The Molecular Basis of Malignant Catarrhal Fever

Colin Peter Sharp

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Declaration

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

Colin Sharp

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Laboratory for Clinical and Molecular Virology
Department of Veterinary Biomedical Sciences
Royal (Dick) School of Veterinary Studies
University of Edinburgh
Summerhall Square
Edinburgh, EH9 1QH

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Abstract

Alcelaphine herpesvirus-1 (AlHV-1) is gammaherpesvirus that can cause the devastating, fatal disease malignant catarrhal fever (MCF) in susceptible ruminant hosts but not in its natural host the blue wildebeest. Despite its scientific and economic importance little is known about the underlying molecular basis of MCF pathogenesis. The purpose of this study is to characterise four unique open reading frames (ORFs) of AlHV-1 and examine their contribution to viral pathogenesis. These ORFs are located at the left hand end of the genome, a region known to contain unique transforming and immunomodulatory genes in other gammaherpesviruses, and are predicted to encode two small gene products with no significant homology to any known proteins (ORF A1 and ORF A4), a transcription factor (ORF A2) and a member of the semaphorin family (ORF A3).

A 6.2 Kb fragment from AlHV-1 containing all four ORFs under their natural promoters was cloned into the left hand end region of murine gammaherpesvirus-76 (MHV-76). This allowed for the study of the *in vivo* contribution to pathogenesis of the gene products in a well characterised small animal model. The recombinant virus showed no difference in its ability to replicate *in vitro*. Viral titres and lung pathology in infected mice were also comparable although the AlHV-1 gene transcripts were detectable.

The ORF A2 gene product expressed as a recombinant fusion protein in mammalian cells consistently showed nuclear localisation, supporting the prediction that this protein functions as a transcription factor. However, attempts to conclusively demonstrate transcriptional activity of this protein were unsuccessful.

The four left hand end genes were also used to screen a novel bovine cDNA library in a yeast two-hybrid system. Although no specifically interacting proteins could be identified and confirmed, analysis of the bait constructs used indicated that there is a domain or domains in the C-terminus of the ORF A2 gene product capable of interacting with DNA or DNA binding proteins, again supporting a role for this protein as a transcription factor.

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Chapter 1: Introduction

1.1 Herpesviruses

The family *Herpesviridae* includes a large number of enveloped, double stranded (ds) DNA viruses that infect a broad range of host species (reviewed in Roizman & Pellett, 2001). Over 130 herpesviruses have currently been isolated from species as diverse as humans and oysters. With newer, more sensitive techniques available for the detection of novel viruses (Ehlers, 2005), the number of known viruses and host range is likely to increase. Herpesviruses are thought to have co-evolved closely with their natural host species. This is supported by the presence of cellular gene homologues in most viruses and the fact that fatal infections in immunocompetent natural hosts are rare.

The range of clinical outcomes resulting from herpesvirus infections can vary greatly from inapparent infection to death. Herpesvirus infections, especially in immunocompromised or heterologous hosts, have been associated with a number of fatal conditions including encephalitis and tumour formation.

A number of biological properties are shared by all herpesviruses (reviewed in Roizman & Pellett, 2001). First, all encode proteins involved in DNA synthesis, nucleotide metabolism and protein processing. Second, viral DNA synthesis and capsid assembly occurs in the nucleus with capsids initially becoming enveloped as they pass through the nuclear membrane. Third, the production of infectious virions is accompanied by the destruction of the infected cell. Finally, all herpesviruses are capable of maintaining a latent infection in their natural host.

In addition to the genes encoding structural proteins and enzymes involved in viral replication, herpesviruses encode an array of proteins involved in virus-host interactions. These include proteins responsible for the modulation of the host cell environment (e.g. interacting with cell cycle machinery) and the subversion of host resistance at both the cellular (e.g. blocking apoptosis) and organism (e.g. immune evasion and immunomodulation) levels.

1.1.1 Herpesvirus Structure

Virion architecture is well conserved among the herpesviruses. The structure of a typical virion is shown in Figure 1.1. The linear dsDNA genome is tightly packed in an icosahedral capsid of approximately 100 to 110 nanometres (nm) in diameter.

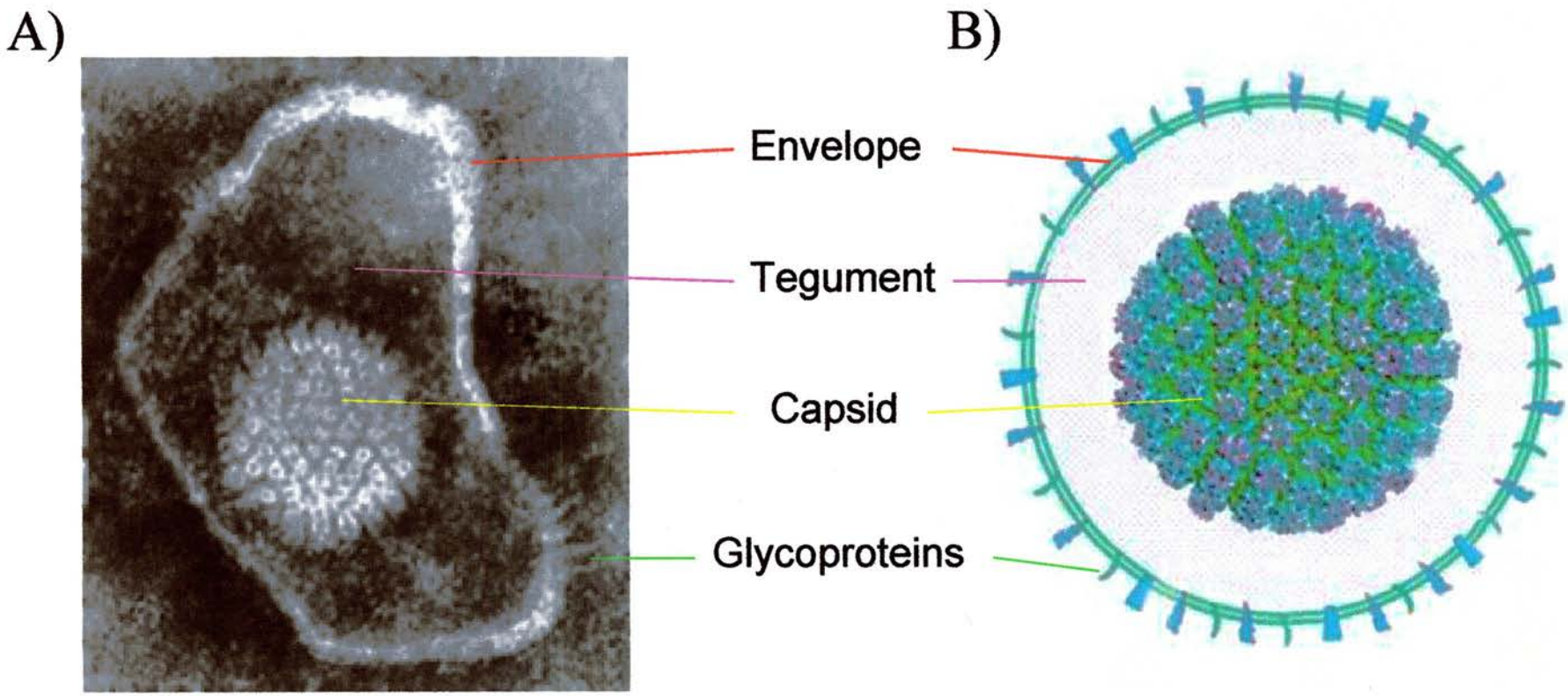


Figure 1.1. A typical herpesvirus virion A) visualised by transmission electron microscopy and B) represented diagrammatically showing the major structural components. Both images taken from http://pathmicro.med.sc.edu/mhunt/dna1.htm.

Between the capsid and the envelope is a substance named the tegument. Although generally amorphous in appearance, cryoelectron tomography and immunoelectron microscopy have shown the presence of filament structures and polarity across the tegument indicating that it does possess some order (Grunewald *et al.*, 2003; Stefan *et al.*, 1997). Surrounding the tegument is a lipid envelope with a typical trilaminar appearance (Epstein, 1962). The cellular origin of the virion envelope will be discussed further in Section 1.3.1. Protruding from the envelope are a number of viral glycoproteins. Cryoelectron tomography has shown that these glycoproteins are distributed non-randomly in the envelope, suggesting functional clustering (Grunewald *et al.*, 2003). Due to variation in the tegument thickness, the overall size of herpesvirus virions can range from 120 to nearly 300 nm.

1.1.2 Herpesvirus Genomes

The genome length of herpesviruses can vary from approximately 120 to 250 kilobase pairs (kb). Internal and terminal reiterated sequences are commonly found in herpesvirus genomes. In some cases, the copy number of these repeat sequences can lead to variation in the genome length of individual viruses of up to 10 kb. Herpesviruses can be divided into six groups, designated by the letters A to F, based upon the presence, number and location of these reiterated sequences. Diagrammatic representations of the sequence arrangements of these six groups adapted from Roizman *et. al.* (1992) are shown in Figure 1.2. In group A viruses, a large region from one terminus of the genome is directly repeated at the other terminus. These regions are designated the left terminal repeat (LTR) and right terminal repeat (RTR). The genomes of group B viruses contain a variable number of directly repeated sequences at both termini. Similar direct repeats are present at the termini of group C virus genomes although these are generally fewer in number. In addition, the unique sequences of group C viral genomes are subdivided by other, unrelated, directly repeated sequences of greater than 100 base pairs (bp). The unique sequences of group D viral genomes are separated into unique long (or large, U_L) and unique short (or small, U_S) domains by an inverted repeat of the sequence from the other terminus of the U_S domain. The U_S domain can invert relative to the U_L domain. As such, DNA extracted from virions or infected cells contains two equimolar populations differing only in the U_S orientation. The unique sequences of group E viral genomes are also divided into U_L and U_S domains. Each of these domains is flanked by its own

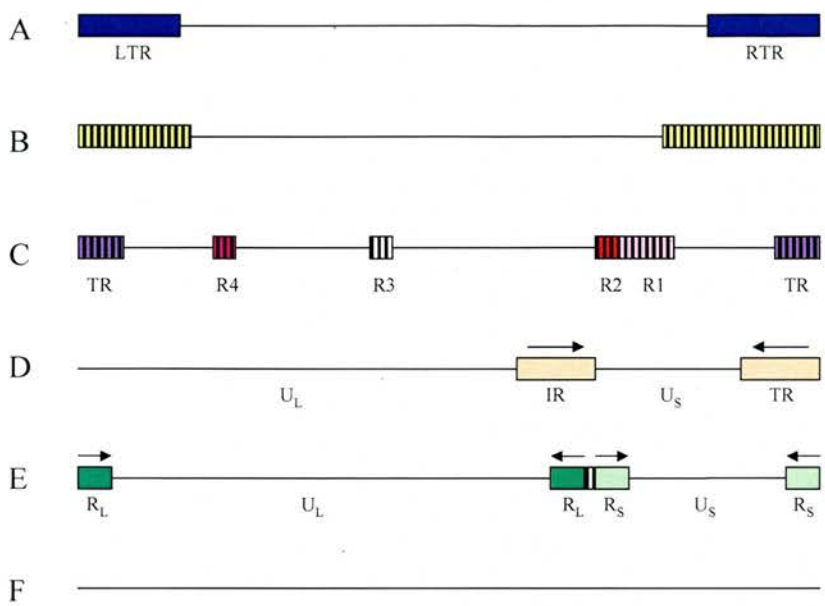


Figure 1.2. Diagrammatic representations of the sequence arrangements of the six classes of viral genomes comprising the family *Herpesviridae* with unique sequences represented by lines and repeat sequences represented by rectangles. Where appropriate, the relative orientations of repeat sequences are indicated with arrows. The genomes of group A viruses (eg. channel catfish herpesvirus) contain a large sequence from one terminus directly repeated at the other terminus. The genomes of group B viruses (eg. herpesvirus saimiri) contain a variable number of directly repeated sequences at both termini. The genomes of group C viruses (eg. Epstein-Barr virus) contain both terminal repeats and other unrelated repeat sequences that subdivide the unique sequence. The unique regions of the genomes of group D viruses (eg. varicella-zoster virus) are divided into two segments separated by an inverted repeat of the terminal region from the short (U_S) segment. The unique regions of the genomes of group E viruses (eg. herpes simplex virus and human cytomegalovirus) are similarly divided with inverted repeats flanking both the long (U_L) and U_S regions. No repeat regions have been described in the genomes of group F viruses (eg. tupaia herpesvirus). Adapted from Roizman *et. al.* 1992. Details in text Section 1.1.3.

inverted repeat sequence, referred to as the R_L (long or large) and R_S (short or small) repeat. Like the U_S domains of group D viruses, both the U_L and U_S domains of group E viruses can invert relative to each other meaning that four possible isomers are found. No repeated sequences have been described in the genomes of group F viruses.

1.2 Herpesvirus Classification

The family *Herpesviridae* is divided into three subfamilies: *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. Classification of viruses into these subfamilies was originally performed on the basis of biological properties of the virus such as host range, reproductive cycle kinetics and the site of establishment of latent infection (Roizman *et al.*, 1973; Roizman, 1979). Classification is now performed on the basis of DNA and protein sequence properties including viral DNA polymerase and glycoprotein B (gB) sequences as well as gene content and genome arrangement (Roizman *et al.*, 1992). In most cases, this new basis for classification has supported previous system although it has resulted in the re-classification of Marek's disease virus (MDV) as an alphaherpesvirus rather than a gammaherpesvirus. In addition to the three main subfamilies, the genus *Ictalurivirus* has been established to contain channel catfish herpesvirus (Ictalurid herpesvirus 1) and related viruses that do not fit neatly into any of the other subfamilies.

1.2.1 Alphaherpesviruses

Alphaherpesviruses classically have a wide host range, short reproductive cycle and rapid spread in culture. Infection of most cell types with alphaherpesviruses leads to productive lytic infection and the establishment of latency occurs in only a few cell types, usually neuronal cells. Members of the *Alphaherpesvirinae* are subdivided into four genera: *Simplexvirus* (including herpes simplex virus 1, HSV-1), *Varicellovirus* (including varicella-zoster virus, VZV), *Mardivirus* (including MDV) and *Iltovirus* (including infectious laryngotracheitis virus).

HSV-1 and HSV-2

HSV-1 (human herpesvirus 1, HHV-1) and HSV-2 (human herpesvirus 2, HHV-2) are two closely related, widespread human pathogens. HSV-1 and HSV-2 are generally associated with oral and genital infections, respectively, although this distinction is far from absolute. HSV infections have a broad spectrum of disease associations ranging from mild cutaneous lesions to potentially fatal encephalitis. Primary HSV infection is generally asymptomatic and is followed by the establishment of latency in the ganglia innervating the site of infection. Reactivations are associated with episodes of

disease, the frequency of which can vary depending on virus subtype and site of infection.

Lesions on the lip (cold sores) are the most common manifestation of HSV-1 reactivation and are estimated to occur in 20-40% of adults (Bader *et al.*, 1978; Embil *et al.*, 1975). Similarly, genital vesicular lesions are caused by reactivations of HSV-2 and, based on viral seroprevalence, are estimated to occur in 5-25% of the adult populations of Western countries (Smith & Robinson, 2002).

A number of other conditions are associated with HSV infections. For example, HSV-1 can cause ocular infections and is a major cause of vision loss (Darougar *et al.*, 1985). HSV infection can cause potentially serious complications of atopic eczema (Bork & Brauninger, 1988) and can also trigger the severe cutaneous conditions erythema multiforme and Stevens-Johnson syndrome (Schofield *et al.*, 1993). The most serious manifestation of HSV infection is herpes simplex encephalitis which can cause neurological impairment and death, particularly in neonates (Ferrante *et al.*, 2000).

VZV

VZV, or human herpesvirus 3 (HHV-3), is another widespread, highly transmissible human alphaherpesvirus. Primary infection usually occurs during childhood via the inhalation of infectious droplets. Virus is disseminated via the blood and viraemia is normally accompanied by the appearance of widespread skin lesions known as "chicken pox" or varicella (Asano *et al.*, 1990; Ito *et al.*, 2001; Mainka *et al.*, 1998). Primary VZV infection can also be associated with pneumonia and other serious systemic infections (Rawson *et al.*, 2001).

VZV establishes latency in sensory ganglia (Kennedy *et al.*, 1998; Lungu *et al.*, 1995). In contrast to the frequent reactivations seen with HSV, reactivation of VZV normally occurs only once. This reactivation results in herpes zoster, the appearance of a vesicular rash generally restricted to a single dermatome and accompanied by sharp radicular pain. Following resolution of the zoster rash itself, pain and sensitivity can persist in the affected dermatome for months or even years in a condition known as post herpetic neuralgia.

MDV

Marek's disease is a condition of chickens originally associated with polyneuritis and visceral lymphoma (Marek, 1907; Pappenheimer *et al.*, 1929a; Pappenheimer *et al.*, 1929b). The aetiological agent of this disease, MDV (or gallid herpesvirus 2, GaHV-2), was originally classified as a gammaherpesvirus based on its oncogenic potential and slow growth in tissue culture but has since been classified as an alphaherpesvirus (Fukuchi *et al.*, 1984).

The tissue culture isolation of MDV in the 1960s allowed for the production of vaccine strains of the virus (Churchill *et al.*, 1969). Inoculation with these vaccine strains initially reduced the incidence of disease by 99%. However, the use of such vaccines led to the emergence of strains that were both resistant to vaccination and induced a more severe acute disease referred to as virulent MDV (vMDV). The production of new vaccine strains to immunise against emerging resistant strains has influenced the evolution of the virus such that very virulent (vv) and vv+ strains have since emerged (Witter, 1997). The nature of the disease induced by these emerging strains has also changed into an acute or peracute condition in which peripheral neuritis and lymphoma can be absent and neurological signs including paralysis and massive brain lesions dominate (Gimeno *et al.*, 1999; Gimeno *et al.*, 2001; Witter *et al.*, 1999).

1.2.2 Betaherpesviruses

The host range for the members of the subfamily *Betaherpesvirinae* is more limited than that of alphaherpesviruses. Betaherpesviruses have a long reproductive cycle and grow slowly *in vitro*. Like alphaherpesviruses, infection of most cell types with betaherpesviruses leads to productive lytic infection although latent infection can be established, predominantly in cells of the monocyte/macrophage lineage. This subfamily is subdivided into three genera: *Cytomegalovirus* (including human cytomegalovirus, HCMV), *Muromegalovirus* (including murine cytomegalovirus, MCMV) and *Roseolovirus* (including human herpesvirus 7, HHV-7).

HCMV

HCMV is a ubiquitous human pathogen infecting between 50 and 90% of the adult population, depending on geographic location and other socio-economic factors (Gandhi & Khanna, 2004). Primary infection is generally asymptomatic but can lead to the development of an infectious mononucleosis similar to that caused by EBV (Stern, 1968). Latent infection is established in mononuclear cells and reactivations are associated with shedding of the virus from mucosal sites (Mocarski, 2004). Although normally asymptomatic, the presence of HCMV has been linked to a number of chronic inflammatory conditions.

Complications with HCMV infection are a major problem for neonates and immunosuppressed individuals. Congenital HCMV infection is the leading infectious cause of mental retardation and a number of other birth defects including deafness (Boppana *et al.*, 1999; Pass *et al.*, 2006). HCMV is also the most important viral pathogen related to opportunistic infections following both solid organ and stem cell transplantation with potentially fatal pneumonitis being the most serious manifestation of disease (Gandhi *et al.*, 2003). The severity and incidence of these infections has led to the categorisation of HCMV as a Level 1 vaccine candidate by the Institute of Medicine (Arvin *et al.*, 2004) and several vaccine studies are currently underway.

1.2.3 Gammaherpesviruses

The subfamily *Gammaherpesvirinae* contains a number of viruses of importance to human and veterinary health. Gammaherpesviruses are classically perceived to have a narrow host range although this is not altogether accurate as the natural host range of some members can extend across an entire animal order. Unlike alpha- and betherpesviruses, the lytic replication of gammaherpesviruses is generally supported only by a subset of cell types and they tend to favour latent infection, particularly in their natural hosts and usually in lymphocytes. As such, many gammaherpesviruses encode proteins involved in the maintenance of latent genomes in dividing cells. In addition, the genomes of gammaherpesviruses contain more homologues of cellular genes than do those of the alpha- and betaherpesviruses. These genes tend to be located at the terminal regions of the genome and a number of specific examples are discussed in Section 1.5. The Herpesvirus Study Group of the International

Committee on Taxonomy of Viruses currently has the subfamily *Gammaherpesvirinae* subdivided into two genera: *Lymphocryptovirus* or gamma-1, (including Epstein Barr virus, EBV) and *Rhadinovirus* or gamma-2 (including herpesvirus saimiri, HVS).

EBV

EBV, or human herpesvirus 4 (HHV-4), is the prototypic lymphocryptovirus and one of the best studied of the gammaherpesviruses. EBV is highly prevalent and infects the vast majority of adults worldwide. Primary infection usually occurs during childhood and is asymptomatic. However, primary infection during adolescence can lead to the development of an acute infectious mononucleosis (IM) (McCollum, 1970) characterised by fever, lymphadenopathy, tonsillitis, pharyngolaryngitis and skin eruptions.

Latent EBV infection in B lymphocytes is associated with the formation of a number of neoplastic conditions including Burkitt's lymphoma, Hodgkin lymphoma, and nasopharyngeal carcinoma (Henle & Henle, 1970). These and other neoplasms often present in patients with immunodeficiencies arising from age, human immunodeficiency virus (HIV) infection or immunosuppressive drug treatment (Ambinder & Mann, 1994; Hanto *et al.*, 1981; Shibata *et al.*, 1991b; Shibata *et al.*, 1991a). In certain geographic areas, however, EBV-associated cancers can occur in the absence of immunodeficiency (Harabuchi *et al.*, 1990; Kawa-Ha *et al.*, 1989; Magana *et al.*, 1998; Su *et al.*, 1995) indicating a role for host genetic background or environmental co-factors in their development.

HVS

HVS is the prototypic rhadinovirus. The natural host of HVS is the squirrel monkey (*Saimiri sciureus*) which becomes infected early in life and maintains a lifelong persistent infection. No apparent disease is caused in the natural host during either acute infection or persistence (Melendez *et al.*, 1968). In contrast, infection of other New World and Old World monkeys has been shown to cause lymphomas and can induce T cell leukaemia.

HVS strains have been classified based on their left hand end sequence similarities and their pathogenicity in different hosts (Medveczky *et al.*, 1989; Medveczky *et al.*, 1984). Subgroup B viruses have the lowest oncogenic potential and subgroup C viruses have the highest. *In vitro*, representative strains from all three subgroups have been shown to immortalise peripheral blood mononuclear cells (PBMC) from common marmosets (Szomolanyi *et al.*, 1987), although those immortalized by the subgroup B virus (SMH1) still required supplemental interleukin-2 (IL-2) for growth.

Although all three subgroups sub-clinically infect the HVS natural host, they differ in their abilities to cause disease in other species. Cottontop tamarins (*Saguinus oedipus*) are highly susceptible to HVS and infection by any of the subgroups can induce disease (Falk *et al.*, 1972; Hunt *et al.*, 1970a; Knappe *et al.*, 1998). Disease caused by all three subgroups has also been reported in another new world species the common marmoset (*Callithrix jacchus*) (Duboise *et al.*, 1998; Laufs & Fleckenstein, 1973; Wright *et al.*, 1977), although there have been reports that this species is not susceptible to infection with subgroup B viruses (Laufs & Melendez, 1973). Disease in Old World primate species, such as rhesus monkeys (*Macaca mullata*) and cynomolgus macaques (*Macaca fascicularis*), has only been reported for subgroup C viruses (Alexander *et al.*, 1997; Knappe *et al.*, 2000) as has the *in vitro* transformation of human T cells (Biesinger *et al.*, 1992).

KSHV

Kaposi's sarcoma-associated herpesvirus (KSHV) or human herpesvirus 8 is the most recently discovered human herpesvirus and, to date, the only human rhadinovirus. Kaposi's sarcoma (KS) is a connective tissue cancer forming highly vascular, dark lesions containing abnormal, elongated tumour cells known as spindle cells. Historically, KS was known to occur rarely in elderly Mediterranean men (classic KS) and in patients receiving immunosuppressive therapy. In the 1980s, a drastic increase in the incidence of KS was seen in HIV patients (epidemic KS). KSHV was discovered in 1994 in the search for an infectious agent related to this outbreak (Chang *et al.*, 1994) and epidemiological evidence has since demonstrated that KSHV is the causative agent of all forms of KS (Birley & Schultz, 1997; Sarid *et al.*, 1999). In addition to KS, KSHV has been associated with a number of other tumours

including primary effusion lymphoma (PEL) (Cesarman *et al.*, 1995) and some forms of multicentric Castleman's disease (Soulier *et al.*, 1995).

MHV-68

Murine gammaherpesvirus 68 (MHV-68), or murid herpesvirus 4 (MHV-4), has proven to be a useful model for the *in vivo* study of gammaherpesvirus infections. As mutagenesis of the virus is relatively straightforward and it is capable of infecting laboratory mouse strains, the influence of both virus and host genetics on viral pathogenesis can be studied.

Transmission of MHV-68 is thought to occur via the intranasal route. Experimental infection of laboratory mouse strains using this route initially leads to a productive lytic infection in the lungs, predominantly in the alveolar epithelial cells but also in mononuclear cells (Sunil-Chandra *et al.*, 1992a). From the lung, the virus is transported to the draining lymph node (the mediastinal lymph node, MLN) where infection of B cells takes place. Latently infected B cells traffic from the MLN to the spleen and undergo a rapid expansion (Sunil-Chandra *et al.*, 1992b). This expansion and resulting increase in spleen size is accompanied by an increase in the number of latently infected cells. This peak of latently infected cells recedes over the course of a few weeks and a latent infection in the spleen is maintained at a fairly constant level for the life of the animal (Flano *et al.*, 2002; Sunil-Chandra *et al.*, 1992a; Usherwood *et al.*, 1996). Although the spleen is the major reservoir of latent virus, latently infected cells can also be found in other tissues including the lungs (Flano *et al.*, 2000; Flano *et al.*, 2003).

Although the vast majority of studies are based upon the infection of laboratory mouse strains, it must be remembered that this does not represent a "natural host". The virus was first isolated from bank voles (*Clethrionmys glareolus*) in Slovakia in 1980 (Blaskovic *et al.*, 1980) and has been shown to be endemic in wood mice (*Apodemus flavicollis*) in the UK (Blasdell *et al.*, 2003). Recent studies have shown that viral pathogenesis is markedly different in experimentally infected wood mice as compared to laboratory strains (Hughes *et al.*, 2005).

MCF Viruses

A number of related gammaherpesviruses have been shown to cause the devastating lymphoproliferative disease known as malignant catarrhal fever (MCF) in several susceptible ruminant species. This disease and its viral agents will be discussed in greater detail in Section 1.4.

1.3 Herpesvirus Life Cycle

To successfully persist within individuals and populations as a whole, herpesviruses have adopted a strategy in which infection at a cellular level can lead to two different outcomes: lytic viral replication or viral latency. Lytic replication produces infectious virus to disseminate infection within the host or infect another individual. During viral latency, however, virus persists within an infected cell as circular episomal DNA and no infectious virus is produced. Latent virus is able to reactivate to produce a lytic infection and it is this ability that differentiates latency from abortive infection.

1.3.1 Lytic Replication

This section describes the processes involved in lytic viral replication. A diagrammatic overview of these processes is also shown in Figure 1.3. The lytic cycle described here is based primarily on that of HSV-1 (reviewed in Roizman & Knipe, 2001) as its replication is well studied and thought to be broadly analogous to that of other herpesviruses.

Attachment and Entry

The attachment of HSV-1 virions to the surface of cells and their subsequent internalisation can effectively be split into three stages. This begins with relatively low-affinity attachment to the cell surface followed by more specific receptor interactions and, finally, fusion of the viral envelope with the cell membrane.

The first stage of attachment involves the interaction of glycoprotein C (gC) and, to a lesser extent, gB on the viral envelope with glycosaminoglycan (GAG) moieties on the cell membrane. The predominant, GAG containing, glycoprotein involved in this process is heparan sulphate (Shieh *et al.*, 1992; WuDunn & Spear, 1989) although binding can occur in its absence utilising other glycoproteins such as chondriotin sulphate. The attachment of gC and gB to these GAG moieties is not essential for infection although, in its absence, the efficiency of infection is reduced (Banfield *et al.*, 1995).

The second stage of attachment is mediated by interactions between viral gD and one of a number of possible cell surface molecules. These coreceptors can be split into three structurally unrelated classes: Tumour necrosis factor receptor (TNFR) family

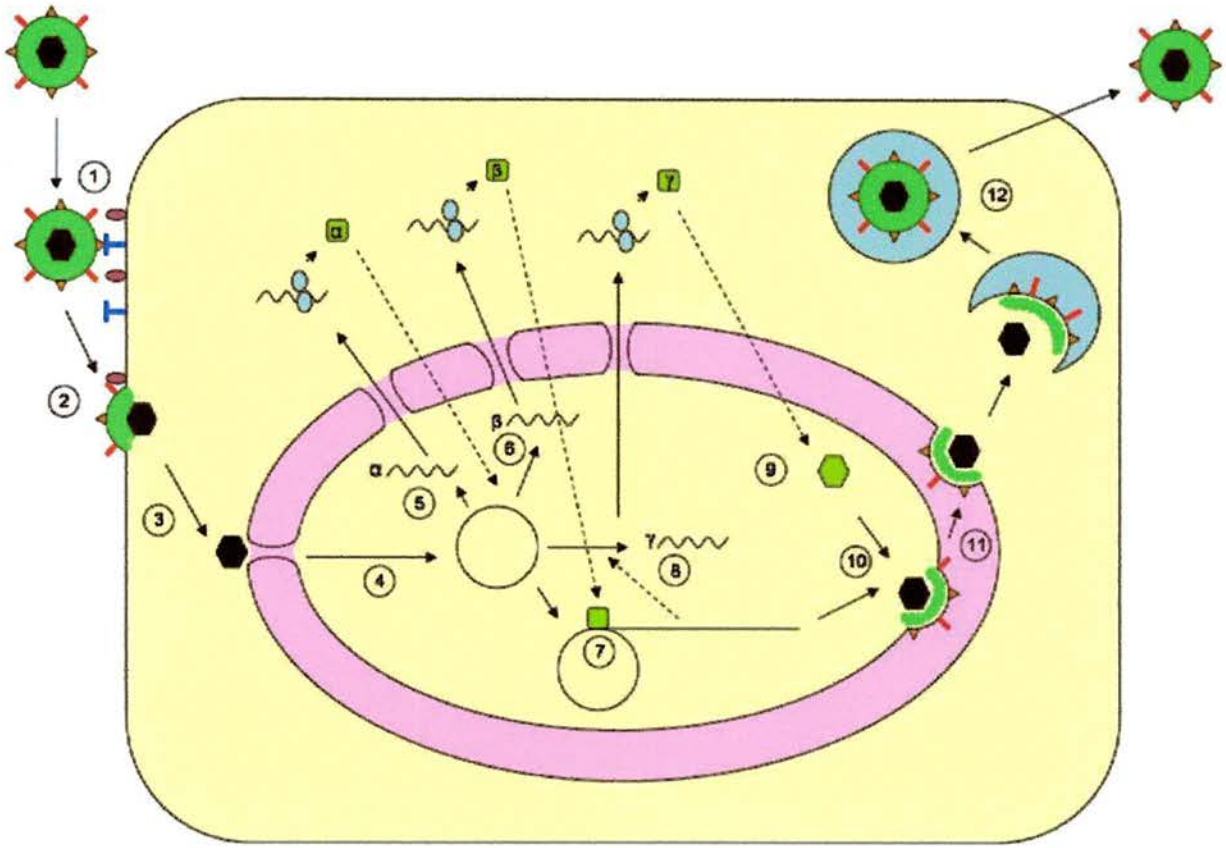


Figure 1.3. Overview of the lytic replication cycle of herpesviruses showing: (1) attachment, (2) membrane fusion, (3) transport of nucleocapsid to the nuclear pore complex, (4) circularisation of DNA, (5) α gene expression, (6) β gene expression, (7) DNA replication, (8) γ gene expression, (9) capsid assembly, (10) packaging of DNA into nucleocapsid, (11) envelopment and de-envelopment of nucleocapsid in nuclear membrane and (12) re-envelopment of nucleocapsid in Golgi apparatus and release of progeny virion. Adapted from Roizman & Pellett (2001) and Mettenleiter (2002). Details in text Section 1.3.1.

molecules, immunoglobulin (Ig) superfamily members called nectins and a third class consisting solely of 3-O-sulphated heparan sulphate. The TNFR family includes HveA (Montgomery *et al.*, 1996; Warner *et al.*, 1998), a molecule that is present predominantly on lymphocytes and is therefore unlikely to be the major mediator of HSV-1 entry *in vivo*. Nectins, including HveB (Warner *et al.*, 1998) and HveC (Geraghty *et al.*, 1998), have a wide tissue distribution and can mediate entry of all HSV-1 strains tested. The 3-O-sulphated heparan sulphates also have a wide tissue distribution and can mediate efficient HSV-1 entry (Shukla *et al.*, 1999).

Studies on the process of fusion between the viral envelope and the cell membrane have demonstrated the involvement of gD, gB and the gL-gH heterodimer as virions lacking any of these proteins show no infectivity (Cai *et al.*, 1988; Forrester *et al.*, 1992; Ligas & Johnson, 1988; Sarmiento *et al.*, 1979). In addition, cultured cells expressing this set of glycoproteins are able to fuse with untransfected neighbouring cells (Muggeridge, 2000; Turner *et al.*, 1998). The precise role of these proteins in this process, however, remains unclear. Recent studies have suggested that unbound gD exists in a closed conformation (Cocchi *et al.*, 2004) and assumes different conformations when bound to one of its receptors (Fusco *et al.*, 2005). It is hypothesised that these changes permit the recruitment of gB, gL and gH leading to the formation of a functional fusogen complex.

The three stage process of viral attachment and entry appears to be similar for all herpesviruses although the molecules involved differ. Like HSV, initial tethering of HCMV is mediated by interactions with cell surface GAG containing glycoproteins (Boyle & Compton, 1998). Both gB and the gM-gN heterodimer are thought to participate in this interaction. The initial attachment of EBV is mediated by interactions of the abundant glycoprotein gp350/220 and cell surface complement receptor 2 (CR2 or CD21) (Carel *et al.*, 1990). Initial attachment interactions are also thought to be carried out by the gp350/220 homologues identified in other gammaherpesviruses including gp150 of MHV-68 (Stewart *et al.*, 2004) and K8.1 of KSHV (Sun *et al.*, 1999) although surface receptors for these molecules have not been identified. No direct counterpart to gD has been found in the beta- and gammaherpesviruses and these viruses employ different molecules to mediate specific attachment. Interactions between cell surface epidermal growth factor receptor

(EGFR) with viral gB have been shown to be involved in the stable attachment of HCMV to cells (Wang *et al.*, 2003). However, the absence of EGFR from haematopoietic cells including monocytes and macrophages indicates that another HCMV receptor exists. In EBV, this interaction involves viral gp42 and cellular class II major histocompatibility complex (MHC) molecules (Borza & Hutt-Fletcher, 2002; Wang *et al.*, 1998; Wang & Hutt-Fletcher, 1998). Homologues of gp42 have also been described in other gammaherpesviruses including K7 of KSHV (Russo *et al.*, 1996). The details of beta- and gammaherpesvirus virion entry is not yet known in detail although, like HSV-1, complexes of gB, gH and gL are thought to be involved in the final fusion event.

Gene Expression and DNA Replication

Following membrane fusion, viral capsids are transported along the microtubule network to the nuclear membrane (Lycke *et al.*, 1984; Lycke *et al.*, 1988; Penfold *et al.*, 1994). During this process, some tegument proteins remain in the cytoplasm while others are transported along with the nucleocapsid. When nucleocapsids reach the nuclear membrane, they associate with the nuclear pore complex (Batterson *et al.*, 1983; Sodeik *et al.*, 1997) through which they release their DNA into the nucleus. The majority of viral DNA circularises upon nuclear entry without prior viral protein synthesis (Garber *et al.*, 1993; Poffenberger *et al.*, 1983).

The expression of viral genes occurs as a highly regulated cascade (Honess & Roizman, 1975). The first viral genes expressed are the α (immediate early or IE) genes. By definition, the transcription of these genes in all herpesviruses occurs initially in the absence of prior viral protein synthesis. However, the precise mechanism by which transcription takes place varies between different viruses.

During HSV infection, the tegument protein VP16 plays an important role in the induction of α gene transcription (Campbell *et al.*, 1984). VP16 is released from the tegument following viral entry into the cell forms a complex first with the cellular protein host cell factor-1 (HCF-1) (Xiao & Capone, 1990) and subsequently with the octamer binding protein Oct-1 (Kristie & Roizman, 1987; Gerster & Roeder, 1988; Kristie *et al.*, 1989). Oct-1 binds 'TAATGARAT' regulatory elements present in the promoter regions of the five HSV α genes ICP0, ICP4, ICP22, ICP27 and ICP47. The

recruitment of the VP16/HCF-1 complex to this protein leads to the transcription of these genes and the initiation of the viral gene cascade.

In gammaherpesviruses, a number of transactiator proteins are involved in the initiation of α gene expression. Of these, the R-transactivator or Rta protein encoded by the BRLF1 gene of EBV (Hardwick *et al.*, 1988) and its homologues encoded by the ORF50 genes of gamma-2-herpesviruses (Whitehouse *et al.*, 1997; Sun *et al.*, 1998; Wu *et al.*, 2000) have been shown to have particular importance. Expression of Rta has been shown to be sufficient for the induction of lytic replication in latently EBV infected cell lines (Ragoczy *et al.*, 1998). Similarly, the ORF50 gene products of MHV-68, HVS and KSHV were also capable of driving lytic replication in latently infected cells (Gradoville *et al.*, 2000; Goodwin *et al.*, 2001; Wu *et al.*, 2000). EBV Rta has been shown to activate the transcription of a number of viral α genes including other transactivators such as Zta and Mta as well as autoregulating its own transcription (Zalani *et al.*, 1992). Mta and its homologues encoded by ORF57 in the gamma-2-herpesviruses are important multifunctional proteins whose functions include the post-transcriptional activation and repression of a number of viral genes and the shuttling of intronless viral mRNAs (Whitehouse *et al.*, 1998; Goodwin *et al.*, 2000; Chen *et al.*, 2001; Williams *et al.*, 2005).

The majority of the α gene products are involved in the transcription of β (early or E) genes. The gene products of the β genes have a wide range of functions including nucleotide metabolism and viral DNA replication. The β gene products involved in DNA synthesis localise to the nucleus where replication of the circular viral DNA molecule begins. A number of these proteins cluster at origins of replication of the viral genome. DNA synthesis initiates in a theta replication mechanism but switches to a rolling circle mechanism in which long "head-to-tail" concatamers of viral genomic DNA are produced. As replication proceeds, accumulation of progeny DNA molecules and replication complexes is seen in globular nuclear structures called replication compartments (Quinlan *et al.*, 1984).

Following the initiation of DNA replication, the transcription of the γ (late or L) genes occurs. The γ genes can be loosely split into the γ_1 (leaky-late or early-late) genes that are expressed relatively early and whose transcription is increased by DNA replication and the γ_2 (true-late) genes that are expressed later. The transcription of

the γ_2 genes is intimately linked to DNA replication and can be effectively blocked in the presence of DNA synthesis inhibitors. The γ genes encode, among other things, the structural proteins necessary for the assembly of progeny virions. The cascade system of gene expression leads to an increased relative abundance of transcripts of these later temporal classes (Zhang *et al.*, 1987) and this is likely to represent the increased need for these structural proteins.

Assembly and Egress

Progeny virus capsids are formed in the nucleus (Gibson & Roizman, 1972) although the assembly of some capsid proteins begins in the cytoplasm (Nicholson *et al.*, 1994; Rixon *et al.*, 1996). Before the insertion of DNA, capsids are supported by an inner protein scaffold. The insertion and packaging of viral genomes into these procapsids is tightly coupled to their cleavage from the long "head-to-tail" concatamers into unit-length molecules (Newcomb *et al.*, 1996; Newcomb *et al.*, 1999; Newcomb *et al.*, 2000).

The majority of current evidence supports a model in which progeny virions mature through a process in which a primary envelope is acquired and subsequently lost by budding through the nuclear membrane and a final secondary envelope is acquired by budding into the trans-Golgi network (reviewed in Mettenleiter, 2002; Mettenleiter, 2004; Mettenleiter, 2006). Following a partial dissolution of the nuclear lamina (Muranyi *et al.*, 2002), nucleocapsids are directed to the inner leaflet of the nuclear membrane through which they bud and acquire a primary envelope (Vlazny *et al.*, 1982). Membrane fusion occurs between the enveloped particle and the outer leaflet of the nuclear membrane, releasing the nucleocapsid into the cytoplasm. In contrast to the membrane fusion mediating viral entry into the cell, this process occurs in the absence of viral glycoproteins but does require the viral protein US3 (Klupp *et al.*, 2001; Reynolds *et al.*, 2002). Tegumentation of the capsid in the cytoplasm occurs through a complex series of interactions involving at least fifteen viral tegument proteins (reviewed in Mettenleiter, 2006). The acquisition of a secondary envelope takes place as the viral particle buds into the trans-Golgi network (Gershon *et al.*, 1994; Granzow *et al.*, 2001; Jones & Grose, 1988; McMillan & Johnson, 2001; Severi *et al.*, 1988; Whealy *et al.*, 1991; Whiteley *et al.*, 1999; Zhu *et al.*, 1995) in a process requiring the presence of the viral membrane glycoproteins gE and gM (Brack *et al.*,

1999). Mature progeny virions reach the cell surface by vesicular movement through the Golgi apparatus. In addition to viral spread from the release of progeny virions into the extracellular space, HSV has been shown to be capable of direct cell to cell spread in a process requiring a complex of gE and gI (Dingwell *et al.*, 1994; Dingwell & Johnson, 1998) that most likely occurs at cell junctions.

1.3.2 Herpesvirus Latency

The ability to establish latent infection is an important feature common to all herpesviruses. The mechanisms adopted for the establishment, maintenance and reactivation from latency by different viruses in different cell types show a great deal of variation. Described below are the strategies employed by HSV-1 and EBV.

HSV-1

At some point during the primary lytic infection of epithelial cells with HSV-1, virus enters the nerve endings of neurons innervating the mucosal epithelium. Viral entry is followed by retrograde transport of the capsid to the cell body in the sensory ganglion. Like all herpesviruses, latent HSV-1 is maintained in the cell as circular episomal DNA.

The only viral transcripts regularly detected in latently infected neurons are the latency associated transcripts (LATs) (Stevens *et al.*, 1987). The LATs are a family of one large (8.3 kb) transcript that maps to the inverted repeats flanking the U_L domain of the HSV-1 genome and two smaller (2.0 and 1.5 kb), relatively more abundant, introns derived from the larger transcript (Devi-Rao *et al.*, 1991; Krause *et al.*, 1988; Wagner *et al.*, 1988a; Wagner *et al.*, 1988b). Despite being the focus of a great deal of investigation, the function of the LATs remains unclear. One thought is that the LATs may play a role in the down-regulation of lytic gene expression, possibly through the expression of a protein product, structural effects on the viral genome or antisense down-regulation of transcripts of α genes involved in lytic replication such as ICP0 and ICP4 (Chen *et al.*, 1997; Garber *et al.*, 1997).

LATs have also been shown to protect infected neurons from apoptosis (Perng *et al.*, 2000). This function of the LATs has been mapped to a region within the first exon (Ahmed *et al.*, 2002; Inman *et al.*, 2001). Recently, it has been shown that this region

encodes a microRNA (miRNA) and it is this miRNA that confers resistance to apoptosis (Gupta *et al.*, 2006). miRNAs are a class of small 21-23 nucleotide non-coding RNAs that can direct cleavage or regulate transcription of mRNAs containing complementary sequences. A number of miRNA sequences have been found in other herpesviruses including the human herpesviruses EBV, KSHV and CMV. The anti-apoptotic properties of the LAT encoded miRNA was shown to be mediated by the downregulation of transforming growth factor-β 1 (TGF-β1) and SMAD3 expression (Gupta *et al.*, 2006).

A number of physical and chemical stimuli are known to be associated with the reactivation of HSV-1 from latency including injury, hypothermia, UV irradiation, hormonal imbalance and stress. The biochemical basis for reactivation is less clear. Attempts to identify viral genes involved in this process have been hampered by the fact that many of these same genes are likely also involved in lytic replication and mutant viruses will therefore be defective other areas.

EBV

Rather than the relatively stable environment of non-dividing neurons, latent EBV infection occurs in dividing B cells and there are therefore a different set of requirements placed upon the virus for its continued survival. In order to persist within this cellular population, the virus must maintain its genome and promote the survival and proliferation of the host cell by affecting signalling pathways, manipulating the immune response and preventing apoptosis. Up to eleven viral genes can be expressed during latency in order to carry out some or all of these functions. Three states of EBV latency can be seen in infected cells: Latency I, II and III (Rowe *et al.*, 1992). These distinct forms are defined by the set of viral transcripts present. Recently, a fourth state of latency in which all latent transcripts are down regulated has been described in resting peripheral memory B cells (Hochberg *et al.*, 2004). It has been proposed that signals from the viral genes expressed in the other states of latency function to drive the differentiation of infected B cells into this long-lived memory cell type (Thorley-Lawson, 2001; Thorley-Lawson & Gross, 2004).

Latency I can be seen in EBV infected cells derived from patients with Burkitt's lymphoma. The only viral transcripts from these cells are the nuclear antigen EBNA-

1 and three untranslated transcripts, one from the *Bam*HI A region (BARTs) and the two EBV-encoded small nuclear RNAs (ERER-1 and EBER-2). EBNA-1 plays a crucial role in the faithful partitioning of the EBV genome into daughter cells during mitosis via its interaction with the OriP element of the viral genome (Yates *et al.*, 1984; Yates *et al.*, 1985). The EBERs are the most abundant RNA population in latently infected cells (Howe & Shu, 1989) but, like the HSV-1 LATs, their functions are not well understood although a recent study has demonstrated their involvement in the transformation of B cells (Yajima *et al.*, 2005). Additional possible functions of these transcripts may include inactivation of the interferon-induced protein kinase PKR and RNA splicing. Like the HSV LATs, the BARTs have been shown to encode two miRNAs with predicted cellular targets involved in cell proliferation and apoptosis, B cell cytokines and chemokines, transcriptional regulators and components of signal transduction pathways (Pfeffer *et al.*, 2004). Cells infected with EBV in the Latency I stage are protected from immune detection as there is no mechanism for the efficient detection of foreign nucleic acids and there does not appear to be a cell mediated response directed at EBNA-1.

EBV infected cells expressing EBNA-1, the untranslated RNAs and the three latent membrane proteins (LMP-1, LMP-2A and LMP-2B) are classed as Latency II. Neoplastic cells with this expression pattern can be derived from patients with nasopharyngeal carcinoma, Hodgkin's lymphoma and T cell lymphomas. LMP-1 has been shown to have transforming effects (Kaye *et al.*, 1993) and can prevent apoptosis by the induction of bcl-2 (Kingma *et al.*, 1996). LMP-2A and LMP-2B are involved in preventing the induction of lytic replication by inhibiting B cell activation (Longnecker, 2000).

Latency III can be seen in cultured human B cells infected with EBV as well as cells derived from B cell lymphomas in immunocompromised patients whose CTL responses have been exhausted. In addition to the seven genes seen in Latency II, these cells express another five nuclear antigens: EBNA-2, EBNA-3A, EBNA-3B, EBNA-3C and EBNA-LP. These five proteins have a variety of roles in the up-regulation and down-regulation of viral and cellular gene expression as well as transformation of B cells.

EBV periodically reactivates to lytic replication. These reactivations appear to occur in memory B cell populations recirculating through the lymphoid tissue associated with the oropharyngeal mucosa (Faulkner *et al.*, 2000) upon the terminal differentiation of these cells into plasma cells (Laichalk & Thorley-Lawson, 2005). The precise stimuli leading to reactivation are not clearly understood although the process does involve the viral proteins BZLF1 and BRLF1. BZLF1 alone is capable of transactivating a number of other EBV α and β genes and thereby initiating the cascade of lytic gene transcription (Hammerschmidt & Sugden, 1988). Reactivation leads to the production of infectious virions that can be detected in oral secretions through which the virus can be transmitted.

1.4 Malignant Catarrhal Fever

Malignant catarrhal fever (MCF) is a devastating lymphoproliferative and necrotic disease of cattle and other susceptible ruminant species. The disease can follow an extremely short time course following the initial presentation of symptoms. MCF was originally thought to be invariably fatal although a few cases of recovery have since been reported (Milne & Reid, 1990; O'Toole *et al.*, 1997). MCF was originally reported in 1923 as a disease of African cattle that was associated with contact with periparturient wildebeest herds (Mettam, 1923) and this form is often referred to as wildebeest associated (WA-) or 'African' MCF. The identification of the disease in European cattle was reported in 1930 (Goetze, 1930) and was associated with sheep contact (sheep associated or SA-MCF). MCF in cattle is an economically significant problem, particularly in sub-Saharan African and Indonesia.

The problem of WA-MCF in sub-Saharan Africa is of particular importance to Maasai pastoralist communities, to whom livestock play a central economic and social role. A recent study was undertaken to evaluate the impact of MCF on these communities (Cleaveland *et al.*, 2001). In high-risk villages, MCF was considered to be the second most important cattle disease and considered to be the fourth most important cattle disease in low-risk villages. In high-risk areas, 86% of cattle herds were affected by MCF with losses of between 5.6 and 6.2% in 2000. The Maasai are forced to abandon grazing ground during wildebeest calving season to avoid MCF and this confinement has caused an indirect increase in the burden of vector-borne and directly-transmitted diseases. The increase in wildebeest numbers in the Serengeti over recent years has only served to exacerbate the problem.

The preferred livestock in Indonesia, Balinese cattle (*Bos javanicus*) and the water buffalo (*Bubalus bubalis*), are particularly susceptible to MCF (Wiyono *et al.*, 1994b). These animals are a major source of draught power and economic wealth to small-holder farmers. The disease in these animals was thought to be sheep associated, a finding that was confirmed by detection of the causative agent of SA-MCF, ovine herpesvirus 2 (OvHV-2) in the peripheral blood of diseased animals (Wiyono *et al.*, 1994a).

Precise figures for the impact of MCF are difficult to obtain as suspected MCF cases are usually terminated prior to confirming the diagnosis. A recent report from the

Veterinary Laboratories Agency has suggested that there is an increasing incidence of MCF in the UK (Veterinary Laboratories Agency, 2006) although this may be due to more efficient detection of disease.

Several ruminant species other than cattle are susceptible to MCF and disease in these animals is also of great economic and scientific importance. Deer species are particularly susceptible to MCF. Several reports on MCF outbreaks in farmed deer populations have been published (Denholm & Westbury, 1982; Reid *et al.*, 1979; Reid *et al.*, 1987; Westbury & Denholm, 1982a) including one in which losses of over 50% of a Pere David's deer (*Elaphurus davidianus*) herd were reported (Orr & Mackintosh, 1988). Similarly, three farmed bison herds have been reported as sustaining losses between 33% and 95% from SA-MCF where very rapid clinical disease occurred (Li *et al.*, 2006; Schultheiss *et al.*, 2000). Losses due to disease outbreaks in zoological and wildlife parks has interfered with conservation and breeding programs (Bridgen *et al.*, 1989; Hanichen *et al.*, 1998) but has also led to the identification of novel MCF viruses (Flach *et al.*, 2002; Klieforth *et al.*, 2002). The recent discovery of MCF in pigs (Loken *et al.*, 1998) has expanded the range of susceptible hosts of MCF viruses to include non-ruminant species.

1.4.1 MCF Viruses

A number of different but closely related gammaherpesviruses have been shown to cause MCF.

AlHV-1

Alcelaphine herpesvirus 1 (AlHV-1) was shown to be the causative agent of WA-MCF (Plowright *et al.*, 1960). This virus inapparently infects its natural host the blue wildebeest (*Conochaetes taurinus taurinus*) and all wildebeest calves over the age of seven months are positive for virus (Plowright, 1967).

Studies on AlHV-1 have predominantly focused on two viral isolates, WC11 and C500. WC11 was isolated in 1965 from a blue wildebeest (Plowright *et al.*, 1965) and has cell-free infectivity. The more virulent C500 was isolated from an ox with MCF in 1975 (Plowright *et al.*, 1975) and the infectivity of this virus is cell-associated. Following serial passage in cultured cells, the WC11 isolate became

attenuated and could no longer induce symptoms of MCF in cattle (Plowright *et al.*, 1975). Similarly, after around 30 rounds of serial passage in cultured cells the infectivity of cell-associated virulent C500 becomes cell-free (Russell, 1980). This adaptation in culture was also accompanied by a loss of pathogenicity in cattle. It has since been shown that the conversion from cell-associated virulent (CAV) to cell-free attenuated (CFA) forms of the virus is associated with genome re-arrangements (Handley *et al.*, 1995; Wright *et al.*, 2003). Although the precise nature of these re-arrangements is not consistent between different clones, they did all involve the translocation of a region from around the centre of the genome (containing ORF 50 and ORF A6) to areas at the right or left hand ends of the genome (Wright *et al.*, 2003). In some cases, the translocation event can cause truncations of ORF 50 and ORF A6 and nearby genes including ORF 48 and ORF A7 could also be affected.

Preliminary characterisation of the AlHV-1 genomes (Bridgen *et al.*, 1989) showed that the unique region of low G + C content (L-DNA) of WC11 was approximately 130 kb in length. The high G + C content (H-DNA) region was approximately 30 kb in length and consisted of a major repeat sequence of 950 bp interspersed with related sequences of varying length. Analysis of the C500 genome demonstrated that it contained a similar L-DNA region but the H-DNA was composed of a single repeated sequence of around 1050 bp. Full sequence analysis of the C500 (CAV) genome (Ensser *et al.*, 1997) confirmed the presence of a 130,608 bp L-DNA unique region with 46.17% G + C content flanked by 20-25 H-DNA repeats of 1,113 to 1,118 bp. As shown in Figure 1.4, the L-DNA sequence encodes homologues of 60 conserved herpesvirus ORFs, arranged in four blocks collinear to HVS, interspersed with ten ORFs unique to AlHV-1.

OvHV-2

The isolation of the causative agent of SA-MCF proved more challenging than that of AlHV-1. Until recently, most evidence for a virus closely related to AlHV-1 being responsible for SA-MCF relied on the cross reaction of sera from adult sheep (Rossiter, 1981), cattle with MCF (Rossiter, 1983) and experimentally infected animals (Reid *et al.*, 1989b). Lymphoblastoid cell lines grown from established cases of MCF were found to contain DNA sequences that hybridised to AlHV-1 DNA and the infecting virus was tentatively classified as caprine herpesvirus-3 (Bridgen &

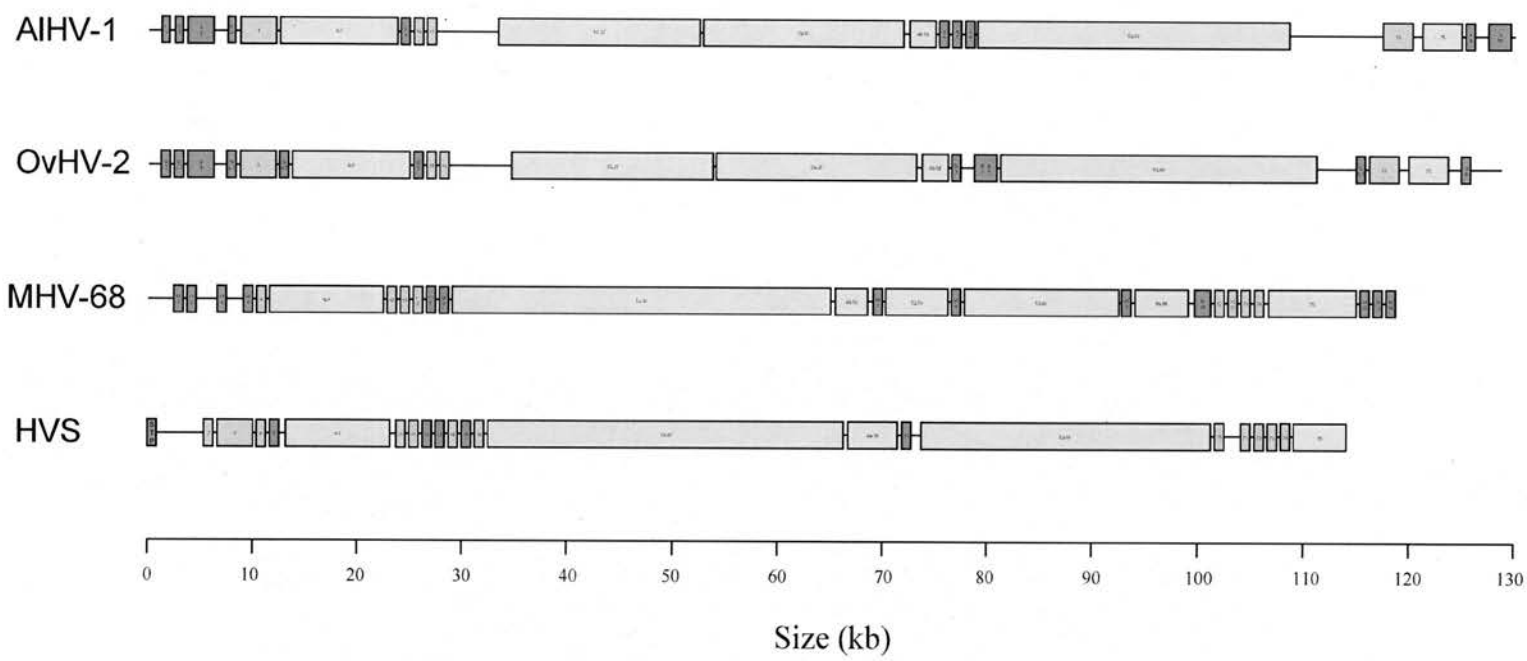


Figure 1.4. Genomic organization of the L-DNA sequences from the MCF associated viruses AlHV-1 and OvHV-2 shown relative to MHV-68 and the prototypic rhadinovirus HVS. ORFs, or blocks of ORFs, conserved among all gammaherpesviruses are shown in yellow, ORFs conserved among some but not all gammaherpesviruses are shown in green and unique ORFs are shown in purple. Conserved ORFs are labelled according to the system used for HVS. Adapted from published genome sequences (Albrecht *et al.*, 1992; Ensser *et al.*, 1997; Rosbottom, 2003; Virgin *et al.*, 1997).

Reid, 1991b) but was later re-named ovine herpesvirus-2 (OvHV-2). A fragment of OvHV-2 DNA was isolated from OvHV-2 infected LCLs and used to develop a PCR test specific for OvHV-2 (Baxter *et al.*, 1993) that has subsequently been employed to demonstrate the presence of this virus in several cases of suspected SA-MCF in a wide range of species (Baxter *et al.*, 1993; Ellis & Masters, 1997; Li *et al.*, 1995; Loken *et al.*, 1998; Mirangi & Kang'ee, 1997; Muller-Doblies *et al.*, 1998; Wiyono *et al.*, 1994a).

The genome of OvHV-2 has recently been cosmid cloned and sequenced (GenBank, Accession # NC_007646). Analysis of an approximately 125 kb region of the L-DNA has identified at least 70 predicted ORFs (Figure 1.4), 60 of which are conserved between all herpesviruses and ten of which are unique to OvHV-2 or have homologues only in AlHV-1 (Rosbottom, 2003). The nature of terminal repeat sequences is not well characterised although the H-DNA is approximately 25-30 kb in length.

Other MCF Viruses

In addition to AlHV-1 and OvHV-2, two other closely related gammaherpesvirus are generally regarded as causative agents of MCF. One virus is known to cause classic MCF disease in white-tailed dear (*Odocoileus virginianus*) and is currently termed MCFV-WTD (MCF virus of white tailed deer) (Kleiboeker *et al.*, 2002; Li *et al.*, 2000). The carrier species of this virus has yet to be identified. The other virus, caprine herpesvirus 2 (CpHV-2) is endemic in domestic goats (*Capra hircus*) (Li *et al.*, 2001b) and has been shown to cause disease in at least two species of deer (Crawford *et al.*, 2002; Keel *et al.*, 2003; Li *et al.*, 2003b).

Other closely related viruses have been identified in ruminant species. Sequences from alcelaphine herpesvirus 2 (AlHV-2) in hartebeest (*Alcelaphus buselaphus cokei*) and topi (*Damaliscus korrigum*) (Mushi *et al.*, 1981), hippotragine herpesvirus 1 in roan antelope (*Hippotragus equinus*) (Reid & Bridgen, 1991), MCFV-Muskox in muskox (*Ovibos moschatus*), MCFV-Oryx in gemsbok (South African oryx, *Oryx gazelle*) and MCFV-Ibex in Nubian ibex (*Capra nubiana*) (Li *et al.*, 2003a) have all been identified using a combination of PCR with degenerate primers and serological analysis. Although there is no conclusive evidence for these viruses causing disease

in either their natural hosts or other ruminant species, a virus closely related to AlHV-2 has been identified in a case of MCF-like disease in a group of Barbary red deer (*Cervus elaphus barbarus*) housed with clinically normal Jackson's hartebeest (*Alcelaphus buselaphus jacksoni*) at a wild animal park in California (Klieforth *et al.*, 2002), suggesting that they may cause disease under certain circumstances.

1.4.2 Clinical Forms of MCF

MCF cases can be broadly classified into four clinical forms of the disease: peracute MCF, intestinal MCF, head and eye form and mild MCF. The clinical symptoms are essentially identical whether the disease is sheep (Selman *et al.*, 1974) or wildebeest associated (Piercy, 1952; Plowright, 1968). Although classification of the clinical manifestations of MCF is helpful in the understanding of the disease, there is significant overlap between the forms and they are of little practical value.

Peracute MCF is the most severe form of the disease. Symptoms include fever and depression and occasionally inflammation of the oral and nasal mucosas. Affected animals can succumb to disease within a few hours of initially displaying symptoms, often due to severe hemorrhagic gastroenteritis.

The intestinal form of MCF is similar to the peracute form only with symptoms becoming more pronounced as disease progresses and lymphadenopathy becoming detectable. Animals usually die of intestinal MCF within four to nine days.

The head and eye form of MCF is the typical form seen in most instances of disease. As the name suggests, symptoms are predominantly seen, but not restricted to, the head and neck regions of the animal. Generalised lesions are frequently seen in the oral mucosa accompanied by hyperaemia and slight to marked drooling. The muzzle of the infected animal invariably has a dry, cracked and encrusted appearance. Nasal discharge progressing from serous to mucopurulent and purulent is also invariably seen. Mucopurulent discharge from the ocular mucosa is common and the eyelids are often swollen. The eyes themselves show progressive corneal opacity and animals are photophobic.

In addition to the symptoms seen in the head mucosa, several other clinical signs are commonly seen in the head and eye form. Lymph nodes, especially those of the head

and neck can be severely enlarged. Diseased animals can have a high fever, with rectal temperatures of 41 to 42°C, but often become hypothermic prior to death. In some cases, nervous disorders are apparent including head shaking, incoordination and muscle tremors. The course of this form of MCF is usually seven to eighteen days.

Mild MCF is classically associated with the disease induced by experimental infection of susceptible species with attenuated viruses and is usually non-fatal. Reports of cattle found to be seropositive for MCF viruses suggests that undiagnosed mild MCF may occur naturally (O'Toole *et al.*, 1997).

1.4.3 Gross Pathology

The severity of gross lesions found following post-mortem examination of MCF cases can vary depending on the course of disease. Animals that die of peracute MCF can show few lesions other than hemorrhagic enterocolitis. On the other hand, animals afflicted with the more protracted head and eye form can show involvement of several organs and tissues including: kidneys, lymph nodes, joints, liver, brain and alimentary, urinary and respiratory tracts.

Typical findings (Liggitt *et al.*, 1978; Piercy, 1952; Plowright, 1968; Selman *et al.*, 1974) include erosions at all levels of the oropharynx. This can include the oesophagus, tongue and occasionally the forestomachs although it is common to find no major macroscopic changes in the intestine. Lesions in the upper respiratory tract, predominantly in the nasal passages and turbinates occasionally extending to the frontal sinuses are also commonly found. Erosions and ulceration of the pharyngeal and laryngeal mucosas is seen and ulceration can also be found in the tracheobronchial mucosa. The enlarged lymph nodes are usually congested and oedematous. Haemorrhagic infarcts are commonly seen in the kidney as well as grey/white foci of up to 5 mm in diameter in the cortical regions. Signs of arthritis such as accumulation of synovial fluid and synovial membrane reddening and swelling with accompanying fibrin deposition are seen in the joints. The liver is often swollen and displays a reticular pattern.

1.4.4 Microscopic Pathology

The microscopic pathology of MCF can be summarised as cellular infiltrates and tissue necrosis (Liggitt *et al.*, 1978; Selman *et al.*, 1974). The predominant histopathological finding is a lymphocytic infiltrate into a large number of affected tissues. Such infiltrates are seen in the oral and nasal mucosa, ducts of endocrine organs, gallbladder, urinary bladder, skin, joints and lungs. The infiltrates generally consist of small to large lymphocytes and macrophages. Plasma cells can be found but are uncommon. Infiltrates are localised around blood vessels and vasculitis is consistently observed. Marked infiltrates are frequently associated with epithelial necrosis and sloughing, the second major histopathological change found in MCF affected tissues.

Lymphocytic infiltrates are also commonly found in the alimentary tract. Portions of the ileum, cecum and colon can show very marked lymphoid hyperplasia. In severe cases, this can result in an almost total loss of crypt architecture.

Lymphocyte hyperplasia can be seen in the paracortical region of enlarged lymph nodes. There is also marked activity of the mononuclear phagocytic system with sinuses packed with macrophages. In severely affected nodes, foci of necrosis are detectable.

In the kidney, foci of mononuclear cells are found corresponding to the grey/white foci seen macroscopically as well as lymphocytic vasculitis.

Microscopic examination of the eyes revealed a cellular infiltration consisting of neutrophils, macrophages and lymphocytes. Occasionally, the corneal epithelium can appear flattened and show signs of superficial ulceration.

1.4.5 Transmission of AlHV-1 and OvHV-2 Infection

Despite epizoological evidence for the acquisition of AlHV-1 by cattle from inapparently infected wildebeest being recognised for over 80 years (Mettam, 1923; Plowright *et al.*, 1960), many details concerning the transmission of this virus remain unclear. Most evidence suggests that the wildebeest calves are the source of natural infection. Firstly, there is a seasonal occurrence of MCF outbreaks at the time of annual calving or in the succeeding three to four months that has long been recognised

by the Maasai herdsmen. Secondly, the presence of infectious virus has been reported in the blood (Plowright, 1965a; Plowright, 1965b) and, importantly, in nasal and ocular secretions (Barnard *et al.*, 1989a; Mushi *et al.*, 1980) of wildebeest calves. Viraemia can be detected in calves less than one week old and persist for up to 36 weeks (Plowright, 1965b). Under normal circumstances, viraemia cannot be detected in adult wildebeest although the reactivation of virus has been reported. In these cases, the reactivation appeared to be associated with stress, either brought on by confinement and betamethasone treatment (Rweyemamu *et al.*, 1974) or by the endocrine and metabolic demands associated with late-stage pregnancy (Plowright, 1965a). Indeed, the presence of virus in pregnant wildebeest as well as in foetal spleens and young calves suggests that vertical transmission of the virus occurs in wildebeest populations, possibly via *in utero* infection (Plowright, 1965a; Plowright, 1965b).

Although wildebeest calves are generally accepted as the source of virus, the physical route of transmission is unknown. Transmission to cattle housed with viraemic calves can occur (Plowright, 1965b), probably by virus shed through nasal and ocular secretions. In the wild, however, transmission appears to occur over fairly large distances (Barnard *et al.*, 1989b) without any direct contact between wildebeest and cattle herds. The Maasai herdsmen believe that contamination of grazing grounds with wildebeest calf hair coats or the afterbirth of wildebeest cows to be the source of infection although failure to detect virus from these sources suggests that this is not the case. In addition, contamination of grazing grounds by saliva and urine has generally been ruled out as a source of transmission as attempts to detect infectious virus from these fluids have been unsuccessful (Mushi *et al.*, 1980), although detection of viral DNA in the urine of one calf has been reported (Michel, 1993). It has been proposed that transmission of virus from blood or ocular secretions may be carried out via an arthropod vector (Barnard *et al.*, 1989b; Goetze, 1930). However, a number of considerations including the small amount of material transferred, the lack of cattle-to-cattle transmission and the lack of any precedent for arthropod transmission of a herpesvirus make this possibility unlikely. Additionally, an experimental set up to test the possibility of transmission by the African face fly (*Musca xanthomelas*) showed that infectious virus was detectable for only 30 minutes following feeding on infective tears or cell culture medium and the flies were

reluctant to take another meal during this time window and, therefore, no transmission could be achieved (Barnard *et al.*, 1990).

Natural transmission of MCF between cattle is generally not thought to occur (Goetze, 1930). It is thought that this may be related to the lack of detectable cell-free virus in the secretions of MCF affected cattle (Plowright, 1965b). Although horizontal transmission is unlikely, a report of an outbreak in a cattle herd in north-western Transvaal indicated that vertical transmission may occur between cattle and their calves (Barnard *et al.*, 1989b; Barnard, 1990).

The natural transmission of OvHV-2 parallels that of AlHV-1 in many ways. Firstly, transmission of the virus appears to occur between sheep and cattle or deer but not amongst these susceptible species (Blood *et al.*, 1961; Goetze, 1930). Secondly, high levels of virus can be detected in nasal secretions (Baxter *et al.*, 1997; Li *et al.*, 2001a) and this virus is infectious (Taus *et al.*, 2005; Taus *et al.*, 2006). Finally, although SA-MCF can occur in all seasons, there does appear to be some seasonal association of disease outbreaks with lambing. However, there is some disagreement as to whether perinatal lambs are the source of infectious virus and whether they account for the seasonal rises in the occurrence of MCF. Virus can indeed be found in the nasal secretions of perinatal lambs (Baxter *et al.*, 1997) but also in adult sheep (Li *et al.*, 2001a). It has been suggested that other factors could contribute to the apparent seasonality of SA-MCF including climatic factors influencing the survival of virus in the environment, variation in stocking densities and the presence of potential vectors (Li *et al.*, 2001a).

Experimental transmission of WA-MCF can be achieved via the transfer of intact blood cells or crude cell suspensions derived from the spleen, lymph node, liver or brain tissue of infected animals (Mettam, 1923; Plowright *et al.*, 1960; Plowright, 1965a; Plowright, 1965b). Infection can also be established in calves via intranasal inoculation of cultured cell-free virus or suspensions of lymph node cells (Plowright, 1964). Transmission of SA-MCF from affected to healthy cattle can also be achieved via transfer of blood or lymph node cell suspensions (Piercy, 1954; Reid *et al.*, 1986; Selman *et al.*, 1978), although irregularly. The transmission of SA-MCF from affected to healthy deer however is far more efficient (Buxton & Reid, 1980; Oliver *et al.*, 1983; Westbury & Denholm, 1982b). It has recently been shown that SA-MCF

can be transmitted to bovine calves via intranasal inoculation with OvHV-2 positive nasal secretions (Taus *et al.*, 2006).

Transmission of both WA-MCF and SA-MCF to rabbits has been a useful tool in the study of the disease and passage of the disease in rabbits was instrumental in the original isolation of AlHV-1 (Plowright *et al.*, 1960). The pathogenesis of MCF in rabbits is very similar to that seen in other susceptible species (Buxton *et al.*, 1984) and has allowed for further investigation into the basis of disease (see Sections 1.4.7 and 1.4.8).

Attempts to transmit MCF to small laboratory rodent species have been described (Buxton *et al.*, 1988; Jacoby *et al.*, 1988a; Jacoby *et al.*, 1988b). Outbred albino mice, Syrian golden hamsters, Wistar rats and outbred Hartley albino guinea-pigs were inoculated with lymph node cells from rabbits infected with either the C500 strain of AlHV-1 or an SA-MCF isolate derived from a red deer. No transmission to mice was seen in any experiments. Disease was transmitted from AlHV-1 infected rabbits to rats, hamsters and guinea-pigs when inoculation was performed on neonates, but only to guinea-pigs when inoculation was performed on weanlings. Of the species tested, only hamsters were susceptible to rabbit adapted SA-MCF. In all cases, attempts to transmit disease back to healthy rabbits were unsuccessful suggesting that virus expression in the rodent system is defective or incomplete. The pathology of WA-MCF seen in rats, hamsters and guinea-pigs was similar to that seen in infected cattle and rabbits with pronounced lymphoproliferation, multisystemic mononuclear cell infiltrates, vasculitis and necrosis in all species (Jacoby *et al.*, 1988a). The pathological changes seen in hamsters with SA-MCF were essentially identical to those seen in hamsters with WA-MCF (Buxton *et al.*, 1988).

1.4.6 Immunisation

Treatment of MCF with a range of antiviral and immunosuppressive drugs can be effective (Milne & Reid, 1990) but the expense and duration of treatment mean that this is not a viable option for the control of disease, even in developed countries. It has therefore been attempted to produce a protective vaccine against the disease. It has been reported that the small percentage of animals that naturally survive MCF are protected from parenteral challenge with virulent material (Plowright, 1964) implying that vaccination should be possible. Attempts have been made to reproduce the

protection seen in recovered animals by inoculation with either inactivated viral material or live attenuated viruses and have had mixed success.

Plowright *et al.* used formalinised WC11 strain of AlHV-1 in incomplete Freund's adjuvant to inoculate groups of naïve cattle (Plowright *et al.*, 1975). This treatment resulted in the induction of a good neutralising antibody response in the experimental animals, particularly following booster inoculation. Despite the strong humoral response, all laboratory cattle succumbed to MCF following challenge with virulent cell-free or cell-associated AlHV-1. The disease in the animals progressed with the same kinetics of incubation and death times as in untreated animals. The same two-dose treatment was given to 1471 cattle in Kenya Maasailand. The incidence of MCF in treated herds was the same as that of placebo control groups although, again, neutralising antibody could be detected in the inoculated animals.

Slightly different results were found using an inactivated virus in rabbits (Edington & Plowright, 1980). Rabbits inoculated with inactivated cell-free C500 strain of AlHV-1 in complete Freund's adjuvant were protected against challenge with virulent cell-free but not cell-associated virus. The difference in protection between these and the previous experiments in cattle may be due to the virus strain used, the presence of killed *Mycobacterium tuberculosis* in the adjuvant or the susceptibility of the host animals used.

Vaccination attempts have also been made using live viruses isolated from hartebeest (Reid & Rowe, 1973) and from sick cattle in America (Mirangi, 1991). The hartebeest virus (K30) was attenuated by tissue culture passage and had become cell-free. Inoculation with this virus did not induce disease but induced an antibody response that neutralised AlHV-1 WC11. Two out of three inoculated cattle were protected from challenge with a virulent cell-associated form of the hartebeest virus and from subsequent re-challenge with a virulent wildebeest isolate. No protection against the virulent wildebeest isolate was seen in cattle that received the initial cell-free inoculation but were not re-challenged with the cell-associated hartebeest virus. Similar results were found using the AlHV-1 related virus isolated from sick American cattle (agent 707K). Two consecutive weekly inoculations with agent 707K were sufficient to protect cattle from challenge with the virulent C500 isolate of

AlHV-1. One inoculation did not protect against such challenge and repeated inoculations carried a risk of inducing disease themselves.

These studies suggest that a neutralising antibody response is not sufficient for protection, with the possible exception of cell-free viral challenge in rabbits, and that the immunity seen in recovered cattle (Plowright, 1964) is most likely due to a cell-mediated immune response. The production of an attenuated AlHV-1 strain that remains cell-associated for use as a candidate vaccine has been hampered by the genomic instability of the virus in culture. It is hoped that the recent cloning of the AlHV-1 genome into a bacterial artificial chromosome (BAC) (Dewals *et al.*, 2006) will now allow for the generation of specifically attenuated mutant viruses capable of inducing strong cell-mediated immunity but not disease.

1.4.7 Large Granular Lymphocyte Cell Lines

One feature of MCF is the ability to establish lymphoblastoid cell lines from infected animals. The first of these cell lines was derived from a rabbit infected with the 57th serial passage of an SA-MCF isolate (Reid *et al.*, 1983). Mesenteric lymph node cells were cultured and although fusion with foetal ovine kidney cells was attempted, the cells retained the rabbit cell karyotype as well as a lymphocyte surface antigen phenotype. The cells contained electron dense granules within their cytoplasm and were classified as large granular lymphocytes (LGL). The LGLs exhibited non-specific cytotoxicity in a ^{51}Cr release assay reminiscent of natural killer cells. Importantly, as few as 10^2 LGL could transfer disease to naïve rabbits.

LGL cell lines have also been propagated from naturally infected cattle and deer (Cook & Splitter, 1988; Reid *et al.*, 1989a; Schock *et al.*, 1998). Cook and Splitter (1988) were able to culture lymphocyte cell lines from the lymph node cells of both an MCF-affected and a healthy cow and these were maintained by the addition of interleukin-2 (IL-2). Cell lines from both animals showed non-specific killer activity and a T cell surface phenotype but only the lines derived from the MCF-affected cow could be maintained past five months. Reid *et al.* (1989a) were also able to establish LGL cell lines from 20 out of 21 SA-MCF affected cattle as well as red and Père David's deer. Cell lines were derived not only from lymphoid tissue including spleen, lymph nodes and thymus but also from cerebrospinal fluid and corneal tissue. The cell lines initially required the addition of exogenous IL-2 or feeder cell lines for

maintenance although the requirement on these factors for growth was lost by some of the cattle but not deer derived lines. Consistent with previous findings, these LGL cell lines exhibited non-specific cytotoxicity *in vitro*. The deer derived lines were able to transfer disease to rabbits and to other deer. The cattle derived lines, however, were unable to transfer disease to rabbits, deer or other cattle.

LGL cell lines derived from naturally and experimentally infected animals have since been further characterised. Bridgen and Reid were able to demonstrate the presence of viral DNA that hybridised to an AlHV-1 probe not only in LGL cell lines derived from rats and rabbits experimentally infected with AlHV-1 but also from rabbits, cattle and deer naturally or experimentally infected with SA-MCF (Bridgen & Reid, 1991a). The surface phenotype of previously studied (Reid *et al.*, 1989a) cattle and deer derived LGLs was investigated using cross-reactive monoclonal antibodies against leukocyte surface antigens (Burrells & Reid, 1991). Three out of four cattle derived lines had the $CD4^-CD8^+$ phenotype of cytotoxic T cells and one had the $CD4^+CD8^-$ phenotype of helper T cells. Both deer derived lines also tested were $CD4^-CD8^-$ but stained positive for the T19 antigen also found on bovine $CD4^-CD8^-$ T cells. The phenotype of another group of LGL lines derived from SA-MCF affected cattle was studied by Schock *et al.* (1998). Three of the five lines studied were $CD4^+CD8^-$, one was $CD4^-CD8^+$ and one was a mixture of $CD4^+CD8^-$ and $CD4^-CD8^+$ cells. In addition, these cells were shown to contain transcripts for the cytokines interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α) and interleukin-10 (IL-10). It was not determined whether the IL-10 transcripts seen were of viral or bovine origin. The cell lines still required exogenous IL-2 for growth and no IL-2 transcription was seen.

The constitutive activation of the *src* family kinases Lck and Fyn has been demonstrated in a cell line derived from an SA-MCF affected cow as well as in two lines derived from AlHV-1 infected rabbits (Swa *et al.*, 2001). These kinases normally link cell surface receptors to downstream signalling pathway genes controlling T cell activation, proliferation and differentiation and their constitutive activation could therefore demonstrate a mechanism underlying the activity of LGL cell lines.

1.4.8 Proposed Model for MCF Pathogenesis

Histological examination of MCF lesions indicates that direct viral cytotoxicity is not involved in the disease pathogenesis. Several alternative hypotheses have been proposed to account for the pathogenesis of MCF including a mechanism similar to graft rejection (Liggitt & DeMartini, 1980), transformation of lymphoid cells (Denholm & Westbury, 1982; Edington *et al.*, 1979; Hunt & Billups, 1979) and cell-mediated responses to infected vascular endothelium (Liggitt & DeMartini, 1980; Selman *et al.*, 1974). It is now generally accepted that viral infection leading to a generalised cell-mediated immune dysfunction is the basis for disease, although many of the details remain unclear.

Although it has been possible to establish LGL cell lines from MCF lesions and demonstrate the presence of virus within these cells following growth in culture, the presence of viral antigens in the lesions themselves has not been detected. The presence of viral DNA in these lesions has been demonstrated by *in situ* hybridisation and, more recently, by *in situ* PCR. Using *in situ* hybridisation, Bridgen *et al.* (Bridgen *et al.*, 1992) were able to demonstrate the presence of viral DNA positive cells in the lesions of AlHV-1 infected rabbits but only in the lung and submandibular lymph node and only at the extremely low frequency of approximately one in 10^4 cells. The more sensitive *in situ* PCR technique has recently been employed (Simon *et al.*, 2003) to show the presence of OvHV-2 DNA in the infiltrating $CD8^+$ lymphocytes in the vascular brain lesions of a bison and a cow with SA-MCF. Taken together, the difficulty of identifying viral DNA and the absence of viral antigen in MCF lesions, but not following culture of cells from these lesions implies a host mediated suppression of viral replication and gene expression.

The nature of the lymphoproliferation in MCF and its role in disease pathogenesis has also been the subject of much investigation. Lymphoproliferative disorders are associated with a number of other herpesvirus infections including EBV (Epstein *et al.*, 1964), MDV (Churchill & Biggs, 1968) and HVS (Hunt *et al.*, 1970b). As in MCF, the proliferating lymphocytes found in these disorders do not commonly contain virions but recovery of virus following *ex vivo* growth of these cells is seen. In contrast to these other conditions, the lymphoproliferation seen in MCF is thought to be hyperplastic rather than neoplastic in nature as the proliferating cells do not

appear to give rise to discrete tumours and the affected lymphoid organs generally maintain their architecture with necrosis only occurring at late stages of disease (Buxton *et al.*, 1984). Several studies (Ellis *et al.*, 1992; Nakajima *et al.*, 1992; Schock & Reid, 1996) have implicated CD8+ T cells as the main cell type involved in hyperplasia although other cell types can be involved. The finding of Burrells and Reid (1991) that the LGL cell lines derived from different tissues of the same animal were similar was taken to imply that cells of one phenotype capable of immortalisation were widely distributed throughout affected animals. It was also hypothesised, based on this finding, that the predominant infection of one cell type in different cases may determine the clinical response to natural infection. It has also been suggested that only a minority of cells accumulating in MCF lesions may be infiltrating and that the majority of cells arise from the multiplication of resident T cell populations within the tissues (Schock & Reid, 1996). Although extensive lymphoproliferation is one of the hallmarks of MCF, doubt remains as to its importance in disease pathogenesis. Treatment of SA-MCF infected rabbits with the potent T cell suppressing agent cyclosporin A (Cs-A) had no effect on the incubation period or outcome of disease despite the effective suppression of the lymphoproliferative response (Buxton *et al.*, 1984). This finding implies that the terminal necrosis and tissue destruction is caused by cells other than those involved in proliferation, most likely the natural killer (NK) cells.

1.5 Left Hand End Genes of Gammaherpesviruses

Gammaherpesviruses usually encode between around 70 to 85 genes or open reading frames (ORFs). ORFs conserved between all or most herpesviruses are arranged in five blocks and are interspersed with ORFs unique to each virus or conserved only between closely related viruses. It is these unique ORFs that are of particular interest to study as they are related to the specific biological characteristics of the virus. Many of the gammaherpesviruses encode a cluster of unique genes at the left hand end (LHE) of their L-DNA, between the terminal repeat regions and the first block of conserved herpesvirus genes (ORFs 6-9). Diagrammatic representations of the LHE of select gamma-2-herpesviruses are shown in Figure 1.5, adapted from their published viral genome sequences (Albrecht *et al.*, 1992; Ensser *et al.*, 1997; Rosbottom, 2003; Russo *et al.*, 1996; Telford *et al.*, 1995; Virgin *et al.*, 1997). In addition to the virus specific genes, the left hand ends of these often contain homologues of the purine biosynthetic enzyme formylglycineamide ribotide amidotransferase (FGARAT, ORF 3) and/or complement control protein homologue (ORF 4) of HVS.

1.5.1 HVS

The LHE regions of HVS show a great deal of variation and are used as the basis for subgroup classification (Biesinger *et al.*, 1990; Medveczky *et al.*, 1989; Medveczky *et al.*, 1984). Albrecht *et al.* (1992) published the genome sequence of HVS strain 11 of subgroup A. The LHE of this virus was shown to contain four ORFs including ORF O1 encoding the saimiri transforming protein (STP-A) and the seven previously described herpesvirus saimiri untranslated RNAs (HSURs). ORF 2 of this virus encodes a dihydrofolate reductase protein (DHFR) with 83% amino acid identity to human DHFR. Subgroup C viruses have also been shown to contain DHFR and HSUR genes but contain two oncogenic genes positionally equivalent to STP-A, STP-C and a tyrosine kinase interacting protein Tip (Biesinger *et al.*, 1990).

<u>The STP Genes and Tip</u>

STP genes are encoded by all three subgroups of HVS and are named based on their strain as STP-A, STP-B and STP-C. These genes show weak sequence homology to each other but do have structural similarities. All three genes encode transmembrane

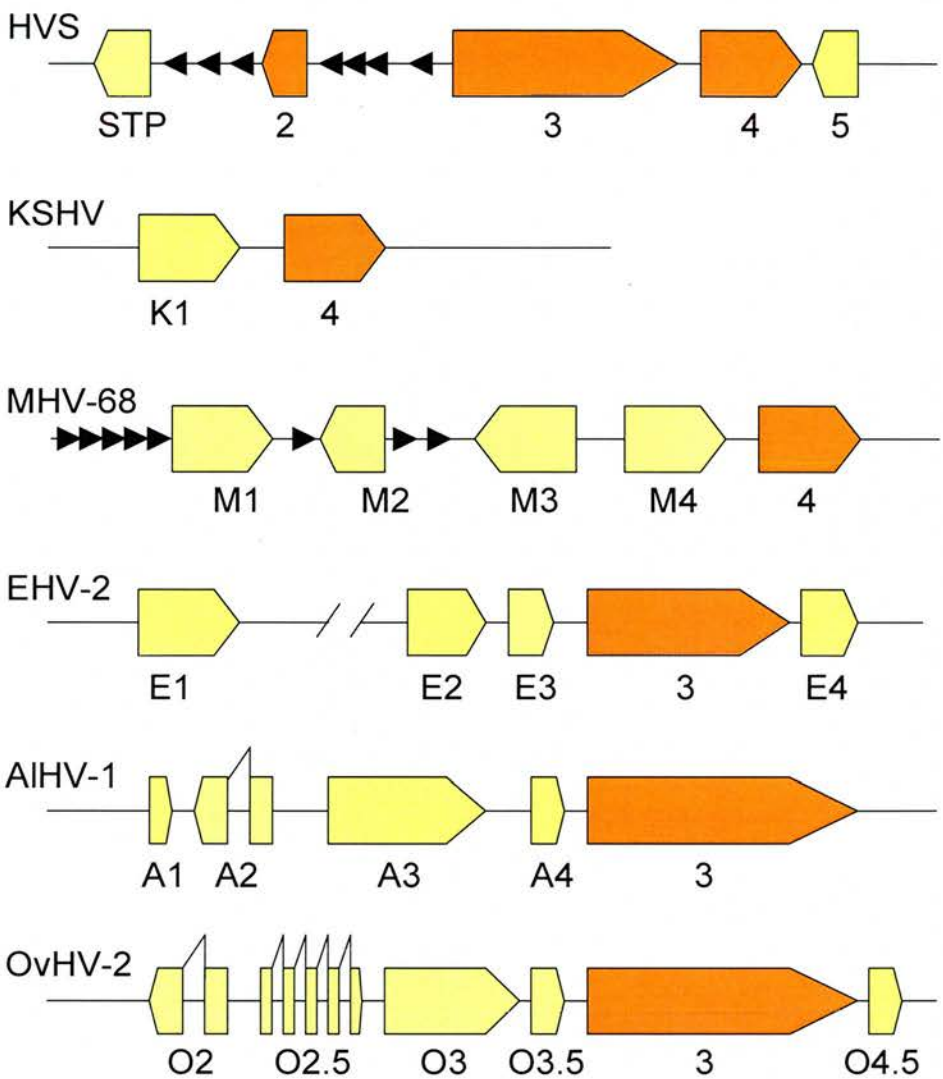


Figure 1.5. Diagrammatic representations (not to scale) of the ORFs contained within the regions right of the terminal repeat regions and left of the first block of conserved genes (ORF 6 – ORF 9) in selected gammaherpesviruses. ORFs unique to each virus are shown in yellow and those shared between HVS and other members of the *Rhadinovirus* genus are shown in orange. Black arrows represent the uRNAs of HVS and tRNA like molecules of MHV-68.

proteins with tumour necrosis factor receptor associated factor (TRAF) binding sites (Choi *et al.*, 2000; Lee *et al.*, 1999). The Tip ORF also encodes a transmembrane protein with intracellular signalling domains (Biesinger *et al.*, 1995).

As previously mentioned in Section 1.2.3, subgroup A viruses are highly oncogenic in New World monkey species. STP-A has been shown to be required for the transformation of murine embryo 3T3 cells *in vitro* (Chung *et al.*, 2004) and the induction of lymphomas in owl monkeys *in vivo* (Desrosiers *et al.*, 1985). Similarly, studies have shown that both STP-C and Tip are required for the transformation of common marmoset primary T cells *in vitro* and the induction of lymphomas in common marmosets *in vivo* by subgroup C viruses (Duboise *et al.*, 1998). In addition, the transgenic expression of STP-C in mice induced the formation of tumours in the salivary gland, pancreas, thymus and liver within the first few weeks of life (Murphy *et al.*, 1994). The absence from STP-B of the signal transduction modules found in other STP proteins is likely to be the cause of the poor oncogenicity of subgroup B viruses. The N termini of STP-A and STP-C contain nine and eighteen collagen repeats, respectively, that are thought to be important in transformation as none are found in STP-B. Indeed, insertion of eighteen collagen repeats into STP-B allowed the protein to oligomerise, activate NF-κB and transform rodent fibroblasts (Choi *et al.*, 2000).

HSURs

Two groups of HSURs are found at the LHE of HVS strain A11, one between STP-A and ORF 2 containing HSURs 1-3 and one between ORF 2 and ORF 3 containing HSURs 4-7 (Albrecht *et al.*, 1992). These U RNAs are the most abundant viral transcripts in HVS-transformed, latently infected T cells but do not appear to be required for transformation or viral replication (Cook *et al.*, 2005). The HSURs have been shown to be expressed and assemble into small nuclear ribonucleoproteins (snRNPs) with Sm proteins (Lee *et al.*, 1988; Lee & Steitz, 1990; Myer *et al.*, 1992). HSURs 1 and 2 expressed in latently HVS-infected marmoset T cells have recently been shown to upregulate a small set of host cell genes, including a number of genes linked to T cell and NK cell activation, indicating that these small RNA molecules are involved in the persistence of virus in the host by regulating T cell activation and proliferation.

1.5.2 KSHV

The LHE of KSHV contains two ORFs, an HVS ORF 4 homologue and the unique ORF K1 (Russo *et al.*, 1996). The K1 ORF encodes a transmembrane protein encoding an immunoreceptor tyrosine–based activation motif (ITAM) analogous to those found in the signalling components of the T- and B-cell receptor complexes (Lee *et al.*, 1998). Unlike other ITAM-based signal transduction events, K1 signalling occurs constitutively in the absence of any crosslinking ligands (Lagunoff *et al.*, 1999). ORF K1 is co-linear with STP of HVS and, although the two proteins share no amino acid similarity, ORF K1 can substitute for the activities of STP to transform primary T cells and induce lymphomas in common marmosets in the context of a recombinant virus (Lee *et al.*, 1998). ORF K1 was also found to induce tumours independently when expressed as a transgene in mice (Prakash *et al.*, 2002). The role of the ORF K1 gene product in normal KSHV biology remains unclear, although it is thought that through its signal transducing abilities it can provide conditions favourable to viral replication.

1.5.3 MHV-68

The left hand end of the MHV-68 genome contains four unique ORFs, ORF M1, ORF M2, ORF M3 and ORF M4, as well as eight viral tRNA like molecules (vtRNAs) and a homologue of the HVS complement control protein ORF 4 (Virgin *et al.*, 1997). A second strain of MHV, MHV-76, was found to be genetically identical to MHV-68 with the exception that the left hand end region encoding the vtRNAs, ORF M1, ORF M2, ORF M3 and approximately half ORF M4 was absent and there was a single nucleotide substitution in the coding region of ORF 6 (Macrae *et al.*, 2001). Comparison of this virus with MHV-68 highlighted the role for these left hand end genes in viral pathogenesis (Macrae *et al.*, 2001). The *in vitro* replication and cell to cell spread of these viruses was found to be identical but major differences were seen following infection of mice, showing that these genes are dispensable for the completion of lytic replication but may be involved in immune evasion and/or the establishment of latency. Mice infected intranasally with MHV-76 replicated to significantly lower titres and induced more severe pathology in the lung early after infection compared to those infected with MHV-68. At later time points, virus induced splenomegaly was significantly reduced. The transient peak of latently

infected splenocytes, usually seen between about 10 and 30 days post infection with MHV-68, was also drastically reduced following infection with MHV-76 although the virus was able to establish long term persistence.

Clambey *et al.* (Clambey *et al.*, 2002) also investigated the role of the MHV-68 LHE genes during infection using a spontaneous mutant virus with a 9,473 bp deletion almost identical to that of MHV-76, γHV-68Δ9473. Similar to the MHV-76 findings, this study also demonstrated a role for the LHE genes during acute lytic replication following both intranasal and intraperitoneal infection with significantly reduced viral titres seen in the lungs and spleens, respectively, of infected mice. The frequencies of viral genome positive (vgp) splenocytes and peritoneal exudate cells (PECs) were reduced by 24- and 4-fold, respectively, compared to MHV-68 following intranasal infection. Interestingly, the levels of reactivatable latent virus were reduced by only 13-fold from splenocytes and increased two-fold from PECs. Following intraperitioneal infection, no change was seen in the frequency of vgp splenocytes, the level of reactivation from splenocytes or the frequency of vgp PECs, however, the level of reactivatable virus from PECs was again increased. These findings suggest a role for one or more of the LHE genes in the reactivation of virus from latency.

The relative ease of inserting and deleting genes from the LHE of MHV-68 has allowed for the specific roles of these genes to be investigated in greater detail.

ORF M1

ORF M1 encodes a 420 amino acid protein with homology to a poxvirus serine inhibitor SPI-1 and some similarities to ORF M3 (Virgin *et al.*, 1997). The disruption of the M1 ORF from MHV-68 by the insertion of a LacZ gene cassette (M1.LacZ) resulted in an increase in the frequency of reactivation from both splenocytes and PECs (Clambey *et al.*, 2000). Following analysis of the viral genome frequency in the PECs, it was determined that virus reactivated *ex vivo* from approximately one in three of the M1.LacZ vgp PECs compared to one in fifteen of the MHV-68 vgp PECs. Although it does not ascribe a specific function to the ORF M1 gene product, this finding does demonstrate the contribution of this protein to the phenotype seen with MHVΔ9473 following intraperitoneal infection.

ORF M2

The M2 ORF encodes a 193 amino acid protein with expression restricted to latently infected B cells (Bougeret *et al.*, 1992; Husain *et al.*, 1999) and localises to the cytoplasm and plasma membrane (Macrae *et al.*, 2003). This gene product contains a classic H-2K^d epitope and the recognition of this epitope by specific CD8+ T cells has been shown to be important in the control of initial latent viral load (Husain *et al.*, 1999; Usherwood *et al.*, 2000). Independent studies using recombinant viruses with disruptions in the M2 ORF have shown the importance of this gene in producing the initial peak of latent infection following intranasal infection (Jacoby *et al.*, 2002; Macrae *et al.*, 2003). The ORF M2 gene product has been shown to bind to Vav signalling proteins in infected B cells (Madureira *et al.*, 2005). These interactions were found to promote B cell proliferation and survival which may play a role in the dissemination of the latent virus and maintenance of latency.

ORF M3

ORF M3 encodes a 44 kilodalton (kD), secreted, broad-spectrum chemokine binding protein. Chemokines are a family of small, structurally related proteins involved in directing the homing of leukocytes to specific areas and have been shown to play important roles in processes such as inflammation, tumour formation, angiogenesis and hematopoiesis. Chemokines are classified into four subgroups (XC, CC, CXC and CX_3C) based on their conserved cysteine motif. These proteins are secreted, with the exception of fractalkine (the only member of the CX_3C subgroup), and form gradients via electrostatic interactions with glycosaminoglycans.

The ORF M3 gene product was found in the supernatants of MHV-68 infected cell cultures and could bind radioiodinated human IL-8, RANTES, MIP-1α and fractalkine (Parry *et al.*, 2000; van Berkel *et al.*, 2000). The binding to labelled IL-8 and RANTES could be inhibited in the presence of a wide variety of chemokines from the CC (human MIP-1α, murine MIP-1α, human RANTES, murine RANTES, viral (v)MIP-2 from KSHV, human MCP-1, human MCP-4 and murine SLC), CXC (human IL-8, human GRO-α, human IP-10 and murine BCA-1), XC (human lympotactin) and CX_3C (fractalkine) families. No inhibition or weak inhibition was seen in the presence of human B cell-specific (BCA-1 and SDF-1) or murine

neutrophil-specific (KC, MIP-2 and LIX) CXC chemokines. Consistent with these findings, the presence of M3 protein in cell culture supernatants was able to inhibit the mobilization of calcium in murine neutrophils in response to MIP-1α but not KC and also inhibit the mobilization of calcium in murine macrophages in response to MCP-1 but not SDF-1 (van Berkel *et al.*, 2000). The M3 protein was also able to inhibit the migration of cells expressing the chemokine receptor CCR7 in response to MIP-3β and SLC (Jensen *et al.*, 2003). These studies demonstrate a functional role for chemokine binding by this protein.

The role of the M3 protein *in vivo* was assessed using a recombinant MHV-68 virus in which the M3 ORF had been disrupted by the insertion of a LacZ gene cassette (M3LacZ) and therefore lacks secreted chemokine binding activity (Bridgeman *et al.*, 2001). The disruption of the M3 ORF had no effect on the acute lytic replication of this virus *in vitro* or in the lungs of infected mice. However, mice infected with M3LacZ showed greatly reduced levels of latent virus in their spleens as measured by infectious centre assay and vtRNA *in situ* hybridisation. This reduction reflects a change in immune evasion rather than disruption of the ORF interfering with normal establishment of latency as mice infected with either M3LacZ or wild type virus that subsequently had their CD8+ cells depleted showed equal levels of latent virus.

ORF M4

The M4 ORF encodes a secreted glycoprotein (Evans *et al.*, 2006) and is expressed immediate early/early during lytic replication (Ebrahimi *et al.*, 2003; Townsley *et al.*, 2004). Townsley *et al.* found that a recombinant virus expressing ORF M4 in MHV-76 (MHV-76inM4) replicated to higher titres in the lung than either MHV-76 or MHV-68 at very early time points (day 1) post intranasal infection, suggesting that the ORF M4 gene product targets an innate immune mechanism. The absence of this phenotype in MHV-68 implies that this effect is somehow masked in the presence of the other LHE genes. At later time points, increased latent virus as measured by infectious centre assay and viral DNA load was found in the spleens of MHV-76inM4 mice compared to those from mice infected with MHV-76 which was unexpected as ORF M4 transcripts were not detected during latency (Virgin *et al.*, 1999).

Two independent studies using recombinant viruses in which the ORF M4 gene from MHV-68 had been disrupted by the insertion of a stop codon cassette found conflicting results (Evans *et al.*, 2006; Geere *et al.*, 2006). Both studies demonstrated a role for the ORF M4 gene product in the establishment of acute phase latency, finding lower levels of reactivatable virus and viral genome copies in infected spleens. However, in the study conducted by Evans and colleagues, the recombinant virus was found to replicate to marginally higher titres in the lungs of infected mice at day 9 post infection and induced significantly less splenomegaly at later time points but these findings were not reproduced in the later study. The reason for the discrepancy between these studies is not clear although the use of different mouse strains may be partially responsible.

Viral tRNA Like Molecules

Eight sequences with the characteristics of tRNAs are encoded at the LHE of the MHV-68 genome (Bowden *et al.*, 1997). Five of these sequences are located between the terminal repeats and ORF M1 (vtRNAs 1-5), one between ORF M1 and ORF M2 (vtRNA 6) and two between ORF M2 and ORF M3 (vtRNAs 7 and 8). The vtRNA sequences have a high level of identity (up to 75%) to some published tRNAs, are capable of forming cloverleaf-like secondary structures and contain RNA polymerase III (pol III) promoter elements. Unlike cellular tRNAs, however, the vtRNAs of MHV-68 are not amino-acylated.

The vtRNAs are encapsulated within progeny MHV-68 virons (Cliffe, 2006) and abundantly expressed during both lytic infection and latency (Bowden *et al.*, 1997). Indeed, the detection of vtRNAs by *in situ* hybridisation has been used as a tool for the detection of latent infection. The role of vtRNAs during infection, however, remains unclear. Recently, studies using recombinant MHV-76 viruses in which vtRNAs 1-5 had been replaced showed that these sequences had no effect on infectious viral titres in the lung or levels of latent virus in the spleen of infected mice following intranasal infection compared to wild-type MHV-76 (Cliffe, 2006).

A recent study has shown that up to fourteen miRNA sequences are embedded within the MHV-68 vtRNA transcripts (Pfeffer *et al.*, 2005). Unlike typical miRNAs, the MHV-68 miRNAs appear to be driven by the vtRNA pol III promoters rather than by

pol II. Nine of the fourteen predicted miRNAs were identified by cloning but their cellular targets and biological significance remain unclear.

1.5.4 EHV-2

In contrast to the other gammaherpesviruses sequenced, analysis of the EHV-2 strain 86/67 genome revealed that the unique region is flanked by two copies of a relatively large direct repeat of approximately 18 kb rather than multiple copies of a smaller repeat (Browning & Studdert, 1989). Contained within this direct repeat region is a unique ORF, ORF E1, meaning that two copies of this gene are encoded by the virus. Between the direct repeat and the first block of conserved genes, in the left hand end proper, EHV-2 encodes three further unique ORFs (ORF E2, ORF E3 and ORF E4) and a homologue of HSV ORF 3 (Telford *et al.*, 1995). ORF E2 and ORF E3 are predicted to encode proteins of 307 and 174 amino acids, respectively, that have not currently been assigned functions.

ORF E1

The E1 ORF of encodes a 383 amino acid protein with seven transmembrane domains which has significant homology to human CC chemokine receptors including CCR3 (47% identity), CCR1 (44%), CCR5 (40%) and CCR8 (35%) and to the HCMV US28 protein, which has also been shown to bind CC chemokines (Telford *et al.*, 1993). Cells expressing the ORF E1 gene product were found to increase intracellular calcium concentrations and migrate in response to the CC chemokine eotaxin but not in response to a number of other CC and CXC chemokines including RANTES, MIP-1α, MIP-1β, MCP-3, I-309, BCA-1, IP-10 and Mig (Camarda *et al.*, 1999).

ORF E4

ORF E4 encodes a homologue of the anti-apoptotic cellular protein Bcl-2 (Marshall *et al.*, 1999a). Bcl-2 homologues have been found in all sequenced gammaherpesviruses. HVS, KSHV, RRV and AtHV-3 each have one Bcl-2 homologue encoded by the conserved ORF 16 (Albrecht *et al.*, 1992; Albrecht, 2000; Russo *et al.*, 1996; Searles *et al.*, 1999). AlHV-1 and MHV-68 also contain one Bcl-2 homologue encoded by the unique ORFs A9 and M11, respectively (Ensser *et al.*, 1997; Virgin *et al.*, 1997). OvHV-2 encodes two Bcl-2 homologues (ORF O4.5 and

ORF O9) (Rosbottom, 2003) as does EBV (BALF-1 and BHRF-1) (Cleary *et al.*, 1986; Marshall *et al.*, 1999b).

Apoptosis, programmed cell death, can occur in response to a number of stimuli including death receptor signalling, DNA damage and virus infection. Cell death in response to virus infection is an innate mechanism to prevent the completion of the viral replication cycle and therefore the spread of infectious virus to neighbouring cells. Apoptosis is controlled by levels of pro-apoptotic (Bax and Bak) and anti-apoptotic (Bcl-2 and Bcl-X_L) members of the Bcl-2 family of proteins. It was originally proposed that apoptosis is induced by homodimerisation of pro-apoptotic Bcl-2 family members and that the anti-apototic family members functioned by forming heterodimers with these proteins, therefore preventing their homodimerisation (Korsmeyer *et al.*, 1993). Recent studies showing the non-overlapping localisation of Bax and Bcl-2 (Hsu & Youle, 1997) and the importance of post-translational modification on Bcl-2 family member function (Cheng *et al.*, 1997; Clem *et al.*, 1998; Fannjiang *et al.*, 2003; Kerr *et al.*, 2002; Lewis *et al.*, 1999) highlight the oversimplification of this model.

Bcl-2 homologues encoded by gammaherpesviruses were predicted to function during acute replication by preventing premature cell death in infected cells in a manner analogous to the viral Bcl-2 homologue encoded by adenovirus, E1B 19K (Pilder *et al.*, 1984; Subramanian & Chinnadurai, 1986; White *et al.*, 1986; White *et al.*, 1991). However, the M11 ORF of MHV-68 was found to be dispensable during lytic replication but involved in the reactivation of the virus from latency (Gangappa *et al.*, 2002). It has also been suggested that viral Bcl-2 homologues, in particular those of EBV and KSHV, play a role in tumour formation.

1.5.5 AlHV-1 and OvHV-2

Analysis of the complete genome sequence of AlHV-1 revealed four unique ORFs (ORF A1, ORF A2, ORF A3 and ORF A4) between the terminal repeat regions and the HVS ORF 3 homologue (Ensser *et al.*, 1997). Later analysis of the left hand end of OvHV-2 (Rosbottom, 2003) found homologues of two of these ORFs (ORF O2 and ORF O3). No homologues of ORF A1 and ORF A4 were found in OvHV-2 although the region was found to contain an HVS ORF 3 homologue and three further

unique ORFs, ORF O2.5, ORF O3.5 and the Bcl-2 homologue ORF O4.5 mentioned in Section 1.5.4.

ORF A1

ORF A1 is a very small ORF encoding only 97 amino acids and shows no significant sequence or structural homology to any known proteins (Ensser *et al.*, 1997) and has no homologue in OvHV-2. Although this ORF has a TATA box downstream and a polyadenylation site upstream, Northern blot analysis indicates that it may in fact be an exon of a larger gene (Dr. David Haig, Personal Communication).

ORF A2 and ORF O2

ORF A2 is predicted to encode a protein with some local similarities to the transcription factor ATF3 (Ensser *et al.*, 1997). The 199 amino acid gene product is encoded by a spliced transcript and contains a predicted nuclear localisation sequence and a basic leucine zipper motif (Ensser *et al.*, 1997). ORF O2 of OvHV-2 encodes a slightly smaller gene product of 186 amino acids, also from a spliced transcript, and shows 71% sequence homology and 56% sequence identity to ORF A2 (Rosbottom, 2003). It is therefore likely that this protein is also a transcription factor of similar function to the ORF A2 gene product.

ORF O2.5

ORF O2.5 encodes a 182 amino acid homologue of the cytokine IL-10 over five distinct exons (Rosbottom, 2003). IL-10 is generally regarded as an immunosuppressive or regulatory cytokine involved in the control of immune responses and the resolution of inflammation. Viral IL-10 homologues are also found in EBV (BCRF-1) (Hsu *et al.*, 1990; Moore *et al.*, 1990), CMV (UL111a) (Kotenko *et al.*, 2000) and EHV-2 (ORF E7) (Rode *et al.*, 1993).

ORF A3 and ORF O3

ORF A3 shows homology to genes encoding members of the semaphorin family of proteins (Ensser & Fleckenstein, 1995). The semaphorin family includes secreted and surface bound proteins that all contain a conserved cysteine-rich 'sema' domain. ORF O3 of OvHV-2 also encodes a semaphorin with 50% amino acid identity and

63% similarity to ORF A3 although is shorter by 200 amino acids at the C-terminus (Rosbottom, 2003). Semaphorin proteins were originally found to play a role in neural development (Kolodkin *et al.*, 1993) but recently several immunological semaphorins have been found, such as CD100 (SEMA4D) which has been shown to play a role in the activation of T and B lymphocytes (Bougeret *et al.*, 1992; Hall *et al.*, 1996; Herold *et al.*, 1996). Virally encoded semaphorins have also been found in members of the pox virus family including mousepox virus Ectromelia (ORF A39R) (Comeau *et al.*, 1998), vaccinia virus (ORF A39R) (Amegadzie *et al.*, 1992;Johnson *et al.*, 1993; Kolodkin *et al.*, 1993), fowlpox virus (ORF FPV047) (Afonso *et al.*, 2000) and variola virus (a composite ORF of ORFs A41L, A42R, A43R and A44R) (Amegadzie *et al.*, 1992; Kolodkin *et al.*, 1993; Shchelkunov *et al.*, 1993). These pox virus genes are more closely related to AlHV-1 ORF A3 than to genes encoding other semaphorins.

A novel cellular receptor for the mousepox virus Ectromelia A39R protein was found and called virus-encoded semaphorin receptor (VESPR) (Comeau *et al.*, 1998). This transmembrane glycoprotein has a wide tissue distribution and expression was found in heart, brain, lung, spleen and at high levels in the placenta of primary human tissues. Importantly, A39R binding was seen on T and B lymphocytes, monocytes and dendritic cells. The binding of A39R to monocytes was found to increase the surface expression of CD54 (intracellular adhesion molecule-1, ICAM-1) and production of interleukin-6 (IL-6) and interleukin-8 (IL-8) (Comeau *et al.*, 1998). Binding of the ORF A3 gene product to human VESPR was also seen, albeit at a much lower affinity, even though ORF A3 and A39R share only 29% amino acid identity (Comeau *et al.*, 1998).

A functional study on the role of vaccinia virus A39R protein *in vivo* was performed using an intradermal murine infection model (Gardner *et al.*, 2001). This study used the Western Reserve (vWR) strain of vaccinia virus which contains a 13 bp deletion inducing a premature stop in the coding region of the A39R gene. The truncated A39R protein encoded by this virus is not secreted from infected cells. The pathogenesis of the wild-type WR virus following infection of the ear pinnae of mice was compared to that of recombinant virus (vWR COPA39R) expressing the full-length secreted form of the A39R protein encoded by the Copenhagen strain of

vaccinia virus. The vWR COPA39R virus induced larger lesions that took longer to resolve than those induced by wild-type or revertant viruses. The finding that similar titres of infectious virus were found in the infected ears suggests that the increased lesion severity associated with the expression of full-length secreted A39R protein is an effect of increased immunopathology rather than direct viral cytopathology.

ORF O3.5

ORF O3.5 encodes a protein of 163 amino acids with a predicted signal peptide sequence (Rosbottom, 2003). This ORF shows no homology to any known genes and no potential function has been assigned to the gene product.

ORF A4

ORF A4 encodes a small protein of 121 amino acids with predicted signal peptide (Ensser *et al.*, 1997). Although this ORF is positionally homologous to ORF O3.5 of OvHV-2 and also encodes a predicted secreted protein, no homology between the two ORFs can be found. This ORF shows no homology to any known genes and no potential function has been assigned to the gene product.

1.5.6 Project Aims

The aim of this project is to characterise the four left hand end ORFs of AlHV-1. To study the contribution of these genes to pathogenesis in a small animal model, a 6.2kb region from the left hand end of the AlHV-1 genome was cloned and inserted into the MHV-68 deletion mutant, MHV-76. In addition to the *in vivo* pathogenesis studies using the recombinant virus, supernatants from cell cultures infected with this virus were used to assess the effects of secreted proteins on lymphocytes. Several *in vitro* studies were performed using the individual ORFs. All four ORFs were cloned for their expression as fusion proteins in mammalian cells and ORF A2 was also cloned for bacterial expression. Finally, a yeast two-hybrid system was used to screen a novel bovine cDNA library for proteins interacting with the gene products of ORF A2, ORF A3 and ORF A4.

Chapter 2: Materials and Methods

2.1 Molecular Techniques

2.2 Bacterial Techniques

2.3 Yeast Techniques

2.4 Southern Blots

2.5 Western Blots

2.6 Tissue Culture and Virus Growth

2.7 *In Vivo* Experiments

2.8 Other Methods

2.9 Recipes

2.1 Molecular Techniques

2.1.1 Polymerase Chain Reaction

Polymerase chain reactions (PCR) were carried out using the reaction mixtures and thermal cycler conditions listed in Appendix 1. Expand Long Template PCR System (Roche) was used for high fidelity amplification of DNA fragments for cloning. Taq DNA Polymerase (Roche) was used for routine screening and RT-PCRs. Taq DNA Polymerase (Invitrogen) was used for screening yeast colonies. Reactions were set up using buffers supplied with the enzyme or enzyme mixture. All samples were made up to a final volume of 50µl in 0.2ml thin-walled PCR tubes and reactions were carried out in a PCR Sprint thermal cycler (Hybaid). Primers were supplied by MWG Biotech and were made up to a working concentration of 0.1µg/µl. 10mM dNTPs were obtained from Sigma.

2.1.2 Cloning of PCR Products

PCR products were cloned using the pGEM-T Easy Vector System (Promega) according to manufacturer's instructions following confirmation of amplification of an appropriately sized product. A 3µl sample of the PCR product was mixed with 1µl of vector, 1µl of T4 DNA ligase and 5µl of 2x ligation buffer and incubated overnight at 4°C.

2.1.3 Agarose Gel Electrophoresis

DNA was analysed by electrophoresis in 0.7-1.5% agarose gels containing 0.5µg/ml ethidium bromide in TAE buffer. Samples up to 20µl were mixed with 6x loading buffer. Electrophoresis was carried out in horizontal gel tanks containing TAE at 70-80V for 30ml (8 cm x 6 cm) gels and at 100-120V for 100ml (15 cm x 12 cm) gels. Samples were compared with DNA ladders (1Kb DNA ladder, Promega: 100bp DNA ladder, Promega: 1Kb DNA ladder, New England Biolabs: PCR marker, New England Biolabs: 100bp DNA ladder, Invitrogen: 1Kb plus DNA ladder, Invitrogen: DIG-labelled DNA molecular-weight marker VII, Roche: DIG-labelled DNA molecular-weight marker VIII, Roche) for size estimation. Gels were visualised on a UV transilluminator.

2.1.4 Purification of DNA by Phenol:Chloroform Extraction

Protein was removed from DNA samples by phenol extraction. The sample was made up to at least 100µl before extraction and 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1 v/v) was added and mixed by vortexing. The sample was separated by centrifugation at 17,900 x g in a bench top microcentrifuge for 1 minute and the upper phase was removed to a fresh tube taking care not to disturb the interface. Residual phenol was removed by the addition of 1 volume of chloroform:isoamyl alcohol (49:1 v/v). The sample was vortexed and separated by centrifugation. The upper aqueous phase containing the DNA was carefully removed and transferred to a fresh microcentrifuge tube.

2.1.5 Concentration of DNA by Ethanol Precipitation

DNA was concentrated by adding 0.1 volumes of ammonium acetate (3M, pH 5.2) and 2.5 volumes of 100% ethanol. The sample was mixed by vortexing and incubated at -20°C overnight or on dry ice for 30 minutes. The DNA precipitate was pelleted by centrifugation at 17,900 x g in a bench top microcentrifuge for 15 minutes. The pellet was gently rinsed with ice cold 70% ethanol and allowed to air dry. DNA was resuspended in an appropriate volume of H_2O or Tris buffer (10mM Tris, pH 8.5).

2.1.6 Restriction Digestion of DNA

DNA was digested with restriction endonucleases (*Aat*II, *Bam*HI, *Bgl*II, *Eco*RI, *Hind*III, *Nhe*I, *Not*I, *Pst*I, *Sac*I, *Sal*I, *Xba*I and *Xho*I, New England Biolabs) according to manufacturer's instructions using the supplied buffers at the recommended temperatures. Digests were usually carried out in a total volume of 20-50µl and never using more than 1µl of enzyme per 10µl total volume to reduce the chances of STAR activity being seen. Digests were incubated from 1-16 hours depending on the enzyme activity. When performing restriction digests for cloning purposes, 1µl of Shrimp Alkaline Phosphatase (Roche) was added to the vector digestion reaction to prevent self ligation.

2.1.7 DNA Ligation

For plasmid cloning, a restriction digested insert was combined with restriction digested and phosphatased vector at a molar ratio of approximately 1:1 and H_2O added to a final volume of 8µl. T4 DNA Ligase (1µl, Promega) was added to the

reaction along with 1µl of supplied 10 x Ligase Buffer. Ligation reactions were incubated overnight at 12°C.

2.1.8 Quantitation of Nucleic Acid by Spectrophotometry

DNA and RNA were quantitated on a Cecil CE 2041 spectrophotometer. 2µl of sample was diluted in 98µl of milli-Q filtered H_2O. The absorbance of the diluted sample was read at 260 and 280nm. DNA concentration was calculated using the following equation: A_{260} x Dilution (50) x 50ng/µl. RNA concentration was calculated using the following equation: A_{260} x Dilution (50) x 40ng/µl. Nucleic acid purity was estimated using the A_{260}/A_{280} ratio.

2.1.9 Ethidium Bromide Plate Assay

Petri dishes were prepared containing 0.8% agarose gels with 0.5µg/ml ethidium bromide in TAE buffer. 1µl samples of DNA standards (200, 150, 100, 75, 50, 25 and 10 ng/µl) or test DNA were carefully applied to the surface of the gel. The spots were allowed to absorb into the gel for 10 min at room temperature and the plates were visualised on a UV transilluminator and the test spots were compared with the standards for estimation of DNA concentration.

2.1.10 DNA Extraction from Agarose Gels

DNA fragments were extracted from agarose gels using the QIAquick Gel Extraction Kit (Qiagen). Following separation in agarose gels, the area containing the DNA fragment of interest was excised from the gel and trimmed using a clean, sharp scalpel blade. The gel slice was weighed and solubilised by the addition of 3 volumes of supplied Buffer QG (1µl per 1mg). The sample was incubated at 50°C until the gel slice was completely dissolved. 1 gel volume of isopropanol (1µl per 1mg) was added to each sample and mixed by pipetting. The sample was then applied to a supplied spin column by pipetting. The column was centrifuged at 17,900 x g for 1 minute to bind DNA to the silica-gel membrane and the flow through was discarded. The sample was washed by applying 500µl of Buffer QG to the column and centrifuging at 17,900 x g for 1 min. The flow-through was discarded. A second wash was performed by applying 750µl of Buffer PE to the column and centrifuging as above. The flow through was discarded. The column was centrifuged as above to completely remove residual wash buffer. The column was placed in fresh 1.5 ml

microcentrifuge tube. 30µl of Buffer EB (10mM Tris, pH 8.5) was applied to the column and allowed to stand for 1 minute. The DNA was eluted by centrifugation as above.

2.1.11 Purification of PCR Products

PCR products were purified using the QIAquick PCR Purification Kit. 5 volumes of supplied Buffer PB was added to 1 volume of PCR sample and mixed by pipetting. The Sample was applied to a supplied spin column. The column was centrifuged at 17,900 x g for 1 minute to bind DNA to the silica-gel membrane and the flow through was discarded. The sample was washed by applying 750µl of supplied Buffer PE to the column and centrifuging as above. After discarding the flow through, the column was centrifuged as above to completely remove residual wash buffer. The column was placed in fresh 1.5 ml microcentrifuge tube. 30µl of Buffer EB (10mM Tris, pH 8.5) was applied to the column and allowed to stand for 1 minute. The DNA was eluted by centrifugation as above.

2.1.12 Sequencing of Plasmid DNA

Plasmid DNA sequencing reactions were performed using IRD-800 modified primers on a Li-cor automated DNA sequencer. Sequencing reactions were performed by Ian Bennett (Division of Veterinary Biomedical Sciences, University of Edinburgh).

2.1.13 Direct Sequencing of PCR Products

PCR products were purified by gel extraction or PCR purification (Sections 2.1.10 and 2.1.11). 10-50ng of purified product was made up to a volume of 6.75µl with Milli-Q H_2O in a 0.2ml thin-walled PCR tube. 1µl of sequencing primer (diluted to 3.2pmol/µl), 1.75µl of 5 x BigDye Buffer and 0.5µl of BigDye was added to the sample. Sequencing reactions were carried out in a thermal cycler using the following program:

96°C 10 sec
50°C 5 sec x 30
60°C 2 min

The sample was transferred to a 0.5ml microcentrifuge tube made up to 25µl with Milli-Q H_2O. 2.5µl of ammonium acetate (3M, pH 5.2) and 50µl of 100% ethanol was added and the sample was mixed by vortexing. The sample was incubated at room temperature for 30 minutes and the precipitate was pelleted by centrifugation at 17,900 x g in a bench top microcentrifuge for 15 minutes. The pellet was gently rinsed with ice cold 70% ethanol and allowed to air dry. Dried samples were sent to Ms. Lynne Richardson (Department of Zoology, University of Oxford) for sequencing.

2.1.14 Sequence Analysis

DNA sequences were analysed using NCBI nucleotide-nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and TIGR Cattle Gene Index BLAST software (http://tigrblast.tigr.org/tgi). Jemboss software (UK MRC Human Genome Mapping Project) was used for primer design and restriction digest mapping.

2.1.15 Isolation of RNA (Small Scale)

RNA was isolated from cultured cells or infected tissue using the RNeasy Mini Kit (Qiagen). Approximately $5x10^6$ tissue culture cells or 30mg of infected tissue was used as the starting material. Infected tissue was homogenised using a fresh RNase-free plastic pestle in a 1.5ml microcentrifuge tube prior to extraction. The cultured cell or tissue homogenate sample was resuspended and 600µl of supplied Buffer RLT added. The lysate was mixed by pipetting and passed through a QIAshredder spin column by centrifugation at 17,900 x g for 2 minutes in a bench top microcentrifuge. 600µl of 70% ethanol was added to the homogenised lysate and mixed by pipetting. The sample was transferred to an RNeasy mini column, centrifuged as above and the flow-through was discarded. 700µl of supplied Buffer RW1 was applied to the column. The sample was centrifuged and flow-through discarded as above. The column was washed twice by adding 500µl of supplied buffer RPE and centrifuging as above. The column was placed in a fresh 1.5 ml microcentrifuge tube. The RNA was eluted by adding 30µl of RNase-free H_2O and centrifuging as above.

2.1.16 Isolation of RNA (Large Scale)

Cultured Bovine Turbinate cells or freshly isolated PBMC were counted and collected by centrifugation as described in Section 2.6.18. The supernatant was poured off and

pellets were resuspended in the remaining liquid. 1ml of RNAwiz (Ambion) was added per 10^7 cells (starting volume) in a polypropylene tube and the sample was mixed by inversion. The sample was incubated at room temperature for 5 minutes before adding 0.2 starting volumes of chloroform. The sample was mixed by vigorous shaking and incubated for a further 20 minutes at room temperature in a bench top centrifuge. The lysate was separated by centrifugation at 12,000 x g for 15 minutes at 4°C. The colourless upper phase containing the RNA was carefully transferred to a fresh tube and mixed with 0.5 starting volumes of RNase-free H_2O. One starting volume of isopropanol was added and the sample was incubated at room temperature for 10 minutes. Precipitated RNA was pelleted by centrifugation at 12,000 x g for 15 minutes at 4°C. The RNA pellet was gently rinsed with one starting volume of ice cold 75% ethanol. The pellet was air dried and redissolved in an appropriate volume of RNase-free H_2O.

2.1.17 DNase Treatment of RNA

RNA was treated with RQ1 DNase (Promega) prior to reverse transcription. 1µg of RNA was resuspended in a volume of 8µl with RNase-free H_2O in a 0.2ml microtube. 1µl of RQ1 DNase and 1µl of supplied 10x DNase buffer were added and the sample was incubated at 37°C for 30 minutes. The DNase was inactivated by adding 1µl of supplied Stop solution and incubating at 65°C for 10minutes.

2.1.18 Reverse Transcription of RNA

Reverse transcription was performed using AMV reverse transcriptase (Promega). 2µg of DNase-treated RNA was mixed with 1µg of oligo $dT_{(18)}$ primer (Ambion) and RNase-free H_2O to a final volume of 12.5µl. The sample was heated to 75°C for 5 minutes and rapidly chilled on ice. 5µl of supplied AMV RT reaction buffer, 2.5µl of dNTP mix (10mM), 1µl (40U) of RNasin ribonuclease inhibitor (Promega), 1µl of 100mM sodium pyrophosphate and 3µl (30U) of AMV RT was added and the sample was incubated at 42°C for 1 hour. An RT-minus sample was set up in parallel with each RT sample and processed identically except 3µl of RNase-free H_2O was added in place of enzyme.

2.1.19 Isolation of mRNA from Total RNA

Isolation of mRNA was performed using the Poly(A) Purist mRNA purification kit (Ambion) according to manufacturer's instructions. 500µg of total RNA was made up to a volume of 500µl in nuclease-free H_2O. The dissolved RNA was mixed with 500µl of supplied 2X Binding Solution and mixed thoroughly by pipetting. The RNA sample was added to a supplied tube of Oligo(dT) Cellulose and mixed by inversion. The sample was heated to 75°C for 5 minutes to denature secondary structure. Following heating, the sample was incubated at room temperature for 60 minutes with constant rocking in a Blood Tube Rotator (Stuart Scientific). The Oligo(dT) Cellulose with bound mRNA was collected by centrifugation at 4,000 x g in a bench top microcentrifuge for 3 minutes at room temperature and the supernatant was carefully removed with a pipette.

The Oligo(dT) Cellulose pellet was resuspended in 500µl of Wash Solution 1 and applied to a supplied spin column in a 2ml microcentrifuge tube. The Wash Solution was passed through the sample by centrifugation at 4,000 x g in a bench top microcentrifuge for 3 minutes at room temperature and discarded. A further 500µl of Wash Solution 1 was added to the spin column and the sample was vortexed briefly to resuspend the Oligo(dT) Cellulose. The Wash Solution was passed through the sample and discarded as above. The sample was washed a further three times as described above, using 500µl of supplied Wash Solution 2.

After the final wash, the spin column was placed in a fresh RNase-free microcentrifuge tube. 200µl of supplied THE RNA Storage Solution (pre-warmed to 80°C) was added to the spin column to strip mRNA from the Oligo(dT) Cellulose. The sample was voretexed briefly and mRNA was collected by centrifugation at 5,000 x g for 2 minutes in a bench top microcentrifuge. This procedure was repeated with a second 200µl of THE RNA Storage Solution to elute any remaining mRNA. RNA was precipitated by adding 40µl of 5M Ammonium Acetate (NH_4Ac), 1µl of Glycogen and 1.1ml of 100% Ethanol. The solution was mixed by briefly vortexing and incubated on dry ice for 30 minutes. The RNA was recovered by centrifugation at 12,000 x g for 30 minutes at 4°C. The supernatant was carefully removed and the RNA pellet was air dried. RNA was resuspended in 30µl of THE RNA Storage Solution and stored at -70°C.

2.1.20 Library cDNA Synthesis

Library cDNA synthesis was performed using the cDNA Synthesis Kit (Stratagene). 5µg of mRNA was made up to a volume of 37.5µl with nuclease-free H_2O in an RNase free microcentrifuge tube. 5µl of 10x first-strand buffer, 3µl of first-strand methyl nucleotide mixture (10mM dATP, dTTP and dGTP plus 5mM 5-methyl dCTP), 2µl of linker-primer and 1µl of RNase Block Ribonuclease Inhibitor (all supplied with kit) were added to the sample and mixed by gentle pipetting. The sample was incubated at room temperature for 10 minutes to allow for annealing of primer. 1.5µl (75U) of supplied StrataScript Reverse Transcriptase was added and the reaction was incubated at 42°C for 60 minutes.

The reaction was placed on ice prior to addition of second-strand synthesis components to ensure that the temperature did not exceed 16°C. 20µl of 10x second-strand buffer, 8µl of second-strand dNTP mixture (10mM), 114µl of sterile distilled H_2O, 2µl (3U) of RNase H and 11µl (99U) of DNA polymerase I were added to the tube. The sample was incubated at 16°C for 2.5 hours and then returned to the ice. To blunt the cDNA termini, 23µl of blunting dNTP mix (2.5mM) and 2µl (5U) of cloned *Pfu* DNA polymerase were added to the tube and the sample was incubated at 72°C for exactly 30 minutes. Protein was removed from the sample by phenol:chloroform extraction (as described in Section 2.1.4). The cDNA was precipitated and recovered by ethanol precipitation (as described in Section 2.1.5). The cDNA pellet was resuspended in 9µl of supplied *Eco*RI adapters and incubated for 30 minutes at 4°C. 1µl of 10x ligase buffer, 1µl of 10mM rAMP and 1µl (4U) of T4 DNA ligase (all supplied with kit) were added to the tube and the sample was incubated at 4°C for 2 days.

Following the two day incubation, the ligase was heat-inactivated by placing the reaction tube in a 70°C water bath for 30 minutes. The reaction contents were collected by brief centrifugation and cooled to room temperature. 1µl of 10x ligase buffer, 2µl of 10mM rATP, 5µl of sterile H_2O and 2µl (10U) of T4 polynucleotide kinase was added to the tube and the reaction was incubated at 37°C for 30 minutes to phosphorylate the *Eco*RI ends. The kinase was heat-inactivated by placing the reaction tube in a 70°C water bath for 30 minutes. The reaction contents were collected by brief centrifugation and cooled to room temperature. The cDNA ends

were digested by adding 28µl of *Xho*I buffer supplement and 3µl (120U) of *Xho*I (both supplied with kit) and incubating at 37°C for 90 minutes.

The cDNA was precipitated by adding 5µl of supplied 10X STE buffer and 125µl of 100% ethanol to the sample. Following overnight incubation at -20°C, the sample was centrifuged at 17,900 x g for 60 minutes at 4°C in a bench top microcentrifuge. The supernatant was discarded and the cDNA pellet was resuspended in 14µl of 1x STE buffer and 3.5µl of supplied column loading dye.

Prior to size fractionation, a drip column was assembled from a sterile 1ml plastic pipette (Sterilin) and a sterile 10ml syringe (BD Biosciences) as follows. A cotton plug of the pipette was cut to approximately 4mm. The syringe was attached to the large end of the pipette using a small piece of supplied connection tubing. The cotton plug was forced down to the small end of the pipette by rapidly pushing the plunger into the syringe. The plunger was removed from the syringe and the column assembly was secured to a ring stand by a three-fingered clamp. Supplied Sepharose CL-2B was mixed until the resin was completely resuspended and transferred to the 1ml pipette until the settled bed was just below the lip of the pipette taking care to avoid the inclusion of any air bubbles. The sepharose was washed by adding 10ml of 1x STE to the syringe and allowing it to drip through the column.

The cDNA sample was applied to the column when there was approximately 50µl of wash buffer remaining above the sepharose. After the sample had entered the resin, 3ml of 1x STE buffer was added to the syringe and allowed to drip through the resin. Sample fractions of 4 drops each were collected from the time that the dye front reached the 0.4ml graduation on the pipette until the trailing edge of the dye reached the 0.3ml graduation. A total of 10 fractions were collected and analysed by ethidium bromide plate assay (as described in Section 2.1.9). Fractions containing the first wave of eluted DNA (but not the second wave of free linkers and unincorporated nucleotides) were pooled. The cDNA from the pooled fractions was cleaned by phenol:chloroform extraction (as described in Section 2.1.4) and concentrated by ethanol precipitation (as described in Section 2.1.5). Library cDNA was ligated into pGAD-T7 vector (Clontech) which had been digested with *Eco*RI and *Xho*I and treated with Shrimp Alkaline Phosphatase (see Sections 2.1.6 and 2.1.7). The library

ligation mixture was incubated overnight at 12°C and transformed into XL10-Gold Ultracompetent cells (Stratagene, see Section 2.2.3).

2.1.21 Annealing of Oligonucleotides for Cloning

Long (71bp) oligonucleotides for cloning were synthesised by MWG. Oligonucleotides were resuspended in H_2O to a concentration of 1µg/µl. 5µl of each oligonucleotide were combined in a microcentrifuge tube along with 5µl of 10 x Buffer 2 (supplied with New England Biolabs Restriction Endonucleases) and 35µl of MilliQ H_2O. The sample was heated to 95°C for 4 minutes and transferred to a beaker containing 500ml of H_2O at 70°C. The sample was allowed to cool in the beaker for several hours until it had reached room temperature. Annealed oligonucleotides were ligated into plasmids cut with appropriate restriction endonucleases and transformed into One-Shot competent cells (see Sections 2.1.6, 2.1.7 and 2.2.2).

2.2 Bacterial Techniques

2.2.1 Bacterial Culture

Various laboratory strains of *Escherichia coli* (*E. coli*) were grown in Luria-Bertani (LB) medium or on LB plates with 1.5% agar. Media was sterilised and agar melted by autoclaving. Media was supplemented with appropriate antibiotics (100μg/ml ampicillin or 50μg/ml kanamycin) prior to use. LB/Agar was supplemented with appropriate antibiotic when the gel had cooled to approximately 50°C and mixed by swirling. LB/Agar was poured into 10cm diameter Petri dishes. Bacteria were plated on the surface of LB/Agar by spreading with a glass spreader or streaking with an wire loop and plates were incubated, inverted, at 37°C overnight. Single colonies from agar plates were used to inoculate liquid medium. Liquid cultures were incubated overnight with shaking at 225 rpm in an orbital shaker at 37°C. Plate pouring and all bacterial procedures were carried out using aseptic bacteriological technique.

2.2.2 Transformation of One-Shot Chemically Competent *E. coli*

Three different strains of One-Shot chemically competent *E. coli* were purchased from Invitrogen. The INVαF' strain was used for routine cloning. The INV110 strain was used for cloning plasmids that required digestion with methylation sensitive restriction enzymes. The Top10F' strain was used for cloning, and the inducible expression, of protein from the pTrcHis vector. Cells were transformed according to manufacturer's instructions using aseptic bacteriological technique.

Single aliquots of competent cells were thawed on ice. A 5μl sample of ligation mixture or <1μl of plasmid was added and the cells were incubated, on ice, for a further 30 minutes. The cells were then incubated at 42°C for exactly 30 seconds and returned to the ice. 250μl of SOC medium was added and the samples were shaken in an orbital shaker at 225 rpm for 1 hour at 37°C. Following this incubation, samples were plated on 10cm diameter Luria-Bertani (LB)/Agar plates supplemented with appropriate antibiotic (100μg/ml ampicillin or 50μg/ml kanamycin). If blue/white screening of colonies was required, LB/Agar plates were supplemented with 40μg/ml X-gal (5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside made up to 20mg/ml in dimethylformamide). Plates were incubated, inverted, at 37°C overnight.

2.2.3 Transformation of XL10-Gold Ultracompetent Cells

XL10-Gold Ultracompetent cells (Stratagene) were thawed on ice and 100µl aliquots were gently added to pre-chilled 14ml Falcon tubes (BD). 4µl of β-Mercaptoethanol was added and the cells were incubated on ice for 10 minutes with occasional swirling. 5µl of ligation mixture was added and the cells were incubated on ice for 30 minutes. Following incubation, the tube was heat-pulsed for exactly 30 seconds in a 42°C water bath. Tubes were immediately transferred to ice for 2 minutes. 900µl of pre-warmed SOC medium (Invitrogen) was added to the tube and the sample was shaken in an orbital shaker at 225 rpm for 1 hour at 37°C. Following this incubation, 200µl aliquots were plated on 10cm diameter Luria-Bertani (LB)/Agar plates supplemented with appropriate antibiotic (100µg/ml ampicillin or 50µg/ml kanamycin). Plates were incubated, inverted, at 37°C overnight.

2.2.4 Preparation of Bacterial Stocks for Long Term Storage

A single bacterial colony was isolated from LB/Agar plates and used to inoculate 5 ml of Luria-Bertani medium supplemented with appropriate antibiotic (100µg/ml ampicillin or 50µg/ml kanamycin) using good bacteriological technique. Cultures were shaken overnight at 225 rpm in an orbital shaker at 37°C. The following day 500µl of culture was mixed with 500µl of sterile 50% glycerol in H_2O. The sample was transferred to a 1.8ml cryovial (Nunc) and incubated on ice for at least 2 hours before storage at -80°C.

2.2.5 Plasmid DNA Isolation from Bacteria (Small Scale)

Plasmid DNA was isolated from transformed bacteria by alkaline lysis of bacterial cultures followed by silica-gel adsorbtion of DNA using the QIAprep Spin Miniprep Kit (Qiagen) according to manufacturer's instructions. A single bacterial colony was isolated from LB/Agar plates and used to inoculate 5 ml of Luria-Bertani medium supplemented with appropriate antibiotic (100µg/ml ampicillin or 50µg/ml kanamycin) using good bacteriological technique. Cultures were shaken overnight at 225 rpm in an orbital shaker at 37°C.

The following day, bacteria were collected by centrifugation at 4,000 x g in a bench-top centrifuge for 5 minutes at room temperature. The bacterial pellet was resuspended in 250µl of Buffer P1 and transferred to a 1.5ml microcentrifuge tube.

Cells were lysed by the addition of 250µl of Buffer P2 followed by gentle inversion and allowed to stand for 3 minutes. Lysis was stopped by the addition of 350µl of Buffer N3 followed by gentle inversion. The sample was then centrifuged at 17,900 x g for 10 minutes in a bench top microcentrifuge to pellet the precipitate.

The supernatant was carefully removed and applied to a supplied spin column by pipetting. The sample was centrifuged at 17,900 x g for 1 minute to bind plasmid DNA to the silica-gel membrane and the flow through was discarded. The sample was washed by applying 500µl of Buffer PB to the column and centrifuging at 17,900 x g for 1 minute. The flow through was discarded. A second wash was performed by applying 750µl of Buffer PE to the column and centrifuging at 17,900 x g for 1 minute. The flow through was discarded. The column was centrifuged at17,900 x g for 1 minute to completely remove residual wash buffer. The column was placed in a fresh 1.5 ml microcentrifuge tube. 50µl of Buffer EB (10mM Tris, pH 8.5) was applied to the column and allowed to stand for 1 minute. The plasmid DNA was eluted by centrifugation at 17,900 x g for 1 minute.

2.2.6 Plasmid DNA Isolation from Bacteria (Large Scale)

For large scale isolation of plasmid DNA the QIAGEN Plasmid Maxi Kit (Qiagen) was used following manufacturer's instructions. A single freshly streaked colony was isolated and inoculated into 5ml of Luria-Bertani medium supplemented with appropriate antibiotic (100µg/ml ampicillin or 50µg/ml kanamycin). This culture was grown at 37°C for 6-8 hours in an orbital shaker at 225 rpm and then used to inoculate 500ml of Luria-Bertani medium supplemented with appropriate antibiotic (100µg/ml ampicillin or 50µg/ml kanamycin). These cultures were shaken overnight at 225 rpm in an orbital shaker at 37°C.

Bacteria were harvested by centrifugation at 6,000 x g for 15 minutes at 4°C in a Beckman J2-21 centrifuge. The bacterial pellet was resuspended in 10ml of supplied Buffer P1 and transferred to a fresh 30ml Oakridge tube. Cells were lysed by the addition of 10ml of Buffer P2 followed by gentle inversion and allowed to stand for 5 minutes. Lysis was stopped by the addition of 10ml of Buffer N3 followed by gentle inversion and incubated on ice for 20 minutes. The sample was centrifuged at 20,000 x g for 30 minutes at 4°C in a Beckman J2-21 centrifuge to pellet the precipitate. The

supernatant was then transferred to a fresh Oakridge tube and centrifuged again at 20,000 x g for 15 minutes at 4°C.

A QIAGEN-tip 500 column was equilibrated with 10ml of Buffer QBT, allowed to enter the silica resin by gravity flow. The supernatant sample from the centrifuged cell lysate was then applied to the column. After the entire sample had entered the resin, the column was washed with a total of 60ml of Buffer QC allowed to pass through by gravity flow. Plasmid DNA was eluted with 15ml of Buffer QF and collected in a clean 30ml glass Corex tube. DNA was precipitated by adding 10.5ml of isopropanol at room temperature to the eluate and mixing by inversion. The sample was then centrifuged at 15,000 x g for 30 minutes at 4°C in a Beckman J2-21 centrifuge. Following centrifugation, the supernatant was discarded and the pelleted DNA sample was washed gently with 10ml of ice-cold 70% ethanol. The pellet was allowed to air dry and was then redissolved in 500µl of Tris buffer (10mM Tris, pH 8.5).

2.2.7 Bacterial Expression of Tagged Proteins

The Top10F' strain of *E. coli* was used for the inducible expression of protein from pTrcHis 2A derived vectors. Single colonies from agar plates were used to inoculate LB medium containing 100µg/ml ampicillin. Liquid cultures were incubated overnight shaking at 225 rpm in an orbital shaker at 37°C. 60µl of the overnight culture was used to inoculate 2ml of LB and the culture was incubated at 37°C shaking at 225 rpm in an orbital shaker until the OD_{600} had reached 0.6 (about 2 - 2.5 hours). Isopropyl-beta-D-thiogalactopyranoside (IPTG, Sigma) was added to the culture at a final concentration of 1mM to induce expression of protein. The culture was incubated as before and at the indicated timepoints post-induction, 200µl samples were transferred to 1.5ml microcentrifuge tubes. Samples were centrifuged for 1 minute at 17,900 x g in a benchtop microcentrifuge and cell pellets were resuspended in 20µl of 1 x EDTA Free Protease Inhibitor Coctail (Roche). Samples were stored at -70°C until analysed by Western blot.

2.2.8 Bacterial Lysis for Protein Isolation

Top10F' bacteria expressing protein were grown and induced as described in Section 2.2.7. A 1.5ml sample of the culture was transferred to a 1.5ml microcentrifuge tube.

The sample was centrifuged for 5 minutes at 17,900 x g in a benchtop microcentrifuge and the cell pellet was resuspended in 400µl of CelLytic B bacterial cell lysis reagent (Sigma). The sample was thoroughly mixed by vortexing and subjected to two rounds of freeze/thaw. The sample was centrifuged as above and the supernatant was transferred to a fresh tube (Fraction 1) containing 40µl 10X EDTA-free protease inhibitor cocktail (Roche). The pellet was resuspended in 400µl of CelLytic B supplemented with 0.2mg/ml Lysozyme (Sigma). The sample was shaken gently and incubated at room temperature for 20 minutes to fragment cell walls. 1ml of CelLytic B (diluted 1:10 in H_2O and containing 1X EDTA-free protease inhibitor cocktail) was added and the sample was centrifuged as above. The supernatant (Fraction 2) was transferred to a fresh 1.5ml microcentrifuge tube and the pellet of inclusion bodies and cell debris was resuspended in 50µl of 1X EDTA-free protease inhibitor cocktail.

2.2.9 Immunoprecipitation of Bacterial Lysates

Bacterial lysates containing proteins of interest (Fraction 2, Section 2.2.8) were used for immunoprecipitation using anti-FLAG antibodies. 20µl of Streptavidin-agarose (Sigma) slurry was added to 1ml of supernatant sample. The sample was incubated with constant rocking at 4°C for 1 hour. The sample was centrifuged for 5 minutes at 17,900 x g in a bench top microcentrifuge. The pellet (Pre-Incubation) and a sample of the supernatant (Supernatant 3) were saved for analysis. The remainder of the supernatant was transferred to a fresh microcentrifuge tube and incubated overnight with 4.4µg/ml Anti-FLAG M2 antibody (Sigma). The following day, 40µl of Streptavidin-agarose slurry was added and the sample was incubated with constant rocking at 4°C for 1.5 hours. The sample was centrifuged as above and an aliquot of the supernatant (Supernatant 4) was saved for analysis. The immunoprecipitate pellet was resuspended in 500µl of CelLytic B bacterial cell lysis reagent (Sigma, diluted 1:10 in H_2O) and centrifuged as above. The pellet was washed in this way two more times and all samples were analysed by Western Blot.

2.3 Yeast Techniques

2.3.1 Yeast Culture

The AH109 strain of *Saccharomyces cerevisiae* was used for all experiments. Untransfected yeast cells were grown in YPDA media or on YPDA plates with 2% agar. Transfected yeast cells were grown in SD media containing the appropriate dropout supplement or on SD/dropout plates with 2% agar. Media was sterilised and agar melted by autoclaving on a sugar cycle (121°C for 15 minutes). Yeast were spread on agar plates with a glass spreader or streaked with a wire loop and plates were incubated, inverted, at 30°C until colonies had grown to a diameter of 1-2mm. For analysis of MEL1 reporter gene activity during growth, 100µl of X-α-Gal solution (5-Bromo-4-chloro-3-indolyl-α-D-galactopyranoside, Clontech, made up to 4mg/ml in dimethylformamide) was spread onto the surface of selective agar plates. Liquid cultures were inoculated with a single colony and vortexed to disperse any remaining clumps of yeast. Plate pouring and all yeast procedures were carried out on a cleared bench next to a Bunsen burner.

2.3.2 Transformation of Yeast

Yeast were transformed using the polyethylene glycol/lithium actetate method. Small scale yeast transformations were used to introduce bait vectors into AH109 yeast or introduce isolated prey plasmids into bait vector-containing yeast for confirmation of interactions. Large scale transformations were used to introduce prey library plasmid DNA into bait vector-containing yeast (sequential transformation).

Fresh yeast colonies of 1-2 mm in diameter were isolated from either YPDA/Agar or SD-Trp/Agar and inoculated into 50ml of YPDA or SD-Trp and grown overnight at 30°C in an orbital shaker at 225 rpm. The following day, enough of the overnight culture to produce an OD_{600} of 0.2-0.3 was transferred to 300ml of YPDA or SD-Trp. This culture was incubated at 30°C in an orbital shaker at 225 rpm until the OD_{600} reached approximately 0.5 (usually 3-5 hours). The culture was divided evenly between 50ml falcon tubes (BD) and centrifuged at 1,500 x g for 5 minutes at room temperature in a bench top centrifuge. Supernatants were discarded and cell pellets were pooled and resupended in 50 ml of sterile TE buffer (10mM Tris, 1mM EDTA, pH 7.5). The cells were again centrifuged at 1,500 x g for 5 minutes at room

temperature. The supernatant was discarded and the cell pellet was resuspended in 1.5ml of freshly prepared sterile TE/LiAc (100mM Lithium acetate, 10mM Tris, 1mM EDTA, pH 7.5). For small scale transformations, 100µl of resuspended cells was added to a 1.5ml microcentrifuge tube containing 0.1µg of plasmid DNA and 100µg of sheared salmon sperm carrier DNA. For large scale transformations, 1ml of resuspended cells was added to a 15ml falcon tube (BD) containing 50µg of library plasmid DNA and 2mg of sheared salmon sperm carrier DNA. Samples were mixed by vortexing. 600µl (6ml for large scale transformations) of freshly prepared sterile PEG/LiAc (40% (w/v) polyethylene glycol 3,350, 100mM Lithium acetate, 10mM Tris, 1mM EDTA, pH 7.5) was added and samples were mixed by vortexing. Samples were incubated at 30°C for 30 minutes in an orbital shaker at 225rpm. 70µl (700µl for large scale transformations) of DMSO was added to the samples and gently mixed by inversion. Samples were heat-shocked in a 42°C water bath for 15 minutes and then chilled on ice for 2 minutes.

Cells from small scale transformation samples were collected by centrifugation at 12,000 x g for 30 seconds in a bench top microcentrifuge. Cells were resuspended in 500µl of sterile TE buffer (10mM Tris, 1mM EDTA, pH 7.5) and a 200µl sample was spread on an agar plate containing the appropriate medium using a glass spreader. Cells from large scale transformation samples were collected by centrifugation at 1,500 x g for 5 minutes in a bench top centrifuge and resuspended in 1.5ml of sterile TE buffer. 100µl samples were spread over 15 SD-Ade/-His/-Leu/-Trp/Agar plates using a glass spreader. Plates were incubated, inverted, at 30°C until colonies had grown to 1-2 mm in diameter.

2.3.3 Isolation of DNA from Yeast

Yeast colonies selected for screening were grown in 1ml of selective medium (SD-Trp-His-Ade-Leu or SD-Trp-Leu). Cultures were shaken overnight at 225rpm in an orbital shaker at 30°C. Liquid cultures were transferred to 1.5ml microcentrifuge tubes and yeast were collected by centrifugation at 17,900 x g in a bench top microcentrifuge for 5 minutes at room temperature. Pellets were resuspended in 50µl of TE buffer. Alternatively, selected yeast colonies were grown in ~1cm^3 patches on plates containing selective medium with 2% agar. Plates were incubated at 30°C until abundant yeast growth was seen. Yeast were then scraped from the plate and

resuspended in 50 µl of TE. 10µl of lyticase solution (5units/µl in TE buffer) was added and the sample was shaken at 225rpm in an orbital shaker at 30°C for 1 hour. 140µl of TE buffer was added and the sample was frozen on dry ice. The sample was then thawed and an equal volume (200µl) of phenol:chloroform:isoamyl alcohol (25:24:1 by volume) was added and mixed by vortexing. The sample was separated by centrifugation at 17,900 x g in a bench top microcentrifuge for 1 minute and the upper phase was removed to a fresh tube taking care not to disturb the interface. Residual phenol was removed by the addition of 1 volume of chloroform:isoamyl alcohol (49:1 by volume). The sample was vortexed and separated by centrifugation. The upper phase containing the DNA was carefully removed and transferred to a fresh microcentrifuge tube. DNA was concentrated by ethanol precipitation (Section 2.1.5) and resuspended in 20ul of Milli-Q H_2O for screening by PCR.

2.4 Southern Blot

2.4.1 Probe Labelling

DNA probes were purified by gel extraction and labelled using the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) according to manufacturer's instructions. 1µg of template DNA was made up to a volume of 16µl in a thin-walled 0.2ml microtube. The DNA was denatured by heating to 100°C for 10 minutes and rapidly chilling on ice. 4µl of supplied DIG-High Prime solution was added and the sample was incubated overnight at 37°C. The labelling reaction was stopped by adding 2µl of 0.2M EDTA and heating the reaction to 65°C for 10 minutes. Labelling efficiency was tested by dot blot as follows. Dilutions of probe in H_2O were made from 1ng/µl to 0.01pg/µl. 1µl from each of the diluted probe samples and the control samples was dispensed onto Hybond N+ membrane (Amersham) and fixed by cross linking with a UV Stratalinker 2400 (Stratagene). The probes were detected and developed as described in Section 2.4.4. The probe signal was compared with that of the control DNA.

2.4.2 Capillary Transfer

Digested DNA and DIG-labelled ladders were resolved in an agarose gel. The DNA was denatured with two 20 minute incubations in denaturation buffer (0.5M NaOH, 1.5M NaCl) on a rotary platform and rinsed in distilled H_2O. The gel was then neutralised with two 20 minute incubations in neutralisation buffer (1M Tris-Cl, 1.5M NaCl) on a rotary platform and rinsed in distilled H_2O. The gel was then placed on top of three layers of Whatmann 3MM filter paper allowed to soak in a 10xSSC buffer-filled transfer tank. A gel-sized piece of pre-wetted Hybond N+ membrane (Amersham) was placed on top of the gel. Two pre-wetted sheets of Whatmann 3MM filter paper were placed on top of the membrane followed by a 15cm stack of dry paper towels, a glass plate and a 1kg weight. DNA was allowed to transfer to the membrane overnight by capillary action. The following day, the membrane was removed from the gel and DNA was fixed by cross linking with a UV Stratalinker 2400 (Stratagene).

2.4.3 Probe Hybridization

DNA blots were probed using solutions from the DIG High Prime DNA Labelling and Detection Starter Kit II (Roche) according to manufacturer's instructions. 0.1ml/cm^2 of pre-warmed supplied DIG Easy Hyb solution was added to the membrane and incubated with gentle turning in a roller bottle at 50°C for 30 minutes. DIG-labelled DNA probe (2.5ng/cm^2) was denatured by incubating at 100°C for 5 minutes and immediately cooling on ice. Denatured probe was added to an appropriate volume of pre-warmed DIG Easy Hyb solution. The membrane was incubated with probe solution overnight at 50°C with gentle turning in a roller bottle. The membrane was washed twice in 2xSSC, 0.1% SDS at room temperature for 5 minutes each with gentle turning in a roller bottle. The membrane was then washed twice again in 2xSSC, 0.1% SDS at 68°C for 15 minutes each.

2.4.4 Probe Detection

Prior to immunological detection of DIG labelled probes the membrane was rinsed in washing buffer (0.1M Maleic acid, 0.15M NaCl, 0.3% Tween 20, pH7.5) followed by a 30 minute incubation in 1ml/cm^2 blocking solution (supplied blocking solution diluted in 0.1M Maleic acid, 0.15M NaCl, pH7.5) at room temperature with gentle turning in a roller bottle. Probes were detected by incubating the membrane with 75mU/ml Anti-Digoxigenin-AP antibody in 0.2ml/cm^2 blocking solution for 30 minutes at room temperature with gentle turning in a roller bottle. The membrane was washed twice in 1ml/cm^2 washing buffer for 15 minutes each at room temperature with gentle turning in a roller bottle. The washed membrane was placed on plastic wrap in a development folder. 0.1ml/cm2 of supplied ready to use CSPD was applied to the membrane which was then covered with another sheet of plastic wrap and the folder was closed to spread CSPD evenly across the membrane. The Membrane was exposed to Lumi-Film Chemiluminescent Detection film (Roche) in a dark room for 5-60 minutes at room temperature. Films were developed using an Optimax processor (Jet).

2.5 Western Blots

2.5.1 Protein Electrophoresis

7.3 x 10.2cm and 8.3 x 10.2cm gel plates were assembled with spacers using the Mini-Protean II apparatus (Bio-Rad). Resolving gels containing between 10% and 15% acrylamide (Section 2.9.2) were poured immediately after the addition of ammonium persulphate (APS) and N, N, N', N'-Tetrametylethylenediamine (TEMED). Gels were poured to a level just below the bottom of the comb and overlayed with sec-butanol. Resolving gels were allowed to polymerise for 30 minutes before the butanol was removed and the top surface of the gel was rinsed with stacking gel solution (Section 2.9.2). Stacking gels were poured on top of the resolving gels, combs were added and the gel was allowed to polymerise for 30 minutes. Gels were assembled in the Mini-Protean II vertical gel tank and the reservoirs were filled with SDS running buffer (Section 2.9.2). Combs were removed and wells were rinsed out to remove any unpolymerised acrylamide before use.

Protein samples were mixed 1:1 with 2x boiling buffer (Section 2.9.2) and heated for 5 minutes at 100°C. Samples were loaded into wells and run at 25mA for 1 - 1.5 hours or until the blue dye front had reached the bottom of the gel. SeeBlue Plus2 (Invitrogen) and Full-Range Rainbow Molecular Weight Markers (GE Healthcare) protein standards were used for comparative size estimation.

2.5.2 Transfer of Protein to Nitrocellulose Membranes

Protran BA 85 nitrocellulose membrane (Schleicher and Schuell) was cut to the size of the resolving polyacrylamide gel (approximately 4.5 x 8 cm) and 6 pieces of 3MM Chr chromatography paper (Whatman) were cut slightly larger. The membrane and sheets of blotting paper were soaked in transfer buffer (Section 2.9.2) prior to use. Three sheets of the blotting paper were placed in the centre of the anode of a Semi-Dry Electroblotter A (Ancos). The nitrocellulose membrane was placed on top of these followed by the polyacrylamide gel. The remaining three sheets of blotting paper were placed on top of the gel and any trapped air bubbles were removed from the assembly by gently rolling the side of a pipette over the top sheet. The cathode was placed on top of the assembly and a current of 0.4mA/cm^2 (based on blotting paper area) was applied for 1 hour. Following transfer, the membrane was carefully removed. The membrane was incubated with Ponceau S solution for 1 minute and

rinsed with dH_2O until well defined bands were seen to confirm even transfer of protein.

2.5.3 Immunological Detection of Protein Blots

For GFP and c-myc detection, protein blots were blocked overnight in PBS containing 2% (w/v) BSA and 0.1% (v/v) Tween 20. The following day, membranes were incubated with either Anti-GFP (JL-8, Clontech) or Anti-c-myc (9E10 from mouse ascites fluid, Sigma) primary antibody diluted 1:2,000 (to 0.5µg/ml) or 1:500 in 2% BSA/PBS, respectively, for 1.5 hours. Membranes were washed twice in 0.1% Tween-20/PBS for 15 minutes each. Membranes were then incubated with Anti-Mouse IgG (Fab Specific) Alkaline Phosphatase Conjugate (Sigma) diluted 1:1,000 in 2% BSA/PBS for 30 minutes. Prior to detection, membranes were washed three times in 0.1% Tween-20/PBS for 20 minutes each. Bound antibody was detected by incubating the membrane with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution (Sigma, 1 SIGMA*FAST* BCIP/NBT tablet dissolved in 10ml dH_20) until clear bands were visible. All washes and antibody incubations were carried out at room temperature with constant agitation on an orbital platform.

For 3xFLAG epitope tag detection, the above protocol was used with the following exceptions: biotinylated Anti-FLAG M2 (Sigma) diluted 1:250 to 4.4µg/ml was used as the primary antibody, Streptavidin-AP Conjugate (Roche) diluted 1:1,000 to 1U/ml was used in place of a secondary antibody and TBS was used in place of PBS in all solutions.

2.6 Tissue Culture and Virus Growth

2.6.1 Growth of Established Cell Lines

BHK-21 cells (hamster kidney fibroblasts) were maintained in Glasgow's medium supplemented with 2% (v/v) newborn calf serum, 10% (v/v) tryptose phosphate broth, 2mM L-glutamine and 200U/L penicillin/streptomycin. C127 cells (mouse mammary gland epithelial tumour cells) were maintained in RPMI-1640 medium supplemented with 10% (v/v) foetal calf serum, 2mM L-glutamine and 200U/L penicillin/streptomycin. BT (Bovine turbinate) cells were maintained in Iscove's Minimal Essential Medium (IMEM, Sigma) supplemented with 10% (v/v) foetal calf serum, 2mM L-glutamine and 200U/L penicillin/streptomycin. Cell lines were maintained in $175cm^2$ and $75cm^2$ tissue culture flasks (Nunc). Confluent cell monolayers were removed from the flask surface using trypsin/EDTA. Firstly, the media was poured off and the monolayer was rinsed with approximately 100µl versene solution (0.2g/L EDTA-4Na, Gibco) per cm^2 of cells. The versene was removed and the cells were incubated with approximately 30µl Trypsin/EDTA solution (Gibco) per cm^2 until all cells had detached. The cells were resuspended in 10ml of complete medium and centrifuged for 5 minutes at 500 x g in a bench top centrifuge. The cell pellet was resuspended in complete medium. A sample of the cell suspension was diluted in Trypan Blue solution (Sigma) and live cells were counted in a haemocytometer. Viable cell number was calculated using the following equation: Cells/ml = Number of cells counted in the middle 25 squares x dilution in Trypan Blue x 10^4. $175cm^2$ flasks were seeded with approximately $5x10^6$ cells in 40ml of medium. $75cm^2$ flasks were seeded with approximately 10^6 cells in 18ml of medium. Cells were grown in a 37°C incubator with 5% CO_2.

2.6.2 Preparation of Cell Lines for Long Term Storage

BHK-21, C127 or BT cells were grown and counted as described in Section 2.6.1. Cells were then pelleted by centrifugation at 500 x g for 5 minutes in a bench top centrifuge and resuspended in 1 ml freezing solution (90% foetal calf serum, 10% dimethyl sulphoxide) per $2x10^6$ cells. Aliquots of 1ml were transferred to 1.8ml cryovials (Nunc). Cryovials were stored overnight at -80°C wrapped in cotton wool. Samples were then transferred to liquid nitrogen for long term storage.

2.6.3 Growing Cell Lines from Frozen Stocks

Vials of frozen cells were removed from liquid nitrogen and kept on dry ice until ready to thaw. Cells were thawed rapidly in a 37°C water bath. The thawed sample was transferred to 10ml of prewarmed complete medium. Cells were pelleted by centrifugation at 500 x g for 5 minutes, resuspended in 8ml of fresh medium and transferred to a 25cm^2 flask (Nunc). Cells were grown in a 37°C incubator with 5% CO_2.

2.6.4 MHV-76 and MHV-ALHE DNA Isolation

5x10^7 BHK-21 cells were suspended in 5ml and infected with 5x10^6 PFU (MOI = 0.1) of virus. Cells were incubated for 90 minutes at 37°C shaking at 225 rpm in an orbital shaker. The cells were then plated out in 10 x 175cm^2 flasks in 40ml complete medium per flask and incubated at 37°C.

Flasks were checked daily until complete cytopathic effect (CPE) was seen (usually 4-6 days). Remaining adherent cells were dislodged by shaking and all media from all flasks was pooled. Cells were pelleted by centrifugation at 500 x g for 20 minutes. The cell pellet was resuspended in 4 ml of PBS and disrupted by 30 strokes, on ice, in a dounce homogeniser. The homogenate was centrifuged at 1,700 x g for 20 minutes at 4°C in a bench top centrifuge. The supernatant was collected and the pellet was resuspended in 1ml of PBS and subjected to a further 30 strokes in the dounce homogeniser. The homogenate was centrifuged at 1,700 x g for 20 minutes at 4°C. The supernatant from this sample was pooled with that of the first round of homogenisation.

The pooled supernatants were carefully layered onto the top of 30ml of 20% (w/v) D-sorbitol in PBS in an SW28 tube. The sample was centrifuged at 141,000 x g for 80 minutes in a Beckman L8-70M ultracentrifuge. Pelleted virus was resuspended in 500µl of 50mM Tris, pH 8.0 and added to 5ml of High Molecular Weight DNA Extraction Buffer (0.625% (w/v) Sodium Dodecyl Sulphate, 125mM EDTA, 250mM Tris, pH 8.0). Capsids were digested by adding 550µg PCR grade Proteinase K (Roche) and the sample was incubated overnight at 53°C.

The sample was carefully transferred to a 15ml polypropylene tube (Falcon). An equal volume (5.5ml) of phenol:chloroform:isoamyl alcohol (25:24:1 by volume) was

added and mixed by gentle rocking on a tilting platform for 30 minutes. The sample was centrifuged at 500 x g for 5 minutes in a bench top centrifuge to aid separation. The upper phase was collected taking care not to disturb the interface and transferred to a fresh 15ml tube. Phenol extraction was repeated 4 times. Residual phenol was removed by adding 1 volume of chloroform:isoamyl alcohol (49:1 by volume) and mixed by gentle rocking on a tilting platform for 30 minutes. The sample was centrifuged at 500 x g for 5 minutes to aid separation. The upper phase was collected taking care not to disturb the interface and transferred to a fresh 15ml tube.

Viral DNA was precipitated by adding 1.7ml of 7.5M ammonium acetate and 12ml of 100% ethanol. The precipitate that formed immediately following the addition of the ethanol was removed using a sealed end Pasteur pipette and transferred to a clean Corex tube containing 20ml of 70% ethanol. The sample was centrifuged at 8,000 x g at 4°C for 30 minutes in a Beckman J2-21 centrifuge. Following centrifugation, the supernatant was discarded and the pelleted DNA sample was washed gently with 10ml of ice-cold 70% ethanol. The pellet was allowed to air dry and then redissolved in 500µl of Tris buffer (10mM Tris, pH 8.5).

2.6.5 Transfection of Cell Lines with Viral DNA by Electroporation

BHK-21 cells were grown in 175cm^2 flasks until almost confluent. The cells were trypsinised and counted as described in Section 2.6.1. 5x10^6 cells were resuspended in 800µl of OptiMEM Reduced Serum Medium (Gibco) and transferred to an electroporator cuvette containing 2µg of highly purified viral DNA, 9µg of insert vector and 20µg of salmon sperm carrier DNA. Cells were transfected in an Easyject Plus electroporator (Equibio) using a two pulse protocol (F1= 600V 75µF 99ohms, F2= 250V 1050µF 99ohms, 0.1 seconds delay). Immediately following electroporation, cells were added to 20ml of complete media. Electroporated cells were combined with 1x10^7 untransfected cells to a final volume of 100ml and plated out in five 96-well plates (approximately 3x10^4 cells/well) and incubated at 37°C with 5% CO_2 until viral plaques formed.

2.6.6 Transfection of Cell Lines with Plasmid DNA by Electroporation

BHK-21 cells were grown in 175cm^2 flasks until almost confluent. The cells were trypsinised and counted as described in Section 2.6.1. 2.5x10^6 cells were resuspended

in 200µl of Optimem medium (Gibco) and transferred to a 0.2cm electroporator cuvette containing 10µg of plasmid DNA. Cells were transfected in a Gene Pulser Xcell electroporator (BioRad) using the pre-set BHK-21 square wave protocol. Immediately following electroporation, cells were added to 3ml of complete media in a 6 well plate and incubated at 37°C with 5% CO_2.

2.6.7 Transfection of Cell Lines with Plasmid DNA using Effectene

The day before transfection, a well in a 6 well plate was seeded with 10^5 BHK-21 cells and incubated overnight at 37°C with 5% CO_2. DNA and the Effectene reagent were complexed by first mixing 0.4µg of plasmid DNA, 3.2µl of supplied Enhancer solution and supplied Buffer EC to a final volume of 100µl. The solution was briefly mixed by vortexing and incubated at room temperature for 5 minutes. 10µl of the Effectene reagent was added and the solution was again mixed by vortexing. Complexes were allowed to form by incubating the solution at room temperature for 10 minutes. During this incubation, media was aspirated from the BHK-21 cells. The well was rinsed with 2ml of sterile PBS and 1.6ml of fresh complete Glasgow's medium was added to the cells. The DNA:Effectene solution was diluted in 600µl of complete Glasgows medium and added, drop-wise, to the cells. Plates were incubated at 37°C with 5% CO_2.

2.6.8 Viral Plaque Harvesting

The entire contents of 96-well plate wells containing BHK-21 cells with a single viral plaque were removed by scraping with a Gilson P200 pipette tip and mixed by pipetting. Half of the media volume (100µl) was transferred to a 1.7ml cryovial (Nunc) and stored at -80°C until PCR screening had been performed on the remainder of the sample (Sections 2.1.1 and 2.6.11).

2.6.9 Limiting Dilution Purification of Virus

1µl of freeze/thawed viral plaque sample was diluted 11 times by doubling dilution in complete Glasgow's media in a 96-well plate. $5x10^4$ BHK-21 cells were added to each well and plates were incubated at 37°C with 5% CO_2 until viral plaques formed.

2.6.10 Agarose Overlay Purification of Virus

Freeze/thawed viral plaque sample was diluted 1/200, 1/2,000 and 1/20,000 in 2ml of complete Glasgow's medium. $5x10^5$ BHK-21 cells were added to each dilution and shaken at 225 rpm in an orbital shaker for 1 hour at 37°C in. Each dilution was plated out in a single well of a 6 well plate and incubated overnight at 37°C with 5% CO_2. The following day, complete Glasgow's medium was mixed (1:1 v/v) with 2% Microseive Low Melt Agarose (Flowgen) in sterile PBS which had been allowed to cool to approximately 45°C. 4ml of the media/agarose mixture was added to each well and allowed to set before plates were returned to the incubator. Plates were incubated at 37°C with 5% CO_2 until viral plaques formed. An area containing a single plaque were marked and removed through the agarose using a sterile Pasteur pipette. The plaque sample was mixed by pipetting with compete Glasgow's medium and frozen at -80°C. The sample was rapidly thawed and incubated with $5x10^4$ BHK-21 cells in a 96-well plate well for 48 hours and harvested as described in Section 2.6.8.

2.6.11 Plaque DNA Isolation

The entire contents of 96-well plate wells containing a single viral plaque were removed by scraping with a Gilson P200 pipette tip and mixed by pipetting. Half of the media volume (100µl) was transferred to a 0.5ml microcentrifuge tube. The sample was centrifuged at 1,500 x g for 5 minutes in a bench top microcentrifuge. The cell pellet was washed with 500µl of TE buffer (20mM Tris, 1mM EDTA, pH 8.0) and centrifuged again. The cell pellet was resuspended in 50µl of TE buffer and frozen on dry ice for 5 minutes and rapidly defrosted to disrupt cell membranes. 19µg of PCR grade Proteinase K (Roche) was added and the sample was incubated overnight at 53°C. The Proteinase K was then heat-inactivated by a 15 minute incubation at 90°C and 10µl of sample was used as template DNA for screening PCRs.

2.6.12 Growth of Viral Stocks

$2.4x10^7$ BHK-21 cells were suspended in 5ml and infected with $2.4x10^4$ PFU (MOI = 0.001) of virus. Cells were incubated for 90 minutes at 37°C with shaking at 225 rpm in an orbital shaker. The cells were then plated out in 8 x 175cm^2 flasks in 40ml complete medium per flask and incubated at 37°C with 5% CO_2. Flasks were checked

daily until complete cytopathic effect (CPE) was seen, approximately 6 days post-infection. Remaining adherent cells were dislodged by scraping. Media and cells from all flasks were pooled. Cells were pelleted by centrifugation at 1,700 x g for 20 minutes in a bench top centrifuge. The cell pellet was resuspended in 4 ml of PBS and disrupted by 30 strokes, on ice, in a dounce homogeniser. The homogenate was transferred to a glass universal and sonicated in an ice bath for 15 minutes. The lysate was transferred to a plastic universal and centrifuged at 1,700 x g for 20 minutes at 4°C in a bench top centrifuge. The supernatant, containing the majority of the infectious virus, was saved on ice. The pellet was resuspended in 1ml of PBS, returned to the homogeniser and homogenised for approximately 30 strokes as before. The homogenate was centrifuged at 1,700 x g for 20 minutes at 4°C. The supernatant was pooled with the previous supernatant. Aliquots of this stock were transferred to 1.7ml cryovials and stored at -80°C.

2.6.13 Viral Titration on BHK-21 Cells

Viral samples of 400µl were diluted through a series of 10-fold dilutions in 4ml of complete Glasgow's medium. $2x10^6$ BHK-21 cells were added to each dilution and incubated for 1 hour at 37°C with shaking at 225 rpm in an orbital shaker. Each dilution was made up to 10 ml and plated on two replicate 60mm Petri dishes. Plates were incubated for 4 days at 37°C with 5% CO2. The media from each plate was then discarded, the cells were rinsed with PBS and fixed with 10% neutral buffered formalin for 30 minutes. The fixative was discarded and the cells were stained with 0.1% toluidine blue solution for 10 minutes. The plates were rinsed in gently running H_2O and allowed to air dry. The number of plaques on each plate was counted using a dissecting microscope. The titre of the original stock or tissue was calculated using the average of the replicate plates of the lowest countable dilution and the following equation: Titre = (Number of plaques x Dilution)/2ml.

2.6.14 One-Step Growth Curve

$5x10^6$ BHK-21 cells were suspended in 2.5ml complete medium and infected with $2.5x10^7$ PFU (MOI = 5) of virus. Two samples of $2x10^5$ cells were taken and kept at -80°C as the -1 hour time point. Remaining cells were incubated for 1 hour at 37°C with shaking at 225 rpm in an orbital shaker. Two samples of $2x10^5$ cells were taken and kept at -80°C as the 0 hour time point. Remaining cells were plated out in

duplicate at 10^5 cells/well in 1ml of complete medium in 24 well plates (one plate per time point) and incubated at 37°C with 5% CO_2. At the appropriate time post infection (1, 4, 8, 12, 18, 24, 30, 36, 48, 60 or 72 hours) a plate was removed, sealed with parafilm and stored at -80°C. When all samples were collected, all the plates and the -1 and 0 hour time points were freeze/thawed three times and the samples titrated as described in Section 2.6.13.

2.6.15 Multi-Step Growth Curve

$2.5x10^6$ BHK-21 cells were suspended in 2.5ml complete medium and infected with $2.5x10^4$ PFU (MOI = 0.01) of virus. Two samples of 10^5 cells were taken and kept at -80°C as the -1 hour time point. Remaining cells were incubated for 1 hour at 37°C with shaking at 225 rpm in an orbital shaker. Two samples of 10^5 cells were taken and kept at -80°C as the 0 hour time point. Remaining cells were plated out in duplicate at 10^5 cells/well in 1ml of complete medium in 24 well plates (one plate per time point) and incubated at 37°C with 5% CO_2. At the appropriate time post infection (12, 24, 36, 48, 60, 72, 96 or 120 hours) a plate was removed, sealed with parafilm and stored at -80°C. When all samples were collected, all the plates and the -1 and 0 hour time points were freeze/thawed three times and the samples titrated as described in Section 2.6.13.

2.6.16 Viral Infection for Analysis of Temporal Expression of Genes

$3x10^6$ C127 were suspended in 2ml complete medium and infected with $1.5x10^7$ PFU (MOI = 5) of virus. Cells were incubated for 1 hour at 37°C with shaking at 225 rpm in an orbital shaker. Samples were transferred to 75cm^2 flasks in 18ml of complete medium and incubated at 37°C with 5% CO_2. Cells were collected by trypsinisation after 24 hours. Where indicated, phosphonoacetic acid (PAA, 100µg/ml) or cycloheximide (CHX, 100µg/ml) was added to the medium. PAA was added at the time of viral infection and CHX was added to cells 30 minutes prior to infection. CHX-treated samples were collected 8 hours post infection.

2.6.17 Preparation of Media Containing Secreted Viral Proteins

$2.5x10^6$ BHK-21 cells in a 6 well plate well were infected with $1.25x10^7$ PFU of MHV-ALHE or MHV-76 (MOI = 6) in 3ml of complete DMEM (Dulbecco's Modified Eagle's medium (DMEM, Gibco) supplemented with 10% (v/v) foetal calf

serum, 2mM L-glutamine, 200U/L penicillin/streptomycin and 0.5nM β-mercaptoethanol). 24 hours after infection, supernatants were removed and passed through Minisart 0.22 micron filters (Sartorius) to remove cell debris.

2.6.18 Isolation of Peripheral Blood Mononuclear Cells

Fresh peripheral ovine blood was supplied by Prof. John Hopkins (Department of Veterinary Biomedical Sciences, University of Edinburgh). Fresh peripheral bovine blood was supplied by Dr. Alastair Macrae (Easter Bush Veterinary Centre, University of Edinburgh). Whole heparinised blood was mixed with two volumes of PBS and gently mixed by inversion. The diluted blood was then carefully layered over 1 starting volume of room temperature Histopaque-1077 (Sigma) in either 15 or 50ml conical centrifuge tubes. Blood fractions were separated by centrifugation at 500 x g for 30 minutes at room temperature with no brake in a bench top centrifuge. Following centrifugation, the opaque interface containing the mononuclear cells was transferred to a fresh tube using a Pasteur pipette. The cells were then washed twice using at least 4 starting volumes of sterile PBS and centrifuging at 500 x g for 5 minutes each wash. Cells were resuspended in PBS and counted using trypan blue exclusion (as described in Section 2.6.1).

2.6.19 Culture of Peripheral Blood Mononuclear Cells

Bovine and ovine PBMC, isolated as described in Section 2.6.18, were cultured in 96 well plates ($4x10^5$ cells per well) in 300µl of complete DMEM or with 100µl of complete DMEM and 200µl of filtered medium from virally infected BHK-21 cell cultures (Section 2.6.17). Concanavalin A (ConA, Sigma) was added to cultures to the final concentrations indicated.

2.6.20 Propidium Iodide Staining of Suspension Cells

At the indicated time points during culture, bovine and ovine PBMC samples were transferred from 96 well plates to 0.5ml microcentrifuge tubes. Samples were centrifuged at 1,000 x g for 5 minutes at room temperature in a bench top microcentrifuge. Supernatants were transferred to fresh tubes and stored at -80°C. Cell pellets were resuspended in 50µl of PBS and fixed by adding 50µl of 4% paraformaldehyde. Samples were incubated at room temperature for 20 minutes and centrifuged as before. Supernatants were discarded and cell pellets were resuspended

in 100µl of PBS containing 0.5% (w/v) bovine serum albumin (BSA), 0.25% (v/v) Triton X-100, 1µg/ml propidium iodide (Fluka), 1U/ml RNase A and 40U/ml RNase T1 (RNase Cocktail, Ambion). Samples were incubated in the dark for 20 minutes and centrifuged as before. Supernatants were discarded and cell pellets were resuspended in 200µl of PBS containing 2% paraformaldehyde and 0.5% BSA. Samples were stored at 4°C wrapped in foil until all samples for an experiment had been collected for FACS analysis.

2.6.21 Staining of Transfected Cells

Transfected BHK-21 cells (see Sections 2.6.6 and 2.6.7) were removed from 6 well plate wells using trypsin/EDTA and counted as described in Section 2.6.1. An area of approximately 4cm^2 was marked out on a sterile glass microscope slide (Snowcoat X-tra Micro Slides, Surgipath) using an ImmEdge pen (Vector Laboratories, Inc.). Approximately 4x10^4 cells in 200-300µl of medium were applied to the slide surface. Slides were incubated, inside Petri dishes, at 37°C with 5% CO_2 for 4-8 hours to allow cells to adhere to the slide surface.

Media was poured off the slide and the cells were gently rinsed with PBS. Cells were fixed for 20 minutes in 10% (v/v) neutral buffered formalin (Surgipath), for C23 staining, or 4% (w/v) paraformaldehyde in PBS, for c-myc staining. Slides were washed twice for 5 minutes each in PBS. Cells were permeablised by incubation with PBS containing 0.25% (v/v) Triton X-100 (t-Octylphenoxypolyethoxyethanol, Sigma) for 20 minutes followed by two 5 minute washes in PBS.

Slides were blocked with normal goat serum (Sigma) diluted 1:500 in 3% BSA/PBS (w/v) for 30 minutes. Blocking solution was poured off and cells were stained with either C23 (MS-3, Santa Cruz Biotechnology) diluted 1:5 to 40µg/ml in 3% BSA/PBS (w/v) for 3 hours at room temperature or Anti-c-myc antibody (9E10 from mouse ascites fluid, Sigma) diluted 1:300 in 3% BSA/PBS (w/v) for 1 hour. Slides were washed twice for 5 minutes each in PBS. Bound antibody was detected using Alexa Fluor 594 goat anti-mouse IgG (H+L) (Molecular Probes) diluted 1:500 to 4µg/ml in 3% BSA/PBS (w/v) for 30 minutes at room temperature in the dark. RNase A (Roche, diluted 1:10,000 to 10µg/ml) and either propidium iodide (Fluka, diluted 1:1,000 to 1µg/ml) or TO-PRO-3 iodide (Molecular Probes, diluted 1:1,000 to 1µM) were added to the secondary antibody solution to counterstain for DNA. The

secondary antibody solution was poured off and slides were washed three times in PBS for 5 minutes each. Coverslips were mounted using either DakoCytomation Fluorescent Mounting Medium (Dako) or Mowoil (Calbiochem) and slides were stored, wrapped in foil, at 4°C until viewed.

For cells transfected with GFP constructs that did not require antibody staining, slides were prepared, fixed, permeablised and counterstained as described above.

2.7 *In Vivo* Experiments

2.7.1 Mouse Infection

All animal work was carried out in accordance with Home Office guidelines under project licence numbers 60/2429 and 60/3283 and personal licence number 60/9208. Female, 5-6 week old, Balb/c mice were purchased from Harlan. Mice were anaesthetised with halothane prior to viral infection. Mice were infected either intranasally with $4x10^5$ plaque forming units (PFU) of virus in 50µl of sterile PBS applied to the nose and inhaled or by injection of 10^6 PFU of virus in 500µl of sterile PBS injected intraperitoneally. Mice were euthanised by CO_2 asphyxiation for tissue collection.

2.7.2 Histology

Lungs for histology were inflated via the trachea with 50% Tissue-Tek O.C.T. compound (Sakura) in water and frozen by immersion in isopentane and dry ice. Spleens were collected in 10% neutral buffered formalin. Tissues were sent to the Veterinary Pathology Department Histology facility for processing, sectioning and staining. Hematoxilin and eosin stained sections were examined under a light microscope and assessed by Mr. Babunilayam Gangadharan (Division of Veterinary Biomedical Sciences, University of Edinburgh).

2.7.3 Preparation of Tissues for Viral Titration

Lungs for infectious virus titration were bisected and each half was placed in a 1.7ml cryovial (Nunc) and frozen on dry ice. Spleens for infectious virus titration were placed in a 1.7ml cryovial and frozen on dry ice. Tissues (half lungs or whole spleens) were homogenised using a glass homogeniser in 1ml of complete Glasgow's medium. The homogenate was transferred to a fresh 1.7 ml cryovial and frozen on dry ice for 30 minutes to disrupt cell membranes. The homogenate was thawed and centrifuged at 1,700 x g for 20 minutes at 4°C. Supernatants were titrated on BHK-21 cells as described in Section 2.6.13. Remaining homogenate was stored at -80°C.

Peritoneal exudate cells (PEC) for titration were removed by injection of 5ml of complete RPMI-1640 medium (RPMI-1640 supplemented with 10% (v/v) foetal calf serum, 2mM L-glutamine, 100 U penicillin/streptomycin, 50µM 2-mercaptoethanol and 20mM HEPES) into the peritoneal cavity and collected by draining through a

peritoneal incision. Cells were collected by centrifugation for 5 minutes at 500 x g in a bench top centrifuge. The supernatant was poured off and discarded and the cell pellet was resuspended in the remaining media. Erythrocytes were lysed by the addition of 1ml sterile distilled water. Samples were immediately equilibrated by adding 9ml of sterile PBS. A sample of the cell suspension was diluted in Trypan Blue solution (Sigma) and live cells were counted in a haemocytometer. Viable splenocyte number was calculated using the following equation: Cells/ml = Number of cells counted in the middle 25 squares x dilution in Trypan Blue x 10^4. The cells were transferred to a fresh 1.7 ml cryovial and frozen on dry ice for 30 minutes to disrupt cell membranes. The sample was thawed and volumes equivalent to 10^5 and 10^6 cells were added in duplicate to BHK-21 cells for titration.

2.7.4 Infectious Centre Assay

Spleens for analysis of reactivatable virus were removed and kept on ice in complete RPMI-1640 medium. Spleens were teased into single cell suspensions using a clean scalpel blade and a 60mm Petri dish and suspended in 10ml of complete RPMI-1640. Cells were collected by centrifugation for 5 minutes at 500 x g in a bench top centrifuge. The supernatant was poured off and discarded and the cell pellet was resuspended in the remaining media. Erythrocytes were lysed by the addition of 1ml sterile distilled water. Samples were immediately equilibrated by adding 9ml of sterile PBS. Debris was allowed to settle and the cell suspension was transferred to a fresh universal. The splenocyte suspension was washed once and resuspended in 5ml of complete RPMI-1640. A sample of the cell suspension was diluted in Trypan Blue solution (Sigma) and live cells were counted in a haemocytometer as described in Section 2.6.1. 10^7, 10^6 or 10^5 cells were added to 60mm Petri dishes containing 10^6 BHK-21 cells in 5ml of complete RPMI-1640 and incubated for 5 days at 37°C with 5% CO^2. The media from each plate was discarded. The cells were rinsed with PBS and fixed with 10% neutral buffered formalin for 30 minutes. The fixative was discarded and the cells were stained 0.1% toluidine blue solution for 10 minutes. The plates were rinsed in gently running H_2O and allowed to air dry. The number of plaques on each plate was counted using a dissecting microscope. The number of infectious centres per spleen was calculated using the average plaque number of the 2 plates with the highest splenocyte number added that was countable and the following

equation: Infectious centres/spleen = (Number of plaques x Total splenocyte number) / Splenocyte number added per plate.

Peritoneal exudates cells (PEC) for infectious centre assays were collected as described in Section 2.7.3. Cells were collected by centrifugation for 5 minutes at 500 x g. The supernatant was poured off and discarded and the cell pellet was resuspended in the remaining media. Erythrocytes were lysed by the addition of 1ml sterile distilled water. Samples were immediately equilibrated by adding 9ml of sterile PBS. A sample of the cell suspension was diluted in Trypan Blue solution (Sigma) and live cells were counted in a haemocytometer as described in Section 2.6.1. 10^6 or 10^5 cells were added to 60mm Petri dishes containing 10^6 BHK-21 cells in 5ml of complete RPMI-1640 and analysed after 5 days as described above.

2.8 Other Methods

2.8.1 Luciferase Activity Assays

Luciferase reporter activity assays were performed using the Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's instructions. BHK-21 cells were transfected, in triplicate, with 2µl each of test plasmid, reporter plasmid and *Renilla* luciferase control plasmid using the protocol described in Section 2.6.6. 48 hours post transfection, supernatants were removed from cultures and adherent cells were briefly rinsed with PBS. 500µl of supplied passive lysis buffer (PLB) was added to each sample and plates were incubated on an orbital shaker for 15 minutes at room temperature.

An appropriate number of 100µl aliquots of supplied Luciferase Assay Reagent (LAR) II were dispensed into opaque 96 well microtitre plates. A Luminskan luminometer (Labsystems Research Systems) was programmed for a 10 second read and blanked on the LAR II containing wells. 20µl of cell lysate was added to the LAR II and mixed by pipetting. The sample was then read for 10 seconds in the luminometer and the firefly luciferase activity measurement was recorded. The plate was removed and 100µl of supplied Stop & Glo Reagent was added to the sample and mixed by pipetting. The sample was read again for 10 seconds in the luminometer and the *Renilla* luciferase activity measurement was recorded. The procedure was then repeated for all samples.

2.8.2 Confocal Microscopy

Slides of transfected cells were viewed using a Leica confocal laser scanning microscope at the University of Edinburgh Confocal Microscopy Suite with the assistance of Ms. Linda Wilson. Image analysis was performed using Leica Confocal Software (LCS) Lite vs. 2.051347a.

2.8.3 FACS Analysis

Forward scatter (cell size), side scatter (cell granularity) and FL2 fluorescence (propidium iodide, DNA content) of stained PBMCs was measured on a FACScan flow cytometer (BD Biosciences). Data was analysed using both Cell Quest (BD Biosciences) and WINMDI (http://facs.scripps.edu/software.html) software.

2.8.4 Statistical Analysis

Statistical analysis was performed using the Graphpad Prism 3.02 software. Differences in viral titres, spleen weights, splenocyte numbers, infectious centre numbers and luciferase activity were analysed using the unpaired t test or the Mann-Whitney test for each time point or group.

2.9 Recipes

2.9.1 Commonly Used Solutions

PBS

10mM Sodium Phosphate

140mM Sodium Chloride

pH 7.4

TBS

10mM Tris-Cl

150mM Sodium Chloride

pH 7.5

2.9.2 Protein Electrophoresis

Stacking Gel

4% (w/v) Acrylamide/bis-Acrylamide (37.5:1)

125mM Tris

0.1% (w/v) Sodium Dodecyl Sulphate

0.1% (w/v) Ammonium Persulphate (APS)

0.1% (v/v) N, N, N', N'-Tetrametylethylenediamine (TEMED)

pH 6.8

Resolving Gel

10-15% (w/v) Acrylamide/bis-Acrylamide (37.5:1)

375mM Tris

0.1% (w/v) Sodium Dodecyl Sulphate

0.1% (w/v) Ammonium Persulphate (APS)

0.1% (v/v) N, N, N', N'-Tetrametylethylenediamine (TEMED)

pH 8.8

Boiling Buffer (2x)

125mM Tris (pH 6.7)

5% (w/v) Sodium Dodecyl Sulphate

25% (v/v) Glycerol

12.5% (v/v) β-mercaptoethanol

0.25% (w/v) Bromophenol Blue

SDS Running Buffer

100mM Tris

38mM Glycine

0.1% (w/v) Sodium Dodecyl Sulphate

pH 8.8

Transfer Buffer

25mM Tris

150mM Glycine

10% (v/v) Methanol

pH 8.3

Ponceau S

O.5% (w/v) Ponceau S

1% (v/v) Acetic Acid

2.9.3 Nucleic Acid Electrophoresis

Tris-Acetate EDTA (TAE)

40mM Tris20mM Acetic Acid

1mM EDTA

pH 8.0

DNA Loading Buffer (6X)

15% (w/v) Ficoll

0.25% (w/v) Bromophenol Blue

0.25%(w/v) Xylene Cyanol

0.25% (w/v) Orange G

2.9.4 Bacterial and Yeast Media

Luria-Bertani (LB) Broth

1% (w/v) Tryptone

0.5% (w/v) Yeast Extract

1% (w/v) Sodium Chloride

pH 7.0

YPDA

20g/L Bacteriological Peptone

10g/L Yeast Extract

20g/L Glucose

20mg/L L-Adenine Hemisulfate

pH 6.5

SD-Ade/-His/-Leu/-Trp

6.7g/L Yeast Nitrogen base without amino acids

20g/L Glucose

0.6g/L -Ade/-His/-Leu/-Trp DO Supplement (Clontech)

pH 5.8

SD-Leu/-Trp

1x SD-Ade/-His/-Leu/-Trp

20mg/L L-Adenine Hemisulfate

20mg/L L-Histidine HCl Monohydrate

pH 5.8

SD-Trp

1x SD-Ade/-His/-Leu/-Trp

20mg/L L-Adenine Hemisulfate

20mg/L L-Histidine HCl Monohydrate

30mg/L L-Leucine

pH 5.8

Chapter 3: Recombinant Virus Results

3.1 Recombinant Virus Production

3.2 Recombinant Virus Characterisation *In Vitro*

3.3 Intranasal Infection of Balb/c Mice

3.4 Intraperitoneal Infection of Balb/c Mice

3.1 Recombinant Virus Production

3.1.1 Aims

One of the primary objectives in the characterisation of the four unique left hand end ORFs of AlHV-1 is to assess their contribution to pathogenesis *in vivo*. Ideally, this would be studied by creating AlHV-1 mutant viruses with one or more of these genes knocked out and using these mutant viruses to infect either naturally susceptible hosts, such as cattle, or laboratory model hosts in which MCF is closely mimicked, such as rabbits. There were, however, several limitations that precluded such studies. Firstly, the tissue culture requirements for AlHV-1 growth mean that genetic manipulation would be difficult. Secondly, the genetic instability of AlHV-1 *in vitro* would mean that even if creation of mutant viruses was successful, the exact genotype of such viruses would be questionable as would any data acquired by their use. Thirdly, the use of cattle or rabbits as hosts would be costly and impractical and would therefore severely limit experimental group sizes.

To overcome these restrictions, a mutant virus was created by inserting the genes into a closely related foreign herpesvirus, murine gammaherpesvirus-76 (MHV-76). MHV-76 is a deletion mutant of MHV-68, a well studied rodent herpesvirus that is often used as a model for gammaherpesvirus infection. The genome sequence of MHV-76 has been shown to be almost identical to that of MHV-68 with the exception of a 9,538 bp deletion at the left hand end of the L-DNA (Macrae *et al.*, 2001). This area encodes four unique ORFs (M1-M4) and eight viral tRNA-like molecules. Despite showing similar growth properties *in vitro*, MHV-76 is attenuated *in vivo* (Macrae *et al.*, 2001). This property of MHV-76 makes it a useful tool for the study of genes thought to be involved in pathogenesis. Mutant MHV-76 viruses created by either replacing the missing MHV genes (Cliffe, 2006; Macrae *et al.*, 2003; Townsley *et al.*, 2004) or by the insertion of foreign genes (Brass, 2004; Hindley, 2005; Sacco, 2003) have previously been used to study the specific contribution of these genes to the various stages of infection *in vivo*, such as immune evasion, transformation and reactivation.

Although it is a very artificial system, the use of the MHV model for the study of AlHV-1 genes offers several advantages over the use of AlHV-1 itself. Firstly, the

creation of MHV mutant viruses is well established and relatively straightforward. Secondly, MHV is genetically stable during tissue culture passage. Thirdly, the use of a small animal model allows for *in vivo* experiments to be performed on larger groups of genetically identical hosts, giving more statistically robust results.

The following sections describe the creation of the recombinant virus with an approximately 6.2 kb region of AlHV-1 inserted into the left hand end of MHV-76 and the analysis of the resultant virus's genomic structure using PCR, restriction digestion and Southern blot analysis.

3.1.2 AlHV-1 Left Hand End Amplification

The LHE region of the L-DNA from the AlHV-1 genome was amplified for cloning by PCR. The JEMBOSS EPRIMER3 software was used to identify highly specific primer sites of sufficient distance downstream of the predicted start codon for ORF A1 (nucleotide 918) to include promoter sequences and upstream of the predicted polyadenylation signal for ORF A4 (nucleotide 6,123). The sites chosen were nucleotide 21 to 40 for the forward priming site and nucleotide 6,197 to 6,216 for the reverse priming site. Primers were designed using these sequences with *Sal*I and *Xba*I restriction endonuclease sites added to the forward and reverse primers, respectively, to facilitate later cloning. PCRs were performed using the high fidelity Expand Long Template PCR system as described in Sections 2.1.1 and A1.3.1 and the resulting product of approximately 6.2 kb is shown in Figure 3.1 A, lane 1.

3.1.3 AlHV-1 Left Hand End Cloning and Sequencing

The LHE PCR product was ligated into the T:A cloning vector pGEM-T Easy as described in Section 2.1.2. The resulting plasmid preparation was shown by restriction digest analysis to contain a product of the correct size with the appropriate restriction sites. The insert was partially sequenced using the T7for and BGHrev primer sites in pGEM-T Easy as described in Section 2.1.12. The sequence from the T7for reaction contained the inserted *Sal*I restriction site followed by AlHV-1 nucleotides 21-635 and the sequence from the BGHrev reaction contained the inserted *Xba*I restriction site followed by AlHV-1 nucleotides 6,216-6,058. The insert size and end sequences are consistent with the cloning of an appropriate PCR product.

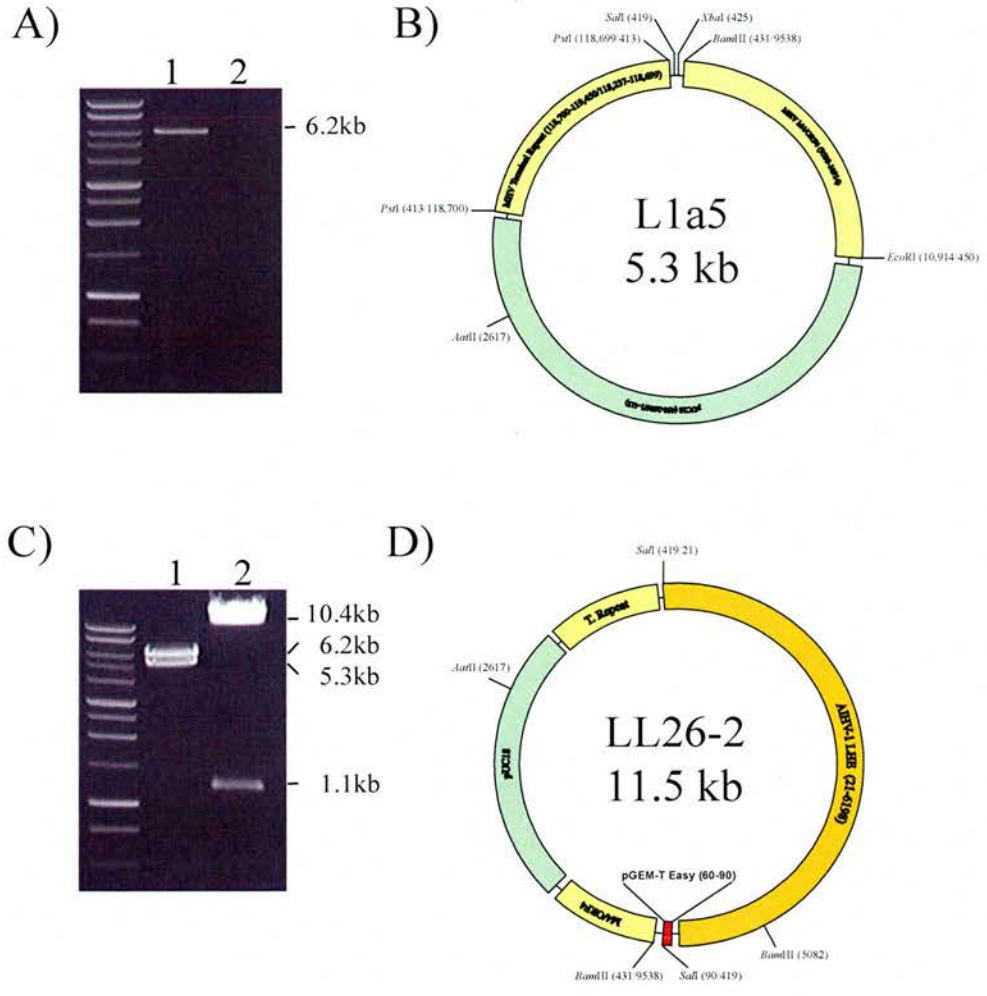


Figure 3.1. A) PCR for AlHV-1 left hand end (lane 1) and negative control (lane 2). B) Map of the L1a5 plasmid indicating restriction sites used for cloning and analysis. C) Restriction digest of LL26-2 plasmid with *Sal*I (lane 1) and *Bam*HI (lane 2). D) Map of the LL26-2 insert vector indicating restriction sites used for cloning and analysis.

To facilitate insertion of the cloned AlHV-1 fragment into MHV-76, it was cloned into the insertion vector L1a5 (kindly provided by Dr. Bernadette Dutia). A vector map of L1a5 adapted from information provided by Dr. Dutia is shown in Figure 3.1 B. L1a5 is a pUC-18 based vector with one complete terminal repeat (MHV-68 nucleotides 118,700-119,450/118,237-118,699) inserted at the *Pst*I restriction site and an M4/ORF4 sequence (MHV-68 nucleotides 9,538-10,914) inserted between the *Bam*HI and *Eco*RI restriction sites of the multiple cloning site. The AlHV-1 insert was intended to be directionally cloned using both the *Sal*I and *Xba*I restriction sites. However, the M4/ORF4 insert was found to contain an *Xba*I site meaning that this strategy was not possible. Instead, the AlHV-1 insert was removed from pGEM-T Easy using *Sal*I alone as the fragment was cloned in an appropriate orientation to use the *Sal*I restriction site in the pGEM-T Easy multiple cloning site. The insert was isolated by gel purification, ligated into *Sal*I digested L1a5 by T4 DNA ligase and cloned. A resulting plasmid (LL26-2) was shown to contain the 6.2 kb insert by *Sal*I digestion and orientation of the insert was shown by digestion with *Bam*HI, which cuts the insert once at position 5082 and once between the L1a5 multiple cloning site and the M4/ORF4 insert, to be consistent with the original design (Figure 3.1 C). A diagram of the LL26-2 insertion vector is shown in Figure 3.1 D. Attempts to remove the inserted 30 pGEM-T Easy nucleotides either by re-cloning the AlHV-1 LHE directly flanked by two *Sal*I sites or by exonuclease removal of the nucleotides from LL26-6 were unsuccessful.

3.1.4 Recombinant Virus Production

The production of recombinant MHV-76 viruses by co-transfection of BHK-21 cells with linearised insert vector and highly purified MHV-76 DNA has been well established. Previous protocols using L1a5 have linearised the vector using *Eco*RI. As the inserted pGEM-T Easy sequence in LL26-2 contained an *Eco*RI restriction site, the *Aat*II restriction site in the pUC18 sequence was used instead (Figure 3.1 C). Linearised vector was purified by phenol:chloroform extraction and concentrated by ethanol precipitation as described in Section 2.1.4.

BHK-21 cells ($2.5x10^6$) were transfected with linearised insert vector and highly purified MHV-76 DNA, mixed with untransfected BHK-21 cells and plated in 96-well plates as described in Section 2.6.5. Five days post-transfection, viral plaques

were seen in four wells. Cells from these wells were collected and split for further purification and PCR screening as described in Sections 2.6.8 and 2.6.11.

Crude lysates from plaque positive wells were screened by PCR for the inserted AlHV-1 ORFs using the programs described in Sections A1.3.2, A1.3.7, A1.3.22 and A1.3.28. Three of the four samples were found to be positive for A2, A3 and A4 but only one of these was also positive for A1. This sample was further purified by limiting dilution of the freeze/thawed sample on fresh BHK-21 cells in 96-well plates as described in Section 2.6.9. After five days, cells were taken from wells with plaques at the highest dilution of virus. These samples were processed as before, screened by PCR and further purified if positive for all inserted ORFs. From the third round of purification onwards samples were also screened for contaminating wild-type MHV-76 using the Trepeat/M-4B PCR. This PCR, detailed in Section A1.3.35, uses a forward primer in the MHV terminal repeat (119,482-119,504) and a reverse primer in the portion of ORF M4 remaining in MHV-76 (9,786-9,760). In the presence of wild-type MHV-76 a product of 293 bp should be amplified but in recombinant virus, with a large insert between the primers, no product should be detected.

After five rounds of limiting dilution purification there was still MHV-76 in the recombinant virus samples. This viral sample was subjected to agarose overlay purification as described in Section 2.6.10. Two plaque samples that were selected following this procedure were both free of contaminating MHV-76. One of these samples, re-named MHV-ALHE, was grown and titrated in BHK-21 cells for use in subsequent experiments.

3.1.5 PCR Screening of Viral Stock DNA

Highly purified viral DNA from MHV-ALHE was isolated, as described in Section 2.6.4, for confirmation of the expected AlHV-1 left hand end insertion. PCRs for A1, A2, A3, A4 and the terminal repeat/M4B junction of MHV-76 were performed on 1-2ng of purified DNA using the programs described in Sections A1.3.3, A1.3.8, A1.3.21, A1.3.29 and A1.3.36. A1, A2, A3 and A4 products were seen in MHV-ALHE but not MHV-76 viral stock reactions (Figure 3.2 A, B, C and D). A Trepeat/M4B product of the appropriate size was seen in MHV-76 but not MHV-ALHE viral stock reactions (Figure 3.2 E).

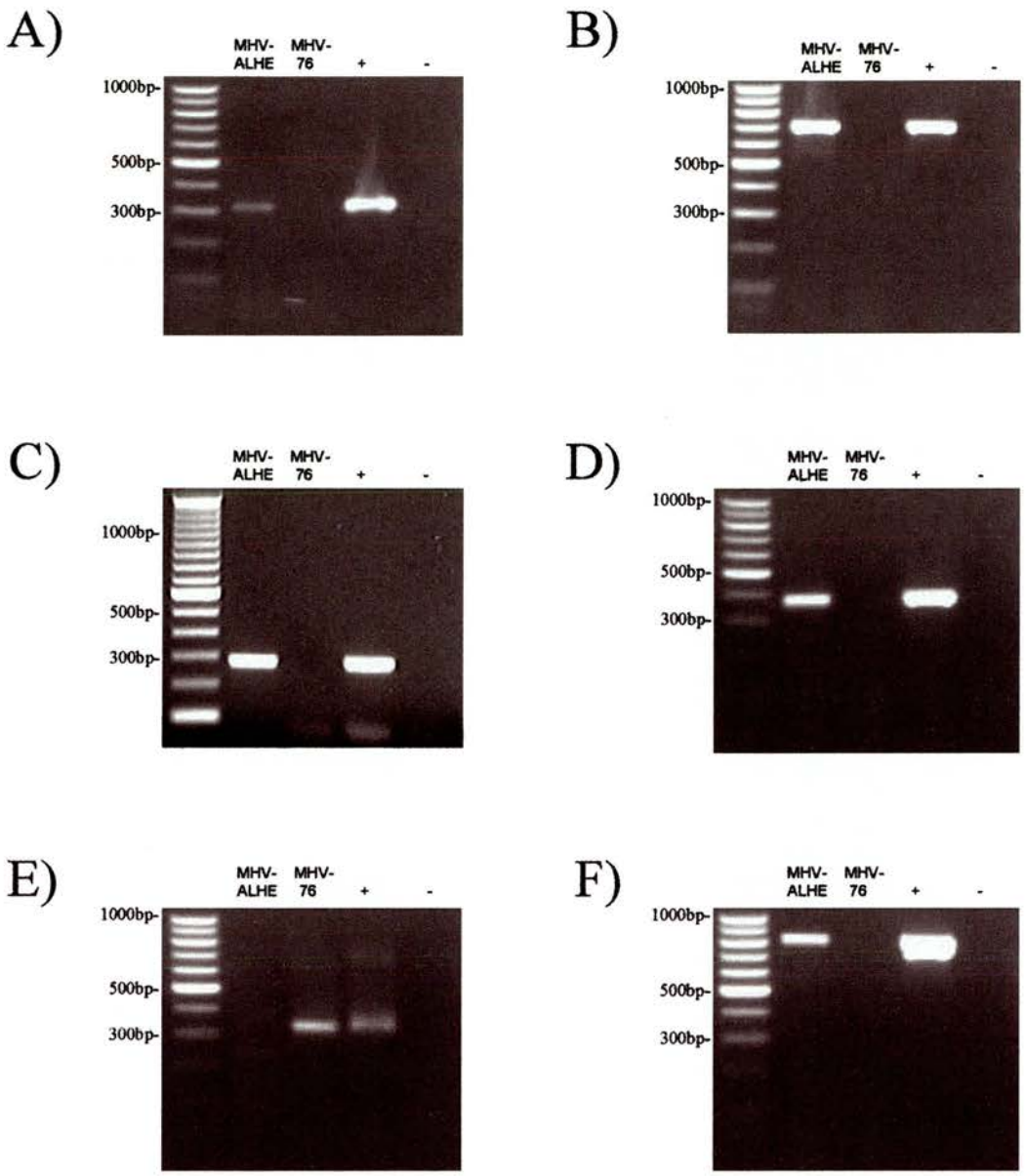


Figure 3.2. PCR screening of recombinant virus. PCRs of A) ORF A1, B) ORF A2, C) ORF A3, D) ORF A4, E) Trepeat/M4B and F) A4for/M4B.

In addition, a PCR was set up to confirm the junction between the insert and the MHV-76 L-DNA using A4 forward and M4 reverse primers. A product of the expected size (780 bp) was seen in the recombinant virus sample (Figure 3.2 F). PCRs with a variety of different parameters were attempted to confirm the junctional region between the MHV terminal repeats and the inserted AlHV-1 sequence but none were successful.

3.1.6 Restriction Digest Analysis of Viral Stock DNA

Highly purified viral DNA was analysed by restriction digest to further confirm appropriate insertion of AlHV-1 sequences. DNA from MHV-ALHE and MHV-76 was digested with either *Sac*I or *Xho*I and separated by agarose gel electrophoresis. These enzymes were selected as they cut within the inserted AlHV-1 sequence but not in the MHV terminal repeats. The MHV-ALHE digest reactions should therefore yield the extra fragments indicated in Figure 3.3 A of approximately 6.1 kb with *Sac*I and 8.9 kb with *Xho*I. The digest patterns were compared with each other and with the published digest patterns for MHV-76. The MHV-76 DNA from the stock used in these experiments showed the same restriction pattern as previously published (Macrae *et al.*, 2001). The predicted additional MHV-ALHE fragments can be seen in Figure 3.3 B, lanes 2 and 4.

3.1.7 Southern Blot Analysis of Viral Stock DNA

To further confirm appropriate insertion of AlHV-1 sequences and clonality of MHV-ALHE, Southern blots were performed on viral stock DNA as described in Section 2.4. A probe consisting of MHV M4/ORF4 sequences (9,538-10,914) was created by removing this region from the L1a5 plasmid by *Eco*RI and *Bam*HI restriction digest and gel extraction of the appropriate fragment. A probe for insert DNA was made by amplifying the full length ORF A3 coding region by PCR using the protocol described in Section A1.3.25 and cloning the resulting product into the T:A cloning vector, pGEM-T Easy. The A3 insert was removed from the vector by *Eco*RI and *Bgl*II restriction digest and gel extracted. Both probes were DIG labelled and labelling efficiency was analysed by dot blot analysis as described in Section 2.4.1.

Highly purified viral DNA was digested using either *Pst*I or a combination of *Not*I and *Hind*III, separated by agarose gel electrophoresis and blotted onto nitrocellulose

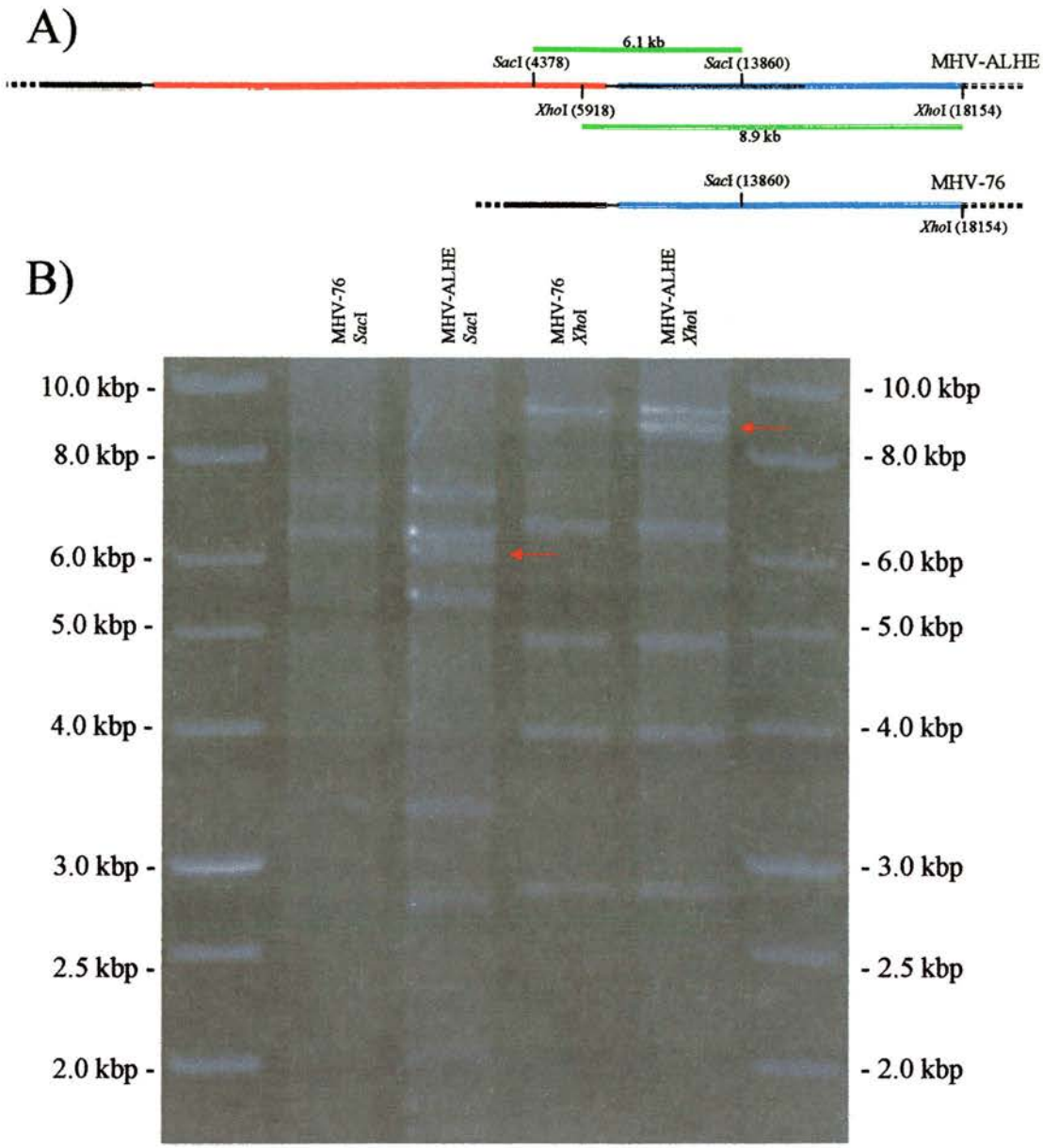


Figure 3.3. A) Restriction digest map showing MHV terminal repeats (black line), MHV-76 L-DNA (blue line), inserted AlHV-1 DNA (red line) and predicted additional digest fragments (green line). B) *Sac*I and *Xho*I restriction digests of MHV-ALHE and MHV-76 genomic DNA. Additional bands in MHV-ALHE digests are indicated with arrows.

membranes by capillary transfer. These restriction enzymes were chosen for various reasons. *Pst*I was used because the terminal repeat of L1a5 is a *Pst*I fragment consisting of nucleotides 118,700-119,450 followed by nucleotides 118,237-118,699 rather than the wild type repeat of nucleotides 118,237-119,450. As such, recombinant viruses created using the L1a5 plasmid should contain a partial repeat (nucleotides 118,237-118,699) followed by a *Pst*I site immediately adjacent to the inserted sequence. In contrast, the internal *Pst*I site in the terminal repeat of wild type MHV-76 is 750 bp downstream of the unique region and therefore a clear difference in restriction fragment lengths should be seen using this enzyme (Figures 3.4 A and 3.5 A). *Not*I was used because it cuts within the MHV terminal repeat and the remaining multiple cloning site of pGEM-T Easy carried into the recombinant virus with the insert. *Hind*III was used to futher digest the *Not*I fragments and allow them to be more easily distinguished.

Following transfer, the membranes were probed with either the M4/ORF4 or the A3 probes and resulting bands were compared after visualisation. As shown in Figure 3.4 B, products of 1640 and 260 bp are detected by the M4/ORF4 probe in *Pst*I digests of both virus samples but a product of 472 bp is only detected in the MHV-ALHE digest. The predicted fragment of 1227 bp is detected in the *Pst*I digest of MHV-76 and there is some signal around that size in the MHV-ALHE sample but this is also seen in the *Not*I and *Hind*III digested samples and is most likely background rather than contamination of the recombinant virus stock. The absence of contamination is also supported by the *Not*I and *Hind*III digests in which there is a fragment of 1571 bp detected the MHV-ALHE sample and not the fragment of 1909 bp as seen in the MHV-76 sample. The A3 probe detected fragments of 3624 and 2591 bp in the *Pst*I digested and fragments of 1828 and 1231 bp in the *Not*I and *Hind*III digested recombinant virus samples (Figure 3.5 B). No binding of this probe to the MHV-76 samples was seen.

3.1.8 Summary

An MHV-76 recombinant virus clone carrying approximately 6.2 kb from the left hand end of AlHV-1 (MHV-ALHE) has successfully been created and purified until free of contaminating wild-type virus. The content, position and orientation of the insert in this clone have been shown by PCR, restriction digest and Southern blot to

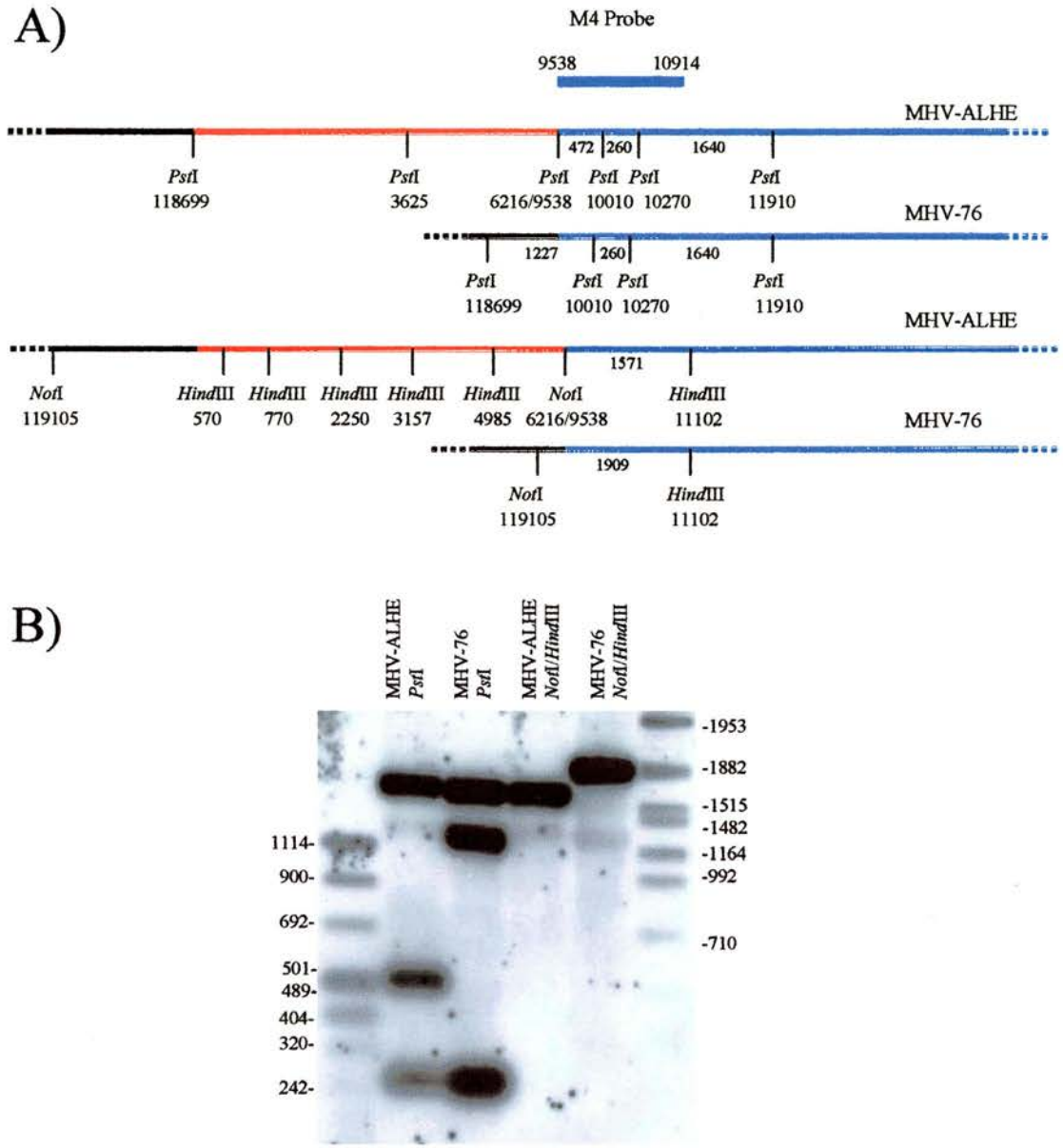


Figure 3.4. A) Restriction digest map of left hand ends of the MHV-ALHE and MHV-76 genomes showing MHV terminal repeats (black line), MHV-76 L-DNA (blue line) and inserted AlHV-1 DNA (red line). B) Southern blot of *Pst*I and *Not*I/*Hind*III restriction digests of MHV-ALHE and MHV-76 genomic DNA. probed with the M4 probe.

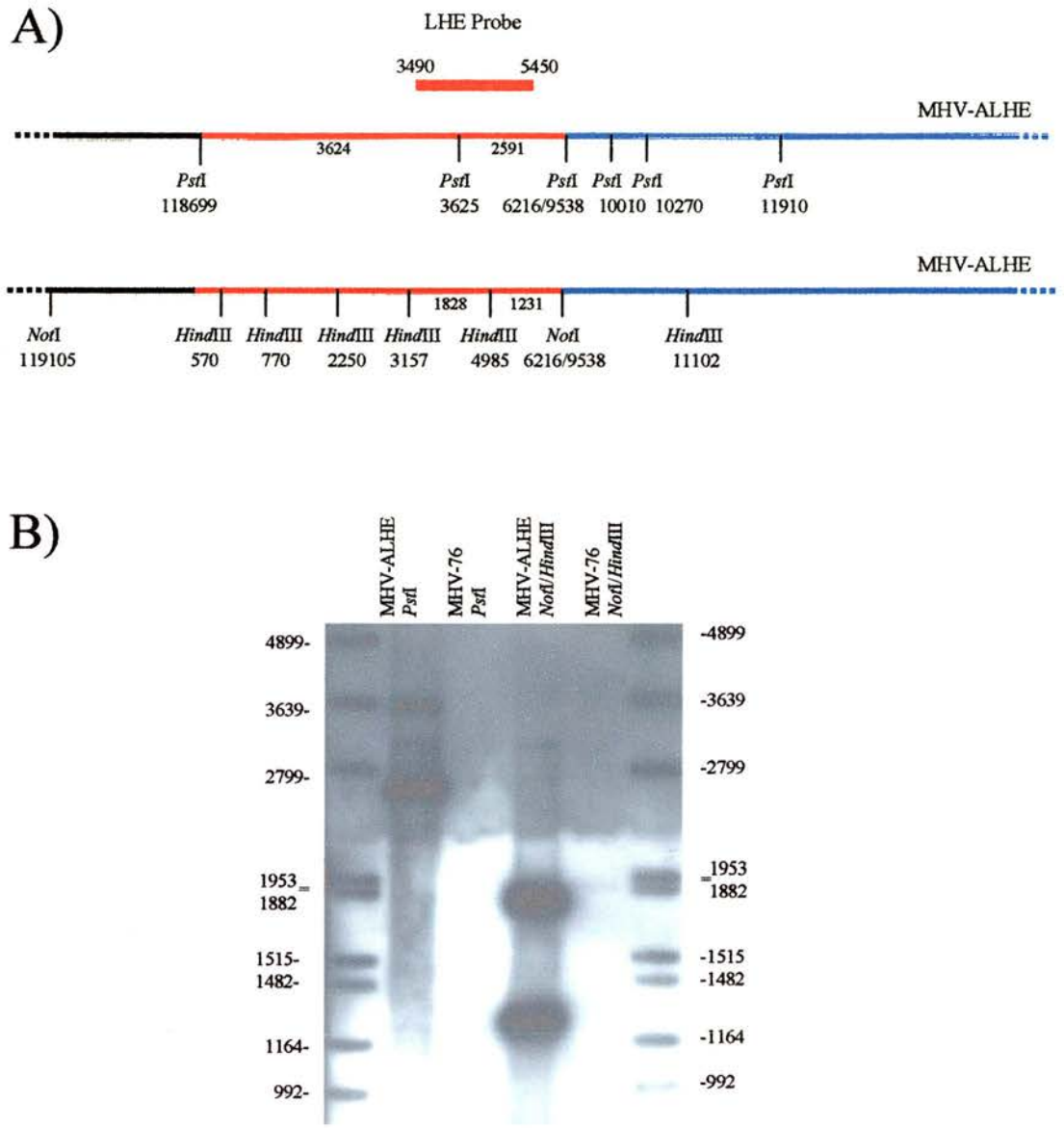


Figure 3.5. A) Restriction digest map of left hand ends of the MHV-ALHE genome showing MHV terminal repeats (black line), MHV-76 L-DNA (blue line) and inserted AlHV-1 DNA (red line). B) Southern blot of *Pst*I and *Not*I/*Hind*III restriction digests of MHV-ALHE and MHV-76 genomic DNA. probed with the A3 probe.

be consistent with the intended genomic structure. This viral clone was used in all further experiments.

3.2 Recombinant Virus Characterisation *In Vitro*

3.2.1 Aims

The biological properties of the recombinant virus were studied *in vitro* to aid in the interpretation of any findings from *in vivo* infection experiments. As the four ORFs inserted in the recombinant virus are under the control of their natural promoters, RNA transcripts were analysed to determine if the ORFs were expressed in a murine host. The growth properties of the virus were studied to determine whether there was any effect on growth rate which would interfere with the interpretation of *in vivo* pathogenesis experiments.

3.2.2 Analysis of AlHV-1 Gene Expression

C127 cells were infected with MHV-ALHE to assess the function of the promoters for the AlHV-1 genes in a murine host. Cells were infected at on MOI of 5 and harvested at 24 hours post infection. Total cellular RNA was extracted and gene expression analysed by reverse transcriptase (RT)-PCR as described in Sections 2.1.15, 2.1.17 and 2.1.18. RNA samples from MHV-76 infected cells and RT-negative assays were used as controls. Successful infection and viral RNA integrity were checked using the PCR described in Section A1.3.38 for MHV ORF 50, an immediate early gene product known to be produced during productive MHV infection, and all samples were found to be positive (Figure 3.6 A). PCRs for the inserted AlHV-1 ORFs, described in Sections A1.3.4, A1.3.9, A1.3.23 and A1.3.30, show that transcripts for all four genes are present in infected murine cells (Figure 3.6 B, C, D and E, lane 1). No products were seen in the negative control samples. In addition, the presence of a second band in the A2 PCR that is about 80bp shorter than the DNA product indicates that A2 message has been spliced as previously reported (Ensser *et al.*, 1997).

In order to determine the temporal class of the inserted genes, infections were performed in the presence of protein synthesis inhibitor cycloheximide (CHX) or the viral DNA replication inhibitor phosphonoacetic acid (PAA) as described in Section 2.6.16.

A1 expression is completely inhibited by CHX and is barely detectable in PAA treated cells (Figure 3.6 B) indicating that it is a late transcript. A second product of

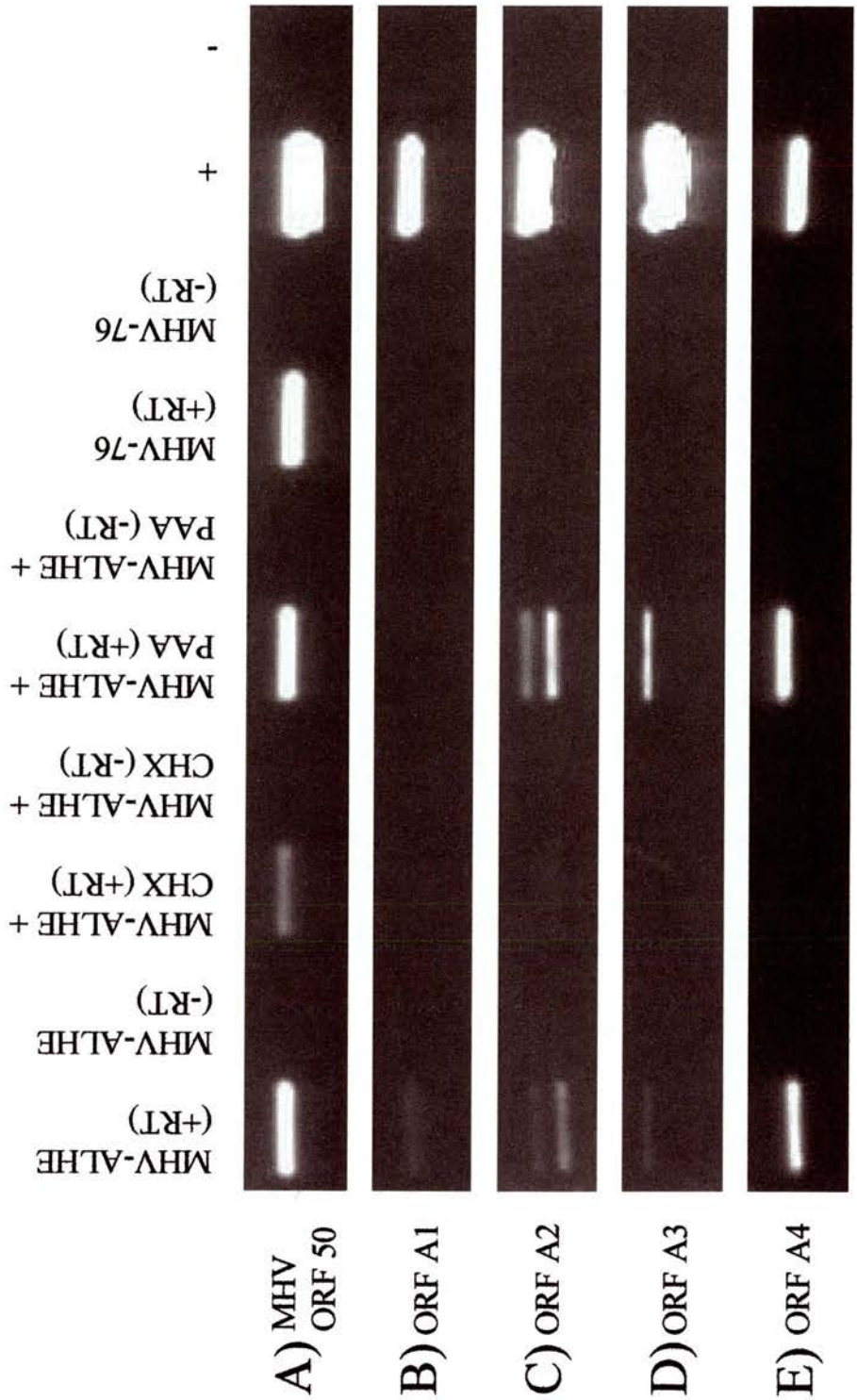


Figure 3.6. *In vitro* RT-PCR results. A) MHV-ORF 50, B) ORF A1, C) ORF A2, D) ORF A3 and E) ORF A4 PCRs. MHV-ALHE infected C127 cells and MHV-ALHE infected C127 cells + phosphonoacteic acid collected 24 hours post infection. MHV-ALHE infected C127 cells + cycloheximide collected at 8 hours post infection.

approximately 150bp is detectable in MHV-ALHE infected cells regardless of treatment (data not shown). This product was cloned and sequenced to determine whether it was a splice variant of A1. Sequence data showed that this product is murine mitochondrial 12S ribosomal RNA with no sequence homology to A1. It is unknown why this product was amplified in MHV-ALHE but not MHV-76 infected samples.

A2, A3 and A4 products were detected as readily in PAA treated cells as untreated (Figure 3.6 C, D and E). An A2 product, the correct size for spliced message, and an A3 product are both present during CHX treatment indicating that although they are predominantly early transcripts there is some immediate early expression. No expression of A4 could be detected in CHX treated cells indicating that it is an early transcript.

BT cells were infected with AlHV-1 and treated with CHX or PAA in the same way as MHV-ALHE infected C127 cells to analyse temporal expression of the left hand end ORFs in a more natural cell type. AlHV-1 is strongly cell associated and *in vitro* infections are carried out by co-culture of uninfected with infected cells rather than incubation with purified virus as is used for MHV infections. Due to this requirement, it was not possible to dissociate newly formed transcripts from those present in the input virus by RT-PCR. Even following freeze/thaw of the inoculum, transcripts for all four left hand end ORFs were seen at the time of infection as well as at later time points (data not shown).

3.2.3 MHV-ALHE Growth *In Vitro*

The growth rate of recombinant virus was compared to that of wild-type MHV-76 using one-step growth curve analysis (Figure 3.7 A) as described in Section 2.6.14. Both viruses adsorbed to the cells similarly as shown by the 0 hour time point. The amount of infectious virus dropped at the 4 and 8 hour time points as expected. The MHV-ALHE titre at 8 hours post infection were lower than wild-type but this difference was not statistically significant. Titres of both viruses grew steadily from 12 to 48 hours post infection and reached a plateau of around 10^7 PFU/ml between 48 and 60 hours. Although the differences in titres at 18, 30 and 36 hours post infection of the two viruses are statistically significant ($p=0.0009$, $p=0.0424$ and $p=0.0036$

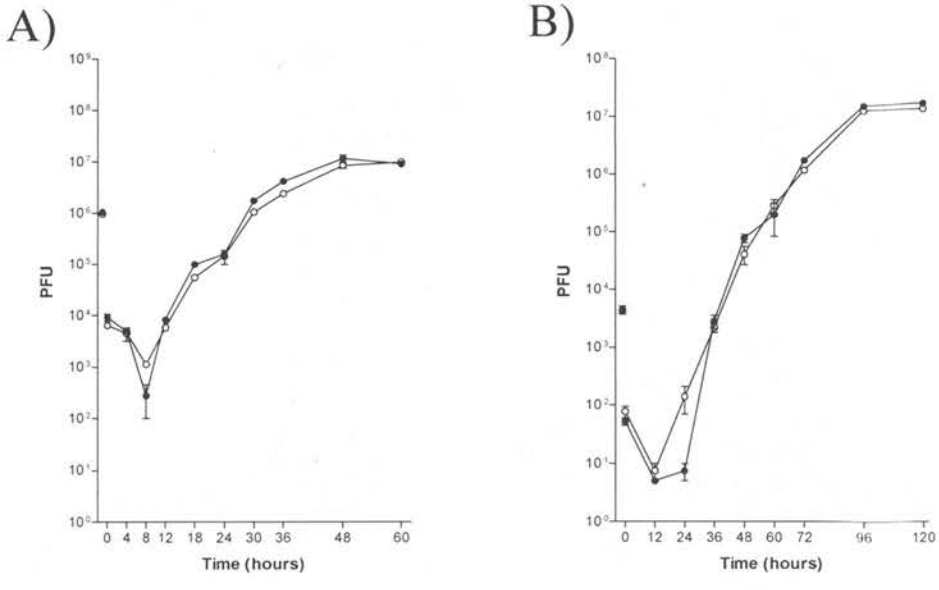


Figure 3.7. A) One-step (MOI = 5) and B) multi-step (MOI = 0.01) growth curve analysis of MHV-ALHE (●) and MHV-76 (○) infected BHK-21 cells.

respectively, using the unpaired t test) due to the small standard deviations, the magnitude of these differences is not biologically significant.

3.2.4 MHV-ALHE Spread *In Vitro*

The growth rate and cell-to-cell spread of virus was compared to that of wild-type MHV-76 using a multi-step growth curve (Figure 3.7 B) as described in Section 2.6.15. As previously seen with the one-step growth curve, both viruses adsorbed to the cells similarly as shown by the 0 hour time point and there was a drop in titre at the 12 hour time point. At 24 hours the production of infectious MHV-ALHE appeared to be delayed but this difference was not statistically significant ($p=0.1991$ using the unpaired t test). By 36 hours, the MHV-ALHE titre was the same as that of MHV-76. The titres for both viruses rose at similar rates from 36 to 96 hours and reached a plateau between 96 and 120 hours of around 10^7 PFU/ml.

3.2.5 Summary

All four of the inserted AlHV-1 left hand end ORFs are expressed from their cognate promoters in murine cells during infection with the recombinant virus MHV-ALHE. Late expression of ORF A1, early expression of ORF A4 and immediate-early/early expression of ORFs A2 and A3 is detectable by RT-PCR. No biologically significant differences were seen in the growth rate and cell-to-cell spread of MHV-ALHE compared to MHV-76 *in vitro*. Taken together, these results indicate that the use of MHV-ALHE is a suitable model to study the effects of the inserted AlHV-1 left hand end ORFs on MHV-76 pathogenesis in a murine host.

3.3 Intranasal Infection of Balb/c Mice

3.3.1 Aims

The ability of the inserted AlHV-1 ORFs to alter the pathogenicity of MHV-76 following intranasal infection was assessed in Balb/c mice. Intranasal infection with MHV-76, which is thought to be the most likely route of natural transmission, causes a phase of acute replication in the lungs followed by latent infection of B cells with transient splenomegaly (Sunil-Chandra *et al.*, 1992a). Mice were infected intranasally with $4x10^5$ PFU of virus and tested at various time points post infection. In the initial experiment, the lungs of four mice per time point per virus were studied at days 3, 5 and 7 post infection to assess any changes in productive viral replication. Also in this experiment, the spleens of four mice per time point per virus were studied at days 7, 10 and 14 post infection to assess any changes in viral induced splenomegaly and latent viral load. Histological studies were also carried out on lungs from infected mice to assess any changes in host response to the infection. These studies were initially performed on two mice per time point per virus at days 3, 5 and 7 post infection but were expanded based on primary results as described in Section 3.3.3.

3.3.2 Productive Infection

Titres of pre-formed infectious virus were studied in the lungs of intranasally infected mice at early time points post infection. The time points selected were before (day 3), around (day 5) and after (day 7) peak titre based on previously published data on intranasal infection of MHV-76 (Macrae *et al.*, 2001). Lungs were collected as described in Section 2.7.3 and titrated on BHK-21 cells as described in Section 2.6.13.

Mean lung titres on days 3 and 5 post infection are slightly higher (approximately 3 fold) in MHV-ALHE infected mice compared to those infected with MHV-76 (Figure 3.8), but these differences are not statistically significant ($p=0.2$ using the Mann-Whitney test). By day 7 post infection, mean titres of both groups are the same.

3.3.3 Lung Histology

MHV-76 has previously been shown to induce a more severe inflammatory response in the lungs of intranasally infected mice than MHV-68 indicating an immunomodulatory role for the deleted ORFs and/or tRNAs (Macrae *et al.*, 2001).

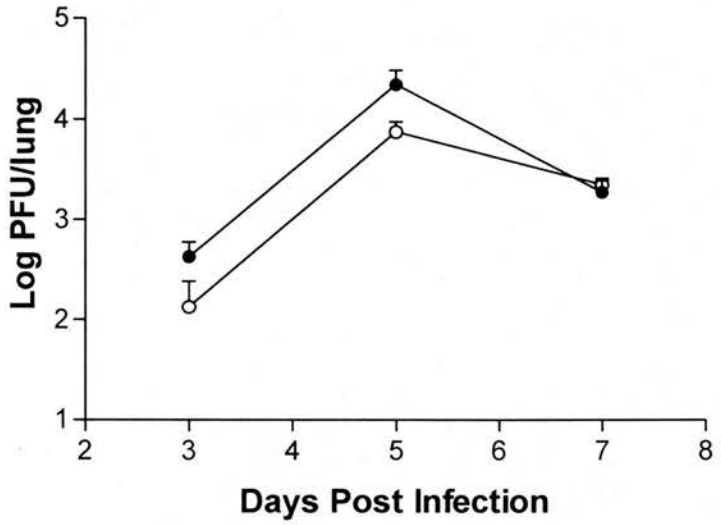


Figure 3.8. Viral lung titres from Balb/c mice infected intranasally with 4 x 10^5 PFU MHV-ALHE (●) and MHV-76 (○).

To determine whether the AlHV-1 ORFs could compensate for the deleted MHV-68 ORFs, hematoxylin and eosin stained sections of inflated lungs of intranasally infected mice were examined under the light microscope as described in Section 2.7.2. Blinded sample were read by experienced pathologist Mr. Babunilayam Gangadharan (Division of Veterinary Biomedical Sciences, University of Edinburgh) and the following results are based on his observations.

In the first experiment, X1 (Table 3.1), two mice per time point per group were studied at 3, 5 and 7 days post infection. Lungs collected from both MHV-ALHE (A5 and A6) and MHV-76 (F5 and F6) infected mice on day 3 post infection showed no indications of inflammation or alveolar thickening. Lung collected from MHV-ALHE infected mice at day 5 post infection (B5 and B6) were beginning to show signs of focal inflammation. However, the lungs from MHV-76 infected mice at this time point (G5 and G6) were clear of inflammation. By day 7 post infection, the inflammation seen in the lungs of MHV-ALHE infected mice (C5 and C6) had become more widespread and was accompanied by alveolar thickening. However, the lungs of MHV-76 infected mice at this time point (H5 and H6) were still clear of inflammation.

As there was a difference seen between the responses to the two viruses and the response to MHV-76 was far weaker than previously reported (Macrae *et al.*, 2001), two further experiments were performed. In the first of these experiments, X3 (Table 3.2), two mice per time point per virus were studied at 7 days post infection to confirm the previous day 7 findings and at 10 days post infection to determine whether the inflammatory response would increase or resolve. In contrast to the previous experiment, lungs from both MHV-ALHE infected mice were relatively clear of inflammation on day 7 post infection (A1 and A2). The lungs from one of the MHV-76 infected mice (B2) was also clear of inflammation but the other sample taken at this time (B1) showed some areas of alveolar thickening, again in contrast to the previous experiment. Lungs collected from both MHV-ALHE (C1) and MHV-76 (D1 and D2) infected mice at day 10 post infection showed areas of alveolar thickening, some of which were quite severe but still milder than expected for MHV-76 infection. The lungs of the second MHV-ALHE infected mouse (C2) were poorly inflated and the degree of inflammation could not be determined.

D3	MHV-ALHE	A5- more or less clear	0
		A6- more or less clear	0
	MHV-76	F5- more or less clear	0
		F6- more or less clear	0
D5	MHV-ALHE	B5- focal inflammation	2
		B6- focal inflammation	2
	MHV-76	G5- more or less clear	0
		G6- more or less clear	0
D7	MHV-ALHE	C5- greater inflammation and alveolar thickening	3
		C6- greater inflammation and alveolar thickening	3
	MHV-76	H5- more or less clear	0
		H6- more or less clear	0

Table 3.1. Lung histology observations and score from experiment X1.

D7	MHV-ALHE	A1- more or less clear	0
		A2- more or less clear	0
	MHV-76	B1- some areas of thickening	3
		B2- more or less clear	0
D10	MHV-ALHE	C1- areas of thickening (some very severe)	3
		C2- unreadable	-
	MHV-76	D1- areas of thickening (some very severe)	3
		D2- areas of thickening (some very severe)	3

Table 3.2. Lung histology observations and scores from experiment X3.

In the third experiment, X5 (Table 3.3), two mice per time point per group were studied at 5, 7 and 9 days post infection and one mouse per group was studied at 15 days post infection to determine whether resolution of inflammation could be seen at this time. Lungs from both MHV-ALHE (A1 and A2) and MHV-76 (B1 and B2) infected mice showed very mild alveolar thickening on day 5 post infection. By day 7 post infection, lungs from both MHV-ALHE (D1 and D2) and MHV-76 (E1 and E2) infected mice showed mild foci of interstitial pneumonia. Occasional foci of interstitial pneumonia were seen in lungs from MHV-ALHE infected mice (G1 and G2) at day 9 post infection. At this time point, more extensive focal inflammation was seen in the lungs of MHV-76 infected mice (H1 and H2). By day 15 post infection, inflammation in the lungs from the MHV-AHLE infected mouse (J1) was resolving with only a small number of very mild inflammatory foci remaining. Similarly, the lungs of the MHV-76 infected mouse at day 15 post infection (K1) were almost entirely clear of inflammation. As the previous two experiments had given unexpected results for MHV-76, a third group was infected using MHV-76 from a stock known to induce the expected inflammatory response in Balc/c mice (MHV-76con, supplied by Miss Anna Cliffe). In this experiment, however, even this virus induced unexpected pathology. On day 5 post infection, lungs from one of the two mice (C2) showed multiple foci of interstitial pneumonia as expected but the lungs of the other mouse (C1) were clear of inflammation. By day 7 post infection, the lungs of the mouse studied (F1) were clear but those of the mouse studied at day 9 (I1) showed multiple foci of interstitial pneumonia.

To simplify the overall analysis of the inflammatory response induced by the different viruses over the three experiments, each sample was assigned a score of 0 to 3 based on the following scale: 0) predominantly clear, 1) a few small foci of mild inflammation, 2) moderate focal inflammation and 3) severe inflammation. The scores for each individual sample are shown in the right hand columns of Tables 3.1, 3.2 and 3.3. The pooled scores from the three experiments are represented, with mean severity, in Figure 3.9. Examples of lungs showing the different grades of inflammation severity are shown in Figure 3.10. Although the group numbers are small and there was a large amount of variability, these experiments show that the time-course of the inflammatory response was similar to previous studies, although

D5	MHV-ALHE	A1- vv mild thickening	1
		A2- vv mild thickening	1
	MHV-76	B1- vv mild thickening	1
		B2- vv mild thickening	1
	MHV-76con	C1- clear	0
		C2- multiple foci of interstitial pneumonia	3
D7	MHV-ALHE	D1- mild foci of interstitial pneumonia	1
		D2- mild foci of interstitial pneumonia	1
	MHV-76	E1- mild foci of interstitial pneumonia	1
		E2- mild foci of interstitial pneumonia	1
	MHV-76con	F1- clear	0
D9	MHV-ALHE	G1- few foci of interstitial pneumonia	2
		G2- occasional foci of interstitial pneumonia	2
	MHV-76	H1- bit more extensive focal interstitial pneumonia	3
		H2- bit more extensive focal interstitial pneumonia	3
	MHV-76con	I1- multiple foci	3
D15	MHV-ALHE	J1- v few foci of interstitial pneumonia	1
	MHV-76	K1- almost clear	0

Table 3.3. Lung histology observations and score from experiment X5.

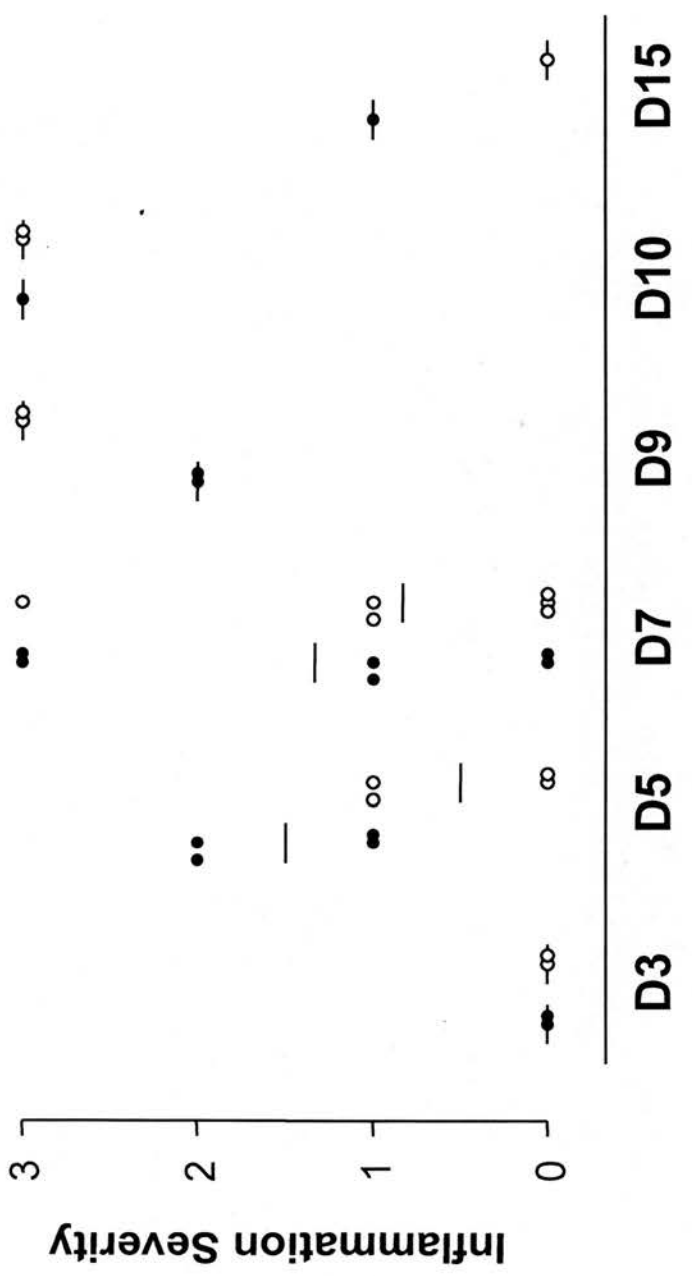


Figure 3.9. Inflammation severity in the lungs of mice infected with MHV-ALHE (●) or MHV-76 (○) pooled from experiments X1, X3 and X5. Bars indicate mean severity.

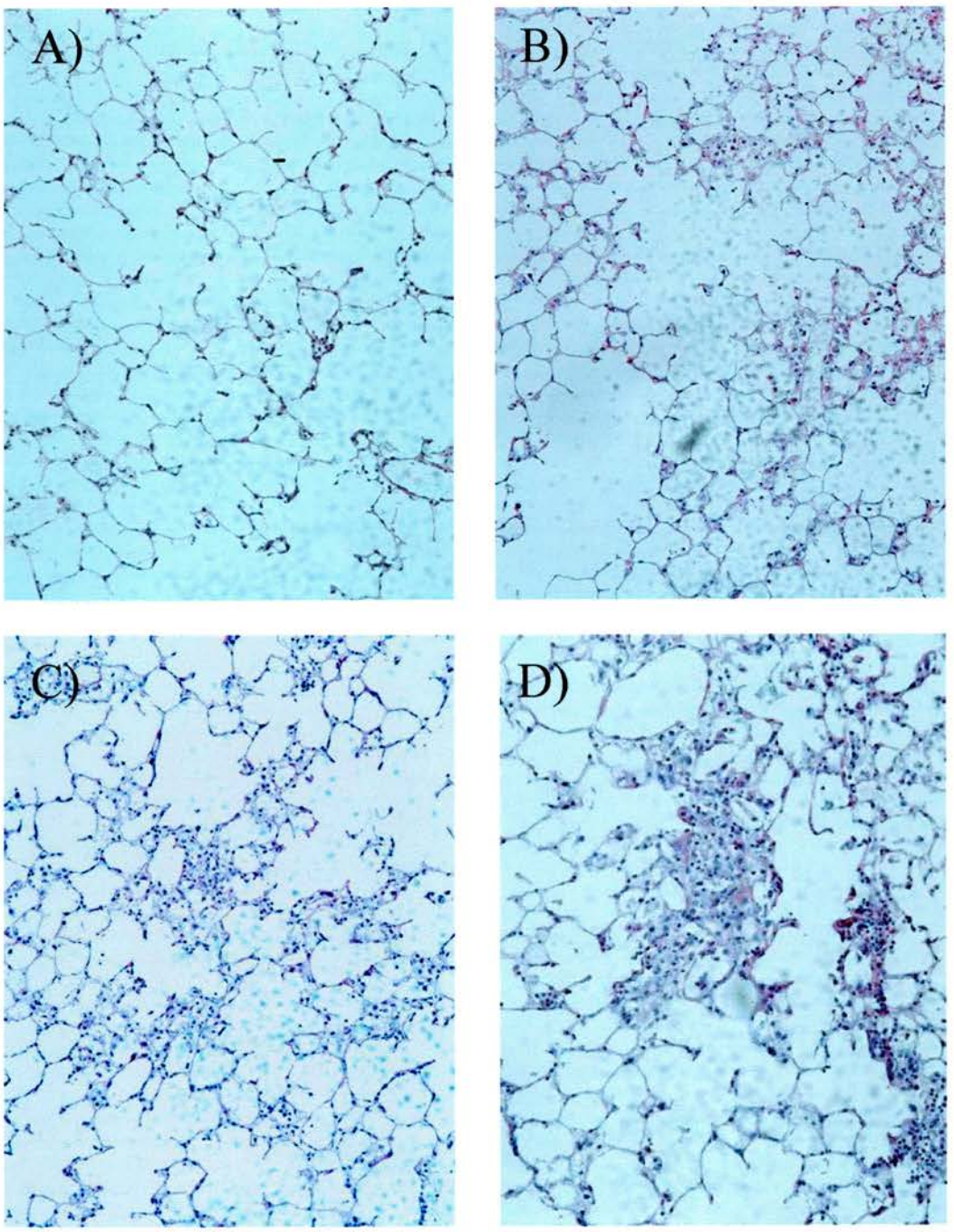


Figure 3.10. Example lung sections showing A) no (X5K1, grade 0), B) mild (X5E1, grade 1), C) moderate (X5G1, grade 2) and D) severe (X5H2, grade 3) inflammation.

with reduced severity, and both MHV-ALHE and MHV-76 elicited a similar response.

As there was an apparent difference in mean inflammation severity in the lungs collected from different groups at day 5 post infection, these samples were sent for independent blinded analysis by a second experienced pathologist, Dr. Susan Rhind (Division of Veterinary Biomedical Sciences, University of Edinburgh). The results of this analysis are shown in Table 3.4. Inflammation severity scores from this analysis are shown in the right hand column, with the previous score shown in parentheses for comparison, and plotted in Figure 3.11. The two MHV-ALHE infected samples previously graded as having moderate inflammation were scored as normal (X1B5) and mild (X1B6), although the faded staining hindered analysis. The MHV-76 infected samples from this experiment (X1G5 and X1G6) were graded as having mild inflammation rather than the previous scoring of more or less clear. Both MHV-ALHE infected samples (X5A1 and X5A2) and one of the MHV-76 infected samples (X5B1) from the second experiment were scored as showing severe inflammation and the remaining MHV-76 infected sample (X5B2) was scored as moderate. Contrary to initial findings, the overall result from the second analysis is that both groups showed the same mean inflammation severity (Figure 3.11).

3.3.4 Latent Infection

MHV-76 is known to establish a latent infection in the spleens of intranasally infected mice albeit at a greatly reduced level compared to that of MHV-68. To assess whether the inserted ORFs had an effect on the establishment of latency, splenocytes from infected mice were used in *ex vivo* reactivation (infectious centre) assays as described in Section 2.7.4. The mean infectious centre number from MHV-ALHE infected mice at days 7 and 21 post infection was found to be extremely low (Figure 3.12 A) and the levels at these time points were the same as those found in MHV-76 infected mice. One of the four MHV-ALHE mice tested at day 14 post infection had an infectious centre number approximately five fold higher than the other three members of this group. An occasional outlier of this type is not uncommon during MHV-76 infection (Dr. Bernadette Dutia, Personal Communication) and the difference in mean infectious centre numbers between this group and the MHV-76

MHV-ALHE	X1B5	Faded	**0** (2)
	X1B6	Mild PB and PV inflammation	**1** (2)
	X5A1	PB and PV inflammation extending to interstitium	**3** (1)
	X5A2	PB and PV inflammation extending to interstitium	**3** (1)
MHV-76	X1G5	Mild predominantly PB inflammation	**1** (0)
	X1G6	Mild focal PB and PV inflammation	**1** (0)
	X5B1	PB and PV inflammation extending to interstitium	**3** (1)
	X5B2	PB and PV inflammation	**2** (1)

Table 3.4. Independent analysis of peribronchiolar (PB) and perivascular (PV) inflammation in lungs samples taken at day 5 post infection. Severity scores are given in the right hand column with previous scores shown in parentheses.

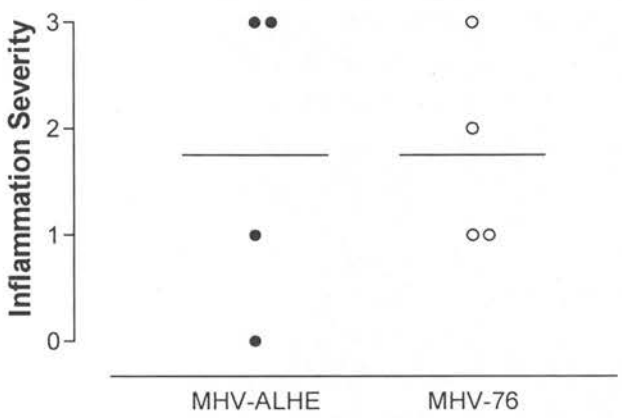


Figure 3.11. Inflammation severity in the lungs taken at day 5 post infection of mice infected with MHV-ALHE (●) or MHV-76 (○) based on second analysis. Bars indicate mean severity.

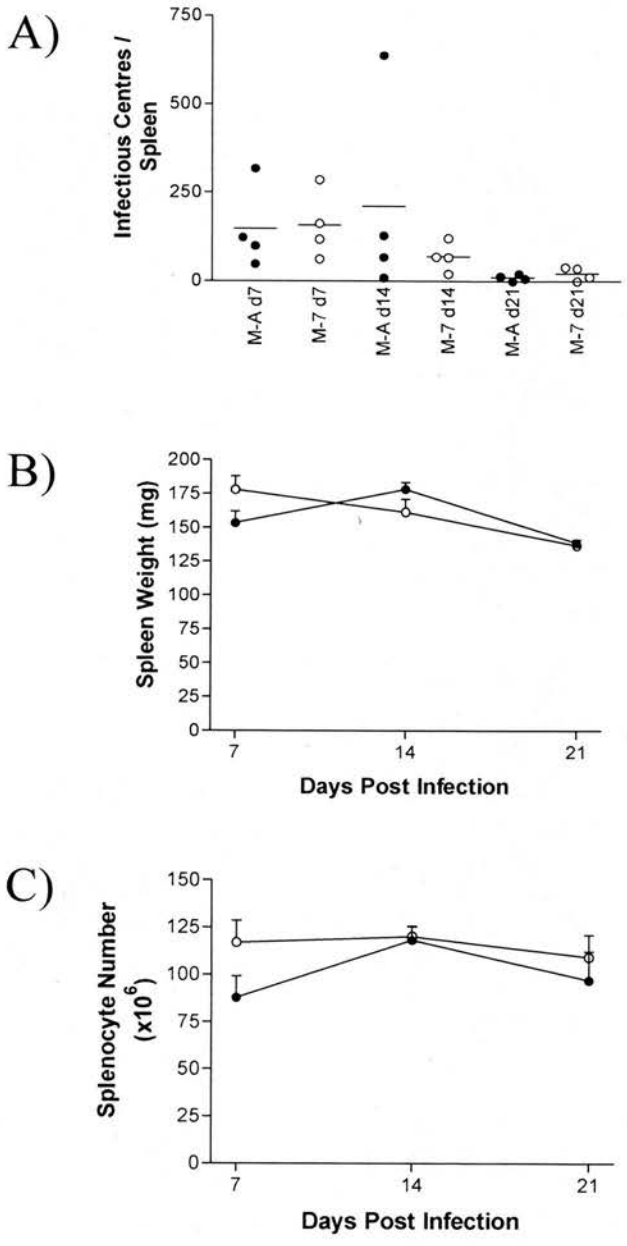


Figure 3.12. A) Spleen infective centres, B) spleen weights and C) spleenocyte numbers from Balb/c mice infected intranasally with 4 x 10^5 PFU MHV-ALHE (●) and MHV-76 (○).

infected group at this time point was not statistically significant (p=0.6857 using the Mann-Whitney test).

3.3.5 Splenomegaly

To determine whether the insertion of AlHV-1 ORFs altered the amount of splenomegaly induced following intranasal infection, spleens from MHV-ALHE and MHV-76 infected mice were removed at various time points after infection and weighed (Figure 3.12 B). Splenocyte numbers were counted following mechanical disruption of the spleen and lysis of erythrocytes (Figure 3.12 C). Again, the time points selected were predicted to be before (day 7), around (day 14) and after (day 21) the peak of splenomegaly based on previously published data for MHV-76 (Macrae *et al.*, 2001). There was no significant difference in the spleen weights or splenocyte numbers between the MHV-ALHE and MHV-76 infected groups at any of the three time points.

3.3.6 Analysis of AlHV-1 Gene Expression

To determine whether the inserted ORFs were expressed during infection, total RNA was extracted from infected lung tissue and screened by RT-PCR. Two separate samples were taken from the lung of an MHV-ALHE infected mouse at day 5 post infection (peak viral titre). One sample of lung tissue from an MHV-76 infected mouse at the same time point was used as a control. All RT+ lung samples were positive for β-actin after one 40-cycle round of PCR and all RT- samples were negative (data not shown) indicating that the RNA was intact and free of cellular DNA contamination.

No expression of the inserted ALHV-1 ORFs or MHV-ORF 50 could be detected after a single 40-cycle round of PCR therefore 1µl of this primary reaction was used as template for a secondary 40-cycle reaction. Using the two round PCR described in Section A1.3.39, MHV-ORF 50 expression was seen in all RT+ samples but not in the RT- samples (Figure 3.13 A) indicating that there was productive infection in the lung areas sampled and the RNA was free from viral DNA contamination. No A1 product was detected in either of the MHV-ALHE infected samples (Figure 3.13 B). Using full-length A2 primers, no products were seen in the MHV-ALHE infected lung samples (data not shown). Internal A2 primers were designed, one of which

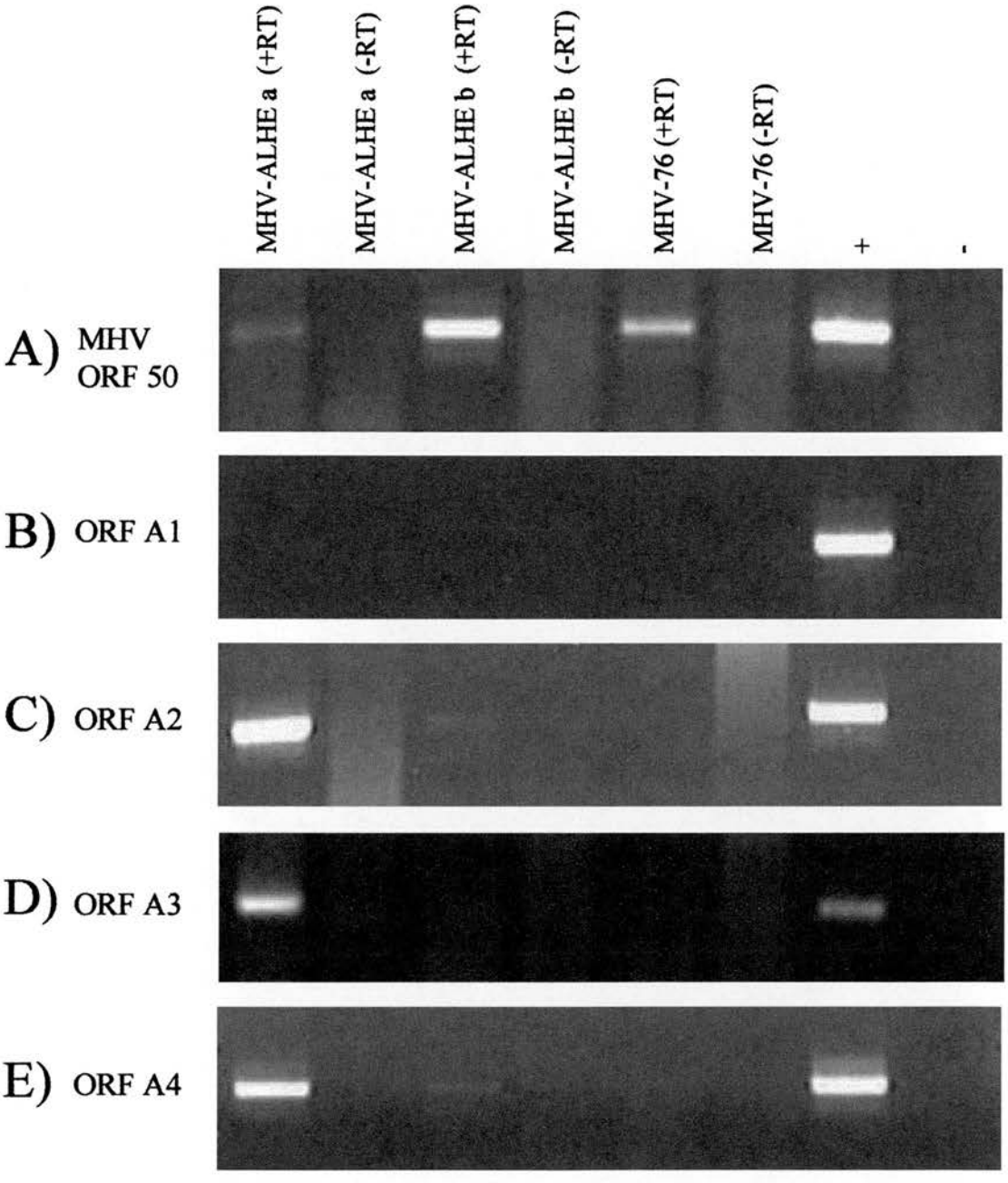


Figure 3.13. *In vivo* RT-PCR. A) MHV-ORF 50, B) ORF A1, C) ORF A2, D) ORF A3 and E) ORF A4 PCRs performed on cDNA from infected lungs at 5 days post infection.

recognised sequences either side the intron so only cDNA made from mature spliced mRNA should be detected. In reactions using these primers, described in Section A1.3.10, expression was detected in both MHV-ALHE infected samples (Figure 3.13 C). No full-length A3 product could be detected in either of the MHV-ALHE infected samples. Again, internal primers were designed to amplify a smaller product. In reactions using these primers, described in Section A1.3.24, expression of A3 could be detected in one of the MHV-ALHE infected samples (Figure 3.13 D). A4 expression, using full-length primers, was detectable in both MHV-ALHE infected samples (Figure 3.13 E). No expression of AlHV-1 ORFs was seen in the MHV-76 infected sample or the RT- reactions.

3.3.7 Summary

Although expression of three of the four inserted ORFs could be detected in the lungs of intranasally infected mice, MHV-ALHE does not show any phenotypic differences from MHV-76 during productive infection at early time points post infection. Studies on changes to the inflammatory response against viral challenge in the lung were difficult to interpret due to the large amount of variation between experiments and in the control groups but there did not appear to be any difference induced by the inserted ORFs. There was also no difference seen between the pathogenesis of the two viruses at later time points in the spleen as measured by reactivatable latent virus, spleen weight and splenocyte number, although expression of the inserted ORFs in infected spleens could not be determined.

3.4 Intraperitoneal Infection of Balb/c Mice

3.4.1 Aims

As there were no significant differences between MHV-ALHE and MHV-76 pathogenesis following intranasal infection, Balb/c mice were infected intraperitoneally to assess any changes in pathogenesis occurring when productive infection occurs in a different location. Intraperitoneal infection is a commonly used route of infection for MHV-68 and related recombinant viruses, including MHVΔ9473 which carries a deletion homologous to MHV-76 (Clambey *et al.*, 2002). Following infection through this route, there is productive replication in the peritoneal cells and spleen rather than the lung epithelia. As the primary productive infection occurs in different cell types and the time course of infection is different, intraperitoneal infection could highlight differences that were not detected following intranasal infection. In the initial experiment, groups of four mice per time point per virus were infected intraperitoneally with 10^6 PFU of virus. Titres of productive virus were measured in the spleens and peritoneal exudate cells (PECs) on days 4 and 9 post infection and reactivatable latent virus was measured in these locations at day 17 post infection. Splenomegaly, as measured by spleen weight was assessed at all time points. A second experiment in which eight mice per virus were infected for study at day 4 post infection was performed based on results of the primary experiment.

3.4.2 Productive Infection

To determine if there is an effect of the inserted AlHV-1 ORFs on the magnitude of productive infection in the intraperitoneal infection model, the titres of pre-formed infectious virus were measured in spleens (Figure 3.14 A) and PECs (Figure 3.14 B) on days 4 and 9 post infection (Figures 3.11 A and B). The titres measured on day 4 post infection were pooled from two independent experiments. There is no statistically significant difference between the titres of MHV-ALHE and MHV-76 at this time point in either spleens or PECs. By day 9 post infection, the productive infection appears to have been completely cleared as all samples for both viruses were below the limit of detection for the assay. This finding is consistent with the published findings for MHVΔ9473 (Clambey *et al.*, 2002).

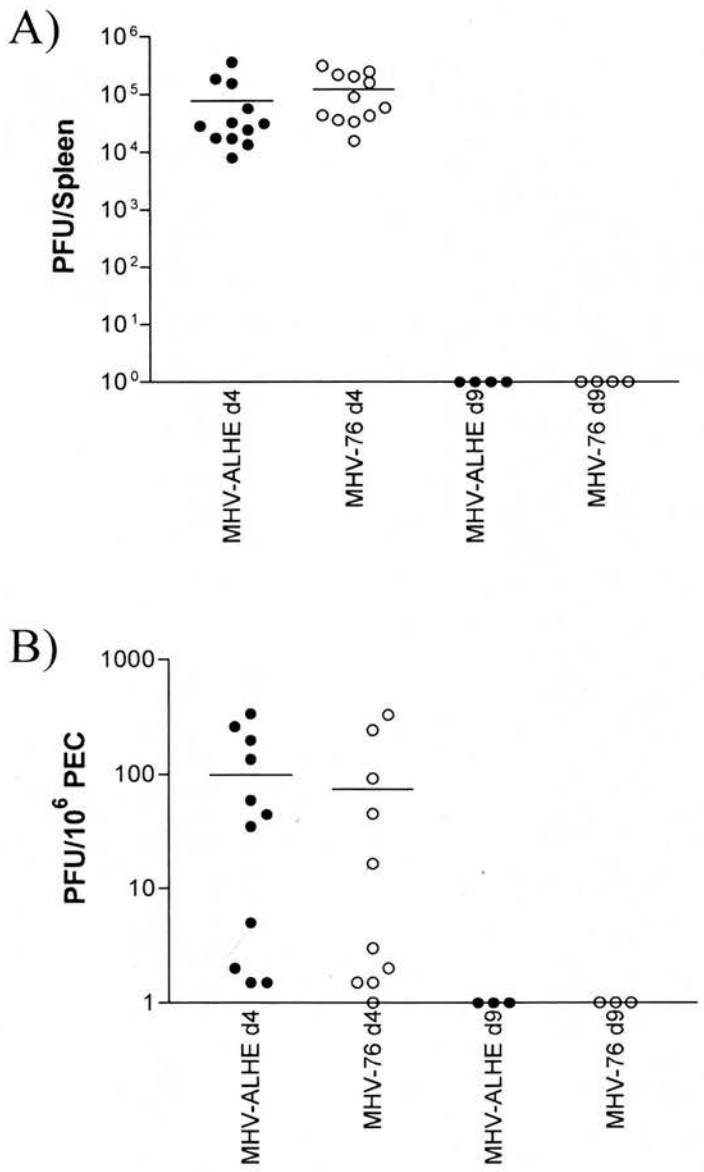


Figure 3.14. Viral titres in the A) spleens and B) peritoneal exudate cells of Balb/c mice infected intraperitoneally with 10^6 PFU MHV-ALHE (●) and MHV-76 (○).

3.4.3 Latent Infection

To assess whether the inserted ORFs had an effect on the establishment of latency in the intraperitoneal infection model, infectious centre assays were performed using splenocytes (Figure 3.15 A) and PECs (Figure 3.15 B) from infected mice at day 17 post infection. The total number of infectious centres in the spleens of both groups at this time point was low and there was no statistically significant difference between MHV-ALHE and MHV-76 infected mice. One of the MHV-ALHE infected mice had an infectious centre number in the PECs much greater than the other three members of the group and the MHV-76 infected mice. It is unclear whether this difference would be significant as sufficient PECs for the assay were obtained from only two of the four MHV-76 infected mice.

3.4.4 Splenomegaly

Spleen weights were measured at days 4, 9 and 17 post infection to determine whether there is an effect of the inserted AlHV-1 ORFs. In both MHV-ALHE and MHV-76 infected mice, there is an increase in spleen weight at day 4 post infection. In the first experiment, this increase was significantly ($p=0.0286$ using the Mann-Whitney test) greater in the MHV-ALHE infected mice. To confirm this finding, a further eight mice per group were infected. The results of the second group of mice alone (data not shown) and the pooled results (Figure 3.15 C) from the two experiments do not show a significant difference ($p=0.5737$ and $p=0.0999$, respectively, using the Mann-Whitney test) between MHV-ALHE and MHV-76 infected mice. By day 9 post infection it appears that the spleen weights of MHV-76 infected mice have dropped back to normal levels and those of MHV-ALHE infected mice remain elevated but this difference is not statistically significant ($p=0.1143$ using the Mann-Whitney test). On day 17 post infection there is no difference between the spleen weights of the two groups and both have returned to a normal level (~150 mg).

3.4.5 Spleen Histology

Although the increased splenomegaly was not found to be reproducible, hematoxylin and eosin stained sections of spleens from infected mice at 4 days post infection were examined microscopically to determine if there was any change in splenic architecture. No obvious differences were seen between spleens of MHV-ALHE and MHV-76 infected mice.

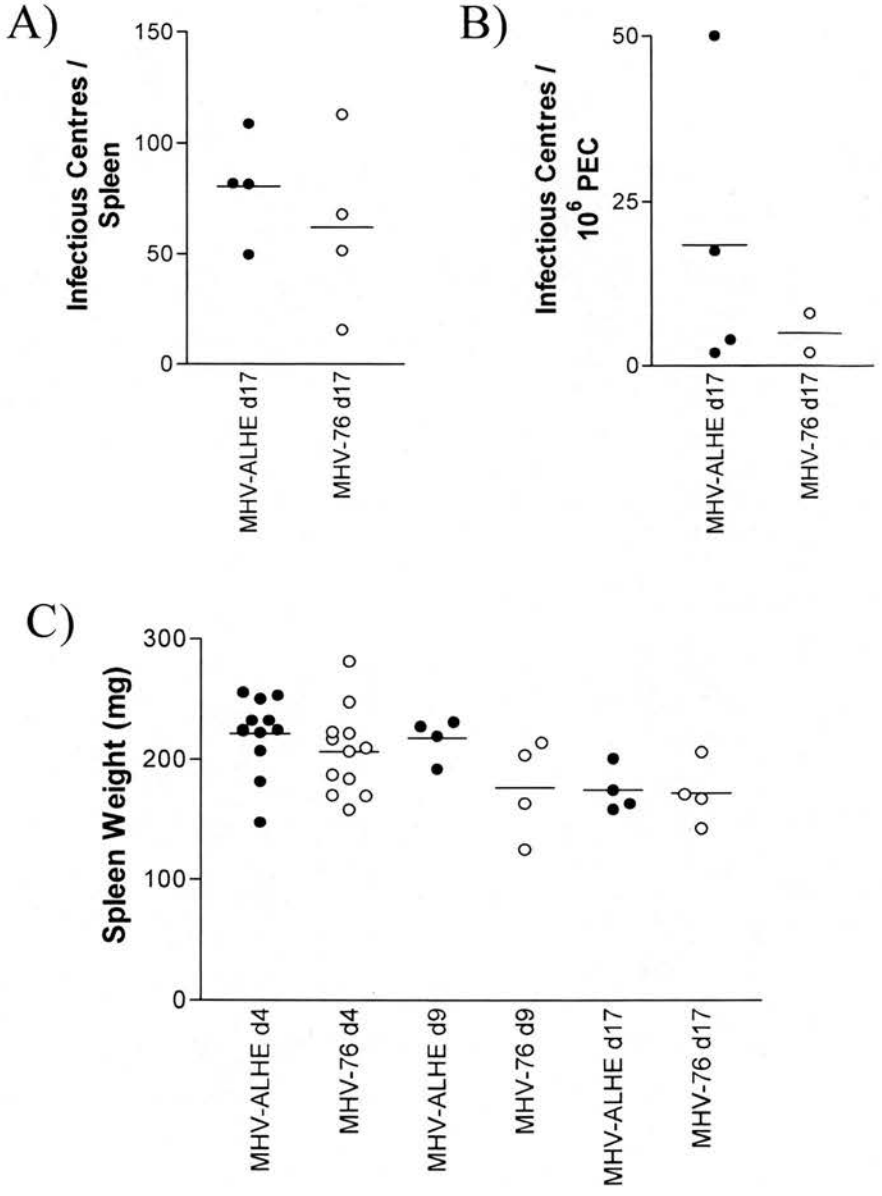


Figure 3.15. Infectious centres in the A) spleens and B) peritoneal exudate cells of Balb/c mice infected intraperitoneally with 10^6 PFU MHV-ALHE (●) and MHV-76 (○). C) Spleen weights of Balb/c mice infected intraperitoneally with 106 PFU MHV-ALHE (●) and MHV-76 (○).

3.4.6 Analysis of AlHV-1 Gene Expression

To determine whether the inserted ORFs were expressed during intraperitoneal infection, total RNA was extracted from infected spleens at 4 days post infection and screened by RT-PCR. One sample from each of the two virus infected groups was taken. Both RT+ assays were positive for β-actin after one 40-cycle round of PCR, described in Section A1.3.40, and both RT- assays were negative indicating that the RNA was intact and free of cellular DNA contamination. No expression of MHV-ORF 50 could be detected even after 2 x 40 cycles. It is possible that there was either no infection in the area of spleen tested or that the level of viral gene expression was below the limit of detection.

3.4.7 Summary

As with intranasal infection, no differences in the pathogenesis of MHV-ALHE and MHV-76 were seen following intraperitoneal infection. The levels of productive infection, reactivatable latent virus and splenomegaly were the same in mice infected with either virus as was splenic architecture. One possible explanation for the lack of differences is that the inserted ORFs are not expressed. However, as transcripts have previously been detected in MHV-ALHE infected murine C127 cells and lungs, it is more likely that the level of expression was low rather than absent.

3.5 Discussion of Recombinant Virus Results

The infection of mice with a recombinant MHV-76 based virus was chosen as the model with which to study the influence on pathogenesis of the four unique left hand end ORFs of AlHV-1. It was decided to create the recombinant by inserting a large AlHV-1 left hand end region of approximately 6.2 kb, containing all four ORFs and their cognate promoter and polyadenylation sequences, rather than creating separate viruses with the individual ORFs under the control of a foreign promoter, a strategy that has also been used for the insertion of foreign herpesvirus genes into MHV-76 (Brass, 2004; Hindley, 2005; Sacco, 2003). There were some drawbacks to creating a single virus but this strategy offered several advantages. Firstly, it was simpler to make one virus versus four and the pathogenesis studies would require fewer animals. Secondly, no artefactual results would arise from either over-expression of the inserted gene due the use of a strong promoter or from the presence of any traceable marker, such as GFP. Approximately 6.2 kb of AlHV-1 DNA was cloned and inserted into the left hand end of MHV-76 as planned with the exception that an additional 30 bp sequence from the cloning vector pGEM-T Easy was inserted along with the AlHV-1 sequence. Although the presence of this short sequence is not ideal, it is very small and does not interrupt any of the inserted coding or promoter sequences and should therefore have no influence on the phenotype of the recombinant virus. Attempts to create a virus without this insert were unsuccessful.

One major disadvantage with the single virus strategy is that the function of the AlHV-1 promoter sequences used had not previously been determined in a murine host and, therefore, it was possible that no expression of the inserted ORFs would be seen. To address this possibility, expression of the genes in murine cells was assessed prior to any *in vivo* experiments. All four inserted ORFs were indeed found to be expressed in C127 cells infected *in vitro* with the recombinant virus. It is interesting that any expression of ORF A1 was seen as other studies have implied that this ORF does not represent an entire gene and may only be an exon as Northern blot analysis detected transcripts larger than 230 bp (Dr. David Haig, Personal Communication). If this is the case, expression of this ORF from the recombinant virus implies that it can be expressed by itself, the other exons are present in the inserted AlHV-1 sequence or MHV-76 sequences can substitute for the other exons. Although the genes were expressed to the RNA level, this does not completely ensure their translation into

protein and, as the ORFs are inserted without any additional epitope tag sequence, the expression of protein cannot be determined at this time due to the lack of specific antibodies.

RT-PCR analysis of cells infected in the presence of cycloheximide or phosphonoacetic acid showed that, in the context of recombinant virus infection, ORF A1 is a late transcript, ORF A4 is an early transcript and ORFs A2 and A3 are immediate early/early transcripts. Detection of immediate early transcription of ORF A2 supports the hypothesis that it encodes a transcription factor. Attempts were made to determine whether this same temporal transcription profile of the left hand end ORFs was seen following AlHV-1 infection of bovine turbinate cells, however, the results were inconclusive due to contamination with transcripts present in the viral inoculum.

Mice were infected with recombinant MHV-ALHE or MHV-76 via either the intranasal or intraperitoneal routes. The intranasal route of infection was chosen for the primary study as this is the most likely route of natural transmission and is well characterised in Balb/c mice. The parameters investigated were based upon previous studies which have shown an acute phase of productive viral infection in the lung followed by splenomegaly and the establishment of a latent infection, predominantly in splenic B cells (Sunil-Chandra *et al.*, 1992b; Sunil-Chandra *et al.*, 1992a). In addition, a cohort of mice was infected intraperitoneally. This artificial route of infection was chosen as a means of studying productive infection in cell types that are more similar to those thought to be involved in MCF pathogenesis, lymphocytes and macrophages, and are therefore more likely to be responsive to the inserted AlHV-1 ORFs. Although lymphocytes and macrophages also become infected following intranasal infection, the attenuation of MHV-76 could mean that too few of these cell types become infected for an effect of the inserted ORFs to be detectable.

Productive recombinant viral titres were the same as those of MHV-76 at early time points in both intranasally and intraperitoneally infected mice. In the lungs of intranasally infected mice, the titres of both MHV-ALHE and MHV-76 rose between day 3 and day 5 post infection and there was some evidence of viral clearance by day 7. As the *in vitro* growth curve analyses of these viruses showed that their growth rates are the same, it can be implied from these results that their clearance rates are

also identical. Similarly, in the spleens and PEC of intraperitoneally infected mice, the titres of both viruses were high at day 4 post infection and completely cleared by day 9.

To determine whether the similarities in viral titres between the two viruses were due to a lack of expression of the inserted ORFs, RT-PCR analysis was preformed on infected tissues. In the lung of an intranasally infected mouse at day 5 post infection, transcripts for ORFs A2, A3 and A4 were detected. The ORF A4 primers used in this experiment are designed to amplify a full-length transcript, however, the ORF A2 and ORF A3 transcripts were detected using internal primers and it is therefore possible that full-length transcripts were not present. Assuming that the transcripts are full-length and expressed to protein level, these results suggest that these three AlHV-1 ORFs do not make the virus more immunogenic or contribute to its ability to evade immune responses in this system. It is also interesting to note that the relative intensities of the PCR products of the inserted ORFs taken from different areas of infected lung appear to correlate inversely with those of MHV ORF 50. This could indicate that the ORF 50 gene product can directly or indirectly down-regulate expression of the inserted ORFs or vice versa, although the PRC used is not quantitative and the sample number used is far too small to draw any conclusions. No transcripts for the inserted ORFs were seen in spleen samples following intraperitoneal infection; however this may simply indicate that the level of infection in that particular area was too low as MHV ORF 50 transcripts were also undetectable.

There was no consistent histological difference seen between the lungs of mice infected intranasally with MHV-ALHE compared to those infected with MHV-76. Data pooled from three experiments (X1, X3 and X5) showed increasingly severe focal inflammation, detectable at day 5 post infection and peaking around day 9 or 10 post infection, following infection with either virus and almost complete resolution of inflammation by day 15 post infection. Based on the results of the first analysis, it was thought that a difference in inflammation severity was detectable on day 5 post infection, however, this difference was not reproducible in a second experiment. In addition, grading of all day 5 post infection samples by a second pathologist showed no difference between the groups, underlining the importance of independent analysis

of experiments where the results are somewhat subjective. The lack of any marked difference in inflammatory response supports the hypothesis that the three expressed AlHV-1 ORFs do not make the virus more immunogenic or contribute to its ability to evade immune responses in this system. Histologically, the spleens of mice infected with either MHV-ALHE or MHV-76 looked normal when studied on day 4 post infection. As with the data on viral titre at this time, the lack of difference may be due to lack of expression.

Latent viral loads were assessed in the spleens of infected mice using the infectious centre assay which measures viral reactivation. Although no significant differences were seen in the number of reactivations of either virus from the spleens of intranasally infected mice at days 7, 10 and 14 post infection or the spleens and PECs of intraperitoneally infected mice at day 17 post infection, there may be a difference in the absolute latent viral load. To address this point, and to determine any difference in reactivation frequency, it would be necessary to determine the viral DNA copy number of the tissue by either limiting-dilution or real-time PCR. Even if differences in DNA load or reactivation frequency were found it would not be possible to definitively attribute these differences to the AlHV-1 genes as transcripts of the inserted ORFs could not be detected.

In summary, a recombinant virus containing four AlHV-1 ORFs was successfully created but showed no biological differences to the parental MHV-76. It is therefore not possible to determine any function of these ORFs from these studies. There are several possible explanations for the lack of phenotypic change conferred by the inserted sequences. Transcripts for all four inserted ORFs were found *in vitro* and at least three are found *in vivo* but, as mentioned above, this does not guarantee that protein is expressed. Even if protein is produced, it is possible that the protein or nucleic acid with which these proteins interact are not sufficiently conserved between the mouse and ruminant species (or between AlHV-1 and MHV-76) for interaction, or are completely absent from this model system, and therefore no effect would be seen. It is also possible that the proteins are having an effect on an aspect of infection that has simply not been assessed in these studies. If this is the case, the recombinant virus model may still be a useful in the future to support further any studies that determine a possible function for any of the four inserted ORFs.

Chapter 4: *In Vitro* Characterisation of LHE ORFs

4.1 Mammalian Expression of LHE ORFs

4.2 Bacterial Expression of LHE ORFs

4.3 Effects of Secreted Proteins on Ruminant Lymphocytes

4.4 Yeast Two-Hybrid Screening

4.1 Mammalian Expression of LHE ORFs

4.1.1 Aims

In addition to the *in vivo* characterisation studies using the recombinant virus, a number of experiments were designed to characterise the LHE ORFs *in vitro*. In the first group of experiments, the individual ORFs were expressed as tagged fusion proteins in mammalian cells. These experiments were carried out in baby hamster kidney (BHK)-21 cells as this cell type is easy to handle in tissue culture, can transfect at higher efficiencies than the available bovine cell lines and is biologically relevant as previous studies have shown that this cell type is permissive for AlHV-1 infection (Frame, 2006). The primary objective of these studies was to determine the localisation of the proteins within cells or whether they were secreted as this would give some indication of function. In the case of ORF A2, further experiments were carried out using one of the tagged proteins to investigate whether this construct could affect transcription from a number of reporter plasmids containing representative AlHV-1 promoter sequences.

4.1.2 Expression of c-myc/6xHIS Tagged LHE ORFs

Initial localisation experiments were performed using sequences of ORFs A1, A2 and A4 cloned into pcDNA3.1/myc-His(-)A. This vector is used for the production of inserted genes with a small C-terminal c-myc/6xHIS tag of 21 amino acids (aa) under the control of the cytomegalovirus (CMV) immediate early promoter for high level constitutive expression. The 10 aa c-myc epitope (EQKLISEEDL) can be used for immunological detection of fusion protein and the 6 histidine epitope can be used for both metal-affinity purification and for metal-affinity or immunological detection. The predicted coding regions of ORFs A1 (912-1208), A2 (2490-1799) and A4 (5732-6094) were amplified by PCR using the reactions described in Sections A1.3.6, A1.3.11 and A1.3.32. Products were cloned into the T:A cloning vector pGEM-T Easy and sequenced before being inserted in frame into pcDNA3.1/myc-His(-)A. Diagrams of the resultant vectors are shown in Figure 4.1 A, B and C. Expression vectors were transfected into BHK-21 cells either by electroporation as described in Section 2.6.6 or by creating DNA:lipid complexes with Effectene as described in Section 2.6.7. Transfected cells were transferred to microscope slides 48 hours post transfection for c-myc staining as described in Section 2.6.21. Supernatants from

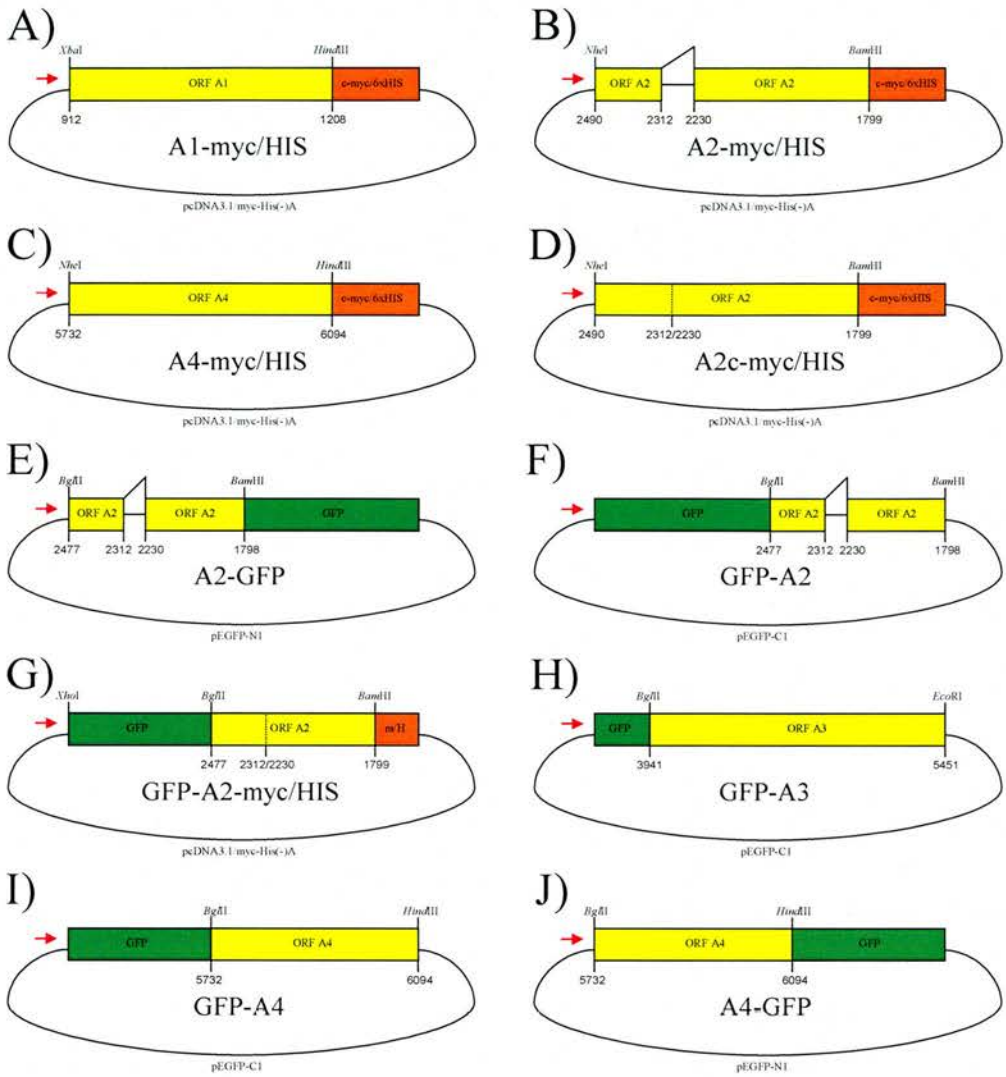


Figure 4.1. Diagrammatic representations of constructs used for the expression of tagged AlHV-1 ORF gene products in BHK-21 cells. Not to scale. Red arrows indicate relative position and orientation of CMV IE promoters. Yellow blocks indicate inserted AlHV-1 sequences and the relative positions of GFP and c-myc/6xHIS tags are indicated by green and red blocks, respectively.

transfected cultures were collected 48 hours post transfection and analysed by Western blot as described in Sections 2.5.1-2.5.3.

In cell cultures transfected with the ORF A1 and ORF A4 constructs, no cells were found to be positive for staining with the anti-c-myc antibody (data not shown). Western blot analysis of concentrated supernatants from these cultures also showed no obvious staining above background (data not shown). Based on the low transfection efficiencies seen in cultures transfected in parallel with positive control and ORF A2 constructs, it is likely that any secreted protein would be too dilute within the supernatant to be detected.

In cells transfected with the ORF A2 construct, c-myc staining was found to localise within the nucleus (Figure 4.2). In the majority of cells, staining co-localised with DNA and was not seen in distinct areas within the nucleus where DNA was also absent (Figure 4.2 A, C and E). It was hypothesised that these areas were nucleoli. This hypothesis was tested in the experiments detailed in the following section. It was also observed that a disproportionately large percentage of transfected cells had an unhealthy nuclear morphology (as represented by the cell on the left in Figure 4.2 B, D and F as compared to the non-transfected cells on the right) and were most likely undergoing apoptosis. The presence of the intron (2312-2231) within ORF A2 did not affect the localisation of the gene product as the same pattern of staining was seen following transfection with a construct containing ORF A2 cloned from cDNA of infected cells rather than viral genomic DNA (diagram shown in Figure 4.1 D, data not shown).

4.1.3 Localisation of ORF A2 GFP Fusion Proteins within Nuclei

In order to determine the nature of the areas from which ORF A2 protein was excluded, transfected cultures were stained using the C23 antibody specific for nucleolin. As both the C23 antibody and the anti-c-myc antibody are murine IgG, simultaneous staining of cells was not straightforward. Instead, ORF A2 (2477-1798, Section A1.3.12) was cloned into the pEGFP-N1 and pEGFP-C1 vectors to allow its production a fusion protein with Enhanced Green Fluorescent Protein (GFP). The use of the GFP tag has the advantage that no staining procedure is required for the visualisation of recombinant protein. However, the use of such a large tag could interfere with the normal behaviour of the ORF A2 protein and constructs were

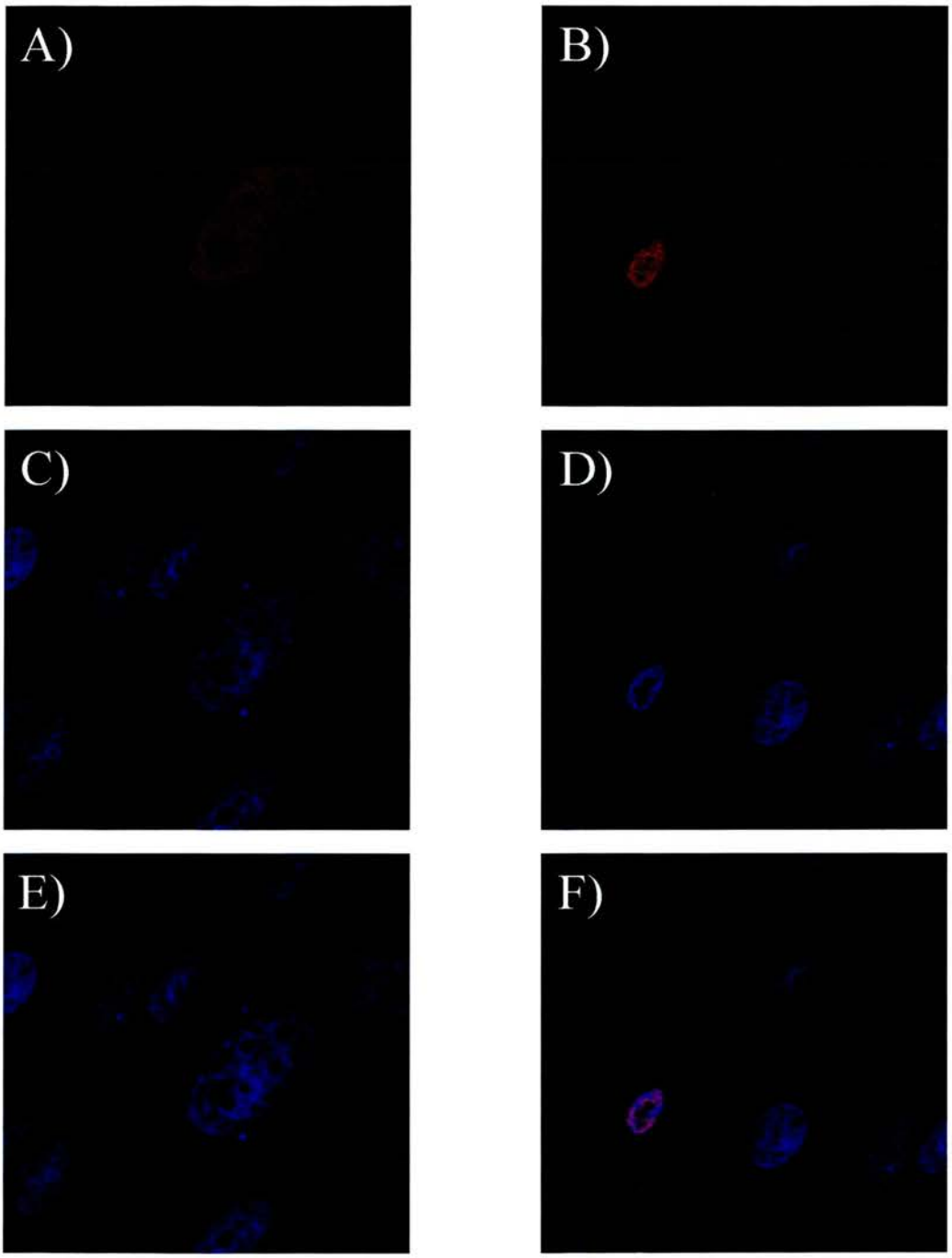


Figure 4.2. A and B) Localisation of c-myc/6xHis tagged ORF A2 protein (red) expressed in BHK-21 cells 48 hours post transfection. C and D) Nuclei counterstained with TO-PRO-3 (blue). E and F) Overlay images.

therefore made with either N- or C-terminal GFP. Digrams of the N- and C-terminally GFP tagged ORF A2 vectors (pE-GFP-A2 and pE-A2-GFP, respectively) are shown in Figure 4.1 E and F.

BHK-21 cell cultures were transfected with the GFP tagged ORF A2 and stained using the C23 antibody 48 hours post transfection as described in Section 2.6.21. The GFP signal in cells transfected with the C-terminally tagged ORF A2 vector (Figure 4.3) showed a similar pattern of distribution as c-myc staining of cells transfected with A2-mycHis, with signal present in the nucleus but preferentially excluded from distinct areas. As hypothesised, these areas were positive for C23 staining indicating that they are indeed nucleoli. In contrast, the GFP signal in cells transfected with the N-terminally tagged ORF A2 vector was still nuclear but more intense in distinct areas (Figure 4.4). Although these areas of more intense GFP signal co-localise with C23 staining, they do not appear to be nucleoli as there is also co-localisation with TO-PRO-3 and are most likely nuclear aggregates. Two possible explanations for the finding that C- and N-terminally tagged ORF A2 proteins are either preferentially excluded from nucleoli or included in nuclear aggregates are that 1) the recombinant ORF A2 protein is being cleaved and the different termini are localising independently or 2) the large GFP tag is interfering with the normal localisation of the protein. Experiments to address these hypotheses are described in the following section.

4.1.4 Localisation of N- and C-Termini of ORF A2

In order to differentiate between the cleavage and altered localisation hypotheses mentioned above, a construct was produced encoding both N- and C-terminal epitope tags. The expression plasmid was produced by cloning a GFP-A2 PCR product into pcDNA3.1myc/His(-)A. The GFP-A2 PCR was performed using the pTH-GFPA2c vector (Section A1.3.17) as a template and the product was first cloned into the pGEM-T Easy cloning vector before being inserted into pcDNA3.1myc/His(-)A. A diagram of the final construct is shown in Figure 4.1 G.

The vector for the dual-tagged construct was tranfected into BHK-21 cells and transferred to microscope slides 48 hours post transfection for c-myc staining as previously described. Cells representative of the majority of strongly GFP positive cells are shown in Figure 4.5. In such cells, the c-myc (C-terminal) localisation

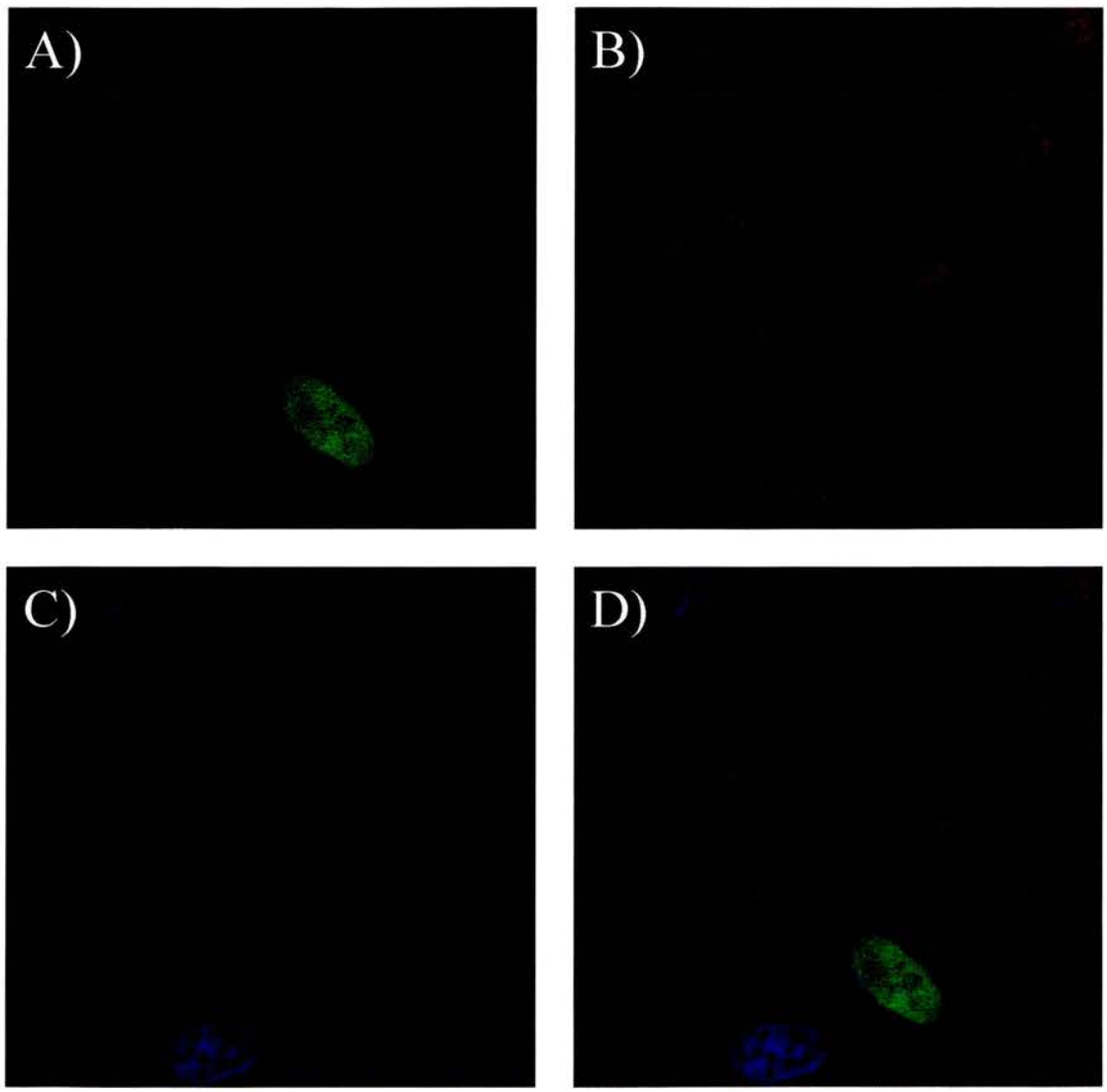


Figure 4.3. Co-localisation of A) C-terminal GFP tagged ORF A2 (green) expressed in BHK-21 cells 48 hours post transfection with B) C23 staining of nucleoli (red). C) Nuclei are counterstained with TO-PRO-3 (blue). D) Overlay image.

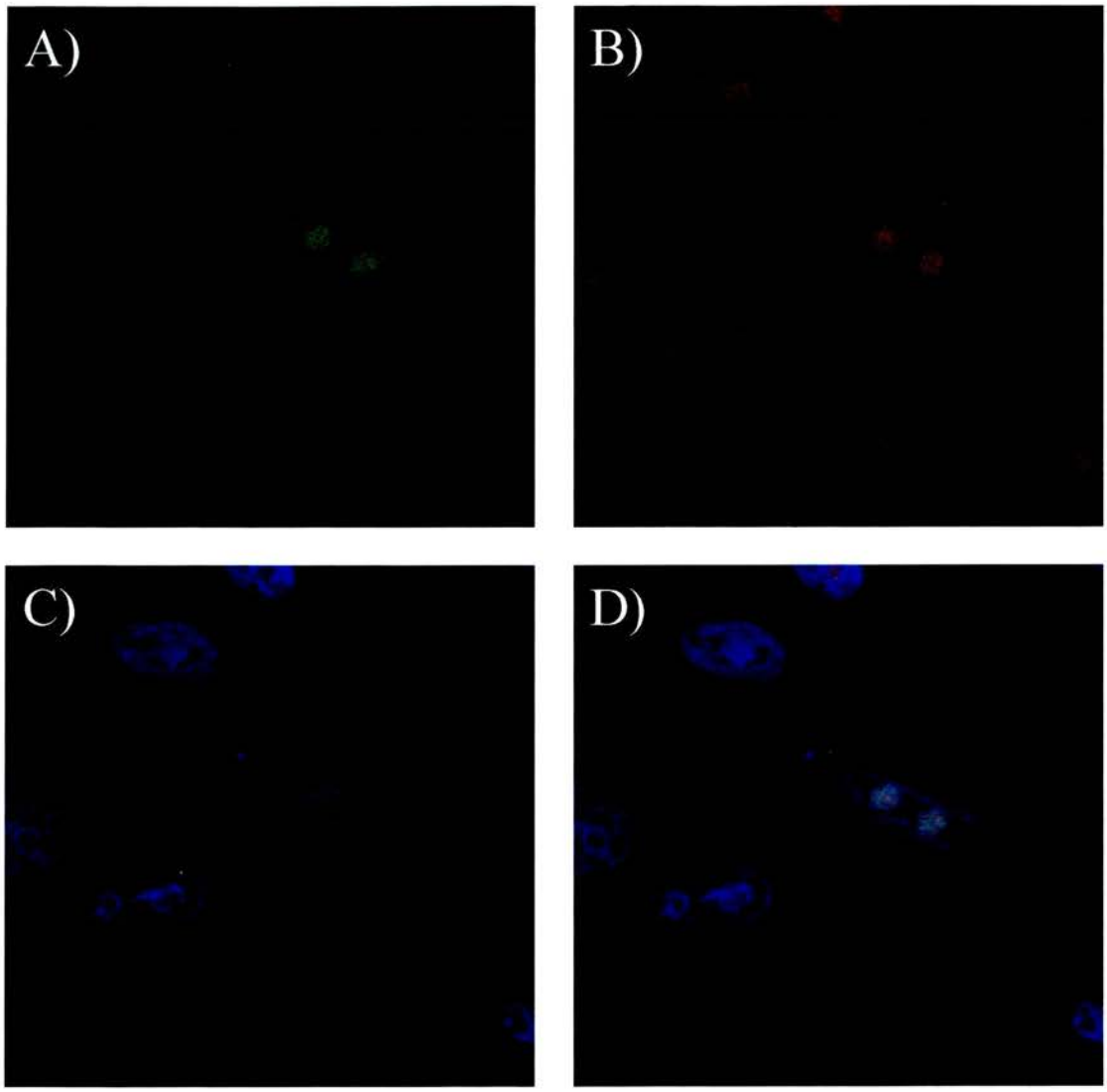


Figure 4.4. Co-localisation of A) N-terminal GFP tagged ORF A2 (green) expressed in BHK-21 cells 48 hours post transfection with B) C23 staining of nucleoli (red). C) Nuclei are counterstained with TO-PRO-3 (blue). D) Overlay image.

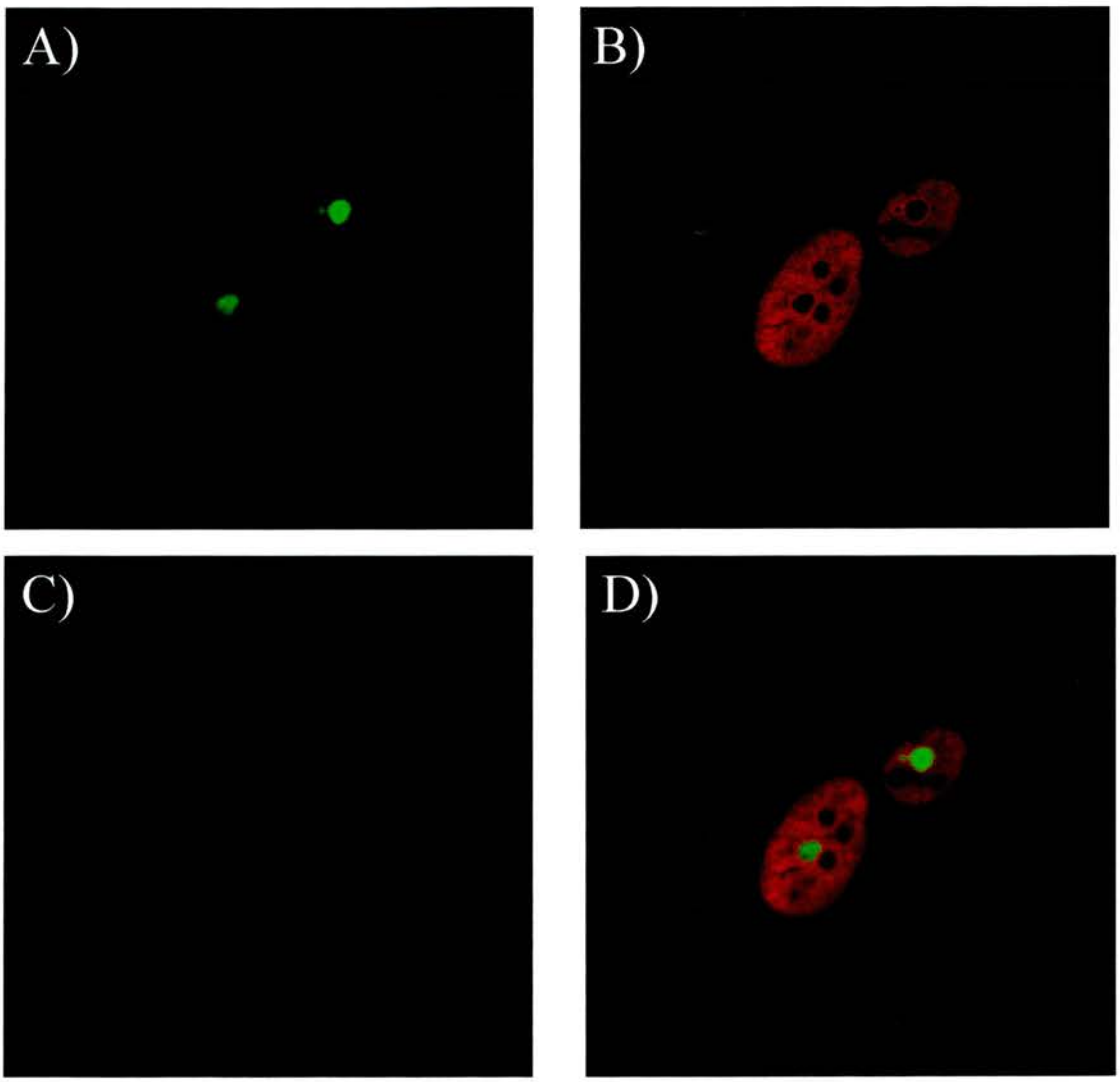


Figure 4.5. Localisation of A) GFP fused to the N-terminus (green) and the B) c-myc tag fused to the C-terminus (red) of ORF A2 protein expressed in BHK-21 cells 48 hours post transfection. C) Nuclei are counterstained with TO-PRO-3 (blue). D) Overlay image.

pattern was similar to that previously shown for C-terminal myc/His and C-terminal GFP tagged ORF A2 products, showing co-localisation with DNA but absence from distinct areas within the nucleus. In contrast, the GFP (N-terminal) signal is present in some but not all of the areas where c-myc staining is absent. Based on the previous findings using the GFP-A2 construct, the area to which the GFP signal localises is probably a nuclear aggregate as there is co-localisation with the TO-PRO-3 stain. Images were further analysed using the Leica Lite software to better represent the distribution pattern. Signal intensities along an arbitrary line drawn across a representative cell (Figure 4.7 A) were plotted in the histograms shown in Figures 4.7 C (GFP) and E (c-myc staining). These plots conclusively demonstrate that the signals are in different areas. This analysis also shows that there is an increase in c-myc signal immediately adjacent to GFP, although the significance of this finding is unclear. These results support the hypothesis that the ORF A2 protein is cleaved and the two termini localise independently.

Another population of cells with a markedly different pattern of c-myc and GFP localisation was also seen in the transfected culture. Examples of these types of cells are shown in Figure 4.6. In these cells, the signal intensities for both c-myc staining and GFP are lower and both localise to the same nuclear compartment. However, the signals do not completely overlap as there would be yellow signal throughout this compartment rather than distinct red and green in the overlaid image (Figure 4.6 D). Similar to the findings previously seen with C-terminal myc/His and C-terminal GFP tagged ORF A2 products, the signals are absent from distinct areas in the nucleus. Again, images were analysed using the Leica Lite software. Signal intensity histograms along the line of the representative cell in Figure 4.6 B are shown in Figures 4.7 D (GFP) and F (c-myc staining). Both histograms have a similar overall pattern but, as expected, do not completely overlap, again indicating that the protein is cleaved. As at least two major patterns of distribution are seen with the same construct, some factor must be affecting the localisation of the protein (or its cleavage products) other than either the ORF A2 sequence or the addition of the traceable markers.

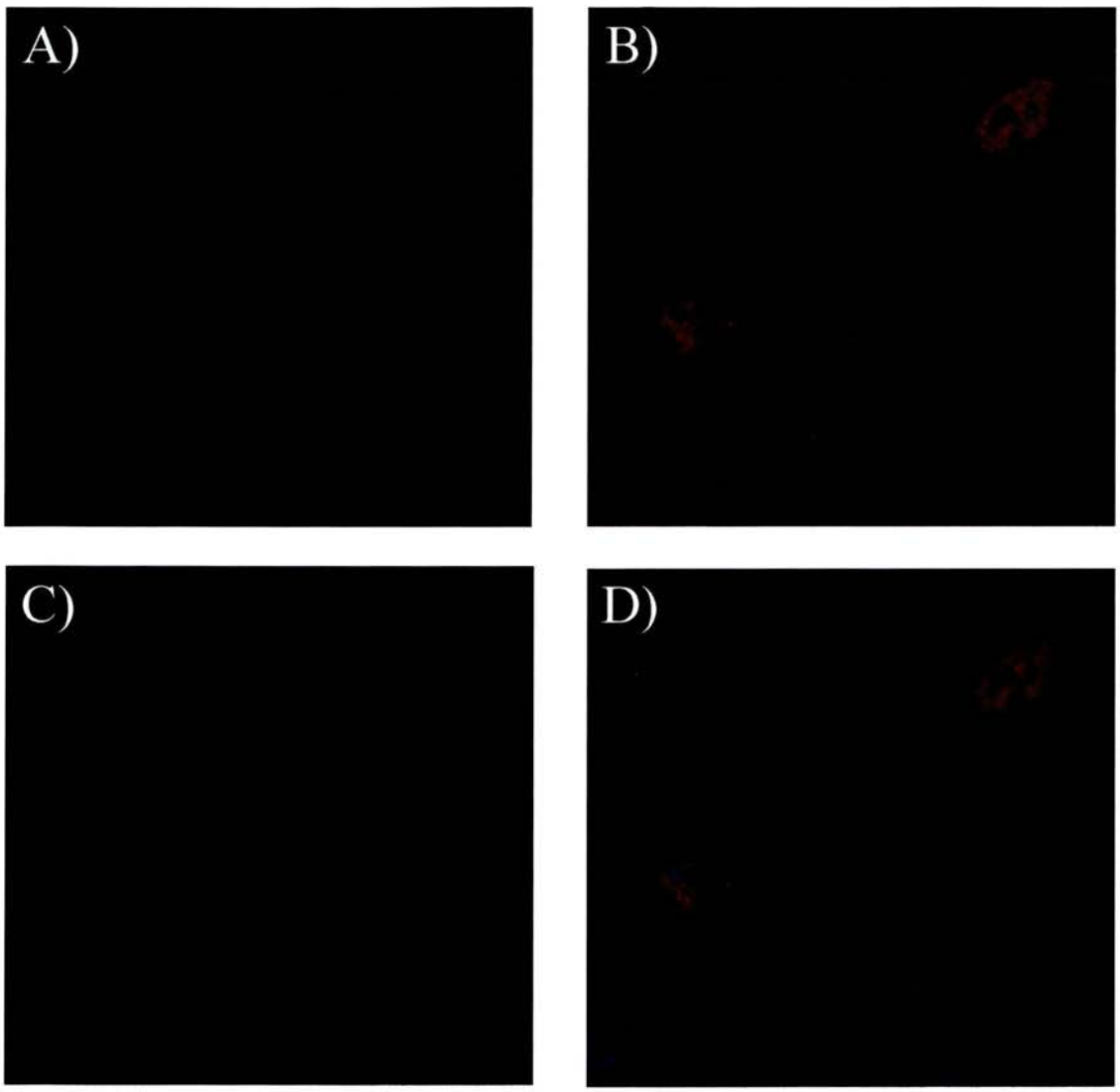


Figure 4.6. Localisation of A) GFP fused to the N-terminus (green) and the B) c-myc tag fused to the C-terminus (red) of ORF A2 protein expressed in BHK-21 cells 48 hours post transfection. C) Nuclei are counterstained with TO-PRO-3 (blue). D) Overlay image.

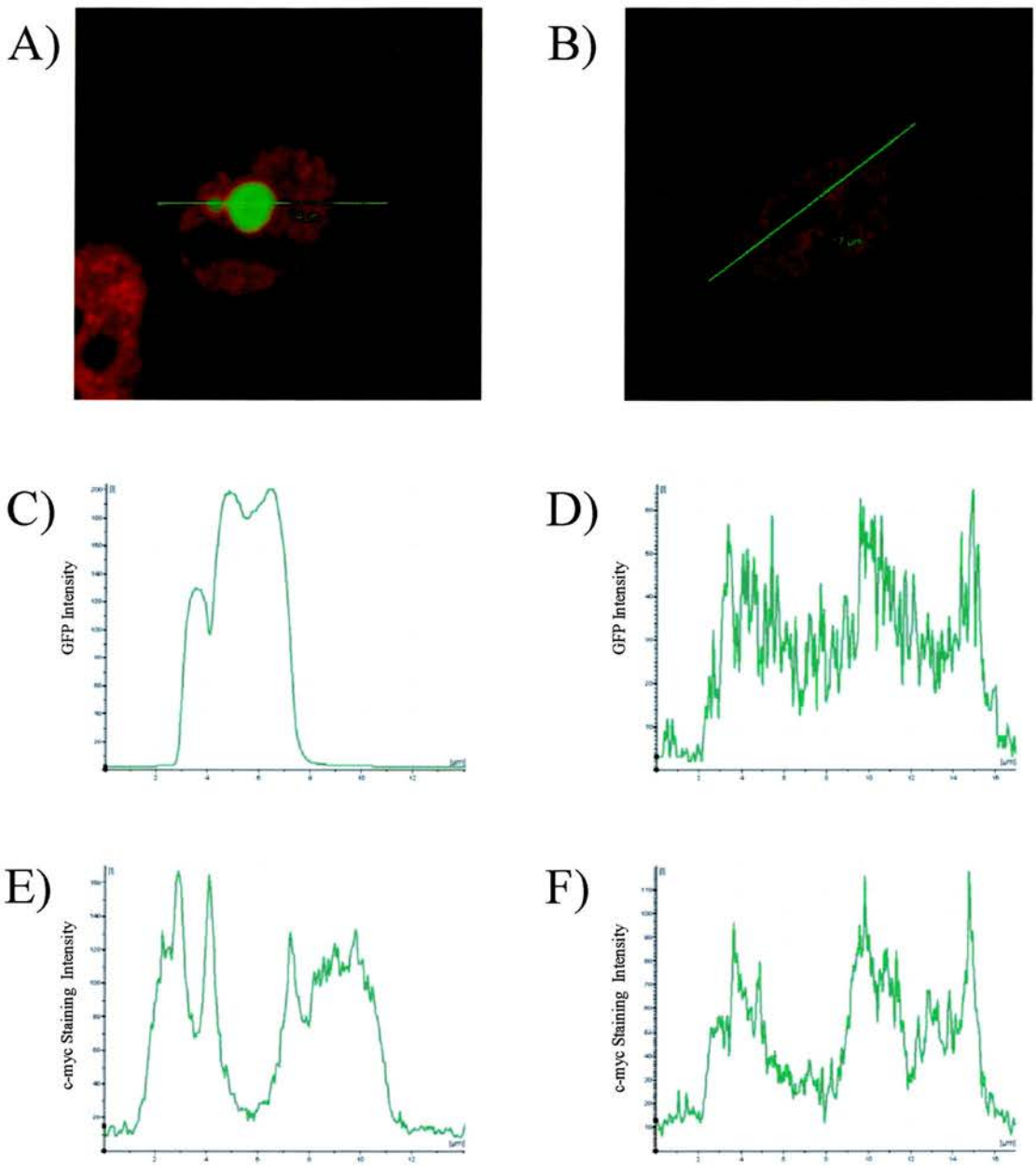


Figure 4.7. A and B) Representative BHK-21 cells expressing GFP fused to the N-terminus (green) and the c-myc tag fused to the C-terminus (red) of ORF A2 protein 48 hours post transfection. C-F) Histograms representing the signal intensity of GFP (C and D) and c-myc staining (E and F) across the lines shown in the example cells in A (C and E) and B (D and F).

4.1.5 Transcriptional Activity of ORF A2 Protein

The nuclear localisation of the ORF A2 gene product in transfected cells is consistent with its predicted role as a transcription factor (Ensser *et al.*, 1997). To further investigate this prediction, studies were undertaken using a promoter-reporter assay to look for transcriptional activity of this protein. BHK-21 cells were co-transfected with the ORF A2myc/His construct (see Section 4.1.2) or empty pcDNA3.1/myc-His(-)A along with one of a number of previously constructed promoter-reporter constructs. The promoter-reporter constructs were based on pGL3-Basic (Promega). This plasmid contains a multiple cloning site, into which a promoter region of interest can be inserted, upstream of the firefly luciferase reporter gene. Plasmids were previously constructed by Dr. Thonur Leenadevi and Dr. Fiona Frame containing AlHV-1 promoter sites. Constructs containing promoter regions of ORF 6 (major single stranded DNA binding protein) as a representative late gene, ORF 21 (thymidine kinase) as an early-late gene and ORF A9 (Bcl-2 homologue) as an early gene were used. Constructs with ORF 50 (Rta homologue) and ORF 57 (Mta homologue) were used as representative immediate early genes and because of their importance in the control of viral gene transcription. Firefly luciferase expression driven by the cytomegalovirus immediate-early promoter was used as a positive control for Firefly activity. A plasmid containing *Renilla* luciferase driven by the herpes simplex virus thymidine kinase promoter for low to moderate constitutive expression was used to control for transfection efficiency.

Transfections were performed in triplicate and 48 hours post transfection, luciferase activity was measured as described in Section 2.8.1. Raw luciferase readings are shown in Appendix 2 and the mean results of the triplicate assays are shown in Figure 4.8, represented as the ratio of Firefly to *Renilla* luciferase measurements. The presence of the ORF A2 vector (grey bars) in transfected cultures neither increased nor decreased the levels of transcription from any of the promoter constructs tested, compared to cultures co-transfected with empty pcDNA3.1/myc-His(-)A (black bars). Although these results show no transcriptional activity of the ORF A2, the promoters tested represent only a small fraction of the potential targets for a transcription factor.

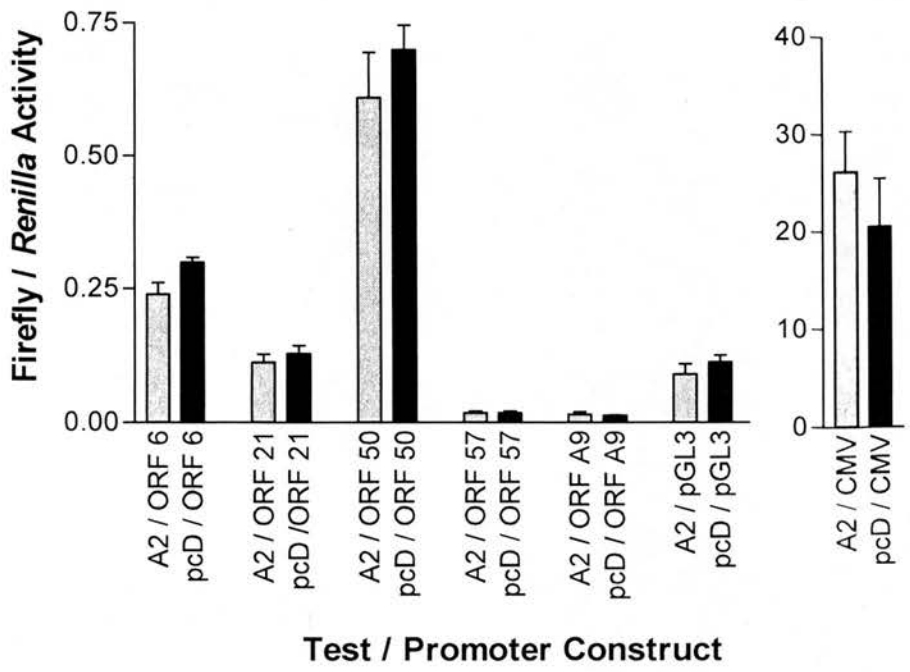


Figure 4.8. Ratios of firefly to *Renilla* luciferase activity in BHK-21 cells transfected with promoter construct and either A2 expressing plasmid (grey bars) or control vector (black bars). Error bars indicate standard deviation of triplicate readings.

4.1.6 Expression of ORF A3 and A4 GFP Fusion Proteins

The products of ORFs A3 and A4 contain sequences consistent with known signal peptides and are therefore predicted to be secreted (Ensser *et al.*, 1997). Previous experiments using c-myc/6xHIS tagged ORF A4 failed to show any evidence that this gene product was secreted, however, this may have been due to the low transfection efficiency or the insensitivity of the detection system used. To facilitate detection, ORF A3 (3491-5451) and ORF A4 (5732-6094) were amplified by PCR using the reactions described in Sections A1.3.26 and A1.3.33 for cloning into pEGFP-N1 and pEGFP-C1. These vectors are used for the production of the inserted genes as C- or N-terminal GFP fusion proteins, respectively. Vectors for N-terminally tagged ORF A3 (GFP-A3) and for N- and C-terminally tagged ORF A4 (GFP-A4 and A4-GFP) were successfully created and diagrams of these constructs are shown in Figure 4.1 H, I and J. Several attempts to clone a C-terminally tagged ORF A3 were unsuccessful.

Vectors were transfected into BHK-21 cells as previously described. Eight hours post-transfection, culture media was replaced with Optimem (Invitrogen) low-serum media to reduce background protein staining in Western blots. Cell and supernatant samples were collected 48 hours post-transfection for Western blot analysis and cell samples were transferred to slides for nuclear counterstaining and microscopic analysis. In cells transfected with vectors for GFP-A3 or GFP-A4, green fluorescent signal was seen throughout the cell in a pattern indistinguishable from empty pEGFP-C1 (Figure 4.9). An anti-GFP Western blot of cell and concentrated supernatant samples is shown in Figure 4.10. No obvious products are seen in either cells or supernatants from GFP-A4 or A4-GFP. The product seen in the cells and supernatant of GFP-A3 transfected cultures is the same size as that of GFP alone indicating that the fusion protein is incompletely produced or cleaved. That product is seen in the supernatant of pEGFP-C1 transfected cultures as well as the cells, despite there being no reason for secretion of GFP, suggests that there is contamination of the supernatant samples, most likely from dead or dying cells. This is also the likely cause of product presence in GFP-A3 supernatants.

4.1.7 Summary

All four LHE ORFs of AlHV-1 were cloned for their expression as fusion proteins in mammalian cells. The results of ORF A1, ORF A3 and ORF A4 were inconclusive.

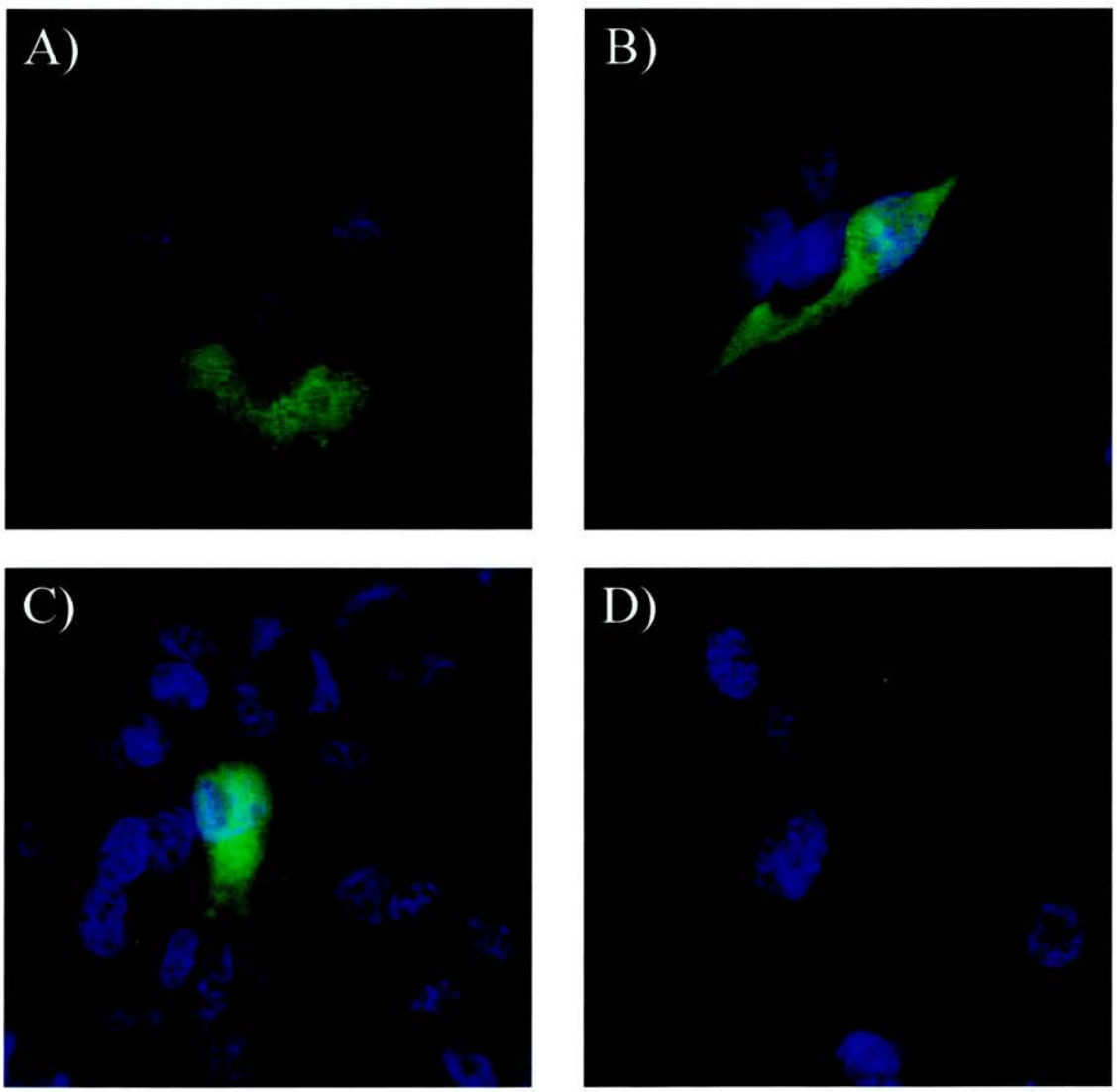


Figure 4.9. Localisation of GFP (A), GFP-A3 (B), GFP-A4 (C) and A4-GFP (D) in BHK-21 cells 48 hours post transfection. Cells are counterstained with TO-PRO-3 iodide.

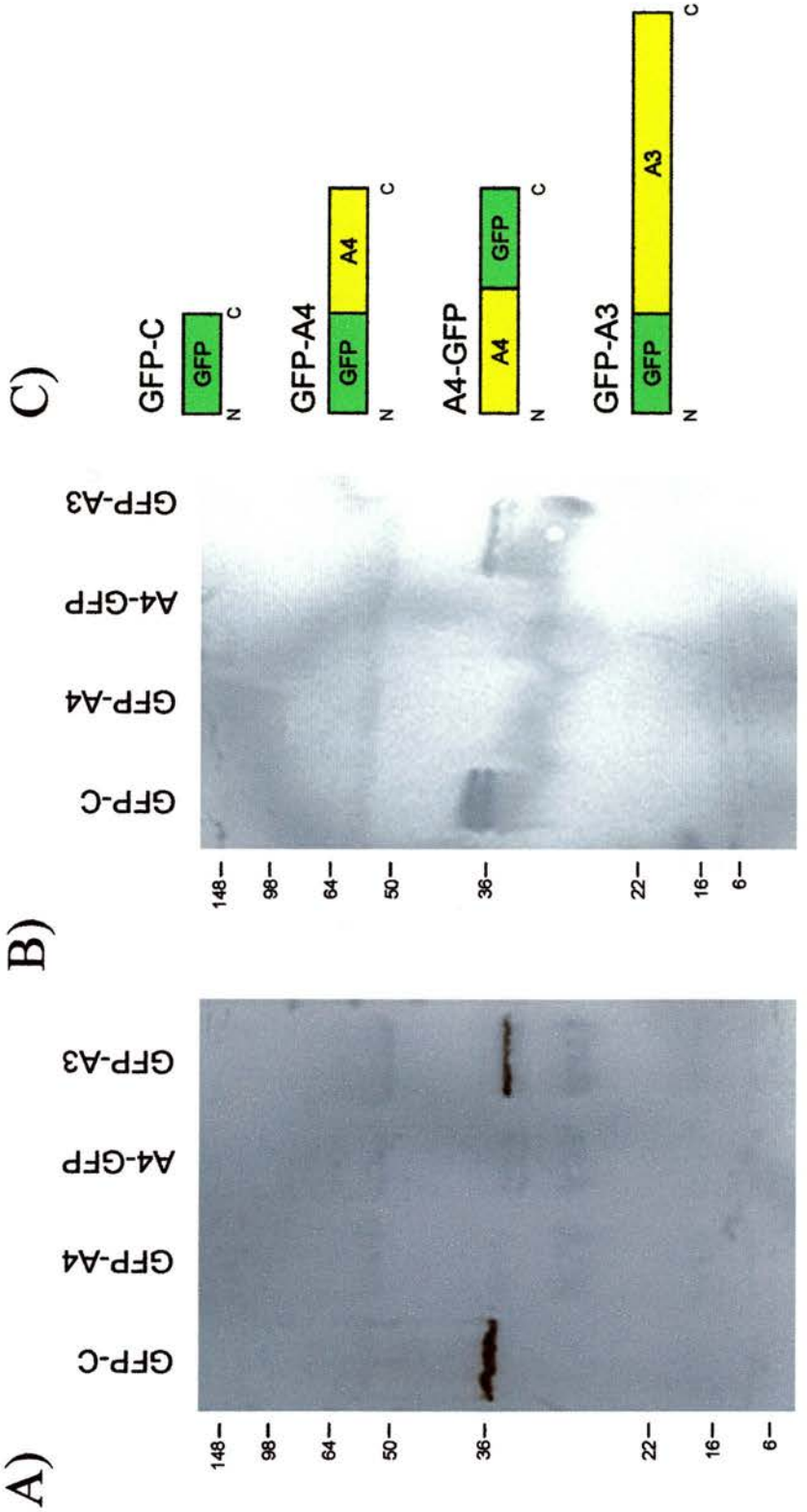


Figure 4.10. Anti-GFP Western blot of (A) cell and (B) supernatant samples from BHK-21 cell cultures transfected with the indicated construct. Positions of molecular weight markers (in kD) shown on the left. Diagrams of predicted fusion proteins are shown (C) indicatinga relative position of GFP tag.

No c-mys/6xHIS tagged ORF A1 was found in transfected cells or in the supernatants of transfected cultures. No evidence to support the predicted secretion of either ORF A3 or ORF A4 could be found. Green signal was seen in cells transfected with N-terminally GFP tagged ORF A3 and ORF A4 although this is most likely due to interference of GFP or incomplete expression of fusion protein. Neither C-terminally c-myc/6xHIS nor C-terminally GFP tagged ORF A4 fusion proteins were detected in culture supernatants or in transfected cells. It is possible that these fusion proteins were present in supernatants but the sensitivity of detection assays was simply too low.

As predicted, the gene product of ORF A2 was found to localise to the nucleus. The precise localisation appeared to vary based upon the location of the tag within the fusion protein. Based on data obtained using a dual-tagged fusion protein, the variation in localisation of protein was most likely due to cleavage, although this cannot be confirmed without Western blot analysis. The transcriptional activity of ORF A2 fusion proteins was tested by co-transfection of BHK-21 cells with A2myc/His vector along with various luciferase promoter-reporter constructs. The presence of ORF A2 gene product neither increased nor decreased the basal transcription levels from any of the constructs tested.

4.2 Bacterial Expression of LHE ORFs

4.2.1 Aims

To follow on from previous data supporting the role of ORF A2 product as a transcription factor, it was hoped that sufficient quantities of protein could be produced for purification and biochemical analysis. However, levels of protein produced by transfection of mammalian cells were insufficient even for detection by Western blot and certainly not enough for purification. Instead, constructs were designed for the production of protein in bacteria using the pTrcHis2 A vector system (Invitrogen). This system is designed for high level inducible expression of recombinant protein with C-terminal c-myc/6xHIS epitope tags.

4.2.2 Expression of Single-Tagged ORF A2 Constructs

ORF A2 was cloned for expression using the pTrcHis2 A vector system. No gene splicing occurs in bacteria and it was therefore necessary to clone the coding region of ORF A2 without the intron (2312-2231). This was accomplished by PCR amplification of the selected ORF A2 sequence (2474-1799, Section A1.3.15) using cDNA produced from BHK-21 cells infected with the recombinant MHV-ALHE virus rather than AlHV-1 viral DNA as the template. The resulting PCR product was cloned first into pGEM-T Easy and then into the pTrcHis2 A vector using methods previously described. A diagram of this construct is shown in Figure 4.11 A.

Cultures of TOP10F' cells containing the pTrcHis2 A/ORF A2 cDNA construct (pTH-A2c) or empty pTrcHis2 A vector as a control were grown to the appropriate optical density and recombinant protein production was induced by the addition of IPTG as described in Section 2.2.7. Bacterial samples were collected prior to the addition of IPTG and at 1, 2, 3, 4 and 5 hours post induction. Samples were analysed by Western blot using antibodies specific for the c-myc epitope (9E10) or the 6xHIS epitope as described in Section 2.5. Neither blot showed any specific staining at the predicted molecular weight of around 27 kD in any of the induced samples (data not shown).

The lack of product may due to the low sensitivity of the detection system or a problem with protein synthesis. To determine whether the lack of product seen was

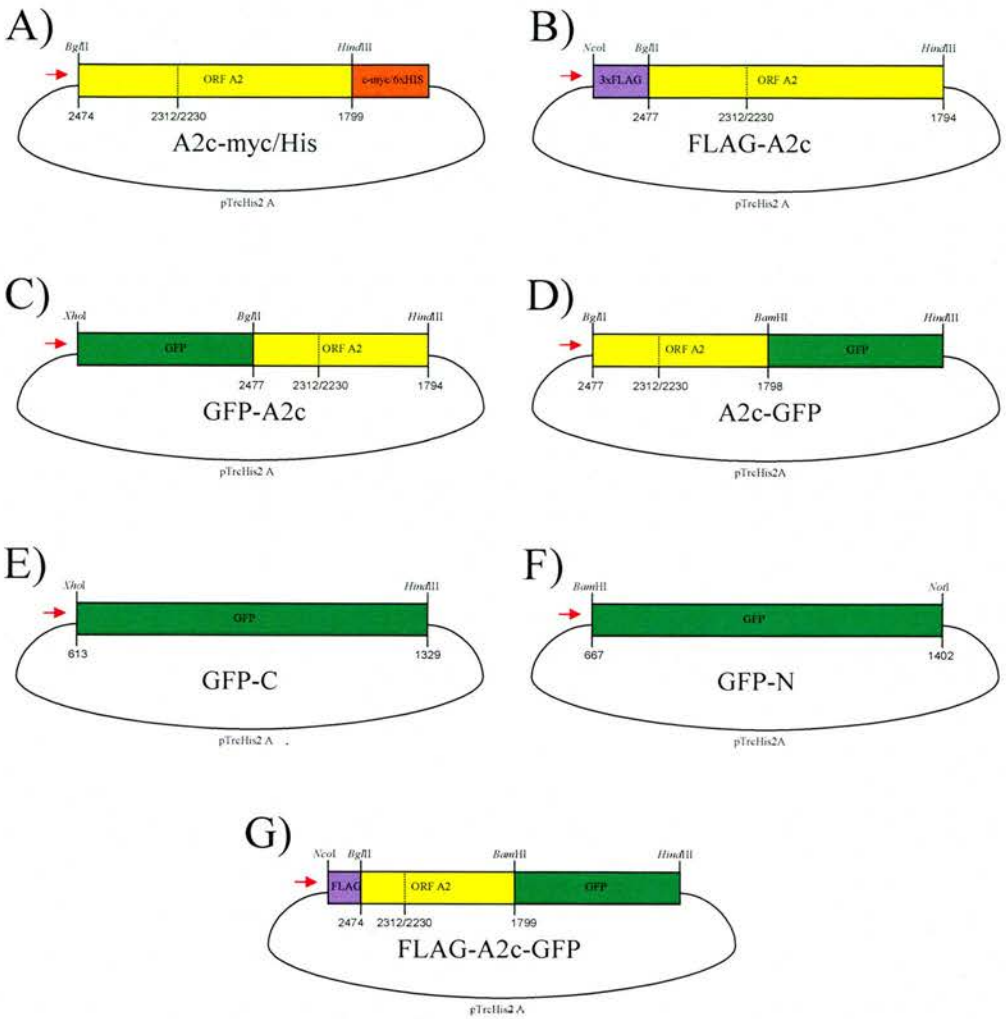


Figure 4.11. Diagrammatic representations of constructs used for the expression of tagged ORF A2 gene products in *E. coli*. Not to scale. Red arrows indicate relative position and orientation of inducible *trc* promoters.

due to a problem with the detection of the c-myc/6xHIS epitope tag rather than a problem with protein synthesis, ORF A2 constructs were made containing either 3xFLAG (DYKDHDGDYKDHDIDYKDDDDK) or GFP tags.

The 3xFLAG epitope was added to an existing pTHA2c construct (containing an ORF A2 cDNA construct cloned using the PCR protocol described in Section A1.3.14) by the insertion of synthesised oligonucleotides that were annealed and ligated into the vector as described in Section 2.1.21. A diagram of the resultant vector (pTH-FLAG-A2c) is shown in Figure 4.11 B. Cultures of TOP10F' *E. coli* transformed with this construct or empty pTrcHis2 A vector as a control were grown and protein production was induced as before. Bacterial samples were collected prior to the addition of IPTG and at 30 minutes and 3 hours post induction. Figure 4.12 A shows the Western blot of these samples stained with the Anti-FLAG M2 antibody. A product of the predicted size of full-length protein (approximately 30 kD, indicated by the blue arrow) is present in the sample taken after 30 minutes of induction. The staining intensity of this product is reduced in the 3 hour sample indicating that degradation of the protein is occurring. Supporting this hypothesis, two smaller products of approximately 18 and 21 kD (indicated by red arrows) were also seen in the 30 minute sample and were far more intense after 3 hours. Any cleavage seen is likely to have occurred during culture as a protease inhibitor cocktail was added to the samples at the time of collection.

The A2 cDNA was also cloned into the bacterial expression vector with both N- and C-terminal GFP tags. A2 sequences (2477-1794 and 2477-1798 for N- and C-terminal fusions, respectively) were amplified using cDNA from MHV-ALHE infected BHK-21 cells as template using the protocol described in Sections A1.3.13 and A1.3.14 and cloned into pGEM-T Easy before ligation into pEGFP-C1 or –N1. A2c-GFP and GFP-A2c products were amplified from these vectors by PCR using the protocols described in Sections A1.3.16 and A1.3.17 incorporating appropriate restriction endonuclease sites for cloning into pTrcHis2 A and cloned as previously described. As controls, GFP alone was inserted into pTrcHis2 A either by cloning directly from pEGFP-N1 (pTH-GFP-N) or by PCR amplification (Section A1.3.41) using pEGFP-C1 as a template and subsequent pGEM-T Easy cloning (pTH-GFP-C). Diagrams of the pTH-GFP-A2c, pTH-A2c-GFP, pTH-GFP-C and pTH-GFP-N

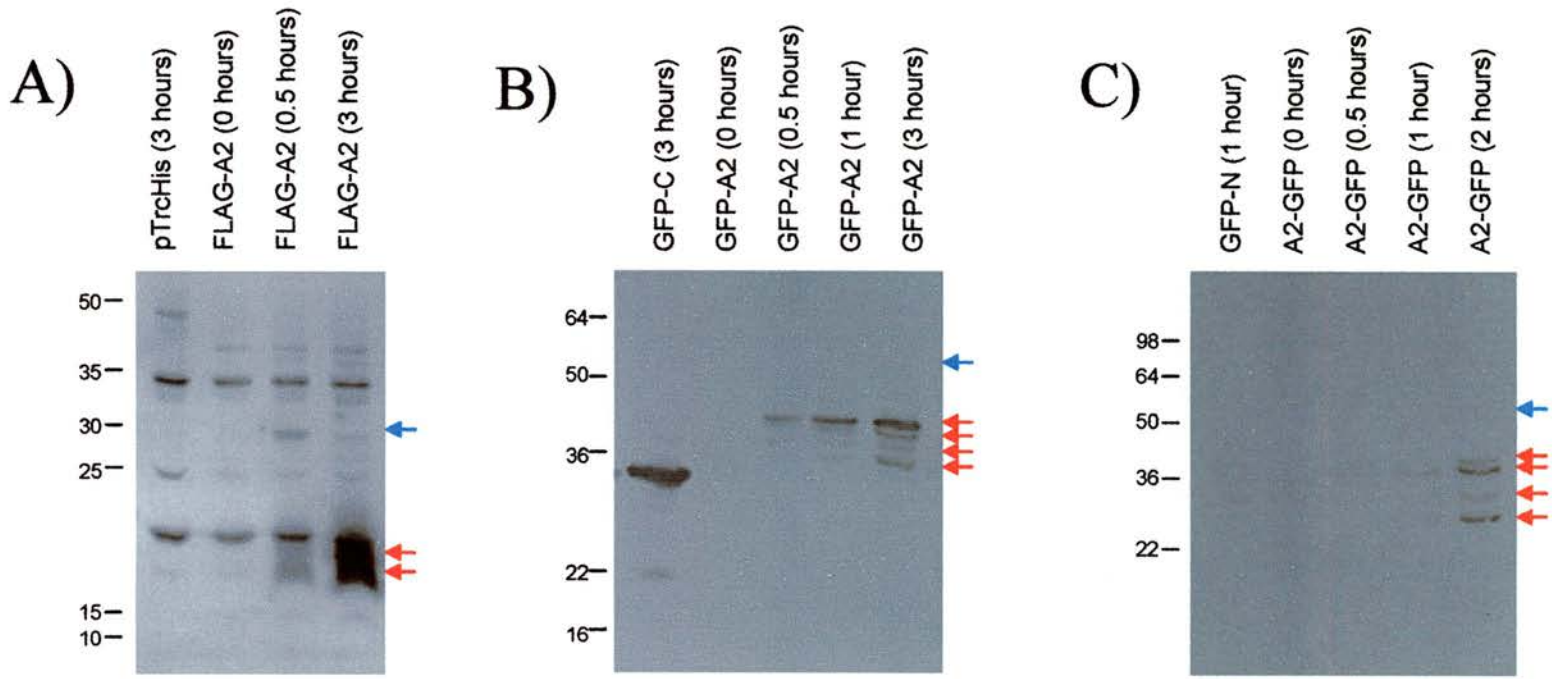


Figure 4.12. Anti-FLAG (A) and anti-GFP (B and C) Western blots of crude lysates from bacteria transformed with the indicated plasmid and induced with IPTG for the indicated amount of time. Positions of molecular weight markers (in kD) shown to the left of each blot. Blue arrows indicate approximate predicted molecular weight for full length recombinant protein. Red arrows indicate probable cleavage products.

vectors are shown in Figure 4.11 C, D, E and F. Bacterial cultures transformed with these constructs were grown and protein production was induced as before.

A Western blot of cultures transformed with either pTH-GFP-A2c or pTH-GFP-C and stained using the anti-GFP (JL-8) antibody is shown in Figure 4.12 B. No obvious product of the predicted molecular weight for full-length GFP-A2c protein (approximately 50 kD) can be seen in any of the three samples taken at 30 minutes, 1 hour and 3 hours following the induction of protein production with IPTG. A number of smaller products (at least four, indicated with red arrows) can be seen in these samples. The smallest of these products is not much larger than GFP alone suggesting that very little ORF A2 gene product remains in the construct. Similar results were seen using the C-terminally tagged ORF A2 construct. Figure 4.12 C shows an anti-GFP stained Western blot of pTH-A2c-GFP and pTH-GFP-N transformed cultures. Again, no obvious product of the predicted molecular weight for full-length A2c-GFP protein was seen in induced samples although several smaller products (indicated with red arrows) were. These results support the hypothesis that the ORF A2 protein produced in this system is rapidly degraded.

4.2.3 Expression of Dual-Tagged ORF A2 Constructs

To better assess the nature of the degradation of the A2 protein, another vector was constructed to produce A2 protein with both N- and C-terminal epitope tags. This would allow for the simultaneous analysis of both termini from the same transformed culture to determine whether complimentary fragments representing full-length protein could be seen. The new vector (pTH-FA2G) was designed to have both N-terminal 3xFLAG and C-terminal GFP tags and was created by the insertion of A2c-GFP sequence from pTH-A2c-GFP into pTH-FLAG. A diagram of this vector is shown in Figure 4.11 G. Bacterial cultures transformed with this construct were grown and protein production was induced as before.

Figure 4.13 A shows the anti-FLAG Western blot stained of bacterial cultures transformed with pTH-FA2G, pTH-FLAG-A2c or empty pTrcHis2 A vector induced with IPTG for one hour. Full-length products of the predicted sizes for both dual- and FLAG-tagged A2 protein (approximately 56 and 31 kD respectively, indicated with red arrows) are seen in the appropriate samples. In addition, smaller products of equal sizes (blue and purple arrows) are seen in both samples indicating that A2 portion of

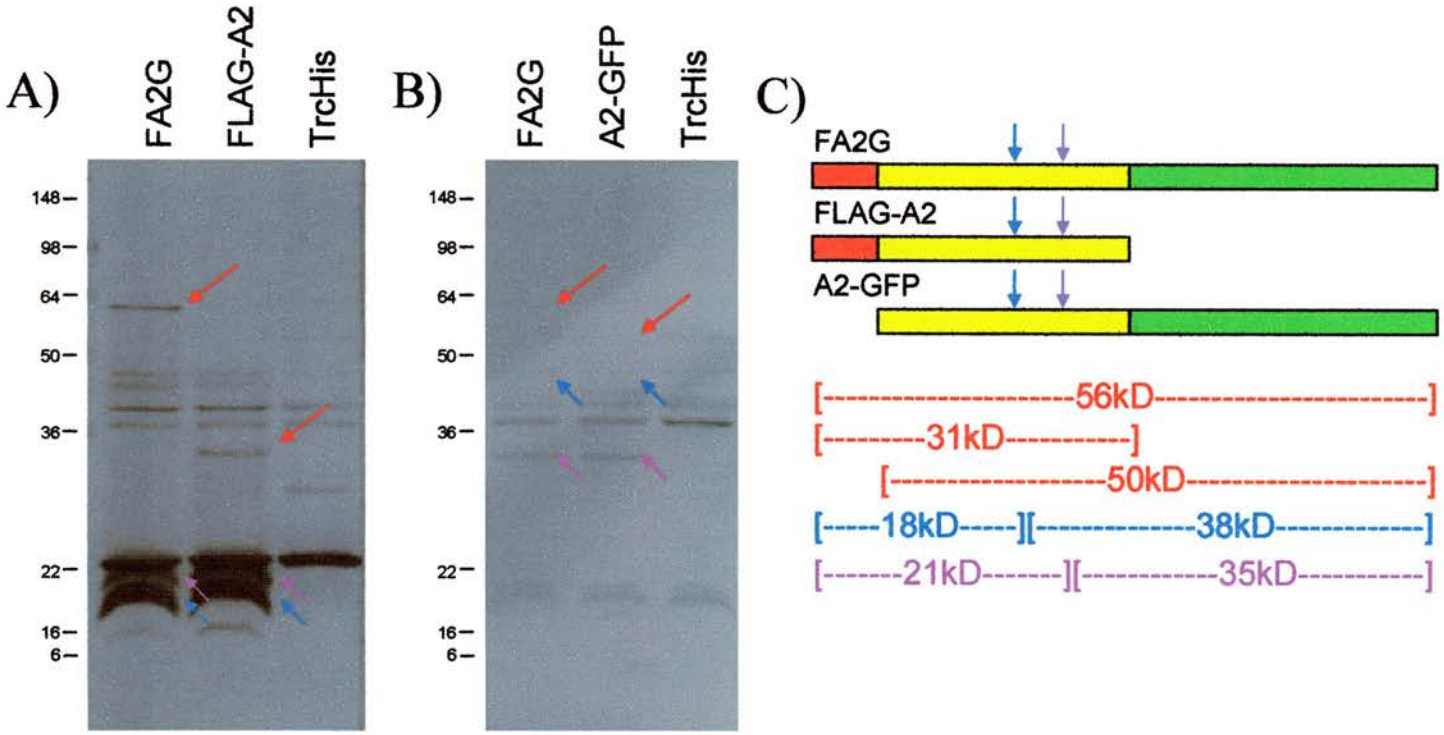


Figure 4.13. Anti-FLAG (A) and anti-GFP (B) Western blots of crude lysates from bacteria transformed with the indicated plasmid and induced with IPTG for one hour. Positions of molecular weight markers (in kD) shown to the left of each blot. Red arrows indicate approximate predicted molecular weight for full length recombinant protein. C) Schematic representations of recombinant proteins indicating possible cleavage sites (blue and purple arrows). Below are show predicted molecular weights for full length proteins and possible cleavage products colour matched to arrows on blots.

the fusion proteins is being cleaved at the same place. Figure 4.13 B shows the anti-GFP Western blot of a bacterial culture transformed with pTH-A2c-GFP induced with IPTG for one hour along with the same pTH-FA2G and pTrcHis2 A transformed samples analysed in Figure 4.13 A. No obvious products of the predicted molecular weight for full-length dual- and GFP-tagged proteins (approximately 56 and 50 kD respectively, indicated with red arrows) are visible despite the presence of full-length dual-tagged protein in the anti-FLAG blot, implying that the anti-FLAG staining protocol is more sensitive. Smaller products of equal sizes are seen in both the pTH-FA2G and pTH-A2c-GFP transformed samples (blue and purple arrows), again indicating that A2 portion of the fusion proteins is being cleaved at the same place.

A diagrammatic representation of a cleavage model fitting this data is shown in Figure 4.13 C. Cleavage of full-length dual- and FLAG-tagged A2 fusions at the position indicated by the blue arrow would result in a product of approximately 18 kD with an N-terminal 3xFLAG tag (as seen in Figure 4.13 A, blue arrows). A corresponding product of approximately 38 kD with a C-terminal GFP tag would also be produced from cleavage of the dual- or GFP-tagged fusion proteins at this site (Figure 4.13 B, blue arrows). Similarly, cleavage of full-length dual- and FLAG-tagged A2 fusions at the position indicated by the purple arrow would result in a product of approximately 21 kD with an N-terminal 3xFLAG tag (as seen in Figure 4.13 A, purple arrows). A corresponding product of approximately 38 kD with a C-terminal GFP tag would also be produced from cleavage of the dual- or GFP-tagged fusion proteins at this site (Figure 4.13 B, purple arrows). In order to support these findings the ORF A2 protein sequence was analysed using the ExPASy PeptideCutter software (http://us.expasy.org/cgi-bin/peptidecutter) to detect all putative protease cleavage sites. The full results of this analysis are shown in Appendix 3 but no obvious single candidate protease is consistent with the proposed model above.

4.2.4 Immunoprecipitation of ORF A2 Constructs

Attempts were made to purify bacterially expressed ORF A2 protein and its cleavage products for further biochemical analysis. In order to perform the purification by immunoprecipitation, it was first necessary to lyse bacteria and obtain a suspension of protein. The ORF A2 fusion containing the N-terminal 3xFLAG tag was chosen for precipitation for several reasons. Firstly, the 3xFLAG tag is much smaller than GFP

and therefore less likely to interfere with downstream analysis of protein function. Secondly, the anti-FLAG detection system appears to be more sensitive than the anti-GFP and full-length FLAG-A2 fusion protein has been detected. Lastly, the cleavage model supported by the experiments detailed above suggests that cleavage is occurring towards the C-terminus of the ORF A2 product and therefore a larger portion of the protein would remain in the cleavage products precipitated with an N-terminal tag.

Bacteria transformed with pTH-FLAG-A2 plasmid were grown as previously described and harvested at one hour after induction of protein expression with IPTG as this is the time at which the largest proportion of full-length product was detected in previous experiments. Bacterial lysis into two separate fractions was performed using the CelLytic B (Sigma) reagent using the protocol described in Section 2.2.8 based on manufacturer's recommendations. Fraction 1 contains soluble proteins extracted by freeze/thaw of bacterial pellets and lysis with the CelLytic B reagent. The insoluble material from the first lysis step was treated with CelLytic B containing 0.2 mg/ml of lysozyme to digest bacterial cell wall components. Proteins solubilised during this step are represented in the Fraction 2 sample with all remaining insoluble material in the pellet.

An anti-FLAG stained Western blot of whole bacteria and lysate fractions is shown in Figure 4.14. Products consistent in size with full-length FLAG-A2 fusion protein (blue arrow) as well as two cleavage products (red arrows) were found in the pTrcHis-FLAG-A2 (Whole Cell +) but not pTrcHis2 A whole cell sample (Whole Cell -) confirming previous findings. The majority of the smaller (approximately 18 kD) and some of the larger (approximately 21 kD) cleavage products but no full-length protein were seen following disruption of the cells by freeze/thaw and lysis with CelLytic B alone (Fraction 1). Following lysozyme treatment, full-length fusion protein and more of both cleavage products were found in solution (Fraction 2) however, some of the full-length protein remained insoluble (Pellet). Based on these findings, Fraction 2 was chosen for use in immunoprecipitation.

Bacterial cultures transformed with pTrcHis-FLAG-A2 or pTrcHis2 A were grown as above. The pTrcHis-FLAG-A2 sample was lysed as before and Fraction 2 was used for immunoprecipitation as described in Section 2.2.9. An anti-FLAG stained

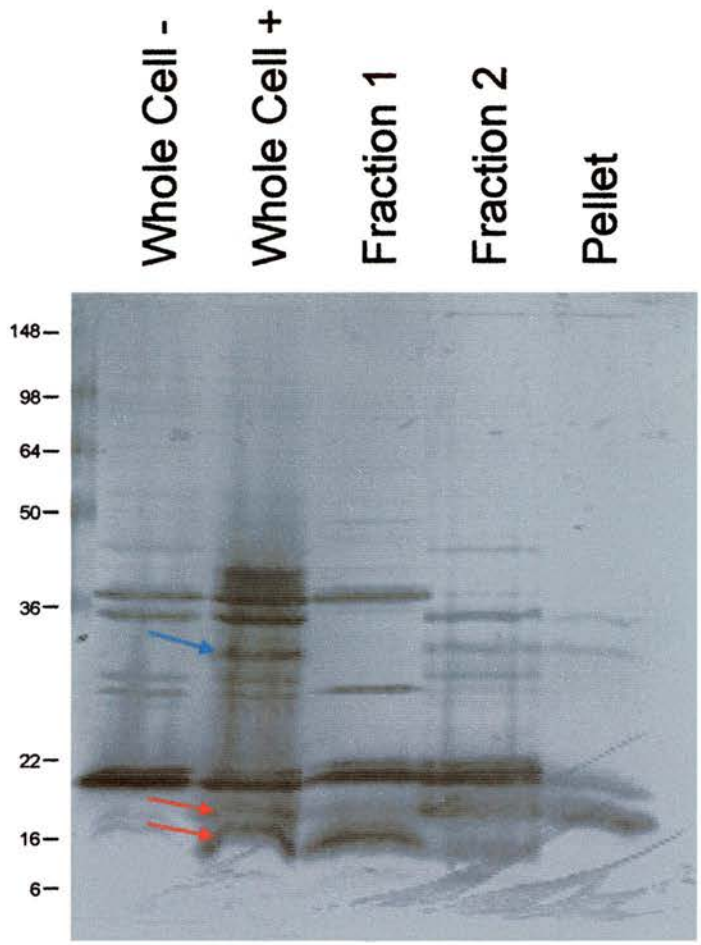


Figure 4.14. Anti-FLAG Western blot of bacterial samples transformed with either pTrcHis2 A (Whole Cell -) or pTrcHis-FLAG-A2 (Whole Cell +) and lysate fractions of pTrcHis-FLAG-A2 transformed cells. All samples were taken from cultures harvested one hour after induction of protein production with IPTG. Blue arrow indicates approximate predicted molecular weight for full length recombinant protein. Red arrows indicate probable cleavage products.

Western blot of whole bacteria, lysate fractions and immunoprecipitate samples is shown in Figure 4.15. The presence of full-length ORF-A2 fusion protein (blue arrow) and the two cleavage products (red arrows) is clearly seen in the whole cell samples of pTrcHis-FLAG-A2 transformed (Whole Cell +) but not pTrcHis2 A transformed (Whole Cell -) bacteria. The lysate samples show a similar distribution of protein as the previous experiment with the majority of the smaller cleavage product in Fraction 1 and presence of full-length fusion protein and the larger cleavage product in Fraction 2. In this experiment, however, a larger portion of the full-length protein remained insoluble in the final Pellet sample. In addition, an unknown product of approximately 14 kD was also seen in Fraction 2 that was not present in the whole cell sample.

Prior to immunoprecipiation, Fraction 2 was pre-incubated with the streptavidin-agarose slurry to remove any proteins that would non-specifically bind. The pre-incubation slurry and supernatant samples shown in Figure 4.15 show that none of the full-length fusion protein or large cleavage product was removed during this step. Following immunoprecipiation, two products of approximately 50 and 25 kD (the heavy and light chains, respectively, of the immunoglobulin used in precipitation) and the larger fusion protein cleavage product are present. No full-length product could be detected in the precipitate although it was still present in the supernatant indicating that it had not been degraded. The supernatant from the immunoprecipitate also contains products consistent with the immunoglobulin heavy and light chains indicating that insufficient streptavidin-agarose was used for the amount of antibody. Even if complete precipitation could be achieved, immunoprecipitated preparations using this protocol would be no more suitable than crude whole cell lysates for further studies as the purity of recombinant protein was not complete as demonstrated by the presence of other products in the sample including the major background protein of approximately 22 kD present in all anti-FLAG stained samples as well as products of approximately 6 and 14 kD.

4.2.5 Summary

In an attempt to produce sufficient quantities of recombinant ORF A2 fusion protein for purification and biochemical analysis, a bacterial expression system was used. Fusion proteins with either GFP or the 3xFLAG tag were produced in sufficient

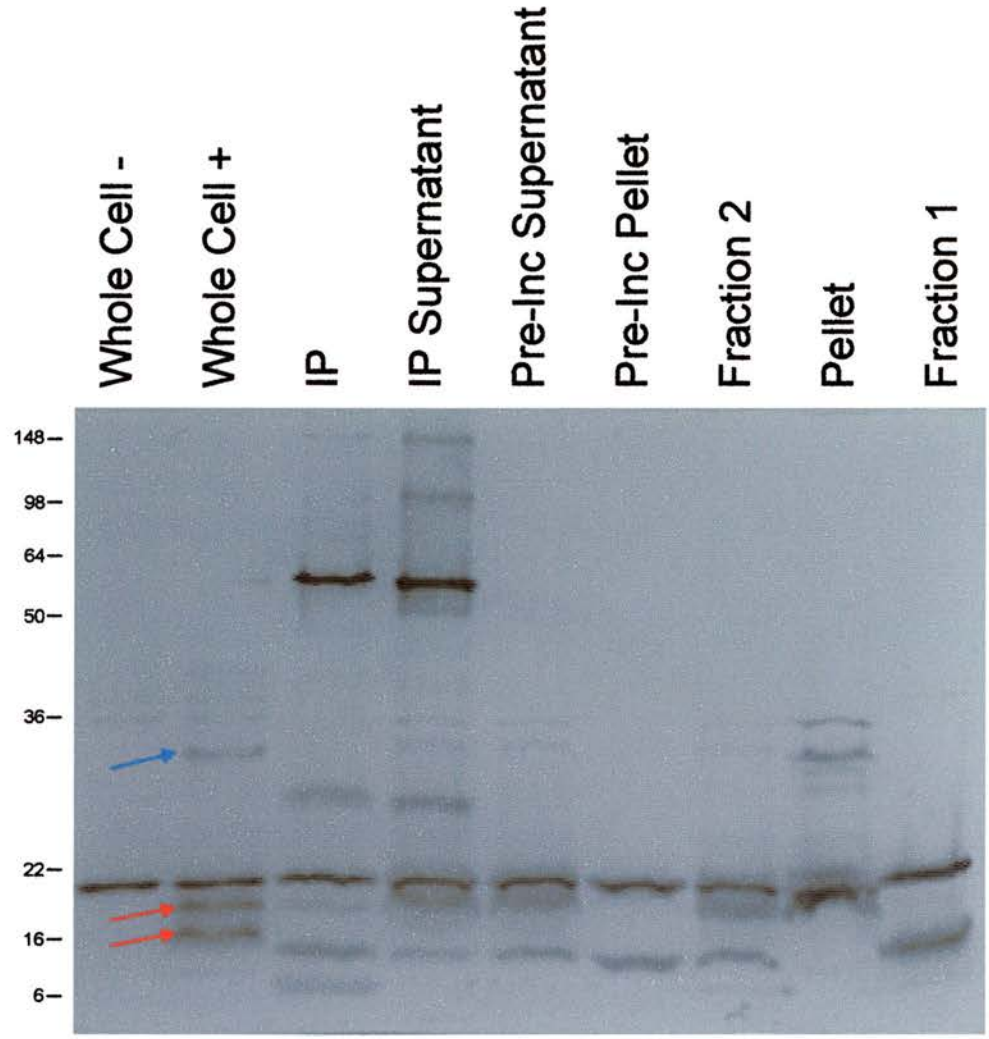


Figure 4.15. Anti-FLAG Western blot of whole cell, lysate and immunoprecipitate samples form pTrcHis-FLAG-A2 transformed cells. Positions of molecular weight markers (in kD) shown to the left of each blot. Blue arrow indicates approximate predicted molecular weight for full length recombinant protein. Red arrows indicate probable cleavage products. Details in text Section 4.2.4.

quantities for detection although the majority of product appeared to be cleaved. To further characterise this finding, experiments were performed using a dual-tagged construct. Results from these experiments were consistent with the hypothesis that cleavage is occurring within the ORF A2 protein, although no clear candidate protease could be determined. Attempts to immunoprecipitate full-length fusion protein or the cleavage products were unsuccessful.

4.3 Effects of Secreted Proteins on Ruminant Lymphocytes

4.3.1 Aims

The marked expansion of lymphocytes in an infected host is one of the hallmarks of MCF. A soluble factor secreted from infected cells is thought to be involved in this process as the majority of studies have found that most of the proliferating lymphocytes are uninfected (Bridgen *et al.*, 1992; Edington *et al.*, 1979; Patel & Edington, 1980). Two of the ORFs studied in this work are predicted to encode secreted proteins and therefore may contribute to lymphoproliferation. This is especially true of ORF A3 as it is predicted to encode for a member of the semaphorin family (Ensser & Fleckenstein, 1995) which includes several members involved in immune regulation. The following sections describe studies addressing the effects of these proteins on cultured lymphocytes.

In order to overcome the problems of low transfection efficiency and the possible prevention of secretion by epitope tags seen using the mammalian expression methods described in Sections 4.1.2 and 4.1.6 for the production of ORF A3 and ORF A4 proteins, lymphocytes were grown in media taken from BHK-21 cells infected with the recombinant MHV-ALHE virus. This strategy should ensure that the genes of interest are delivered to almost every cell and therefore lead to the highest possible level of protein production. The presence of ORF A3 and ORF A4 products in this media could not be confirmed as the proteins are untagged and no specific reagents are available. However, the results in Section 3.2.2 suggest that protein is produced as mRNAs for both ORF A3 and ORF A4 are seen in MHV-ALHE infected cultures. In addition to the proteins of interest, several other factors are expected to be present in the media from infected cells. These factors could either be produced from MHV-76 sequences or of cellular origin, induced or released in response to infection. To account for the effects these additional secreted factors, lymphocytes grown in media from MHV-76 infected BHK-21 cells were used as a control.

Lymphocytes of ovine and bovine origin were used in these studies as both were available and it is thought that they may react differently as these hosts respond differently to infection with members of the MCF virus family.

4.3.2 Establishment of Assays and Controls

Peripheral blood mononuclear cells (PBMC) were isolated from ovine blood as described in Section 2.6.18. Cells were cultured in triplicate in complete DMEM (negative control) or DMEM containing various concentrations of Concanavilin A (ConA) as described in Section 2.6.17. to establish assays for measurement of activation and an approximate time course for study using virally infected media. The number of viable cells in each culture sample was measured by trypan blue exclusion. The results of viable cell counts taken at the time of isolation (time 0) and after 24, 48, 72 or 144 hours in culture are shown in Figure 4.16 A. The number of viable cells per well dropped from time 0 to 72 hours in culture in all samples but most substantially in those samples containing high concentrations of ConA (10, 20 and 40μg/ml). From 72 to 144 hours, proliferation was seen in samples cultured with 1, 5 and 10 μg/ml ConA whereas the cell numbers in samples cultured with 20 and 40 μg/ml continued to decline as did those of negative controls.

In addition to viable cell counts, activation was measured by size and DNA content of cells using FACS analysis. Following trypan blue counts, cells from negative controls and samples containing 1 or 5 μg/ml of ConA were collected, fixed, permeablised and stained with propidium iodide as described in Section 2.6.20. Cells from samples containing 10, 20 and 40 μg/ml of ConA were not studied as viable cell counts indicated that these concentrations may be toxic. Samples were acquired using a FACScan flow cytometer with the forward scatter, side scatter and FL2 fluorescence all set on linear scales. The mean forward scatter (size) and propidium iodide fluorescence of all cells within an arbitrary viable lymphocyte gate were measured for each sample. The mean size of viable cells from cultures containing 1 and 5 μg/ml of ConA showed a steady increase over the course of the experiment whereas the size of the negative control cells showed a slight reduction (Figure 4.16 B). A representative histogram showing the mean forward scatter readings of samples from negative control (red fill) and 1 μg/ml ConA (black line) cultures taken at 72 hours is shown in Figure 4.16 D. The mean DNA content of cells from cultures containing 1 and 5 μg/ml ConA showed a sharp increase between 48 and 72 hours, just before proliferation is seen by trypan blue exclusion, and remains high at 144 hours (Figure 4.16 C). There is also a transient increase in the mean DNA content of negative control samples at 72 hours although no proliferation of these samples was seen by

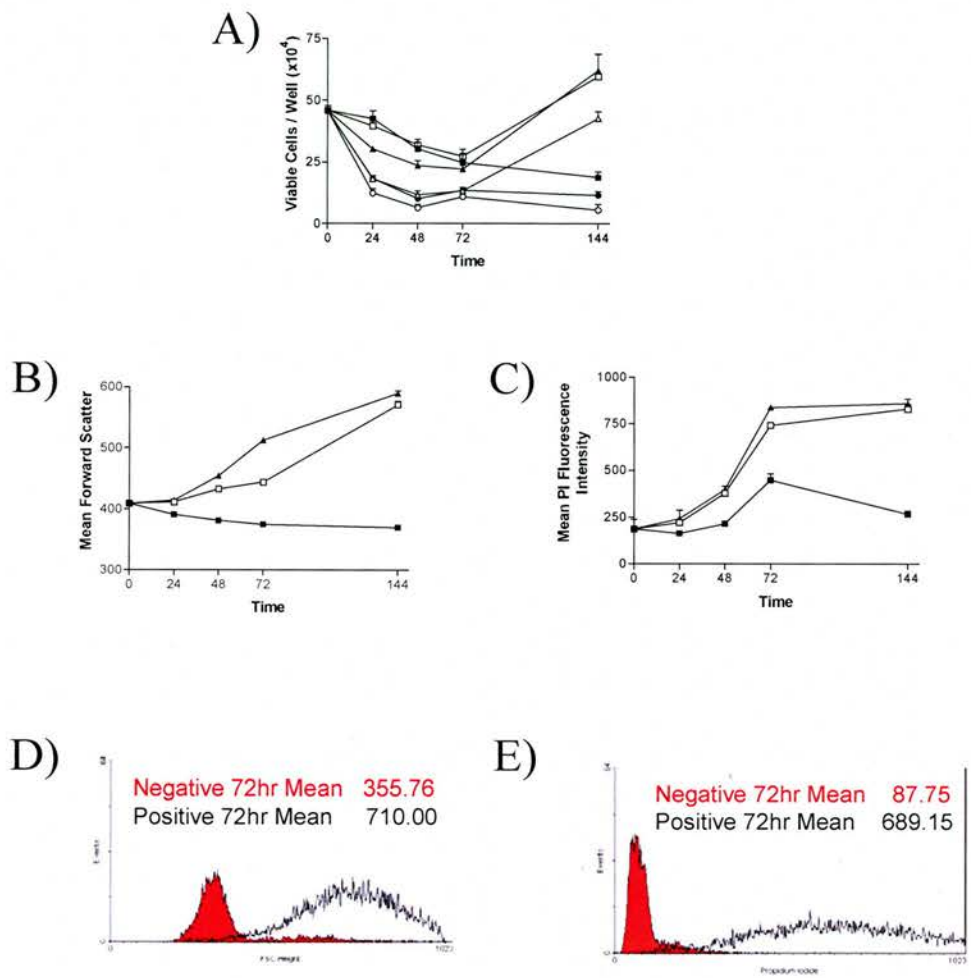


Figure 4.16. A-C) *Ex vivo* activation of ovine peripheral blood mononuclear cells in media containing 0 (■), 1 (□), 5 (▲), 10 (Δ), 20 (●) or 40 (○) µg/ml of Concanavalin A (ConA) represented by viable cell number (A), cell size (B) and DNA content (C). D and E) Representative FACS plots showing the cell size (D) and DNA content (E) of samples cultured with 0 (Negative, red fill) or 1 (Positive, black line) µg/ml of ConA collected at 72 hours.

viable cell counts. A representative histogram showing the propidium iodide fluorescence readings of samples from negative control (red fill) and 1 μg/ml ConA (black line) cultures taken at 72 hours is shown in figure 4.16 E. Although a clear difference between the samples can be seen, no clear peak of tetraploid cells is visible. These results demonstrate that activation of PBMCs can be detected using these assays and occurs over the course of about 6 days.

4.3.3 Ovine Lymphocyte Cultures

Ovine PBMC were isolated as before and cultured in duplicate in complete DMEM (Negative), media containing 1 μg/ml ConA (Positive), media from MHV-ALHE infected BHK-21 cells or media from MHV-76 infected BHK-21 cells. Viable cell numbers, mean cell size and mean cellular DNA content in all samples were measured using previously described methods at the time of isolation and after 72 or 120 hours of incubation.

Viable cell counts (Figure 4.17 A), mean cell size (Figure 4.17 C) and mean cellular DNA content (Figure 4.17 E) in positive (filled circles) and negative (filled squares) controls are consistent with the findings of previous experiments. The samples incubated in MHV-ALHE infected BHK-21 cell media (open circles) and MHV-76 infected BHK-21 cell medium (open squares) behaved the same as negative controls for all parameters measured. This indicates that no factors, or insufficient concentrations of factors, are present in the supernatants of MHV-ALHE or MHV-76 infected BHK-21 cell cultures that are able to activate ovine lymphocytes.

4.3.4 Bovine Lymphocyte Cultures

Bovine PBMC were isolated, cultured and analysed as described above for ovine PBMC. Positive control samples (filled circles) showed a similar increase in viable cell numbers (Figure 4.17 B), mean cell size (Figure 4.17 C) and mean cellular DNA content (Figure 4.17 E) as ovine PBMC although the increases in mean cell size and mean cellular DNA content were more transient and had returned close to baseline levels by 120 hours. No increases in any of the parameters measured were seen in either the samples incubated in MHV-ALHE infected BHK-21 cell media (open circles) or those incubated with MHV-76 infected BHK-21 cell medium (open squares) which both behaved the same as the negative controls (filled squares)

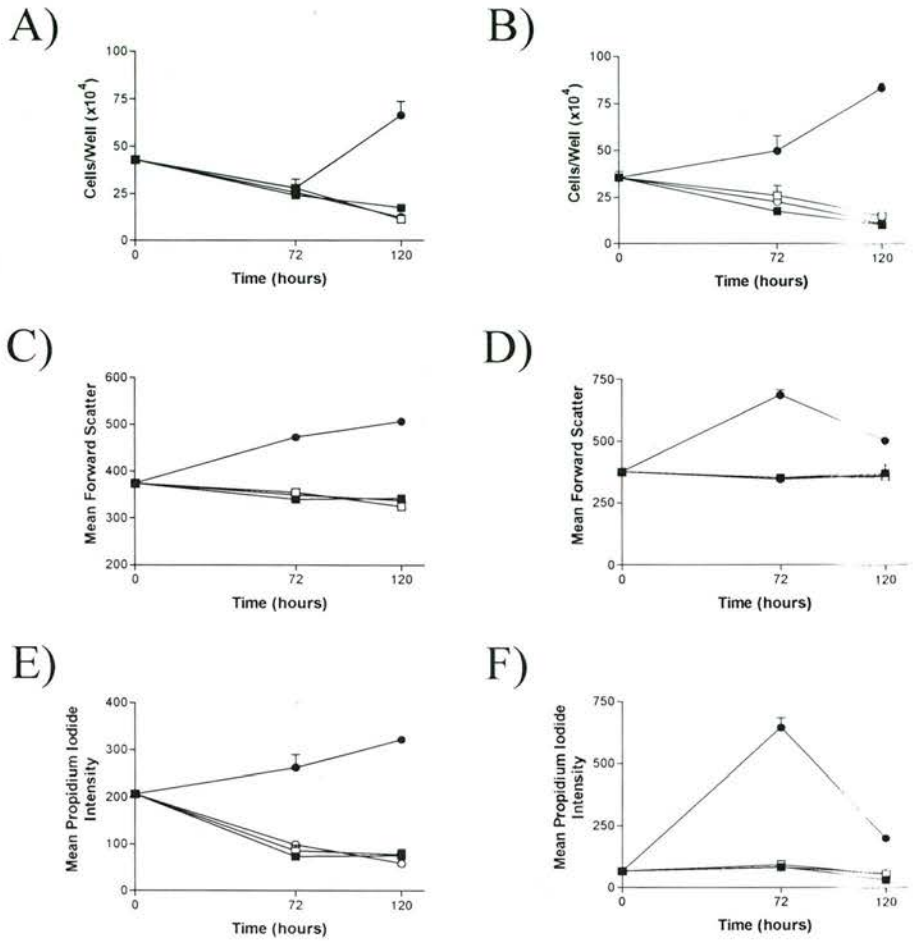


Figure 4.17. *Ex vivo* activation of ovine (A, C and E) and bovine (B, D and F) peripheral blood mononuclear cells cultured in complete DMEM (■, negative control), complete DMEM containing 1 μg/ml ConA (●, positive control), media from MHV-ALHE infected BHK-21 cells (○) or media from MHV-76 infected BHK-21 cells (□). Activation represented by viable cell number (A and B), cell size (C and D) and DNA content (E and F).

throughout the experiment. Like the ovine PBMC experiment, this indicates that no factors, or insufficient concentrations of factors, are present in the supernatants of MHV-ALHE or MHV-76 infected BHK-21 cell cultures that are able to activate bovine lymphocytes.

4.3.5 Summary

To determine whether the left hand end region of AlHV-1 encodes any proteins involved in the activation of lymphocytes, ovine and bovine PBMC were cultured in media from MHV-ALHE infected BHK-21 cell cultures. No activation as measured by proliferation, cell growth and increase in DNA content was seen in either ovine or bovine samples incubated with this media. These results suggest that proteins encoded by the in the left hand end of AlHV-1 do not activate lymphocytes, however without specific reagents, it is not possible to confirm whether these proteins are even present in the culture media.

4.4 Yeast Two-Hybrid Screening

4.4.1 Aims

One of the main objectives of this study was the functional characterisation of the protein products of the four left hand end ORFs of AlHV-1. It was hoped that further experiments could be performed on purified recombinant proteins expressed either in mammalian or bacterial cells. However, as detailed above, problems with low expression and/or degradation of these proteins precluded such studies. Instead, attempts were made to identify proteins interacting with the ORF products using the yeast two-hybrid system. This system allows for the screening of a large number of target proteins without the need for purification and its high sensitivity means interactions should be detected even if the ORFs are poorly expressed.

Experiments were performed using the Matchmaker 3 Two-Hybrid system (Clontech). This system employs four separate reporter genes (*HIS3* and *ADE2* for nutritional selection and *MEL1* and *lacZ* for blue/white galactosidase selection) under the control of three distinct GAL4 upstream activating sequences (UASs) and TATA boxes (GAL1 UAS/GAL1 TATA for HIS3, GAL2 UAS/GAL2 TATA for ADE2 and MEL1 UAS/MEL1 TATA for MEL1 and lacZ). Expressed sequences from AlHV-1 ORFs A2, A3 and A4 were cloned into the pGBK-T7 (GAL-4 binding domain, bait) vector and used to screen a novel bovine cDNA library cloned, as described below in Section 4.4.2, into the pGAD-T7 (activation domain, prey) vector.

4.4.2 Library Construction and Testing

A cDNA library was constructed using RNA extracted from freshly isolated bovine PBMC and cultured bovine turbinate (BT) epithelial cells. These cell types were selected as they are representative of the different cell types most likely to be involved in MCF pathogenesis and are readily available. To account for the possibility that the gene products would interact with other AlHV-1 proteins, half of the BT cells used were infected with the pathogenic C500 strain of AlHV-1 48 hours prior to harvesting. RNA was extracted separately from 10^8 freshly isolated PBMC and from $3.5x10^7$ mixed infected and uninfected BT cells with RNAwiz (Ambion) as described in Section 2.1.16. 40 µg of total RNA was isolated from the PBMC and 490 µg was isolated from the mixed BT cells. The RNA samples were pooled and mRNA was

purified from a 500 µg sample using the Poly(A) Purist Kit (Ambion) as described in Section 2.1.19. 5.52 µg of mRNA was collected from this procedure and the entire sample was used for cDNA synthesis and ligated into the pGAD-T7 vector following the protocol described in Section 2.1.20.

XL-10 Gold Ultra competent cells (Stratagene) were transformed with the library ligation mixture using the protocol described in Section 2.2.3 and plated onto fifteen 26.7 cm^2 LB/Agar plates supplemented with 100 µg/ml of ampicillin. Following overnight incubation at 37°C colonies had grown to a density of approximately 100 per cm^2 and, therefore, approximately 13,500 total colonies.

Twelve individual colonies were selected for screening to determine the quality of the library. The colonies were grown in LB broth overnight and plasmid DNA was extracted using the protocol described in Section 2.2.5. To determine the presence and size of inserted sequences, plasmid DNA was used as template for the pGAD-T7 insert PCR reaction described in Section A1.3.42 and also analysed by restriction digestion using *Eco*RI and *Xho*I restriction endonucleases. The results of colony screening are summarised in Table 4.1. Plasmids from only 6 of the 12 colonies selected were positive by PCR. By restriction digest analysis, the plasmid from one of these colonies (colony 7) contained a fragment of the appropriate size for pGAD-T7 (approximately 800 bp) but no insert. Another of the PCR positive plasmids (colony 1) contained a fragment of approximately the same size as the PCR product but the other fragment was not the correct size for pGAD-T7. Overall, 4 of the 12 colonies (colonies 2, 5, 10 and 11) contained appropriate library vectors. The insert sizes of these colonies ranged from approximately 700 to 4000 bp. By extrapolation, the library represented approximately 4,500 (one third of 13,500) good sequences. Assuming all of these sequences have inserted in the correct orientation, it is likely that only one in three will be inserted in frame to make with the GAL-4 activation domain sequence and many of these will be duplicates. Taken together, this leaves the final complexity of the library at below 1,500 different fusion proteins. All remaining colonies were pooled and library plasmid DNA was extracted using the protocol described in Section 2.2.6.

Colony	1	2	3	4	5	6	7	8	9	10	11	12
PCR Product Size (bp)	1500	-	700	-	1000	-	1400	-	-	4000	2200	-
Restriction Fragment Sizes (bp)	7500 1500	8000 650	8000 700	5800 1000 600	8000 600 400	7000 900 800	8000	8000	8000	8000 2200 1800	8000 1900 300	11000 7000 4500
Note	Unknown	Unknown	Good	Unknown	Good	Unknown	Unknown	Empty	Empty	Good	Good	Unknown

Table 4.1. Summary of library colony plasmid screening showing approximate sizes of PCR products and restriction digestion fragments. The notes in the bottom row indicate whether the colony contained an appropriate library vector (Good), empty pGAD-T7 (Empty) or a plasmid (or plasmids) with a restriction profile inconsistent with pGAD-T7 or showing unexplained PCR results (Unknown). Details in text Section 4.4.3.

4.4.3 Bait Construct Production and Testing

Coding sequences from ORFs A2, A3 and A4 were cloned into the pGBK-T7 vector for expression as GAL4 binding domain fusion proteins to use for screening the bovine cDNA library. The full length coding sequence of ORF A2 (2477-1799) was amplified by PCR from AlHV-1 genomic DNA and cloned into pGEM-T Easy, but all attempts to clone the sequence into pGBK-T7 were unsuccessful. To determine whether the presence of the intron was responsible for the cloning difficulties, the PCR was repeated using cDNA from MHV-ALHE infected BHK-21 cells as template. Again this PCR product cloned into pGEM-T Easy but all attempts to clone the sequence into pGBK-T7 failed. The next strategy was to clone overlapping truncations of the ORF rather than the entire sequence. Sequences lacking 201 bp (2000-1799) coding for 67 C-terminal amino acids (A2ΔC) or 280 bp (2477-2197) coding for 66 N-terminal amino acids and the 82 bp intron (A2ΔN) were amplified using the programs detailed in Sections A1.3.19 and A1.3.20. Again, cDNA from MHV-ALHE infected BHK-21 cells was used as template as truncation of the sequence could interfere with proper splicing of the A2ΔC product. Both sequences were successfully cloned into pGBK-T7.

To increase the likelihood of ORF A3 and ORF A4 GAL4 binding domain fusion proteins localising appropriately to the yeast nucleus, the coding sequences were cloned lacking their predicted signal peptides. To determine the predicted location of the signal peptides, ORF A3 and ORF A4 amino acid sequences were analysed using the SignalP 3.0 software (http://www.cbs.dtu.dk/services/SignalP). The results for ORF A3 and ORF A4 are shown in Figure 4.18. The s-score (green line) represents the relative probability that the amino acid at that position is part of a signal peptide. The c-score (red lines) represents the likelihood of cleavage occurring at that position. The y-score (blue line) is derived from the c-score combined with the s-score to give the best prediction for cleavage.

As shown in Figure 4.18 A, the ORF A3 amino acid sequence based on the originally predicted start codon (Ensser & Fleckenstein, 1995) does not show a clear signal peptide location. It has now been shown that translation of the protein begins at what was thought to be the second methionine (Dr. Armin Ensser, Personal Communication) and ORF A3 therefore shows greater N-terminal homology to the

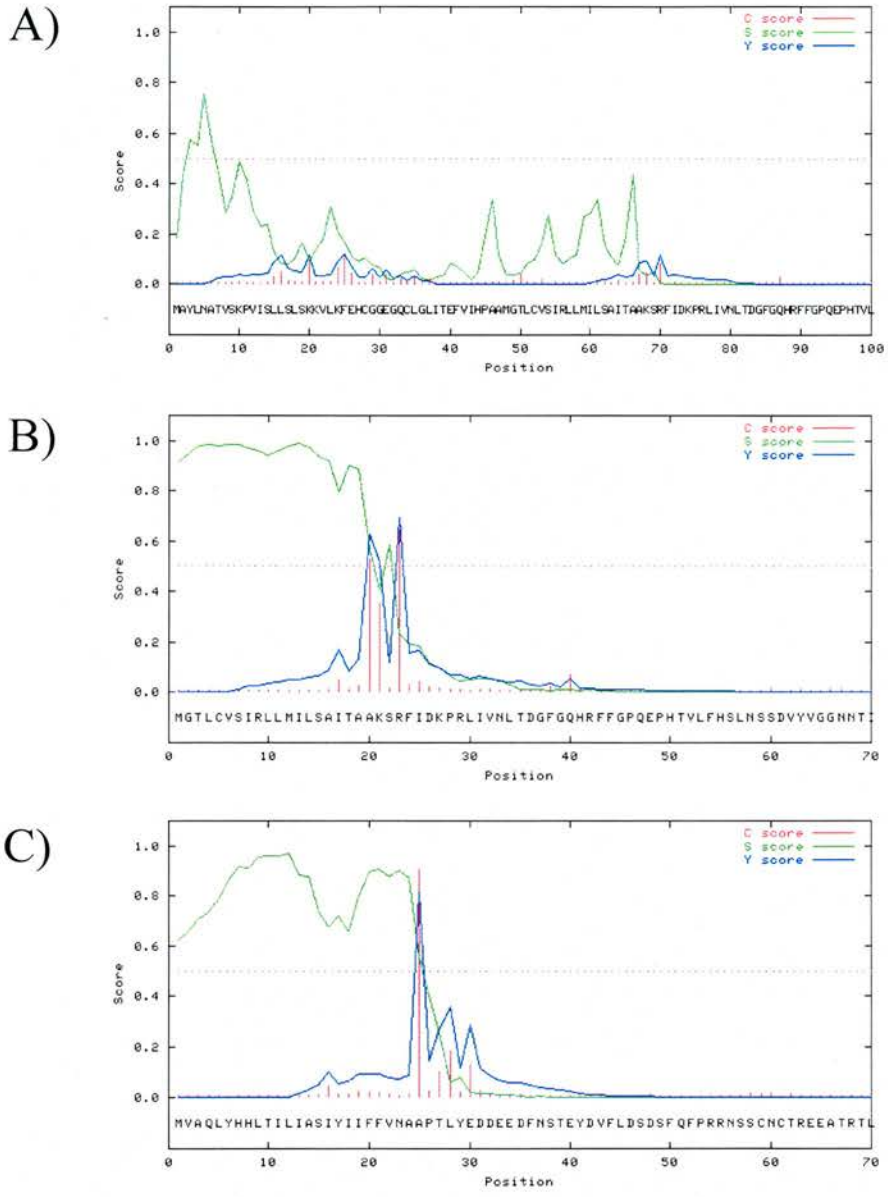


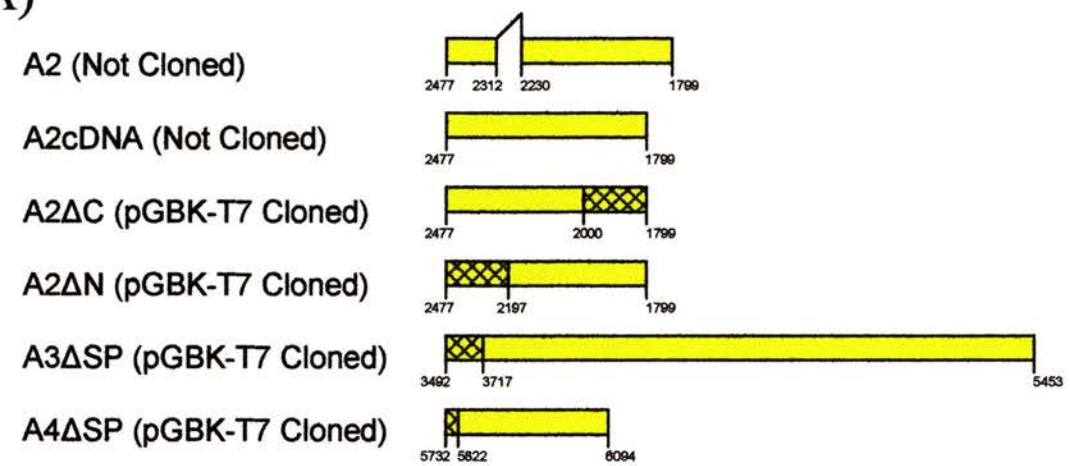
Figure 4.18. Signal peptide prediction results using SignalP 3.0 software for the N-terminal of A) ORF A3 beginning with the methionine encoded by nucleotides 3492-3494, B) ORF A3 beginning with the methionine encoded by nucleotides 3633-3635 and C) ORF A4.

other members of the semaphorin family than originally predicted (Ensser & Fleckenstein, 1995). The sequence was resubmitted beginning instead with this start codon. As shown in Figure 4.18 B, a high probability signal peptide is predicted followed by three possible cleavage sites, the most likely occurring between S22 and R23. To exclude this region, the ORF A3 sequence was amplified for cloning by PCR using a forward primer with sequences from nucleotides 3717-3736 (A3ΔSP) as detailed in Section A1.3.27. The PCR product was successfully cloned into pGBK-T7.

The protein sequence encoded by ORF A4 beginning with the predicted start codon (Ensser *et al.*, 1997) (encoded by nucleotides 5732-5734) was also submitted for signal peptide prediction. As shown in Figure 4.18 C, a high probability signal peptide followed by a high probability cleavage between A24 and A25 was predicted. To remove this region from the fusion protein, the ORF A4 sequence was amplified for cloning by PCR using a forward primer with sequences from nucleotides 5822-5839 (A4ΔSP) as detailed in Section A1.3.32. The PCR product was successfully cloned into pGBK-T7.

Diagrammatic representations of the coding sequences from ORF A2, ORF A3 and ORF A4 that were used for the creation of GAL4 binding domain fusion proteins are shown in Figure 4.19 A with cross-hatched regions representing coding sequences that were excluded from the final vectors. The A2ΔN, A2ΔC, A3ΔSP and A4ΔSP vectors were transformed into AH109 yeast using the protocol described in Section 2.3.2 for the first step of sequential library transformation and grown on SD-Trp selection plates until colonies appeared. To determine whether any of the four binding domain fusion proteins possessed any intrinsic GAL4 activating activity, colonies were streaked onto SD-Trp plates supplemented with X-α-Gal. As shown in Figure 4.19 B, growth of all transformed yeast was seen. A blue colour, indicating α-galactosidase activity, was seen in the A2ΔN transformed yeast. This shows that the C-terminal portion of the ORF A2 gene product has the ability to activate transcription of the MEL1 reporter gene in the absence of an interaction with the GAL4 activation domain and therefore was unsuitable for further use in the library screening.

A)



B)

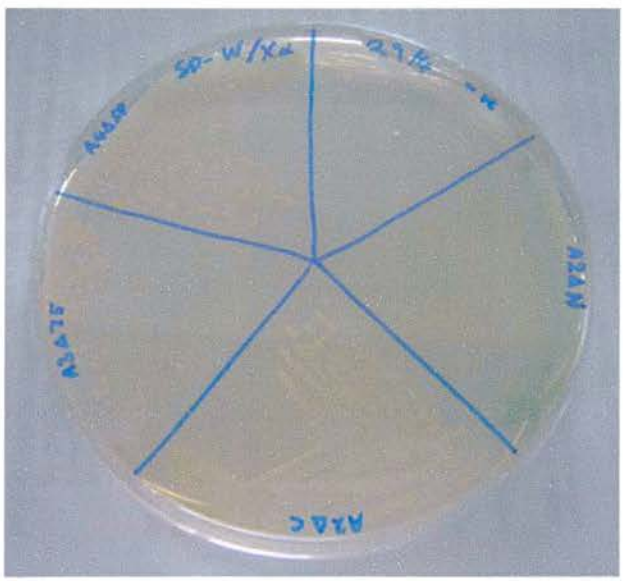


Figure 4.19. A) Diagrammatic representations of the coding regions of ORF A2, ORF A3 and ORF A4 with nucleic acid coordinates. Hatched areas represent regions not included in bait constructs. B) Growth of yeast transfected with indicated bait constructs on SD-Trp/Agar plates supplemented with X-α-Gal.

4.4.4 Library Screening

Library pGAD-T7 vectors were introduced to cultures of A2ΔC, A3ΔSP and A4ΔSP transformant yeast colonies as described in Section 2.3.2. Library transformations were plated on 15 high stringency (SD-Ade/-His/-Leu/-Trp) agar plates and incubated at 30°C until clear colonies had formed. Colonies were subsequently grown for DNA extraction either by replating an approximately 1 cm^2 area on a high stringency plate or by overnight growth in 2 ml of high stringency broth. If sufficient secondary growth was seen, DNA was isolated from the yeast as described in Section 2.3.3. The DNA was then used as a template for the pGAD-T7 PCR detailed in Section A1.3.42 to amplify sequences inserted into the pGAD-T7 vector. PCR products from positive reactions were sequenced either directly using the forward primer from the pGAD-T7 PCR as described in Section 2.1.13 or by first cloning the product into pGEM-T Easy and subsequently sequencing from plasmid DNA as described in Section 2.1.12. Direct sequencing has the advantages that it is less time consuming than first cloning the PCR product and allows for the orientation of the product relative to the pGAD-T7 vector but the disadvantage that a large proportion of the reactions returned sequences that were unreadable. Returned sequences were submitted for analysis using the NCBI nucleotide-nucleotide BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and TIGR Cattle Gene Index BLAST software (http://tigrblast.tigr.org/tgi). In most cases, the interacting protein was assumed to be the product of the bovine gene with the highest homology to the query sequence. Complete sequences returned from successful sequencing reactions are presented in Appendix 4 and are summarised below.

A2

A total of five colonies grew from the A2ΔC library screen. Of these, only three showed sufficient secondary growth for DNA extraction. One of the three PCRs gave no product (A2B3), one gave a single strong product (A2B4) and one gave multiple weak products implying that it was a mixed colony (A2B5). Direct sequencing reactions from the three products of the A2B5 PCR returned unreadable results. The sequence returned from the A2B4 PCR product sequencing reaction identified the gene as bovine amyloid beta precursor protein. However, this protein would not be produced as a fusion with the GAL4 activation domain as the gene was inserted in the

antisense orientation. No genes in the sense orientation with high homology to the query sequence were found and the true amino acid sequence encoded was not determined.

A3

Three colonies grew from the A3ΔSP library screen, all of which showed sufficient secondary growth for DNA extraction. Two of the three PCRs gave strong single products of unique molecular weights (A3B8 and A3B9) and one gave no product (A3B7). The positive reactions were sequenced by first cloning into pGEM-T Easy as the reactions were performed prior to use of the direct sequencing method. The returned sequences were found to encode bovine type III pro-collagen (A3B8) and a bovine gene similar to tenascin precursor. Collagen and tenascin are related structural proteins and collagen-like proteins have been found to be a common class of false positives in yeast two-hybrid screens owing to their ability to bind non-specifically to a wide range of proteins (www.biologicalprocedures.com/bpo/arts/1/16/m16.htm). Although the orientation of these sequences relative to the GAL4 activation domain could not be determined due to the non-directional cloning of PCR products into pGEM-T Easy, it is likely that these are the proteins encoded based on the large number of similar sequence found in the correct orientation in the A4ΔSP screening described below.

A4

Unlike the A2ΔC and A3ΔSP screens, a large number (over 160) of colonies grew in the A4ΔSP library screen. Of the 160 colonies selected, 139 showed sufficient secondary growth for DNA extraction and from these 67 PCRs gave at least one product. From the first 58 colonies screened (A4B1 to A4B60), 15 PCRs gave positive products. Restriction digest analysis using the frequent cutting Sau3AI restriction endonuclease was used to eliminate replicate inserts. It was found that the products amplified from colonies A4B5, A4B8 and A4B9 had identical restriction patterns as did those of colonies A4B27, A4B30 and A4B32. All unique products and one product from each replicate group (A4B5 and A4B30) were cloned into pGEM-T Easy for sequencing. The genes corresponding to the returned sequences are shown in Table 4.2. From the remaining 81 colonies screened (A4B61 to A4B160), 52 PCRs

Colony	Result
A4B1	Bos taurus COL1A2 mRNA for alpha2(I) collagen
A4B5	Bos taurus similar to tenascin
A4B7	Bos taurus retina G protein-coupled receptor kinase 7 mRNA
A4B8	Bos taurus similar to tenascin
A4B9	Bos taurus similar to tenascin
A4B16	Bos taurus similar to pro alpha 1(I) collagen
A4B27	Bos taurus similar to tenascin
A4B28	Cloning vector psiLentGene Basic
A4B30	Bos taurus similar to tenascin
A4B32	Bos taurus similar to tenascin
A4B34	Bos taurus low molecular mass ubiquinone-binding protein mRNA
A4B40	Bos taurus similar to 40S ribosomal protein S16
A4B41	Bos taurus similar to eIF-3 p110 subunit
A4B50	Bos taurus similar to tenascin
A4B52	Bos taurus similar to Ubiquitin-like 1 activating enzyme E1A

Table 4.2. A4ΔSP library screening results obtained by pGEM-T Easy cloning of pGAD-T7 Insert PCR products.

gave positive products. Direct sequencing reactions were performed on all of these products. Of the returned sequences, 21 were unreadable. The genes corresponding to the returned sequences of the remaining 31 reactions are shown in Table 4.3.

The results of the A4ΔSP library screen are summarised in Table 4.4. The vast majority of the returned sequences corresponded to tenascin (20 of 46) or collagen (10 of 46) related proteins. A number of other proteins belonging to groups that have previously been shown to give false positive results (www.biologicalprocedures.com/bpo/arts/1/16/m16.htm) were also found. These include three members of the ribosomal protein family (A4B40, A4B101 and A4B138), two proteins involved in the ubiquitin/proteasome pathway (A4B34 and A4B52) and a matrix protein (matrix metalloprotease, A4B116). One sequence was a contaminant (pSilenciA4B) most likely acquired while attempting to clone the PCR product into pGEM-T Easy.

The nine remaining sequences were considered genes of interest. Four of these sequences encoded nucleic acid binding proteins including three sequences from a poly(rC) binding protein (A4B123, A4B134 and A4B151) and one from another nucleic acid binding protein (A4B142). The other sequences showed homology to retina G protein-coupled receptor kinase 7 (A4B7), a bovine gene similar to the eIF-3 p110 subunit (A4B41), tumor necrosis factor-receptor (TNFR) 1 (A4B82), GPAA1P anchor attachment protein (A4B98) and the Ras related protein Rab-8A (A4B126). Further study was performed on four of these colonies (A4B82, A4B98, A4B126 and A4B151) as described in the following section. Although the sequence from A4B82 is 5' of the coding region for the TNFR-1 protein and the sequence for coding GPAA1P in A4B98 is in the wrong orientation relative to GAL4 activation domain, it was possible that novel amino acid sequences that bind the ORF A4 gene product could be determined by their use.

4.4.5 Confirmation Experiments

The pGAD-T7 based vectors in colonies A4B82, A4B98, A4B126 and A4B151 were isolated for use in confirmation experiments. Chemically competent *E. coli* were transformed using the crude DNA preparations from these colonies and incubated on LB/Agar plates supplemented with 100μg/ml ampicillin. Colonies were selected for plasmid DNA extraction. The presence of a plasmid consistent with the PCR screening results was determined by restriction digest analysis using *Eco*RI and *Xho*I

Colony	Result	Orientation
A4B62	Bos taurus similar to Tenascin precursor	+
A4B80	Bos taurus collagen alpha 2(I) chain precursor	+
A4B82	Bos taurus tumor necrosis factor-receptor I	+
A4B83	Bos taurus similar to Tenascin precursor	+
A4B86	Bos taurus COL1A2 mRNA for alpha2(I) collagen	+
A4B96	Bos taurus similar to pro alpha 1(I) collagen	+
A4B98	Bos taurus GPAA1P anchor attachment protein 1 homolog	-
A4B101	Bos taurus similar to 60S ribosomal protein L22	-
A4B102	Bos taurus similar to pro alpha 1(I) collagen	+
A4B111	Bos taurus COL1A2 mRNA for alpha2(I) collagen	+
A4B114	Bos taurus similar to Tenascin precursor	+
A4B116	Bos taurus matrix metalloproteinase 2	+
A4B118	Bos taurus similar to pro alpha 1(I) collagen	+
A4B123	Bos taurus similar to nuclear poly(C)-binding protein	+
A4B126	Bos taurus Ras-related protein Rab-8A	+
A4B129	Bos taurus similar to Tenascin precursor	+
A4B131	Bos taurus similar to Tenascin precursor	+
A4B132	Bos taurus similar to Tenascin precursor	+
A4B134	Bos taurus similar to nuclear poly(rC)-binding protein	+
A4B135	Bos taurus similar to Tenascin precursor	+
A4B138	Bos taurus similar to 40S ribosomal protein S26	+
A4B142	Bos taurus similar to nucleic acid binding protein	+
A4B143	Bos taurus similar to pro alpha 1(I) collagen	+
A4B145	Bos taurus COL1A2 mRNA for alpha2(I) collagen	+
A4B147	Bos taurus similar to Tenascin precursor	+
A4B148	Bos taurus similar to Tenascin precursor	+
A4B150	Bos taurus similar to Tenascin precursor	+
A4B151	Bos taurus similar to nuclear poly(rC)-binding protein	+
A4B154	Bos taurus similar to Tenascin precursor	+
A4B157	Bos taurus similar to Tenascin precursor	+
A4B158	Bos taurus similar to Tenascin precursor	+

Table 4.3. A4ΔSP library screening results obtained by direct sequencing of pGAD-T7 Insert PCR products. Orientation of insert relative to the GAL4 activation domain is indicated in the right hand column.

Tenascin and Related	20
Collagen and Related	10
Nuclear Poly(rC)-Binding Protein	4
Ribosomal	3
Ubiquitin Pathway	2
Retinal G-Protein Coupled Receptor	1
Cloning vector psiLentGene Basic	1
eIF-3 p110 Subunit	1
TNF Receptor 1	1
GPAA1P Anchor Attachment Protein	1
Matrix Metalloprotease	1
Ras-related Protein Rab-8A	1

Table 4.4. Summarised results of the A4ΔSP library screen indicating probable false positives (blue), a contaminant (red) and genes of interest (black).

restriction endonucleases and by repeating the pGAD-T7 insert PCR with plasmid DNA as template. Using this cloning strategy, the pGAD-T7 based plasmid from A4B98 (pGAD98) was successfully isolated but attempts to directly isolate the other three vectors were unsuccessful. Instead, the pGAD-T7 based vectors from A4B82, A4B126 and A4B151 (pGAD82, pGAD126 and pGAD151, respectively) were recreated by cloning PCR products from the pGAD insert PCR screening of the colonies into pGAD-T7 using *Eco*RI and *Xho*I restriction endonucleases. All three vectors were successfully recreated using this strategy.

Inserts in the pGAD82, pGAD98, pGAD126 and pGAD151 plasmids were sequenced from the T7 promoter upstream of the multiple cloning site to reconfirm previous findings and also to determine the reading frame relative to the GAL4 activation domain and therefore the precise amino acid sequence translated. All four reactions returned sequences consistent with previous results. Translated sequences of the inserts determined from the reading frame of the GAL4 activation domain are shown in Table 4.5 with the amino acids encoded by the vector shown in red and those encoded by the insert shown in black. Although the inserted sequence of pGAD82 appeared to originate from mRNA for bovine TNFR1, it only encoded a further fourteen amino acids when read in frame with the GAL4 activation domain and this amino acid sequence showed no homology to know proteins using Protein-Protein BLAST (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) analysis. Similarly, the inserted sequence of pGAD98 encoded only five amino acids. In contrast, the inserts in the pGAD126 and pGAD151 vectors encode fairly long polypeptides of 121 and 175 amino acids, respectively. Although a high degree of homology was found between the nucleic acid sequence inserted in pGAD126 and the Rab 8A gene, very little alignment between the translated sequence of the insert and any of the predicted ORFs of Rab 8A was seen using the SIM Alignment software (http://www.expasy.ch/tools/sim-prot.html) and no other proteins of high homology to the translated sequence were found using Protein-Protein BLAST. As shown in Figure 4.20, there is an almost total alignment between the translated insert of pGAD151 and the bovine poly(rC) binding protein 2 with a four amino acid insertion in pGAD151 being the only difference.

Colony	Translated Sequence	
82	MAMEASEFGTRGELSKATWGTYVGP*	
98	EASEFGTRELFIS*	
126	XSLIRLXYRASAAMEYPYDVPDYAHMAM EASEFGTRQPALRQGARSSSSSSSSPVVSK LNVLARDFYFSRSVVSGGDSVFDLQWQA QVIYREHTHTLPAPRARGGLQGLAVEQSG WVFNCRHRQLLFQQLLSSWRDGSQDQVA RSEGCHQGAFLFLF*	
151	XFFNTTHYRASAAMEYPYDVPDYAHMA MEASEFGTRSSSMTNSTAASRPPVTLRLV VPASQCGSLIGKGGCKIKEIRESTGAQVQV AGDMLPNSTERAITIAGIPQSIIECVKQICV VMLETLSQSPPKGVTIPYRPKPSSSPVIFAG GQDRYSTGSDSASFPHTTPSMCLNPDLEG PPLEAYTIQGQYAIPQPDLTKLHQLANAT VSFFP*	

Table 4.5. Translated sequences of four inserts based on sequencing data using the T7 primer site upstream of the multiple cloning site with vector encoded amino acids shown in red and those encoded by the insert shown in black.

```
pGAD151          1 SSSMTNSTAASRPPVTLRLVVPASQCGSLI
Poly(rC)BP      84 SSSMTNSTAASRPPVTLRLVVPASQCGSLI
                   ******************************
pGAD151         31 GKGGCKIKEIRESTGAQVQVAGDMLPNSTE
Poly(rC)BP     104 GKGGCKIKEIRESTGAQVQVAGDMLPNSTE
                   ******************************
pGAD151         61 RAITIAGIPQSIIECVKQICVVMLETLSQS
Poly(rC)BP     144 RAITIAGIPQSIIECVKQICVVMLE----S
                   *************************    *
pGAD151         91 PPKGVTIPYRPKPSSSPVIFAGGQDRYSTG
Poly(rC)BP     170 PPKGVTIPYRPKPSSSPVIFAGGQDRYSTG
                   ******************************
pGAD151        121 SDSASFPHTTPSMCLNPDLEGPPLEAYTIQ
Poly(rC)BP     200 SDSASFPHTTPSMCLNPDLEGPPLEAYTIQ
                   ******************************
pGAD151        151 GQYAIPQPDLTKLHQLA
Poly(rC)BP     230 GQYAIPQPDLTKLHQLA
                   *****************
```

Figure 4.20. Protein sequence alignment comparing the translated insert sequence from the pGAD151 plasmid with the sequence from the bovine gene similar to nuclear poly(rC)-binding protein.

To confirm the interactions of the pGAD82, pGAD98, pGAD126 and pGAD151 encoded proteins with the ORF A4 product, AH109 yeast containing the pGBKA4ΔSP plasmid were transfected with the appropriate vector or empty pGAD-T7 as a negative control. To test the specificity of the interactions, the pGAD vectors were also transfected into AH109 yeast containing empty pGBK-T7. Transfected yeast were initially plated on low stringency (SD-Leu/-Trp) agar plates to allow for growth of yeast containing both a bait and a prey plasmid regardless of interaction. Colonies were then transferred to high stringency (SD-Ade/-His/-Leu/-Trp/+X-α-gal) agar plates to determine whether any interaction between bait and prey proteins could be detected. As shown in Figure 4.21 A, colonies containing all the bait/prey combinations tested could be established and grown on low stringency plates. Growth and α-galactosidase activity of the A4/151 yeast was seen on the high stringency plates (Figure 4.21 B), but was also seen in the BK/151 yeast and is therefore a false positive interaction. The other interactions tested were not reproducible as no growth of the A4/82, A4/98 or A4/126 yeast was seen on the high stringency plate. The lack of growth was not due to absence of the appropriate pGAD plasmid as pGAD-T7 Insert PCR products of the appropriate sizes for pGAD82 (~400 bp), pGAD98 (~850 bp) and pGAD126 (~450 bp) could be amplified from DNA extracted from the colony used to inoculate the high stringency plate (Figure 4.21 C)

4.4.6 Summary

Bait constructs containing sequences from ORF A2 (A2ΔC), ORF A3 (A3ΔSP) and ORF A4 (A4ΔSP) were produced and used to screen a novel bovine prey construct library representing approximately 4,500 genes. Few colonies grew in the A2ΔC and A3ΔSP library screens but a large number grew in the A4ΔSP screen. Of those colonies from which the insert in the pGAD-T7 plasmid could be determined, the majority contained sequences encoding proteins known to produce false positive results. Four of the interactions from the A4ΔSP screen were carried forward for further analysis and confirmation. One of these four was found to be a false positive interaction and the remaining three interactions were not reproducible.

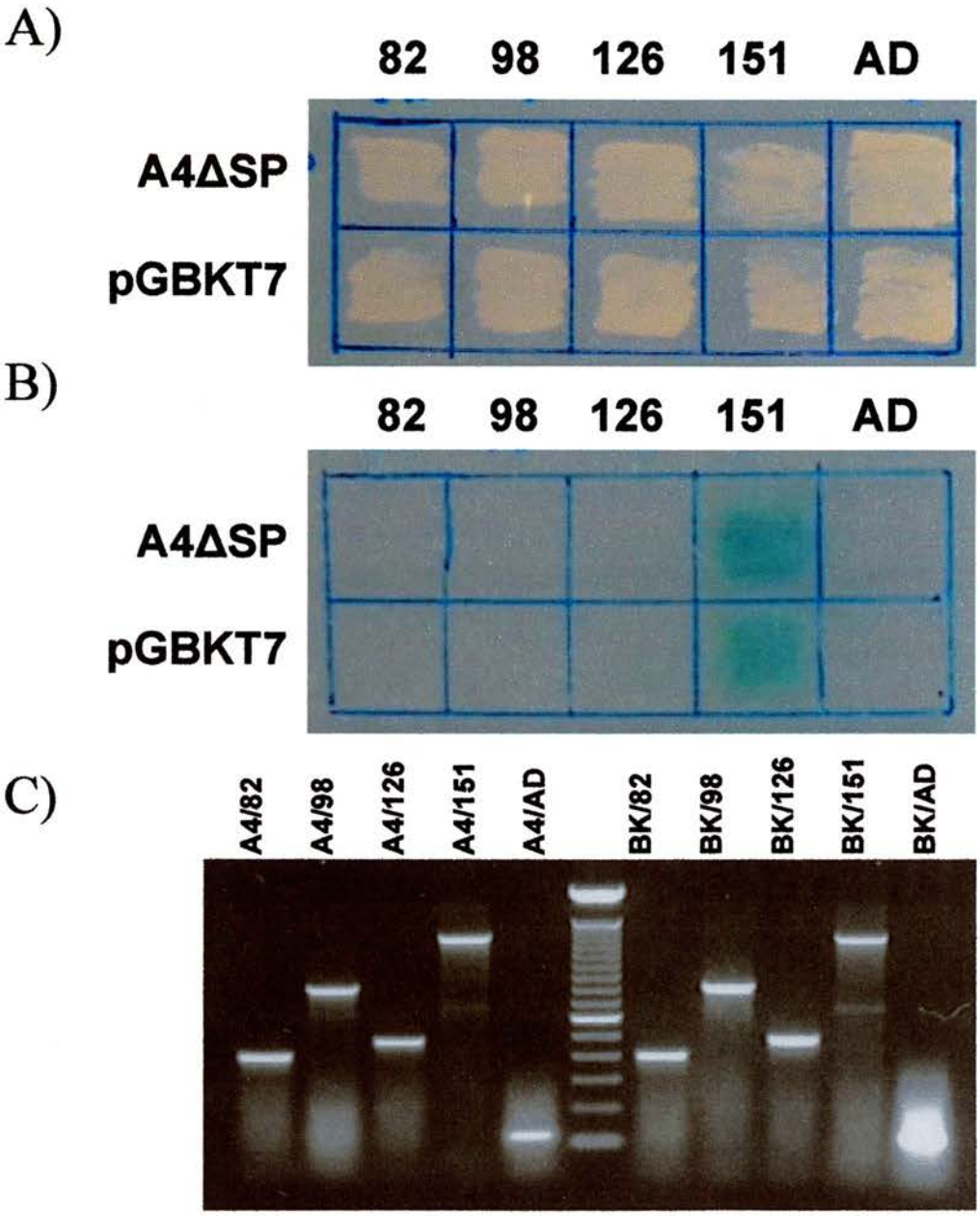


Figure 4.21. Growth of yeast colonies transfected with the indicated bait and prey vectors on A) low stringency (SD-Leu/-Trp) and B) high stringency (SD-Ade/-His/-Leu/-Trp/+Xgal) agar plates. C) pGAD-T7 Insert PCR screening of DNA extracted from yeast colonies transfected with the indicated bait and prey vectors.

4.5 Discussion of *In Vitro* Experiment Results

A number of different experiments were undertaken to assess various biological properties of the gene products of the LHE ORFs of AlHV-1. The expression of the ORF products as fusion proteins in mammalian cells was used in an attempt to determine the intracellular localisation or secretion of the proteins. The mammalian cell expression system was also employed to assess the potential transcriptional regulatory activity of the ORF A2 gene product. A bacterial system was also used for the expression of ORF A2 as a means of producing large amounts of recombinant protein and, although unsuccessful in this objective, provided data of possible relevance to the mammalian expression results. Supernatants from BHK-21 cell cultures infected with the recombinant MHV-ALHE virus were used to study the effects of secreted viral proteins, predicted to include the ORF A3 and ORF A4 gene products, on bovine and ovine PBMCs. Finally, a yeast two-hybrid system was used to screen a novel bovine cDNA library for proteins interacting with the LHE ORF gene products.

ORF A1 was successfully cloned for expression in mammalian cells as a C-terminally c-myc/6xHIS tagged fusion protein. No clear signal could be detected in transfected cell cultures immunostained using the anti-c-myc antibody. There are several possible reasons for the lack of detection. It is possible that the protein is not produced or is secreted, but these are unlikely as the CMV IE promoter should ensure a high level of constitutive expression and no conserved signal sequences were found in the gene product. A more likely explanation is that protein is present in some of the cells but at a low level, possibly due to degradation or poor translation of mRNA or protein instability, and is therefore below the sensitivity of the detection protocol.

Mammalian expression of ORF A2 was more successful. ORF A2 gene products C-terminally tagged with either c-myc/6xHIS or GFP showed localisation within the nucleus of transfected cells but were preferentially excluded from nucleoli. N-terminally GFP tagged ORF A2 gene products also showed nuclear localisation but appeared to localise into nuclear aggregates. It was initially thought that the reason for this apparent discrepancy was the presence of a large tag interfering with appropriate localisation considering that the molecular weight of GFP is similar to that predicted for the ORF A2 gene product. As the results obtained from bacterial

expression of ORF A2 indicated that the gene product was rapidly cleaved, attempts were made using a dual-tagged fusion protein to assess whether this was also the case in mammalian cells and whether such cleavage could account for the discrepancy between the localisation of N- and C-terminally tagged fusion proteins. The results of this study also supported a model in which proteolytic cleavage was involved. In transfected cells expressing relatively low amounts of protein, both N-terminal GFP signal and C-terminal c-myc staining localised within the nucleus but were excluded from distinct areas, most likely nucleoli based on the findings of C-terminally GFP-tagged fusion protein. Importantly, signals from the two termini did not completely overlap, indicating cleavage. The difference in localisation of the GFP signal and c-myc staining became more pronounced as the signal intensities increased, with the GFP signal localising preferentially to areas within the nucleus from which the c-myc staining was excluded. Although no direct evidence for proteolytic cleavage was obtained due to the insufficient expression for detection by Western blot of any of the ORF A2 fusion proteins, a cleavage model is the most rational explanation for the observed results. Assuming that cleavage is involved, there remain several issues to be addressed. Firstly, there is no indication of where and when the cleavage is occurring. The issue of how the cleavage products localise separately also raises several questions. For example, do the different cleavage products contain their own localisation signals? As two distinct localisation patterns were seen using the same construct, the possibility of a time or protein level effect on localisation would also have to be addressed possibly using a construct under the control of an inducible promoter. It is also possible that no cleavage of the ORF A2 gene product itself is occurring and only the N-terminal GFP tag is cleaved and sequestered in nuclear aggregates. However, without antibodies directed against specific epitopes of the ORF A2 gene product it is not possible to analyse the true localisation of this protein in the absence of any potentially artefactual results.

As the ORF A2 gene product was found to have nuclear localisation and has a predicted basic leucine zipper motif (L23-Y76), it is likely to be a DNA binding protein and may function as a transcription factor, as originally predicted (Ensser *et al.* 1997). The possibility that this protein affects transcription from viral promoter sequences was tested using a luciferase reporter system. The ORF A2 c-myc/6xHIS construct was used for the production of ORF A2 gene product in BHK-21 cells and

cultures were co-transfected with one of five reporter constructs containing promoter sequences from selected immediate-early, early and late genes encoded by AlHV-1. No change in transcription of the reporter gene was seen with any of the constructs in the presence of ORF A2 gene product. This finding was not unexpected as it is not confirmed that the protein is indeed a transcription factor and, assuming it is, the promoter sequences tested represent a very small fraction of the sequences potentially targeted including the remaining AlHV-1 promoters as well as those of Wildebeest and species susceptible to MCF. As immediate-early ORF A2 transcripts were seen in the context of the recombinant virus (Section 3.2.2), the most likely candidate promoter sequences are those of the AlHV-1 early genes and these could serve as the basis for future experiments.

It was hoped that the DNA binding ability of the ORF A2 gene product could be assessed using purified recombinant protein. Unfortunately, insufficient protein could be produced using the mammalian expression system and most of the protein expressed in bacteria was cleaved and could not be cleanly immunoprecipitated from cell lysates. One attempt was made to perform South-Western blot analysis using DIG-labelled DNA to probe crude bacterial lysates electrophoresed and blotted under both denaturing and non-denaturing conditions. No obvious binding was seen in samples from pTH-FLAG-A2c transformed cultures versus those transformed with empty pTrcHis2 A (data not shown), although the quality of the assay could not be established as appropriate controls were not readily available. To overcome some of the problems experienced during these experiments, the production of protein could in future be carried out in bacterial strains better optimised for recombinant protein production (such as BL21 or Rosetta) and express a known DNA binding fusion protein in parallel as a control. With sufficient protein amounts expressed, problems arising from the low sensitivity of detection and purification should be more easily resolved.

Mammalian expression experiments failed to demonstrate the predicted secretion of either ORF A3 or ORF A4. The GFP signals in cells transfected with N-terminal fusions of both the ORF A3 and ORF A4 gene products were seen throughout the cell in a pattern indistinguishable from that of GFP expressed alone. Western blot analysis showed that, in cells transfected with the ORF A3 construct, the expressed

fusion protein is of a molecular weight identical to GFP alone. Possible explanations for this finding include the incomplete translation of the fusion protein or cleavage of the GFP tag from the ORF A3 gene product, possibly occurring during secretion. Some of this product was also found in concentrated supernatant samples but this is likely to be indicative of cellular contamination or release from dead or dying cells rather than true secretion as the same was seen using GFP alone. N-terminally tagged ORF A4 was not detected by Western blot most likely due to lower transfection efficiency although it would probably give results analogous to ORF A3 if the fusion protein were similarly cleaved or incompletely translated as is suggested by the localisation. One would predict that C-terminal fusion proteins of theses two gene products would localise more appropriately as the tags would be more distant from the predicted signal sequences and therefore less likely to interfere with secretion or be cleaved. Unfortunately, attempts to clone C-terminally tagged ORF A3 fusion proteins were unsuccessful. C-terminally GFP and c-myc/6xHIS tagged ORF A4 constructs were successfully cloned but no signal or staining could be detected in transfected cells or their culture supernatants although this may simply represent a lack of sensitivity of the detection assays used.

As potentially secreted proteins, it was thought that the ORF A3 and ORF A4 gene products could exhibit biological effects on lymphocytes, contributing to the lymphoproliferation and immune disregulation seen in MCF. To test this hypothesis, PBMCs of ovine and bovine origin were cultured in media predicted to contain these proteins. As insufficient protein levels could be produced and secretion could not be demonstrated using plasmid vector based expression of tagged fusion proteins, the recombinant MHV-ALHE virus was used for highly efficient gene delivery to BHK-21 cells for expression. This system had the advantage that the efficiency of gene delivery should be almost 100% but did have some drawbacks. The major disadvantage is that there was no way to confirm the presence of the gene products in the supernatants without specific reagents, although previous experiments had demonstrated expression to the mRNA level in similar cultures. To address the possibility that MHV encoded genes or other factors secreted by infected cells could also have effects on the PBMCs, supernatants from MHV-76 infected BHK-21 cell cultures were used as controls. No change in the proliferation of PBMC cultures of ovine or bovine origin were seen in the presence of supernatants from cell cultures

infected with either MHV-ALHE or MHV-76, as measured by cell number, cell size and DNA content. It is possible that the lack of an effect is due to the absence of ORF A3 and ORF A4 gene products in the supernatants as the translation and secretion of these proteins could not be conclusively demonstrated. Assuming the proteins are present, it is entirely possible that they would have an effect on the PBMCs other than the induction of proliferation or have effects on entirely different cell types.

In an attempt to identify proteins interacting with the ORF A2, ORF A3 and ORF A4 gene products, the yeast two-hybrid system was used for several reasons. Firstly, it requires no purification of recombinant proteins which had proven difficult for the gene products to be analysed. Secondly, due to the high sensitivity of this assay, protein interactions should be detectable even if fusion proteins are poorly expressed or rapidly degraded as had been suggested by previous findings. Lastly, as little is known about the ORF gene products in question, it provided a useful tool for screening a large number of potential interactions without the need for prior prediction of possible functions. In order to perform these screens, it was first necessary to produce a prey library and bait constructs. The prey library was composed of cDNAs from different bovine cell types that may be involved in MCF pathogenesis i.e. turbinate cells and PBMCs. As it is possible that the gene products of interest interact with other virally encoded proteins, half of the BT cells used were infected with AlHV-1. It was hoped that a 1:1 mixture of PBMC and BT RNA would be used for construction of the library. However, using the RNA isolated this would have resulted in a maximum starting sample of only 80 ng which, based on pilot mRNA isolation, would have yielded insufficient mRNA for cDNA synthesis. Therefore, an approximately 1:11 mixture of PBMC and BT RNA was used.

Approximately 13,500 colonies grew from the library transformation but analysis of plasmid DNA extracted from randomly selected colonies indicated that a high percentage contained either empty vector (~17%) or plasmids having a restriction digest profile inconsistent with that of pGAD-T7 (~50%). Taking together with the with the fact that about two thirds of these inserts will not be in frame and there is likely to be a large proportion of duplicate sequences, this implied that only around 750-1,500 genes were appropriately represented by this library. This number is very

small compared to commercially available libraries and although sufficient for a small pilot study was unlikely to identify any specific interactions.

Several issues complicated the construction of an ORF A2 bait vector with which to screen the library. Numerous attempts to clone the full length ORF as a GAL4 binding domain fusion protein, either from viral genomic DNA or cDNA lacking the intron were unsuccessful. It was initially thought that the cloning difficulty may be due to incompatibility between insert and plasmid DNA sequences; however, this is unlikely as two overlapping truncations of this ORF cloned successfully on the first attempt. Of these two constructs, the one producing a fusion protein lacking 66 N-terminal amino acids of the ORF A2 gene product (A2ΔN), but not the one producing a fusion protein lacking 67 C-terminal amino acids (A2ΔC), showed auto-activation of the *lacZ* reporter gene indicating possible transcriptional activity in the C-terminal portion of the gene product. It is known that highly acidic proteins can show such activity, however, analysis of the protein sequence indicated that the C-terminal portion does not contain a very large percentage of acidic residues or at least no higher than the N-terminal portion. Taken together, these results suggest that the C-terminal portion of the ORF A2 gene product contains a domain, or domains, capable of specifically interacting with DNA or DNA binding proteins, a finding consistent with the predicted role of this gene product as a transcription factor. The construction of ORF A3 and ORF A4 bait vectors was comparatively straightforward. Both were successfully cloned using primers designed to exclude the predicted signal peptides, so as to minimize the possibility of cleavage from the GAL4 binding domain or inappropriate localisation.

Library screening transformations were carried out using the A2ΔC, A3ΔSP and A4ΔSP construct bait vectors. Compared with the library co-transfection using the A4ΔSP construct, very few colonies grew from those using the A2ΔC and A3ΔSP constructs. It is possible that this difference was simply due to a much higher transfection efficiency being achieved with the A4ΔSP containing yeast. It is also possible that the ORF A4 gene product exhibits more non-specific binding than those of ORF A2 and ORF A3. The vast majority of interacting inserts sequenced in all library screens were for collagen and tenascin related structural proteins. This finding may be indicative of over-representation of these genes in the library, possibly due to

high levels of expression in source cells, most likely BTs. Of the potentially interesting proteins interacting with the ORF A4 construct, many were intracellular proteins which were unlikely to be true positives as the ORF A4 gene product is predicted to be secreted. Indeed, one of these interacting proteins, a nuclear poly(rC) binding protein, was shown to be a clear false positive in follow-up experiments. Three other interactions were found to be irreproducible. One possible explanation for this finding is that the colonies containing these plasmids that grew in the original library screen contained other prey vectors responsible for the growth, either through auto-activation or a true interaction with the ORF A4 gene product, but present at low levels and, therefore, not detected by insert screening PCR or rescued by bacterial transformation with extracted yeast DNA. It is also possible that proteins produced from other prey vectors could have interacted with both the ORF A4 fusion protein and the cloned prey fusion protein and without this binding intermediate, the interaction could not be reproduced. For either of these occurrences to have taken place in three out of the four colonies tested does seem unlikely although no other rational explanation can be found.

In summary, experiments using tagged ORF A2 gene product consistently found nuclear localisation in mammalian cells. This finding supports the prediction that this protein is a transcription factor although attempts to verify this were unsuccessful. Cleavage of this protein was seen during bacterial expression and is also likely to occur in mammalian cells. Attempts to demonstrate the secretion of ORF A3 and ORF A4 gene products were unsuccessful and hindered by the low transfection efficiency of plasmids, insensitivity of detection assays and inability to clone select constructs. Assuming that these proteins are secreted in sufficient quantities from BHK-21 cells infected with the recombinant MHV-ALHE virus, they have no effect on the proliferation of ovine and bovine PBMCs. A yeast two-hybrid system was employed to screen a novel bovine cDNA library for proteins interacting with the gene products of ORF A2, ORF A3 and ORF A4. Although no specifically interacting proteins could be identified and confirmed, analysis of the bait constructs used indicated that there is a domain or domains in the C-terminus of the ORF A2 gene product capable of interacting with DNA or DNA binding proteins.

Chapter 5: Conclusions

The primary objective of this study was the production of a recombinant virus containing the LHE ORFs of AlHV-1 in MHV-76 to use as a tool for the study of the role of these genes in viral pathogenesis *in vivo*. This virus was successfully created and upon initial investigation appeared to be appropriate for *in vivo* study as the ORFs were expressed from their cognate promoters in the context of murine cells and the recombinant virus did not show any overt changes in its ability to replicate. However, no differences in viral pathogenesis between the recombinant and wild-type viruses were seen in mice infected via either the intranasal or intraperitoneal route. Without specific antibodies to the AlHV-1 gene products, there is no way to conclusively determine whether lack of protein is responsible for the lack of phenotype although this is unlikely as transcripts for three of the genes could be found in infected lungs. Assuming that the gene products are present, it is unknown whether these proteins are able to interact with the murine or MHV counterparts of their natural targets found in wildebeest or AlHV-1. A lack of such interaction may also account for the inability of AlHV-1 to infect mice (Jacoby *et al.*, 1988b).

The lack of phenotype is similar to the findings of other studies using recombinant MHV-76 viruses into which genes from other herpesviruses had been inserted (Brass, 2004; Hindley, 2005; Sacco, 2003). These studies also found little difference between viral pathogenesis of the recombinant and wild-type or marker control viruses. Taken together, the results of this and previous studies suggest that the use of MHV-76 recombinants, while a powerful strategy in theory, may not be the best approach to studying the pathogenesis genes of foreign herpesviruses.

Although not possible at the outset of this project, the creation of a deletion mutant lacking the LHE ORFs using the recently created AlHV-1 BAC (Dewals *et al.*, 2006) would be a much better system to assess the contribution of these genes to pathogenesis. Being of AlHV-1 background, such a virus could be used in more biologically relevant animal species that are known to develop MCF, ideally cattle but also small laboratory animals such as rabbits and hamsters.

One notable finding from this part of the study was the expression of ORF A1 in the context of a recombinant virus. If this gene is indeed part of a larger transcript, as has been suggested (Dr. David Haig, Personal Communication), then this finding indicates that either it can be expressed alone or the other sequences to which it is

associated are present in the recombinant virus or can be substituted for by sequences present in MHV-76. It may be of interest in the future to further analyse the ORF A1 transcripts produced from the recombinant virus, possibly by Northern blot and eventually cloning, to determine their precise nature.

The secondary objective of this project was to gain functional information about the LHE gene products from their expression in a variety of *in vitro* systems. This proved to be less straightforward than initially predicted due to complications arising from low expression, inefficient detection and the apparent instability of certain fusion proteins. Although no conclusive functional data was generated from these studies, a number of interesting properties of the ORF A2 gene product were found.

The results from several of the studies using the ORF A2 gene product are consistent with the possibility that this protein is able to interact with DNA or DNA binding proteins. It is of interest to note that the C-terminal portion of the protein which had transcriptional activity in the yeast two-hybrid system is likely to be same portion of the protein which consistently localised to the area of the nucleus, outside the nucleolus, containing the DNA where transcription occurs. This finding should assist with functional mapping of the protein and provide an initial target around which to design truncated or mutated recombinant proteins for future investigation.

In conclusion, while this study has not provided any conclusive data on the functions of the AlHV-1 LHE ORFs and their role in viral pathogenesis, it has raised a number of interesting issues concerning these genes and generated results that may support or provide a starting point for future experiments. In addition, a number of the reagents generated throughout this study, including the recombinant virus, the bovine cDNA library and a number of the expression vectors, could prove useful in other investigations.

Appendix 1: PCR Programs

A1.1 Recipes

A1.2 Reaction Conditions

A1.3 Specific Reaction Details

A1.4 Primer Sequences

A1.1 Recipes

A. Expand Long-Template PCR System

10x Buffer 3	5µl
dNTP (10mM)	1.75µl
Primer 1 (0.1µg/µl)	1µl
Primer 2 (0.1µg/µl)	1µl
Template DNA	* µl
Enzyme Mix	0.75µl
Milli-Q H_2O	to 50µl

B. Taq DNA Polymerase (Roche)

10x Buffer + $MgCl_2$	5µl
dNTP (10mM)	1µl
Primer 1 (0.1µg/µl)	1µl
Primer 2 (0.1µg/µl)	1µl
Template DNA	* µl
Taq DNA Polymerase	1µl
Milli-Q H_2O	to 50µl

C. Taq DNA Polymerase (Invitrogen)

10x Buffer	5µl
dNTP (10mM)	1µl
$MgCl_2$	1.5µl
1% W-1	2µl
pGADfor Primer (0.1µg/µl)	1µl
pGADrev Primer (0.1µg/µl)	1µl
Template Yeast Colony DNA	5µl
Taq DNA Polymerase	0.5µl
Milli-Q H_2O	33µl

Note: Template DNA volumes (*) varied according to template concentrations and the requirements of specific reactions and are listed below. Milli-Q H_2O volumes were adjusted accordingly.

A1.2 Reaction Conditions

A.	Pre-Heat	94°C	10 sec	
		94°C	Hold	
	Initial Denaturing	94°C	2 min	
	Denaturing	94°C	10 sec	
	Annealing	* °C	30 sec	x * cycles
	Extension	68°C	* min	
	Final Extension	68°C	7 min	
		4°C	Hold	

B.	Pre-Heat	95°C	10 sec	
		95°C	Hold	
	Initial Denaturing	95°C	3 min	
	Denaturing	94°C	10 sec	
	Annealing	55°C	45 sec	x * cycles
	Extension	72°C	45 sec	
	Final Extension	72°C	7 min	
		4°C	Hold	

Note: Annealing temperatures, extension times and cycle numbers (*) varied according to the requirements of specific reactions and are listed below.

A1.3 Specific Reaction Details

A1.3.1
AlHV-1 Left Hand End (21-6216, Cloning and Virus Production)
Recipe: A
Product Size: 6212
Primer 1: AHV_LHEfor+Sal
Primer 2: AHV_LHErev+Xba
Template DNA: 1µl AlHV-1 genomic DNA
Reaction Conditions: A
Annealing Temperature: 60°C
Extension Time: 4 min 20 sec
Cycle Number: 30

A1.3.2
ORF A1 (912-1208, Viral Plaque Screening)
Recipe: B
Product Size: 230
Primer 1: A1_for+Xba
Primer 2: A1_rev+Hind
Template DNA: 10µl crude plaque lysate
Reaction Conditions: A
Annealing Temperature: 58°C
Extension Time: 1 min
Cycle Number: 35

A1.3.3
ORF A1 (912-1208, Recombinant Virus Analysis)
Recipe: B
Product Size: 230
Primer 1: A1_for+Xba
Primer 2: A1_rev+Hind
Template DNA: 1µl genomic viral DNA
Reaction Conditions: A
Annealing Temperature: 58°C
Extension Time: 1 min
Cycle Number: 35

A1.3.4
ORF A1 (912-1208, in vitro RT-PCR)
Recipe: B
Product Size: 230
Primer 1: A1_for+Xba
Primer 2: A1_rev+Hind
Template DNA: 1µl infected BHK-21 cDNA
Reaction Conditions: A
Annealing Temperature: 58°C
Extension Time: 1 min
Cycle Number: 35

A1.3.5
ORF A1 (912-1208, in vivo RT-PCR)
Recipe: B
Product Size: 230
Primer 1: A1_for+Xba
Primer 2: A1_rev+Hind
Template DNA: 1µl lung or spleen cDNA (first round), 1µl first reaction (second round)
Reaction Conditions: A (2 Rounds)
Annealing Temperature: 58°C
Extension Time: 1 min
Cycle Number: 40

A1.3.6
ORF A1 (912-1208, pcDNA3.1/myc-His(-)A Cloning)
Recipe: A
Product Size: 230
Primer 1: A1_for+Xba
Primer 2: A1_rev+Hind
Template DNA: 1µl AlHV-1 genomic DNA
Reaction Conditions: A
Annealing Temperature: 58°C
Extension Time: 1 min
Cycle Number: 30

A1.3.7
ORF A2 (2490-1799, Viral Plaque Screening)
Recipe: B
Product Size: 707
Primer 1: A2_for+Nhe
Primer 2: A2_rev+Bam
Template DNA: 10µl crude plaque lysate
Reaction Conditions: A
Annealing Temperature: 50°C
Extension Time: 1 min
Cycle Number: 35

A1.3.8
ORF A2 (2490-1799, Recombinant Virus Analysis)
Recipe: B
Product Size: 707
Primer 1: A2_for+Nhe
Primer 2: A2_rev+Bam
Template DNA: 1µl genomic viral DNA
Reaction Conditions: A
Annealing Temperature: 50°C
Extension Time: 1 min
Cycle Number: 35

A1.3.9
ORF A2 (2490-1799, in vitro RT-PCR)
Recipe: B
Product Size: 707 (625 spliced)
Primer 1: A2_for+Nhe
Primer 2: A2_rev+Bam
Template DNA: 1µl infected BHK-21 cDNA
Reaction Conditions: A
Annealing Temperature: 50°C
Extension Time: 1 min
Cycle Number: 35

A1.3.10
ORF A2 (2324-1932, in vivo RT-PCR)
Recipe: B
Product Size: 310
Primer 1: A2_for_RT
Primer 2: A2_rev_RT
Template DNA: 2µl lung or spleen cDNA (first round), 1µl first reaction (second round)
Reaction Conditions: A (2 Rounds)
Annealing Temperature: 50°C
Extension Time: 1 min
Cycle Number: 40

A1.3.11
ORF A2 (2490-1799, pcDNA3.1/myc-His(-)A Cloning)
Recipe: A
Product Size: 707
Primer 1: A2_for+Nhe
Primer 2: A2_rev+Bam
Template DNA: 1µl AlHV-1 genomic DNA
Reaction Conditions: A
Annealing Temperature: 50°C
Extension Time: 1 min
Cycle Number: 30

A1.3.12
ORF A2 (2477-1798, pEGFP-N1 and pEGFP-C1 Cloning)
Recipe: A
Product Size: 695
Primer 1: A2_for+Bgl
Primer 2: A2_rev2+Bam
Template DNA: 1µl AlHV-1 genomic DNA
Reaction Conditions: A
Annealing Temperature: 50°C
Extension Time: 1 min
Cycle Number: 30

A1.3.13
ORF A2 cDNA (2477-1794, pEGFP-C1 Cloning)
Recipe: A
Product Size: 699 (617 spliced)
Primer 1: A2_for+Bgl
Primer 2: A2_rev2+Hind
Template DNA: 1µl infected BHK-21 cDNA
Reaction Conditions: A
Annealing Temperature: 48°C
Extension Time: 1 min
Cycle Number: 35

A1.3.14
ORF A2 cDNA (2477-1798, pEGFP-N1 Cloning)
Recipe: A
Product Size: 695 (613 spliced)
Primer 1: A2_for+Bgl
Primer 2: A2_rev2+Bam
Template DNA: 1µl infected BHK-21 cDNA
Reaction Conditions: A
Annealing Temperature: 48°C
Extension Time: 1 min
Cycle Number: 35

A1.3.15
ORF A2 cDNA (2474-1799, pTrcHis2 A Cloning)
Recipe: A
Product Size: 691 (609 spliced)
Primer 1: A2_for_TH-Xho
Primer 2: A2_rev_TH-Hind
Template DNA: 1µl infected BHK-21 cDNA
Reaction Conditions: A
Annealing Temperature: 50°C
Extension Time: 1 min
Cycle Number: 35

A1.3.16
A2 cDNA-GFP (2477-1798, pTrcHis2 A Cloning)
Recipe: A
Product Size: 1353
Primer 1: A2_for+Bgl
Primer 2: GFP_rev+Hind
Template DNA: pEGFP-N1-A2cDNA plasmid
Reaction Conditions: A
Annealing Temperature: 48°C
Extension Time: 1 min
Cycle Number: 40

A1.3.17
GFP-A2 cDNA (2477-1799, pcDNA3.1/myc-His(-)A Cloning)
Recipe: A
Product Size: 1388
Primer 1: GFP_for+Xho
Primer 2: A2_rev+Bam
Template DNA: pTH-GFPA2c plasmid
Reaction Conditions: A
Annealing Temperature: 56°C
Extension Time: 1 min
Cycle Number: 35

A1.3.18
GFP-A2 cDNA (2477-1794, pTrcHis2 A Cloning)
Recipe: A
Product Size: 1383
Primer 1: GFP_for+Xho
Primer 2: A2_rev2+Hind
Template DNA: pEGFP-C1-A2cDNA plasmid
Reaction Conditions: A
Annealing Temperature: 48°C
Extension Time: 1 min
Cycle Number: 40

A1.3.19
ORF A2ΔC (2477-2000, N-term pGBK-T7 Cloning)
Recipe: A
Product Size: 411
Primer 1: A2_for+Nde
Primer 2: A2i_rev+Bam
Template DNA: 1µl infected BHK-21 cDNA
Reaction Conditions: A
Annealing Temperature: 50°C
Extension Time: 1 min
Cycle Number: 35

A1.3.20
ORF A2ΔN (2197-1799, C-term pGBK-T7 Cloning)
Recipe: A
Product Size: 332
Primer 1: A2i_for+Nde
Primer 2: A2_rev+Bam
Template DNA: 1µl infected BHK-21 cDNA
Reaction Conditions: A
Annealing Temperature: 50°C
Extension Time: 1 min
Cycle Number: 35

A1.3.21
ORF A3 (4061-4350, Recombinant Virus Analysis)
Recipe: B
Product Size: 289
Primer 1: A3scr_for
Primer 2: A3scr_rev
Template DNA: 1µl genomic viral DNA
Reaction Conditions: A
Annealing Temperature: 58°C
Extension Time: 1 min
Cycle Number: 35

A1.3.22
ORF A3 (4061-4350, Viral Plaque Screening)
Recipe: B
Product Size: 289
Primer 1: A3scr_for
Primer 2: A3scr_rev
Template DNA: 10µl crude plaque lysate
Reaction Conditions: A
Annealing Temperature: 58°C
Extension Time: 1 min
Cycle Number: 35

A1.3.23
ORF A3 (4061-5455, in vitro RT-PCR)
Recipe: B
Product Size: 1402
Primer 1: A3scr_for
Primer 2: A3C_rev+Sal
Template DNA: 1µl infected BHK-21 cDNA
Reaction Conditions: A
Annealing Temperature: 58°C
Extension Time: 1 min 30 sec
Cycle Number: 35

A1.3.24
ORF A3 (5184-5455, in vivo RT-PCR)
Recipe: B
Product Size: 279
Primer 1: A3CTscr_for
Primer 2: A3C_rev+Sal
Template DNA: 1µl lung or spleen cDNA (first round), 1µl first reaction (second round)
Reaction Conditions: A (2 Rounds)
Annealing Temperature: 58°C
Extension Time: 1 min
Cycle Number: 40

A1.3.25
ORF A3 (3491-5451, Southern Blot Probe Cloning)
Recipe: A
Product Size: 1976
Primer 1: A3_for+Bgl
Primer 2: A3_rev+Eco
Template DNA: 1µl AlHV-1 genomic DNA
Reaction Conditions: A
Annealing Temperature: 56°C
Extension Time: 2 min
Cycle Number: 30

A1.3.26
ORF A3 (3491-5451, pEGFP-C1 Cloning)
Recipe: A
Product Size: 1976
Primer 1: A3_for+Bgl
Primer 2: A3_rev+Eco
Template DNA: 1µl AlHV-1 genomic DNA
Reaction Conditions: A
Annealing Temperature: 56°C
Extension Time: 2 min
Cycle Number: 30

A1.3.27
ORF A3ΔSP (3717-5455, pGBK-T7 Cloning)
Recipe: A
Product Size: 1754
Primer 1: A3_forΔ75
Primer 2: A3C_rev+Sal
Template DNA: 1µl AlHV-1 genomic DNA
Reaction Conditions: A
Annealing Temperature: 56°C
Extension Time: 1 min 30 sec
Cycle Number: 30

A1.3.28
ORF A4 (5732-6094, Viral Plaque Screening)
Recipe: B
Product Size: 380
Primer 1: A4_for+Nhe
Primer 2: A4_rev+Hind
Template DNA: 10µl crude plaque lysate
Reaction Conditions: A
Annealing Temperature: 62°C
Extension Time: 1 min
Cycle Number: 35

A1.3.29
ORF A4 (5732-6094, Recombinant Virus Analysis)
Recipe: B
Product Size: 380
Primer 1: A4_for+Nhe
Primer 2: A4_rev+Hind
Template DNA: 1µl genomic viral DNA
Reaction Conditions: A
Annealing Temperature: 62°C
Extension Time: 1 min
Cycle Number: 35

A1.3.30
ORF A4 (5732-6094, in vitro RT-PCR)
Recipe: B
Product Size: 380
Primer 1: A4_for+Nhe
Primer 2: A4_rev+Hind
Template DNA: 1µl infected BHK-21 cDNA
Reaction Conditions: A
Annealing Temperature: 62°C
Extension Time: 1 min
Cycle Number: 35

A1.3.31
ORF A4 (5732-6094, in vivo RT-PCR)
Recipe: B
Product Size: 380
Primer 1: A4_for+Nhe
Primer 2: A4_rev+Hind
Template DNA: 1µl lung or spleen cDNA (first round), 1µl first reaction (second round)
Reaction Conditions: A (2 Rounds)
Annealing Temperature: 62°C
Extension Time: 1 min
Cycle Number: 40

A1.3.32
ORF A4 (5732-6094, pcDNA3.1/myc-His(-)A Cloning)
Recipe: A
Product Size: 378
Primer 1: A4_for+Nhe
Primer 2: A4_rev+Hind
Template DNA: 1µl AlHV-1 genomic DNA
Reaction Conditions: A
Annealing Temperature: 62°C
Extension Time: 1 min
Cycle Number: 30

A1.3.33
ORF A4 (5732-6094, pEGFP-N1 and pEGFP-C1 Cloning)
Recipe: A
Product Size: 378
Primer 1: A4_for+Bgl
Primer 2: A4_rev+Hind
Template DNA: 1µl AlHV-1 genomic DNA
Reaction Conditions: A
Annealing Temperature: 58°C
Extension Time: 1 min
Cycle Number: 35

A1.3.34
ORF A4ΔSP (5822-6097, pGBK-T7 Cloning)
Recipe: A
Product Size: 291
Primer 1: A4_forΔSP
Primer 2: A4_rev+Bam
Template DNA: 1µl AlHV-1 genomic DNA
Reaction Conditions: A
Annealing Temperature: 56°C
Extension Time: 1 min
Cycle Number: 30

A1.3.35
Trpt/M4B (Viral Plaque Screening)
Recipe: B
Product Size: 316
Primer 1: Trepeat
Primer 2: M4B
Template DNA: 10µl crude plaque lysate (first round), 1µl first reaction (second round)
Reaction Conditions: B (2 Rounds)
Cycle Number: 40

A1.3.36
Trpt/M4B (Recombinant Virus Analysis)
Recipe: B
Product Size: 316
Primer 1: Trepeat
Primer 2: M4B
Template DNA: 1µl genomic viral DNA (first round), 1µl first reaction (second round)
Reaction Conditions: B (2 Rounds)
Cycle Number: 40

A1.3.37
ORF A4/M4B (Recombinant Virus Analysis)
Recipe: B
Product Size: 788
Primer 1: A4_for+Nhe1
Primer 2: M4B
Template DNA: 1µl genomic viral DNA
Reaction Conditions: A
Annealing Temperature: 62°C
Extension Time: 1 min 30 sec
Cycle Number: 35

A1.3.38
MHV ORF 50 (in vitro RT-PCR)
Recipe: B
Product Size: 292
Primer 1: MHV_ORF_50_F
Primer 2: MHV_ORF_50_R
Template DNA: 1µl infected BHK-21 cDNA
Reaction Conditions: B
Cycle Number: 35

A1.3.39
MHV ORF 50 (in vivo RT-PCR)
Recipe: B
Product Size: 292
Primer 1: MHV_ORF_50_for
Primer 2: MHV_ORF_50_rev
Template DNA: 1µl lung or spleen cDNA (first round), 1µl first reaction (second round)
Reaction Conditions: B (2 Rounds)
Cycle Number: 40

A1.3.40
β-actin (in vivo RT-PCR)
Recipe: B
Product Size: 810
Primer 1: B-actin-for
Primer 2: B-actin-rev
Template DNA: 1µl (lung) or 5µl (spleen) cDNA
Reaction Conditions: B
Cycle Number: 40

A1.3.41
GFP-C (pTrcHis2 A Cloning)
Recipe: A
Product Size: 751
Primer 1: GFP_for+Xho
Primer 2: GFP_rev+Hind
Template DNA: pEGFP-C1 plasmid
Reaction Conditions: A
Annealing Temperature: 56°C
Extension Time: 1 min
Cycle Number: 30

A1.3.42
pGAD-T7 Insert PCR
Recipe: C
Reaction Conditions: A
Annealing Temperature: 60°C
Extension Time: 4 min
Cycle Number: 35

A1.4 Primers

Primer	Sequence
A1_for+Xba	GGT CTA GAC CTT CAT GAG GCT ACA TTT TC
A1_rev+Hind	GGA AGC TTT TTT GTG GTG TCT TCA TTT GC
A2_for+Bgl	GAA GAT CTA TGT CCC AAA ACT CAA ATT C
A2_for+Nde	GGC ATA TGA TGT CCC AAA ACT CAA ATT C
A2_for+Nhe	GGG CTA GCC AAA TCC TCA AAA ATG TCC C
A2_for_RT	CAA CAA GAA AAA GGG CTG
A2_for_TH+Xho	GGC TCG AGA TCC CAA AAC TCA AAT TCT GA
A2i_for+Nde	GGC ATA TGC TGC GAT GCC AAG TGG
A2_rev+Bam	GGG GAT CCT AAA TTG TAC AAG TTG TTC C
A2_rev2+Bam	GGG GAT CCC ATA AAT TGT ACA AGT TGT TC
A2_rev2+Hind	GGA AGC TTT TAT AAA TTG TAC AAG TTG T
A2_rev_RT	GTG TGT AAG GTA GCT GTT GC
A2_rev_TH+Hind	GGA AGC TTT AAA TTG TAC AAG TTG TTC C
A2i_rev+Bam	GGG GAT CC TTT AAG TTG TTA GTT GTA TAC
A3_for+Bgl	GGA GAT CTA TGG CCT ACT TAA ATG CTA C
A3_forΔ75	GGC ATA TGA GGC TGA TTG TTA ACC TAA C
A3CTscr_for	CAC TAT GCT CCA ACT GTG G
A3scr_for	CTT AGC CCC CTT CTC ACC AT
A3_rev+Eco	GGG AAT TCA CAT CAG TGT CAC ATT CAC CTC
A3C_rev+Sal	GGG TCG ACT GTT ACA TCA GTG TCA CAT TC
A3scr_rev	CCT GCC CCA CTC TAG GTA CA
A4_for+Bgl	GGA GAT CTA TGG TTG CTC AGC TCT ACC
A4_for+Nhe	GGG CTA GCC ATG GTT GCT CAG CTC TAC C
A4_forΔSP	GGC ATA TGG ATG ATG AAG AGG ACT TC
A4_rev+Bam	GGG GAT CCT TAT GGT ATT GCT GAT GGT AG
A4_rev+Hind	GGA AGC TTT GGT ATT GCT GAT GGT AGT AC
AHV_LHE_for+Sal	GGG TCG ACG CAC TTT TCT GGC TCT CTG G
AHV_LHE_rev+Xba	GGT CTA GAT ACT GTG GCC TGT GGT TAG C
B-actin-for	CGA GCA CAG CTT CTT TGC AG
B-actin-rev	GCC AAT AGT GAT GAC CTG GC
GFP_for+Xho	GGC TCG AGA ATG GTG AGC AAG GGC GAG G
GFP_rev+Hind	GGA AGC TTT TAC TTG TAC AGC TCG TCC A
MHV_ORF_50_for	AGG GCT AAT GGG TGA AAA TGG C
MHV_ORF_50_rev	AAA AGT TCT GCA TCC CAG ACC C
M4B	CGC GGA ATT CGG TTC TAG AAA GTC ATA AAT CTC AAT ACC
pGAD-for	TAC GAC GTA CCA GAT TAC GC
pGAD-rev	TCA GTA TCT ACG ATT CAT CTG C
Trepeat	GCG CAA GGA GGA GCT AGG CCA CG

Sequences in red indicate inserted restriction endonuclease sites.

Appendix 2: Luciferase Readings

	Firefly	Renilla	Compensated
A2/ORF 6	62.57	230.6	0.271336
	49.06	245.9	0.199512
	24.9	100.3	0.248255
pcDNA/ORF 6	45.96	156.8	0.293112
	31.58	107.4	0.294041
	39.27	126.8	0.3097
A2/ORF 21	7.455	52.31	0.142516
	8.12	84.45	0.096152
	10.89	111.8	0.097406
pcDNA/ORF 21	7.234	48.25	0.149927
	7.962	79.52	0.100126
	9.764	72.06	0.135498
A2/ORF 50	138.1	177.2	0.779345
	60.71	114.4	0.530682
	55.75	108.1	0.515726
pcDNA/ORF 50	128.5	166.1	0.77363
	99.33	161.3	0.615809
	117.2	166	0.706024
A2/ORF 57	1.752	73.66	0.023785
	1.988	138.9	0.014312
	0.923	58.32	0.015826
pcDNA/ORF 57	1.672	80.74	0.020708
	1.979	95.72	0.020675
	1.195	98.2	0.012169
A2/ORF A9	2.258	103.8	0.021753
	2.047	141.2	0.014497
	0.888	88.54	0.010029
pcDNA/ORF A9	1.518	113.2	0.01341
	1.63	153.7	0.010605
	1.564	115.5	0.013541
A2/pGL3	3.908	32.03	0.122011
	4.584	81.1	0.056523
	8.273	90.48	0.091435
pcDNA/pGL3	8.579	62.46	0.137352
	11.89	129.1	0.092099
	12.81	117.4	0.109114
A2/CMV	2661	80.32	33.12998
	1698	90.8	18.70044
	1825	68.68	26.57251
pcDNA/CMV	1867	86.77	21.51665
	2488	100.1	24.85514
	1621	107.1	15.13539

Appendix 3: ExPasy Peptide Cutter Results

The sequence to investigate:

```
        10         20         30         40         50         60
MSQNSNSESP SPRKKRYVKM CDLTEEQKER RRSINRRASK NFLKRRRIFE EQQEPQEIRI

        70         80         90        100        110        120
LREWLNYHKC TTLQNYNTGP PEPRVKVENS LEMQCATAFL NLDQQYTTNN LNIPETVSGN

       130        140        150        160        170
NTTNGFAAAT ATLHTNCYEK TLANNTNNFE AKLNCEVLPS FTSALDDLLS IDWIINLYNL
```

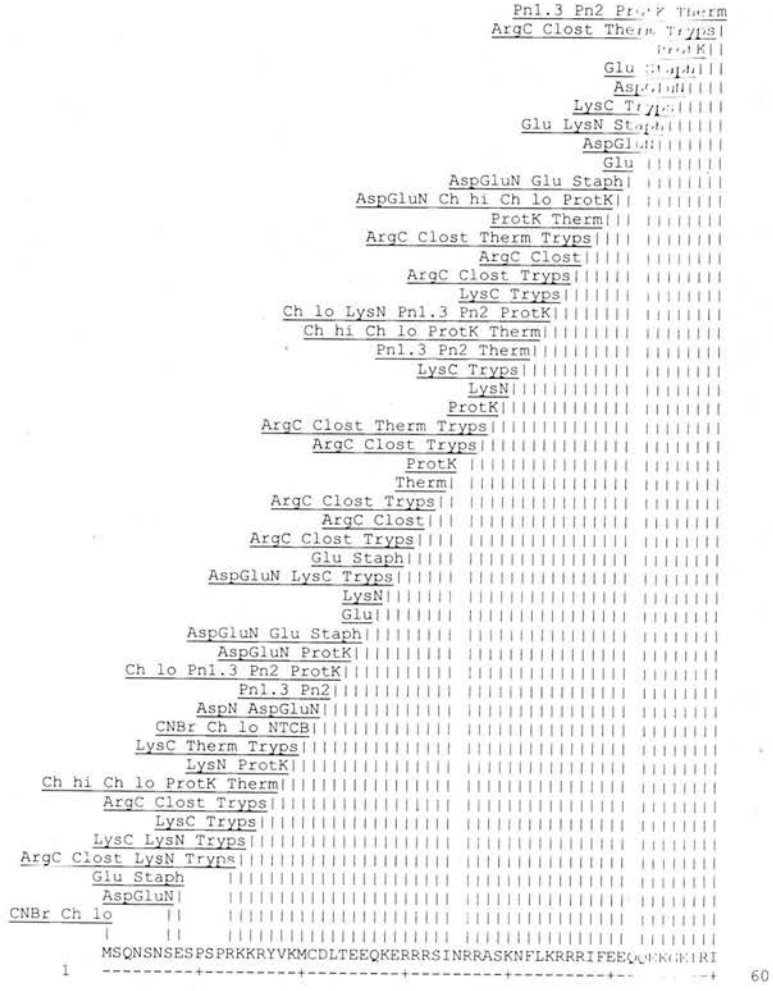
These chosen enzymes do not cut:

Caspase1
Caspase10
Caspase2
Caspase3
Caspase4
Caspase5
Caspase6
Caspase7
Caspase8
Caspase9
Enterokinase
Factor Xa
Formic acid
GranzymeB
Proline-endopeptidase
Thrombin

These enzymes cleave the sequence:

Arg-C proteinase	13	13 16 30 31 32 36 37 45 46 47 59 62 84
Asp-N endopeptidase	5	21 102 165 166 171
Asp-N endopeptidase + N-terminal Glu	21	7 21 24 25 28 49 50 53 56 62 81 87 91 102 114 138 149 155 165 166 171
BNPS-Skatole	2	64 173
CNBr	3	1 20 93
Chymotrypsin-high specificity (C-term to [FYW], not before P)	14	17 42 49 64 67 76 99 106 126 138 149 161 173 177
Chymotrypsin-low specificity (C-term to [FYWML], not before P)	36	1 17 20 23 42 43 49 61 64 65 67 68 73 76 91 93 99 100 102 106 111 126 133 134 138 142 149 153 161 165 168 169 173 176 177 179
Clostripain	13	13 16 30 31 32 36 37 45 46 47 59 62 84
Glutamyl endopeptidase	16	8 25 26 29 50 51 54 57 63 82 88 92 115 139 150 156
Hydroxylamine	1	124
Iodosobenzoic acid	2	64 173
LysC	11	14 15 19 28 40 44 55 69 86 140 152
LysN	11	13 14 18 27 39 43 54 68 85 139 151
NTCB (2-nitro-5-thiocyanobenzoic acid)	5	20 69 94 136 154
Pepsin (pH1.3)	52	22 23 41 43 60 63 65 66 67 72 73 75 76 90 91 98 99 99 100 101 102 105 106 110 111 125 126 132 133 137 138 141 148 149 152 153 158 161 164 165 167 168 168 169 172 173 175 176 176 177 178 179
Pepsin (pH>2)	39	22 23 41 43 60 65 72 73 90 91 98 99 99 100 101 102 110 111 125 126 132 133 141 148 149 152 153 158 161 164 165 167 168 168 169 175 176 178 179
Proteinase K	69	17 18 23 24 34 38 42 43 48 49 58 60 61 64 65 67 71 72 73 76 78 85 87 91 96 97 98 99 100 102 106 107 108 111 113 116 117 122 123 126 127 128 129 130 131 132 133 135 138 141 142 143 146 149 151 153 157 158 161 162 164 165 168 169 171 173 176 177 179
Staphylococcal peptidase I	14	8 25 29 50 54 57 63 82 88 92 115 139 150 156
Thermolysin	39	17 19 33 37 41 42 47 48 59 60 64 72 84 86 90 95 97 98 99 101 110 116 125 126 127 128 130 132 141 142 148 152 160 163 164 168 170 175 178
Trypsin	22	13 14 15 16 19 28 30 32 36 37 40 44 45 47 55 59 62 69 84 86 140 152

Digest Map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ProtK
Glu Staph
AspGluN
LysC Tryps
Glu LysN Staph
AspGluN
Glu
AspGluN Glu Staph
AspGluN Ch hi Ch lo ProtK
ProtK Therm
ArgC Clost Therm Tryps
ArgC Clost
ArgC Clost Tryps
LysC Tryps
Ch lo LysN Pn1.3 Pn2 ProtK
Ch hi Ch lo ProtK Therm
Pn1.3 Pn2 Therm
LysC Tryps
LysN
ProtK
ArgC Clost Therm Tryps
ArgC Clost Tryps
ProtK
Therm
ArgC Clost Tryps
ArgC Clost
ArgC Clost Tryps
Glu Staph
AspGluN LysC Tryps
LysN
Glu
AspGluN Glu Staph
AspGluN ProtK
Ch lo Pn1.3 Pn2 ProtK
Pn1.3 Pn2
AspN AspGluN
CNBr Ch lo NTCB
LysC Therm Tryps
LysN ProtK
Ch hi Ch lo ProtK Therm
ArgC Clost Tryps
LysC Tryps
LysC LysN Tryps
ArgC Clost LysN Tryps
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CNBr Ch lo
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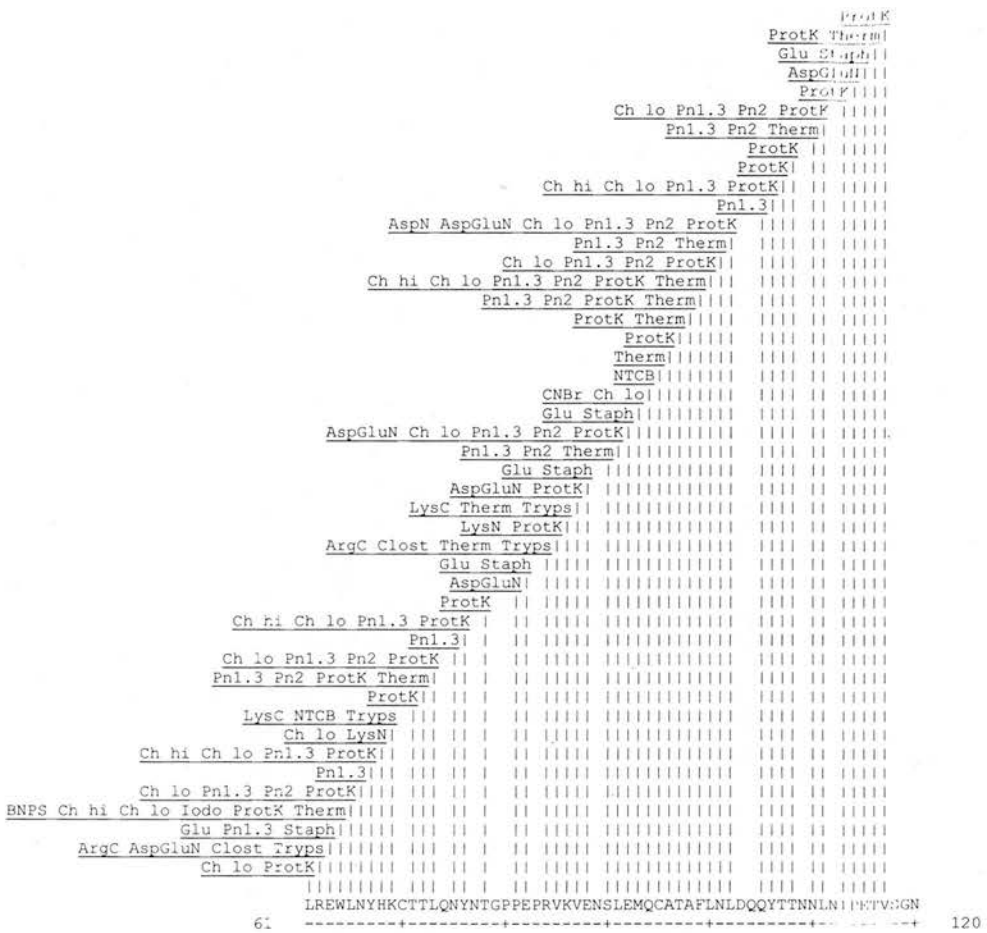
1
60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ProtK Therm
Glu Staph
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ProtK
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ProtK
ProtK
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Pn1.3
AspN AspGluN Ch lo Pn1.3 Pn2 ProtK
Pn1.3 Pn2 Therm
Ch lo Pn1.3 Pn2 ProtK
Ch hi Ch lo Pn1.3 Pn2 ProtK Therm
Pn1.3 Pn2 ProtK Therm
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ProtK
Therm
NTCB
CNBr Ch lo
Glu Staph
AspGluN Ch lo Pn1.3 Pn2 ProtK
Pn1.3 Pn2 Therm
Glu Staph
AspGluN ProtK
LysC Therm Tryps
LysN ProtK
ArgC Clost Therm Tryps
Glu Staph
AspGluN
ProtK
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Ch lo Pn1.3 Pn2 ProtK
Pn1.3 Pn2 ProtK Therm
ProtK
LysC NTCB Tryps
Ch lo LysN
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Pn1.3
Ch lo Pn1.3 Pn2 ProtK
BNPS Ch hi Ch lo Iodo ProtK Therm
Glu Pn1.3 Staph
ArgC AspGluN Clost Tryps
Ch lo ProtK
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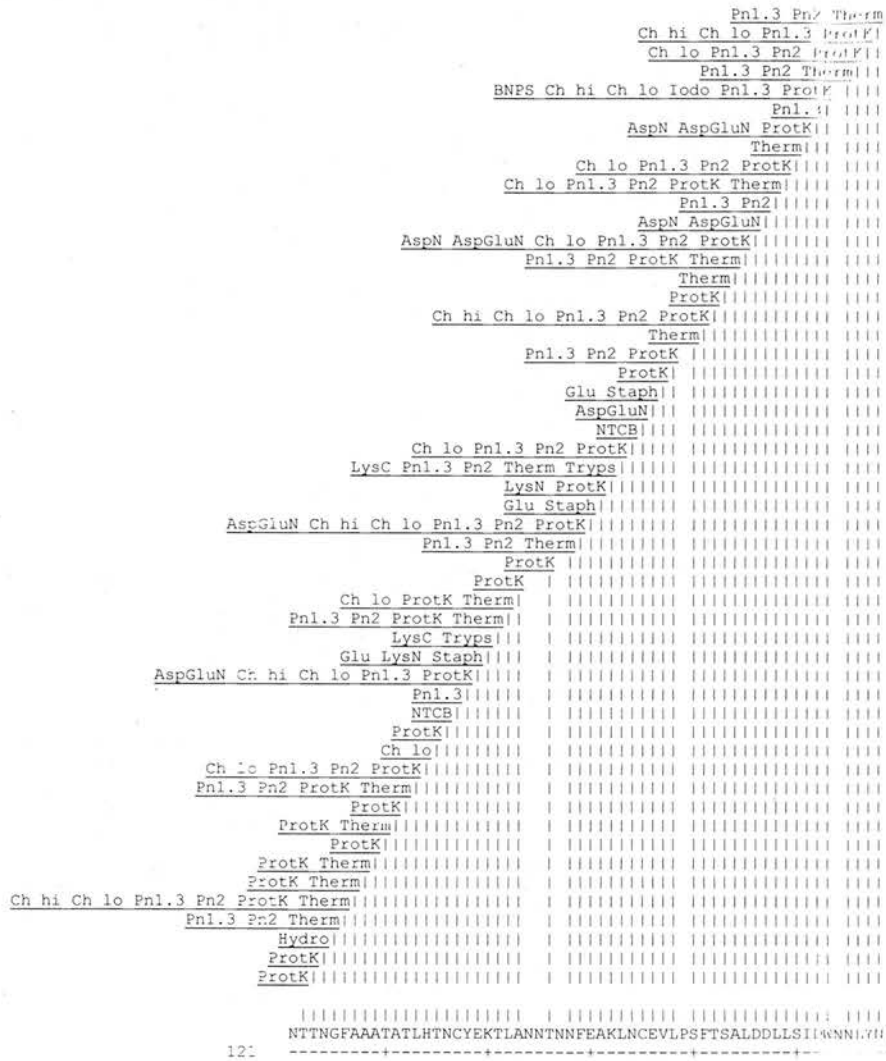
61
120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Ch hi Ch lo Pn1.3 ProtK
Ch lo Pn1.3 Pn2 ProtK
Pn1.3 Pn2 Therm
BNPS Ch hi Ch lo Iodo Pn1.3 ProtK
Pn1.3
AspN AspGluN ProtK
Therm
Ch lo Pn1.3 Pn2 ProtK
Ch lo Pn1.3 Pn2 ProtK Therm
Pn1.3 Pn2
AspN AspGluN
AspN AspGluN Ch lo Pn1.3 Pn2 ProtK
Pn1.3 Pn2 ProtK Therm
Therm
ProtK
Ch hi Ch lo Pn1.3 Pn2 ProtK
Therm
Pn1.3 Pn2 ProtK
ProtK
Glu Staph
AspGluN
NTCB
Ch lo Pn1.3 Pn2 ProtK
LysC Pn1.3 Pn2 Therm Tryps
LysN ProtK
Glu Staph
AspGluN Ch hi Ch lo Pn1.3 Pn2 ProtK
Pn1.3 Pn2 Therm
ProtK
ProtK
Ch lo ProtK Therm
Pn1.3 Pn2 ProtK Therm
LysC Tryps
Glu LysN Staph
AspGluN Ch hi Ch lo Pn1.3 ProtK
Pn1.3
NTCB
ProtK
Ch lo
Ch lo Pn1.3 Pn2 ProtK
Pn1.3 Pn2 ProtK Therm
ProtK
ProtK Therm
ProtK
ProtK Therm
ProtK Therm
Ch hi Ch lo Pn1.3 Pn2 ProtK Therm
Pn1.3 Pn2 Therm
Hydro
ProtK
ProtK
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121
179

Appendix 4: Yeast Two-Hybrid Sequences

Colony: A2B4
Sequence:
GGNGCNTGGANTCGGCCGAGGGTTTTTTTCATTTTATTTAATTTATTTATGT
AATACAGTGTAGAACGCTATCATGGTCATACGCAATGATCCTGTACAATC
ATCCTGCAGAAAAGTTTTTTGGGAGAATTCTTGGTAAATTGAGACCAGCA
GAGCGCTCCCACCCCCACCCTCCCCAACCCGTGAAAGTGCTTACAATGAA
CAGGGATTCTTTTCTTTATCAAAGATCTGAAGATACATGGACGAAAAAAG
CTTCAATTCTCAATGCCTAATGTGTGCACATAAAACAGGCACGAAGAAAT
AAATGTCTATCCTCATACTTCCTATATCACACATATAGCAGGAGCAGCAAT
CTGTACAGTAAAATGCAATTGTGGAAAAAAAATTCCTCTAACTCATTTGG
ATTACATAAAAACGTTGAACTTTAAAATGCATAGTGATCAGGAAAAGAAT
ACTTAGGCAAGAGAAGCAGCTGCAGATGAATCGANNATACTGAA

Colony: A3B8
Sequence:
GCGAATTCACTAGTGATTCAGTATCTACGATTCATCTGCAGCTTTTTATTG
GAAGAGTGTTGAACGTTTATTTATTATAGCACACTTGAGACATTTTGAAAT
TGAAAGTTGGTAAAAAAATAGAACAATAACGATTTGAACTGTATTTGGTG
TAATACGAAAAACAAATATGCGTTTTTTGGTCAGCTTACACTTTTTTCAGA
AGAACTTTTGGAAATAAATAACTGGAAATTGGTCACTTGTACTGGCTGAC
AAGATTAGAATAAAACGGAACATATGGAGTTGAAGCTTTTTCTGGGACTT
TTGGTAGAAGTTGG

Colony: A3B9
Sequence:
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GCTGGGATGCCCTCAGACTGCACTGGACCAGCCCCGATGGGATCTATGAA
CGGTTTGTCATTAAGATCCGGGAGACTGACCAGCCCCAAGAAGTTCACAG
TCTCACGGTTCCTGGCAGCCAGCACTCCGTGGAGATTCCAGGCCTCAAGG
CTGGTACCTCTTACACAATCACCCTGCGTGGCGAGGTCAGGGACCACAGC
ACTCAACCCCTTGCTGTGGAGGTCATCACAGCGGAGcTCCCCCCAGCTGGG
AGACTTAGTCGTGACTGAGGCTGGCTGGGATGGCCTCAGACTCAACTGGA
CCGCAGCTGATCAGGCCTTTGAGCACTTTGGTCATTCAGGCGCAGGAGGC
CAACAGGGTGGAGGCGGCTCAGAACTCACGGTGCCAGGGAACATGCGGG
CTGTGGACATCCCGGGCCTGAAGGCCGCCACCCCTTTACAGAGTCACATC
TA

Colony: A4B1
Sequence:
GCGAATTCACTAGTGATTTACGACGTACCAGATTACGCTCATATGGCCAT
GGAGGCAGTGAATTCGGCACGAGGGGCCCACAAGGTATTCGAGGTGACA
AGGGAGAGCCTGGTGATAAGGGTCCCAGAGGTCTTCCTGGCTTAAAGGGA
CACAATGGGTTGCAAGGTCTCCCGGGTCTTGCTGGTCATCATGGCGATCA
AGGTGCTCCCGGTGCTGTGGGTCCCGCTGGTCCCAGGGGCCCTGCTGGTC
CTTCTGGCCCCGCTGGCAAAGACGGTCGCATTGGACAGCCTGGTGCAGTC
GGACCTGCTGGCATTCGTGGCTCTCAGGGTAGCCAAGGTCCTGCTGGCCC
TCCTGGTCCCCCTGGCCCTCCTGGACCTCCTGGCCCAAGTGGTGGTGGTTA
CGAGTTTGGTTTGATGGAGACTTCTACAGGGCTGACCAGCCTCGCTCACC
AACTTCTCTCAGACCCAAGGATTATGAAGTTGATGCTACTCTGAAATCTCT
CAACAACCAGATTGAGACCGTTCTTACTCAGAAGGCTCTAGGAAGAACCA

AGCTCGACATGCCGAGACTTGAGACTCAGCAACCAGGATGGAGCAGTGGT
TACT

Colony: A4B5
Sequence:
GCGAATTCACTAGTGATTTCAGTATCTACGATTCATCTGCAGCTCGAGATT
GGTCAGTGCATACTCCACCGTGTTCCCGGACACCGTGCGGGTAATTTCTGG
CACTCTCTCCCCTGTGTAGGAGATGACGTaNTTATCCACAGGGGCGATGGC
TGGCTGCCACATGGCCAAGGCTTCTGAGTCAGTGATGTTGGCTGTCACCA
AGCTAGATGGCCATCCAGAGCTGTGGTGAGCGTCCCCGAGACAGGCTCGC
TTTCCTCAAAGCCCTTCAGGGCGATGACGCTGACCAGGTAATCCGCTCCA
GGTAAGAGTCTCAGCAGCCTGGTCTGGGTCTTGGTCCCATCCACAGTCAC
CGCTGAGGGCGCACCTCCTGCAATGGGCACATAGGTGACCCGGAAGCTCT
CCACCTGGGTAGTGGGTGCCATCCAGCTGACACTGGCTGCGTTTTCAGTG
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Colony: A4B7
Sequence:
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TTGGCATTTGTATTAATAGTTAAAGTTCACTTCCAAATAAAACTCCCATAA
TGCTAGATTTGATGTGTGCCCAATTTTGAACAAGGGTTGATTGACACCTGT
AAAATTTGTTAAAACGTTCCTCTTCTAAGGAAATACAATAATCTTAAATTA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
AA

Colony: A4B16
Sequence:
GCGAATTCACTAGTGCATTTACGACGTACCAGATTACGCTCATATGGCCAT
GGAGGCAGTGAATTCGGCACGAGGCAAGGCTGCAACCTGGATGCCATTA
AGGTCTTCTGCAACATGGAAACCGGTGAGACCTGTGTATACCCCACTCAG
CNCAGCGTGGCCCAGAAGAACTGGTATATCAGCAAGAACCCCAAGGAAA
AGAGGCACGTCTGGTACGGCGAGAGCATGACCGGCGGATTCCAGTTCGAG
TATGGCGGCCAGGGGTCCGATCCTGCCGATGTGGCCATCCAGCTGACTTT
CCTGCGCCTGATGTCCACCGAGGCCTCCCAGAACATCACCTACCACTGCA
AGAACAGCGTGGCCTACATGGACCAGCAGACTGGCAACCTCAAGAAGGC
CTGCTCCTCCAGGGCTCCAACGAGATCGAGATCCGGGCCGAGGGCAACAG
CCGCTTCACCTACAGCGTCACCTACGATGGCTGCACGAGTCACACCGGAG
CCTGGGGCAAGACAGTGATCGAATACAAACCACCAAGACCTCCGGTTGCC
ATCATCGATGTGGCCCCTTGGACGTTGGCGCCCAGACCAGGATTTCGCTTC
G

Colony: A4B28
Sequence:

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AAATCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAGC
TCCCAACGCGTTGGATGCATAGCTTGAGTATTCTATAGTGTCACCTAAATA
GCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAATTGTTATCCGCT
CACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGG
GGTGCCTAATGAGTGAGCTAACTCACATTAATTGGCGTTGCGCTCACTGCC
CGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGGAATCGGC
CAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCT

Colony: A4B30
Sequence:
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GTGCATACTCCACCGTGTTCCCGGACACCGTGCGGGTAATTTCTGGCACTC
TCTCCCCTGTGTAGGAGATGACGTAATTATCCACAGGGGCGATGGCTGGC
TGCCACATGGCCAAGGCTTCTGAGTCAGTGATGTTGGCTGTCACCAAGCT
AGATGGGCCATCCAGAGCTGTGGTGAGCGTCCCCGAGACAGGCTCGCTTT
CCTCAAAGCCCTTCAGGGCGATGACGCTGACCAGGTAATCCGCTCCAGGT
AAGAGTCTCAGCAGCCTGGTCTGGGTCTTGGTCCCATCCACAGTCACCGCT
GAGGGCGCACCTCCTGCAATGGGCACATAGGTGACCCGGAAGCTCTCCAC
CTGGGTAGTGGGTGCCATCCAGCTGACACTGGCTGCGTTTTCAGTGATGTC
TGAGAAAATGATTTCCTTCGGGGAGCCCATGGCTGTTGTTGCTATGGCAGT
GACTGGCTGGGATCGCCTTCCACTGCTTATTCCATAGAGTTCAATTTCATA
TTCAGTGGCCTCTCTGAGACCTGTAATGTCCCTGGTTCGTTCGGGGGCAAG
GAGGGTTATCTCCAGTGGCTCGGACTGCTTTTTGGTATCTCTGATTTTGAG
AACAAAACTGTCGAAGCTCCTTCATCAGCTGTCCAGGCAGACGGAACCAT
CTGGGGTGGCATCTG

Colony: A4B34
Sequence:
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GGTGTGGCGGCGAGGTGACCTCGAGGCTGCGGTGACCATGGGCCGCCAGT
TTGGGCATCTGACACGGGTGCGGCATGTGATCACCTACAGCTTGTCGCCCT
TCGAGCAGCGCGCCTTCCCGCACTACTTCAGCAAGGGCATCCCCAACGTT
CTGCGCCGAACTCGGGCGTGCATCCTTCGCGTCGCGCCGCCGTTCGTAGC
GTTTTATCTTGTCTACACATGGGGAACGCAGGAGTTTGAGAAATCGAAGA
GGAAGAATCCAGCTGCCTATGAAAATGACAGATAAGCAGCTCATCTGGAT
AATGGTTCCTTGTCTCTGAAAGACCCTTCTCTGGGAGAGGAGTGTATATTG
TAGTGTCTTGAAGACACAATAAACTTATGGGCTTCAAAAAAAAAAAAAAAA
AAAAAAAAACCTCGTGCCGAATTCACTGGCCTCCATGGCCATATGAGCGT
AATCTGGTACGTCGTAAATCACTAGTGAATTCGCGGCCGCCTGCAAGGTC
GACCATAT

Colony: A4B40
Sequence:
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GCTGGGCTTCATGACGGGCTTATCGGTAGGATTTCTGGTAGCGGGCACGG
GCACCAGGACCTCCAAACTTCTTGGATTCGCAGCGACGGGGATCGGCTAC
CAGCAGGGTCCGGTCATACTGGATGAGGATGTCTTTGATCTCCTTCTTGGA
AGCCTCATCCACATATTTCTGGTAATAGGCCACCAAGGCTTTGGAGATGG

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TTCCAGTAGCTTGTATTGCAGCGTGCGCGGTTCGATCATCTCCAGGGGTCG
TACGTTCACCTTGATGAGGCCGTTACCTCGTTTGCAGTGCGCCACGGTGTG
GCCGTCTTCTTACGTCCGAAGACTTGCACCGACTGAAGAGGGCCCTTGGA
CGGCATGGCTCAAGGCGCAGAGACGAGATCCTCACTGCGCGCCTCGTGCC
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TCACTAGTGAATTCGCGGCCGCCTGCAGGTCGACCATATGGGAGAG

Colony: A4B41
Sequence:
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ATCAGCAAGCAGTTCCATCACCAGC

Colony: A4B50
Sequence:
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GCCACATGGCCAAGGCTTCTGAGTCAGTGATGTTGGCTGTCACCAAGCTA
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Colony: A4B52
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TCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATACCTGTCC
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TATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAA
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TCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAA
CAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGT

GGTGGCCTAACTACGGCTACACTAGAAGAACAGTATTTGGTATCTGCGCT
CTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTGGATCCGG
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Colony: A4B62
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GGGATGGCCTCAGACTCAACTGGACCGCAGCTGATCAGGCCTTTGAGCAC
TTTGTCATTCAGGCGCAGGAGGCCAACAGGGTGGAGGCGGCTCAGAACCT
CACGGTGCCAGGGAACATGCGGGCTGTGGACATCCCGGGCCTGAAGGCC
GCCACCCCTTACAGAGTCACCATCCACGGGGTGATCCGGGGCTATAGGAC
CCCAGTGCTCTCTGCTGAGGCCTCCACAGCCAGAGAACCTGAACTTGGAA
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CTACTGATGGTGCCTTCGAGACCTTTACCGTTGAAATTATTGATTCCAATA
GGTTCCTGGAGACCATGGAATATAACGTCTCTGGTGCTGAACGAACAGCC
CACATCTCAGGGCTCCGCCCTAGTAATGACTTTATTGTCTACCTCTCTGAN
TCGCTCCAGCATGCANACAAAACATCAGTGCACGGCAGCACAATGCACCC
AAAGGNTCCTCTGTCTGAAAGTGAGAAGATCTCAAAGTTTGTCTCAATCA
AAAACAAAACATCNANCNTGGAANACCTCTGCCCAAAACAGATTNGGTT
NANGCCTGANNATTACTTTGANACNNGAGNNCCCCTCTGCN

Colony: A4B80
Sequence:
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CCTGGTGATAAGGGTCCCAGAGGTCTTCCTGGCTTAAAGGGACACAATGG
GTTGCAAGGTCTCCCGGGTCTTGCTGGTCATCATGGCGATCAAGGTGCTCC
CGGTGCTGTGGGTCCCGCTGGTCCCAGGGGCCCTGCTGGTCCTTCTGGCCC
CGCTGGCAAAGACGGTCGCATTGGACAGCCTGGTGCAGTCGGACCTGCTG
GCATTCGTGGCTCTCAGGGTAGCCAAGGTCCTGCTGGCCCTCCTGGTCCCC
CTGGCCCTCCTGGACCTCCTGGCCCAAGTGGTGGTGGTTACGAGTTTGGTT
TTGATGGAGACTTCTACAGGGCTGACCAGCCTCGCTCACCAACTTCTCTCA
GACCCAAGGATTATGAAGTTGATGCTACTCTGAAATCTCTCAACAACCAG
ATTGAGACCCTTCTTACTCCAGAAGGCTCTAGGAAGAACCCAGCTCGCAC
ATGCCGAGACTTGAGACTCAGCCACCCAGAATGGAGCAGTGGTTACTACT
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AGTCAAGAACTGGTACAGAATTCCAAGGNCAANAACATGTTTGGGTAGG
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Colony: A4B82
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TNTGNTTNNNCNGNNTNTNANTNCNANNNTNNTTTANNGNNNGNGGNGTT
ANNNATGGTTTNNTANGCNGGATNGGCNNNGNTAGNANCATNGNGNGAT
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GTGTNNGCTGNATAGGNGGTTNGGNANGTTNNTAGTTNGANNNNNCTTGG
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Colony: A4B83
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GCNNNNGTNTNNGTAGCNNATNNNANCATANNTGTANNGGCCATANTNA
GNCNTCNTNTANTTNCTCTCTCTTCCGTATNCCNTNATNGNGTCNCGTCAN
TNCGTNTTNANCNNNGNTTANNTGACCTTNTATAGTCNTATANTGTNNNG
TNTG

Colony: A4B86
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GCCTGGTGATAAGGGTCCCAGAGGTCTTCCTGGCTTAAAGGGACACAATG
GGTTGCAAGGTCTCCCGGGTCTTGCTGGTCATCATGGCGATCAAGGTGCTC
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CCTGGCCCTCCTGGACCTCCTGGCCCAAGTGGTGGTGGTTACGAGTTTGGT
TTTGATGGAGACTTCTACAGGGCTGACCAGCCTCGCTCACCAACTTCTCTC
AGACCCAAGGATTATGAAGTTGATGCTACTCTGAAATCTCTCAACAACCA
GATTGAGACCCTTCTTACTCCAGAAGGCTCTAGGAAGAACCCAGCTCGCA

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NAGGANTGGCTACCAATTGCNTTATGNTTTGTTGGCAANCTGCTTTNAAAT
TCNTANNTTGAANAAGCTGCNNNNTGTTGANAAATGAACTNAAAAGTGNT
TTTNAGAACAANNNTAATNTTCAANNAAAAATTNTNANNTTTTGAATNNT
TTAAAAAATANNAAAA

Colony: A4B96
Sequence:
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GGCTGCAACCTGGATGCCATTAAGGTCTTCTGCAACNTGGAAACCGGTGA
GACCTGTGTATACCCCACTCAGCCCAGCGTGGCCCAGAAGAACTGGTATA
TCAGCAAGAACCCCAAGGAAAAGAGGCACGTCTGGTACGGCGAGAGCAT
GACCGGCGGATTCCAGTTCGAGTATGGCGGCCAGGGGTCCGATCCTGCCG
ATGTGGCCATCCAGCTGACTTTCCTGCGCCTGATGTCCACCGAGGCCTCCC
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ACTGGCAACCTCAAGAAGGCCCTGCTCCTCCAGGGCTCCAACGAGATCGA
GATCCGGGCCGAGGGCAACAGCCGCTTCACCTACAGCGNTCACCTACGAT
GGCTGCACGANTCACACCGGAGCCTGGGGCAAGACAGTGATCGAATACA
AAAACCACCAAGACCTCCCGCTTGCCCATCATCGATGTGGCCCCCTTGGA
CGTTGGCGCCCCAGACCAGGAATTCGGTTTCGACGTTGGCCCTGCCTGCTT
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NCNCCTGACTTTGGNAACAGACAAACAACCCAAACTGAAACCCCGNAAA
GCCAAAAATGGGAGACAATTTNCCATGGACTTGGAAAANATTTTTTTCTTT
TGCATTCNTCTNTCAAACTTAGNTTTTNTCTTTGACCAACTGANATGACCA
AAAACCAAAAGTGCTATNTANCTTACCAAAAAAAAAAAAAAANNNNGNT
TTGGAANNTTNAATTNNAAANNNTAATNNNTTTTGTTNGGTTNNNTTTNAT
NTAGNGNTNCTTTTTNTTTG

Colony: A4B98
Sequence:
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CGTGGGGAACTCCCACNCTGNCTCCGTCACTTCCAGAAGAGCACGTTCCA
NAAAAAAACCCAGCAGGGGTAGAGGCCCAGAGCCAGCAGTGGGAAGAGC
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Colony: A4B101
Sequence:
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GCCGNCCAGGTTGNNAGNTTTTCCATTCACCTTGANCCTCTCCNGAANAA
NGTGCTCANAANNGGCAGGTCCCNTGATCCCATCTACNACAGGGNGGGNT
NCACGGCNAGAAAGGACATNCGGANNTGCTTNNTNGGAATNCGCCNCNC
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GNNAGNCNNTTGAGTANAAATNGGACANTCNNNNCGANGAGATNNCACT
TNNTNNNATNANNNNNGNANNCNAANGCAAGNCGANTTNNNCAAATNTG
CGCGNGNNNCCNNNCTANNTGNACGAGNNNTNTNNGCTNNNNGAGNGNN
AAAAGNANCNAANGCNNNNCNNNGANAAAAGNCTCTCNNNGNCANCGT
NNGNANNNGGANGNANAAATNAANNCGGNNCNGCANCNNNGNNGGGTG
CCAGTCANCNCCAGTANGNNNNCGTAAGNNNCACCCNNTCNTCCNCNTA
NCNNTCNNNNNTCCNCT

Colony: A4B102
Sequence:
GNNTGAAANATTCGGCCGGGGGGTTGNTGGATNACTCGTACNTNCTNGAA
TCNCNCCTGGGCATCAANNTCACATTGTGAATGCTGGTCCTGCTGGTCCTC
CCGGCCCTCCTGGACCCCCTGGTCCCCCAGGTCCTCCCAGCGGCGGCTAC
GACTTGAGCTTCCTGCCCCAGCCACCTCAAGAGAAGGCTCACGATGGTGG
CCGCTACTACCGGGCTGATGATGCCAATGTGGTCCGTGACCGTGACCTCG
AGGTGGACACCACCCTCAAGAGCCTGAGCCAGCAGATCGAGAACATCCG
GAGCCCTGAAGGCAGNCNCAAGAACCCCNCCCGCACCTGCCGTGACCTCA
AGATGTGCCACTCTGACTGGAANAGCGGGAGAATACTGGATTGACCCCAA
CCAAGGCTGCNACCTGGATGCNATTAAGGTCTTCTGCAACATGNAAACCG
GTGAGANCNTGTGTATACCCCNCTTCANNACNAGCGTGGCCCATAAGAAC
TGGTNTATNATTCNAGANANCCCAANGAAAANAGGCNCNTCTTGGTACTN
CGAAGANCNTTACCGGCNGAATTCNCANTTCNNGATNTNGGCNGNGCNN
NGTGNTCCCGAATNCTGNCCGATNGTTNCCNNNNCAAGNTGANNTTTTCN
TGNNGCTTGNANNTNCACCNAGGNCNTCNTANATANTTCACCCTANNANT
GNTAAAAACAGNCTNGTCCTTTATGGTANNNCCATATTNNTNNCCTTAGTT
AAGGGCTTGTTCCNTCCANGGAGTCNACCAATNTAGANNATCNTNTCGNG
GTANATCTGCTTNATTNANTAGTCANTTTCNNTTNNTGNTTANTTTNTTCT
NCANCTGNTTATTNNTGTTNTTNATANAAANNTTNNNANTTCTNCNTGTCC
TTTTNTNTGTCTNNTTNTTTGT

Colony: A4B111

Sequence:
ANNTNGCAAGANTCNGCCGAGGCTCCCGGNTCNTGCTGGTNCATCATNGC
GATCAAGGTGCTCCCGGTGCTGTGGGNCCCGCTGGTCCCAGGGGCCCTGC
TGGTCCTTCTGGCCCCGCTGGCAAAGACGGTCGCATTGGACAGCCTGGTG
CAGTCGGACCTGCTGGCATTCGTGGCTCTCAGGGTAGCCAAGGTCCTGCT
GGCCCTCCTGGTCCCCCTGGCCCTCCTGGACCTCCTGGCCCAAGTGGTGGT
GGTTACGAGTTTGGTTTTGATGGAGACTTCTACAGGGCTGACCAGCCTCGC
TCACCAACTTCTCTCAGACCCAAGGATTATGAAGTTGATGCTACTCTGAAA
TCTCTCAACAACCAGATTGAGACCCTTCTTACTCCAGAAGGCTCTAGGAA
GAACCCAGCTCGCACATGCCGAGACTTGAGACTCAGCCACCCAGAATGGA
GCAGTGGTTACTACTGGATTGACCCTAACCAAGGATGTACTATGGATGCT
ATCAAAGTATACTGTGATTTCTCTACTGGCGAAACCTGCATCCGGGCTCAA
CCTGAAGACATCCCAGTCAAGAACTGGTACAGAAATTCCAAGGCCAAGA
AGCATGTCTGGGTAGGAGAAACTATCAACGGTGGTACCCAGTTTGAATAT
AATGTTGAAGGAGTAACCACCAAGGAAATGGCTACCCAACTTGCCTTCAT
GCGTCTGCTGGCCAACCATGCCTCTCAGAACATCACCTACCATTGCAAGA
ACAGCATTGCATACATGGGATGANGAAACTGGCAACCTGAAAAAGGCTG
TCATTCTGCAAGAATCCAATGATGTCNAACTTGTTGCCGANGGCANCAGC
ANATCCCTTACCCTGTTCTTGTAANNGCTGCTCTAAAANAAAATGAATGG
CAAAANAA

Colony: A4B114
Sequence:
GTGNAATCGGCCGAGGCTCACAGTGACCAGGGTTAGCTGGGATGCCCTCA
GACTGCACTGGACCAGCCCCGATGGGATCTATGAACGGTTTGTCATTAAG
ATCCGGGAGACTGACCAGCCCCAAGAAGTTCACAGTCTCACGGTTCCTGG
CAGCCAGCACTCCGTGGAGATTCCAGGCCTCAAGGCTGGTACCTCTTACA
CAATCACCCTGCGTGGCGAGGTCAGGGACCACAGCACTCAACCCCTTGCT
GTGGAGGTCATCACAGCGGAGCTCCCCCAGCTGGGAGACTTAGTCGTGAC
TGAGGCTGGCTGGGATGGCCTCAGACTCAACTGGACCGCAGCTGATCAGG
CCTTTGAGCACTTTGTCATTCAGGCGCAGGAGGCCAACAGGGTGGAGGCG
GCTCAGAACCTCACGGTGCCAGGGAACATGCGGGCTGTGGACATCCCGGG
CCTGAAGGCCGCCACCCCTTACAGAGTCACCATCCACGGGGTGATCCGGG
GCTATAGGACCCCAGTGCTCTCTGCTGAGGCCTCCACAGCCAGAGAACCT
GAACTTGGAAACTTAAGTGTTTCCGACATCACTTCTGAGAGTTTCAATCTC
TCCTGGACCGCTACTGATGGTGCCTTCGAGACCTTTACCGTTGAAAATTAT
TGATTCCAATAGGTTCNTGGAGACCATGGAATATAACGTCTCTTGGTGCTG
AACGAACAGCCCACATNTTCAGGGNTTCCGCCCTANTAATGANTTTTATT
GTCTACCTCTCTNGACTCGCTCCCAGCATGCAAAACCAANACCATNAATG
CCACGGGCNNCACAANGNCAANCCNAGATGGNTTCNNTCTGTTCNTGGAA
AGNTNATNAAGGANTTTTCAANTTTTNTTTCCAAANCAAAAACCAAAAGA
TNTTCCGACN

Colony: A4B116
Sequence:
CGTGAATCGGNCGAGGAGCATTCTCCCCCCACCTGGTAATTTAAGAAACC
CTAGAGTGCCCCTTTNANNNGNGCTCAAGTAAAGGTGCTGACTGTACTCC
TCCNGNNTGNNCCTCCCCCTCCATATCTCACCGGCCCTCCAGAGCCACCCC
CAAAGAGATGCCTTGAGATTCTCAATGCAGCCATGCCTTGGGGCTGCCTT
GGTGCTGCCACACTTCAGGCTCTCCTCCTTCCACAACTTTCTGTGGNTTTA

CNGCACCTTGGGAGCCAACNGAGACTACNTCGAGAGGGCACCGGTGGNN
CGACNGNNTGGTCNGGCATGGGGNAGTGNGAGNGGGGCATNNATCTGGC
GAGNACCCCNGATGNTTGNGNTTTCTCNCTGANGGTTGCNTTAGANGTAT
TGNTANGNNGNNTNTTGNTTTGNANGGNCTTAGTTCNTGTTTGNGNAANT
TTNAGCTNNTTNNCTTTTCCCNATTNNANNGNATTTNGNGAGGAGANNTN
TCACGTNGTTGGACTANNNNATACNANTANTCTCNTTGNNTNNTGNANGG
TNCTAGGGGCNNTTNTTTTANAGTGNCGNTNTGGTNNNNTCAGGAGGTGG
GAGANNCTAGGNTTTTTAGGGGATANNGTNGTTTCNTNTNTNTAGTATTN
CNTGGANGAANANNNNGTANANGCNAGAACNTGNNNTTCATNNNNGNGG
TNNTGNTAATAAGNAAAANNNCCCGGNANTTTTNNATNCANNTNNATANT
GGNNANCGTTGNATNAAATTNGGNNTNTCNNGAAGTNNNNNAGNCTTNT
NANTCGNNTTNNNANTGTGNNNTTCTNGGGNNGNNTNNNTNNNNANNGN
ACNTCTNNGNGNGGNATTNNNNGGNGATATNCGTCTNNTCTNATTAG

Colony: A4B118
Sequence:
ANNTANNAAGAATCGGCCGAGGGGGCCTCGTAGGATCGCACTGGTGATG
CTGGTCCTGCTGGGTCCTCCCTTAAATCCTGGACCCCCTGGATCCCCCAGG
TCCTCCCAGCGCCNGCTACNACTTGAGCTTCCTGCCCCAGCCACCTCAAGA
GAAGGCTCACGATGGTGGCCGCTACTACCGGGCTGATGATGNCAATGTGG
TNCGTGACCGTGACCTCNAGGTGGACACCACCCTCAAGAGCCTGAGNCAN
CAGATCTAGAACATCCTGAGCCCTGAAGGNAGCCGCAAGAACCCCGCCCG
NANCTGNNGTGANNTCNANATGTGNCACTCTGACTGGAAGANCGGANAA
TACTGCNATTGACCCCGANCAAGGNNGTNACCTGGGANGNTATTNAGGTC
TTCTGGGACATGGAAACCNGAGAGATNTGTNGATACCCNANTCAGNCAA
GNGTTGGTCCAGAAGAAATGGTNTATTNAGCAAGAANCTCNANGGANAA
AATANGCACNTCNGGTATNNNNANAGNNTGAACCGGNGNAANANNNNCG
TANANNAATGGTTGGCNANNGGGTCCNNATNNTGNNCATGTGTNNCANTC
CNAGNTGAANNTTCTTCGTGNCCTNNAANNTCCGANCGATGTTCACNTCG
AAANAATCACNTNCNANCTTGNAGTTAANANANTGGNCANNACAANNGA
ATTATTNGNATNGGNGCTNTTTTTATNAAGGNNNTNGTNGATNTNCGNNT
GCCTANCNANATCNNAAAGAGNNGCCNGNANGGNAGCTACCNNCTTTNA
TCNANATCGTNTNNCNTANAATNGATTNNTNCATTTATNTCCGGATCTCTN
NTGTGATNATTTNGTNNANNATNAAACANCTNGATTCCNGTCGNNCNNNA
TTTATTGGCNCNCCGNNNTNTNNNCCNNGACNTNNNATTNGTTNNAGTTG
NCCTNCNNNNNTCCNTANNNNNNCCTGTGAGANGNNANGTTCCCTNTCAA
TTTCCNGNGNNGNAANNNNAAATCNGCNNNNNATNGANNTT

Colony: A4B123
Sequence:
GANGGAANGNATTCGGCCGAGGGACAGCTCTATGAACCAATAGCACAGC
TGCCAGTAGAACCCCCAGCTCACCCTGAAGGCTGGTGGTCCCTGCTAGTC
AGTGTGGCTCTCTCATTGGGAAAGGTGGTTGCAAGATCAAGGAAATACGA
GAGAGTACAGGGGCTCAGGTCCAAGTGGCAGGGGATATGCTCCCCAACTC
AACTGAGCGGGCCATCACTATTGCTGGCATTCCGCAATCCATCATTGAGT
GTGTGAAACAGATCTGCGTGGTTATGTTGGAGACTCTCTCCCAGTCCCCCC
CGAAGGGCGTGACCATCCCGTACCGGCCCAAGCCGTCCAGTTCTCCGGTC
ATCTTTGCAGGTGGTCAGGACAGGTACAGCACAGGCAGCGACAGTGCGA
GCTTTCCCCACACCACCCCGTCCATGTGCCTCAACCCTGACCTGGAGGGAC
CACCTCTAGAGGCCTATACCATTCAAGGACAGTATGCCATTCCACAGCCA

GATTTGACCAAGCTGCACCAGTTGGCAATGCAACAGTCTCATTTTCCCATG
ACGCATGGCAACACCGGATTCAGTGGCATTGAATCCAGCTCTCCAGAGGT
GAAAGGCTATTGGGGTTTGGATGCATCTGCTCAGACTACTTCTCATGAACT
CACCATTCCAAACGATTTGATTGGCTGCATAATCGGGCGTCAAGGCGCCA
AAATCAATGAGATCCGTCAGATGTCTGGGGCGCAGATCAAAATTGCGAAC
CCAGTGGAAGGATCTACTGATAGGCAGGTTACCATCACTGGATCTGCTGC
CAGCATTANCCTGGCTCAATATCTAATCAATGTCAGGNTTTCCTCGGAAA
ANGGTGGCNNGGGANCANCTAAAACAATGCAAATTCNTCNTATCTTNTGT
GTCNCACACCATGATCATCTGGTATTTTGANNTCACGATCNGGTTAANATT
GAATTCNTTNAANATTTT

Colony: A4B126
Sequence:
NGGCGCAAGAATTCGGCAGAGGCAGCCCGCCCTCCGCCAGGGCGCCCGCT
CCTCCTCCTCCTCCTCCTCCTCCCCCGGTCGTCTCCAAATTGAACGTTCTTG
CTAGAGATTTTTATTTTTCCAGATCTGTAGTGTCAGGAGGTGATTCCGTCT
TTGATTTACAATGGCAGGCCCAGGTGATCTATCGGGAACACACGCACACC
CTTCCCGCCCCGAGAGCTCGGGGTGGCCTGCAGGGCCTGGCTGTAGAGCA
GTCTGGGTGGGTTTTCAACTGCCGTCATCGCCAACTGCTGTTTCAACAGCT
CTTAAGTTCCTGGAGAGACGGCAGTCAGGACCAGGTCGCCAGGTCAGAGG
GGTGTCACCAGGGAGCCTTTCTCTTTCTTTTTTGAATTAAAACCAACATAC
CAGCAAAAAAAAAAAAAAAAACCCNGNNNGGNAAAAAANNCAAAAANN
NAAAAA

Colony: A4B129
Sequence:
GANNGAAANNATTCGGCACGAAGGCTCACAGTGACCAGGGTTAGCTGGG
ATGCCCTCAGACTGCACTGGACCAGCCCCGATGGGATCTATGAACGGTTT
GTCATTAAGATCCGGGAGACTGACCAGCCCCAAGAAGTTCACAGTCTCAC
GGTTCCTGGCAGCCAGCACTCCGTGGAGATTCCAGGCCTCAAGGCTGGTA
CCTCTTACACAATCACCCTGCGTGGCGAGGTCAGGGACCACAGCACTCAA
CCCCTTGCTGTGGAGGTCATCACAGCGGAGCTCCCCCAGCTGGGAGACTT
AGTCGTGACTGAGGCTGGCTGGGATGGCCTCAGACTCAACTGGACCGCAG
CTGATCAGGCCTTTGAGCACTTTGTCATTCAGGCGCAGGAGGCCAACAGG
GTGGAGGCGGCTCAGAACCTCACGGTGCCAGGGAACATGCGGGCTGTGG
ACATCCCGGGCCTGAAGGCCGCCACCCCTTACAGAGTCACCATCCACGGG
GTGATCCGGGGCTATAGGACCCCAGTGCTCTCTGCTGAGGCCTCCACAGC
CAGAGAACCTGAACTTGGAAACTTAAGTGTTTCCGACATCACTTCTGAGA
GTTTCAATCTCTCCTGGACCGCTACTGATGGTGCCTTCGAGACCTTTACCG
TTGAAATTATTGATTCCAATAGGTTCCTGGAGACCATGGAATATAACGTCT
CTGGTGCTGAACGAACAGCCCACATCTCAGGGCTCCGCCCTAGTAATGAC
TTTATTGTCTACCTCTCTGGACTCGCTCCAGCATGCANACCAAAACATCAG
TGCCACGGCCAGCACAGATGCCACCCCAGATGGNTTCGTCTGTCCTGGAA
GCTGATAAAGATTCTTCAAAGTTTGTTTCAAATCAAGATACCAAAGCANT
CNAGCCNTGGNATACCTCTTGCCCNAAAAACAGGAATNAAGGTTCAAAGC
CNTGATNNATTATT

Colony: A4B131
Sequence:
GCATGAATTCGGCCGAGGCTNNANCTGTANCATNGGTTANCTGGANATGC

CCTCAGACTGCACTGGACCANCCCCNATGGGATCTATGAACGGTTTGTCA
TTAAGATCCGGGAGACTGACCAGCCCCAAGAAGTTCACAGTCTCACGGTT
CCTGGCAGCCAGCACTCCGTGGAGATTCCAGGCCTCAAGGCTGGTACCTC
TTACACAATCACCCTGCGTGGCGAGGTCAGGGACCACAGCACTCAACCCC
TTGCTGTGGAGGTCATCACAGCGGAGCTCCCCCAGCTGGGAGACTTAGTC
GTGACTGAGGCTGGCTGGGATGGCCTCAGACTCAACTGGACCGCAGCTGA
TCAGGCCTTTGAGCACTTTGTCATTCAGGCGCAGGAGGCCAACAGGGTGG
AGGCGGCTCAGAACCTCACGGTGCCAGGGAACATGCGGGCTGTGGACATC
CCGGGCCTGAAGGCCGCCACCCCTTACAGAGTCACCATCCACGGGGTGAT
CCGGGGCTATAGGACCCCAGTGCTCTCTGCTGAGGCCTCCACAGCCAGAG
AACCTGAACTTGGAAACTTAAGTGTTTCCGACATCACTTCTGAGAGTTTCA
ATCTCTCCTGGACCGCTACTGATGGTGCCTTCGAGACCTTTACCGTTGAAA
TTATTGATTCCAATAGGTTCCTGGAGACCATGGAATATAACGTCTCTGGTG
CTGAACGAACAGCCCACATCTCAGGGCTCCGCCCTAGTATGACTTTATTGT
CTACCTCTCTGGACTCGCTCCCAGCATGCAGACAAAACNATCAGTGCCAC
GGCCAGCACAGATGCCACCCCAGATGGTTTCGTCTGTCNTGAAAGCTATG
AAGGANTCTTCCACGTTTTGTTTCAANTCAAGATACAAAAGCANTCGACC
CTGAAAAACCTCTTGCCCNAACANCAGGATTAAGGTTCAAAGCNTGAT

Colony: A4B132
Sequence:
ATGGATTCGGCACGAAGGCTNNNANCNGTANCATCGGATTATCCTGGANA
TGCCCTCAGACTGCACTGGTACCANCCCCAATGGGATCTATGAACGGTTT
GTCATTAAGATCCGGGAGACTGACCAGCCCCAAGAAGTTCACAGTCTCAC
GGTTCCTGGCAGCCAGCACTCCGTGGAGATTCCAGGCCTCAAGGCTGGTA
CCTCTTACACAATCACCCTGCGTGGCGAGGTCAGGGACCACAGCACTCAA
CCCCTTGCTGTGGAGGTCATCACAGCGGAGCTCCCCCAGCTGGGAGACTT
AGTCGTGACTGAGGCTGGCTGGGATGGCCTCAGACTCAACTGGACCGCAG
CTGATCAGGCCTTTGAGCACTTTGTCATTCAGGCGCAGGAGGCCAACAGG
GTGGAGGCGGCTCAGAACCTCACGGTGCCAGGGAACATGCGGGCTGTGG
ACATCCCGGGCCTGAAGGCCGCCACCCCTTACAGAGTCACCATCCACGGG
GTGATCCGGGGCTATAGGACCCCAGTGCTCTCTGCTGAGGCCTCCACAGC
CAGAGAACCTGAACTTGGAAACTTAAGTGTTTCCGACATCACTTCTGAGA
GTTTCAATCTCTCCTGGACCGCTACTGATGGTGCCTTCGAGACCTTTACCG
TTGAAATTATTGATTCCAATAGGTTCCTGGAGACCATGGAATATAACGTCT
CTGGTGCTGAACGAACAGCCCACATCTCAGGGCTCCGCCCTAGTAATGAC
TTTATTGTCTACCTCTCTGGACTCGCTCCCAGCATGCAGACCAAACCATCA
GTGCCCCGGCCAGCACAGATGCCACCCANATGGGTTCGTCTGTCTGGAAG
CTGATGAAGATCTTCNAAGTTTTGTNNCAAANTCAANATNCCAAAAGCAT
CCGAGCCCTGAAATAACCTCTTGCCCCAAAANCAGGAATNANGNTTNAAA
AGCCNTGAATTNATNAATTTG

Colony: A4B134
Sequence:
TNNNNAGGAACNAATTCGCCCNNATCCTGACTCTNTGACCCNTATTCACA
GACTGGCCAGTAGACCCCCACCCCTTNNAAGNGGNTGGTGGTCCCTGCTA
GTCAGTGTGGCTCTCCCTTNNNGAAAGGTGGTTGCAAGATCAAGGAAATA
CGAGAGAGTACAGGGGCTCAGGTCCAAGTGGCAGGGGATATGCTCCCCA
ACTCAACTGAGCGGGNCATCACTATTGCTGGNATTCCGNAATCCATCATT
GAGTGTGTGAAACAGATCTGCGTGGNTATGTTGGAGACTCTCTCCCAGTC

CCCCCCGAAGGGNGTGACCANCCCGTACCGGCCCAAGCCGTNNAGTTCTC
CGGTCATCTTTGCAGGTGGGCAGGACAGGTACAGCACAGGCAGCGACAGT
GCGAGCTTTCCCCACACCACCCCGTCCATGTGCCTCAACCCTGACCTGGAG
GGACCACCTCTAGAGGNNTATACCATTCAAGGACAGTATGCCATTCCACA
GCCAGANTTGACCAAGNTGCNCCAGNTGGGAATGCAANAGNCTCATTTTC
CCATGANGCATGGCAACACCGGATTCAGTGGNATTGAAACCAGCTCTCCA
NAGGNTGNANAGGCTATTGGGGNTTGNATGNACNGCTCNAACAACNTCTT
CATGANATCACCATTCAAANGANGNGATNGGGGNANATANAGNGGCANG
GNGCCAAATNATNAANCCGTCAAAANCANGGGGNGNGAATAAAANTGNG
AANCACGGGAGGANNGNCNGAAGGCAGGAANNNCACTGGGNTGCGGNA
GAATAANNNGGCCANAAAAAAAAAAGGCNGGATTTCCCAAAAAGGGGG
NANGGGANNAAGNAAAAAAGNAAATTTNCANATTCNTTTTGCTGTCNCAC
CCCAAAACCATGTGGNNGTTTNAAAGNNNATCCCGGNTAAAAGNNGAAA
TAAGTTANNNCTTTNNCCNNNGGATNNAGGAGG

Colony: A4B135
Sequence:
NTTNAAAGGAAAGAATTCGGCACGAGGGCTNAAACTGTACCANGGATTA
TCCTGGAAATGCCCCTCAGACNGCACNCCTNTTANACCNAATGGGATCTA
TGAACGGTTTGTCATTAANACCTTGGAGACTGACCAGCCCCAAGAAGTTC
ACAGTCTCACGGTTCCTGGCAGCCAGCACTCCGTGGAGATTCCAGGCCTC
AAGGCTGGTACCTCTTACACAATCACCCTGCGTGGCGAGGTCAGGGACCA
CAGCACTCAACCCCTTGCTGTGGAGGTCATCACAGCGGAGCTCCCCCAGC
TGGGAGACTTAGTCGTGACTGAGGCTGGCTGGGATGGCCTCAGACTCAAC
TGGACCGCANCTGATCAGGCCTTTGAGCACTTTGTCATTCAGGCGCAGGA
GGCCAACAGGGTGGAGGCGGCTCANAACCTCANGGTGCCAGGGAACATG
CGGGCTGTGGACATNCCGGGGCTGAAGGCCGCCACCCCTTACAGAGTCAC
CATCCACGGGGTGATCCGGGGCTATAGGGANCCCAGNGCTCTCTGCTGAG
GCCTCCACAGCCAGAGAACCNGAAANTTGGAAACTTTAAGTGTNTTCCNN
AANCACTTCTGANAGNTTCAATCTCTCCTGGANCNCTANTGNNNGGNGCC
TTCGAGAACTTTACCGGTTGGAANTANTGATTCCNATANGGTTNCNGGAG
ACCATGGAAAAANAAANTCTCNGGGGGGAAAGGAAAANCCCACNNNTAA
GGGGTCCCCCCCCAANTAANAANTTNATTGTNTACNTCCCTGGANTCGCNC
CCAGAANGNAGANAAAACCATCANGCNANGGCNGNACAAAGNGACCCA
AANNGNTCGNNCTNNNTGGNNNNCNGAGAAGGGNCTTNNAANNNTNTTT
NNNNNAAAAAANAANANCATCGNNGGGGGAAAAANNNTTGCCCTACNAC
GGNNNTNGGTNGANGCNNAATNNNNNNAACTGCANNNCGGAGGGGG

Colony: A4B138
Sequence:
ANNGGNTGCAAGAATTCGGCCGAGGGANAGAAGGAACAACGGTCGTGCC
AAAAAGGGCCGCGGCCATGTGCAGCCTATTCNCTGCACCAACTGGTGCCC
GATGTGTGCCCAAGGATAAGGCCATTAAGAAGTTTGTCATTCGGAACATC
GTAGAGGCCGCAGCCGTCAGGGACATTTCTGAAGCGAGTGTTTTCGATGC
CTATGTACTTCCCAAGCTGTATGTGAAACTACATTACTGCGTGAGTTGTGC
CATTCACAGCAAGGTAGTCAGGAATCGCTCTCGGGAAGCCCGTAAGGACC
GAACACCTCCACCCCGATTTAGACCTGCGGGTGCTGCCCCACGTCCTCCAC
CAAAGCCCATGTAAGGATCCAAGTCCTTAAAGACTGAAGAATATTGGTTT
CTCTGAAAAGACAATAAAATGGAAATTATACTTTAAAAAAAAAAAAAAAA
AANNNNGGGNNGNAANGNATTNCTAAAATNNGNAANTNTNTTTTTTTNTT

CTTTTGTNATNTTNNNNGATNTTATTNATTCNCTTTATTTTNTNTTTTATTN
GTTTCTNTATTANTTTTTGCTTTNNNNNGNTNNNNTNNCTTCNTGNTNNTG
TATTTTTATNTCTNCTNTTNNNTGTTTTNTTTNTNGGTGTNTNTATGNTTTT
TTANCTTTTNNTTGTNCTNTGCGNTTTTTTTAGGNTGNNNTNTTNNTNGAT
ATNNTTGTTTNNNTCTCTTNNNTTTTNTNTTTNTGTTTTGTNTNTANTNNTT
TTNTNNTTATGCNTTTANNGATNTTNNNNNGTTNTTCNTTNTTNTTATNNG
NNTNTTNAGTNCTTTTNGTNANTTNTTNGCTTTTTNTTTATTNGTNNTCTTT
ATNTNTTTTTTTATTNTTTTNTCATTCTTNNNTTGNTTTTCTTNTTT

Colony: A4B142
Sequence:
AGCGGGGNAGGGAGTCAGNCGAGGCAGCAGCTCTATGACCNGGAGCGGA
GGCTGGGNGTAGACACCCCCCCNTAAAAAANGNTGGTGGNNCCTGCTAGT
CAGTGNGGCTCNCCNTTANNGANNGGTGGNTGCAAGATCAAGGAAATAC
NANAGAGGACAGGGGCTCAGGACCAAGNGGGAGGGGATATGCTCNNNAA
CTNAACTGAGCGGGCCANCACTATTGNTGGGGTNNGCANNCCATCANTGA
GTGTGTGAAACAGATGGTGGGGGGATGANGTTGGAGACTCTNTCAAGTAN
NNCCNGAAGGGGGNGACNNTNNCGGGACGNGNNNAAGNCGGNNNGAGN
GNTNCGGAGAANNATGAAGGNGGNCAGGANAGGNANAGNANAGGCAGC
GAAGAGAGNGAGATTNNCCCGCACNAANGGGNCGATGNGGNNNNANNC
NCTGACGGAGAGGGANCACNGCNNGGNGGGGGNGGGNANNAGAAGGAC
AGGNANGAGGNANCCGNNGCNCNGAGTNGGANCGGGGGNGNACGGGGT
NGGANAAGGAAANAGANNCAAGNAGCGCNGATAGANNGGAGGGGATNG
GAGGGGANNANGGGANANAGGAAANNNNGCANCTAGAGNGNNGANNGG
NAAGCAGGAGGANNGGGAANGAATGANTNTAGNAAGGAGAGTNNAGGA
GGTNNAGAGGGAAGAGGGNANGGNNNGGGANGAAGNAAGGGGNGGNCA
GGGAAGGGAAAGNNANNGNAGGGAGGCNNANNNNNGNGGGGCGNAATN
NAAANNGNGNNTCANAGNGNGNTAGATANCGNGNGNNANGGNAAAACG
ACAGGGGAGAGNGGNNGNNGNGACAGGCAGAAAANGNAAAGAGGGGNG
AAGNGGTNAGGAAAGAGNNAGTAGAGNGAGGANNGAAAAGAAATAGNG
AGGGAGNNAANGNANGNNANGGNGGNAGGGAANNNGTANANCGCGAAG
CGCTGANGANNCNANCAGG

Colony: A4B143
Sequence:
TNNAANGAAANNNATTCGGCCGAGGGGGCCTCNNAGGATCGCACTGGAT
GTATGCTGGATCCTGCTGGTCCTCCCTTTACCTCCTGGACCCCCTGGTCCC
CCAGGTCCTCCCAGCGGCNGCTACGACTTGAGCTTCCTGCCCCAGCCACCT
CAAGAGAAGGCTCACGATGGTGGCCGCTACTACCGGGCTGATGATGCCAA
TGTGGTCCGTGACCGTGACCTCGAGGTGGACACCACCCTCAAGAGCCTGA
GCCAGCAGATCGAGAACATCCGGAGCCCTGAAGGCAGCCGCAAGAACCC
CGCCCGCACCTGCCGTGACCTCAAGATGTGCCACTCTGACTGGAAGAGCG
GAGAATACTGGATTGACCCCAACCAAGGCTGCAACCTGGATGCCATTAAG
GTCTTCTGCAACATGGAAACCGGTGAGACCTGTGTATACCCCACTCAGCC
CAGCGTGGCCCAGAAGAACTGGTATATCAGCAAGAACCCCAAGGAAAAG
AGGCACGTCTGGTACGGCGAGAGCATGACCGGCGGATTCCAGTTCGAGTA
TGGCGGCCAGGGGTCCGATCCTGCCGATGTGGCCATCCAGCTGACTTTCCT
GCGCCTGATGTCCACCGAGGCCTCCCAGAACATCACCTACCACTGCAAGA
ACAGCGTGGCCTACATGGACCAGCAGACTGGCAACCTCAAGAAGGCCCTG
CTCCTCCAGGGCTCCAACGAGATCGAGATCCGGGCCGAGGGCAACAGCCG

CTTCACCTACAGCGTCACCTACGATGGCTGCACGAGTCACACCGGANCCT
GGGGCAAGAAGTGATCGAATACAAAACCACCAAGACTCCNGTTGCCCATC
ATCGATGTGGCCCNTTGNAGTNGNGCCCAAACAAGANTCGTTTCNANTTG
CCTNCTGNTNCTGTAAATCTTCNACCCAACTGGTCCTCCNCCAACCATTGC
CNTNATTTGAAAAAAAAAAACNAAN

Colony: A4B145
Sequence:
GAAAANGAAAGAANTCGGCCGAGGGGCCACAAGGTATTCNAGGTGACAG
GGAGAGCCTGGTGATNCCTTTANAGANGTCTTCCTGGCTTAAAGGGACAC
AATGGGTTNCTAGGTCTCCCGGGTCTTGCTGGTCATCATGGCGATCAAGGT
GCTCCCGGTGCTGTGGGTCCCGCTGGTCCCAGGGGCCCTGCTGGTCCTTCT
GGCCCCGCTGGCAAAGACGGTCGCATTGGACAGCCTGGTGCAGTCGGACC
TGCTGGCATTCGTGGCTCTCAGGGTAGCCAAGGTCCTGCTGGCCCTCCTGG
TCCCCCTGGCCCTCCTGGACCTCCTGGCCCAAGTGGTGGTGGTTACGAGTT
TGGTTTTGATGGAGACTTCTACAGGGCTGACCAGCCTCGCTCACCAACTTC
TCTCAGACCCAAGGATTATGAAGTTGATGCTACTCTGAAATCTCTCAACA
ACCAGATTGAGACCCTTCTTACTCCAGAAGGCTCTAGGAAGAACCCAGCT
CGCACATGCCGAGACTTGAGACTCAGCCACCCAGAATGGAGCAGTGGTTA
CTACTGGATTGACCCTAACCAAGGATGTACTATGGATGCTATCAAAGTAT
ACTGTGATTTCTCTACTGGCGAAACCTGCATCCGGGCTCAACCTGAAGAC
ATCCCAGTCAAGAACTGGTACAGAAATTCCAAGGCCAAGAAGCATGTCTG
GGTAGGAGAAACTATCAACGGTGGTACCCAGTTTGAATATAATGTTGAAN
GAGTAACCACCANGNAATGGCTACCNACTTGCCTTCATGCGTCTGCTGGC
CAACATGCCTCTCAAAAATCACCTACCATTGCANAAAGCATNNATANTGG
NGAGAANTGGCACCTGAAAAGGTGTCNTNGCAGGACCATGATGCAANTG
TGCGAGGCAAACAATNNTNAACTGTCTGAAGGTGTTTAAAAAAAGAAGN
AAAAANCTTGANAANAAAACCTNC

Colony: A4B147
Sequence:
GAANAAGNAAGAATCGGCAGAGGCTCCANTGACCAGGGATTANCTGGNA
ATGCCCTCNNACTGCACNCCNTTTAAACNNNATGGGATCTATGAACGGTT
TGTCATTAAGATCNTTGNANACTGACCAGCCCCAAGAAGTTCACAGTCTC
ACGGTTCCTGGCAGCCAGCACTCCGTGGAGATTCCAGGCCTCAAGGCTGG
TACCTCTTACACAATCACCCTGCGTGGCGAGGTCAGGGACCACAGCACTC
AACCCCTTGCTGTGGAGGTCATCACAGCGGAGCTCCCCCAGCTGGGAGAC
TTAGTCGTGACTGAGGCTGGCTGGGATGGCCTCAGACTCAACTGGACCGC
AGCTGATCAGGCCTTTGAGCACTTTGTCATTCAGGCGCAGGAGGCCAACA
GGGTGGAGGCGGCTCAGAACCTCACGGTGCCAGGGAACATGCGGGCTGT
GGACATCCCGGGCCTGAAGGCCGCCACCCCTTACAGAGTCACCATCCACG
GGGTGATCCGGGGCTATAGGACCCCAGTGCTCTCTGCTGAGGCCTCCACA
GCCAGAGAACCTGAACTTGGAAACTTAAGTGTTTCCGACATCACTTCTGA
GAGTTTCAATCTCTCCTGGACCGCTACTGATGGTGCCTTCGAGACCTTTAC
CGTTGAAATTATTGATTCCAATAGGTTCCTGGAGACCATGGAATATAACGT
CTCTGGTGCTGAACGAACAGCCCACATCTCAGGGCTCCGCCCTANTAATG
ACTTTATTGTCTACCTCTCTGGACTCGCTCCCAGCATGCANACCAAAACCA
TCAGTGCCACGGCCAGCACAGATGCCACCCCAGAAGGGTTCCGTCTGTCT
GGANAGCTGANGAAGATNTCCACANTTTGTTCNCAAATCANAGANCCAA

AANAGTCNNANCCTGAAANACCTCTTGCCCCNACAACAGGAATAAGNTN
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Colony: A4B148
Sequence:
GAAAATGAAAGANTCGGCCGAGGCTACAGTGACCAGGGATTAGCTGGNA
TGCCCTCANACTGCACNCCNNTTANACCCNATGGGATCTATGAACGGTTT
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CGGTTCCTGGCAGCCAGCACTCCGTGGAGATTCCAGGCCTCAAGGCTGGT
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ACCCCTTGCTGTGGAGGTCATCACAGCGGAGCTCCCCCAGCTGGGAGACT
TAGTCGTGACTGAGGCTGGCTGGGATGGCCTCAGACTCAACTGGACCGCA
GCTGATCAGGCCTTTGAGCACTTTGTCATTCAGGCGCAGGAGGCCAACAG
GGTGGAGGCGGCTCAGAACCTCACGGTGCCAGGGAACATGCGGGCTGTG
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GGTGATCCGGGGCTATAGGACCCCAGTGCTCTCTGCTGAGGCCTCCACAG
CCAGAGAACCTGAACTTGGAAACTTAAGTGTTTCCGACATCACTTCTGAG
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GACTTTATTGTCTACCTCTCTGGACTCGCTCCAGCATGCNAACAAAACATN
CAGTGCACGGCCAAACAAATGCCCCCNAAATGGTTCGTTTTCTGGAAACT
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Colony: A4B150
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GNANACTNAGTNNTGANTGAGGCTGGCTGGNATGNNNTATANACTCCAN
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CTCNTTAANAAGTNGANTNATNACTGGGNGTGNTCAGGGGTNAGNGGAN
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ANNANNTNNNGGTNTTCNTNNTACGAGAAAAAANAGTTGANGTNATGNA
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Colony: A4B151
Sequence:
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TGGTCNANACNAATNNANTTNANGTTTNNCTCAAAGNANGNNGTGGAAT
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Colony: A4B154
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GGGTGATCCGGGGCTATAGGACCCCAGTGCTCTCTGCTGANGCCTCCACA
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Colony: A4B157
Sequence:
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GNCAACANGGTGGAGGNGGCTCAAAACCTNNNGGNGNCAGGGAANNAG
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TCNAANNGNNGNAANATCCNGCGNNNAAANANGACCCGAGGNTGGTNGC
NGGCGANNAGGGNTNANTNTGTTNGNTAANAATAAATCCNNNNGGANAN
NNTGNCAACCNGGANGNNGGTGNANNNGNCNNTATTTNANCTG

Colony: A4B158
Sequence:
TNGNGNCNNTTTGCTNTNNNTNNTTTNNTTNNNCNTTTNNNNNNNGGATA
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GTTCCTGGCAGCCAGCACTCCGTGGAGATTCCAGGCCTCAAGGCTGGTAC
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NACCTTTGNCGGNGGAAANTNATTGATTCCANTANGGCNTCNTGGAGACC
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GNGGCNNACCTAAACCATCAATGCCNNGGNCAGTNTANANGCANCCCAA
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TGCATCNNNNANCNAAAACNATCCNNNATTGAGAGNAACTTNTNNCCNA
NTANCGGGNTTTTGGGTCTCNNGGCATGGAGTGATTGNTNTTTGATTNCCG
TNGNTTCCCCNTG

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