Identification of Proteins Interacting with the Polymerase (L) Protein of Rinderpest Virus

by

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Declaration

I declare that all the work submitted in this thesis has been carried out solely by myself, unless acknowledged in the text.

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Abstract

Rinderpest virus (RPV) is a morbillivirus which causes a highly contagious disease affecting members of the order *Artiodactyla*. The viral L protein is the catalytic subunit of the RNA-dependent RNA polymerase, but requires the P protein for activity. In previous studies it was found that, in addition to a direct L-P interaction, both the C and V non-structural proteins bind to L. The L proteins of morbilliviruses consist of three long highly conserved domains separated by short unconserved sequences. The interaction of P, C and V with these three domains was studied. Using co-immunoprecipitation, it was shown that P interacts with the first domain, whilst C and V were each shown to interact with the central domain. Further mutational analysis using the yeast two-hybrid system (Y2HS), showed that the P binding site lies in the amino-proximal domain of L, between amino acids 1 and 233, which fits with the co-immunoprecipitation data. However, the Y2HS suggested that the binding site for C and V includes a region between amino acids 1 and 363 of L, i.e. within the first domain. These data indicate (i) that the P binding site is distinct from that of C and V, and (ii) that the C and V binding site(s) may be complex.

To search for host cell proteins with which L interacts, a library screen was performed using the Y2HS and a porcine macrophage cDNA library. Three host cell proteins were recovered from the library screen as putative L interactors. The interaction with one of these, striatin, was confirmed by co-immunoprecipitation, and co-localisation of the two proteins was observed by confocal microscopy. The L sequence with which striatin interacts was investigated. Like the C and V proteins, striatin was shown to interact with the second conserved domain of L by co-immunoprecipitation and Y2HS data indicated that a possible second binding site for striatin includes a region of L sequence between amino acids 1 and 363.

List of Abbreviations

α	- Anti (as applied to target of antibodies/antisera)
APS	- Ammonium persulphate
Amp	- Ampicillin
ATP	- Adenosine tri-phosphate
bp	- Base pairs
BSA	- Bovine serum albumin
cDNA	- Complementary deoxyribonucleic acid
CDV	- Canine distemper virus
CMV	- Cytomegalovirus
CNS	- Central nervous system
Co-ip	- Co-immunoprecipitation
CPE	- Cytopathic effects
CSU	- Central service unit
DAPI	- 4', 6-Diamidino-2-phenylindole hydrochloride
DDW	- Double distilled water
DEPC	- Diethyl pyrocarbonate
DMEM	- Dulbecco's modified Eagle's medium
DMSO	- Dimethyl sulfoxide
DMV	- Dolphin morbillivirus
DNA	- Deoxyribonucleic acid
DNAse	- Deoxyribonuclease enzyme
dNTPs	- 2'-deoxynucleotide 5'-triphosphates
dsRNA	- Double stranded ribonucleic acid
DTT	- Dithiothreitol
EDTA	- Ethylenediaminetetra-acetic acid
EF-1	- Translation elongation factor-1
F	- Fusion protein
FCS	- Fetal calf serum
FITC	- Fluorescein isothiocyanate

FPT7	- Fowl pox T7 virus (a recombinant virus expressing T7 polymerase)
GE	- Gene end
GFP	- Green fluorescent protein
GS	- Gene start
GST	- Glutathione-S-transferase
H	- Haemagglutinin protein
HA	- Haemagglutinin tag (from influenza virus)
HEPES	- N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid
HeV	- Hendra Virus
hPIV-1	- Human parainfluenza virus type 1
hPIV-2	- Human parainfluenza virus type 2
hPIV-3	- Human parainfluenza virus type 3
HSV	- Herpes simplex virus
IF	- Immunofluorescence
IFN	- Interferon
Ig	- Immunoglobulin
IPTG	- Isopropyl-3-D-galactopyranoside
Kan	- Kanamycin
kbp	- Kilobase pairs
kDa	- Kilo Daltons
L	- Large (polymerase) protein
LB	- Luria Bertani medium
M	- Matrix protein
MeV	- Measles virus
MOI	- Multiplicity of infection
mRNA	- Messenger ribonucleic acid
MTHS	- Mammalian two-hybrid system
N	- Nucleocapsid protein
NDV	- Newcastle disease virus
NiV	- Nipah virus
NS	- Non-structural
OD	- Optical density

ORF	- Open reading frame
P	- Phosphoprotein
PBS	- Phosphate buffered saline
PBS-E	- Calcium and magnesium free phosphate buffered saline
PBS-G	- PBS-E containing 0.2% gelatin
PCR	- Polymerase chain reaction
PDV	- Phocid distemper virus
PFA	- Paraformaldehyde
PHA	- Phytohaemagglutinin
PMA	- Phorbol myristyl acetate
PMSF	- Phenylmethylsulphonylfluoride
PMV	- Porpoise morbillivirus
PPRV	- Peste des petits ruminants virus
P/S	- Penicillin/Streptomycin
RBOK	- Rinderpest of bovine origin Kabete
RNA	- Ribonucleic acid
RNase	- Ribonuclease
rpm	- Revolutions per minute
RPV	- Rinderpest virus
RSV	- Respiratory syncytial virus
RT	- Room temperature
SAP	- Shrimp alkaline phosphatase
SDS	- Sodium dodecyl sulphate
SeV	- Sendai virus
SLAM	- Signalling lymphocyte activation molecule
SSPE	- Sub-acute sclerosing panencephalitis
ssRNA	- Single stranded ribonucleic acid
STAT	- Signal transducer and activator of transcription
SV5	- Simian virus 5
SV40	- Simian virus 40
TAE	- Tris-acetate EDTA
TBS	- Tris buffered saline
$TCID_{50}$	- 50% tissue culture infective dose

TE_8	- Tris-EDTA pH 8.0
TEMED	- N,N,N',N'-tetramethylethenediamine
TF	- Transfast
Tris	- Tris-(hydroxymethyl)-aminomethane
VSV	- Vesicular stomatitis virus
VTF7-3	- Recombinant vaccinia virus expressing T7 polymerase
X-gal	- 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Y2HS	- Yeast two-hybrid system
YNB	- Yeast nitrogen base

Chapter One
Introduction

1.1 Rinderpest

1.1.1 The Disease

Rinderpest or "Cattle Plague" is an acute, highly contagious viral disease affecting even-toed ungulates of the order *Artiodactyla*, most commonly cattle and buffalo. Infections are characterised by the sudden onset of clinical symptoms manifested in high fever, nasal and ocular discharge, severe gastroenteritis with inflammation and haemorrhage, resulting in constipation followed by diarrhoea. The disease has a high morbidity rate and an associated mortality rate of 90-100% with the more virulent strains, falling to around 20% with milder strains. Rinderpest was one of the first diseases shown to be caused by a filterable agent in 1902 by Nicolle and Adil Bey (Rossiter, 1994) and the infectious agent, rinderpest virus (RPV), was first grown in bovine tissue culture by Plowright and Ferris (Plowright & Ferris, 1957).

The incubation period of rinderpest varies with virus strain and dosage, in addition to the route of exposure, and individual strains of rinderpest vary in their pathogenicity depending on host species and breed (Rossiter, 1994). The molecular factors determining variations in virulence remain unknown. Animals infected with rinderpest shed virus one-two days prior to the onset of clinical symptoms, and up to ten days following the start of pyrexia, in their nasal, oral, ocular and vaginal secretions and faeces (Rossiter, 1994). Rinderpest is spread almost exclusively by contact between infected and susceptible animals. RPV may also be transmitted by aerosol and it has been shown experimentally that RPV can be transmitted by aerosol over several hundred metres (Hyslop, 1979). Nevertheless, droplets are large and are only infectious for a short period of time thus close contact is necessary for infection (Reid, 1981). RPV has been isolated

from a variety of insects: yet there is no evidence for them playing a role in the transmission of the virus (Kahrs, 2001).

Although rinderpest can be prevented with vaccination, it spreads easily among non-vaccinated herds, through livestock trade and pastoral migrations, causing economic hardship and widespread famine. Animals vaccinated with vaccine strains of rinderpest do not shed virus and this is most likely due to the low levels of viraemia.

Extensive clinical depictions of rinderpest exist in the literature with the course of disease in both cattle and buffalo similar. In the classic acute form of the disease, sudden onset of fever usually occurs one to four days post exposure and lasts between six and eight days, known as the "prodromal phase". The affected animal's temperature gradually climbs over the first two to four days to between 40 and 41.2°C. The animal is unwell, yet it lacks any symptoms of diagnostic value. The animal may also exhibit conjunctivitis, clear serous nasal and ocular discharge, anorexia, slowed rumination causing constipation and depression. Visible mucous membranes are hyperaemic and may be abnormally dry. It is in the "erosive" phase where the specific signs of RPV are seen, characterised by the appearance of necrotic oral lesions lasting about five days, which start to appear around day four or five, after the onset of pyrexia and accompanied by excessive salivation. One to two days after the onset of oral lesions, when the fever begins to fall, diarrhoea starts. The animal has no appetite, a difficulty in breathing, is thirsty, restless, depressed and has a dried out muzzle. Ocular and nasal discharges become mucopurulent, accompanied by fetid breath. The fate of the animal is soon decided. It collapses, dying immediately, or remains the same for a few days and then dies, or the diarrhoea rapidly progresses resulting in death due to dehydration and malnutrition. If the animal has enough resistance to recover, the fever recedes, returning to normal in around two days. The oral lesions heal, the diarrhoea ceases and the animal regains its appetite. Abortion occurs in recovering pregnant animals.

Two variants of the disease also occur. Peracute infections are rare and usually occur in young calves, with the death of the infected host occurring in about three days (Anderson *et al.*, 1996). Subacute reactions tend to have a longer incubation period of up to fifteen days and clinical signs are usually milder than those seen with the classic form of disease. Most animals with this type of infection survive and are normally the young and the immature adults in areas where rinderpest is enzootic.

Both mild and highly pathogenic strains of RPV share the same tissue tropism *in vivo*; however, highly pathogenic isolates such as Saudi 1/81 replicate more rapidly in permissive cells resulting in a faster rate of disease progression and a wider distribution when compared to milder strains (Wohlsein *et al.*, 1995). Although RPV grows in cells of very different tissue origin, it is primarily lymphotropic and epitheliotropic in its host (Wohlsein *et al.*, 1993). It is the destruction of lymphocytes by RPV that causes the affected animal to become immunosuppressed, leading to secondary bacterial infections that can often be fatal and cause mis-diagnosis. Unlike most morbilliviruses, RPV has never been detected in the brain of infected animals.

1.1.2 History of the Disease

Rinderpest was for centuries the most feared bovine plague known, capable of destroying entire populations of cattle and buffalo and responsible for changing the course of history. Although the distribution of RPV may now be limited, at the start of the twentieth century it still affected cattle populations throughout Asia, Africa and parts of Europe, and it was the fear generated by rinderpest that brought about the first European veterinary colleges. The disease is thought to be of ancient Asiatic origin with recognisable descriptions of rinderpest dating back to the European epizootic of 376-386AD (Reid, 1981). Historically, outbreaks of rinderpest follow wars and periods of civil disturbance where there is uncontrolled movement of people and troops along with live food animals, which can carry the virus. A recent example is that of Turkey, where the virus

reappeared in September 1991 as a consequence of the Gulf War and the displacement of the Kurdish people (Anderson *et al.*, 1996).

The Great Rinderpest Pandemic of 1889-97 is widely thought to be the first major introduction of rinderpest into Africa, yet there still remains confusion surrounding the exact source of the virus that started this pandemic. The more popular opinion is that the virus was introduced to Eritrea by the cattle that accompanied the Italian army in 1887. The virus then proceeded to spread rapidly through Ethiopia in 1888, along the Rift Valley and into West Africa via Sudan and Chad. Within five years the virus had reached the Atlantic and in less than a decade, the virus had crossed into South Africa. Mortality rates in cattle and wildlife exceeded 90%, although waterbuck and alcelaphines, such as wildebeest, remained relatively unsusceptible to the disease (Rossiter, 1994). The great pandemic was responsible for the deaths of over 5.25 million cattle and uncountable wildlife. Humans were affected not only financially, but also through collapse of the transport system and widespread famine.

The last reported outbreaks of rinderpest in Europe were in Belgium in 1920 and in a Rome zoo in 1949 (Rossiter, 1994); the cause in both cases was the importation of infected livestock.

1.1.3 The Eradication of Rinderpest

Rinderpest never established itself in the Americas or Australia/New Zealand and its distribution in other parts of the world is now restricted due to internationally co-ordinated vaccination programs. As a result, rinderpest is currently targeted for global eradication by 2010 (Anderson *et al.*, 1996, Reid, 1981). If this goal is achieved then it will be the first time that an animal disease will have been eliminated worldwide and, after smallpox, the second disease eradicated in history.

In 1961 Joint Project 15 (JP15) was founded, an internationally funded and co-ordinated mass vaccination campaign to control rinderpest in Africa using a tissue-culture attenuated virus vaccine. The attenuated RBOK vaccine strain of RPV was derived in Kenya from a virulent strain of RPV (Kabete 'O') (Plowright & Ferris, 1962). Kabete 'O' was passaged 90 times in primary calf kidney cells, losing the ability to induce a febrile response in all classes of cattle inoculated with the tissue culture adapted form. Passages 41 and 90 were tested for reversion to virulence by back passage in cattle. Passage 41 reverted to virulence by passage 2, whilst passage 90 of the attenuated virus showed no reversion to virulence. Virus passages 90-100 in primary calf kidney cells are used for vaccine production. JP15 was not only responsible for a dramatic reduction in the incidence of rinderpest, but in some countries the disease was even eliminated for several years. By the time the campaign ceased in 1976, more than 70 million cattle in 22 countries had been vaccinated (Masiga *et al.*, 1988, Rossiter, 1994). However, due to a recession in the world economy, many countries could no longer afford to vaccinate their herds against rinderpest. This resulted in a gradual increase in the incidence of rinderpest as it spread from its endemic strongholds. Following the resurgence of rinderpest in Africa from 1979, a new internationally co-ordinated scheme was introduced to Africa in 1981 – the Pan-African Rinderpest Campaign (PARC). The aim of this was to re-establish control of rinderpest, to assist veterinarians and to eventually rid Africa of this disease (Masiga *et al.*, 1988, Rossiter, 1994).

The Global Rinderpest Eradication Programme (GREP) was founded in the 1980s and works together with eradication campaigns in Africa and Asia, in addition to other regional, national and international organisations to co-ordinate rinderpest eradication efforts. The GREP strategy is again based on mass vaccination, in addition to movement controls, surveillance and training to improve veterinary support services. The aim of GREP was to eradicate rinderpest by the end of 2003 and to achieve full Office International des Épizooties (OIE) accreditation of freedom from rinderpest by 2010. As it currently stands, the target year of 2010 remains achievable, with the last remaining reservoir of rinderpest thought

to be limited to the Somali pastoral ecosystem spanning the south and south-east of Somalia and north-eastern Kenya (Mariner & Roeder, 2003).

Despite the huge successes seen over the past few years with the progress of eradication, caution is now necessary, as the major fatal epizootics that were such a feature of the nineteenth century and the start of the twentieth century could now return. This is due to the fact that they occurred in naïve populations, much like those seen today where vaccination has ceased in accordance with eradication guidelines. Such a fear is understandable, as many cattle no longer have any immunity/resistance to the disease leaving numerous vulnerable herds and wildlife.

1.2 Rinderpest – the Virus (RPV)

1.2.1 Classification

Rinderpest virus (RPV), which is closely related to the human *Measles virus* (MeV), is a member of the genus *Morbillivirus*, family *Paramyxoviridae* in the order *Mononegavirales* (Pringle, 1991, Schneemann *et al.*, 1995); for classification see figure 1.2.1. In addition to RPV, the morbillivirus genus includes several viral pathogens of veterinary and medical importance, *Canine distemper virus* (CDV), *Dolphin morbillivirus* (DMV), MeV, *Peste des petits ruminants virus* (PPRV), *Phocine distemper virus* (PDV) and *Porpoise morbillivirus* (PMV) (Barrett *et al.*, 1993, Cosby *et al.*, 1988, Mahy *et al.*, 1988). RPV has been proposed as the archevirus of the morbillivirus group as it reacts with a wide range of monoclonal antibodies produced against other morbilliviruses (Norrby *et al.*, 1985).

Over recent years the number of members of the morbillivirus genus has increased. Until 1988, there were only four members, CDV, MeV, PPRV and RPV. Since then three new morbilliviruses, DMV, PDV and PMV have emerged causing epizootics in aquatic mammals (Barrett *et al.*, 1993), although DMV and

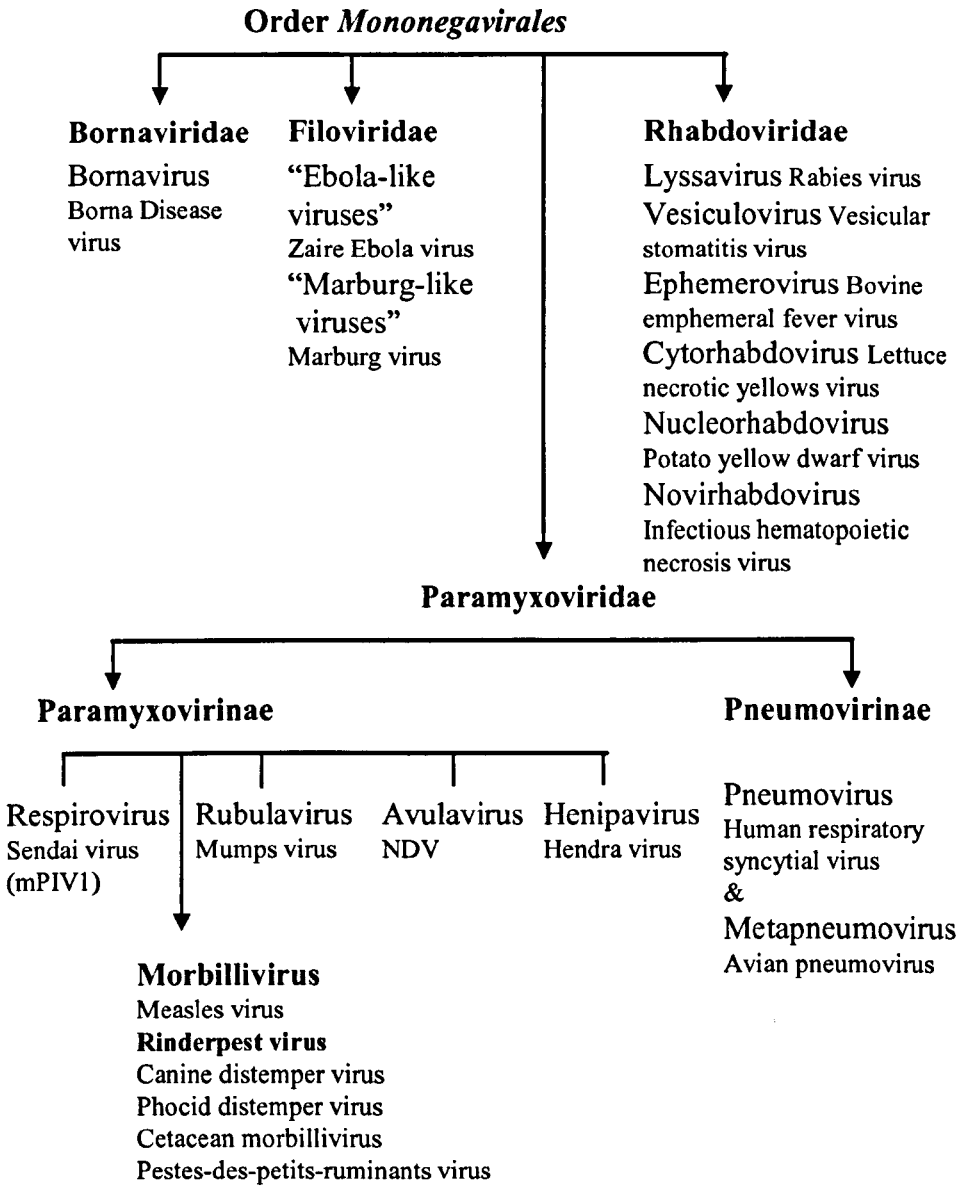

Figure 1.2.1. Classification of single-stranded monopartite negative sense RNA viruses. Showing the type species for all genera, with the exception of the genus *Morbillivirus*, where all known members are listed. Sendai virus is also known as murine parainfluenza virus type 1 (mPIV1) and NDV corresponds to Newcastle disease virus.

PMV are now considered to be the same virus, *Cetacean morbillivirus*. Further to this, in 1994 a virus isolated from horses in Australia, originally called "Equine Morbillivirus", was tentatively classified within the genus *Morbillivirus* (Murray *et al.*, 1995). This virus is now known as Hendra virus (HeV) and following genetic analysis of its entire genome is classified within a new genus, the Henipavirus, together with Nipah virus (NiV), within the family *Paramyxoviridae* (Harcourt *et al.*, 2001).

All known morbilliviruses are capable of causing severe disease in their respective hosts, with each virus species in general having its own host range. Morbilliviruses do not persist in an infectious form following an acute infection and recovery of the host results in life-long immunity. The virus thus relies on a constant supply of new susceptible hosts for its maintenance.

1.2.2 Virus Structure and Genome Organisation

Morbilliviruses are single-stranded monopartite negative sense RNA viruses, replicating in the host cell cytoplasm; they are not robust, being sensitive to heat, light and ultrasonic waves. Denaturation of the virus occurs in both high and low pH conditions and also in the presence of lipid solvents due to the fact that virions are enveloped (Anderson *et al.*, 1996). The large pleomorphic virions are 150-300nm in diameter and obtain their lipid bilayer envelope from the host cell during the budding process. Projecting from this envelope are the two surface glycoprotein spikes, F and H. The fusion (F) protein plays an important role in virus infectivity; it not only mediates the pH independent fusion between the host cell plasma membrane and the viral envelope, but F proteins expressed at the surface of virally-infected cells can also mediate cell-cell fusion resulting in the formation of syncytia, or giant multi-nucleated cells (Evans *et al.*, 1990). To be in an active state the fusion protein must be cleaved by a host cell trypsin-like protease from its inactive F_0 precursor state into the biologically active disulfide-linked F_1 and F_2 heterodimer. The new amino terminus of F_1 generated by this cleavage reveals the highly conserved hydrophobic fusion peptide, which is

believed to be involved in the fusion event. Like other paramyxoviral attachment proteins, the morbillivirus haemagglutinin (H) proteins of both RPV and PPRV have neuraminidase activity (Langedijk *et al.*, 1997, Seth & Shaila, 2001b). MeV H (Ohgimoto *et al.*, 2001) and RPV H (Yoneda *et al.*, 2002) have both been shown to play important roles in determining tissue tropism and recent studies with CDV indicate that the H protein is the main determinant of the fusion efficiency of the virus (von Messling *et al.*, 2001).

For the majority of paramyxoviruses, both the F and H proteins are required for the induction of cell-cell fusion, as is the case for RPV (Das *et al.*, 2000, Seth & Shaila, 2001a, Wild *et al.*, 1991) and CDV (Bar-Lev Stern *et al.*, 1995). The PPRV F protein however can alone induce fusion, in the absence of the haemmaglutinin protein (Seth & Shaila, 2001a), as has been shown for some strains of SV5 (Ito *et al.*, 1997). Unlike the majority of paramyxoviruses, where there exists a requirement for homotypic F and H proteins to generate cell-cell fusion, MeV and CDV F and H proteins are able to form heterotypic interactions both for generating cell-cell fusion and forming viable viruses (Bar-Lev Stern *et al.*, 1995, Nussbaum *et al.*, 1995, von Messling *et al.*, 2001). The same has been more recently shown for the two glycoproteins of NiV and HeV (Bossart *et al.*, 2002). RPV and PPRV F and H proteins could not be mixed to create viable virus (Das *et al.*, 2000), but in transfected cells heterotypic interactions can occur between the glycoproteins of the two viruses (Seth & Shaila, 2001a). It has been shown that the strength of the interaction between F and H is responsible for modulating viral fusogenicity; the stronger the interaction, the less fusogenic the virus is and vice versa (Plemper *et al.*, 2002). Only these two surface proteins stimulate the virus neutralising antibody response (Belsham *et al.*, 1989, Giavedoni *et al.*, 1991, Schlereth *et al.*, 2000, Yamanouchi *et al.*, 1993, Yilma *et al.*, 1988) and neutralising antibodies are primarily against H (Giraudon & Wild, 1985).

The matrix (M) protein forms a paracrystalline layer under the viral lipid bilayer envelope (Bachi, 1980) and is widely considered to be the main organiser of viral morphogenesis. M functions to stabilise the viral structure by interacting with the

cytoplasmic tails of both the F and H proteins, with the lipid bilayer and with the nucleocapsid (N) protein (Mottet *et al.*, 1999, Stricker *et al.*, 1994). Also contained within the viral membrane is the nucleocapsid core, which contains the single-stranded RNA genome (see figure 1.2.2.1). Until recently it was thought that morbilliviruses, like the majority of viruses, packaged a single copy of the viral RNA genome into each virus particle. However, a stable MeV mutant that efficiently packages at least two genomes has been characterised and found to be fully functional, growing with comparable efficiency to an unmodified control MeV (Rager *et al.*, 2002).

The nucleocapsid protein enwraps the genomic RNA to form the core structure to which the phosphoprotein (P) and large (L) proteins are attached during transcription and replication. It is this helical, nuclease-resistant, nucleocapsid, rather than naked RNA, that acts as the template for all RNA synthesis. N is the most abundant viral protein in RPV infected cells and the principal component of the helical nucleocapsids (Grubman *et al.*, 1988), which have a characteristic herringbone appearance when viewed through the electron microscope (Kingsbury, 1990). Using immunogold labelling, it has been shown that there are at least 60-70 molecules of P and 30-35 molecules of L clustered together along paramyxoviral nucleocapsids (Portner *et al.*, 1988). The helical nucleocapsid core of Sendai virus (SeV), a related paramyxovirus, is composed of approximately 2,600 nucleocapsid (N) proteins, 300 P proteins, and 50 L proteins associated with the viral RNA (Lamb *et al.*, 1976).

N proteins of paramyxoviruses self-associate, forming organised nucleocapsid structures. Such interactions have been characterised for numerous paramyxoviruses including SeV (Buchholz *et al.*, 1993, Horikami *et al.*, 1996), MeV (Fooks *et al.*, 1993, Spehner *et al.*, 1991), human parainfluenza virus type 2 (hPIV-2) (Nishio *et al.*, 1999), Newcastle disease virus (NDV) (Kho *et al.*, 2003) and human respiratory syncytial virus (RSV) (Murphy *et al.*, 2003). The self-assembly domain for SeV (Buchholz *et al.*, 1993, Myers *et al.*, 1999), MeV (Bankamp *et al.*, 1996, Liston *et al.*, 1997), hPIV-2 (Nishio *et al.*, 1999) and

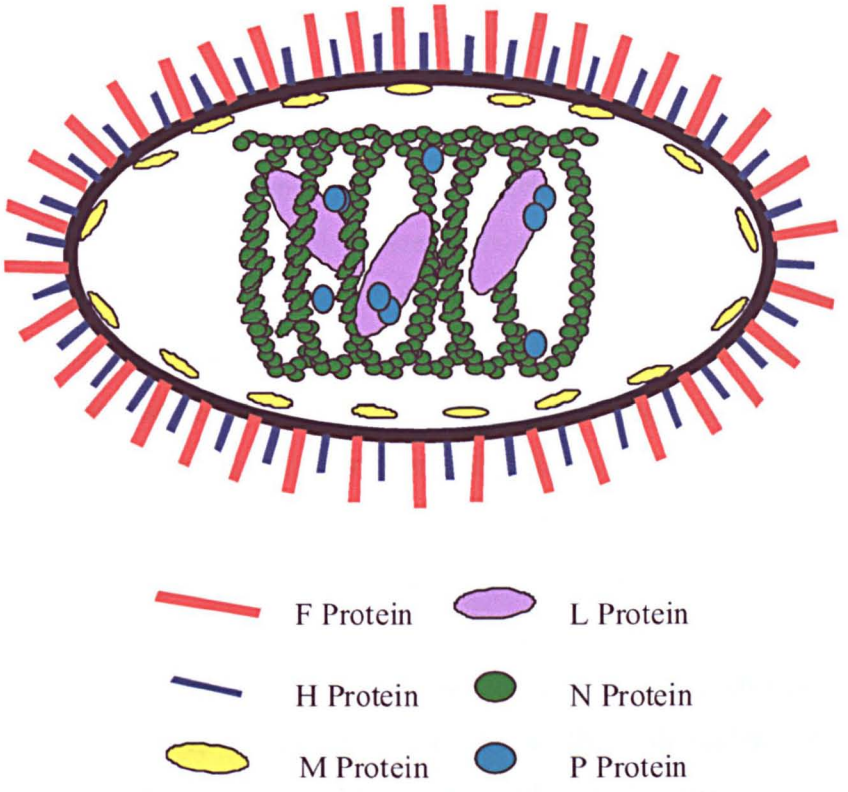

Figure 1.2.2.1 Schematic representation of a rinderpest virion. The two surface glycoproteins, the fusion (F) protein and haemagglutinin (H) protein are shown protruding from the host derived membrane envelope, which is represented as a black border. Located just below the membrane envelope, the matrix (M) protein is thought to be attached to the cytoplasmic tails of F and/or H. The RNA is depicted as being encapsidated within numerous copies of the nucleocapsid (N) protein, which is associated with both the large (L) protein and the phospho- (P) protein.

NDV (Kho *et al.*, 2003) N proteins has been mapped to the amino-terminal two-thirds. It is a possibility that the self-interactions of N may form the RNA binding site. The carboxy-terminal region of NDV N was found to be dispensable for the formation of the nucleocapsid particles and was postulated to play a regulatory role in N polymerisation (Kho *et al.*, 2003), as seen for SeV (Curran *et al.*, 1993) and recently with RSV (Murphy *et al.*, 2003).

The RPV genome is 15 882nt in length and consists of a short 3' "leader" RNA region followed by coding regions for the six structural proteins. Each transcription unit is separated by defined stop-start sequence motifs and the genome ends with a short 5' "trailer" RNA region. The genome encodes, in a 3' to 5' order, the nucleocapsid protein, phosphoprotein, matrix protein, fusion protein, haemagglutinin protein and the large protein, (Baron & Barrett, 1995b), and this gene order is the same for all morbilliviruses (Rima *et al.*, 1986), as outlined in figure 1.2.2.2. The F 5' untranslated region (UTR) located between the M and F open reading frames (ORFs) has been found to be GC-rich in MeV and RPV (Evans *et al.*, 1990, Richardson *et al.*, 1986). Furthermore, in morbilliviruses, this region has been shown to be specific to each virus type and is thought to be host specific (Meyer & Diallo, 1995), with evidence that it may function as a virulence factor in MeV (Valsamakis *et al.*, 1998).

In MeV, there is a transcription polarity which has been observed *in vitro* from the N gene towards the L gene meaning more copies of N are produced than of L (Horikami & Moyer, 1991); this has also been observed for RPV both *in vivo* and *in vitro* (Ghosh *et al.*, 1996). Thus it is the position of the gene within the genome that determines the transcriptional level of each gene.

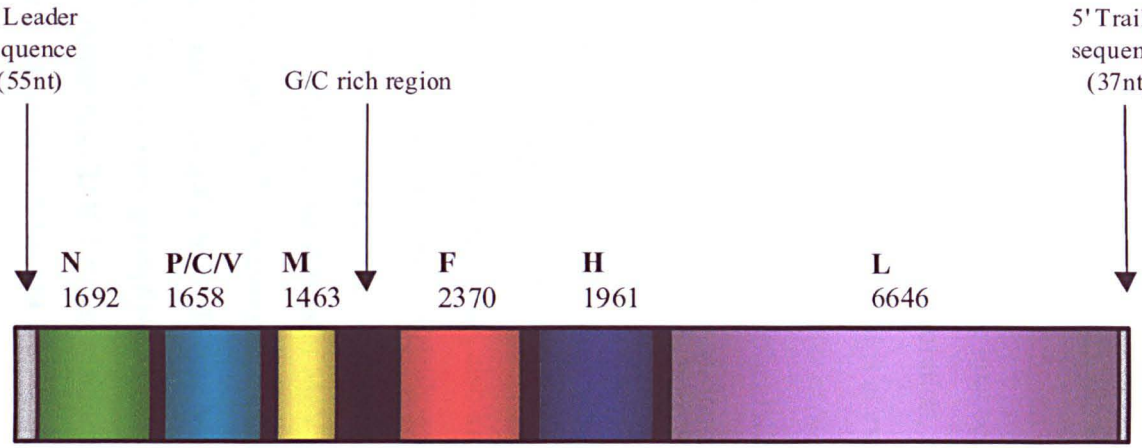

Figure 1.2.2.2. Diagrammatic representation of the genome organisation of rinderpest virus. The length of each gene in nucleotides is given. The second gene is labelled P/C/V, as the P gene encodes several proteins; the P core protein and two non-structural proteins C and V. It should be noted that the black regions correspond to the untranslated sequence, which includes gene start, gene stop and intergenic motifs, these conserved motifs consist of at least "AAAACTTAGG(A/G)". The black regions also include poorly conserved untranslated regions at the 5' or 3' ends of each open reading frame.

1.3 Viral Replication

1.3.1 Virus Entry

Upon attachment of the virion to the host cellular receptor, mediated by the haemmaglutinin protein (Scheid *et al.*, 1972), the viral membrane fuses with the cellular plasma membrane at the neutral pH found at the cell surface, resulting in the release of helical nucleocapsids into the host cell cytoplasm. The H protein is a class II glycoprotein of tetrameric configuration, with a single N-terminal hydrophobic membrane anchor domain that is at exactly the same position for all morbillivirus H proteins (Blixenkrone-Møller *et al.*, 1996), a short cytoplasmic tail, and a large unconserved C-terminal extracellular domain, which is thought to play a role in host specificity (Blixenkrone-Møller *et al.*, 1996).

Until recently, little was known about the host cellular receptors of morbilliviruses, only the receptors for MeV were identified. SLAM (human signalling lymphocytic activation molecule, also known as CDw150), has been shown to be a cellular receptor for MeV (Erlenhoefer *et al.*, 2001, Ono *et al.*, 2001, Tatsuo *et al.*, 2000), whilst CD46, a complement regulatory protein, is the acknowledged receptor for vaccine strains of MeV such as the Edmonston and Halle strains (Dorig *et al.*, 1993). In contrast, recent evidence suggests that CD46 can also act as the natural receptor for wild type MeV as primary clinical isolates were found to use CD46 as a receptor (Manchester *et al.*, 2000). The H protein of wild type MeV possesses CD46 binding residues and it has been postulated that wild type MeV interacts with SLAM with high affinity, but that it uses CD46 as an additional low affinity receptor (Massé *et al.*, 2002). In a further development, recently published data show that MeV has as yet an unidentified third cellular receptor (Andres *et al.*, 2003, Hashimoto *et al.*, 2002); wild type MeV was shown to infect cells in the absence of SLAM and in the presence of CD46-blocking antibodies. In addition, subacte sclerosing panencephalitis (SSPE) strains of MeV have also been shown to enter cells in a SLAM- and CD46-independent manner (Shingai *et al.*, 2003).

It has been shown that a single codon in MeV H, an asparagine at residue 481, is important in determining the virus' ability to utilise CD46 as a receptor; when this residue changes from asparagine to tyrosine, an improvement is seen in the ability of the virus to utilise CD46 for entry (Schneider *et al.*, 2002).

Further research from the Japanese group that identified SLAM as a cellular receptor for MeV suggests that SLAM may act as common cellular receptor for the morbillivirus genus (Tatsuo *et al.*, 2001). Three strains of canine distemper virus (CDV), including the Onderstepoort vaccine strain, and a strain of RPV, have been shown to use SLAM as a cellular receptor. Furthermore, MeV, CDV and RPV were able to use SLAM of non-host species for virus entry (Tatsuo *et al.*, 2001). However, data presented by this group is controversial, as wild type virus has not been used in these receptor studies. To truly identify uncharacterised receptors for viruses such as RPV and CDV, primary isolates of these viruses need to be studied, not viruses that have been passaged repeatedly in the laboratory to gain attenuation. It will be interesting to see if these results can be duplicated with wild type RPV and CDV.

1.3.2 Transcription and Replication

Both vesicular stomatitis virus (VSV) and Sendai virus (SeV) are model viruses used to study the transcription and replication of negative sense monopartite RNA viruses and are similar to RPV, except where noted.

The initiation of both transcription and replication of negative sense monopartite RNA viruses requires the presence of promoter regions, which are usually untranslated regions at the termini of the viral genomes. Such promoter sequences are able to act in *cis*- with viral proteins of the polymerase complex to direct transcription and replication. The extragenic sequence at the 3' end of the viral genome is referred to as the leader region, whilst the sequence at the 5' end of the viral genome is termed the trailer region. RNA synthesis begins once an

active initiation complex is established at the polymerase entry site on the 3' terminal genomic leader region; transcription commences with the synthesis of a short unmodified (+)- strand leader RNA (Ghosh *et al.*, 1996, Horikami & Moyer, 1991). The minimum unit with transcriptional activity is the encapsidated genome associated with the P and L polymerase complex for both VSV and RPV (Emerson & Schubert, 1987, Ghosh *et al.*, 1995). The (+)- leader RNA is released at the junction of the leader region and the 3' proximal gene; and the polymerase resumes synthesis at the following gene start signal (Ghosh *et al.*, 1996, Horikami & Moyer, 1991). However recent studies with SeV and VSV have provided evidence to suggest that *in vitro* transcription and replication may initiate at separate sites on the viral genome (Whelan & Wertz, 2002, Vulliémoz & Roux 2002).

Approximately the first sixteen nucleotides of the leader and the last sixteen nucleotides of the trailer are complementary (Baron & Barrett, 1995a), this is most likely to be due to the fact that the leader contains the viral genome promoter, whilst the trailer region contains the anti-genome promoter. In addition to these sequences, the related paramyxovirus SeV, also contains a promoter sequence element which is outside of the leader and trailer region, the "BBbox"(Blumberg *et al.*, 1991), containing the motif $(GNNNNN)_3$ which is essential for replication (Tapparel *et al.*, 1998). A similar motif has been shown to be important in Simian virus 5 (SV5) (Murphy & Parks, 1999) and RPV (Mioulet *et al.*, 2001).

Viral transcription units are transcribed sequentially by a stop-start mechanism that is guided by the conserved regions found at the start and the end of each transcription unit (Dickens *et al.*, 1984); and it is the resulting mRNAs which direct the synthesis of each of the virus proteins. These elements include the gene start (GS) motif at the upstream border, the gene end (GE) motif at the downstream border of each gene, and the intergenic region located between GE

and GS. Located between the gene* boundaries are intergenic regions, which are highly conserved only in parainfluenzaviruses and morbilliviruses (Lamb & Kolakofsky, 1996) and for the genus *Morbillivirus* are three nucleotides in length and are either GAA or GCA or GAT (Baron & Barrett, 1995b). Studies using several members of the order *Mononegavirales* have shown that GE signals direct polyadenylation and termination of each mRNA, whilst GS signals direct initiation and capping (Barr *et al.*, 1997, Hwang *et al.*, 1998, Kuo *et al.*, 1997, Kuo *et al.*, 1996, Rassa & Parks, 1999, Stillman & Whitt, 1997, Stillman & Whitt, 1999). It is possible for the polymerase to detach from the template when a gene end signal is reached. The polymerase complex must then re-attach at the promoter (3' end of the genome). This results in the transcription gradient seen in infected cells where products of distal viral genes are produced in lower quantity than those from genes located in closer proximity to the 3' genome terminus.

Paramyxovirus mRNAs are capped at their 5' end and polyadenylated at the 3' end like eukaryotic mRNAs. Capping and polyadenylation are performed co-transcriptionally by the VSV polymerase (Hercyk *et al.*, 1988, Hunt *et al.*, 1988, Hunt *et al.*, 1984); such specific functions for morbillivirus polymerases remain to be identified. Polyadenylation occurs through a stuttering mechanism, in which the polymerase slips back and copies the short polyuridylate tract at the end of each gene reiteratively. Reiterative copying or pseudotemplated transcription by the viral polymerase not only accounts for the synthesis of poly (A) tails but also for the different versions of P mRNA which occur in all the paramyxoviruses except human parainfluenza virus 1 (hPIV-1) and the pneumoviruses (Hausmann *et al.*, 1999a, Hausmann *et al.*, 1999b, Lamb & Kolakofsky, 1996, Vidal *et al.*, 1990), see section 1.5.

It was previously thought that the same polymerase complex was active in both transcription and replication, however it is now a more accepted view that the

* Although it is common usage to use the term gene with these viruses for each transcription unit, some may find the term inaccurate as it may be thought that a gene should include a promoter/enhancer element, in addition to the coding region/transcription unit, while these viruses have a single promoter and multiple transcription units.

transcriptase and replicase comprise two distinct complexes composed of L-P_{2-3} or L-P_4 or L_2-P_8, and L-(N-P) (Curran *et al.*, 1995a, Das *et al.*, 1997, Tarbouriech *et al.*, 2000a, Tarbouriech *et al.*, 2000b).

Once in replication mode, the polymerase synthesises without break, ignoring the gene stop signals, until the 5' end of the genome is reached and the polymerase complex is then released. Entry of the polymerase complex then occurs within the (+) – trailer region (i.e. the 3' end of the newly synthesised (+) (sense) copy of the genome) and this anti-genome serves as template for multiple rounds of genome synthesis. Newly synthesised genomic and anti-genomic RNA are never found as free RNA, but are always co-transcriptionally encapsidated with the nucleocapsid protein, forming RNAse resistant nucleocapsids (Baker & Moyer, 1988).

As in mRNA transcription, the minimum unit for replication is the nucleocapsid with attached P and L proteins (Horikami & Moyer, 1991). It has been found with Sendai virus (SeV) that the L does not directly interact with the nucleocapsid (Horikami & Moyer, 1995, Ryan & Portner, 1990), but that the P protein of SeV binds to the C-terminal domain of N in order to position the polymerase onto the template (Buchholz *et al.*, 1993, Curran *et al.*, 1994, Ryan *et al.*, 1993, Smallwood *et al.*, 1994). The domains of MeV N required for interaction with P have been identified as two non-contiguous regions, amino acids 4-188 and amino acids 304-373, while deletion of amino acids 189-239 were found not to affect N-P binding activity (Bankamp *et al.*, 1996). Thus for MeV, the N oligomerisation and N-P interaction map to the same domain of N, perhaps explaining why morbillivirus N proteins are so highly conserved over the first 400 amino acids (Baron & Barrett, 1995a, Baron & Barrett, 1995b, Diallo *et al.*, 1994, Kamata *et al.*, 1991, Rozenblatt *et al.*, 1985). P is thought to unwind the N-RNA template, thereby facilitating the movement of L along the template RNA. Although recent research using the rubulavirus, hPIV-2, has shown both *in vivo* and *in vitro* that the N and L proteins of this virus do indeed interact with one another, the function of this complex remains unclear (Nishio *et al.*, 2000).

Studies using SeV mini-genome templates revealed that only RNA templates which were an exact multiple of six nucleotides in length were efficiently replicated (Calain & Roux, 1993). This provided functional evidence for the physical properties observed earlier by Egelman and colleagues (Egelman *et al.*, 1989), who by analysing negatively stained SeV nucleocapsids concluded that each N protein subunit was associated with approximately 6 nucleotides. "The rule of six" (Calain & Roux, 1993) predicts that each nucleocapsid protein associates with precisely six nucleotides and that templates are efficiently replicated only when their 3' ends are completely covered by terminal nucleocapsid proteins. Only Sendai virus genomes which are multiples of six nucleotides in length are found in nature (Hausmann *et al.*, 1999a, Hausmann *et al.*, 1996, Kolakofsky *et al.*, 1998).

The "rule of six" is also adhered to by MeV (Radecke *et al.*, 1995, Sidhu *et al.*, 1995) and less stringently by the rubulavirus, Simian virus 5 (SV5) (Murphy & Parks, 1997). An exception to the rule for paramyxoviruses is that of respiratory syncytial virus (RSV), as non-hexamer genome analogs of RSV are efficiently replicated by RSV polymerase (Samal & Collins, 1996).

Encapsidation of nascent leader RNA is proposed to be the anti-termination mechanism that causes the polymerase to ignore the stop signal at the junction between the leader and the first gene (Blumberg *et al.*, 1981). It is thought that the initiation of encapsidation of viral RNA by N probably involves the recognition of a specific leader RNA sequence (Das & Banerjee, 1992). It has been proposed that when unassembled N protein, which exists as a soluble N-P complex, accumulates in the cell the polymerase "switches" from mRNA transcription to replication mode (Banerjee & Barik, 1992, Blumberg *et al.*, 1981, Patton *et al.*, 1984). This idea of a "switch" is controversial and there is evidence with studies using RSV that an excess of unassembled N, or N and P complexed together had no detectable effect upon the ratio of mRNA transcription to replication (Fearns *et al.*, 1997).

Recent work with rabies virus has suggested that it may be the M matrix protein that may regulate the balance between transcription and replication modes (Finke *et al.*, 2003), which contradicts a previous report of work with rabies virus that suggested it is N that regulates viral RNA transcription and replication, depending on its phosphorylation state (Yang *et al.*, 1999). However, Wu and co-workers have recently shown that both transcription and replication levels are reduced when rabies N is not phosphorylated (Wu *et al.*, 2002). When expressed alone, rabies N is phosphorylated and this is due to phosphorylation by cellular casein kinase II (Wu *et al.*, 2003), which also phosphorylates VSV P (Barik & Banerjee, 1992). Another recently described possible mechanism for controlling the switch between transcription and replication involves the P protein of a rhabdovirus (Chandipura virus) that is closely related to VSV (Basak *et al.*, 2003). The proposal here is that as unphosphorylated P protein binds the nascent leader RNA it actively promotes readthrough of transcription termination signals and provides a bias for replication. This was shown when unphosphorylated P was overexpressed and the level of replication increased. When phosphorylated, the P protein is no longer able to bind to the leader RNA. The exact mechanism by which mRNA synthesis switches to genomic replication remains to be identified.

1.3.3 Virus Assembly and Exit

Both the F and H proteins are synthesised in the endoplasmic reticulum and transported via the exocytic pathway to the plasma membrane where the virus is assembled and from where it buds (Ali & Nayak, 2000). However, it has been demonstrated with MeV that the neither of these two surface glycoproteins are required for the budding process, with such a role proposed to be played by either the L, P, N or M proteins (Maisner *et al.*, 1998). The M protein is now widely thought to be critical for virus assembly and budding and an important role for the morbillivirus M protein has been hinted at with studies using SeV. SeV M and hPIV-1 M are known to interact with newly assembled nucleocapsids via the N subunit (Coronel *et al.*, 2001, Mottet *et al.*, 1999, Stricker *et al.*, 1994), a

requirement for the incorporation of the nucleocapsid into the virion (Coronel *et al.*, 2001). It has recently been shown that the M proteins of both SeV (Ali & Nayak, 2000) and MeV (Noll *et al.*, 2001) interact with the glycoproteins F and H in the absence of any other viral proteins, demonstrating that the viral nucleocapsid is not an obligatory factor in M/glycoprotein interaction. These data indicate that M could function as the central organiser of viral morphogenesis (Sanderson *et al.*, 1994). This has now been confirmed, as mutations in cysteine residues of SeV M have been linked to altered viral morphology and disruption of the incorporation of the viral genome (Sakaguchi *et al.*, 2003). Studies with SeV and MeV have shown that both F and M are able to drive the budding process (Cathomen *et al.*, 1998b, Spielhofer *et al.*, 1998, Takimoto *et al.*, 2001) and that M is necessary for maturation of virus into particles (Inoue *et al.*, 2003). Furthermore, it has been shown with MeV, that viruses lacking the M protein are severely disabled when it comes to forming MeV particles containing the RNP complex (Cathomen *et al.*, 1998a), providing further evidence that MeV M interacts with the ribonucleocapsid, as previously proposed by Hirano and co-workers (Hirano *et al.*, 1993). In addition, more extensive cell-cell fusion was seen with cells infected with the M-less MeV indicating that M may play a role in regulating cell to cell fusion (Cathomen *et al.*, 1998a). MeV M is thought to exert this effect through its interaction with the cytoplasmic tail of F, an interaction which may regulate the fusogenicity of F (Cathomen *et al.*, 1998b).

The budding of the new virus particle occurs from the apical surface of the host cell and it has been known for sometime that this process utilises the host cell protein tubulin (Moyer *et al.*, 1986). In addition there is evidence to suggest that both SeV (Takimoto *et al.*, 2001) and MeV (Tyrrell & Norby, 1978) use actin during the budding process. Most other cellular proteins are selectively excluded from the matured virions during budding, indicating that specific protein:protein interactions occur.

1.4 Phospho- (P) Protein

The P gene of RPV encodes several proteins (Baron *et al.*, 1993, Ghosh *et al.*, 1996, Haas *et al.*, 1995, Yamanaka *et al.*, 1992); the P core protein and two non-structural proteins, C and V, which are discussed in sections 1.5 and 1.6. The P protein is the only P gene product essential for viral RNA synthesis (Baron & Barrett, 2000, Radecke & Billeter, 1996, Schneider *et al.*, 1997) being very versatile and playing many roles within the life cycle of the virus. The paramyxovirus P protein is a modular protein with two distinct functional domains; the carboxy-terminal domain, which is highly conserved, possesses all regions required for viral transcription, whilst the amino-terminal domain provides additional functions required for replication (Curran, 1996, Curran *et al.*, 1995b). Paramyxoviral P proteins are highly phosphorylated, with the majority of the phosphorylation sites of MeV P residing within the amino-terminal portion of the protein (Das *et al.*, 1995).

The P protein of SeV has been shown to be a tetramer (Tarbouriech *et al.*, 2000a) and RPV P is known to self-associate, to bind both monomeric and polymeric N protein to form the nucleocapsid complex (Shaji & Shaila, 1999), in addition to forming the polymerase with L (Sweetman *et al.*, 2001). The interaction of P with unassembled N protein prevents N from assembling into nucleocapsids in the absence of the nascent RNA and this function has been mapped to nine amino acids at the amino-terminus of SeV P (Curran *et al.*, 1995b). Furthermore, it is the interaction of P from the polymerase complex with the N component of the nucleocapsid that enables the binding of the polymerase to the RNA template (Horikami & Moyer, 1995). Many of the functions of P are thus mediated through its interaction with N. The P protein of MeV has been shown to regulate the efficiency with which the N protein assembles into nucleocapsids (Spehner *et al.*, 1997), and N-P and P-P interactions have also been confirmed in other paramyxoviruses (Bankamp *et al.*, 1996, Harty & Palese, 1995, Paul *et al.*, 1988, Slack & Easton, 1998, Takacs *et al.*, 1993). The carboxy-terminal half of the

morbillivirus P protein is far more conserved than the amino-terminal domain (Baron *et al.*, 1993). It is therefore interesting that both the amino- and carboxy-terminal domains of RPV P are required for interactions with N, but only the carboxy-domain is required for interactions with itself (Shaji & Shaila, 1999), mimicking earlier findings of MeV P (Harty & Palese, 1995, Liston *et al.*, 1995). The carboxy terminus of MeV P is known to bind L for the formation of the polymerase (Liston *et al.*, 1995) and it is thought that one of the functions of P is to ensure the correct folding of the L protein. A similar finding was recently observed for the pneumovirus, *Bovine respiratory syncytial virus* (Khattar *et al.*, 2001) and the rubulavirus hPIV-2 (Nishio *et al.*, 2000). In contrast, the L binding site of rabies virus is located in the first 19 residues of P (Chenik *et al.*, 1998). Furthermore, it is known that the P, N and L binding domains of SeV P do not overlap (Bowman *et al.*, 1999, Smallwood *et al.*, 1994).

An additional function of MeV P is that it has been found to retain N in the cytoplasm and prevent its transport to the nucleus, as a result of specific interactions between itself and N; co-expression of N with either M or H resulted in no such cytoplasmic retention of N (Huber *et al.*, 1991). SeV P is thought to act as a chaperone for the monomeric N protein during the nascent chain assembly step of genome replication (Curran *et al.*, 1995b).

1.5 Production of Non-structural Proteins, C, V and W

The P genes of many paramyxoviruses are known to encode multiple proteins. In addition to the P protein, the morbillivirus P gene also produces three additional proteins, C, V and W. The C protein is generated from a separate translation start site. This is as a result of leaky scanning by ribosomes, which enables the first AUG codon to be skipped and the second AUG of the mRNA to be accessed, allowing the initiation of the translation of the C protein (Baron *et al.*, 1993, Bellini *et al.*, 1985) (see figure 1.5.1). The V and W proteins are generated by a pseudotemplated transcription mechanism which allows access to alternate reading frames within the P coding region (Cattaneo *et al.*, 1989, Thomas *et al.*,

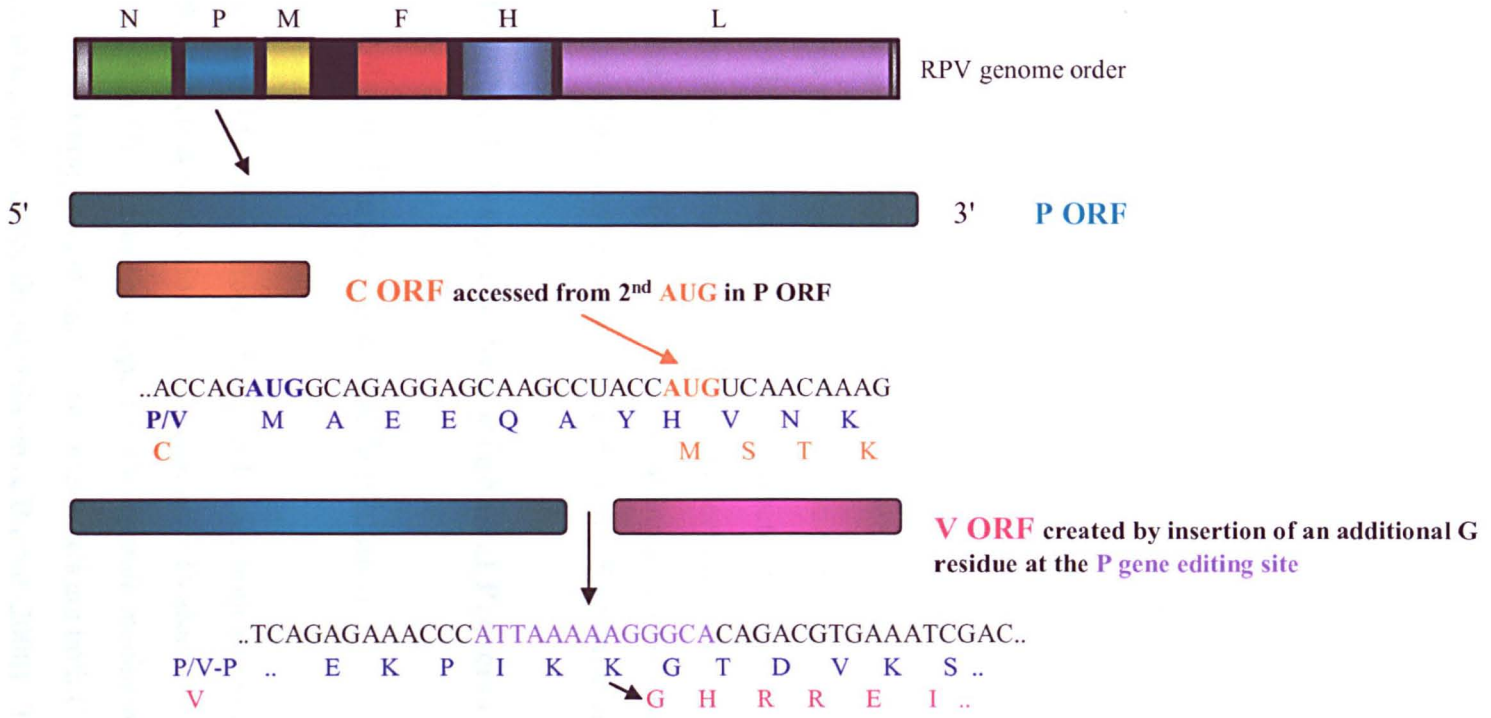

Figure 1.5.1. The generation of the non-structural proteins, C and V, from the P ORF. The C ORF (shown in orange) is accessed by the start of translation from the second AUG in the P ORF, due to "leaky" ribosomal scanning. The V ORF (shown in pink) is generated by the co-transcriptional insertion of an additional non-templated G residue at the highly conserved P gene editing site (highlighted) resulting in a frameshift producing the V mRNA. The V protein thus has an identical amino-terminus to that of P, and a different cysteine rich carboxy-terminus.

1988). The co-transcriptional addition of one non-templated G residue at the editing site in the mRNA causes a frameshift within the P ORF resulting in the synthesis of the V protein, whilst the addition of two non-templated G residues results in the synthesis of the W protein (Baron *et al.*, 1993, Ghosh *et al.*, 1996). The editing site is a highly specific, conserved region of sequence located at approximately the centre of the P ORF, that is identical for RPV, CDV, MeV, DMV and PDV (Baron *et al.*, 1993, Blixenkrone-Møller *et al.*, 1992, Cattaneo *et al.*, 1989, Ghosh *et al.*, 1996). The V protein thus has an amino-terminus which is identical to that of the P protein, and a different cysteine rich carboxy-terminus (Baron *et al.*, 1993) (see figure 1.5.1). The W protein, which is present in only 1-2% of transcripts (Baron *et al.*, 1993) consists essentially of the amino-terminal half of P.

The C, V and W proteins are termed non-structural as they have yet to be found in purified virions, but they could still be critical for viral replication. The V protein of RPV has been shown to be distributed uniformly throughout the cytoplasm and nucleus in infected cells (Sweetman *et al.*, 2001), while the C proteins of both MeV and RPV have been shown to co-localise with N in the cytoplasm of infected cells (Bellini *et al.*, 1985, Sweetman *et al.*, 2001).

1.6 Possible Roles of the Non-structural Proteins

1.6.1 Roles in Transcription and Replication

Studies with MeV have shown that C and V are both accessory factors required for efficient virus replication in transgenic mice (Valsamakis *et al.*, 1998), with evidence that they are likely to operate via separate mechanisms (Patterson *et al.*, 2000). In addition, mutant rinderpest viruses lacking both C and V have been shown to replicate sub-optimally (Baron & Barrett, 2000). The RPV C knockout virus showed impaired growth *in vitro* in addition to a reduction in viral mRNA synthesis, whilst the V knockout resulted in a virus with a large syncytium-

inducing phenotype and it also displayed increased levels of both viral genome and anti-genome RNAs (Baron & Barrett, 2000).

Previous studies have found that RPV C self-associates in high molecular weight aggregates independent of other viral proteins and, in addition, RPV C and V proteins were found to associate with L (Sweetman *et al.*, 2001), the significance of which remains to be determined. Interestingly, the SeV C protein has been shown to inhibit viral replication *in vitro*, by complexing with the L protein subunit of the polymerase (Tapparel *et al.*, 1997). It is therefore possible that the interaction between RPV C and L may play a similar role; however, it should be noted that there is a total lack of sequence similarity between SeV C and RPV C, suggesting that they may interact with L in different ways. SeV C functions as a promoter-specific inhibitor of viral RNA synthesis and as a fidelity factor by increasing the specificity of the 3' genomic promoter and, by doing so, limits the extent of replication (Cadd *et al.*, 1996, Horikami *et al.*, 1997, Tapparel *et al.*, 1997). More selectivity is generated as C proteins accumulate during infection, although this inhibitory effect of C can be reversed by the overexpression of P (Tapparel *et al.*, 1997). The first 98 amino acids of SeV C have been shown to be unnecessary for this down-regulation of RNA synthesis (Kato *et al.*, 2001). The binding of C to L does not affect L-P binding and indicates that not only are the C and P binding sites on L different, but that C is able to interfere with RNA synthesis by direct action on L (Cadd *et al.*, 1996, Horikami *et al.*, 1997). To date no specific functional region of C has been identified, although recent sequence analysis of SeV C indicates that the carboxyl two thirds of the C protein are of functional importance (Fujii *et al.*, 2001).

The V protein is expressed in almost all members of the sub-family paramyxovirinae, unlike the C protein, which is not expressed in the rubulaviruses, as reviewed in (Lamb & Kolakofsky, 1996). Another distinction between the V and C proteins is the fact that, unlike the V proteins, which share a highly conserved cysteine-rich carboxy terminal domain, the C proteins share no conserved motifs or regions of sequence. The only exception to this appears to

be the C proteins of morbilliviruses which possess a conserved motif of approximately 15 amino acids in the central portion of the protein (M. Baron, personal communication). The V proteins of RPV (Baron & Barrett, 2000), MeV (Patterson *et al.*, 2000) and SeV (Kato *et al.*, 1997) are known to be non-essential for virus replication and are thought to encode a "luxury function" required for *in vivo* pathogenicity. Alignment of the carboxy-terminal sections unique to paramyxovirus V proteins revealed that seven cysteine residues, whose positions are well conserved among all the V proteins, form a "zinc-finger"-like motif (Liston & Briedis, 1994, Nakamura *et al.*, 1999). Further, it has been shown that this carboxy-terminal domain of the MeV V protein binds zinc and this metal binding activity is highly specific to zinc (Liston & Briedis, 1994). RPV V has been shown to interact with N (Sweetman *et al.*, 2001), the function of which remains to be determined, but may be similar to that of SeV V (Horikami *et al.*, 1996) and MeV V (Tober *et al.*, 1998) which are known to interact with unpolymerised N to regulate viral RNA synthesis. This interaction is hardly surprising as the amino terminal domain of P, which is shared with V, contains the N binding site for MeV (Harty & Palese, 1995). V has been proposed to inhibit genome replication by interfering with either the formation or the use of the N-P encapsidation complex (Horikami *et al.*, 1996), whilst Kato and co-workers (Kato *et al.*, 1997) have proposed that SeV V protein may function in conjunction with host cellular factors to regulate transcription. Alternatively, it may be that the recently discovered interaction of V with L is responsible for regulating viral transcription (Sweetman *et al.*, 2001).

Data therefore suggests that the roles of these accessory proteins in the viral life cycle are specific to each genera of virus within the sub-family paramyxovirinae.

1.6.2 Roles in Innate Immune Resistance

The C and V proteins of paramyxoviruses are known to interact with several host cellular proteins to modulate the host cell environment and interfere with the host immune response and, again, the roles of these proteins varies with the genus. The V proteins of the rubulaviruses SV5 (Didcock *et al.*, 1999b) and mumps virus (Kubota *et al.*, 2001) are known to target STAT-1 (signal transducer and activator of transcription-1) for proteasome-mediated degradation, whilst the V protein of hPIV-2 targets STAT-2, rather than STAT-1 for degradation (Parisien *et al.*, 2001). The V protein of MeV also functions to inhibit the host cell IFN response (Takeuchi *et al.*, 2003), by actively inhibiting the phosphorylation of both STAT-1 and STAT-2, suggesting that the effect is on a particular kinase rather than the STATs. Furthermore, the V protein of the recently emergent NiV was shown to counteract the host cell interferon (IFN) response (Park *et al.*, 2003b), with V acting to inhibit cellular IFN responses by forming aggregates with, and retaining in the cytoplasm, both STAT-1 and STAT-2 (Rodriguez *et al.*, 2002). More recently it has been shown that the V protein of NDV not only functions as an alpha/beta interferon antagonist (Park *et al.*, 2003b), but that it also plays an important role in host range restriction by blocking innate host defences in a species specific manner (Park *et al.*, 2003a). This corroborated previous work with SV5 which revealed that a single amino acid substitution at residue 100 in V, from asparagine to aspartic acid, determined the virus' ability to block the IFN response in either human or mouse cells (Young *et al.*, 2001). Wild type SV5 is unable to block the IFN response in murine cells, but is able to block IFN signalling in monkey, human and canine cells (Didcock *et al.*, 1999a).

On the other hand, for SeV, it has been found that it is the C protein which interacts with cellular proteins to interfere with the interferon response produced by SeV infected cells (Garcin *et al.*, 2000, Gotoh *et al.*, 1999, Kato *et al.*, 2001). Furthermore, Kato and co-workers recently demonstrated that the amino-terminal portion of SeV C is not required for this function (Kato *et al.*, 2002), providing experimental data to support the hypothesis of Fuji and co-workers that the

carboxyl two thirds of the C protein are of functional importance (Fujii *et al.*, 2001). The four C proteins of SeV all interact with STAT-1 to prevent IFN signalling through the JAK/STAT pathway (Garcin *et al.*, 2000). In addition to the V protein, the putative C and W proteins of NiV are also known to block the host cell IFN response (Park *et al.*, 2003b).

The V protein of Simian virus 5 (SV5) is known to slow progression of the cell cycle, via interaction with a host cell protein (DDB1, Damaged DNA Binding Protein 1), showing that V, like C, appears to be a multifunctional protein (Lin & Lamb, 2000, Lin *et al.*, 1998).

1.7 Large (L) Protein

The RNA-dependent RNA polymerase of monopartite negative-strand RNA viruses consists of two virus-encoded subunits, the P protein and the L protein, the latter being the largest virus structural protein. L is approximately 240 kDa in size and is the least abundant component in the nucleocapsid, with approximately 50 copies per virion (Lamb *et al.*, 1976), correlating with the fact that it is encoded by the most distal gene from the promoter. All L proteins of nonsegmented negative strand RNA viruses have a large net positive charge, are large in size (~245 kDa), have an unusually high leucine and isoleucine compostition and short regions of high basicity which may be vital for the interaction of L with the RNA template (Barik *et al.*, 1990). Low levels of L expression are necessary for efficient viral replication, with high levels of L inhibiting replication *in vitro* for both VSV (Meier *et al.*, 1987) and SeV (Gotoh *et al.*, 1989).

The L gene of RPV is identical in length to that of the MeV L, and sequences of L are highly conserved amongst members of the genus *Morbillivirus* (McIlhatton *et al.*, 1997). Recent work with MeV L proteins from both wild type virus and vaccine strains of virus indicate that amino acid substitutions in the L protein may play a role in attenuation of the virus (Bankamp *et al.*, 2002). This confirms

earlier work which looked at MeV attenuation, where they found that amino acid changes in the L protein and the C and V proteins were responsible for tissue culture attenuation resulting in impaired levels of transcription (Takeda *et al.*, 1998).

The L subunit of the polymerase complex acts as a multifunctional enzyme and is thought to be responsible for all catalytic activities necessary for viral RNA synthesis (Horikami *et al.*, 1994), including initiation, elongation, termination, capping, methylation and polyadenylation. Studies with VSV and SeV have confirmed that polyadenylation, methyltransferase, capping and kinase activities are associated with the L protein (Einberger *et al.*, 1990, Gupta *et al.*, 2002, Hammond *et al.*, 1992, Hercyk *et al.*, 1988, Horikami & Moyer, 1982, Hunt & Hutchinson, 1993). Formation of a complex between L and P is vital for these processes (Hamaguchi *et al.*, 1983). Furthermore, a 2'-O-ribose (cap 1) methyltransferase domain has recently been identified in the L protein of all mononegavirales (Bujnicki & Rychlewski, 2002, Ferron *et al.*, 2002).

A difference in the properties of the polymerase complexes of paramyxoviruses and rhabdoviruses is emerging in the literature. In the case of SeV (Horikami *et al.*, 1992, Smallwood *et al.*, 1994) and MeV (Horikami *et al.*, 1994), co-expression of P and L in the same cell must occur for the polymerase complex to form, yet this is not necessary for either VSV (Canter & Perrault, 1996) or for rabies virus (Chenik *et al.*, 1998). The L proteins of SeV (Smallwood *et al.*, 1994) and MeV (Horikami *et al.*, 1994) are unstable unless they are co-expressed with P. To form the stable polymerase complex, the amino-proximal domain of L is involved in the interaction with P in both MeV and SV5 (Horikami *et al.*, 1994, Parks, 1994), while for VSV, the carboxy-terminal domain of L is required for interaction with P (Canter & Perrault, 1996).

It is widely acknowledged that there are several motifs that are unique to polymerases. All polymerases which exhibit RNA template specificity have been shown to possess four highly conserved motif sequences designated A, B, C and D which define the "polymerase module" (Poch *et al.*, 1989) and this has been

confirmed for the RPV L protein (Baron & Barrett, 1995b). These major motifs include a proposed RNA binding region (Motif A), the QGDNQ-containing region proposed to be the active site of the polymerase and the proposed purine nucleotide binding site (both found in Motif C) (Poch *et al.*, 1990, Poch *et al.*, 1989, Tordo *et al.*, 1988). The structural shape of a polymerase is often thought of as a right hand with domains that resemble the finger, palm and thumb regions of a human hand. The four conserved A, B, C and D motifs of polymerases are thought to constitute the palm and part of the fingers of the polymerase active site, into which the template locks. As the template binds the polymerase it is thought that the finger and thumb regions clamp the template into position, as reviewed by O'Reilly and Kao (O'Reilly & Kao, 1998).

Analysis of available RNA-dependent-RNA polymerase sequences show that they possess the same conserved motifs in the same order, even though the degree of sequence conservation along their length is poor (Barik *et al.*, 1990, Feldhaus & Lesnaw, 1988, Poch *et al.*, 1990, Poch *et al.*, 1989, Tordo *et al.*, 1986, Tordo *et al.*, 1988, Volchkov *et al.*, 1999), thus indicating a common evolutionary ancestor. There exists a high degree of sequence conservation between paramyxoviral and filoviral L proteins (Galinski *et al.*, 1988, Volchkov *et al.*, 1999), which is seen to a lesser extent with members of the rhabdoviridae family (Barik *et al.*, 1990, Le Mercier *et al.*, 1997), yet apart from these conserved motifs, no homology exists between the polymerases of the segmented bunyaviruses and the non-segmented paramyxoviral or rhabdoviral L proteins (Elliott, 1989). The QGDNQ motif is found in almost all mononegavirales polymerases; in NiV, HeV and Tupaia paramyxovirus (TPMV) the Q following the GDN tripeptide is replaced by an E (Harcourt *et al.*, 2001) while the rhabdovirus infectious hematopoietic necrosis virus (IHNV) has a V residue at this position (Bjorklund *et al.*, 1995).

Building on previous observations (Barik *et al.*, 1990, Feldhaus & Lesnaw, 1988), a sequence alignment and comparison study of L proteins of five monopartite negative-strand RNA viruses within the paramyxovirus and rhabdovirus families revealed highly conserved amino acids clustered into six

domains along the length of L, joined by variable regions (Poch *et al.*, 1990), as outlined in figure 1.7.1 on seven aligned sequences. The enzymatic functions of the polymerase are thought to be located within the conserved domains of L, thereby resembling a "chain of enzymes".

Domain I is mainly composed of hydrophobic residues, but most importantly it contains a very rare invariant tripeptide, "GHP", at positions 428-430, its rarity is due to its low frequency in the protein database, according to the Claverie and Bougueleret cataloguing method (Poch *et al.*, 1990). The significance of this remains unknown, although the histidine residue is positioned in such a way that it may be involved in some enzymatic activity (McIlhatton *et al.*, 1997, Poch *et al.*, 1990).

Domains II and III are dominated by the presence of charged amino acids and it is thought that this is where the polymerase module of the L protein is located (Muller *et al.*, 1994, Poch *et al.*, 1990). Domain II commences at position 578 and from position 618 onwards it contains a central highly conserved stretch very rich in basic amino acids, which form a "KEKE (hydrophobic) K" motif. It is thought that this region may contain the RNA binding site in the form of an amphiphilic α-helix (Poch *et al.*, 1990) and it is found in all L proteins of paramyxovirinae and filoviridae (Mühlberger *et al.*, 1992, Takimoto *et al.*, 2000).

Domain III is the most highly conserved out of the six proposed domains and includes the four highly conserved motifs as identified in Poch *et al.*, 1989, thus indicating a key functional role for domain III. The conserved pentapeptide "QGDNQ" is known to be required for cation (Mg^{2+} or Mn^{2+} or Ca^{2+}) co-ordination and is similar to the "GDD" motif of other such RNA dependent polymerases (Blumberg *et al.*, 1988, McIlhatton *et al.*, 1997, Muller *et al.*, 1994, Poch *et al.*, 1990). GDD and LDD motifs are highly conserved amongst DNA/RNA polymerase proteins and have been postulated to form, or be part of, the active site of an ancestral RNA polymerase from which other polymerases have evolved (Kamer & Argos, 1984).

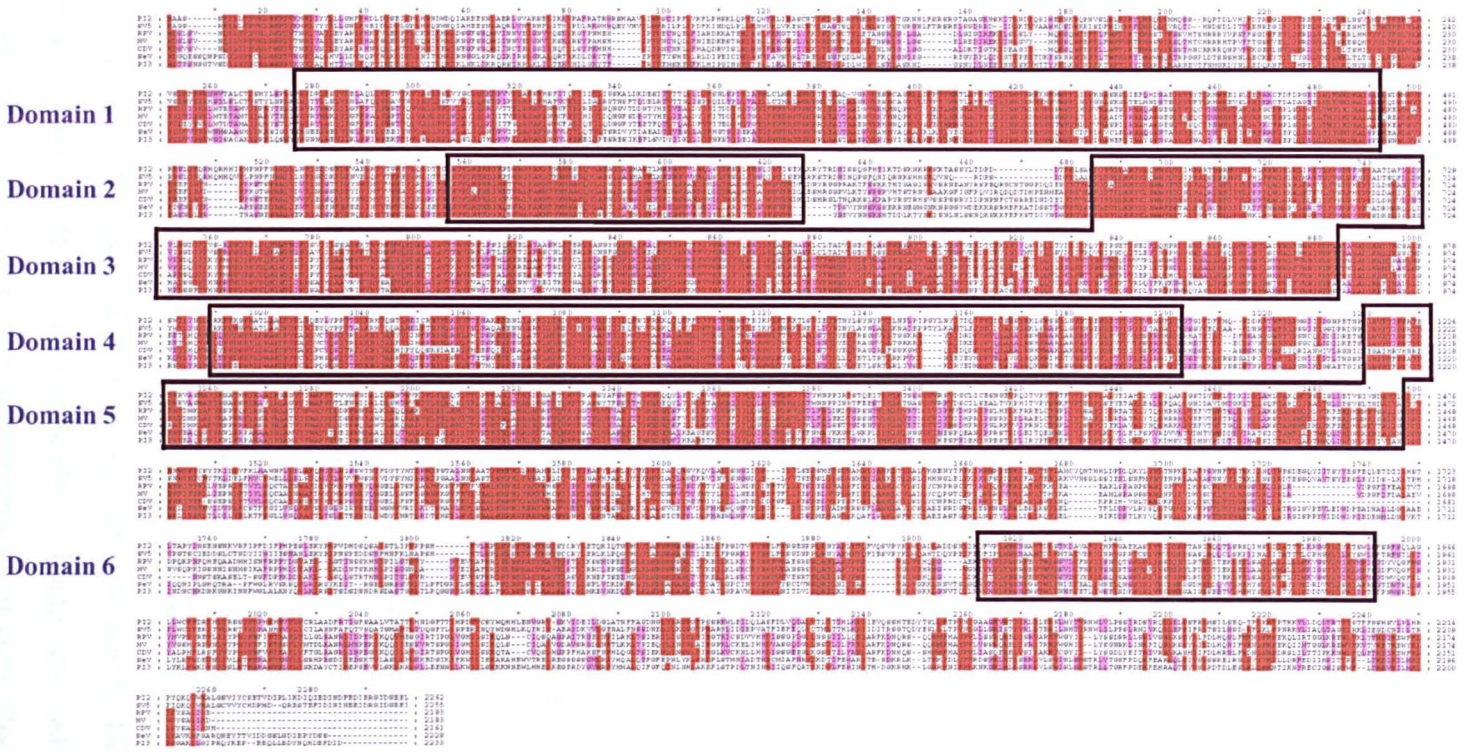

Figure 1.7.1. Representation of the six conserved domains of the L protein, as proposed by Poch et al., 1990. Positions of identity in all or most sequences are shaded red. Positions of similarity are shaded pink. The sequences shown are of parainfluenza virus type 2 (PI2), simian virus 5(SV5), RPV, measles virus (MV), CDV, SeV, human parainfluenza virus type 3 (PI3).

Domain IV is very rich in invariant proline residues and contains four of these, whilst domain V contains numerous cysteine and histidine residues, which are generally associated with metal-binding sites, though it has no recognisable motifs (Cortese *et al.*, 2000, Poch *et al.*, 1990). It has recently been shown that the amino acid residues located in domain V of SeV L are important for various stages of viral transcription and replication (Cortese *et al.*, 2000).

Domain VI is the more variable of these identified clusters and possesses a "GXGXG" motif, which is conserved and located at an approximate position of amino acids 1951-1955. Upstream of this motif there is a lysine-rich sequence that together with the GXGXG motif forms part of an ATP-binding site, which is common in protein kinases (Poch *et al.*, 1990). It has recently been postulated that domain VI contains a methyltransferase domain rather than an NTP binding site (Ferron *et al.*, 2002). In addition, this more variable region of the L protein has been proposed to contain the sequence for specialised functions developed by individual viruses to increase their host cell specificity and individual characteristics. Recent studies with SeV have shown that even a 10 amino acid deletion in the carboxy terminal domain of L results in defective RNA synthesis, with longer truncations in this region further abolishing all activity, thus demonstrating the essential role of the carboxy terminal domain of L for both RNA transcription and replication (Smallwood *et al.*, 2002).

The variable regions located between some of these domains are thought to have functions also. The region located between domains II and III, and the region between domains III and IV have been postulated by Poch *et al.* (Poch *et al.*, 1990) to mediate an optimal co-operation between the functional domains. It has also been recognised that these variable regions of L proteins in both paramyxoviruses and rhabdoviruses are very similar in length. However, the region between domains II and III, despite always being very hydrophilic, varies considerably in its composition in these two families of virus (Poch *et al.*, 1990), thus conservation of a function becomes less likely. Point mutations in the unconserved region between domains V and VI result in a series of unexpected

defects in the function of the polymerase (Horikami & Moyer, 1995, Hunt & Hutchinson, 1993).

Extensive domain mutation analyses in SeV L have failed to identify the function of any individual domain (Chandrika *et al.*, 1995, Cortese *et al.*, 2000, Feller *et al.*, 2000, Horikami & Moyer, 1995). Recent studies with SeV have shown that mutations of conserved residues in domains II and III of L do not affect P binding and the majority resulted in the inactivation of SeV mRNA synthesis and leader mRNA synthesis (Smallwood *et al.*, 2002). Two of the mutations, at positions 736 and 741, resulted in no protein being expressed at all, demonstrating that the stability of the protein was compromised (Smallwood *et al.*, 2002).

McIlhatton and co-workers (McIlhatton *et al.*, 1997) investigated the L proteins of morbilliviruses and rejected Poch *et al.*'s (Poch *et al.*, 1990) six domain hypothesis, proposing that, for this genus, there were in fact two regions of poorly conserved sequence along the length of L, which separated three highly conserved domains. These two regions were referred to as "hinges" and, like the variable regions located between Poch's six domains, were thought to be flexible and enable optimal co-operation between the conserved domains, see figure 1.7.2.

In addition to the catalytic activities related to RNA synthesis, the polymerase of VSV possesses two blocks of sequence, one in domain II and the other in domain V, which show significant similarity to the catalytic domains of tyrosine-specific protein kinases (McClure & Perrault, 1989). Furthermore, the VSV New Jersey L protein has been shown by the analysis of ATP-binding sites present in transcription complexes of VSV to possess Mg^{2+}- dependent ATP-utilising serine/threonine protein kinase activity (Hammond *et al.*, 1992). Like VSV L (Sanchez *et al.*, 1985), SeV L has been shown to be a specific kinase for the phosphoprotein (Einberger *et al.*, 1990), with SeV L also phosphorylating N (Einberger *et al.*, 1990). VSV L of the Indiana serotype has been shown to possess two consensus ATP binding sites similar to the motif found in protein kinases (Massey & Lenard, 1987).

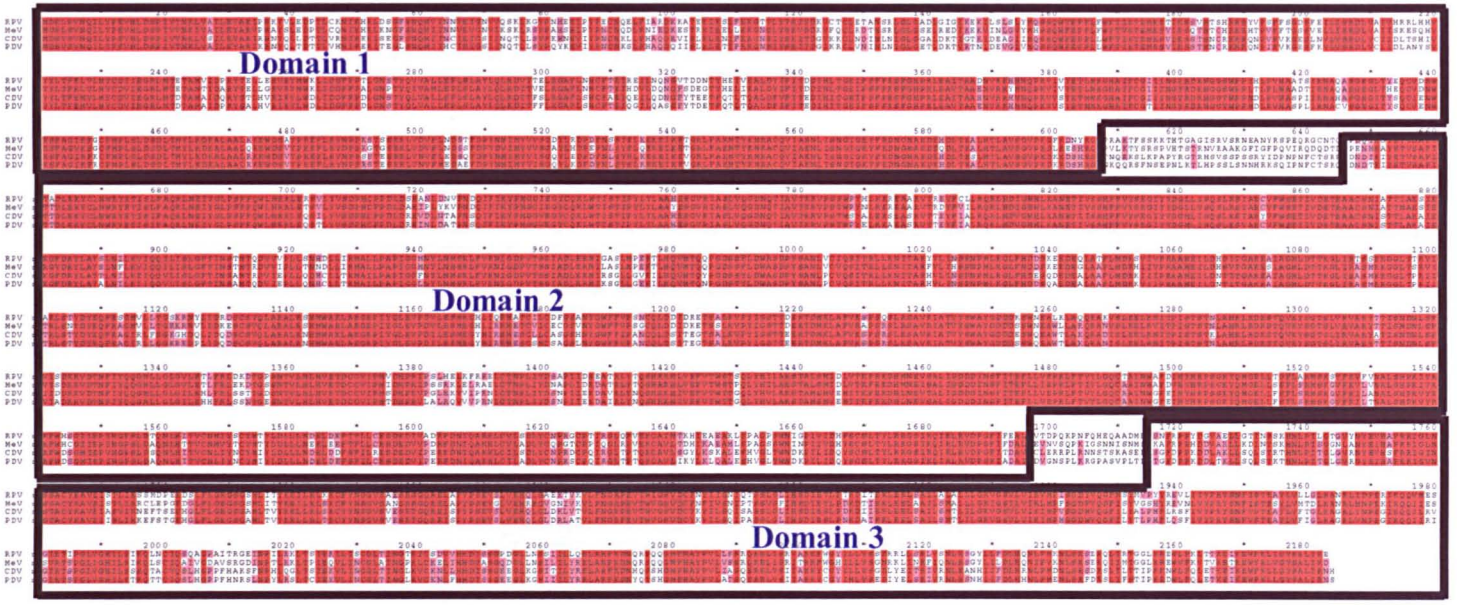

Figure 1.7.2. Representation of the three conserved domains of the *Morbillivirus* L protein as proposed by McIlhatton *et al.*, 1997. Mapped on to Morbillivirus sequence alignments.

1.7.1 Interaction of L with Host Cell Proteins

Both MeV L and VSV L have been shown to interact with the host cell protein tubulin, which is known to be required for RNA synthesis of MeV, SeV and VSV (New Jersey strain) (Moyer *et al.*, 1990, Moyer *et al.*, 1986). In contrast no such interaction was observed in similar studies using SeV L (Moyer *et al.*, 1990). Actin is known to be required for the transcription of not only hPIV-3 (De *et al.*, 1993), but also RSV (Burke *et al.*, 1998). More recent work with RSV has demonstrated that another host cellular protein, profilin, is required for the optimal transcription of RSV (Burke *et al.*, 2000), but this function of profilin required the presence of actin.

In addition to binding tubulin, the L protein of the prototype of non-segmented negative strand RNA viruses, VSV, has recently been found to bind another host cell protein. The RNA-dependent RNA polymerase of VSV associates with translation elongation factor- 1 $\alpha\beta\gamma$, via the L protein, an association that is required for its activity (Das *et al.*, 1998). Prior to this finding only tubulin was known to be required for the RNA polymerase activity of any monopartite negative sense RNA viruses.

1.8 Aims of Project

The aims of this study are to:

1. Determine which of the three conserved "domains" of L interact with proteins P, C and V of RPV. By determining the regions of L to which the P, C and V proteins bind, it is hoped that the functional regions of L will be further elucidated. In addition, such binding studies will serve to provide information of the functional basis of the interaction of both C and V with L, depending upon with which region of L they associate.

2. Investigate the interaction of L with host cell proteins suspected to be involved in viral transcription (actin, tubulin, profilin, elongation factor-1-alpha, guanyl-transferase). It has been postulated that the L proteins of members of the order monegavirales interact with host cell proteins. To determine if this is the case for RPV, a yeast two-hybrid library screen will be undertaken using L as the bait plasmid and a porcine macrophage cDNA library. It is hoped that any such interactions will thus be detected using this popular system for identifying novel protein:protein interactions.

Chapter Two
Methods

For a list of all solutions used in this chapter, please refer to Appendix 1, and for a list of all primary antibodies used, please refer to Appendix 2. In addition, all primers utilised can be found in Appendix 3 and plasmid maps are located in Appendix 4.

2.1 Cell Lines

2.1.1 Tissue Culture

Vero (African green monkey kidney) cells were passaged when confluent and split 1/6. Cells were maintained using Dulbecco's modified Eagle's medium (DMEM) with 25mM HEPES buffer (Central Service Unit - CSU) containing 5% fetal calf serum (FCS), penicillin (100U/ml) and streptomycin sulphate (100µg/ml).

Cos-1 (African green monkey kidney, expressing the simian virus 40 (SV40) large T antigen) cells were passaged before confluency was achieved and split 1/6. Cells were maintained as described for Vero cells.

A549 (immortalised human type II alveolar epithelial) cells were passaged when confluent and split 1/10. Cells were maintained as described for Vero cells and Cos-1 cells.

B95a (Marmoset B-lymphoblastoid cells transformed with Epstein Barr virus (EBV)) cells were passaged when confluent and split 1/3. Cells were maintained using RPMI 1640 medium with Glutamax-1 and 25mM HEPES buffer (GIBCO-Invitrogen), containing 10% FCS, penicillin (100U/ml) and streptomycin sulphate (100µg/ml).

2.1.2 Bacterial Cells

Either *E.coli* JM109 (genotype: *e14-*, *(McrA-)*, *rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, (rK- mK+), *sup*E44, *rel*A1, *Δ(lac-proAB)*, [F', *tra*D36, *proAB*, *lacI*^{q}*Z*ΔM15]) or *E.coli* DH5α (genotype: *deo*R, *end*A1, *gyr*A96, *hsd*R17*(*$r_k^- m_k^+$*)*, *rec*A1, *rel*A1, *sup*E44, *thi*-1, *Δ(lacZYA-arg*FV169*)*, *φ80δlacZ*ΔM15, F$^-$, λ$^-$) bacterial cells were used for all transformations except where specified, and cultured in Luria Bertani broth (LB). *E.coli* BNN132 (genotype: (*Cre*$^+$ strain), *end*A1, *gyr*96, *thi*, *hsd*R17, *sup*E44, *rel*A1, *Δ(lac-proAB)*, (F', *tra*D36, *proAB*$^+$, *lac*I^{q}ZΔM15), (λ, *Kan-Cre*)) bacterial cells were used to prepare the plasmid form of the pig macrophage cDNA library by *in vivo* recombination. Bacterial cells were stored as glycerol stocks at –70°C.

Either *E.coli* KC8 (genotype: *hsd*R, *leu*B600, *trp*C9830, *pyr*F::Tn5, *his*B463, *lacΔφ*74, *str*A, *gal*U, *gal*K) electrocompetent bacterial cells (BD-Clontech) or *E.coli* HB101 (genotype: F$^-$, *hsd*S20, (r_B^-, m_B^-), *thi*-1, *sup*E44, *ara*14, *gal*K2, *lac*Y1, *pro*A2, *rps*L20, (StrR), *xyl*-5, *mtl*-1, *rec*A13, *mcr*B, *leu*B6) were used for attempts on the cloning of plasmid DNA isolated from yeast AH109 cells. The HB101 cells were stored as glycerol stocks at –70°C, whilst electrocompetent KC8 cells were stored at -70°C in 100μl aliquots, as supplied by Clontech.

2.1.3 Yeast Cells

Either the Y190 (genotype: MATa, *ura*3-52, *his*3-D200, *lys*2-801, *ade*2-101, *trp*1-901, *leu*2-3, 112, *gal*4Δ, *gal*80Δ, URA3 : : $GAL1_{UAS}$-$GAL1_{TATA}$-*lacZ*, *cyh*^{r}2, LYS2 : : GAL_{UAS}-$HIS3_{TATA}$-HIS3) (Flick & Johnston, 1990, Harper *et al.*, 1993) or AH109 (genotype: MATa, *trp*1-901, *leu*2-3, 112, *ura*3-52, *his*3-200, *gal*4Δ, *gal*80Δ, LYS2 : : $GAL1_{UAS}$-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3 : : $MEL1_{UAS}$-$MEL1_{TATA}$-*lacZ*) (James *et al.*, 1996) strains of *Saccharomyces cerevisiae* cells were used for all yeast transformations and

cultured using YEPD broth. Yeast strains were stored as glycerol stocks at -70°C and propagated on YEPD agar plates at 30°C when required for use.

2.2 DNA Techniques

Standard DNA techniques were performed according to methods found in Sambrook *et al.*, third edition (Sambrook & Russell, 2001), except as specified here.

2.2.1 Polymerase Chain Reaction (PCR)

2.2.1.1 *Pfu* PCR

Pfu, a proof-reading enzyme, was the enzyme of choice when the PCR product generated was to be used in the production of a new construct. The exact sequence was therefore vital and no errors were permitted.

Into a sterile 0.5ml microfuge tube, the following components were placed and the final volume made up to 50µl with DEPC-treated water:
5µl of 10X *Pfu* buffer (Stratagene)
1µl of each of the required primers (10pmol/µl)
1µl of 10mM dNTP's (10mM in each dNTP)
0.5µl of *Pfu* enzyme (Stratagene)
10ng of template DNA

The program below was followed for the generation of desired coding regions of RBOK sequence.
Step 1
Initial denaturation at 95°C for 1 minute 30 seconds
Step 2
Denaturation and disruption at each cycle at 95°C for 30 seconds
Step 3

Annealing of the melted primers to the template DNA at 50°C for 30 seconds

Step 4

Extension time at 72°C. The time depended upon the length of the desired sequence being amplified (approximately 1 minute 30 seconds for each kb).

Step 5

29 times to step 2

Step 6

Cooling of PCR product to 4°C for 10 minutes

Step 7

End of PCR run

PCR products were checked on an analytical 0.8% agarose gel in TBE buffer (45mM tris-borate, 2mM EDTA), to ensure that the correct size of product had been amplified. Reaction products were stored at -20°C until required.

2.2.1.2 *Taq* PCR

Taq polymerase was used for PCRs where the absolute accuracy of the product generated was not critical, for example during the screening of yeast colonies from the yeast two-hybrid system library screen. In this instance we were only verifying that there was a plasmid present in the crude DNA preps. In addition to this, PCR with *Taq* is more resistant to any contaminants present in the DNA preps and also to a higher load of DNA than PCR with *Pfu*.

For all *Taq* reactions the same conditions were used as for *Pfu* reactions, the only difference being the actual components within the reaction tube and an annealing temperature of 55°C was used in place of the 50°C used with *Pfu*; this was due to the different primers being used. Into a sterile 0.5ml microfuge tube, the following components were placed and the final volume made up to 50µl with DEPC-treated water:

5µl of 10X *Taq* buffer (Invitrogen)

1.5µl 50mM $MgCl_2$ (Invitrogen)

2µl of each of the required primers (10pmol/µl)
1µl of 10mM dNTP's (10mM in each dNTP)
0.5µl of *Taq* enzyme (Invitrogen)
1µl yeast plasmid DNA (prepared as in section 2.4.5)

The PCR products were then checked on an analytical 0.8% agarose gel in TBE buffer, to determine the size of product amplified. Reaction products were then stored at -20°C until required.

2.2.2 Restriction Digests

For each analytical digest 2µl of "mini-prep" DNA was taken and digested by 1 unit of the appropriate restriction enzyme(s) in a final volume of 10µl, using an appropriate buffer for the enzyme or enzyme combination being used. The reaction mix was incubated for a period of 1 hour at the optimum temperature for the restriction enzyme used, normally 37°C. The total digest volume, with 2µl of 5X loading buffer (20% (w/v) Ficoll 400, 5mM EDTA, 0.05% bromophenol blue, 0.03% xylene cyanol), was then run on a 0.8% agarose gel in TBE buffer for 1 hour at 120 volts to analyse the products of digestion.

For each preparative enzyme digest, 3-5µg of DNA were taken into a final volume of 50µl containing 10 units of the appropriate restriction enzyme, the appropriate enzyme buffer (1X) and BSA (1µg/µl). The reaction mix was incubated for a period of at least 3 hours at the optimum temperature for the restriction enzyme(s) used. After this incubation period, 1.5µl of the digest were run on a 0.8% agarose gel in TBE buffer for 1 hour at 120 volts to confirm complete digestion. Digested DNA fragments, with shrimp alkaline phosphatase (2.2.3) treatment where required, were purified on a 0.8% low melting point (LMP) agarose gel in TAE buffer (40mM tris-acetate, 1mM EDTA) overnight at 20 volts.

2.2.3 Shrimp Alkaline Phosphatase (SAP) Treatment

Plasmid vectors required for ligations needed to be treated with shrimp alkaline phosphatase after digestion. This was to prevent re-circularisation of the plasmid by removing the phosphate group present at the 5' ends of the freshly digested DNA. After the preparative digest was checked on a 0.8% agarose/TBE gel, there were 48.5µl of the digest left. To this remaining volume 1µl of SAP (1 U) and 5.5µl of 10X SAP buffer were added. The reaction mix was incubated at 37°C for 20 minutes and then at 65°C for 20 minutes. The vector was purified on a 0.8% LMP agarose gel in TAE buffer overnight at 20 volts.

2.2.4 Extraction of DNA from Agarose Gels

Restriction digests of DNA were run on a preparative 0.8% LMP gel. The required DNA band was excised from the gel, using a sterile scalpel, and transferred into a sterile microfuge tube. DNA was extracted from the agarose using one of the two methods described below.

2.2.4.1 "Freeze-Squeezing" DNA

The bands of DNA were placed at -70°C for at least 20 minutes. The gel slice was "smashed" using an oocyte smasher and 400µl of buffer-saturated phenol (BioGene Limited) added. The sample was vortexed for at least 30 seconds to produce an emulsion. The gel matrix had to be thoroughly collapsed at this stage. The mixture was quick frozen at -70°C for at least 10 minutes and centrifuged for 15 minutes at room temperature at 13 200rpm (using an Eppendorf 5415D centrifuge with a F45-24-11 rotor). The top aqueous layer was next transferred to a fresh microfuge tube and phenol extracted once more, by adding an equal volume of buffer-saturated phenol. The sample was then extracted once using an equal volume of phenol-chloroform. The DNA was precipitated by adding $1/10^{th}$ volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of ethanol (-20°C) and leaving on ice for at least 10 minutes. The sample was centrifuged for 15 minutes

as before and the pellet washed with 1ml of 70% ethanol, before being re-spun for 5 minutes. The ethanol was removed and the pellet dried. The pellet was resuspended in ~20µl TE_8 (10mMTris/Cl pH 8.0, 1mM EDTA pH 8.0) and the DNA quantitated, as in section 2.2.5, on an analytical 0.8% agarose gel in TBE buffer.

2.2.4.2 DNA Extraction Using Purification Columns

DNA was extracted from agarose using GFX columns from the "GFX PCR DNA and Gel Band Purification Kit" (Amersham Biosciences). Extractions were carried out according to the manufacturer's protocol and guidelines.

2.2.5 Ligations in Agarose

LMP agarose slices containing the vector and insert required were melted at 70°C for 10 minutes in a waterbath. 2µl of molten agarose from each sample was run on a 0.8% agarose/TBE gel and quantitated by comparing the brightness of bands achieved alongside λIII DNA markers (*Eco*RI/*Hin*DIII digested). A molar ratio of 3:1, insert:vector, of the DNA fragments in molten agarose was combined at 37°C, with sterile distilled water where required, in a total volume of 10µl. This was then made up to a final volume of 20µl in 1X ligase buffer (Promega) containing 1 unit of T4 DNA ligase. These 20µl ligations were then incubated overnight in a 15°C waterbath.

2.2.6 Ligations of Extracted DNA

DNA was extracted from LMP agarose as described above. Eluted DNA (2µl) was quantitated on a 0.8% agarose/TBE gel and quantitated by comparing the brightness of bands achieved alongside λIII DNA markers (*Eco*RI/*Hin*DIII digested). A molar ratio of 3:1, insert:vector of the DNA fragments was combined, with sterile distilled water where required, in a total volume of 10µl.

This was then made up to a final volume of 20µl in 1X ligase buffer (Promega) containing 1 unit of T4 DNA ligase. These 20µl ligations were then incubated overnight in a 15°C waterbath.

2.2.7 Preparation of Competent Bacterial Cells

A modification of the technique described by Chung and Miller (Chung & Miller, 1988), was used for this method.

A 2ml culture of bacterial cells was grown up overnight in SOB at 37°C. This culture was then diluted 1/200 with LB and incubated until the required OD_{600} was achieved (0.1-0.15 for DH5αs and HB101s, and 0.25-0.35 for JM109s). Cells were harvested by centrifugation at 2 500rpm for 10 minutes (using an IEC Centra GP8R centrifuge with a 218 rotor), the supernatant discarded and the pellet resuspended in $1/10^{th}$ original volume of TSS (LB broth containing 10% PEG 3350 or 8000, 20mM $MgSO_4$, 5% tissue culture grade DMSO). The bacterial cells were left on ice for at least an hour, after which they were ready for transformation.

2.2.8 Transformation of Competent Bacterial Cells

The 20µl ligations were heated at 70°C for 10 minutes (if ligations were performed in agarose), diluted to 200µl with 1.1X TCM (11mM Tris/Cl pH 7.5, 11mM $MgCl_2$, 11mM $CaCl_2$) and chilled on ice. Competent cells (200µl) were added to each ligation. The ligation components were mixed gently by flicking the microfuge tube and incubated on ice for one hour after which time the cells were heat shocked for 2 minutes in a 45°C waterbath. The samples were returned to ice for 5 minutes before adding 0.6ml SOC (SOB containing 20mM glucose) to each tube. The tubes were then incubated at 37°C, with shaking, for one hour and the 1ml final volumes were plated out on LB/amp (100µg/ml ampicillin) selection plates and incubated at 37°C overnight.

2.2.9 Transformation of *E.coli* Bacterial Cells with Extracted Yeast Plasmid DNA

2.2.9.1 HB101 Cells

Glass bead/phenol-chloroform extracted AH109 DNA (5µl), as described in section 2.4.5, or "Yeastmaker" extracted AH109 DNA (1µl), as in section 2.4.6, was placed in a sterile ice-cold 15ml centrifuge tube (Falcon). To each tube, 100µl of competent HB101 cells (prepared as 2.2.7 or gift of L. Goatley) were added and mixed gently by pipetting up and down. Samples were incubated on ice for 30 minutes and then heat shocked for 90 seconds at 42°C, before incubating on ice for a further 2 minutes. SOC (900µl) was added to each tube and samples incubated at 37°C, on a shaker set at 200rpm, for 1 hour. Samples were then spun for 5 minutes at 2 500rpm (using an Eppendorf 5415D centrifuge with a F45-24-11 rotor), the supernatant discarded and cells were resuspended in 200µl of 1X M9 salts, thereby removing all residual traces of leucine. The total volume was plated out onto M9 salts –Leucine/amp (ampicillin at 50µg/ml) agar selection plates and incubated for 2-5 days at 37°C.

2.2.9.2 DH5α Cells

Either 5µl of glass bead/phenol-chloroform extracted AH109 DNA or 1µl of "Yeastmaker" extracted AH109 DNA were transformed using chemically competent (TSS method) DH5α cells. The yeast plasmid DNA was made up to 200µl with 1.1X TCM and the transformation protocol followed was identical to that described in section 2.2.8. The DH5α's were prepared as described in section 2.2.7.

2.2.10 Preparation of Electrocompetent Bacterial Cells

Two 250ml cultures of the required cell type were grown up in LB at 37°C on a shaker until an OD_{600} of 0.5-0.6 was obtained. Cells were chilled on ice for 15 minutes and spun in a pre-chilled centrifuge at 6 000rpm for 20 minutes (using a Sorvall SLA 1500 GSA rotor). The supernatant was discarded and cell pellets washed twice with 250ml ice cold double distilled water (DDW). Cell pellets were harvested by discarding the supernatants and resuspended in 50ml ice cold sterile 10% glycerol and spun as above for 10 minutes only. The supernatant was then discarded and final cell pellets resuspended in 500µl ice cold 10% sterile glycerol. The final cell mixtures were aliquoted out into 40µl volumes and stored at -70°C until required.

2.2.11 Transformation of Yeast Plasmid DNA Using Electrocompetent Bacterial Cells

The required number of electrocompetent cell aliquots were thawed on ice, 1 aliquot was required for each transformation for the home-made electrocompetent cells. For the electrocompetent KC8 cells (Clontech), 1 aliquot was sufficient for 5 transformations and the Clontech electroporation protocol guidelines were followed in full. Final cell/DNA mixes were plated out on LB/amp (ampicillin at 100µg/ml). To electroporate the HB101 cells we used a BioRad electroporator set at 25µF, 150Ohm and 2.5kV using cuvettes with a 2mm gap. The cuvettes and pipette tips used were all pre-chilled. To the aliquot of HB101s, the required amount of DNA was added and mixed by gently pipetting up and down. The contents were then electroporated and immediately after pulsing, 0.9ml of SOC was added to the cuvette, which was placed on ice. Total transformation mixes were incubated for one hour on an orbital shaker set at 37°C, before being plated out onto M9 salts –Leucine/amp (ampicillin at 50µg/ml) plates and incubated for 2-5 days at 37°C.

2.2.12 Plasmid Mini-Preps

The technique used here was a modification of the technique described by Zhou, (Zhou *et al.*, 1990).

Using a sterile cocktail stick, a single colony was picked from the LB/Amp agar selection plate and used to inoculate a 2ml LB/amp overnight culture (ampicillin at 100µg/ml). One ml of this overnight culture was transferred to a sterile microfuge tube and spun at 13 000rpm for 30 seconds (using an Eppendorf 5415D centrifuge with an F45-24-11 rotor). All but approximately 80µl of the supernatant was removed and the bacterial cell pellet resuspended by vortexing. After this, 300µl of TENS solution (10mM Tris/Cl, 1mM EDTA, 0.5% SDS, 0.1M NaOH) were added and the samples were vortexed for at least 5 seconds. This was followed by the addition of 150µl of 3M sodium acetate (pH 5.2), after which the sample was again vortexed for at least 5 seconds. The samples were centrifuged at 13 000rpm for 3 minutes, as above, and the supernatant transferred to a fresh microfuge tube. One ml of ethanol (-20°C) was added and the samples were re-spun at 13 000rpm for 3 minutes. The supernatant was removed and the resulting pellet washed with 70% ethanol and vacuum dried. The final pellet was dissolved in 40µl of TE_8 containing RNAse A at a final concentration of 25µg/ml and either analysed immediately or stored at -20°C until required.

2.2.13 Plasmid Midi-Preps

30ml cultures of the required transformed bacteria were grown up in selective medium (LB/Amp – ampicillin at a concentration of 100µg/ml) on a 37°C shaker overnight. Cultures were then harvested and the "Qiagen midi-prep kit" used according to the manufacturer's protocol and guidelines.

2.2.14 Plasmid Maxi-Preps

200ml cultures of the required transformed bacteria were grown up in selective medium (LB/Amp - ampicillin at a concentration of 100µg/ml) overnight. The following morning, cultures were centrifuged in 250ml Sorvall pots for 10 minutes at 7 000rpm (using a Sorvall RC5B+ centrifuge and a Sorvall SLA 1500 GSA rotor). The supernatant was discarded and the resulting bacterial cell pellet resuspended in 10ml of maxi-prep solution 1 (TE_8) and left at room temperature for 5 minutes. Maxi-prep solution 2 (0.2M NaOH, 1% SDS) was then added (20ml), the preparation mixed gently and incubated on ice for 5 minutes. After this, 15ml of maxi-prep solution 3 (for 100ml: 60ml 5M potassium acetate, 11.5ml glacial acetic acid, 28.5ml DDW) were added, followed by incubation on ice for 15 minutes. Preparations were centrifuged at 15 000rpm for 10 minutes (using a Sorvall RC5B+ centrifuge and a Sorvall SLA 1500 GSA rotor). The supernatant was decanted through a layer of Miracloth (Calbiochem) into a fresh centrifuge pot and 40ml of isopropanol added. After being left at room temperature for 20 minutes, DNA was pelleted by centrifugation for 10 minutes at 15 000rpm, as before. Supernatants were discarded and the resulting pellets washed with 70% ethanol. The wash was then removed and the pellet re-dissolved in 8ml of TE_8.

To this TE/DNA mix, exactly 8g of CsCl were added and dissolved. Ethidium bromide (400µl at 10mg/ml - Sigma) was then added to each preparation and the whole volume transferred to a 5/8" x 3" polyallomer "QuickSeal" tube (Beckman). The DNA solutions were overlayed with mineral oil, balanced and heat-sealed. The tubes were then either centrifuged overnight at 55 000rpm, at 20°C, or they were centrifuged over the weekend at the same temperature, but at a speed of 50 000rpm (using a Beckman L8-70N ultrafuge and a Beckman 70.1ti rotor).

After ultracentrifugation the CsCl gradients were unloaded by clamping the tube and using an 18g needle and a 2ml syringe to remove the lower red band of DNA.

This band of DNA was transferred to a 15ml Falcon tube and made up to a volume of 2ml with TE_8. DNA was extracted 4-6 times, or until the aqueous phase was clear, with equal volumes of isoamyl alcohol to remove the ethidium bromide. After the last extraction, the aqueous layer was removed from under the isoamyl alcohol layer and transferred to a 50ml Falcon tube. DDW (6ml) and ethanol (16ml) were then added to each sample. Samples were then left at -20°C for at least an hour before being centrifuged at 2 500rpm for 30 minutes (using an IEC Centra GP8R centrifuge with a 218 rotor). The supernatant was discarded and the resulting pellet washed with 70% ethanol and re-centrifuged at 2 500rpm for 30 minutes, as before. The supernatant was again discarded, the pellet dried and re-dissolved in 200µl of TE_8.

The concentration and purity of the DNA produced was then measured by taking a series of OD readings on the spectrophotometer, at wavelengths of 260, 280 and 320nm. The 260:280 ratio provided a measure of the purity of the DNA obtained, whilst the 260 reading provided the amount of DNA produced, once multiplied by the dilution factor, and then multiplied by 50 (as 1 OD_{260} is equal to 50µg/ml of DNA).

2.2.15 Sequencing

Sequencing of DNA was performed using either T7 polymerase or by cycle sequencing.

2.2.15.1 Sequencing Using T7 Polymerase

Sequencing of DNA with T7 polymerase was performed using the dideoxy-mediated chain termination method as originally described in (Sanger *et al.*, 1977). This technique was then modified as described in (Kristensen, 1988) and as outlined below.

For each reaction 2-3μg of plasmid DNA, in a volume of 18μl, were denatured using 2μl of 2M NaOH at room temperature for 5 minutes. DNA was precipitated by the addition of 75μl 100% ethanol and 3μl 4M NH_4Ac (pH 5.2). The sample was incubated on ice for a minimum of 10 minutes and centrifuged for 10 minutes in a microcentrifuge at 4°C, at 13 000rpm. The supernatant was aspirated and the pellet washed with 100μl 70% ethanol. The sample was centrifuged for 5 minutes in a microcentrifuge at 4°C, at 13 000rpm. The supernatant was then aspirated and the DNA pellet dried. The pellet was dissolved in a mix of 10μl water, 2μl annealing buffer (1M Tris/Cl pH7.6, 100mM $MgCl_2$, 160mM DTT) and 2μl primer (5pmol/μl) and incubated for 30 minutes at 37°C to anneal the primer and DNA. The sample was then briefly centrifuged to collect all of the reaction in the bottom of the tube. To the annealed primer/DNA, 6μl of the following reaction mix were added: 2μl diluted T7 polymerase (1:4 dilution using manufacturer's enzyme dilution buffer), 3μl 'A' labelling mix (1.375μM each dCTP, dGTP, and dTTP, 333.5mM NaCl) and 1μl diluted [^{35}S] dATPαS label (approximately 5μCi/μl, a 1:1 dilution with water) and incubated at room temperature for 5 minutes. During this 5 minute incubation period, 4 x 4.5μl of the reaction mix were transferred into separate wells on a U-bottomed 96 well plate. Then 2.5 μl of the appropriate termination mix (either A, C, G or T) were placed on the side of each well. The plate was sealed and centrifuged until a speed of 2 000rpm was reached and then immediately stopped. The plate was incubated for 5 minutes at 37°C. Then, to each well, 5μl of stop solution (0.3% each bromophenol blue and xylene cyanol FF, 10mM EDTA pH 7.5, 97.5% deionised formamide) were added and the plate tapped gently on the bench surface to ensure that the contents were mixed and the reaction stopped.

Samples were either stored at –20°C until required, or immediately heated at 75°C for a period of 3 minutes prior to being loaded onto a 6% acrylamide/8M urea gel. For each sequencing reaction, 1.5μl were loaded per 3mm well and 1X

TBE was used as buffer. Electrophoresis was carried out at 72 watts for 2-3 hours. The gel was dried and exposed directly to X-ray film.

2.2.15.2 Cycle Sequencing

These sequence reactions were performed using the "CEQ 8000 Dye Terminator Cycle Sequencing with Quick Start kit" according to the manufacturer's protocol and guidelines (Beckman Coulter). Reactions were analysed on a CEQ 8000 Capillary sequencer, using the Beckman Coulter software for base calling.

2.2.16 Transfections

Eukaryotic cells were grown overnight in six well plates at the required seeding density (Vero cells at $3x10^5$/well, Cos-1 and A549 cells at $5x10^5$/well and B95a cells at a 1/3 split from confluent).

2.2.16.1 Using Transfast Transfection Reagent

For each 35mm well, 0.7ml of Optimem I (GIBCO) was used in addition to the relevant amount of plasmid DNA being transfected and the corresponding quantity of Transfast (TF) reagent. TF reagent was used according to the manufacturer's protocol (Promega). For Cos-1, A549 and B95a cells, 3µl of TF reagent were used for every microgram of plasmid DNA used, whilst for Vero cells, 6µl of TF were used for every microgram of plasmid DNA. TF/DNA/Optimem I mixes were made up in 7ml polystyrene bijoux, vortexed thoroughly and incubated at room temperature for 15 minutes. During this incubation period, the cells were washed twice with 1ml per well of Optimem I. After the removal of the final wash, the TF/DNA/Optimem I mix was added to the cells and incubated at 37°C for 2 hours. After this, 1.3ml of pre-warmed complete medium were added to each 35mm well and the cells incubated at 37°C for 48 hours.

2.2.16.2 Using Lipofectin Transfection Reagent

This protocol was usually used after the cells had first been infected for one hour with either fowlpox T7 virus or vaccinia vTF7-3 virus, see 2.2.17.

For each 35mm well, 10µl of lipofectin were combined with 90µl of Optimem I and incubated at room temperature for 45 minutes. The relevant amount of plasmid DNA was then diluted in Optimem I to a final volume of 100µl. The DNA mixture was combined with an equal volume of Lipofectin/Optimem I mix, mixed thoroughly by pipetting up and down, and then incubated for 10 minutes at room temperature. After this incubation period, the mixture was added to the required well and 0.5ml of Optimem I added. Cells were incubated at 37°C for between 5 and 6 hours after which time 2ml of the relevant pre-warmed medium were added to each 35mm well. Cells were then incubated for either 18 or 42 hours at 37°C.

2.2.17 Transient Expression System

Cells were grown overnight in six well plates at their required seeding densities. The cells were washed once using 1ml Optimem I per 35mm well. Each well was infected using 40µl of fowlpox T7 virus (FPT7: titre $10^{-7.9}$ TCID50/ml on chick embryo fibroblasts) in 400µl of Optimem I or 55µl vaccinia vTF7-3 (10^{8} $TCID_{50}$/ml on baby hamster kidney cells) in 500µl of serum free DMEM and incubated at 37°C for 1 hour, with rocking at 10 minute intervals. The inoculum was removed from the cells, which were then treated as in the transfection protocol, except that cells were used after overnight incubation where vTF7-3 had been used.

2.3 Protein Techniques

2.3.1 Immunofluorescence

The required cells were grown overnight on coverslips, seeded at their relevant density. Cells were transfected using the desired method with the constructs of interest. Either one or two days after transfection, the cells were washed three times using 2ml PBS-E (Ca/Mg free PBS) and fixed using 2ml 3% paraformaldehyde (PFA), incubating for a period of 20 minutes at room temperature in a tissue culture cabinet. Cells were washed a further three times with 2ml PBS-E. At this point, the PFA was quenched with 2ml 50mM NH_4CL in PBS-E for 10 minutes at room temperature. Again the cells were washed three times using 2ml PBS-E. After this stage, the cells were opened for internal staining using 2ml 0.5% Triton X-100 in PBS-E (5 minutes at room temperature). The cells were then washed three times with 2ml PBS-E and either incubated in 2ml 0.2% PBS-gelatin for a period of 5 minutes, or 2ml 0.2% PBS-gelatin containing 5% normal goat serum for 30 minutes, to block non-specific binding.

The cells were now ready for staining with the primary antibody, which was first diluted to the correct concentration in blocking solution. The diluted primary antibody (25µl) was placed on a strip of parafilm, which was located in a moist chamber. The coverslip was then placed cell side down on top of this drop of antibody for 30 minutes at room temperature. At the end of this incubation period, excess PBS was pipetted under the coverslip in order to break the surface tension thereby allowing the coverslip to float up. The coverslip was transferred to either fresh PBS-gelatin or PBS-E and left for 5 minutes at room temperature. Cells were washed twice for a period of 5 minutes each using 2ml PBS-E before a final 5 minute wash using 2ml PBS-gelatin, or washed for 5 minutes four times with 2ml PBS-E. The secondary antibody was then diluted to the correct working concentration in blocking solution, and the coverslip was incubated in the moist chamber as described for the primary antibody.

After this incubation with the secondary antibody, cells were washed twice for 5 minutes with PBS-E. Again the cells were washed, using 2ml PBS-E containing 2µl Hoechst 33258 stain (0.5mg/ml) before being washed once more using 2ml PBS-E for 5 minutes.

Coverslips were rinsed in DDW, excess water removed with pieces of Whatman No 1 filter paper and the coverslips mounted on slides using 20µl of Mowiol (Calbiochem). Alternatively, after the secondary antibody incubation, cells were washed four times for 5 minutes each with PBS-E and mounted using Vectashield mounting medium with DAPI stain (Vector Labs).

Mowiol mounted slides were allowed to set at room temperature for at least 45 minutes before being viewed under the microscope using the relevant magnification and filters. For epifluorescence microscopy, images were captured using a Nikon Eclipse E800 microscope together with Openlab software (Improvision) and for confocal microscopy, single slice images were captured using a Leica SP2 confocal microscope and the Leica software provided with the microscope.

2.3.2 Preparation of SDS-PAGE Gels

2.3.2.1 Laemmli Gels

These gels were run using the buffer system as described in (Laemmli, 1970).

	Resolving Gel		Stacking Gel
	8%	12%	
40% (w/v) Acrylamide/bis 37:1 ratio (Sigma)	8ml	12ml	2ml
3M Tris pH 8.8	5.6ml	5.6ml	-
1M Tris pH 6.8	-	-	1ml
Water	26ml	22ml	12.8ml
TEMED	20µl	20µl	8µl
10% SDS	0.4ml	0.4ml	160µl
20% APS	100µl	100µl	60µl

Using the above table, gel mixes (acrylamide/water/buffer/TEMED) for the relevant percentage gel and the stacking gel were made simultaneously. APS and SDS were added to the resolving gel mix and poured into a casting tank to the desired level. Water-saturated N-butanol (100µl) was added to each side of the gel to form an overlay, which kept the surface of the resolving gel flat. The gel was allowed to set at room temperature for one hour. Once set, the N-butanol was poured off and the gel surface rinsed thoroughly with water. SDS was then added to the stacking gel mix, swirled to mix, and 500µl of this were used to give the top of each running gel a final rinse. APS was added to the stacking gel mix, the solution swirled once more to mix contents and the gel mix added to fill the space above the running gel to the top of the casting tank. Combs were inserted and the gel left to set for a further hour at room temperature.

Once set, the combs were removed and excess stacking gel removed with a scalpel. Wells were rinsed thoroughly with water and the gel(s) placed in a running stand and the running buffer (0.1% SDS (w/v), 1.44% glycine (w/v), 0.3% Tris base (w/v)) added. Gels were run at a constant current of 18mA per gel until the dye front had run off the bottom of the gel.

2.3.2.2 Tricine Gels

Tricine gels were run using the buffer system as described in (Schagger & von Jagow, 1987).

	Resolving Gel	Stacking Gel
40% (w/v) Acrylamide/bis 37:1 ratio (Sigma)	7.625ml	1.25ml
3M Tris/Cl buffer pH 8.45	10ml	3.1ml
Water	8.015ml	7.965ml
TEMED	10µl	10µl
Glycerol	4g	-
10% SDS	0.3ml	0.125ml
20% APS	50µl	50µl

Using the above table, gel mixes (acrylamide/water/buffer/TEMED) for a 10% tricine resolving gel and stacking gel were made simultaneously and gels were made as described in section 2.3.2.1. Gels were run using different buffers for the anode (0.2M Tris, pH 8.9) and the cathode (1% SDS (w/v), 0.1M Tris, 0.1M Tricine, pH 8.25). Gels were first run for one hour at 30 volts and 70mA, and then at 150 volts and 30mA until the dye front was about a cm from the bottom of the gel.

2.3.3 Western Blot

Prepared protein samples were run on either Laemmli gels or Tricine gels as described above. After running the relevant gel, the stacking gel was removed from the resolving gel using a scalpel and the proteins transferred to Immobilon-P membrane (Millipore) using the method of (Towbin *et al.*, 1979). Following transfer, membranes were blocked in blocking solution (5% (w/v) Marvel in Tris-buffered saline containing 0.1% Tween 20 (w/v) (TBS-T)) either at 4°C overnight, or for 1 hour at room temperature on a rocker in an open topped box. The membranes (protein side in) were then placed in a 50ml Falcon tube with the relevant primary antibody at the current working concentration in 5ml blocking solution and incubated for 1 hour at room temperature on a Spiramix (Denley).

After this incubation period with the primary antibody, membranes were washed twice for 30 seconds and twice for 10 minutes in TBS-T in an open box with agitation at room temperature. Membranes were then incubated with the required secondary antibody, as described for the primary antibody. Secondary antibodies were HRP-conjugated sheep anti-mouse, or donkey anti-rabbit (Amersham/Pharmacia). At the end of this one hour incubation period, membranes were washed as before in TBS-T. The membranes were then incubated for 5 minutes at room temperature with "SuperSignal West Pico Chemiluminescent Substrate" (Pierce), before being exposed to photographic film.

2.3.4 Metabolic Labelling of Transfected Cells and Immunoprecipitation

Transfected cells were washed once using Met/Cys-free Eagle's medium and starved for two periods of 30 minutes using 2ml pre-warmed Met/Cys-free Eagle's medium at 37°C. Cells were labelled at 37°C, for one hour, in 0.5ml (per 35mm well) of Met/Cys-free Eagle's medium containing 5-10µl [^{35}S]-Promix (Amersham). Label was removed from the cells and the cells washed once with

Met/Cys-free medium without label. All liquid waste was disposed of as radioactive waste in the appropriate manner. Labelled cells were lysed for 5 minutes in 0.5ml per well NP40 lysis buffer (1% NP40, 0.15M NaCl, 50mM Tris/Cl pH 7.5, 2mM EDTA) containing either 0.5mM PMSF or protease inhibitor cocktail set III (Calbiochem) at a final dilution of 1/200. Cell lysates were transferred to sterile microfuge tubes and spun at 13 000rpm for 30 minutes at 4°C. Supernatants were then transferred to fresh tubes and incubated with the required amount of the appropriate antibody overnight at 4°C, with rotation.

The following morning, 30µl of protein G-Sepharose were added to each tube and incubated at 4°C, with rotation for 30 minutes. All tubes were then briefly microfuged at 6 500rpm (Eppendorf 5415D centrifuge, F45-24-11 rotor), to harvest the agarose. Supernatants were stored at -20°C in fresh tubes for possible further extractions. The resulting agarose pellets were washed four times with 0.5ml wash buffer A (0.2% NP40, 10mM Tris/Cl pH 7.5, 150mM NaCl, 2mM EDTA) and once with 0.5ml of wash buffer C (10mM Tris/Cl pH 7.5).

After the final washing, 40µl of SDS-PAGE sample buffer were added to each sample. This buffer consisted of: 16.5µl 3X sample buffer (New England Biolabs), 5µl 0.5M DTT (dithiothreitol) and 18.5µl DDW, per sample. Samples were immediately vortexed and heated for 5 minutes at 95-100°C. All samples were re-vortexed and microfuged briefly at 6 500rpm (as before), to pellet the agarose.

At this stage samples were either stored at -20°C until required for loading onto a gel, or 15µl of each sample was immediately run on the appropriate type of SDS-PAGE gel (Laemmli or Tricine) as described above. The SDS-PAGE gel was then soaked in 1M sodium salicylate (50ml) for 20 minutes and then placed on 3MM Whatmann filter paper and dried. Once dry, the gel was exposed either to photographic film (Kodak) at –70°C overnight in a lightproof cassette, or to a phospho-imaging screen in a lightproof cassette for 3 hours at room temperature. After exposure, either the film was developed, using a Compact X4 X-Ograph, or

the phospho-imaging screen scanned with a personal molecular imager FX (Bio-Rad). If weak signals were obtained then the exposure times were increased accordingly.

2.3.5 Yeast Protein Isolation

Transformed yeast cells were set up as 1.5ml cultures, under selection for the plasmid of interest, for 2-3 days at 30°C, on a shaker set at 250rpm. Cells were microfuged at 2 600rpm for 5 minutes, and the resulting cell pellet resuspended in 0.25M NaOH/1% 2-mercaptoethanol (1ml) and chilled on ice for 10 minutes. Trichloroacetic acid (0.16ml of a 50% stock solution) was then added to each sample, the contents mixed by vortexing and the samples were again chilled on ice for 10 minutes. Samples were then centrifuged for 5 minutes at 14 000rpm (Eppendorf 5415R centrifuge, F45-24-11 rotor) and the supernatant discarded. Ice cold acetone (1ml) was then added to each sample and the cell pellet resuspended by vigorous vortexing. The samples were microfuged once more at 14 000rpm for 5 minutes, the supernatant was removed and the pellet left to air dry. The resulting pellets were resuspended in 200µl of SDS sample buffer (114µl DDW, 66µl 3X SDS sample buffer (New England Biolabs), 20µl 0.5M DTT). Samples were immediately vortexed and heated for 15 minutes at 95-100°C. All samples were re-vortexed and microfuged briefly at 6 500rpm, to place the contents at the bottom of the tube. Samples were then used for Western blot analysis, as in section 2.3.3.

2.3.6 *In vitro* Transcription/Translation

These reactions were performed using the "TNT Quick Coupled Transcription/Translation System" kit (Promega) according to the manufacturer's protocol and guidelines.

2.3.7 Dual-Luciferase Reporter Assay System

Reactions were performed using this reporter assay kit (Promega) according to the manufacturer's protocol and guidelines.

2.4 Yeast Two-Hybrid System

2.4.1 Growing Stocks of Pig Macrophage cDNA Library

The porcine macrophage cDNA library was converted from λ ACT into plasmid form by automatic subcloning (*in vivo* recombination) using loxP/cre-recombinase technology (Durfee *et al.*, 1993, Elledge *et al.*, 1991). Cre recombinase fuses together loxP sites (cre recognition sites) that flank the plasmid portion of λ ACT when transferred to cre-expressing E.coli BNN132 cells.

BNN132 cells were plated on LB/Kanamycin (Kanamycin at 25μg/ml) and incubated overnight at 28°C. The next day, a 20ml culture of the fresh BNN132 cells in LB + 40μg/ml kanamycin, 0.2% maltose, 10mM $MgCl_2$, was set up and incubated on a shaker at 28°C overnight. The overnight culture was spun down at 1 800rpm for 5 minutes (using an IEC Centra GP8R centrifuge with a 218 rotor) and cells re-suspended in 1ml 10mM $MgCl_2$. The OD_{600} was measured and the culture diluted with 10mM $MgCl_2$ to an OD_{600} of 0.5. The main part of the porcine macrophage library (341μl) and 198μl of the second part of the library, were placed in a 50ml Falcon tube and 4ml diluted cells added. This was incubated at 30°C, without shaking, for 30 minutes. LB (4ml) was added and the mix incubated at 30°C, with shaking, for 1 hour. The mix was diluted with LB to produce a total volume of 24ml. To check the transformation efficiency a serial dilution up to 10^{-7} was made from this 24ml and plated out on LB/Amp plates (ampicillin at 50μg/ml), after which 1.5ml of the remaining 24ml culture was

plated out onto each of 16 large (245 x 245 x 25mm) LB/Amp plates (ampicillin at a final concentration of 50μg/ml) and incubated overnight at 37°C.

Cells were scraped from each of the large LB/Amp plates and the harvested cells combined and added to a total of eight x 500ml of terrific broth (450ml terrific broth base, 50ml terrific broth buffer), so that every 500ml of terrific broth contained cells harvested from two plates. Each of the preparations was then shaken at 37°C, at 200rpm, for 5-6 hours until stationary phase was achieved. The 500ml cultures, in 250ml Sorvall pots, were then centrifuged at 7 000rpm for 10 minutes (using a Sorvall RC5B+ centrifuge and a Sorvall SLA 1500 GSA rotor), and pellets resuspended with 20ml TE_8 containing 5mg/ml lysozyme and incubated at room temperature for 5 minutes. Maxi-prep solution 2 (as in section 2.2.14) (40ml) was added, the bacterial suspension mixed by repeated inversion and incubated for 5 minutes on ice. Maxi-prep solution 3 (as in section 2.2.14) (30ml) was added, the contents mixed by inversion and incubated for 15 minutes on ice. Preparations were centrifuged at 15 000 rpm for 10 minutes (using the same rotor and centrifuge as before), the supernatant decanted into a clean pot through a layer of Miracloth (Calbiochem) and 80ml of isopropanol added. After being left at room temperature for 20 minutes, the DNA was pelleted by being centrifuged at 15 000rpm for 10 minutes (as before). Supernatants were then discarded and the resulting pellets washed with 70% ethanol. The final DNA pellet was re-dissolved in 8ml of TE_8.

The rest of the protocol followed was identical to that of the maxi-prep of plasmid DNA, as previously described in section 2.2.14, from the set up of CsCl gradients onwards.

2.4.2 Preparation of Competent Yeast Cells and Small-Scale Transformation

A culture of either Y190 or AH109 yeast cells was set up using 3-5 single colonies to inoculate 50ml YEPD broth. The culture was vortexed briefly to disperse any clumps and incubated overnight at 30°C. The next morning the OD_{600} of the yeast culture was measured and the culture diluted to produce an OD_{600} of 0.2 in 300ml YEPD broth. The culture was incubated at 30°C, on a shaker set at 250rpm, for 3 hours. Cells were centrifuged at 1 800rpm for 5 minutes (using an IEC Centra GP8R centrifuge with a 218 rotor), the supernatant discarded and the cells resuspended in 25ml sterile DDW. The cells were re-centrifuged and resuspended in 1.5ml 1X TE/LiAc (0.1M lithium acetate (LiAc), 10mM Tris pH 7.5, 1mM EDTA pH 8.0) and kept on ice until required. Prior to use, carrier DNA (sheared salmon sperm DNA (GIBCO)) was boiled for 7 minutes and chilled on ice for 2 minutes. For each transformation, 200ng of each of the required plasmid DNAs and 0.1mg carrier DNA were placed in a microfuge tube. One hundred µl of the TE/LiAc/cell mix were added to each tube and mixed well with the DNA by vortexing. Sterile LiPEG (0.1M LiAc, 40% PEG_{3350}) (600µl) was then added to each tube and vortexed at high speed to mix. Each tube was incubated at 30°C, on a shaker set at 250rpm, for 30 minutes. To each tube, 70µl of sterile DMSO was added and the contents mixed by gently swirling the tube. The samples were heat shocked for 15 minutes at 42°C and kept on ice for 2 minutes. After chilling, samples were microfuged for 5 seconds at 14 000rpm and the supernatant removed. Cells were re-suspended in 250µl 1X TE_8 and the total volume was plated on the relevant type of synthetic complete (SC) selection plate and incubated at 30°C.

For selection of double transformants, cells were plated out on synthetic complete plates lacking leucine and tryptophan (SC-L-T plates). For selection of cells positive for the *ADE2* gene product (adenine biosynthesis), on SC plates lacking leucine, tryptophan and adenine (SC-L-T-Ade), and for selection of cells positive for the *HIS3* gene product (histidine biosynthesis), cells were plated out on SC

plates lacking leucine, tryptophan, histidine, and containing 15mM 3-AT (3-amino-1, 2, 4- triazole) (SC-L-T-H+15mM 3-AT). 3-AT is used to counteract leaky expression from the *HIS3* reporter gene by competitively inhibiting the *HIS3* gene product. The amount of 3-AT used is dependent upon both the strain of yeast used and the bait protein used. The lowest possible concentration of 3-AT was used to minimise any negative effect that the 3-AT may exert on the transformation efficiency (Bai & Elledge, 1997).

2.4.3 Large-Scale Yeast Transformation for Library Screen

A 150ml culture of AH109 yeast cells in YEPD broth was set up and incubated overnight at 30°C. The next morning the OD_{600} of the yeast culture was measured. The culture was diluted to produce an OD_{600} of 0.2 in 1litre YEPD broth and incubated at 30°C, on a shaker set at 250rpm, for 3 hours. Cells were centrifuged at 1 800rpm for 5 minutes (using an IEC Centra GP8R centrifuge with a 218 rotor), the supernatant discarded and pellets resuspended in 500ml sterile DDW. The cells were re-centrifuged and resuspended in 8ml sterile 1X TE/LiAc and kept on ice until required. For each transformation, 1mg bait plasmid DNA (pAS2/L), 500µg library plasmid DNA and 20mg sheared salmon sperm DNA (GIBCO) were placed into a sterile 500ml conical flask (prior to use, the carrier DNA was boiled for 7 minutes and then chilled on ice for 2 minutes). To this flask, 8ml of the TE/LiAC/cell mix were added and the contents mixed well by vortexing. Sterile LiPEG (60ml) was then added to the flask and the contents vortexed at high speed to mix. The flask was incubated at 30°C, on a shaker set at 250rpm, for 30 minutes. After this incubation period, 7ml of sterile DMSO were added to the flask and the contents mixed by gentle swirling. The contents were then divided into 2 sterile 50ml Falcon tubes, heat shocked for 15 minutes at 42°C and chilled on ice for 2 minutes. During the heat shock period, the tubes were mixed occasionally by gentle swirling. Samples were centrifuged at room temperature for 5 minutes at 1 800rpm (as before) and the supernatant removed. Cells were re-suspended in 10ml 1X TE_8.

One hundred µl of the final cell suspension were used for a serial dilution (10^{-1} – 10^{-6}), with which to determine the transformation efficiency, to be calculated on day five. A 100µl aliquot of each dilution was plated out on small SC-T-L selection plates and incubated at 30°C. Of the remaining cell suspension, 1ml was plated out onto each of 10 large SC-T-L-H+15mM 3-AT bio-assay selection plates and incubated at 30°C for a period of 10 days.

2.4.4 Beta-Galactosidase Assay

Colonies resulting from co-transformations plated out on SC-L-T plates were picked, resuspended in DDW and used to inoculate SC-L-T grids. SC-L-T grids were SC-L-T plates with a grid drawn on the bottom of the plate. This enabled a large number of colonies to be cultured and assayed at any one time. Grids were incubated overnight at 30°C.

The following morning, colonies were adsorbed to Hybond-C Extra nitrocellulose membrane (Amersham) by placing the membrane on top of the colonies, and then lysed by placing the membranes colony-side up into a liquid nitrogen bath for a period of 30 seconds. A piece of filter paper (Whatman 3MM) was pre-soaked in developing solution (3ml buffer Z (60mM Na_2HPO_4, 40mM Na_2PO_4, 10mM KCl, 1mM $MgSO_4$), 8.1µl 2-mercapto-ethanol and 30µl 100mg/ml X-gal stock solution). The frozen membrane was then placed on top of the pre-soaked filter paper, colony side up, in a petri dish and allowed to thaw at room temperature. The lid was then placed on top of the petri dish, which was placed in a 30°C incubator. The membrane was checked every hour until blue colour had developed on the positive controls.

2.4.5 Isolation of Yeast Plasmid DNA Using Glass Beads and Phenol-Chloroform

SC-L cultures (5ml) were set up from positive colonies from the library screen and incubated at 30°C, on a shaker set at 250rpm, for 3-5 days. One and a half ml of culture were transferred to a microfuge tube and centrifuged at 14 000rpm for 1 minute. The supernatant was removed and the cell pellet resuspended in the residual liquid by vortexing. To each tube, 200µl of yeast lysis solution (2% Triton X-100, 0.1M NaCl, 10mM Tris-Cl pH8.0, 2.5mM EDTA pH8.0), 200µl of phenol-chloroform and 0.3g of acid-washed glass beads were added and the tubes vortexed for 2 minutes. The samples were centrifuged at 14 000rpm for 5 minutes and the aqueous phase transferred to a fresh microfuge tube. DNA was precipitated by adding 0.6 volumes of isopropanol and incubating at room temperature for 20 minutes. Samples were centrifuged at 14 000rpm for 20 minutes to collect the DNA, and the DNA pellet was washed with 70% ethanol. The final DNA pellet was left to air dry and was resuspended in 10µl of sterile DDW. Yeast DNA was stored at -20°C until required.

2.4.6 Isolation of Yeast Plasmid DNA Using "Yeastmaker" Yeast Plasmid Isolation Kit

DNA was isolated using the "Yeastmaker" yeast plasmid DNA isolation kit (Clontech) according to the manufacturer's protocol and guidelines.

Chapter Three

Investigating Protein:Protein Interactions of RPV L with Rinderpest Viral Proteins P, C and V using the Mammalian Two-Hybrid System (MTHS)

3.1 Introduction

3.1.1 The Mammalian Two-Hybrid System (MTHS)

The mammalian two-hybrid system, a recent modification of the yeast two-hybrid system, is used to assess protein:protein interactions in transiently transfected mammalian cells and, like the yeast two-hybrid system (Fields & Song, 1989), artificially mimics the natural process of transcription, building on observations that transcriptional activators can be separated into discrete regions corresponding to DNA-binding domains and transcriptional activation domains (Brent & Ptashne, 1985, Hope & Struhl, 1986). Therefore like the yeast two-hybrid system, the MTHS analyses interactions between proteins that are localised in the nucleus.

The MTHS works by expressing each of the two proteins of interest 'X' and 'Y' as fusions with a GAL4 DNA binding domain and the mammalian transcriptional activation domain of the VP16 protein from *Herpes simplex virus I.* In both types of fusion protein, there is an in-built nuclear localisation signal. The reporter vector, pG5luc, is co-transfected with both of the fusion constructs into mammalian cells and contains 5 GAL4 DNA binding sites upstream of a minimal TATA box which, in turn, is upstream of the reporter gene. If there is any interaction between 'X' and 'Y', the DNA binding domain of GAL4 and the transcriptional activation domain of VP16 will be brought into close proximity and the reporter gene will be expressed from pG5luc (see figure 3.1.1). In this case the reporter gene is that of firefly luciferase, expression of which can be easily assayed. The luciferase assay is known to be highly sensitive and thus allows analysis of weak promoters and the use of smaller amounts of DNA and

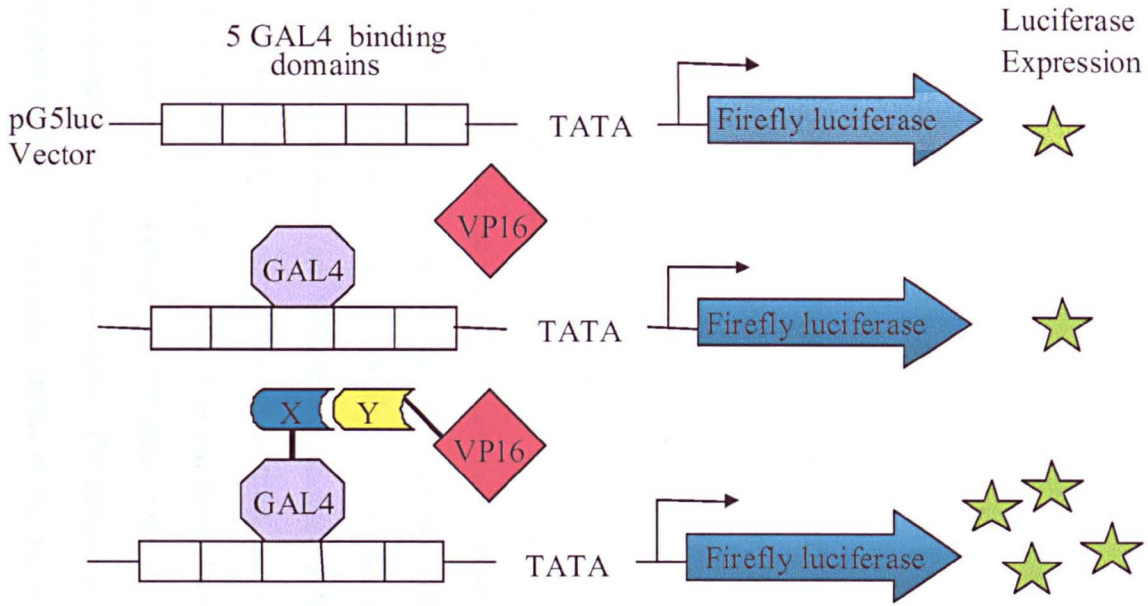

Figure 3.1.1. Schematic representation of the mammalian two-hybrid system. The pG5luc vector contains five GAL4 binding sites upstream of a minimal TATA box which, in turn, is upstream of the firefly luciferase gene. In negative controls, the background level of luciferase is measured in the presence of GAL4 (from pBIND) and VP16 (from pACT). Interaction between the two suspect proteins, as GAL4-X and VP16-Y fusion constructs, results in an increase in luciferase expression over the negative controls.

cells in transfection studies. In order to normalise transfection efficiency and minimise experimental variability, an internal control reporter gene is expressed from the pBIND vector, that of renilla luciferase. Measurements of both types of luciferase can be made in the same reaction tube.

In order to study protein:protein interactions of specific "domains" of L with P, C and V, it was initially proposed to use the Promega Checkmate mammalian two-hybrid system. This should have allowed an understanding of the way these proteins interact with each other during infection due to the fact that the proteins of interest are expressed in mammalian cells, so the intracellular environment will be more like that normally encountered by the viral proteins.

There are several possible advantages that the MTHS has over other methods which are used to detect protein:protein interactions. For example co-immunoprecipitations, GST pull-down assays and far-Western blotting all enable the assessment of the ability of one protein to interact with another; however, these methods do not show under which conditions these interactions occur and require relatively stable interactions. Weak or unstable interactions may therefore not be detected and such techniques also require additional manual processing, which would be time-consuming when compared to the MTHS luciferase assays. Although the Y2HS also enables the identification of protein:protein interactions, any interaction detected within this system must also be confirmed using at least one additional method, due to the fact that the Y2HS is known to generate false protein-binding partners. Furthermore any interactions characterised using the Y2HS are hard to quantitate, as a result of the type of reporter genes utilised within the system. The MTHS however permits the analysis of such protein:protein interactions in the more physiological context of the intact cell and allows the quantification of results obtained in the form of firefly luciferase readings, thus enabling the determination of the relative strength of protein:protein interactions investigated within the system. Another theoretical advantage of the MTHS over the Y2HS is that interactions can be confirmed rapidly, with the luciferase assays ready in minutes. The Y2HS however is a time-consuming method with which to investigate protein:protein interactions

due to the nature of the yeast cells themselves which require several days to grow up post-transformation and which then have to be re-cultured under the relevant selective conditions for the reporter gene being used.

The MTHS was thought to be suitable for this part of my PhD project as the physical interaction of native L with P, C and V was already proven (Sweetman *et al.*, 2001). A rapid system was therefore required with which to map the interacting domains of L with P, C and V. Since sub-domains of a protein may interact with binding partners more weakly than the whole protein, a sensitive system such as the MTHS was an ideal choice.

3.2 Results

3.2.1 Preparation of MTHS Constructs

PCR amplification using a proof-reading polymerase with mutagenic primers, using specific restriction sites (see appendix 3) generated the appropriate coding regions from existing laboratory plasmids, enabling production of protein encoding constructs (pACT and pBIND). The N coding region was obtained from pKSN (Baron & Barrett, 1997), the P and C coding regions from pKSP (Baron & Barrett, 1997) and the coding region for V from pKSV (pBluescript KS(+) containing a cDNA of V-type mRNA). The ORFs for the N, P, C and V proteins were all cloned into both pACT and pBIND vectors (*Mlu*I/*Not*I cut) and the 5' sequences at the fusion protein joins confirmed. The production of these constructs was fairly easy, but this was not true of the two constructs containing L. Due to its large size, it was difficult to produce a large yield of L PCR product, despite adaptations to the PCR program. Eventually L was removed from an existing laboratory plasmid, pGempol (Baron & Barrett, 1997), by designing a set of overlapping linker primers which created a new *Sal*I site just upstream of L. This allowed the L ORF to be excised as a *Sal*I- *Eag*I fragment and should have enabled easy ligation into the pACT and pBIND vectors (*Sal*I/

*Not*I cut). However before this was possible, problems became apparent with this system (as described in sections 3.2.3 and 3.2.4).

3.2.2 Investigating Known RPV Protein:Protein Interactions Using the MTHS

Experiments could now be undertaken to reconfirm findings of (Sweetman *et al.*, 2001) with initial experiments focusing on N-N and N-P interactions in order to get the system established. Transfections using Vero cells were set up using 1μg of each plasmid DNA per 35mm well, as in section 2.2.16.1. The cells were then incubated at 37°C for two days and luciferase measurements taken (as described in section 2.3.7). Renilla luciferase measurements were used to normalise transfection efficiency.

Two positive control vectors, pBIND-Id and pACT-MyoD, containing GAL4:Id and VP16:MyoD respectively, are fusion proteins known to interact with each other and were used as a positive control. To enable background levels of firefly luciferase to be determined, pBIND and pACT vectors were co-transfected.

N-N and N-P interactions were investigated, and each of the plasmid constructs tested was also co-transfected with its relevant negative counterpart. For example for N-N interactions, 3 reactions would be set up: pACTN co-transfected with pBINDN, pACTN co- transfected with pBIND and pBINDN co-transfected with pACT, thus showing that any increase in firefly luciferase expression was as a result of genuine interactions between fusion proteins and not the insertion of a particular coding region into pACT or pBIND. Despite the fact that both the positive and negative controls worked well, no interaction between N and P was observed. There was also poor reproducibility of transfection efficiency between the various reactions (data not shown).

To assess control parameters, transfections were set up which examined concentrations of the reporter plasmid pG5luc at 0.35μg, 0.7μg and 1.05μg

amounts of plasmid DNA with the two positive control vectors, pACTmyoD and pBINDId. No significant difference was observed in the results, thus concluding that the amount of reporter gene used is not a limiting factor of luciferase expression (data not shown).

After optimisation, it was decided that 0.35μg of pG5luc would be used and 0.4μg of each of the two plasmids being tested for interactions would be used. This yielded similar results to those of previous experiments. A strong positive control was obtained, but no N-N, N-P, or N-V interactions were seen, all of which are known rinderpest protein interactions and the N-N and N-P interactions are required for viral nucleocapsid formation (figure 3.2.2.1). The renilla luciferase measurements showed that the transfections had worked in each case, although there was still considerable variability in what should have been a more or less constant value.

The amount of pG5luc used in transfections was increased to 1μg and in addition, a new molar ratio of 2:1:1 was used (pACT: pBIND:pG5luc) as described by (Fotin-Mleczek *et al.*, 2000). Despite a stronger positive control being produced (figure 3.2.2.2), it was still not possible to detect the expected N-N and N-P interactions with this system. Renilla luciferase measurements, meant to allow normalisation of transfection efficiency, were still problematic due to the amount of variability observed between pairings of constructs.

After several sets of transfections and luciferase assays, no N-N, N-P, P-N or N-V interactions were seen with the pACT and pBIND constructs, despite obtaining good interaction with the two positive control constructs, pACTmyoD and pBINDId. Rather than simply abandon this work, efforts were made to determine why the system was failing to show even known protein:protein interactions. This was in case some simple problem existed with the pACT and pBIND constructs that had been made, which could then be corrected.

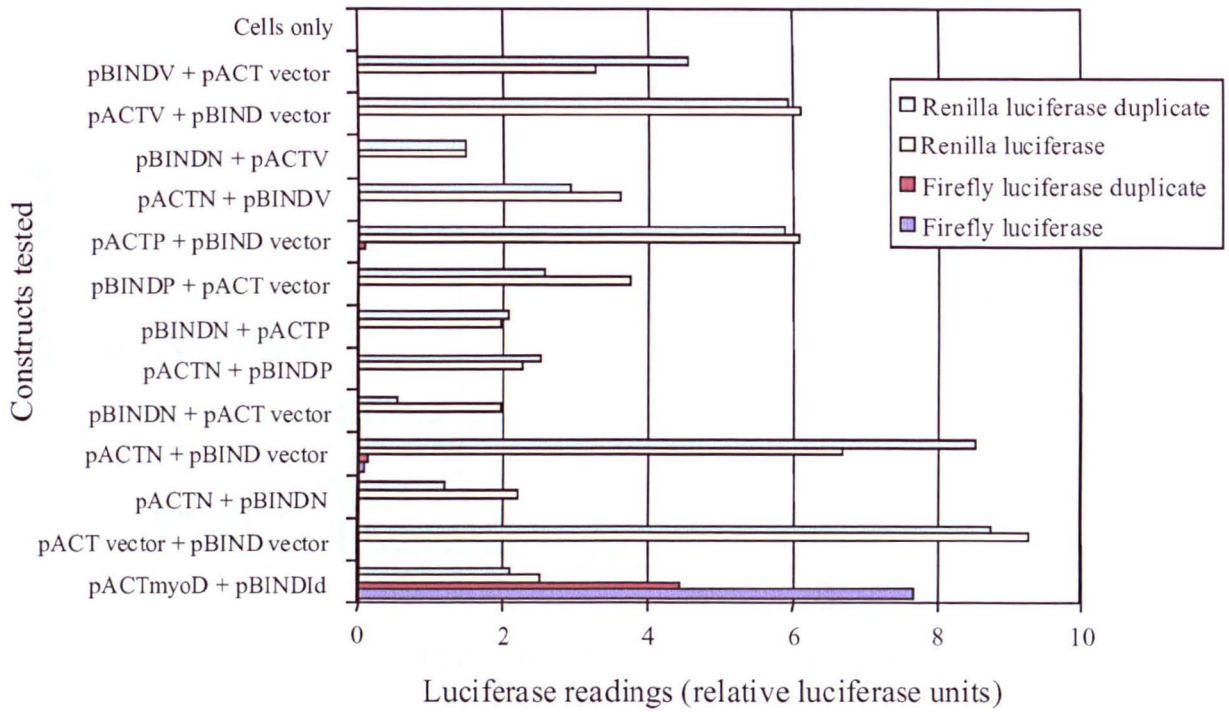

Figure 3.2.2.1. Dual-Luciferase assays for the confirmation of N-N, N-P and N-V rinderpest protein:protein interactions using the MTHS. Vero cells were transfected with 0.4µg of each of the indicated plasmids, plus 0.35µg pG5luc. Two days post transfection, cells were lysed and both firefly and renilla luciferases were assayed as described in 2.3.7. The results from duplicate transfections are shown.

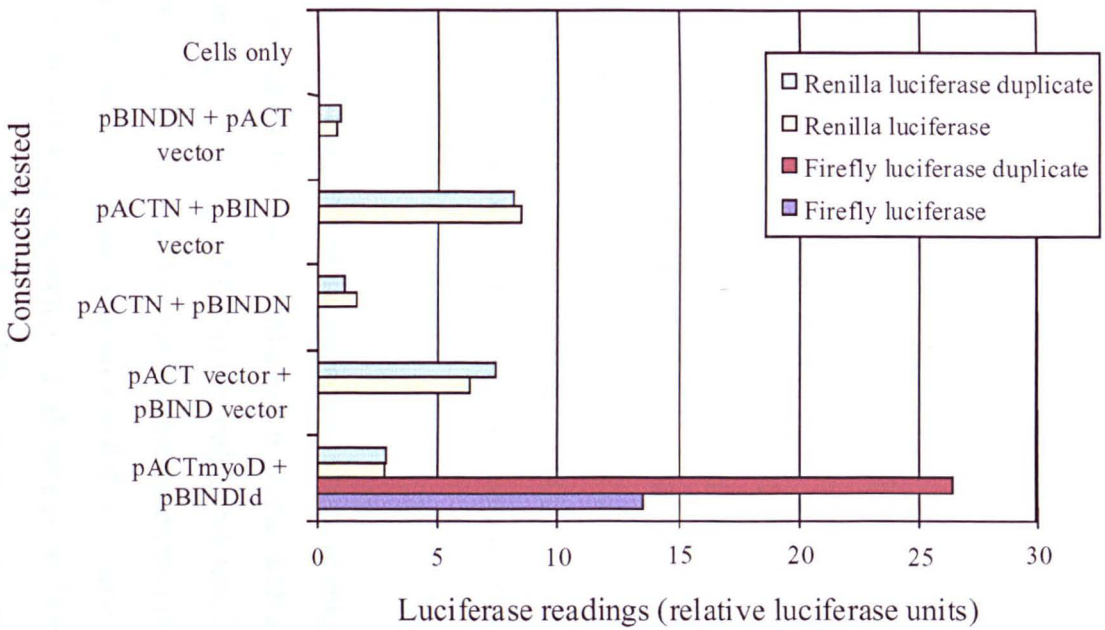

Figure 3.2.2.2. Optimisation for the detection of N-N protein:protein interactions using an increased amount of pG5luc and altered plasmid ratios. Vero cells were transfected with the indicated plasmids (at a molar ratio of 2:1:1, pACT:pBIND:pG5luc), plus 1µg pG5luc. Two days post transfection, cells were lysed and both firefly and renilla luciferases were assayed as described in 2.3.7. The results from duplicate transfections are shown.

3.2.3 Expression of Fusion Proteins from MTHS Constructs

There are several possible reasons for the MTHS constructs appearing not to interact as expected. It was possible that, although the sequence at the fusions of the GAL4 DNA binding domain and the VP16 transcription activation domain to the RPV sequences had been checked, that there was a problem with the expression of one or more of the constructs. In order to try and eliminate this possible cause of failure, it was decided to assess whether the expected proteins were being expressed from the MTHS constructs.

Constructs of interest were transfected into Cos-1 cells (as in section 2.2.16.1), cells were metabolically labelled with [^{35}S] amino acids and cell lysates immunoextracted using the appropriate rabbit polyclonal antibody (as described, 2.3.4). MB2 was raised against a mixture of peptides covering the carboxy-terminal 100 amino acids of the RPV N protein, MB18 against a peptide representing the carboxy-terminal 15 amino acids of the RPV P protein, while MB38 was raised against a bacterially expressed GST fusion protein containing the whole of the RPV C protein. The samples were run on a 12% acrylamide Laemmli gel (figure 3.2.3.1).

The expected sizes of the protein fusion constructs were calculated and not all matched the size of the proteins pulled down with the antibody used. ACTN (the fusion protein expressed from pACTN) was calculated to migrate at a size of 67.2 kDa, but was shown instead to have an increased mobility, migrating further distance than the 66 kDa marker, at approximately the same size as the BINDN fusion. Yet the BINDN fusion, with a calculated size of 74 kDa, was expected to migrate even less distance than the 66 kDa marker. In addition ACTC was expected to migrate at a size of 29 kDa, but was actually shown to migrate with a reduced mobility when compared to the 30 kDa marker. BINDC was expected to run at an approximate size of 35.5 kDa and this does look like the case on the SDS-PAGE gel (figure 3.2.3.1). ACTP was calculated to migrate at an approximate size of 65 kDa, whilst BINDP was expected to show a size of

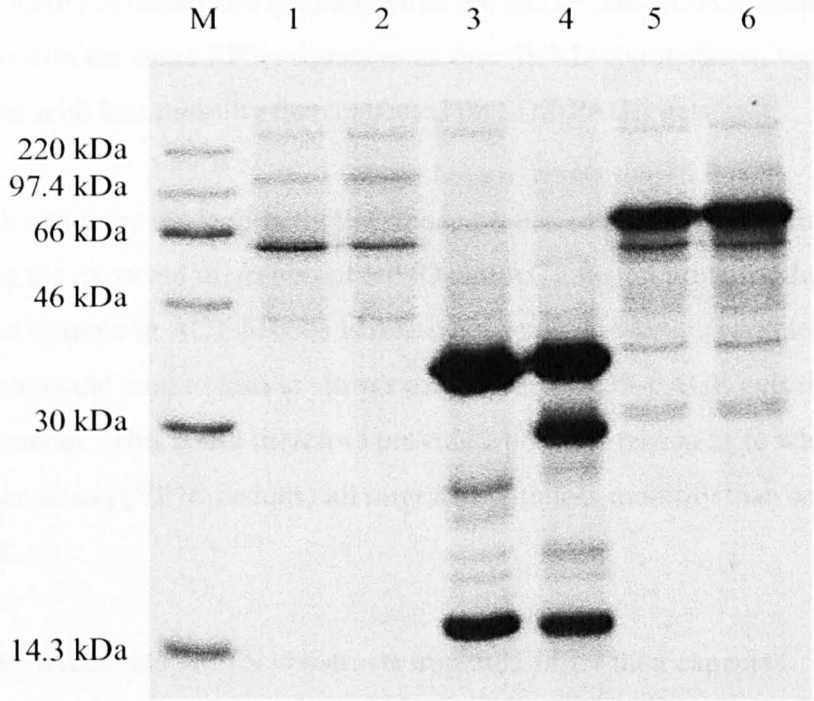

Figure 3.2.3.1. Immunoextraction of N, P and C fusion proteins from Cos-1 cells transfected with mammalian two hybrid constructs. Cells were transfected with 5µg of the relevant MTHS construct; two days post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with 1µl of the appropriate rabbit polyclonal antibody and immunoprecipitated proteins were analysed on a 12% acrylamide Laemmli gel alongside [^{14}C] labelled molecular weight markers (M). The size of each expected fusion protein is given in brackets. Track 1= pACTN (67.2 kDa), 2= pBINDN (73.8 kDa), 3= pACTC (28.9 kDa), 4= pBINDC (35.5 kDa), 5= pACTP (65.3 kDa) and 6= pBINDP (71.9 kDa). ACTN and BINDN were immunoextracted with MB2; ACTP and BINDP with MB18; ACTC and BINDC with MB38.

approximately 72 kDa, both fusions however migrated at the same size, just above the 66 kDa molecular weight marker.

From these observations it would appear that the BINDP and BINDC fusion proteins were expressed as expected, while the ACTP and ACTC constructs, which contain the same RPV sequences as their BIND counterparts, were shown to migrate with less mobility than expected on SDS-PAGE gels.

Although enquiries made directly to Promega failed to provide any information regarding the expected migration of BIND and ACT fusion proteins, the VP16 activation domain in ACT fusions is relatively acidic (14 acidic residues out of 46) which would tend to lead to slower migration on SDS-PAGE gels than for a neutral protein. This could therefore provide a possible reason as to why the pACT constructs (VP16 fusions) all migrated with less mobility than was expected.

Both the BINDN and ACTN constructs migrated faster than expected. DNA sequencing showed that the amino-terminus of the N ORFs in each construct was correct, and correctly joined to the ACT and BIND components. In addition, since the proteins are precipitated by MB2, they must contain at least part of the carboxy-terminal 20% of the N protein. The fusion proteins were therefore thought to be either truncated at their extreme carboxy-terminus (due to cellular proteases, or PCR error) or to have an internal deletion (due to a PCR error). While PCR error was unlikely based on previous laboratory experience with the *Pfu* proof-reading polymerase, it was only possible to eliminate this option by sequencing these constructs in their entirety.

The N ORF of the pBINDN construct was therefore sequenced and found to be correct (data not shown). There were therefore no mutations present due to PCR error, neither were there any internal deletions within the N ORF itself. The anomalous migration of these fusion proteins is thus not due to an error with the construct itself. Only the pBINDN construct was sequenced due to the fact that the fusion protein expressed from this construct migrated the furthest away from

its expected size, when compared to pACTN. BINDN should have migrated at 73.8 kDa and ACTN was expected to migrate at 67.2 kDa, however as previously mentioned both fusion proteins migrated faster than the 66 kDa marker.

In addition, the possibility of degradation by cellular proteases was tested by expressing the proteins *in vitro,* using a coupled transcription/translation system, since the pACT and pBIND plasmids have T7 RNA polymerase promoter sequences upstream of the fusion proteins, no difference in the size of fusion proteins expressed from any of the MTHS constructs was detected (data not shown).

Since there did not seem to be any errors in the sequence of the N plasmids, or a problem with either degradation or mutation of the expressed proteins, the anomalous migration of BINDN and ACTN must be due to some property of the N protein sequence.

3.2.4 Detection of the Cellular Distribution of MTHS Fusion Proteins by Immunofluorescence

From the immunoprecipitation data, it was clear that all the constructs were expressing fusion proteins of at least approximately the expected size. It was therefore necessary to determine whether or not these fusion proteins were in fact being localised to the nucleus as is a requirement for the functionality of the MTHS. This is due to the fact that the MTHS, like the YTHS, requires nuclear localisation of protein constructs in order for the reporter gene to be expressed. It could be that this was not happening, or was inefficient with these MTHS constructs. It is important to note that each of the two fusion proteins for the MTHS possess inbuilt nuclear localisation signals (NLS); ACT has the simian virus 40 (SV40) large T antigen nuclear targeting sequence fused downstream of the VP16 domain (Promega) and BIND (the GAL4 DNA binding domain) has been found to possess a NLS 74 amino acids in length (Chan *et al.*, 1998).

Another possible explanation could be that steric hindrance might play a role in preventing interactions between these viral proteins.

Immunofluorescence of the MTHS constructs was used as a quick method to determine where the proteins localise once made in the cell. MTHS constructs were transfected (as in 2.2.16.1), using 5µg of plasmid DNA, into Cos-1 cells growing on glass coverslips in 35mm wells, incubated for 2 days and the cells fixed and then treated with the appropriate antibodies at the current working dilution (section 2.3.1). Cos-1 cells were used in place of Vero cells as it was thought that these would be good for immunofluorescence studies and that increased levels of protein expression would be seen in the transfected cells.

This experiment yielded interesting data. Both pACTC and pBINDC (figure 3.2.4.1) showed good expression of C fusions and, more importantly, images showed clear nuclear localisation of the C protein fusions. Whilst C was distributed to a certain extent throughout the cell, it was definitely concentrated within the nucleus, as it should be. pACTP and pBINDP (figure 3.2.4.2) produced P fusion proteins which were found mainly in the host cell cytoplasm, with possibly a low level of expression in the nucleus which was just detectable over background staining of untransfected cells. This would require confocal microscopy to confirm, as it might just be protein in the cytoplasm above or below the nucleus.

Constructs pACTV and pBINDV produced differing results (figure 3.2.4.3). Whilst ACTV was found in the majority of transfected cells to be localised in the cytoplasm of the cell, BINDV was distributed throughout the cell and in some cases was found to be more concentrated in the nucleus than in the cell cytoplasm.

ACTN and BINDN (figure 3.2.4.4), like the P protein fusions, appeared to be excluded from the host cells' nuclei. In only one pACTN transfected cell, was there a slight speck of N protein within the nucleus of a cell and this was observed on the confocal microscope, so it can be said with certainty that the

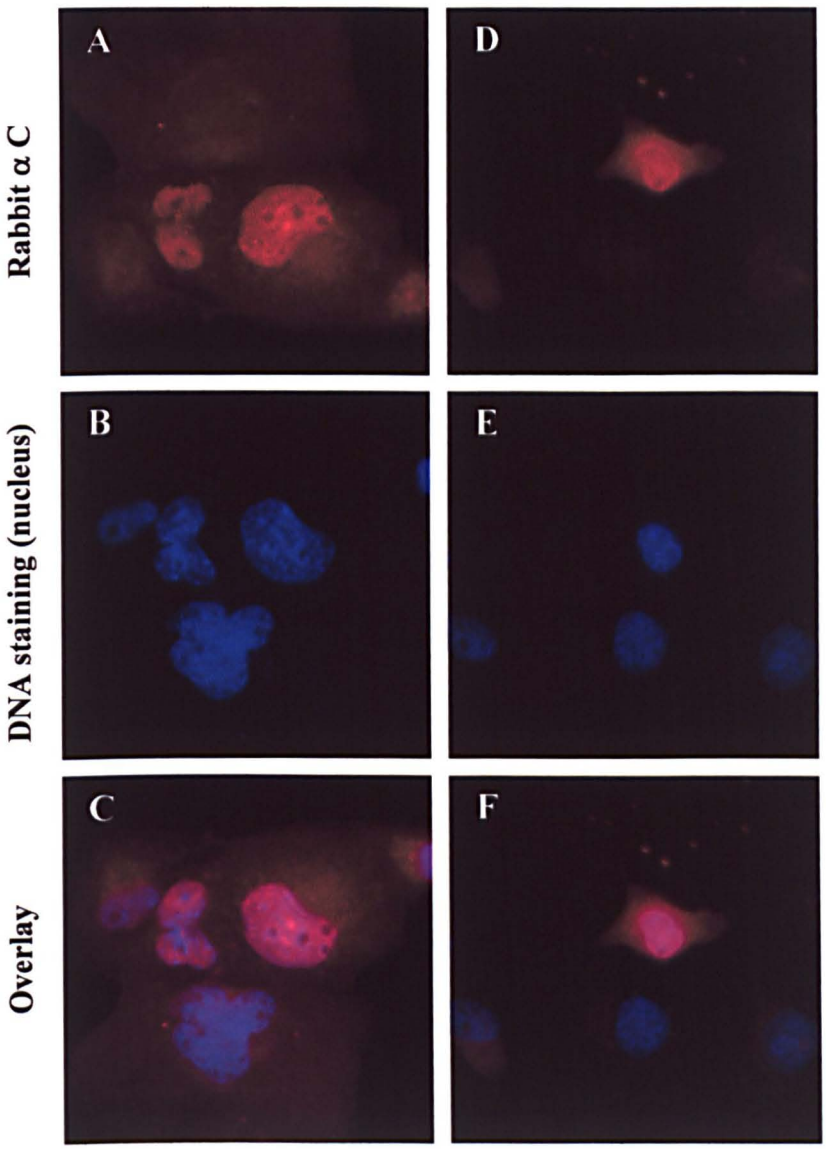

Figure 3.2.4.1 Immunofluorescence microscopy of cells transfected with MTHS C constructs. Cos-1 cells were transfected with pACTC (A, B, C) or pBINDC (D, E, F). Two days post transfection, cells were fixed, and permeabilised with 0.5% Triton X-100 before staining with antibody. Cells were reacted with rabbit α -RPVC (MB38, at 1/400), and the primary antibody visualised with Texas Red α -rabbit IgG (at 1/200, A & D). B and E show nuclei of cells counter-stained using Hoechst 33258. Overlay of the two stains is shown in C and F.

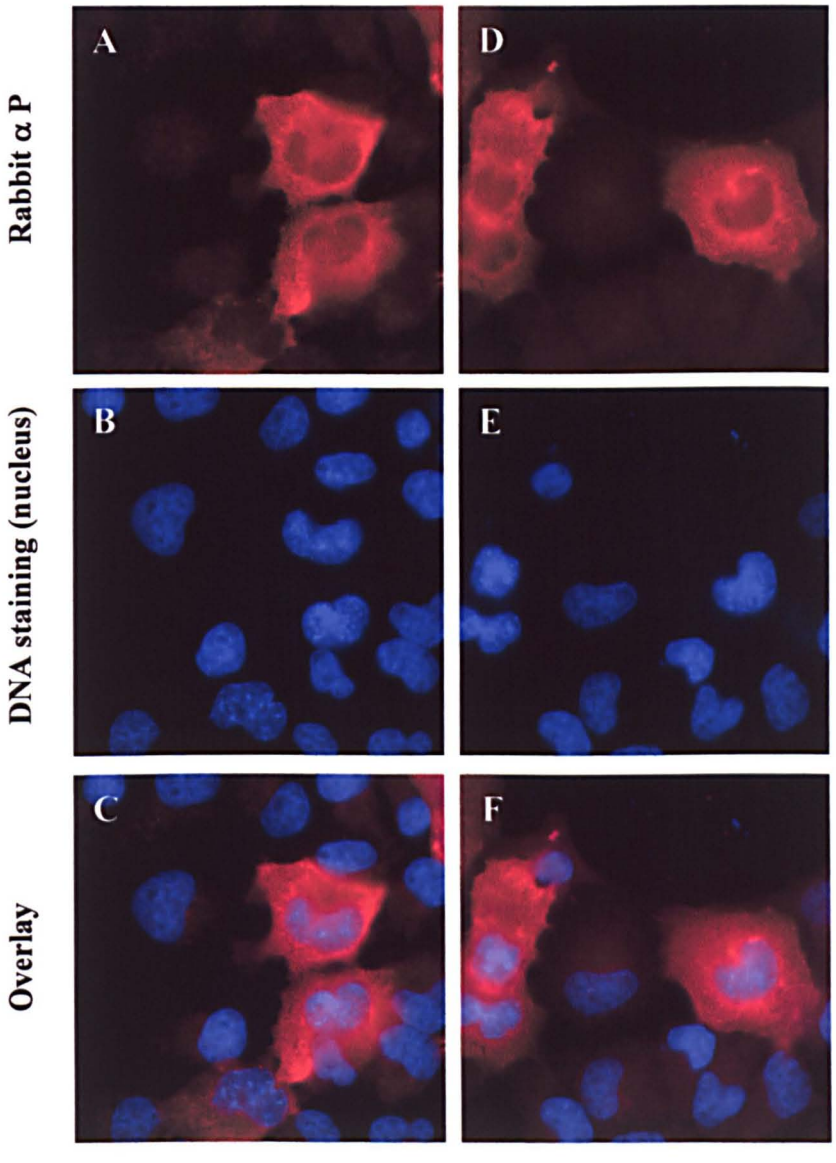

Figure 3.2.4.2 Immunofluorescence microscopy of cells transfected with MTHS P constructs. Cos-1 cells were transfected with pACTP (A, B, C) or pBINDP (D, E, F). Two days post transfection, cells were fixed, and permeabilised with 0.5% Triton X-100 before staining with antibody. Cells were reacted with rabbit α -RPVP (MB18, at 1/300), and the primary antibody visualised with Texas Red α-rabbit IgG (at 1/200, A & D). B and E show nuclei of cells counter-stained using Hoechst 33258. Overlay of the two stains is shown in C and F.

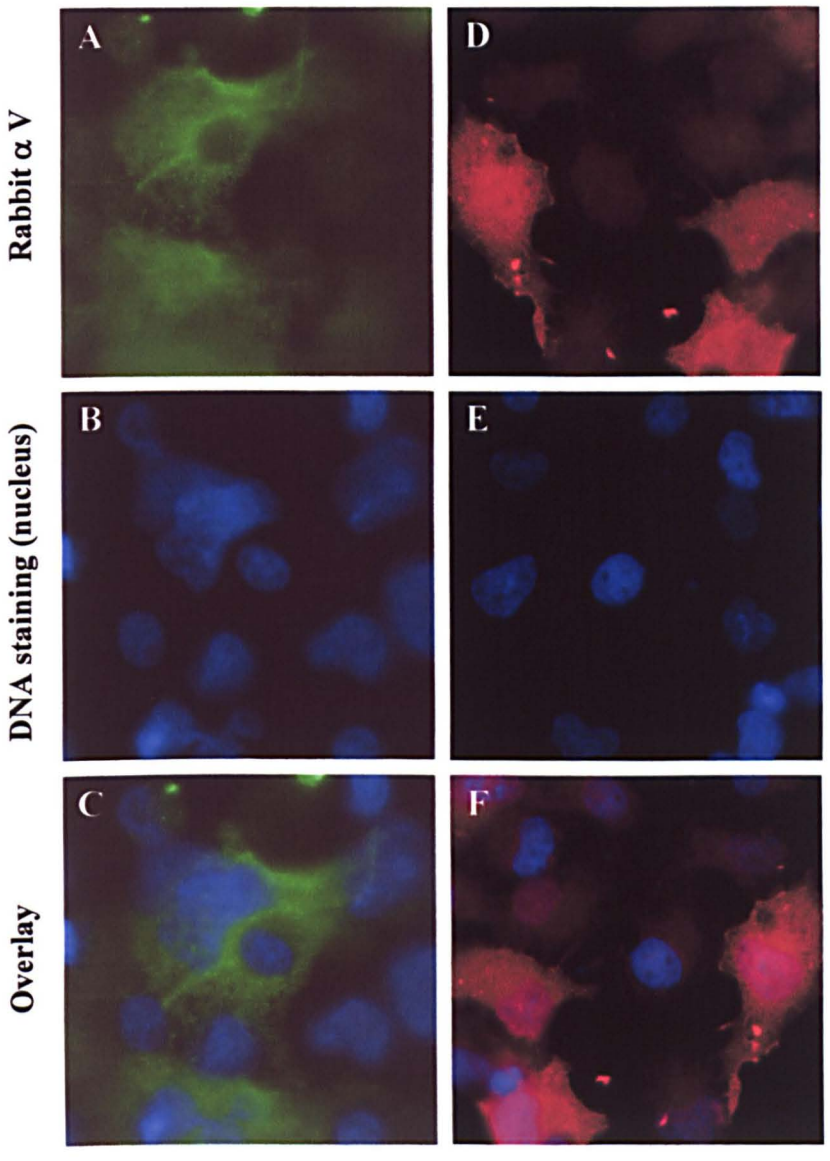

Figure 3.2.4.3 Immunofluorescence microscopy of cells transfected with MTHS V constructs. Cos-1 cells were transfected with pACTV (A, B, C) or pBINDV (D, E, F). Two days post transfection, cells were fixed, and permeabilised with 0.5% Triton X-100 before staining with antibody. Cells were reacted with rabbit α -RPV V (α -V_2, at 1/600), and the primary antibody visualised with FITC α -rabbit IgG (at 1/220, pACTV) (A) or Texas Red α -rabbit IgG (at 1/200, pBINDV) (D). B and E show nuclei of cells counter-stained using Hoechst 33258. Overlay of the two stains used is shown in C and F.

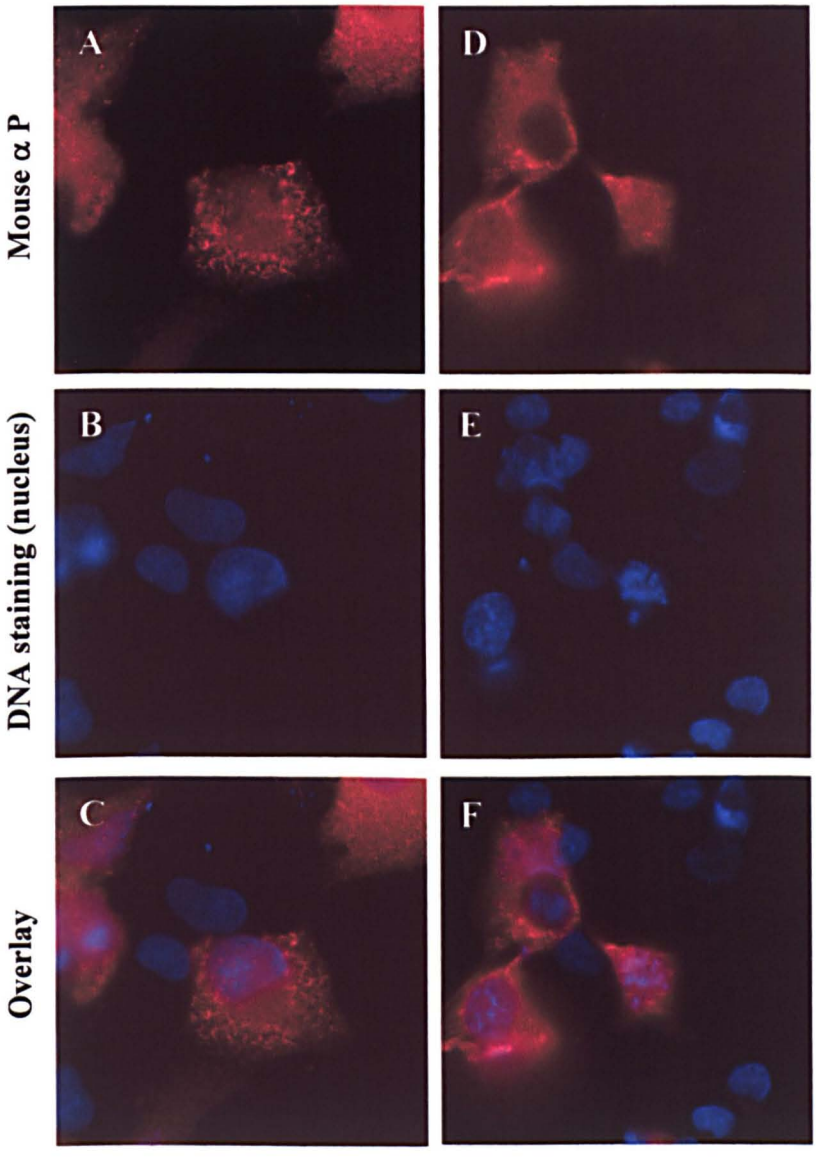

Figure 3.2.4.4 Immunofluorescence microscopy of cells transfected with MTHS N constructs. Cos-1 cells were transfected with pACTN (A, B, C) or pBINDN (D, E, F). Two days post transfection, cells were fixed, and permeabilised with 0.5% Triton X-100 before staining with antibody. Cells were reacted with mouse α-RPVN (C39, at 1/50), and the primary antibody visualised with Texas Red α - mouse IgG (at 1/200, A & D). B and E show nuclei of cells counter stained using Hoechst 33258. Overlay of the two stains is shown in C and F.

protein was in the nucleus, though in extremely limited quantities. Further, the N protein which was found within the cytoplasm of transfected cells had formed aggregates which were "worm-like" in appearance. Thus, it would appear that this aggregation somehow overrides the nuclear localisation signals, as the small quantity of N found within the nucleus of that one cell did not appear to be in an aggregate.

It is interesting to note that the expression levels of the N fusion proteins seen in the immunofluorescence images appear to be a lot higher than the amount of expression suggested by immunoprecipitation. A possible reason for this observation could be that aggregates of N seen in the cytoplasm of transfected cells, by immunofluorescence, could somehow block the binding domain of the anti-N antibody used for the immunoprecipitation work. It is also possible that aggregates of N, due to their presumably large size, are pelleted with cell debris from lysates prior to being immunoextracted. It is therefore also possible that, during immunoprecipitation, full-length N fusions were not being immunoextracted.

3.2.5 Investigation of RPV C-C Interaction

As the two C fusion proteins were shown to localise to the nuclei of transfected cells, experiments could be performed using these constructs. Earlier work has shown that RPV C self-associates (in the Y2HS) and forms high molecular weight aggregates even in the absence of other viral RPV proteins (Sweetman *et al.*, 2001). On the other hand, GST-C failed to bring down C when the two proteins were co-expressed (Sweetman, 2000). As this interaction had already been seen in infected mammalian cells (Sweetman *et al.*, 2001), the MTHS was used to potentially further characterise this interaction.

Transfections (2.2.16.1) were performed using Cos-1 cells and the previously used 2:1:1 ratio of pACT: pG5luc: pBIND was used, plus pG5luc (0.8µg) per 35mm well. Transfast (6µl) was used per µg plasmid DNA to be transfected. The

cells were then incubated at 37°C for two days and luciferase measurements taken (as described in section 2.3.7). As previously described, Renilla luciferase measurements were used to normalise transfection efficiency (see figure 3.2.5.1).

Despite strong positive controls no C-C interaction was found within this system (see figure 3.2.5.2). It could be that the C-C interaction seen during previous work in the laboratory was an aggregation rather than a functional interaction. If this is the case then such aggregates may not allow the function of VP16 and GAL4, which would explain why this interaction remains undetected in the MTHS.

3.3 Discussion

The MTHS should have allowed the rapid and quantitative analysis of RPV protein:protein interactions within a cellular environment that closely mimics the native protein environment. However, this did not prove to be the case with the constructs produced for this study. To investigate possible causes for the failure of this system, the size and cellular distribution of fusion proteins was investigated. The MTHS constructs used were found to generate fusion proteins that migrated approximately as expected on SDS-PAGE gels. However, both the N fusions were found to migrate in an anomalous fashion. ACTN and BINDN, which were expected to migrate at around 67.2 kDa and 74 kDa respectively, were both found to migrate faster than the 66 kDa marker, at approximately the same size as each other. To determine if any mutations or internal deletions were present in the N ORF of the MTHS N constructs, the N ORF of the pBINDN construct was sequenced in its entirety and found to be correct. The anomalous migration of the N fusion proteins was therefore found to be not due to an error with the construct itself.

When the nuclear localisation of these constructs in transfected cells was investigated, not all the fusion proteins were found to nuclear localise. In particular, the N fusions were all cytoplasmic, and initial characterisation of the

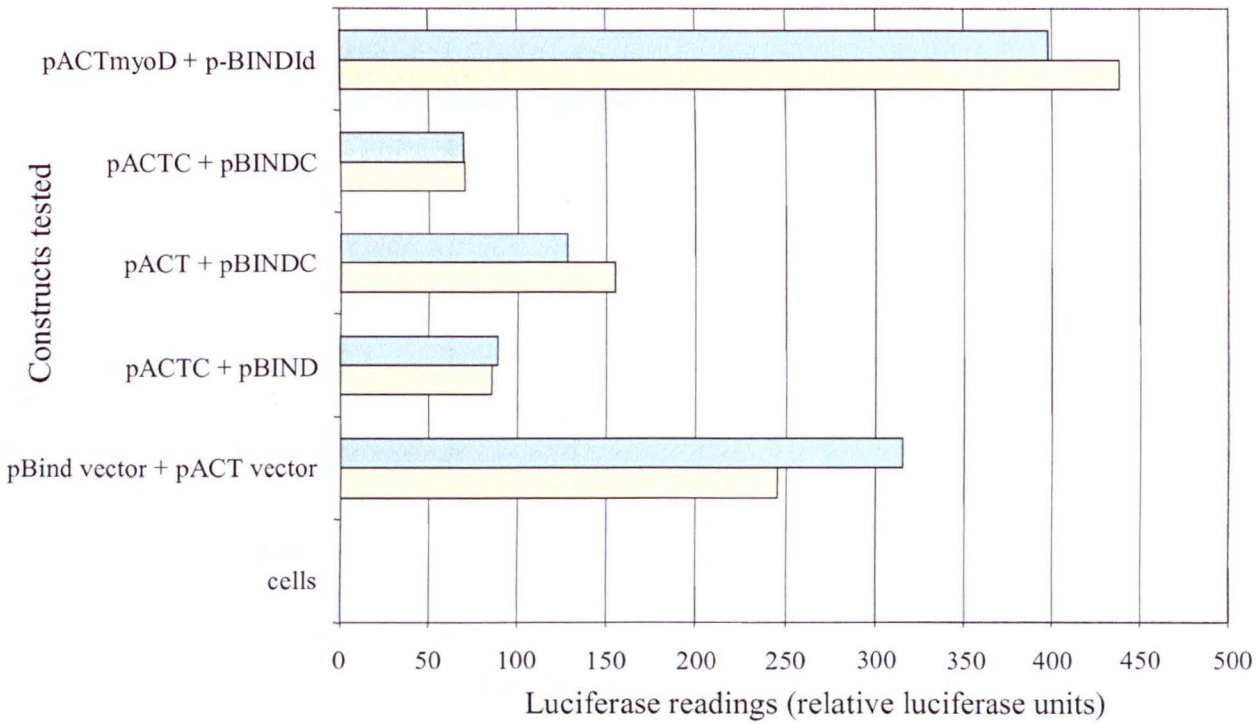

Figure 3.2.5.1. Renilla luciferase data from dual-Luciferase assays for the detection of C-C protein:protein interactions using the MTHS. Cos-1 cells were transfected with the indicated plasmids (at a molar ratio of 2:1:1, pACT:pBIND:pG5luc. Two days post transfection, cells were lysed and both firefly and renilla luciferases were assayed as described in 2.3.7. The results from duplicate transfections are shown.

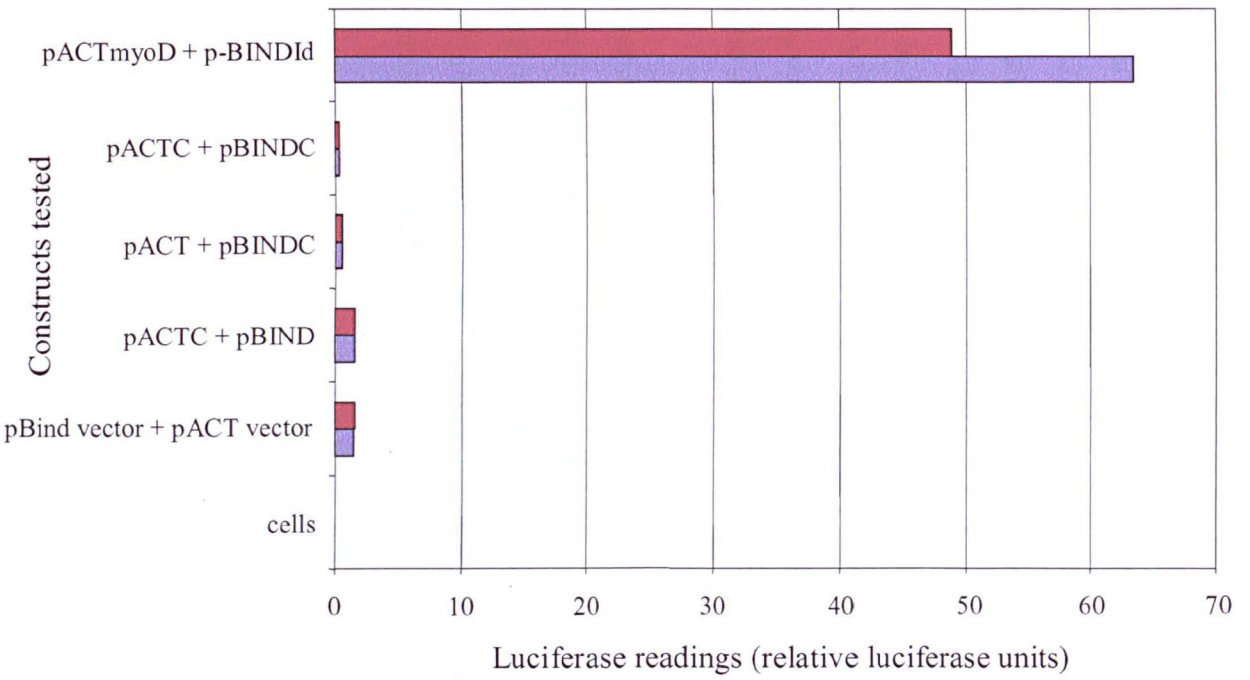

Figure 3.2.5.2. Firefly luciferase data from dual-Luciferase assays for the detection of C-C protein:protein interactions using the MTHS. Cos-1 cells were transfected with the indicated plasmids (at a molar ratio of 2:1:1, pACT:pBIND:pG5luc). Two days post transfection, cells were lysed and both firefly and renilla luciferases were assayed as described in 2.3.7. The results from duplicate transfections are shown.

system had been carried out with several N-X pairs of proteins. Although the N constructs were not necessary for this study, both the P fusions and the ACTV fusion, which were vital for this work, were found to be very inefficient in going to the nucleus of transfected cells. If the L constructs had been made and tested than it may very well have been that these constructs might also not have gone to the nucleus. This is despite the fact that each of the vector-encoded fusion proteins for the MTHS possesses in-built nuclear localisation signals. These signals must somehow be over-ridden or be inefficient with the constructs generated using the RPV coding sequences. It is interesting to note that in a recent literature search only one group has published work using the Promega Checkmate MTHS (De Jong *et al.*, 2002), which could indicate that this has not been one of Promega's more successful kits. Furthermore, in previous work using the Y2HS, the majority of the constructs containing the same RPV coding sequences were found to go to the nucleus, indicating that the Y2HS is less sensitive to difficulties in translocation than the MTHS.

After several unsuccessful attempts to confirm previously characterised rinderpest protein:protein interactions within this system, efforts were channelled towards setting up another system. To determine which conserved domain of the L protein interacts with P, C and V it was decided to next use co-immunoprecipitation studies.

Chapter Four

Investigating the Interaction of RPV L with Rinderpest Viral Proteins P, C and V using Co-Immunoprecipitation studies

4.1 Introduction

Due to the problems encountered with the MTHS, co-immunoprecipitation studies were used to evaluate the interactions of L with the viral proteins P, C and V. Even if the MTHS had worked, further investigations with another technique such as co-immunoprecipitation would have been required to confirm any preliminary data. Co-immunoprecipitations (co-ips) have been in use for over two decades and provide a direct method of establishing protein:protein interactions both *in vitro* and *in vivo*. Co-ips may be used to not only prove if a specific interaction occurs between two known proteins, but to also isolate novel protein-binding partners of a known protein of interest. In either case, the principle behind co-immunoprecipitation is the same and straightforward: an antibody for a known protein is used, resulting in the immunoprecipitation of that protein along with any associated proteins, from either virally infected cells, or cells transfected with plasmids expressing proteins of interest.

However, like all systems, there are some disadvantages to using co-ips for the analysis of protein:protein interactions, for example an interaction needs to be stable if it is to be successfully detected by co-ip. Furthermore, it is not possible to quantitate the strength of an interaction demonstrated by co-ip and another possible drawback is that the process is time consuming, with a lot of "hands on time" required following the point of transfection.

For this part of the study, constructs encoding the desired proteins were transfected into mammalian cells. The transfected cells were then radiolabelled, harvested and lysed under non-denaturing conditions, thereby preserving any protein:protein interactions which may occur within the intact cell. Using an antibody specifically raised against one of the proteins of interest, the protein is

immunoextracted from the cell lysate and analysed on an SDS-PAGE gel. Any other proteins associated with the protein of interest may be visualised on the gel, either by Western blot or, in the case of radiolabelled cells, by autoradiography (see figure 4.1.1. for a diagrammatic overview of the system).

4.2 Results

4.2.1 Preparation of Constructs for Co-Immunoprecipitation Studies

In order to perform co-immunoprecipitation studies, new constructs were made containing the desired protein ORFs, including an HA-tagged version of L (HAL), which was made due to the lack of a suitable anti-L antibody. The L protein was HA-tagged at its amino terminus, thus enabling L, and any deleted forms of L, to be recognised by commercially available monoclonal antibodies raised against the HA tag from influenza virus. The other advantage of using an epitope tag is that anti-tag antibodies should not interfere with protein:protein interactions, whereas an antibody to the protein of interest may bind to and block the binding/interaction site. The L coding region was cut from an existing laboratory plasmid, pGempol (Baron & Barrett, 1997), using *Eco*52I and then inserted into a modified pcDNA/HA plasmid (*Not*I cut). This modified plasmid was derived from pcDNA/HA/Nac, which was a kind gift from L. Goatley (Department of Molecular Biology, Institute for Animal Health, Pirbright, Surrey, UK). Prior to the insertion of RPV L, the plasmid was digested with *Eco*RI and *Xho*I to remove the unnecessary coding sequence for the Nac protein, and a *Not*I site was re-introduced using a set of specifically designed overlapping primers (Hains 1 and Hains 2, see appendix 3). It was this plasmid (pcDNA/HA/Not) which was then digested with *Not*I and into which the RPV L ORF was cloned as described above.

Constructs containing N, P, V and C were also made, each being inserted into pcDNA3.1+ (Invitrogen), a plasmid containing both the CMV and T7 promoters. It was originally intended to use solely the CMV promoter of this plasmid, as

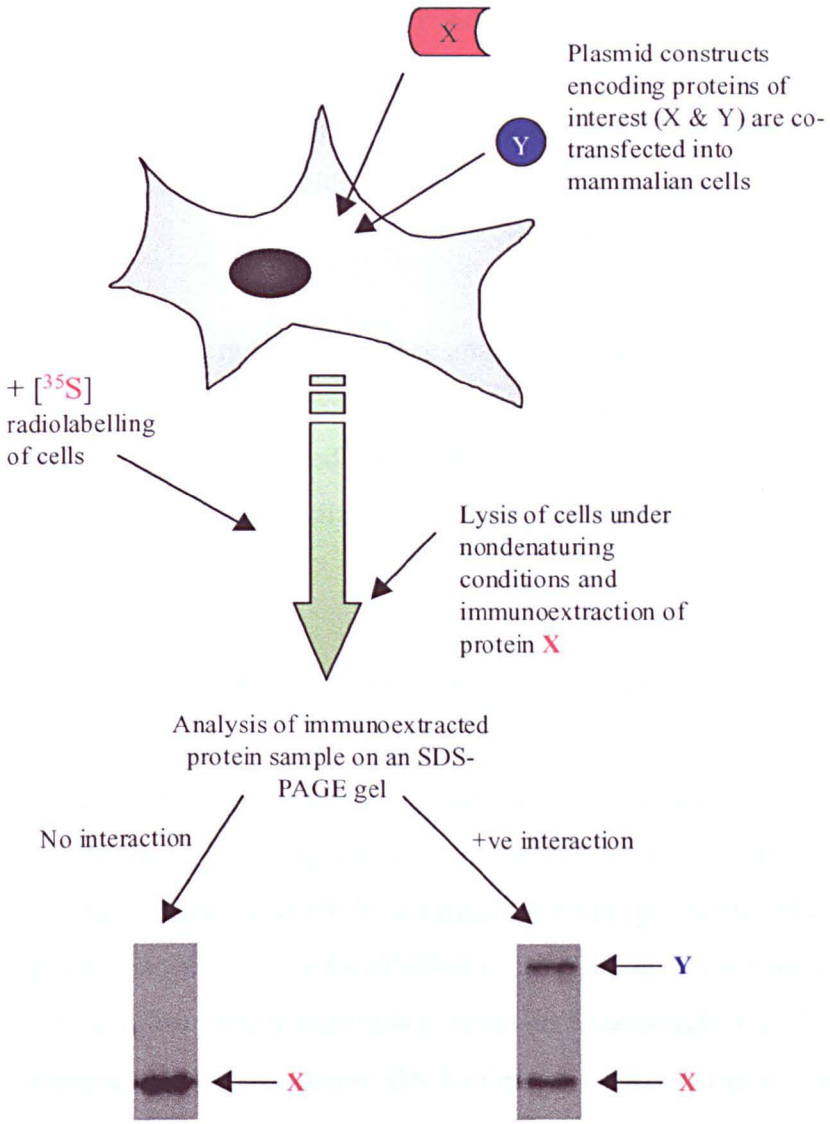

Figure 4.1.1. Diagrammatic representation of the analysis of protein:protein interactions using co-immunoprecipitation.
Mammalian cells are transfected with plasmid constructs encoding the proteins of interest. One or two days post transfection, transfected cells are labelled with [^{35}S] amino acids and lysed. Cell lysates are immunoextracted with the relevant antibody and immunoprecipitated proteins analysed on SDS-PAGE gels.

previous studies using vaccinia T7/T7 promoter-driven expression resulted in the degradation of the C protein (Sweetman *et al.*, 2001). The coding regions for C and V were obtained from existing laboratory plasmids, C from pC2C and V from pKSV, whilst the coding region for P was acquired from pKSP (Baron & Barrett, 1997), all of which were digested with *Eco*RI and then cloned into pcDNA3.1+ (*Eco*RI cut/SAP). The N coding region was obtained by digesting pKSN (Baron & Barrett, 1997) with *Not*I and was then cloned into pcDNA3.1+ (*Not*I cut/SAP).

All constructs were confirmed by sequence analysis of the 3' and 5' ends of the inserted sequence. A functional assay of HAL was not performed, as a recombinant RPV has been rescued containing an HA-tag at exactly the same location as the pcDNA/HAL construct used during these studies (M. Baron, personal communication).

4.2.2 Optimisation of Protein Expression from Constructs

Following production of these constructs, protein expression was analysed using immunoprecipitation studies. Expression of proteins from the majority of these constructs was easy to obtain when driven from the CMV promoter. However, the HA-tagged L construct took a lot of effort to get good levels of protein expression. To confirm protein expression from these constructs, Cos-1 cells were transfected using 5µg of plasmid DNA of each of the constructs of interest (as in section 2.2.16.1), radiolabelled, lysed and then immunoextracted using 1µl of the appropriate antibody (figure 4.2.2.1). Clear expression of N (expected size ~60.5 kDa), P (expected size ~59 kDa, P is known to migrate slower than expected) and C proteins (expected size ~22 kDa) was seen. However it was noticed that degradation of the C protein still occurred, despite using the CMV promoter in place of vaccinia T7/T7 promoter-driven expression. Although C should migrate at ~22 kDa, the majority of protein expressed from pcDNA/C appeared to be in a degraded form migrating at ~15 kDa.

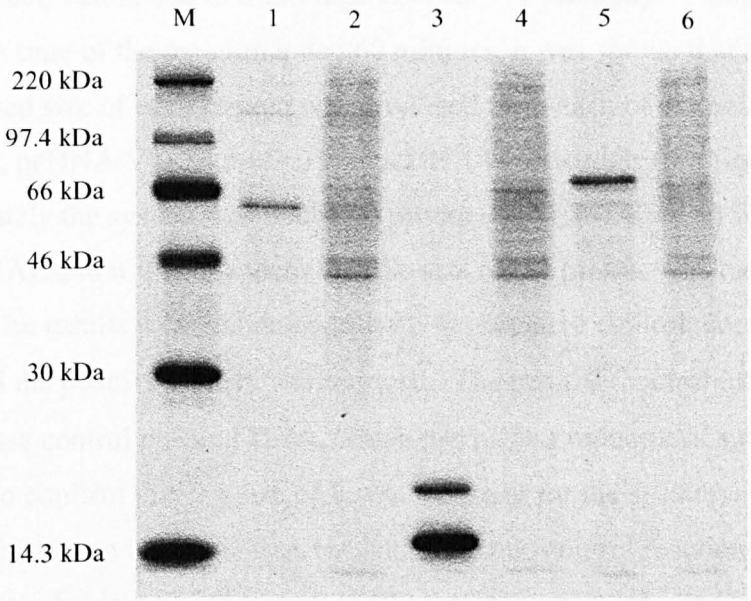

Figure 4.2.2.1. Immunoprecipitation of proteins expressed from pcDNA constructs. Cos-1 cells were transfected with pcDNA/N (1), pcDNA/C (3) or pcDNA/P (5), or left untransfected (2, 4, 6). Two days post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with MB2 (anti-N) (1&2), MB38 (anti-C) (3&4) or MB18 (anti-P) (5&6). Immunoprecipitated proteins were analysed on a 12% acrylamide Laemmli gel alongside [^{14}C] labelled molecular weight markers (M).

To further check the expression of the pcDNA constructs and to determine if co-immunoprecipitations could be performed using TNT lysates, *in vitro* transcription-translation reactions were set up. This also enabled the expression of V from pcDNA/V to be confirmed, as this construct was not tested by immunoprecipitation due to a shortage of α-RPV V antibody. Using an incubation time of the recommended 60 minutes, it was shown that a product of the expected size of each protein was produced from each of the pcDNA/C, pcDNA/P, pcDNA/V (~34.5 kDa) and pcDNA/N constructs (see figure 4.2.2.2). Unfortunately the system was unable to produce HAL (~240 kDa) from pcDNA/HAL and it would appear that the size of the protein was too great for the system. The results were validated as both the negative control, containing no DNA, and the positive control worked well. The positive control included 0.5μg of luciferase control plasmid DNA, which produces a monomeric protein 61 kDa in size. To confirm that the size of L was too great for the system, a further TNT reaction was set up using pcDNA/HAL and the two control reactions, but this time an extended incubation period of 120 minutes was used. The same result was obtained as with the 60 minute incubation period (data not shown). The TNT system was therefore not useable for co-expression/co-immunoprecipitation studies.

It was noticed when the pcDNA/P and pcDNA/V TNT reactions were analysed on Laemmli gels that they appeared to produce a protein the same size as C, in addition to P or V (see figure 4.2.2.2). Furthermore, all three plasmids (P, C or V) produced a protein larger than C (~24 kDa) (figure 4.2.2.2). To investigate whether C was being expressed from the pcDNA/P and -/V constructs, an experiment was set up to try to immunoprecipitate C from cell lysates transfected with pcDNA/P using MB38 (a rabbit polyclonal α-C antibody). A series of controls were also performed; pcDNA/N was used as a negative control to show that MB38 is specific to C, while pcDNA/C was used as a positive control (figure 4.2.2.3). As the figure shows, pcDNA/P does also produce C, and it is therefore reasonable to conclude that the small protein seen expressed from pcDNA/V by TNT was also C. The ~24 kDa protein was not seen in immunoprecipitation with

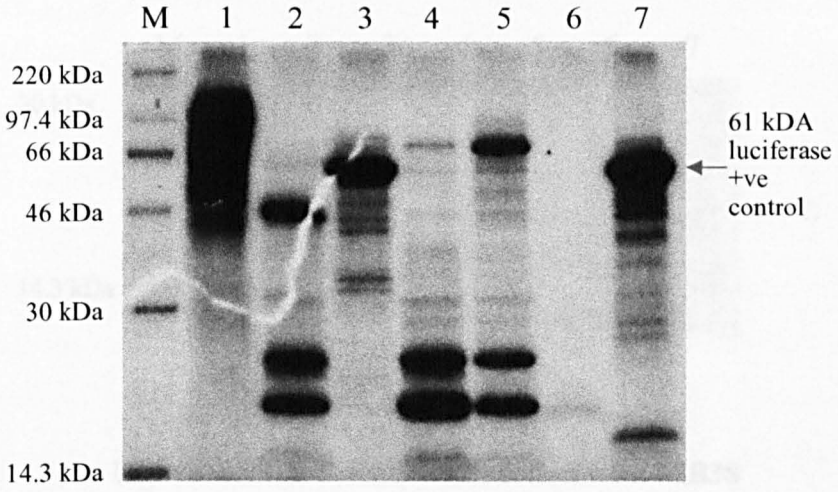

Figure 4.2.2.2. *in vitro* transcription-translation reactions of all pcDNA constructs. TNT reactions were set up using 0.5µg plasmid DNA and incubated for 60 minutes at 30°C and products analysed on a 12% acrylamide Laemmli gel with [^{14}C] labelled molecular weight markers (M). Track 1= pcDNA/HAL, track 2= pcDNA/V, track 3= pcDNA/N, track 4= pcDNA/C, track 5= pcDNA/P, track 6= negative control containing no DNA and track 7= the positive control containing luciferase control plasmid DNA.

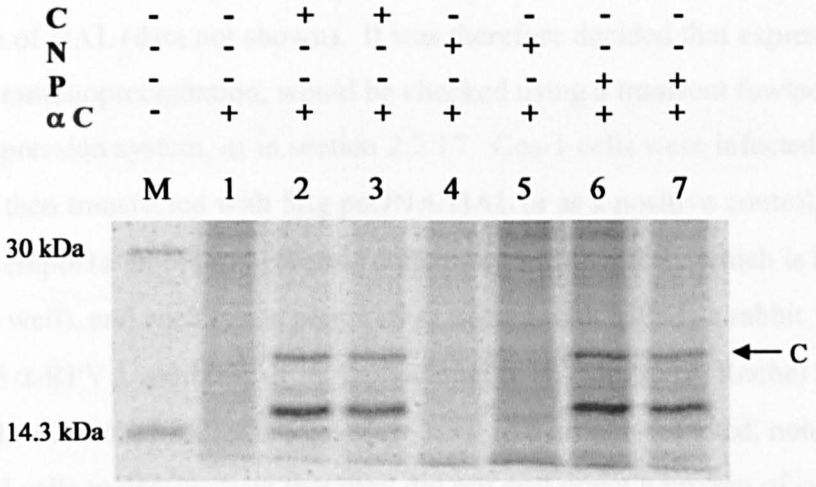

Figure 4.2.2.3. Immunoextraction with MB38 (α-C) of cell lysates transfected with either pcDNA/C, pcDNA/N or pcDNA/P constructs.
Cos-1 cells were transfected with different pcDNA constructs. Two days post infection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Lysates were then treated with rabbit polyclonal α-C (MB38) and analysed on a 12% acrylamide Laemmli gel alongside [^{14}C] labelled molecular weight markers (M). Lysate from cells transfected with nothing (1); 5μg pcDNA/C (2); 10μg pcDNA/C (3); 5μg pcDNA/N (4); 10μg pcDNA/N (5); 5μg pcDNA/P (6) and 10μg pcDNA/P (7). The position of full-length C protein is arrowed.

anti-C antibody in these or any other experiments. The origin of this protein in the TNT reactions is unclear.

Initial experiments resulted in a failure to detect HAL expression from the pcDNA/HAL construct, despite previous immunofluorescence data confirming expression of HAL (data not shown). It was therefore decided that expression of HAL, by immunoprecipitation, would be checked using a transient fowlpox T7 (FPT7) expression system, as in section 2.2.17. Cos-1 cells were infected with FPT7 and then transfected with 5μg pcDNA/HAL or as a positive control, with 5μg of pGempol (a laboratory plasmid containing full-length L, which is known to express well), and each lysate preparation treated with MB41, a rabbit polyclonal α-RPV L antibody or rat monoclonal α-HA antibody (Roche) (figure 4.2.2.4). In addition, both antibodies were used to treat non-infected, non-transfected cells to demonstrate that they did not pull down a protein of similar size to L from the untransfected cell lysate. All controls worked as expected and good expression of HAL was shown from pcDNA/HAL when driven by the T7 promoter (figure 4.2.2.4). It was now clear that HAL could be detected by immunoprecipitation, as it was being expressed, just in very low amounts when governed by the weaker CMV promoter.

A series of experiments were set up in Cos-1 cells to optimise HAL expression when driven by the CMV promoter. To begin with 5μg, 7.5μg and 10μg amounts of pcDNA/HAL were transfected but produced no detectable levels of HAL expression (data not shown). Less DNA was then transfected into Cos-1 cells, resulting in the observation that either 1μg or 2μg of transfected plasmid DNA produces a higher level of HAL expression than 3μg, 4μg or 5μg of transfected plasmid DNA (figure 4.2.2.5). It is unclear why HAL should express efficiently when less DNA is transfected.

In an effort to increase the levels of HAL protein expression when driven from the CMV promoter, several CMV promoter enhancers were investigated. Some evidence exists in the literature that sodium butyrate can act as a chemical

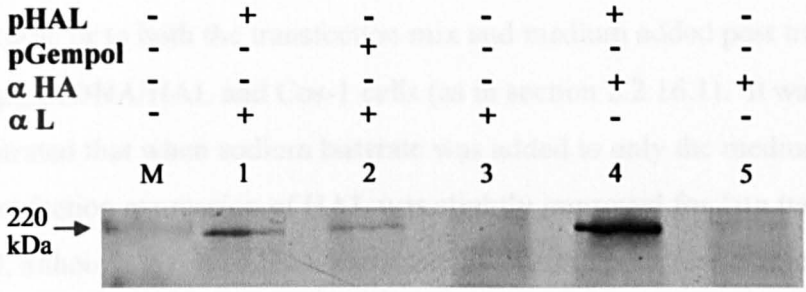

Figure 4.2.2.4. Transient expression of pcDNA/HAL in Cos-1 cells. Cos-1 cells were infected with fowl pox T7 for one hour and transfected with 5μg pcDNA/HAL (1 & 4), or 5μg pGempol (2). Two days post transfection, cells were labelled with [^{35}S] amino acids for one hour and cells were lysed with 1% NP40. Samples of lysates were immunoextracted using rat monoclonal α-HA (Roche) (4 & 5) or rabbit polyclonal α-L, MB41 (1, 2 & 3). Immunoprecipitated proteins were analysed on an 8% acrylamide Laemmli gel. Where M= [^{14}C] labelled molecular weight markers, 1= pcDNA/HAL, MB41, 2= pGempol, MB41, 3= untransfected, MB41, 4= pcDNA/HAL, rat α-HA and 5= untransfected, rat α-HA.

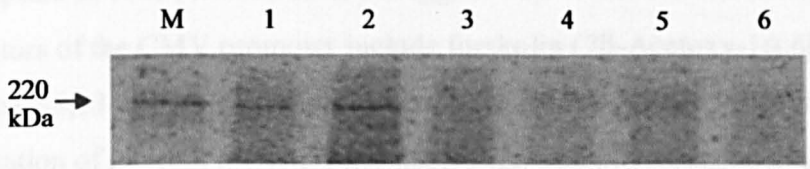

Figure 4.2.2.5. Optimisation of pcDNA/HAL expression. Cos-1 cells were transfected with varying amounts of pcDNA/HAL; two days post transfection, the cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Cell lysates were then treated with 1μl rat monoclonal α-HA antibody (Roche). Immunoprecipitated proteins were analysed on an 8% acrylamide Laemmli gel, alongside [^{14}C] labelled molecular weight markers (M). Track 1= 1μg pcDNA/HAL, 2= 2μg pcDNA/HAL, 3= 3μg pcDNA/HAL, 4= 4μg pcDNA/HAL, 5= 5μg pcDNA/HAL and 6= lysate from untransfected cells.

enhancer of CMV promoter activity (Gorman & Howard, 1983). At first sodium butyrate was used at a final concentration of 1.5mM, and was either added to the Transfast transfection mix only, or to the pre-warmed medium added post transfection, or to both the transfection mix and medium added post transfection using 2µg pcDNA/HAL and Cos-1 cells (as in section 2.2.16.1). It was demonstrated that when sodium butyrate was added to only the medium added post transfection expression of HAL was slightly improved for 1µg transfected plasmid, although levels of HAL expression were still poor (see figure 4.2.2.6). The experiment was then repeated using final concentrations of sodium butyrate at 1.5mM, 5mM, 7.5mM, 10mM and 12.5mM in only the medium added post-transfection. However, no improvement was seen in the level of HAL protein expression as detected by immunoprecipitation (data not shown).

Protein expression driven by the CMV promoter can be increased by a number of chemical stimuli. These function by activating the transcription factor nuclear-factor-kappa B (NFκB). NFκB then binds to four NFκB binding sites present in the CMV promoter sequence which activates CMV promoter dependent transcription in cells (Wilkinson & Akrigg, 1992). Other known chemical stimulators of the CMV promoter include forskolin (7β-Acetoxy-1α,6β,9α-trihydroxy-8,13-epoxy-labd-14-en-11-one), from *Coleus forskohlii*, and a combination of phorbol myristate acetate (PMA) and phytohaemagglutinin (PHA) (Clesham *et al.*, 1996, Wilkinson & Akrigg, 1992). Therefore, forskolin (Sigma) was used (at a final concentration of 10µmol/L), or PMA (gift from M. Denyer, Department of Immunology, Institute for Animal Health, Pirbright, Surrey, UK) (at 50ng/ml) and PHA (Sigma) (at 4µg/ml) were used together, or alternatively, all three were combined and utilised simultaneously. To determine if HAL expression could be increased in the presence of any of these known CMV promoter enhancers, each of the above combinations were included in the pre-warmed medium added to the cells post transfection, as described in (Wilkinson & Akrigg, 1992) and (Clesham *et al.*, 1996). In each case 1µg pcDNA/HAL plasmid DNA was transfected using Transfast (as in section 2.2.16.1) and, as a control, pcDNA/HAL was transfected in the absence of any

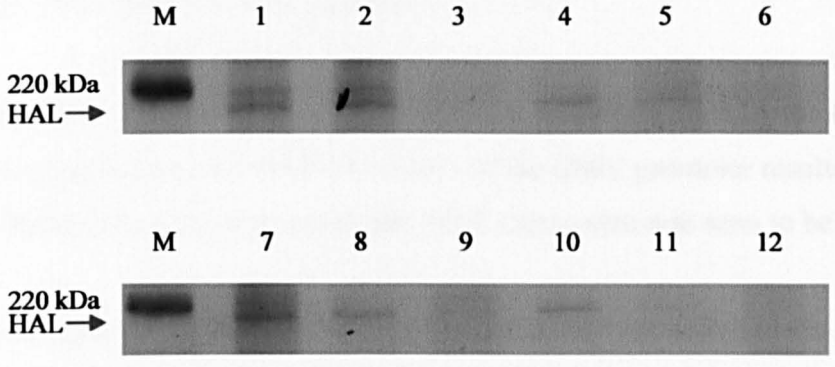

Figure 4.2.2.6. The effect of sodium butyrate (1.5mM) on HAL expression. Cos-1 cells were transfected with either 1μg or 2μg pcDNA/HAL and the cells were treated with sodium butyrate as described below; two days post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with 1μl rat monoclonal α-HA antibody (Roche) and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels alongside [^{14}C] labelled molecular weight markers (M). Where 1 = 1μg pcDNA/HAL, no sodium butyrate, 2= 2μg pcDNA/HAL, no sodium butyrate, 3= untransfected cell lysate, no sodium butyrate, 4= 1 μg pcDNA/HAL, sodium butyrate in transfection mix only, 5= 2μg pcDNA/HAL, sodium butyrate as for 4, 6= untransfected cell lysate, sodium butyrate as for 4, 7= 1μg pcDNA/HAL, sodium butyrate in medium added post transfection, 8= 2μg pcDNA/HAL, sodium butyrate as for 7, 9= untransfected cell lysate, sodium butyrate as for 7, 10= 1μg pcDNA/HAL, sodium butyrate in transfection mix and medium added post transfection, 11= 2μg pcDNA/HAL, sodium butyrate as for 10, 12= untransfected cell lysate, sodium butyrate as for 10.

chemical enhancers of the CMV promoter to establish the level of HAL protein expression in the absence of any enhancers. The level of HAL expression from pcDNA/HAL was shown to decrease in the presence of forskolin alone, but was shown with PMA and PHA, or in the presence of all three chemicals, to be the same as pcDNA/HAL transfected alone, that is, in the absence of any chemical enhancer of the CMV promoter (see figure 4.2.2.7).

Therefore efforts to boost HAL expression levels when governed by the CMV promoter using known chemical stimulators of the CMV promoter resulted in no increase being observed, and in one case HAL expression was seen to be reduced.

As none of the known enhancers of CMV promoter activity tested produced an increase in the level of HAL expression, another possibility was explored. A commercially available plasmid, pAdVAntage (Promega), was tested next as it had previously been shown to enhance levels of protein expression from transfected cells (Terenzi *et al.*, 1999). This plasmid encodes the gene for the Adenovirus virus-associated I (VAI) RNA; when co-transfected with plasmid constructs of interest, it acts to enhance transient protein expression by increasing translation initiation within mammalian cells. This is possibly due to the fact that VAI RNA is able to block the activation of the cell protein kinase R (PKR) by complexing with it. PKR is an enzyme involved in the host cell's antiviral defence system which phosphorylates the translation initiation factor eIF-2, thereby preventing host cell translation and consequently protein production. PKR is thought to be activated by dsRNA generated as a result of the transfection process. To determine if pAdVAntage could increase HAL expression in transfected Cos-1 cells, 1μg of pAdVAntage was co-transfected with 1μg pcDNA/HAL; in addition to the co-transfection of pcDNA/HAL with pAdVAntage, the media added post transfection contained PMA and PHA. When pcDNA/HAL and pAdVAntage were co-transfected in the absence of PMA and PHA, a minor increase in HAL expression was observed (figure 4.2.2.8).

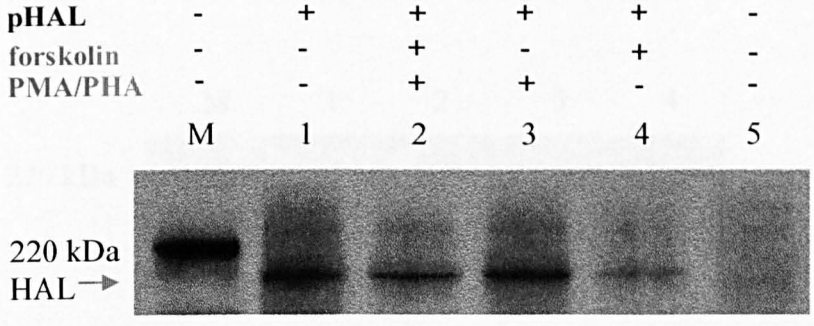

Figure 4.2.2.7. Effect of CMV promoter chemical enhancers on the expression level of HAL from pcDNA/HAL. Cos-1 cells were transfected with 1μg pcDNA/HAL; two days post transfection, the cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Cell lysates were then immunoextracted with 1μl of rat α-HA monoclonal antibody (Roche) and immunoprecipitated proteins were analysed on an 8% acrylamide Laemmli gel alongside [^{14}C] labelled molecular weight markers (M). Track 1= 1μg pcDNA/HAL, 2= 1μg pcDNA/HAL with PMA, PHA and forskolin, 3= 1μg pcDNA/HAL with PMA and PHA, 4= 1μg pcDNA/HAL with forskolin, 5= lysate from untransfected cells.

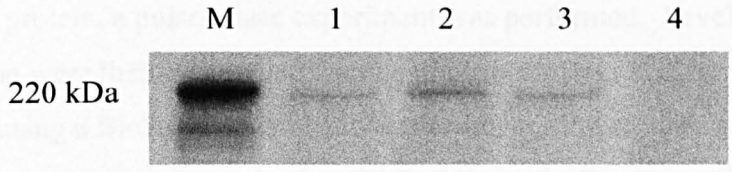

Figure 4.2.2.8. pAdVAntage increases the level of HAL expression in transfected cells. Cos-1 cells were transfected with the constructs on interest; two days post transfection, the cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Cell lysates were then treated with 1µl of rat α-HA monoclonal antibody (Roche) and immunoprecipitated proteins were analysed on an 8% acrylamide Laemmli gel alongside [^{14}C] labelled molecular weight markers (M). Track 1= 1µg pcDNA/HAL, 2= 1µg pcDNA/HAL with 1µg pAdVAntage, 3= 1µg pcDNA/HAL with 1µg pAdVAntage, with PMA and PHA and 4= lysate from untransfected cells.

Before the utilisation of pAdVAntage could be optimised, it was observed that co-transfection of HAL with RPV N resulted in significantly boosted levels of HAL protein expression, even more than that seen with pAdVAntage (figure 4.2.2.9; note in tracks 2, 3 and 6 there is a background band which migrates just above the expected position of N, see tracks 4 and 5). This apparent increased level of HAL protein expression was not due to the co-transfection with an additional pcDNA construct, as co-transfection of pcDNA/HAL with pcDNA/P did not result in an increased level of HAL expression (data not shown). To determine if N was exerting this effect on HAL expression by acting to stabilise the HAL protein, a pulse-chase experiment was performed. Levels of protein expression were then quantitated using a phosphorimager screen, which was scanned using a BioRad Personal Molecular Imager FX scanner and protein quantification was performed using BioRad Quantity One Quantification software. N was demonstrated not to stabilise HAL, as HAL was shown to degrade at the same rate in the presence of N, as when HAL was expressed alone (see figures 4.2.2.10 and 4.2.2.11).

In addition to obtaining good expression of HAL, undegraded expression of C was also problematic. Previous work in the laboratory had shown that transiently transfected cells using vaccinia T7/T7 promoter-driven expression resulted in the majority of the C protein being expressed in a degraded form, unlike C expressed in infected cells (Sweetman *et al.*, 2001), which is why the CMV promoter was utilised. Despite driving the expression of C from pcDNA/C using the CMV promoter, the majority of C was still expressed in a degraded form (see figure 4.2.2.1). To address this problem several different cell lines were utilised to determine if the degradation of transiently expressed C protein was a common event in transfected cells. In addition to Cos-1 cells, Vero cells and A549 cells (a gift from Dr. S. Moyer, Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, Florida, USA) were also tested, however none of these cell lines conferred any protection to the expressed C protein. Lysis buffers with varying concentrations of protease inhibitors were tested also and cells were lysed on ice to minimise any degradation. All failed to yield undegraded C protein (data not shown).

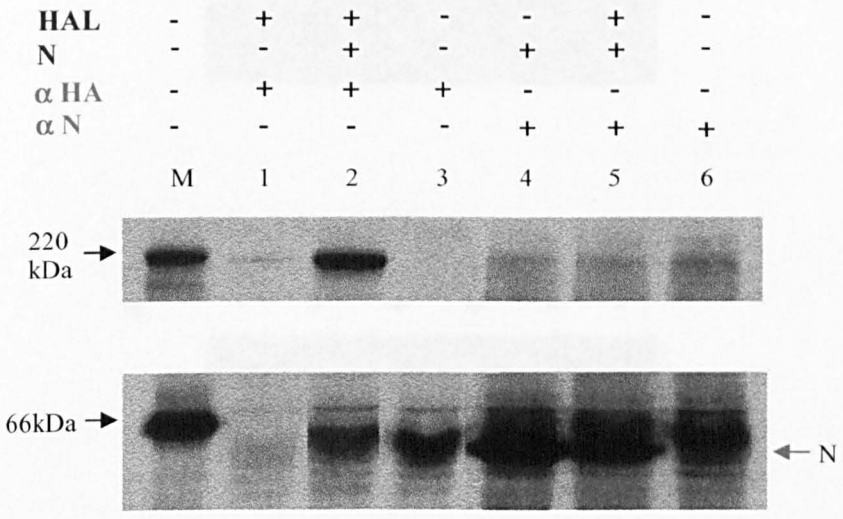

Figure 4.2.2.9. The effect of RPV N on HAL expression.
Cos-1 cells were transfected with 2μg of each of the constructs indicated. Two days post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with the relevant antibody and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels alongside [^{14}C] labelled molecular weight markers (M). Track 1= pcDNA/HAL, rat monoclonal α-HA antibody (Roche), 2= pcDNA/HAL and pcDNA/N, with rat α-HA, 3= untransfected cell lysate, rat α-HA, 4= pcDNA/N, rabbit polyclonal α-RPV N antibody (MB2), 5= pcDNA/HAL, pcDNA/N, MB2 and 6= untransfected cell lysate, MB2.

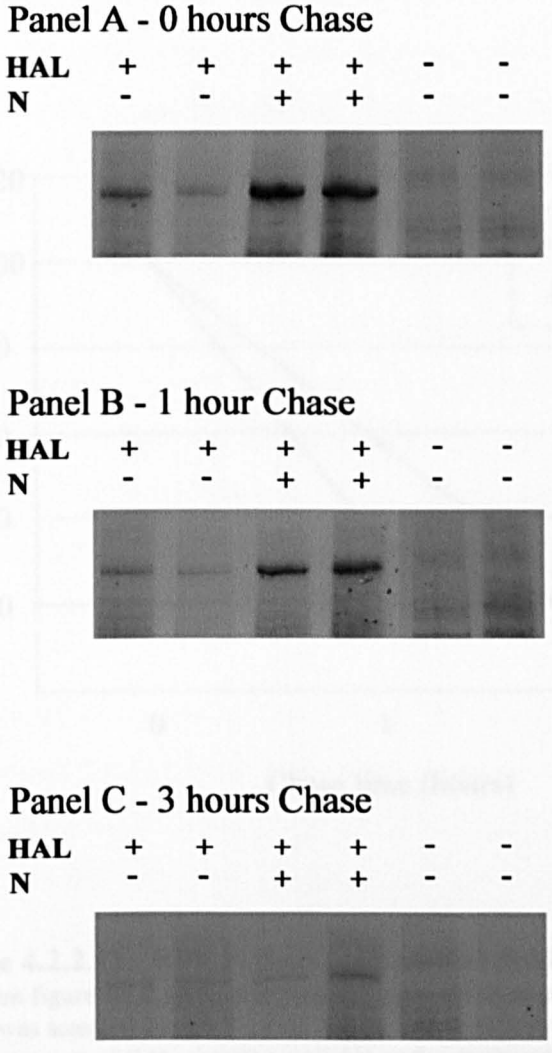

Figure 4.2.2.10. RPV N does not appear to stabilise HAL.
Three batches of Cos-1 cells were transfected with 2μg of each of the desired constructs (as indicated) in duplicate; two days post transfection cells were labelled with [^{35}S] amino acids for one hour. After labelling, one batch of cells was lysed with 1% NP40 (panel A), the two remaining batches of cells had 2ml of whole medium added to each 35mm well and were incubated at 37°C for either 1 hour (panel B) or 3 hours (panel C) before lysis with 1% NP40. Samples of lysates were immunoextracted with 1μl rat anti-HA monoclonal antibody (Roche) and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels.

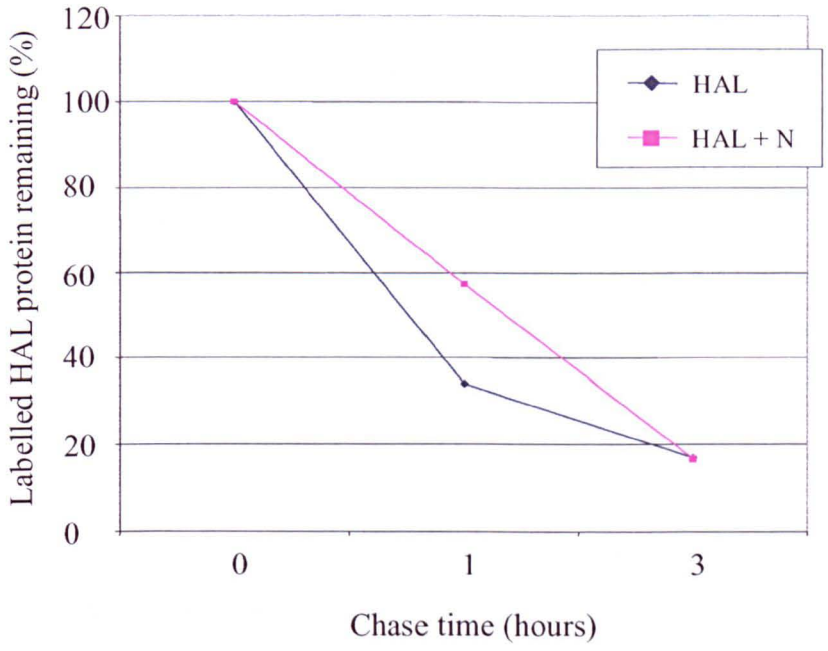

Figure 4.2.2.11. RPV N does not stabilise HAL. The Laemmli gels from figure 4.2.2.10 were exposed to a phosphorimager screen and the screen was scanned using a BioRad Personal Molecular Imager FX scanner and the amount of HAL protein present in each sample was quantitated using BioRad Quantity One Quantification software. The amount of labelled HAL is expressed as a percentage of that present at time=0. Each data point represents the average value of each set of duplicates.

The coding sequence for RPV C was examined in an attempt to explain the results obtained to date with the pcDNA/C construct. It was thought possible that the 15 kDa band seen in both immunoprecipitations and TNT reactions could be an additional small protein produced from an extra ATG which resides in the C ORF and therefore not actual degradation of the C protein. It could be that this smaller protein was being preferentially made over the full-length C protein and to determine if this was the case, a new pcDNA/C construct, pcDNA/newstartC, was made (using *Pfu* PCR and the primers NewCstart2 and C end primer, see appendix 3). This created a construct which changed the C ATG to the optimal sequence for initiation by eukaryotic ribosomes, as identified by Kozak (Kozak, 1986). This new C construct was transfected alongside pcDNA/C in Vero cells and expression tested by immunoprecipitation. No difference was observed in the level of full-length C protein seen and it is therefore likely that the C protein really was being degraded (figure 4.2.2.12).

Previous work in the laboratory had shown that full-length C protein could be obtained from infected cells (Sweetman, 2000, Sweetman *et al.*, 2001). In order to test the lysis and immunoprecipitation reagents, expression of C in Vero cells and B95a cells infected with RPV (RBOK strain) was compared. B95a cells were chosen due to the fact that this is the cell line normally used in the laboratory for studies of RPV. RBOK infected Vero cell lysates produced degraded C protein; however, under identical conditions, undegraded C protein was obtained from B95a cells (see figure 4.2.2.13). These observations suggested that it was not the expression method that was leading to C degradation, but the cell line. To confirm this, B95as were infected with vaccinia vTF7-3 and pcDNA/C (5μg) was transfected, using either Transfast or lipofectin as the transfection reagent. As was seen in infected cells, undegraded expression of C was obtained (figure 4.2.2.14). Consequently the optimisation of HAL expression from the CMV promoter was no longer necessary and all further transient protein expression work was performed using the vaccinia T7/T7 promoter-driven system using B95a cells.

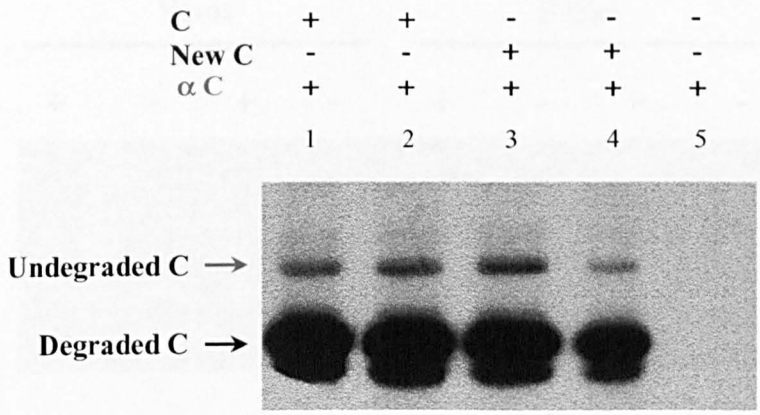

Figure 4.2.2.12. Comparison of C protein expression by immunoprecipitation from pcDNA/C and pcDNA/newstartC constructs. Vero cells were transfected with either pcDNA/C (tracks 1 and 2) or pcDNA/newstartC (tracks 3 and 4); one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Track 5 represents non-transfected (no DNA) control cell lysates. Samples of lysates were immunoextracted with rabbit α-C antibody (MB38) and immunoprecipitated proteins analysed on 12% acrylamide Laemmli gels.

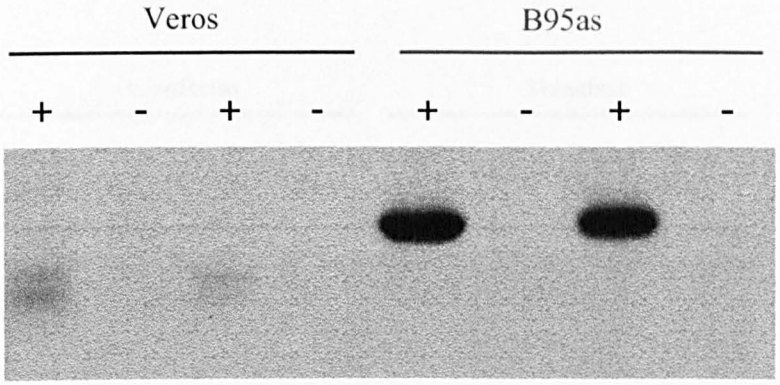

Figure 4.2.2.13. Undegraded expression of C protein from RPV infected cells. In duplicate, either Vero cells were infected using 25µl of RBOK per 35mm dish (+) or B95a cells were infected using 40µl of RBOK per 35mm dish (+) and noninfected cells (-) were used as a negative control; 3 days post infection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40 buffer containing protease inhibitor cocktail set III (Calbiochem) at a final concentration of 1/200. Samples of lysates were immunoextracted with MB38, a rabbit polyclonal α-RPV C antibody, and immunoprecipitated proteins were analysed on 12% acrylamide Laemmli gels.

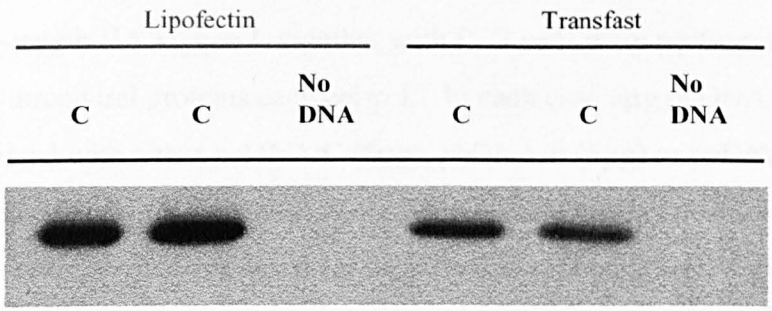

Figure 4.2.2.14. Undegraded expression of C protein from cells transfected with pcDNA/C using vaccinia T7/T7 promoter expression. B95a cells were infected with vTF7-3 and transfected with 5μg pcDNA/C in duplicate, using either Transfast or lipofectin as the transfection reagent. In both cases a negative control sample was included which contained no DNA. One day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with MB38, a rabbit polyclonal α-RPV C antibody, and immunoprecipitated proteins were analysed on 12% acrylamide Laemmli gels.

For the remainder of these studies, transfections were performed using lipofectin transfection reagent (section 2.2.16.2) and B95a cells, and proteins were expressed using the vaccinia T7/T7 promoter-driven expression system (section 2.2.17).

4.2.3 Co-Immunoprecipitation of Full-Length L with P, C and V

To show that this system was fully functional, co-immunoprecipitations (co-ips) using full-length HA-tagged L together with P, C or V were performed to confirm that these three viral proteins each co-ip L. In each case 5μg pcDNA/HAL was co-transfected with either pcDNA/C (5μg), pcDNA/P (5μg) or pcDNA/V (5μg) and proteins transiently expressed using B95a cells. One day post transfection, cells were radiolabelled and lysed (as described in section 2.3.4). Cell lysates were immunoextracted using the rat α-HA monoclonal antibody, MB38, a rabbit polyclonal α-RPV C, 2-1, a mouse monoclonal α-RPV P or U32, a mouse monoclonal α-RPV P antibody which had been found to recognise an epitope common to P and V (for the detection of V). Immunoprecipitated proteins were analysed on either 8% or 12% acrylamide Laemmli gels. The C and P proteins were both shown to co-immunoprecipitate full length HA-tagged L, expressed from pcDNA/HAL (figure 4.2.3.1), showing that this system was functional and confirming these previously identified interactions. However, the V protein failed to co-ip HAL (see figure 4.2.3.2).

To confirm the L-V interaction by co-ip, an existing laboratory construct expressing a glutathione-S-transferase (GST)-tagged version of V, pGSTV (Sweetman *et al.*, 2001) (5μg), was co-transfected with pcDNA/HAL (5μg) into B95a cells. The transfected cell lysates were extracted using either rat α-HA or MB38 since this antibody was raised against a GST-fused C protein and will thus recognise all GST fusions and can be used as an α-GST antibody. This GST-tagged form of the V protein migrates at ~66 kDa (true size 59 kDa) and was shown to co-ip full length L in this system (figure 4.2.3.2). Earlier work in the laboratory had shown that GST did not interact with full-length L (Sweetman,

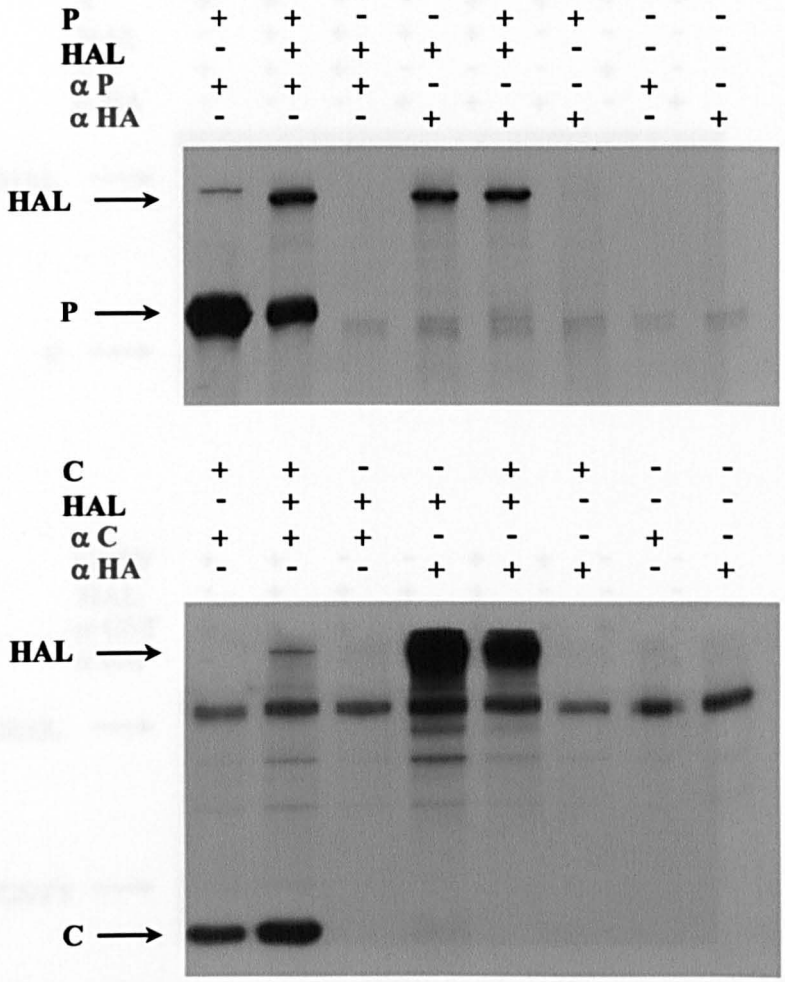

Figure 4.2.3.1. P and C co-immunoprecipitate full-length L expressed from pcDNA/HAL. B95a cells were infected with vTF7-3 and transfected with the desired constructs (as indicated); one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with the appropriate antibody (see above) and immunoprecipitated proteins were analysed on either 8% (for pcDNA/P and pcDNA/HAL) or 12% (for pcDNA/C and pcDNA/HAL) acrylamide Laemmli gels.

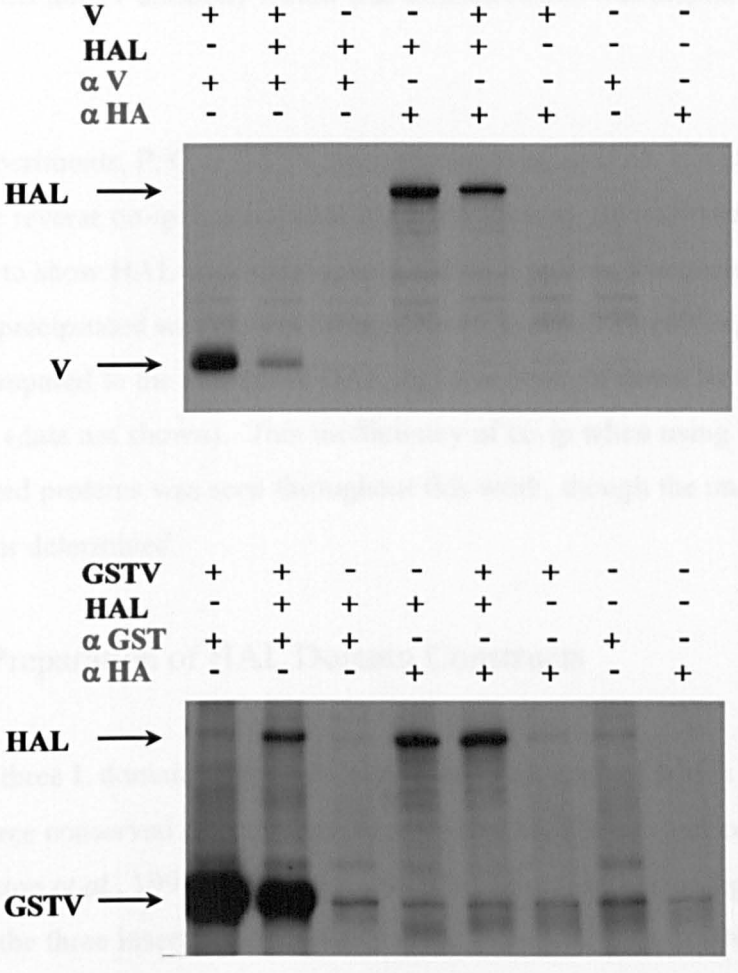

Figure 4.2.3.2. V-L co-immunoprecipitation could be shown using GST-tagged V, but not V itself. B95a cells were infected with vTF7-3 and transfected with the desired constructs (as indicated); one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with the appropriate antibody (see above) and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels.

2000); pGSTV was therefore used in place of pcDNA/V for the remainder of these L binding studies. The most likely explanation for the failure of V to co-ip L is that the anti-V antibody which was utilised (U32) was displacing the L protein.

In all experiments, P, C or GSTV were shown to co-ip HAL but in the majority of cases the reverse co-ip was not seen (data not shown). In addition when it was possible to show HAL co-immunoprecipitating a protein, the protein co-immunoprecipitated was always brought down in small quantities, especially when compared to the amount of HAL that was brought down by the protein in question (data not shown). This inefficiency of co-ip when using HAL or other HA-tagged proteins was seen throughout this work, though the underlying cause was never determined.

4.2.4 Preparation of HAL Domain Constructs

Initially three L domain constructs were generated, each of which contained one of the three conserved domains, as identified by McIlhatton and co-workers (McIlhatton *et al.*, 1997), with the relevant hinge region(s) (see figure 4.2.4.1). Each of the three inserts was produced using *Pfu* PCR, with pcDNA/HAL as the template DNA. The first construct, pcDNA/HAL1, encompasses the first of the three conserved domains of L in addition to the first of the two proposed hinge regions, spanning the first 1950 base pairs (bp) of L. pcDNA/HAL2 (3333bp in size) consists of the second of the three conserved domains of L and each of the two proposed hinge regions, covering 1820-5152bp of L. pcDNA/HAL3 (1527bp in size) comprises the third and final conserved domain of L as well as the second of the two hinge regions, covering 5084-6610bp of L. pcDNA/HAL1 was generated using the primers HAL1start and HAL1end, pcDNA/HAL2 using primers HAL2start and HAL2end, whilst pcDNA/HAL3 was made using the primers HAL3start and Lend (from the MTHS work, see appendix 3 for primer sequences). Each domain was cloned into pcDNA/HA/Not with the re-introduced *Not*I site (*Not*I/ *Xho*I cut) (as described in section 4.2.1.) for co-

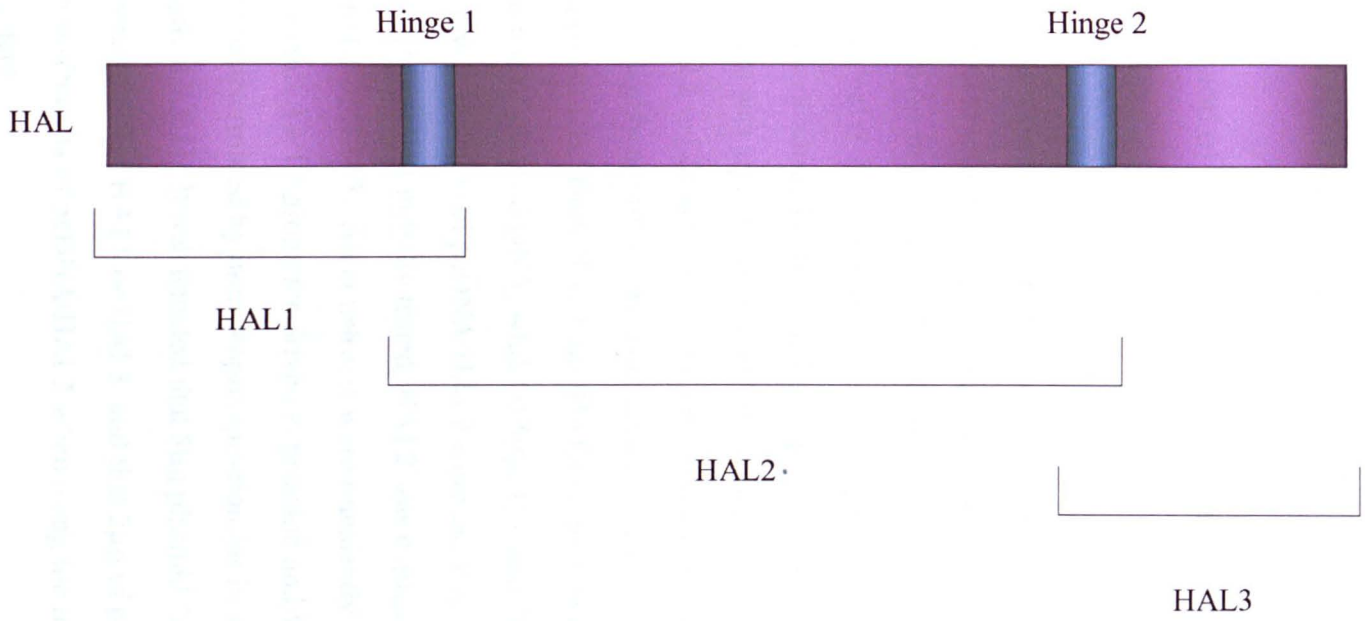

Figure 4.2.4.1. Diagrammatic representation of the three initial L domain constructs prepared for co-immunoprecipitation studies. The L proteins of morbilliviruses have been shown to consist of two regions of poor sequence identity (hinge regions), which separate three highly conserved domains along the length of the L protein. To identify which domain of L interacts with P, C and V these three sections were cloned separately, fusing their coding sequence to that of the HA epitope tag, as described in the text.

immunoprecipitation studies. Thus each domain was HA-tagged at the amino-terminus of the inserted region of the L protein, enabling the use of the rat α-HA monoclonal antibody from Roche, as previously used in these studies.

All three constructs were sequenced at the 5' and 3' ends of the inserted coding region, for confirmation purposes.

4.2.5 Verification of Protein Expression from HAL Domain Constructs

To confirm protein expression from each of these three constructs, 2μg plasmid DNA was transfected into B95a cells using the vaccinia T7/T7 promoter-driven expression system (as in section 2.2.17). Cell lysates were immunoextracted using rat monoclonal α-HA antibody and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels. Expression of each of the three domain constructs was seen, with proteins migrating as expected, HAL1 (~71.5 kDa), HAL2 (~122 kDa) and HAL3 (~56 kDa). Expression of these constructs was roughly optimised by transfecting varying quantities of each of the three domain constructs. Both HAL1 and HAL3 were transfected using 3μg, 5μg or 7.5μg amounts of plasmid DNA, whilst 0.5μg, 1μg and 2μg amounts of HAL2 were used. Lower amounts of pcDNA/HAL2 were used as, when expression of these three constructs was initially tested, HAL2 was expressed at a higher level than either HAL1 or HAL3. All constructs were transiently transfected into B95a cells using vaccinia T7/T7 promoter-driven expression and levels of protein expression were determined by immunoprecipitation (as in sections 2.2.17 and 2.3.4, see figure 4.2.5.1). It was decided that 5μg plasmid DNA would be used for transfections of either HAL1 or HAL3, and that 2μg of plasmid DNA would be used for transfections of pcDNA/HAL2 when using the transient T7 expression system.

During the optimisation of protein expression from these L domain constructs, it was observed that the HAL1 protein migrated as a doublet. However, this

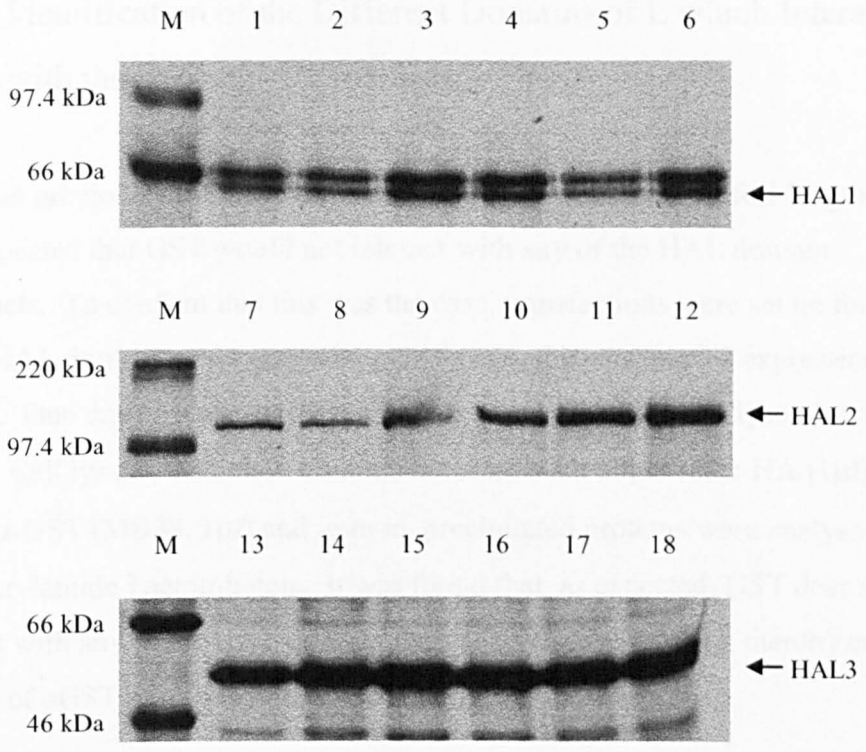

Figure 4.2.5.1. Expression optimisation of pcDNA/HAL domain constructs. B95a cells were infected with vTF7-3 and transfected with the desired constructs; one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with 1µl rat monoclonal α-HA antibody and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels alongside [^{14}C] labelled molecular weight markers (M). pcDNA/HAL1 was transfected using either 3µg (1, 2), 5µg (3, 4) or 7.5µg (5, 6) amounts of plasmid DNA. pcDNA/HAL2 was transfected using either 0.5µg (7, 8), 1µg (9, 10) or 2µg (11, 12) amounts of plasmid DNA. pcDNA/HAL3 was transfected using either 3µg (13, 14), 5µg (15, 16) or 7.5µg (17, 18) amounts of plasmid DNA.

appears to have been an artefact of some kind, as, in all later experiments, HAL1 was seen to migrate as a single band.

4.2.6 Identification of the Different Domains of L which Interact with the P, C and V Proteins

As it had previously been shown that GST did not interact with full-length L, it was expected that GST would not interact with any of the HAL domain constructs. To confirm that this was the case, transfections were set up for each of the HAL domain constructs with pGST using the vaccinia T7 expression system. One day post transfection, cells were radiolabelled and lysed (section 2.3.4). Cell lysates were then immunoextracted with either rat α-HA (1μl) or rabbit α-GST (MB38, 1μl) and immunoprecipitated proteins were analysed using 10% acrylamide Laemmli gels. It was found that, as expected, GST does not interact with any of the HAL domain constructs (figure 4.2.6.1), thereby enabling the use of pGSTV for this part of the study.

To identify the region of the L protein to which the P, C and V proteins bind, each HAL domain construct was co-transfected with pcDNA/P, pcDNA/C, or pGSTV and transfected cells radiolabelled and lysed (as in section 2.3.4) one day post transfection. Cell lysates were then immunoextracted with the relevant antibodies and immunoprecipitated proteins analysed on the relevant percentage acrylamide Laemmli gel. For pcDNA/P, pcDNA/C and pGSTV, 5μg plasmid DNA was co-transfected with 5μg of HAL1 or HAL3, or with 2μg of HAL2.

When pcDNA/P was co-transfected with each of the three HAL domain constructs, it was found that P co-immunoprecipitated HAL1, but not HAL2 or HAL3 (see figure 4.2.6.2). Unfortunately the reverse co-ip (that is, HAL1 co-immunoprecipitating P) could only be shown on one occasion out of four (data not shown). P therefore interacts with the first conserved domain of the L protein. The C protein on the other hand was shown to co-immunoprecipitate

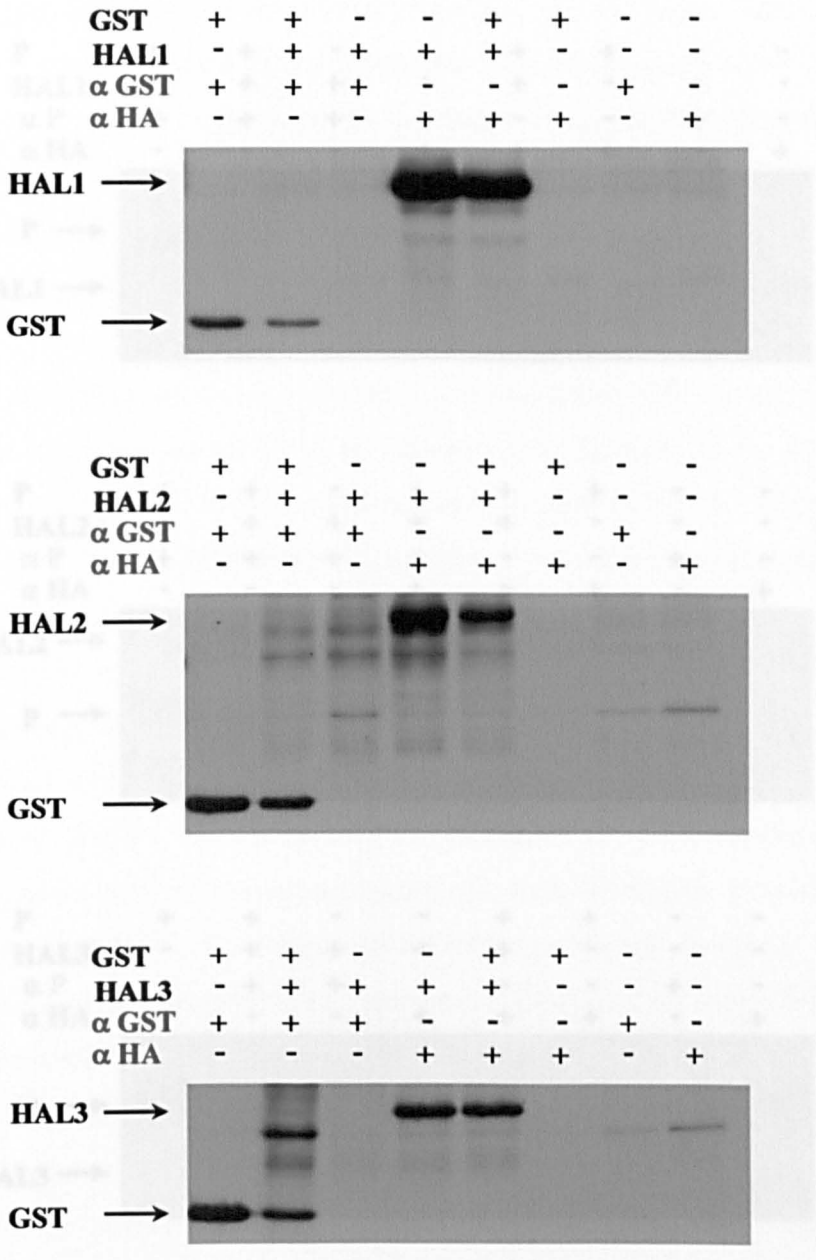

Figure 4.2.6.1. GST does not bind to any of the L subdomains. B95a cells were infected with vTF7-3 and transfected with the desired constructs (as indicated); one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with the appropriate antibody (see above) and immunoprecipitated proteins were analysed on 10% acrylamide Laemmli gels.

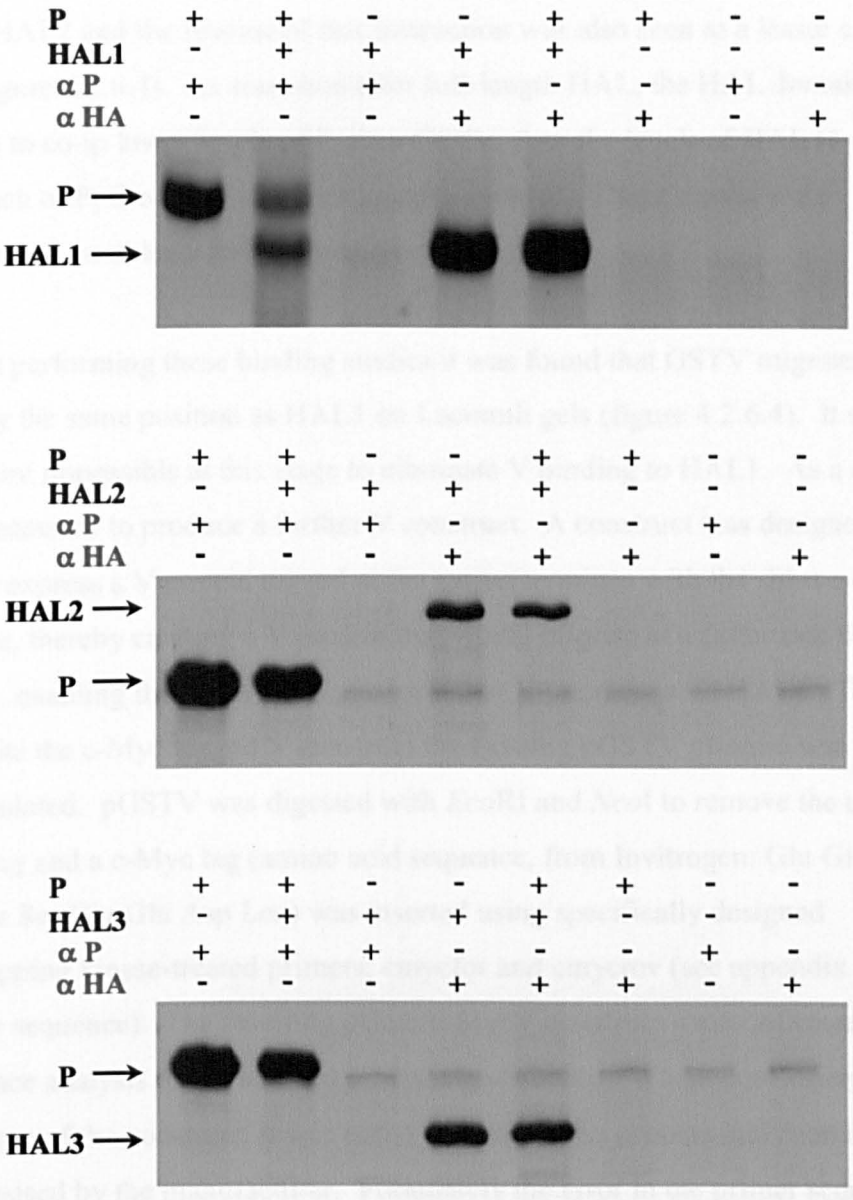

Figure 4.2.6.2. Co-immunoprecipitations of P with pcDNA/HAL domain constructs. B95a cells were infected with vTF7-3 and transfected with the desired constructs (as indicated); one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with the appropriate antibody (see above) and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels.

HAL2, and not HAL1 or HAL3 (see figure 4.2.6.3) and the reverse co-ip was demonstrated, that is HAL2 was shown to co-ip C. GSTV, like C, was shown to co-ip HAL2 and the reverse of this interaction was also seen to a lesser extent (see figure 4.2.6.4). As was shown for full-length HAL, the HAL domains were shown to co-ip lower levels of P, C or GSTV, than the levels of HAL (1 or 2), that each of P, C or GSTV co-immunoprecipitated. These results were reproduced on at least three separate occasions.

Whilst performing these binding studies it was found that GSTV migrates at exactly the same position as HAL1 on Laemmli gels (figure 4.2.6.4). It was therefore impossible at this stage to eliminate V binding to HAL1. As a result, it was necessary to produce a further V construct. A construct was designed that would express a V protein tagged at the amino terminus with the short c-Myc epitope, thereby creating a V protein that would migrate at a faster rate than HAL1, enabling the determination of whether V interacts with HAL1. To generate the c-Myc tagged V construct the existing pGSTV plasmid was manipulated. pGSTV was digested with *Eco*RI and *Nco*I to remove the unwanted GST tag and a c-Myc tag (amino acid sequence, from Invitrogen: Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu) was inserted using specifically designed overlapping kinase-treated primers, cmycfor and cmycrev (see appendix 3 for primer sequence). The resulting pGem/c-MycV construct was confirmed by sequence analysis of the inserted c-Myc tag sequence. Whilst confirming the sequence of the construct, it was noted that one of the primers had been mis-synthesised by the manufacturer. Fortunately the error in the primer sequence did not affect the c-Myc tag, nor the reading frame of the construct (see figure 4.2.6.5).

To confirm that the construct was expressing a c-Myc tagged version of V, expression was checked by immunofluorescence microscopy. pGem/c-MycV (5μg) was transiently expressed using vaccinia T7 in B95a cells (as in section 2.2.17). One day post transfection, cells were fixed and permeabilised with 0.1%

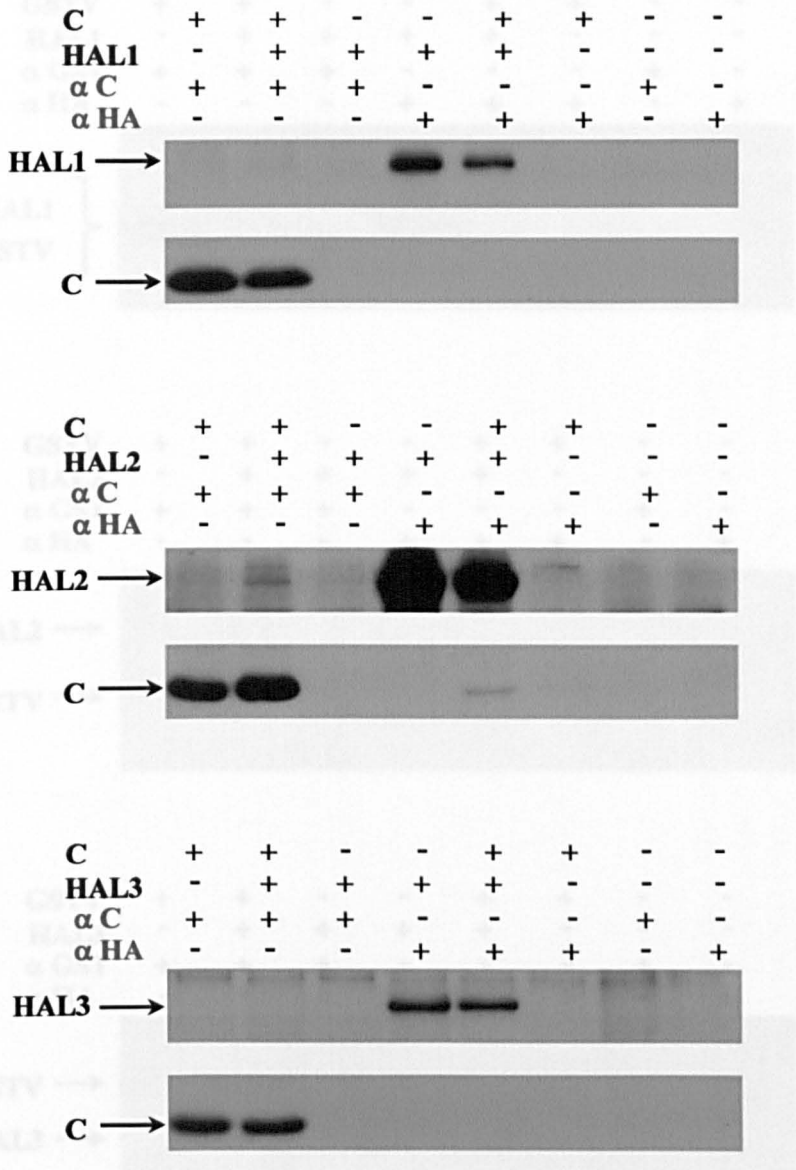

Figure 4.2.6.3. Co-immunoprecipitations of C with pcDNA/HAL domain constructs. B95a cells were infected with vTF7-3 and transfected with the desired constructs (as indicated); one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with the appropriate antibody (see above) and immunoprecipitated proteins were analysed on 12% acrylamide Laemmli gels. Gels shown have been split to save space.

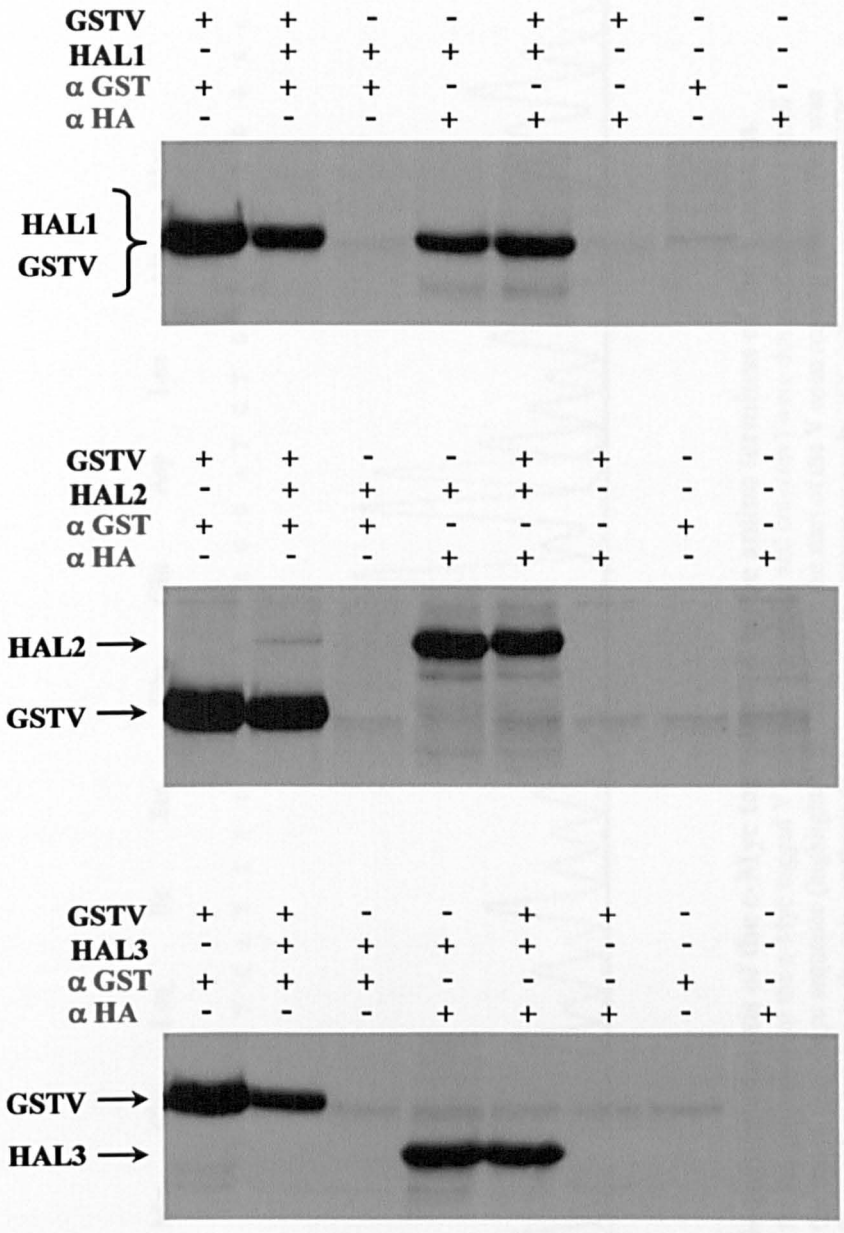

Figure 4.2.6.4. Co-immunoprecipitations of GSTV with pcDNA/HAL domain constructs. B95a cells were infected with vTF7-3 and transfected with the desired constructs (as indicated); one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with the appropriate antibody (see above) and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels.

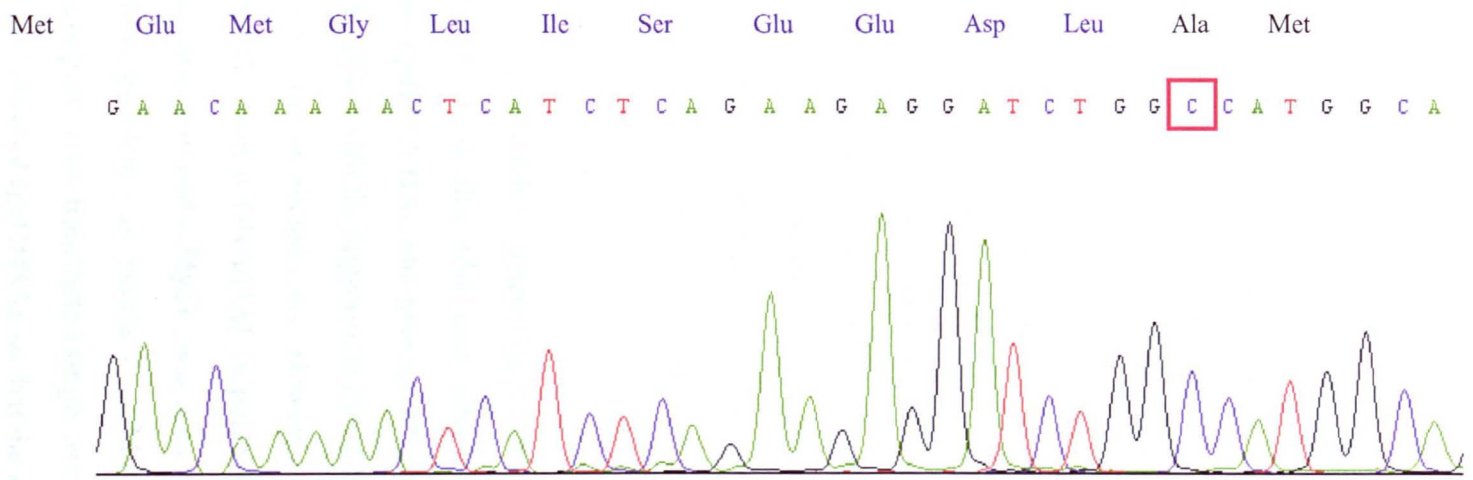

Figure 4.2.6.5. Sequence analysis of the c-Myc tag inserted at the amino terminus of the V protein.
The primers ordered for the generation of the c-Myc tagged V construct (cmycfor and cmycrev) were designed to insert a small spacer amino acid at the end of the c-Myc sequence (highlighted in blue) before the start of the V open reading frame. This was designed to be a glycine residue, but due to the mis-synthesis of the primer(s) the sequence reads GCC (alanine) instead of GGC (glycine), as highlighted by the red box. This error in the primer sequence was not in a critical region of sequence, so neither the reading frame of the construct, nor the c-Myc tag were affected.

Triton X-100 before labelling with antibody. Cells were labelled using either 9E10, a monoclonal mouse α-c-Myc antibody (Santa Cruz Biotechnology) or a monoclonal rat α-c-Myc antibody (Serotec) (figure 4.2.6.6). Clear expression of c-MycV was observed.

However, it was not possible to immunoprecipitate c-MycV using 9E10. It was confirmed by immunoprecipitation that the pGem/c-MycV was expressing the V protein as lysate of cells transfected with pGem/c-MycV immunoextracted with U32 (α-V antibody) pulled down the V protein (see figure 4.2.6.7). The problem was therefore with the α-c-Myc antibody being utilised. Due to time constraints, cell lysates transfected with pGem/c-MycV were immunoextracted using 1μl of each of the following α-c-Myc antibodies simultaneously; A-14 (rabbit α-c-Myc, Santa Cruz Biotechnology), 9E10 and rat α-c-Myc. As a control, cell lysate transfected with pGem/c-MycV was also immunoextracted, using 9E10. Using the combination of the three antibodies raised against the c-Myc epitope, c-MycV was shown to be immunoprecipitated from cells transiently transfected with pGem/c-MycV (figure 4.2.6.8).

It should now have been possible to determine if V interacts with the first conserved domain of L. To do this A549 cells were co-transfected with a combination of either pcDNA/HAL and pGem/c-MycV, or with pcDNA/HAL1 and pGem/c-MycV, along with the appropriate controls of the singly transfected plasmids, in addition to untransfected cells. However, whenever pGem/c-MycV was co-transfected with either pcDNA/HAL or pcDNA/HAL1, the level of protein expression, particularly of c-MycV, was seen to be reduced (figure 4.2.6.9). To see if this problem was specific for c-MycV and HAL or HAL1, all plasmids which were previously transfected singly as controls were now co-transfected with a null plasmid (pcDNA3), so that the same amount of DNA was being transfected in all cases. These experiments showed that, when transfected with any other plasmid, the expression of c-MycV was almost eliminated (figure 4.2.6.10). The same experiments were performed using separate CsCl maxi-

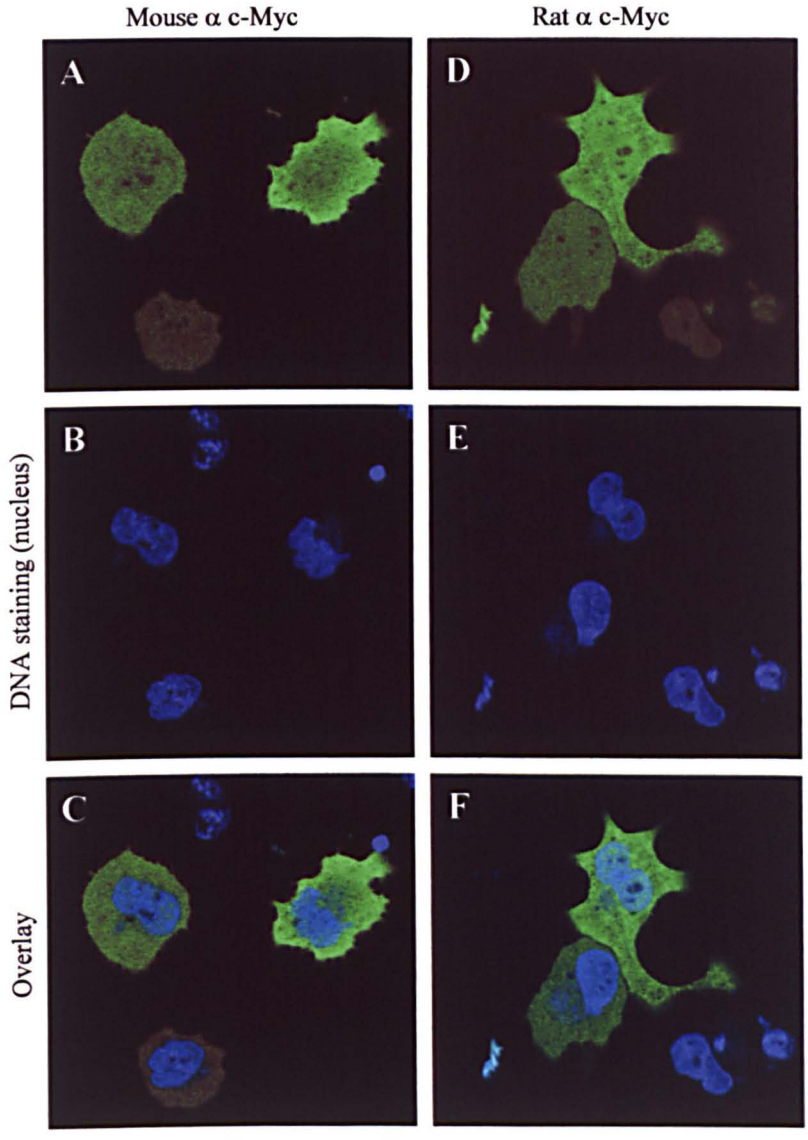

Figure 4.2.6.6. Immunofluorescence confocal microscopy of cells transfected with pGem/c-MycV. B95a cells were infected with vTF7-3 and transfected with pGem/c-MycV. One day post transfection, cells were fixed and permeabilised with 0.1% Triton X-100 before staining with antibody. Cells were reacted with either mouse α-c-Myc (9E10, Santa Cruz Biotechnology), at a dilution of 1/10 and the primary antibody visualised with Alexa Fluor 488 α-mouse IgG, at a dilution of 1/200 (A), or with rat α-c-Myc (Serotec), at a dilution of 1/200 and the primary antibody visualised with Alexa Fluor 488 α-rat IgG, at a dilution of 1/200 (D). B and E show nuclei of cells counter-stained using DAPI and overlay of the stains used is shown in C and F.

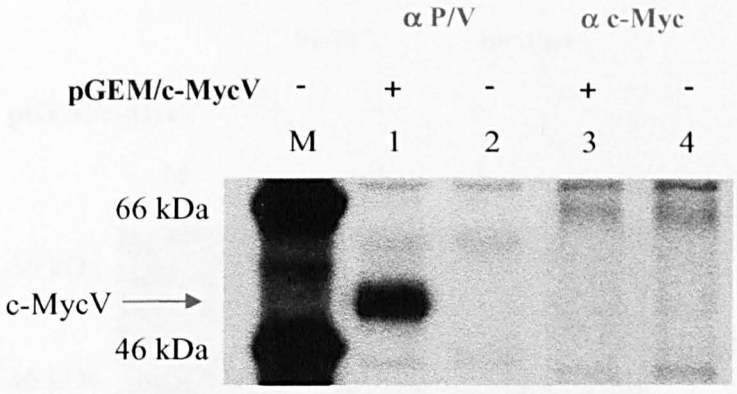

Figure 4.2.6.7. Verification of V protein expression from pGem/c-MycV. B95a cells were infected with vTF7-3 and transfected with 5μg of pGem/c-MycV; one day post transfection, the cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Cell lysates were then treated with either 0.5μl of U32 (mouse α-P/V monoclonal antibody) (tracks 1 and 2) or with 1μl 9E10 (mouse α-c-Myc monoclonal antibody, Santa Cruz Biotechnology) (tracks 3 and 4) and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels, alongside [^{14}C] labelled molecular weight markers (M). Track 1= 5μg pGem/c-MycV, U32, 2= untransfected cell lysate, U32, 3= 5μg pGem/c-MycV, 9E10 and 4= untransfected cell lysate, 9E10.

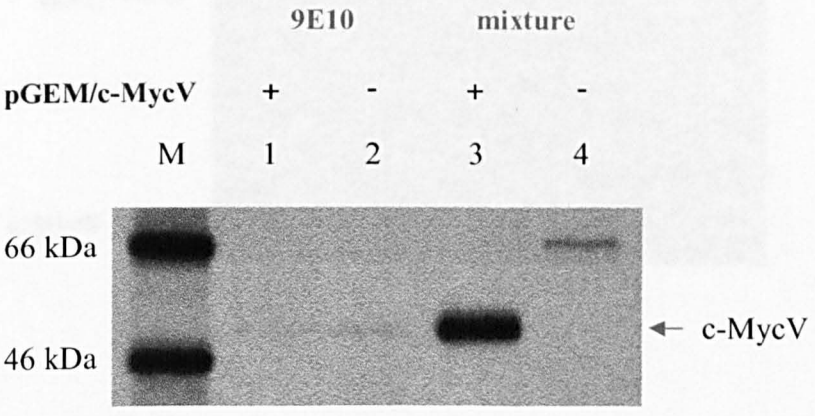

Figure 4.2.6.8. Immunoprecipitation of c-MycV protein expression from pGem/c-MycV using a combination of anti-c-Myc antibodies. A549 cells were infected with vTF7-3 and transfected with 5µg of pGem/c-MycV; one day post transfection, the cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Cell lysates were then treated with either 2µl of a previously tested aliquot of 9E10 (mouse α-c-Myc monoclonal antibody, Santa Cruz Biotechnology) (tracks 1 and 2) or with 1µl of each of 9E10 (mouse α-c-Myc monoclonal antibody, Santa Cruz Biotechnology), A-14 (rabbit α-c-Myc antibody, Santa Cruz Biotechnology) and rat α-c-Myc antibody (Serotec) (tracks 3 and 4) and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels, alongside [^{14}C] labelled molecular weight markers (M).

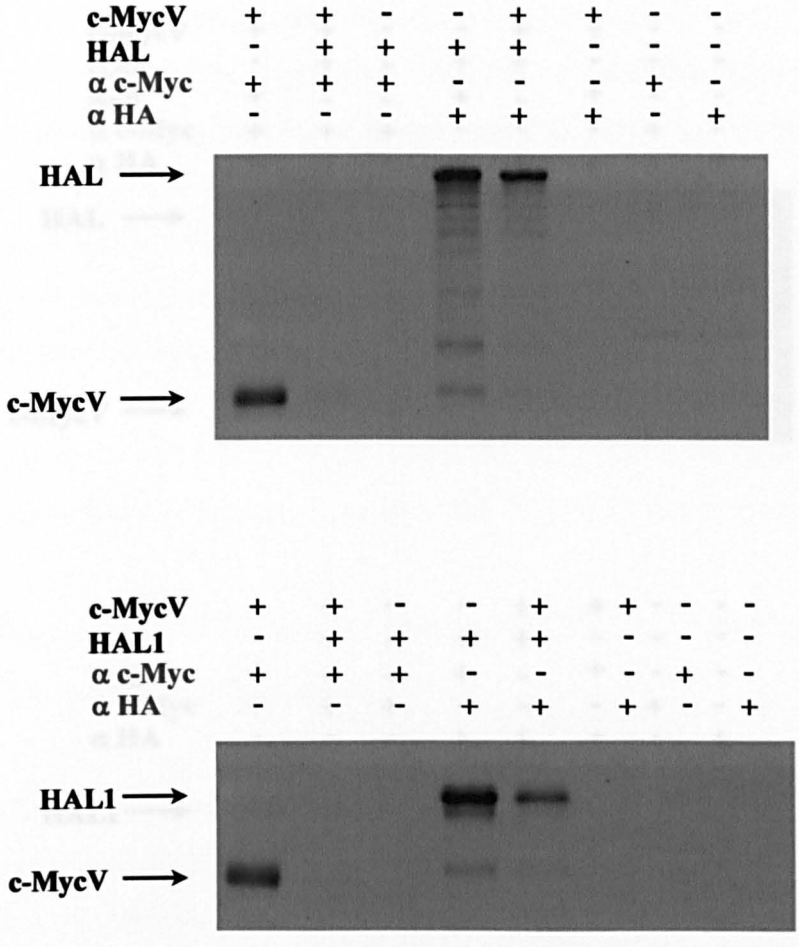

Figure 4.2.6.9. Co-transfection with pGem/c-MycV reduces levels of c-MycV expression. A549 cells were infected with vTF7-3 and transfected with the desired constructs (as indicated); one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with either 1µl rat anti-HA (Roche) or a mixture of anti-c-Myc antibodies (1µl of each of 9E10, mouse α-c-Myc (Santa Cruz Biotechnology), A-14 (rabbit α-c-Myc (Santa Cruz Biotechnology) and rat α-c-Myc (Serotec)),as indicated above, and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels.

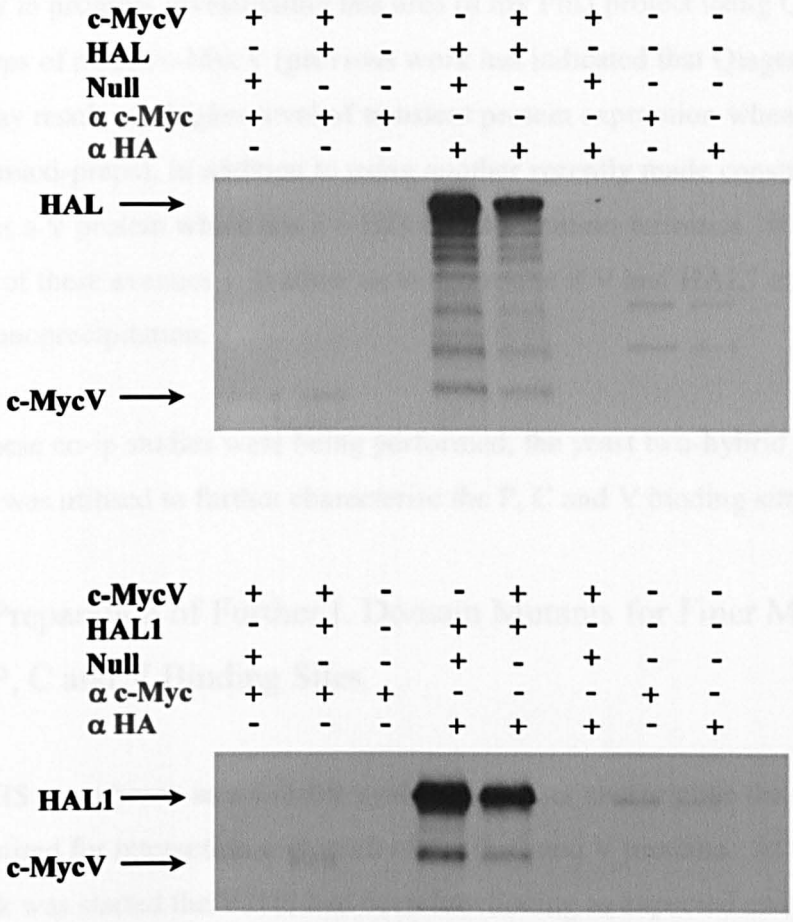

Figure 4.2.6.10. Co-transfection with a null plasmid virtually eliminates c-MycV expression. A549 cells were infected with vTF7-3 and transfected with the desired constructs (as indicated); one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with either 1µl rat anti-HA (Roche) or a mixture of anti-c-Myc antibodies (1µl of each of 9E10, mouse α-c-Myc (Santa Cruz Biotechnology), A-14 (rabbit α-c-Myc (Santa Cruz Biotechnology) and rat α-c-Myc (Serotec)),as indicated above, and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels.

preps prepared from two different clones of the pGem/c-MycV construct, but no difference was observed in the expression of c-MycV. Due to time constraints the problem of whether or not V interacts with HAL1 remains unsolved. Work is currently in progress investigating this area of my PhD project using Qiagen midi-preps of pGem/c-MycV (previous work has indicated that Qiagen midi-preps may result in a higher level of transient protein expression when compared to CsCl maxi-preps), in addition to using another recently made construct which expresses a V protein which has a 6-HIS tag at its amino-terminus. It is hoped that one of these avenues will allow us to determine if V and HAL1 interact by co-immunoprecipitation.

While these co-ip studies were being performed, the yeast two-hybrid system (Y2HS) was utilised to further characterise the P, C and V binding sites on L.

4.2.7 Preparation of Further L Domain Mutants for Finer Mapping of P, C and V Binding Sites

The Y2HS was chosen as a suitable system to further characterise the sequences of L required for interaction with each of the P, C and V proteins. At the time this work was started the Y2HS had been functioning as expected and it was thought that, when compared to co-ip analysis of protein:protein interactions, the Y2HS would require less "hands on time", which was a critical factor at this stage of the research.

To enable more exact identification of the sequences of L interacting with P, C and V, a panel of carboxy-terminal domain deletions of L was made from an existing laboratory yeast plasmid, pAS2/L. Using a combination of naturally occurring restriction sites within the L protein and *Pfu* PCR, L deletion mutants were prepared, each starting at the beginning of the L protein (figure 4.2.7.1).

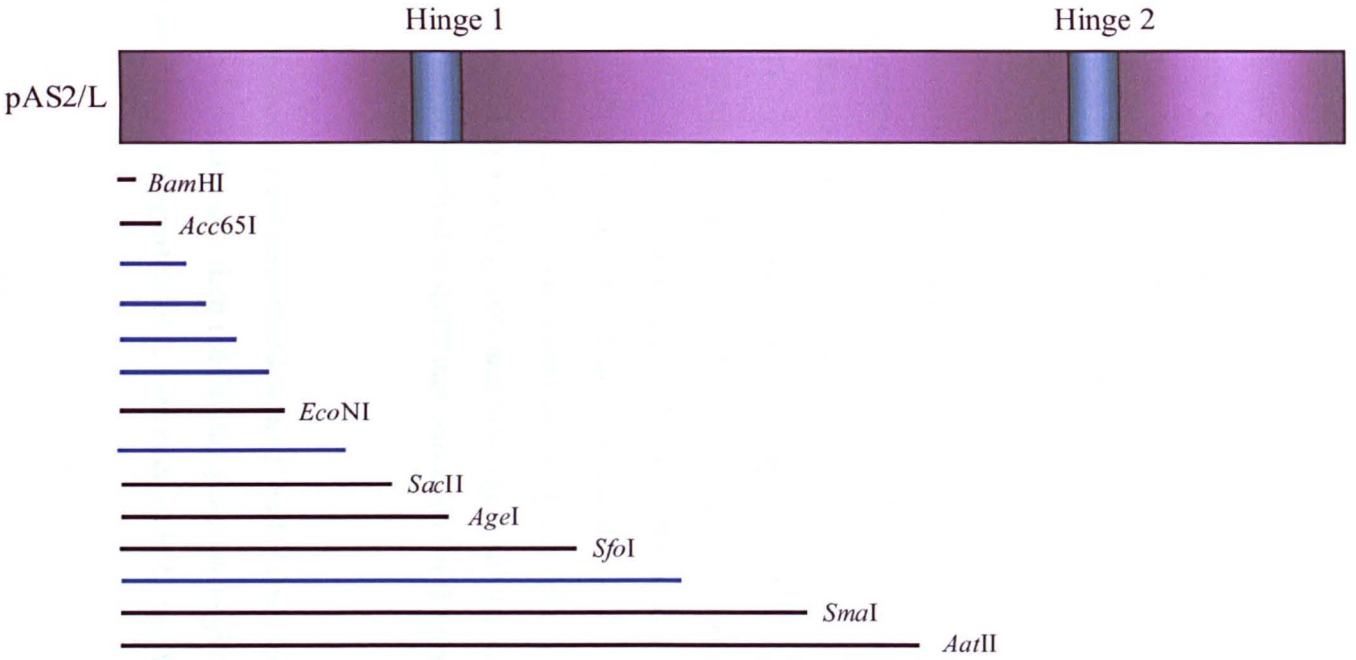

Figure 4.2.7.1. Diagrammatic representation of pAS2/L carboxy-terminal domain deletions.
Mutants denoted in black were created using restriction sites naturally occurring in L. Mutants denoted in blue were created by amplifying the coding region of interest from pAS2/L, using *Pfu* PCR, and cloning the resulting insert into a suitable pAS2 vector.

Regions of the L protein covered by each mutant and the method of construction can be seen in table 4.2.7.I. All carboxy-terminal domain deletions generated by *Pfu* PCR (see table 4.2.7.I) consisted of *Nco*I/*Sma*I inserts, which were then cloned into pAS2 (*Nco*I/*Sma*I cut). All reactions used the HAL1 start primer in addition to a specific primer designed for each mutant, and pcDNA/HAL was used as the template for each reaction performed. Deletion mutants generated by restriction enzyme digestion were digested with the enzyme indicated (see table 4.2.7.I) in addition to digestion with *Sal*I, prior to treatment with T4 DNA polymerase, which blunt-ended the batch of prepared digested plasmid. Addition of T4 DNA ligase was all that was required for a simple re-ligation.

Further to these carboxy terminal deletion mutants of L, Y2HS constructs were made which corresponded to each of the three L domain constructs prepared previously for the co-immunoprecipitation studies (see section 4.2.6), L1 (pcDNA/HAL1), L2 (pcDNA/HAL2) and L3 (pcDNA/HAL3). L1 was generated by digesting pT7blue/HAL1 with *Nco*I and *Sal*I, and ligating the resulting insert into pAS2 (*Nco*I/*Sal*I cut). The insert for L2 was produced by digesting pcDNA/HAL2 with *Eco*RI and *Xmn*I and this was then cloned into pGBT9 (Clontech) (*Eco*RI/*Sma*I cut). The insert for the L3 construct was obtained by digesting pcDNA/HAL3 with *Eco*RI and *Stu*I, the resulting insert was then cloned into the same batch of pGBT9 that was used for the production of the L2 construct.

In all cases, constructs were sequenced at the 5' and 3' ends for confirmation purposes. Each L mutant was then used in the yeast two-hybrid system for the analysis of the binding properties of L with each of P, C and V.

Clone Name	Region of L covered (bp)	Region of L covered (amino acids)	Method of Construction
L1-122	1-126	1-42	Restriction digest (*Bam*HI)
L1-360	1-336	1-112	Restriction digest (*Acc*65I)
L1-500	1-519	1-173	*Pfu* PCR L1-500rev primer
L1-700	1-699	1-233	*Pfu* PCR L1-700rev primer
L1-900	1-897	1-299	*Pfu* PCR L1-900rev primer
L1-1100	1-1089	1-363	*Pfu* PCR L1-1100rev primer
L1-1200	1-1158	1-386	Restriction digest (*Eco*NI)
L1-1500	1-1497	1-499	*Pfu* PCR L1-1500rev primer
L1-1800	1-1839	1-613	Restriction digest (*Sac*II)
L1-1900	1-1935	1-645	Restriction digest (*Age*I)
L1-2100	1-2115	1-705	Restriction digest (*Sfo*I)
L1-2400	1-2400	1-800	*Pfu* PCR L1-2400rev primer
L1-3100	1-3213	1-1071	Restriction digest (*Sma*I)
L1-3640	1-3639	1-1213	Restriction digest (*Aat*II)

Table 4.2.7.1. Carboxy-terminal domain deletions of full-length L. A panel of carboxy-terminal domain deletions of L were made using a combination of *Pfu* PCR to generate the coding regions of L required and then cloning the resulting insert into the yeast expression vector pAS2 or, using unique naturally occurring restriction sites found along the length of L, an existing full-length L yeast construct, pAS2/L, was digested with a specific enzyme, in addition to digestion with *Sal*I. Digested preparations of pAS2/L were then blunt-ended using T4 DNA polymerase and re-ligated using T4 DNA ligase.

4.2.8 Identification of More Exact Binding Regions on L for P, C and V

Once these L deletions were confirmed by sequence analysis, each of the constructs was then co-transformed using AH109 yeast cells with yeast constructs expressing each of P (pACT2/P), C (pACT2/C) and V (pACT2/V). To test for autoactivation properties, each mutant was also co-transformed with empty pACT2 vector. In all cases 200ng of each plasmid used was transformed and interactions were screened for by using two of the three available reporter genes; adenine biosynthesis and histidine biosynthesis. If a negative result was obtained for either assay, then that particular pairing of constructs was scored as not interacting with each another. None of the L deletions generated were found to autoactivate when transformed using AH109s.

Co-transformation of each of the carboxy-terminal deletions of L with P revealed that the P binding site requires the first 700bp of L (see table 4.2.8.I), mapping to between amino acids 1 and 233, which fit with the co-ip of P and HAL1 (4.2.6).

The same batch of L deletions when co-transformed with each of the non-structural proteins C and V, identified the region of L required for interaction with these two proteins lies in the first 1100bp of L, which is the equivalent to somewhere between amino acids 1 and 363 of L (see table 4.2.8.I). This was a surprising result as this region of L is well inside the first conserved domain of L. Using co-immunoprecipitations it had been revealed that the L binding sites of these two proteins lie somewhere in the second of the three conserved domains, with no hint of an interaction occurring between HAL1 and C. The interaction between HAL1 and V remains untested.

To determine which domain of L each protein bound to within the yeast two-hybrid system, the yeast L domain mutants (L1, L2 and L3) were each co-transformed with P, C and V (data not shown). As indicated from the yeast experiments with the carboxy terminal deletions of L, all three viral proteins were

RPVL Yeast Mutant	P	C	V
L1-122bp	✗	✗	✗
L1-360bp	✗	✗	✗
L1-500bp	✗	✗	✗
L1-700bp	✓	✗	✗
L1-900bp	✓	✗	✗
L1-1100bp	✓	✓	✓
L1-1200bp	✓	✓	✓
L1-1500bp	✓	✓	✓
L1-1800bp	✓	✓	✓
L1-1900bp	✓	✓	✓
L1-2100bp	✓	✓	✓
L1-2400bp	✓	✓	✓
L1-3100bp	✓	✓	✓
L1-3640bp	✓	✓	✓

Table 4.2.8.I. Interaction of carboxy-terminal deletions of full-length L with P, C and V in the yeast two-hybrid system. Using the yeast two-hybrid system each of the above L deletions were co-transformed with each of P, C and V into AH109 cells and interactions investigated. If an interaction was scored as positive, then a positive result was obtained for beta-galactosidase activity and adenine biosynthesis. If one of these assays produced a negative result, then the pairing was deemed to yield no interaction.

shown to interact with L1. In addition, no interaction was detected between L2 and any of P, C or V. This was another unexpected result, as both C and V were expected to interact with L2, based on the co-ip data. On a more positive note none of the three proteins were found to interact with L3, which was as expected. To confirm that L1 was just not interacting with any protein sequence inserted into pACT2, L1 was co-transformed with pACT2N. RPV N is known to not interact with RPV L and so no interaction between the two was expected. Co-transformants were screened for both adenine biosynthesis in addition to histidine biosynthesis, as with all the previous pairings. No interaction was detected between L1 and N, suggesting that the interaction observed between L1 and both C and V is genuine within this system.

The lack of an interaction between L2 and either C or V was an obvious problem in these mapping studies. It remains unknown why the L2 mutant was defective within this system.

Each of the co-transformations described here was performed on three separate occasions, with the same results and conclusions being drawn.

4.2.9 Demonstration of RPV L Self-Association

Early work during the start of this project involved investigating the possibility of an RPV L self-association using the Y2HS. However, despite numerous attempts to uncover an L-L interaction, no such interaction was observed within this system (data not shown). The results obtained in 4.2.8 suggested that some clear interactions (such as C and L2 or V and L2) might not be detected by the Y2HS, so co-immunoprecipitation studies were used to look for an L-L interaction. Using the vaccinia transient expression system as previously described, pGempol (5μg) and each of the three HAL domain constructs were co-transfected into B95a cells. It was clearly shown that HAL1 co-immunoprecipitates full-length L, revealing that L self-associates through interaction with the first of the three conserved domains (see figure 4.2.9.1). Unfortunately the reverse of this co-ip

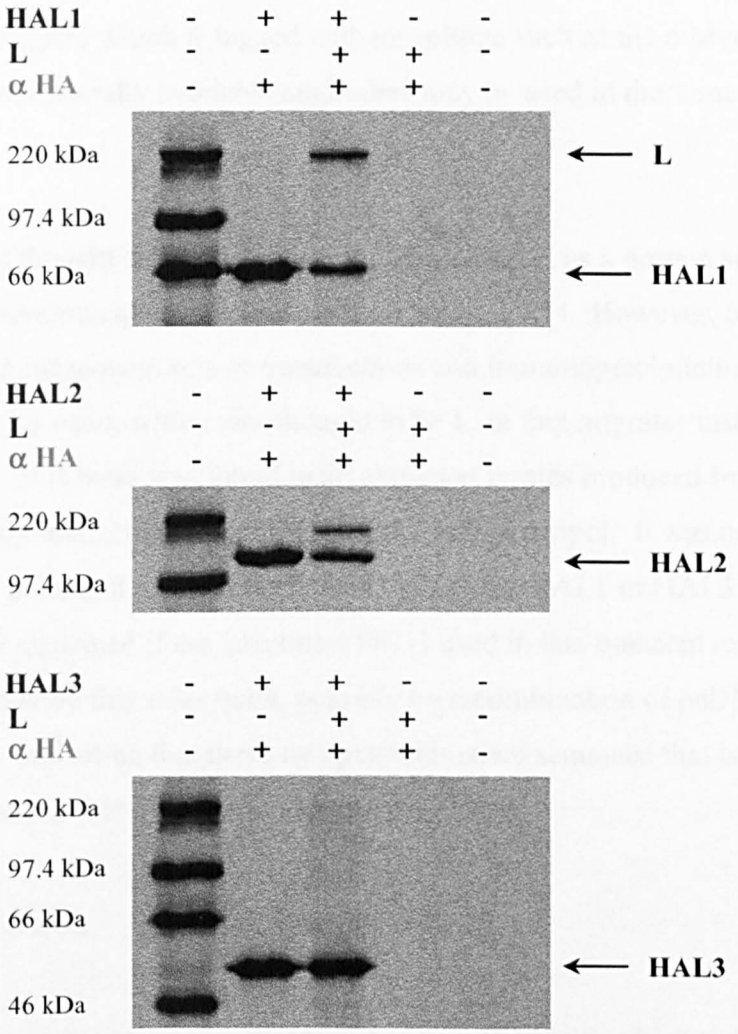

Figure 4.2.9.1. Co-immunoprecipitations of L with pcDNA/HAL domain constructs. B95a cells were infected with vTF7-3 and transfected with the desired constructs (as indicated); one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with 1µl monoclonal rat α-HA antibody (Roche) and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels, alongside [^{14}C] labelled molecular weight markers (the first track on each gel).

could not be demonstrated due to the fact that the only antibody available against the L protein, MB41, is unsuitable for immunoprecipitations as it generates a lot of background. To confirm this interaction, a further full-length L construct needs to be designed which is tagged with an epitope such as the c-Myc epitope, so that two commercially available antibodies may be used in the same experiment.

Initially it was thought that L also interacted with HAL2, as a protein similar to L was co-immunoprecipitated with HAL2 (figure 4.2.9.1). However, on closer inspection and subsequent sets of transfections and immunoprecipitations, it was revealed that this band, which was thought to be L, in fact migrates faster than full-length L. This band was found in all extracted lysates produced from cells which were co-transfected with pcDNA/HAL2 and pGempol. It was not seen in lysates where pGempol was co-transfected with either HAL1 or HAL3. These data would be explained if the vaccinia vTF7-3 used in this transient expression system is producing this extra band, possibly by recombination of pcDNA/HAL2 and pGempol, indicating that these two plasmids share sequence that is particularly susceptible to recombination.

4.3 Discussion

Co-immunoprecipitation studies were used as a method with which to further characterise known protein:protein interactions of the rinderpest L protein. Before any immunoprecipitations could be performed it was necessary to make new protein expressing constructs in the pcDNA vector. Expression of the P and N constructs was easily optimised, but good levels of L and C protein expression were difficult to obtain. Expression levels of L were extremely low when driven by the CMV promoter, but when the T7 promoter was used a good level of L protein expression was seen. Attempts to increase the level of protein expression included the utilisation of known chemical stimulators of the CMV promoter, in addition to a commercially available plasmid, pAdVAntage, which acts to increase the levels of transient protein expression in transfected mammalian cells.

In an effort to achieve expression of the C protein, several combinations of lysis buffers and protease inhibitors were tried without success, even when expression was driven from the CMV promoter. Previous work in the laboratory had shown that when C protein expression was performed using the vaccinia T7/T7 promoter system, the protein was found in a predominantly degraded form, which is why the CMV promoter was initially used for this work. However, a change of cell line resulted in good expression of C, even when driven off the T7 promoter using Vaccinia T7, which also eliminated the problem of poor L expression.

During the course of these studies it was noticed that co-expression of RPV N with pcDNA/HAL resulted in an increase in the level of HAL protein expressed from transiently transfected cells. The precise reason for this remains unclear. It was shown that N is not exerting this effect on HAL expression by stabilising the HAL protein. N could however be stimulating plasmid uptake to the nucleus, or acting to upregulate transcription levels within the transfected cell. Further work could include investigating whether or not this effect of N on protein expression is specific to HAL. Preliminary data has indicated that N also boosts the level of P protein expression (data not shown). It would be interesting to see if this was an effect confined to members of the genus *Morbillivirus*, or if RPV N could act to increase the level of protein expression from a totally unrelated virus, such as African swine fever virus (ASFV).

To confirm the previously established interactions of L with P, C and V (Sweetman *et al.*, 2001), co-immunoprecipitations were performed with full-length L and each of P, C and V. Each of these three interactions were confirmed, although a GST-tagged version of the V protein had to be used, as a pcDNA construct expressing the V construct failed to co-ip full length L within this system. The most likely explanation for this unexpected observation is that the anti-V antibody, which was being used for the immunoextractions of cell lysates transfected with pcDNA/V, was displacing the L protein. Due to the large size of the L protein, it was first decided to determine with which of the three conserved domains of the L protein P, C and V interact. To facilitate this, three initial domain mutants of L were designed and constructed, HAL1, HAL2 and

HAL3. Each of these three constructs contained one of the conserved domains, as determined by McIlhatton and co-workers (1997) (McIlhatton *et al.*, 1997), and the appropriate hinge region(s).

Using co-immunoprecipitation studies P was found to interact with the first conserved domain of the L protein, whilst the two non-structural proteins, C and V, were found to interact with the second of the three conserved domains of L. Using the Y2HS, further deletion mutants of L were investigated for their binding properties with the viral proteins P, C and V. These yeast studies should have confirmed the preliminary binding site data obtained by co-immunoprecipitations (section 4.2.6). Experiments using the Y2HS confirmed that P binds to the first conserved domain of L, as identified by co-immunoprecipitation. However, unlike the co-immunoprecipitation data, which identified C and V as interacting with HAL2, the second conserved domain of L, the Y2HS showed that C and V both interact with the L1 yeast mutant and not with L2. One explanation for these data might be the existence of a secondary binding site for these two non-structural proteins within the first conserved domain of the L protein, somewhere between amino acids 1 and 363 (and therefore distinct from the P binding domain).

It is difficult to explain why the C-L2 and V-L2 interactions were not picked up within this system. It could be due to the fact that one or both of the proteins are being mis-folded, or that they are not being taken to the yeast nucleus, which as mentioned in chapter three, is a requirement for the functionality of the Y2HS. The problems encountered with the L2 yeast construct failing to show an interaction with either C or V, together with the fact that both the C and V L binding sites were mapped to a region within the first domain of L suggest that the Y2HS is not a reliable system with which to map protein:protein interactions. The Y2HS is known in some cases to not detect previously characterised protein:protein interactions. An example of this is that an interaction between RPV N and P was never shown in the Y2HS, despite numerous attempts (Sweetman, 2000), although this interaction is necessary for the formation of viral nucleocapsid complexes.

The accessory proteins C and V both appear to have an additional L binding site, somewhere in the first 1100bp of L. This interaction was picked up by the Y2HS only and could indicate that this interaction is weak. The Y2HS is known to be very sensitive and is capable of detecting both weak and transient interactions. Co-ips on the other hand are known to detect physiologically relevant protein:protein interactions, and it is acknowledged that low affinity or transient protein:protein interactions may not be detected. It is therefore possible that the interactions seen in the Y2HS, between L1-1100 and C and V are either artefacts of the highly artificial nature of the system, or evidence of a secondary L interaction site for these two proteins.

As it was recently shown that the L protein of SeV oligomerises (Smallwood *et al.*, 2002), an RPV L-L interaction was investigated. To determine if such an interaction exists for the rinderpest L protein, a combination of the Y2HS and co-immunoprecipitation studies were performed. Once again conflicting results were obtained from these two systems. The Y2HS failed to provide any evidence for an L-L interaction (data not shown), whilst clear co-immunoprecipitation of L with HAL1 was observed. As previously mentioned, it is more likely that the interaction seen by co-immunoprecipitation is genuine, rather than the negative result obtained with the Y2HS. Further work now needs to be carried out to further map the binding region for this L-L self-association.

Chapter Five

Identification of interactions of RPV L with Host Cell Proteins using the Yeast Two-Hybrid System (Y2HS)

5.1 Introduction

To enable the identification of novel host cell proteins that interact with the L protein of rinderpest virus, the yeast two-hybrid system was utilised. The Y2HS, originally developed nearly fifteen years ago by Fields and Song (Fields & Song, 1989), has been widely used to identify novel protein:protein interactions, in addition to enabling the further characterisation of known interactions between proteins. The Y2HS allows the identification of novel protein binding partners of a known protein by the screening of expression libraries and it is this function which was to be exploited in this part of my PhD project. This system should therefore allow a large number of host cell proteins to be screened for an interaction with L in a short period of time.

The functionality of the Y2HS relies on the fact that transcriptional activators can be separated into discrete regions corresponding to DNA-binding domains and transcriptional activation domains (Brent & Ptashne, 1985, Hope & Struhl, 1986). To assess protein:protein interactions, the Y2HS uses two protein fusions, which are each expressed from a separate plasmid vector, and which are co-expressed in a suitable host strain of yeast containing inducible reporter genes. The protein of interest, the "bait" protein, is encoded as a fusion to a DNA-binding domain, and for the purpose of these studies the RPV L protein is the bait. The "prey" protein, on the other hand, is fused to a transcriptional activation domain and in this case the "prey" corresponds to proteins encoded by the porcine macrophage cDNA library. If an interaction occurs between the bait and the prey, then the transcriptional activator will be reconstituted, leading to the transcription of the reporter genes present within

the host strain, thereby generating a phenotypic signal. Reporter gene products can be easily assayed on the basis of nutritional requirements of the transformed yeast cells, or on the basis of a colour change, such as that scored in a beta-galactosidase activity assay.

At the onset of this work the Y2HS was thought to be a reliable and robust system with which to study protein:protein interactions. However, during a late stage of the screening process, problems became apparent with this system.

5.2 Results

5.2.1 Performing Large-Scale Library Screen

Initial experiments with the Y2HS were performed using the Y190 strain of yeast cells, which had previously been used in the laboratory (Sweetman *et al.*, 2001) however, this particular stock of yeast cells started to exhibit the unusual characteristic of growing very slowly. This resulted in the purchase of a more recent yeast strain from Clontech, the AH109 strain of yeast. AH109's are considered to be a superior strain of yeast when compared to the Y190's, as it has three selectable markers, one more than the Y190's. More importantly, each of the three reporter genes are under the control of different GAL4-inducible promoters, thereby theoretically eliminating virtually all false positives specific to the promoter element in the GAL4 system (James *et al.*, 1996). It was therefore expected that the number of false positives obtained with this library screen would be dramatically reduced, from an early stage of the screening process. However, before a library screen could be performed, the transformation efficiency had to be optimised for AH109's, as a high number of transformants are necessary for a successful library screen. To optimise the transformation efficiency, a known interactor of L was used (C) and varying quantities of plasmid DNA were co-transformed. Either 50ng, 100ng, 200ng, 300ng or 500ng of each of the two plasmids being co-transformed was

utilised. Clontech recommend that 100ng of plasmid be used for each transformation. However, due to the large size of L, the transformation efficiency of pAS2/L alone was found to be significantly lower than that of any other pAS2 construct which contained the coding sequence for a protein considerably smaller than L, for example pAS2/C (data not shown). Following optimisation it was decided that 200ng of each plasmid being co-transformed would be utilised for small-scale transformations, this figure was then adjusted according to the scale of the transformation being undertaken.

As the transformation efficiency had been optimised, a full-scale library screen could now be performed (as in section 2.4.3), see figure 5.2.1.1. The L protein was provided by the existing laboratory pAS2/L construct (Sweetman *et al.*, 2001), as used in chapter four. The porcine macrophage cDNA library was a kind gift from Dr. L. Dixon (Department of Molecular Biology, Institute for Animal Health, Pirbright, Surrey, UK), and stocks were grown as in section 2.4.1. For the library screen approximately 1mg of the porcine macrophage cDNA library was used along with 600µg of pAS2/L.

Initial co-transformants were screened for cells positive for the *HIS3* gene product (histidine biosynthesis), as described in section 2.4.2. After five days the transformation yield of the library screen was calculated to be 2.6×10^5, meaning that 260, 000 clones of the library had been screened. After ten days the number of putative L interactors obtained from the library screen was 57. Each of these 57 colonies was picked with a sterile P10 tip and used to inoculate 20µl of SC-L-T liquid medium and 5µl of this was used to inoculate each of two SC-L-T grids, and an SC-L-T-Ade grid. The grids referred to here are just agar plates each with a grid drawn on the back. Each square of the grid was inoculated with a single colony, therefore allowing a large number of colonies to be screened at any one time. Two SC-L-T grids were required as one acted as a master plate containing all colonies under selection for both interacting plasmids, and also to confirm the presence of two

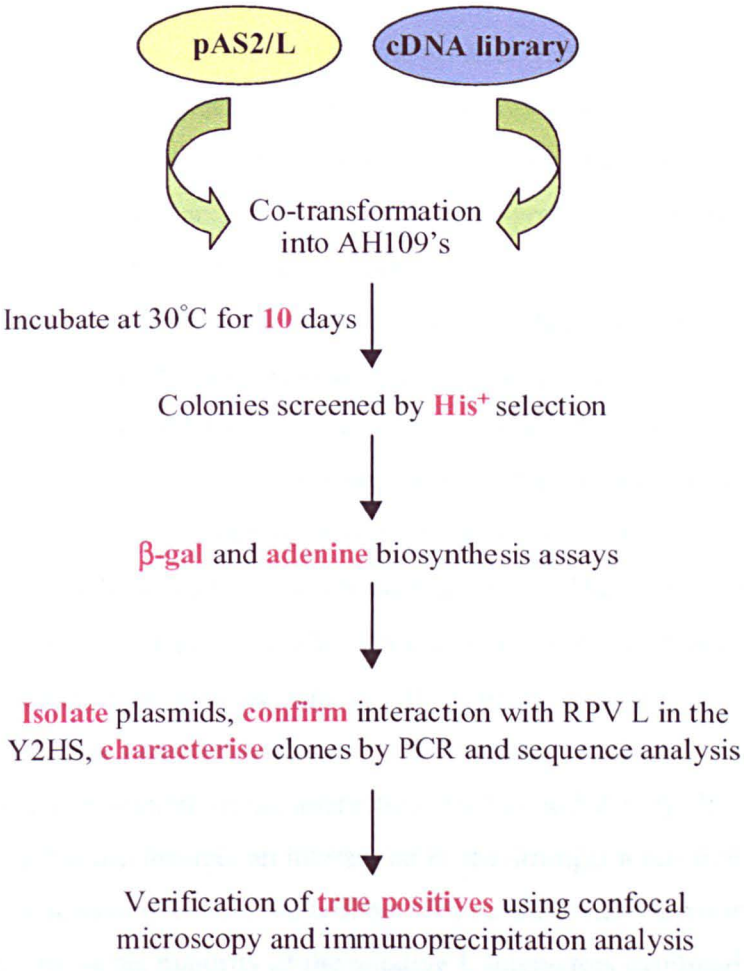

Figure 5.2.1.1. Diagrammatic representation of a library screen using RPV L as the "bait" protein. AH109 yeast cells were co-transformed with pAS2/L and a porcine macrophage cDNA library. Transformants were plated out on minimal agar lacking histidine and incubated for a period of 10 days at 30°C. Resulting colonies were then cultured and further screened using both adenine biosynthesis and beta-galactosidase assays as indicators of a functional interaction. If the library clones isolated provided positive scores under all three selectable reporter genes tested, then they were taken through to the next stage of the screening process. Prey plasmids were then isolated and put back into the Y2HS to confirm an interaction with L. cDNAs of positive plasmids were then characterised by PCR and sequence analysis. To determine if a genuine interaction with RPV L occurs outside of the yeast two-hybrid system, other methods were employed.

plasmids; the second of the SC-L-T grids was used for the beta-galactosidase assay which was performed at a later stage. The SC-L-T-Ade grid was to select for transformed cells positive for the *ADE2* gene product (required for adenine biosynthesis) and was considered to be the most stringent selection assay available in determining a true interaction between L and the isolated library clone. After an incubation period of 24 hours, all 57 colonies had re-grown on both SC-L-T grids, as expected. One of the inoculated SC-L-T grids was then used for beta-galactosidase assays (as described in section 2.4.4, for results see table 5.2.1.I). Colonies that turned blue after an incubation period as long as overnight were still scored as positive, due to the fact that the negative controls remained unchanged in colour. In addition, growth on the SC-L-T-Ade grids was scored after 24hrs, 48hrs and 72hrs (see table 5.2.1.I). Only colonies positive for all three reporter genes tested were taken through to the next stage of the screening process. Therefore at this stage, 12 colonies were eliminated as they scored a negative result for either adenine biosynthesis or beta-galactosidase activity. The remaining 45 colonies were then divided into 4 groups based on the growth characteristics displayed, which served to indicate the relative strength of the interactions (see table 5.2.1.II). It is generally acknowledged that the stronger an interaction is, the stronger a positive result obtained under screening conditions will be. The results, when taken in this context, looked promising as the majority of the putative L interactors exhibited a strong beta-galactosidase activity score in addition to strong growth under adenine biosynthesis selection.

Clone	SC-L-T regrowth	SC-L-T-Ade	Beta-galactosidase activity
1	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓
2	✓ ✓ ✓	✓ ✓ ✓	✓ ✓
3	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
4	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
5	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
6	✓ ✓ ✓	×	✓
7	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓
8	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
9	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
10	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓
11	✓ ✓ ✓	✓ ✓ ✓	×
12	✓ ✓ ✓	✓ ✓ ✓	✓
13	✓ ✓ ✓	✓	✓ ✓
14	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
15	✓ ✓ ✓	✓ ✓ ✓	✓ ✓
16	✓ ✓ ✓	×	✓ ✓ ✓ ✓
17	✓ ✓ ✓	✓ ✓	✓ ✓ ✓
18	✓ ✓ ✓	✓ ✓	✓ ✓
19	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓
20	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
21	✓ ✓ ✓	✓ ✓ ✓	✓
22	✓ ✓ ✓	✓ ✓ ✓	✓ ✓
23	✓ ✓ ✓	✓ ✓ ✓	×
24	✓ ✓ ✓	✓ ✓	✓ ✓ ✓
25	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓
26	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓
27	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
28	✓ ✓ ✓	×	×
29	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓
30	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓
31	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
32	✓ ✓ ✓	✓ ✓ ✓	×
33	✓ ✓ ✓	✓ ✓ ✓	×
34	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
35	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓

Clone	SC-L-T regrowth	SC-L-T-Ade	Beta-galactosidase activity
36	✓ ✓ ✓	✓	✗
37	✓ ✓ ✓	✓ ✓ ✓	✗
38	✓ ✓ ✓	✓ ✓ ✓	✓ ✓
39	✓ ✓ ✓	✗	✗
40	✓ ✓ ✓	✗	✓
41	✓ ✓ ✓	✓ ✓ ✓	✓ ✓
42	✓ ✓ ✓	✓ ✓ ✓	✓
43	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
44	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓
45	✓ ✓ ✓	✗	✓
46	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
47	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
48	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
49	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
50	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓
51	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓
52	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
53	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓
54	✓ ✓ ✓	✓ ✓ ✓	✓ ✓
55	✓ ✓ ✓	✓ ✓ ✓	✓ ✓
56	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓ ✓ ✓
57	✓ ✓ ✓	✓ ✓ ✓	✓ ✓ ✓

Table 5.2.1.I. Growth characteristics of isolated library clones. Co-transformants selected for by histidine biosynthesis were further screened by re-culturing colonies on media maintaining the selection for both plasmids (SC-L-T). Resulting colonies were then assayed for both adenine biosynthesis (SC-L-T-Ade) and β-galactosidase activity. SC-L-T and SC-L-T-Ade key: ✓ ✓ ✓ indicates strong growth after 24hrs, ✓ ✓ growth after 48hrs and ✓ strong growth after 72hrs, ✗ no growth at 72hrs. Beta-galactosidase activity key: ✓ ✓ ✓ ✓ ✓ blue coloration after 2hrs, ✓ ✓ ✓ ✓ blue after 3hrs, ✓ ✓ ✓ blue after 5hrs, ✓ ✓ blue after 7hrs, ✓ blue after overnight incubation.

Group	Adenine biosynthesis	β-galactosidase activity	Number of positive clones
1	✔ ✔ ✔ ✔ ✔	Blue within 3hrs	25
2	✔ ✔ ✔ ✔	Blue within 5hrs	8
3	✔ ✔	Blue within 7hrs	9
4	✔	Blue after overnight incubation	3

Table 5.2.1.II. Positive library clones divided into four individual groups based on growth characteristics. (Which may indicate the relative strength of the interaction between the isolated library clone and RPVL).

5.2.2 Recovery of Putative L Interactors

To facilitate the retrieval of possible L interactors, yeast plasmid DNA was isolated from yeast cultures. Colonies cultured on SC-L-T agar were picked with a sterile P10 tip and used to inoculate 5ml SC-L liquid medium and incubated for 3-5 days at 30°C on an orbital shaker set at 250rpm. SC-L (synthetic complete medium lacking leucine) medium was used to culture the isolated library plasmid as this maintained selection for only the library plasmid, which contains the *LEU2* gene. Yeast plasmid DNA was then extracted using a combination of either acid washed glass beads and phenol chloroform (as in section 2.4.5) or the "Yeastmaker" yeast plasmid isolation kit (Clontech, as in section 2.4.6). To confirm the successful extraction of yeast plasmid DNA from the cultures, plasmid inserts were amplified using *Taq* PCR (as in section 2.2.1.2) with primers (pACT23' and pACT25'), specific for the yeast library plasmid, pACT2 (see appendix 3 for primer sequences). When PCR products were analysed on agarose gels, it was noted that several of the isolated colony cultures contained more than one library plasmid (figure 5.2.2.1). Despite three separate attempts, from three separate cultures, no PCR product was ever isolated from library clones 5, 9, 17, 21, 22, 26, 27, 31 or 56, taking the number of putative L interactors obtained from the library screen down to 36 (see table 5.2.2.I). However, due to the fact that some of the cultures contained more than one isolated library plasmid, the number of plasmids requiring confirmation rose to a total of 46 clones. The presence of multiple library plasmids in a single culture is not uncommon at this stage of a library screen.

Once the presence of a library insert was confirmed in each DNA preparation, it was necessary to transform the yeast plasmid DNA into bacteria to permit cloning of the library plasmid and identification by DNA sequence analysis. To enable isolation of the library plasmid it is widely thought that *E. coli* strains carrying a *leuB* gene

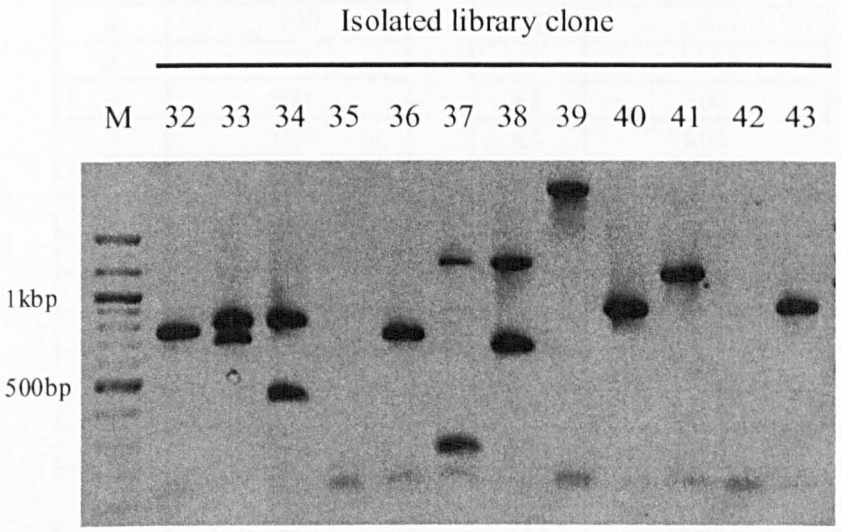

Figure 5.2.2.1. Examples of *Taq* PCR products generated from isolated positive yeast plasmids from a yeast-two hybrid system library screen. Samples of *Taq* PCR products generated from isolated yeast plasmid DNA were analysed on 0.8% agarose gels in TBE buffer alongside 100bp ladder molecular weight markers (M, Roche). It was noted that several of the DNA preparations contained more than one yeast plasmid, for example clones 33, 34, 37 and 38.

Clone	*Taq* PCR product size (bp)
1T	~900
1B	~650
2	~400
3	250
4	500
7	1000
8	200
10	~450-500
12	~1050
13	1200
14	200
15	650
18T	~1100
18B	~950
19T	700
19B	200
20	200
24	200
25	200
29	200
30	500
34T	800
34B	500

Clone	*Taq* PCR product size (bp)
35	250
38T	1100
38B	~700
41	1050
42	200
43	800
44	200
46T	300
46B	200
47T	300
47B	200
48	200
49	250
50T	600
50B	500
51T	1900
51B	800
52T	1901
53	250
54T	1150
54B	850
55	~550
57	1180

Table 5.2.2.I. Size of *Taq* PCR products amplified from isolated positive yeast library plasmids from a yeast two-hybrid system library screen. Cultures that were found to contain more than one plasmid resulted in the clones being named as follows: the number of the clone was suffixed with either a T (e.g. 34T) or a B (e.g. 34B). The T refers to the larger of the two products produced by PCR, whereas the B denotes the smaller product amplified from any one yeast clone.

mutation are required. This is due to the fact that the library plasmid, pACT2, contains the *LEU2* gene which complements the *leuB6* mutation seen in *E. coli* strains such as HB101 and KC8. This is important, as it was the prey that is being selected and not the bait (RPV L). It is also generally accepted that yeast plasmid DNA requires electroporation for successful transformation to occur. Taking this information into account, competent HB101s (a kind gift from L. Goatley, Department of Molecular Biology, Institute for Animal Health, Pirbright, Surrey, UK) and DH5αs (used as a positive transformation control), were used for electroporation (as in section 2.2.11). However, no transformants were obtained. To determine if home-made electrocompetent cells were at fault, commercially available electrocompetent KC8 cells (Clontech) were next tried. However a change of cell line did not alter the outcome and no electroporation was successful.

Attempts were next made to transform the isolated yeast plasmid DNA using chemically competent HB101 and KC8 cells (cells prepared as in section 2.2.7, transformations as in section 2.2.9). Despite several efforts this approach remained unsuccessful, due to the low level of transformation obtained with these cells using our standard method of making competent cells. It was then decided to try transforming chemically competent DH5α's (as in sections 2.2.7 and 2.2.8) which do not solely select the library plasmid. It was therefore feared that the majority of transformants recovered would consist of the "bait" L protein only. For each transformation using the DH5α's, 2μl of isolated yeast plasmid DNA was used and final transformation mixes were plated out on the usual LB/Amp plates and incubated overnight at 37°C. This method proved to be reasonably successful with approximately 50% of the transformants containing the prey plasmid. Although transformation efficiency did vary considerably between yeast plasmid DNA samples, this variability is most likely due to the crude DNA preparations being used. Once this transformation difficulty was overcome, the majority of putative L interactor plasmids were retrieved. Out of the remaining 46 isolated library clones, I was unable to recover 9 using this system (clones 3, 7, 8, 10, 29, 47T, 47B, 48 and

49). Due to time constraints and the large number of clones that were recovered, these samples were stored as -70°C glycerol stocks. The final 37 recovered library clones were all grown up and CsCl maxi-preps were made for each one (as in section 2.2.14). CsCl DNA preparations were necessary as maxi-preps purified using Qiagen columns were found not to function in the Y2HS during earlier studies as part of this project (data not shown).

5.2.3 Identification and Classification of L Interactors

The majority of the isolated putative L interactors were sequenced and this was done using either sample from PCR products, or sample from DNA maxi-preps of recovered library plasmids. Isolated library clones were sequenced by either T7 polymerase sequencing (as described in section 2.2.15.1) or by cycle sequencing (as in section 2.2.15.2). In all cases the ACT25' primer was used (for primer sequence see appendix 3).

Once obtained, nucleotide sequences were submitted to an NCBI BLAST search to identify the putative L interacting partner (see table 5.2.3.I). As a result of the sequence analysis, many of the remaining clones were theoretically eliminated due to published literature on common false positives found using yeast library screens (http://chaos.fccc.edu/research/labs/golemis/main_false.html accessed on 27/11/2002 at 12:37pm, http://chaos.fccc.edu/research/labs/golemis/Table1.html, accessed on 27/11/2002 at 12:41pm). According to the data compiled by that laboratory, ferritin, cytochrome oxidase C (COX proteins) and zinc-finger proteins are amongst those which most commonly occur as false positives when performing yeast two-hybrid system library screens. The following clones were therefore considered to be probable false positives at this stage; clones 19T, 34T, 38B, 41 (this protein has a zinc-finger domain), 52 and 57. Clones 4, 14, 20, 24, 30, 34B, 51T and 51B were also considered to be probable false positives as they were all identified to be either GAL4 gene sequences or parts of cloning vectors.

Clone	Identity
1T	mouse thymus cDNA
1B	no significant matches
2	no significant matches
3	no significant matches
4	Drosophila GAL4 gene
12	human cDNA clone HEP08253
13	human cDNA
14	pACT2 MCS
15	human DNA sequence from RP11-54A4
18T	Numerous porcine clones
18B	no significant matches
19T	cox subunit VIIb
19B	no significant matches
20	pACT2 MCS
24	pHELLSGATE cloning vector
25	no significant matches
30	GAL4 ACTivation Domain
34T	bovine/pig ferritin heavy chain
34B	*S. cerevisiae* GAL4 gene

Clone	Identity
35	human striatin
38T	human helicase mRNA
38B	human cox VIIb mRNA
41	dorfin-double ringfinger protein
42	human ARX protein mRNA
43	pig platelet binding protein
44	short porcine type I IFN related protein
46T	no significant matches
46B	no significant matches
50T	sheep beta actin
50B	no significant matches
51T	*S. cerevisiae* GAL4 Gene
51B	GAL4 ACTivation domain
52	cox subunit VIIb
53	Sequence 21 from patent WO0208456
54T	Human hexokinaseII mRNA
54B	sequence 81 from patent WO0210453
55	human TRAF6 binding protein
57	human coxII protein/homologue

Table 5.2.3.I. Identification of the remaining putative L interactors as determined by DNA sequencing. Library clones were sequenced using either T7 polymerase sequencing or cycle sequencing. Sequences obtained were submitted to a nucleotide NCBI blast search; the match with the highest score for each clone is shown. In some instances no significant matches were found on the databases searched. Highlighted clones were **not** taken through to the next stage.

5.2.4 Determination of "True" Interactors within the Y2HS

All recovered yeast plasmids were put back into the yeast two-hybrid system to not only confirm an interaction with RPV L in yeast, but to also check the library clones for auto-activation properties. Each library plasmid (200ng) was therefore co-transformed with pAS2/L and, to test for auto-activation properties, each library plasmid was also co-transformed with empty pAS2 vector. In addition, the specificity of the interaction of each library clone with L was tested by co-transformation with pAS2/C. All interactions were screened using the three separate reporter genes of AH109s. The result obtained for each co-transformation was reproduced on three separate occasions. The majority of clones were found to either no longer interact with L when put back into the Y2HS, or be auto-activators (see table 5.2.4.I).

Library clones shown to be non-auto-activators and which failed to interact with L when put back into the Y2HS are difficult to explain. Experiments were set up on two separate occasions using fresh stocks of AH109 cells, but the results obtained were the same. In all cases the positive control pairing of pAS2/L and pACT2/C continued to work, although the strength to the interaction, as measured by the speed of colony growth on SC-L-T-Ade media, was found to be erratic. All colonies containing both L and C should have provided a positive result, yet this was not seen. Co-transformants isolated from SC-L-T plates were found to only provide a positive result in either adenine biosynthesis or beta-galactosidase activity about 50% of the time. It had been noticed that the AH109s grew at unreliable rates when cultured for any period of time; as a result experiments were set up using both Y190s and AH109s in duplicate. Results obtained were inconclusive, as the positive control of pAS2/L and pACT2/C proved to be unreliable in the reporter assays.

Clone	Auto-activator?	Interaction with L?
1T	yes	N/A
1B	yes	N/A
2	yes	N/A
4	yes	N/A
12	no	yes
13	no	yes
14	yes	N/A
15	no	no
18T	no	no
18B	no	no
19T	no	no
19B	no	no
20	Not tested	Not tested
24	Not tested	Not tested
25	no	no
30	yes	N/A
34T	yes	N/A
34B	no	no
35	no	yes

Clone	Auto-activator?	Interaction with L?
38T	no	no
38B	no	no
41	no	no
42	yes	N/A
43	yes	N/A
44	no	no
46T	no	no
46B	no	no
50T	no	no
50B	no	no
51T	yes	N/A
51B	no	no
52T	yes	N/A
52	no	no
53	no	no
54T	yes	N/A
54B	no	no
55	yes	N/A
57	yes	N/A

Table 5.2.4.1. Yeast two-hybrid system analysis of the specificity and auto-activation properties of recovered putative L interactors. Recovered library clones were tested for auto-activation properties in addition to confirming an interaction with L within the yeast two-hybrid system. Highlighted clones were confirmed to interact with L and found not to auto-activate.

Despite these problems, three library plasmids, clones 12, 13 and 35, were identified as interacting with L and were therefore taken through to the next stage of analysis. Sequence analysis of the clones (table 5.2.3.I), identified only clone 35 as a known protein; it was shown to be 92% similar to the human striatin protein after an NCBI BLAST nucleotide search (see figure 5.2.4.1 for sequence alignment). Striatin was also confirmed to be expressed from clone 35, in the correct reading frame (see figure 5.2.4.2). To date little is known about the host cell protein striatin. It is however known that striatin is a highly conserved intracellular protein which is mostly membrane associated. Striatin is mainly expressed in the central nervous system (Castets *et al.*, 1996). The interaction between L and striatin was further characterised.

5.2.5 Investigating the Interaction of L with Striatin Using Co-Immunoprecipitation Studies

Co-immunoprecipitation studies were performed as a method with which to characterise the potential interaction of L and striatin in a system outside of the Y2HS. To determine if L co-ips full length striatin, a construct was produced by manipulating the construct pSVLHAStriatin (a kind gift from Francis Castets, FRE 9041, CNRS, 31 Chemin Joseph Aiguier, Université de la Méditerranée, Marseille, 13402 Marseille Cedex 20, France), which contains the rat striatin ORF. pSVLHAStriatin was digested with *Nco*I and *Bam*HI to produce a full length striatin insert, which was then ligated into pGST(*Nco*I/*Bam*HI cut) to produce pGST/striatin. The resulting construct was confirmed by sequence analysis and expression verified by immunofluorescence (data not shown). It was decided to use GST as a way to detect the striatin protein as it had been previously shown that full-length L does not interact with GST (Sweetman *et al.*, 2001) and it was demonstrated in section 4.2.6 that none of the three pcDNA/HAL constructs interact with GST. Using A549 cells, pGST/striatin and pcDNA/HAL were transfected either singly or together, in duplicate, and the proteins expressed using the vaccinia T7/T7 expression system (as

```
>gi|4507282|ref|NM_003162.1| Homo sapiens striatin, calmodulin binding protein (STRN), mRNA
          Length = 2790

 Score =  319 bits (161), Expect = 4e-84
 Identities = 209/225 (92%)
 Strand = Plus / Plus


Query: 324 agcccagtacagcctcccgggaatcctgcacttccttcagcacgagtgggcccgcttcga 383
           ||||||||||||| ||||||||| |||||||||||||| |||||||||||||||||||||
Sbjct: 141 agcccagtacagtctcccggggatcctgcacttcctgcagcacgagtgggcccgcttcga 200


Query: 384 agtggagagagcccagtgggaagtagagcgggcggagctgcaggctcagattgccttcct 443
            |||||||||||||||||||| || |||||||||||||||||||| ||||||||||||||
Sbjct: 201 ggtggagagagcccagtgggaggtggagcgggcggagctgcaggcccagattgccttcct 260


Query: 444 tcagggggacaggaaaggtcaagaaaatttgaagaaggatcttgtgaggcggatcaaaat 503
            ||||| || ||||| || ||||||||||||||||||||||||||||| ||||||||||
Sbjct: 261 gcagggagaaaggaagggccaagaaaatttgaagaaggatcttgtgaggaggatcaaaat 320


Query: 504 gctggagtgtgctctcaaacaggaaagagccaaataccacaagtt 548
           | |||||| |||||| |||||||||||||||||||||||||||||
Sbjct: 321 gttggagtatgctcttaaacaggaaagagccaaataccacaagtt 365
```

Figure 5.2.4.1. NCBI blast search result for library clone 35. The sequence obtained for pACT2/clone35 was submitted to an NCBI blast nucleotide search. Here the Query sequence is that of pACT2/clone35 and the subject (Sbjct) sequence is the sequence identified as a match from the BLAST search.

```
>gi|6755686|ref|NP_035630.1| striatin, calmodulin binding protein [Mus musculus]
 gi|17368003|sp|O55106|STRN_MOUSE Striatin
 gi|2828301|emb|CAA11545.1| striatin [Mus musculus]
          Length = 780

 Score =  166 bits (419), Expect = 5e-40
 Identities = 85/120 (70%), Positives = 87/120 (72%)
 Frame = +1

Query: 193 MDEQAGPGVFFSNNHPGASXXXXXXXXXXXXXXXXXXXXXXXXXQYSLPGILHFLQHEW 372
           MDEQAGPGVFFSNNHPGA                          QYSLPGILHFLQHEW
Sbjct: 1   MDEQAGPGVFFSNNHPGAGGAKGLGPLAEAAAAGDGAAAAGAARAQYSLPGILHFLQHEW 60

Query: 373 ARFEVE*AQWEVERAELQAQIAFLQGD*KGQENLKKDLV*RIKMLECALKQE*AKYHKFE 552
           ARFEVE AQWEVERAELQAQIAFLQG+ KGQENLKKDLV RIKMLE ALKQE AKYHK +
Sbjct: 61  ARFEVERAQWEVERAELQAQIAFLQGERKGQENLKKDLVRRIKMLEYALKQERAKYHKLK 120
```

Figure 5.2.4.2. Striatin is expressed from the isolated pACT2 library clone 35. In addition to screening the DNA sequence of library clones, the expressed open reading frame of the pACT2 constructs was calculated and the translated protein sequence submitted to an NCBI protein search. Clone 35 again matched striatin (note that the BLAST search engine automatically replaces repetitive sequences with runs of X). The query sequence is the sequence obtained for pACT2/clone35 and the subject sequence is the sequence retrieved using the BLAST search.

in section 2.2.17). One day post transfection, transfected cells were labelled with [^{35}S] amino acids and lysed. Cell lysates were then harvested and immunoextracted with either rabbit anti-GST antibody (MB38), or rat anti-HA antibody (Roche). GST/striatin was shown to co-ip full length L (figure 5.2.5.1) and this result was shown on three separate occasions. However it was not possible on any of these occasions to show HAL co-immunoprecipitating full length GST/striatin due to the presence of background bands migrating at the same location as GST/striatin in cells transfected with pcDNA/HAL and immunoextracted with rat anti-HA antibody. By demonstrating GST/striatin co-immunoprecipitating L, the interaction between the two proteins was proven to be genuine and not an artefact of the Y2HS.

The region of L with which striatin interacts was narrowed down by co-transfecting pGST/striatin with each of three HAL domain constructs used in section 4.2.6. Again, each plasmid construct (pcDNA/HAL1, 2 or 3) was either transfected alone or with pGST/striatin into A549 cells using the vaccinia T7/T7 expression system (as in section 2.2.17). GST/striatin was shown only to co-ip HAL2 (figure 5.2.5.2), indicating that striatin may interact with L in a similar region to that of the two non-structural proteins C and V. Once again this interaction was reproducible, confirming that striatin interacts with the second conserved domain of L.

In an attempt to further map the striatin binding site on the L protein, the panel of pAS2/L carboxy-terminal domain deletions as used in section 4.2.8 were each co-transformed with pACT2/clone 35 using AH109 cells and 200ng of each plasmid. As described previously interactions were screened using *HIS3* and *ADE2* reporter genes. Using the Y2HS, striatin was shown to bind to L in a region including the first 900 and 1100bp of L. This demonstrates that the striatin binding site resides somewhere between base pairs 1 and 1100 of L which is the equivalent to somewhere between amino acids 1 and 363 (see table 5.2.5.I). Striatin was also shown to interact with the yeast construct L1, but not L2 or L3. This mimicked the data obtained for both the C and V proteins in chapter 4. Once again the data

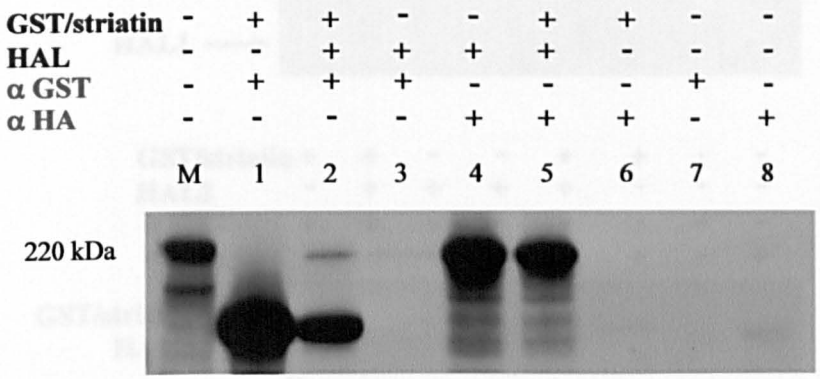

Figure 5.2.5.1. Co-immunoprecipitation of pGST/striatin with pcDNA/HAL. A549 cells were infected with vTF7-3 and transfected with the desired constructs; one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with 1µl of the appropriate antibody and immunoprecipitated proteins were analysed on an 8% acrylamide Laemmli gel, alongside [^{14}C] labelled molecular weight markers (M). Track 1= pGST/striatin, α-GST, 2= pGST/striatin and pcDNA/HAL, α-GST, 3= pcDNA/HAL, α-GST, 4= pcDNA/HAL, α-HA, 5= pcDNA/HAL and pGST/striatin, α-HA, 6= pGST/striatin, α-HA, 7= untransfected cells, α-GST, 8= untransfected cells, α-HA.

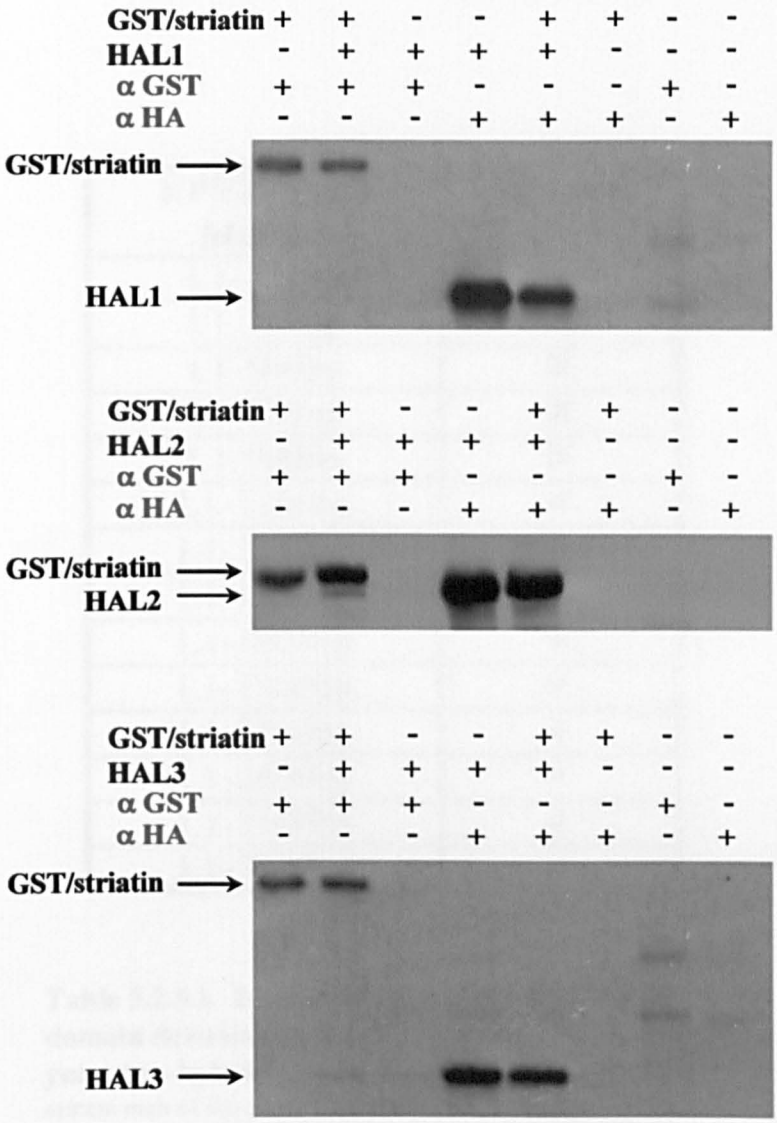

Figure 5.2.5.2. Co-immunoprecipitations of pGST/striatin with pcDNA/HAL domain constructs.
A549 cells were infected with vTF7-3 and transfected with the desired constructs (as indicated); one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Samples of lysates were immunoextracted with the appropriate antibody (see above) and immunoprecipitated proteins were analysed on 8% acrylamide Laemmli gels.

RPVL Yeast Mutant	Striatin
L1-122bp	✗
L1-360bp	✗
L1-500bp	✗
L1-700bp	✗
L1-900bp	✗
L1-1100bp	✓
L1-1200bp	✓
L1-1500bp	✓
L1-1800bp	✓
L1-1900bp	✓
L1-2100bp	✓
L1-2400bp	✓
L1-3100bp	✓
L1-3640bp	✓

Table 5.2.5.I. Interaction of carboxy-terminal domain deletions of full-length L with striatin in the yeast two-hybrid system. Using the yeast two-hybrid system each of the above L deletions were co-transformed with straitin into AH109 cells and interactions investigated. If an interaction was scored as positive, then a positive result was obtained for both beta-galactosidase activity and adenine biosynthesis. If either of these assays produced a negative result, then the pairing was deemed to yield no interaction.

obtained with the Y2HS contradicted that obtained with the (possibly more reliable) data obtained with co-ips.

5.2.6 Investigating the Interaction of L with Striatin Using Confocal Microscopy

To determine if striatin and L co-localise in transiently transfected cells, GST/striatin and HAL were expressed either singly or together in A549 cells using the vaccinia T7/T7 expression system (as in section 2.2.17). When cells were transfected with pcDNA/HAL alone a diffuse cytoplasmic distribution of the protein was seen, with aggregates of L forming in the cytoplasm and perinuclear regions of the cell (figure 5.2.6.1). When GST/striatin was expressed alone, it was found at the outer edge of the cell, with a low level of cytoplasmic labelling, as is the normal cellular distribution of striatin (figure 5.2.6.2). When the two proteins were co-expressed in the same cell co-localisation was not seen on a large scale in cells where there was a low level of expression of the two proteins (see figure 5.2.6.3), yet the cellular distribution of the two proteins was clearly altered. The GST/striatin protein was no longer found almost exclusively around the perimeter of the cell, but had adopted an almost diffuse cytoplasmic distribution. In addition, the aggregates of L which were seen when HAL was expressed alone were no longer visible. However, in cells where high levels of protein expression were seen, the two proteins could clearly be seen to co-localise (figure 5.2.6.4). These effects were seen in all cells which were co-transfected with the two plasmids expressing the constructs of interest, and co-localisation was only seen in those cells where the levels of protein expression were high. To demonstrate that it was the striatin protein itself, and not the GST tag that was having these effects on the HAL protein, an identical experiment was performed using pGST in place of pGST/striatin. When pGST was transfected alone, expression of GST was not confined to the outer edge of the cell as was seen for pGST/striatin, instead GST was shown to be universally distributed throughout the cell, with low levels of protein expressed in the nucleus (figure 5.2.6.5). Moreover,

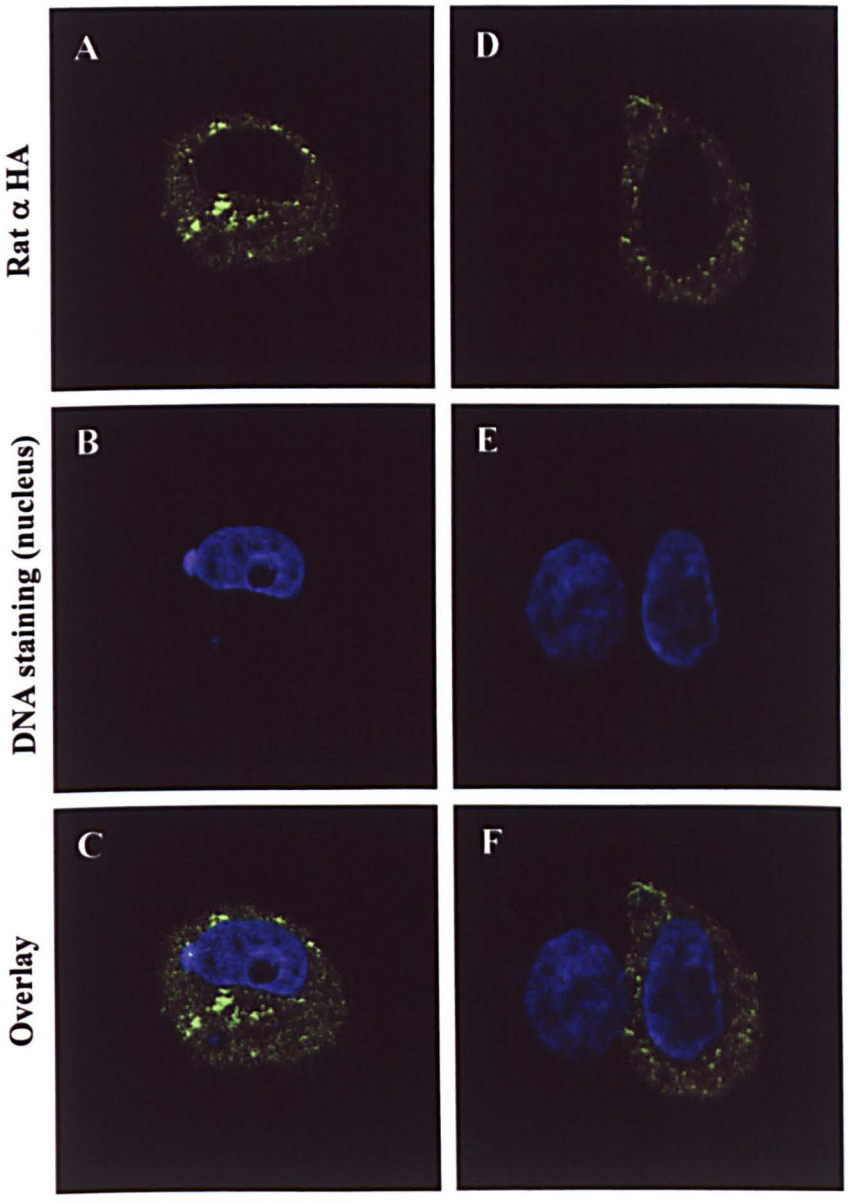

Figure 5.2.6.1. Immunofluorescence microscopy of cells transfected with pcDNA/HAL. A549 cells were infected with vTF7-3 and transfected with pcDNA/HAL. One day post transfection, cells were fixed, and permeabilised with 0.1% Triton X-100 before staining with antibody. Cells were reacted with rat α-HA (at 1/300, Roche) and the primary antibody visualised with Alexa Fluor 488 α-rat IgG (at 1/200, A&D). B and E show nuclei of cells counter-stained using DAPI and overlay of the stains used is shown in C&F.

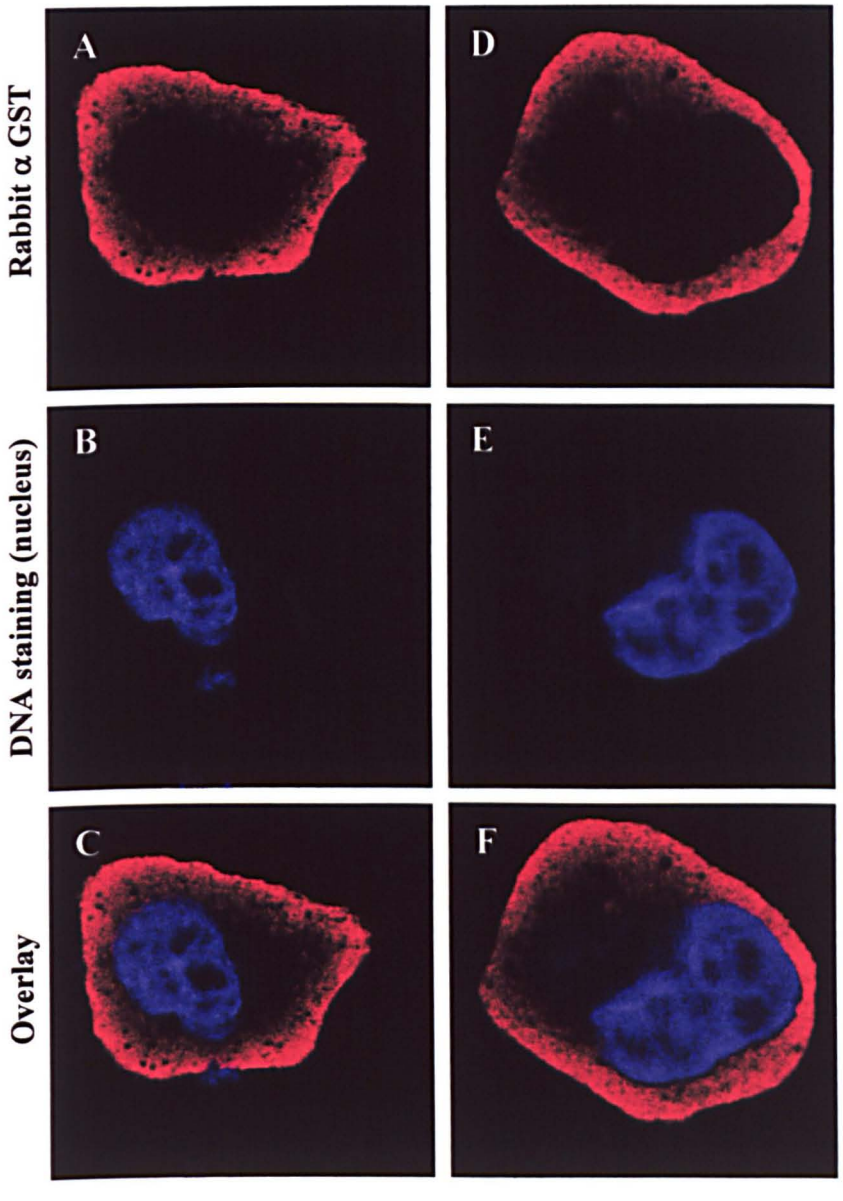

Figure 5.2.6.2. Immunofluorescence microscopy of cells transfected with pGST/striatin. A549 cells were infected with vTF7-3 and transfected with pGST/striatin. One day post transfection, cells were fixed, and permeabilised with 0.1% Triton X-100 before staining with antibody. Cells were reacted with rabbit α-GST (MB38, at 1/400) and the primary antibody visualised with Alexa Fluor 568 α-rabbit IgG (at 1/200, A&D). B and E show nuclei of cells counter-stained using DAPI and overlay of the stains used is shown in C&F.

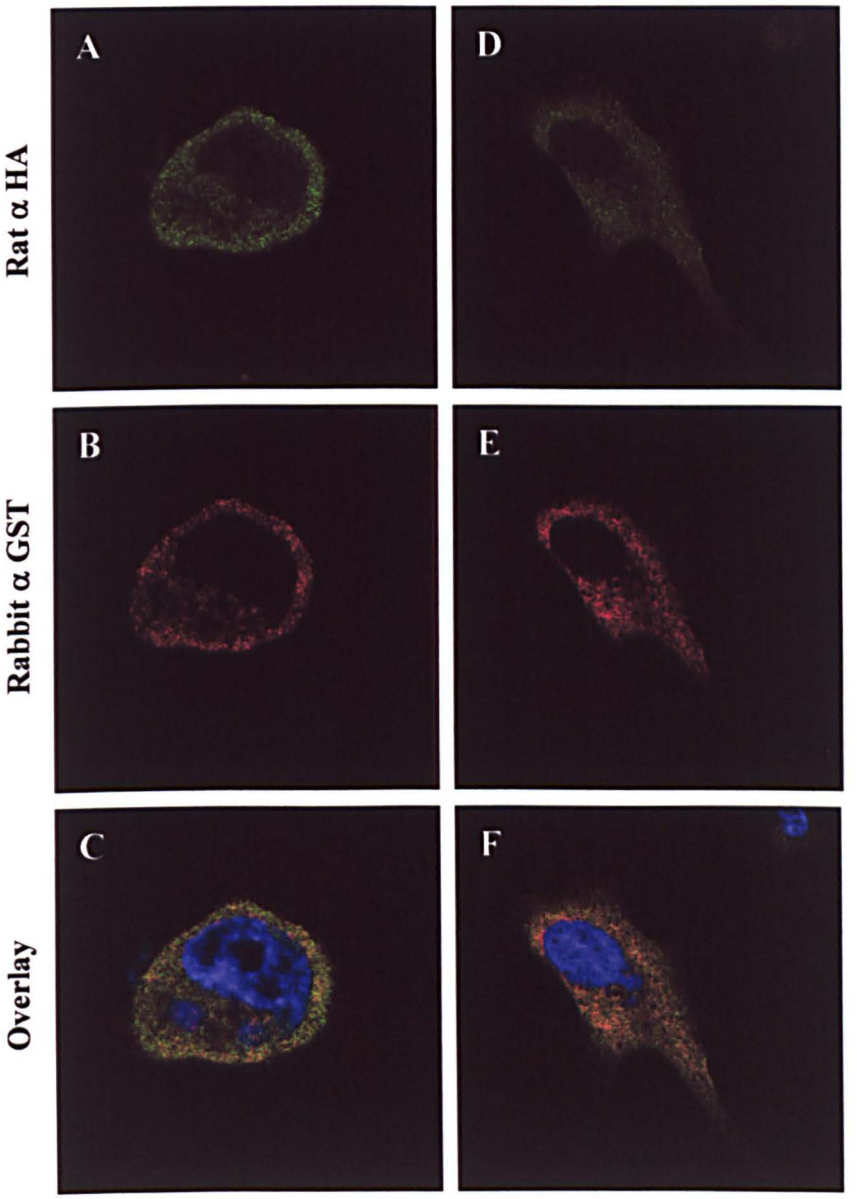

Figure 5.2.6.3. Immunofluorescence microscopy of cells co-transfected with pcDNA/HAL and pGST/striatin. A549 cells were infected with vTF7-3 and co-transfected with pcDNA/HAL and pGST/striatin. One day post transfection, cells were fixed, and permeabilised with 0.1% Triton X-100 before staining with antibody. Cells were reacted with rat α-HA (at 1/300, Roche), and the primary antibody visualised with Alexa Fluor 488 α-rat IgG (at 1/200, A&D), and with rabbit α-GST (MB38, at 1/400), and the primary antibody visualised with Alexa Fluor 568 α-rabbit IgG (at 1/200, B&E). C and F show overlay of the stains used (nuclei of cells counter-stained using DAPI).

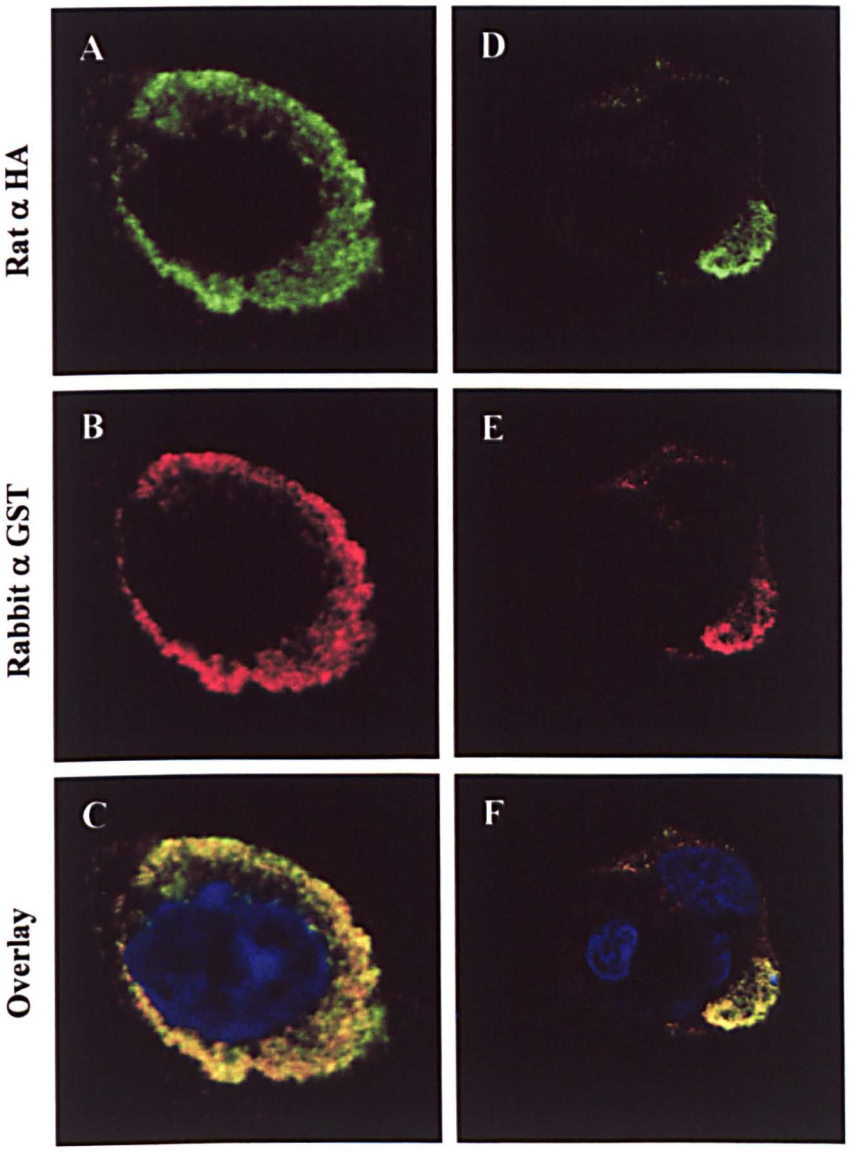

Figure 5.2.6.4. Immunofluorescence microscopy of cells co-transfected with pcDNA/HAL and pGST/striatin. A549 cells were infected with vTF7-3 and co-transfected with pcDNA/HAL and pGST/striatin. One day post transfection, cells were fixed, and permeabilised with 0.1% Triton X-100 before staining with antibody. Cells were reacted with rat α-HA (at 1/300, Roche), and the primary antibody visualised with Alexa Fluor 488 α-rat IgG (at 1/200, A&D), and with rabbit α-GST (MB38, at 1/400), and the primary antibody visualised with Alexa Fluor 568 α-rabbit IgG (at 1/200, B&E). C and F show overlay of the stains used (nuclei of cells counter-stained using DAPI).

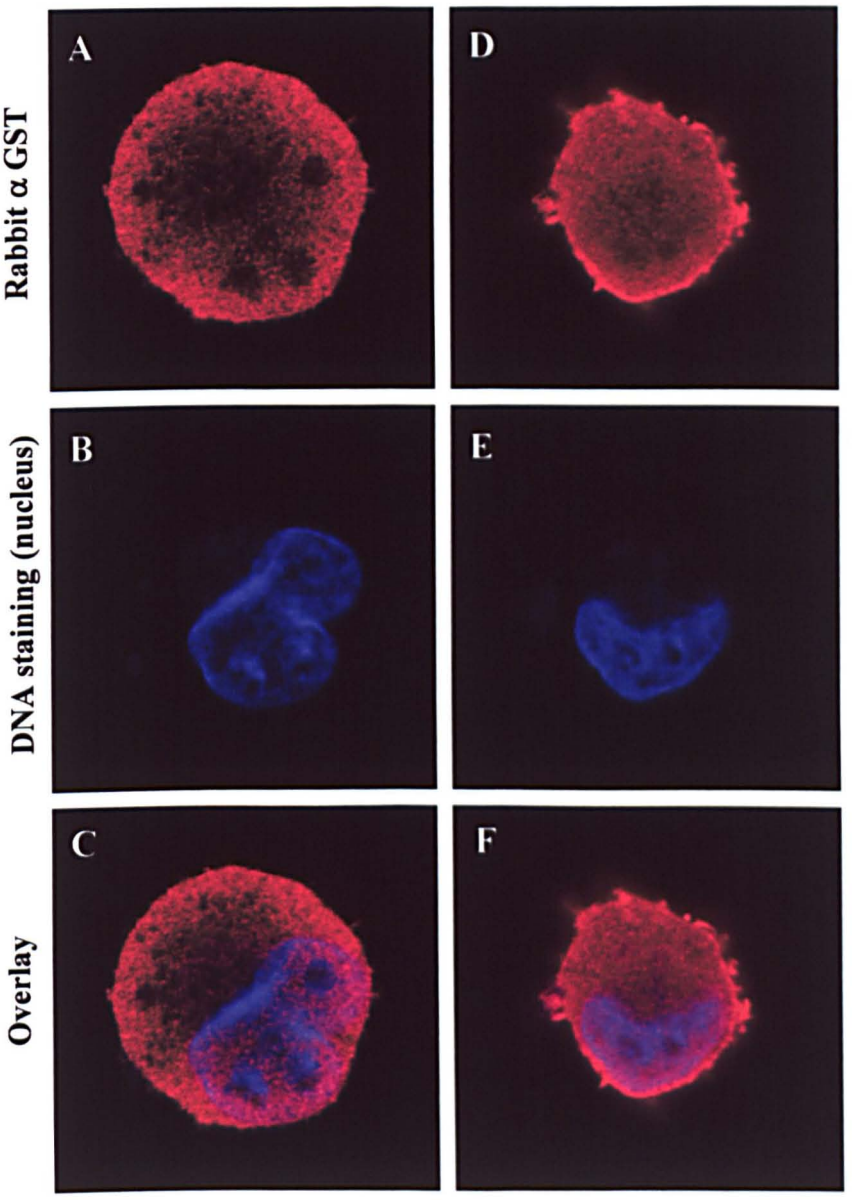

Figure 5.2.6.5. Immunofluorescence microscopy of cells transfected with pGST. A549 cells were infected with vTF7-3 and transfected with pGST. One day post transfection, cells were fixed, and permeabilised with 0.1% Triton X-100 before staining with antibody. Cells were reacted with rabbit α-GST (MB38, at 1/400) and the primary antibody visualised with Alexa Fluor 568 α-rabbit IgG (at 1/200, A&D). B&E show nuclei of cells counter-stained using DAPI and overlay of the stains used is shown in C&F.

when the two proteins (GST and HAL) were co-expressed there was no alteration of the cellular distribution of either protein and no evidence of co-localisation was seen (figure 5.2.6.6). These data clearly demonstrate that the cellular distribution of both GST/striatin and HAL is altered when the two proteins are co-expressed. In addition the two proteins have been shown to co-localise in cells where the level of protein expression is high. All the images shown in figures 5.2.6.1 through to figure 5.2.6.6 were taken using a confocal microscope, using sequential scanning. These results have been reproduced on two separate occasions.

5.3 Discussion

A large-scale library screen was performed using a porcine macrophage cDNA library and RPV L as the "bait" protein. From this library screen 46 possible L interactors were obtained and the majority of prey plasmids were recovered using DH5αs. After recovery plasmids were sequenced for identification purposes and then put back into the Y2HS to confirm an interaction with L and check for auto-activation. A large proportion of the remaining library clones was found to either auto-activate, or to no longer interact with the L protein in this system. The reason for this remains unclear; it could be that the initial preparation of pAS2/L which was used for the library screen contained a contaminant, or that the stock of AH109s used contained a mutation. To try and eliminate the yeast cells as the cause of these observations, fresh batches of AH109 were co-transformed with the library plasmids and pAS2/L, however the majority of library plasmids still failed to interact with L. The same positive control is always used for the co-transformations, that of pAS2/L and pACT2/C. It was found that this positive control only produces a positive result under the *ADE2* reporter gene ~50% of the time using the stock of AH109 cells we had. The AH109 strain of yeast has therefore been shown to be unreliable in nature and not as useful as expected with regards to eliminating the majority of false positives. It was hoped that by using this strain of *S. cerevisiae* that the number of false positives encountered when performing the library screen would be limited due

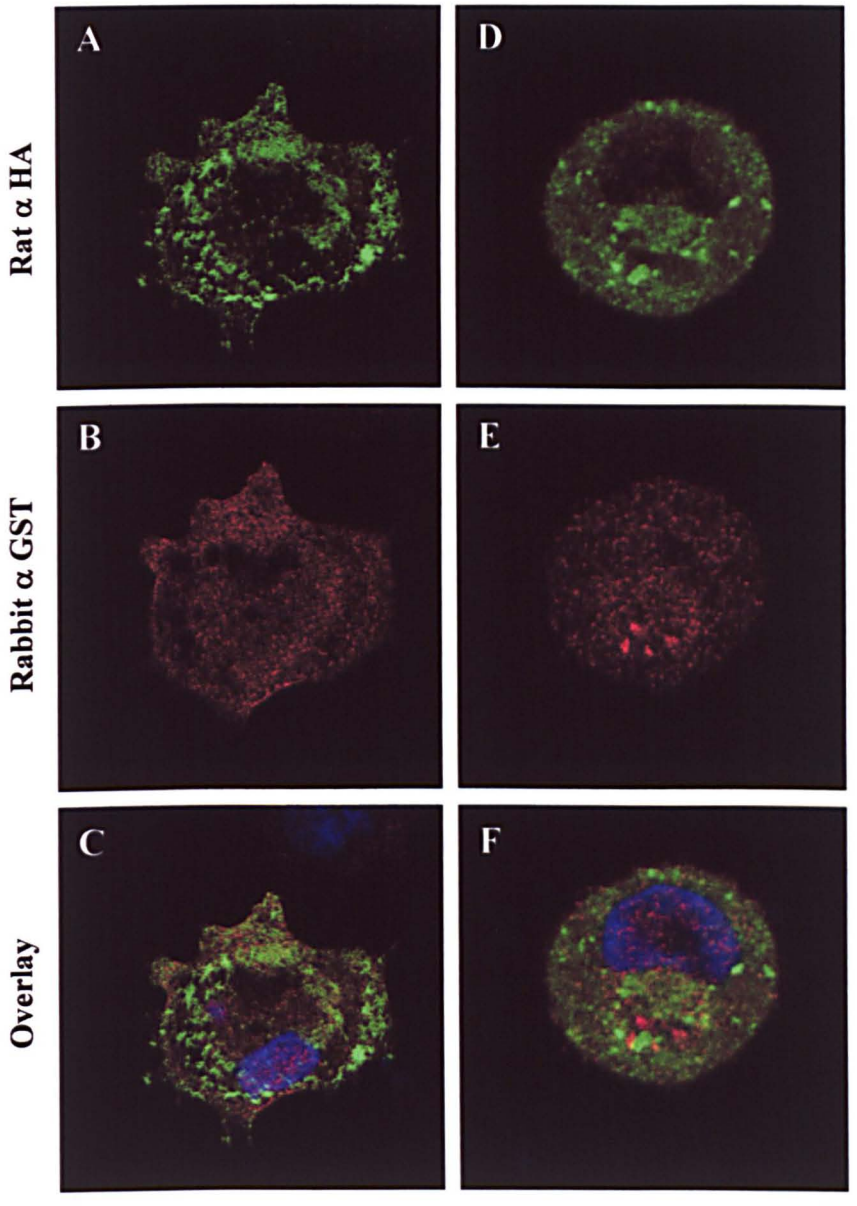

Figure 5.2.6.6. Immunofluorescence microscopy of cells co-transfected with pcDNA/HAL and pGST. A549 cells were infected with vTF7-3 and co-transfected with pcDNA/HAL and pGST. One day post transfection, cells were fixed, and permeabilised with 0.1% Triton X-100 before staining with antibody. Cells were reacted with rat α-HA (at 1/300, Roche), and the primary antibody visualised with Alexa Fluor 488 α-rat IgG (at 1/200, A&D), and with rabbit α-GST (MB38, at 1/400), and the primary antibody visualised with Alexa Fluor 568 α-rabbit IgG (at 1/200, B&E). C&F show overlay of the stains used (nuclei of cells counter-stained using DAPI).

to the fact that the strain possesses three reporter genes which are all under the control of different GAL4-inducible promoters. However, in actual fact it was found that a very high level of false positives relative to previous screens carried out in other laboratories at the Institute of Animal Health using Y190s were obtained.

Despite the problems with auto-activators and clones that were found to no longer interact with the L protein, three clones were shown to interact with L and demonstrated to not auto-activate. One of these clones was sequenced and shown to be 92% similar to the human striatin protein. Due to time constraints it was decided to only take this protein through to the next stage. A construct expressing full-length striatin was a generous gift from Francis Castets and this construct was manipulated to produce a striatin construct that was GST-tagged. This construct was then used in co-immunoprecipitation studies with pcDNA/HAL and each of the three pcDNA/HAL domain constructs as used in chapter 4. An interaction between L and striatin was confirmed outside of the Y2HS as GST/striatin was shown to co-ip full-length L. Moreover, the binding site of striatin on L was shown to be located somewhere in the second conserved domain of the L protein as GST/striatin co-immunoprecipitated HAL2, but not HAL1 or HAL3. Striatin was shown by Y2HS analysis to interact with L somewhere in the first 1100bp of L, the equivalent to between amino acids 1 and 363; striatin was also shown to interact with the yeast construct L1, but an interaction with L2 or L3 was not detected. The same results were obtained for the two non-structural proteins C and V in chapter 4, where the data obtained using co-ips contradict the data obtained with the Y2HS.

Confocal microscopy was next utilised and it was demonstrated that in cells with high levels of expression of GST/striatin and HAL, co-localisation of the two proteins was seen. Even in co-transfected cells where levels of protein expression are too low to demonstrate co-localisation, the cellular distribution of each of the two proteins was shown to be altered when compared to the cellular distribution when each one is expressed alone. Clear evidence was therefore obtained for a novel

interaction between a host cellular protein and RPV L. The possible role(s) of an L-striatin interaction in the viral life cycle are considered in the final discussion (chapter 7).

Chapter Six

Interaction of RPV L with Host Cell Proteins known to Interact with Polymerases of other members of the order *Mononegavirales*

6.1 Introduction

Several host cell proteins are known to interact with viral proteins from members of the order *Mononegavirales*, for example the L protein of VSV (Indiana serotype) associates with translation elongation factor-1 α (Das *et al.*, 1998), while the L proteins of both MeV and VSV interact with tubulin (Moyer *et al.*, 1990, Moyer *et al.*, 1986). Furthermore, the amino terminal domain of the phosphoproteins of two lyssaviruses, rabies virus and Mokola virus, was recently found to interact with the dynein light chain, LC8 (Jacob *et al.*, 2000).

The dynein light chain, LC8, is a small protein with extremely high sequence conservation and is a component of both cytoplasmic dynein and myosin-V, an actin-based motor. Cytoplasmic dynein, a microtubule-associated motor protein complex, is involved in numerous motile events including retrograde movement of organelles along microtubules, nuclear migration, axonal transport and mitotic spindle alignment, as reviewed by Janmey, 1998 (Janmey, 1998), and references therein.

A number of diverse cellular and viral proteins that interact with LC8 have now been identified and two distinct types of LC8 binding motifs have been characterised (Rodríguez-Crespo *et al.*, 2001). The neuronal nitric oxide synthetase (nNOS) and several other proteins use the binding sequence, "MKDTGIQV" (Liang *et al.*, 1999), while the apoptosis regulator, bim, amongst others, uses the binding motif "SCDKSTQT" (Puthalakath *et al.*, 1999). An nNOS-type motif was identified in the lyssavirus P protein that mediates the interaction of P and LC8 (Poisson *et al.*, 2001). Following sequence analysis, a putative LC8 binding motif was identified within the L protein of rinderpest virus (see figure 6.1.1). This theoretical LC8 binding motif appears to be conserved

Cellular proteins	LC8 binding motif	Viral proteins	LC8 binding motif
nos peptide	MKDTGIQV	Rabies phosphoprotein	DKSTQT
bim	SCDKSTQT	Rotavirus VP4	DKSTQL(I)
dynamin	DSWLQV	Coxsackievirus & poliovirus VP2	DRVL(M)QL
		African swine fever virus p54	NTASQT

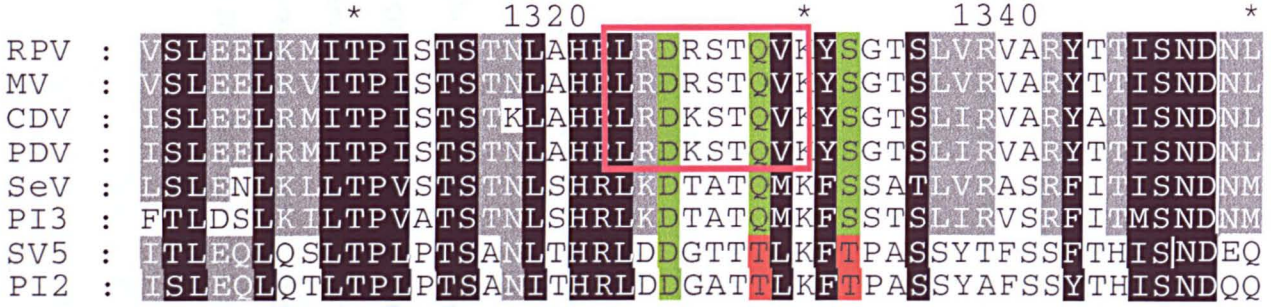

Figure 6.1.1. An apparently conserved putative LC8 binding motif in RPVL. Examples of known cellular and viral LC8 interactors are shown together with their respective LC8 binding motifs. Highlighted by the blue shaded box are the two identified types of LC8 binding motif; the nNOS type and the bim type. Sequence analysis showed that the L proteins of morbilliviruses may possess a conserved LC8 binding motif, which appears to be conserved amongst morbilliviruses, but not in paramyxoviruses generally.

amongst the L proteins of the genus *Morbillivirus*, indicating that if an interaction does occur between L and LC8, it may play an important role within the viral life cycle. Only the DXXXQ part of the motif is conserved to a lesser extent throughout the paramyxoviruses as a whole. This part of the motif is not enough for LC8-binding, as it does not contain other critical residues from the nNOS and bim motifs.

As I am interested in identifying host cell proteins with which rinderpest virus interacts, it was decided to investigate whether translation elongation factor-1 α, a component of a group of soluble proteins that function in the chain elongation process during polypeptide synthesis at the ribosome, or LC8 interacted with the L subunit of the rinderpest polymerase, using the yeast two-hybrid system.

6.2 Results

6.2.1 Preparation of Yeast Two-Hybrid System Constructs

Yeast constructs were made to express translation elongation factor- 1 (EF-1) subunits α, β and γ from the yeast expression vector, pACT2. EF-1 subunits α and β were cut from existing laboratory pT7blue clones using *Sal*I and *Eco*RI and ligated to pACT2 (*Xho*I/*Eco*RI cut). EF-1 subunit γ was excised from another existing pT7blue clone using *Xma*I and *Sal*I and inserted into pACT2 (*Xma*I/*Xho*I cut). Resulting pACT2 constructs were then sequenced to confirm correct insertion and reading frames of each of the EF-1 subunits.

6.2.2 Investigation of Interactions with Translation Elongation Factor- 1($\alpha\beta\gamma$)

Two hundred ng of pAS2/L with each of pACT2/EF-1α, pACT2/EF-1β and pACT2/EF-1γ were co-transformed using either competent Y190 yeast cells or competent AH109 yeast cells, as in section 2.4.2. As a positive control, pAS2/L was co-transformed with pACT2/C and 200ng of each plasmid used. To check for autoactivation properties each of the three pACT2/EF-1 constructs were co-transformed with empty pAS2 vector. Co-transformants were selected for by plating out on minimal medium lacking leucine and tryptophan. Resulting colonies derived from the Y190 transformations were then picked and assayed for histidine biosynthesis in addition to beta-galactosidase activity, whilst colonies derived from the AH109 transformations were assayed for both histidine and adenine biosynthesis on the relevant selection plates. Only the positive control of pAS2/L and pACT2/C grew under either of the selection conditions and provided a positive beta-galactosidase result. In addition, none of the three pACT2/EF-1 constructs were found to autoactivate.

To confirm that the pACT2 constructs were expressing the EF-1 subunits, 200ng of each of the three constructs was transformed using either AH109 cells or Y190 cells (as in section 2.4.2) and each transformation mix plated out on SC-L plates and incubated at 30°C to select cells transformed with the pACT2 plasmid. A single colony was then picked and used to inoculate liquid cultures. After 2-3 days protein samples were harvested (as in section 2.3.5) and expression tested by Western blot analysis, as in section 2.3.3. It was demonstrated that only pACT2/EF-1γ expressed a fusion protein of the expected size (~63 kDa) (see figure 6.2.2.1). pACT2/EF-1α and pACT2/EF-1β were expected to express fusion proteins of ~63 kDa and ~26.8 kDa respectively, yet no proteins of this size were detected. The same results were obtained for both strains of yeast tested (data not shown) and a varying amount of expression was seen between isolated colonies.

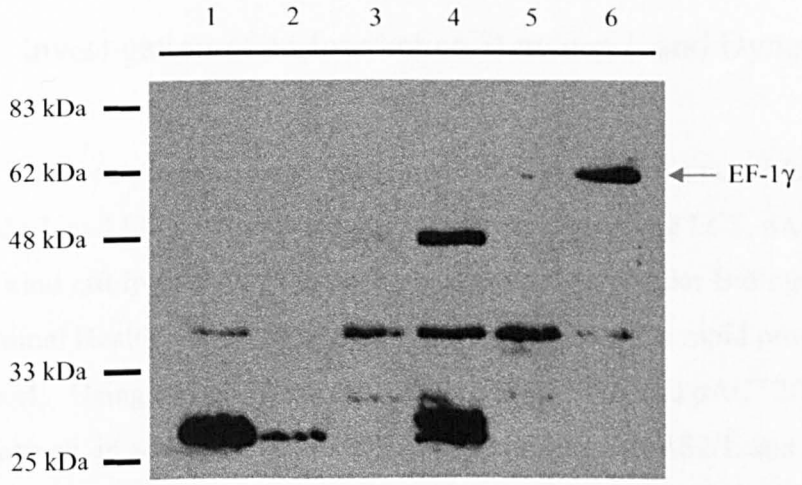

Figure 6.2.2.1. Western blot analysis of EF-1 subunit expression by protein samples derived from transformed AH109 yeast cell lysates. Prepared protein samples from AH109 yeast cell lysates transformed with the construct of interest were run on 10% Laemmli SDS-PAGE gels, alongside prestained protein broad range marker (New England Biolabs). After running, the gel was transferred to membrane and probed using rat α-HA-Peroxidase linked antibody (Roche) at 1/1000. Tracks 1&2 = pACT2/EF-1α, tracks 3&4 = pACT2/EF-1β and tracks 5&6 = pACT2/EF-1γ, where each track represents individual clones. Note: replicate clones showed considerable variation in protein expression.

It was thus concluded that RPV L does not interact with the translation elongation factor-1 γ subunit within this system. However an interaction between L and EF-1α or EF-1β may still be possible due to the lack of protein expression from pACT2/EF-1α and pACT2/EF-1β in transformed yeast cells.

6.2.3 Investigation of an Interaction Between L and Dynein LC8

The Y2HS was used as a method with which to search for a possible interaction between L and LC8. A yeast plasmid construct expressing LC8, pACT2/LC8, was a kind gift from Dr. L. Dixon (Department of Molecular Biology, Institute for Animal Health, Pirbright, Surrey, UK) and enabled the rapid progression of this work. Using AH109 yeast cells, 200ng of pAS2/L and pACT2/LC8 were co-transformed, in addition to a positive control pairing of pAS2/L and pACT2/C (as in section 2.4.2). Previous work using the pACT2/LC8 construct had revealed that it did not autoactivate in Y190 cells, but the construct was nonetheless tested for autoactivation properties in AH109 cells. pACT2/LC8 was shown not to autoactivate in AH109s, by co-transforming 200ng of the plasmid together with 200ng of empty pAS2 vector. Double transformants were selected for using minimal agar lacking leucine and tryptophan and an L-LC8 interaction confirmed by selection for adenine biosynthesis and histidine biosynthesis, in addition to demonstrable β-galactosidase activity, as assayed in section 2.4.4. As a novel interaction had been found within this system, it was necessary to confirm the interaction in a system outside of the Y2HS. This was to verify that the interaction was genuine and not an artefact of the Y2HS. The Y2HS is known to throw up spurious interactions that cannot be corroborated with other systems. To confirm the interaction between L and LC8, co-immunoprecipitation studies and co-localisation studies using immunofluorescence were employed.

6.2.4 Co-Immunoprecipitation Studies of L and Dynein LC8

Before this work could be undertaken, it was first necessary to set up a gel system to enable LC8 to be resolved. This is due to the fact that LC8 is such a small protein (despite its name, LC8 is actually a 10 kDa protein). The tricine buffer system as described by Schagger and von Jagow (Schagger & von Jagow, 1987) was utilised as it has been shown to have superior resolution for proteins <20 kDa. The ability to detect LC8 was first tested by Western blot. Vero cell lysates were prepared by resuspending 10^6 cells in 100µl of SDS loading buffer and incubating at either 90°C or 40°C for thirty minutes. A series of cell lysate samples were run on a 10% acrylamide tricine gel (as in section 2.3.2.2) and analysed by Western blot (as in section 2.3.3); using this system it was possible to detect LC8 (figure 6.2.4.1). The normal denaturation temperature (90°C) was found to be superior to the temperature used by Schagger and von Jagow (40°C) (Schagger & von Jagow, 1987), as can be seen in figure 6.2.4.1.

As it was possible to resolve LC8 by Western blot it was decided to try demonstrating pcDNA/HAL bringing down LC8 using this system. Immunoprecipitated HAL was analysed by 10% acrylamide tricine gels and Western blot, using the monoclonal mouse α-PIN antibody used in the successful Western blot. However, possibly due to the fact that only a small amount of HA-tagged L is produced in transfected cells (probably due to its size), no LC8 was detected in the HAL fraction (see figure 6.2.4.2). The same experiments were performed with pcDNA/HAL1, -/HAL2 and -/HAL3, but again no LC8 was detected (data not shown).

Since it was possible that the Western blot was not sensitive enough to detect co-precipitated LC8, direct co-immunoprecipitation studies of metabolically labelled proteins were next performed. Again, cells transfected with pcDNA/HAL were used and lysates examined for evidence that LC8 co-precipitated with the full length L (HAL). However, possibly due to the low levels of LC8 present in

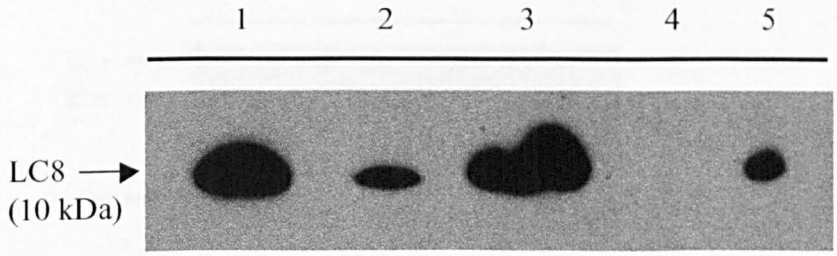

Figure 6.2.4.1. Detection of LC8 by Western blot.
Vero cell lysates were prepared by the centrifugation of 1 x 10^6 trypsinised cells at 500rpm for four minutes. The cell pellet was resuspended in 100µl SDS loading buffer and incubated at either 90°C (tracks 2 and 3) or 40°C (tracks 4 and 5) for thirty minutes. Either 5µl (tracks 2 and 4) or 20µl (tracks 3 and 5) of sample was loaded onto a 10% tricine gel, alongside 5µl of prestained protein broad range marker (New England Biolabs) and 5µg of rat cerebrum brain lysate (provided with the α-PIN antibody), as a positive control (track 1). After running the gel under conditions described by Shagger and von Jagow (1987), the gel was transferred to membrane and probed using mouse monoclonal α-PIN at 1/1 000 (BD Transduction Laboratories) and with α-mouse Ig-HRP linked whole antibody from sheep at 1/10 000(Amersham Pharmacia Biotech).

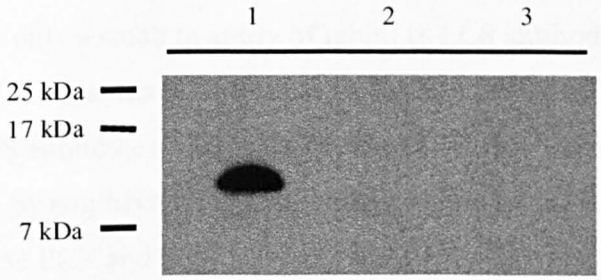

Figure 6.2.4.2. Analysis of immunoprecipitated HAL by Western blot using anti-LC8 antibody. Lysate from Vero cells transfected with pcDNA/HAL was immunoextracted using rat monoclonal anti-HA antibody (Roche) and immunoprecipitated proteins analysed on 10% acrylamide tricine gels. Either 10µl (track 2) or 20µl (track 3) of sample was loaded onto the gel alongside 5µl of prestained protein broad range marker (New England Biolabs) and 5µg of rat cerebrum brain lysate (provided with the α-PIN antibody), as a positive control (track 1). After running the gel under conditions described by Schagger and von Jagow (1987), the gel was transferred to membrane and probed using mouse monoclonal α-PIN at 1/1000 and with α-mouse Ig-HRP linked whole antibody from sheep (Amersham Pharmacia Biotech).

Vero cells, A549 cells and B95a cells, the natural LC8 present in these cell types could not be detected when labelled and immunoprecipitated using either the mouse monoclonal α-PIN antibody, or a rabbit α-LC8 antibody (a kind gift from Dr. Stephen M. King, Department of Biochemistry, University of Connecticut Health Centre, Farmington, Connecticut, 06030-3305, USA) (data not shown). An α-PIN antibody was utilised for the detection of LC8, as LC8 is also referred to as PIN (protein inhibitor of nNOS – neuronal nitric oxide synthase). A construct was therefore generated that expressed LC8 from a T7 promoter; since there was only a small quantity of rabbit α-LC8 antibody available, a 6-HIS tagged LC8 construct was designed, enabling the use of commercially available anti-6-HIS antibodies. The LC8 construct with a C-terminal 6-HIS tag was produced by amplifying the LC8 coding region from pACT2/LC8 using *Pfu* polymerase PCR and the LC8hisfor and LC8hisrev primers (see appendix 3). The resulting PCR product was cloned into pT7blue, excised by digestion with *Eco*RI and *Sal*I and cloned into pcDNA3 (*Eco*RI/*Xho*I cut). To confirm correct insertion of the 6-HIS tag and the LC8 coding sequence, the resulting pcDNA3 construct was sequenced.

To confirm expression of LC8 from the pcDNA/LC8/HIS construct and to ensure that the 6-HIS tag was functional, A549 cells were transfected using 2μg or 5μg amounts of plasmid DNA, as in section 2.2.17. One day post transfection, the cells were fixed, permeabilised and stained using a mouse monoclonal α-6-HIS antibody (Sigma) and the rabbit polyclonal α-LC8 antibody. As expected, a higher transfection efficiency was seen in cells transfected with the 5μg amount of plasmid DNA than with cells transfected with the lower amount of DNA. The immunofluorescence confirmed the expression of LC8 from pcDNA/LC8/HIS and the 6-HIS tag was shown to be fully functional (figure 6.2.4.3).

Despite the production of pcDNA/LC8/HIS, the levels of LC8 remained undetectable by immunoprecipitation within transiently transfected cells, even when other transfected constructs expressed proteins which were detected from

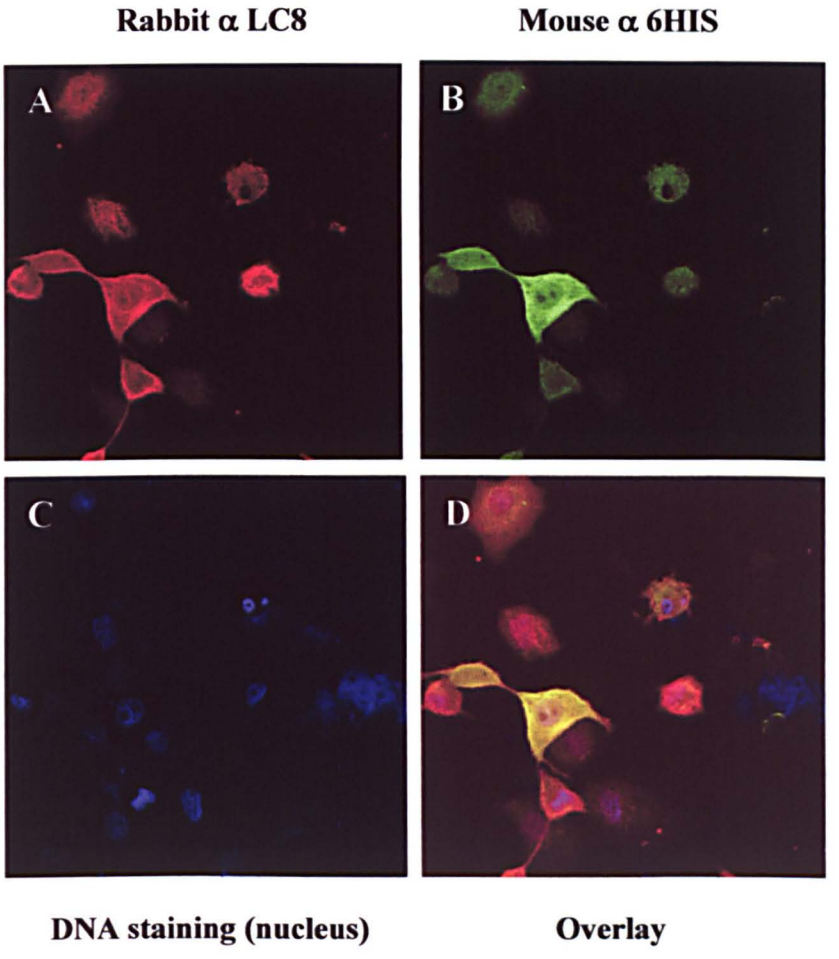

Figure 6.2.4.3. Immunofluorescence microscopy of cells transfected with pcDNA/LC8/HIS. A549 cells were infected with vTF7-3 and transfected with pcDNA/LC8/HIS. One day post transfection, cells were fixed, and permeabilised with 0.1% Triton X-100 before staining with antibody. Cells were reacted with either rabbit α-LC8 (at 1/10, a kind gift from S. King), and the primary antibody visualised with Alexa Fluor 568 α-rabbit IgG (at 1/200, A), or with mouse α-6 HIS (at 1/100, Sigma), and the primary antibody visualised with Alexa Fluor 488 α-mouse IgG (at 1/200, B). C shows nuclei of cells counter-stained using DAPI and overlay of the stains used is shown in D.

the same batch of transfections, see figure 6.2.4.4, where HAL is efficiently expressed from pcDNA/HAL. LC8/HIS should migrate at approximately 11 kDa, and as can be clearly seen in figure 6.2.4.4, no protein of this size was detected in cells transfected with pcDNA/LC8/HIS, even when the gel was greatly overexposed. In addition to the usual NP40 lysis buffer, a high salt lysis buffer was also tried, where the concentration of NaCl was increased from the usual 0.15M to 0.5M. This lysis buffer breaks open the nucleus and improves the dissociation of cytoskeletal proteins, which may have improved the solubility of LC8. However, the change in lysis buffer salt concentration did not alter the fact that LC8 was undetectable. The failure to detect LC8 in lysates of radiolabelled transfected cells could be due to there not being enough methionine/cysteine residues in this small protein. LC8 ([^{35}S] labelled) could not be detected by immunoprecipitation from labelled cells, even though LC8 was being immunoprecipitated, as shown by subsequent Western blot of the immunoprecipitated material with anti-LC8 antibody (M. D. Baron, personal communication).

6.2.5 Immunofluorescence Studies on the Interaction of L and Dynein LC8

One possible explanation for the L-LC8 interaction being observed in the yeast two-hybrid system, but not in co-immunoprecipitation, would be if the interaction is transient or unstable under the conditions of immunoprecipitation. Such an interaction, however, should lead to significant co-localisation of the two proteins when co-expressed in cells. To clarify if an interaction between L and LC8 does in fact exist, co-localisation studies were performed using constructs expressing L and LC8. pcDNA/HAL and pcDNA/LC8/HIS (5μg of each) were transiently transfected into B95a and A549 cells, as in section 2.2.17. One day post transfection the cells were fixed and labelled with rabbit α-HA (Santa Cruz Biotechnology) and mouse α-6-HIS (Sigma). In the majority of transiently transfected cells of either cell type, no co-localisation was observed between these two proteins (see figure 6.2.5.1); however, in a few transfected cells of both

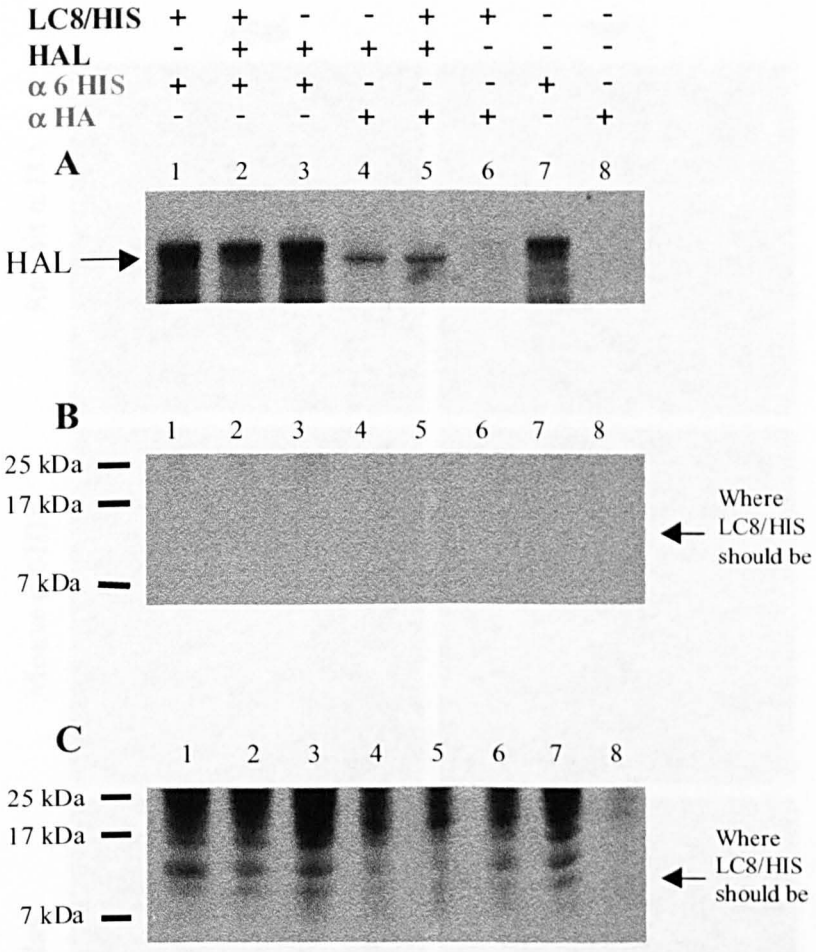

Figure 6.2.4.4. Failure to immunoprecipitate LC8/HIS from cells transfected with either pcDNA/LC8/HIS alone, or pcDNA/LC8/HIS co-transfected with pcDNA/HAL, despite the immunoprecipitation of HAL. B95a cells were infected with vTF7-3 and transfected with either pcDNA/LC8/HIS only (tracks 1 and 6), or pcDNA/HAL only (tracks 3 and 4), or both constructs were co-transfected (tracks 2 and 5); one day post transfection, cells were labelled with [^{35}S] amino acids for one hour and lysed with 1% NP40. Tracks 7 and 8 represent non-transfected (no DNA) control cell lysates. Samples of lysates were immunoextracted with either mouse α-6 HIS monoclonal antibody (Sigma) (tracks 1, 2, 3 and 7), or rat α-HA monoclonal antibody (Roche) (tracks 4, 5, 6 and 8) and immunoprecipitated proteins analysed on both 8% acrylamide Laemmli gels and 10% acrylamide Tricine gels. Panel A shows an overnight exposure of the 8% Laemmli gel, panel B an overnight exposure of the 10% Tricine gel and panel C a 10 day exposure of the 10% Tricine gel.

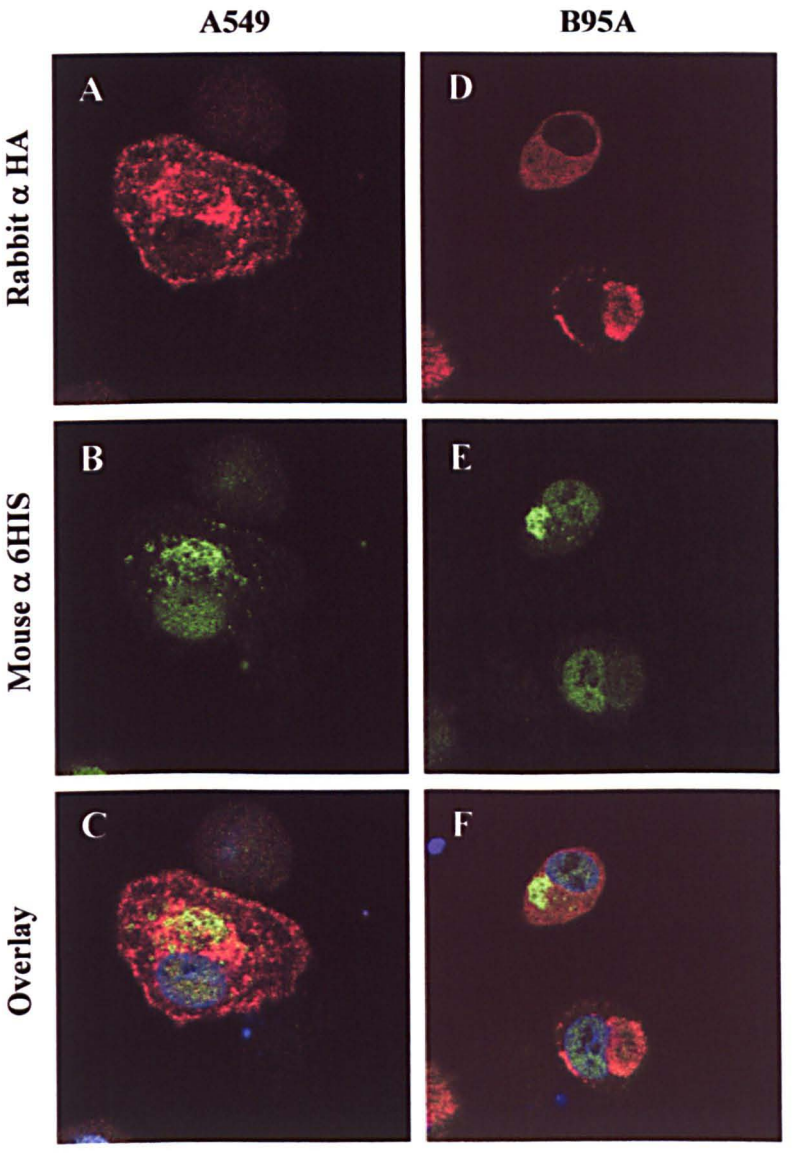

Figure 6.2.5.1. Immunofluorescence microscopy of cells co-transfected with pcDNA/HAL and pcDNA/LC8/HIS. A549 cells (A, B and C) or B95a cells (D, E and F) were infected with vTF7-3 and co-transfected with pcDNA/HAL and pcDNA/LC8/HIS. One day post transfection, cells were fixed, and permeabilised with 0.1% Triton X-100 before staining with antibody. Cells were reacted with either rabbit α-HA (at 1/200, Santa Cruz Biotechnology), and the primary antibody visualised with Alexa Fluor 568 α-rabbit IgG (at 1/200, A&D), or with mouse α-6 HIS (at 1/100, Sigma), and the primary antibody visualised with Alexa Fluor 488 α-mouse IgG (at 1/200, B&E). C and F show overlay of the stains used (nuclei of cells counter-stained using DAPI).

cell types, it did appear as though L and LC8 did in fact co-localise (see figure 6.2.5.2). It is not clear why apparent co-localisation was seen in these cells, but not in others. It may indicate an interaction that depends on cell cycle state, or it may be that the rare co-localisation seen was an artefact.

Although it is unlikely that L interacts with dynein LC8, it was observed in the course of the studies that in RPV infected cells not only were the levels of LC8 increased, but that LC8 was also re-organised within infected cells (M. D. Baron, personal communication). It is possible that another component of the viral nucleocapsid may be interacting with either LC8 or another component of the dynein complex, thus accounting for the re-organisation of LC8 seen in RPV infected cells. To investigate if this was the case, A549 cells were transfected using pcDNA constructs expressing N, P or C, together with the pcDNA/LC8/HIS construct. From these studies using confocal microscopy it was clear that LC8 did not interact with N, as N was shown to form aggregates and did not share a cellular distribution with LC8/HIS (data not shown). It was impossible to determine if LC8 interacted with either P (figure 6.2.5.3) or C (figure 6.2.5.4) by this technique; the apparent co-localisation (yellow in overlays) is due to the extensive diffuse cytoplasmic distribution seen with each of these proteins.

6.3 Discussion

Recent work with VSV has shown that the L protein subunit of the polymerase interacts with EF-1 α for its activity (Das *et al.*, 1998). Using the Y2HS, such an interaction was explored with the RPV L protein. Despite numerous attempts to identify an interaction between L and the EF-1 subunits, no such interaction between these proteins was seen. When the expression of fusion proteins from each of the pACT2 constructs made was tested, it was found that only pACT2/EF-1γ expressed a fusion protein of the expected size. It was therefore only possible to conclude that RPV L does not interact with EF-1γ within the

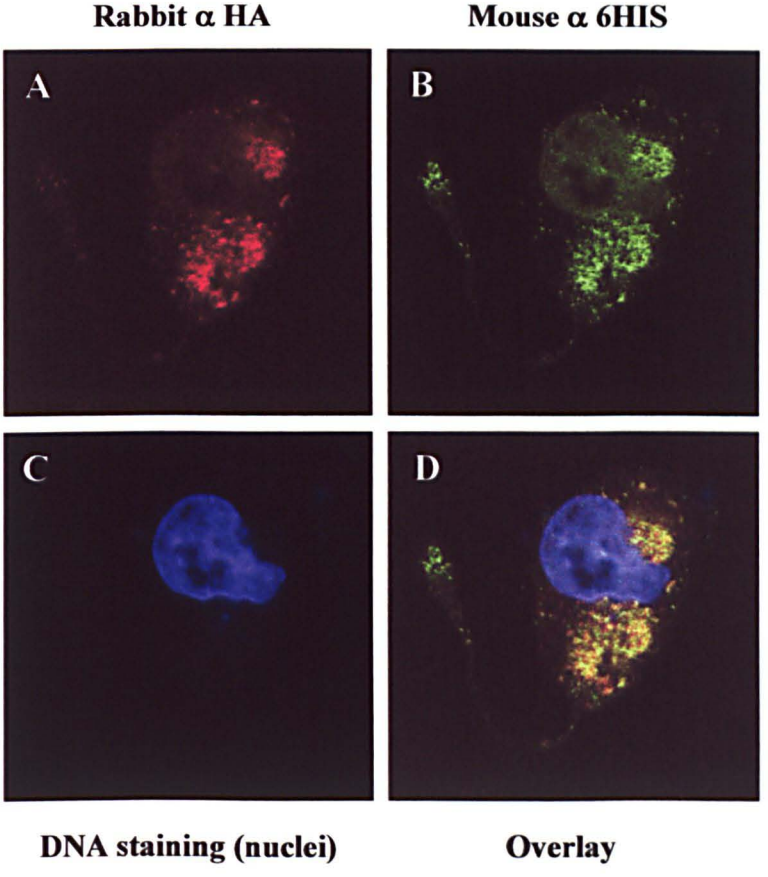

Figure 6.2.5.2. Immunofluorescence microscopy of cells co-transfected with pcDNA/HAL and pcDNA/LC8/HIS. A549 cells were infected with vTF7-3 and co-transfected with pcDNA/HAL and pcDNA/LC8/HIS. One day post transfection, cells were fixed, and permeabilised with 0.1% Triton X-100 before staining with antibody. Cells were reacted with either rabbit α-HA (at 1/200, Santa Cruz Biotechnology), and the primary antibody visualised with Alexa Fluor 568 α-rabbit IgG (at 1/200, A), or with mouse α-6 HIS (at 1/100, Sigma), and the primary antibody visualised with Alexa Fluor 488 α-mouse IgG (at 1/200, B). C shows nuclei of cells counter-stained using DAPI and D shows an overlay of the stains used.

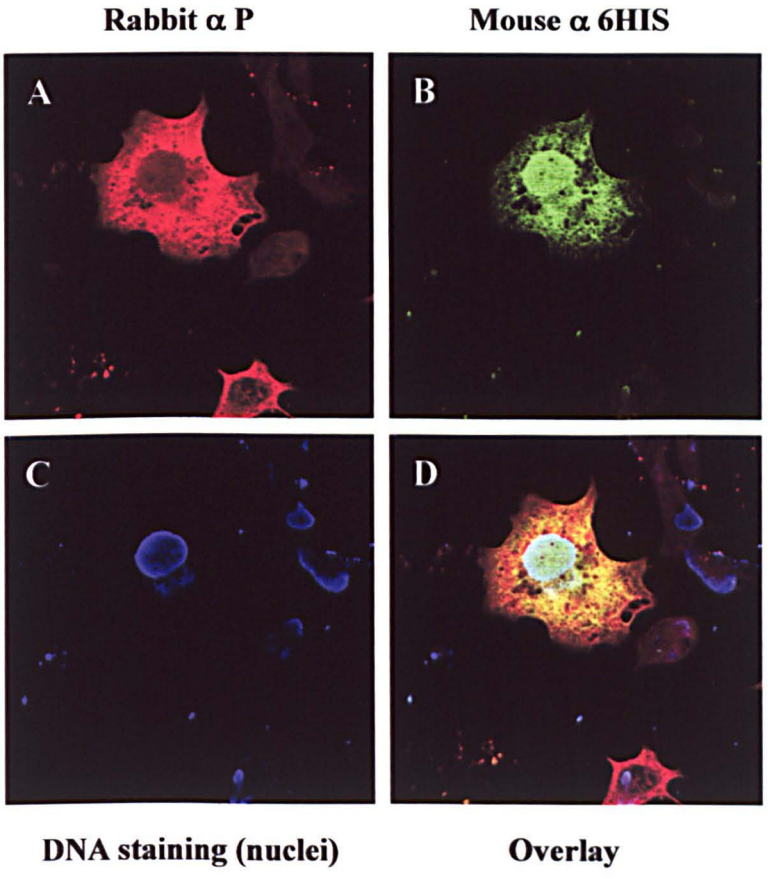

Figure 6.2.5.3. Immunofluorescence microscopy of cells co-transfected with pcDNA/P and pcDNA/LC8/HIS. A549 cells were infected with vTF7-3 and co-transfected with pcDNA/P and pcDNA/LC8/HIS. One day post transfection, cells were fixed, and permeabilised with 0.1% Triton X-100 before staining with antibody. Cells were reacted with either rabbit α-RPVP, and the primary antibody visualised with Alexa Fluor 568 α-rabbit IgG (A), or with mouse α-6 HIS (Sigma), and the primary antibody visualised with Alexa Fluor 488 α-mouse IgG (B). C shows nuclei of cells counter-stained using DAPI and D shows an overlay of the stains used.

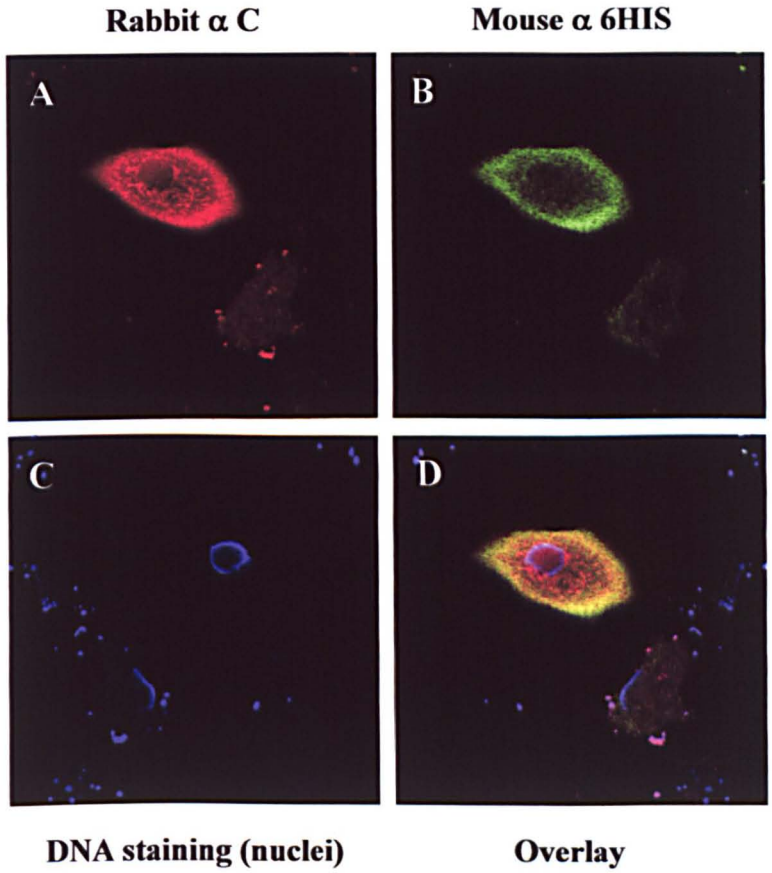

Figure 6.2.5.4. Immunofluorescence microscopy of cells co-transfected with pcDNA/C and pcDNA/LC8/HIS. A549 cells were infected with vTF7-3 and co-transfected with pcDNA/C and pcDNA/LC8/HIS. One day post transfection, cells were fixed, and permeabilised with 0.1% Triton X-100 before staining with antibody. Cells were reacted with either rabbit α-RPVC (MB38), and the primary antibody visualised with Alexa Fluor 568 α-rabbit IgG (A), or with mouse α-6 HIS (Sigma), and the primary antibody visualised with Alexa Fluor 488 α-mouse IgG (B). C shows nuclei of cells counter-stained using DAPI and D shows an overlay of the stains used.

Y2HS. Each of the EF-1 subunits should now be cloned into pcDNA and an interaction with L investigated using co-immunoprecipitation.

More interestingly, further studies using the Y2HS revealed a direct interaction between RPV L and LC8. However attempts to confirm this interaction outside of the Y2HS resulted in no such interaction being observed. This was despite the fact that the interaction was observed on numerous occasions within the Y2HS and was selected for using all the available reporter genes.

Co-immunoprecipitations were utilised but LC8 failed to co-ip L and vice versa. Initially it was thought that this lack of co-ip was due to the low levels of LC8 present in the B95a cells and Vero cells, which were used for this work. To get around this problem a construct was made which expressed a 6-HIS tagged version of LC8 from a T7 promoter. Despite boosted levels of LC8 expression in transfected cells, no direct interaction between the two proteins of interest could be confirmed, because labelled LC8/HIS was never detected by immunoprecipitation from transfected cell lysates. The α-6-HIS antibody was known to be functional as it had previously been used successfully in the laboratory for immunoprecipitation studies, and it was unlikely that the antibody had deteriorated as it was used in the same time period for accompanying immunofluorescence studies, where it was shown to be fully functional. The co-immunoprecipitation data therefore failed to confirm the observations seen with the yeast two-hybrid system, but this was largely due to problems with the labelling and detection of LC8. It would be useful to use purified LC8 protein, as was used to study the interaction of this protein with the African swine fever virus (ASFV) protein p54 (Alonso *et al.*, 2001).

Confocal microscopy was next used and again the evidence was inconclusive as to an interaction between L and LC8. In a population of A549 cells transfected with L and LC8 there did appear to be co-localisation between the two expressed proteins, but only in a small proportion of the total cell number. These data, together with those obtained from the co-immunoprecipitation studies outlined in section 6.2.4, would indicate that there is little evidence to suggest that a genuine

interaction exists between L and LC8, leading to the conclusion that the interaction found in the Y2HS may either be an artefact of the system within which it was found, or that it may be a weak or transient interaction which will be difficult to confirm.

As it had been shown that LC8 is re-organised in RPV infected cells, in addition to there being more LC8 present in RPV infected cells, it was decided to test if any of the other viral proteins present in the nucleocapsid complex co-localised with LC8 in transfected cells. Using confocal microscopy the N protein was shown not to co-localise with LC8. However, P and C could not be eliminated as they each share a diffuse cytoplasmic distribution with LC8. This remains an interesting area to be investigated further.

It has been proposed that the interaction of viral capsid proteins with the LC8 dynein light chain may be a common virus event (Poisson *et al.*, 2001). Many viruses are now known to interact with LC8 for intracellular movement of viral components along microtubules during virus infection, including the ASFV protein p54 (Alonso *et al.*, 2001). More recently several viral proteins from a wide variety of families have been shown to bind LC8 by a pepscan technique (Martinez-Moreno *et al.*, 2003). These proteins include the attachment protein of RSV, the polymerase protein of vaccinia virus, two herpes simplex virus proteins and a human coxsackievirus capsid protein, providing evidence that the hijacking of the dynein motor complex by viruses could be a common virus event.

Chapter Seven
Discussion and Future Work

The L proteins of paramyxoviruses are known to interact with the P protein to form the functionally active RNA dependent RNA polymerase complex (Hamaguchi *et al.*, 1983) and recently the L protein of rinderpest virus was demonstrated to interact with the two non-structural proteins (C and V), in addition to P (Sweetman *et al.*, 2001). One of the major aims of this study was to map the region of the L protein to which each of these three viral proteins binds.

Initial attempts to determine the sequences of L which interact with the P, C and V proteins were performed using the Promega Checkmate MTHS. However, this system was shown to be unsuitable as the majority of constructs generated using RPV coding sequence failed to localise to the nuclei of transfected cells; nuclear localisation of protein encoding constructs is essential for the functionality of the MTHS, so that the reporter gene may be transcribed. Using the MTHS, it was however possible to further investigate a putative C-C interaction, previously seen in the Y2HS but not directly in any other system (Sweetman *et al.*, 2001). Such an interaction was not confirmed using the MTHS. This does not mean that this C-C interaction does not occur, it may just be that is unable to be detected within this system. There are several possible reasons for the MTHS not detecting a genuine interaction, including the proteins being investigated not being correctly folded within this system or interactions being blocked as a result of steric hindrance.

To study the domain of L that interacts with the P, C and V proteins, co-immunoprecipitation studies were utilised. P and C were both shown to co-ip full length L. The V protein, however, failed to co-ip L yet, when expressed as a GST fusion, an L-V interaction was confirmed. A possible explanation for this result is that the monoclonal anti-V antibody (U32), which was being used to immunoprecipitate V, was in some way displacing the L protein. Previous work

had shown that L does not interact with GST (Sweetman *et al.*, 2001) an observation that was reconfirmed in these studies.

Using co-immunoprecipitation studies it was demonstrated that the P protein co-immunoprecipitates HAL1. That is, P interacts with the first of the three conserved domains of the L protein, as defined by McIlhatton and co-workers (McIlhatton *et al.*, 1997). In an effort to further characterise the L binding site for the P protein, a series of L deletions were generated to enable binding studies to be performed using the Y2HS (as in section 4.2.7 and 4.2.8). Using the Y2HS, it was confirmed that the P protein binds to the first conserved domain of the L protein. The P binding region, or at least an essential part of that binding region, was further mapped to sequence found in the first 700bp of L, corresponding to amino acids 1-233. Amino-terminal deletions are still required to define the precise boundary of the P-binding domain.

The P-L binding data obtained during the course of these studies ties in with the literature published to date as, like the RPV P protein, the P proteins of MeV (Horikami *et al.*, 1994), SV5 (Parks, 1994), hPIV-3 (Malur *et al.*, 2002) and SeV (Chandrika *et al.*, 1995, Horikami *et al.*, 1997) are all known to interact with the amino-terminal domain of the L protein. The binding site for MeV P has been mapped to the first 408 amino acids of L (Horikami *et al.*, 1994), that of SV5 has been mapped to the N-terminal 1247 amino acids of L (Parks, 1994), for hPIV-3 the first 15 amino acids of L are required (Malur *et al.*, 2002) and for SeV the P binding site has been shown to reside within the first 350 amino acids of L (Holmes & Moyer, 2002). For the rhabdoviruses VSV and rabies virus, on the other hand, the carboxy-terminal domain of L is required for interaction with P (Canter & Perrault, 1996, Chenik *et al.*, 1998). For RPV, the region of L sequence required for P binding confirms the difference seen in the binding properties of the L-P interaction between members of the rhabdoviridae family and members of the sub-family paramyxovirinae.

In contrast to the co-ip data obtained for P, each of the two non-structural proteins, C and V were shown to co-ip HAL2, demonstrating an interaction with

the central conserved domain of the L protein. At this stage however, it was not possible to rule out that the V protein does not also interact with HAL1 since the GST tagged form of the V protein migrated at exactly the same position as the HAL1 protein on Laemmli gels. To determine if V interacts with HAL1, a c-Myc tagged version of V was made, enabling the V protein to migrate at a faster rate than the HAL1 protein when analysed on Laemmli gels. However, it was found that when the c-Myc tagged V construct was co-transfected with another plasmid, the level of c-Myc V expression was dramatically reduced. Expression levels of this construct at this time are too poor to determine if HAL1 and V interact. This work is ongoing and due to time constraints could not be completed in time for inclusion in this thesis.

When the L binding characteristics of C and V were further investigated using the Y2HS, conflicting data were produced. Using the Y2HS, the C and V proteins were both shown to interact with the first conserved domain of the L protein and not the second domain of L, as was identified using co-ip studies (section 4.2.6). The C and V proteins were implied to interact with L somewhere between amino acids 1 and 363, which is well within the first conserved domain of the L protein. Taken together, these results may therefore suggest that the two non-structural proteins have an additional binding site within in the first conserved domain of the L protein that is distinct from the P binding sequence. No interactions were detected between either C or V and the domain 2 L construct using the Y2HS, which was surprising given the clear interaction demonstrated by co-ip between C and V and the second domain of L. At the start of this work, it was thought that the P and V proteins might bind to the same region of L as a result of the shared domain between P and V. However, this was shown not to be the case by both co-ip and the Y2HS. To date little is known about the regions of L required for C and V binding. It is however known that the amino-terminal half of SeV L contains the C binding site (Holmes & Moyer, 2002). Despite the fact that the C proteins of RPV and SeV are very dissimilar, it remains possible that each may bind to the same domain of L.

This part of the project is ongoing and further work will involve the confirmation of the P, C and V binding sites on L. To do this both amino- and carboxy-terminal deletions of the L protein will be made, resulting in constructs which can be utilised for protein expression in eukaryotic cells, and thus enable the use of co-ips for the remainder of these protein binding studies. Such constructs will determine whether the regions of L sequence identified by the Y2HS, or those sequences identified by co-ip studies so far, are required for L-P, L-C and L-V interactions. The existing panel of L deletions that were generated for the production of the constructs utilised for the Y2HS studies will be used for the generation of the deletions required for co-ip studies. Further Domain 2 L deletions will also need to be synthesised to ascertain which region of domain 2 is necessary for interaction with each of the non-structural proteins, C and V.

In addition to mapping the domains required for the interaction of L with P, C and V, co-immunoprecipitations were also used to reveal that the L protein of RPV interacts with itself. It was demonstrated that the region of L sequence required for self-association resides within the first conserved domain of the L protein, as full-length L was shown to co-ip HAL1. Further work now needs to be carried out to map the binding region for this novel RPV interaction. This work will be performed using the L deletions, which are currently being prepared for use in co-immunoprecipitations to further characterise the P, C and V binding sites on L. The L protein of SeV was recently demonstrated to form an oligomer in the polymerase complex (Smallwood *et al.*, 2002b). The region of SeV L required for this self-association has now been mapped to somewhere in the first 174 amino acids of the L protein (Cevik *et al.*, 2003). It has also been shown with SeV that pairs of different mutant L proteins, each defective for RNA synthesis, can be co-expressed to restore viral RNA synthesis on an added template, by complementing each other, providing further evidence that the L-L interaction is of functional importance (Smallwood *et al.*, 2002b). Furthermore, analysis of RNA-dependent RNA polymerase structures and predicted secondary structures of positive strand RNA viruses has revealed the conservation of a unique region at the amino terminus of the polymerase, which is thought to be

involved in oligomerisation, as reviewed by O'Reilly and Kao (O'Reilly & Kao, 1998).

Like the RPV L self-association, the oligomerisation of SeV L is now known to be independent of the P protein (Cevik *et al.*, 2003). Furthermore, it has been proposed that for SeV, the L protein self associates, forming an oligomer, prior to binding the P protein (Cevik *et al.*, 2003) as, in the case of SeV, P functions to stabilise the L protein (Smallwood *et al.*, 1994). This would therefore mean that the minimal constitution of the polymerase complex would be L_2:P_8, as SeV P has been shown to be a tetramer (Tarbouriech *et al.*, 2000a, Tarbouriech *et al.*, 2000b). RNA polymerase proteins of the positive sense RNA viruses, poliovirus (Hobson *et al.*, 2001, Pata *et al.*, 1995) and hepatitis C virus (Qin *et al.*, 2002, Wang *et al.*, 2002) are also known to require oligomerisation for functionality.

It is of interest to note that neither of the two hinge regions of L, as proposed by McIlhatton and co-workers (McIlhatton *et al.*, 1997), appear to be required for either the oligomerisation of L, or for the interaction of L with P, C or V. These studies may therefore provide evidence to support the claims that these two regions of sequence, which share less identity with related viruses than the three domains of highly conserved sequence, may function to mediate the optimal co-operation between each of the three conserved domains, in addition to mediating the conformation of L required for binding to some of its functional partners. Recent research with MeV has indicated that only the first of the two proposed hinge regions has either spatial or size constraints for its functionality. This is due to the fact that the c-Myc tag could be inserted into the second hinge region of MeV L without affecting the function of the polymerase (and it was subsequently shown that even with the insertion of the green fluorescent protein (GFP) ORF at this site in L, the polymerase remained functional, although polymerase activity was shown to have reduced by over 40% (Duprex *et al.*, 2002)). However, not even the insertion of the epitope tag c-Myc was tolerated in the first of the two hinge regions (Duprex *et al.*, 2002), indicating that only the second of the two hinge regions is completely flexible. These findings, together with the fact that domain 3 has been shown to be unnecessary for L

oligomerisation, or for P, C or V binding, may suggest that the third conserved domain of the L protein can be spatially re-orientated with respect to the other two domains. The fact that the first hinge region was unable to tolerate an increase in size, together with the C and V binding data, may suggest that the C and V proteins have complex L binding sites which span the first and second domains of L. Any disruption to the spatial arrangement of these domains may abolish C-L or V-L interactions. Despite the fact that domain 3 of L has been shown to be unnecessary for the self-interaction of L, or the binding of P, C or V, it has been demonstrated for SeV that the C-terminal region of L is essential for both viral transcription and replication (Smallwood *et al.*, 2002a).

Using a minigenome system and an experimental approach similar to that used by Duprex and co-workers (2002), parallel observations with RPV L would be possible. It would be interesting to determine if the L proteins of MeV and RPV are comparable with respect to the functional spatial requirement of each domain. It has, indeed, already been found that RPV L, like MeV L remains functional *in vitro* after the insertion of the GFP ORF into the second of the two hinge regions (D. Brown, personal communication). One major difference between the L proteins of MeV and RPV, which share a high level of sequence conservation, is already known; as unlike RPV L, MeV L requires co-expression with MeV P for stability (Horikami *et al.*, 1994).

Another major aim of this project was to determine if the L protein of RPV interacts with host cellular proteins. The Y2HS was used to enable a porcine macrophage cDNA library to be screened for proteins that may interact with L. After an enormous amount of effort and time, a novel interaction between L and the host cell protein, striatin, was confirmed, not only in the Y2HS, the system within which the interaction was isolated, but also confirmed by co-immunoprecipitation studies and confocal microscopy. Furthermore, it was demonstrated that striatin, when expressed as a GST fusion, co-ips HAL2, that is, it interacts with the second of the three conserved domains of the L protein. Using the Y2HS, it was shown that striatin binds L in a similar way to that of the non-structural proteins, C and V, somewhere in the first 363 amino acids of L,

indicating that striatin may also have an additional binding site located in the first conserved domain of L. However, like the P, C and V binding sites, the interacting sequences of L with striatin need to be confirmed. Co-immunoprecipitation studies will be performed using the same batch of L deletions, which are currently being cloned for use in the identification of the exact sequences of L required for self-association, in addition to interaction with P, C and V.

After a recent literature search it would appear that this is the first described interaction of a viral protein with striatin. Unfortunately, little is known is about this protein. However, striatin is known to be a highly conserved intracellular protein of 780 amino acids (86,225 kDa), belonging to the family of WD-repeat proteins (Castets *et al.*, 1996, Moqrich *et al.*, 1998), containing 8 WD-repeats at its carboxy- terminal domain (Castets *et al.*, 1996), although recent analysis has suggested that striatin may only possess 7 WD-repeats (Castets *et al.*, 2000). The large family of WD-repeat proteins constitutes a group of proteins that are specific to eukaryotes, are regulatory in function though none are known to possess enzymatic activity (Neer *et al.*, 1994). WD proteins are so-called due to the fact that they consist of between four and eight copies of a highly conserved unit – the WD-repeat, which is commonly found to start with the amino acids Gly-His (GH), followed by a core that varies in length from 23 amino acids to 41 amino acids and ends with the amino acids Trp-Asp (WD), each WD-repeat being separated by short variable sequences (Neer *et al.*, 1994).

Striatin is known to be expressed almost exclusively in the CNS, with the brain striatum being the richest source of this protein (Castets *et al.*, 1996), although striatin has also been found in kidney and placenta samples (Moqrich *et al.*, 1998), in addition to the lungs and spleen (Castets *et al.*, 2000). As a striatin clone was found in the porcine macrophage library used in this study it can be concluded that striatin is expressed in macrophages. The distribution of striatin therefore encompasses the main tissue targets of RPV which is lymphotrophic and epitheliotrophic. Striatin has been demonstrated, by subcellular fractionation, to be a mostly membrane-associated protein (Castets *et al.*, 1996). Striatin is

known to bind calmodulin in a calcium-dependent manner and was the first WD-repeat protein identified to bind calmodulin (Castets *et al.*, 1996). This interaction is known to be mediated by amino acids 149 to 166 of striatin, revealing that it is not the WD-repeats of this protein that are necessary for this interaction (Bartoli *et al.*, 1998). It is thought that striatin acts as both a calcium dependent signalling protein and as a scaffolding protein (Bartoli *et al.*, 1998, Castets *et al.*, 1996, Castets *et al.*, 2000). Striatin is known to interact with the host cell proteins phocein, a protein that is thought to play a role in membrane traffic and membrane budding reactions (Baillat *et al.*, 2002) and caveolin-1, an integral membrane protein that is a main component of caveolae, the specialised domains of rafts (Gaillard *et al.*, 2001). Furthermore, striatin is known to associate with protein phosphatase 2A, a vital serine/threonine protein phosphatase found in all eukaryotic cells (Moreno *et al.*, 2000). More recently, striatin was also shown to associate with mMOB1, a mammalian homologue of the MOB yeast protein (Moreno *et al.*, 2001). It has been proposed that striatin may interact with PP2A and mMOB1 to regulate interactions of, or re-organise, the cytoskeleton and membrane structures (Moreno *et al.*, 2001). Although the precise function of striatin remains unknown, the fact that its sequence is conserved throughout the animal kingdom, even down to *Drosophila* and *Caernorhabditis elegans*, suggests that the function of striatin is basic and well-conserved (Moqrich *et al.*, 1998).

From the literature available it is possible to postulate why L may be interacting with striatin. L may be interacting with striatin when it is complexed with phocein, in order to "piggy back" a ride from the cytoplasm to the cell membrane where viral assembly may then occur. The striatin may then dissociate from its complex with phocein and form a new association with caveolin-1 as a mechanism with which to anchor itself onto the membrane structure. MeV is already known to assemble on lipid rafts and it is known that F glycoprotein is targeted to the rafts independently of the other viral proteins (Manie *et al.*, 2000, Vincent *et al.*, 2000). Furthermore, the pneumovirus RSV assembles on the host cell surface membrane in areas rich with caveolin-1, which is known to be incorporated into mature RSV particles during virus assembly; however,

caveolin-1 was found not to associate with the viral nucleocapsids (Brown *et al.*, 2002a, Brown *et al.*, 2002b, McCurdy & Graham, 2003). This may indicate the presence of intermediary proteins, which may act as a bridge between RSV proteins and caveolin-1. Another interesting prospect is that L may be interacting with striatin in order to regulate the activity of PP2A, which may function to dephosphorylate RPV P, thereby regulating the levels of RNA synthesis.

In addition to a cDNA library screen, direct protein:protein interactions of L with host cell proteins known to interact with components of the polymerase complex of members of the order *Mononegavirales* were also investigated. These included the three subunits of translation elongation factor- 1 (EF-1 $\alpha/\beta/\gamma$), as the L protein of VSV is known to interact with the EF-1 α subunit for its activity (Das *et al.*, 1998). Using the Y2HS, no such interaction was demonstrated for the L protein of RPV, with the EF-1 γ subunit. Analysis of the EF-1 α and β yeast plasmid constructs used for the investigation of an interaction with L revealed that the EF-1 α and β subunits were not expressed in transformed yeast cells from the constructs made. It is therefore still possible that an interaction like that demonstrated for VSV L may still exist for RPV L. To confirm this, it would now appear that the method of choice would be co-ip analysis. The EF-1 subunits could be cloned into a suitable expression vector and once expression was confirmed in mammalian cells, immunoprecipitations could be performed to determine if L interacts with EF-1 $\alpha/\beta/\gamma$.

Due to the fact that the phosphoprotein component of the RNA-dependent polymerase complex of rabies virus and Mokola virus was recently found to interact with the dynein light chain, LC8 (Jacob *et al.*, 2000), an interaction between LC8 and RPV L was searched for using the Y2HS. A direct interaction between these two proteins was demonstrated within the Y2HS, but to date this has yet to be confirmed in a system outside of the Y2HS. Some evidence was obtained using confocal microscopy that would suggest these two proteins may co-localise in a small number of co-transfected cells. Additionally, in RPV infected cells, the levels of LC8 are known to have increased when compared to

non-infected cells and LC8 is also shown to be re-organised in RPV infected cells (M. Baron, personal communication). These data would suggest that a component of the viral nucleocapsid is exerting these effects on LC8. Further investigation into the mechanism of LC8 reorganisation seen in cells during RPV infection is required. Preliminary confocal microscopy findings have indicated that the N protein is not responsible (data not shown). However, it was not possible to rule out the involvement of the P, C or L proteins.

It is entirely plausible that L is interacting with LC8 in order to transport itself and other components of the viral nucleocapsid from the host cell plasma membrane to the cytoplasm where RPV is known to replicate. Several viruses are already known to utilise dynein for the transport of viral capsids including, adenovirus (Suomalainen *et al.*, 1999), herpes simplex virus type 1 (Dohner *et al.*, 2002, Ye *et al.*, 2000), African swine fever virus (Alonso *et al.*, 2001), canine parvovirus (CPV) (Suikkanen *et al.*, 2003). For hPIV-3 (Bose *et al.*, 2001) and rotaviruses (Nejmeddine *et al.*, 2000) it is known that the microtubules are used for transport, but it remains unclear whether they target a component of the dynein motor complex. Taken together, it would appear that the interaction of viral capsid proteins with the dynein motor complex might be a common exploitation of the host cell machinery by viruses.

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Appendix 1- Media, solutions and reagents

1.1 Bacterial culture media and reagents

Ampicillin (Sigma)

Standard anhydrous form (sodium salt) made up using 50% ethanol to a stock concentration of 100mg/ml. Store at -20^0C.

1.2 Buffers and Reagents used for PCR

10mM dNTP Mix

5µl 100mM dATP

5µl 100mM dTTP

5µl 100mM dCTP

5µl 100mM dGTP

Make up to 50µl with distilled deionised water.

1.3 DNA sequencing with T7 polymerase

Short sequencing termination mixes

'A' short mix	840µM each of dCTP, dGTP and dTTP; 93.5 µM dATP; 14µM ddATP; 40mM Tris-HCL (pH 7.6) and 50mM NaCl.
'C' short mix	840µM each of dATP, dGTP and dTTP; 93.5 µM dCTP; 17µM ddCTP; 40mM Tris-HCL (pH 7.6) and 50mM NaCl.

'G' short mix	840µM each of dATP, dCTP and dTTP; 93.5 µM dGTP; 14µM ddGTP; 40mM Tris-HCL (pH 7.6) and 50mM NaCl.
'T' short mix	840µM each of dATP, dCTP and dGTP; 93.5 µM dTTP; 14µM ddTTP; 40mM Tris-HCL (pH 7.6) and 50mM NaCl.

1.4 Buffers and reagents used for yeast two-hybrid analysis

Terrific Broth Base

48g Tryptone

96g Yeast extract

pH to 7.0

Add 16ml glycerol and make up to 3.6 litres with double distilled water and autoclave to sterilise.

Terrific Broth Buffer

11.5g Di-hydrogen potassium phosphate

62.7g Di-potassium hydrogen phosphate

pH to 7.0

Make up to 500ml with double distilled water and autoclave to sterilise.

Drop out amino acids (10X) (DOAA) (without histidine, tryptophan or leucine)

300mg isoleucine

1.5g valine

200mg adenine hemisulphate salt

200mg arginine

300mg lysine HCL

200mg methionine

500mg phenylalanine

2g threonine

300mg tyrosine

Make up to 1 litre with double distilled water and autoclave in 100ml volumes to sterilise.

Drop out amino acids (10X) (DOAA) (without adenine)

As above, but minus adenine hemisulphate salt.

Stock amino acids (100X)

200mg Histidine HCL in 100ml

1g leucine in 100ml

200mg tryptophan in 100ml

Filter sterilise or autoclave and store at 4°C.

Synthetic complete plates minus tryptophan (SC-T plates)

170ml molten YNB agar

20ml 10X DOAA

10ml 40% dextrose

2ml histidine

2ml leucine

Synthetic complete plates minus tryptophan and leucine (SC-T-L plates)

170ml molten YNB agar

20ml 10X DOAA

10ml 40% dextrose

2ml histidine

Synthetic complete plates minus tryptophan, leucine and adenine (SC-T-L-Ade plates)

170ml molten YNB agar

20ml 10X DOAA (without adenine)

10ml 40% dextrose

2ml histidine

Antibody	Target	Antibody Type	Source
MB2	RPV N: peptides based on N carboxy terminus	Rabbit PAb	(Baron *et al*., 1999)
MB18	RPV P: single carboxy terminal peptide of P	Rabbit PAb	(Baron & Barrett, 1997)
MB38	RPV C and GST: GST/C protein fusion	Rabbit PAb	(Baron & Barrett, 2000)
α-V_2	RPV V: against MeV V	Rabbit PAb	D. Briedis (Wardrop & Briedis, 1991)
C39	RPV N	Mouse Mab	Fleming & Barrett, unpublished
MB41	RPV L: GST fusion of carboxy terminal third of L	Rabbit PAb	(Sweetman *et al*., 2001)
α-HA	HA tag	Rat Mab	Roche
2-1	RPV P	Mouse MAb	Dr. Sugiyama (Sugiyama *et al*., 1989)
U32	RPV P/V	Mouse MAb	Dr. Sugiyama (Sugiyama *et al*., 1989)
9E10	c-Myc tag	Mouse MAb	Santa Cruz Biotechnology
α-c-Myc	c-Myc tag	Rat MAb	Serotec
A-14	c-Myc tag	Rabbit PAb	Santa Cruz Biotechnology
α-PIN	LC8	Mouse MAb	BD Trandsuction Laboratories
α-LC8	LC8	Rabbit PAb	S. King, University of Connecticut, USA
α-6-HIS	poly HIS tag	Mouse MAb	Sigma
α-HA	HA tag	Rabbit PAb	Santa Cruz Biotechnology

Appendix 2: A list of all primary antibodies utilised during this project. PAb= polyclonal antibody and MAb=monoclonal antibody.

Appendix 3- Primer Sequences

3.1 Primers designed for the generation of MTHS constructs

Primer Name	Primer Sequence (5' - 3')
KatNstart	TCTCTACGCGTAAATGGCTTCTCTCTTGA
KatNend	CTCTTGCGGCCGCTCAGTTGAGAATATCCTTGT
KatLstart	TATCTCGTCGACCCATGGACTCCCTCTCA
KatLend	TCTCGCGGCCGCCAGTTGCAGAATCACTCC
KatCstart	TCTCTACGCGTCCATGTCAACAAAGGCCTG
KatCend	TCTCGCGGCCGCCTTCTACTGTTTCAACATC
KatP/Vstart	ACCGTACGCGTAGATGGCAGAGGAGCAAG
KatPend	TCTCGCGGCCGCCTAGTTCTTTAGAATTTTGACC
KatVend	TCTCGCGGCCGCTCAATGTTACTCTGGGATAT

3.2 Primers designed to insert a new *Sal*I site downstream of L in pGempola

Primer Name	Primer Sequence (5'-3')
KatL1	CGTCGACAGGCC
KatL2	TGTCGACGGGCC

3.3 Overlapping primers designed to re-introduce a *Not*I site into pcDNA/HA

Primer Name	Primer Sequence (5'-3')
HAins1	AATTCGATATCGCGGCCGC
HAins2	TCGAGCGGCCGCGATATCG

3.4 Primers designed for the generation of pcDNA/HAL domain constructs

Primer Name	Primer Sequence (5'-3')
HAL1Start	ATATGCGGCCGCCATGGACTCCCTCTCAGT
HAL1End	TTATGTCGACTCATATCTGATCGGGGCCAC
HAL2Start	ATATGCGGCCGCTGCTAAGACTTTCTCCTCCAAG
HAL2End	ATATGTCGACTCAATTGCTTATGTGCATATCAG
HAL3Start	ATATGCGGCCGCTCTAACTGTAACAGATCC
Lend	TCTCGCGGCCGCCAGTTGCAGAATCACTCC

3.5 Primer designed for the generation of the newstartC pcDNA construct

Primer Name	Primer Sequence (5'-3')
NewCstart2	CAGCAGAATTCACCATGGGGTCAACAAAGGCCTG--GAAT

3.6 Primers designed for the insertion of a 6-HIS tag into pcDNA/LC8

Primer Name	Primer Sequence (5'-3')
lc8hisrev	CCCCGAGTCGACTTAATGATGATGATGATGATGA--CCAGATTTGAACAGAAG
lc8hisfor	CCCCTAGAATTCACCATGTGTGACCGAAAG

3.7 Primers designed for the insertion of a c-Myc tag into pcGem/V

Primer Name	Primer Sequence (5'-3')
Vmycfor	AATTATGGAACAAAAACTCATCTCAGAAGAGGAT--CTGGGCATG
Vmycrev	CATGCCCAGATCCTCTTCTGAGATGAGTTTTTGT--TCCAT

3.8 Primers designed for the generation of pAS2/L deletion mutants

Primer Name	Primer Sequence (5'-3')
L1-500rev	ATATCCCGGGTTATGTCTTGATCGTGAACCAG
L1-700rev	ATAACCCGGGTTAGATATCACAGTACATCAACAC
L1-900rev	ATATCCCGGGTTATAATTCTGTTGTTACATCTCTCA
L1-1100rev	ATATCCCGGGTTAGATTGCCTCAAGCCTGGG
L1-1500rev	ATATCCCGGGTTAATCGACTAGCCTCCGTGAA
L1-2400rev	TTATCCCGGGTTATGTCACGCGTGCAGCTTC

3.9 Universal M13 sequencing primers

Primer Name	Primer Sequence (5'-3')
Forward	GTAAAACGACGGCCAGT
Reverse	CAGGAAACAGCTATGAC

3.10 pcDNA sequencing primers

Primer Name	Primer Sequence (5'-3')
T7promoter	TTAATACGACTCACTATAGGG
SP6promoter	AGCATTTAGGTGACACTATA

3.11 NewstartC pcDNA sequencing primer

Primer Name	Primer Sequence (5'-3')
KATcpr1a	TCTGGGGAGGAGATGAGG

3.12 pACT2 sequencing primers

Primer Name	Primer Sequence (5'-3')
act25'	CTATTCGATGATGAAGATACCCCACCAAACCC
act23'	GAAGTGAACTTGCGGGGTTTTTCAGTATCTACG
pACT25'A	AGAGATCTGTATGGCTTAC
pACT23'A	ATAGATCTCTCGAGCTCG
pACT23'B	GATGTATAAATGAAAGAA

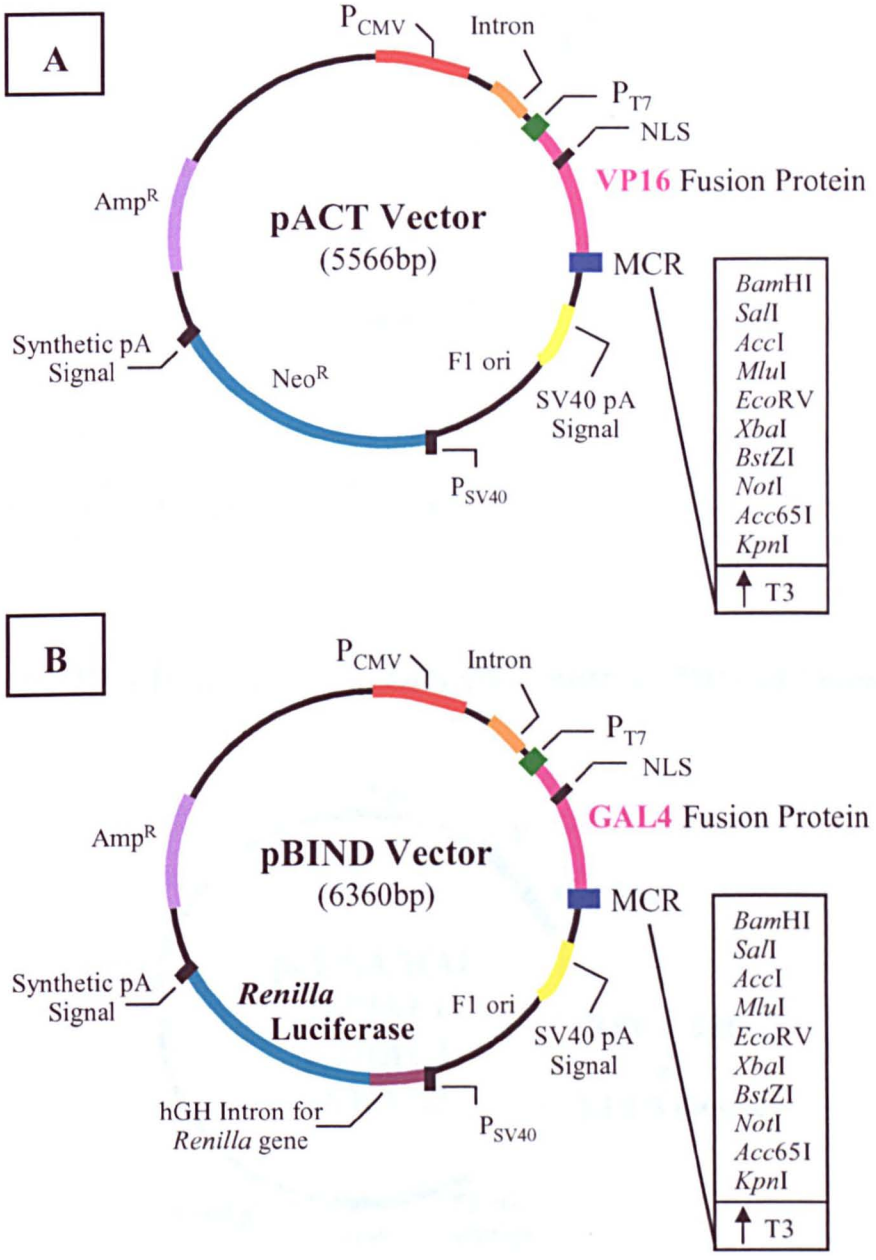
3.13 pAS2 sequencing primers

Primer Name	Primer Sequence (5'-3')
pas2rseq	CATGCCGGTAGAGGTGTGGTCA
pAS2F	GACTGTATCGCCGGTATTGCAA
pAS2R	ATTCGCCCGGAATTAGCTTGG

Appendix 4- Plasmid Maps

The relative positions of all major features of plasmids are shown. For clarity, the size of features in the maps are not shown to scale.

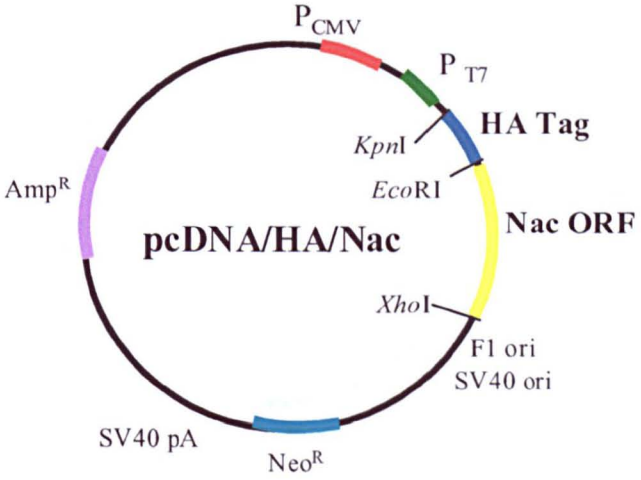
4.1 MTHS pACT and pBIND Plasmid Maps



KEY: P_{CMV}= CMV immediate-early promoter, P_{T7}= T7 RNA polymerase promoter, P_{SV40}= SV40 early promoter, T3= T3 RNA polymerase promoter, pA= polyadenylation signal, Amp^R= ampicillin resistance gene, Neo^R= neomycin resistance gene.

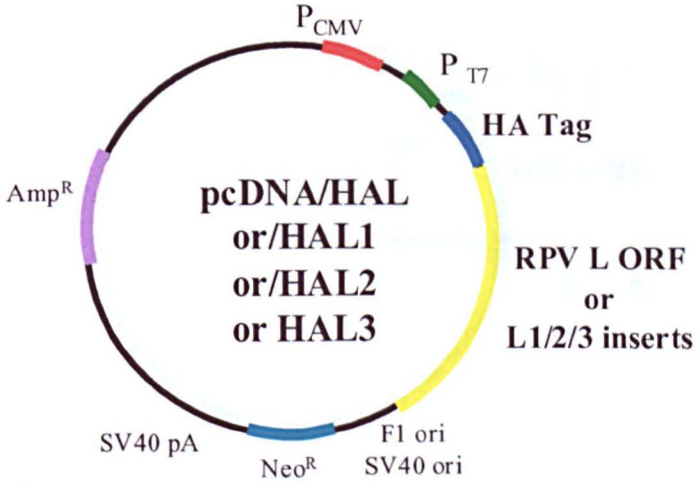
4.2 pcDNA/HA/Nac Plasmid Map

This plasmid, a derivative of pcDNA3 (Invitrogen), was used in the construction of pcDNA/HAL and the pcDNA/HAL domain mutants.



KEY: P_{CMV}= CMV immediate-early promoter, P_{T7}= T7 RNA polymerase promoter, F1 ori= phage F1 origin of replication, SV40 ori= SV40 origin of replication, pA= polyadenylation, Amp^R= ampicillin resistance gene, Neo^R= neomycin resistance gene.

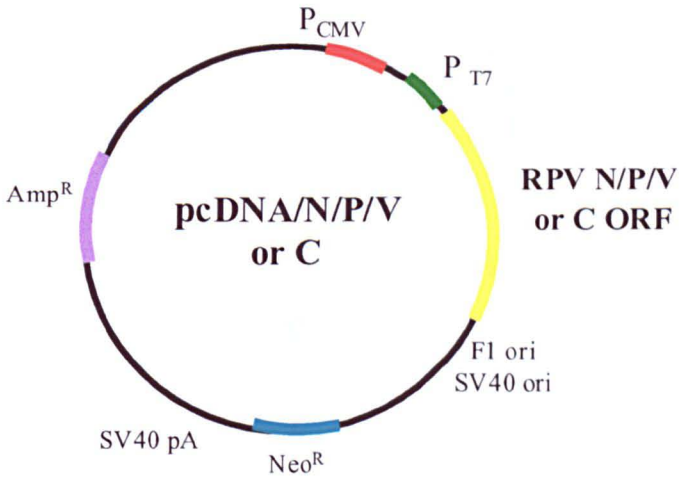
4.3 pcDNA/HAL and HAL Domain Construct Plasmid Maps



KEY: P_{CMV}= CMV immediate-early promoter, P_{T7}= T7 RNA polymerase promoter, F1 ori= phage F1 origin of replication, SV40 ori= SV40 origin of replication, pA= polyadenylation, Amp^R= ampicillin resistance gene, Neo^R= neomycin resistance gene.

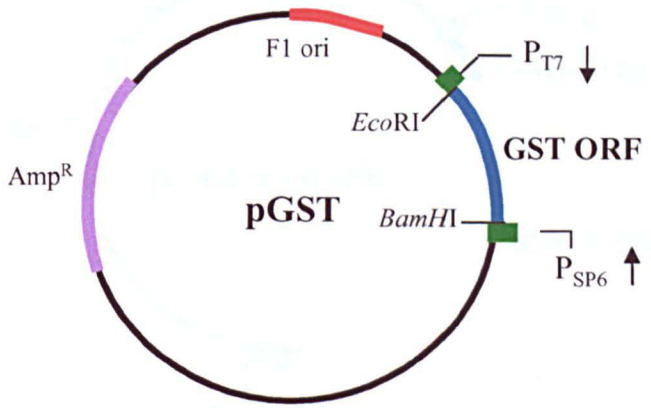
4.4 pcDNA/N/P/V or C Plasmid Map

These plasmids are all derivatives of pcDNA3.1 (Invitrogen).



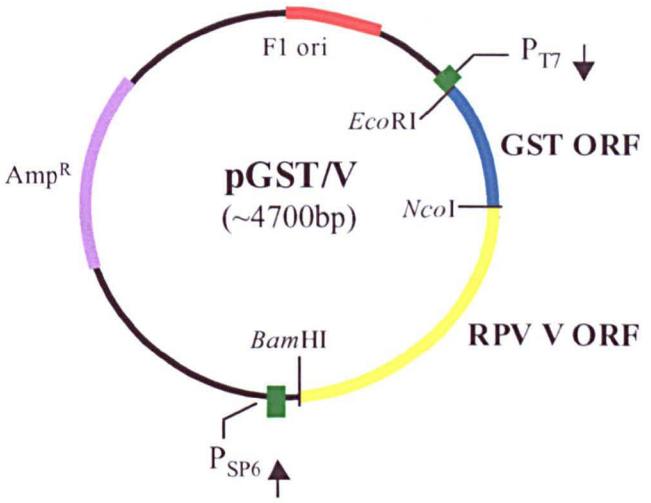
KEY: P_{CMV}= CMV immediate-early promoter, P_{T7}= T7 RNA polymerase promoter, P_{SV40}= SV40 early promoter, F1= T3 RNA polymerase promoter, pA= polyadenylation, Amp^R= ampicillin resistance gene, Neo^R= neomycin resistance gene.

4.5 pGST Plasmid Map



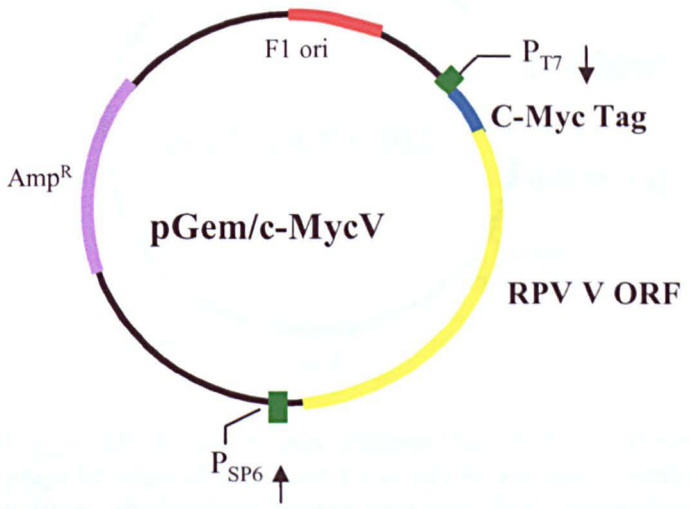
KEY: P_{T7}= T7 RNA polymerase promoter, P_{SP6}= SP6 promoter, Amp^R= ampicillin resistance gene, F1 ori= phage F1 origin of replication.

4.6 pGST/V Plasmid Map



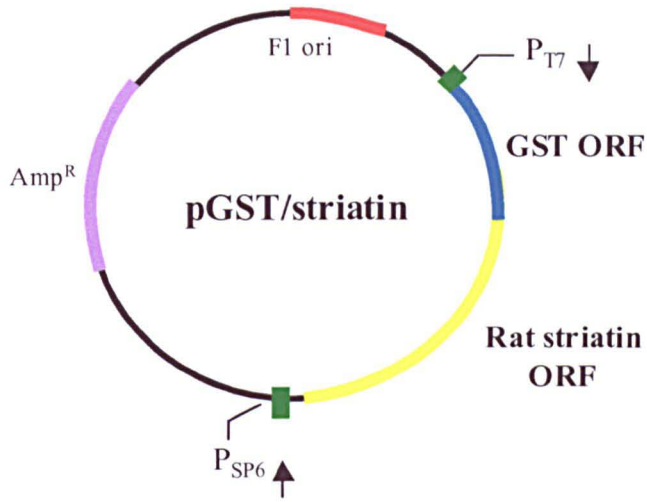
KEY: P_{T7}= T7 RNA polymerase promoter, P_{SP6}= SP6 promoter, Amp^R= ampicillin resistance gene, F1 ori= phage F1 origin of replication.

4.7 pGem/c-MycV Plasmid Map



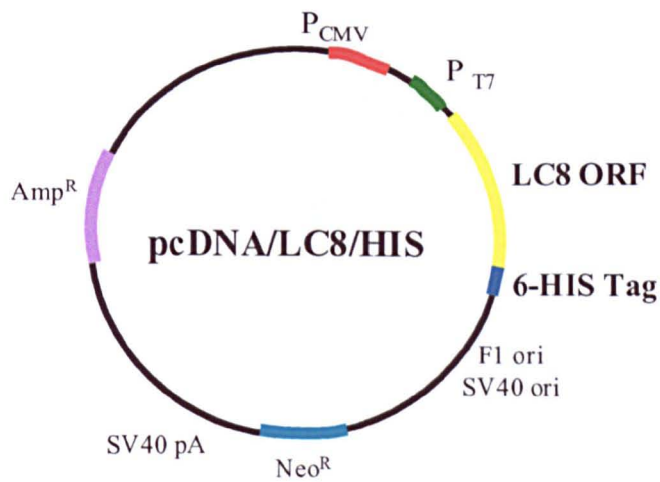
KEY: P_{T7}= T7 RNA polymerase promoter, P_{SP6}= SP6 promoter, Amp^R= ampicillin resistance gene, F1 ori= phage F1 origin of replication.

4.8 pGST/striatin Plasmid Map



KEY: P_{T7}= T7 RNA polymerase promoter, P_{SP6}= SP6 promoter, Amp^R= ampicillin resistance gene, F1 ori= phage F1 origin of replication.

4.9 pcDNA/LC8/HIS Plasmid Map



KEY: P_{CMV}= CMV immediate-early promoter, P_{T7}= T7 RNA polymerase promoter, F1 ori= phage F1 origin of replication, SV40 ori= SV40 origin of replication, pA= polyadenylation, Amp^R= ampicillin resistance gene, Neo^R= neomycin resistance gene.