEASYAMVQH IAKGIRILEA RVARVEALVD xMMVYQELDC WHYQHYCVTS
      EV1 LEATYAMVQH VAKGVRILEA RVARVEVLVD RMMVYQELDC WHYQHYCVTS

        100                                                 149
Consensus TrsEVAxYVN WTRykDNCTW QQWEEEIEQH EaNLSlLLrE AALQVhIAQR
   Saomvv TRTEVAQYVN WTRYKDNCTW QQWEEEIEQH EANLSLLLKE AALQVQIAQR
     1514 TRSEVANYVN WTRFKDNCTW QQWEEEIEQH EGNLSLLLRE AALQVHIAQR
      EV1 TKSEVAKYVN WTRYRDNCTW QQWEEEIEQH EANLSQLLRE AALQVHIAQR

        150                                                 199
Consensus DAqRIPDvWk AlQeAFdWsg WFsWLKYIPW IvmgIvGliC FRilMCVism
   Saomvv DAQRIPDVWK ALQEAFDWSG WFSWLKYIPW IVVCIVGVIC FRLLMCVITM
     1514 DARRIPDAWK AIQEAFNWSS WFSWLKYIPW IIMGIVGLMC FRILMCVISM
      EV1 DAQRIPDVWT ALQGAFDWAG WFAWLKYIPW IVMGIIGLIC FRIIMCVVSL
```

```
        200                                                        249
Consensus CLQAYkQvxq IRYTqVTvVI EaPVdLEEKq xeexDGxnGx exLehEkRTS
   Saomvv CLQAYRQVRE IRYTRVTLVI EAPVDLEEKQ REERDGSSGS ENLEHEKRTS
     1514 CLQAYKQVKQ IRYTQVTVVI EAPVELEEKQ KRNGDGTNGC ASLEHERRTS
      EV1 CLQAYKQIQQ IRYTQVTVVI EEPVDLEEKP GEEKDGLNGF EDLRQEKRTS

        250                                                        299
Consensus xRsFiQIWRA TwqAWKtSPW xkxWrxxlYm tLLPlLxxxq WmEEtGWnGx
   Saomvv PRSFIQIWRA TVQAWKTSPW GKGWKKILYM TLLPLLTLQI WMEETGWNGD
     1514 HRSFIQIWRA TWWAWKTSPW RHNWRTMPYI TLLPILVIWQ WMEENGWNGE
      EV1 RRTFTQIWRA TWQAWKNSPW KKSWRRSLYM SLLPLLAVHQ WLEETGWIGG

        300                              329
Consensus nqxKkKKERV DCQdREqMPx iENdYVElx*
   Saomvv KRCKKKKERV DCQDRESMPA IENEYVELS*
     1514 NQHKKKKERV DCQDREQMPT LENDYVEL
      EV1 NQLKNKKERV DCQGREQMPM IENDYVEMK*
```

conservative change.

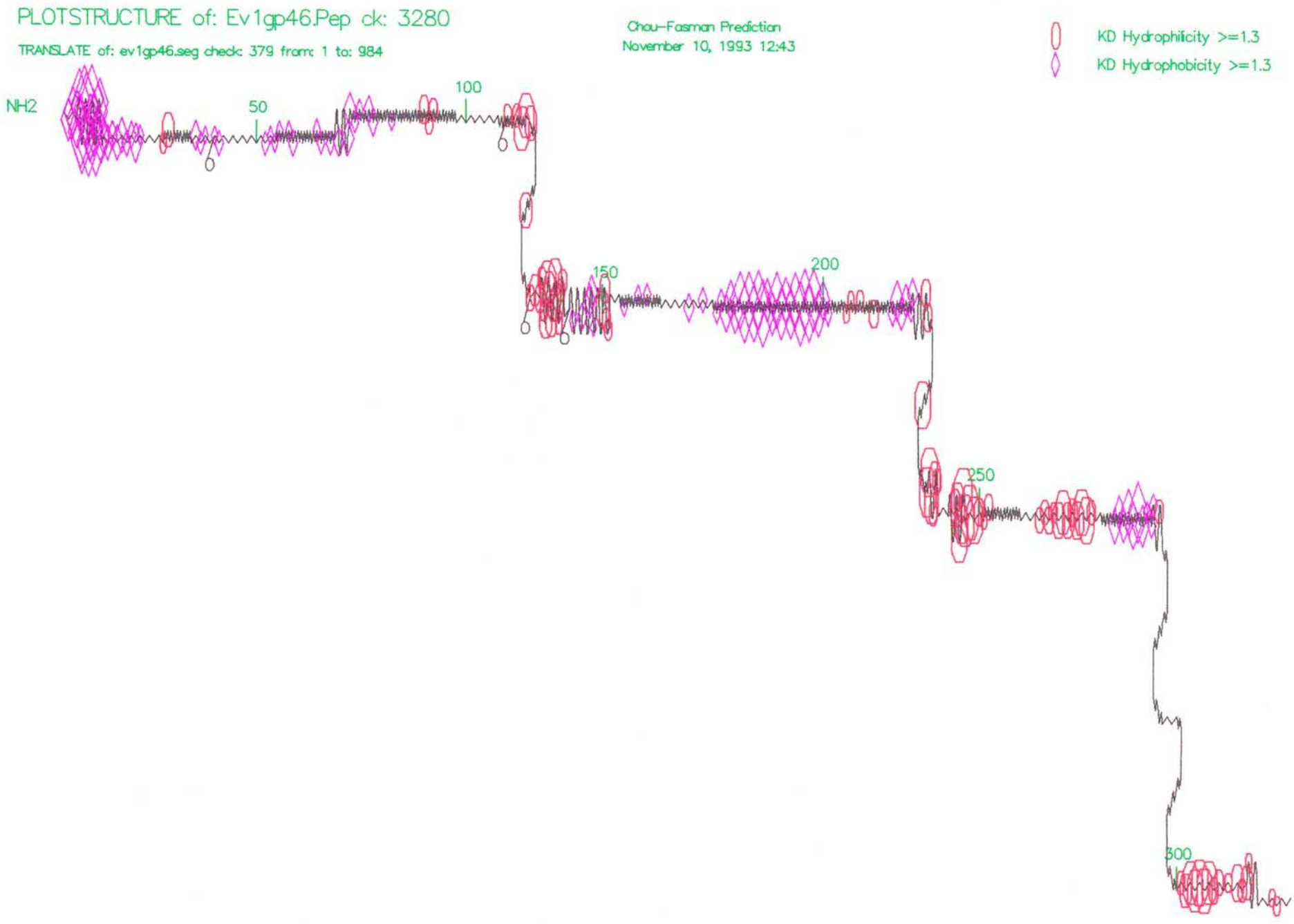
In contrast, the carboxy-terminal region of *gp46*, encoding the cytoplasmic tail of the gp46 protein, is very variable; the longest run of amino-acids conserved in all three strains in this region is only seven residues, and at many positions each of the sequences have different amino acids. This is the region of the MVV genome where the *env* and rev genes overlap but are translated in different reading frames.

Figure 2.7 shows a secondary structure prediction for gp46. Superimposed on the structural backbone are hydrophobic and hydrophilic domains. The profile shows two particularly hydrophobic areas; one at the extreme N-terminal of the protein, the other covering the probable membrane spanning region.

In the amino terminal half of gp46 the majority of sequence changes between the three isolates substitute one amino acid for another of similar charge or polarity. This together with the conserved cysteine residues and potential glycosylation sites, suggests conservation of the secondary structure of the external part of the native protein, whilst the carboxy terminal region, predicted to lie to the cytoplasmic side of the membrane, is much more variable.

Figure 2.7: Predicted secondary structure of gp46

The secondary structure of Ev1 gp46 was predicted using the UWGCG programs "peptidestructure" and "plotstructure". Alpha helices are shown as tight zig-zags, whilst beta-pleated sheets are shown as wide zig-zags. Predicted hydrophobic and hydrophilic regions are shown in pink and red, respectively.


PLOTSTRUCTURE of: Ev1gp46.Pep ck: 3280
TRANSLATE of: ev1gp46.seg check: 379 from: 1 to: 984
Chou-Fasman Prediction
November 10, 1993 12:43
KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
NH2
50
100
150
200
250
300

Chapter 2 :

DISCUSSION

2.4.1: **Cloning**

It was decided to attempt to amplify EV1 *gp46* by PCR in order to clone it. A sequence from the 3' end of *gp46* in pV1LTR was chosen for the complementary PCR primer. At this time the only known sequence for the 5' end of *gp46* was from the 1514 MVV strain. To give the best chance of the primer annealing with the EV1 gene the primer was chosen from the sequence of 1514 but including dITP at some of the third base positions to allow for codon ambiguities. A mixture of dATP, dCTP, dGTP or dTTP was used for the 3' end of the primer where the use of dITP is not recommended. An *EcoRI* recognition sequence was added at the 5' and of each primer in the hope of amplifying a PCR product with *EcoRI* ends to make cloning easier. The primers amplified a product of the expected size from EV1 Hirt DNA template and this was shown to be MVV specific by blotting with a probe derived from a full-length MVV-1514 clone. However the yield from the PCR was poor. This may have been partly due to poor primer design, however the choice of primers was restricted by the need to be as close as possible to the ends of the gp46 coding region. Several books published since the time this work was done suggest typical primers should be 18-28 nucleotides long, 50-60% G+C, and with a balanced Tms for a given primer pair.

```
3' PCR primer
140D-   5'-   GAA TTC TCA ATC ATG GGC ATC TGT TCT C   -3'
            16 A/T, 12 G/C, Tms approx. 80°C
5' PCR primer                                      A
141D-   5'-   GAA TTC ATG GGI ATA GGI TTG GTI ATA GT T G   -3'
                                                     C
      14 or 15 A/T, 3 I, 10 or 11 C/G, Tms approx. 70-72°C
```

This suggests the percentage of G and C nucleotides was low thus decreasing the annealing efficiency of the primers. However in the initial rounds of amplification the extra bases forming the *EcoRI* recognition sites at the 5' end would not be involved in pairing and therefore may be discounted in calculating the Tms.

There did not appear to be amplification of any non-specific products so it may have been possible to increase the yield of *gp46* PCR product by reducing the annealing temperature and/or by increasing the number of cycles.

Nevertheless the PCR did amplify sufficient amounts of the required

fragment to enable cloning. EV1, unlike 1514, was found to have an *EcoRI* site near the middle of *gp46* which meant that the terminal *EcoRI* sites which had been added could not be utilised for cloning the entire product and instead, blunt-ended PCR product was ligated into pTZ19R.

2.4.2: **Sequencing**

As *Taq* polymerase has no editing function there is always a possibility that PCR amplification will introduce base changes. The error rate of *Taq* polymerase is affected by a number of factors including enzyme, substrate and dNTP concentrations (Innis *et al.*, 1988). The estimates for the error rate vary from 1 in 300 (Meyerhans *et al.*, 1989) to 1 in 1500 (Perrin and Gilliland, 1990) to 1 in 9000 for single base pair substitutions and 1 in 41000 for frameshift mutations (Tindall and Kunkel, 1988). Although several clones were isolated and sequenced these were all obtained from the same ligation and transformation. The transformation protocol allows a period of growth in LB prior to plating for single colonies on selective agar but this period is insufficient to allow more than one cell division and therefore the 4 clones are unlikely to have been the result of a single ligation/transformation event. Although each of the four clones contained insert in the same orientation this is not sufficient to confirm or deny the above suggestion.

A single PCR reaction may also by chance amplify one variant preferentially from another. As the error rate for reverse transcriptase has been estimated at approx. 10^{-7} there are likely to be some variants within any population of MVV, so the particular sequence amplified and cloned will always represent only a quasispecies within a population except if a full-length infectious clone is being used.

The high rate of variation is due to the high error rate of reverse transcriptase which lacks a proof-reading activity. However the difference in mutation/variation rate between the different genes shows other factors must also be important. Studies by Hahn *et al.*, (1985), on HIV, showed 10^{-2}-10^{-3} changes per base per year for *env* but tenfold less for *gag*.

2.4.3: **Sequence comparisons**

If reverse transcriptase is considered to introduce base changes at random throughout the retroviral genome then sequences which remain conserved between different strains are likely to be maintained by functional constraints. Meanwhile other areas may appear hypervariable if there are no functional constraints on them. Indeed there may be positive advantages for the virus in evading the immune system

by introducing changes e.g. Braun *et al.*, (1987), described sequence differences between 1514 and a sequential isolate LV1-1 which was resistant to neutralisation antibodies directed against strain 1514. This may be one reason why *env* is the least homologous gene between MVV isolates (Querat *et al.*, 1987).

It has been shown that passage of plaque purified 1514 produced neutralisation resistant variants in absence of selective antibody (Staskus *et al.*, 1991). Variants had different sequence to 1514 and the changes were not spread evenly throughout the genome. This shows that even in the absence of a selective immune response *env* is the region of the genome most permissive for variation

Sequence comparisons between the *gp46* sequence of the three different strains of MVV revealed regions of relatively conserved or of variable sequences. This is expected to be related to both the error rate of RT and negative selection of any mutations which result in loss of infectivity/replication ability of the virus. Thus some regions are conserved probably because a particular sequence or structure is required for infection/replication.

2.4.4: **Prediction of functional regions of gp46**

Structure of gp46: Conserved cysteines and potential glycosylation sites suggest some requirement for maintenance of a particular secondary structure. This may be required for interaction with other gp46 molecules to form functional trimers (or tetramers).

Fusion: The extreme amino terminal end of gp46 was first identified as a fusion peptide sequence in sequence comparison studies (Gallaher, 1987). The high degree of conservation at the amino terminal end of the protein is as might be expected for a predicted functional region. MVV does not include the Phe-X-Gly motif found in the paramyxovirus fusion domains and also in SIV and HIV, but it does exhibit the characteristic highly hydrophobic amino-terminal region typical of other fusion-mediating viral envelope proteins. The amino acid sequence 12-29 within the N-terminal region has some homology with the predicted fusogenic epitope of ortho- and paramyxoviruses.

Immunosuppression: Ruegg *et al.*, (1990) identified a sequence within the MVV transmembrane which had (limited) sequence homology to the region of HIV which had been identified as having immunosuppressive properties. This in turn had been identified by sequence similarity with an immunosuppressive peptide

conserved in the transmembrane protein, p15E, of the oncornavirus subgroup of retroviruses. The peptide 64-78 (GvRILEARVARVEal) has been reported to be immunosuppressive. When conjugated to carrier protein the peptide specifically inhibited T-cell proliferation at micromolar concentrations. This peptide as well as those of HIV and the oncornaviruses appear to exert their immunosuppressive effects by inhibition of protein kinase C (Ruegg *et al.*, 1990, Gottlieb *et al.*, 1989). As shown by the small letters in the sequence above there are changes between MVV isolates at either end of this peptide but the central 11 residues are conserved. This suggests the sequence may be functional even though immunosuppression is not generally considered to be an outcome of MVV. It may be that the peptide is effective at limited immunosuppression and may help MVV to persist. The peptide may be more potent in its native form as part of the TM gp, so lower concentrations may be effective *in vivo* than measured *in vitro* using synthetic peptides. However there appears to be no difference between the mitogen stimulation or mixed lymphocyte responses of peripheral blood leukocytes of experimentally MVV infected compared to mock infected sheep (Griffin *et al.*, 1978) suggesting that MVV-infected sheep are not immunosuppressed. It may be that any immunosuppressive effects of gp46 act only at a local level, or that as part of gp46 the peptide is not presented in such a way as to be immunosuppressive.

Contact with the surface envelope glycoprotein, gp110: The 11-base sequence ELDCWHYQHYCVTS, (aa 87-100) was conserved for all three MVV isolates. This sequence has been proposed as the immunodominant region of MVV gp46 by analogy with a similar sequence in the TM glycoprotein of HIV and of other retroviruses (Schulz *et al.*, 1992). Di-sulphide bonding between the two cysteines is thought to form a finger or knob-like structure which interacts with a pocket-like structure in the external membrane glycoprotein (by analogy with HIV). This region was identified as a gp120 contact region because it binds a monoclonal antibody which is an anti-idiotypic antibody against another monoclonal which reacts with the carboxyterminal end of gp120 (Schulz *et al.*, 1991). Mutations in this region of gp120 abrogate its binding to gp41, (Helseth *et al.*, 1991).

CHAPTER 3: Expression of recombinant gp46 in yeast

3.1: INTRODUCTION

Investigations into the structure, function and antigenicity of MVV envelope glycoproteins has been extremely limited because of the difficulty in purifying sufficient yields from viral preparations. Stanley *et al.*, (1987) used lectin based affinity chromotography to prepare gp135 from MVV-infected cells, but there are no reports of purification of gp46 from infected cells and because it is a transmembrane protein it would be extremely difficult to separate it from other membrane components. As well as being difficult, preparation from large-scale virus cultures would include variants introduced during virus culture whereas a recombinant system will express a defined product.

The yeast Ty-VLP expression system was chosen as it has previously been reported to produce high yields of recombinant protein in a non-denatured immunogenic form. Fragments of HIV-1 gp41 and HIV-2 gp36 (Gilmour *et al.*, 1989 and 1990), and fragments of HIV-1 gp120 (Adams *et al.*, 1987) are among the proteins which have been expressed in this system.

The Ty element is a yeast retrotransposon. It consists of long terminal repeats (LTRs) at either end, and two genes TyA and TyB analogous to retroviral *gag* and *pol* genes (Kingsman *et al.*, 1981). It encodes proteins that self-assemble in the cytoplasm of yeast cells into virus-like particles (VLPs) containing Ty RNA, reverse transcriptase, a protease and probably integrase. A truncated form of the Ty retrotransposon of yeast can be utilised as an expression system for recombinant protein (Adams *et al.*, 1987). The TyA gene encodes the protein p1 which in the complete Ty VLP is the precursor of the four structural proteins constituting the capsid of the VLP. However p1 alone can self-assemble in the yeast cytoplasm to form virus-like particles. These are approximately 60nm in diameter and comprised of about 300 p1 monomers. Another coding sequence fused in frame to the TyA encodes a hybrid p1 fusion protein that may assemble into hybrid Ty-VLPs. The particulate nature of the product enables purification by centrifugation on sucrose gradients. In addition the polyvalency of the product and the immunogenic nature of p1 accounts for it being recommended as a good recombinant antigen. The system may be further modified by including an Xa protease recognition sequence to allow proteolytic cleavage of the protein from p1 (Braddock, *et al.*, 1989). Some but not all of the recombinant proteins expressed in the Ty-VLP system have been shown to be glycosylated (Carey, 1992), although glycosylation is unlikely to be normal.

Plasmids pOGS40 and pMA5620 were obtained from British Biotechnology, Oxford. Both are E.coli/yeast shuttle vectors encoding 381 codons of TyA followed by a *BamHI* linker, stop codons in each of the three reading frames, and a transcription terminator. pMA5620 expresses p1 from the constitutive yeast phosphoglycerate kinase (PGK) promoter (Adams *et al.*, 1987), whilst pOGS40 expresses p1 from the hybrid PGK/GAL promoter which is galactose (or IPTG) inducible (Gilmour *et al.*, 1989).

Expression from the pOGS40 PGK-GAL promoter is dependent on the relative levels of (host-encoded) GAL4 and GAL80 proteins. GAL4 activates expression from the GAL promoter by binding to a specific DNA site, whilst its antagonist, GAL80, blocks activation by binding to GAL4 and/or its DNA binding site. Excess GAL4 over GAL80 is thus required to promote expression. The helper plasmid pUG41S expresses GAL4 protein under the control of the *GAL1* promoter which is galactose inducible and also activated by GAL4 (positive feedback). Therefore co-transformation with pUG41S and pOGS40 should increase the level of expression from the PGK-GAL promoter by overcoming the limiting levels of GAL4, whilst maintaining the galactose inducibility of expression (Schultz *et al.*, 1987).

All the above vectors are selectable by ampicillin resistance in *E.coli*. pOGS40 is selectable in *S. cerevisiae* by transformation of leu$^-$ strains to leucine independence, whilst pMA5620 transforms trp$^-$ *S.cerevisiae* to tryptophan independence, and pUGS 41 transforms to uracil independence. *S.cerevisiae* co-transformed with pUG41S and pOGS40 can be selected by their uracil and leucine independence. The yeast vectors are based on the 2μm plasmid which is normally present at 100-200 copies per yeast cell.

Whereas I had to design PCR primers to specifically amplify the gene fragment in the correct reading frame, a family of pOGS vectors are now available which differ in the reading frame of the *BamHI* cloning site at amino acid 381. Similarly vectors are also now available which contain a factor Xa cleavage site immediately upstream of the *BamHI* cloning site whereas I added the protease recognition site by including its sequence in the 5' PCR primer.

Chapter 3:

METHODS

3.2.1: Plasmid Construction

PCR reactions were set up using approximately 1ng plasmid pCC18.9 as template for primers 095G and 096G, or 096G and 097G, (shown in section 3.3.2). 0.3μg or 0.6μg of each primer, 10μl 10x PCR buffer, 10μl 300μM dNTPs, and 10μl 200μg/ml BSA was used for each reaction and was made up to 100μl with SDW. 35 cycles of melting at 95°C for 0.6 min, annealing at 45°C for 1.0 minute, and extending at 70°C for 4.0 min were completed with a final extension at 70°C for 5.0 min.

The 990bp product amplified by primers 097G and 096G was designated naTF (Transmembrane protein , Full length), whilst the 500bp product amplified by primers 095G and 096G was designated naTH (Transmembrane protein, Half-length).

3.2.2: Ligation of naTH and naTF (PCR products) into plasmid vectors

A series of reactions were set up to try to ligate *BamHI* digested PCR products into pOGS40 or pMA5620 without success. It was therefore decided to ligate in the first place into pTZ18R in order to increase the amount of available product to insert into the larger vectors.

In order to make more product, the PCRs were repeated. After 30 cycles of PCR as described above, a further 0.5μl of each primer, 2μl dNTPs and 0.1μl of Taq polymerase was added to each reaction tube. Three more PCR cycles as before were followed by a final extension of ten min at 70°C. The aim was to try to increase the amount of PCR product with completed ends. The PCR products, naTH or naTF, were phenol extracted, ethanol precipitated and resuspended in 50μl of water.

A sample of each PCR product was treated with Klenow and T4 polynucleotide kinase to ensure blunt ends. The remainder was digested with *BamHI*. All four products were gel purified and recovered from low gelling temperature (LGT) agarose using Geneclean (as manufacturers instructions). The blunt-ended or *BamHI* treated PCR products were ligated into *HincII* or *BamHI* cut CIP treated pTZ18. DNA was from each ligation reaction was phenol/chloroform extracted, ethanol precipitated (Appendix II) and resuspended in 30μl of SDW. 10μl was used to transform competent *E. coli* strain JM83 (Appendix I) .

Transformed bacteria were selected on LB/Amp/X-Gal/IPTG agar (Appendix I). Plasmid mini-preps were prepared from overnight cultures of transformants

which had been inoculated into 6ml LB, 150μg/ml Amp (Appendix II). Samples of super-coiled plasmid were electrophoresed. Plasmids larger than pTZ18R were digested with *BamHI* and analysed by electrophoresis.

Two clones were selected; pF102.7 consisting of naTF in pTZ18R and pH103.11 containing naTH insert in pTZ18R. Plasmid was prepared from 25ml overnight cultures, resuspended in TE/RNase, restriction digested with *BamHI* and electrophoresed in 1% LGT agarose. Bands of ~500bp and ~1Kbp, corresponding to inserts naTH(*BamHI*) and naTF(*BamHI*) respectively, were excised and the DNA extracted by Geneclean (as manufacturers instructions).

Ligation reactions were set up using *BamHI* restricted, CIP treated pOGS40 or pMA5620 vector and naTH(*BamHI*) or naTH (*BamHI*) insert at molar ratios of insert : vector of approximately 1:1, 5:1, 10:1, and 15:1. Vector, insert and SDW were added and the mixture was heated to 65°C, then snap cooled on ice before addition of ligase buffer and enzyme. The ligation reactions were incubated overnight at room temperature. 3μl or 7μl of each ligation was used to transform competent E.coli JM83. Transformants were selected by plating onto LB agar/150μg/ml Amp. Colonies which had grown up overnight incubated at 37°C were picked onto duplicate LB agar/150μg/ml Amp. plates and screened by colony hybridisation on Nylon filters probed with ^{32}P labelled gp46INS (Appendix III).

Blot positive clones were inoculated into 6ml of LB/150μg/ml Amp. Plasmid mini-preps were made, resuspended in TE/RNase, and the desired clones were identified by electrophoresis of plasmid samples digested with *BamHI*, *PstI* or *EcoRI* alongside digested pOGS40 or pMA5620.

3.2.3: **Verification of vectors for expression of EV1 gp46 in yeast**

Large scale plasmid preparations (Appendix II) were made of the three clones selected to use for transformation of yeast.

2μg of each of the three plasmids was digested with *BamHI*, *PstI* or *EcoRI* in a total volume of 20μl. 4μl of each digest was electrophoresed alongside control digests of pMA5620 or pOGS40. The gels were Southern blotted onto nitrocellulose and probed with ^{32}P labelled gp46INS (Appendix III).

The plasmids were sequenced (Appendix IV) across the TyA:gp46 junction using ~0.5pmole of primer 098G (sequence: CAG GAG AAA TCC GAG TG) to anneal to ~1pmole (~6μg) of double-stranded plasmid template.

Expression of EV1 gp46 in yeast

3.2.4: Transformation of Yeast with Expression Vectors

Protease deficient yeast, strain BJ2168 (a, leu2=, trp1-, ura3-52, prb1-1122, pep4-3, pcr1-407, gal2) was transformed with pEV1TH.O by a Lithium acetate transformation method (Appendix V). Transformants, designated BTH.O, were grown up on SC-Glc agar plus tryptophan and uracil. BTH.O cells were again made competent (Lithium acetate method) and transformed with pUGS41 to become uracil and leucine independent. Doubly transformed cells were designated BTH.U.

Subsequently BJ2168 was transformed by a spheroplast method. (Appendix V). Briefly, the yeast cell wall was digested off using the enzyme glusulase and 5μg or 10μg of plasmid DNA was added, with or without 10μg salmon sperm DNA as carrier. Cells which had taken up the plasmid DNA were selected by growth in regeneration agar plus tryptophan and uracil for singly transformed cells or plus tryptophan only for double transformants. BJ2168 transformed with the pMA5620 derivative pEV1TF.A were designated BTF.A. Similarly cells transformed with the pOGS40 derivative pEV1TF.O were designated BTF.O. Double transformants with both pEV1TF.O and pUGS41 were designated BTF.U.

Four colonies of each type of transformant, i.e. BTF.A, BTF.O and BTF.U, were taken from the regeneration plates and streaked onto SC-glc agar plates with appropriate amino acid(s) added.

3.2.5: Preparation of yeast crude cell extract

A single colony from each streaked plate was used to inoculate 50ml of SC-glc medium plus amino acid(s), and incubated at 30°C with shaking. After 45 h The OD_{600} of each culture was measured to estimate cell density using the relationship that OD_{600} 1.0 is equivalent to approximately $3x10^7$ cells/ml. Stocks were stored down by taking 1ml aliquots, adding an equal volume of 40% glycerol in SC-glc and freezing at -20°C for 1 hour then at -70°C.

To prepare crude cell extract approximately $5x10^8$ cells were spun down (1500rpm for 5 min) and resuspended in 1ml TEN (Appendix V). 1g of acid washed glass beads was added and the cells were disrupted by whirlimixing for three one minute periods with one minute cooling on ice in between. Samples were analysed by SDS PAGE and Western blotting (Appendix VI).

3.2.6: **Comparison of protein production from double or single transformed yeast**

Single colonies of BTF.O.6.1 and BTF.U.8.2 were inoculated into 100ml of SC-glc plus uracil and tryptophan or SC-glc plus tryptophan, respectively, and grown at 30°C with shaking. After 2 days aliquots of approximately $5x10^8$ cells were resuspended in 50ml SC-glc/gal plus amino acid(s), and incubated at 30°C with shaking. After various times the OD_{600} was measured, and crude cell extract was prepared. Samples were analysed by SDS- PAGE and Western blotting (Appendix VI).

3.2.7: **Large scale preparations of p1-fusion protein**

Large scale preparations of p1-fusion proteins from BTH.U or BTF.U were made. Incubations were at 30°C with shaking and media contained added tryptophan at 40mg/l. One glycerol stock was inoculated into 100ml of SC-glc and incubated until the cell density had reached $2\text{-}4x10^7$ cells/ml. The culture was divided into two 2l flasks containing 1l of pre-warmed SC-glc. After 24 h incubation the cells were harvested in 250ml Beckman bottles in a J2-21 rotor at 5000 rpm for 5min at 4°C. The cells were washed three times with SDW and once with TEN. To the cell pellet an equal volume of TEN plus protease inhibitors and of acid-washed glass beads was added. The mixture was vortexed for at least ten 30 second periods interspersed with cooling on ice then centrifuged for 5 min at 3000 rpm, 4°C. The supernatant was retained. 4ml of buffer (TEN plus protease inhibitors) was added to the pellet. Vortexing and centrifugation was repeated and the supernatant was retained. 3ml of buffer was added to the pellet and the mixture was vortexed a further four times, centrifuged and the supernatant retained once more. Cell breakage was evaluated by adding a drop of the cell pellet to a drop of water and looking for lysed cells by phase`contrast microscopy. If lysis was less than 70%, further rounds of vortexing etc. were continued. Pooled supernatants were centrifuged at 9K for 20 min to pellet cell debris and whole cells. The supernatant was transferred to a Beckman SW40 tube, underlaid with 2ml 60% sucrose in TEN, and centrifuged for 1 hour at 30K in a Beckman ultracentrifuge (SW40 rotor). The sucrose cushion and the layer immediately above were collected and dialysed overnight against TEN. The dialysate was microcentrifuged for 15 min at 4°C and the supernatant was carefully loaded on top of a 15-45% sucrose gradient (prepared as 10ml layers of 15%, 25%, 35%, and 45% sucrose in TEN, left at 4°C overnight, and underlaid with 3ml of 60% sucrose just before use). After 3 h centrifugation at 25K, 4°C, the gradient was harvested into 2 ml fractions. Fractions containing p1:fusion VLPs were identified

by running 5µl aliquots on SDS-PAGE, and were pooled and dialysed against PBS before use or storage at -20°C.

3.2.8: **Preparation of yeast for electron microscopy** (Adapted from the method of Byers and Goetsch, 1975)

One glycerol stock of BTF.U.8.2 was inoculated into 100ml of SC-glc plus trp and incubated at 30°C with shaking for 20 h until until an OD_{600} of 0.7 was reached. 30ml was added to 100ml SC-gal plus trp. and 20ml was added to 100ml of SC-glc plus trp. After 6 h and 22 h 25ml of culture was harvested at 4°C, resuspended in 5 ml cacodylate buffer (3% gluteraldehyde, 0.1M cacodylate pH6.8, 5mM $CaCl_2$) and incubated at room temperature for 30 min. Cells were pelleted, resuspended in 10ml cacodylate buffer and mixed overnight at 4°C on a rotator. Cells were washed twice with 10ml pre-treatment buffer (0.1M mercaptoethanol, 0.02M EDTA, 0.2M Tris-Cl pH8.1) and resuspended in 5ml citrate-phosphate buffer (0.04M citric acid, 0.06M Na_2HPO_4, pH to 5.8 with NaOH) containing 250µl of glusulase. Following 1 h incubation at 30°C, spheroplasts were washed twice in 10 ml citrate-phosphate buffer and finally resuspended in 5ml citrate-phosphate buffer. Cells were treated for 60 min in 2% osmium tetroxide, rinsed with water then treated with 2% aqueous uranyl acetate at 20°C for 60 min. The cells were then dehydrated and embedded in resin. Blocks were sectioned using a microtome and the sections were fixed to grids for viewing by EM.

Cultures of untransformed BJ2168 were also grown up in a similar manner (except leu, trp and ura was added to media) and cells were prepared for EM.

3.2.9: **Negative staining of VLPs for EM**

A drop of VLP preparation, at a concentration of ~300µg/ml was placed on an EM grid and left for 15 seconds. The edge of a piece of Whatman 1 paper was used to draw off most of the liquid. The grid was washed three or more times by adding a drop of SDW to the grid then drawing the water off with paper. A drop of 2% uranyl acetate in SDW, (filtered through a 0.22 µm filter and stored in the dark at -20°C and microcentrifuged for 5 min immediately prior to use), was added to the grid and then most of the liquid was drawn off . The grid was placed on filter paper to dry, then stored at room temperature prior to EM.

3.2.10: **Transformation of yeast strain MC5**

Diploid yeast strain MC5 (homozygous prb-1, prc-1, pep-4, trp-1, leu-2, ura-3) was transformed with plasmids pTF.O, pTF.O and pUG41S, or pTH.O and

pUG41S for expression of p1-fusion proteins. Transformation was by the spheroplast method, using 5μg or 10μg of each plasmid with or without 10μg of salmon sperm DNA as carrier. Transformed yeast were selected initially in regeneration agar with tryptophan (single transformants) or tryptophan and uracil (double transformants) (Appendix V). After several days incubation at 30°C colonies were picked and streaked onto SC-glc agar plus amino acid(s).

MC5 transformants were validated for production of p1 or p1-fusion protein by running samples of crude lysate on SDS-PAGE. Nomenclature was assigned to the MC5 transformants in a similar way to the BJ2168 transformants e.g., cells transformed with TF.O were designated MTF.O, cells transformed with pTF.O and pUGS41 were designated MTF.U.

3.2.11: **Growth curves of induced MC5 transformants**

A colony from an SC-glc streaked plate was inoculated into 50ml SC-glc plus trp or trp/uracil and incubated for 48 h at 30°C with shaking. The OD_{600} was measured and approximately 3 x 10^8 cells were pelleted and resuspended in 50ml SC-gal plus trp or trp/uracil. Incubation at 30°C with shaking continued and the OD_{600} of each culture was measured at intervals over 28 h.

3.2.12: **Large scale preparation of p1-fusion protein from MEV1TF.U**

p1:TF was prepared from 8l of induced MTF.U using the method described above.

3.2.13: **Factor Xa protease digestion of p1:TF**

Factor Xa protease was added at ratios of 1:10, 1:20, 1:40 and 1:80 w:w enzyme:total protein in buffer to give a final concentration when added to the substrate of 10mM $CaCl_2$, 100mM Tris pH7.5, 0.05% CHAPS, 0.05% DOC. Samples were taken after 1, 2, 4, 8, and 21 h incubation at 25°C. 10μl of each was run on SDS PAGE and detected by silver stain.

Subsequently p1:TF was digested at a ratio of 1:25 Factor Xa protease:total protein at 25°C for 16-24 h.

3.2.14: **Production of polyclonal rabbit serum against p1 or p1:TF**

Rabbit R194 was immunised i/m with ~100μg of p1 protein in complete Freunds adjuvant (CFA) and boosted after 2 weeks with 100μg of p1 in incomplete Freunds adjuvant (IFA). Rabbit R193 was immunised i/m with material derived from induced BTF.U containing ~ 100μg of p1:TF (~250μg total protein) in CFA and

boosted after 2 weeks with the same preparation in IFA. 5ml of blood was taken from each rabbit 2 weeks after the boost.

Pre-immune and post-inoculation sera were reacted, at 1:500 dilution, against Western blots of p1, p1:TF and Factor Xa digested p1:TF. (1μg of antigen per track of a 10% acrylamide reducing gel, blotted onto nitrocellulose) (Appendix VI)

3.2.15: **p1:Sepharose column**

6mg of p1 was dialysed against four changes of 0.1M Sodium Bicarbonate pH9, 0.5M NaCl. 0.7g of Cyanogen bromide activated Sepharose 4B was swollen in 1mM HCl in a sintered glass funnel. p1 protein was added to the Sepharose and mixed on a rotator overnight at 4°C. The coupled Sepharose was recovered by centrifugation at 1000rpm for 5 min. The protein concentration of the supernatant was measured by Biorad protein assay to check coupling efficiency. The Sepharose was rotated in 1M ethanolamine for 3 h, then washed with BBS and allowed to settle in a 1cm diameter column. The column was pre-eluted with 0.1M glycine pH2.7 then equilibrated with BBS. 1ml of serum (pH7-8) was loaded and the flow through collected and loaded through the column once more. 0.5ml fractions were collected. The column was washed with BBS and fraction collection was continued until the effluent was clear. The column was washed extensively before antibodies bound to the column were eluted with 10ml 0.1M glycine pH2.7. 0.5ml fractions were collected into tubes containing 30μl 2M Tris pH9 to neutralise the glycine. The OD_{280} of each fraction was measured. Peak fractions were pooled and dialysed into PBS. The column was washed extensively with BBS before reuse.

Activity of the sera, pooled fractions and column flow-through was measured by ELISA, (Appendix VII), against plates coated with 70μl per well of 10μg/ml p1 or 10μg/ml p1:TF (~25μg/ml total protein). The same samples were also reacted against p1, p1:TF, p1:TF digest and BJ2168 crude lysate on Western blots.

3.2.16: **Western blots with sera from MVV infected or uninfected sheep**

Sera from sheep infected with MVV or from uninfected sheep were reacted against p1, p1:TF, p1:TF digest or EV1 antigen on Western blot (Appendix VI).

3.3.1: Construction of plasmids for expression of recombinant EV1 gp46 in the yeast Ty-VLP system

The aim of this work was to express recombinant EV1 transmembrane glycoprotein in yeast as a fusion protein with p1. A 43kDa polypeptide fused to p1 has been expressed without disrupting VLP formation (Burns *et al.*, 1992) so the size of gp46 was not expected to present a problem, but due to the possibility that the hydrophobic nature of gp46 might have interfered with assembly into VLPs two different products were made; one to encode full length EV1-gp46, bases 1-990, (TF), the other to encode the external domains of gp46, bases 1-530, (TH). Inserts suitable for ligation into pOGS40 or pMA5620 were modified from the EV1 gp46 clones in pTZ using PCR. As shown below additional bases were included in the PCR primers in order to add *BamHI* recognition sites at either end of the coding sequences to make cloning into theses plasmids easier and an Xa protease cleavage site was added at the 5' end to enable cleavage of the fusion protein product from p1.

3.3.2: PCR primers for expression of V1-gp46 in the Ty system

096G- (5' primer)

G GGA TCC ATA GAA GGT AGA TGT GGG ATA GGC TTG GTG ATA GT
[*BamHI*][Factor Xa][5' gp46]

097G- (3' primer for full length construct, TF) :

GGA TCC TCA ATT CTC AAT CAT GGG CAT CTG
[*BamHI*][Stop][Complementary to gp46 bases 970-950]

095G- (3' primer for external half of EV1-gp46 construct, TH) :

G GGA TCC TCA TTT CAA CCA TGC GAA CCA TCC TGC
[*BamHI*][Stop][Complementary to gp46 bases 529-506]

Using pCC18.9 as template primers 096G and 097G amplified a product, (TFna), of about 1kb representing 1-990 of the gp46 insert described in Chapter 2. Primers 096G and 095G amplified bases 1-530 of the gp46 insert, (THna). Initial attempts to ligate *BamHI* treated PCR products directly into the *BamHI* site of either plasmid proved unsuccessful. The PCRs only produced a low yield perhaps because the additional bases on the PCR primers made them sub-optimal. Ligation into the yeast expression plasmids was predicted to be less efficient than into pTZ18R or

pTZ19R because of the larger size, so to increase the yield of naTH and naTF it was decided to first ligate the PCR products into pTZ18R. Additional primers were added for the final few PCR cycles to try to maximise the amount of product with completed ends for ligation. Two clones were selected; pF102.7 and pH103.11 consisting of TF and TH PCR products, respectively, blunt-end ligated into the *HincII* site of pTZ18R. *BamHI* was used to retrieve the inserts from these plasmids, and the gel purified inserts were then ligated into *BamHI* cut, CIP-treated pOGS40 or pMA5620. Cloning the PCR products via pTZ18R not only enabled a larger amount of insert DNA to be prepared for ligation into pMA5620 or pOGS40 but also fortuitously circumvented a problem that had not been recognised at the time, namely that restriction enzymes do not cut efficiently at sites very close to the ends of PCR products.

Recombinant Ty vectors were selected initially by colony hybridisation. Plasmid mini-preps were made from blot positive clones and restriction digested to check the size and directionality of the insert in any positive clones (Figure 3.1). There was quite a high rate of carry over of pTZ18R plasmids demonstrating the inefficiency of gel purification of the inserts. Any pTZ18R in the ligation reaction was not CIP treated and would therefore readily religate and also more easily transfect competent cells than the larger plasmids (Hanahan, 1983). As shown in the predicted restriction maps of the plasmids (Figure 3.1d)*BamHI* digestion could be used to confirm the size of the insert, *EcoRI* digestion allowed pOGS40 and pMA5620 plasmids to be distinguished, and *PstI* digestion determined the orientation of the insert .

Three plasmids were selected for expression in yeast ;. plasmid pTF.O (insert encoding MVV-EV1 transmembrane protein (full-length) in pOGS40), plasmid pTF.A (full-length insert in pMA5620), and plasmid pTH.O (half-length insert in pOGS40).

Restriction digests of these plasmids and Southern blots probed with gp46INS (Figure 3.1), agreed with the predicted results and verified the choice of plasmid. Double stranded sequencing through the TyA:TH or TyA:TF junction region of each of the three correctly orientated constructs confirmed the *BamHI* and Xa protease recognition sequences were intact and that the insert was in the same reading frame as the p1 encoding sequence (Figure 3.2).

Figure 3.1: Restriction fragment analysis of Ty-based expression constructs.

Plasmid DNA extracted from clones 2C6 (pTH.O), 2C2 (pTF.A), and 7A5 (pTF.O) were digested with *BamHI*, *PstI* or *EcoRI*, and were electrophoresed alongside restriction digested control plasmid in order to confirm the size and directionality of the insert. As a further check Southern blots were made and hybridised with gp46INS (the insert from pCC18.9 described in Chapter 2). PCR products naTH and naTF were included as positive controls.

The predicted restrictrion fragment sizes are shown below. Those shown in bold type would contain gp46 sequence and therefore should hybridise with the gp46INS probe. Fragments shown in bold with a * only contain 50 or 66 bases of gp46 and therefore would be expected to hybridise only weakly, if at all.

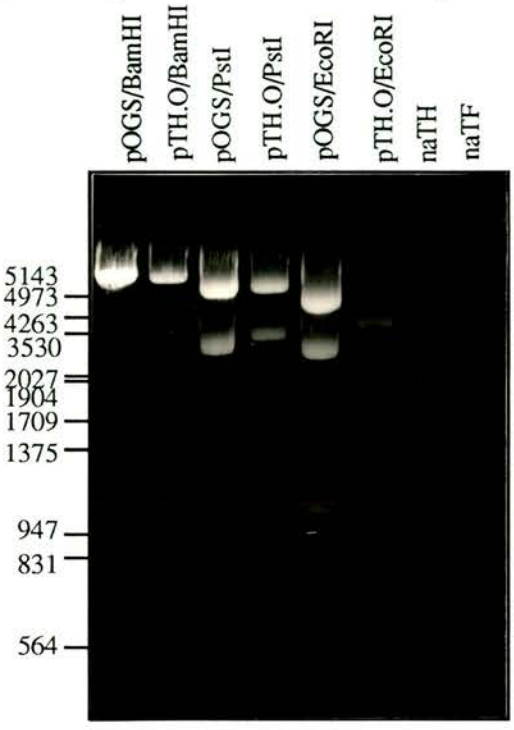
Predicted restriction fragment sizes of Ty constructs

Plasmid	*BamHI* digest	*PstI* digest	*EcoRI* digest
	Restriction fragment size (kb)		
pOGS40	9.42	6.55, 2.87	5.02, 2.55, 1.1, 0.75
pMA5620	9.42	6.55, 2.87	6.12, 2.55, 0.75
pOGS40+ TH insert (correct orientation)	9.42, **0.5**	6.55, **2.75**, 0.35*	3.39*, 2.55, **2.16**, 1.1, 0.75
pOGS40 + TH insert (inverted orientation)	9.42, **0.5**	6.55, 2.32*, **0.78**	**3.78**, 2.55, 1.77*, 1.1, 0.75
pOGS40 + TF insert (correct orientation)	9.42, **0.97**	6.55, **3.19**, 0.35*	**3.83**, 2.55, **2.16**, 1.1, 0.75
pOGS40 + TF insert (inverted orientation)	9.42, **0.97**	6.55, 2.32*, **1.22**	**3.78**, 2.55, **2.21**, 1.1, 0.75
pMA5620 + TF insert (correct orientation)	9.42, **0.97**	6.55, **3.19**, 0.35*	**3.26**,2.55, **3.83**, 0.75
pMA5620 + TF insert (inverted orientation)	9.42, **0.97**	6.55, 2.32*, **1.22**	**3.76**, 2.55, **3.31**, 0.75

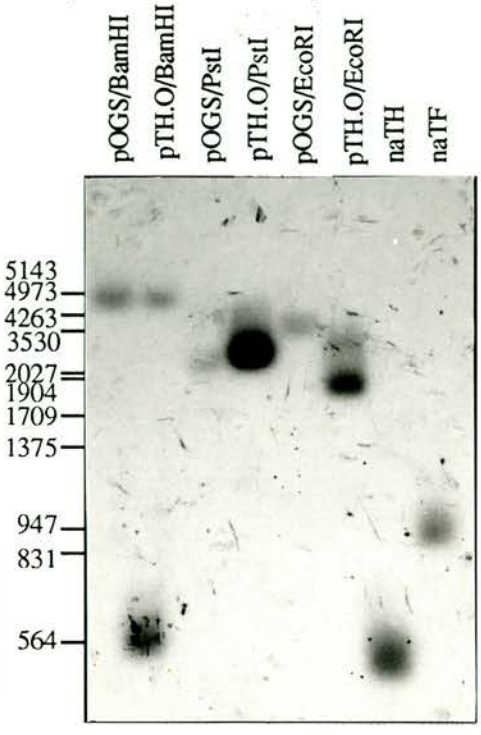
d) shows plasmid maps of the constructs.

a). pTH.O (pOGS40:THinsert)

i) Electrophoresis of restriction fragments

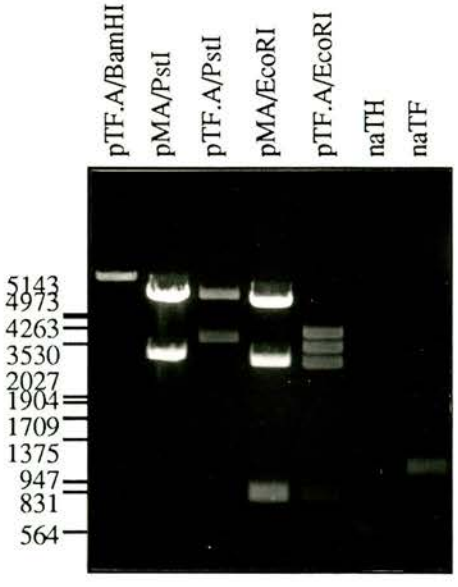


ii) Southern blot of i)

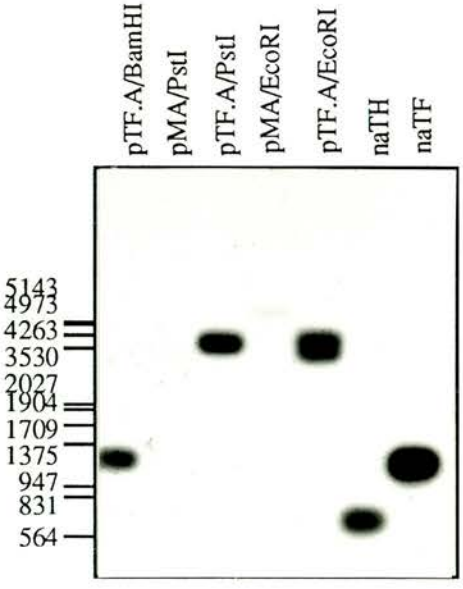


b). pTF.A (pMA5620:TFinsert)

i) Electrophoresis of restriction fragments

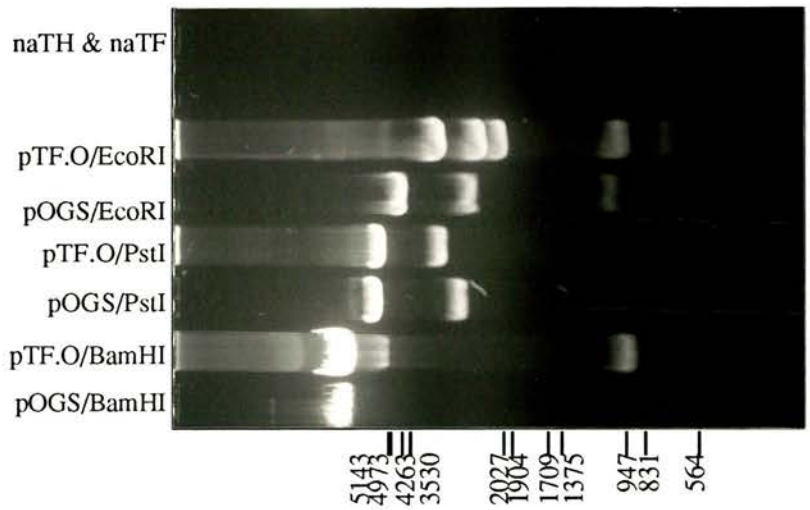


ii) Southern blot

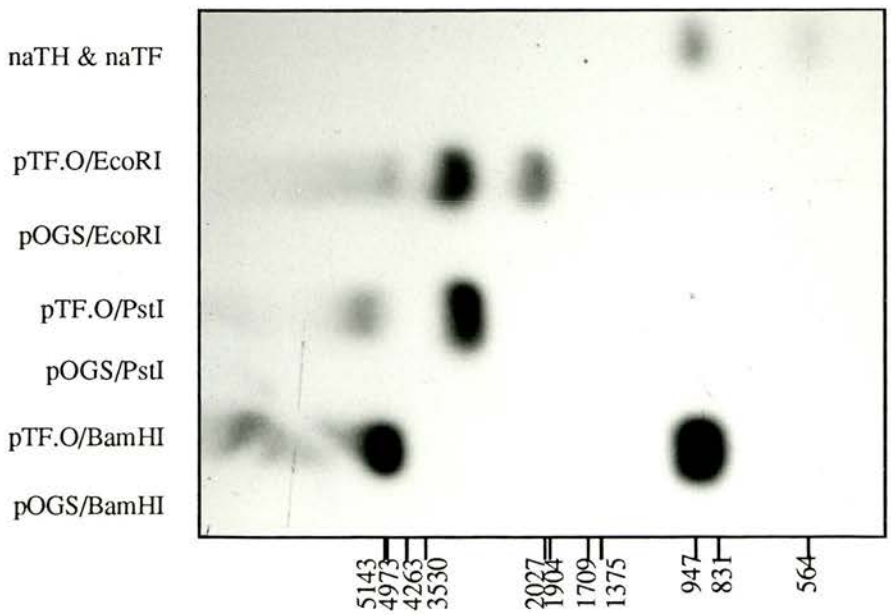


c). pTF.O (pOGS40:TFinsert)

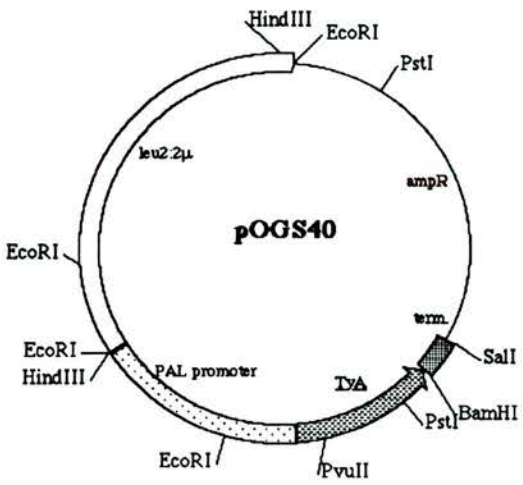
i) Electrophoresis of restriction fragments



ii) Southern blot



d)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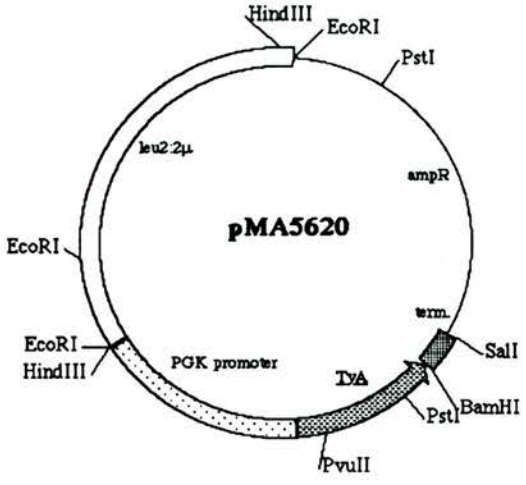
PstI
leu2:2μ
ampR
pOGS40
EcoRI
EcoRI
HindIII
PAL promoter
TyA
term.
SalI
BamHI
PstI
EcoRI
PvuII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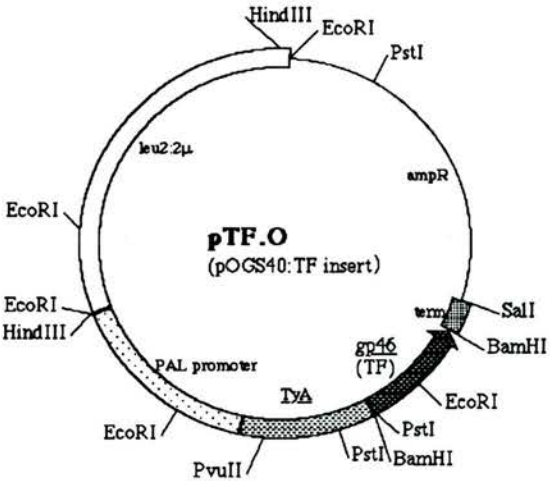
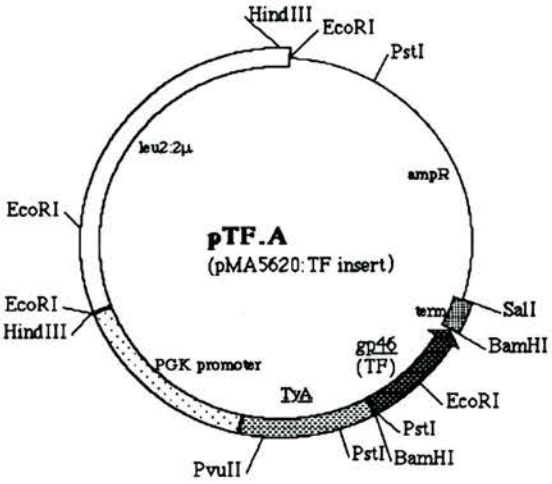
PstI
leu2:2μ
ampR
pMA5620
EcoRI
EcoRI
HindIII
PGK promoter
TyA
term.
SalI
BamHI
PstI
PvuII


HindIII
EcoRI
PstI
leu2:2μ
ampR
pTF.O
(pOGS40:TF insert)
EcoRI
EcoRI
HindIII
PAL promoter
TyA
gp46
(TF)
term
SalI
BamHI
EcoRI
PstI
PstI
BamHI
EcoRI
PvuII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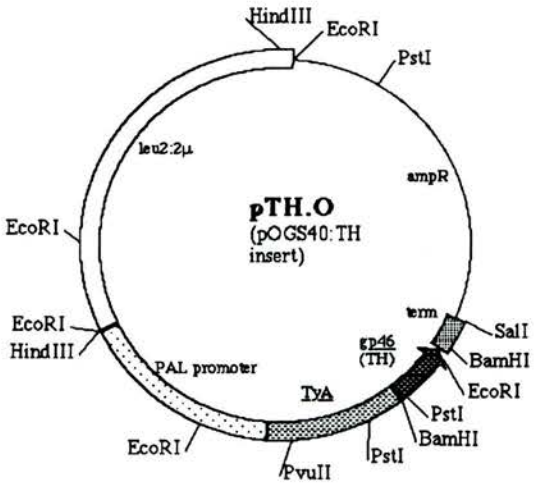
PstI
leu2:2μ
ampR
pTF.A
(pMA5620:TF insert)
EcoRI
EcoRI
HindIII
PGK promoter
TyA
gp46
(TF)
term
SalI
BamHI
EcoRI
PstI
PstI
BamHI
PvuII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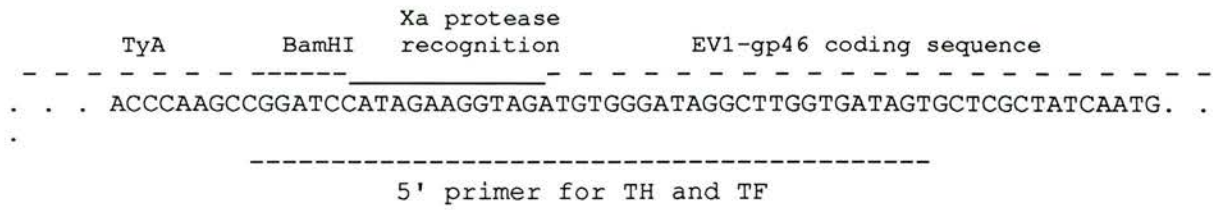
EcoRI
PstI
leu2:2μ
ampR
pTH.O
(pOGS40:TH
insert)
EcoRI
EcoRI
HindIII
PAL promoter
TyA
gp46
(TH)
term
SalI
BamHI
EcoRI
PstI
BamHI
EcoRI
PstI
PvuII

Figure 3.2: Sequence of V1-gp46/Ty constructs through the insert site.



3.3.3: **Transformation of yeast and expression of p1 fusion proteins.**

The first of the expression plasmids constructed was pTH.O. This was used to transform *Saccharomyces cerevisiae* strain BJ2168 and create clone BTH.O. BTH.O was transformed with helper plasmid pUG41S to create clone BTH.U. The same *S. cerevisiae* strain was transformed with pTF.O, or pTF.A, but by a spheroplast transformation method. Selection of transformants was by growth in regeneration agar with tryptophan (trp) and uracil (ura) added, but not leucine, and transformed clones were named BTF.O and BTF.A respectively. Double transformants with both pTF.O and pUGS41, (called BTF.U), were made and selected by transformation to leucine and uracil independence in regeneration agar plus tryptophan.

3.3.4: **Results of spheroplast transformation**

Spheroplast transformation with pTF.A resulted in numerous very small colonies. The size was at first thought to be due to competition by too many colonies. Transformation with pTF.O resulted in hundreds of colonies whilst double transformation with TF.O and pUGS41 together was less efficient producing 10-44 colonies per plate.

As illustrated in Figure 3.3, when streaked onto SC-glc agar plates with appropriate amino acid(s) added, the BTF.O and BTF.U transformants had grown up to give single colonies of about 2mm diameter in 2 days, whilst the BTF.A colonies were still barely visible.

The pMA5620 derived construct, pTF.A had a constitutive promoter whilst the pOGS40 derived construct had a promoter inducible by galactose, so the fact that the BTF.A yeast clones did not grow well suggested immediately that expression of the p1:TF fusion protein might be toxic to the yeast. This was backed up by observation of BTF.O and BTF.U cells after galactose induction in liquid medium. Examination of the cells under a phase contrast microscope showed that initially normal budding cells were visible in the cultures but several hours after

Figure 3.3: Growth of transformed yeast strains on solid medium

Single colonies of yeast BJ2168 transformed with vectors for the expression of p1:gp46 (TF), as shown below, were streaked out onto SC-glc agar plus appropriate amino acids and incubated at 30°C for 44 hours.

The photographs illustrate the variation in growth rate between the different transformed clones.

a) BTF.O 5.1 (transformed with pTF.O)

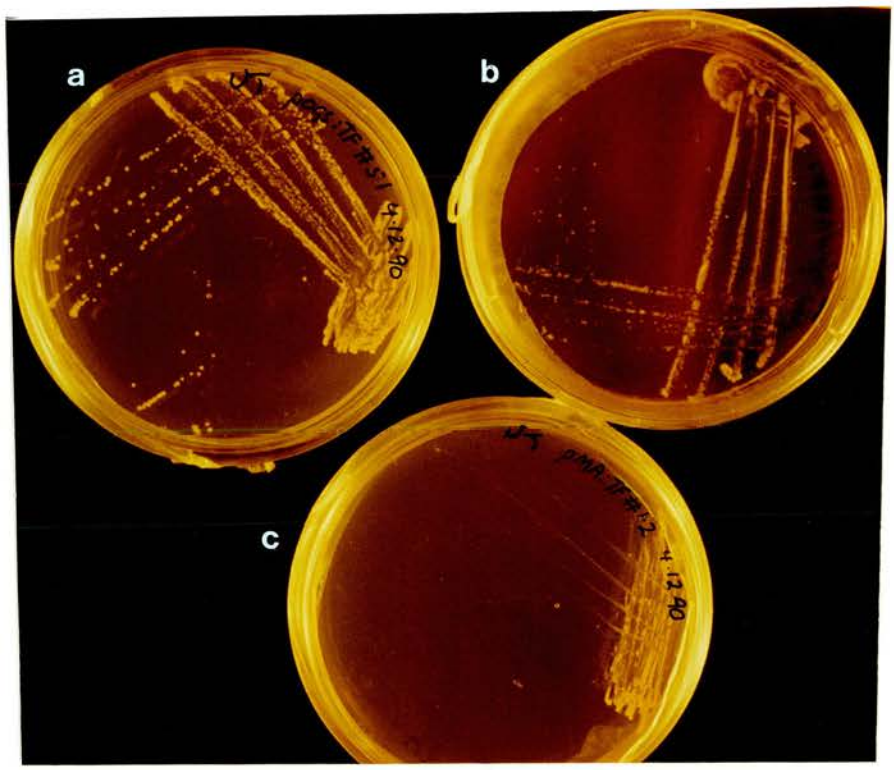
b) BTF.U 8.1 (transformed with pTF.O and pUGS41)

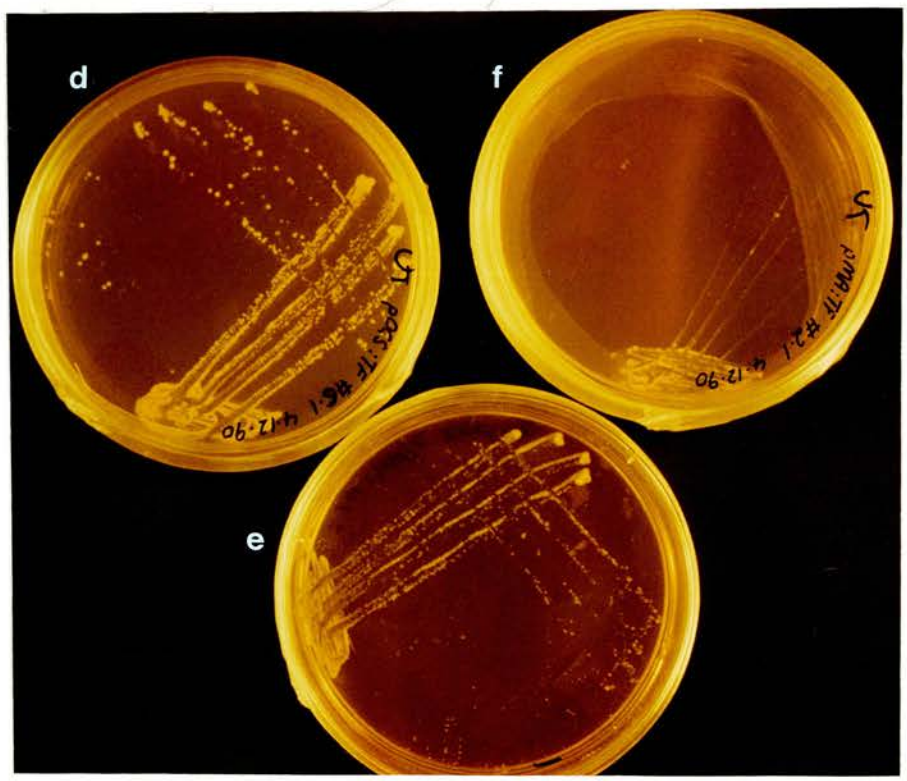
c) BTF.A 1.2 (transformed with pTF.A)

d) BTF.O 6.1 (transformed with pTF.O)

e) BTF.U 8.2 (transformed with pTF.O and pUGS41)

f) BTF.A 2.1 (transformed with pTF.A)


a
b
c


d
f
e

transfer to galactose-containing medium there were many swollen cells which appeared large and dark by phase contrast. The proportion of 'bloated' cells appeared to be greater in the double transformants; 12 hours after induction BTF.U cultures contained many large cells and a few phase bright dead cells, and after 24 hours very many large cells were visible but there were also very many dead and clumped cells, whilst BTF.O cultures had fewer "sick" cells. Budding cells were still present at 24 hours although some aberrantly shaped cells (oval or balloon shaped) were also present at this time. BTF.U was also slower growing than BTF.O in liquid culture; inocula of 10^7 cells/ml into SC-gal reached $3.3x10^7$ cells/ml for BTF.O and $2.4x10^7$ cells/ml for BTF.U. Cultures of four different clones of each all produced very similar results.

3.3.5: **Analysis of p1:fusion protein production**

Crude whole cell extracts, made by breaking the cells open using glass beads, were analysed by SDS-PAGE (Figure 3.4). Samples from wild-type BJ2168, BP1.A (pMA5620 transformed BJ2168), and a transformant which produces p1:p25gag (gifted by H.T.Reyburn) were run alongside as controls. One gel was stained with Coomassie, the other was blotted onto nitrocellulose. The blot was reacted with anti-p1 polyclonal rabbit serum. By comparison with extract from pMA5620 transformed cells, samples from each transformant show an upwards shift in size compatible with the predicted sizes of the p1 fusion products p1:TF (81KDa) and p1:TH (63KDa) as compared with p1 alone (43KDa). (Note that p1 migrates with an apparent molecular weight of 55-60kDa in this gel system. This is consistent with previous data (Adams *et al.*, 1987)). On Coomassie stained gels of the same preps no clear band corresponding to the fusion product is visible. This indicates that the recombinant protein is not produced at high yield. The blots show that p1 protein was produced endogenously by untransformed yeast and that in the transformed yeast strains p1 was present pre-induction but at low levels.

None of the strains of BTF.A produced any detectable p1-fusion protein, but produced only p1, equivalent to that made by wild-type untransformed BJ2168 (Figure 3.5). As the BTF.A strains did not grow well on agar it is likely that they also did not grow well in liquid medium . It may have been that the cells which did grow had lost the insert from the plasmid which would account for detection of only wild-type p1. (This could have been checked either by trying to recover plasmid from the yeast or more simply by Southern blotting of DNA from the yeast). Another explanation could be that a stop codon had been introduced near the start of the gp46 coding region.

Figure 3.4: Analysis of cell extracts from transformed yeast strains

a) Coomassie-stained SDS-PAGE gel

5µl samples of crude yeast lysate were analysed by SDS-PAGE.

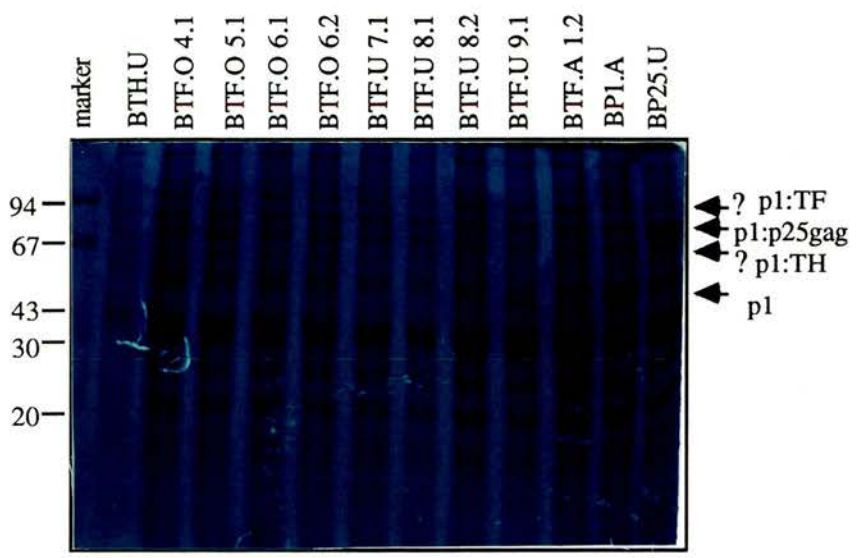
b) **Western blot**

10µl samples of crude cell lysate were run on a 1% polyacrylamide SDS-PAGE gel which was blotted onto nitrocellulose. The blot was reacted with polyclonal anti-p1 rabbit antiserum followed by goat anti-rabbit AP conjugate.

Predicted size of p1:fusion proteins (unglycosylated)

p1:	43kDa
p1:TH:	63kDa
p1:TF:	81kDa

a)


marker
BTH.U
BTF.O 4.1
BTF.O 5.1
BTF.O 6.1
BTF.O 6.2
BTF.U 7.1
BTF.U 8.1
BTF.U 8.2
BTF.U 9.1
BTF.A 1.2
BP1.A
BP25.U
94
67
43
30
20
? p1:TF
p1:p25gag
? p1:TH
p1

b)

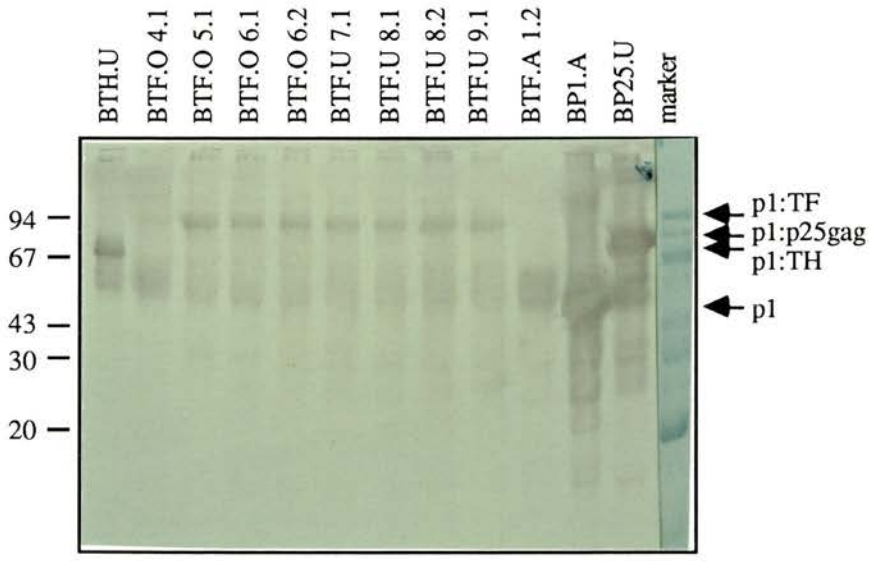

BTH.U
BTF.O 4.1
BTF.O 5.1
BTF.O 6.1
BTF.O 6.2
BTF.U 7.1
BTF.U 8.1
BTF.U 8.2
BTF.U 9.1
BTF.A 1.2
BP1.A
BP25.U
marker
94
67
43
30
20
p1:TF
p1:p25gag
p1:TH
p1

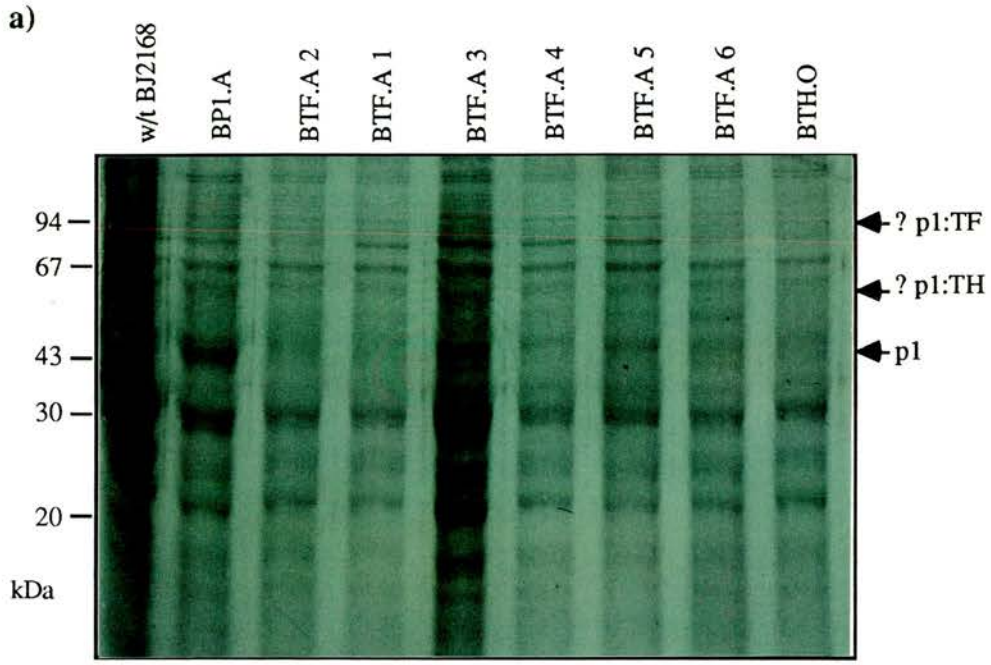
Figure 3.5: Analysis of BTF.A yeast strains

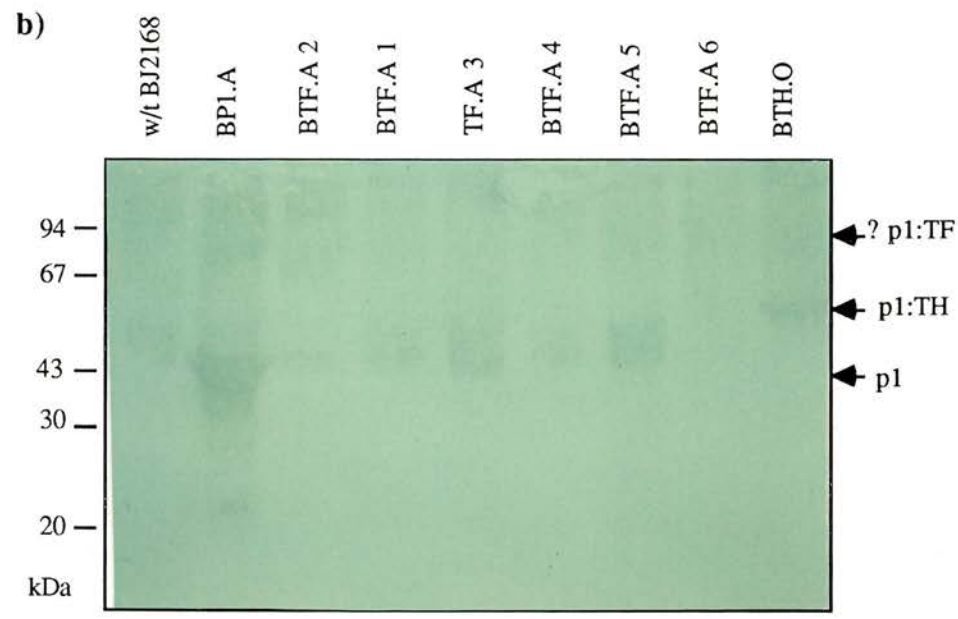
a) Coomassie-stained SDS-PAGE gel

5µl samples of crude yeast lysate were analysed by SDS-PAGE.

b) **Western blot**

10µl samples of crude cell lysate were run on a 1% polyacrylamide SDS-PAGE gel which was blotted onto nitrocellulose. The blot was reacted with polyclonal anti-p1 rabbit antiserum followed by goat anti-rabbit AP conjugate.


a)
w/t BJ2168
BP1.A
BTF.A 2
BTF.A 1
BTF.A 3
BTF.A 4
BTF.A 5
BTF.A 6
BTH.O
94
67
43
30
20
kDa
? p1:TF
? p1:TH
p1


b)
w/t BJ2168
BP1.A
BTF.A 2
BTF.A 1
TF.A 3
BTF.A 4
BTF.A 5
BTF.A 6
BTH.O
94
67
43
30
20
kDa
? p1:TF
p1:TH
p1

3.3.6: **Comparison of protein production between BTF.O and BTF.U**

BTF.O and BTF.U cells grown up in SC-glc media and transferred into SC-gal plus amino acid(s). After various incubation times crude cell extract was prepared and analysed by SDS-PAGE (not shown) and by Western blotting (Figure 3.6). The Coomassie stained gels show that the loading was approximately equal on both gels. However, the blot of BTF.O is clearly less dark than the blot of the doubly transformed BTF.U, indicating that the yield of recombinant protein was greater in the latter strain. The amount of p1:TF appears to peak at about 12 hours after induction in strain BTF.O but peaked earlier in strain BTF.U. On the Western blots of BTF.U reactivity was saturated after 8 hours, so the concentrations can not be compared in this way. There is no evidence of a reduction in the amount of p1:TF at 24 hours or beyond which would indicate spontaneous breakdown of the product, however, 18 hours was chosen as the optimal time for harvesting induced cultures because less dead and dying cells were seen at this time compared to at 24 hours.

Little or no p1:TF was produced pre-induction in BTF.O but it was produced in uninduced BTF.U suggesting that the gal4 promoter on the pUGS41 promoter is leaky.

3.3.7: **Large scale preparation of p1:TF from BTF.U**

Production was scaled up in order to purify recombinant protein. Briefly, 1l galactose-induced cultures were harvested and the cells lysed with glass beads. Cell debris was pelleted and the supernatant was centrifuged onto a 60% sucrose cushion. The VLP containing fraction spanning the interface with the sucrose cushion was collected, dialysed to remove the sucrose and then centrifuged through a 15-45% sucrose gradient.

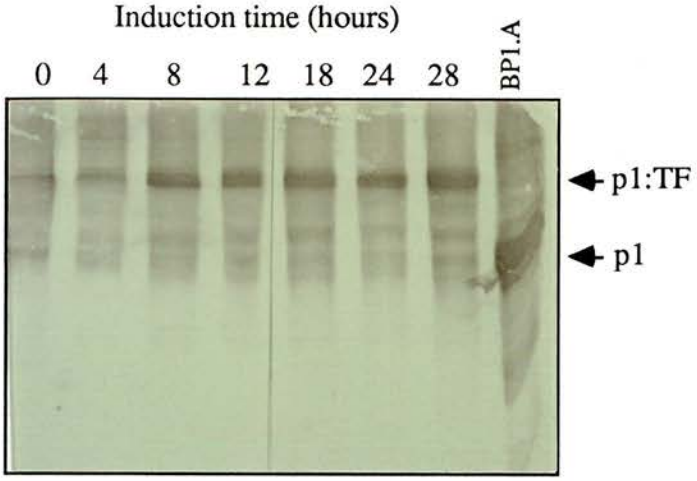
Collection of gradient fractions and analysis by SDS-PAGE (Figure 3.7) showed that the p1:fusion protein did not band tightly in the gradient but was spread over most fractions. Most p1:fusion proteins do not band tightly and different p1:fusion proteins tend to band at different densities in the gradient. The amount and the purity of the protein obtained varied between individual large scale preps. A number of factors could have played a part in this e.g. the percentage cell lysis acheived can significantly affected the yield. The state of the sucrose gradient would also have been important in separating contaminant proteins away from the fusion protein. However the use of a stepped gradient rather than a semi-continuous one made little difference to the spread of p1:TF across many fractions (results not shown).

Figure 3.6: Comparison of P1:TF fusion protein production between BTF.O and BTF.U

To show the effect of co-transformation of pTF.O with or without pUG41S Western blots were made of crude extracts from samples taken at intervals following galactose induction of p1:TF expression in transformed yeast strains BTF.O and BTF.U.

Samples were subjected to (10% polyacrylamide) SDS-PAGE and blotted onto nitrocellulose. The blot was reacted with polyclonal anti-p1rabbit antiserum (1:2000) followed by goat anti-rabbit IgG:AP conjugate (1:1000).

Strain BTF.U 8.2



Strain BTF.O.6.1

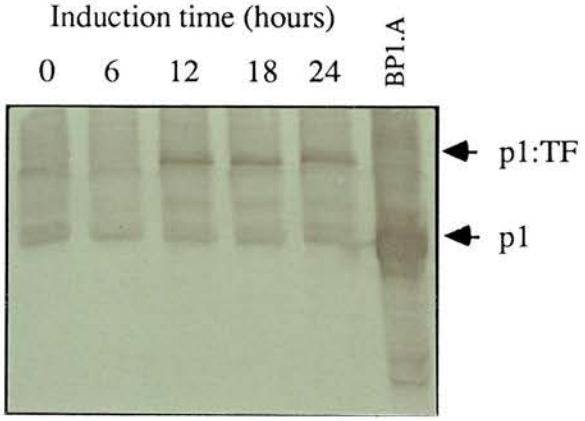


Figure 3.7: Fractions from sucrose gradient purification of p1:TF fusion protein

a) Coomassie-stained SDS-PAGE gel

20µl of samples of fractions from sucrose density gradient centrifugation were analysed by (10% polyacrylamide) SDS-PAGE. 1ml fractions were collected numbered beginning from the top of the gradient.

b) Western blot

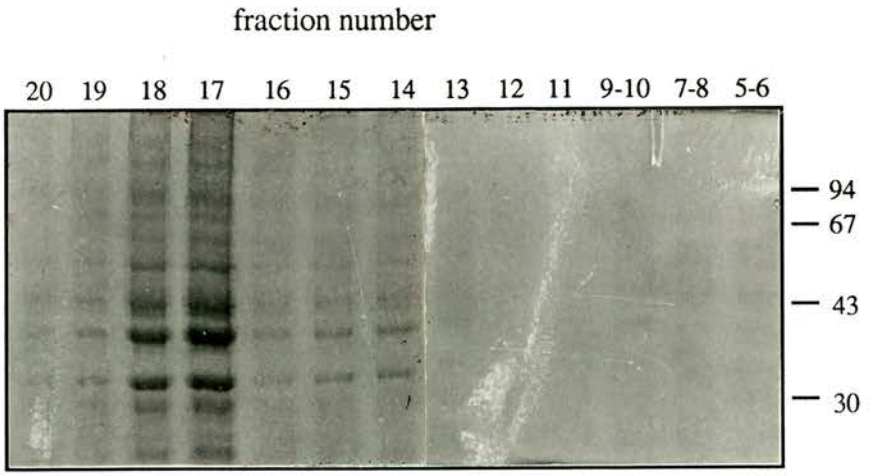
Duplicate gels to the ones shown in a), above, were blotted onto nitrocellulose. The blot was reacted with polyclonal anti-p1rabbit antiserum (1:2000) followed by goat anti-rabbit IgG:AP conjugate (1:1000).

(Note that the lanes in the western blot are shown back-to-front relative to the Coomassie stained gel).

c) Final product of large scale preparations of p1 and p1:TF

The products of large scale preparations were analysed by (10% polyacrylamide) SDS-PAGE.

a)


fraction number
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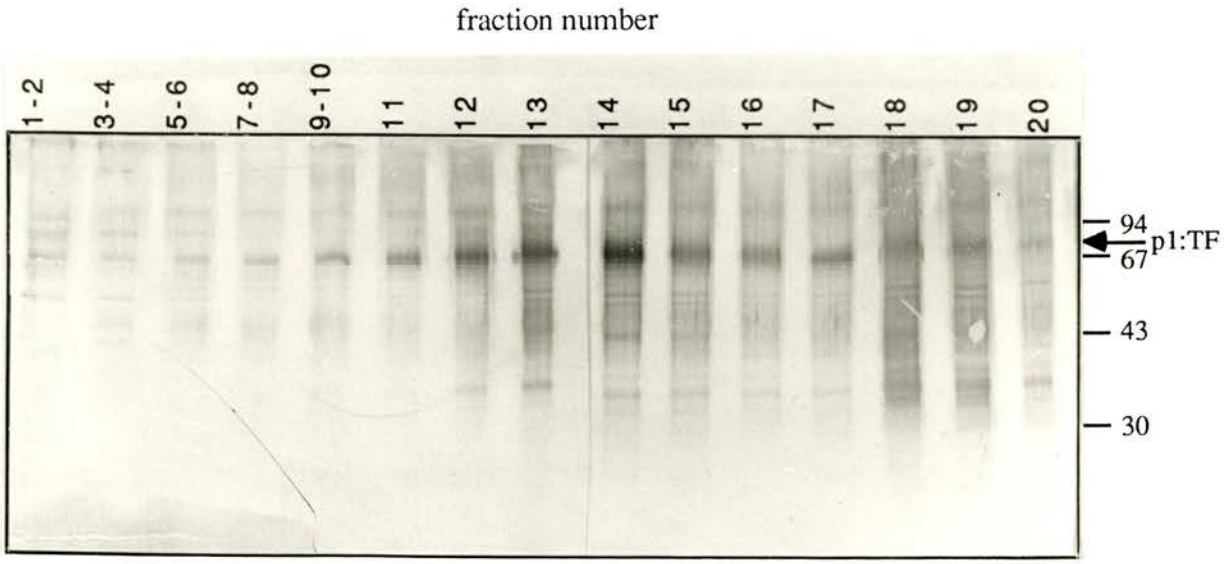
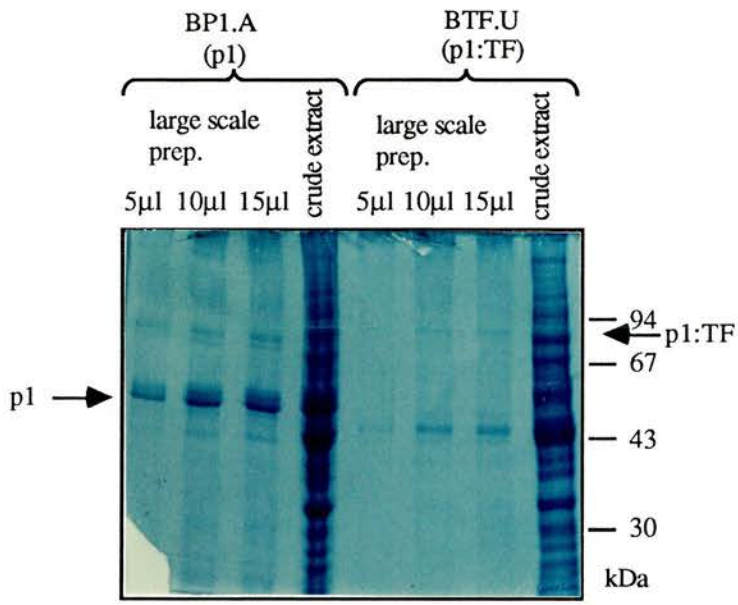
94
67
43
30

b)


fraction number
1-2 3-4 5-6 7-8 9-10 11 12 13 14 15 16 17 18 19 20
94
67
p1:TF
43
30

c) Final products of p1 and p1:TF large scale preps.
Coomassie stained SDS-PAGE gel.



p1:TF was never obtained at high yield in any of the fractions and as a consequence the relative amount of contaminating proteins was very high. Other p1 fusion proteins produced in this laboratory at much higher yield tended to contain almost as much contaminant protein in real terms but far less relative to the amount of p1 fusion product. The p1 preparation from BP1.A, (BJ2168 transformed with pMA5620), also contained contaminating proteins. A major contaminating band in the p1:TF preparation at ~43kDa may represent endogenous VLPs, but as the same band is not present in the p1 preparation this band may instead be a protein which associates with p1:TF.

The western blot (Figure 3.7b) reacted with anti-p1 serum suggests p1 at many molecular weights. This may be due to induction of antibodies to contaminating yeast proteins in the p1 preparation used for immunisation. Proteolysis or instability of the fusion protein cannot be ruled out, although protease inhibitors were included during the preparation to try to limit proteolysis.

A large scale VLP preparation from 10l of induced BTF.U gave a total yield of approximately 5mg protein of which less than 20% was p1:TF, the rest being contaminant yeast proteins. Large scale preparation of p1:TH gave a similar yield and purity. In contrast strain BP1.A yielded over 10mg of p1 per litre.

As the yield of p1:TH was not noticeably better than the yield of p1:TF it was decided to use the latter for the subsequent studies.

3.3.8: **Visualisation of VLPs by electron microscopy**

Electron micoscopy of the p1:TF product confirmed the formation of virus-like particles. (Figure 3.8) These were much more heterogeneous than VLPs formed by p1 alone.

Electron micrographs of whole yeast cells expressing p1 protein often show the cytoplasm to be absolutely full of VLPs. By contrast it was very hard to find anything resembling VLPs in the yeast cells expressing p1:TF. Many of the cells had vacuoles in their cytoplasm, a condition which is often seen in unhealthy cells. Particles in the vacuoles had the appearance of VLPs but untransformed BJ2168 grown in galactose for a similar length of time contained similar particles in their vacuoles. The only things resembling VLPs in induced BTF.U cells but not in uninduced or untransformed controls are arrowed in Figure 3.8. These appear in the cytoplasm and seem to be of a similar size and structure to the purified particles seen in Figure 3.7, and to published EMs of yeast cells expressing hybrid VLPs. These putative VLPs were visible in only a few BTF.U cells, and very few were present in

Figure 3.8: Electron micrograph of p1:TF preparation

A sample of the product of a large scale preparation of p1:TF was negatively stained with uranyl acetate in order to visualise VLPs by electron microscopy.

Magnification: x 60 000

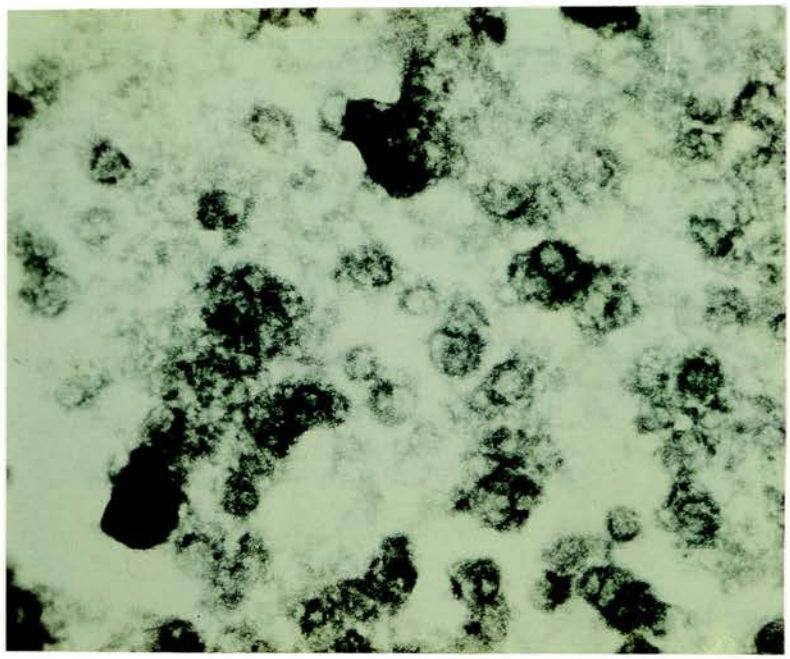
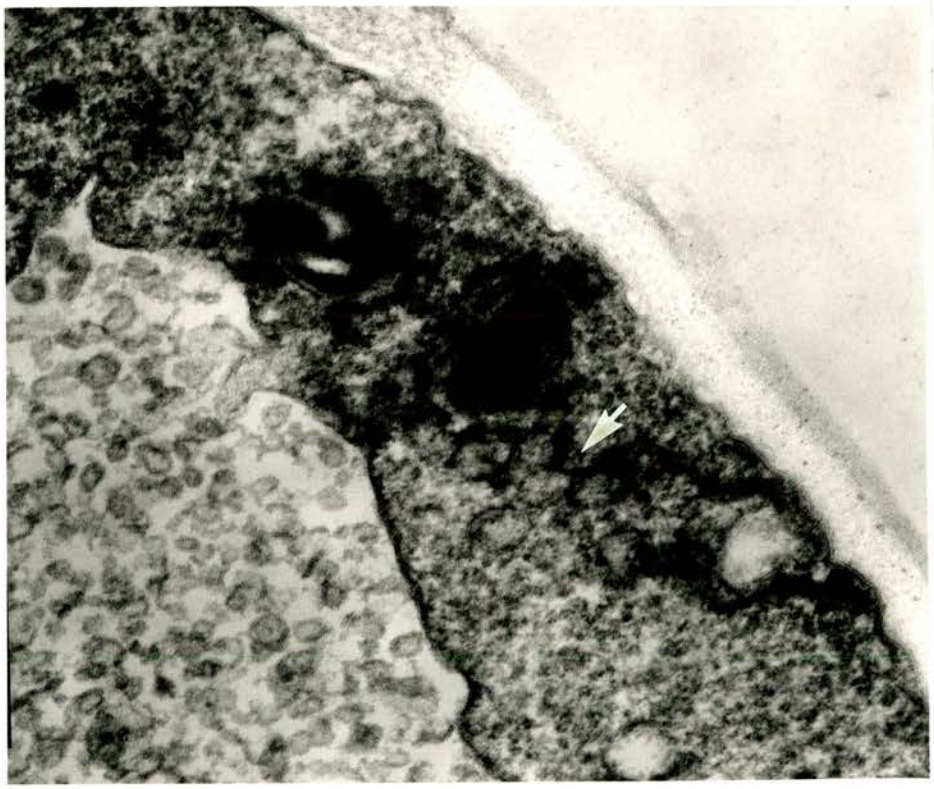


Figure 3.9: Electron micrographs of yeast cells

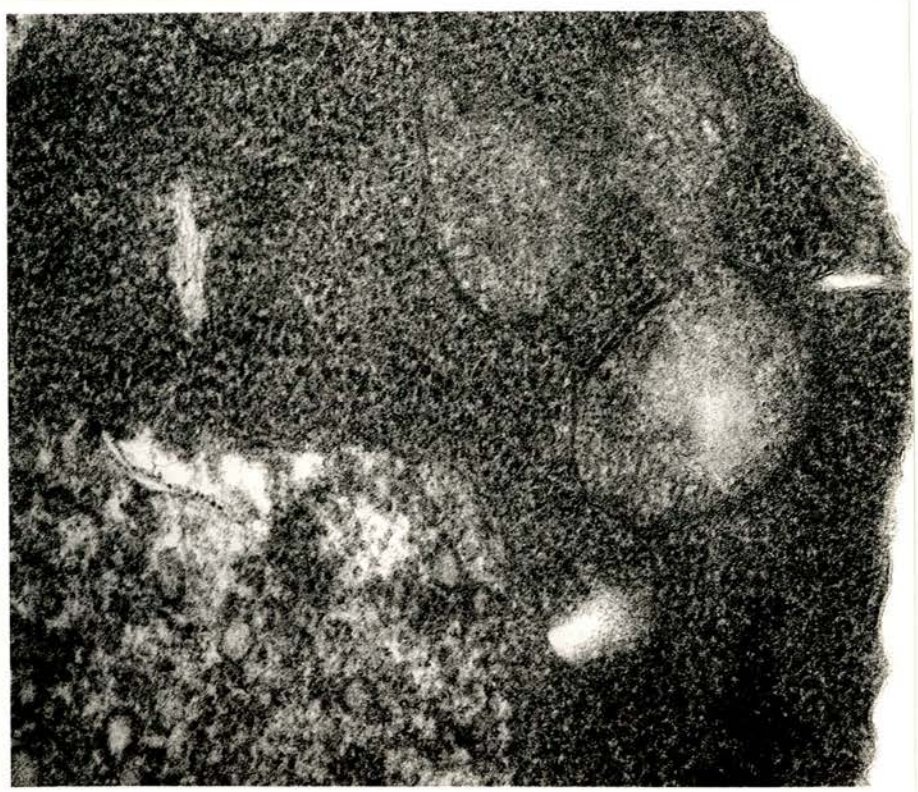
a) BTF.U cell prepared for EM after 22 hours culture in (SC-gal plus trp).
Possible p1:TF VLPs are shown by an arrow.
Magnification: x 60,000

b) Untransformed BJ2168 cell after 22 hours culture in SC-gal (plus trp, leu and ura).
Magnification: x 60,000

a)



b)



each cell. In contrast, the cytoplasm of transformed yeast expressing p1 as a fusion with visna-EV1 gag protein, was packed with VLPs when examined by EM and p1:gag protein could be purified from them at a yield of 2 mg from 1l of cells (Personal communication H. T. Reyburn).

The scarcity of VLPs in induced BTF.U cells may explain why the yields of p1:TF acheived from large scale preparations were so low despite the fact that the cells were expressing p1:TF as detected by analysis of crude lysate. Consideration of Figures 3.8 and 3.9 demonstrates the efficiency of the large scale preparation method to purify the small number of VLPs present.

3.3.9: **Transformation of *S. cerevisiae* strain MC5**

MC5 is a homozygous diploid strain (prb-1, prc-1, pep-4, trp-1, leu-2, ura-3) of *S.cerevisiae*. It was hoped that the larger diploid cells might be able to withstand the apparent toxicity of the recombinant proteins better than strain BJ2168 and therefore improve the yield of protein.

The spheroplast transformation method was used to transform MC5 yeast with pOGS40, pOGS40 and pUGS41 together, pTF.O, pTF.O and pUGS41 together, or with pTH.O. Transformed strains were named MP1.O, MP1.U, MTF.O, MTF.U and MTH.O, respectively. Western blotting of crude lysates (Figure 3.10) confirmed that recombinant proteins were being produced. Coomassie-stained SDS-PAGE gels again indicated the level of production to be low, even though the MC5 transformant cells did look more healthy following induction than their BJ2168 counterparts.

Cell division of the MC5 transformants following galactose induction was monitored by measuring the OD_{600} of culture at intervals following transfer of the cells into SC-gal (Figure 3.11). As the strain cannot utilise galactose, growth would be expected to slow down once the amount of glucose available became limiting. In the strains producing p1 the OD_{600} at least doubled before levelling off after about 24 hours. The OD_{600} of the cultures expressing recombinant gp46 did not even double and levelled off rapidly following induction. The strain potentially producing the most p1:TF, i.e. MTF.U stopped growing most quickly.

From these growth curves, and taking into account the low yields of recombinant proteins produced compared to the yield of p1, it may be concluded that full-length MVV EV1 transmembrane protein (TF) is toxic to yeast cells and that the half-length product (TH) is also toxic but to a slightly lesser extent.

Figure 3.10: Analysis of cell extracts from transformed MC5 strains

a) Coomassie stained SDS-PAGE gel

4μl samples of crude cell lysate from MC5 transformants grown in SC-glc (uninduced) or in SC-gal (induced) were analysed by SDS-PAGE.

b) Western blot

A western blot of samples of crude extracts, as above, was developed with 1:2000 dilution of rabbit anti-p1 serum and and 1:2000 dilution of goat anti-rabbit alkaline phosphatase conjugate.

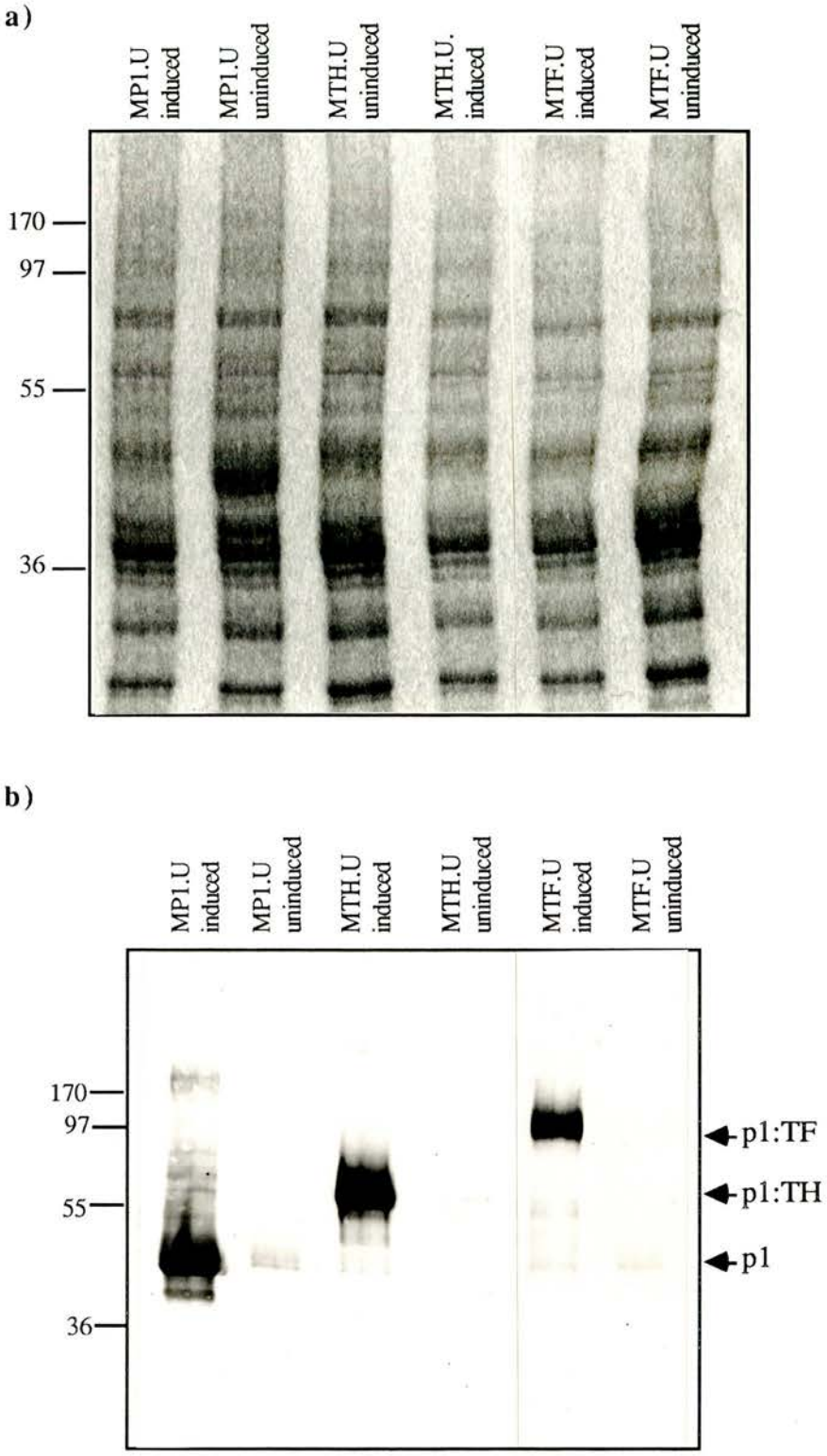
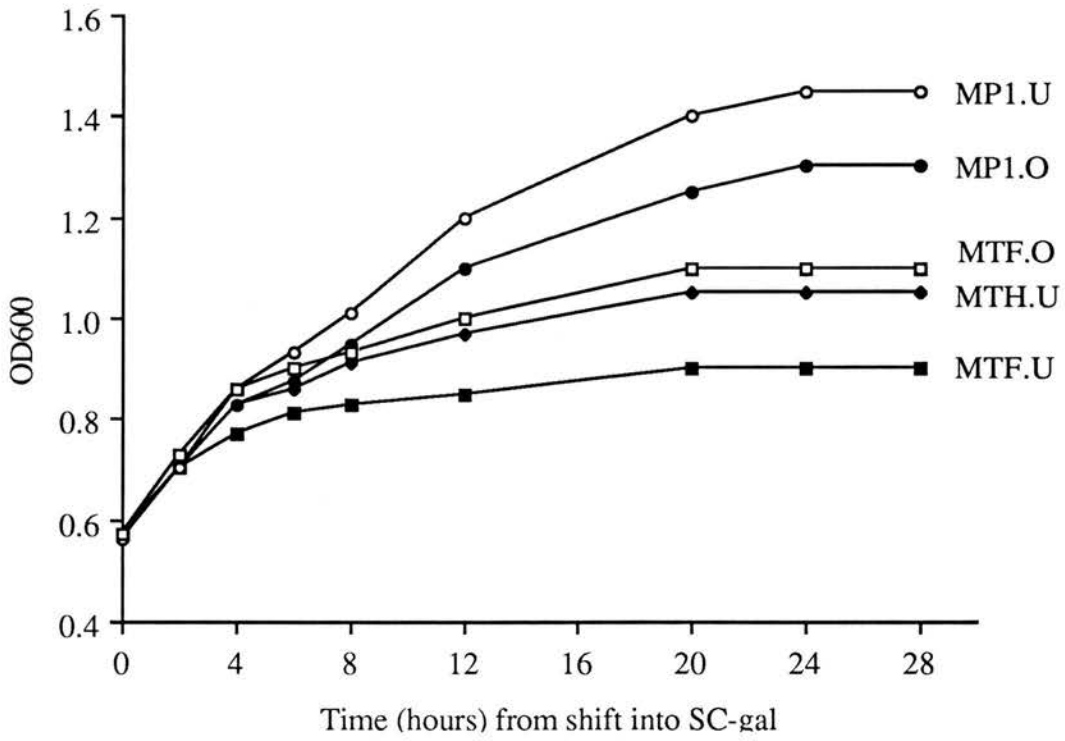

a)
MP1.U induced
MP1.U uninduced
MTH.U uninduced
MTH.U. induced
MTF.U induced
MTF.U uninduced
170
97
55
36
b)
MP1.U induced
MP1.U uninduced
MTH.U induced
MTH.U uninduced
MTF.U induced
MTF.U uninduced
170
97
55
36
p1:TF
p1:TH
p1

Figure 3.11: Growth curves of MC5 transformants in galactose containing medium.

Log phase cultures of transformed MC5 yeast strains in SC-glc medium were used to inoculate SC-gal medium to give an initial concentration of approximately 1.7 x 10^7 cells /ml. The growth of each strain was followed by measuring the OD_{600} at intervals over the 28 hours following transfer to SC-gal medium.



3.3.10: **Factor Xa digestion of p1:TF**

Initial experiments suggested many sheep sera tested on western blots reacted with p1 and with p1:TF therefore it was desirable to separate the TF component from p1 in order to screen sera from MVV-infected sheep against recombinant gp46. (However, in retrospect, the amounts of antigen used in these blots was too high and may have resulted in false positives).

p1:TF was incubated with the protease Factor Xa at various ratios of enzyme to substrate for various incubation times. Western blots of digested p1:TF developed with anti-p1 sera (Figure 3.12) showed a size shift back to that of p1, indicating that p1:TF was being cleaved. The amount of p1:TF detected decreased whilst p1 increased with higher concentrations of Factor Xa and longer incubation times.

Cleaved TF was expected to be approximately 38kDa in size. Unfortunately, it was not possible to identify a band corresponding to cleaved TF on a Coomassie or silver stained gel due to the other contaminants in the p1:TF preparation. No antibody against MVV gp46 was available to identify TF by Western blot. However, two out of six sera from MVV-infected sheep tested did react with a protein band of approximately the predicted size for cleaved TF (Figure 3.13); sera ShM7 and ShM19 both reacted with a band running just ahead of the 36kDa marker in the track of Factor Xa-digested p1:TF but not in any other track. Normal sheep sera did not react with this band. The seropositivity of the six MVV-infected sheep was confirmed by their reactivity with various EV1 proteins especially bands indicative of p25 and gp160.

Unfortunately the gel shown in Figure 3.13 had run too far to illustrate that the polyclonal rabbit anti-p1:p25 serum reacts with MVV-EV1 p25. The serum also reacts strongly with p1. However only a faint band representing p1 is visible in the p1 and Factor Xa digested p1:TF tracks and no p1:TF band is visible in either the digested or undigested track. This shows that the amount of antigen used for these blots was low. This adds credence to the specificity of the putative TF band which reacted with S30 and S31.

Ideally TF protein would have been purified, following digestion with Factor Xa, by spinning down the VLPs leaving cleaved off recombinant protein in solution. However, with the extremely low amount of TF present, even before digestion, it was not possible to recover acceptable amounts.

Figure 3.12: Factor Xa protease digestion of p1:TF

p1:TF was incubated with four different concentrations of Factor Xa. Samples were taken after 1, 2, 4, 8, and 21 hours and analysed by SDS-PAGE (10% acrylamide) and western blotting. Mock-digested p1 and p1:TF were included as controls.

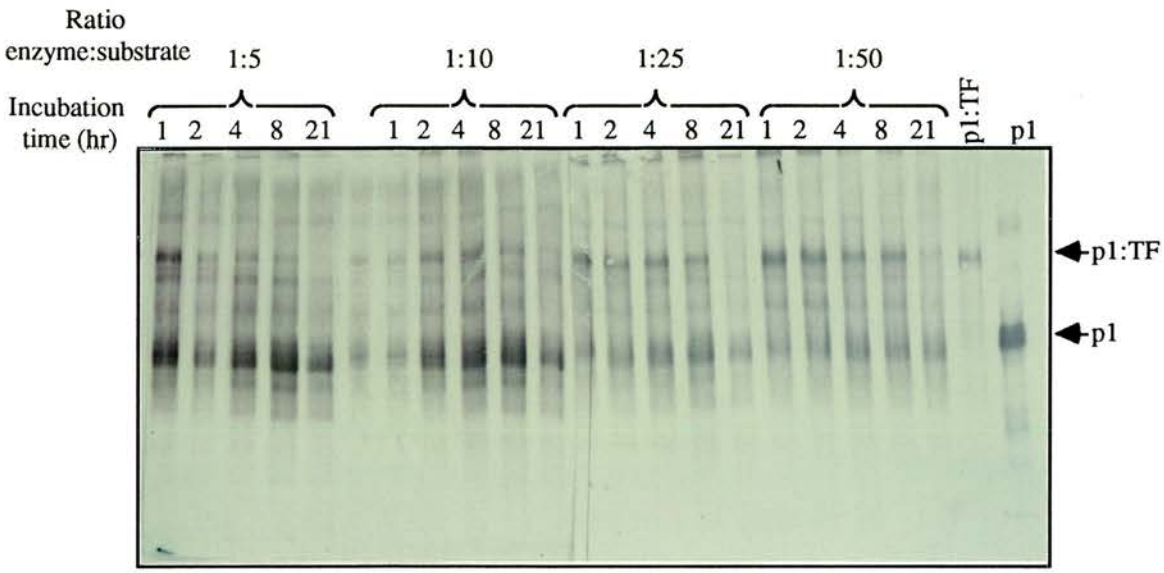
a) Western blot

The blot was developed with 1:200 rabbit anti-p1 serum and 1:1000 goat anti-rabbit -AP conjugate.

b) Silver stained SDS-PAGE gel

The p1:TF digests shown here were incubated with Factor Xa at a ratio of approximately 1:25 Factor Xa:total protein. About 1.5μg of total protein was loaded per track.

a)


Ratio
enzyme:substrate
1:5
1:10
1:25
1:50
Incubation
time (hr)
1 2 4 8 21
1 2 4 8 21
1 2 4 8 21
1 2 4 8 21
p1:TF
p1
p1:TF
p1

b)

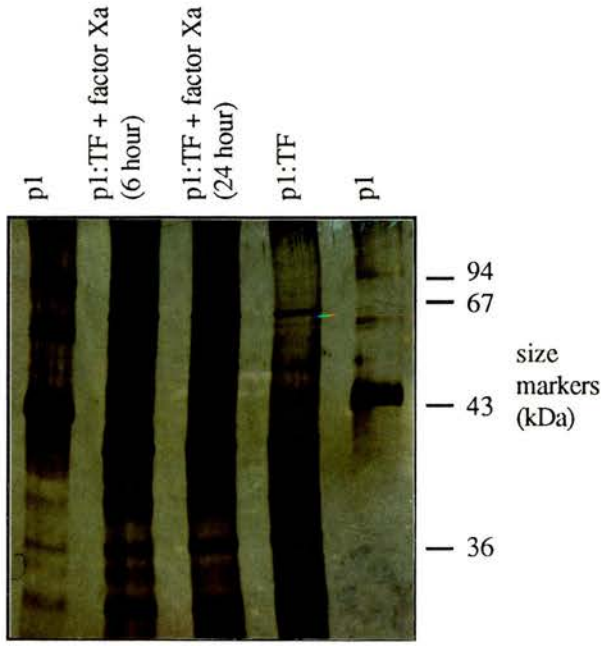
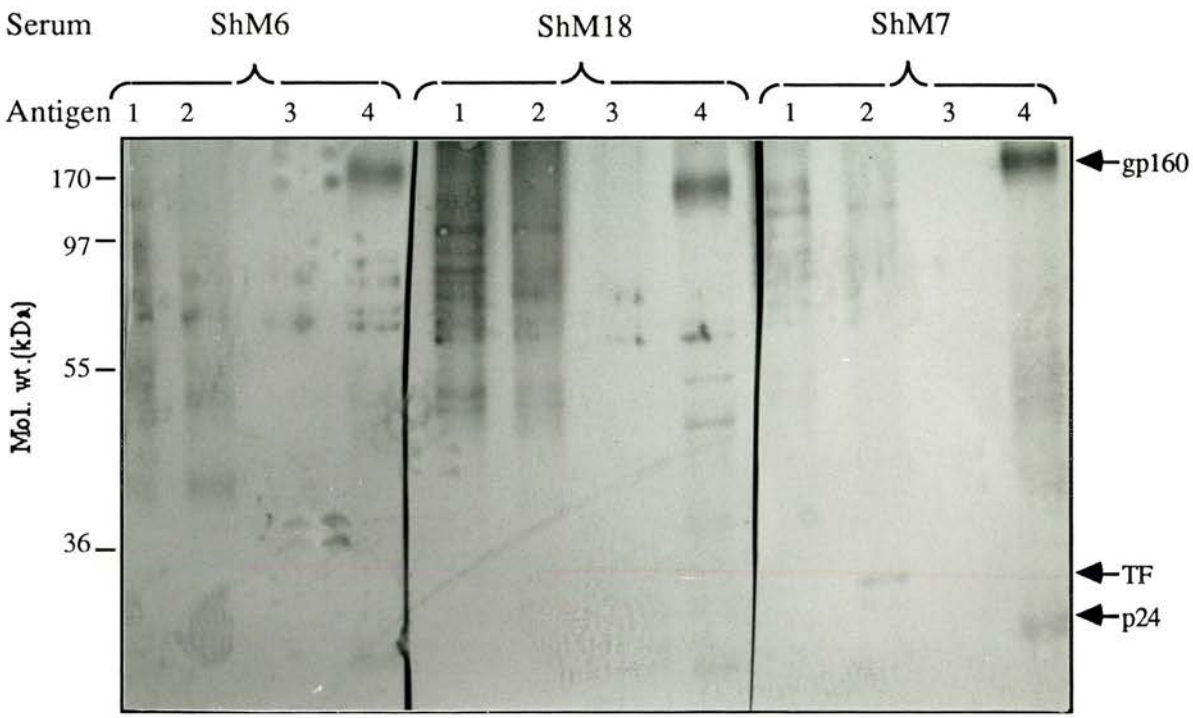

p1
p1:TF + factor Xa
(6 hour)
p1:TF + factor Xa
(24 hour)
p1:TF
p1
94
67
43
36
size
markers
(kDa)

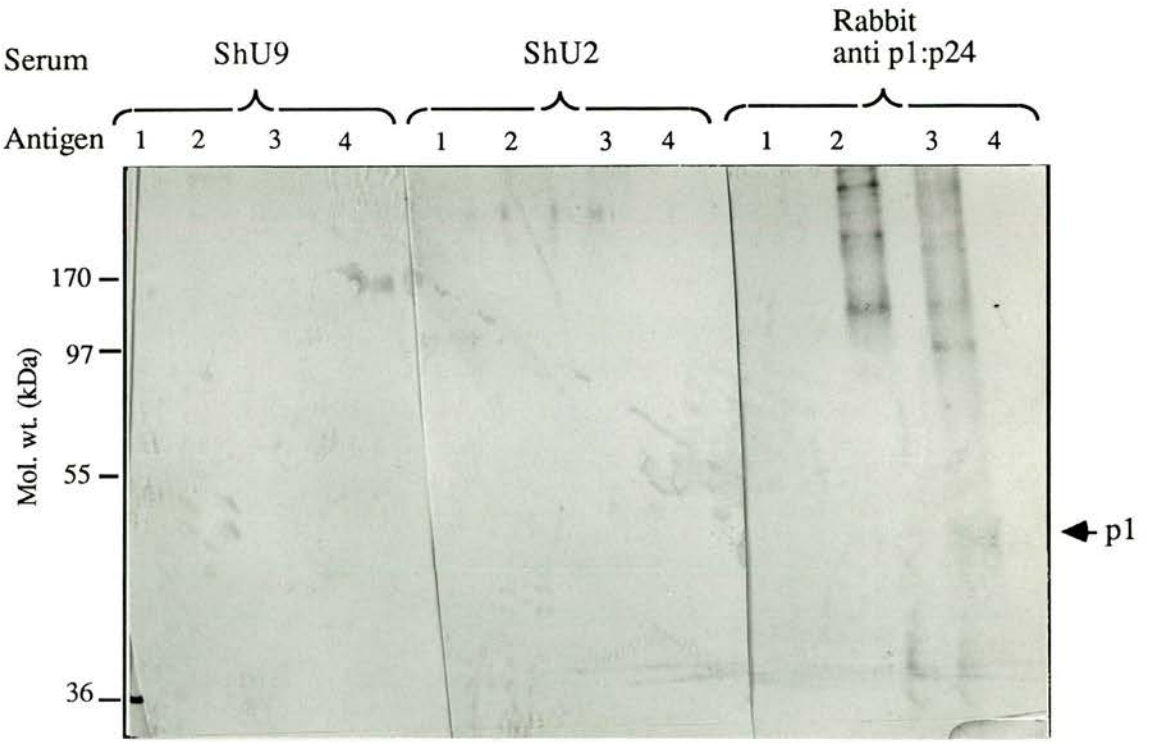
Figure 3.13: Western blots of p1, p1:TF and EV1 developed with sheep sera.

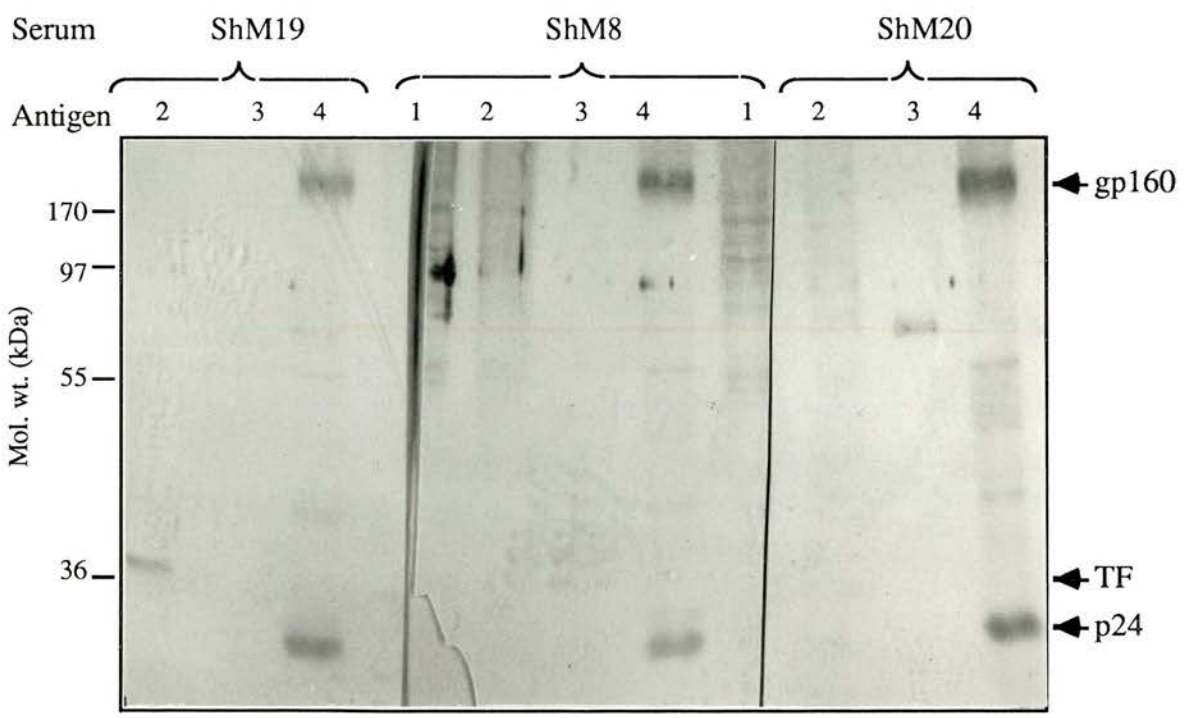
Antigen: 1: p1:TF
2: p1:TF after Factor Xa digestion
3: p1
4: EV1 antigen prepared from infected cells *in vitro*.

Western blots of the antigens listed above were developed with 1:100 dilution of sheep serum and 1:2000 anti-sheep-AP conjugate, or 1:500 rabbit serum followed by 1:2000 anti-rabbit-AP conjugate.

Sheep ShM were infected with MVV. Normal sheep ShU were uninfected controls. The rabbit had been immunised with recombinant p1:p24 (MVV gag) protein.




Serum
ShU9
ShU2
Rabbit
anti p1:p24
Antigen
1 2 3 4
1 2 3 4
1 2 3 4
170
97
55
36
Mol. wt. (kDa)
p1


Serum
ShM19
ShM8
ShM20
Antigen
2 3 4
1 2 3 4 1
2 3 4
170
97
55
36
Mol. wt. (kDa)
gp160
TF
p24

3.3.11: **Immunisation of rabbits with p1 or p1:TF**

Rabbits R194 and R193 were immunised with p1 and p1:TF, respectively. The preparations used for immunisation were from large scale preparations, as shown in Figure 3.7c. Serum collected before and after immunisation was tested against p1 and p1:TF on Western blots (Figure 3.14) and by ELISA (Figure 3.15).

R194 sera reacted with p1 on Western blots, but R193 had little reactivity against p1 or p1:TF (Figure 3.14 and Figure 3.15 where a greater amount of antigen was used). Pre-immune sera had low levels of activity against a number of yeast proteins. Surprisingly pre-immune R193 serum reacted against a band similar in size to p1:TF in the BTF.U crude extract track but no similar reactivity was seen against BJ2168 crude extract or against the p1:TF preparation. Immunisation increased the response to several proteins including p1/p1:TF. However it was not possible to detect any activity against the TF component of Factor Xa digested P1:TF.

To try to enhance the detectable anti-TF activity it was attempted to remove as much as possible of the anti-p1 component of the R193 serum by binding it to a column of p1 cross-linked to Sepharose. The p1 affinity-purified antibody peak eluent contained increased activity against p1 as expected; bands representing p1 and p1:TF became more prominent on Western blots (Figure 3.15). The size shift of Factor Xa digested p1:TF on this blot showed that p1:TF had been cleaved but that the amount of antigen present was low. The column flow-through reacted with a number of bands of smaller size than p1 in the Factor Xa digested p1:TF track. Although it is not certain which, if any, of these bands may represent TF protein a likely candidate is the protein running just below the 36kDa marker which may be the same band as was recognised by sera from MVV-infected sheep, ShM7 and ShM19 (Figure 3.13).

Figure 3.14: Western blots of p1:TF or p1 developed with serum from rabbits immunised with p1:TF or p1

Antigen :

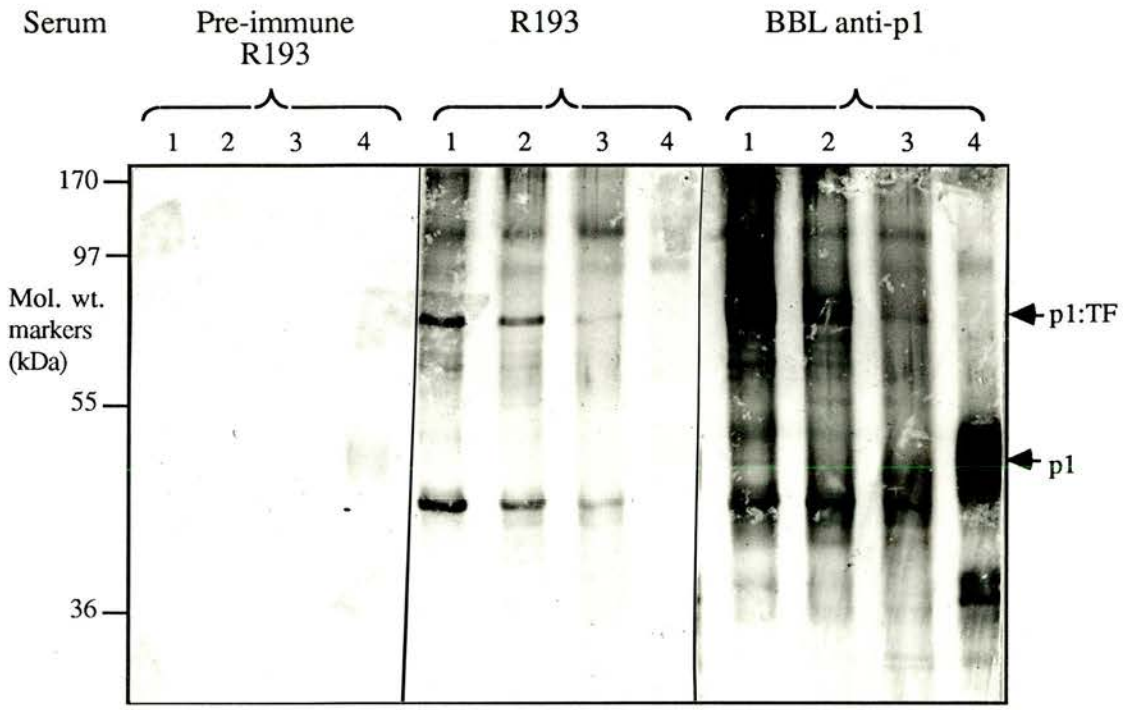
1: p1:TF

2: p1:TF incubated with Factor Xa for 2 hours.

3: p1:TF incubated with Factor Xa for 22 hours.

4: p1

Rabbit R193 was immunised with p1:TF. R194 was immunised with p1. BBL anti-p1 rabbit serum was a gift from British Biotechnology Limited. Western blots of the antigens listed above were developed with serum or pre-immune serum. 1:200 dilution of serum and 1:2000 dilution of goat anti-rabbit AP was used.


Serum
Pre-immune R193
R193
BBL anti-p1
1 2 3 4 1 2 3 4 1 2 3 4
170
97
Mol. wt. markers (kDa)
55
36
p1:TF
p1

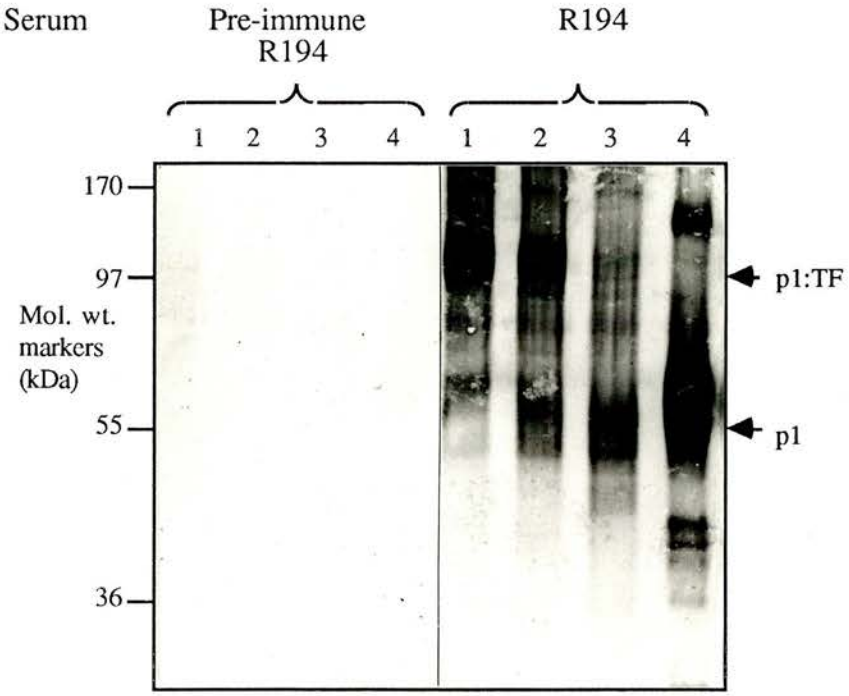

Serum
Pre-immune R194
R194
1 2 3 4 1 2 3 4
170
97
Mol. wt. markers (kDa)
55
36
p1:TF
p1

Figure 3.15: Western blots: p1:TF antiserum (R193) against p1:TF

Antigen :

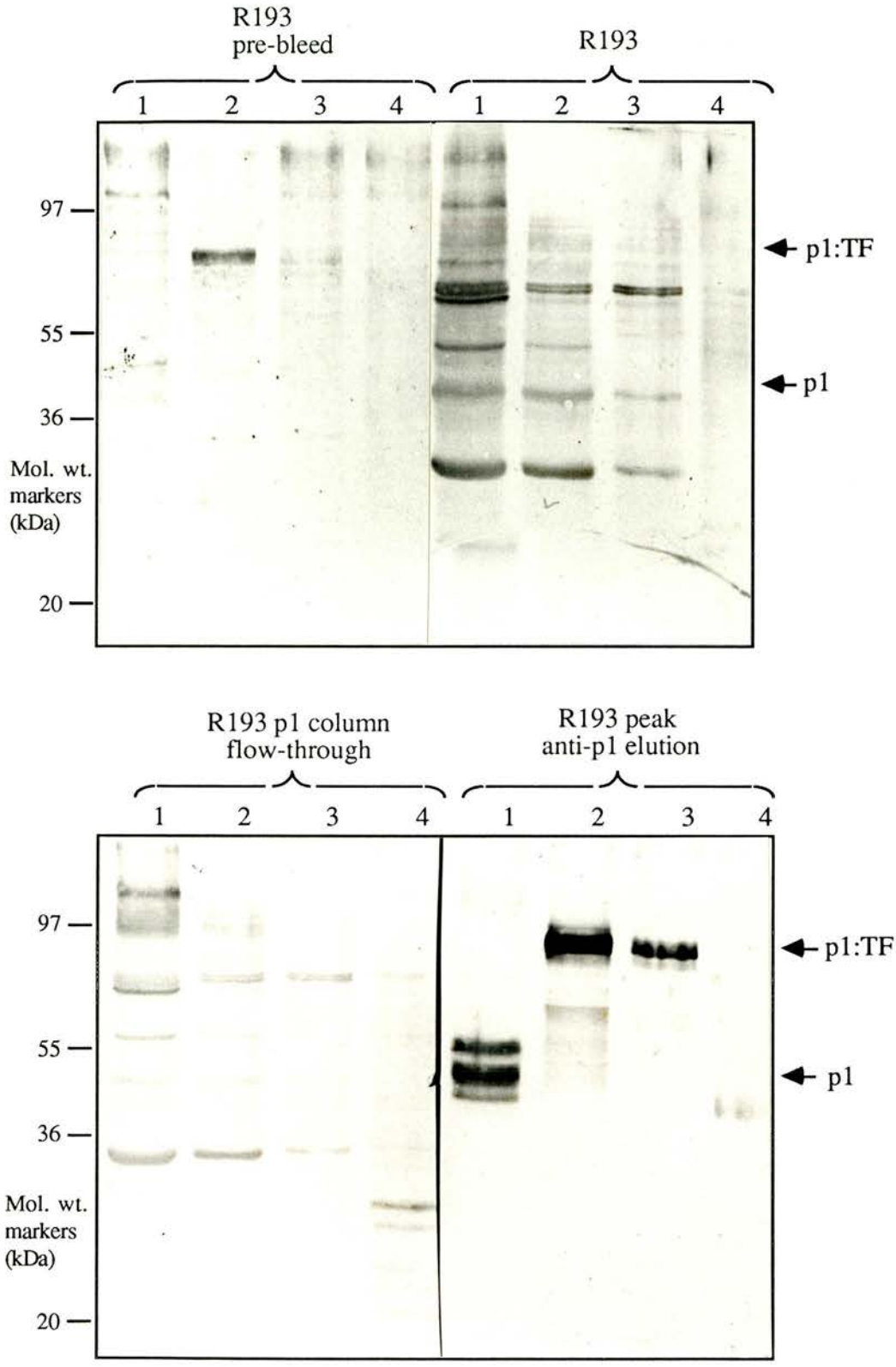
1: BJ2168 (untransformed) crude extract

2: BTF.U crude extract

3: p1:TF

4: Factor Xa treated p1:TF

Rabbit R193 was immunised with p1:TF. R193 serum was passed through a p1:Sepharose column to try to separate anti-p1 activity from other activities in the serum. Western blots of the antigens listed above were developed with flow-through or peak antibody eluent from the p1 column or with R193 whole serum or pre-immune serum. 1:500 dilution of serum and 1:2000 dilution of goat anti-rabbit AP was used.


R193
pre-bleed
R193
1
2
3
4
1
2
3
4
97
55
36
Mol. wt.
markers
(kDa)
20
p1:TF
p1
R193 p1 column
flow-through
R193 peak
anti-p1 elution
1
2
3
4
1
2
3
4
97
55
36
Mol. wt.
markers
(kDa)
20
p1:TF
p1

3.3.12: **ELISA of serum from p1:TF immunised rabbit**

Pre-immune serum from R193 was negative in ELISAs against p1 or p1:TF (Figure 3.15). After immunisation with p1:TF R193 serum showed positive reactivity in the ELISAs. (A negative result was taken to be near background and a positive ELISA was taken to be a reading of greater than twice background).

The results of R193 serum, of p1 column flow-through and of the antibody peak eluted from the p1-column in ELISA, with p1 as antigen, were compared. R193 serum had a titre of 1:400-1:800 against p1 and flow-through had a titre of 1:200. There was no dilution factor to take into account, therefore 50-75% of the anti-p1 activity had bound to the column, whilst 25-50% remained unbound and was present in the column flow-through. The peak was eluted in 1ml and the anti-p1 titre was 1:800 (but some dilution had occurred as antibody was also present in non-peak aliquots). The anti-p1 activity coming out of the column was thus greater than that going in. This suggests the column was incompletely eluted during its previous use and so some anti-p1 activity was carried forward to this elution. As the same p1 preparation was used for the p1-Sepharose column and as antigen in the ELISA the effect of the presence of small amounts of contaminating proteins should cancel out and not alter the conclusions above.

The results of ELISA against p1:TF showed that passing the serum through the p1 column reduced the anti-p1:TF titre by a factor of about two. Even taking into account that 25% or more of the anti-p1 activity remained in the flow-through it does appear that there was reactivity to proteins/epitopes in the p1:TF preparation which were not present in p1:preparation. This is backed up by the difference between the results of the p1 and p1:TF ELISAs; the titre of R193 serum was about 16 times higher against p1:TF than against p1 and the titre of the flow-through was also much higher against p1:TF than against p1. It was not possible to determine from these results whether the activity was directed against TF or against contaminating yeast proteins, particularly as the amount of p1:TF present in the ELISA was shown to be lower than the amount of p1 used for ELISA: the anti-p1 peak titred out at 1:200-1:400 against p1:TF compared to 1:800 against p1. As the amount of total protein used was similar the result confirms what was already clear i.e. that the amount of contaminating proteins in the p1:TF preparation was considerably higher than in the p1 preparation.

In summary, immunisation with p1:TF had induced detectable antibodies to p1 protein, and also to components found in the p1:TF preparation but not in the p1 preparation, although specific anti-TF activity could not be determined due to the difficulties in preparation of TF.

Figure 3.16: Results of ELISA of R193 serum against p1 or p1:TF antigen

Twofold dilutions of serum were made starting from 1:50 and added to ELISA plates coated with 10μg/ml p1 or p1:TF. 1:4000 dilution of anti-rabbit horse radish peroxidase was used as second antibody. After development with OPD results were read by measuring the OD_{492} of each well.

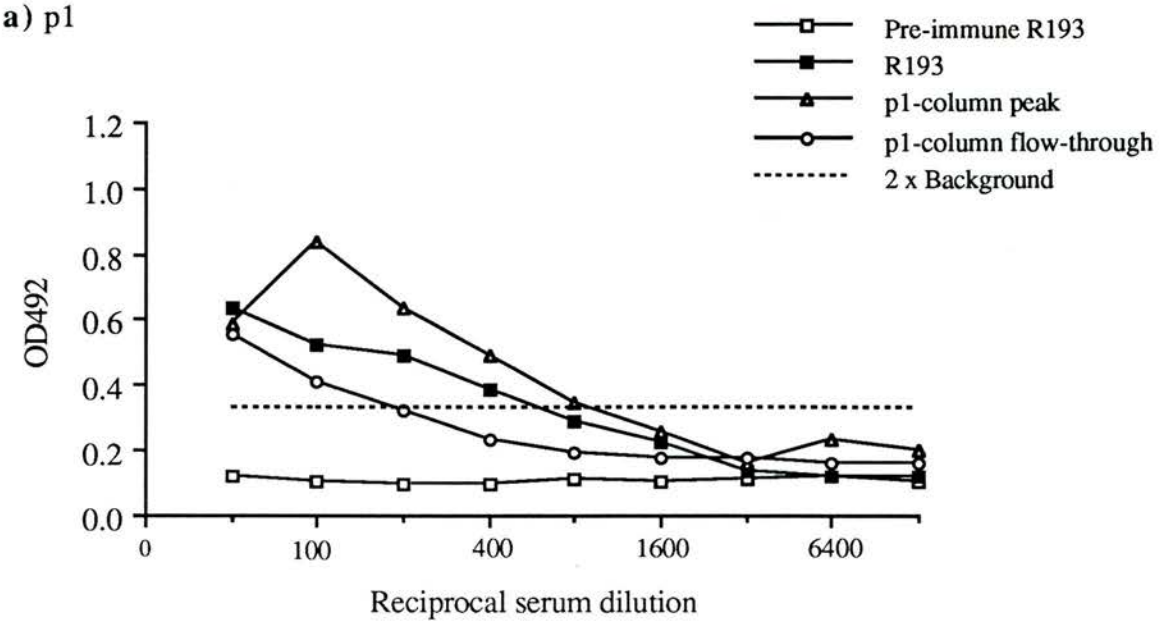
The results of duplicate wells were averaged and are shown in the graphs opposite. The results from 8 negative control wells containing buffer but no antibody were averaged then doubled to give a figure for 2xbackground

The serum used was pre-immune serum from R193, serum collected following immunisation of R193 with p1:TF and derivatives of the latter serum depleted of anti-p1 activity after passing through a p1-Sepharose column (flow-through), or enriched for anti-p1 activity after binding to and elution from the p1 Sepharose column (column peak).

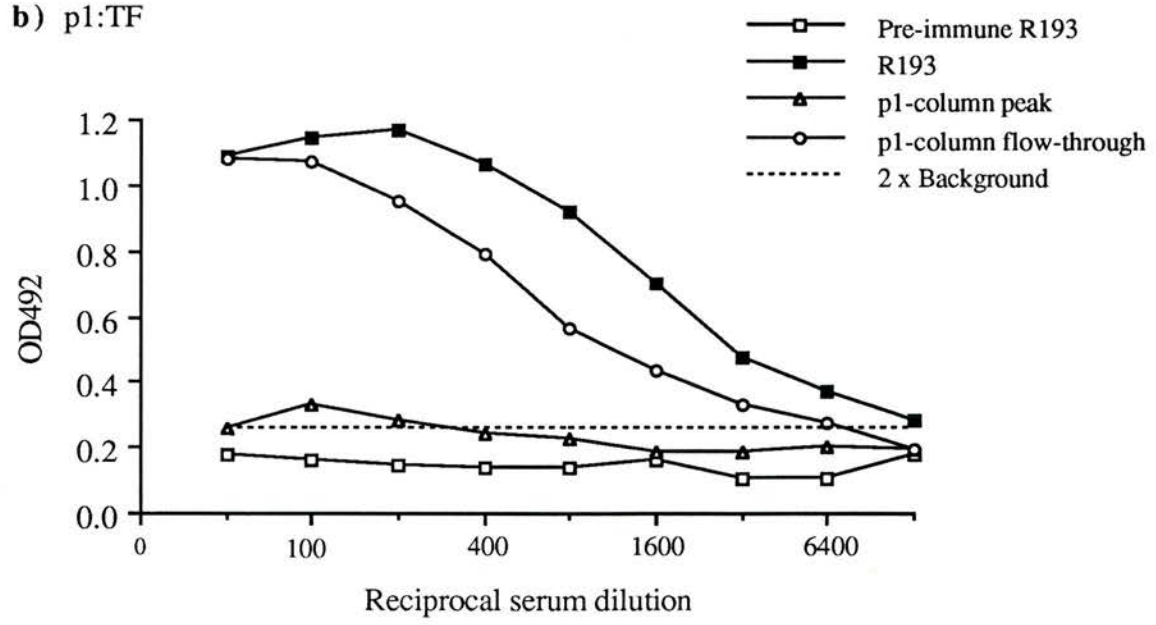
a) Results of ELISA against p1

b) Results of ELISA against p1:TF

a) p1


Pre-immune R193
R193
p1-column peak
p1-column flow-through
2 x Background
OD492
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
100
400
1600
6400
Reciprocal serum dilution

b) p1:TF


Pre-immune R193
R193
p1-column peak
p1-column flow-through
2 x Background
OD492
1.2
1.0
0.8
0.6
0.4
0.2
0.0
0
100
400
1600
6400
Reciprocal serum dilution

Chapter 3:

DISCUSSION

3.4.1: **Production of recombinant gp46 polypeptides**

These studies showed that the recombinant full-length and half-length gp46 fusion proteins with p1, i.e. p1:TF and p1:TH, respectively, were expressed in transformed yeast but that their expression was toxic to the yeast. They also showed the necessity of using an inducible promoter for expression of toxic proteins as no transformants expressing p1:TF from the constitutive promoter could be found.

The toxicity of the expressed proteins to the host cells is particularly clear from the growth curve which shows an almost immediate cessation of cell division upon induction of p1:TF at a time before much recombinant protein is detectable, e.g. p1:TF is barely detectable on western blots of crude lysates of BTF.O cells 6 hours after induction (Figure 3.6), yet cell division has almost stopped at this time in MTF.O (Figure 3.11). Unfortunately the two figures concern different yeast strains, nevertheless the data suggests that expression of even small amounts of recombinant protein has a profound effect on the cell metabolism. A similar though slightly reduced effect upon growth was seen following induction of p1:TH expression. Since expression of p1, which is produced at a much higher yield than either p1:TH or p1:TF does not have as rapid an effect on cell division, diversion of host cell enzymes and energy reserves would not account for the apparent toxicity.

The toxicity of the recombinant proteins caused great difficulty in their purification. The yield of recombinant protein was low and there was a relatively high level of contamination with other yeast proteins. The large scale purification method is dependent upon the formation of VLPs. It may be that the hydrophobicity of the TF portion makes it bind other cell proteins reducing the ability of the p1 portions to interact with other p1 molecules and to form VLPs. This may also be the mechanism by which the protein is toxic to the cell. Nevertheless given the EM evidence that very few VLPs were present in the cells the fact that even a small amount of recombinant protein was recoverable shows that the purification method must be very efficient.

If the ability to form VLPs was the problem rather than the total yield of p1:recombinant protein it should be possible to purify it using an anti-p1 affinity column rather than the method reliant on VLP purification. An anti-p1 affinity column may not be so efficient for purification of recombinant VLPs as the non-p1 moiety is thought to lie to the outside of the VLP whilst the p1 subunits interact with each other on the inside of the VLP.

p1:TH preparations also yielded very small amounts of recombinant protein suggesting that the amino-terminal hydrophobic region, in the absence of the transmembrane hydrophobic region is sufficient to cause this problem.

An alternative reason for a low yield of recombinant fusion protein compared to the fusion partner alone is sometimes due to more rapid turnover of mRNA encoding the fusion protein (Hoekema *et al.*, 1987). There may be some influence of codon usage on the rate of turnover of mRNA.

Asenjo *et al.*, (1993), report an alternative purification method for yeast proteins using purified glucanase for cell lysis instead of mechanical lysis. This appears to reduce degradation of VLPs. Although they report that this method also gives selective product with less contaminants they present no evidence to support this as only Western blots probed with anti-p1 antibody, but not Coomassie or Silver stained PAGE gels, were published.

The yields of heterologous proteins can be markedly influenced by the host strain (Kingsman *et al.*, 1990). The best strain varies for different proteins. The reason for using BJ2168 was that this is the strain used routinely at British Biotechnology Ltd. (BBL) for production of hybrid VLPs. This strain has the advantage of being protease deficient and is therefore less likely to degrade the recombinant protein. However, BJ2168 gave a very poor yield of recombinant protein so strain MC5 was tried, in the hope that this diploid strain might be better able to withstand the toxicity of the expressed proteins. Unfortunately the yield from MC5 transformants was not significantly better than for BJ 2168 transformants.

Factor Xa protease clearly cleaved the recombinant protein as shown by the shift from p1:TF to p1 on Western blots probed with anti-p1 antiserum. The lack of any defined anti-gp46 antiserum and the presence of contaminating proteins in the preps made it impossible to be certain which band on SDS-PAGE gels or western blots corresponded to TF. Nevertheless the reactivity of sera from some MVV-infected sheep, and not from uninfected sheep, indicated that the recombinant gp46, TF, was a band running just ahead of the 36 kDa marker on SDS-PAGE.

3.4.2: **Antibody responses to recombinant p1:TF**

Although it has been reported that a range of hybrid VLPs have been used to elicit antibodies against the non-Ty components in rabbits (Adams *et al.*, 1987) we have found that the Ty component is often immunodominant. It is possible that the rabbits used have previously encountered yeast infections and therefore when immunised with Ty-VLPs mount a secondary response to the p1 component but only a primary response to the recombinant component. Alternatively recombinant gp46 may be poorly immunogenic. Serum from MVV-infected sheep does not generally react with gp46 on Western blots of MVV infected cell lysate. This deficiency is often considered to be due to the presence of only very low amounts of the membrane protein in the MVV infected cell lysate preparation whereas it may actually be due to the failure to mount an antibody response to gp46.

A problem with analysis of antibody response by Western blots is not only that the antigen is denatured but also that the results are critical upon the amounts of antigen and antibody used. Testing of a series of antibody dilutions against a fixed amount of antigen may have better determined the reactivity of the sera.

The rabbit immunised with p1:TF (R193) raised antibodies to p1 as well as to components present in the p1:TF preparation but not in the p1 preparation. Because of the contaminant proteins in the p1:TF preparation and the low yield of TF produced by cleavage of p1:TF it was not possible to determine whether antibodies to TF itself had been raised. Western blots showed that pre-immune sera reacted with some of the contaminating proteins (Figure 3.15 shows this more clearly than Figure 3.14 probably due to the amount of antigen on the blot). The activity against the contaminants was significantly increased following immunisation of R194 with p1 which highlights the fact that the p1 preparation also includes contaminant proteins. Thus the p1-Sepharose column was only partly able to separate out antibodies against p1 from the rest. Nevertheless "anti-p1" antibodies eluted from the column should have been used to immunopurify p1:TF which might have resulted in a much cleaner preparation for use as an immunogen and as antigen for ELISA.

CHAPTER 4: Expression of recombinant EV1 *gp46* in a bacterial expression system and by recombinant vaccinia virus

INTRODUCTION

4.1.1: The pGEX bacterial expression system

pGEX2T (Smith and Corcoran, 1990) is a bacterial expression vector that encodes the glutathione-S-transferase (GST) from *Schistosoma japonicum* which can be expressed as a fusion protein with the product of a gene inserted into the multiple cloning site. pGEX2T was chosen as the vector to express gp46 as GST fusion proteins are generally soluble and easily purifiable: mg quantities of GST fusion proteins have been purified from 1l cultures (Smith and Johnson, 1988). As a fusion partner for expression of recombinant protein GST has been reported to be more successful than LacZ or MalE in producing soluble protein. The pGEX system has been used successfully to produce recombinant protein for a wide range of studies including immunological studies (Toye *et al.*, 1990) and vaccine production (Fikrig *et al.*, 1990).

The use of pGEX2T to express MVV gp46 was hoped to improve upon the production of recombinant gp46 in the Ty system, in terms of purity and yield.

The GST moiety can be exploited as an affinity tag enabling purification of fusion protein from crude bacterial lysate under non-denaturing conditions by affinity chromotography on immobilised glutathione (glutathione-agarose beads). Bound proteins can be eluted from the matrix by competition with free glutathione under mild non-denaturing conditions. This helps preserve the antigenicity and functional activity of the recombinant protein.

pGEX2T has an advantage over earlier pGEX1 vectors in that it includes a thrombin cleavage site between the GST gene and the multiple cloning site to enable the recombinant protein to be cleaved from the GST component. After thrombin digestion, it should be possible to remove the GST carrier and uncleaved product by binding it to immobilised glutathione whilst leaving recombinant protein in solution.

As expression is IPTG-inducible the pGEX system enables production of proteins which are toxic to the bacteria. The *tac* promoter (P*tac*) upstream of the GST gene controls expression. P*tac* is a hybrid derived from the *trp* and *lac* promoters (Amann *et al.*, 1983, De Boer *et al.*, 1983). The *lac* repressor (*lacI* product) binds to P*tac* repressing expression of the GST fusion protein. When IPTG is added the *lacIq* gene encoded within pGEX2T is switched off and the GST fusion protein is derepressed.

4.1.2: **Expression of rgp46 by recombinant vaccinia virus**

Vaccinia expression constructs were made in order to generate recombinant vaccinia virus expressing gp46. The aim was to use the recombinant virus to generate antiserum against gp46 and also for use in the analysis of the cell mediated immune response to gp46 by MVV infected sheep.

Recombinant vaccinia virus has several advantages as a eukaryotic expression vector (reviewed by Mackett and Smith, 1986); It infects a wide range of host species at high infectivity, it has a high capacity to incorporate foreign genetic elements (25kb has been inserted without affecting growth or infectivity), there is no requirement for helper virus and most importantly for our purposes it has the ability to elicit humoral and cellular immune responses in experimental animals. Cells infected with recombinant vaccinia virus present foreign antigen in association with MHC and may therefore be used as targets for autologous cytotoxic T-lymphocytes directed against the foreign antigen (Yewdell *et al.*, 1985). Recombinant vaccinia virus is also proposed to be particularly good for raising antisera against glycoproteins as they are expressed in their glycosylated form. A number of virus glycoproteins expressed by recombinant vaccinia viruses have been shown to elicit neutralising antibodies and to protect experimental animals against subsequent challenge with the virus from which the glycoprotein was derived (reviewed by Mackett and Smith, 1986) e.g. recombinant vaccinia viruses containing HTLV-1 *env* inserted into the vaccinia *HA* gene have been shown to induce humoral antibodies to HTLV-1 and are protective against infection with HTLV-I.

Recombinant virus is created by homologous recombination *in vivo* between a plasmid constructed to contain the foreign gene linked to a vaccinia virus promoter and the vaccinia genome. Cells are infected with wt vaccinia virus at low m.o.i. (0.05 p.f.u./cell) and transfected with calcium phosphate precipitated plasmid DNA. At best only 0.1% of total progeny virus from a transfection experiment are recombinants so screening or selection of recombinants is required. Insertion into the TK gene locus allows selection for recombinant (TK-) viruses by plaquing on a TK- cell line in the presence of 5-bromodeoxyuridine (Mackett *et al.*, 1982). To confirm the nature of the TK- recombinants the genomic DNA is analysed by restriction endonuclease digestion and Southern blotting. The virus is routinely plaque purified twice before larger stocks are grown up.

Recombinant vaccinia expressing MVV EV1 gp46 was made in this project with the aim of using it for generating anti-sera against gp46 and for analysis of the cell mediated immune responses of MVV infected sheep to this protein.

4.2.1: **Construction of pGEX-derived Expression Vectors**

Construction of the expression vectors to express gp46 as a fusion with glutathione S-transferase (GST) was helped by the fact that the *BamHI* insert from the vectors which had been constructed for expression in yeast provided a coding fragment in the correct reading frame for pGEX2T, with the added benefit of a second protease specific site, Factor Xa.

Plasmids pEV1TH.O and pEV1TF.O were digested with *BamHI*. Inserts of approximately 0.5Kb or 1Kb, respectively, were gel purified by electrophoresis on a 1% LGT agarose gel, followed by extraction with hot phenol/chloroform or by microcentrifugation through 3MM paper. Insert (either naTH or naTF) was added to 300ng of *BamHI* cut, CIP-treated pGEX2T at approximate molar ratios insert:vector of 3:1, 6:1, 9:1. The mixture was ethanol precipitated, washed twice with 70% ethanol and resuspended in 8µl TE pH8.0. 1µl 10 x ligase buffer and 1µl of ligase were added. The ligation reaction was incubated at 16°C overnight before being used to transform competent *E.coli* JM83. Transformants were selected by plating onto LB/Amp agar incubated overnight at 37°C. Colonies were picked onto duplicate LB/Amp plates and grown up. Lifts were made onto nylon filters (Buluwela *et al.*, 1989) and, after fixing, the filters were probed with ^{32}P labelled gp41INS (Appendix 3).

4.2.2: **Selection of transformants for expression of GST fusion proteins**

Bacteria from blot positive cells were inoculated into 6ml L-broth/10µg/ml Amp and incubated with shaking at 37°C for 5 hours. Filter sterilised IPTG was added to a final concentration of 0.1mM and incubation was continued for an hour. The bacteria were harvested by centrifugation and resuspended in 600µl PBS. 300µl was used to prepare *BamHI* or *PstI* digested plasmid mini-preps (Appendix 2). The remainder was used for small-scale preparation of glutathione-binding protein; The cells were lysed by ultra-sonication for three 10 second pulses with cooling on ice in between. The lysate was microcentrifuged for 5 min and the supernatant was transferred to a tube containing 50µl of a 50% slurry of glutathione-agarose beads (Sigma). After gentle mixing for one hour, on ice, the beads were washed three times as follows; 1ml of ice-cold PBS was added and mixed by vortexing, beads were collected by 5 seconds microcentrifugation, and the supernatant was discarded. The beads were resuspended in 25µl of reduced sample buffer (RSB), boiled for 3 min

then electrophoresed on a 10% acrylamide SDS PAGE gel (Appendix 6).

Stocks of selected transformants were stored at -70°C in 20% glycerol.

4.2.3: **Large scale preparation of GST or GST:fusion proteins**

A loopful of bacteria from a single colony on an LB/Amp plate was inoculated into 100ml of L-broth/10μg/ml Amp and incubated overnight at 37°C with shaking. 1.5ml of overnight culture was used to prepare a *PstI* digested plasmid miniprep. The rest of the overnight culture was added to 1l of L-broth/10μg/ml Amp at 37°C and incubated with shaking for 1.5 hours. 24mg/ml IPTG was added to give a final concentration of 0.1mM IPTG. After a further 3 hours incubation, cells were harvested at 5.5K for 5 min (Beckman JA-2 rotor). Bacteria from 1l of culture were resuspended in 18ml of ice-cold PBS. 9ml of suspension was stored at -20°C. The remainder was transferred to a glass Universal and the cells were lysed by three 1-minute pulses of ultrasonication on ice. 10% Triton X-100 was added to a final concentration of 1% and the lysate was microcentrifuged for 5 min. The supernatant was added to 1ml 50% slurry of glutathione agarose beads in a 15ml tube and mixed for 30 min on ice. The supernatant was added to a second 1ml of glutathione-agarose beads and again mixed for 30 min on ice. Both lots of beads were washed four times with 15ml PBS. The beads were resuspended in 0.5ml PBS and transferred to an Eppendorf tube. Protein was eluted by resuspending the beads in 1ml of 50mM Tris (pH8.0), 5mM reduced glutathione and mixing gently for 5 min at room temperature. The beads were spun down by microcentrifugation and the eluate was collected. Elution was repeated three times. Fractions were analysed by SDS-PAGE.

The total protein concentration was estimated by Biorad protein assay using ovalbumin as a standard.

4.2.4: **Preparation of inclusion body material**

Cells derived from 500ml of IPTG induced culture stored at -20°C were lysed in 8ml PBS/1mM EDTA by ultrasonication. Triton X-100 was added to a final concentration of 1% and the lysate was microcentrifuged for 5 min. The supernatant was used to prepare GST-fusion protein by binding to glutathione agarose beads as described in the previous paragraph. The pellet from the equivalent of 20ml starting culture was used to prepare insoluble inclusion body material. The pellet was resuspended in 300μl of lysis buffer and 700μl of TNE, 12 μg/ml DNaseI, and incubated at 37°C for 45 min with occasional mixing. The material was microcentrifuged for ten min. The pellet was resuspended in 1ml of lysis buffer/0.2mg/ml lysosome for 15 min at room temperature. Insoluble material was

pelleted by microcentrifugation for 10 min, resuspended in lysis buffer plus 0.2% DOC, for 10 min at RT. Insoluble material was pelleted and resuspended in 50mM Tris, 0.05% Triton X-100, 10mM EDTA, 100mM NaCl at room temperature for 10 min. The final pellet was washed three times with water and resuspended in 1ml of water. Samples were analysed by 10% acrylamide SDS-PAGE.

4.2.5: **Growth curves of induced cultures**

Cultures of the recombinant clones selected, LGPG1, LG7H19, LG2H11, LG7F21 and LG7F22, were prepared by inoculating 10 ml L-broth plus 50µg/ml Amp with a single colony from a plate, and incubating overnight at 37°C with shaking. 2ml of each overnight culture was added to two flasks containing 50ml L-broth, 10µg/ml Amp. After 1h incubation at 37°C with shaking, 59µl of 100mM IPTG was added to one flask of each of the clones. The growth of uninduced or IPTG-induced cultures was followed by measuring the OD_{600} at 30 or 60 minute intervals for 6 hours.

4.2.6: **Protease cleavage of GST:TH**

80µl of eluted fusion protein (~4µg of GST or ~3µg GST:TH) in 50 mM Tris-Cl pH8.0, 5mM glutathione was added to 20µl 50mM Tris-Cl. pH7.0, 750mM NaCl, 12.5mM $CaCl_2$.0µl, 1µl or 3µl of thrombin (0.5U/µl, 125ng/µl) was added to each tube. A control tube contained thrombin cleavage buffer and enzyme but no substrate. A second set of tubes contained exactly the same reagents with the addition of 0.05% CHAPS and 0.05% DOC. All ten tubes were incubated at 25°C. 20µl samples were removed immediately after addition of thrombin, after 1 hour, 2 hours, 5 hours and 24hours. An equal volume of RSB was added immediately after sampling and boiled for 3 min. 30µl was analysed by SDS-PAGE/silver-stain.

4.2.7: **Construction of Vaccinia expression vector**

PCR primers were designed to amplify the EV1-gp46 coding region from the pTZ based clone, pCC18.9, and to add enzyme sites in order to give directionality of insert, to add an ATG in frame at the 5' end, and to add a stop codon in frame at the 3' end. PCR reactions were set up using primers 763F (C CCG GGA TCC ATG GGG ATA GGC TTG GTG ATA GTG CTC GC) and 761F (C CCG GGT ACC TCA ATT CTC AAT CAT GGG CAT CTG TTC) and ~100pg of pCC18.9 (pTZ18R plus EV1*gp46* insert, described in Chapter 2) as template. PCR conditions were 30 cycles of melting at 95°C for 0.6 min, annealing at 45°C for 1 minute and extension at 70°C

for 5 min, with a final extension at 70°C for 5 min.

The PCR product was digested with *BamHI* and *KpnI*, and gel purified by electrophoresis on a 1% agarose gel. The DNA was recovered and ligated into *BamHI/KpnI* cut, CIP-treated pRK194B. Ligation reactions were set up at molar ratios of insert:vector of 3:1 or 10:1. The ligation product was used to transform *E.coli* JM101 cells. Plasmid mini-preps were prepared from transformants selected for ampicillin resistance. Large scale plasmid preps were made (Appendix 2) of plasmids pV769 and pV752, and pRK194B and were digested with a number of restriction enzymes in order to map the plasmids. Sequence from plasmids pV769 and pV752 was obtained by double stranded sequencing (Appendix 4) using primer 923G, (5' - ATT GGA TAT TAA AAT CAC GC - 3'), which is complementary to -48 to -29 with respect to ATG of the 4b promoter.

4.2.8: **Analysis of recombinant vaccinia viruses**

pV769 was used to generate recombinant vaccinia by the method of Smith, Mackett and Moss (1982). Cells infected with recombinant vaccinia expressing EV1 gp46, as judged by Southern and Northern blots (Dr B.A. Blacklaws and Dr D.R.Sargan, respectively) were harvested with glass beads, resuspended in DME and stored at -20°C. Recombinants were also made with plasmid constructs to express the herpes gD protein and MVV EVI p25 protein. African green monkey fibroblasts, CV-1, were used for the transfections. Recombinant vaccinia, designated Vgp46, VgD or Vp25, respectively, were grown up in TK-143 cells (TK- human fibroblasts).

Mice were inoculated with cells infected with recombinant vaccinia diluted in PBS.

1st inoculation:	5 mice inoculated I/P with 10^6 p.f.u. of VgD
	4 mice inoculated I/P with 10^6 p.f.u. of Vp24
	3 mice inoculated I/P with $5x10^5$ p.f.u. of Vgp46
2nd inoculation	same dose of virus I/P 24 days later
1st bleed	12 days later. Samples were collected into potassium EDTA and the plasma from each group was pooled and stored at -20°C
3rd inoculation	same dose I/P 92 days after last inoculation
2nd bleed	14 days later. Individual samples were collected and stored at -20°C.

Western blots of MVV EV1-infected-cell lysate and of recombinant gp46 TH:GST fusion protein were developed with sera from the inoculated mice.

RESULTS

4.3.1: **Construction of pGEX expression vectors**

Plasmids vectors for the expression of recombinant gp46 as a fusion with GST were made by ligating fragments naTH and naTF from the yeast expression plasmids pTH.O and pTF.O into the *BamHI* site of pGEX2T. From the known restriction sites in the plasmid and inserts it was possible to use *BamHI* digestion to determine the plasmid and insert size and to use *PstI* digestion to determine orientation. Restriction fragment analysis of one clone of each sort in either orientation is shown in Figure 4.1a.

Blot positive clones were used to prepare glutathione-binding protein on a small scale from lysate of induced cells. However SDS-PAGE of these crude preparations did not help in selection of the desired clones. Control clones containing pGEX2T produced a prominent band of protein running a little below the 30KDa marker (GST is predicted to be 27.5KDa). Clones containing pTZ18R produced a protein of the same size, but at a considerably lower yield, suggesting that GST is produced in absence of pGEX2T, i.e. that GST is also chromosomally encoded and is made endogenously in *E.coli* . None of the crude lysates prepared contained sufficient GST fusion protein to be visible on Coomassie stained SDS-PAGE gels (data not shown). Thus clones contained the desired plasmid construct, as shown by plasmid minipreps made concurrently, although the above assay for GST fusion protein was too insensitive to confirm its expression.

On the basis of the information gained from the plasmid digests, five different JM83 transformants were selected; GPG1(transformed with pGEX no insert),GH11-(pGEX.TH insert in inverted orientation), GH19+ (pGEX.TH insert correct orientation), GF21- (pGEX.TF insert inverted) and GF22+ (pGEX.TF insert correct orientation). Maps of the plasmids are shown in Figure 4.1b.

4.3.2: **Preparation of GST:fusion proteins**

Recombinant protein was prepared from transformants GPG1, GH19+, GF22+ and GF21-. Briefly, lysate derived from 500 ml of culture 1 hour after IPTG addition was incubated with glutathione-agarose beads. After washing, bound protein was eluted from the beads with 50 mM Tris-Cl (pH 8.0), 5mM reduced glutathione. Samples were run on SDS-PAGE (Figure 4.2). The three tracks on each gel show a sample of product from the first second and third elutions. Clearly a large amount of elutable protein still remained on the beads even after several incubations with elution buffer.

Figure 4.1: Restriction digests of plasmid mini-preps of clones selected for expression of rgp46 in *E.coli*

Predicted restriction fragment sizes of pGEX constructs

Plasmid	*BamHI* digest	*PstI* digest	(clone selected)
pGEX2T	4900	4900	GPG1
pGEX2T + TH insert (correct orientation)	530,4900	1410,4020	GH19 (+)
pGEX2T + TH insert (inverted orientation)	530,4900	980,4450	GH11 (-)
pGEX2T + TF insert (correct orientation)	970,4900	2100,4020	GF22 (+)
pGEX2T + TF insert (inverted orientation)	970,4900	980,4890	GF21 (-)

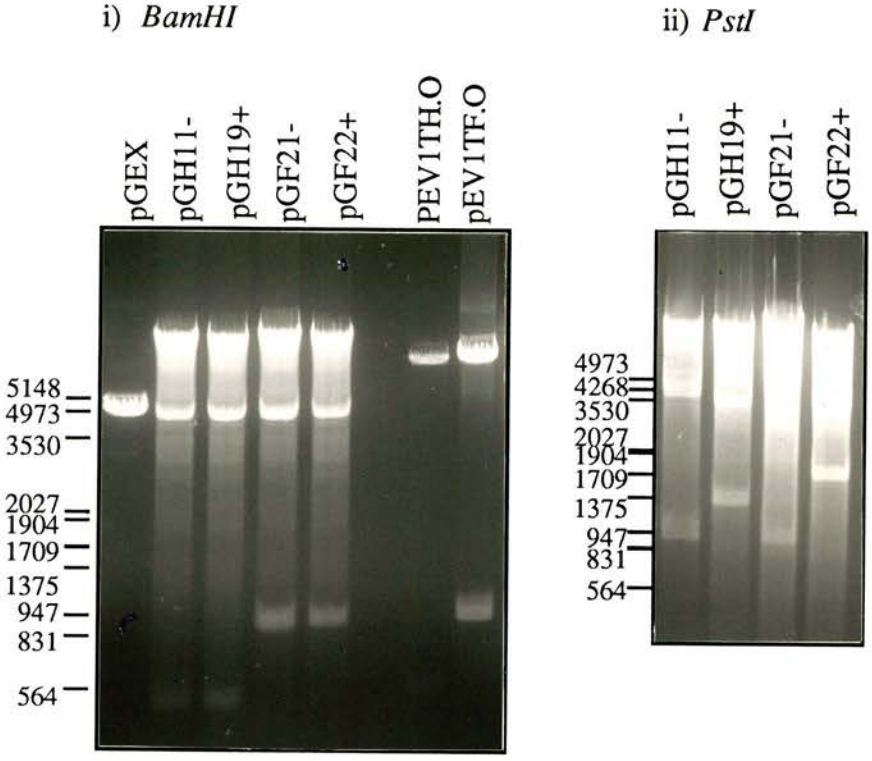

i) BamHI
pGEX
pGH11-
pGH19+
pGF21-
pGF22+
PEV1TH.O
pEV1TF.O
5148
4973
3530
2027
1904
1709
1375
947
831
564
ii) PstI
pGH11-
pGH19+
pGF21-
pGF22+
4973
4268
3530
2027
1904
1709
1375
947
831
564

Figure 4.1b: Plasmid maps of pGEX-derived GST:gp46 expression vectors

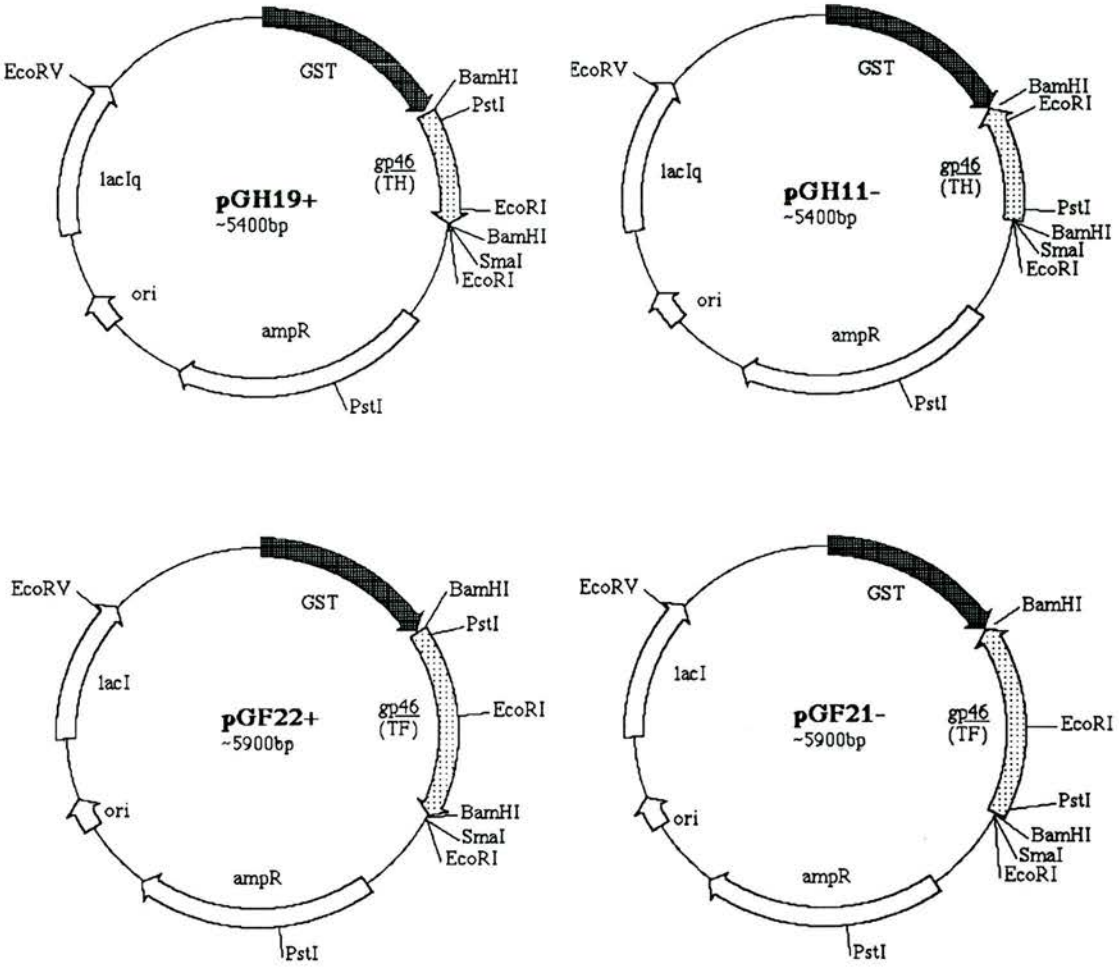
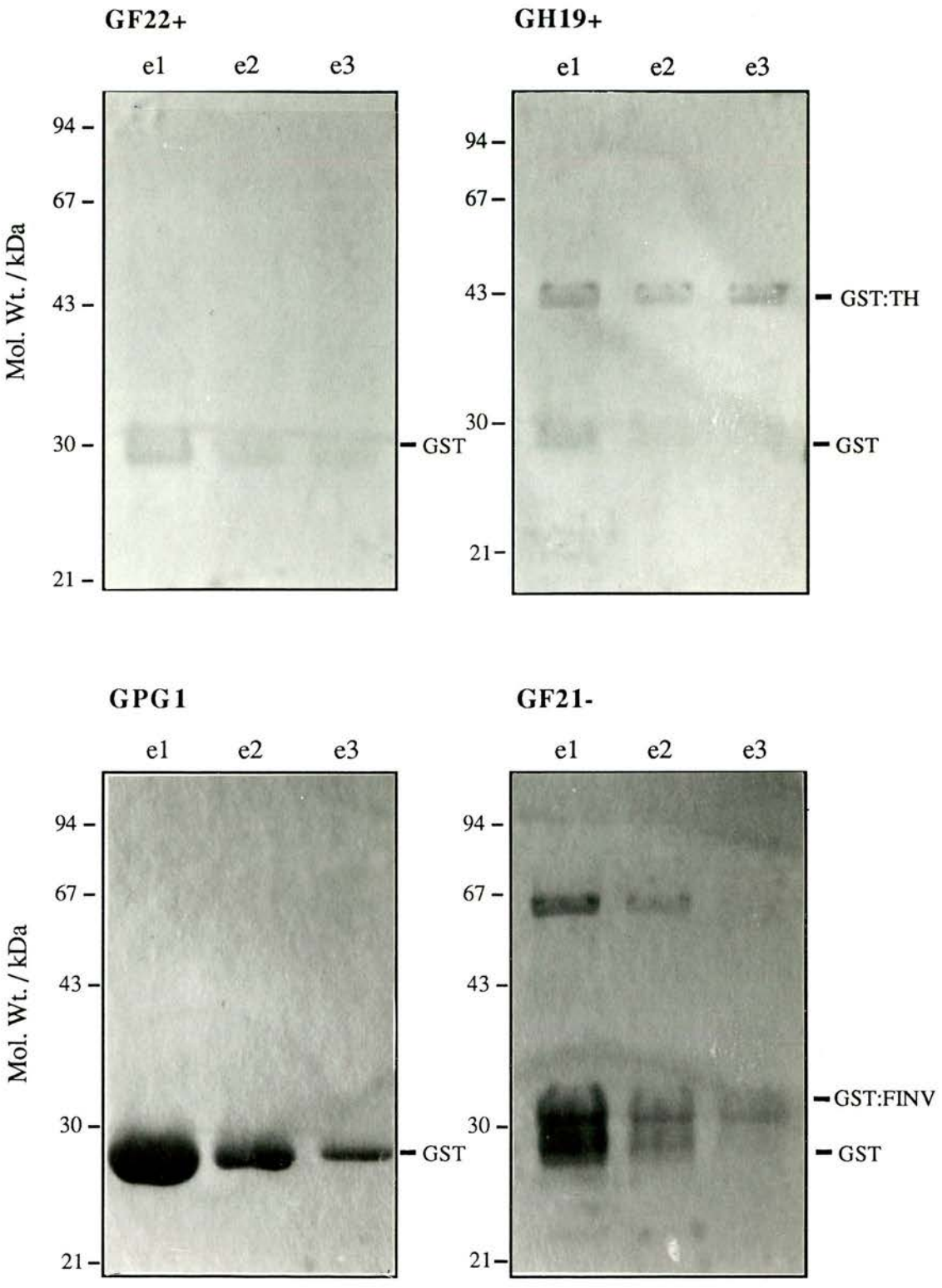


Figure 4.2: Large scale preparations of GST:fusion proteins

10% acrylamide SDS-PAGE was used for analysis of sequential elutions, e1, e2 and e3, from glutathione agarose beads which had been incubated with lysate from 500ml IPTG-induced cultures. 5
µl samples from GH19+, GF22+, or GF21+, and 1µl samples from LGPG1 were loaded. The gels were stained with Coomassie blue.

Predicted size of GST:fusion proteins
(Predicted using UWGCG Peptidesort)

GST	27.5KDa	
GST + TF	(27.5KDa + 38KDa)	65.5KDa
GST + TH	(27.5KDa + 20KDa)	47.5KDa
GST + TF Inverted (FINV)	(27.5KDa + 4.5KDa)	32KDa
GST + TH Inverted	(27.5KDa + 5KDa)	32.5KDa


GF22+
GH19+
GPG1
GF21-
e1
e2
e3
Mol. Wt. / kDa
94
67
43
30
21
GST
GST:TH
GST:FINV

Thenceforth, several elutions were done routinely in the preparation method and the eluates were pooled.

This procedure yielded about 8mg GST per litre of GPG1, 1.0 mg GST:TH from a litre culture of GH19+, but no detectable GST:TF product from GF22+.

Plasmid pGF21 contains the gp46 coding region in inverted orientation yet GF21- produced a protein of approximately the predicted size of GST:TF (65.5 kDa), as well as a protein just larger than p1 as predicted for this clone. Restriction fragment analysis of plasmid prepared from the same cultures as GST:fusion protein confirmed that this was not due to mixing or contamination of the clones. Therefore the protein of about 65kDa seems to be bacterial rather than plasmid encoded. The purity of the product obtained from GH19 varied depending on induction time. In particular the prominent band seen at ~67kDa from GF21- also appeared in GH19+ after 3 hours IPTG induction but was barely visible from culture harvested after only 1 hour induction.

From Figure 4.2 it can be estimated that GH19+ prep contains about 60-70% GST:TH and about 30% GST. However estimates from a Coomassie stained gel may be very inaccurate because individual proteins present at low concentrations will not be visualised although cumulatively they might represent a significant percentage of the preparation. As mentioned above, the proteins produced by the preparation technique varied depending on the induction time.

4.3.3: **Inclusion body preparations**

Some recombinant proteins produced in E.coli are partitioned into insoluble inclusion bodies in the cell cytoplasm. In order to determine whether rgp46 proteins expressed in *E.coli* formed inclusion bodies, preparations were made using a quick but relatively crude method (Bowden *et al.*, 1991). As can be seen from Figure 4.3 the inclusion preparation method (tracks iii and iv) provides a degree of purification when compared to crude lysate (track i). However the amount of contaminating bacterial proteins still present is significantly greater than by affinity purification with glutathione agarose beads (track v). Although GST:TH was present in the insoluble fraction as well as in the soluble fraction the amounts in the insoluble fraction did not warrant changing to this method of protein preparation. However, a combination of the inclusion body preparation followed by purification using glutathione beads may have improved upon the use of either method singly.

Figure 4.3: Inclusion body preparations of GST:fusion proteins

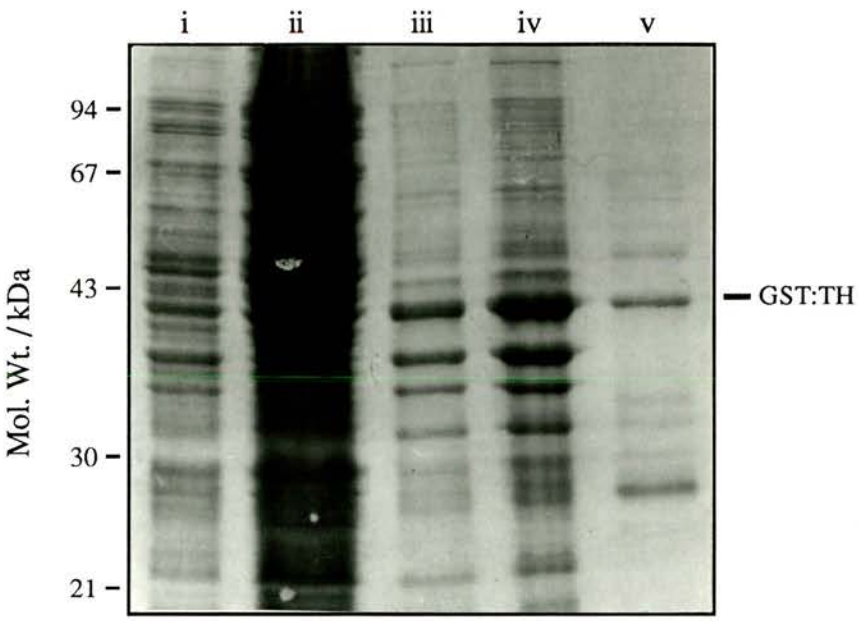
500ml IPTG-induced cultures of GH19+ or GF21- were used to prepare GST:fusion protein for comparison of preparation by binding to glutathione agarose beads or by a crude inclusion body preparation method. The products were analysed by 10% acrylamide SDS-PAGE. The gels were stained with Coomassie blue.

a) GST:TH preparations from GH19+

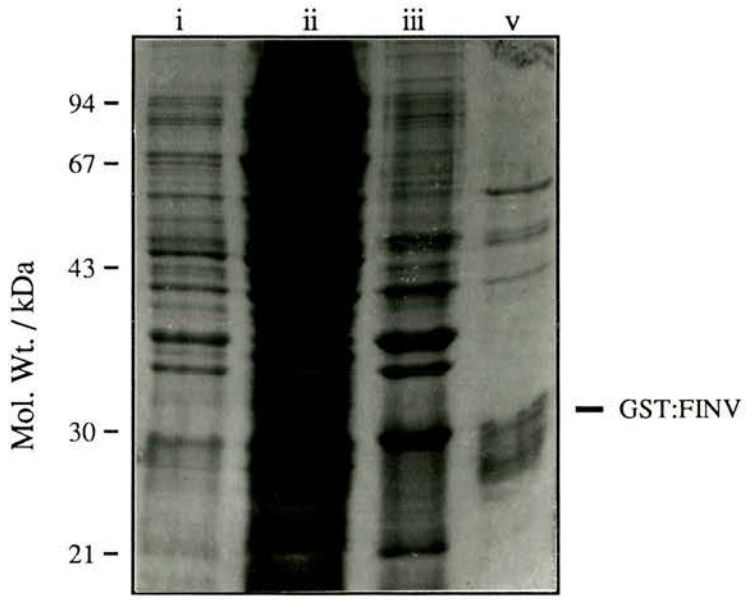
b) GST:FINV preparations from GF21-

i Crude lysate (from 312µl starting culture).
ii Supernatant after incubation with glutathione agarose beads (from 1.25ml starting culture).
iii Inclusion body preparation (from 2.5ml starting culture).
iv Inclusion body preparation (from 5ml starting culture).
v Eluate from glutathione agarose bead preparation (from 2.5ml starting culture).

a)


i
ii
iii
iv
v
94
67
43
30
21
Mol. Wt. / kDa
GST:TH

b)


i
ii
iii
v
94
67
43
30
21
Mol. Wt. / kDa
GST:FINV

4.3.4: **Growth of transformants following induction of GST:fusion protein production.**

Growth of the transformants with or without the addition of IPTG was measured by OD_{600} over 6 hours (Figure 4.4). The growth of GPG1 was slowed down after induction in comparison to uninduced culture probably due to the burden of producing such large amounts of GST protein. There was a dramatic change following induction of GF22+ culture; growth ceased within 30 minutes of adding IPTG. This indicates that recombinant protein must indeed have been produced in order to have such an effect. The difficulty encountered in purifying GST:TF protein, as described above, suggests it may only have been produced at low levels. It follows then that the GST:TF was extremely toxic to the cells as it exerted an effect at low concentration, and very quickly stopped cell division and further protein production following induction. Production of TH protein also appeared to be toxic to the cells. IPTG induction of GH19+ rapidly halted growth of the culture. The effect was only a little less rapid than that seen upon induction of GST:TF. It is likely that this difference enables more GST:TH to be produced before cell death occurs, thus enabling its extraction, albeit at low levels, from induced cell lysate. pGH11- and pGF21- which encode peptides of 5KDa and 4KDa, respectively, added onto GST had no effect on the growth of transformants after IPTG induction. It is unclear why the growth of these cultures was not affected, whilst the growth of GPG1 did slow down after induction of GST. The yield of GST fusion protein from GH11- or GF21- was significantly less than.the yield of GST from GPG1. One possible explanation is that the mRNAs encoding these proteins are unstable so that less protein was produced which was less demanding of cellular resources so that cell growth and division was able to continue.

4.3.5: **Protease digestion of GST:TH**

Attempts to digest GST:TH with Factor Xa proved unsuccessful. Secondary structure due to the hydrophobicity of the N-terminal region of gp46 immediately adjacent to the Factor Xa site may account for this.

Digestion of GST:TH with thrombin (Figure 4.5) was very inefficient in that digestion of GST:TH did not near completion, as judged by the continued presence of a visible GST:TH band even after overnight incubation with thrombin. However, incubation of GST:TH with thrombin did lead to the appearance of a protein running just below the 20 KDa marker. This band was not present in mock digested controls or in GST or GST:FINV treated with thrombin showing it was specific to thrombin treated GST:TH.

Figure 4.4: Growth of transformed cultures after induction with IPTG

Log phase cultures of the five different pGEX-derived transformed clones were diluted to approximately the same cell density in pre-warmed medium with or without IPTG. The OD_{600} of each culture was measured at intervals over 6 hours in order to determine the effect of induction of GST:fusion protein expression on growth of the different clones.

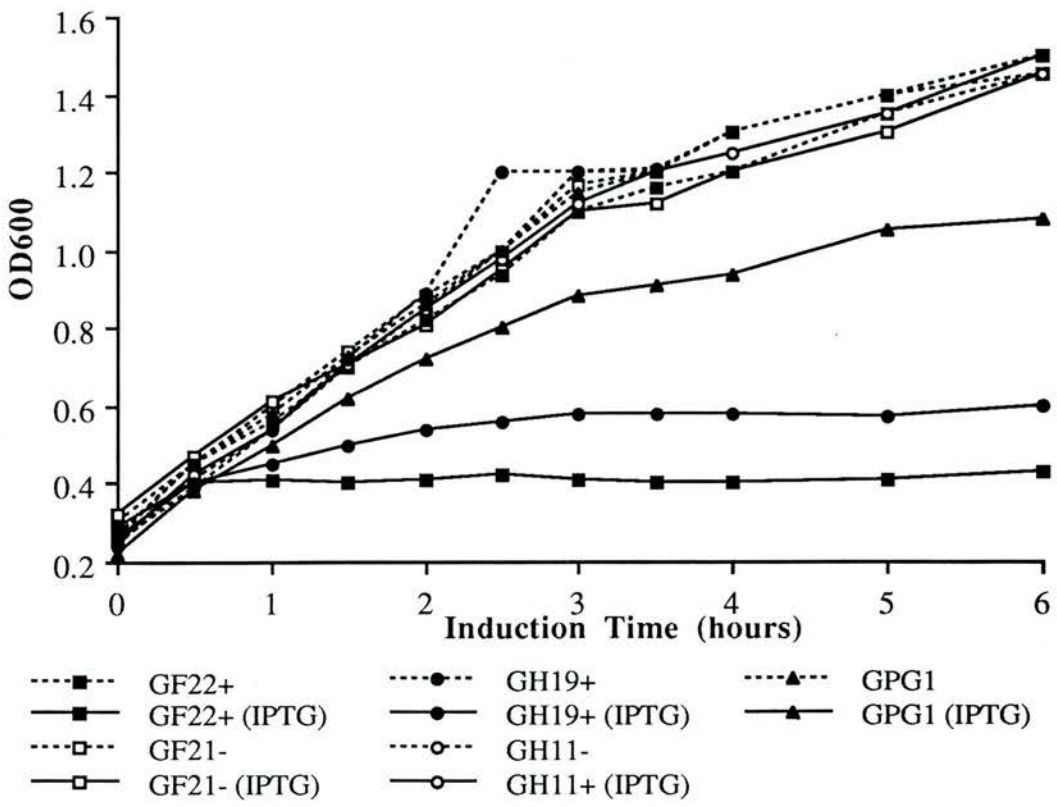


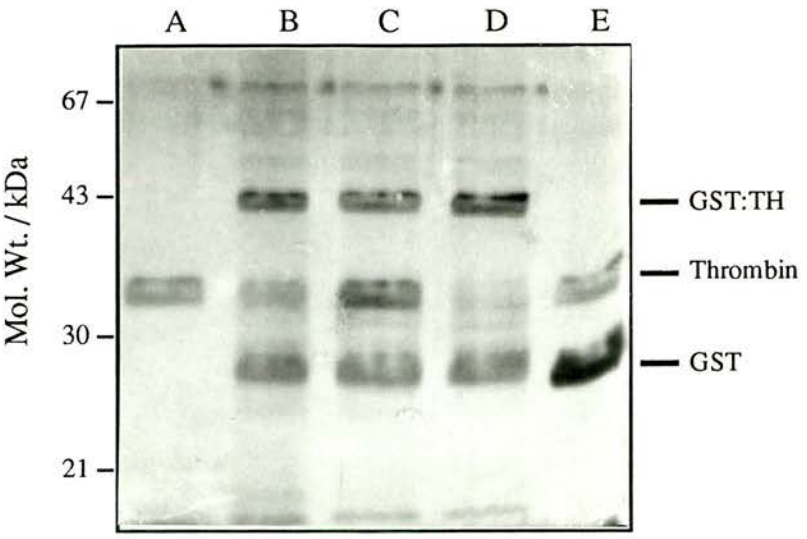
Figure 4.5: Thrombin digestion of GST:TH

GST or GST:TH was incubated with thrombin at 25°C. Samples were taken after 0, 1, 2, 5 or 24 hours and analysed by SDS-PAGE on a 5-20% polyacrylamide gradient gel. Proteins were visualised by silver staining the gel.

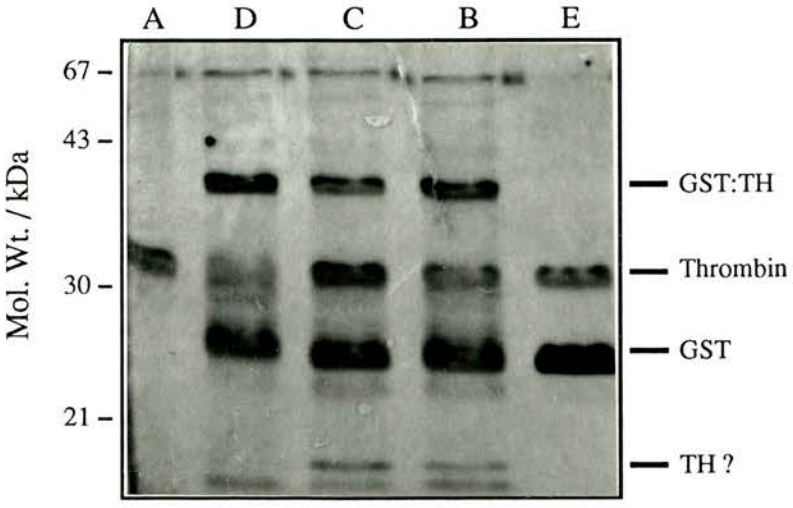
Track A - thrombin
Track B - GST:TH plus 125ng thrombin
Track C - GST:TH plus 375ng thrombin
Track D - GST.TH mock digestion
Track E - GST plus thrombin

(250ng of thrombin is 1 Unit of enzyme which, in theory, should be able to cleave 24μg of fusion protein).

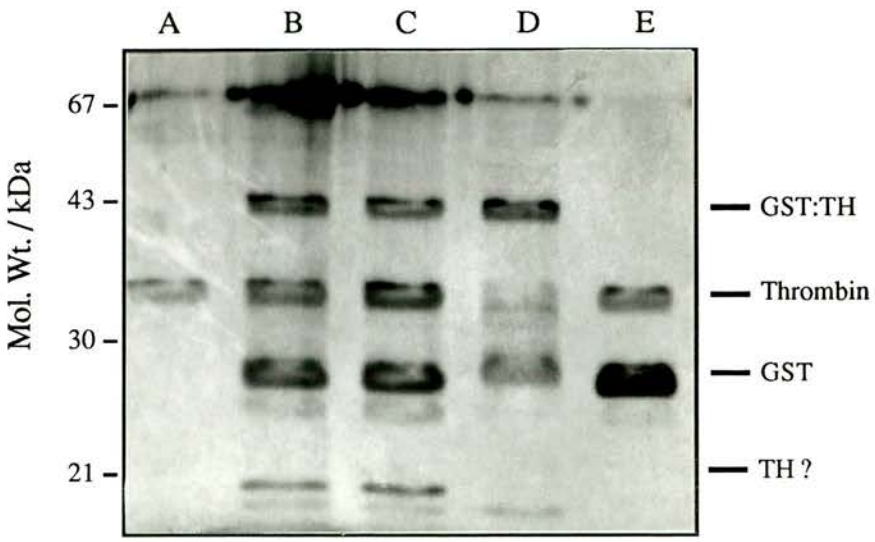
0 hour incubation



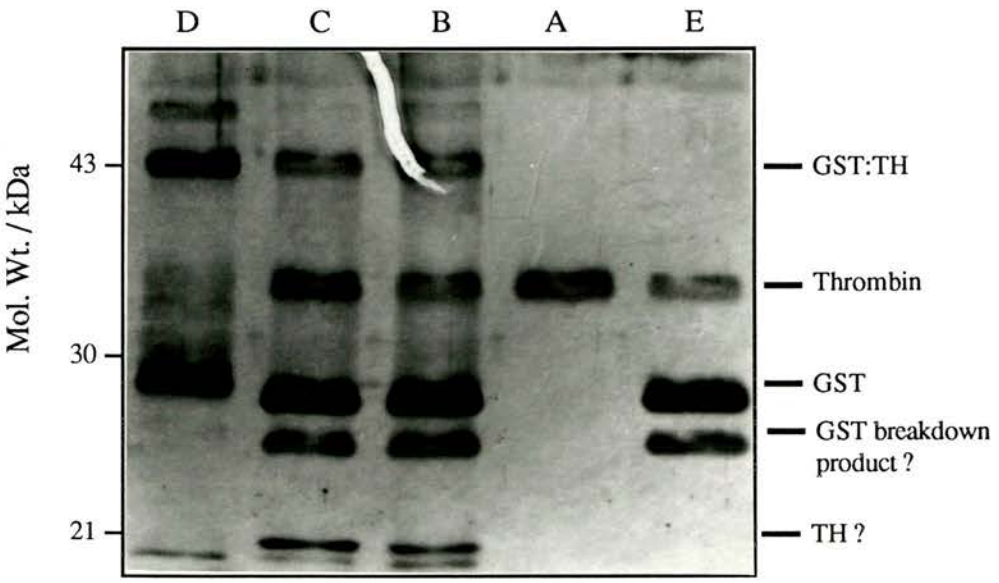
2 hour incubation



5 hour incubation



24 hour incubation



A band does begin to appear running below GST and is particularly noticeable after 24 hours incubation. This appears to be a thrombin cleaved breakdown product of the GST moiety as it was present after incubation of both GST or GST:TH with thrombin but not after incubation without thrombin or of thrombin alone

The inclusion in the digestion buffer of 0.1% CHAPS and 0.1% DOC, was tried to see if the detergents would improve the efficiency of the cleavage reaction perhaps by partially "unfolding" the proteins. However there was no difference in the results with or without the detergents.

4.3.6: **Construction of Vaccinia expression vectors.**

The pRK194B vector enables production of recombinant Vaccinia virus expressing a foreign gene from a strong late promoter. Immediately upstream of the plasmid promoter is a *BamHI*, *SmaI*, *KpnI*, *EcoRI* polylinker enabling insertion of a foreign gene. PCR primers, 763F and 761F, were designed in order to amplify EV1 *gp46* with restriction sites at either end to enable insertion into pRK194B in the correct orientation for expression of rgp46. In addition the primers encoded a 5' methionine in frame and a 3' stop codon, respectively.

763F - 5' primer :

```
          BamHI  Start          EV1 gp46
                 ---.....................................
5'-C CCG GGA TCC ATG GGG ATA GGC TTG GTG ATA GTG CTC GC
-3'
```

761F - 3' primer :

```
             KpnI  Stop  Complementary to 3' end of EV1 gp46
                   ---  .....................................
5'- C CCG GGT ACC TCA ATT CTC AAT CAT GGG CAT CTG TTC -3'
```

PCR with primers 763F and 761F and pCC18.9 template resulted in ~ 100ng/µl (total 10µg) of a 900-1000 base product.

The PCR product was digested with *KpnI* and *BamHI* and ligated into *BamHI/KpnI* cut, CIP-treated pRK194B. Plasmid minipreps were made from Ampicillin resistant transformants. Two clones, V752 and V769 which contained plasmids larger than pRK194B, as judged by electrophoresis of uncut plasmid on a 1% agarose gel, were selected. Restriction fragment analysis of the two plasmids is shown in Figure 4.6. Neither of the plasmids exactly matched the predicted restriction map for the PCR product inserted into pRK194B (Figure 4.6b). pV769

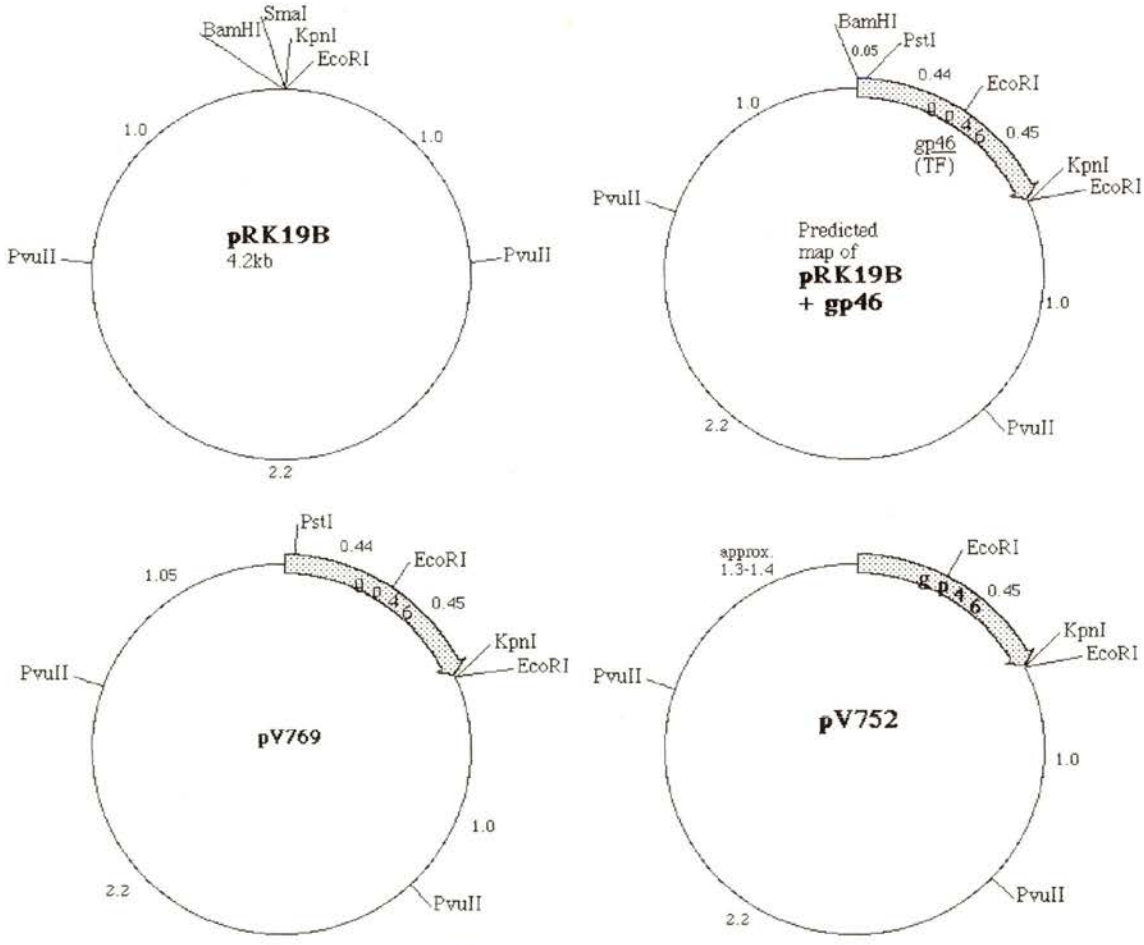
Figure 4.6: Restriction fragment analysis of pV752 and pV769

Predicted restriction fragment sizes of pRK194B plus gp46 insert

Restriction enzyme(s)	pRK194B	pRK194B plus gp46 insert
EcoRI	4.2 (linear)	**4.69**, **0.45**
PvuII	2.2, 2.0	2.2, **2.95**
KpnI/BamHI	4.2 (linear)	4.2, **0.95**
PvuII/EcoRI	2.2, 1.0, 1.0	2.2, **1.5**, 1.0, **0.45**
PvuII/BamHI	2.2, 1.0, 1.0	2.2, **1.95**, 1.0
PstI/BamHI	4.2 (linear)	**5.1**, **0.05**
PstI	4.2 (closed circular)	**5.15** (linear)
PvuII/PstI	2.2, 2.0	2.2, 1.05, **1.89**
KpnI/PstI	4.2.(linear)	4.26, **0.89**
KpnI/PvuII	2.2, 1.0, 1.0	2.2, **1.95**, 1.0

Restriction fragment sizes are given in kilobases and assume all restriction sites to be intact. Figures in bold type represent fragments which should hybridise with a gp46 probe.

Plasmid maps deduced from restriction fragment analysis



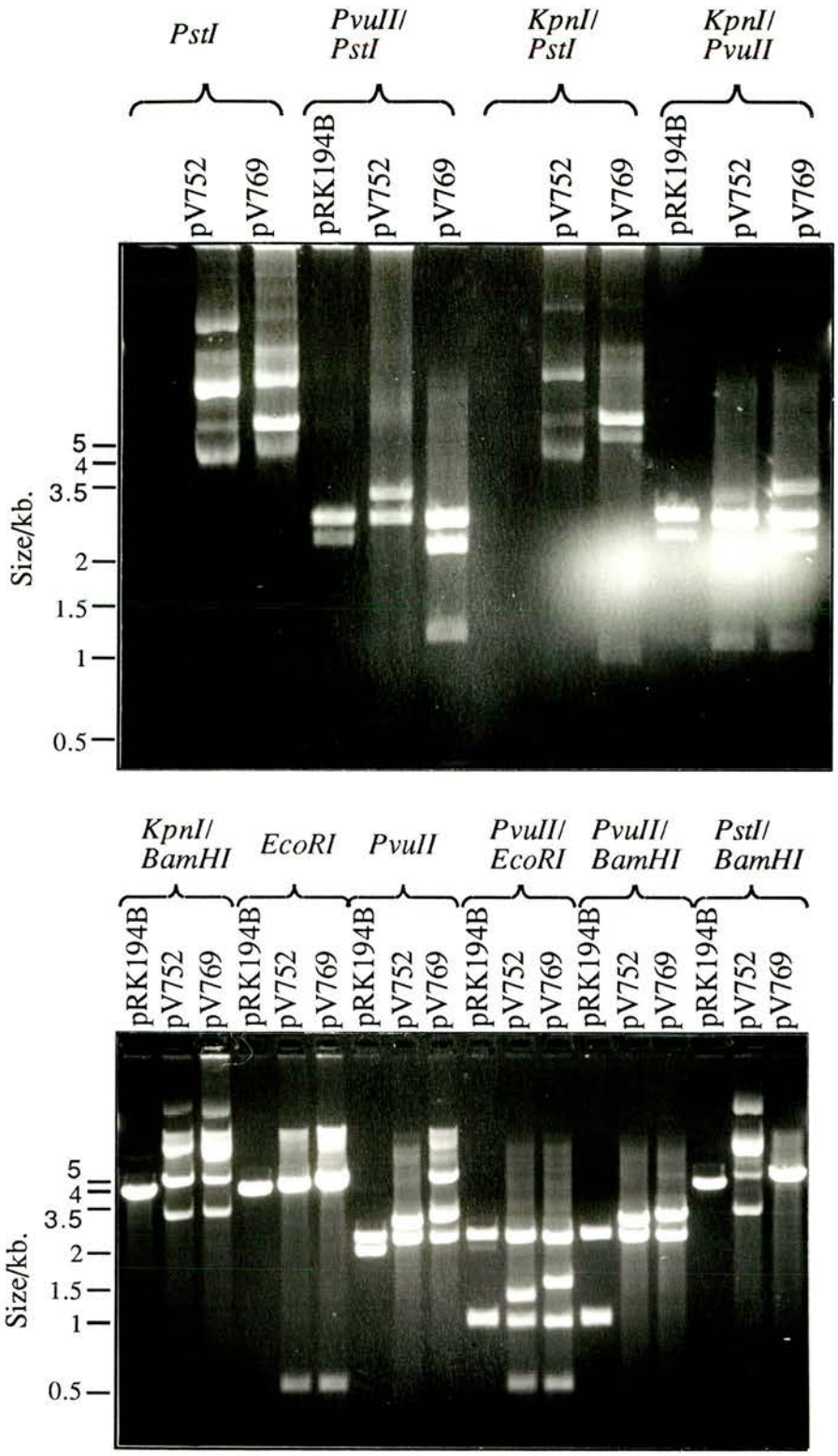

PstI
PvuII/ PstI
KpnI/ PstI
KpnI/ PvuII
pV752
pV769
pRK194B
pV752
pV769
pV752
pV769
pRK194B
pV752
pV769
Size/kb.
5
4
3.5
2
1.5
1
0.5
KpnI/ BamHI
EcoRI
PvuII
PvuII/ EcoRI
PvuII/ BamHI
PstI/ BamHI
pRK194B
pV752
pV769
Size/kb.
5
4
3.5
2
1.5
1
0.5

Figure 4.7: Results from sequencing of pV752 and pV769.

a) Sequence obtained from pV769 - "Bestfit" with EV1 *gp46*.

```
   [  5' PCR  primer (763F)   ]
           .         .         .   .         .         .
 9 GGGATAGGCTTGGTGATAGTGCTCGCTATCATGGCAATAATAGCTGCTGC 58
   ||||||||||||||||||||||||||||||||||||||||||||||||||
 5 GGGATAGGCTTGGTGATAGTGCTCGCTATCATGGCAATAATAGCTGCTGC 54
           .         .         .         .
59 AGGAGCTGGTCTCGGTGTTGCAAATGCCGTGCAGCAGTCCTATAC 104
   |||||||||||||||||||||||||||||||||||||||||||||
55 AGGAGCTGGTCTCGGTGTTGCAAATGCCGTGCAGCAGTCCTATAC 99
```

b) Sequence obtained from pV752 - "Bestfit" with EV1 *gp46*.

```
           .         .         .         .         .
  1 CCAAANNNANTAANAATATTANANNCAANANTTNCCCGAGTGGAACTCCTG 50
    ||||||:|:|||:|||||||:|::|||:|:||:|||||||||||| |||||
186 CCAAAGGAGTAAGAATATTAGAGGCAAGAGTTGCCCGAGTGGAAGTCCTG 235
           .         .         .         .         .
 51 GTAGATAGGATGATGGTATATCAGGAATTAGATTGTTGGCATTATCAGCA 100
    ||||||||||||||||||||||||||||||||||||||||||||||||||
236 GTAGATAGGATGATGGTATATCAGGAATTAGATTGTTGGCATTATCAGCA 285
           .         .         .         .         .
101 CTATTGTGTAACCTCAACAAAAAGTGAAGTAGCAAAGTATGTGAATTGGA 149
    ||||||||||||||||||||||||||||||||||||||||||||||||||
286 CTATTGTGTAACCTCAACAAAAAGTGAAGTAGCAAAGTATGTGAATTGGA 335
           .
150 CAAGATACAGGGATAATT 166
    ||||||||||||||||||
336 CAAGATACAGGGATAATT 353
```

was not cut by *BamHI* but was otherwise the same as expected. pV752 was not cut by *BamHI* or by *PstI*. The fact that pV752 was not restricted by *PstI* suggests a deletion inclusive of at least fifty bases had occurred at the 5' end of *gp46* in pV752. This deletion was confirmed by double-stranded sequencing (Figure 4.7).

The pV769 sequence obtained included a large part of the 763F primer sequence and was equivalent to nucleotides 5-99 of gp46 showing that the insert had been ligated in the right orientation. Unfortunately the sequence obtained began one base short of the complete *BamHI* site. Nevertheless, the sequencing did confirm that the AUG start codon was intact and in frame with the gp46 sequence following on from it. In retrospect the sequencing primer chosen was not ideal, as a primer from further upstream of the insert site may have enabled the complete sequence through the *BamHI* site to be read.

The pV752 sequence obtained using the same primer and the same reaction conditions, resulted in sequence equivalent to nucleotides 186-353 of EV1-gp46. This suggests that the deletion at the 5' end of the gp46 insert is of about 180 bases but not more than 186 bases.

4.3.7: **Generation of recombinant vaccinia virus**

pV769 was recombined with vaccinia virus to produce recombinant vaccinia with gp46 integrated into its genome, Vgp46. This was confirmed by Southern blotting of cells infected with recombinant Vgp46 , and transcription of gp46 was shown by Northern blotting. Vgp46 was slow growing compared to other recombinant vaccinia strains generated at the same time. It also grew only to low titre; about 1 x 10^8 pfu/ml for Vgp46 compared to about 3 x 10^9 pfu/ml for the other recombinant strains. The cytopathic effect produced by all the strains was the same, i.e. plaque formation. Vgp46 did not induce formation of syncytia.

4.3.8: **Generation of anti-sera against recombinant vaccinia virus**

Vgp46 was used to inoculate mice. Additional mice were inoculated with recombinant vaccinia VgD, expressing the gD protein of herpes virus, or Vp25, expressing MVV p25 protein.

Plasma samples were tested against lysate of MVV EV1 infected cells by Western blot (Figure 4.8d). A number of non-specific bands were recognised by Vgp46 inoculated mice in common with bands recognised by some of the VgD or Vp25 inoculated controls. The pooled Vp25 plasma recognised a band corresponding to p25 and to what are thought to be p25 precursors. There was no evidence of activity against a gp46 band, however no positive control serum was available to show

Figure 4.8: Reactivity of plasma from mice inoculated with recombinant Vaccinia virus with EV1 antigens and with GST:TH.

Western blots were developed with mouse plasma at 1:125 and rabbit anti-mouse alkaline phosphotase conjugate at 1:1000

Antigen

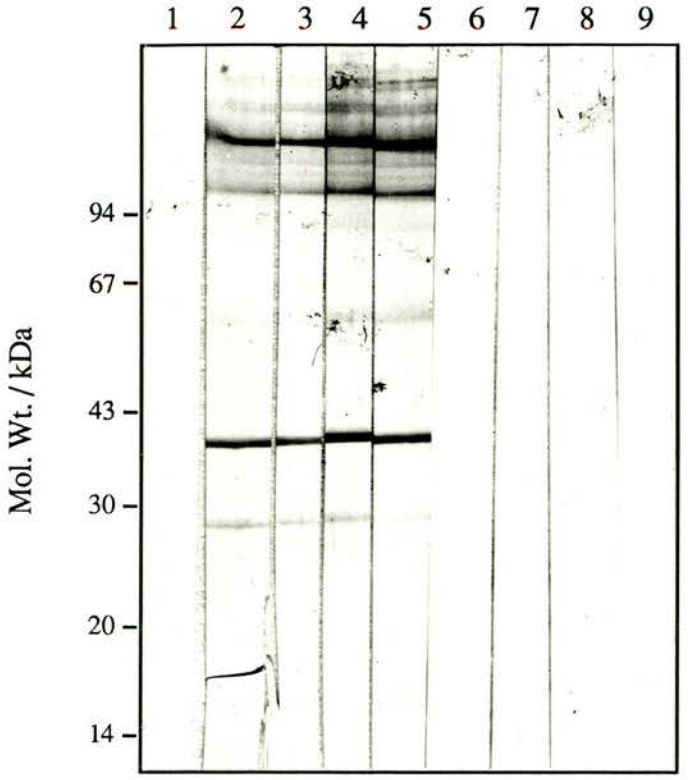
a) EV1 infected cell lysate.
b) GST:TH.
c) GST:TH digested with thrombin.
d) GST:FX treated with thrombin.

Proteins in a) were separated by SDS-PAGE on a 5-20% polyacrylamide gradient, b)c) and d) were electrophoresed on 10% acrylamide SDS-PAGE gels.

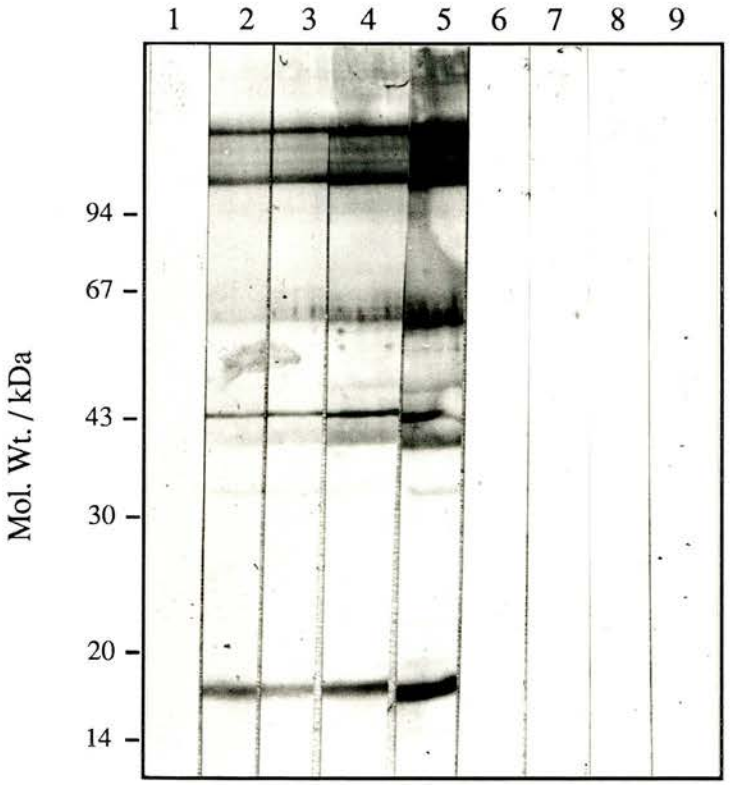
Antisera

1. Pooled plasma from mice inoculated with VgD.
2. Plasma from mouse 1 inoculated with Vgp46.
3. Plasma from mouse 2 inoculated with Vgp46.
4. Plasma from mouse 3 inoculated with Vgp46.
5. Pooled plasma from mice inoculated with Vgp46.
6. Plasma from mouse 4 inoculated with VgD.
7. Plasma from mouse 5 inoculated with VgD.
8. Plasma from mouse 6 inoculated with VgD.
9. Pooled plasma from mice inoculated with Vp25.

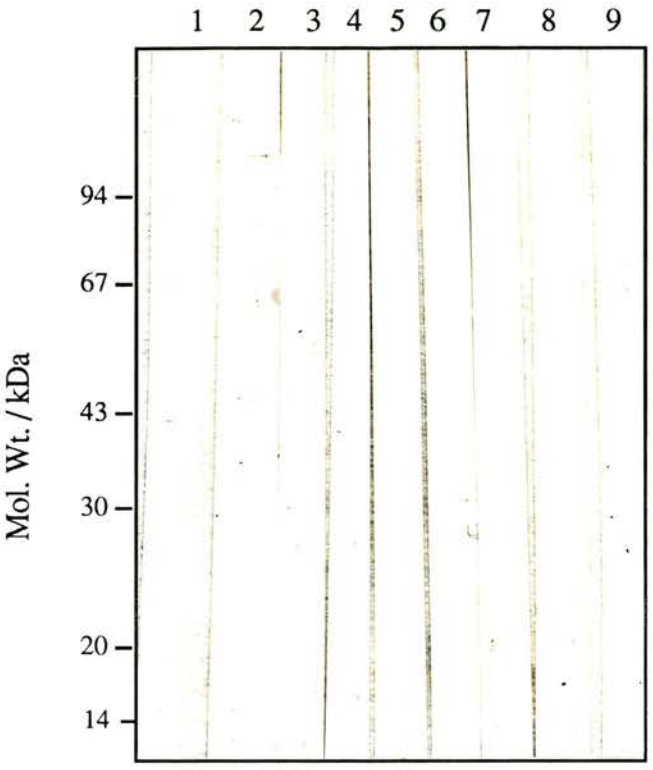
a) GST:TH



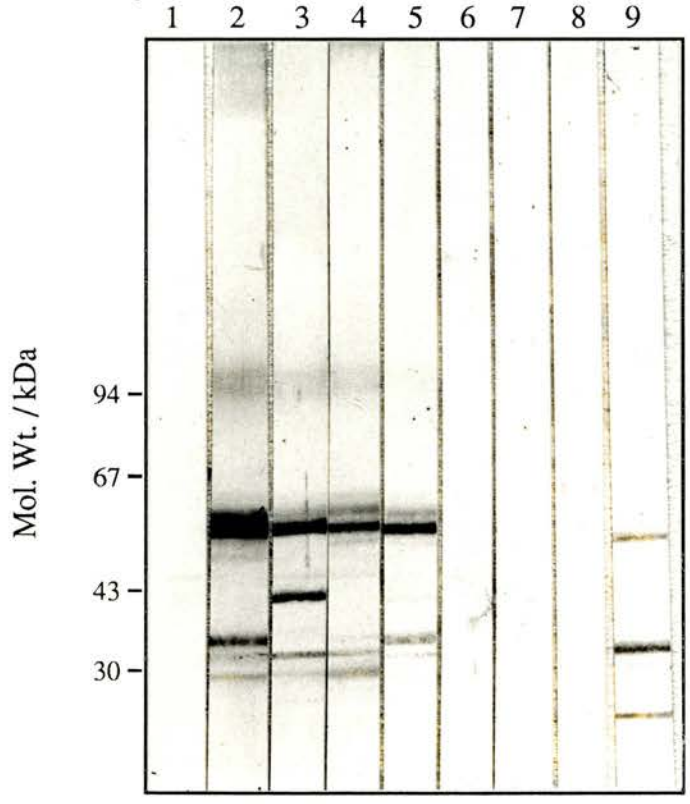
b) GST:TH thrombin digest



c) GST:FINV thrombin digest



d) EV1 lysate



whether gp46 was present on the Western blots. The most striking difference between the reactivity of the Vgp46 inoculated mice compared to the others was against smears at just above the 94kDa marker, and near the top of the gel (perhaps the envelope precursor).

Western blots of GST:TH, thrombin digested GST:TH or thrombin digested GST:FINV were developed with sera from mice inoculated with Vgp46 (Figure 4.8a, b, and c). Sera from mice inoculated with recombinant vaccinia expressing herpes gD protein (VgD) or MVV p25 (Vp25) were tested as controls. Samples from the VgD or Vp25 inoculated mice recognised very little on these blots whilst the Vgp46 plasma reacted with GST:TH and against TH after digestion of GST:TH. This confirms the expression of rgp46 by the recombinant virus which enables its potential use to produce T-cell targets for cytotoxic T-cell assays in studies on T-cell responses to MVV in experimentally infected sheep. It also shows that rgp46 expressed in vaccinia is immunogenic.

Chapter 4:

DISCUSSION

4.4.1: **Expression of recombinant gp46 in *E.coli***

As gp46 is very difficult to purify from whole virus, perhaps due to its association with membranes, recombinant gp46 (rgp46) was required in order to generate specific antisera and for studies on the immune response of MVV-infected sheep to gp46. Recombinant gp46 was expressed in yeast but the protein preparations contained a large amount of contaminants which were found to interfere with interpretation of the immunoreactivity of sheep sera against rgp46 (Chapter 3). The pGEX expression system was chosen as an alternative because of the potential specificity and ease of the technique for purification of the recombinant GST:fusion protein product.

As with expression in yeast, recombinant TH and TF proteins (half- and full-length gp46, respectively) were found to be toxic to the host cell. In fact, expression of TF in these cells was so toxic that cell division ceased after half an hour which suggests cells which were already dividing completed the cell cycle but no new cell division occurred. This extreme toxicity may be the reason that no GST:TF was recoverable from the transformed cells. In contrast GST:TH was recoverable at a better yield and purity than recovery of p1:TH or p1:TF from yeast.

A large proportion of the GST:TH produced by the bacteria was lost during the purification procedure. Comparison of Coomassie stained bands after electrophoresis suggests that the amount of GST:TH in crude lysate from the equivalent of only 312µl of induced GH19+ culture was similar to the amount of GST:TH recovered from 2.5ml of the same culture by affinity purification. This indicates a recovery of only about 12%. Loss of GST:TH may have occurred at a number of stages in the purification procedure; it may have pelleted with insoluble material during the centrifugation steps, some will have remained in solution instead of becoming bound to the glutathione agarose beads and elution of GST:TH from the beads may have been incomplete. Nevertheless, the method does significantly enrich for the recombinant protein: The amount of GST:TH present in unpurified crude lysate from induced GH19+ culture, estimated from SDS-PAGE gels (Figure 4.3), was about 10-20% of total protein. After affinity purification with glutathione agarose beads the GST:TH preparation contained about 60% GST:TH and about 30% GST. This estimation is not very accurate since it depends upon the amount of sample loaded and the quality of the gel and staining: Figure 4.2 shows the result of electrophoresis of ~200ng of GST:TH preparation. In this case only GST:TH and GST protein appeared to be

present. In contrast, the gel in Figure 4.3 revealed small amounts of numerous other proteins in ~400ng of GST:TH preparation.

The physical characteristics of recombinant proteins have been known to cause problems if glycosylation and disulphide cross linking are required for stability and solubility. Glycosylation does not occur in the bacterial expression system and protein folding and disulphide cross-linking are likely to be different from the native protein. For this reason heterologous proteins, expressed in bacteria may occur as insoluble aggregates known as inclusion bodies. The native form of gp46 is glycosylated and traverses the membrane so its folding is likely to be entirely different in the bacterially expressed form. Despite this, and the hydrophobicity of the recombinant protein, GST:TH did not appear to be partitioned into inclusion bodies. This was shown by comparison of a method to purify inclusion bodies with the glutathione affinity preparation method. The purification of inclusion bodies yielded more GST:TH than the glutathione affinity preparation method, (perhaps twice as much from the same amount of culture), but contained a much higher proportion of bacterial proteins (Figure 4.3). The pursuit of further methods, such as sucrose density gradients, might have improved inclusion body purification but would not necessarily have produced any better final yield than the affinity purification method.

4.4.2: **Protease Cleavage of GST:TH**

GST:TH contains specific protease cleavage sites for recognition by Factor Xa or thrombin. The results shown are for thrombin treatment of GST:TH (Figure 4.5), but were similar for Factor Xa. Protease treatment of GST:TH did not result in efficient cleavage of TH from its GST fusion partner, despite attempts to improve cleavage by increasing the incubation time and the amount of enzyme The GST:TH protein did not disappear but a protein similar to the predicted size of TH did appear. Serum from mice immunised with recombinant vaccinia expressing gp46 (TF) reacted with this protein whilst control sera did not, thus confirming it to be TH.

The N-terminal end of gp46 is joined to the C-terminal end of GST. This means that the hydrophobic region of TH lies close to the junction site encoding the protease recognition sequences. Protein folding might internalise this hydrophobic region and therefore may make the cleavage sites inaccessible to protease. The addition of detergents to the buffer was tried to see whether partial "unfolding" of the fusion protein could increase cleavage. The anionic detergent deoxycholic acid (DOC) and the zwitterionic detergent CHAPS were added at 0.1% and 0.2% respectively. The concentrations chosen were approximately half the critical micelle concentration. It is recommended that detergents should be used at their critical micelle concentration

for solubilisation of proteins and membrane components (Womack *et al.*, 1983). Half this value was chosen arbitrarily to try to balance the fact that the detergents might make the cleavage site more accessible, but may, at the same time, reduce the activity of the enzyme by denaturing. Titration of the detergents in the cleavage reaction would perhaps have been better. At the concentration chosen there was no apparent difference in the efficiency of protease cleavage in the presence or absence of detergent.

Investigations upon the immunoreactivity of sera from MVV-infected or uninfected sheep against GST:TH are described in Chapter 5.

4.4.3: **Expression of EV1 gp46 by recombinant vaccinia virus**

Two potential vectors for the production of recombinant gp46 expressing vaccinia were isolated, and checked by restriction fragment analysis and by partial sequencing. pV769 was selected for use as pV752 had a deletion within the gp46 coding region. One explanation for the deletion in *gp46* in pV752 may be that PCR amplification of *gp46* introduced a mutation that gave rise to a new *BamHI* site. The sequence of gp46 182-187 is GTAGCC which requires two base changes to become the *BamHI* recognition sequence GGATCC. Alternatively *BamHI* star activity may have been responsible for cutting at this sequence. The use of this junction site fits in with the deletion size of 180-186 bases estimated from the results of parallel sequencing reactions for pV752 and pV769.

Recombinant vaccinia virus, Vgp46, was generated and shown to express gp46 mRNA. Vgp46 grew only slowly and to low titre suggesting gp46 decreased virus production. Whether the reduction in virus production was due to a direct interaction of gp46 with other virus components, or by interference with cellular metabolism, is not known. The toxicity of gp46 when expressed in yeast and bacterial systems suggests that the latter may occur. Nevertheless, gp46 protein was produced by Vgp46 cells. This was shown by the activity of anti-sera, from mice immunised with Vgp46, against bacterially-derived TH protein.

Due to lack of time I was unable to conduct the studies on cell mediated immune responses of MVV-infected sheep against gp46. However, subsequent studies by Dr B.A.Blacklaws have demonstrated cytotoxic activity against Vgp46-infected cells by T-cells from MVV-infected sheep. Cytotoxicity was not shown against Vgp46-infected target cells by immune cells from uninfected sheep, nor against control VgD-infected target cells by T-cells from MVV-infected sheep. This shows that the Vgp46 infected cells present gp46 peptides in association with MHC class I, and that MVV infected sheep have T-cells that recognise and respond to gp46.

CHAPTER 5: Effect of serum from MVV-infected or uninfected sheep on fusion by MVV and reactivity of sera with MVV gp46

INTRODUCTION

5.1.1: **Fusion of cells by MVV**

When MVV is added to sheep skin cell fibroblasts at low m.o.i. CPE in the form of syncytia appears after 7-14 days caused by fusion of infected cells with neighbouring uninfected cells. This is known as fusion from within. If MVV virus is added at high m.o.i. (>10) cells fuse to form syncytia in less than 6 hours. This is too short a time for viral replication to have taken place and is thought to be due to fusion of viral particles with more than one cell at once. This is known as fusion from without. Fusion-from-without (FFWO) by virus at a high m.o.i. *in vitro* occurs rapidly and reproducibly and has been used in previous studies to look at virus fusion. The mechanism by which the actual fusion event occurs during FFWO is not known but has been assumed to be the same as during fusion of the virus with the host cytoplasmic membrane at initial infection. Thus the results of an FFWO-based assay may reflect the ability of the serum, antibody, or other factors to affect virus infectivity by acting on the fusion event at initial entry into the host cell.

5.1.2: **Fusion Assay**

The first target of the work described in this chapter was to develop an assay to measure modulation of MVV-mediated cell fusion by serum. This assay was used to determine whether there was a difference between the effect on fusion by sera from MVV infected or from uninfected sheep, and also whether fusion modulatory activity was IgG mediated. The 37°C fusion assay which has been used here, and similar assays used by previous researchers, can not distinguish between fusion modulatory activity which is due to a factor affecting the step upstream of the fusion event, i.e. virus:cell binding, and factors which act upon the fusion event itself. For this reason a novel fusion assay, the 4°C assay, was devised to assay fusion modulatory activity occurring after virus binding.

5.1.3: **Immunoreactivity of sheep serum with MVV gp46**

The aim of the second aspect of work described in this Chapter was to investigate whether sheep make a detectable anti-gp46 response using the recombinant protein, the production of which is described in Chapter 4. Both ELISAs and western blots were tried as each method has different advantages and disadvantages. Western blots enable activity against a particular protein to be distinguished from activity against other proteins present in the antigen preparation. However the antigen on a western blot is in a highly denatured form, and therefore some antibodies particularly those directed against conformational epitopes cannot be detected by western blotting. Conversely, ELISAs are considered better for detecting antibodies directed against conformational epitopes as the protein folding is believed to be more like the native form than in western blots, but denaturation may still occur upon binding of the antigen to the plastic plates. The disadvantage of ELISAs is that activity against contaminating proteins cannot be distinguished from activity against the antigen of interest.

5.1.4: **Correlation of results**

As gp46 is believed to play a critical role in the fusion process, a hypothesis was made that immunoreactivity against gp46 could alter the ability of the virus to mediate membrane fusion. To test this hypothesis the results of both the groups of experiments outlined above were examined to see if fusion modulatory activity correlated with immuno-reactivity against gp46.

Finally data on the clinical status of some of the MVV-infected animals was obtained to see if fusion modulatory activity and/or immuno-reactivity against gp46 could be used to give any indication of the prognosis for an MVV infected animal.

Chapter 5:

METHODS

5.2.1: **Cells for propagation of virus and for fusion assay.**

YT40 cells were derived by culture of an ovine skin explant and have fibroblastic morphology. YT40 cells were grown in T225 flasks in 45-60 ml of DME6 (Dulbecco's MEM (Gibco Biocult, Uxbridge) supplemented with 100U/ml benzyl penicillin, 100U/ml streptomycin, 2mM L-glutamine and 6% foetal calf serum(Azo-Tek)), at 37°C in a CO_2 incubator. When the cells reached confluence they were split into three flasks using trypsin/versene. For seeding 96-well plates cells were taken off with trypsin/versene and approximately $5x10^4$ cells in 100µl DME6 were added to each well.

5.2.2: **Fixing and staining cells in 96-well plates.**

Cells were fixed overnight at 4°C in buffered neutral formalin (BNF) or for 10 min in 50% acetone, 50% methanol (100µl per well). The fixative was removed. 1% potassium dichromate was added. After 30 min at room temperature it was replaced with ice cold 50% acetone, 50% methanol for 5 min. Plates were allowed to drain dry for at least 10 min. A drop of Gurr's Giemsa, freshly diluted 1:1 in tap water was added to each well for 5 min. Cells were differentiated for 1-2 min with water until the nucleus and cytoplasm were clearly distinguishable, (pale mauve cytoplasm, deep pink/purple nucleus).

5.2.3: **Production of EV1 stocks for fusion assays**

400µl of EV1 stock culture, (~10^5 $TCID_{50}$/ml), in 10ml of medium was added to T225 flasks containing 100% confluent YT40 sheep skin fibroblasts, and allowed to adsorb at 37°C for 1h. A further 90ml of medium containing only 2% FCS was added and the cells were incubated at 37°C until nearly 100% CPE was seen. Virtually all the cells had come off the plastic by 6-7 days after addition of virus. The medium was clarified by centrifugation in 250 ml Beckman bottles in a J2-21 rotor for 30 min at 5000g. The virus was concentrated by a factor of about ten using Amicon filtration through a filter with a 300kDa cut off point. The virus was titred for $TCID_{50}$ and to estimate the ability to cause fusion-from-without of sheep skin cells within 6-24 hours incubation.

5.2.4: **Titration of cells and virus for fusion assay**

Each well of four flat-bottom 96-well tissue culture plates (Technicon) was inoculated with 100µl of YT40 ovine skin cell fibroblasts in DME5 at concentrations of 8 x 10^5 cells/ml, 5 x 10^5 cells/ml, 2.5 x 10^5 cells/ml or 5 x 10^4 cells/ml. (Two rows of each cell density per plate). Plates were incubated at 37°C overnight in a CO_2 incubator.

Concentrated EV1 virus, (as described in the previous paragraph), was serially diluted twofold from 1:2 to 1:32 in DME5. The medium was removed from three plates of cells and replaced with 50µl of one dilution of virus in each well of two columns per plate. Cells plus virus were incubated at 37°C with CO_2. After 7, 23, and 31h one plate was fixed with BNF and stored at 4°C until staining. A fourth plate was cooled to 4°C for 30 min, diluted virus was added to the cells, and the plate was placed at 4°C for 1h. Cells were then washed twice with cold DME5 and incubated at 37°C with 50µl per well DME5. After 23 hours the cells were fixed in BNF. The four plates of BNF-fixed cells were stained with Giemsa and scored for the presence or absence of syncytia.

5.2.5: **Fusion assay (37°C)**

Dilutions of complement inactivated sera were made in DME5 in 96-well round-bottomed plates. EV1(concentrated stock) was diluted 1:8 in DME5. 60µl was added to each serum dilution and incubated at room temperature for 1 hour.

5 x 10^4 YT40 sheep skin cells in 100µl DME5 were inoculated into 96-well tissue culture plates and grown to confluence overnight. The medium was removed and 50µl of each virus/serum dilution was transferred to duplicate plates of monolayered YT40 cells. The cells plus serum/virus were incubated overnight at 37°C with CO_2, then fixed and stained with Giemsa.

Control wells containing monolayered cells without virus added, and cells with virus but no serum, were included on every plate.

Wells were scored from -2 to +2 on comparison with the control wells containing virus without added serum; a score of 0 was ascribed to wells with similar number and size of syncytia to the control, -1 to wells with significantly fewer/smaller syncytia, -2 to wells where syncytium formation was completely blocked and no syncytia were visible, +1 where syncytium formation was enhanced with respect to number of cells involved, and +2 where syncytium formation was enhanced to the extent that virtually all the cells in the monolayer had become fused. The results from duplicate tests were averaged.

5.2.6: **Fusion assay (4°C)**

EV1 stock virus was diluted in DME5. 50µl/well replaced the medium on monolayered YT40 sheep skin cells in a 96-well tissue culture plate. The cells/virus were incubated at 4°C for 1hour. Virus was removed and the cells were washed three times on ice with cold DME5. 50µl/well of serum diluted in DME5 (4°C) was added. Plates were incubated at 37°C for 24 hours before fixing and staining. The wells were scored as described for the 37°C assay, above.

5.2.7: **Effect of rgp46 on syncytium formation.**

Recombinant gp46 (N-terminal half) produced as a fusion protein with glutathione S-transferase, GST:TH, (as described in chapter 4) could be cleaved from its fusion partner using the enzyme thrombin. To investigate whether the rgp46(TH) could itself mediate membrane fusion or enhance fusion by MVV virus it was added to *in vitro* YT40 skin cell monolayers as used for the fusion assay described above. A number of control solutions were also tested; thrombin digestion buffer alone, buffer plus thrombin, GST in digest buffer, GST plus thrombin, GST:FINV plus thrombin, GST:TH, and GST:TH digested with thrombin. Two-fold serial dilutions of the above preparations in DME5 were added to monlayered YT40 cells in 96 well plates with or without sufficient virus to cause fusion-from-without overnight.

5.2.8: **ELISA:Sheep sera reacting with GST or GST fusion proteins**

Approximately 50µg/ml GST, GST:TH, or GST:FINV (as described in Chapter 4) was used as antigen to coat 96-well plates for ELISAs performed as described in Appendix VII.

5.2.9: **Western blots:Sheep sera reacting with GST or GST fusion proteins**

Approximately 150-200ng of GST, GST:TH, or GST:FINV (as described in Chapter 4) was used per strip as antigen for Western blots performed as described in Appendix VI.

MVV EV1 antigen was prepared from infected cells as described in Appendix VI. 5µl of antigen per well was loaded and electrophoresed on a 5-20% polyacrylamide gradient gel for subsequent western blotting (also described in Appendix VI).

5.2.10: **Source of sheep sera**

The MVV-infected sheep were from a flock from a farm in the south of Scotland. All the sheep were Texel breed apart from ShM19 which was a Bleu de Maine and ShM11 which was a Blue-faced Leicester. They were all female and aged between 1 and 5 years of age at the time when the serum samples were collected. The year of birth of individual sheep is shown in Figure 5.14.

Sheep had been judged to be MVV-infected by a positive result in the agar gel immunodiffusion test (AGIDT) (Winward *et al.*, 1979) which detects antibodies against MVV. The sheep were naturally infected so the date of infection is unknown.

The uninfected control sheep were from a flock belonging to the Edinburgh Centre for Genome Research. None of the sheep in this flock have ever tested MVV-positive by AGIDT.

Serum was complement inactivated by incubation at 56°C for 30 min and was stored at -20°C.

Chapter 5:

RESULTS

MVV virus was grown *in vitro* in sheep skin cells derived from skin explants (Sargan *et al.*, 1991). Sheep skin cells YT40 were passaged 4 times before stocks were laid down in 20% glycerol in liquid nitrogen. When revived the YT40 cells grew for at least another 16 passages providing they were split as soon as a 100% monolayer was formed and cells were not left to overgrow.

5.3.1: **Effect of sheep serum on fusion by MVV**

A fusion assay was developed to investigate whether serum from MVV infected sheep could modulate cell fusion by MVV. For clarity, sheep from an MVV-infected flock are numbered ShM, whilst control sheep from an uninfected flock are numbered ShU. Titration of MVV and YT40 skin cell fibroblasts showed that the optimum density of cells that would reliably produce CPE with the minimum amount of virus was as follows; inoculation of a 96 well plate with approximately 5 x 10^4 YT40 cells per well resulted in a complete monolayer after overnight incubation, inoculation of one of these wells with 50µl of a 1:16 dilution of EV1 stock consistently produced 10-20 visible syncytia (with three or more nuclei) after 20-24 hours.

As illustrated in Figure 5.1, incubation of serial dilutions of complement inactivated sheep serum with the virus before addition to the YT40 cell monolayer produced a range of effects when compared with addition of virus alone, from complete inhibition of syncytium formation, to no difference compared to control, to greatly enhanced syncytium formation.

The results for a panel of sera are shown in Figure 5.2. The sera were tested blind i.e. before knowing which ones were MVV seropositive to avoid any unintentional bias in reading the results.

As mentioned above, a range of results were seen. One serum, ShM9, was able to inhibit syncytium formation at low serum dilutions and had no effect at higher dilutions. Six of the fourteen sera from sheep from the MVV-infected flock inhibited syncytium formation at low dilutions but enhanced at higher dilutions of serum. ShM17 had little effect on syncytium formation compared to the control. A further six of the ShM sera enhanced syncytium formation and did not exhibit any syncytium inhibitory activity. Of these, five had enhancing activity even at dilutions down to 1:500. In particular, serum ShM14 greatly enhanced syncytium formation

Figure 5.1: Effect of serum from MVV-infected sheep on fusion from without by MVV on YT40 sheep skin cells

Sufficient MVV to cause fusion-from-without (FFWO) of sheep skin fibroblasts was incubated with dilutions of sheep sera for 1 hour at 37°C. Virus plus serum was then added to the cells, incubated for 20 hours then fixed and stained to determine whether the serum had any effect upon FFWO by MVV.

Examples of the results are shown opposite:

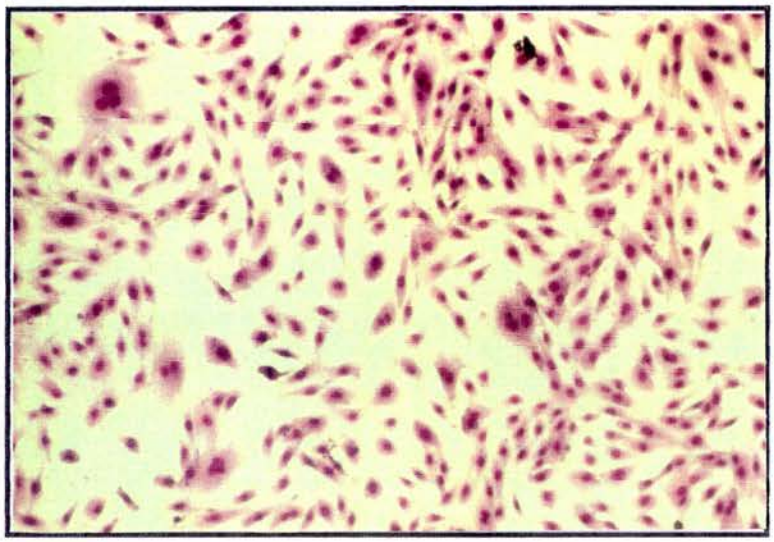
a) Serum ShU6 at 1:8 dilution; no effect on syncytium formation (relative to control).

b) Serum ShM4.1 at 1:8 dilution; inhibition of syncytium formation.

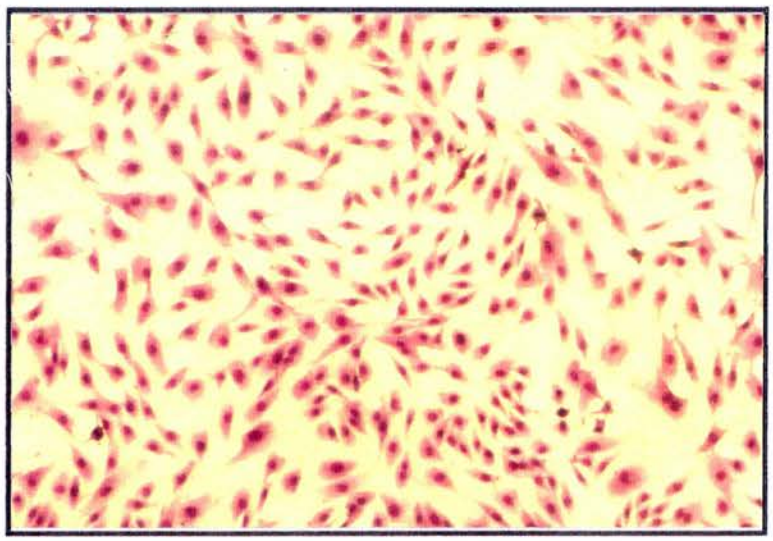
c) Serum S14.1 at 1:8 dilution; enhancement of syncytium formation.

In the absence of virus, the addition of serum to the cells has no apparent affect, i.e. the appearance of the cells is the same as with medium alone.

a)



b)



c)

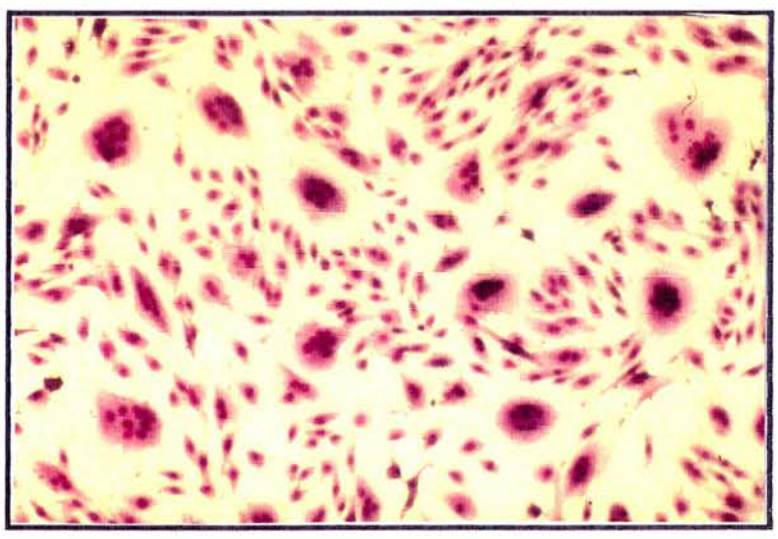


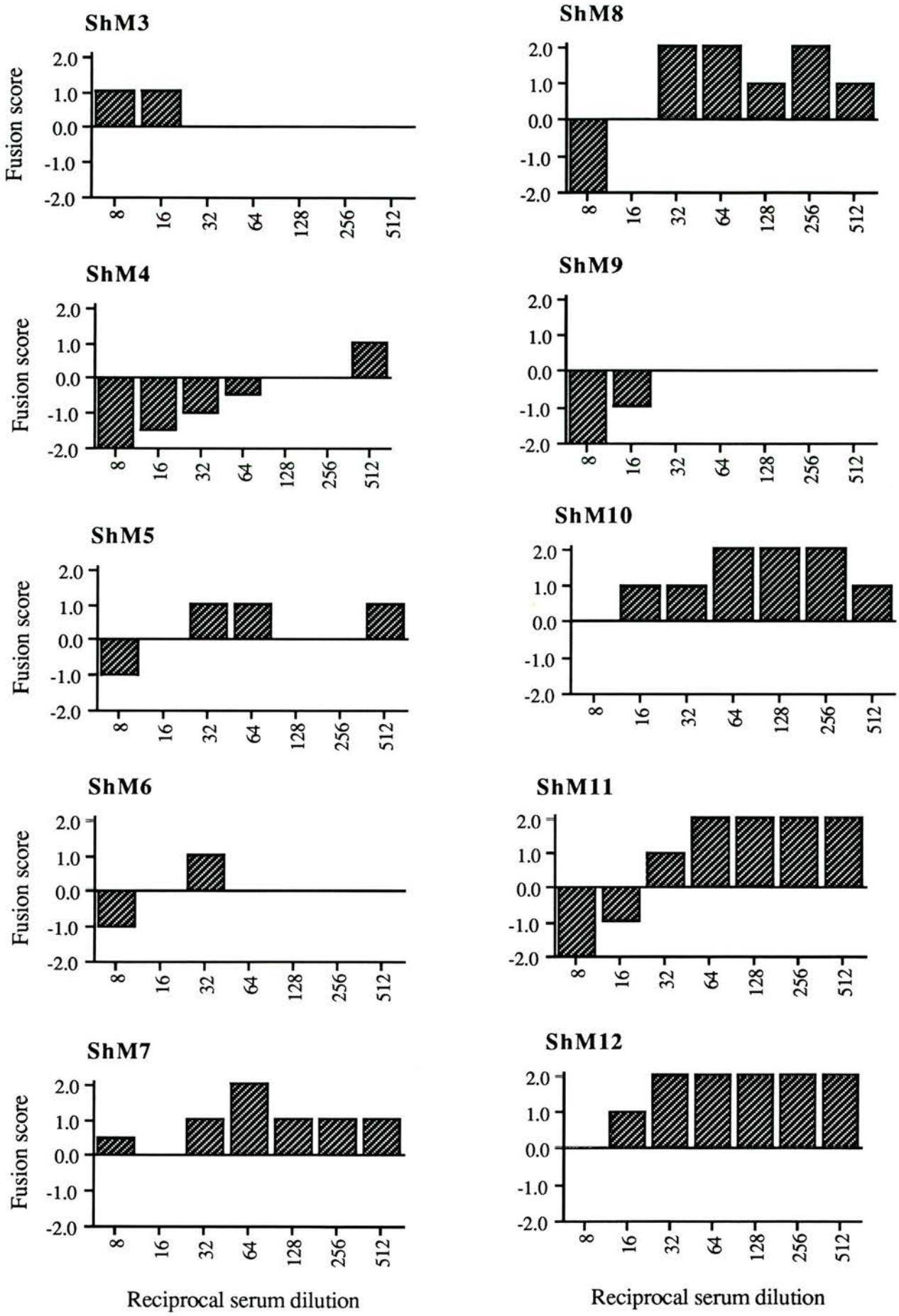
Figure 5.2 Fusion assay results - Blind trial on sera from MVV-infected flock and from MVV-negative flock.

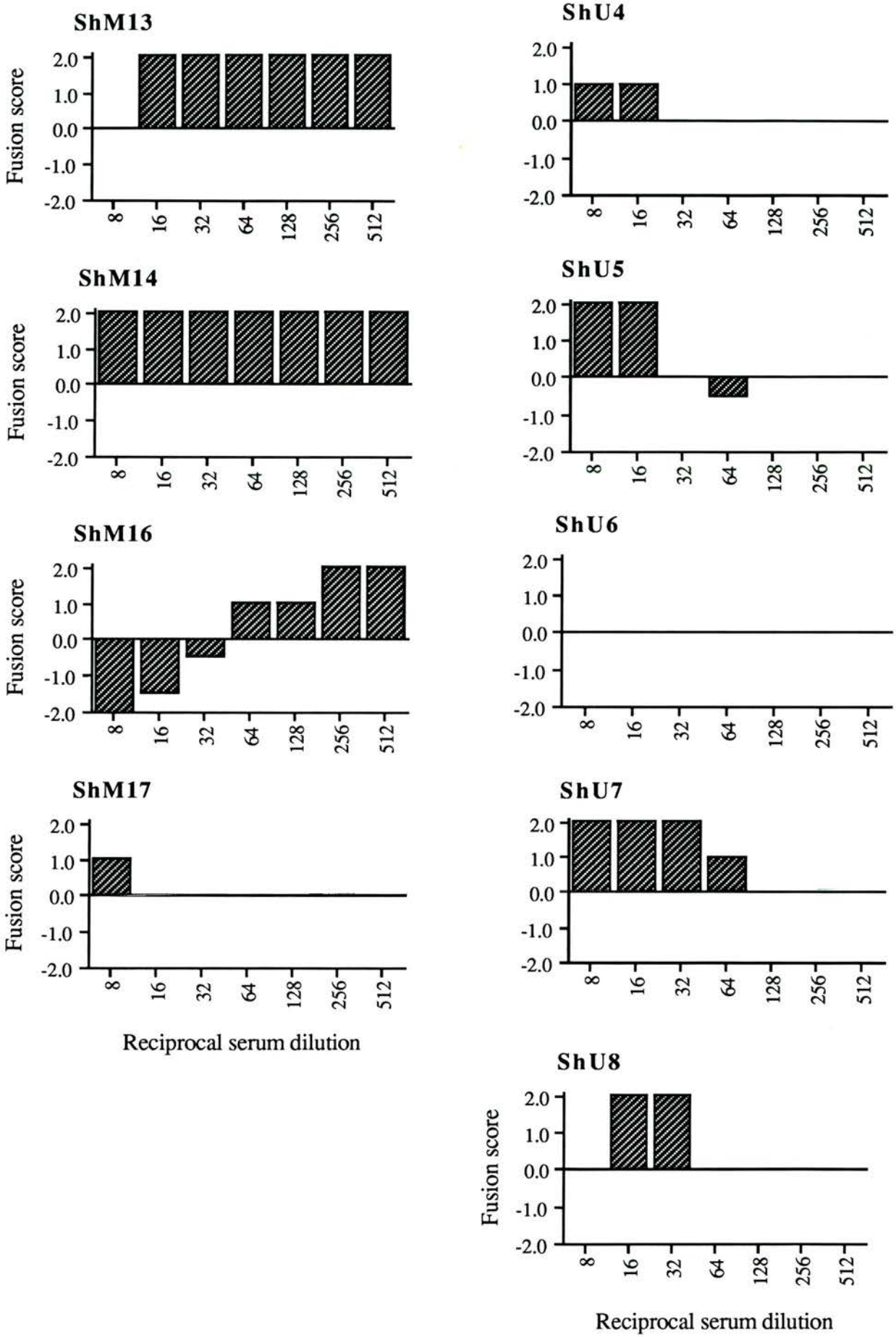
Serum	Reciprocal serum dilution 8	16	32	64	128	256	512
ShM3.1	1	1	0	0	0	0	0
ShM4.1	-2	-1.5	-1	-0.5	0	0	1
ShM5.1	-1	0	1	1	0	0	1
ShM6.1	-1	0	1	0	0	0	0
ShM7.1	0.5	0	1	2	1	1	1
ShM8.1	-2	0	2	2	1	2	1
ShM9.1	-2	-1	0	0	0	0	0
ShM10.1	0	1	1	2	2	2	1
ShM11.1	-2	-1	1	2	2	2	2
ShM12.1	0	1	2	2	2	2	2
ShM13.1	0	2	2	2	2	2	2
ShM14.1	2	2	2	2	2	2	2
ShM16.1	-2	-1.5	-0.5	1	1	2	2
ShM17.1	1	0	0	0	0	0	0
ShU4	1	1	0	0	0	0	0
ShU5	2	2	0	-0.5	0	0	0
ShU6	0	0	0	0	0	0	0
ShU7	2	2	2	1	0	0	0
ShU8	0	2	2	0	0	0	0

Sheep ShU are from an uninfected flock. Sheep ShM are from a MVV-infected flock.

The scores are the average from four plates.

The results are also shown opposite in a graphical form. Bars above the zero line represent enhancement of virus mediated cell fusion. Bars below zero represent inhibition of virus mediated cell fusion.


ShM3
ShM8
ShM4
ShM9
ShM5
ShM10
ShM6
ShM11
ShM7
ShM12
Fusion score
2.0
1.0
0.0
-1.0
-2.0
8
16
32
64
128
256
512
Reciprocal serum dilution


ShM13
ShU4
ShM14
ShU5
ShM16
ShU6
ShM17
ShU7
ShU8
Fusion score
2.0
1.0
0.0
-1.0
-2.0
8
16
32
64
128
256
512
Reciprocal serum dilution

at all the dilutions tested. Of the negative controls, ShU, four of the five had some enhancing activity, although only at higher serum concentrations. The highest dilution which mediated enhancement was 1:64.

The results of the two groups of sera, ShU and ShM, from sheep from an uninfected or MVV-infected flock respectively, were compared to see if there was any significant difference in the way they acted in the fusion assay. The null hypothesis is that the two populations are the same with respect to this assay. The calculated values of t when comparing the results of ShU with ShM are listed below. The formulae for calculating t are shown in Appendix IX.

Comparison of sera from MVV infected sheep and uninfected sheep in the fusion assay

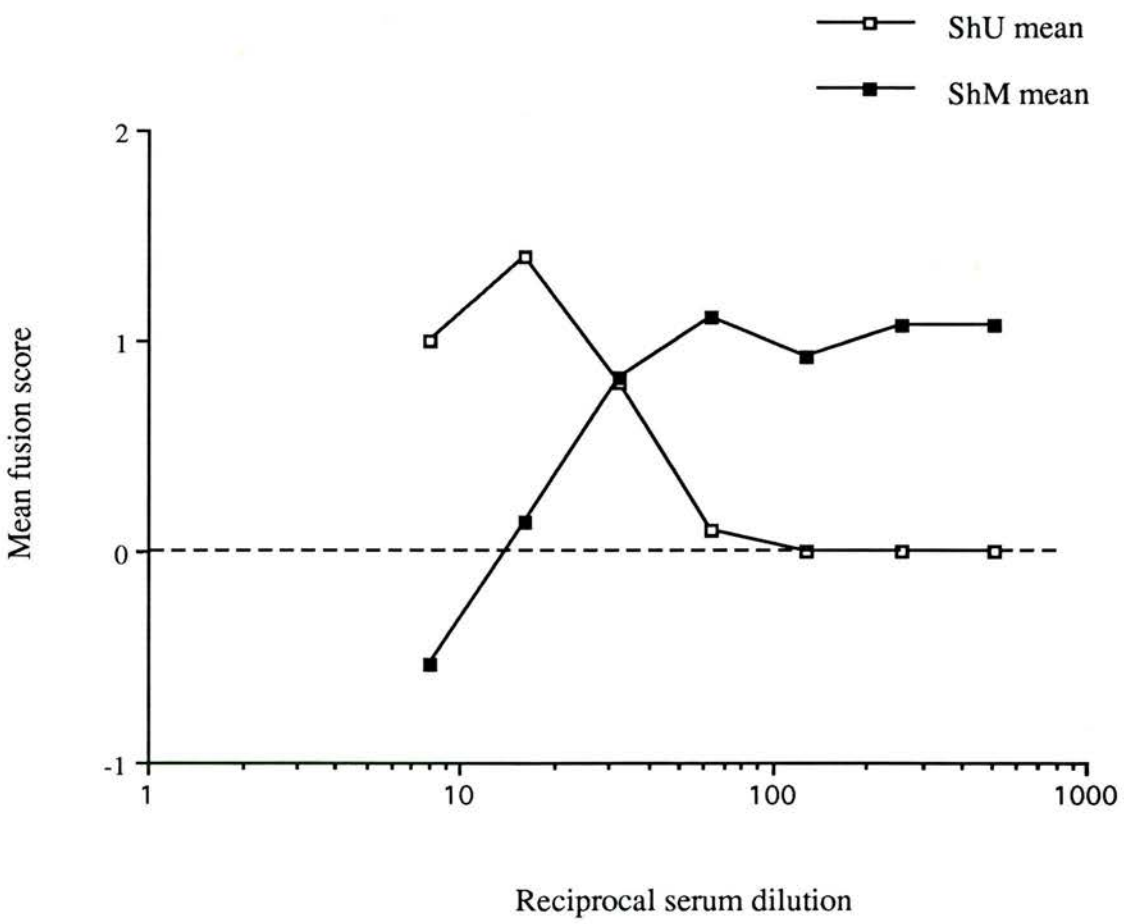
Serum dilution factor	ShU mean value	ShM mean value	t value
8	1	-0.54	2.228
16	1.4	0.14	2.144
32	0.8	0.82	0.036
64	0.1	1.11	2.138
128	0	0.93	2.159
256	0	1.07	2.290
512	0	0.687	2.754

The critical value for $t_{0.05}$ with 17 degrees of freedom is 2.11. For all dilutions except 1:32 the calculated value of t is greater than the critical value. Thus the null hypothesis can be rejected with 95% confidence, i.e., the two populations are significantly different in the way the sera react in the fusion assay.

A graph of the mean fusion scores for the ShM and ShU groups is shown in Figure 5.3. This shows that on average sera from MVV-infected sheep inhibit fusion at the highest concentration and enhance at lower concentrations. In contrast sera from uninfected sheep tend to enhance fusion at higher concentrations but enhancement titres out by 1:64. The dilution of 1:32 at which there is no significant difference between the two populations is where the average values cross-over.

Figure 5.3: Mean fusion scores for sera from MVV-infected sheep and uninfected sheep.

The mean score at each dilution in the 37°C fusion assay shown in Figure 5.2 was calculated for sera from MVV-infected sheep (ShM) and uninfected control sheep (ShU).



Serial bleeds taken from sheep in the MVV-infected flock at 6 weekly intervals were tested in the 37°C fusion assay to see if the activity remained consistent. The results, shown in Figure 5.4, were broadly similar for the serial samples from any one sheep, except in the case of ShM2 where the first 2 samples had no effect on syncytium formation whilst the third and fourth bleeds had enhancing activity and ShM8 where the results were variable.

5.3.2: **Reproducibility of the 37°C fusion assay**

Ten sera were common to both the assays whose results are shown in Figure 5.2 and Figure 5.4. Clearly there are some discrepancies between the results of the different assays. There is inherent variability in this assay despite the fact that the same cells (but different passages), aliquots of the same virus stock, and the same serum samples were used on each occasion. Although the fusion scores showed some variation at all dilutions tested, direct comparison of the sera common to both assays showed the results were not as dissimilar as they at first appeared: 29 of the fusion scores were the same for the same serum at the same dilution factor, 29 differed by 0.5-1, and 12 differed by more than 1. Thus 82% of the individual scores did not differ by more than 1 point, indicating that most results were similar if not the same, in both assays. In most cases the overall pattern of activity for each individual serum was conserved. Comparison of the fusion assay results of the ShM sera in Figure 5.2 with those of the ShMn.1 sera in Figure 5.4, bearing in mind that three sera in each assay were not used in the other, shows that they were not significantly different since at each dilution the calcualted t value was less than the critical value.

Comparison of results of different fusion assays

Serum dilution factor	ShM (Fig 5.2) mean value	ShM (Fig 5.4) mean value	t value
8	-0.54	-0.57	0.09
16	0.14	-0.04	0.67
32	0.75	0.50	1.17
64	1.11	0.89	1.37
128	0.93	1.18	1.92
256	1.07	1.32	1.85
512	1.07	1.32	1.85

n=14 The critical value for $t_{0.05}$ with 13 degrees of freedom is 2.16.

Figure 5.4: Fusion assay results- Serial bleeds from sheep from an MVV-infected flock.

Blood samples were taken at 6 weekly intervals. Sample n.1 represents the first sample, (taken at week 0), n.2 the second, (taken at week 6), etc.

	Reciprocal serum dilution						
Serum	8	16	32	64	128	256	512
ShM1.1	-1.5	-0.5	2	2	2	2	2
ShM1.2	-2	-1.5	0	1	2	2	2
ShM1.3	-2	-1	0	0	2	2	2
ShM2.1	-1	0	0	0	0	0	0
ShM2.2	0	0	0	0	0	0	0
ShM2.3	0	1	1	1	2	1	0
ShM2.4	0	2	2	1	2	1	0
ShM3.1	-1.5	0	0	0	0	0	0
ShM3.2	-1	0	0	0	0	0	0
ShM3.3	-1.5	1	0	0	0	0	0
ShM4.1	-1.5	0	1	1	1	2	2
ShM4.2	0	0	0	1	1	1	2
ShM5.1	-2	-1.5	-0.5	0	2	2	2
ShM5.2	-2	-1.5	1	1	2	1	2
ShM5.3	-2	-1.5	0	1	2	2	2
ShM5.4	-2	-1.5	-0.5	0	1	1	1
ShM6.1	-2	-2	-1	0	1	1	1
ShM6.2	-2	-0.5	0	1	1	1	2
ShM6.3	-1	0	0	0	1	1	1
ShM6.4	-2	-0.5	1	1	1	1	2
ShM8.1	-1	0	0	1	1	1	1
ShM8.2	-2	-2	0	0	0	0	2
ShM8.3	0	1	1	1	1	2	2

Serum	Reciprocal serum dilution 8	16	32	64	128	256	512
ShM9.1	-2	-2	-1	1	1	1	1
ShM9.2	-2	-2	-1	0	0	0	1
ShM9.3	-2	-2	-2	-0.5	0	0	1
ShM10.1	2	2	2	2	2	2	2
ShM10.2	2	2	2	2	2	2	2
ShM10.3	2	2	2	2	2	2	2
ShM10.4	2	2	2	2	2	2	2
ShM12.1	-1	0	1	1	2	2	2
ShM12.2	-1	0	1	1	1	1	1
ShM12.3	-0.5	0	0	1	1	1	2
ShM12.4	0	0	0	1	1	1	1
ShM13.1	1	2	2	2	2	2	2
ShM13.2	1	2	2	2	2	2	2
ShM13.3	1	2	2	2	2	2	2
ShM14.1	2	2	2	2	2	2	2
ShM14.2	2	2	2	2	2	2	2
ShM14.3	2	2	2	2	2	2	2
ShM14.4	1.5	2	2	2	2	2	2
ShM15.1	-1.5	-1.5	-1.5	-1.5	-1.5	-0.5	-0.5
ShM15.2	-1.5	-1.5	-1.5	-1.5	-0.5	-0.5	0
ShM15.3	-2	-1.5	-2	-1.5	-0.5	-0.5	0
ShM15.4	-2	-2	-2	-1	-1	-0.5	-0.5
ShM17.1	2	2	2	2	2	2	2
ShM17.2	1	1	1	1	1	1	1
ShM17.3	2	2	2	2	0	0	0

5.3.3: **Effect of IgG and IgG-depleted serum in the 37°C fusion assay**

Eight sera were selected covering the range of activities seen in the fusion assay and including two MVV negative controls. They were recoded and shuffled in order to screen them blind.

1- MVV positive, neutral in fusion assay (ShM2.1)

2- MVV positive, blocked syncytium formation (ShM10.3)

3- MVV positive, enhanced syncytium formation (ShM13.3)

4- MVV negative, neutral in fusion assay (ShU4)

5- MVV negative, neutral in fusion assay (ShU6)

6- MVV positive, blocked syncytium formation at low dilutions, enhanced at higher dilutions (ShM5.4)

7- MVV positive, blocked syncytium formation at low dilutions, enhanced at higher dilutions (ShM1.3)

8- MVV positive, blocked syncytium formation at low dilutions, neutral at higher dilutions (ShM3.3)

IgG was prepared from these sera using a Sepharose-G column. The approximate protein concentrations of the pooled peak IgG fractions, (B), and the column flow through (C), are shown in Figure 5.5. The IgG concentration in the flow-through shows that the Sepharose G column had removed the majority of the IgG from the serum. The IgG eluted from the column had been diluted compared to the expected IgG concentration in serum (~10mg/ml) during elution from the column.

The sera, IgG preparations and column flow-through (serum with most of the IgG removed) were tested alongside each other in the fusion assay to investigate which components of the serum might be exerting an effect on syncytium formation by MVV virus (Figure 5.6). The control for comparison with the IgG preparation, (which had been eluted in Tris, glycine before dialysis into PBS), was Tris, glycine pH7.0. This had no effect on syncytium formation. Control wells with the test samples at the highest concentrations used in the assay showed that serum, IgG or column flow-through did not cause fusion or have a visible detrimental effect on the cells in the absence of MVV.

As predicted a range of results were seen in the fusion assay with whole serum. The IgG preparations all showed fusion enhancing activity but the titres varied, whereas the IgG depleted sera had very little fusion enhancing activity. This indicates that fusion enhancing activity was mediated principally by the IgG fraction.

Figure 5.5: Fractions prepared from sheep sera.

IgG was prepared using a Sepharose-G column. The protein concentration in the pooled IgG fractions eluted from the column was measured using a colourimetric assay (Biorad protein assay). The approximate dilution factor in the pooled fractions compared to serum assumes a concentration of approx. 10mg/ml IgG in serum. The IgG concentration in the column flow-through was measured using an IgG capture ELISA.

Pooled IgG fractions	Protein concentration (mg/ml)	Approximate IgG dilution factor
ShM1.3B	1.43	7
ShM2.1B	2.62	4
ShM3.3B	1.58	6
ShM5.4B	0.86	11
ShM10.3B	1.84	5
ShM13.3B	2.47	4
ShU4B	0.75	13
ShU6B	1.15	9

Column flow-through	Protein concentration (mg/ml)	IgG concentration (µg/ml)
ShM1.3C	3.5	15
ShM2.1C	2	<8
ShM3.3C	1.3	8
ShM5.4C	2.6	<8
ShM10.3C	1.5	<8
ShM13.3C	2.6	8
ShU4C	4.9	<8
ShU6C	3.1	<8

Figure 5.6: Fusion assay results - Ig preparations from selected sera.

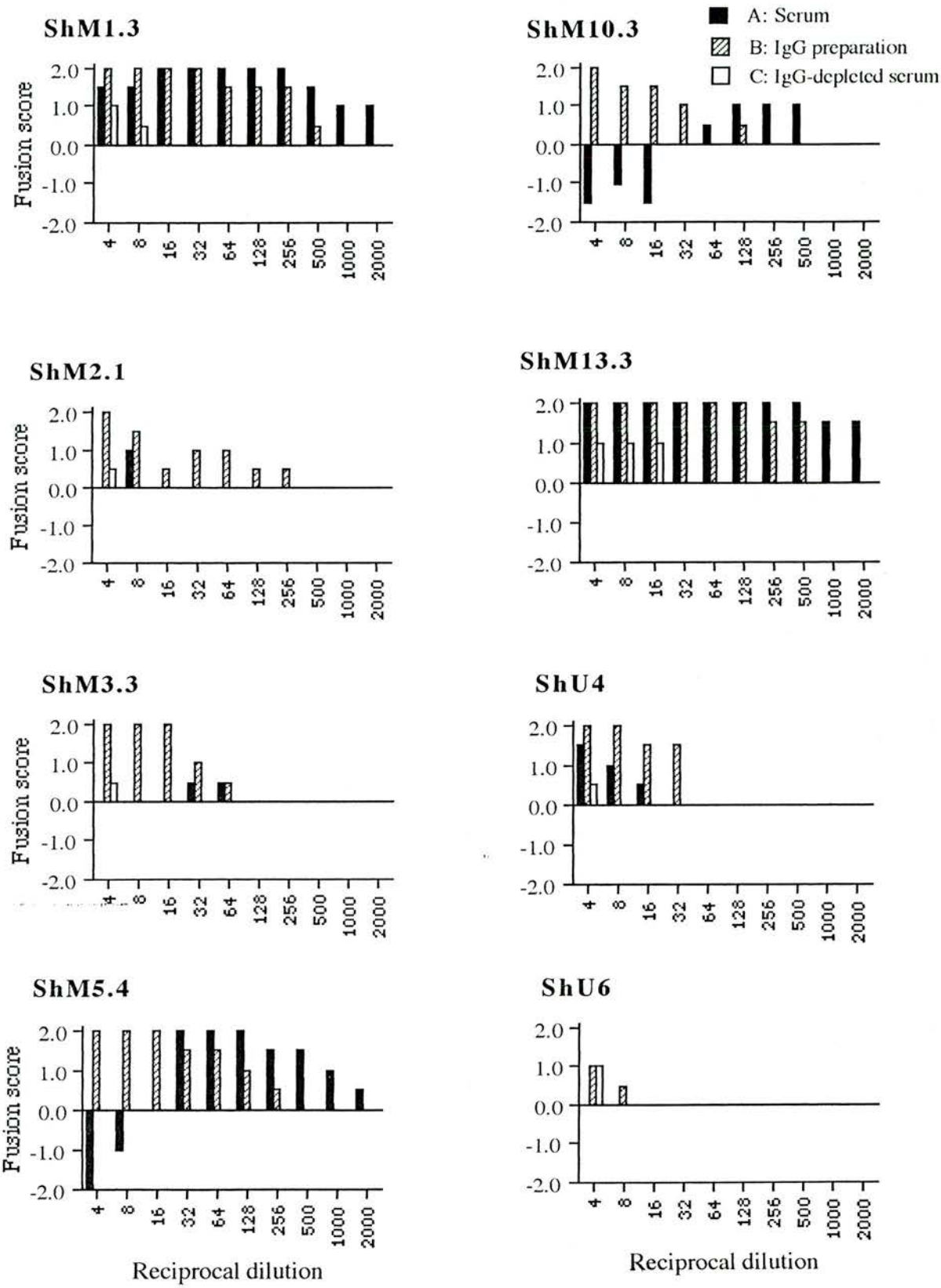
The results are tabulated below and illustrated as bar charts opposite.

KEY:- A-Serum.

B-Ig eluted from Sepharose-G column.

C-Serum depleted of IgG (i.e. flow-through from Sepharose-G column)

	Reciprocal dilution									
	4	**8**	**16**	**32**	**64**	**128**	**256**	**500**	**1000**	**2000**
ShM1.3A	1.5	1.5	2	2	2	2	2	1.5	1	1
ShM1.3B	2	2	2	2	1.5	1.5	1.5	0.5	0	0
ShM1.3C	1	0.5	0	0	0	0	0	0	0	0
ShM2.1A	0	1	0	0	0	0	0	0	0	0
ShM2.1B	2	1.5	0.5	1	1	0.5	0.5	0	0	0
ShM2.1C	0.5	0	0	0	0	0	0	0	0	0
ShM3.3A	0	0	0	0.5	0.5	0	0	0	0	0
ShM3.3B	2	2	2	1	0.5	0	0	0	0	0
ShM3.3C	0.5	0	0	0	0	0	0	0	0	0
ShM5.4A	-2	-1	0	2	2	2	1.5	1.5	1	0.5
ShM5.4B	2	2	2	1.5	1.5	1	0.5	0	0	0
ShM5.4C	0	0	0	0	0	0	0	0	0	0
ShM10.3A	-1.5	-1	-1.5	0	0.5	1	1	1	0	0
ShM10.3B	2	1.5	1.5	1	0	0.5	0	0	0	0
ShM10.3C	0	0	0	0	0	0	0	0	0	0
ShM13.3A	2	2	2	2	2	2	2	2	1.5	1.5
ShM13.3B	2	2	2	2	2	2	1.5	1.5	0	0
ShM13.3C	1	1	1	0	0	0	0	0	0	0
ShU4A	1.5	1	0.5	0	0	0	0	0	0	0
ShU4B	2	1.5	0.5	0	0	0	0	0	0	0
ShU4C	0.5	0	0	0	0	0	0	0	0	0
ShU6A	0	0	0	0	0	0	0	0	0	0
ShU6B	1	0.5	0	0	0	0	0	0	0	0
ShU6C	1	0	0	0	0	0	0	0	0	0


ShM1.3
ShM10.3
A: Serum
B: IgG preparation
C: IgG-depleted serum
ShM2.1
ShM13.3
ShM3.3
ShU4
ShM5.4
ShU6
Fusion score
2.0
1.0
0.0
-1.0
-2.0
4
8
16
32
64
128
256
500
1000
2000
Reciprocal dilution

It is interesting to note that ShM13.3C and ShM1.3C, which were the flow-through fractions with the most enhancing activity in the fusion assay, were two of the three samples which still contained measurable amounts of IgG.

IgG prepared from ShU4 and ShU6 exhibited fusion enhancing activity only down to dilutions of 1:16 and 1:8 respectively, whilst enhancement by the ShM sera titred out at 1:128-1:500. Although the ShU results suggest that specific anti-MVV antibodies are not necessarily required for fusion enhancement, the difference in titres between ShU and ShM does indicate that antibodies against MVV are important. The lowest concentration of IgG required to enhance fusion can be estimated by dividing the protein concentration in the IgG preparation by the highest dilution which had a positive score in the fusion assay. For the MVV seropositive group this figure ranged from 3-24μg/ml, compared to 50-140μg/ml for the uninfected group. Admittedly the numbers tested here were too small for the difference to be statistically significant.

The fact that syncytium inhibition was seen by sera but not by IgG may be explained by the dilution factor between the amount of IgG normally found in serum and the concentration at which it could be purified. Since the sheep sera only have fusion blocking activity at low dilutions a good recovery of IgG from the serum is neccessary to attempt to show a similar effect. if the activity is indeed IgG mediated. The concentration of IgG eluted from the column varied between 1/3 and 1/13 of the concentration of IgG in normal serum.

There are some unexpected results in Figure 5.6: ShM2.1A serum has little effect in the assay whilst the IgG prep ShM2.1B enhances fusion. The same is seen for ShU6 and ShM3.3. Since IgG-depleted serum does not inhibit fusion, it does not appear to contain factors acting directly with cells or virus to inhibit fusion. Thus it appears that factors in serum may interact with IgG and block the activity that enhances syncytium formation when IgG is tested in the fusion assay in the absence of other serum factors.

ShM1.3 serum was previously shown to block syncytium formation at high concentrations and to enhance at low concentrations, yet in this assay only enhancing activity was seen. This is an extreme case in the variability of the fusion assay.

5.3.4: **The 4°C fusion assay**

The 4°C fusion assay was devised to try to distinguish between inhibition of MVV-mediated fusion by prevention of binding of the virus to the cell surface or by a mechanism which specifically blocks the fusion event. The 37°C assay where virus is preincubated with antibody cannot distinguish between the two, and at 37°C the interval between virus binding to the cell and membrane fusion taking place may be to short to assay for factors acting upon the post-binding fusion event. At 4°C cell membranes lose their fluidity and membrane fusion is prevented, or at least greatly slowed, but binding of virus to the cell surface can happen at this temperature. The hypothesis is that by binding virus at 4°C and removing any unbound virus before adding serum and shifting to 37°C that factors which affect fusion should be detectable which may otherwise be masked by factors which affect viral binding.

In the 4°C fusion assay monolayered YT40 cells were incubated with high titre MVV at 4°C for 60 min. Unbound virus was washed off using cold DME2. Dilutions of serum were added initially at 4°C then the plates were incubated at 37°C overnight. The effect of the serum on fusion-from-without was scored by comparison with wells to which virus but no serum was added. Preliminary experiments were done to optimise the cell density and virus concentrations. It was neccessary to have the cells slightly overconfluent as they "shrank back" during incubation at 4°C so that a less mature monolayer did not form syncytia during subsequent incubation at 37°C. In addition the concentration of MVV virus used had to be increased to allow for the fact that much of the virus remained unbound and was removed from the culture.

As can be seen in Figure 5.7 the results of the 4°C assay were different from the results of the 37°C assay. For example ShM4.1 blocked fusion in the 37°C assay but enhanced in the 4°C assay, whilst ShMU7 and ShMU8 were both neutral at 37°C but enhanced at 4°C. ShU6 inhibited syncytium formation in the 4°C assay.

The results showed that enhancement of syncytium formation can occur after the virus has already been bound to the cell surface. Sera that inhibit fusion in the 37°C but not in the 4°C assay probably act by blocking binding of the virus to the cell.

Statisical analysis revealed a significant difference between sera from the ShU and ShM populations with respect to fusion modulatory activity at dilutions 1:32 and 1:128, but not at 1:8, 1:16, nor 1:64. The sera from MVV-infected sheep tended to have greater enhancing activity in the 4°C fusion assay than sera from uninfected controls.

Figure 5.7: Comparison of results from the 4°C and 37°C fusion assays.

The table below shows the results of the 4°C fusion assay for seven sera from sheep from an MVV-infected flock and four sera from uninfected control sheep.

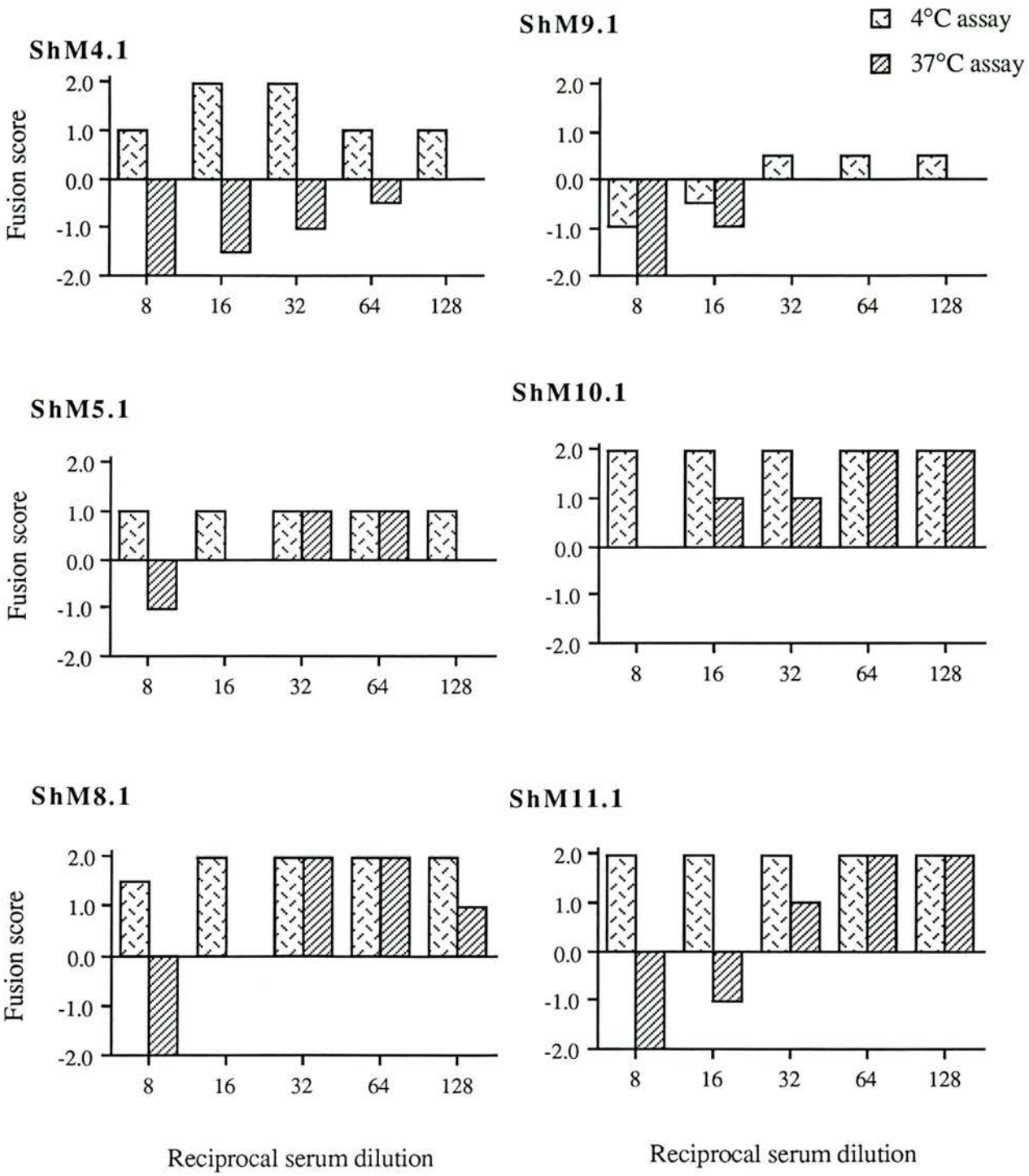
The bar graphs opposite illustrate the results of the 4°C fusion assay alongside the results of the 37°C fusion assay (shown in figure 5.2).

Serum	Reciprocal serum dilution				
	8	16	32	64	128
ShM4.1	1	2	2	1	1
ShM5.1	1	1	1	1	1
ShM8.1	1.5	2	2	2	2
ShM9.1	-1	-0.5	0.5	0.5	0.5
ShM10.1	2	2	2	2	2
ShM11.1	2	2	2	2	2
ShM14.1	-1	0	0	0	1
ShU5	-1.5	0	0	0	0
ShU6	1	1	0	1	0
ShU7	0	0	0	1	1
ShU8	0	0	0	0	0

Statistics

	Reciprocal serum dilution				
	8	16	32	64	128
ShU mean	-0.125	0.25	0	0.5	0.25
ShU variance	0.797	0.187	0	0.25	0.187
ShM mean	0.785	1.214	1.357	1.214	1.357
ShM variance	1.418	0.990	0.622	0.561	0.336
t value	1.244	0.055	3.17	1.58	3.109

Critical value $t_{0.05}$ (9 degrees of freedom) = 2.26


ShM4.1
ShM9.1
4°C assay
37°C assay
ShM5.1
ShM10.1
ShM8.1
ShM11.1
Fusion score
2.0
1.0
0.0
-1.0
-2.0
8
16
32
64
128
Reciprocal serum dilution

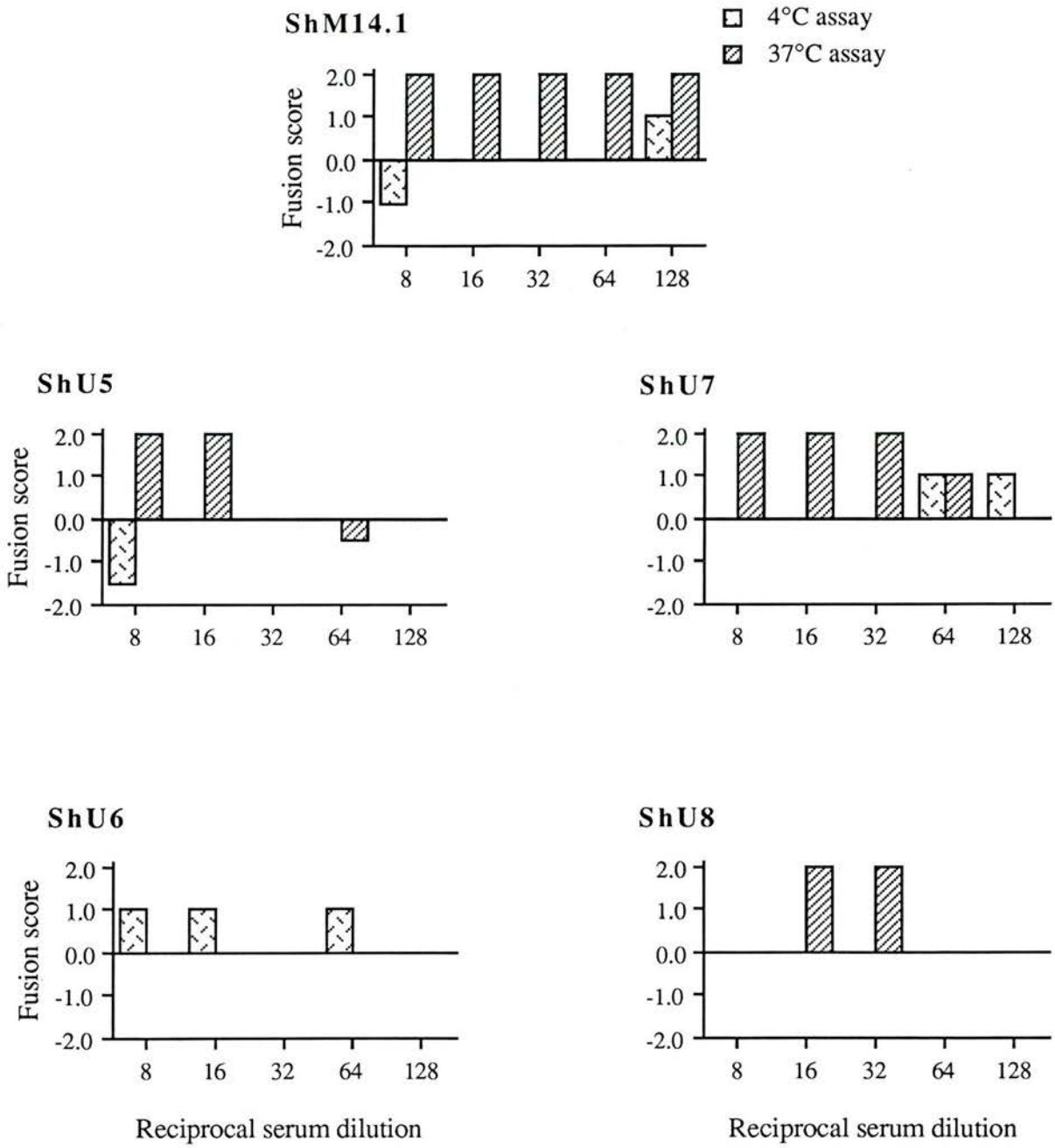

ShM14.1
4°C assay
37°C assay
Fusion score
2.0
1.0
0.0
-1.0
-2.0
8
16
32
64
128
ShU5
ShU7
ShU6
ShU8
Reciprocal serum dilution
Reciprocal serum dilution

Figure 5.8: 4°C fusion assay results- selected sera Ig preparations.

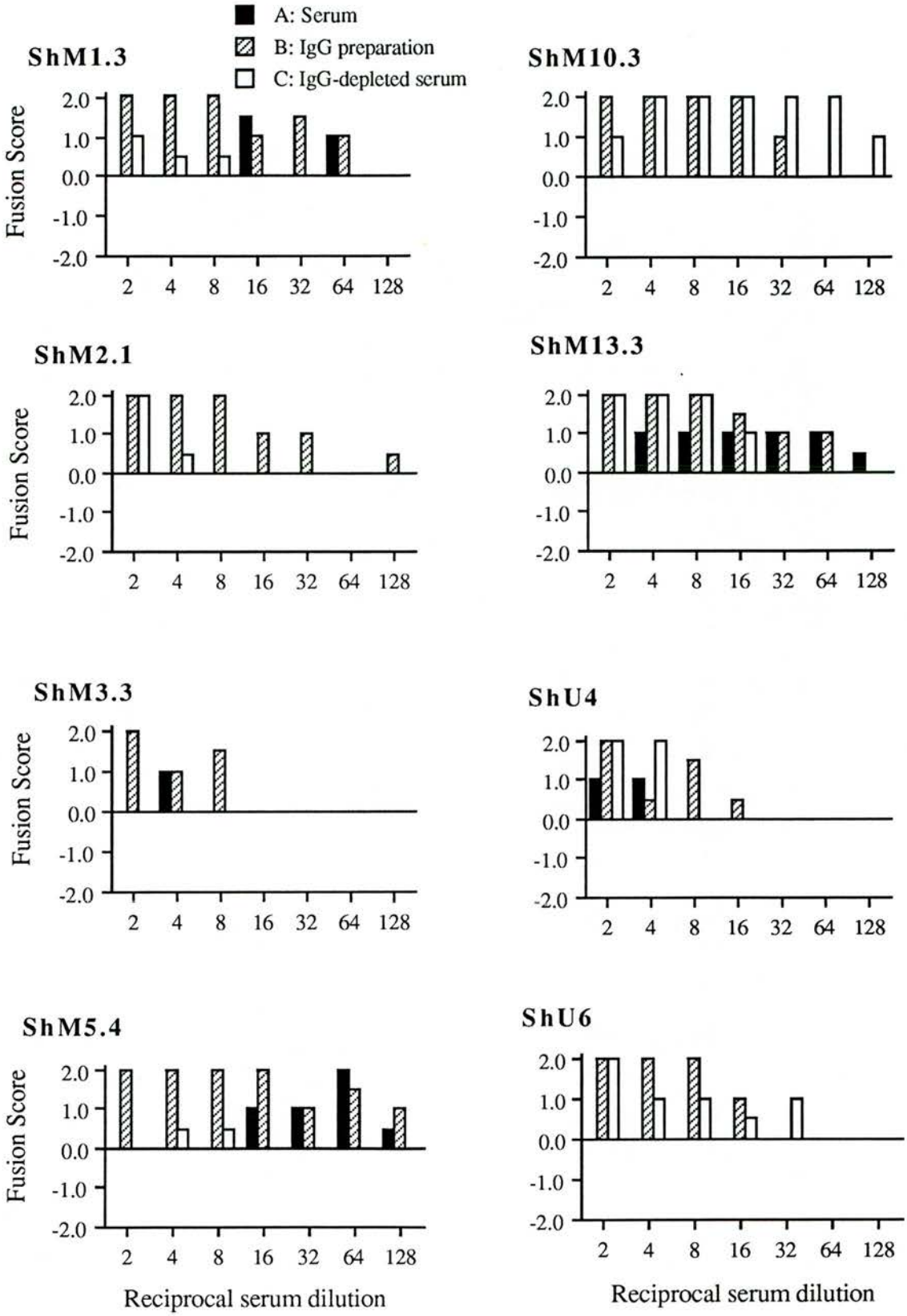
The results are tabulated below and illustrated as bar charts opposite.

KEY:- A-Serum.

B-Ig eluted from Sepharose-G column.

C-Serum depleted of IgG (i.e. flow-through from Sepharose-G column)

	Reciprocal Dilution						
	2	4	8	16	32	64	128
ShM1.3A	0	0	0	1.5	0	1	0
ShM1.3B	2	2	2	1	1.5	1	0
ShM1.3C	1	0.5	0.5	0	0	0	0
ShM2.1A	0	0	0	0	0	0	0
ShM2.1B	2	2	2	1	1	0	0
ShM2.1C	2	0.5	0	0	0	0	0
ShM3.3A	0	1	0	0	0	0	0
ShM3.3B	2	1	1.5	0	0	0	0
ShM3.3C	0	0	0	0	0	0	0
ShM5.4A	0	0	0	1	1	2	0.5
ShM5.4B	2	2	2	2	1	1.5	1
ShM5.4C	0	0.5	0.5	0	0	0	0
ShM10.3A	1	2	2	2	2	2	1
ShM10.3B	2	2	2	2	1	0	0
ShM10.3C	1	0.5	1	0	0	0	0
ShM13.3A	0	1	1	1	1	1	0.5
ShM13.3B	2	2	2	1.5	1	1	0
ShM13.3C	2	2	2	1	0	0	0
ShU4A	1	1	0	0	0	0	0
ShU4B	2	0.5	1.5	0.5	0	0	0
ShU4C	2	2	0	0	0	0	0
ShU6A	0	0	0	0	0	0	0
ShU6B	2	2	2	1	0	0	0
ShU6C	2	1	1	0.5	1	0	0


A: Serum
B: IgG preparation
C: IgG-depleted serum
ShM1.3
ShM10.3
ShM2.1
ShM13.3
ShM3.3
ShU4
ShM5.4
ShU6
Fusion Score
2.0
1.0
0.0
-1.0
-2.0
2
4
8
16
32
64
128
Reciprocal serum dilution

Eight IgG preparations, as described previously, were tested in the 4°C assay alongside whole serum and IgG depleted serum (Figure 5.8). All eight of the IgG preparations enhanced fusion whilst only three of the eight serum samples showed enhancing activity at more than two different dilutions. This suggests that, as in the 37°C fusion assay, other factors in serum may mask the enhancing activity of the IgG which it contains.

5.3.5: **Effect of rgp46 on syncytium formation.**

Two-fold serial dilutions of GST:TH, GST:TH digested with thrombin, or a range of appropriate controls were added to monlayered YT40 cells with or without sufficient virus to cause fusion-from-without overnight, in order to investigate whether recombinant gp46(TH) could itself mediate membrane fusion or enhance fusion at 37°C by MVV virus . TH, at the concentration available, (about 50µg/ml GST:TH), had no apparent affect on the cells without virus, when compared to control wells incubated with medium alone. In the presence of MVV, the recombinant GST:TH appeared to neither enhance nor reduce syncytium formation.

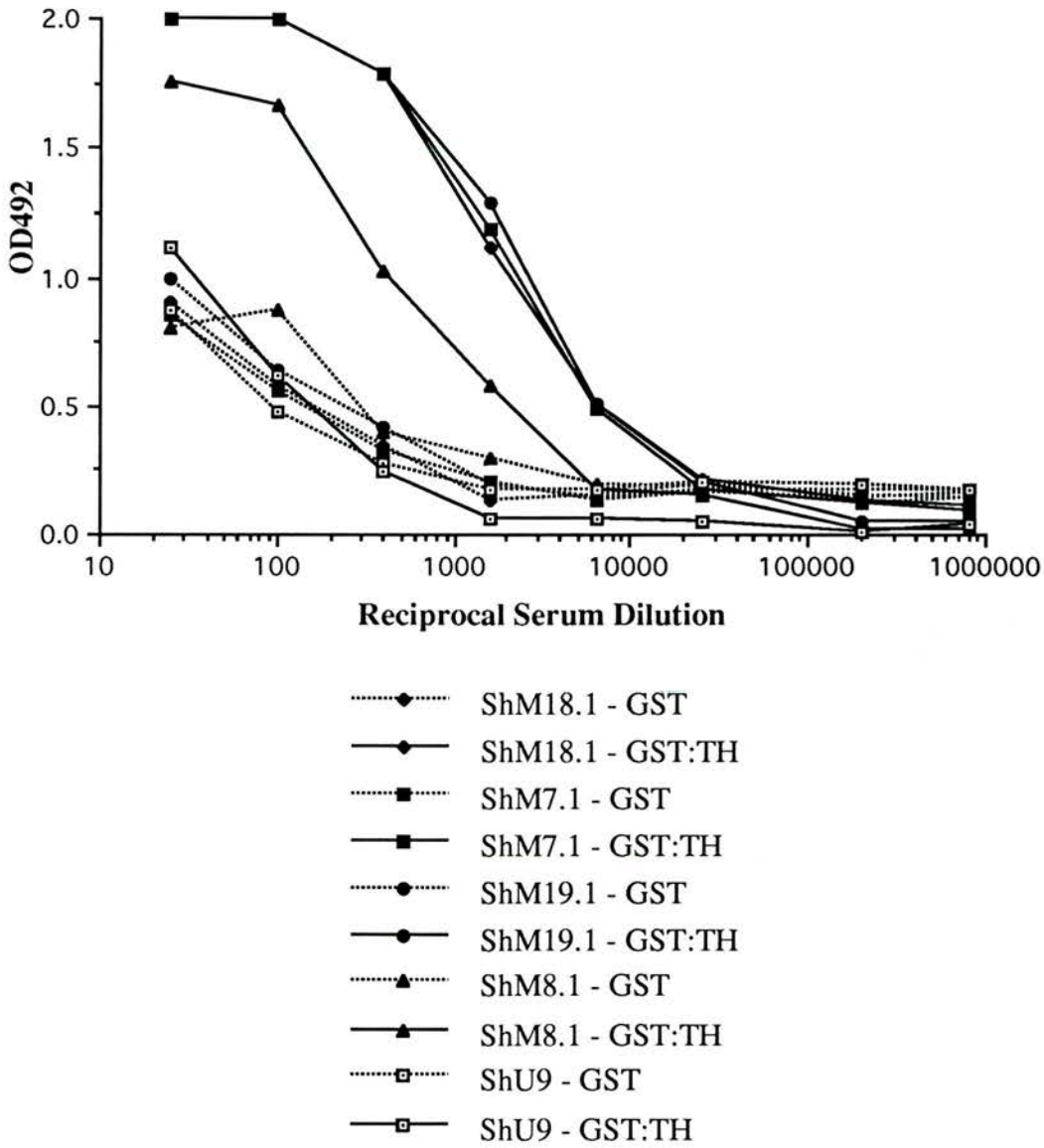
Ideally a higher concentration would have been used for this experiment. Larger amounts of recombinant gp46 would also have enabled an experiment to mop up anti-gp46 activity from sheep sera/IgG preparations to see how this affected the results of the fusion assays.

5.3.6: **Immunoreactivity of sheep sera against recombinant gp46 ;- ELISA against GST:TH**

The results of an ELISA of sera from four MVV-infected sheep (as assessed by AGIDT) and one negative control against GST or GST:TH antigen is shown in Figure 5.9. The MVV negative serum reacted to a similar low titre with GST as with GST:TH which confirmed the amount of antigen in each preparation was similar. The MVV-positive sheep had antibodies reactive more strongly with GST:TH than with GST suggesting a specific reactivity with the TH moiety by the sheep with antibodies against MVV.

Figure 5.9: ELISA of sheep sera against GST:TH or GST

As an initial assessment of the ELISA, sera from a small number of MVV-infected (ShM) and one uninfected sheep (ShU) were tested for reactivity against recombinant gp46 TH by comparison of ELISA against GST or GST:TH fusion protein.



A larger group of sheep sera, including some serial samples taken at 6 weekly intervals, from 18 sheep from a MVV-infected flock and 7 sheep from a negative control flock were tested against GST and GST:TH by ELISA (Figure 5.10). None of the sheep had high levels of activity against GST and all were more reactive against GST:TH than against GST. The difference between reactivity against GST and GST:TH ELISA varied from sheep to sheep. If the OD_{460} values for ELISA against GST are subtracted from those for ELISA against GST:TH, to compensate for any reactivity against GST, then the ShM and ShU sheep form two overlapping groups with the majority of the ShM sheep having higher reactivity against TH than sheep from the uninfected group.

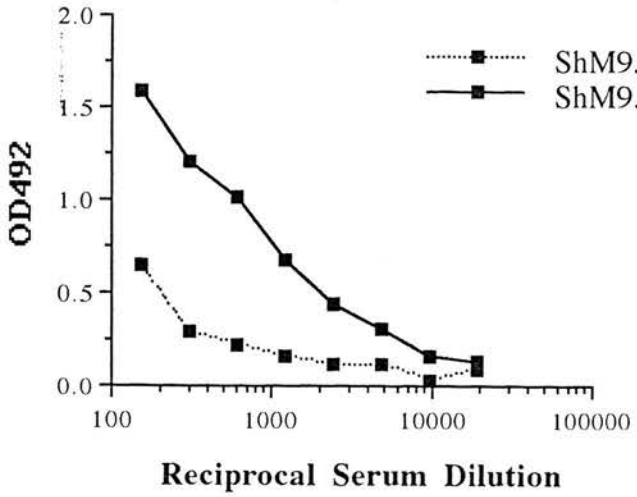
5.3.7: **Correlation of ELISA and fusion assay results**

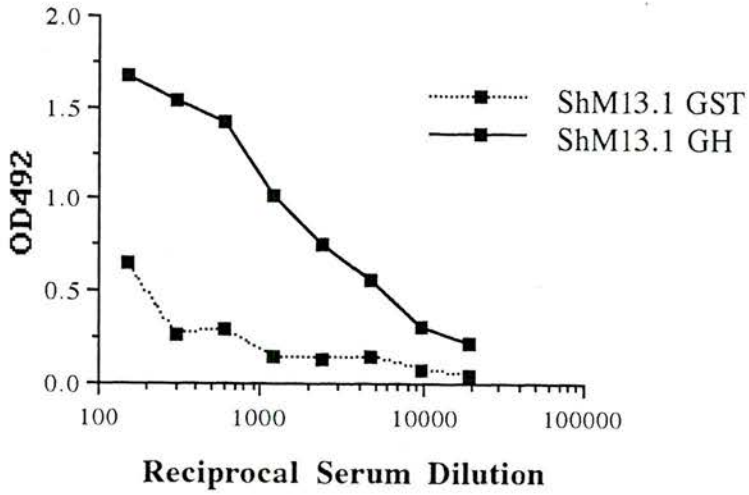
A scatterplot (Figure 5.11) was drawn to see if the ELISA results correlated with results of the fusion assay. The difference between the ELISA OD_{460} against GST:TH and against GST at a serum dilution of 1:300 was calculated. (This value is described below as the "ELISA value"). This value was plotted against the fusion scores obtained with the same serum at 1:8 or 1:128 dilution (Figure 5.2). 1:300 dilution was chosen as the differences between GST and GST:TH ELISA results were greatest at this dilution. Fusion assay results for 1:8 and 1:128 dilutions were chosen since they were both values at which there was a significant difference between the uninfected and MVV-seropositve groups.

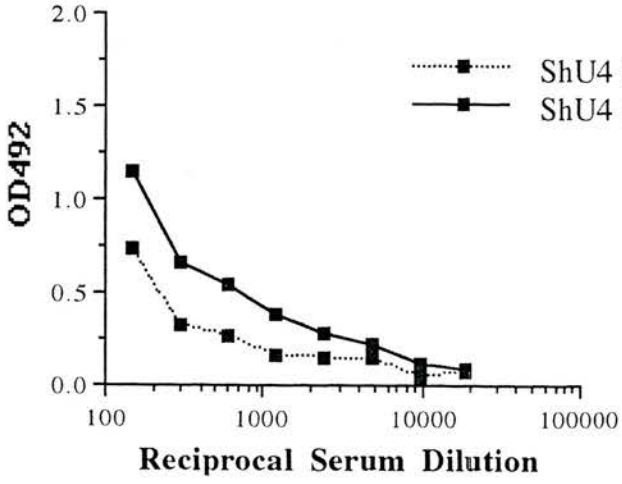
Sera which enhanced fusion at 1:128 dilution all had relatively high ELISA values in the range 0.7-1.4 whilst sera which had no effect in the fusion assay at 1:128 spanned a range from 0.1-1.4. In contrast, sera which enhanced fusion at 1:8 dilution had low ELISA values whilst sera which inhibited fusion had high ELISA values. Thus high ELISA values tend to correlate with fusion inhibition at high serum concentrations and fusion enhancement at lower serum concentrations. The scattergraphs also show that, although there were some exceptions, sera from uninfected sheep tended to have low ELISA values, have enhancing activity in the fusion assay at high serum concentrations, but no effect upon fusion at low serum concentrations., whilst sera from MVV seropositive sheep spanned a broader range of results in both assays.

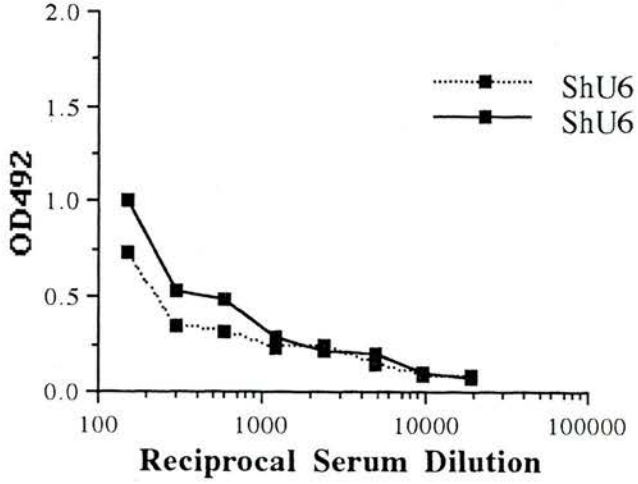
Figure 5.10: ELISA of sheep sera against GST:TH or GST (ii).

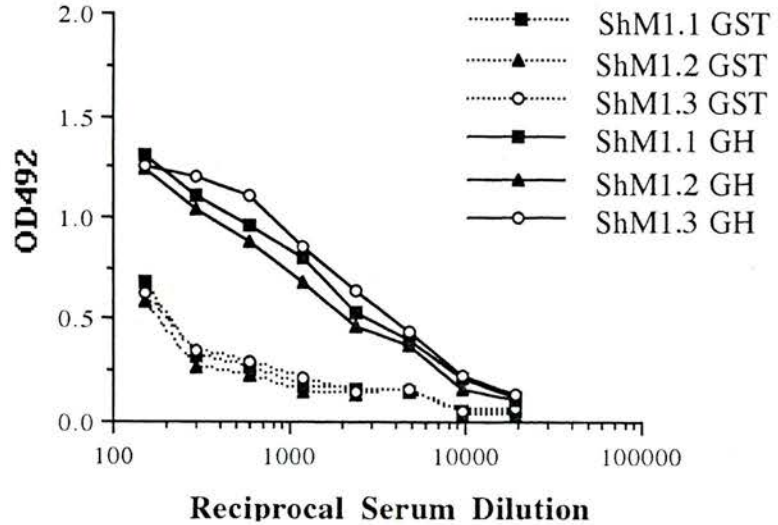
The reactivity of a larger group of sheep sera was tested against GST or GST:TH by ELISA. Eighteen sera tested were from sheep from the MVV-infected flock and seven were from sheep from the uninfected control flock. The results for sera from the six MVV-positive and two uninfected controls used in the previous experiments on fractionated sera are shown here.

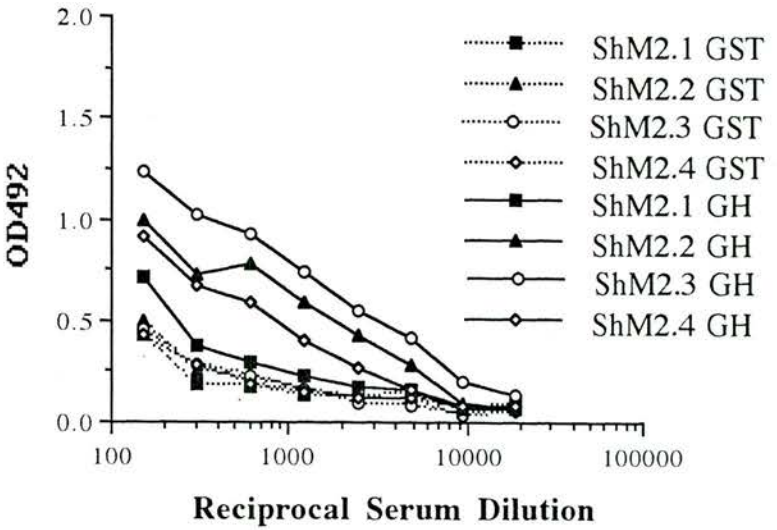

ShM9.1 GST
ShM9.1 GH
OD492
2.0
1.5
1.0
0.5
0.0
100
1000
10000
100000
Reciprocal Serum Dilution

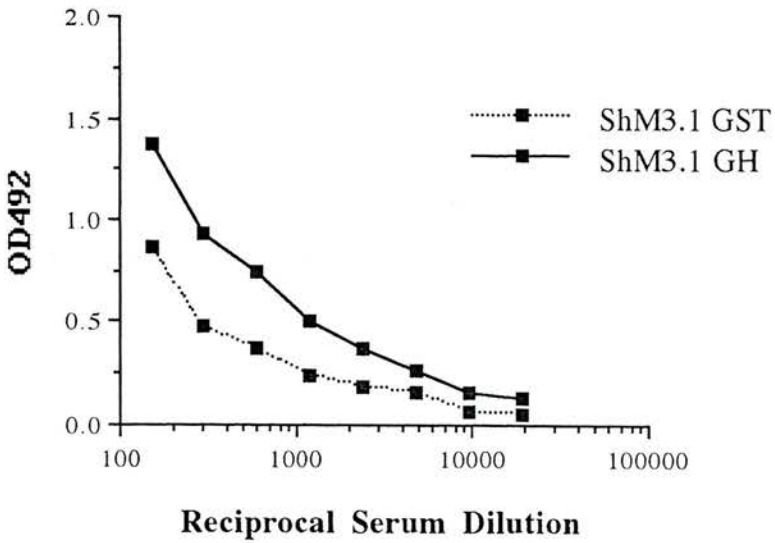

ShM13.1 GST
ShM13.1 GH
OD492
2.0
1.5
1.0
0.5
0.0
100
1000
10000
100000
Reciprocal Serum Dilution


ShU4 GST
ShU4 GH
OD492
2.0
1.5
1.0
0.5
0.0
100
1000
10000
100000
Reciprocal Serum Dilution


ShU6 GST
ShU6 GH
OD492
2.0
1.5
1.0
0.5
0.0
100
1000
10000
100000
Reciprocal Serum Dilution


ShM1.1 GST
ShM1.2 GST
ShM1.3 GST
ShM1.1 GH
ShM1.2 GH
ShM1.3 GH
OD492
2.0
1.5
1.0
0.5
0.0
100
1000
10000
100000
Reciprocal Serum Dilution


ShM2.1 GST
ShM2.2 GST
ShM2.3 GST
ShM2.4 GST
ShM2.1 GH
ShM2.2 GH
ShM2.3 GH
ShM2.4 GH
OD492
2.0
1.5
1.0
0.5
0.0
100
1000
10000
100000
Reciprocal Serum Dilution


ShM3.1 GST
ShM3.1 GH
OD492
2.0
1.5
1.0
0.5
0.0
100
1000
10000
100000
Reciprocal Serum Dilution

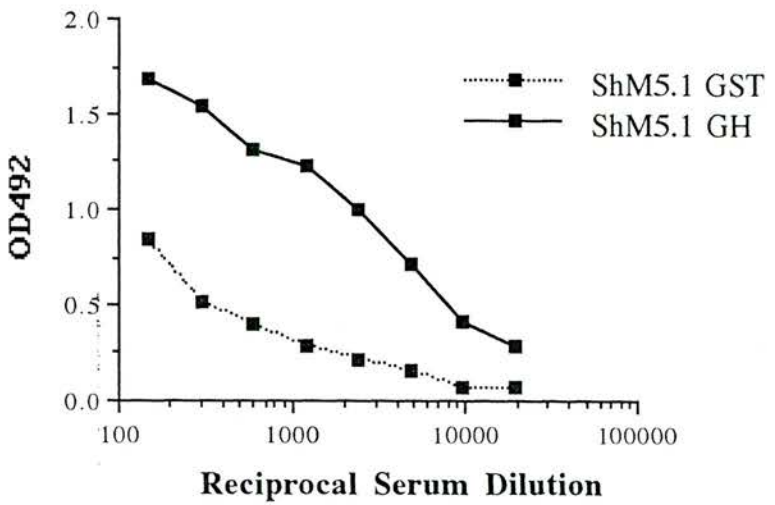
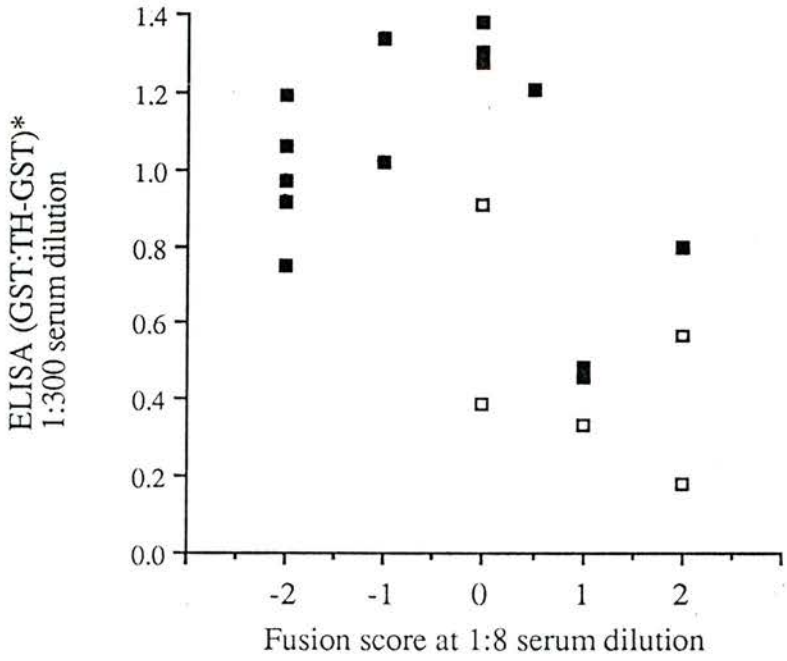

ShM5.1 GST
ShM5.1 GH
OD492
2.0
1.5
1.0
0.5
0.0
100
1000
10000
100000
Reciprocal Serum Dilution

Figure 5.11: Correlation of the results of ELISA against GST:TH with the results of the fusion assay.

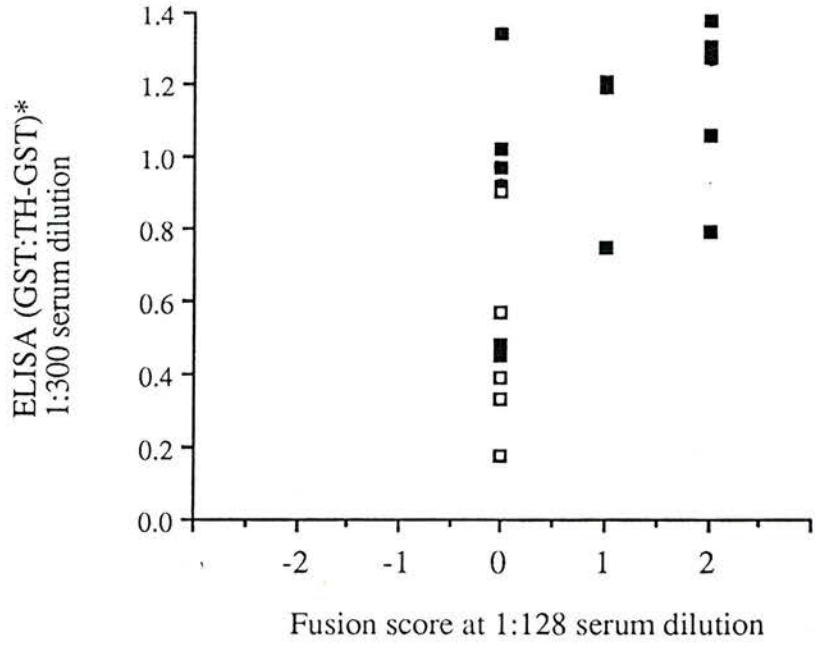
The anti-GST:TH activity of each sera was estimated by deducting the ELISA result against GST from the ELISA result against GST:TH: for each individual sheep an "ELISA value" was calculated by deducting the result (OD_{460}) of ELISA against GST at a serum dilution of 1:300, from the result of ELISA against GST:TH (1:300 serum dilution). This value was plotted against the fusion scores obtained with the same serum at 1:8 or 1:128 dilution (from Figure 5.2).

Open squares on the scatterplots represent sera from uninfected sheep, black squares represent sera from MVV-infected sheep.

Scatterplot: ELISA results at 1:300 serum dilution against fusion assay results at 1:8 dilution



Scatterplot: ELISA results at 1:300 serum dilution against fusion assay results at 1:128 dilution



5.3.8: **Immunoreactivity of sheep sera sheep against recombinant gp46, GST:TH, on western blots**

Sheep sera were reacted with Western blots of GST or GST:FINV. As can be seen from Figure 5.12, nearly all the sera reacted against a protein running just below the 67kDa marker in both GST and GST:FINV preparations. This appears to be the bacterial protein identified as a major contaminant in preparations made from GF21- (see Figure 4.2) and which was also present under some conditions in preparations from other clones. Only sera from MVV-positive sheep reacted with the 43kDa protein predicted to be GST:TH, with one MVV-negative exception, ShU3. ShU3 also reacted with a band below 30 kDa which was probably endogenous GST. Thus activity by ShU3 against the GST:TH product can be explained by recognition of the GST moiety. This is a clear indication that recognition of recombinant gp46 (TH) is specific to sheep seroreactive with MVV. Seven of the nine ShM sera tested reacted with GST:TH, which suggests that a high proportion of MVV-infected sheep raise antibodies against the external half of gp46. The recombinant protein is probably unglycosylated suggesting these antibodies are not dependent upon glycosylation of the protein for their reactivity.

There was also some minor antibody reactivity against other bands which merely confirms the presence of contaminating bacterial proteins in the GST:TH preparation, as was visualised on silver stained SDS-PAGE gels (Figure 4.3a).

5.3.9: **ELISA of sheep sera against GST:TH with GST:FINV as control antigen**

Western blotting against GST and GST:FINV, (Figure 5.12), showed that sera from both ShU and ShM groups reacted with some of the contaminating bacterial proteins in the preparations. The GST preparation contained very little contaminating E.coli protein whereas GST:FINV contained a similar proportion of contaminating bacterial proteins to the GST:TH preparation. Therefore GST:FINV is a better negative control antigen for ELISA than GST.

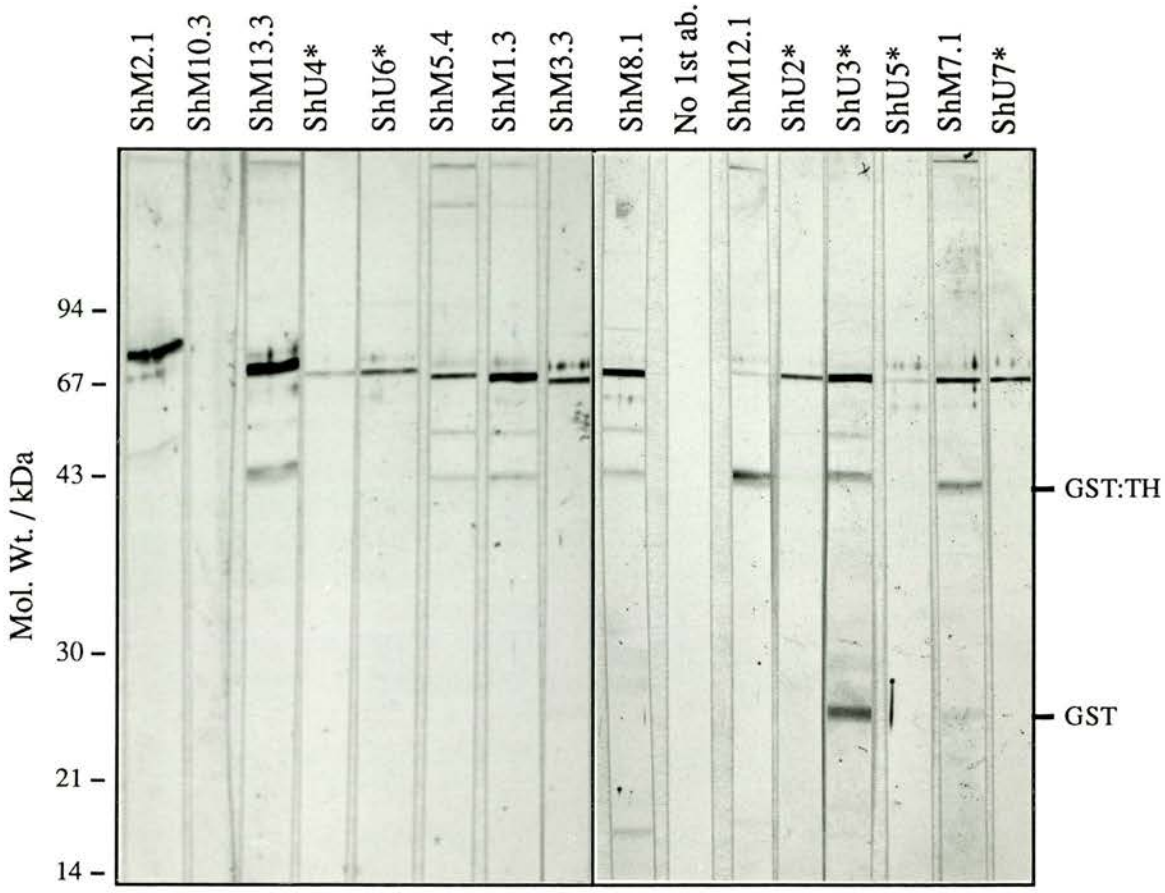
The eight IgG preparations, described in Figure 5.5 were assayed by ELISA against GST:TH and GST:FINV (Figure 5.13). The results for each serum differed little between the two antigens. Consequently the difference found in ELISA activity against GST:TH compared to GST cannot be concluded to be necessarily due to activity against the TH moiety as reactivity against the additional bacterial proteins present in the GST:TH preparation cannot be ruled out. This does not negate the correlation between ELISA and fusion assay results described in Figure 5.11, but does alter the interpetation of the results in that a high ELISA value cannot

Figure 5.12: Reactivity of sheep sera with western blots of GST:TH.

Sera from MVV-infected sheep, (ShM), or from uninfected sheep, (ShU),were reacted with Western blots of a) GST:TH or b) GST:FINV. (Sera from uninfected sheep are further emphasised with a *).

The constituents of GST:TH or GST:FINV preparations were separated by SDS-PAGE on a 10% polyacrylamide preparative gel and western blotted onto nitrocellulose. Strips were developed with sheep sera at 1:200 and anti-sheep alkaline phosphatase conjugate at 1:1000.

a)


ShM2.1
ShM10.3
ShM13.3
ShU4*
ShU6*
ShM5.4
ShM1.3
ShM3.3
ShM8.1
No 1st ab.
ShM12.1
ShU2*
ShU3*
ShU5*
ShM7.1
ShU7*
Mol. Wt. / kDa
94
67
43
30
21
14
GST:TH
GST

b)

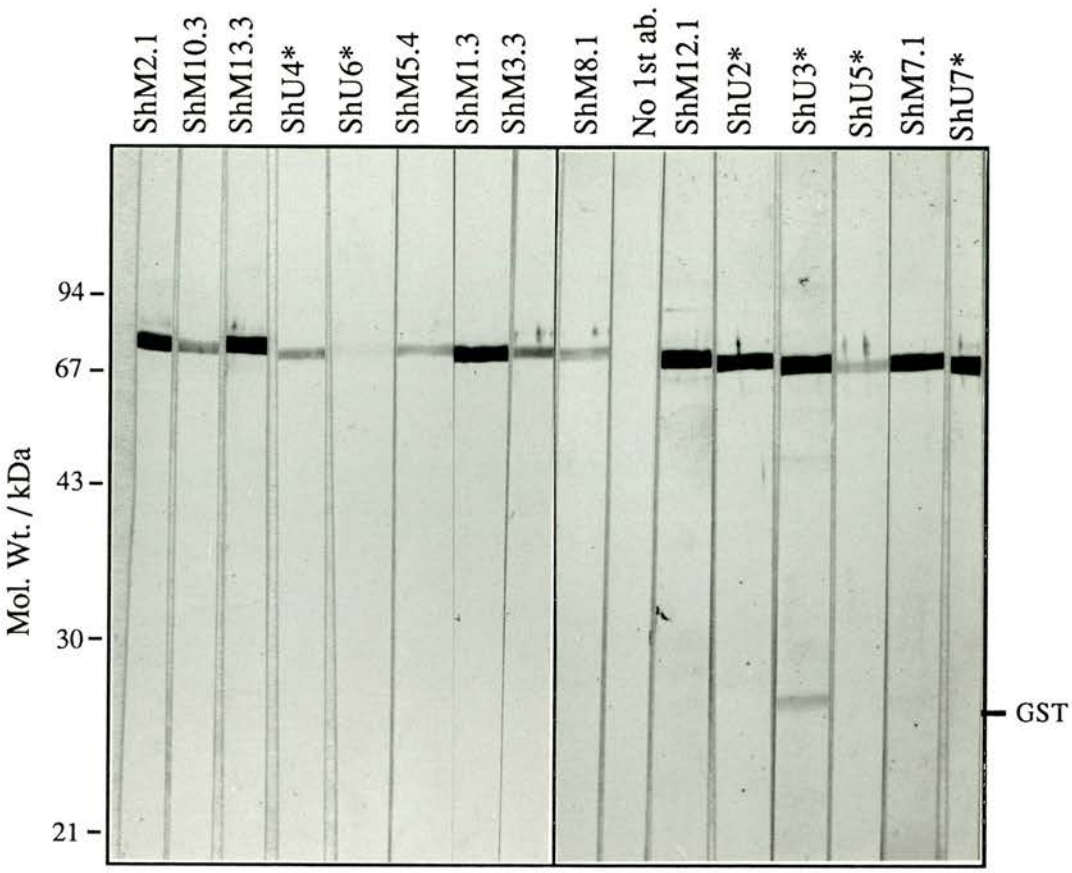
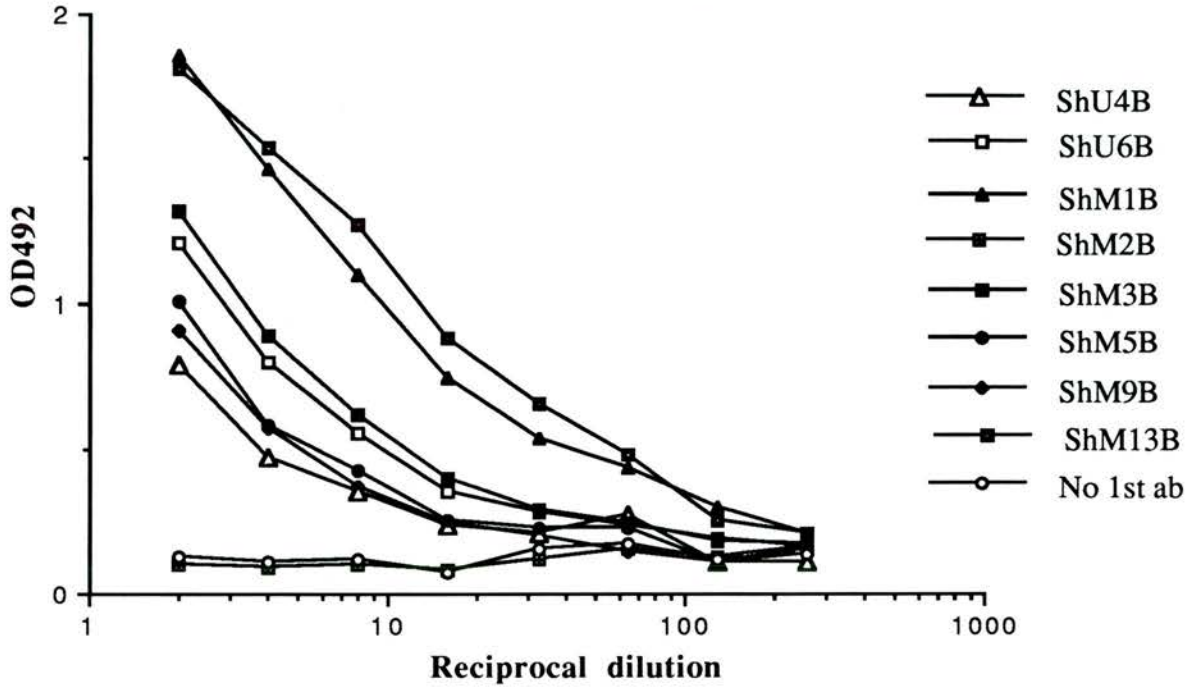

ShM2.1
ShM10.3
ShM13.3
ShU4*
ShU6*
ShM5.4
ShM1.3
ShM3.3
ShM8.1
No 1st ab.
ShM12.1
ShU2*
ShU3*
ShU5*
ShM7.1
ShU7*
Mol. Wt. / kDa
94
67
43
30
21
GST

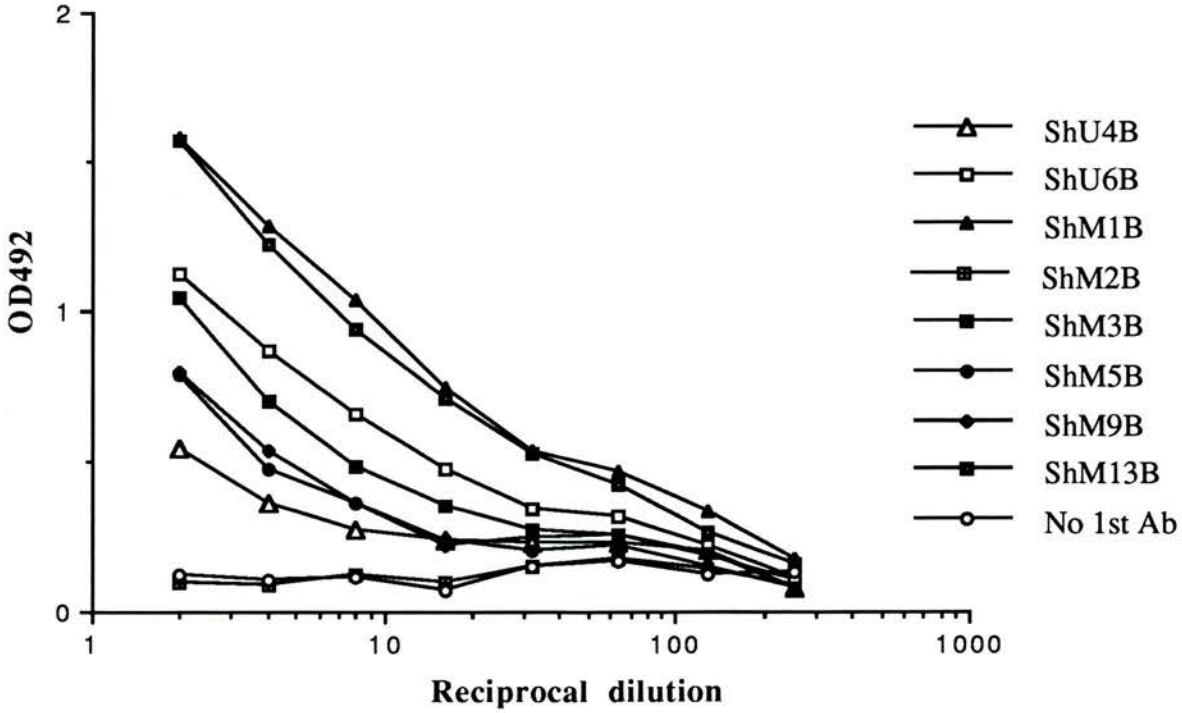
Figure 5.13: ELISA of IgG from MVV-infected or uninfected sheep against GST:TH or GST:FINV

IgG prepared from the serum of six MVV-infected (ShM) and two uninfected sheep (ShU) were tested for reactivity against recombinant gp46 TH by comparison of ELISA against GST:TH fusion protein or GST:FINV as control.

a) GST:TH



b) GST:FINV (Control)



be assumed to be due solely to anti-rgp46 activity. If the main reactivity measured by the ELISA was directed against contaminating bacterial proteins then it could be concluded that, in general, sera from MVV-infected sheep had greater reactivity with bacterial proteins than sera from uninfected sheep. Another explanation would be that the first ELISA did indeed detect substantial anti-TH activity but that much of this could have been mediated by IgM rather than IgG.

5.3.10: **Summary of results from fusion assays and antibody detection.**

Figure 5.14 summarises the analysis of activity of serum from a small group of sheep from an MVV-infected flock and two sheep from an uninfected flock. It is clear that the antibody reactivity against whole MVV as measured in the Ministry of Agriculture AGIDT correlates with activity in the fusion assay when comparing seropositive to seronegatives. However, the degree of positivity in the AGIDT, i.e., +/-, +, or ++, is not predictive of what activity the individual serum will have in the fusion assay.

Since antibodies against the external envelope protein are also known to interfere with virus fusion it is not surprising that although the majority of ShM sera are reactive against rgp46 TH on Western blots, (Figure 5.11), this does not show a complete correlation with activity in the fusion assays.

5.3.11: **Correlates of clinical status**

Figure 5.16 summarises the results from the larger group of sheep sera. Unfortunately the data is incomplete in places especially concerning reactivity to EV1-lysate on Western blots. The final column gives a crude indication of the clinical status of the sheep and is derived from data compiled by Dr N.J. Watt and shown in Figure 5.15. A +/-scoring system was used to grade the severity of pathological lesions found at post-mortem in the lung, CNS, and mammmary tissue of each sheep. The score for pathology status was derived by adding the total number of +s, thus a higher pathology score indicates more serious disease. There is no apparent correlation between fusion activity and disease in individual sheep. However this is by no means automatic e.g. sera from sheep ShM13 and ShM14 both have high enhancing activity (+2) right down to a 1:500 dilution in each of the serial samples yet at post-mortem ShM14 had no significant clinical pathology (score = 0) whilst ShM13 had pathology of maedi in the lung and also had mastitis (score = 4).

Figure 5.14 Clinical-pathological status of MVV-infected sheep *

Sheep No.	Year of Birth	Date of PM†	Respiratory Maedi	Respiratory Other	CNS Visna	Mammary Mastitis	PATH. SCORE
ShM1	1986	6.5.91	++	-	-	++	4
ShM2	1989	A					
ShM3	1986	7.3.91	+	-	-	++	3
ShM4	1986	22.1.91	-	-	-	++	2
ShM5	1986	A					
ShM6	1986	31.5.93	(sudden death, no PM).				
ShM7	<1985	29.5.92	+++	+	-	+	5
ShM8	1986	11.12.92	-	-	meningioma	-	1
ShM9	1986	17.6.91	+	fibrosis	-	-	2
ShM10	1986	A					
ShM11	<1985	24.6.91	-	-	-	ND	0
ShM12	1986	24.6.91	-	-	-	ND	0
ShM13	<1985	24.4.91	+++	-	-	+	4
ShM14	1986	16.9.91	-	-	-	-	0
ShM15	1987	9.9.92	+++	-	-	+	4
ShM16	1986	18.12.90	+	-	-	+++	4
ShM17	1987	<1.11.90	++	-	+	+	4
ShM18	1986	29.5.92	+	++	-	++	5
ShM19	1987	17.3.93	+	-	-	-	0
ShM20	<1985	12.6.92	-	oedema	-	-	1

* Score of disease severity from clinical data compiled at post-mortem by Dr N.J.Watt.

† Date of post-mortem. Sheep not yet post-mortemed are shown as A (Alive).

Figure 5.15: Summary of activity of sheep sera.

	AGIDT*	Western blots				Fusion assay†		Pathology
		GST	GST:TH	TH	MVV	1:8	1:256	score∞
ShM1	+		-	-	p24+	b	e	4
ShM2	+/-	-	-	-		0	0	ND
ShM3	+/-					b	0	3
ShM4	++	-	?	+		b	e	2
ShM5	+				p24+	b	e	ND
ShM6	+				+	b	e	ND
ShM7	++	-	+	+		0	e	5
ShM8	++	-	+	+		b	e	1
ShM9	+		+			b	e	2
ShM10	+		+	+		e	e	ND
ShM11	++			+		b	e	0
ShM12.1	+			+		b	e	0
ShM13	++					e	e	4
ShM14	+			+		e	e	0
ShM15						b	b	4
ShM16	?			+		b	e	4
ShM17	?			-		e	0	4
ShU2	-	-	-	+		e	0	
ShU4	-	-	-	-	-	e	0	
ShU5	-	-	-	-		e	0	
ShU6	-	-	-		-	0	0	
ShU7	-	-	-	-		e	0	
ShU8	-		+	-		0	0	

* AGIDT Agar gel immunodiffusion test for seroreactivity against whole MVV

† Fusion assay (37°C) results are shown for 1:8 and 1:256 serum dilutions and are summarised as e (fusion enhancing), b (fusion blocking), and 0 (no effect on MVV induced fusion).

∞ Pathology scores are taken from Figure 5.14

Chapter 5:

DISCUSSION

5.4.1: **The fusion assay**

The fusion assay was devised as a read out for virus/cell fusion to ask whether serum from infected sheep could modulate cell fusion by MVV. Although fusion-from-without (FFWO) by virus at high m.o.i. has been used in previous studies of viral fusion the mechanism by which the actual fusion event occurs is not known. It has been assumed to be the same mechanism as for initial viral entry. The assumption may be correct if syncytia are formed during FFWO by individual virus particles fusing with more than one cell at the same time. This could explain why confluent cells and high concentrations of virus are necessary for FFWO as this would maximise the chances of virus particles landing between two adjoining cells. An alternative mechanism for FFWO may be that the virus binds to and fuses first with one cell and subsequently mediates fusion with a neighbouring cell. Fusion with the first cell would release the capsid into the cytoplasm and the viral envelope would become incorporated into the cell cytoplasmic membrane. If the envelope proteins remain functional and are laterally mobile within the membrane they may move to an area of contact with an adjacent cell and may be able to mediate fusion between the two membranes. High concentrations of virus could enhance this process by increasing the chances of multiple infection so that the concentration of envelope proteins on the infected cell surface would be increased thereby increasing the probability of fusion with a neighbouring cell. The latter hypothetical pathway is likely to be a slower mechanism than the former.

5.4.2: **Effects of sheep serum on fusion-from-without by MVV**

The fusion assay showed that the individual pattern of fusion modulatory activity was variable from sheep to sheep but taken overall there was a significant difference between the effect of sera from uninfected sheep compared to sera from MVV-seropositive sheep upon cell fusion mediated by MVV. Serum from uninfected sheep generally enhanced fusion at low dilutions of serum (<1:64) and had no effect at higher dilutions, whereas serum from MVV infected animals tended to inhibit fusion at low serum dilutions (<1:16) and enhance at higher dilutions.

The effect of purified IgG compared with the corresponding serum or serum depleted of IgG, in the fusion assay showed that enhancement of MVV syncytium formation was mediated by antibody. IgG from MVV infected sheep was shown to enhance fusion down to concentrations ranging from 3-24μg/ml (total IgG) whereas

the IgG from uninfected sheep enhanced fusion only at greater than 50-140µg/ml. This shows that although non-specific IgG can enhance fusion, IgG which includes antibodies against MVV has greater enhancing activity than non-specific IgG.

Although inhibition of fusion by IgG could not be shown directly this may have been due to dilution of IgG relative to the concentration in serum. As fusion inhibition was not shown by IgG depleted serum where there was no dilution factor relative to whole serum, it may be concluded that IgG is required for fusion inhibitory activity. A requirement for IgG in association with some other serum components cannot be ruled out.

Clearly the effect in the fusion assay was dependent on the dilution of the serum. The relative amount of antibody to virus probably determines the outcome of the of the fusion assay. Sera which inhibited at higher concentrations almost always enhanced fusion at lower concentrations of serum. This is in agreement with reports in the HIV literature that when antibody was in excess fusion/infection could be blocked, whilst low amounts of antibody could actually facilitate infection (Takeda, 1988, Perno *et al.*, 1990).

The 37°C fusion assay could not differentiate between inhibition by blocking of virus attachment or by blocking of fusion. The 4°C fusion assay was designed to try to detect only antibodies which block fusion after binding of the virus to the cell surface. In this assay virus was bound to the cells at 4°C and free virus was removed before addition of serum and shifting to a temperature permissive for membrane fusion. This differs from the 37°C assay where antibody was reacted with virus before adding both to the cells. In the 4°C assay serum was shown also to have either an inhibitory or enhancing effect on FFWO. The fact that enhancement can occur after virus has bound to the cell suggests that increasing binding to the cell surface is not the only mechanism of enhancement . Similarly it is clear prevention of virus binding to the cell surface is not the only mechanism of fusion inhibition.

Possible mechanisms of fusion enhancement and inhibition by serum are discussed in Section 6.7 and 6.8.

5.4.3: **Problems and possible improvements to the fusion assay**

Despite care being taken to try to make the assays reproducible repeated assays did not produce exactly the same results on different occasions, although the general trends remained the same. There are a number of reasons why results may have varied. Although the same cell line was used throughout, the passage number of the cells increased over time. Changes in the cells such as time from previous passage possibly had some effect on the rate of fusion. The results of fusion assays

with serum with the majority of the IgG removed shows that serum factors other than IgG can have an effect. Different batches of FCS may contain different cellular growth factors. However, a single batch of FCS was used for the media throughout these experiments and therefore should not have contributed to variation. The degree of syncytium formation in the control wells remained fairly constant suggesting the amount of virus in different aliquots did not vary significantly.

The relatively subjective scoring system used in this chapter only noted striking effects upon virus mediated syncytium formation: Scoring was by comparison with control wells containing virus without added serum and a score of 0 for similar number and size of syncytia to the control, -1 where the syncytia were significantly fewer/smaller, -2 where no syncytia were visible, +1 where there were larger and more numerous than in the control, and +2 where syncytium formation was enhanced to the extent that virtually all the cells in the monolayer had become fused. (Scores of + or - 0.5 and 1.5 sometimes resulted due to averaging the score from duplicate wells). An assay which measured fusion index by counting the number of nuclei and number of cells in a given field of view could have improved the sensitivity of the assay by detecting more minor effects on fusion. However, given the variability of the assay a fusion index would probably have been no more informative than the +/- system of scoring and the conclusions from the assays would have remained the same, i.e, that sera from MVV-infected and uninfected sheep have different fusion modulatory activities, (as described above).

5.4.4: **Alternatives to the fusion assay**

I devised a method based on FACScan analysis and anti-MVV antibodies to differentiate infected and/or cell with virus bound or fused from uninfected cells Unfortunately the anti-MVV polyclonal rabbit anti-sera tried had anti-cellular activity and therefore the background against uninfected cells was too high for this method to be successful. Now that recombinant MVV envelope proteins are available it should be possible to generate anti-sera or monoclonal antibodies which could be used for this method.

A system which has been used to study membrane fusion is resonance energy transfer or flourescence dequenching. This assay takes advantage of the resonance energy transfer between two fluorescent lipids (N-NBD-PE and N-Rh-PE). When they are both present in the labelled membrane, fusion with an unlabelled membrane causes dilution of the fluorescent lipids resulting in decreased energy transfer and an increase in fluorescence emission from the energy donor N-NBD-PE. This

technique has been used to monitor fusion between membrane vesicles (liposomes), or between membrane vesicles and virions, erythrocyte ghosts or living cultured cells (Struck *et al.*, 1981, Chejanovsky and Loyter, 1986, Chejanovsky *et al.*, 1986). This technique requires sophisticated equipment so a simpler method was tried which involved labelling of the viral envelope or cell membrane with a fluorescent marker, NBD, detectable by FACScan. However, this marker did not remain stably bound to the cell membrane. When an NBD-labelled population of uninfected cells was mixed with an unlabelled population of cells all the cells became labelled. Even extensive washing after labelling did not prevent this problem. Therefore the method was not pursued. Better fluorescent labels now on the market, which are reported to remain stably bound to labelled cells, may have enabled a successful assay to have been developed along these lines.

5.4.5: **Recombinant gp46 TH**

The effect of rgp46 TH on cells and on FFWO by MVV was tested. No conclusions can be drawn from the fact that it did not induce cell-to-cell fusion nor have any apparent modulatory effect on MVV mediated fusion. Firstly the concentration and purity of the recombinant protein was very poor. Secondly, if, as predicted, the secondary structure of the protein is important for fusion function such recombinant protein would be unlikely to be functional as both protein folding and oligomerisation might be very different from native protein.

The problems with expression of recombinant gp46 meant that many of the planned experiments were not possible. These included an experiment to see if rgp46 could bind to cellular receptors and block virus fusion, and experiments to remove or extract anti-gp46 antibodies from sera to determine whether such antibodies are important in fusion modulation.

5.4.6: **Immunoreactivity of sheep sera to rgp46**

The sheep in the ShM group had been judged to be MVV-infected by the AGIDT (agar gel immunodiffusion test) which detects antibody reactivity to MVV proteins but does not differentiate between activity against individual viral proteins (Winward *et al.*, 1979). Western blots with MVV-infected-cell lysate as antigen detects antibodies against some MVV proteins but no protein corresponding to gp46 can be identified on such blots. An example is included in Figure 3.13, which shows that all six of the MVV-infected sheep had antibodies reactive with gp160 and four of these also reacted with p24. It is not clear whether the failure to detect good gp46 reactivity on western blots is due to a lack of gp46 in the antigen preparation or to

non-reactivity of infected sheep to this protein. Thus recombinant gp46 was necessary to determine whether an antibody response to gp46 is elicited in the MVV-infected animal.

Recognition of recombinant gp46 (TH) was shown to be specific to MVV-infected sheep. 78% of the MVV seropositive sera tested reacted with GST:TH. Although the number of sera tested was small, the result nevertheless suggests that a high proportion of MVV-infected sheep raise antibodies against the external domain of gp46.

ELISA against GST:TH, with GST as control antigen suggested that sera from MVV-infected sheep had greater activity against GST:TH than sera from uninfected sheep, and that the ELISA could be used for screening for antibodies to the recombinant protein. In contrast when ELISA of IgG prepared from sheep sera against GST:TH was compared with GST:FINV as control there was no apparent difference between reactivity of each individual sheep to either antigen preparation nor between MVV-infected and uninfected. This can be partly explained by re-examination of the western blots (Figure 5.12) which showed that nearly all the sheep sera reacted with a bacterial protein of about 67kDa which was present in both the GST and GST:TH, but not GST, protein preparations.

The higher ELISA reactivity of sera from MVV-infected sheep compared to uninfected controls may therefore not be entirely TH specific, but may also reflect greater reactivity with bacterial proteins. This might suggest that the ShM group had been subjected to more bacterial infections than the other, perhaps because of a lower non-specific immunity; a higher incidence of various infections is apparent in MVV-infected flocks although specific immune deficiencies have not been reported (discussed in Chapter 1).

Another explanation for higher reactivity by MVV-infected animals could be a cross reaction autoimmune reactivity. Recent experiments by G. Harkiss and colleagues (personal communication) showed that sheep infected with MVV had increased seroreactivity to heat shock protein HSP65 from *Mycobacterium leprae* and *Mycobacterium bovis*. Heat shock proteins are well conserved across all phyla, thus the bacterial protein, running level with the 67kDa marker on western blots (Figure 5.12) could be the *E.coli* homologue of HSP65. If so, the fact that Harkiss *et al.*, found that IgM but not IgG reactive with HSP65 was elevated in MVV-infected sheep might explain why the ELISA using whole sera detected a significant difference between ShU andShM whereas the ELISA using IgG preparations (lacking IgM) did not (Figures 5.11 and 5.13, respectively). However this is not a very convincing explanation since both ShU and ShM sera reacted with this 67kDa

protein on western blots.

5.4.7: **Correlation of fusion assay results with other data**

If considering the uninfected sheep (ShU) and MVV seropositive sheep (ShM) as two groups rather than as individuals some clear correlations do emerge from the data. The ShM group tend to have high ELISA values against GST:TH antigen preparation and to inhibit MVV-mediated fusion at high serum concentrations whilst enhancing fusion at lower serum concentrations. In contrast ShU sera tended to have low ELISA values, and had enhancing activity in the fusion assay at high serum concentrations, but no effect upon fusion at low concentrations.

5.4.8: **Relevance to MVV infection *in vivo***

There was no apparent correlation between either fusion modulatory activity or the presence of antibodies to rgp46TH and prognosis for the infected animal. However the sheep in this study had been infected for at least 1 year and probably for several years. Antibodies against gp46 may not protect from disease pathology late in infection, although the hope is that they could protect from the establishment of MVV infection if they were present at the time of viral challenge. *In vivo* the relative amounts of virus:antibody are likely to be quite different from the fusion assay in which a very high infectious dose of virus is used. At initial infection in the non-immune sheep, even if the viral challenge is very low, virus will be in vast excess to specific antibody. As the fusion assay showed, at this antibody:virus ratio non-specific antibodies may actually enhance the ability of the virus to fuse with target cells. If instead vaccinated animals could raise specific antibodies to MVV, (but preferably not strain specific), then when challenged with virus, antibodies would be in excess and fusion of the virus with the target cell might be halted.

CHAPTER 6: Final Discussion

6.1: INTRODUCTION

MVV is an important sheep disease internationally and in some flocks in this country . MVV in sheep has also been proposed as an animal model for some aspects of HIV infection in that MVV infects macrophage/monocytes but not lymphocytes whereas HIV infects both these cell types. In lentiviral infections the host mounts an immune response yet this is not effective in eliminating the infection. Much effort is going into trying to find ways to prevent HIV infection or halt the spread of disease. The results from studies on MVV may be applicable to HIV. The principal problem seems to be induction of a protective immune response which is effective and also broadly reactive rather than type-specific. The transmembrane protein mediates an essential virus function and as such is a candidate target for a protective immune response. A number of questions need to be answered to determine the suitability of gp46 as a vaccine component.: e.g. Is the protein immunogenic? Are antibodies against gp46 strain specific? Can antibodies against gp46 control virus infection/spread?

MVV does not grow to high titre in culture making it very difficult to purify gp46 in quantity from virus preparations. Therefore recombinant gp46 was required for studies to answer the questions posed above. Prior to this the region of *env* encoding the transmembrane subunit had to be cloned. Nucleic acid sequencing of the clone enabled comparison with other strains of MVV and with other viruses.

The aim of the final section of work was to see whether serum from MVV-infected sheep had any affect on fusion by MVV to determine whether induction of antibodies which affect fusion could potentially alter the progression or outcome of maedi visna disease.

6.2: THE TRANSMEMBRANE ENVELOPE GLYCOPROTEIN, gp46, OF MVV

Cloning of *gp46*, i.e. the 3' part of *env* that encodes gp46, enabled its sequence to be determined for MVV strain EV1. Comparison with the nucleic acid or amino acid sequences of other MVV strains revealed conserved and variable regions. Overall there was about 20% divergence within *gp46* between strain EV1, 1514 and SAOMVV. This compares to approximately 10% for *gag*, 15% for *pol*, 20% for *vif*, 21% for *tat* and 35% for *rev* (Sargan *et al.*, 1992).

Comparison of the amino acid translations of gp46 between different MVV isolates revealed both conserved and variable sequences. Most of the conserved regions can be linked with a particular function either directly from mutational studies on MVV or by analogy with regions identified in studies on other viruses. In particular the amino-terminal end of gp46 is conserved in all MVV isolates sequenced to date which suggests that most changes in amino-acid sequence and tertiary structure in this region are disadvantageous. In contrast some regions of the surface envelope glycoprotein are very variable and antibody resistant viruses can be generated rapidly. Studies have shown that HIV gp120 induces protective neutralising antibodies only against homologous strains of virus. The same is likely to be true of MVV gp110. The sequence conservation of parts of gp46 suggests that these may be candidate epitopes for induction of cross-protective rather than type specific immunity.

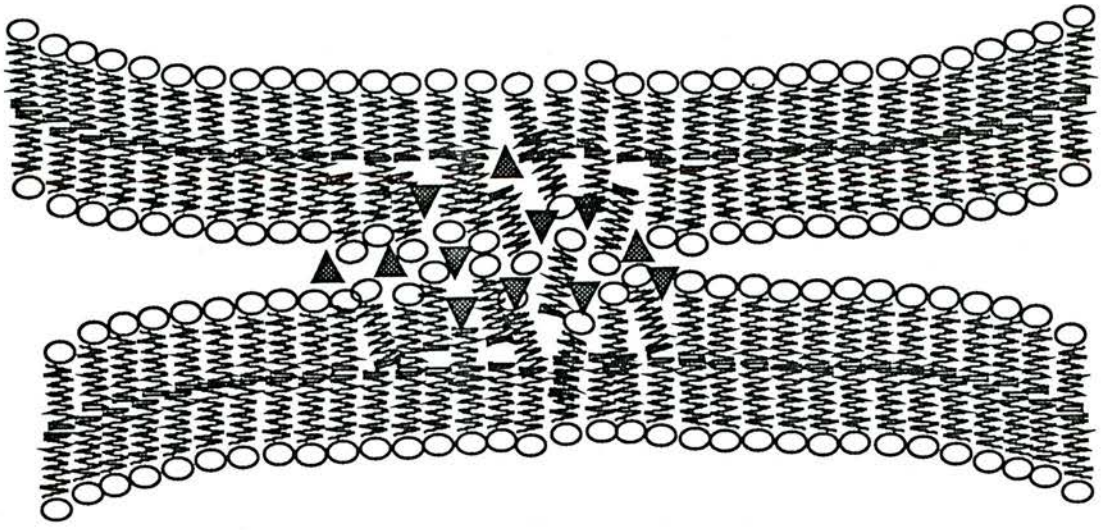
By analogy with other retroviruses, gp46 has been assumed to be important in fusion of the viral and host cell membrane to facilitate infection. Hydrophobicity and secondary structure plots, derived using the sequence data, showed that gp46 is a typical fusion protein; It has a hydrophobic alpha-helical region which probably traverses the cell membrane/virus envelope, and it has an alanine/glycine rich hydrophobic region at the amino-terminal end. The latter is analogous to fusion peptides from other enveloped viruses and is conserved between different MVV isolates. The extracellular domain has potential glycosylation sites. gp46 has a leucine-zipper like motif carboxy-terminal of the fusion peptide and has conserved cysteine residues, as have other viral fusion proteins. Conservation of the cysteines is thought to be necessary in maintaining the required secondary structure for functionality.

The function of the MVV fusion peptide has been confirmed by Crane *et al.* (1991); a peptide GIGLVLAIMAIIAAAGAGLGVA, which is conserved between the sequenced isolates of MVV, was used to immunise 4 rabbits. One of the rabbits produced antibodies against this peptide. The serum blocked MVV-induced syncytium formation by 64% at 1:10 and by 50% at 1:160. The fusion mediating role of this region was further confirmed by site-directed mutagenesis; when two hydrophobic amino acids within this region were replaced with non-hydrophobic amino acids fusion was ablated.

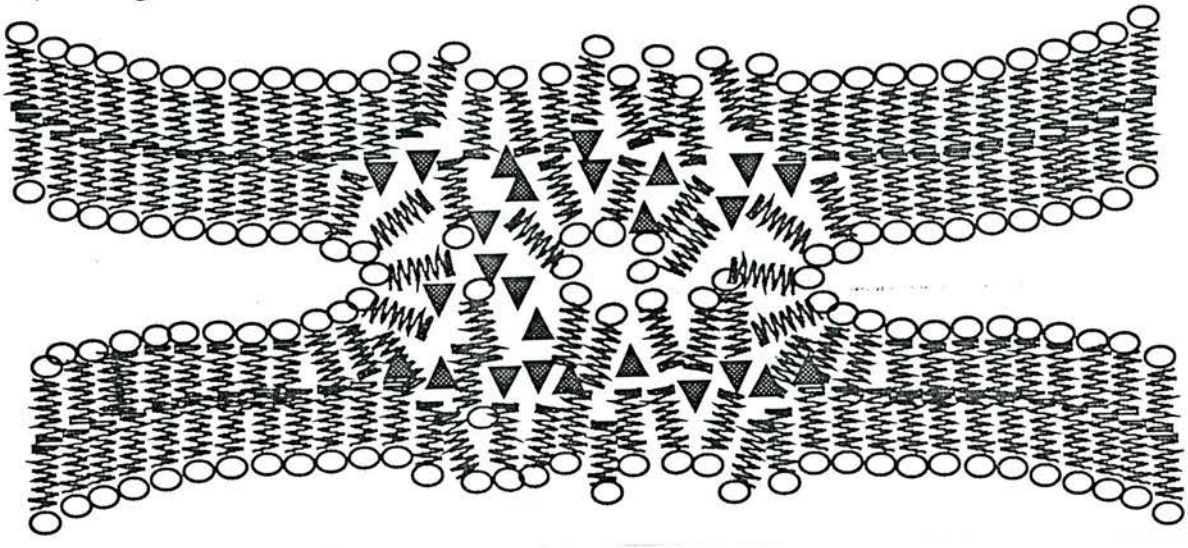
The authors also showed that the peptide described above could itself induce cell fusion in the absence of virus. The rabbit antiserum against the fusion peptide blocked the peptide-mediated cell fusion. It has been shown that hydrophobic peptides spontaneously insert into membranes (Engelman and Steitz, 1981) and that viral fusion proteins may interact directly with phospholipid components of the cell

Figure 6.1: Hypothetical stages in peptide mediated membrane fusion

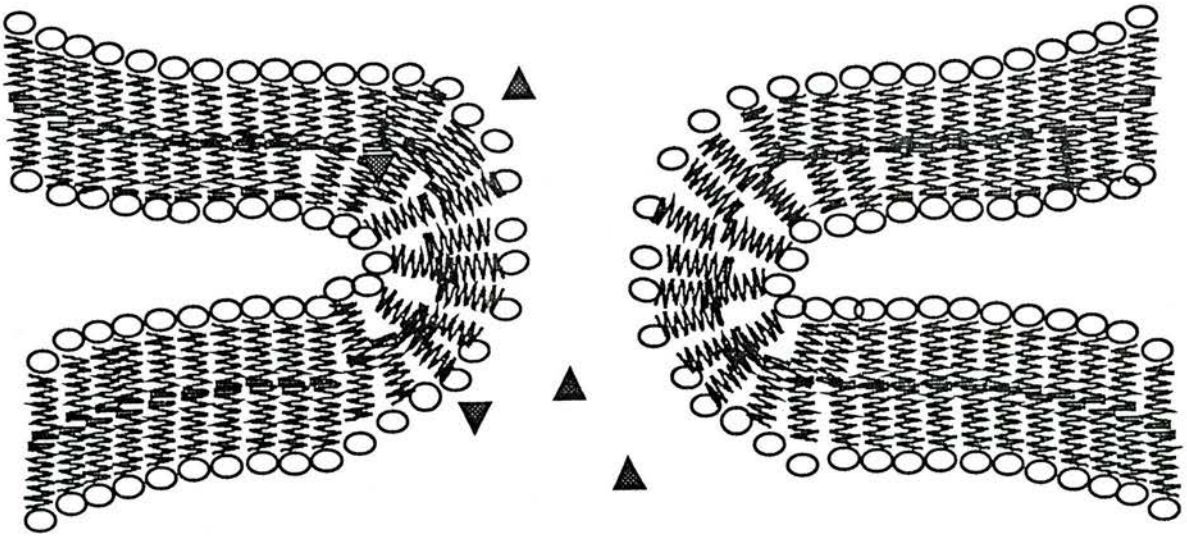
a) Destabilisation of opposing phospholipid bilayers



b) Mixing of membrane components



c) Reformation of lipid bilayers in a new plane resulting in cell fusion



membrane. This leads to the hypothesis that the hydrophobic peptides insert into and perturb the bilayer structures of closely opposed membranes resulting in mixing and then resolution with the re-formation of stable bilayers (Figure 6.1). In peptide-mediated fusion "abortive fusion reactions", i.e. reformation of the original bilayers, may well be occurring although only the forward reaction, fusion of cells, is measurable. In virus-mediated fusion the fusion peptide most probably initiates fusion by destabilising the opposing bi-layer whilst the envelope glycoproteins may be important in providing a bias towards the forward reaction, e.g. the influenza fusion model described in Chapter 1 envisages the opening of a fusion pore.

6.3: EXPRESSION OF RECOMBINANT gp46 PROTEIN

The cloned *gp46* coding sequence was used to generate vectors to express recombinant gp46 which was required as antigen to study the immune response to gp46. Yeast and bacterial expression systems were tried. In the absence of gp46 specific antiserum the criteria for the production of the required protein had to be verification of the expression vector, identification of the protein fusion partner and a shift in the size of the fusion partner. The exact size of the recombinant protein was difficult to determine because mobility on SDS-PAGE gels is not an exact relationship with molecular weight. Finally the fact that most MVV seropositive but no MVV seronegative sera recognised the recombinant proteins confirmed their identification.

Vectors were constructed for expression of two different forms of recombinant gp46; TH, the external region of the transmembrane protein, and TF the full-length TM sequence, in recognition of the potential problems that expression of the hydrophobic transmembrane domain might cause upon cytoplasmic expression.

Expression of rgp46 in yeast, as a fusion protein with p1, enabled recovery of p1:TF and p1:TH although only at low yield and very poor purity. In yeast p1:TF could be recovered nearly as well as p1:TH so production of p1:TF was concentrated upon. Unfortunately the poor yield and purity made it impossible to recover TF after cleavage from p1. This is why an alternative system was tried, namely, expression of rgp46 in*E.coli* as a fusion with GST. The *E.coli* expression system did enable production of GST:TH at a better yield than p1:TH, but GST:TF was not recoverable at all. Fewer contaminating proteins were present in the GST:TH preparation than in p1:TH or p1:TF. The introduction of extra steps, such as a further round of binding to glutathione agarose beads or purification through a Sepharose size exclusion column might have improved purification further but probably with a concomitant loss

of yield.

gp46 (TF) was expressed in cells using recombinant vaccinia virus. Although the amount of rgp46 was very low, it was sufficient as immunogen in the form of whole recombinant vaccinia virus and also for the use of infected cells as targets in cytotoxic T cell assays. Whether the recombinant gp46 was expressed in transmembrane form by Vgp46 is not clear: Recognition of specific leader "signal" sequences on a nascent polypeptide is required to initiate translocation of the ensuing protein domains across the lipid bilayer (Walter *et al.*, 1984) and the transmembrane domain acts as a "stop transfer" signal. As neither TK nor *gp46* included a leader signal sequence, rgp46 was probably not expressed in a transmembrane form. However it is possible that the hydrophobic N-terminal of gp46 could act as a signal sequence since signal sequences consist of hydrophobic amino acids but do not have conserved primary structures or lengths (Blobel and Dobberstein, 1975). Signal sequences are subsequently cleaved from the rest of the protein so this would result in an aberrant transmembrane form of gp46. More detailed studies would be required to determine whether gp46 expressed by recombinant vaccinia virus took this form. Antibodies elicited by this gp46 could cross-react with TH, so the fact that sera from Vgp46 immunised mice reacted with TH on western blots does not indicate whether or not the protein was expressed in a transmembrane form.

Recombination of *gp46* into the HA gene of vaccinia virus, which does include a leader sequence may have enabled expression of gp46 in nearer native form, probably glycosylated and as a transmembrane protein, as was described for expression of bovine PIV-3 envelope glycoproteins by recombinant vaccinia viruses (Sakai and Shibutah, 1989). Unfortunately plasmids for generation of HA gene recombinants were not available to us at the time.

The amount of recombinant gp46 expressed in each of the three of the systems tried was very poor and thus limited the experiments for which rgp46 could be used. Previous use of each of the expression systems has shown that the vector promoters etc are not at fault. There are a number of other reasons for poor yields of recombinant proteins: Low yields of fusion proteins are sometimes found to be due to more rapid turnover of the recombinant protein than of the fusion partner alone, or due to more rapid turnover of mRNA, since there is some influence of codon usage on the stability of mRNA (Hoekema *et al.*, 1987). The activity of host cell proteases may also lead to low yields of heterologous proteins (Gottesman, 1989). Although such factors may have contributed to the problem of low yield, toxicity of the expressed protein was probably the major reason.

Even at very low or undetectable levels of recombinant protein, induction of its expression had a profound inhibitory or toxic effect on growth of the host cells. Other hydrophobic proteins have also been shown to be toxic when expressed intracellularly, although the mechanism for this toxicity has not been ascertained. One possibility is that interaction of the hydrophobic regions of the protein with cellular proteins may disrupt various cellular biochemical pathways resulting in cell death. Interaction with cellular proteins could also account for the high level of contaminating proteins carried through the purification procedures.

An alternative/additional mechanism of rgp46 toxicity is the potential immunosuppressive peptide which has been identified as amino acids 64 to 79 (Ruegg *et al.*, 1990). It is present in both TH and TF recombinant proteins. The immunosuppressive peptide is thought to inhibit the enzyme protein kinase C which is involved in signalling pathways. Thus rgp46 may be toxic to the cells which express it by inhibiting of protein kinase C and disrupting the pathways in which it is involved. A related sequence found in HIV gp41 inhibits NK cell function, monocyte chemotactic responses and proliferation in response to mitogens or specific antigens (Harris *et al.*, 1987, Miedema *et al.*, 1988).

6.4: IMMUNE RESPONSES TO rgp46

Mice immunised with Vgp46 raised antibodies specific to rgp46 produced by *E.coli*. A rabbit immunised with p1:TF raised specific antibodies to TF as well as to p1 and other yeast protein contaminants. Although none of these sera appeared to react with gp46 on western blots of EV1 lysate, there was no positive control to confirm the presence of EV1 gp46 on the blots and it cannot be concluded that they did not recognise native gp46.

A peptide corresponding to amino acids 116-134 of EV1 gp46 and linked to ovalbumin was used to immunise two rabbits. Both rabbits raised antibodies to the peptide but the antibodies did not react against native protein (neither GST:TH nor p1:TF by ELISA and western blot nor EV1 lysate by western blot), nor did they have any effect in the fusion assay.

78% of sera tested from MVV infected sheep and none of the sera from uninfected sheep reacted with GST:TH but not GST on Western blots. This shows that gp46 is immunogenic in the infected animal, and that the majority of MVV-infected animals generate antibodies against the external domain of gp46. There are a number of possible reasons why serum from some MVV infected sheep failed to react

with the TH on western blots. i) The animal may not have raised antibodies to gp46. ii) Any antibodies raised may have been unreactive with denatured proteins, or been glycosylation dependent. iii) Some antibodies against gp46 may be type specific. If the most variable epitopes are immunodominant testing of sera from sheep infected with field strains of MVV against EV1 protein may not be ideal. Later in infection the humoral immune response is thought to be more broadly reactive to the envelope epitopes. Thus the sera reacting with EV1 rgp46 may be reacting to conserved epitopes recognised late in infection. Unfortunately the time of infection of individuals in this flock is unknown.

ELISA may be preferable to western blots as a way to measure antibody responses because proteins are not denatured as much by coating onto ELISA plates. Western blots detect primarily antibodies against linear epitopes but are better at separating out reactivity with the protein in question from reactivity with contaminating proteins. The high levels of contaminating yeast or bacterial proteins in the preparations of recombinant gp46 makes the ELISA results difficult to interpet.

6.5: THE FUSION ASSAY AND THE EFFECT OF SERUM ON MVV FUSION-FROM-WITHOUT

The fusion assay was devised as a method to determine whether MVV-infected animals mount an immune response which can alter the ability of MVV to fuse with host cells. It basically involved preincubation of MVV with serum, prior to addition to cells. The fusion assay demonstrated that some sera can dramatically alter the ability of the virus to fuse cells. Both enhancement and inhibition of MVV mediated cell fusion was demonstrated. Surprisingly, it was clear that the same serum could inhibit fusion at high concentrations of serum yet enhance when diluted.

It was initially assumed that the mechanism of FFWO in the assay would be the same as for fusion of virus with cell at initial infection. The results of my studies, and of others, suggest this is not necessarily so. As discussed in Chapter 5 the actual mechanism of FFWO is not known. Two alternative hypotheses were proposed; i) A virus happens to land between two cells so that it is able to bind and fuse with two cells at the same time, thus fusing the two cells. ii) The virus fuses first with one cell, but envelope proteins remaining on the cell surface mediate fusion with a neighbouring cell. Both mechanisms would require high concentrations of virus to maximise the chances of cell fusion.

Sera from uninfected sheep had some effect on MVV fusion in that preincubation of MVV with low dilutions of serum increased the number and size of syncytia formed compared to MVV preincubated with medium alone. More dilute serum from uninfected sheep did not affect syncytium formation. There was a significant difference between the pattern of activity by sera from MVV-infected (ShM) or uninfected (ShU) sheep in the fusion assay. The activity of ShM sera ranged from fusion inhibition at low serum dilutions and enhancement at high dilutions of the same serum to fusion enhancement at all serum dilutions down to the lowest tested which was 1:512. Averaging all the ShM fusion assay results showed that the typical pattern of activity of ShM sera was fusion inhibition at dilutions ≤1:8 and enhancement of fusion at dilutions ≥ 1:16.

6.5.1: **Hypothetical mechanisms of fusion inhibition in the fusion assay**

Most of the mechanisms of virus neutralisation could, if antibody is present at sufficient concentration, lead to inhibition in the fusion assay. Examples include aggregation of virus particles, blocking of binding to receptor, and blocking of epitopes necessary for fusion including prevention of conformational changes.

Fusion efficiency may also be affected by alterations in the cell rather than the virus. A candidate factor which could be active in this assay, as it is not affected by heat treatment, is the unique form of interferon induced by lentiviruses (Narayan *et al.*, 1985). This "lentiferon" has been reported to block fusion of infected macrophages with synovial membrane cells and to cause a dramatic reduction in the fusion function of CAEV (Zink and Narayan, 1989). Although fusion inhibition activity was detected in serum, it was not detected in serum depleted of IgG i.e.the fraction in which LV-IFN would be expected. This suggests that LV-IFN was not a significant factor in fusion inhibition in these assays. It could be that LV-IFN was not present at sufficient concentration in the sera for its fusion inhibitory activity to be detectable or it may be that fibroblasts are not affected by LV-IFN.

6.5.2: **Hypothetical mechanisms of enhancement in the fusion assay**

The fusion assay described in Chapter 5 clearly showed that high concentrations of normal serum from most of the uninfected sheep tested enhanced fusion-from-without by MVV. This titred out quite quickly (1:64 maximum) whereas sera from some of the MVV infected sheep enhanced fusion at least to 1:512.

The mechanism by which fusion enhancement occurs *in vitro* has important consequences for assessing how relevant the fusion assay result is to enhancement of infection or cell-to-cell fusion (FFWI) *in vivo*. Several hypothetical mechanisms of

enhancement of fusion in the assay are described below.

i) Formation of fusogenic immune complexes:

At a given ratio of antibody to antigen the multi-valency of antibodies allows antibody:antigen aggregates or immune complexes to form. *In vivo* this generally aids the clearance of antigen by phagocytic cells. In the *in vitro* fusion assay aggregation of virus particles may enhance fusion purely physically; larger, heavier particles may settle more rapidly and also may be more likely to end up lying between cells in the monolayer. If this is so, it may actually be aiding delivery of virus to a site where a single virus or more than one in the complex may be able to fuse with two cells simultaneously.

ii) Cross-linking of envelope on the cell surface:

If FFWO occurs by fusion of the virus with first one cell, leaving functional envelope proteins on the cell surface to subsequently mediate fusion with a neighbouring cell, then antibodies could conceivably enhance fusion by cross-linking envelope proteins, effectively increasing the surface density of envelope proteins. Low affinity antibodies would probably be better at aiding formation of fusion complexes without blocking fusion.

Another potential enhancement mechanism also assumes the two-step model of FFWO; Capping is a phenomenon whereby antibodies bound to cell surface proteins tend to move together in the membrane so that an area with a high concentration of bound antibodies is formed and this is pinched off to remove it from the cell surface. If in fibroblasts capping occurs at the margins of the cells i.e. where they are in closest contact with their neighbours then low affinity (or gp110 stripping) antibodies might help to deliver the envelope proteins to regions proximal with an opposing membrane and thus enhance fusion.

IgG cross-linking of cellular bound virus to incoming virus may enhance virus/cell interaction and therefore increase fusion.

iii) Antibody enhancement via Fc receptors:

Antibody enhancement of infection generally occurs via Fc receptors (FcR) or complement receptors. Skin-derived fibroblastic cells are not normally thought to express such receptors, but the YT40 skin cell fibroblast line used in these experiments had been extensively passaged and aberrant expression of surface molecules cannot be ruled out. Also some viruses can upregulate FcR, including on fibroblastic cells, but no studies have been reported on upregulation of FcR by MVV. Antibodies specific for ovine FcR were not available to check this simply, although rosetting with RBCs coated with anti-RBC sheep antibody could have been used as a

crude test for FcRs. Fab fragments of the IgG from the enhancing sera would have been useful to determine whether or not FcRs were implicated in antibody enhancement of FFWO.

iv) "Stripping" of gp110 from virus particles:

Antibodies against gp110 could alter its interaction with gp46, perhaps to induce dissociation, although, because of the lack of appropriate antisera, it is not known how readily MVV gp110 dissociates from gp46. If antibodies could induce dissociation of the MVV envelope glycoproteins then stripping of gp110 from the viral membrane might expose gp46 enabling it to interact with the cell membrane. Such a mechanism is more likely to enhance fusion if an optimal SU:receptor interaction is not possible. MHCII has been identified as a receptor for MVV, yet is undetectable on YT40 fibroblast cells, so that in the fusion assay the virus may be making use of a sub-optimal receptor on fibroblast cells. Thus a case can be made for enhancement by increasing the ability of gp46 to interact directly with the cell membrane. The same hypothesis can be proposed if anti-envelope antibodies can mediate a change in the conformation of the envelope proteins without actually dissociating gp110. Antibodies binding to the SU glycoprotein may "activate" or expose the fusion peptide, in the same way that binding of the SU to its receptor might do.

There are arguments both for and against exposure of the fusion peptide as a mechanism of fusion enhancement. Acid pre-treatment of influenza HA irreversibly renders it incapable of subsequently mediating membrane fusion apparently by overshooting the conformational change normally required for fusion (Wilson *et al.*, 1981). On the other hand acid pre-treatment of another pH-dependent virus, VSV, enhances subsequent fusion (Puri, *et al.*, 1988). Soluble CD4 has been shown to block HIV infection by removing the gp120 protein from the viral envelope (Moore *et al.*, 1990), while SIV infection was enhanced in the presence of sCD4 perhaps by exposure of the fusogenic domains (Allan, 1991).

The hypothesis that in sheep skin cell fibroblasts the virus is using an alternative receptor to the one it normally recognises might be important in this. It would be interesting to see if the sera used in the fusion assay described here would have similar effects on MVV fusion of macrophages.

v) Cross-linking of cellular membrane proteins

It has been shown that antibodies directed against the host cells can enhance virus mediated fusion; anti-cellular antibodies enhanced FFWI by Newcastle disease virus (NDV) but did not alter the fusion inhibition by antibodies against NDV F protein (Ito *et al.*, 1987). The enhancement was narrowed down to antibodies against cell surface proteins, called fusion regulatory proteins (FRPs) (Ito *et al.*, 1992).

Monoclonal antibodies against FRPs, like antibodies against other cell surface proteins, can cross-link and aggregate cells. However, fusion enhancement appears to be specific to anti-FRP antibodies. It was suggested that antibody cross-linking of FRPs could trigger disturbance of the cytoskeleton and thereby enhance fusion.

There is some evidence for the generation of autoreactive antibodies during MVV infection. Such antibodies could be involved in mediating enhancement in the fusion assay by sera from MVV infected sheep. One way to determine whether this is the case would be to test the same sera in a fusion assay using a different fusogenic virus which could fuse sheep cells.

vi) If non-Ig serum factors affected FFWO, perhaps by altering expression of cellular receptors, then this could only be a minor component of fusion enhancement since serum with IgG removed had a much lower fusion enhancing effect than sera containing IgG.

6.5.3: **The balance between fusion inhibition and enhancement**

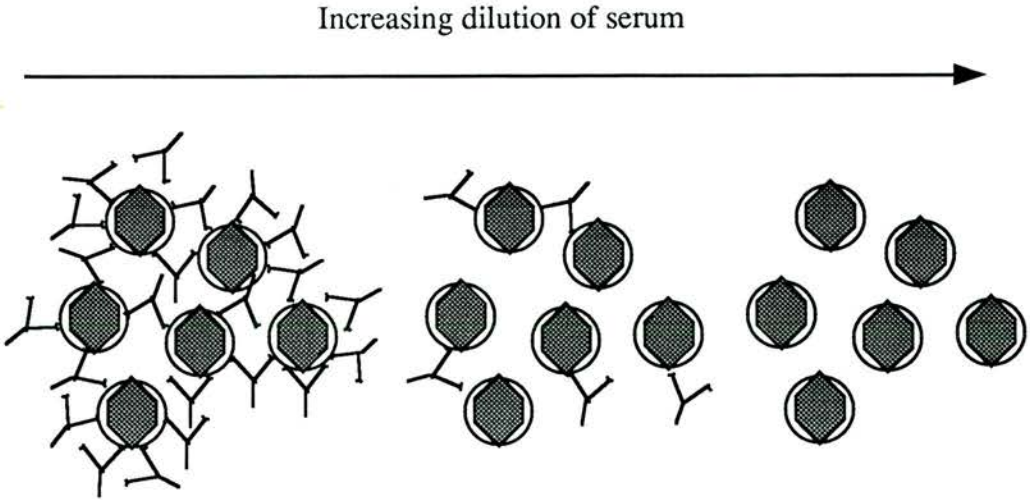
The effects seen in the fusion assay were dependent upon serum concentration since the same serum at low dilution can inhibit fusion yet at higher dilution enhanced fusion. Thus there appears to be a balance between fusion inhibition and enhancement. This balance could either be due to different factors with positive and negative effects on fusion or due to the same factors having different effects when present at high or low concentration.

A model to explain the balance between fusion inhibition and enhancement which fits with the results obtained is shown in Figure 6.2.: Virus fusion is blocked when antibody is in excess and binds to the virus thus preventing interaction with the cell surface. At low proportions of virus only a few sites on each virion bind virus, leaving most of the envelope glycoproteins free to mediate fusion which is enhanced by by cross-linking of virus particles or virus to cell incorporated viral proteins, or via FcRs.

This is no doubt an over-simplification; the results of fusion assays with IgG preparations and IgG depleted sera suggested that other serum factors can affect fusion either independently or by interactions with IgG.

Serial serum samples taken at 6 weekly intervals were tested in the fusion assay. For most sheep the activity in the assay was the same for each serum sample. However in ShM2.1 the first sample was blocking, the second was neutral and the third and fourth were enhancing. This suggests that over the course of twelve weeks the balance of activity in this sheep had changed from fusion inhibiting to fusion

Figure 6.2: Hypothesis to explain the balance between enhancement and inhibition in the fusion assay



High ratio of antibodies:virus (and/or high affinity)

Low ratio of antibodies:virus (and/or low affinity)

No specific antibodies

Inhibit fusion (interfere with interactions with cell surface?)

Enhance fusion (cross-linking ? FcRs?)

No effect on fusion

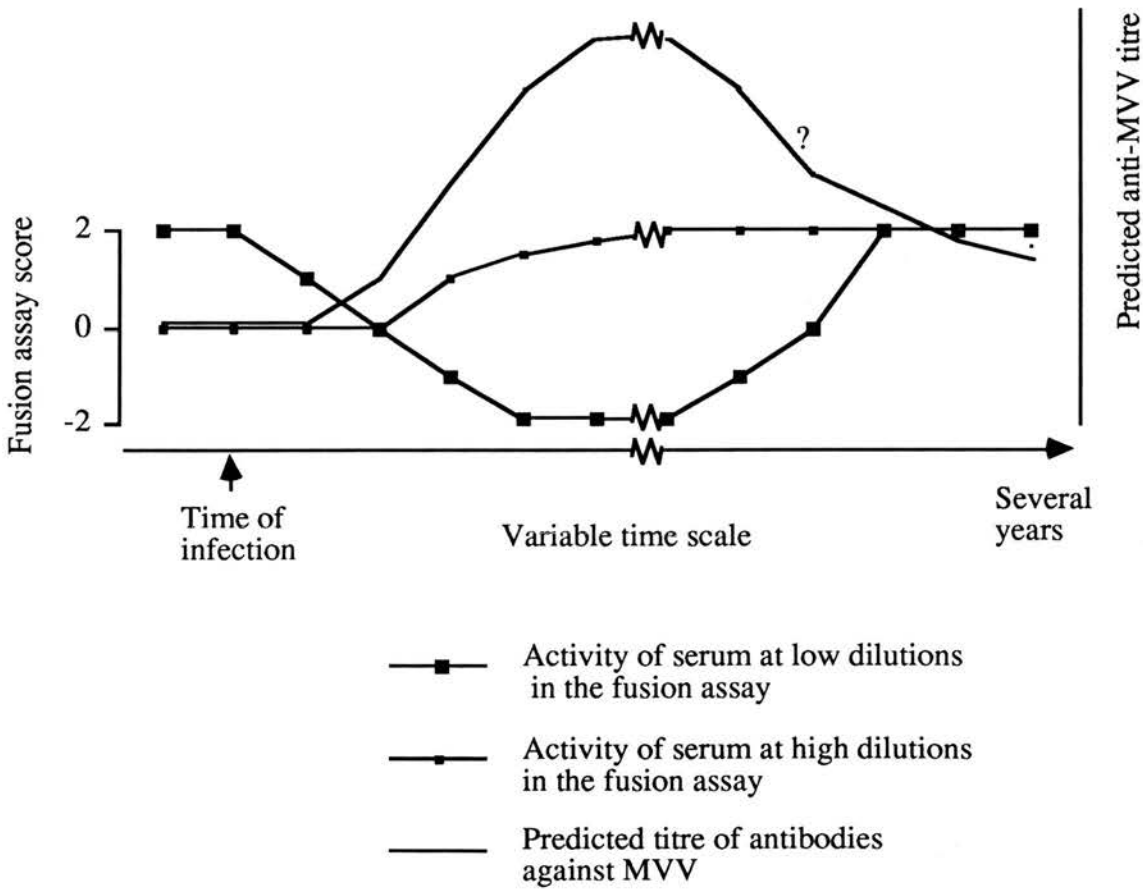
low dilutions in fusion assay **ShU** high dilutions in fusion assay

low dilutions in fusion assay **ShM** high dilutions in fusion assay

Figure 6.3: Hypothesis for a balance between fusion enhancement and fusion inhibition over the course of MVV infection

The diagram below is an attempt to illustrate the hypothesis that the pattern of activity in the fusion assay may change as disease progresses. It requires the acceptance of the hypothesis illustrated in Figure 6.2 that whether fusion is enhanced or inhibited is dependent on the ratio of virus to antibody.

It is postulated that fusion modulatory activity changes from blocking to enhancing after a variable period of infection. A decrease in MVV-specific antibodies might lead to such a change, although there is as yet no evidence to support this..



enhancing. The fact that the activity moved through zero shows that this is probably a real effect. Sampling may have fortuitously caught the window in time when the shift of balance occured in this sheep. Sheep M4, M8 and M12 also appeared to shift away from fusion inhibition in the later serum samples. None of the serial assays changed from fusion enhancement to fusion inhibition or even neutrality. Also the shift is to fusion enhancement at high and low dilutions, so it is not the same as the fusion activity exhibited by sera from uninfected sheep.

A pattern of inhibition/enhancement over the course of MVV infection can be postulated from the fusion assay results and the hypothesis that fusion enhancement results from low concentration/ low affinity antibodies and fusion inhibition results from high concentration / high affinity antibodies: Before production of anti-MVV antibodies some cross-reactive antibodies in preimmune serum may mediate fusion enhancement. Development of a specific anti-viral response produces antibodies at higher concentration and affinity which inhibit MVV fusion but when diluted down in the assay enhance fusion. If a shift towards fusion enhancement in the assay is indeed a consequence of continuing MVV infection, this suggests that the amount of MVV specific antibody decreases.

It would be very interesting to assay serial samples over a longer time course, preferably from before and after experimental infection right through to disease appearance, to see if the pattern of fusion inhibition/enhancement during MVV infection fits the prediction above.

In HIV infection a decrease in antibodies reactive with peptides representing HIV envelope has been shown to coincide with progression to clinical AIDS (Neurath *et al.*, 1990). This decrease appears to be HIV-specific as immunoglobulin levels are not decreased between asymptomatic and AIDS patients and the antibody response to another viral protein, hepatitis B surface protein did not decrease. In particular a relationship has been shown between disease and declining levels of antibodies to regions of gp120 and to the C-terminal end of gp41 (Neurath *et al.*, 1990).

6.5.4: **Comparison to results of related studies**

The strain of virus used in the assay and the strain against which antibodies were generated may affect the results of the fusion assay. The sheep sera tested in the fusion assay described in Chapter 5 were from a flock infected with an unclassified field isolate(s) and strain EV1 was used in the fusion assay. In contrast, Crane *et al.* (1988) tested sera from sheep which had been hyperimmunised with MVV strain 1514 for fusion inhibitory activity against 1514 or its antigenic variant LV-1. The results differ in that Crane *et al.* (1988) detected syncytium inhibition but do not describe

fusion enhancement. This can be extrapolated to suggest that the difference in results may be due to type-specificity such that antibodies against a different strain from the challenge may enhance fusion. This fits with the model proposed for the balance between inhibition and enhancement; different variants of MVV are likely to have epitopes in common as well as some type-specific epitopes so testing serum against a heterologous variant effectively reduces the concentration of virus specific (high affinity) antibodies and shifts the balance towards fusion enhancement. The suggestion that sera appears to have greater fusion enhancement activity against heterologous strains of MVV demonstrates a way in which the emergence of a new variant might not only allow neutralisation escape but also actually enhance spread of infection within the host. Further experiments are required to clarify the MVV strain specificity of fusion enhancement and inhibition.

Similar results as was shown here for MVV fusion have been reported in experiments with assays of the effect of immune sera on HIV infection. Significant enhancement of HIV infection was reported with higher dilutions of HIV antibody positive sera whilst neutralising activity was detected at lower dilutions (Takeda, 1988). Enhancing activity of antibody is HIV isolate/strain dependent. (Prince *et al.*, 1987, Cheng-Mayer *et al.*, 1988, Homsy *et al.*, 1990,). It was shown that sera from some HIV infected patients enhanced infection of macrophages by a homotypic strain of virus, that is virus isolated from the same patient as the serum tested, whilst neutralising another strain of virus (Homsy *et al.*, 1990). This is in contrast to the hypothesis made above, that sera from MVV seropositive sheep may enhance fusion by heterotypic strains, but may reflect more rapid appearance of variants in HIV, such that the antiserum was generated against variants circulating before the virus isolated for the assays.

6.5.5: **Relationship between neutralisation and fusion inhibition**

Fusion inhibition may occur with occupation of a smaller number of critical sites by antibody than is sufficient for neutralisation. If most of the envelope glycoprotein spikes on the incoming virus are coated with antibody, any remaining free sites may be able to mediate fusion and therefore enable infection whilst the chances of the virus being able to fuse with two cells at once (single event model of FFWO) would be very low. Thus fusion-blocking antibodies may block FFWO but still allow a rare fusion event. If the two step model of FFWO is considered then antibodies may block fusion by cross-linking proteins and preventing their movement within the membrane or by denaturing them without actually being able to block fusion by incoming virus. Alternatively, in the presence of fusion blocking antibodies entry

may be via the endocytic route instead of by fusion with the cytoplasmic membrane, which would allow infection .

Immune sera from sheep hyperimmunised with MVV-1514 were shown to neutralise virus infectivity and prevent cell fusion, suggesting a linkage between these phenomena (Crane *et al.*, 1991). LV-1 was a variant isolated from a sheep which had originally been inoculated with MVV 1514. Monospecific sera which neutralised 1514 but not LV-1 prevented FFWO by both. They suggested their results showed that different epitopes are responsible for fusion and neutralisation. The difference in kinetics between the two assays may be a better explanation, in that the fusion assay read-out requires very many fusion events whereas the neutralisation read-out requires very few infection events, so lower affinity and/or lower titre antibodies may block fusion in the FFWO assay than are necessary to block infection.

Dalziel *et al.*, (1991), demonstrated that soluble MHCII could block fusion but not infection of skin cell fibroblasts (YT40 cells as used in my experiments). Again this may be explained by the kinetics for virus fusion being much greater than that required for infection. Only a small number of free receptor binding sites, sufficient to form one fusion complex, may be required for infection whereas at least enough for two complexes (one per cell) are required for the virus to bind two cells at once and thereby initiate fusion (assuming this is the mechanism of cell fusion in the assay). Thus binding of sMHCII to MVV may block the viral binding site for the cellular receptor to an extent whereby visible cell-to-cell fusion does not occur but infection may still be possible.

6.5.6: **Relevance of fusion assay results to *in vivo* viral fusion**

One would predict that inhibition of the fusion assay would reflect the ability of the antiserum to reduce viral infectivity *in vivo*, perhaps reducing the rate of initial infection as well as limiting subsequent spread of virus to new cells or tissues. In addition, fusion inhibiting antibodies might reduce syncytium formation in the infected tissues thus minimising cell-to-cell spread as a way to elude the immune system. Functional antibodies which inhibit this process could potentially reduce cytopathic damage and perhaps reduce disease severity. Enhancement of FFWO *in vitro* may correspond to enhancement of viral infectivity in the host animal.

Fusion inhibiting antibodies in HIV infected children have been shown to correlate with a more benign outcome with respect to lung disease (Brenner *et al.*, 1991) whilst the appearance of increased enhancing antibodies/enhanced strains of HIV was associated with progression towards disease suggesting that enhancement may contribute to the severity of infection (Homsy *et al.*, 1990). The results of the

fusion assay did not appear to have any relationship with the disease state of the sheep except that sera from infected sheep, which are therefore those that eventually succumb to disease, tend to differ in serum fusion-modulatory activity compared to uninfected sheep. However this does not rule out an association being found if larger numbers were tested and if different criteria for disease state were chosen.

The fusion assay results for sera from uninfected sheep suggest that free virus may be helped to fuse with and infect cells by components in pre-immune sera. As sera from uninfected sheep varied in the ability to enhance MVV fusion, it would be interesting to see if fusion enhancement by pre-immune serum has any correlation with susceptibility of the sheep to MVV infection.

As discussed above, the results of the MVV fusion assay show that the relative amounts of antibody and virus may be crucial in determining enhancement or inhibition of fusion, and potentially of infection. However, the considerable differences between the *in vitro* fusion assay and the situation occuring in tissues of the infected animal should be taken into account when extrapolating from one to the other. In the seropositive animal there is probably more fusion inhibition and less fusion enhancement than is suggested from the fusion assay results: Very high concentrations of virus were used in the assay so the ratio of virus to antibody was much higher than would be found *in vivo* where MVV has only been detected at relatively low levels. According to the model proposed this would shift the balance in the assay towards fusion enhancement. In addition, some of the potential mechanisms of fusion enhancement proposed *in vitro* may not be applicable *in vivo* where cells of the immune system and also complement are present. For example, limited aggregation *in vitro* might enhance in the fusion assay by forming larger particles which sink to the bottom of the well more quickly thus increasing virus:cell contact, whereas *in vivo* such aggregates (immune complexes) should be cleared rapidly by phagocytic cells.

The balance between fusion enhancement and inhibition may have important implications for the use and design of vaccines; Infecting virus entering a new host is most likely to be at very low titre either in the form of cell-free virus or virus produced by a small number of transferred infected cells. Non-specific crossreactive antibodies may facilitate or enhance infection, whereas specific antiviral antibodies may be able to inhibit fusion and possibly prevent infection.

The fusion assay suggests that induction of type specific antibodies may protect against one variant but act as lower affinity fusion enhancing antibodies if

challenged with a different strain of virus. This is of importance to consideration of potential vaccines as induction of a type specific immune response might do more harm than good. The mechanism of enhancement needs to be elucidatedin order to determine whether some epitopes would be better for induction of protective as opposed to enhancing antibodies, e.g., enhancement might be mediated by activity against MVV epitopes other than the fusion peptide, but immunisation with the conserved fusion peptide might be protective.

6.6: CONCLUSIONS

Sera from most MVV-infected sheep recognised recombinant gp46 (EV1). The difficulty of producing rgp46, due to its toxicity to the host cell, meant that some of the planned experiments could not be done in the time available. However the project generated reagents which could be used to further define the relationship between antibodies to gp46 and fusion modulatory activity.

MVV-seropositivity appeared to be associated with antibodies to recombinant gp46 and with a particular pattern of activity in the *in vitro* MVV FFWO assay. However, it was not possible to show conclusively that the increased ELISA reactivity of MVV infected sheep to GST:TH was entirely against TH or whether it was partly due to reactivity with bacterial proteins contaminating the GST:TH preparation. There was insufficient time to test all the sheep sera on western blots of GST:TH, but of those tested there was no apparent relationship to fusion assay activity. This may be at least partly because antibodies directed against the external envelope glycoprotein, gp110, as well as antibodies against gp46, would affect fusion. Although the 4°C fusion assay attempted to separate the effect of antibodies which block binding of virus to the host cell from those that subsequently inhibit fusion, the reproducibility of this assay was very poor, so the 37°C assay was used more extensively.

Assays of the effect of sheep serum upon fusion by MVV produced some interesting results: The same MVV-seropositive serum could inhibit fusion when at low dilutions yet enhance fusion when diluted further. This led to the hypothesis that the balance between fusion inhibition and enhancement is determined by the relative amount of virus and anti-viral antibody. Non-specific antiserum from uninfected sheep could also enhance fusion perhaps due to the presence of cross-reactive

antibodies which suggests that pre-immune serum may actually help initial MVV infection. MVV FFWO was inhibited by serum from most of the MVV-seropositive sheep when at low dilutions. Since in the assay virus was in vast excess to antibody, it is likely that *in vivo*, antibodies would be in sufficient excess to MVV to be able to inhibit its fusion with host cells. Nevertheless the virus persists and the sheep still succumb to disease showing that a fusion inhibitory response generated after infection is not protective. Whether such a response could be protective if generated before challenge has yet to be determined. Comparison with similar studies suggested that antiserum to a different MVV strain from that used in the assay may be more likely to enhance than to inhibit fusion. Further studies are required to elucidate the mechanisms of inhibition and enhancement in the assay as the results of this assay may have important implications in design of potential vaccines (and perhaps also for HIV vaccine design).

APPENDICES

APPENDIX I: Bacterial culture medium and transformation of *E.coli*

Preparation of competent *E.coli*

Solutions:

Psi broth

Tryptone 10g	}	
Yeast extract 2.5g	}	Autoclave
SDW 490ml	}	
1M $MgSO_4$ (24.6g)	}	
0.5M NaCl (2.9g)	}	Filter sterilise and add 10ml
0.25M KCl (1.86g)	}	to 490ml tryptone/yeast extract
SDW (100ml)		}

TfBI

NaOAc (1.43g)	}	
$CaCl_2.6H_2O$ (1.1g)	}	
Glycerol (75g)	}	Filter sterilise
pH to 5.9 with acetic acid.	}	
Make up to 500ml then add	}	
RbCl (6g)	}	
$MnCl_2$ (4.95g)	}	

TfBII

MOPS (0.209g)	}	pH to 6.8 with KOH
RbCl (0.12g)	}	Make up to 100ml
$CaCl_2.6H_2O$ (1.64g)	}	Filter sterilise
Glycerol (15g)	}	

Method:

A colony from an LB agar plate was inoculated into 10ml L-broth and grown overnight at 37°C with shaking. 1ml of overnight culture was added to 50ml of Psi broth and incubated until an OD_{550} of ~0.48 was reached. (Takes about 3h). The culture was cooled briefly on ice and then the cells were spun down by centrifugation (2,000rpm bench centrifuge). Cells from 100ml of culture were resuspended in 33ml of ice-cold TfBI and incubated on ice for 10-15 min. The cells were spun down and resuspended in 4ml TfBII on ice for 20 min. 0.2ml amounts were aliquoted into 1.5ml Eppendorfs, frozen rapidly on dry ice and stored at -20°C.

Transformation of competent cells

Competent cells were thawed on ice. DNA was added (in small volume if possible). The tubes were placed on ice for 30 miniutes. The cells were heat-shocked by transferring the tubes to a 42°C water bath for 60-90 sec. After cooling briefly on ice, 0.8ml of L-broth was added to each tube. The tubes were incubated at 37°C for 60 min. Cells were spun down by 30 sec microcentrifugation and resuspended in 200µl of L-broth. 100µl aliquots were spread on selective agar plates (LB/Amp, or LB/Amp/X-Gal/IPTG) which were then incubated overnight at 37°C.

LB/Amp agar plates

15g of agarose was added per litre of L-broth. The agar was placed in a steamer until it had dissolved then it was autoclaved. The agar was cooled to 55°C before addition of Ampicillin to 150µg/ml. About 20ml of agar was used for each plate. LB/Amp plates were stored at 4°C for up to 2 weeks.

LB/Amp/X-Gal/IPTG plates

Base agar was made by dissolving 15gof bacteriological agar per litre of L-broth. Top agar contained 8g of agar per litre of L-broth. The agar was dissolved by placing in a steamer before autoclaving. It was then cooled to 55°C. Ampicillin was added to 150µg/ml. About 20ml of base agar was poured per plate. When the base agar had set it was overlaid with about 6ml per plate of top agar containing 150µg/ml of ampicillin, 20µg/ml X-Gal (made up in dimethyl formamide) and 17µg/ml of IPTG (in SDW).

Alternatively, 25µl of 24mg/ml IPTG (in SDW) and 40µl of 20mg/ml X-Gal (in DMF) were spread directly onto LB/Amp plates using a glass spreader.

LB/Amp/X-Gal/IPTG plates were stored at 4°C for up to 4 days.

Storage of bacterial stocks

An overnight culture was grown up in L-broth (plus 150µg/ml ampicillin if required). The bacteria were spun down by 5 min at 6,00rpm in a bench centrifuge. The medium was removed and the cells were resuspended in L-broth/15% glycerol. (3.4ml LB + 0.6ml glycerol) 1ml aliquots were transferred to Eppendorf tubes and snap frozen on dry ice. Stocks were stored at -70°C.

APPENDIX II: Preparation and electrophoresis of DNA

Preparation of Phenol

Redistilled phenol was stored at -40°C. Phenol was melted in a water bath at 68°C then cooled to RT. Hydroxyquinilone was added to 0.1%, i.e. 0.1g to 100ml of phenol. An equal volume of 0.1M Tris pH8.0 was added and mixed well. The two layers were allowed to separate and the aqueous layer was removed. Addition of 0.1M Tris pH8.0, mixing and separation was repeated at least four times, until the pH of the Tris remained at pH8.0 after mixing and separation.
The phenol/Tris was stored in the dark at 4°C.

Preparation of Phenol/chloroform/isoamyl alcohol

Phenol was equilibrated with 0.1M Tris (pH8.0) as described above. Chloroform and isoamyl alcohol were added at a ratio of 25:24:1 of phenol:chloroform: isoamyl alcohol. The solution was stored with Tris in the dark at 4°C.

Phenol extraction and ethanol precipitation

An equal volume of phenol (pH8.0) was added to the solution of DNA and mixed vigorously by vortexing for 30sec. The aqueous and solvent layers were separated by microcentrifugation for 5min. The top layer was collected to a new tube taking care to avoid any material at the interface. An equal volume of phenol/chloroform/isoamyl alcohol was added and mixed by vortexing for 30sec. After microfuging for 5min the aqueous (top) layer was again transferred to a new tube. An equal volume of chloroform was added, mixed and separated by spinning for 2 min. The aqueous layer was collected. 0.1 volumes of 3M sodium acetate and 2.5 volumes of ethanol was added. The tube was placed at -20°C overnight, at -70°C for 30min or on dry ice for 15-20min. The DNA was precipitated by 15min microcentrifugation at 4°C. The pellet was washed twice with 70% ethanol, dried and resuspended in SDW.

Restriction endonuclease digestion

The optimum conditions required for digestion vary between each restriction enzyme. This information was supplied by the manufacturer. Generally 10x concentrated digestion buffers supplied with each enzyme were used to create optimum buffer conditions. One tenth volume of 10x digestion buffer was added to a DNA solution in water or, in the case of plasmid minipreps, in TE/RNase. Excess

restriction enzyme was then added depending on the estimated amount of DNA to be digested. Digestions were incubated in a dry hot block at 37°C for the required amount of time (1 hour or more for most enzymes, overnight for BamHI digestion).

When digestion with two enzymes was required both were added at once if both had similar optimal buffer conditions. Alternatively the enzyme requiring the lower salt/ionic molarities was used first, then the molarity required for the second enzyme was obtained by the addition of appropriate solutions and the second restriction enzyme was added.

CIP treatment

After incubation of plasmid with restriction enzyme for 1h (or as required) at 37°C 1µl (15 Units) of Calf Intestinal Phosphatase (CIP) (Sigma) was added. After 30min a further 1µl of CIP was added and incubation was continued for another 30min.

Agarose gel electrophoresis

Solutions:

50xTAE- 242g Tris, 57.1ml glacial acetic acid, 100ml 0.5M EDTA pH(8.0), made up to 1l with deionised water.

Loading buffer- 100mM EDTA, 30% Ficoll, 0.025% bromophenol blue, 0.025% Xylene cyanol.

Method

Agarose was dissolved by boiling in TAE buffer then allowed to cool a little. Ethidium Bromide was added to a concentration of 0.6µg/ml. The gel was poured and left to set. TAE running buffer was made up and enough was added to the tank to just cover the gel. 0.2 volumes of loading buffer was added to each sample. Samples were loaded and the gel was run at 70-80V (or 40-50V at 4°C for low-gelling temperature (LGT) gels). Nucleic acid in the gels was visualised on a UV transilluminator.

Recovery of DNA from agarose

1. Hot Phenol/chloroform

The agarose slice containing the DNA was melted at 65°C. An equal volume of 65°C phenol/chloroform was added and vortexed. The aqueous and solvent phases were seperated by microcentrifugation for five min. The aqueous phase was collected then extracted once with phenol, once with phenol/ chloroform (RT), once

twice with 70% ethanol, dried and resuspended in water or buffer.

2. Geneclean.

DNA was recovered from LGT agarose by binding to then elution from silica. "Geneclean" reagents were used according to the manufacturers instructions. Briefly, a slice of gel containing the DNA of interest was excised and weighed to estimate its volume. 2.5-3 volumes of NaI stock solution was added and the gel was melted by heating to 60°C. Glassmilk suspension (silica) was added (5µl for 5µg or less) and placed on ice for 5 min with mixing every 1-2 min. The silica matrix and bound DNA were pelleted by microcentrifuging for 5 sec. The supernatant was removed. The pellet was washed three times with "NEWWASH". After the third wash as much supernatant as possible was removed. The DNA was eluted from the glassmilk by resuspending the pellet in 10µl of SDW and incubating at 50°C for 2-3 min. The supernatant was collected after microcentrifuging for 30 sec. The elution was repeated once more and the supernatants were pooled. The DNA concentration in the supernatant was estimated by running a sample on an agarose gel alongside markers.

3. Centrifugation through 3MM paper

DNA was recovered from agarose (not LGT) as described by Weichenman, (1991). Briefly 3MM paper was formed into a beaker inside a 0.5 ml Eppendorf tube with a hole pierced in the bottom. The agarose slice was placed into the paper beaker. The first tube is mounted inside a 1.5ml Eppendorf tube and microcentrifuged for 1 minute. The buffer and DNA are collected in the second tube leaving behind dehydrated agarose in the blotting buffer. Recovery of DNA from the gel is predicted to be greater than 50%.

Minipreps for the isolation of plasmid DNA (Adapted from Serghini et al., 1989)

Transformants grown on selective agarose plates were inoculated into L-broth/Amp and grown up overnight at 37°C with shaking. 3ml of overnight culture was pelleted by 30s microcentrifugation and resuspended in 100µl of TNE. 100µl of phenol/chloroform was added. The mixture was vortexed for 60 sec. After 5 min microcentrifugation the aqueous (top) layer was transferred to a fresh Eppendorf. An equal volume of phenol/chloroform was added. The mixture was vortexed for 60 sec. Again the aqueous and solvent layers were separated by microcentrifugation for 5 min. The aqueous layer was collected and heated to 70°C for 5 min to denature any DNAses. One tenth volume of 3M NaOAc and 2.5 volumes of ethanol was

added. After one hour at -20°C or 5 min on dry ice precipitated DNA was pelleted by microcentrifugation for 15 min at 4°C. The pellet was washed twice with 70% ethanol, dried and resuspended in TE/25µg/ml of RNAseA. If restriction digestion was required the DNA was resuspended in TE/RNAse plus the approriate buffer and enzyme. The resuspended DNA was incubated at 37°C for 1 hour. Samples were run on a 1% agarose gel alongside DNA size markers and the appropriate control plasmids.

Large Scale plasmid preparation

20ml of overnight culture was inoculated into 1l of L-broth/150µg/ml ampicillin. The 1l culture was incubated overnight then harvested in 250ml Beckman bottles in J2-21 rotor at 15K for 15 min. The cells were washed once in STE (0.1M NaCl, 10mM Tris-Cl pH8.0, 1mM EDTA) The pellet from 500ml of culture was resuspended gently in 10ml of cold solution I plus 5mg/ml lysosyme and placed at RT for 15 min. 20ml of 0.2M NaOH, 1% SDS was added, mixed gently and placed on ice for 10 min. 15ml 5M Potassium acetate was added mixed and placed on ice for a further 10 min. The mixture was centrifuged for 20 min at 11K. The supernatant was recovered into a fresh 250ml Beckman bottle taking care to avoid any floating or pelleted material. 0.6 volumes of isopropanol was added. The mixture was incubated at RT for 20 min. DNA was precipitated by centrifuging for 10 min at 10K at 4°C. The pellet was washed once with 30ml 70% ethanol, air dried, resuspended in 5.0ml TE/10µg/ml RNAse A and incubated at 37°C for 1 hour. The DNA was extracted 3 times with phenol/chloroform and once with chloroform. The supernatant was transferred to a Corex tube. 10M ammonium acetate was added to a final concentration of 2M. 0.6 volumes of isopropanol was added. The tube was placed at RT for 20-30 min then centrifuged at 8K for 10 min. The pellet was washed with 70% ethanol, air dried and resuspended in exactly 1.8ml of 10mM Tris-Cl pH8.0, 1mM EDTA, 1M NaCl. A PZ523 (5'-3' Inc.) column was prepared by removing the top and bottom closures and spinning for 1 minute at 1500rpm to remove the storage buffer. 1.8ml of sample was loaded and spun for 12 min at 1500rpm. The collected solution was extracted twice with phenol chloroform, once with chloroform . The DNA was precipitated by adding 0.6 volumes of isopropanol, mixing, incubating for 20 min at RT, then microfuging for 15 min. The precipitate was washed twice with 70% ethanol, air dried and resuspended in 200µl of SDW. (The resulting plasmid concentration was normally in the range of 2-8µg/µl).

APPENDIX III: DNA blotting and hybridisation protocols

Labelling probe DNA with ^{32}P

50ng of probe DNA (plasmid insert) was made up to 26µl with SDW and boiled for 5 min. The solution was cooled to 37°C. 10µl 5 x OLB buffer, 1µl BSA (1mg/ml), 10µl (100µCi) ^{32}PdCTP and 3µl Klenow DNA polymerase was added. The mixture was incubated for 4 hours at 37°C. The reaction was stopped by adding 50µl of 10xTE. The radioactivity incorporation was measured.

Southern blotting onto nitrocellulose

After electrophoresis to separate the DNA fragments the gel was washed for 15min in 0.2M HCl, washed in two changes of denaturing solution (0.5M NaOH, 1.5M NaCl) for 15 min each, and in several changes of neutralising solution (0.5M Tris-HCl pH7.0, 1.5M NaCl) for a total of 45 min. The gel was rinsed in waterand was placed on top of two pieces of 3MM paper acting as a wick to draw up 10 x SSC buffer from the trough below. Nescofilm was placed around the gel. A piece of nitrocellulose cut to size and soaked in 10 x SSC was placed on the gel. 8-10 pieces of 3MM paper soaked in 10 x SSC were placed on top of this, followed by a stack of dry paper towels. A glass plate and a weight were placed on top. The assembly was left overnight in which time the towels had drawn up buffer by cappillary action and transferred the DNA to the nitrocellulose. The nitrocellulose was air dried and then baked at 60°C under vaccuum for 2h to fix the DNA to it. Once baked the blot could be stored at room temperature until required.

Southern blotting onto nylon filters

After running the gel it was soaked in 10 x SSC. The apparatus was set up as above but using a nylon filter instead of nitrocellulose, and left to blot overnight. The nylon filter was fixed and denatured by placing on top of 3MM paper soaked in 10 x SSC and microwaving for 2.5 min at maximum power.

Prehybridisation buffer

10ml 10% Marvel

4ml 1M $NaHPO_4$ pH7 (Made by adding 1M NaH_2PO4 to 1M Na_2HPO_4 until the pH was 7.0)

28.5ml 20xSSC

0.8ml 0.25M EDTA

2ml 10% SDS

2ml 10mg/ml yeast RNA

152.7ml SDW

Filters were prehybridised for at least 4 hours at 65°C in prehybridisation buffer. Most of the buffer was removed leaving just enough to cover the filters. Labelled probe was boiled for 2 min then added to the filters (about 2 x 10^6 counts/minute per filter) and incubated overnight at 65°C. The filters were washed at least 6 times for 10 min with 3xSSC, 0.1%SDS, at room temperature, then twice for 30 min with 0.3xSSC, 0.01% SDS, at 65°C. The filters were dried at room temperature then put down against XS autoradiography film and stored at -20°C for 1h to several days as required depending on the signal intensity.

Colony screening using nylon filters (Adapted from Buluwela et al, 1989)

Transformants selected by spread plating on LB agar 150μg/ml Amp were picked onto duplicate fresh plates. When these had grown sufficiently lifts were made onto nylon filters (Hybond-N) by placing dry filters onto plates to contact colonies then peeling off the filter. Filters were laid colony side up on a piece of 3MM paper soaked in 2 x SSC, 5%SDS, and microwaved (650W) on a rotating turntable for 4 min. The filters were prehybridised for 2 hours at 65°C then labelled probe was added directly to the prehybridisation buffer and incubated at 65°C overnight with mixing. Filters were washed 6 times with 3 x SSC, 0.1%SDS at room temperature and twice with 0.3 x SSC, 0.01%SDS at 65°C, then dried and placed against XAR film for 3 days.

Production of single stranded pTZ derivatives

JM101 cells transformed with recombinant pTZ were grown in 2 x YT medium plus 150μg/ml ampicillin at 37°C to OD_{660} 0.5-0.8. 2ml of this culture was inoculated with phage M13K07 at a multiplicity of infection of 10 (assuming 1 OD_{660} to be equivalent to $8x10^8$ cells/ml). The culture was incubated with vigorous shaking at 37°C for 1 hour. 400μl of these infected cells was added to 10ml 2xYT medium plus 70μg/ml kanamycin and incubated at 37°C for 14-18 hours with good aeration. The culture was centrifuged to remove cells. The supernatant was collected and recentrifuged until a cell pellet was no longer observed. 0.25 volumes of 20% polyethylene glycol in 3.5M NaCl was added and placed on ice for 30 min to precipitate the phage containing single strand pTZ DNA (as well as a small amount of M13K07 helper phage). The precipitate was pelleted by centrifugation at 11,000g for 30 min at 4°C for 30 min. The supernatant was removed and the pellet was resuspended in 0.4ml of 20mM Tris-HCl (pH7.5), 20mM NaCl, 1mM EDTA. The DNA was phenol/chloroform extracted and ethanol precipitated. The precipitate was washed with 70% ethanol, dried and dissolved in 50μl of 10mM Tris-HCl (pH7.5), 1mM EDTA. Samples were electrophoresed on a 1% agarose gel to check concentration and purity prior to sequencing.

Sequencing gels

The gel plates were cleaned thoroughly, siliconised, and polished before assembling the apparatus. 120μl TEMED and 110μl 25% ammonium persulphate (freshly made) was added to 40ml 6% acrylamide/urea/TBE and used to seal the base. 110ml of 6% acryl./urea/TBE was filtered and de-gassed then 110μl TEMED and 110μl 25% APS was added. The gel was poured and the comb was put in place. 100ml. 10 by TBE was made up to 2l. with millipore water and used to fill the running buffer tank. After removing the comb, the gel was prewarmed by running at 90W for 20-30 mins. The wells were flushed out with buffer immmediately before loading. Samples were heated at 75°C for 2-3 min and 5μl of each was loaded. Sequencing gels were run at at 90W or 2950V for 2-8 hours. The apparatus was disassembled and the gel was transferred onto 3M paper and dried on a vacuum drier at 80°C for 1-2 hours. The dried down gel was placed against X-ray film in an autorad casette and stored at room temperature. The film was developed 24h to several weeks later.

Sequencing

Sequencing reactions were conducted as shown below using the Sequenase Version 2.0 kit (United States Biochemical Corporation).

Single-stranded sequencing reactions

Annealing: 2µl (~2µg) single-stranded DNA, 1µl reverse primer, 1µl DMSO, 2µl reaction buffer and 4µl SDW were added together, heated to 65°C for 2 min and then cooled slowly to room temperature in a beaker of water.

Labelling: 5µl DTT, 10µl dGTP labelling mix (diluted 1:5 in SDW), 2.5µl 35SdATP, and 10µl sequenase enzyme (diluted 1:8 in enzyme dilution buffer) was added and incubated at room temperature for 2-5 min.

Termination: 3.5µl of each labelling reaction was added to pre-warmed tubes each containing 2.5µl of one of the four ddNTP termination mixes, ddATP, ddCTP, ddGTP or ddTTP. The termination reactions were incubated at 37°C for 5 min, before addition of 4µl of stop buffer to each tube.

Double-stranded sequencing reactions

~0.5pmole of sequencing primer was annealed to ~1pmole of double-stranded DNA, in annealing buffer, by boiling for five min then snap cooling on ice. 5µl of elongation mix (made up of 4µl DTT, 4µl DMSO, 1µl dGTP mix, 2µl 35SdATP, 7µl sequenase buffer, and 1µl of sequenase enzyme) was added to each tube. The elongation reaction was allowed to proceed for ten min on ice. 3.5µl of each reaction was added to 2.5µl of each of the four ddNTP termination mixes in seperate pre-warmed tubes, and incubated at 37°C for 5 min. 4µl of stop buffer was then added.

APPENDIX V: Yeast transformationand yeast culture media

Culture media for yeast

YEPD: peptone 20g/l, yeast extract 10g/l, glucose 20g/l

SC-glc: yeast nitrogen base (without amino acids) 6.7g/l, glucose 10g/l, amino acids added as required after autoclaving.
For SC-glc plates 20g/l Bacto agar was added before autoclaving.

SC-Gal: yeast nitrogen base (without amino acids) 6.7g/l, glucose 3g/l, galactose 10g/l, amino acids added as required after autoclaving.

Amino acid stocks: 10mg/ml L-tryptophan, 10mg/ml uracil, 10mg/ml leucine
10mg/ml L-tryptophan, 10mg/ml uracil
10mg/ml L-tryptophan
Amino acid stocks were dissolved by boiling briefly, filter sterilised and stored at 4°C in the dark. 2ml of amino acid stock was added per litre of medium

Regeneration agar: 5g ammonium sulphate, 1.7g yeast nitrogen base, 20g dextrose, 30g agar, 1 pellet NaOH, 500ml 2M sorbitol, 20ml YPD medium, 480ml water.
The mixture was melted in a steamer and 20 ml aliquots were transferred to Universals. After autoclaving, the agar was cooled to 55°C and amino acids were added as appropriate.

Solutions for yeast transformation (All filter sterilised)
$CaCl_2$ solution: 0.1M Tris-Cl pH7.4, 0.1M $CaCl_2$
Lithium acetate solution: 0.1M lithium acetate, 10mM Tris-Cl, pH8.0, 1mM EDTA
PEG solution: 40% wt/vol polyethylene glycol 3350, 0.1M lithium acetate, 10mM Tris-Cl, pH8.0, 1mM EDTA
PEG/$CaCl_2$ solution: 40% wt/vol polyethylene glycol 3350,10mM Tris-Cl, pH7.4, 10mM $CaCl_2$
Sorbitol/$CaCl_2$ solution: 1M sorbitol, 10mM Tris-Cl, pH7.4, 10mM $CaCl_2$

Lithium acetate transformation method

50ml YEPD was inoculated with 25, 50 or 100µl of a saturated of yeast and incubated at 30°C with shaking for 16 hours. The OD_{600} was measured and cultures were checked for contamination. 50ml of whichever culture had an OD_{600} of 0.5-1 was spun down at 1100g for 10 min. The pellet was washed in 10ml 10mM Tris(pH8), 1 mM EDTA then resuspended in 10ml lithium acetate solution. Cells were spun down for 10 min at 1100g, resuspended in 500µl of lithium acetate solution, and incubated for 1 hour at 30°C with gentle shaking. 100µl aliquots were transferred to sterile Eppendorfs. 1-10µg of donor DNA with 0-20µg of carrier DNA (salmon sperm DNA) was added. The tubes were incubated at 30°C for 30 min without shaking. 0.7ml of PEG solution was added and incubated at 37°C for a further 45 min. The tubes were placed at 42°C for 5 min without shaking. For each transformation four 200µl aliquots were mix plated onto selective agar and incubated at 30°C for several days until transformant colonies were visible.

Spheroplast transformation

A 10ml culture of yeast was grown overnight at 30°C. 5, 10, 25, or 50µl was used to inoculate 50ml of YPD to achieve an OD_{600} of 0.5-1 (equivalent to $1\text{-}2\text{x}10^7$ cells/ml) after overnight culture. The cells were pelleted by 5 min centrifugation at 1100g and resuspended in 5ml of 1M sorbitol. This was repeated twice then 150µl of glusulase was added and incubated at 30°C with occasional gentle shaking for 60 min. Spheroplasts were pelleted for 4 min at 400g. The supernatant was carefully removed and the pellet was gently resuspended in 10ml 1M sorbitol. Pelleting and resuspension in 10ml Sorbitol was repeated. The spheroplasts were pelleted and resuspended in 9ml of sorbitol plus 1ml of $CaCl_2$ solution, mixed gently by swirling, and pelleted again at 400g. The pellet was resuspended in 1ml sorbitol/$CaCl_2$ solution. 200µl aliquots were transferred to sterile Eppendorfs. Plasmid DNA with or without carrier DNA was addedand incubated for 10 min at room temperature. 10 volumes of PEG/$CaCl_2$ solution was added to each aliquot and incubated at room temperature for 10 min. Spheroplasts were pelleted at 400g and the supernatant was removed. The pellet was gently resuspended in 0.5ml of sorbitol/$CaCl_2$ solution, mixed with to 20 ml of 55°C regeneration containing the appropriate amino acids and poured into a sterile petri dish. The plates were incubated at 30°C until small colonies were visible. Individual colonies were then picked and streak plated on selective SC-glc agar.

APPENDIX VI: SDS-PAGE and Western blotting

SDS-PAGE protein gels

The Biorad Mini Protean II slab gel apparatus was used. Either 10% or 5-20% gradient separating gels were used, using a 30% acrylamide/0.8% bis stock solution and a final concentration of 0.38M Tris-Cl pH8.8, 2mM EDTA, 0.1% SDS, 0.08% APS, 0.05% TEMED. The stacking gel was 3.48 % acrylamide/0.093% bisacrylamide, 0.145M Tris-Cl pH6.8, 0.12% SDS, 0.12% APS, 0.17% TEMED. Running buffer contained 0,05M Tris, 0.37M glycine, 19.5μM EDTA, 0.1% SDS

Samples were boiled for 2-3 min with an equal volume of reducing sample buffer (0.1M Tris pH6.8, 2% 2-mercaptoethanol, 30% glycerol, 1% SDS, 0.001% bromophenol blue) before being loaded into the wells using a Hamilton micro-syringe. Molecular weight markers (Pharmacia) were run in at least one well of each gel. Electrophoresis was performed at 150V for approximately 45 min. or until the dye front had reached the bottom of the gel.

Coomassie Staining

The PAGE gel was placed in a tray of 0.2% Coomassie stain (0.2% Coomassie blue R250 (Sigma, UK), 20% methanol, 5% acetic acid), for 20 min followed by extensive destaining with 5% acetic acid, 20% ethanol. The gel was dried down under vacuum at 80°C for 1hr.

Silver staining

All solutions for silver staining were made using Milli-Q deionised water. The gel was fixed for 15 min in 50%methanol, 10% acetic acid, for 30 min in 5% methanol, 7% acetic acid, and then for 30 min in 10% formaldehyde. The gel was rinsed overnight in several changes of water. 0.1% silver nitrate was added and incubated with the gel for 15 min. The gel was rinsed briefly with water then 500ml 3% Na_2CO_3 and 250μl 37% formaldehyde was added. When the colour had developed sufficiently, solid citric acid was added to stop the reaction. The gel was rinsed for 30 min in water, fixed for 1 minute in 10% Ilfofix, and rinsed thoroughly in water before drying down onto paper.

Western blotting

The polypeptides separated by SDS-PAGE were transferred from the gel onto nitrocellulose (Hybond-C, Amersham, UK),using a semi-dry electroblotter (Ancos) with 25mM Tris, 20% methanol, 123mM glycine as buffer. A current of 120mA was applied across the blotter for 1 hour. The marker track was cut off, stained with 0.5% Amido black for 30 min and destained in several changes of 50% methanol, 5% acetic acid . The rest of the nitrocellulose blot was blocked with PBS, 5% milk powder (Marvel), 0.5% Tween 20 (Sigma UK) for 1 hour at room temperature or overnight at 4°C. Blots were developed at once or stored at -20°C.

Developing a blot

Pre-blocked blots were incubated for 60 mins at room temperature or overnight at 4°C in anti-serum diluted in PBS, 1% marvel, 0.1% Tween 20. After five washes five times over at least 30 min with PBS, 1% marvel, 0.1% Tween 20, the second antibody (alkaline phosphotase conjugated antiglobulin diluted according to the manufacturers instructions in PBS, 1% marvel, 0.1% Tween 20) was added to the blot and incubated for 1 hour at room temperature. The blot was washed at least 5 times in PBS, 1% marvel, 0.1% Tween 20, and twice in 0.1M Tris pH9.5. Blots were developed with 0.1M Tris pH9.5, 0.2 mg/ml nitro blue tetrazolium (NBT), 0.1mg/ml bromo-chloro-indolyl-phosphate toluidine salt (BCIP), 20mM $MgCl_2$. When development was complete the blots were washed with water then dried.

Production of Visna EV1 antigen for Western blots

Sheep skin cells 657 were grown in DME plus 6% FCS , pen/strep, L-glutamine, and fungizone, in T225 flasks. Confluent monolayers were infected with EV1. Initially , 400µl of EV1 stock culture in 10ml of medium was added to each flask and allowed to adsorb at 37°C for one hour. A further 90ml of medium was added and the cells were incubated at 37°C until nearly 100% CPE was seen. Virtually all the cells had come off the plastic by 6-7 days after addition of virus. The medium was then transferred to 250 ml Beckman bottles and centrifuged in a J2-21 rotor overnight at 10,000g. The pellet was resuspended in 12ml PBS then centrifuged for 2 hours at 30K in an SW40 rotor . The pellet was finally resuspended in 1ml PBS per 200ml of starting culture medium, aliquoted and stored at -70°C

APPENDIX VII: ELISA

96-well plates (Falcon) were coated with 75µl of diluted antigen per well at 4°C overnight. Excess antigen was removed and plates were washed at least three times in 0.1% Tween 20 in PBS. 75µl 2% BSA in PBS was added to each well and incubated at room temperature for 30min. Plates were washed at least three times in 0.1% Tween 20 and 100µl of serum or antibody sample, diluted in PBS, was added to each well and incubated at 37°C for 1h. Plates were washed at least three times in 0.1% Tween 20 in PBS. Alkaline phosphatase-conjugated antiglobulin was diluted in 2% BSA/PBS according to the manufacturers instructions. 75µl was added per well and incubated at 37°C for 60min. Plates were washed at least three times in 0.1% Tween 20 in PBS then 75µl of HPX substrate was added to each well. Substrate was allowed to develop in the dark for no more than 10min. 50µl of 2M H_2SO_4 was added to stop the reaction and OD_{492} was read using a Titertek Multiskan blanked against an empty plate.

HPX substrate: (made up fresh each time) 0.8ml OPD mixture, 12.1ml H_2O, 6.2ml 0.2M Na_2HPO_4, 5.8ml 0.1M citric acid, 4µl H_2O_2.

OPD mixture: (Stored in 0.8ml aliquots at -70°C) 0.5g OPD, 10.04ml H_2O, 5.14ml 0.2M Na_2HPO_4, 4.82ml citric acid

APPENDIX VIII: IgG

Preparation of IgG from sheep sera

A 2ml Sepharose-G column (1cm diameter) and a 2ml Sepharose pre-column were pre-eluted with four column volumes of 0.1M glycine/HCl (pH2.7) then re-equilibrated with PBS (pH8.0). 1ml of serum was complement inactivated by heating for 30 min at 56°C. The serum was loaded through the pre-column then through the Sepharose-G column at least four times. Unbound material was washed off by flushing the column through with more than six volumes of PBS (pH8.0). Bound protein was eluted with 0.1M Glycine/HCl (pH2.7) and 0.5ml fractions were collected. The pH of each fraction was adjusted to pH7-8 with 0.5M Tris (pH8.0).

The OD_{280} of each fraction was measured and fractions containing protein were dialysed into PBS overnight at 4°C. Finally the protein concentration in the pooled fractions was estimated from the OD_{280}. 1µl samples were electrophoresed on a 10% acrylamide SDS-PAGE gel which was then silver stained.

The column was washed extensively with PBS (pH8.0) between application of each sample, and was finally washed and stored in PBS/0.01% Sodium azide.

IgG capture ELISA

Falcon PVC plates were coated with 10µg/ml mouse anti-sheep IgG monoclonal antibody (VPM6) in borate buffered saline (BBS), pH8.2, overnight at 4°C. After washing three times with BBS, 0.05% Tween-20, twofold dilutions of the samples were made down the plate. A standard of sheep IgG was included beginning at a concentration of 500ng/ml. The plates were incubated for 1 hour at 37°C then washed with BBS, 0.05% Tween-20. Rabbit anti-sheep horse radish peroxidase conjugate was added at 1:4000 in BBS/1%BSA and incubated for 1 hour at 37°C. The plates were developed with H_2O_2 and orthophenyldiamine (OPD) in citrate phosphate buffer (pH5.2) for fifteen min. The reaction was stopped by adding 2M H_2SO_4 and the optical density was measured at 492nm.

APPENDIX IX: Formulae used for statistical calculations

The variance (s^2) of each sample was calculated using the equation:

$$s^2 = \frac{\sum x^2 - (\sum x)^2/n}{n - 1}$$

The pooled variance, s_p^2, was estimated using the equation:

$$s_p^2 = \frac{(n_1 - 1)\, s_1^2 + (n_2 - 1)\, s_2^2}{(n_1 - 1) + (n_2 - 2)}$$

The pooled variance was used in the formula below for calculating t:

$$t = \frac{x_1 - x_2}{s_p^2 [\, 1/n_1 + 1/n_2 \,]}$$

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