Characterisation of UL102, the helicase-primase associated protein of human cytomegalovirus

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Summary

Amongst the set of 11 proteins which are required for human cytomegalovirus (HCMV) origin-dependent DNA synthesis, are six which are conserved amongst the herpesvirus family and which perform the essential functions required for viral DNA synthesis. In HCMV these functions, a processive DNA polymerase, ss DNA-binding and helicase-primase activities are provided by the UL54, UL44, UL57, UL70, UL102 and UL105 proteins which are referred to as the core replication fork proteins. UL54 and UL44 comprise the catalytic and processivity subunits, respectively, of the DNA polymerase holoenzyme, UL29 functions as the ss DNA-binding protein whilst UL70, UL102 and UL105 associate to form the heterotrimeric helicase-primase complex.

Current models of herpesvirus DNA synthesis are based mostly upon knowledge regarding the herpes simplex virus type 1 (HSV-1) replication fork proteins which have been more extensively studied than any of their counterparts in other herpesviruses. In HSV-1 the replication fork proteins are UL30/UL42 (DNA polymerase), UL29 (ss DNA-binding protein) and UL5/UL8/UL52 (helicase-primase complex). Multiple interactions between the HSV-1 replication fork proteins have been demonstrated and it is thought that these interactions serve to co-ordinate the functions of the HSV-1 replication proteins for efficient viral DNA synthesis.

Unlike the HSV-1 replication proteins, the HCMV replication proteins are not well characterised and therefore their roles in HCMV DNA synthesis have been largely predicted by analogy with their HSV-1 counterparts. The aim of this project was to investigate the properties of UL102, one of the essential HCMV replication fork proteins which forms part of the helicase-primase complex, and compare its characteristics with that of its HSV-1 homologue, UL8, to assess whether it may perform similar functions to UL8 during HCMV DNA synthesis.

The following specific properties of UL102 were investigated to allow direct comparison with HSV-1 UL8:

1) Its ability to interact with the HCMV DNA polymerase catalytic subunit, UL54.
2) Its ability to bind to DNA and DNA/RNA hybrid oligonucleotides representing the forms of nucleic acid present at the replication fork.
3) Its intracellular localisation when expressed alone in mammalian cells and also its ability to alter the intracellular localisations of the other HCMV helicase-primase

subunits, UL70 and UL105, when it is co-expressed with these proteins in mammalian cells.

In order to address these objectives and to enable subsequent structure-function analysis, a variety of UL102 reagents were produced. These included 1) a panel of 51 UL102-specific monoclonal antibodies 2) constructs to express, in *E. coli,* full-length and truncated UL102 GST-fusion proteins 3) constructs to express, in mammalian cells, full-length UL102, UL70 and UL105 proteins and truncated UL102 proteins and 4) constructs which express UL102 and UL54 that permit 2-hybrid analysis in mammalian cells.

Using three different methods, ELISA, co-immunoprecipitation and mammalian-2-hybrid analysis, no evidence for a specific interaction between UL102 and UL54 could be demonstrated. In this respect UL102 may differ from its HSV-1 counterpart, UL8.

In immunofluorescence studies, UL102 efficiently translocated to the nucleus of mammalian transfected cells. In this respect it differs from UL8, which localises to the cytoplasm when expressed alone. Both UL70 and UL105 displayed a cytoplasmic localisation when expressed on their own. UL102 did not influence their cytoplasmic localisations when co-expressed with either protein. Preliminary results indicate that nuclear localisation of the helicase-primase proteins occurs only when all three are co-expressed. In this respect, UL102 behaves similarly to UL8, which is required for efficient nuclear localisation of the HSV-1 helicase-primase proteins but does not influence their intracellular localisation when it is expressed with either protein individually.

Nucleic acid binding experiments were performed either in the presence of 50 mM NaCl or in the absence of any salt. Under both conditions, UL102 did not detectably bind to ss DNA, ds DNA or DNA-RNA hybrids representing the structures of nucleic acid present at the replication fork. Similarly, UL8 did not detectably bind DNA.

The UL102 characterisation studies presented in this thesis indicate that it is unlikely that UL102 performs the same precise set of functions which have been attributed to HSV-1 UL8. As UL102 shares some characteristics in common but also possesses differing characteristics to UL8, it is probable that it performs a subset of the functions carried out by UL8 including a likely role in nuclear translocation of the HCMV helicase-primase complex. Since UL102, like UL8, is dispensable for the enzymatic activities of

the helicase-primase and does not bind DNA, it is possible that it functions to augment the activities of the complex and likely mediates necessary interactions between the helicase-primase complex and other replication fork protein/protein complexes during DNA synthesis, which are established characteristics of UL8.

Conversely, the demonstration that the properties of UL102 and UL8 differ in some aspects suggests that it is unlikely that UL102 performs some of the functions which have been predicted by analogy with UL8. Most notably, a specific interaction between UL102 and UL54 could not be established, and may not exist, in marked contrast to UL8 which has been shown to interact specifically with the HSV-1 polymerase catalytic subunit, UL30. The UL8-UL30 interaction is predicted to facilitate the UL8-mediated increased efficiency in primer utilisation by UL30. If UL102 and UL54 do not interact then it is unlikely that a similar effect of UL102 on primer utilisation by HCMV UL54 would be observed. In addition, the observation that UL102, in contrast to UL8, can localise to the nucleus independent of any other replication proteins suggests that UL102 may perform a function which is not shared by UL8. The reagents produced in this study will be of benefit to any future studies that address the issue of the as yet, undefined role of UL102 in HCMV DNA replication.

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Finally, I send a very special thank you to my family; Mum and Dad for their unerring support over the years and for always taking an interest, Stella for her kindness and encouragement (and provision of much appreciated holidays...!) and to Joe and Anita for their support, encouragement and sympathetic ear. I am grateful to all of you for your amazing generosity and all your help.

Unless otherwise stated, the results presented in this thesis were obtained through my own efforts.

Pamela Hamill **July 2001**

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List of abbreviations

aa	Amino acid
ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
AcNPV	Autographa californica nuclear polyhedrosis virus
AD	Activation domain
AIDS	Acquired immune deficiency syndrome
ATPase	Adenosine 5' triphosphate-ase
BHK	Baby hamster kidney
BMT	Bone marrow transplant
bp	Base pairs
BSA	Bovine serum albumin
CAT	Chloramphenicol transferase
Ci	Curies
CIP	Calf intestinal phosphatase
CNS	Central nervous system
CREB	Cyclic AMP response element binding factor
C-terminal	Carboxy-terminal
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DNA-BD	DNA-binding domain
°C	Degrees celcius
DMSO	Dimethyl sulphoxide
ds DNA	double-stranded DNA
DTT	Dithiothreitol
EBV	Epstein-Barr Virus
E.coli	*Escherichia coli*
ECL	Electro-chemoluminescence
EDTA	ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EtBr	Ethidium bromide
EWB	ELISA wash buffer
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GEB	Glutathione elution buffer
GMP	Guanosine mono-phosphate

GST	Glutathione-S-transferase
GTP	Guanosine tri-phosphate
GTPase	Guanosine 5'-triphosphate-ase
HAART	Highly active anti-retroviral therapy
HCMV	Human cytomegalovirus
HEPES	(N-[2-hydroxyethyl] piperazine-N'-[2-ethane sulphonic acid])
HF	human fibroblast
HFFF	human foetal foreskin fibroblast
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
hpi	hours post-infection
HRP	Horse-radish peroxidase
HSV-1/2	Herpes simplex virus-1/2
ICP8	infected cell protein 8
IE	Immediate early
IP	Immuno-precipitation
IPTG	isopropyl-β-D-thio-galactoside
IRS/L	Inverted repeat short/long
kbp	kilobase pairs
kDa	kilodaltons
KSHV	Kaposi's sarcoma-associated herpesvirus
LB	Luria-Bertani medium
LBA	LB containing ampicillin
M	Molar
MAb	Monoclonal antibody
MCP	Major capsid protein
mCP	Minor capsid protein
mCP-BP	Minor capsid protein-binding protein
MCS	Multiple cloning site
M-2-H	Mammalian-2-hybrid
MHC	Major histocompatibility complex
MIE	Major immediate early
mA	milliampere
ml	millilitre
mm	millimetre
mM	milli-molar

mRNA	Messenger RNA
MW	Molecular weight
ND10	Nuclear domain 10
ng	nanograms
nm	nanometre
N-terminal	Amino terminal
nt	nucleotide
OD	Optical density
ORF	Open reading frame
%	percent
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	Potential of hydrogen
pM	pico moles
PML	Promyelocytic leukemia protein
PODs	PML oncogenic domains
RNA	Ribonucleic acid
rpm	Revolutions per minute
RR	Ribonucleotide reductase
SCP	Smallest capsid protein
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sf	*Spodoptera frugiperda*
SRT	Shortest replicator transcript
ss DNA	single-stranded DNA
SV40	Simian virus 40
TAE	Tris, acetate, EDTA
TBS	Tris-buffered saline
TLC	Thin layer chromatography
Triton-X-100	Octyl phenoxy polyethoxy ethanol
TRS/L	Terminal repeat short/long
UL	Unique long
US	Unique short
UV	Ultraviolet
V	Volts
vRNA	virus-associated RNA

VZV	Varicella-Zoster virus
v/v	volume per volume (ratio)
w/v	weight per volume (ratio)
µg	microgram
µl	microlitre
2-YTA	YT medium containing ampicillin

Amino acids

alanine	A	leucine	L
arginine	R	lysine	K
asparagine	N	methionine	M
aspartate	D	phenylalanine	F
cysteine	C	proline	P
glutamate	E	serine	S
glutamine	Q	threonine	T
glycine	G	tryptophan	W
histidine	H	tyrosine	Y
isoleucine	I	valine	V

Nucleotide Bases

adenine	A
cytosine	C
guanine	G
thymine	T
uracil	U

Chapter 1

Introduction

1.1 The Herpesviruses

1.1.1 General characteristics

The herpesviruses are a large and diverse family of over 100 viruses that share a variety of common characteristics, including their genome type, virion morphology, basic mode of replication and the ability to establish different forms of infection in their natural hosts. The herpesvirus-common characteristics have been summarised by Roizman *et al.*, (1992) and Davison & Clements, (1998).

All herpesviruses possess large, linear double-stranded (ds) DNA genomes and hence have the capacity to encode many viral proteins and enzymes. All specify a variety of enzymes involved in nucleic acid synthesis and metabolism. Herpesvirus virions are correspondingly large and complex with a common morphology consisting of four elements; core, capsid, tegument and envelope. The core consists of the ds DNA genome which is packaged into an icosahedral capsid. The nucleocapsid is surrounded by an amorphous, proteinaceous layer known as the tegument which contains a variety of viral proteins and enzymes. The tegument is, in turn, enclosed within an envelope consisting of a lipid bilayer derived from host nuclear or golgi membranes, which contains several viral glycoproteins. The number of viral glycoproteins on the virus particle varies between different herpesvirus but four, gB, gH, gL and gM appear to be common to all herpesviruses. The generalised herpesvirus particle structure is shown in Figure 1.1.

The general life cycles of the herpesviruses are similar. Virus entry to the host cell is accomplished by glycoprotein-mediated binding and fusion with the cell membrane. The nucleocapsid complex is then transported to the nucleus, into which the viral genome is released. Replication of the viral genome and construction of progeny nucleocapsids then takes place in the nucleus. Virions acquire an envelope by budding through the inner nuclear membrane, but this is followed by de-envelopment at the outer nuclear membrane. It is thought that virions finally acquire an envelope from post-endoplasmic reticulum cytoplasmic compartments. This has been shown for herpes simplex virus (Skepper *et al.*, 2001). Mature virions are thought to exit the cell by a process of exocytosis. Ultimately, virus production results in cell death. Replication is mediated largely by viral enzymes but is dependent on host cell enzymes for several functions such as DNA ligation and synthesis of base precursors.

Herpesvirus genome arrangements consist of various combinations of unique

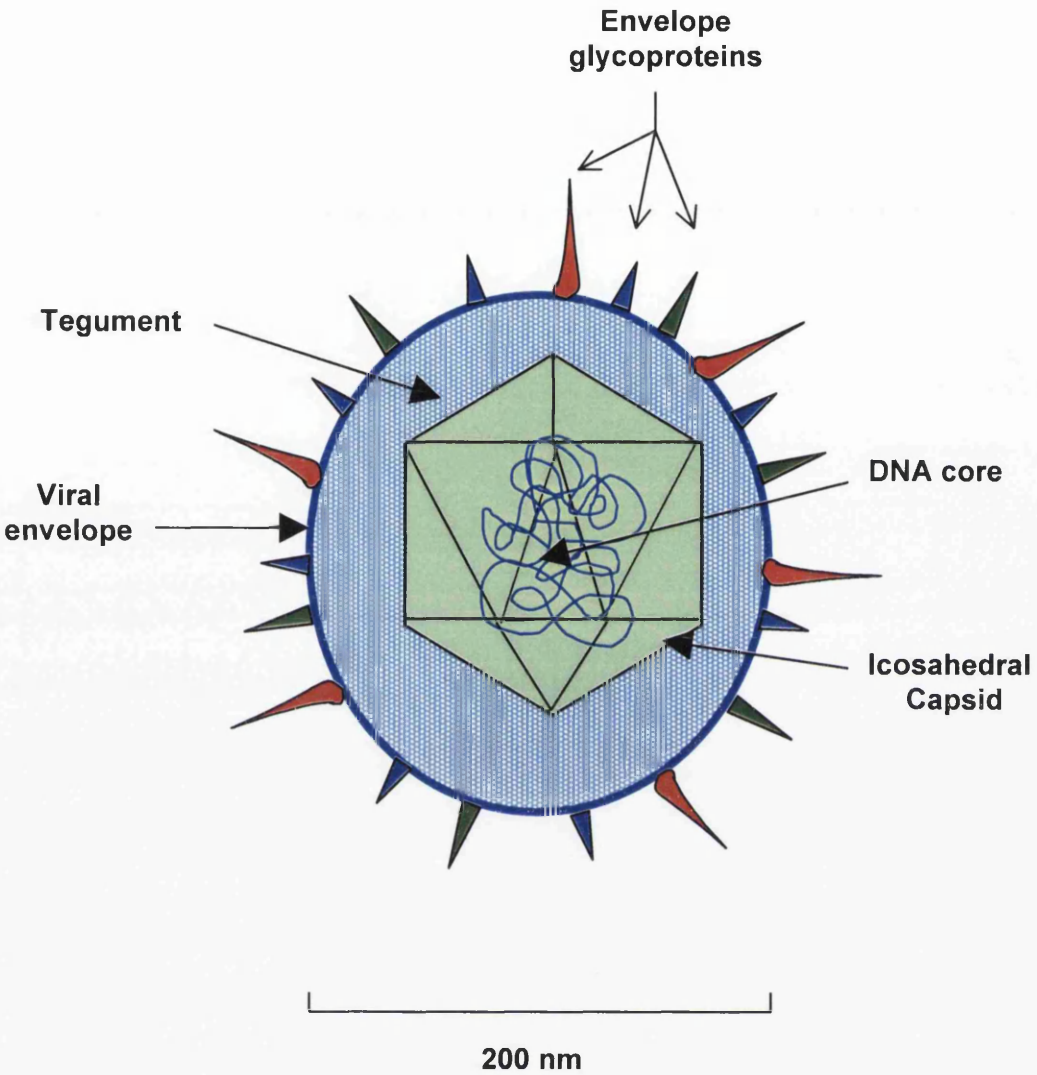


Figure 1.1 Generalised structure of a Herpesvirus virion

A schematic representation of a herpesvirus particle virion is shown, with the DNA core, icosahedral capsid, tegument layer and lipid envelope indicated. The viral envelope also contains various glycoproteins which protrude from the surface. The number of envelope glycoproteins varies amongst the herpesviruses. An indication of the average virion diameter is also given.

sequences and repeated elements, which may be present either internally or at the genome termini. This gives rise to a variety of distinct genome organisations and sizes depending on the sequence arrangements and the copy number of the repeated regions, respectively. Hence, herpesvirus genomes have been classified into seven groups according to the genome arrangement, as described by Roizman *et al.*, (1992) and Davison & McGeoch, (1995). The structures of the seven genome classes are represented in Figure 1.2.

Perhaps the most distinctive feature of the herpesviruses is their ability to establish latent infections in their natural hosts. Following primary infection, herpesviruses can persist in a quiescent state, with strictly limited gene expression and without active replication. Such latent infections typically last for the entire life of the host. The cell type in which latency is established varies between the different herpesviruses, but the exact site has been elucidated in only a few cases. Reactivation to productive infections may occur sporadically throughout the life span of the host. The mechanisms underlying the establishment of latency and reactivation are not completely understood.

Collectively, the herpesviruses have a wide host range, from invertebrates (Comps & Cochennec, 1993) to a wide variety of vertebrate organisms including fish, amphibians, reptiles, birds and mammals. Those which infect humans have been widely studied. Human herpesviruses (HHVs) are ubiquitous agents with large proportions of the world-wide population having been exposed to and latently infected by one or more of these viruses. They are generally spread by close contact via bodily secretions. HHVs are discussed further in section 1.1.3.

1.1.2 Herpesvirus sub-classification

Owing to the large numbers and diversity within the herpes family, it is sub-divided into three subfamilies (Roizman *et al.*, 1981). Sub-classification has traditionally been made on the basis of differing biological properties, such as host range, length of reproductive cycle, cytopathology and site of latent infection. However increasingly, genome sequence data is being used for the purpose of herpesvirus classification (e.g McGeoch *et al.*, 1995). In most cases, the original classifications have been substantiated by the groupings which have now been made on the basis of comparison of sequence data. The updated herpesvirus classifications were published in the current International Committee on Taxonomy of Viruses report (Minson *et al.*, 2000). The herpesvirus sub-families are as follows;

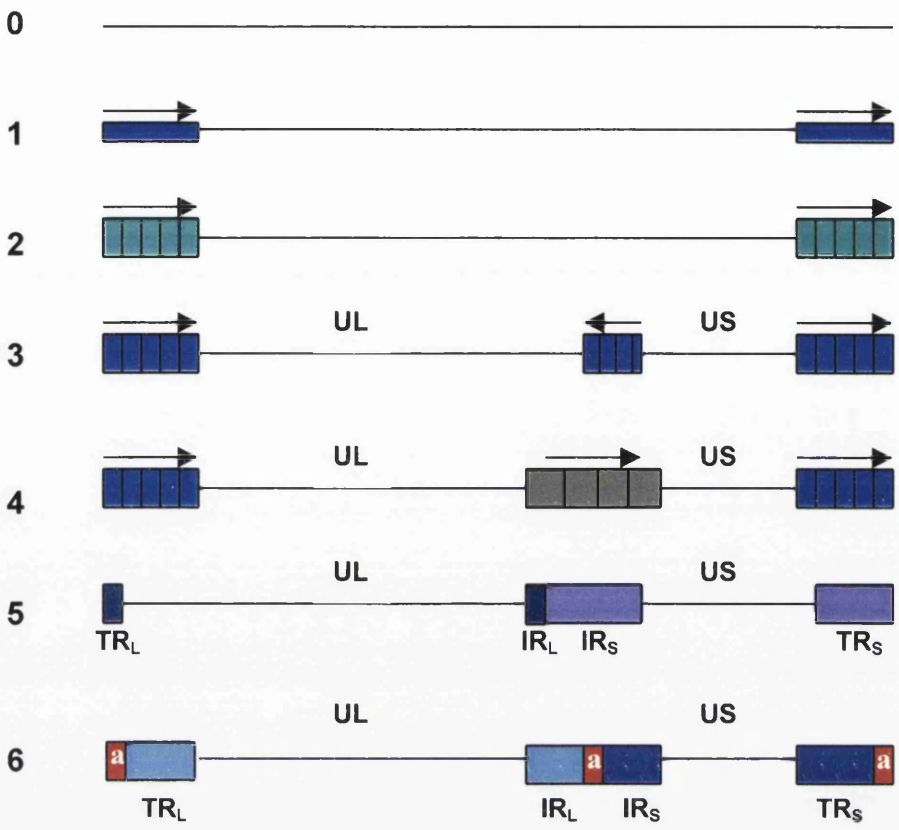


Figure 1.2 Herpesvirus genome arrangements
Schematic representations of the 7 classes of herpesvirus genomes are shown (not to scale). Unique and repeat regions are depicted by horizontal lines and rectangles, respectively. The orientations of unique and repeated sequences are indicated by arrows. Genome structure 0 corresponds to the F group described by Roizman *et al.*, (1992) whilst structures 1-6 correspond to those described by Davison & McGeoch (1995).
Group 0 consist of a single unique sequence e.g tree-shrew herpesvirus.
Group 1 consist of a single unique region flanked by direct terminal repeats e.g HHV-6, HHV-7.
Group 2 consist of a single unique region flanked by groups of direct terminal repeats e.g herpesvirus saimiri.
Group 3 consist of two unique regions (UL and US) flanked and separated by a group of direct repeats e.g cottontail rabbit herpesvirus.
Group 4 consist of two unique regions separated by a group of internal repeats. Each end of the genome is flanked by groups of direct terminal repeats unrelated to the internal repeats e.g EBV
Group 5 consist of two unique regions, each region being flanked by a pair of unrelated inverted repeats. The pair of repeats flanking UL are relatively short. VZV has this genome arrangement.
Group 6 consist of two unique regions, each region flanked by a pair of inverted repeats. The repeats flanking UL and US regions are not related. An additional repeat, called the 'a' sequence is found at the genomic termini and between the internal repeated regions. HSV-1 and HCMV both share this type of genome arrangement.

Alphaherpesvirinae

These are typically neurotropic viruses with a short reproductive cycle and a wide host range *in vitro.* They are highly cytolytic and some members have been shown to establish latent infections in neurones.

Betaherpesvirinae

These are slow-growing viruses with a narrow host range *in vitro* and characteristic cytopathology. Infected cells often become enlarged and fuse to form multinucleate cells called cytomegalia. Latent infections have been associated with cells of the monocyte series.

Gammaherpesvirinae

Gammaherpesviruses are generally lymphotropic and often establish latency in T or B lymphocytes. Host range in cell culture and length of reproductive cycle is variable, as is the resulting cytopathology. Productive infections are associated with the development of lymphoproliferative disorders.

Subfamilies of the herpesvirus are further divided into genera, on the basis of nucleotide or predicted amino acid sequences. Members of a distinct genus are grouped if their sequences form a distinct lineage within the subfamily.

1.1.3 Human herpesviruses (HHVs)

Eight herpesviruses which infect humans have been identified to date (designated HHV-1 to HHV-8). Generally, primary herpesvirus infection in immunocompetent hosts does not result in severe or fatal disease. However, herpesvirus infections in certain susceptible populations, such as immunocompromised individuals, may cause serious illness. In addition, several HHVs are also associated with the development of malignant tumours.

Three alphaherpesviruses are known to infect humans. Herpes simplex viruses 1 (HSV-1 or HHV-1) and 2 (HSV-2 or HHV-2) are closely related. Both are associated with mucosal infections and establish latency in sensory ganglia. HSV-1 is primarily associated with mucosal infections of the mouth and throat and may cause symptoms including fever and more commonly, oral lesions (cold sores), but is also associated to a lesser extent with genital mucosal infections. Conversely, HSV-2 is primarily associated with mucosal infections of the genitalia and to a lesser extent the mouth and throat. Both viruses can reactivate which is manifested by lesions in the skin

served by the sensory ganglia. Varicella-zoster virus (VZV or HHV-3) is the third human alphaherpesvirus and the causative agent of chicken pox during primary infection. VZV establishes latency in sensory neurones and reactivation results in the more severe illness of shingles.

Human cytomegalovirus (HCMV or HHV-5) is a well-studied betaherpesvirus which causes widespread infection in humans. As with other HHVs, primary infection is usually asymptomatic but severe disease can occur in susceptible populations infected with HCMV. The scope of HCMV-associated disease is discussed further in section 1.2.1. HHV-6 and HHV-7 are more recently identified human betaherpesviruses. Both are associated with febrile illnesses in children and post-transplant disease in immunosuppressed transplant recipients. Recent studies have also implicated HHV-6 in the aetiology of multiple sclerosis and chronic fatigue syndrome (Ablashi *et al.*, 2000). HHV-6 has been found in a latent state in macrophages (Levy, 1997) whereas the site of latency for HHV-7 is unclear.

The final 2 HHVs are members of the *gammaherpesvirinae*. Epstein-Barr virus (EBV or HHV-4) was the first human gammaherpesvirus to be identified and is the causative agent of infectious mononucleosis in a proportion of primary infections. EBV establishes latency in B-lymphocytes and has been associated with malignancies including Burkitts lymphoma, Hodgkin's disease and nasopharyngeal carcinoma. HHV-8, also known as Kaposi's sarcoma-associated herpesvirus (KSHV), is a gammaherpesvirus which is the causative agent of Kaposi's sarcoma and is also associated with two other human cancers, primary effusion lymphoma and multicentric Castleman's disease. HHV-8 may also be a co-factor involved in the progression of HIV infection. Kaposi's sarcoma is the most common neoplasm in AIDS sufferers and is a serious complication, being associated with an increased risk of death (Brodt *et al.*, 1998).

1.1.4 Anti-herpetic chemotherapy

As herpesvirus DNA replication is largely autonomous from the host cell replicative machinery, this stage of the life cycle is a suitable target for anti-viral therapies. Herpesvirus DNA replication enzymes are sufficiently distinct from their cellular counterparts to allow specific targeting of viral functions. Hence, the vast majority of current anti-herpetic drugs target herpesvirus DNA polymerase enzymes and many are nucleoside analogues.

Acyclovir is the most widely utilised and successful anti-herpetic drug, used mainly to treat HSV infections. Acyclovir is a guanine nucleoside analogue which is phosphorylated to the mono-phosphate form (acyclo-GMP) by the viral thymidine kinase (tk) and to the triphosphate form (acyclo-GTP), by host cellular kinases. The activated form is incorporated selectively by the viral polymerase into the growing viral DNA chain, where it acts as a terminator of further chain elongation. The success of acyclovir can be attributed to its high specificity for viral enzymes, as it requires phosphorylation by the viral tk, and in addition, incorporation is far more specific to the viral polymerase than cellular counterparts. However, other herpesviruses are less sensitive to acyclovir than HSV.

HCMV-specific chemotherapy became available with the introduction of gancyclovir, which is a guanine analogue similar to acyclovir. It also requires tri-phosphorylation before it is incorporated into the growing DNA chain. The first phosphorylation is catalysed by the HCMV protein kinase UL97 and subsequent phosphorylations are carried out by cellular enzymes. Incorporation of gancyclovir decreases the likelihood of further extension of the DNA chain. Despite its effectiveness against the HCMV polymerase, gancyclovir has unpleasant side effects and is associated with haemotoxicity and nepherotoxicity which precludes its use beyond serious and life-threatening HCMV infection.

Anti-herpetic nucleoside analogues such as acyclovir and gancyclovir suffer from poor bioavailabilty and short half life. Consequently, various other nucleoside analogues which have improved pharmacokinetic properties, such as the pro-drug, valaciclovir, have been developed. Valaciclovir is a valine ester of acyclovir which is better absorbed prior to its cleavage to render acyclovir and the natural amino acid. Non-nucleoside analogues to treat herpesvirus infections have also been developed. Foscarnet is distinct from other herpesvirus drugs as it does not require phosphorylation to an active form. It is a pyrophosphate analogue which functions as a direct DNA polymerase inhibitor, interfering with the binding of incoming nucleotide triphosphates (Coen, 1992). Unfortunately, foscarnet is also associated with side effects including nepherotoxicity which limit its usefulness.

Considering the wide spectrum of human disease associated with herpesvirus infections and the poor bioavailability, undesirable side effects and emergence of resistant strains associated with many current herpesvirus treatments, the need for novel antiviral therapies effective against HHVs is clear. Recently, novel HCMV

therapeutic compounds which target viral proteins other than the polymerase have been developed and some are currently undergoing trials. Benzimidavir is one such drug, which targets the HCMV viral protein kinase UL97 (Davis *et al.,* 1998; Sethna *et al.*, 1998). An antisense oligonucleotide based therapy, Fomiversen, has recently been licensed for intraocular HCMV treatment. Fomiversen works by binding to complementary mRNA sequences transcribed from the major immediate-early gene, which encodes the IE1 and IE2 proteins. As the IE1 and IE2 proteins are essential for transactivation of subsequent classes of genes, viral replication is blocked at this stage through inhibition of translation of the IE1 and IE2 mRNAs (Azad *et al.,* 1993).

1.2 HCMV Biology

1.2.1 HCMV Pathogenesis

HCMV is extremely prevalent amongst all populations world-wide. Infection rates, as defined by seropositivity, range from 50-75% in developed countries to nearly 100% of the population in closely crowded environments and under-developed countries (Haffey & Field 1995). Crowded environments, rather than hygiene or social class, are correlated with higher rates of infection (Britt & Alford, 1996). Transmission occurs via contact with infectious bodily fluids.

In vitro, HCMV has a very restricted host cell range and a slow replicative cycle, however, *in vivo,* HCMV is capable of infecting and replicating in a wide variety of cell types (reviewed by Sinzger & Jahn, 1996), and has been shown to be a quickly-replicating virus (Emery *et al.*, 1999). Major cell types that are susceptible to HCMV infection *in vivo* are the ubiquitously distributed epithelial, endothelial and fibroblast cells. Leukocytes also harbour infectious HCMV, although viral gene expression has so far only been detected in these cells during acute infection (Gerna *et al.*, 1992).

The vast majority of HCMV infections are asymptomatic in immunocompetent hosts. Although rare, symptomatic HCMV infection may take the form of a mononucleosis-like syndrome similar to that caused by EBV. Primary infection with HCMV results in a lifelong latent infection. Granulocyte/monocyte progenitor cells have been proposed as sites of HCMV latency and reactivation of HCMV from these cells has been described (Soderberg-Naucler *et al.*, 1997; Hahn *et al.*, 1998). Stress which stimulates the release of catecholamines is suggested as one pathway of HCMV reactivation in latently infected individuals (Prosch *et al.*, 2000).

HCMV is, however, a medically important virus owing to its propensity to produce serious illness in immuno-compromised individuals, in which it is a common

opportunistic pathogen. The main populations susceptible to severe HCMV-associated disease are infants infected *in utero*, neonates, organ transplant recipients receiving immunosuppressive treatments and AIDS patients.

HCMV is the most common congenital viral infection, with an incidence in the USA of between 0.2-2.2%. Clinical aspects of congenital HCMV infection have been reviewed by Britt & Alford, (1996). Approximately 10-15% of infected babies will develop symptomatic HCMV disease, with the major risk factors associated with severe disease being primary infection of the mother during pregnancy and infection early in gestation. Severe disease can result in a range of symptoms including retinitis, hepatitis, gastroenteritis, pneumonitis and permanent neurological damage such as hearing loss.

HCMV is a consistently prevalent pathogen in transplant recipients with high proportions of patients developing HCMV infections following transplantation. In solid organ transplant recipients, infection rates can be between 60-100% (Pollard, 1988). A wide variety of HCMV disease is observed in such patients, with increased severity in individuals experiencing primary infection. Severe infection of the GI tract, hepatitis and pneumonia are frequent and potentially life-threatening complications. In bone marrow transplant (BMT) recipients, in particular, HCMV infection is associated with high mortality rates, with HCMV-associated pneumonia the major manifestation of disease (Britt & Alford, 1996).

HCMV is one of the most common opportunistic infections in AIDS patients and frequently causes severe and life-threatening disease, either through primary infection or reactivation of productive infection in seropositive individuals. One autopsy study showed that 90% of AIDS patients experience active HCMV infections and up to 40% will develop life- or sight-threatening HCMV disease (Gallant *et al.*, 1992). HCMV disease in AIDS patients can involve multiple organ systems but the most clinically significant occur in the lung, CNS and GI tract (Britt & Alford, 1996). In particular, HCMV pneumonitis and retinitis are associated with poor prognosis and high mortality. The introduction of highly active anti-retroviral therapy (HAART) for AIDS patients has resulted in a decrease in severe disease caused by opportunistic pathogens, such as HCMV. However, HCMV-associated disease is still a concern, especially as drug-resistant HIV and HCMV strains continue to emerge.

In addition to its role as an opportunistic pathogen in HIV-infected patients, HCMV has been implicated as a co-factor in the pathogenesis of AIDS. HCMV seropositivity is associated with increased risk of AIDS development and early death of AIDS patients

(Webster *et al.*, 1989; Gallant *et al.*, 1992). Numerous studies have demonstrated interactions between HCMV and HIV *in vitro*, such as those by Toth *et al.*, (1995) and Lathey *et al.*, (1994) who showed that HCMV could enhance replication and production of infectious HIV-1. However, direct evidence that HCMV contributes to progression of HIV infection *in vivo* has yet to be established (Britt & Alford, 1996). HCMV has also recently been shown to activate lytic replication of latent HHV-8 (Kaposi's sarcoma-associated herpesvirus), suggesting that it may be an "augmenting co-factor" in HHV-8 disease (Vieira *et al.*, 2001).

HCMV has also been implicated in the pathogenesis of other disease states. Hiemstra *et al.*, (2001) presented evidence that HCMV may be involved in the development of autoimmune disease through a mechanism of molecular mimicry of human glutamic acid decarboxylase (GAD65) by the HCMV UL57 protein. HCMV has also been implicated in the development of atherosclerosis (Horvath *et al.*, 2000).

1.2.2 The HCMV Genome

The linear ds DNA genome of HCMV is 230-240 kbp and represents the largest of all the herpesviruses identified to date. HCMV is the only betaherpesvirus to possess a Group 6 genome structure, an arrangement which is also shared by HSV-1 (Figure 1.2). It consists of 2 unique sequences (UL and US), each flanked by a pair of inverted repeats, IRS/TRS and IRL/TRL, which are not related. An additional repeat, known as the *a* sequence, is found at the genomic termini and the junction between the internal repeats (IRL/IRS). This genomic arrangement can give rise to 4 genome isomers, through inversion and differential orientation of the L and S components which is mediated by the *a* sequence (Kemble & Mocarski, 1989). The four isomers appear to be functionally equivalent.

HCMV strain AD169 has been completely sequenced (Genbank accession number X17403) by Chee *et al.*, (1990) and is predicted to encode 208 ORFs. HCMV contains seven conserved sequence blocks shared with other herpesviruses. The gene products of the ORFs contained within these conserved blocks are involved in DNA repair and replication, nucleotide metabolism or virion structure. Many HCMV gene products have yet to be characterised, but of those which have been identified, 41 are dispensible for growth in human fibroblast (HF) cell culture (Mocarski, 1996). It has become evident that there is considerable genome heterogeneity amongst the highly passaged laboratory strains Towne and AD169, and clinical HCMV isolates. Cha *et al.*, (1996) found segments of DNA in the virulent Toledo strain that were absent in Towne

and AD169 strains. Both laboratory strains also lacked up to nineteen reading frames found in clinical isolates. Heterogeneity between different stocks of AD169 strain has also been demonstrated by Dargan *et al.*, (1997) who showed that the published AD169 sequence lacks 1 kbp of DNA present in other AD169 stocks. Hence, as the published sequence was obtained from a highly passaged strain of HCMV, it is perhaps not appropriate to extrapolate the findings to clinical strains. Long-term passage in cell culture is associated with a decrease in virulence which may result from the loss of genetic information during propagation in cell culture (Brown *et al.*, 1995).

The HCMV genes are designated according to the region of the genome where they are positioned (e.g UL, US) and are numbered sequentially. Throughout this thesis, the gene product encoded by a particular HCMV gene is named after the gene itself. This is the generally accepted nomenclature but some proteins are additionally named on the basis of their size and function, for example UL83 is also known as pp65 or the lower matrix protein. A schematic representation of the HCMV genome, with the cis-acting regions and genes involved in DNA replication indicated, is given in Figure 1.3.

1.3 HCMV Lytic cycle

1.3.1 HCMV attachment and penetration of host cells

The primary step in HCMV infection of target cells is virus attachment to the cell surface. Initial interactions between the virus particle and the cell surface are known to involve heparan sulphate (Compton *et al.*, 1993), however the cellular receptor(s) which mediate binding has not yet been identified. Several studies have implicated cellular surface proteins with molecular masses of 30-34 kDa and 92.5 kDa, respectively, as candidate receptors (Adlish *et al.*, (1990); Taylor & Cooper (1990); Keay *et al.*, (1989)). Nowlin *et al.*, (1991) studied the distribution of the putative 30-34 kDa receptor and found it was ubiquitously expressed on a wide range of cell types and that the level of virus attachment to target cells, but not penetration, correlated with the abundance of this receptor. These results are consistent with the ability of HCMV to infect a wide range of cell types *in vivo* (Sinzger & Jahn, 1996).

HCMV gB is thought to mediate the interaction with cellular receptors and binding kinetic experiments have suggested it may associate with two classes of receptor (Boyle & Compton, 1998). In addition, gB-specific MAbs have been shown to block penetration, but not attachment, of HCMV to cells (Navarro *et al.*, 1993). Studies carried out by Keay & Baldwin (1991) suggest that gH may also be involved in fusion of

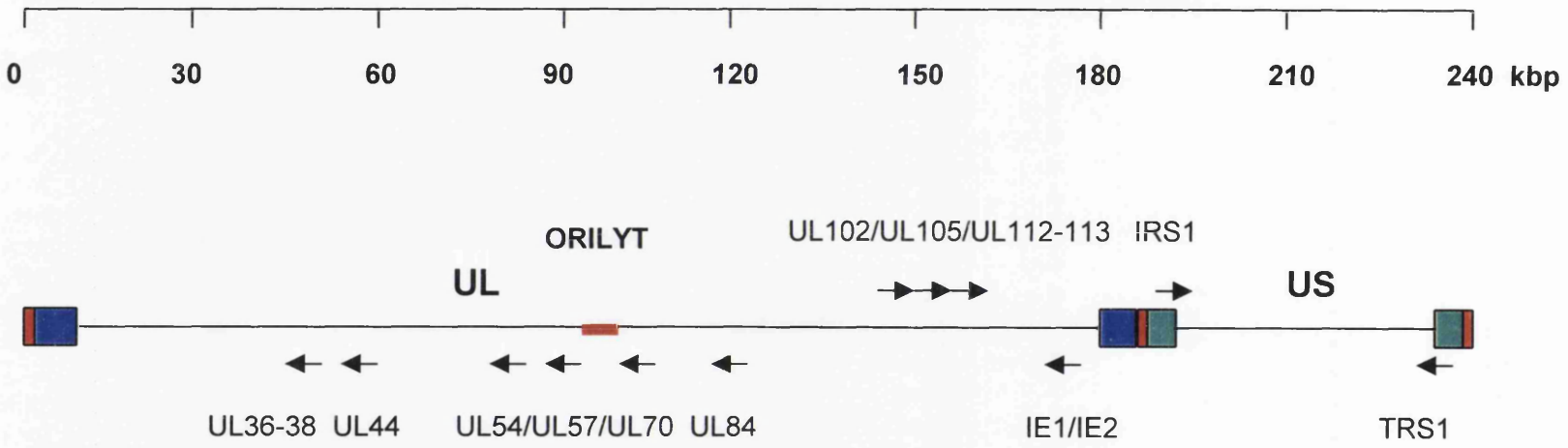


Figure 1.3 The HCMV Genome showing regions involved in DNA replication
The HCMV genome is represented in schematic form, with ORFs and *cis*-acting sequences required for origin-dependent DNA replication indicated. The Unique long (UL) and unique short (US) sequences are represented by lines. The inverted repeats and *'a'* sequence are represented as follows; (TRL/IRL), 'a' sequence, (TRL/IRL). The position and orientation of the ORFs identified by Pari & Anders (1993) as being necessary for origin-dependent DNA replication in a transient assay are indicated by arrows. The origin of replication, ORILYT, located in the centre of UL, is represented by a thick red line.

viral and host cell membranes. Virus entry occurs through direct fusion of the viral envelope with the cellular plasma membrane and is pH independent (Compton *et al.*, 1992).

1.3.2 Early events in HCMV lytic infection/replication

Following penetration, HCMV capsids migrate to the nucleus by an as yet, unknown mechanism. The DNA is released into the nucleus and the genome is circularised prior to replication (LaFemina & Hayward, 1983; McVoy & Adler, 1994). Once inside the nucleus, viral DNA associates with or close to nuclear structures called nuclear domain 10 (ND10), also known as promyelocytic leukemia protein (PML) oncogenic domains (PODs) (Ishov & Maul, 1997). ND10 are punctate nuclear structures that contain several cellular proteins, including PML and transcription factor SP100. Various HCMV encoded proteins subsequently associate with ND10. HCMV immediate-early protein IE1 causes a redistribution of PML from punctate ND10 structures to a diffuse nuclear pattern and IE2 associates with PML without any obvious effect on its localisation (Ahn & Hayward, 1997). The disruption of PML-associated nuclear bodies may therefore be a critical event for efficient lytic replication of HCMV. Ishov *et al.*, (1997) demonstrated that association with ND10 is also necessary for HCMV IE gene transcription and that viral transcripts were directed from ND10 into splicesome assembly factor (SC35) domains through the accumulation of viral IE2 protein at these sites. IE2 then recruits components of the basal transcription machinery, such as TFIIB. Hence, HCMV creates an immediate transcription environment by utilising existing intranuclear structures, facilitating initiation of lytic replication.

The dispersal of ND10-associated proteins is a feature common to several DNA viruses, suggesting this may be an important event in the early stages of lytic infection. This characteristic was first observed in HSV-1 infected cells where the viral Vmw110 protein was found to be responsible for redistribution of PML from ND10 domains (Everett & Maul, 1994). Redistribution of ND10 proteins also occurs in EBV-infected cells (Szekely *et al.*, 1996) and adenovirus-infected cells (Carvalho *et al.*, 1995).

1.3.3 DNA Replication

As with the other herpesviruses, replication of HCMV DNA is initiated at a specific site on the genome, called the origin of replication. In HCMV, this is known as orilyt, which is described further in section 1.5.1. Following the circularisation of viral genomic DNA, synthesis of viral DNA begins at orilyt, although the process of initiation of replication has not yet been elucidated. Initial rounds of replication are thought to proceed via

IE2 uncouples passage through G1 restriction pt with commitment to replicate DNA
So, allows cell to enter S phase but not undergo DNA rep. ∴ IE2 promotes expression of cellular rep. factors but disallows completion cell DNA synth.

theta replication of the circular template followed by rolling circle replication which produces concatemeric products. Viral DNA synthesis takes place in replication compartments which form near the cellular ND10 structures described in section 1.3.2. HCMV IE2 protein and auxiliary replication protein UL112-113 co-localise in close proximity to ND10 at early times in infection and both proteins are subsequently incorporated into large viral replication compartments which form at the periphery of ND10. These are the sites at which core replication proteins accumulate for initiation of viral DNA synthesis (Ahn *et al.*, 1999). Penfold & Mocarski, (1997) have also reported the localisation of UL112-113 and replication fork proteins UL57 and UL44 to subnuclear structures which subsequently develop into replication compartments. HCMV DNA replication is discussed further in section 1.5.

1.3.4 HCMV gene expression

As with the other herpesviruses, HCMV gene expression is highly regulated with genes being sequentially expressed in three temporal classes; immediate early or α, early or β and late or γ, based on the time of synthesis following infection.

Immediate-early gene expression

Immediate early genes are the first to be expressed upon infection and do not require *de novo* protein synthesis for their expression. Four major regions of the HCMV genome are expressed with immediate-early kinetics; the major immediate-early (MIE) region (UL122/UL123), UL36-38, TRS1/IRS1 and US3. All of these gene products are involved mainly in regulating the expression of subsequent classes of genes. The vast majority of IE transcripts originate from the major IE locus spanning the UL122 and UL123 genes, giving rise to two main gene products which are both nuclear phosphoproteins; IE1(IE72) and IE2 (IE86). IE2 is a potent transactivator which can stimulate expression of HCMV early genes by itself or can act synergistically with IE1, or other IE gene products, to activate some early gene promoters (Artl *et al.*, 1994; Schwartz *et al.*, 1994; Scully *et al.*, 1995; Depto *et al.*, 1989; Malone *et al.*, 1990; Colberg-Poley *et al.*, 1992; Kerry *et al.*, 1994;). IE1 and IE2 can also positively and negatively modulate expression from their own promoter, respectively (Cherrington & Mocarski, 1989; Stenberg *et al.*, 1990; Hermiston *et al.*, 1990). Detailed characterisation of IE1 and IE2 has revealed that, in addition to their involvement in gene expression, these proteins can modulate diverse cellular processes. IE1 and IE2 can both inhibit apoptosis and IE2 was recently also shown to block cell cycle progression following transition into the S phase (Zhu *et al.*, 1995; Murphy *et al.*, 2000). The regulation of HCMV IE gene expression has been reviewed by Meier & Stinski,

(1996). Expression of immediate-early genes is required for activation of subsequent early and late gene expression, as well as for modulating cellular processes to promote a favourable environment for viral replication. As the IE proteins are essential for lytic HCMV replication, the ability of a given cell type to support expression of IE gene expression may determine HCMV cell tropism. Expression of IE1 and IE2 does not occur in blood monocytes or granulocyte-macrophage progenitor cells, in which HCMV genomes are found only in a latent state.

The UL36-38 locus gives rise to several proteins which synergise with IE1 in regulating transcription of early genes (Colberg-Poley *et al.*, 1992). Similarily, the IRS1/TRS1 gene products co-operate with IE1/IE2 to activate early gene products. US3 can also transactivate gene expression in conjunction with other HCMV proteins but also functions to aid immune evasion by binding to and retaining MHC class I chains in the endoplasmic reticulum, thereby inhibiting antigen presentation (Colberg-Poley *et al.*, 1992; Ahn *et al.*, 1996).

Early gene expression

The HCMV early genes are the second class of genes to be transcribed and their expression is dependent upon synthesis of functional IE proteins. The early genes encode a wide variety of proteins including many involved in DNA replication and metabolism, and some non-structural proteins.

Late gene expression

The late genes are the final class of genes to be transcribed and their expression is either dependent on or greatly stimulated by viral DNA synthesis. Late genes constitute the majority of the HCMV genome and primarily encode structural proteins. Expression usually occurs at 48 hours post-infection or later.

Temporal distinction of HCMV gene expression is perhaps not always clear-cut as translation of mRNAs does not always immediately follow their transcription and during productive infection, IE, E and L gene expression can all occur simultaneously at late times in infection (Mocarski, 1996).

1.3.5 Capsid assembly and DNA packaging

The process of DNA packaging in herpesviruses involves the cleavage of long linear concatemeric DNA molecules at specific junctions between individual genomes followed by packaging the DNA into pre-formed capsids. HCMV capsid assembly is

not well understood and current models are based on what is known regarding capsid assembly in HSV-1. The structure and assembly of HCMV virions has been reviewed by Gibson, (1996). The four proteins which constitute the capsid shell are the major capsid protein (MCP or UL86), minor capsid protein (mCP or UL85), mCP-binding protein (mCP-BP or UL46) and the smallest capsid protein (SCP or UL48/49). Two other proteins are involved in capsid assembly: the assembly protein (AP) precursor and the proteinase precursor. The capsid shell proteins migrate to the nucleus where they assemble into precursor capsids called preB-capsids. Maturation of the preB-capsids occurs upon proteolytic cleavage of the proteinase and assembly protein precursors, which causes a conformational change in the particle. Cleaved assembly protein is then eliminated, allowing packaging of DNA into the mature capsid.

Cis -acting sequence elements known as *pac*-1 and *pac*-2, located within the genomic *a* sequence repeats, act as signals for DNA packaging in herpesviruses, including HSV-1 and HCMV. These sequences are located near to the cleavage site and are thought to bind viral and possibly, cellular proteins involved in the cleavage process. Individual HCMV genomes are probably packaged into capsids in a process similar to that in HSV-1, supported by data which shows that HSV-1 recognises and packages plasmid DNA bearing the HCMV *a* sequence (Spaete & Mocarski, 1985). However, the process of genome maturation in HSV-1 and HCMV is not well understood.

In HSV-1, seven proteins have been identified that have probable roles in cleavage and packing of newly synthesised viral DNA (reviewed by Homa & Brown, 1997). Homologs of these proteins have also been identified in HCMV. These are UL104, UL89, UL77, UL56, UL52, UL51 and UL93 (Chee *et al.,* 1990). All the genes encoding these proteins have homology with ORFs in other herpesviruses (Mocarski, 1996), suggesting the mechanism of DNA packaging may be a conserved feature within the herpesvirus family.

Direct evidence has been obtained that supports the involvement of UL56 and UL89 in HCMV packaging. This evidence was provided by the demonstration that mutations conferring resistance to benzimidazole ribonucleotides, which inhibit viral DNA packaging, mapped to the UL56 and UL89 ORFs (Krosky *et al.*, 1998). UL89 has subsequently been shown to have some sequence homology with bacteriophage T4 terminase protein and binds to HCMV packaging signals (Underwood *et al.,* 1998; Wang & McVoy, IHW 1999). UL56 is a nuclease which also binds specifically to HCMV packaging signals and cleaves DNA bearing these signals (Bogner *et al.*, 1998). UL56 also localizes to viral replication compartments and interacts with the viral DNA

polymerase processivity sub-unit, UL44 (Giesen *et al.*, 2000). These findings suggest that DNA replication and packaging may be coupled processes.

1.3.6 Virus maturation and egress

Following encapsidation, the next stage in virion assembly is tegument acquisition. Virion assembly is a poorly understood process in the herpesviruses in general and HCMV is no exception. Tegumentation and envelopment of HCMV has been suggested to follow a similar pathway to the model proposed for HSV-1. Originally, it was thought that tegumentation occurs in the nucleus and envelopment takes place at the nuclear membrane (Roizman, 1996). However, recent evidence suggests that virions acquire an envelope at the inner nuclear membrane, but this is lost as the virions traverse the outer nuclear membrane. Final envelopment then occurs in a post-endoplasmic reticulum cytoplasmic compartment (Skepper *et al.*, 2001). Studies on HCMV indicate that virions acquire their envelope by a similar mechanism. Several HCMV tegument proteins, including UL25, UL99 (pp28) and UL32 (pp150), localize exclusively within the cytoplasm of infected cells, which is inconsistent with a model of nuclear tegumentation (Battista *et al.*, 1999; Sanchez *et al.*, 2000a; Sanchez *et al.*, 2000b). These results indicate that acquisition of tegument in HCMV involves a cytoplasmic phase and hence that final envelopment must also take place in the cytoplasm. Sanchez *et al.*, (2000b) further showed that several HCMV tegument proteins (pp65, pp150 and pp28) accumulated in a cytoplasmic juxtanuclear structure along with three HCMV virion glycoproteins (gB, gH and gp65) and propose that this structure may represent a cytoplasmic site of virion assembly.

Egress of HCMV virions from the cell may be mediated by an exocytic pathway as there is evidence that virions are transported in vesicles via the golgi apparatus and is sensitive to Brefeldin A, which is an inhibitor of protein secretion (Mocarski, 1996).

1.4 Herpesvirus DNA replication

1.4.1 Initiation of replication

DNA replication begins at defined regions within herpesvirus genomes known as origins. These *cis*-acting sequences provide all the necessary signals required for initiation of DNA replication and are assumed to be the sites at which viral DNA synthesis commences. There is considerable variation in the size and structure of origins amongst the human herpesviruses, indicating that mechanisms of initiation of replication at these sites, involving unwinding of the DNA duplex to form a replication fork and recruitment of the essential viral DNA synthesis proteins, may differ. Some

herpesviruses, such as HSV, VZV and HHV-6, encode specific origin-binding proteins which are involved in establishing DNA replication, but such proteins have not been identified in other herpesviruses (Elias *et al.,* 1986; Stow *et al.*, 1990; Inoue *et al.*, 1994). This is a further indication of the likely diversity in initiation of herpesvirus DNA synthesis.

Once a replication fork has been established, DNA synthesis is presumed to proceed via a common mechanism as the ORFs encoding essential replication fork proteins are amongst the conserved gene blocks found in all herpesviruses. HSV-1 was the first herpesvirus for which the origins of replication and the essential viral DNA replication proteins were identified. Having been extensively studied, it has become the prototype herpesvirus DNA replication system and much knowledge regarding herpesvirus DNA replication in general has been inferred by analogy to HSV-1.

The seven HSV-1 proteins found to be essential for origin-dependent DNA replication constitute 4 proteins or protein complexes with the following functions; an origin-binding protein, a ss DNA-binding protein, a heterodimeric DNA polymerase holoenzyme and a heterotrimeric complex possessing helicase and primase activity. Homologs of all of these proteins, except the origin-binding protein, have subsequently been identified in all herpesviruses sequenced to date, including the human herpesviruses EBV (Baer *et al.*, 1984), HCMV (Chee *et al.*, 1990) and VZV (Davison & Scott, 1986). Confirmation that the predicted homologues were required for EBV and HCMV origin-dependent DNA synthesis was obtained using transient replication assays (Fixman *et al.*, 1992; Pari & Anders, 1993). Conservation of the six proteins which constitute the basic replicative machinery indicates that the mode of DNA synthesis is shared throughout the herpesvirus sub-families. The names and functions of the six conserved DNA replication proteins in HSV-1, and their proposed homologs in HCMV and EBV, representing the α, β and γ herpesvirus sub-families, respectively, are listed in Table 1.1. The biochemical functions of the essential herpesvirus replication proteins are summarised in Figure 1.4.

1.4.2 HSV-1 origins of replication

HSV-1 contains two different origins of replication, known as ori_S and ori_L (Stow, 1982; Weller *et al.*, 1985). Ori_S is located in the inverted repeat sequence (TRs/IRs) which flanks the Us segment and hence two copies are present within the genome. One copy of ori_L is present in the centre of the U_L segment. Although they vary in size, the sequences of ori_S and ori_L are very similar. Each contain palindromic sequences

Function	HSV-1(α)	HCMV (β)	EBV (γ)
ss DNA-binding protein	UL29	UL57	BALF2
DNA Polymerase catalytic sub-unit	UL30	UL54	BALF5
DNA Polymerase processivity sub-unit	UL42	UL44	BMRF1
Helicase	UL5	UL105	BBLF/4
Primase	UL52	UL70	BSLF1
Helicase-primase accessory protein	UL8	UL102	BBLF2/3

Table 1.1 Conserved Herpesvirus DNA replication proteins
Six core replication fork proteins are conserved throughout the *Herpesviridae*. The table gives the names and functions of the conserved replication fork proteins in HSV-1, HCMV and EBV, which represent the α-, β- and γ-herpesviruses, respectively. HSV-1 was the herpesvirus for which the minimal set of proteins required for origin-dependent DNA synthesis was elucidated (Wu *et al.*, 1988). Homologs of the HSV-1 replication fork proteins were subsequently identified amongst the virus encoded proteins required for DNA synthesis in HCMV and EBV, using transient replication assays (Fixman *et al.*, 1992; Pari & Anders, 1993).

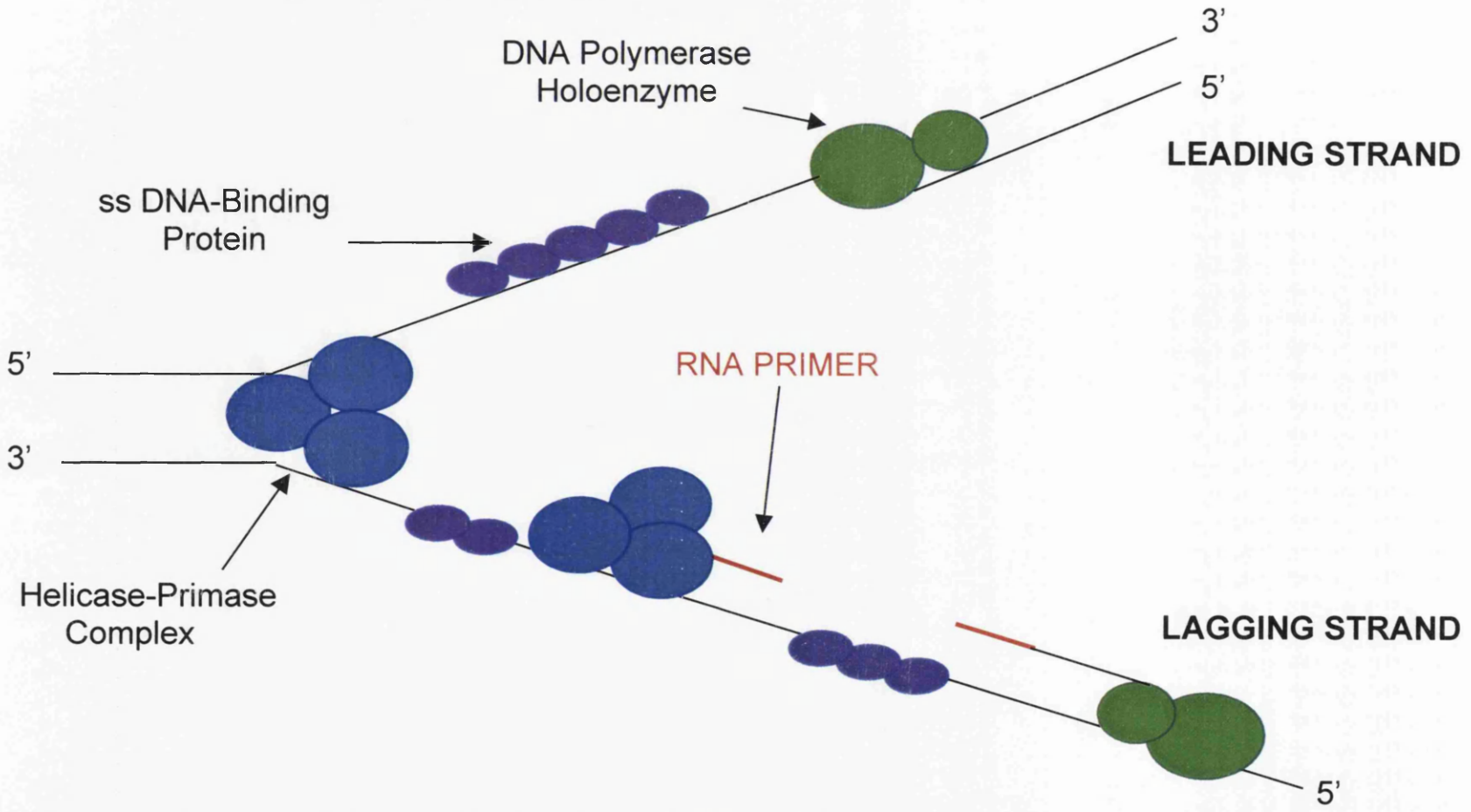


Figure 1.4 Summary of functions of essential herpesvirus replication proteins
The functions of the essential herpesvirus replication proteins are summarised on the above diagram of a replication fork depicting leading and lagging strand synthesis. At the head of the replication fork is the heterotrimeric helicase-primase complex, which functions to unwind duplex DNA and synthesise short RNA primers for elongation by the polymerase during lagging strand synthesis. Once unwound, the DNA strands are stabilised by the ss DNA binding protein, which maintains the strand in a conformation suitable for use as a template by the polymerase holoenzyme.

centered around a region consisting of A and T residues only. The core region of ori_S is approximately 80 bp and contains an imperfect 45 bp palindrome with a central 18 bp A+T region. Within the palindromic sequence flanking the A+T region of ori_s are three related sequences, box I, box II and box III, which are binding sites for the HSV-1 origin-binding protein, UL9. Box I (CGTTCGCACT) has the highest affinity for UL9, with a five-fold higher affinity than box II (TGCTCGCACT) and 1000-fold higher affinity than box III (CGTTCTCACT) (Olivo *et al.*, 1988; Koff & Tegtmeyer, 1988; Elias *et al.*, 1990; Hazuda *et al.*, 1991). Ori_L is larger and more symmetrical than ori_s, spanning a core region of 425 bp and containing a perfect 144 bp palindrome with a 20 bp A+T central region. Ori_L contains one box I and one box III sequence on either arm of the palindromic sequence. Hence, ori_L contains two very high affinity UL9-binding sites.

In addition to the UL9-binding sites, both ori_S and ori_L contain binding sites for a cellular protein, designated OF-1, whose role in HSV-1 replication is not known but appears to be necessary for full origin function (Dabrowski *et al.*, 1994). The A+T regions, UL9-binding sites and OF-1 binding sites represent the minimal core regions of ori_S and ori_L that are essential for optimal efficiency of initiation of DNA replication, however the auxiliary flanking sequences are necessary for maximum origin activity. These regions contain binding sites for cellular transcription factors but the mechanism by which they enhance origin activity is not clear.

The functional significance of the presence of three origins of replication within the HSV-1 genome and the structural differences between ori_S and ori_L is not known. There appears to be a degree of redundancy between ori_S and ori_L as mutant viruses lacking either ori_L or both copies of ori_S grow as well as wild type virus in cultured cells (Polvino-Bodnar *et al.*, 1987; Igarashi *et al.*, 1993).

1.4.3 Essential HSV-1 DNA replication proteins

The identification of the HSV-1 origins of replication enabled investigation of the identity of the viral-encoded proteins involved in DNA synthesis, using a transient complementation assay. A series of cosmids containing cloned fragments of the HSV-1 genome were tested for their ability to support replication of a plasmid containing an HSV-1 origin of replication when all were co-transfected into cells. This led to the identification of six fragments of HSV-1 DNA which supplied the necessary trans-acting functions required to replicate the transfected origin (Challberg, 1986). Systematic sub-cloning of these fragments resulted in the identification of seven viral genes which were necessary and sufficient for origin-dependent replication (Wu *et al.*, 1988). The

results of the transient assay were subsequently confirmed by detailed mapping of DNA negative mutant HSV-1 viruses (reviewed by Boehmer & Lehman, 1997) and by demonstrating that replication of an origin-containing plasmid is supported by infection of *Spodoptera frugiperda*-9 (*Sf*) cells with recombinant baculoviruses expressing the seven replication proteins (Stow, 1992).

The seven HSV-1 genes that are essential for origin-dependent DNA replication are: UL5, UL8, UL9, UL29, UL30, UL42 and UL52. The functions of these gene products elucidated by biochemical analysis are summarised in Table 1.1. The UL9 gene specifies an origin-binding protein, which is involved in initiation of the replication fork. The remaining genes encode proteins which are conserved throughout the herpesviruses and provide the core activities required at the replication fork: a processive DNA polymerase, a ss DNA-binding protein, and a helicase-primase function (Figure 1.4). The characteristics of the HSV-1 replication proteins are described further below.

The HSV-1 DNA polymerase isolated from HSV-1 infected cells is a heterodimer consisting of the UL30 and UL42 proteins (Vaughan *et al.*, 1985). UL30 is the catalytic sub-unit, which has been extensively studied since it is the target for many antiviral drugs. It shares sequence similarity to other DNA polymerases and hence, has also been studied as a model eukaryotic DNA polymerase. In addition to its polymerase function, UL30 possesses a 3'→5' exonuclease activity and RNase H activity (Knopf, 1979; O'Donnell *et al.*, 1987; Crute & Lehman, 1989). These properties are presumed to confer a proof-reading activity and the ability to remove RNA primers during the processing of Okazaki fragments, respectively.

UL42 is a phosphoprotein with sequence-independent ds DNA-binding activity which associates with UL30 and serves to increase its processivity (Gallo *et al.*, 1988; Hernandez & Lehman, 1990; Gallo *et al.*, 1989; Gottlieb *et al.*, 1990). The mechanism by which it retains UL30 on the DNA template is distinct from other processivity factors, which have no intrinsic DNA-binding ability but associate to form a multimeric toroidal structure around the duplex DNA and interact with the polymerase to prevent its dissociation. Instead, it is thought that by interacting with UL30 and ds DNA simultaneously, UL42 tethers UL30 to the template, enabling the synthesis of long DNA chains (Gottlieb & Challberg, 1994). The interaction between UL30 and UL42 is mediated by a short aa sequence in the C-terminus of UL30 and appears to be critical for DNA replication. Deletion of this sequence has no effect on the DNA polymerase

activity of UL30 but abolishes its ability to support long chain synthesis (Digard *et al.*, 1993; Tenney *et al.*, 1993) and origin-dependent DNA replication (Stow, 1993). This finding has identified the UL30-UL42 interaction as a possible novel target for anti-viral intervention, discussed further in section 1.4.5.

UL29 functions as the HSV-1 ss DNA-binding protein (Bayliss *et al.*, 1975), also known as ICP8. It binds more tightly to ss DNA than ds DNA and its binding is co-operative and independent of nucleotide sequence (Ruyechan, 1983; Ruyechan & Weir, 1984). Its main function is to bind the unwound ss DNA at the replication fork and maintain it in a conformation which can be utilised as a template by the polymerase. There is also evidence that UL29 may have additional roles at the replication fork, as it can stimulate the helicase activity of the UL9 origin-binding protein (Boehmer *et al.*, 1993) and the helicase and primase activities of the helicase-primase complex (Hamatake *et al.*, 1997).

Essential helicase and primase functions in HSV-1 infected cells are provided by a heterotrimeric complex comprised of the UL5, UL8 and UL52 proteins (Crute *et al.*, 1989). The 5'→3' helicase activity has both ATPase and GTPase activities (Crute & Lehman, 1991). The primase exhibits strong template sequence preference for the synthesis of short oligoribonucleotide primers of between 8-10 bases (Tenney *et al.*, 1995; Crute & Lehman, 1991). The UL5 protein contains six motifs characteristic of helicases and UL52 contains a sequence motif similar to that found in other DNA primases (McGeoch *et al.*, 1988), hence the helicase and primase functions have been assigned to the UL5 and UL52 sub-units, respectively. However, neither protein can be purified in isolation in a functional form so that assignment of these functions can be proven. A sub-assembly of the UL5 and UL52 proteins displays both helicase and primase functions, indicating strongly that the proposed functions are correct (Calder & Stow, 1990; Dodson & Lehman, 1991). In addition, site-directed mutagenesis studies have demonstrated that the helicase and primase active sites reside within the UL5 and UL52 components, respectively (Zhu & Weller, 1992; Klinedinst & Challberg, 1994).

The role of the UL8 protein was initially unclear, as it does not appear to perform any obvious enzymatic functions and does not bind to DNA (Parry *et al.*, 1993). Several studies have now indicated it is likely that UL8 has several auxiliary roles at the replication fork. It is known to be necessary for efficient primer utilisation by the polymerase in a model of lagging strand synthesis (Sherman *et al.*, 1992), to stimulate

Falkenberg

5' labelled — UL29 coated

UL8 req'd for helicase activity using this temp.
UL29 stimulates helicase activity only in presence of UL8

Helicase — analyse product on a denaturing gel + autoradiography

Sherman – model lagging strand synth.
UL29 coated — (co-upled primase/polym assay)
Incorporated 32P labelled dNTP's into partially or fully duplex
Circular DNA mds by electrophoresis

Primers made by S18/52 or S52 (32P labelled)
- similar as judged by denaturing electrophoresis

Inc rNTP's + dNTP's
products analysed by electrophoresis
- get range of products with S18/52 + 30/42
but S52 only → no lagging strand synth
add UL8 - get synth.
+ Pol I → S18/52 also efficiently primes but S52

No elongation of primers – UL8
They suggests stab of primase mechanism as S18/52 worked with Pol I
using M13 temp.
using simple poly dT template UL8 not req'd by Pol I.
Simple temp → reassociation of prim + temp rapid
Complex " → " slower.
∴ primers stab ↓ problem with simple temp.

Tenney 1994

Coupled primase/Pol assay
S52 gives product but UL8 stimulates by ↑ rate of primer synth
(contradicts Sherman).

UL29 stimulates primer synth of S18/52, not S52

Tenney & Hacker 1994

temp!

UL29 stim S18/52 but not S52 alone, specific

Helicase assay
displacement of strand
Spec assay for ATPase

Coupled assay — S18/52
UL8 stim primase activity
UL29 inhibits S52 atpase but adding 8 reverses inhibition.

Bernard

23ΔN ? absolutely inhibits 37°C ? to SV40 DNA rep. but not due to inability to interact with S52 as still co-IP

primer synthesis (Tenney *et al.*, 1994) and is required for efficient nuclear translocation of the helicase-primase complex (Calder *et al.*, 1992; Marsden *et al.*, 1996). UL8 is known to interact with several other replication proteins, indicating a multifunctional role at the replication fork. These interactions are described further in section 1.4.4.

The seventh essential HSV-1 DNA replication protein is UL9, which functions as an origin-binding protein, involved in the initiation of DNA replication. This function is not one of the core activities conserved throughout the herpesvirus family. Homologues of UL9 have only been identified in the alphaherpesviruses and the roseolavirus genera of the betaherpesvirus sub-family, such as HHV-6. UL9 exists as a homodimer in solution and binds to specific sequences present in HSV-1 origins (Elias & Lehman, 1988; Olivo *et al.*, 1988). It possesses DNA-dependent ATPase and helicase activity (Fierer & Chalberg, 1992; Boehmer *et al.*, 1993). The amino-terminal portion of the protein mediates the helicase, ATPase and dimerisation activities whilst the carboxy-terminal domain is involved in sequence-specific DNA binding. The non-sequence specific helicase activity of UL9 appears to be required for DNA synthesis as the introduction of mutations into the helicase motifs renders the protein unable to support DNA replication (Martinez *et al.*, 1992). There is conflicting evidence as to whether UL9 binds to single origin binding sites as a monomer or dimer (Fierer & Challberg, 1995; Martin *et al.*, 1994). Recent data from surface plasmon resonance studies indicates that UL9 binds to an oligonucleotide template containing the HSV box 1 sequence as a dimer (Graeme Thomson, personal communication). Binding of UL9 to sites I and II in ori_S is co-operative, indicating that an interaction occurs between UL9 protein(s) bound at each site (Elias *et al.*, 1990). The helicase activity of UL9 is stimulated by the ss DNA-binding protein, UL29 (Fierer & Challberg, 1992).

1.4.4 Host cell-encoded functions involved in herpesvirus DNA replication

HSV-1 DNA replication is largely autonomous from the host cell, however, several essential functions are not encoded by HSV-1 and are therefore assumed to be provided by host cell enzymes. A topoisomerase would be required to remove supercoils from replicating DNA and in addition a DNA ligase would be necessary to join the Okazaki fragments produced during lagging strand synthesis. Neither of these functions are thought to be encoded by HSV-1. Other host cell proteins may also be involved in other aspects of HSV-1 DNA replication. A cell line deficient in the cellular regulator of chromosome condensation (Rcc1) is unable to support the circularisation of HSV-1 genome DNA and its subsequent replication, indicating that this protein plays an essential role in HSV-1 replication in BHK cells (Umene & Nishimoto, 1996).

1.4.5 Assembly and co-ordination of replication fork protein functions

The recruitment of the replication fork proteins and subsequent co-ordination of their functions is mediated through a series of protein-protein interactions. Since the essential replication fork proteins were identified, they have been extensively studied to elucidate both their biochemical characteristics and how they interact with each other to perform co-ordinated DNA synthesis at the replication fork. Consequently, multiple interactions between the HSV-1 replication fork protein have now been demonstrated. The nature of these interactions and their significance to HSV-1 DNA replication has recently been reviewed by Stow, (2000). Since the replication fork proteins are conserved amongst the herpesviruses, similar interactions can be predicted to occur amongst the conserved proteins in other herpesviruses. Figure 1.5 summarises the interactions that have been demonstrated amongst the HSV-1 replication proteins, and those that have been demonstrated, or are predicted to occur between HCMV and EBV replication proteins.

HSV-1 replication protein interactions

UL30 and UL42 physically associate to form the DNA polymerase holoenzyme and are co-purified from HSV-1 infected cells (Vaughan *et al*, 1985) and there is strong evidence that the interaction is essential for viral DNA replication (Digard *et al.*, 1993; Stow, 1993; Tenney *et al.*, 1993). The helicase-primase complex is formed through interactions between the UL5, UL52 and UL8 proteins (Crute *et al.*, 1989). The physical interactions between the helicase-primase subunits underlie a strong functional interdependence to the extent that none of these proteins exhibits detectable enzymatic activity in isolation.

In addition to interactions involved in the formation of enzyme complexes, further interactions between individual proteins/enzyme complexes have been described. The origin-binding protein, UL9, interacts with UL29 (Boehmer & Lehman, 1993), UL8 (McLean *et al.*, 1994) and UL42 (Monahan *et al.*, 1998). These interactions may be important for recruitment of essential replication proteins and complexes to the origin for initiation of DNA synthesis. UL29 interacts with the polymerase accessory protein, UL42, (Vaughan *et al.*, 1984), resulting in a stimulation of polymerase activity (Ruyechan & Weir, 1984), although this interaction is not well characterised. UL29 also interacts with the helicase-primase complex, probably mediated by the UL8 subunit (Falkenberg *et al.*, 1997), and stimulates the enzymatic activities of the complex (Hamatake *et al.*, 1997). UL8 also interacts with the catalytic sub-unit of the polymerase holoenzyme, UL30 (Marsden *et al.*, 1997).

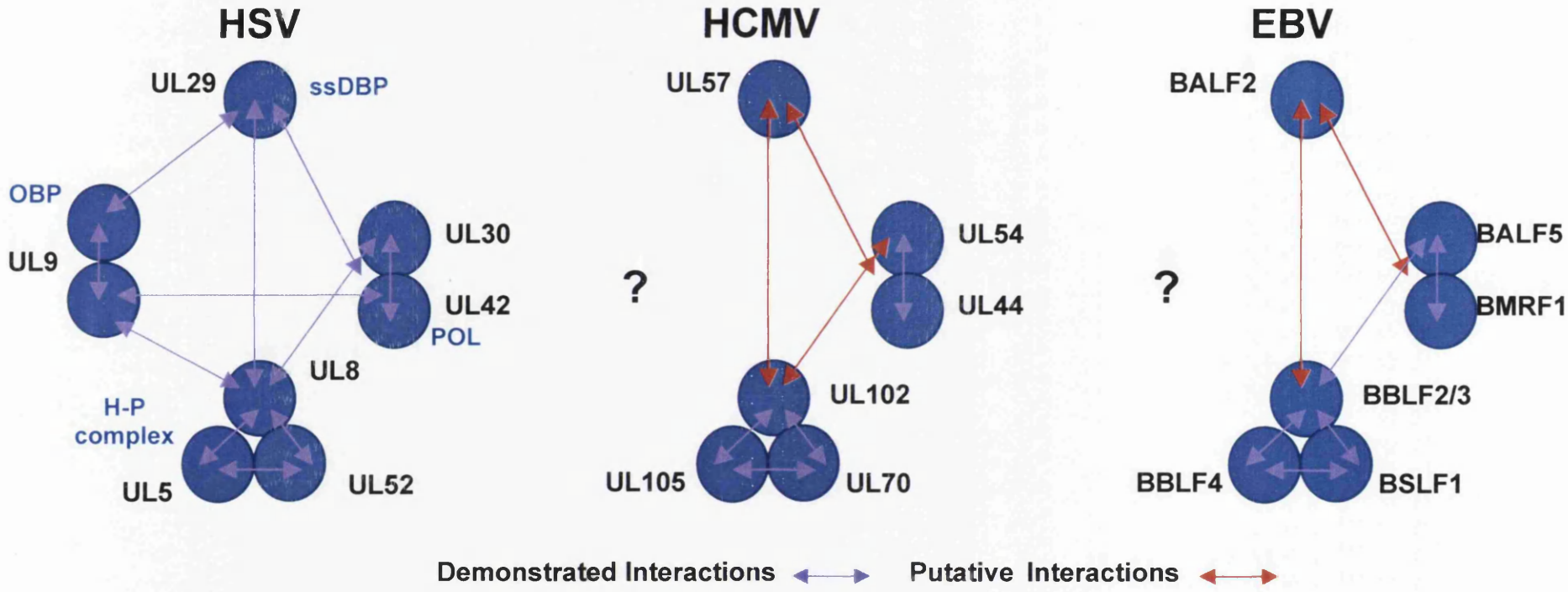


Figure 1.5 Demonstrated and predicted interactions between conserved herpesvirus replication proteins
Multiple interactions between the HSV-1 replication fork proteins have been identified. Both the HSV-1 DNA polymerase (POL) and the helicase-primase (H-P complex) are complexes formed by the close association of their respective subunits, UL30/UL42 and UL5/UL8/UL52 (Vaughan *et al.*, 1985; Crute *et al.*, 1989). In addition, there are interactions between individual proteins and protein complexes. The origin-binding protein, UL9, interacts with UL29, the ss DNA-binding proteins (ss DBP), the UL42 subunit of POL and the UL8 subunit of the H-P complex (Boehmer & Lehman, 1993; Monahan *et al.*, 1998; McLean *et al.*, 1994). UL29 interacts also with UL42 and UL8 (Vaughan *et al.*, 1984; Falkenberg *et al.*, 1997). In addition, UL8 interacts with the catalytic subunit of POL, UL30 (Marsden *et al.*, 1997). In HCMV, POL is formed through the interaction between UL54 and UL44 (Ertl & Powell, 1992). The UL70, UL102 and UL105 proteins associate to form the H-P complex (McCollum *et al.*, 1999). In EBV, BALF5 and BMRF1 interact to form POL (Tsurami *et al.*, 1993) and the H-P compex is formed by association of BBLF2/3, BBLF4 and BSLF1 (Yokoyama *et al.*, 1999). An interaction between the Pol catalytic subunit, BALF5, and the H-P associated protein, BBLF2/3, has also been described (Fujii *et al.*, 2000). Further interactions can be predicted to occur between HCMV and EBV replication proteins, by analogy with HSV-1.

Interactions which are common to HSV-1, HCMV and EBV are those which occur between the three subunits of the helicase-primase complex and also between the two subunits of the DNA polymerase holoenzyme. Interactions between the HCMV replication fork proteins are described further in section 1.5.3. In EBV, the BSLF1, BBLF2/3 and BBLF4 proteins associate to form the helicase-primase complex (Yokoyama *et al.*, 1999), whilst the polymerase holoenzyme is formed by the interaction of the BALF5 and BMRF1 proteins (Tsurami *et al.*, 1993). The interaction between the UL30 and UL8 proteins in HSV-1 (Marsden *et al.*, 1997), is also observed between their counterparts in EBV, the BALF5 and BBLF2/3 proteins, respectively (Fujii *et al.*, 2000). Conservation of the interactions that occur between the core replication fork proteins in different herpesvirus sub-families is a further indication of a common basic mode of herpesvirus DNA synthesis.

Interactions between herpesvirus DNA replication proteins are of importance to study, firstly, as it is clear such interactions are fundamental for co-ordinated DNA synthesis. Hence, dissection of the complex interplay between the replication proteins should result in a better understanding of the process of herpesvirus DNA replication. Secondly, it is likely that many of the interactions between the replication fork proteins are essential for DNA synthesis and therefore they represent potential novel anti-viral targets. The disruption of essential protein-protein interactions as a means of inhibiting viral DNA replication, or indeed any other process necessary for the production of infectious progeny, obviously requires a detailed knowledge of the characteristics of the interaction and the region(s) on one or other of the proteins involved.

The HSV-1 UL30-UL42 interaction is one of the best characterised HSV-1 protein-protein interactions owing to its potential as a target for anti-viral drugs. Several studies initially strongly indicate that the interaction is essential for viral DNA replication, as UL30 proteins with mutations in the region involved in interacting with UL42 retain basal polymerase activity but do not support long chain DNA synthesis or origin-dependent DNA synthesis (Digard *et al.*, 1993; Stow, 1993; Tenney *et al.*, 1993). It was subsequently shown that the interaction can be disrupted by short peptides corresponding to the C-terminus of UL30 (Marsden *et al.*, 1994; Digard *et al.*, 1995) and that this inhibits the ability of UL30 to synthesise long DNA chains, thus identifying the interaction as a target for novel anti-viral drugs aimed at inhibiting the interaction. Loregian *et al.*, (1999) have demonstrated that a peptide corresponding to the C-terminal 27 residues of UL30 fused to the B subunit of *E.coli* enterotoxin can enter the nucleus of HSV-1 infected Vero cells and specifically inhibit viral replication.

The well characterised interaction between the HSV-1 ribonucleotide reductase (RR) enzyme subunits, R_1 and R_2, is another example of a herpesvirus protein-protein interaction whose disruption leads to inhibition of viral replication *in vitro* (Marcello *et al.*, 1994). This antiviral strategy is more likely to result in a lower incidence of drug resistant strains and this was found to be true in the case of inhibition of RR, as drug resistant virus which emerged through mutation was found to have impaired RR activity and consequently was less viable (Bonneau *et al.*, 1996).

1.4.6 Model for origin unwinding and initiation of DNA replication in HSV-1

Using the information that has been gathered regarding the HSV-1 origins, replication proteins and how they interact with each other, a model for the unwinding of the replication origin and establishment of a replication fork has been proposed (Boehmer & Lehman, 1997 and Stow, 2000). Although the initial events in unwinding the replication origins may vary amongst the different herpesviruses, the subsequent recruitment of replication proteins and establishment of a replication fork may follow a similar mechanism to that which is proposed for HSV-1. Figure 1.6 depicts the model that has been proposed, although it should be noted that not all the events proposed have been substantiated experimentally. Each stage is numbered with an explanation for each step given below;

1) The initial step involves the binding of UL9 to recognition sites on either side of the AT region at the centre of ori_S and ori_L. UL9 may bind in the form of a monomer or dimer.
2) Bound UL9 proteins interact with each other, possibly mediated by a leucine zipper motif within the N-terminal region of UL9. This interaction results in a distortion of the DNA helix in the AT region between the binding sites. Specifically, it has been suggested that the intervening DNA is held in a loop configuration as a result of the interaction (Koff *et al.*, 1991).
3) The interaction between UL9 and UL29 serves to recruit UL29 to the origin, where it binds to the distorted DNA in the AT region. UL29 stimulates the helicase activity of UL9 and increases its processivity. The UL9-UL29 complexes then proceed to unwind the duplex DNA adjacent to the origin, utilising the sequence-independent helicase activity of UL9. The UL9-UL9 interaction is maintained during initial unwinding. ss DNA extruded from the UL9-UL29 complex is stabilised by coating with UL29. Sequence specific unwinding of an HSV-1 origin by UL9 has only been demonstrated in the presence of UL29, indicating the importance of the UL9-UL29 interaction to the unwinding process (Lee & Lehman, 1997).

Figure 1.6 Model for unwinding of the HSV-1 origins and initiation of DNA synthesis
A model for the unwinding of the origins of replication and initiation of DNA synthesis has been proposed (Boehmer & Lehman, 1997; Stow, 2000), although not all the events proposed have been demonstrated. The first step involves the binding of the origin-binding protein (UL9) to specific sequences in the viral origin.

1) Two UL9 dimers bind to high-affinity recognition sites in the origin.

2) Interaction between the two bound UL9 dimers results in distortion of the A-T region between the binding sites into a loop structure.

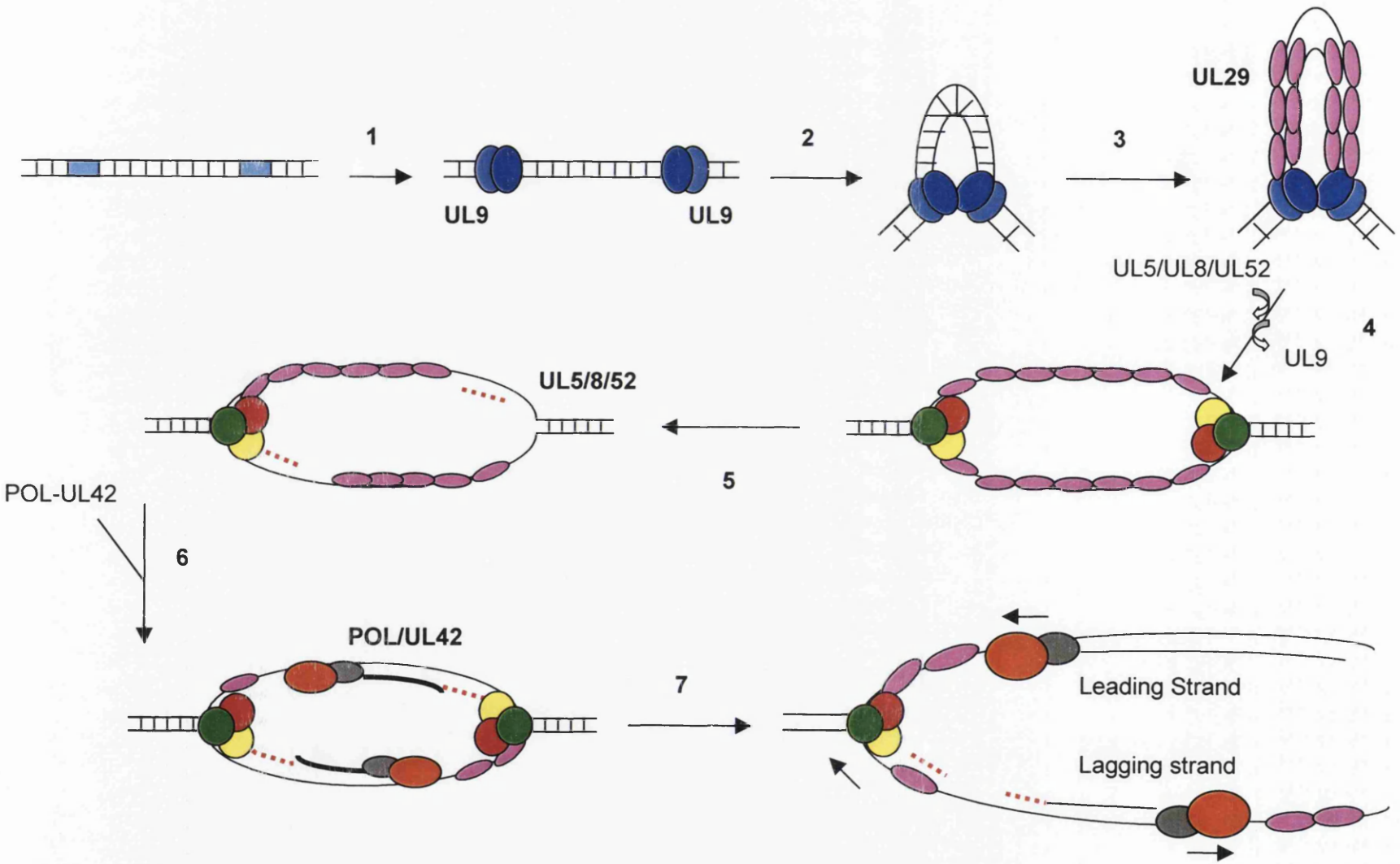
3) UL29, the ss DNA-binding protein, is recruited to the origin, via its interaction with UL9. It stimulates the helicase activity of UL9, which proceeds to unwind the DNA duplex. Unwound ss DNA extruded from the UL9-UL29 complex is immediately stabilised by UL29.

4) The helicase-primase complex (UL5/UL8/UL52) is then recruited, through interactions between UL9 and the UL8 subunit, and/or between UL29 and UL8. The interaction between UL9 and UL8 disrupts the co-operative binding of the UL9 dimers to the origin, and UL9 is displaced, allowing the formation of a replication bubble.

5) The newly recruited helicase-primase complex continues unwinding of the DNA duplex and also synthesises RNA primers so that the polymerase enzyme can initiate DNA synthesis.

6) Recruitment of the polymerase holoenzyme (UL30/UL42) is thought to be achieved through the interaction between UL9 and the UL42 subunit, or alternatively between UL8 and the UL30 subunit.

7) Leading and lagging strand synthesis is established at the replication fork.


1
UL9
UL9
2
3
UL29
UL5/UL8/UL52
4
UL9
5
UL5/8/52
POL-UL42
6
POL/UL42
7
Leading Strand
Lagging strand

4) Once unwinding of the duplex DNA has begun, recruitment of the helicase-primase and polymerase functions is necessary for DNA synthesis to commence. It is postulated that the arrival of the helicase-primase and polymerase enzyme complexes result in either the displacement of UL9, or disruption of the interaction between UL9 proteins, allowing the origin to take the form of a replication bubble. It is likely that the helicase-primase heterotrimer is probably recruited via protein-protein interactions involving the UL8 component, as UL8 physically interacts with UL9 (McLean *et al.*, 1994) and an interaction with UL29 is strongly suggested (Falkenberg *et al.*, 1997; Tanguy Le Gac *et al.*, 1996). Either of these interactions may serve to recruit the helicase-primase complex to the unwound DNA at the origin, although it seems more likely that the UL8-UL9 interaction may be the mechanism of recruitment. This is supported by the fact that the same N-terminal regions of UL9 involved in co-operative binding to the origin (Hazuda *et al.*, 1992) are also necessary for its interaction with UL8 (McLean & Stow, unpublished data). Hence, the interaction would not only recruit UL8 to the origin but would also disrupt the UL9-UL9 interaction, allowing the formation of a replication bubble. Subsequently, the UL8-UL29 interaction may serve to direct the helicase-primase complex to regions of ss DNA and to modulate the enzymatic activities of the complex. UL29 stimulates the DNA-dependent ATPase and helicase functions of the complex (Hamatake *et al.*, 1997) and efficient helicase-primase activities on UL29-coated templates are dependent on the presence of UL8 (Falkenberg *et al.*, 1997; Tanguy Le Gac *et al.*, 1996).
5) The helicase-primase complex then directs the synthesis of short RNA primers on both unwound DNA strands to enable elongation of complementary strands by the polymerase holoenzyme.
6) Recruitment of the polymerase holoenzyme to the sites of unwound and RNA-primed DNA is possibly mediated through interactions between the polymerase holoenzyme and UL9 or the UL8 subunit of the helicase-primase complex. At present, it is not known whether recruitment of polymerase occurs before or after recruitment of the helicase-primase complex and RNA priming. The polymerase accessory protein, UL42, specifically interacts with UL9 (Monahan *et al.*, 1998). The N-terminal region of UL9 is involved in binding to UL42, hence this interaction may also contribute to the proposed dissociation of UL9 to allow formation of a replication bubble. Another interaction which may be involved in recruitment of polymerase occurs between UL30 and the UL8 subunit of the helicase-primase complex. Characterisation studies indicate that the region spanning the C-terminal 32 amino acids of UL8 is involved in the interaction (Marsden *et al.*, 1997). It is

interesting to note, therefore, that a UL8 mutant lacking the C-terminal 33 residues does not support origin-dependent replication (Barnard *et al.*, 1997), supporting the hypothesis that the UL8-UL30 interaction may be crucial for DNA synthesis. However, whether or not the loss of viability of the UL8 mutant was directly related to a disruption of the interaction with POL cannot be ascertained, as other functions may be affected by the truncation also. In addition to involvement in recruitment of the polymerase, the UL8-UL30 interaction likely has a role in co-ordination of DNA unwinding and leading strand synthesis and also the synthesis of RNA primers with lagging strand synthesis.

7) Once polymerase has been recruited to the replication fork, the RNA primers are extended and bi-directional DNA synthesis is established. The helicase-primase complex proceeds to further unwind and prime the duplex DNA to allow lagging strand synthesis to continue.

1.4.7 Pathway of DNA replication in HSV-1

Whilst much knowledge has been accumulated regarding the HSV-1 origins of replication and the essential replication proteins, the pathway and forms of replicative intermediates which are utilised remain unclear. Current models for HSV-1 DNA replication propose that initial rounds of replication proceed via a theta-like mechanism, followed by a rolling-circle mode of replication at later times in infection (reviewed by Boehmer & Lehman, 1997). It is known that linear HSV-1 genomes are rapidly circularised following infection, probably via direct ligation of the complementary single overhanging nucleotides at the genomic termini (Mocarski & Roizman, 1982). Genomes remain circularised throughout the replicative cycle, a strategy that overcomes the problems of replicating the genomic termini. A theta-like mechanism is indicated by the rapid non-linear accumulation of viral DNA during early stages of DNA synthesis, which is inconsistent with rolling-circle replication. (Zhang *et al.*, 1994).

The final products of HSV-1 DNA replication, however, are long head-to tail genome concatemers, indicated by sedimentation and restriction fragment analysis of newly synthesised DNA (Jongeneel & Bachenheimer, 1981). These findings are consistent with a rolling-circle mode of DNA replication, but how the proposed switch from theta to rolling-circle replication occurs is not known. In model systems for rolling-circle replication (reviewed in Kornberg & Baker, 1992), initiation occurs at a free 3'-OH end on one of the DNA molecules, which requires a 'nicking' of one of the DNA strands. The viral and/or cellular proteins which may be involved in nicking the DNA remain unknown. DNA synthesis is likely to proceed in the same way as during theta

replication, but whilst the uncleaved circular DNA strand would act as the template for leading strand synthesis, the displaced concatemeric single-stranded product of leading strand synthesis would act as the template for lagging strand synthesis.

It has recently been shown that the HSV-1 replication proteins are capable of directing rolling-circle DNA synthesis *in vitro* on a circular DNA molecule partially annealed to a complementary strand with a free 5'-poly-dT tail (Falkenberg *et al.*, 2000). Using this system, it was demonstrated that the product of synthesis was double-stranded concatemeric DNA, and that leading and lagging strand synthesis were concurrent. Interestingly, the only HSV-1 proteins that were absolutely required were the polymerase holoenzyme and the UL5 and UL52 subunits of the helicase-primase complex. Whilst the use of this artificial *in vitro* system demonstrates that the HSV-1 proteins can direct rolling-circle DNA synthesis, it does not provide any information regarding the initiation of replication or recruitment of the enzyme complexes.

1.5 HCMV DNA replication

1.5.1 HCMV Origins of replication

A region of the HCMV genome which serves as an origin of DNA replication was first identified by Hamzeh *et al.,* (1990) and Anders & Punturieri, (1991), using a novel gancyclovir-induced chain terminating method and a transient replication assay, respectively. Hamzeh *et al.,* (1990) observed that DNA synthesis was detectable in one genomic region only in cells treated with gancyclovir, which acts as a chain terminator when incorporated into a growing viral DNA chain. A transient replication assay, involving the transfection of plasmids containing cloned regions of the HCMV genome into cells followed by superinfection with HCMV, revealed that sequences upstream of the ORF encoding the ss DNA-binding protein, UL57, and including the amplified region in gancyclovir treated cells, provided the *cis*-acting signals required to mediate DNA replication. The boundaries and structure of this origin of lytic-phase DNA replication, designated orilyt, were further defined by Anders *et al.*, (1992). They found that sequences spanning approximately 2.4 Kbp near the centre of the U_L segment, upstream of the gene encoding the UL57, contributed to orilyt function. This region contains the highest content of inverted and direct repeats in the HCMV genome (Masse *et al.*, 1992) and is comprised of multiple components required for orilyt function and regulation. These include various repeated sequence motifs, transcription factor binding sites, including ATF, CREB and Sp-1, and an A-T rich segment (Anders *et al.*, 1992).

The presence of transcription factor binding sites is a common feature of eukaryotic and viral replication origins. The possible role of transcription units in HCMV orilyt function was investigated by Huang *et al.*, (1996), who demonstrated that several transcripts originate within or span regions of orilyt. Of particular interest was the identification of a series of short, non-polyadenylated transcripts, collectively known as the smallest replicator transcript (SRT), which originate in the centre of the core orilyt sequence and terminate within an oligopyrimidine sequence, known as the Y block, which is essential for orilyt function. Spanning approximately 0.22 Kbp, with a common 5' terminus and heterogeneous 3' ends, SRTs are early transcripts, as defined by sensitivity to cyclohexamide, and are driven by a candidate upstream promoter. The structure of SRT suggests that it is not an mRNA, leading to suggestion that it may have a role in initiation of DNA synthesis. This possibility is discussed further in section 1.5.4. Another feature of HCMV orilyt which may provide clues to the mechanism of orilyt activity is the presence of persistent RNA-DNA hybrid structures covalently linked to DNA-DNA segments, which are integrated into the genomes of a proportion of HCMV virions (Prichard *et al.*, 1998). Two virus-associated RNAs (vRNAs) were identified; vRNA 1 lies within the region between nt 93799 and nt 94631 and is approximately 500 bp long, whereas vRNA 2 maps to a 300 bp sequence between nt 92636 and nt 93513.

Further analysis of orilyt defined a minimal core region of approximately 1.5 Kbp, between nt 91751 and 93299 (Zhu *et al*, 1998). Deletion analysis of this sequence defined two essential regions of 364 bp and 166 bp, respectively. Essential region I contains the Y-block element which consists of an oligopyrimidine tract. The Y block has a homologous counterpart in EBV, which is also essential for EBV orilyt function. In addition, the heterogeneous 3' ends of the SRT overlap the Y-block (Huang *et al.*, 1996). Region I also contains various repeated sequences, including inverted pairs of ATF-CREB binding sites, reported to be the most important component of the left half of this region (Zhu *et al*, 1998). Region II contains consensus SP-1 binding sites and sequences with potential SRT promoter function (Huang *et al.*, 1996; Zhu *et al*, 1998). vRNA 2 also overlaps essential region II (Zhu *et al*, 1998). A schematic representation showing structural features of HCMV orilyt is shown in Figure 1.7.

1.5.2 HCMV replication proteins

A set of 11 loci encoding functions that are necessary and sufficient to direct HCMV origin-dependent DNA replication were elucidated by Pari & Anders, (1993), who used a transient replication assay similar to that utilised to investigate the essential HSV-1

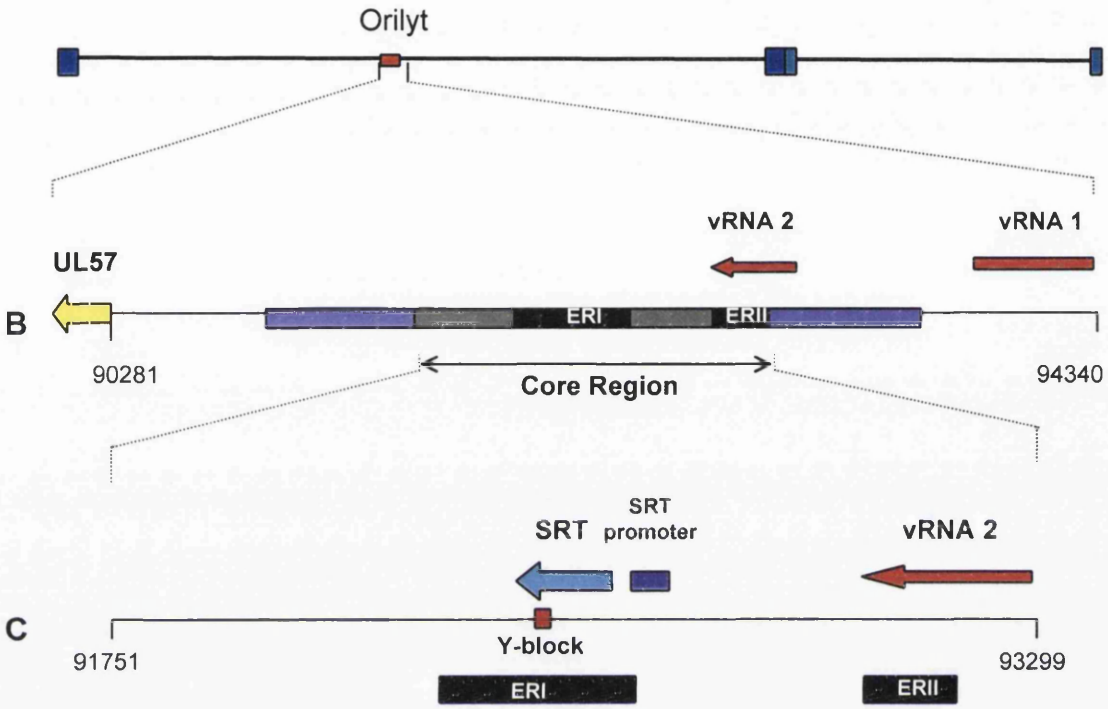


Figure 1.7 Structural Features of HCMV oriLyt

A) A schematic diagram of the HCMV genome showing the location of oriLyt.

B) The region encompassing oriLyt and flanking sequences. The position of the UL57 ORF and the stably incorporated viral RNA molecules (vRNAs) (Prichard *et al.*, 1998) are indicated. The lilac box represents the 2.4 Kbp region which contributes to orilyt function (Anders *et al.*, 1992), containing the 1.5 Kbp oriLyt 'core' sequence (Zhu *et al.*, 1998). The positions of essential regions I and II (ERI and ERII) are indicated.

C) The core region of HCMV oriLyt lies between nt 91751-93299 (Zhu *et al.*, 1998). It contains both the essential regions, I and II and vRNA 2. The smallest replicator transcript (SRT) refers to a series of 0.22 Kbp RNA molecules which are transcribed from nt 92686 and terminate in the region of the Y block (Huang *et al.*, 1996). The Y-block is an essential 45 nt long oligopyrimidine tract (Huang *et al.*, 1996).

replication proteins (Wu *et al.*, 1988). Amongst the 11 loci were ORFs which encode predicted homologues of the six core, conserved herpesvirus replication fork proteins; the DNA polymerase catalytic (UL54) and accessory (UL44) sub-units, a ss DNA-binding protein (UL57) and a heterotrimeric helicase-primase complex (UL70, UL102 and UL105). The other five loci encode auxiliary proteins; UL36-38, UL84, IRS1/TRS1, UL122-123 (MIE) and UL112-113. In contrast to their HSV-1 counterparts, the HCMV replication fork proteins have not been extensively characterised.

Core Replication Fork Proteins

Similar to HSV-1, DNA polymerase activity in HCMV-infected cells was originally found to be associated with two proteins (Huang, 1975), which were subsequently identified as UL54 and UL44. The catalytic sub-unit, UL54, is the most extensively characterised of all the HCMV replication fork proteins, being the target for the majority of currently licensed anti-HCMV drugs. UL54 shares considerable homology with the HSV-1 DNA polymerase and contains a set of six sequence motifs in common with HSV-1 Pol and other members of the α-like DNA polymerase family (Wang, 1991). UL54 likely possesses the 3'→5' proofreading exonuclease function associated with the holoenzyme purified form infected cells (Nishiyama *et al.*, 1983) and, on the basis of sequence homology, possibly a 5'→3' exonuclease/Rnase H-like function also (Marcy *et al.*, 1990). Analysis of the arrangement of the conserved motifs in UL54 suggests that the exonucleolytic activities are mediated by the amino half and the polymerase functions in the central domain. The interaction between UL54 and UL44, the polymerase accessory protein, is mediated by the C-terminus of UL54, as is found in their HSV-1 counterparts, UL30 and UL42. A peptide corresponding to the 21 C-terminal amino acids of UL54 disrupts the physical interaction between the proteins and the stimulatory effect of UL44 (Roberto Rigatti & Howard Marsden, personal communication). UL44 is an abundant 53 kDa DNA-binding phosphoprotein, which has only limited aa sequence homology with its HSV-1 counterpart, UL42. UL44 physically associates with UL54 and serves to stimulate its polymerase activity and its processivity also (Ertl & Powell, 1992; Weiland *et al.*, 1994;). The DNA-binding domain of UL44 resides in the N-terminal two thirds of the protein whereas C-terminal regions do not contribute to stimulation of UL54 or DNA-binding properties (Weiland *et al.*, 1994).

The HCMV ss DNA-binding protein is encoded by ORF UL57. It has not been well studied, however sequence alignment with other herpesvirus counterparts reveals it contains several conserved aa sequence blocks (Anders, 1990). The limited

characterisation carried out so far indicates that UL57 has similar properties to its HSV-1 counterpart, UL29 and hence is predicted to fulfill the same role of stabilising single-stranded DNA unwound at the replication fork (Anders *et al.,* 1990; Kemble *et al.*, 1987).

Helicase-primase activity in HCMV-infected cells is associated with a 1:1:1 heterotrimeric complex comprised of the UL70, UL102 and UL105 proteins, which can be purified from the nuclei of HCMV-infected cells (McCollum *et al.*, 1999). The purified complex possesses DNA-dependent ATPase, DNA helicase and RNA primase activities. The UL70, UL102 and UL105 components also stably associate when co-expressed in both mammalian and insect cells (McCollum *et al.*, 1999; Anders & McCue, 1996). Immunoprecipitation experiments indicate that each of the helicase-primase subunits is able to form pair-wise interactions with all other members of the complex, and that interacting sequences of each subunit reside in the N-terminal domain (McMahon *et al.*, 1999). On the basis of sequence homology with their HSV-1 counterparts and the presence of conserved functional sequence motifs, helicase and primase functions are attributed to the UL105 and UL70 proteins, respectively (Chee *et al.,* 1990; Martignetti *et al.*, 1991). Analysis of the recombinant complex, expressed in insect cells, demonstrated that a sub-assembly comprised of the UL70 and UL105 proteins retains helicase and primase activities (McCollum *et al.*, 1999). This feature is also observed with a heterodimer comprised of the UL5 and UL52 subunits from the HSV-1 helicase-primase complex (Calder & Stow, 1990; Dodson & Lehman, 1991). However, in contrast to its HSV-1 counterpart, UL5, the HCMV helicase subunit, UL105, retains detectable ATPase activity when purified in isolation (McCollum *et al.*, 1999).

UL102 is an essential HCMV replication protein which is proposed to be analogous to the HSV-1 helicase-primase associated protein, UL8, with which it shares only limited sequence homology (Chee *et al.*, 1990; Smith & Pari, 1995a). No characterisation studies of UL102 have been carried out. Like its HSV-1 counterpart, UL102 does not appear to possess any detectable enzymatic activity. Hence, its role as part of the helicase-primase complex at the replication fork is unclear, but several functions can be predicted by analogy with its HSV-1 counterpart, UL8.

Auxiliary replication proteins

In addition to the six proposed replication fork proteins, five additional loci were required for origin-dependent HCMV DNA replication; UL36-38, UL84, UL112-113,

UL122-123 (MIE region) and IRS1/TRS1 (Pari & Anders, 1993). None of these auxiliary loci encode proteins which are analogous to the HSV-1 origin-binding protein, UL9. Subsequent studies have shown that UL36-38, UL112-113, UL122-123 and IRS1/TRS1 have roles in transactivating expression of the replication fork proteins from their native promoters (Iskenderian *et al.*, 1996). The functions of the MIE region (UL122-123), UL36-38 and IRS1/TRS1 have been reviewed (Colberg-Poley, 1996; Stenberg,1996).

The UL122-123 locus, or MIE region, encodes four immediate-early proteins and one late protein, via differential splicing. The major products are the IE1 and IE2 proteins, both of which have been the subject of extensive characterisation. IE1 and IE2 play key roles in regulating both viral and cellular promoters, functioning at the level of the transcription complex. IE2 alone is a potent transcriptional modulator which can activate early viral genes and also auto-regulate the MIE promoter by repressing its transcription. IE1, in contrast, activates the MIE promoter and acts synergistically with IE2 to activate a variety of early viral promoters. In addition, IE1 and IE2 co-operate with other IE regulatory proteins (UL36-38, IRS1/TRS1 and UL112-113) to enhance expression from early promoters (Iskenderian *et al.*, 1996).

IE2 interacts with the UL84 protein and this complex can be co-immunoprecipitated from HCMV-infected HF cells (Spector & Tevethia, 1994). IE2 is thought to exert its regulatory effects through a variety of mechanisms. These include binding to DNA directly, illustrated by studies which demonstrated IE2 binds to its own promoter (Lang & Stamminger, 1993), by interaction with cellular transcription factors, e.g. CREB (Lang *et al.,* 1995) or by interactions with components of the basal transcription machinery e.g, TFIIB (Caswell *et al.*, 1993). The importance of IE2 to the HCMV replicative cycle was recently highlighted by studies using a bacterial artificial chromosome (BAC) clone of the HCMV Towne strain genome with the IE2 gene deleted (Marchini *et al.*, 2001). The growth cycle of the IE2 mutant was arrested due to a failure in expression of early genes. IE1, however, is non-essential for growth in tissue culture, as a mutant HCMV virus lacking IE1 grows comparably to wild-type when used to infect cells at a high multiplicity (Greaves & Mocarski, 1998).

The UL36-38 locus encodes the IE proteins UL36, UL37 and UL37-exon1 (UL37x1) as well as the early temporal class UL38 protein. UL36 is a member of the US22 gene family which regulates viral gene expression (Colberg-Poley *et al.*, 1992). There have been conflicting results regarding the necessity of UL36 for growth of HCMV in cell

culture. In one study, the use of antisense nucleotides to inhibit splicing of UL36 and UL37 transcripts resulted in inhibition of HCMV DNA replication in HF cells (Smith & Pari, 1995b). However, more recently, an HCMV recombinant virus lacking UL36 was shown to display wild-type growth kinetics in HF cells (Patterson & Shenk, 1999). UL37 is a transmembrane glycoprotein which shares its first 162 aa's with UL37x1. Both are reported to have transcriptional regulatory activity (Colberg-Poley *et al.*, 1992). An acidic domain is common to both proteins, however, in UL37, this has been shown to be dispensable for its transcriptional regulatory activity (Zhang *et al.,* 1996). The function of UL38, an early protein, is unknown, however it does not appear to possess any regulatory activity (Colberg-Poley *et al.*, 1992).

The IRS1/TRS1 loci are contained within repeated elements flanking the short genome segment and encode highly homologous proteins which are members of the US22 gene family. Their homology is such that either protein can support DNA replication (Pari *et al.*, 1993; Pari & Anders, 1993) by transactivating DNA replication protein promoters in transient assays (Iskenderian *et al.*, 1996). IRS1/TRS1 co-operate with other IE proteins to transactivate the early gene promoters. TRS1 acts in conjunction with IE1/IE2 to transactivate the UL44 gene promoter (Stasiak & Mocarski, 1992).

The UL112-113 locus specifies a family of differentially spliced transcripts which encode four early temporal class DNA-binding phosphoproteins. Proteins specified from this locus co-operate with IE1/IE2, IRS1/TRS1 and UL36-38 proteins to activate transcription of early genes encoding DNA replication fork proteins (Iskenderian *et al.*, 1996). UL112-113 gene products co-localise with viral DNA prior to and during replication in infected cells and expression of antisense UL112-113 RNA blocks DNA replication (Yamamoto *et al.*, 1998). In addition to its role in transactivation of early viral genes, UL112-113 gene products are involved in recruitment of replication fork proteins to viral pre-replicative compartments (Ahn *et al.*, 1999).

UL84 is an early protein which interacts with IE2 in HCMV-infected cells (Spector & Tevethia, 1994). Although the significance of this interaction is not fully understood, the interaction may serve to regulate IE2 function, as UL84 can inhibit IE2-mediated transactivation (Gebert *et al.*, 1997) and it may also be a means by which UL84, which lacks an obvious nuclear localisation signal, to translocate to the nucleus. It has also been suggested that UL84 may possess RNA endonuclease activity (Sarisky & Hayward, 1996b).

Roles of the HCMV auxiliary replication proteins

The findings of Iskenderian *et al.*, (1996) indicate that the UL36-38, IRS1/TRS1, IE1/IE2 and UL112-113 auxiliary proteins are required for adequate expression of the essential replication fork proteins. They found that the gene products of each of these loci co-operatively promote expression of the replication fork genes, with each contributing to activation to varying degrees. The MIE proteins were found to be the most potent activators of transcription. Despite testing for an effect upon activation of all the early replication fork promoters, no clear evidence was found to support a role for the fifth auxiliary protein, UL84, in modulation of expression of these genes.

The role of the auxiliary proteins in HCMV DNA replication was further investigated by Sarisky & Hayward, (1996a), who carried out transient replication assays in Vero and HF cells. These experiments involved co-transfection of a HCMV orilyt-containing plasmid and plasmids expressing all 11 HCMV auxiliary proteins under the control of constitutive heterologous promoters. The requirement for the individual auxiliary proteins was investigated by omitting each, in turn, from the replication assay. Whilst omission of plasmids encoding other auxiliary proteins, in particular IE1/IE2, resulted in a reduction in replication efficiency, UL84 was the only protein whose omission completely abrogated replication of the origin-containing plasmid. UL84 was the only auxiliary protein which was found to be indispensable for replication of orilyt, when all the other auxiliary proteins were present. Although UL36-38 was also found to be necessary initially, it was subsequently found that the HCMV UL69 protein could substitute for the UL36-38 expressing plasmid, indicating that UL36-38 has an indirect role in DNA synthesis. UL69 is not one of the originally identified essential HCMV replication proteins, but is known to possess transactivation properties (Winkler *et al.*, 1994).

Sarisky & Hayward (1996a) also demonstrated functional complementation of the HCMV core proteins by substituting them with the EBV replication fork proteins. The EBV core proteins were capable of directing replication of an HCMV orilyt-containing plasmid, when supplemented with the UL84-expressing plasmid alone. No other auxiliary proteins could substitute for UL84 under these conditions, indicating that UL84 is the only auxiliary protein absolutely necessary for orilyt-dependent replication in the transient assay. In addition, the formation of intranuclear replication compartments in HF cells, which is observed upon co-transfection of the 11 essential HCMV replication proteins, was absolutely dependent on the presence of UL84.

When taken together, the results of Iskenderian *et al.,* (1996) and Sarisky & Hayward (1996a) strongly implicate UL84 as serving an essential origin-specific function. Whilst four of the five auxiliary proteins are required for adequate expression of the core replication proteins, no such role for UL84 was substantiated (Iskenderian *et al.,*1996). However, UL84 is the only auxiliary protein which is a) absolutely required for HCMV orilyt-dependent replication; b) able to mediate replication of HCMV orilyt by the EBV replication fork proteins and c) indispensable for the formation of viral replication compartments in HF cells (Sarisky & Hayward, 1996a). Hence it was concluded that UL84 was the most likely candidate to perform an origin-specific function in HCMV although this does not necessarily imply that UL84 binds the HCMV origin directly (Sarisky & Hayward, 1996a). No further work on the role of UL84 in HCMV DNA replication has been published.

Recent work carried out in the Institute of Virology (Ellsmore, (2000) has provided an alternative insight into the roles of the HCMV auxiliary DNA replication proteins. A novel transient replication systems was utilised to investigate the HCMV-encoded proteins which provide origin-specific functions. The HSV-1 replication fork proteins were used to replicate an HCMV origin-containing plasmid in Vero cells, in the presence of the HCMV auxiliary proteins. No absolute requirement for UL84 was demonstrated, as omission of the UL84-expressing plasmid did not significantly reduce replication of the origin-containing plasmid when the four remaining auxiliary loci were present. IE2 was found to be the sole auxiliary protein required to direct replication of the HCMV origin by the HSV-1 replication proteins. UL36-38 was not absolutely required but was found to increase the efficiency of replication. These findings are in contrast to those of Sarisky & Hayward (1996a), who reported that UL84 was the only auxiliary protein absolutely required for origin-dependent DNA replication.

Whilst the available data indicates that the UL112-113, IE2/IE1, UL84 and UL36-38 proteins have roles in transactivation of early genes involved in DNA replication, they may also have additional roles in DNA replication during viral infection. IE1 is involved in the redistribution of PML from ND10 structures in early stages of replication (Ahn & Hayward, 1997). In addition, UL112-113 has been shown to be necessary for the recruitment of core replication proteins to viral replication compartments (Ahn *et al.*, 1999).

1.5.3 Interactions between HCMV replication proteins

The HCMV core replication proteins have not been as well characterised as their

counterparts in HSV-1, and consequently far fewer interactions between them have been reported. Figure 1.5 summarises the interactions which have been described so far and those which are predicted to occur by analogy with the HSV-1 replication proteins.

The HCMV DNA polymerase holoenzyme is formed by a close association of the UL54 and UL44 proteins (Huang, 1975; Ertl & Powell, 1992). Similarily, the UL70, UL102 and UL105 proteins associate to form the helicase-primase complex (McCollum *et al.*, 1999). The interactions between subunits of the DNA polymerase and helicase-primase enzyme complexes are conserved amongst the mammalian herpesviruses. So far, none of the inter-enzyme-complex interactions that occur between HSV-1 and EBV DNA replication enzyme complexes have been described for HCMV (Figure 1.5). The results of an investigation into the putative interaction between the HCMV DNA polymerase, UL54, and the helicase-primase accessory subunit, UL102 are presented in Chapter 5 of this thesis.

1.5.4 Models for initiation of HCMV DNA replication

Whilst the overall mechanism of HCMV DNA synthesis is likely to be similar to that in HSV-1, the information which has been gathered so far regarding the HCMV origin of replication and essential HCMV replication proteins suggests that HCMV employs a mechanism of initiation of DNA replication distinct from that of HSV-1. Firstly, HCMV orilyt bears little resemblance to the HSV-1 origins, ori_S and ori_L - HCMV orilyt is distinctive due to its far greater size and structural complexity. Secondly, no known homolog of the HSV-1 origin-binding protein, UL9, is encoded by HCMV.

Several features of HCMV orilyt have been reported which may provide an insight into the possible mechanism of initiation of DNA synthesis. Huang *et al.*, (1996) reported a series of short replicator transcripts, or SRT, which originate in orilyt and terminate in the essential Y-block element. Subsequently, stable RNA-DNA hybrid structures within orilyt were identified (Prichard *et al.*, 1998). Together with the identification of numerous transcription factor binding sites in orilyt (Anders *et al.*, 1992), these findings are consistent with models in which transcription elements and/or transcription across the origin are involved in the initation of DNA synthesis.

The covalently integrated RNA within orilyt is a key feature in one such model. If cleaved by an RNase H-like enzyme, the free 3'-OH end of the RNA moiety could then act as a primer from which DNA synthesis could be initiated. This model requires an,

as yet unidentified, RNA endonuclease function, however UL84 is a possible candidate for this role. An RNA endonuclease function has been attributed to UL84 (Sarisky & Hayward, 1996b) and Prichard *et al.*, (1998) also comment that UL84 has an effect on RNA stability.

In another model, proposed by Huang *et al.*, (1996), initiation would occur via a mechanism similar to that of mitochondrial DNA (mtDNA) heavy strand replication, which has been reviewed by Shadel & Clayton (1997). The two strands of mtDNA are designated as heavy and light, due to a strand bias in G+T content, and are not replicated simultaneously. Transcription of each strand is initiated from either the heavy strand promoter (HSP) or the light strand promoter (LSP), respectively. Initation of mtDNA replication begins with transcription from the LSP, involving the mtRNA polymerase and one or more transcription factors. As transcription proceeds, the newly synthesised transcript forms a RNA-DNA hybrid with the light template strand, and the heavy strand is displaced. The hybrid adopts a stable configuration which is then processed to form RNA primers. DNA synthesis can then be initiated by extension of the processed RNA primer by DNA polymerase γ.

By analogy with the mtDNA heavy strand replication mechanism, Huang *et al.*, (1996) have proposed that transcription of the HCMV SRT would commence from the SRT promoter, possibly mediated by cellular RNA polymerase III and transcription factors with binding sites located in the vicinity of the SRT promoter. The SRT transcript would subsequently form a stable hybrid with the Y-block element, causing displacement of the opposite DNA strand and creating a locally unwound region. Processing of the RNA component of the hybrid region, likely by RNase-H activity, would provide a free 3'-OH tail from which DNA synthesis could commence. The known characteristics of UL84 are consistent with a role in initiation of DNA replication by this mechanism.

The relationship, if any, between the SRT, which is not embedded in the HCMV genome, and the two vRNAs which are incorporated into orilyt, is unclear. It has been suggested that the SRT may represent a portion of vRNA2 which is not integrated into the genome (Prichard *et al.*, 1998). However, as not all HCMV virions appear to contain vRNA in their genomes, this feature cannot be essential for initiation of DNA replication in HCMV.

Considering the overall complexity of HCMV orilyt, and the differing requirements for trans-acting factors which have been observed in different cell lines (Sarisky &

Hayward, 1996a) and in different transient replication assays (Sarisky & Hayward, 1996a; Ellsmore, 2000), it is possible that initiation of replication can proceed by more than one mechanism and may therefore depend on the availability of the necessary trans-acting factors in a given cell type and possibly the stage of the cell-cycle. If transcription and/or transcription elements play a role in initiation, then different factors may be utilised in different cell lines, as orilyt contains a variety of cellular transcription factor binding sites and also potential IE2-binding sites (Anders *et al.*, 1992; Huang *et al.*, 1996).

1.6 Aims of this project

Current models for HCMV DNA replication are based on what is known about the prototype herpesvirus replication system, HSV-1. Since many of the details of the proposed model for HCMV have yet to be demonstrated and, unlike their HSV-1 counterparts, the HCMV replication proteins have not been well studied, further characterisation of the replication proteins and how they interact with each other is required to fully understand the mechanism of HCMV DNA replication. As well as furthering overall understanding of the biology of HCMV, this may also lead to the identification of novel targets for the development of anti-HCMV treatments.
In HSV-1, the helicase-primase associated protein, UL8, appears to have multiple roles in DNA replication. It enhances the functions of the helicase-primase complex, it is required for nuclear translocation of the complex, and also interacts with all of the other core replication proteins. These interactions are probably important for the recruitment of other essential protein complexes to the replication fork and the co-ordination of both leading and lagging strand DNA synthesis. Its HCMV counterpart, UL102, has not been characterised although by analogy with UL8, it is likely to perform several essential functions during HCMV DNA replication. The gene encoding UL102 had been cloned and expressed using the baculovirus system prior to the start of this project. The overall aim of this project was to characterise UL102 and establish whether it shares common properties with the HSV-1 UL8 protein. The specific aims were;

a) To generate a variety of reagents enabling structure-function analysis of UL102. This included the generation of monoclonal UL102-reactive antibodies and constructs expressing truncated versions of UL102 in a variety of systems.

b) To establish whether UL102 shares common characteristics with that of the HSV-1 UL8 protein. The specific aspects of UL102 behaviour to be investigated were its;

- DNA-binding properties.
- Ability to interact with the HCMV DNA polymerase catalytic subunit, UL54.
- Ability to influence the intracellular localisation of the other HCMV helicase-primase complex proteins.

Chapter 2

Materials and General Methods

2.1 General materials

2.1.1 Chemicals and reagents

All chemicals and solutions were obtained from Sigma Chemicals Company, Mo, USA or BDH Laboratory Supplies, Poole, UK with the following exceptions;

Amersham Pharmacia Biotech (APB)	ECL western blotting detection reagents, Rainbow protein MW markers
Boehringer Mannheim, Germany	Tris base, Nonidet-P-40, complete EDTA-free protease inhibitor tablets
Bio-Rad Laboratories, CA, USA	Ammonium persulphate, 30 % acrylamide/Bis solution, Protein assay dye reagent concentrate
Calbiochem Corporation, CA, USA	Cleland's reagent
Kirkegaard & Perry Laboratories, USA	ABTS-peroxidase substrate
Prolabo, Fontenay Sur Bois	Glycerol, Chloroform, Methanol
Life Technologies, UK	1Kb DNA ladder markers
Smithkline Beecham Research	Ampicillin

2.1.2 Enzymes

Restriction endonucleases were obtained from Boehringer Mannheim. Other enzymes were obtained from;

Boehringer Mannheim, GmbH, Germany	T4 DNA Ligase, Alkaline Phosphatase
New England Biolabs, Massachusetts, USA	T4 Polynucleotide Kinase

2.1.3 Antibodies

UL54-specific monoclonals were made by Dr Susan Graham (MRC Virology Unit, Glasgow). Other antibodies were supplied by the following;

Sigma Chemical Company, MO, USA	Anti-mouse-HRP, Anti-FLAG monoclonal, Anti-c-myc monoclonal, Anti-mouse-FITC
Amersham Pharmacia Biotech (APB)	Anti-rabbit-Cy5 conjugate

2.1.4 Radiochemicals

γ-^{32}P-ATP, ^{35}S-Methionine and ^{14}C-Chloramphenicol were supplied by NEN, MA, USA.

2.1.5 Miscellaneous materials

Other materials used in experimental work were obtained from the following;

Schleicher & Schuell	Protran BA 85 Nitrocellulose,
Macherey-Nagel GmbH & Co	Polygram 0.2mm sil G TLC sheets
Medicell International Ltd	Dialysis membrane
Dynex Technologies Inc, USA	Immulon 1microtitre Plates
Whatman International Ltd	3mm filter paper
Kodak Ltd	X-omat Film
Citifluor	UKC Chemical Laboratories

2.1.6 Plasmids

Commercial plasmids were obtained from;

Amersham Pharmacia Biotech	pGEX-2T, pGEX-4T2, pGEX-4T3
Stratagene, CA, USA	pCMV-Tag2, pCMV-Tag3
Clontech Laboratories, CA, USA	Mammalian-2-hybrid vectors: pM, pVP16, pM3-VP16

Dr Marion McElwee (MRC Virology Unit, Glasgow) made and supplied plasmids pTZ18u-102, pacCL29.105 and pacCL29.70. pCMV10 was supplied by Dr Nigel Stow, (MRC Virology Unit, Glasgow) (Stow *et al*, 1993). Plasmid PY-POL was made by Dr Sun Yi, (MRC Virology Unit, Glasgow).

2.1.7 Viruses

HCMV strain AD169 was obtained from Dr Derrick Dargan, MRC Virology Unit, Glasgow. Recombinant baculoviruses AcNPV-UL102 and AcNPV-UL54, which express the HCMV UL102 and UL54 proteins under the control of the polyhedrin promoter, were made by Dr Sun Yi and Mary Murphy (MRC Virology Unit, Glasgow).

2.1.8 Oligonucleotides

Oligonucleotides used for PCR amplification and sequencing of UL102 gene fragments (primers 4166, 4641, 4643 and 250C) were made by Mr D McNab in the Division of Virology. Universal and reverse sequencing primers for pUC-derived plasmid sequencing were obtained from New England Biolabs, Inc. DNA oligonucleotides ϕX174A and ϕX174B, used for DNA binding studies, were obtained from MWG Biotech. RNA olionucleotide primers RNA1 and RNA2, also used in DNA binding experiments, were synthesised and purified by Cruachem Ltd.

2.2 LIST of SOLUTIONS

AE buffer	100 mM Tris base (pH 8), 100 mM NaCl, 2 mM EDTA, 0.5% (v/v) deoxycholate, 1% (v/v) NP-40, 10% (v/v) glycerol, 1 complete protease inhibitor tablet per 50 ml
Cell Fix solution	19 ml PBS, 1 ml formaldehyde, 0.4g sucrose
Cell freezing Mix	Appropriate cell medium + 10% DMSO
Cell Permeabilisation Solution	19 ml PBS, 1 ml 10% NP40, 2 g sucrose
Coomassie blue gel stain	5% (v/v) methanol, 7% (v/v) acetic acid, 0.2 % Coomassie brilliant blue in 1 L H_2O
DNA loading buffer (5x)	4% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol
DNA binding buffer (+ salt)	50 mM Hepes, 10% (v/v) glycerol, 0.1 mM EDTA, 0.5 mM DTT, 50 mM NaCl
DNA binding buffer (- salt)	50 mM Hepes, 10% (v/v) glycerol, 0.1 mM EDTA, 0.5 mM DTT
ELISA washing buffer (EWB, 10x)	1.45 M NaCl, 75 mM Na_2HPO_4, 28 mM NaH_2PO_4, 0.5% (v/v) Tween-20
Gel destain	5% (v/v) methanol, 7% (v/v) acetic acid, 88% (v/v) water
IP wash	100 mM Tris (pH 8), 100 mM NaCl, 2 mM EDTA
PBS	170 mM NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 6.8 mM $CaCl_2$, 4.9 mM $MgCl_2$ (pH 7.2)
PBS-Tween	PBS with 0.001% Tween-20
Resolving gel buffer	0.74M Tris-HCL (pH 8.0), 1% SDS
SDS-PAGE sample buffer	6% SDS, 30% stacking gel buffer, 30% glycerol, 210 mM β-mercaptoethanol, 0.3% bromophenol blue
SDS-PAGE tank buffer	52 mM Tris, 53 mM Glycine, 0.1% SDS
Stacking gel buffer	0.122M Tris-HCL (pH 6.7), 0.1% SDS
TAE	40 mM Tris.acetate, 1 mM EDTA
TBS	20 mM Tris-HCL (pH 7.5), 500 mM NaCl
Trypsin	0.25% (w/v) trypsin in tris-saline containing phenol red (pH 7.5)
Tryptose Broth	29.5 g Difcobacto tryptose phosphate broth in 1L H_2O
Towbin transfer buffer	25 mM Tris base, 192 mM glycine, 20% methanol (v/v), 0.01% SDS (w/v)
Versene	0.6 μM EDTA, 0.02% phenol red in PBS

2.3 Chromatography materials and buffers

All chromatography buffers were made using sterilised distilled water.

2.3.1 UL102 Purification

DEAE-sepharose CL-6B was obtained from Amersham Pharmacia Biotech.

Hydroxyapatite pre-packed 5 ml columns were obtained from Bio-Rad.

Buffer G extraction buffer	20 mM Triethanolamine, 10% (v/v) Glycerol, 10 mM KCl, 1.5 mM MgCl, 1 mM DTT, 1 complete protease inhibitor tablet per 50 ml.

DEAE-sepharose chromatography buffers:

Triethanolamine Buffer	20 mm Triethanolamine, 10 % (v/v) Glycerol, 100 mM NaCl.
Low-salt NaCl	20 mM Tris (pH 8), 10 % (v/v) Glycerol, 100 mM NaCl.
High-salt NaCl	20 mM Tris (pH 8), 10 % (v/v) Glycerol, 2M NaCl.

Hydroxyapatite chromatography buffers:

NaCl Loading buffer	20 mM Hepes (pH 7.6), 10% (v/v) Glycerol, 1 mM DTT, 1 mM EDTA, 50 mM NaCl.
Low Salt $(NH_4)_2SO_4$	20 mM Hepes (pH 7.6), 10% (v/v) Glycerol, 1 mM DTT, 1 mM EDTA, 50 mM $(NH_4)_2SO_4$.
High Salt $(NH_4)_2SO_4$	20 mM Hepes (pH 7.6), 10% (v/v) Glycerol, 1 mM DTT, 1 mM EDTA, 500 mM $(NH_4)_2SO_4$.

2.3.2 UL54 Purification

Cellulose phosphate was obtained from Whatman International. ss DNA cellulose was obtained from Sigma.

NSC extraction buffer	20 mM Hepes (pH 7.9), 1.5 mM MgCl, 0.2 mM EDTA, 1 mM DTT, 600 mM NaCl, 25% (v/v) glycerol, 0.5% NP-40, 1 complete protease inhibitor tablet per 50 ml.
POL dialysis buffer	20 mM Hepes (pH 7.5), 50 mM NaCl, 10% (v/v) glycerol, 0.5 mM EDTA, 1 mM DTT.

Phosphocellulose chromatography buffers

Low-salt NaCl	20 mM Hepes (pH 7.5), 50 mM NaCl, 10% (v/v) glycerol, 0.5 mM EDTA, 1 mM DTT.
High-salt NaCl	20 mM Hepes (pH 7.5), 0.85 M NaCl, 10% (v/v) glycerol, 0.5 mM EDTA, 1 mM DTT.

ss DNA cellulose chromatography

Low-salt buffer	20 mM Hepes (pH 7.5), 50 mM NaCl, 10% (v/v) glycerol, 0.5 mM EDTA, 1 mM DTT.
High-salt buffer	20 mM Hepes (pH 7.5), 0.65 M NaCl, 10% (v/v) glycerol, 0.5 mM EDTA, 1 mM DTT.

2.4 Cell culture

2.4.1 Bacterial cells and culture

E. coli strain DH5α was used for the maintenance and propogation of plasmid DNA. Bacterial cells were grown in L-broth (LB) (10g NaCl, 10g bactopeptone, 5g yeast extract in 1 litre of water at pH 7.5) or 2YT for protein expression from pGEX plasmids (5g NaCl, 16g bactopeptone, 10g yeast extract in 1 litre of water at pH 7.5). Agar plates were made using 1.5 % (w/v) agar in LB. When necessary, media and agar plates were supplemented with the appropriate antibiotics.

2.4.2 Eukaryotic cells and cell culture

All cell media and supplements were obtained from Gibco, BRL except tryptose broth which was produced by Media services, Institute of Virology. The following cell lines were used in experimental work. All were obtained from the Cytology department in the Institute of Virology.

Spodoptera frugiperda-9 *(Sf)*	Insect cell line derived from worm ovarian tissue (Vaughn *et al.*, 1977).
BHK 21 C13	Syrian hamster kidney fibroblasts (MacPherson & Stoker, 1962)
HFFF	Human foetal foreskin fibroblasts
Hela	Human epithelial cell line derived from cervical carcinoma
Vero	African green monkey kidney fibroblasts

2.5 General Methods

2.5.1 DNA Manipulation

2.5.1.1 Restriction enzyme digestion of DNA

Restriction enzyme digestions were carried out using commercial enzymes and corresponding buffers. Typically, 0.5-2μg of DNA was digested in a final volume of 10-20μl using an excess of enzyme (5-10 units/digest) and the corresponding buffer at the recommended temperature for 1-2 hours.

2.5.1.2 Agarose gel electrophoresis of DNA

DNA samples were mixed with a 1/5 volume of DNA loading buffer and loaded into wells in horizontal 1% agarose gels made in 1x TAE containing 0.5 μg/ml EtBr. A 1 kb DNA ladder size marker was also loaded onto the gel. Electrophoresis was carried out using BIO-RAD sub-cell DNA gel electrophoresis systems with the gel submerged in 1x TAE also containing EtBr for approximately 40 minutes at 70 V. DNA was visualised using either a short wave or long wave UV transilluminator, as appropriate.

2.5.1.3 Purification of DNA from non-denaturing agarose gels

DNA fragments resolved by agarose gel electrophoresis were visualised under long-wave UV illumination and the required bands were cut from the gel. Gel slices were melted and the DNA subsequently purified using the Geneclean kit by Bio 101 inc, according to the manufacturer's instructions and using supplied materials. The volume of the gel slices was determined and 3x volumes of NaI was added. They were melted by incubating at 45°C for 5-10 minutes. A 10 μl volume of DNA-binding glassmik was added and the solution was incubated at room temperature, with mixing, for approximately 15 minutes. The glassmilk was pelleted at 13 000 rpm for 5 secs and washed using 400 μl of wash. This wash was repeated twice. After the final wash, tubes were left open at room temperature for 10 minutes to ensure ethanol from the wash solution had evaporated. The DNA was re-suspended in dH_2O, approximately 20 μl for < 5 μg of DNA.

2.5.1.4 Quantification of plasmid DNA

Plasmid DNA was quantified by UV absorption in a spectrophotometer at 260 nm. Concentrations were calculated assuming that an absorbance value of 1.0 corresponds to 50 μg/ml ds DNA.

2.5.1.5 DNA Ligation reactions

Ligation of DNA fragments and linearised plasmid DNA was carried out as follows. Fragment and plasmid DNA were mixed such that the fragment or insert DNA was in molar excess of the plasmid DNA by approximately 3-fold. One unit of bacteriophage T4 DNA ligase enzyme was added along with an appropriate amount of 10X ligase enzyme buffer in a final volume of 10-20 μl. Reactions were incubated overnight at room temperature. Where the insert was to be ligated into the plasmid by means of one restriction enzyme site only, the plasmid was subjected to treatment using calf intestinal phosphatase (CIP) enzyme prior to the ligation, in order to prevent

recircularisation during the ligation reaction. Typically, 10 units of CIP along with phosphatase buffer was added to 1 μg of restriction enzyme-digested plasmid DNA in a final volume of 10-20 μl and incubated at 37°C for one hour.

2.5.1.6 Preparation of competent DH5α E.coli cells

A single colony of DH5α *E.coli* cells was used to inoculate 5ml of LB and cells were grown overnight at 37°C in an orbital shaker. The next day, 1 ml of the overnight culture was diluted in 50 ml LB and allowed to grow for 2 hours at 37°C, with shaking. Cells were chilled on ice for 20 minutes and then pelleted at 3000 rpm (microfuge) for 5 minutes at 4°C. The pellet was resuspended in 25 ml 0.1M $CaCl_2$ and incubated on ice for 30 minutes. The cells were pelleted again at 3000 rpm (microfuge) for 5 minutes at 4°C and finally resuspended in 4 ml $CaCl_2$. Cells were stored at 4°C for at least one hour before use in transformation reactions.

2.5.1.7 Transformation of competent DH5α E.coli cells

Approximately 100 ng of DNA (either unmodified plasmid DNA or that from ligation reactions) was mixed with 200 μl of competent cells and incubated on ice for 30 minutes. Competent cell/DNA mixtures were then subjected to "heat-shock" by incubating at 42°C for 90 seconds. 800 μl of LB was added immediately and samples were incubated at 37°C for 1 hour, with shaking. Cells were then pelleted at 13000 rpm in a microfuge for 30 seconds and 800 μl of the supernatant discarded. The cell pellet was resuspended in the remaining 200 μl of media and then spread onto LB agar plates, containing appropriate antibiotics (100 μg/ ml ampicillin or 25 μg/ ml kanamycin) using a sterile plastic spreader. Plates were incubated overnight at 37°C.

In the case of cells transformed using DNA from ligation reactions, individual bacterial colonies that had grown were picked from agar plates into 5 ml of sterile LB containing the appropriate antibiotic (to which the transformed plasmid had conferred resistance) at the afore-mentioned concentrations, and shaken overnight at 37°C. The following day, small-scale preparations of plasmid DNA were made.

2.5.1.8 Small scale preparation of plasmid DNA (mini-prep)

Bacteria contained in 1.5 ml of overnight culture were pelleted by centrifugation at 13000 rpm (microfuge) for 30 seconds and the supernatant discarded. Plasmid DNA was then isolated using a 'Perfect Prep' kit (5'→3', Inc) according to the manufacturers

instructions and using reagents as supplied. Plasmid DNA was eluted using 60µl of dH_2O at 65°C, and stored at -20°C.

2.5.1.9 Large-scale preparation of plasmid DNA (midi-prep)

A single colony or glycerol stock of bacteria was used to inoculate 100 ml of LB containing the appropriate antibiotic, using a sterile loop. Cultures were grown in 500 ml flasks. The cells were pelleted by centrifugation at 3000 rpm for 15 minutes and the supernatant was discarded. The DNA was extracted using a Qiagen midi-prep kit following the manufacturers instructions.

2.5.1.10 Propagation of plasmid DNA

Stocks of plasmid DNA were stored long-term in glycerol at -70°C. To prepare these stocks, 0.5 ml of an overnight culture of the plasmid-containing bacteria was mixed with 0.5 ml glycerol. When new preparations of plasmid DNA were required, a small sample of glycerol stock was streaked out onto an LB agar plate (containing appropriate antibiotics, where necessary) using a sterile loop and incubated at 37°C overnight. The following day, an individual bacterial colony was picked into a suitable volume of LB containing the necessary antibiotics and grown at 37°C, with shaking, for the required time. Cells were then harvested and the DNA isolated using a Qiagen midi-prep kit as described previously.

2.5.1.11 DNA sequencing

An ABI automated sequencer was used for sequencing of double-stranded recombinant plasmid DNA, using the dideoxy method of Sanger, (1977).

2.6 Analysis of Proteins

2.6.1 SDS-PAGE

Complex protein mixtures were resolved using SDS-PAGE. Bio-Rad Mini Protean II gel apparatus was used to prepare and run mini gels. Usually 7.5% polyacrylamide resolving gels (acrylamide:bisacrylamide 37.5:1)were prepared in 1x running gel buffer and poured between vertical mini-gel plates. A 5% polyacrylamide stacking gel (acrylamide:bisacrylamide 19:1) prepared in 1x stacking gel buffer was polymerised on top of this. Prior to loading into gel wells, protein samples were mixed with 1/3 volume of SDS-PAGE sample buffer and boiled for 5 minutes. Gels were electrophoresed at 180 mA for 40-60 minutes, until the dye front reached the bottom of the gel. Proteins could then be detected by staining gels in 0.2 % Coomassie blue stain for 10 minutes followed by de-staining or transferred onto nitrocellulose membrane by western blotting for detection using antibodies.

2.6.2 Western Blotting

Proteins were transferred from polyacrylamide gels onto nitrocellulose membranes according to the method of Towbin *et al*, (1979). Gels were places on top of Whatman 3 mm paper presoaked in Towbin transfer buffer. A sheet of nitrocellulose membrane followed by another sheet of 3 mm paper (both pre-soaked) was placed on top of the gel. This assembly was transferred into a Bio-Rad mini trans-blot cartridge and tank as instructed. Electro-blotting was carried out in Towbin transfer buffer for 1 hour at 200 mA.

2.6.3 Detection of proteins on nitrocellulose membrane using antibodies

Nitrocellulose membranes were immersed in blocking buffer, consisting of 5% dried milk in PBS, and agitated for 1 hour at room temperature or overnight at 4°C. Blocking buffer was rinsed off using PBS containing 0.001% Tween-20 (PBS-Tween) and then the membranes were incubated with primary antibody, either in sealed plastic bags or suitable plastic containers, at room temperature for 1-2 hours. After six 5 minute washes in PBS-Tween, the membranes were incubated with either anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugate antibody (depending on the source of primary antibody), diluted 1:1000 in PBS-Tween 20, at room temperature for 1 hour, with agitation. Unbound secondary antibody was removed by six 5 minute washes using PBS-Tween. Membranes were then transferred onto glass plates and treated using Amersham ECL western blotting reagents according to the manufacturer's instructions. After a 1 minute incubation, the nitrocellulose was covered with

transparent film and then exposed to Kodak X-Omat film, which was processed using a Kodak X-Omat film developer.

2.6.4 Quantification of proteins

Protein concentrations were determined using the Bio-Rad protein assay kit (micro-assay), which is based on the Bradford dye-binding protein assay (Bradford, 1976), according to the manufacturers instructions. A standard curve of protein concentration against absorbance at 595 nm was produced, using BSA as the standard protein at the following concentrations (μg/ml); 50, 100, 200, 400, 800 and 1000. The absorbance of the sample proteins was measured and protein concentration calculated from the standard curve.

2.7 Cell Culture

2.7.1 Insect cell culture

Sf cells were grown in 600 ml plastic flasks containing 50 ml of medium or alternatively in 2 L plastic roller bottles containing 250 ml of medium. They were grown in TC-100 medium supplemented with 5% FCS, 1% penicillin-streptomycin and 0.5% neomycin and incubated at 28°C without CO_2. Cells were passaged by removing the existing medium then tapping the cells into a small amount of fresh *Sf* medium added to the vessel. Harvested cells were typically split 1:4 into fresh flasks.

2.7.2 Mammalian cell culture

Mammalian cells were grown in 600 ml plastic flasks containing 50 ml medium and maintained in a humidified incubator at 37 °C with 5% CO_2. Cells were passaged by washing the monolayer with 10 ml of versene and then removing the cells by trypsinisation using 10 ml of trypsin/versene (1:6). Once dispersed, 10 ml of the appropriate fresh medium was added and cells were split into fresh flasks. Where necessary, cells were counted using a Neubauer haemocytometer. The media composition for each cell type is listed below;

BHK cells: BHK cells were grown in BHK-21 medium supplemented with 10% newborn calf serum, 10% tryptose broth and 1% penicillin-streptomycin.

Vero cells: Vero cells were grown in DMEM supplemented with 5% FCS, 1% penicillin-streptomycin and 1% non-essential amino acids.

HFFF-2 cells: HFFF-2 cells were grown in DMEM supplemented with 10% FCS, 1% L-glutamine, and 1% penicillin-streptomycin.

Hela cells: Hela cells were grown in DMEM supplemented with 10% FCS and 1%

penicillin-streptomycin.

2.8 Transfection protocols

2.8.1 Preparation of cationic liposomes

DDAB (dimethyldioctadecyl ammonium bromide) was made up to a concentration of 4 mg/ml using 1 ml of chloroform. The DDAB solution was mixed with 1 ml DOPE (dioleoyl-α-phoshatidyl ethanolamine) which was already suspended in chloroform. The chloroform was evaporated by passing under a stream of nitrogen. The resulting white solid was lyophilised overnight in a freeze dryer. The dried liposomes were resuspended in 10 ml sterile distilled water by sonication. The liposome suspension was further sonicated, on ice, using a soniprobe intermittently until the suspension cleared. Liposomes were stored at 4°C for up to three months.

2.8.2 Transfection of mammalian cells using cationic liposomes

24-well plates containing 13 mm glass coverslips were seeded with mammalian cells at a concentration of 0.4x 10^5 cells/well, unless otherwise stated. The following day, provided cells were between 50-70% confluent, they were transfected with 2 μg/well of plasmid DNA as follows. DNA was mixed with 250 μl of serum-free Optimem medium (Gibco-BRL). 10 μl of liposome suspension, vortexed immediately prior to use, was also mixed with 250 μl of optimem in a separate tube. The Optimem/ DNA solution was added dropwise to the Optimem/liposome solution. The mixed solutions were vortexed for 30 seconds and then incubated at room temperature for 15 minutes. The existing medium was removed from the cells to be transfected and they were washed twice using PBS. The 500 μl transfection mixtures were added to each well and the plates were rocked gently to ensure the mix was evenly distributed. Cells were incubated at 37°C for 3 hours, after which the transfection mixtures were removed. Cells were washed once using PBS and then 1 ml of fresh medium was added to each well. Cells were replaced at 37°C for the required period of time.

2.9 Purification of recombinant insect cell-expressed proteins

2.9.1 Preparation and purification of recombinant UL102

2.9.1.1 Infection of Sf cells with baculovirus AcNPV-UL102

Typically, 12 large flasks of *Sf* cells at approximately 70% confluency (3.5×10^7 cells/ flask) were infected with recombinant baculovirus AcNPV-UL102 at a MOI of 5-10 in 2mls medium. Virus was allowed to adsorb for 1 hour, then 20 ml medium was added

to flasks which were then incubated for 3 days at 28°C. Cells were harvested 3 days post infection by shaking into the medium. The contents of each flask was then transferred to a roller bottle of *Sf* cells at a confluency of 1×10^6 cells/ml. Cells were incubated at 28°C for 28 hours.

2.9.1.2 Harvesting of infected Sf cells and extraction of protein

Cells from the roller bottles were harvested into the existing cell medium by shaking and transferred to Falcon 225 ml conical centrifuge bottles. Cells were pelleted by centrifugation at 3000 rpm for 7 minutes at 4°C and the supernatant discarded (Sorvall RT-7 Benchtop centrifuge). Cells were washed 3 times by resuspension in 80 ml ice-cold TBS followed by centrifugation at 3000 rpm for 7 minutes at 4°C, each time the supernatant being discarded. Cells were kept on ice throughout this procedure. Following the final wash, cell pellets were resuspended in 5 ml cold Buffer G then transferred to a dounce homogeniser. Cells were lysed by 12-15 strokes in the homogeniser. The lysed cell suspension was transferred to Sorvall 35 ml centrifuge tubes and centrifuged at 40000 rpm for 1 hour at 4°C using a Sorvall T865 ultracentrifuge rotor (Sorvall OTD-50B Ultracentrifuge). The final supernatant was carefully decanted and kept on ice if the purification was being carried out immediately or alternatively stored at -70°C.

2.9.1.3 Purification of UL102

Recombinant UL102 was purified from insect cell lysate by a two-step chromatographic process. UL102 was initially captured from insect cell lysate onto a column consisting of the anion exchange resin, DEAE-Sepharose CL-6B. A 100 mM-2M gradient of NaCl was then applied to the column, causing elution of UL102 at about 200 mM NaCl. Fractions containing UL102 from this stage were identified by SDS-PAGE followed by both Coomassie blue staining and western blotting using a UL102-specific antisera. The second stage of purification utilises an Hydroxyapatite column. Hydroxyapatite ($Ca_5(PO_4)_3OH)_2$ is a form of calcium phosphate which is thought to act as a "mixed-mode" ion exchanger, as it contains both positively and negatively charged ions. Peak fractions from DEAE-separation were then pooled and applied to a pre-packed commercial Hydroxyapatite column in low-salt NaCl loading buffer. UL102 does not bind to this column but instead flows straight through, separating it cleanly from the mixture of other proteins in the preparation. The contaminating proteins bind to the column and are eluted by applying a 50-500 mM gradient of ammonium sulphate salt. All chromatography was carried out using a Pharmacia Biotech 'AKTA *purifier*'.

DEAE-Sepharose column purification of sf cell extract containing UL102

Column preparation: DEAE-Sepharose CL-6B beads were packed to a final volume of 12 ml inside a Pharmacia glass chromatography column then equilibriated using low-salt triethanolamine buffer overnight at a flow rate of 0.2 ml/minute.

This first stage of UL102 purification consisted of 3 steps as follows;

1. *Loading Run;* Clarified *sf* cell extract was injected onto the column at a flow rate of 0.5 ml/minute using triethanolamine buffer. Many of the insect cell proteins do not attach to this column but UL102 binds and remains bound until eluted using NaCl buffer.
2. *Wash Run;* The purpose of this stage was to wash away proteins that were not tightly bound to the column. The column was washed using 90 ml of low-salt (100 mM) NaCl buffer at a flow-rate of 0.5 ml/minute.
3. *Elution Run*; In this stage a salt gradient was applied to the column, causing elution of the bound proteins on the basis of their charge. The gradient applied had two parts. In the first, the salt concentration in the buffer was increased from 100 mM to 260 mM NaCl salt (0-13%) over 26 ml buffer volume. The second part of the gradient consisted of a steep increase in salt concentration applied to the column, from 260 mM to 2M NaCl (13-100%) over a volume of 2.4 ml. UL102 was eluted during the first part of the gradient, at approximately 200 mM NaCl salt concentration. The flow rate was 0.5 ml/minute, and fractions of 1.5 ml volume were collected. Small samples of 50 µl volume were taken from each fraction to be used in subsequent analysis. The remainder of each fraction was stored at -70°C.

Fractions eluted from the DEAE-Sepharose column were analysed using SDS-PAGE. The 50µl samples were split equally between two gels. One was stained with coomassie blue and the other was used for western blotting analysis as described in section 2.6.3 using a UL102-specific antisera to allow unambiguous identification of the peak fractions containing UL102.

Hydroxyapatite Column Purification of UL102 from DEAE-Sepharose fractions

Column preparation: A Bio-Rad Econo-Pac CHT-II 5 ml pre-packed hydroxyapatite column was equilibriated using 2 ml of 50 mM ammonium sulphate buffer followed by a gradient from 50-500 mM ammonium sulphate salt over 20 ml buffer volume. The column was washed with a further 2 ml high salt buffer before the salt concentration was reduced to 50 mM again over 3 ml buffer volume. The flow rate used was 1 ml/minute.

Sample loading and run details; Following equilibriation, the column was washed using

low-salt (50 mM) NaCl for 30 minutes at 1ml/minute. Pooled fractions from the DEAE-Sepharose purification were mixed 1:1 by volume with NaCl buffer then injected onto the column in volumes not exceeding 9 ml. Samples were loaded and resolved using the same ammonium sulphate wash and gradient run conditions described above. 1ml fractions were collected throughout, with 50µl samples removed from each for analysis before storing at -70°C.

The purity of the UL102 preparation was determined by SDS-PAGE analysis of the peak fractions followed by coomassie blue staining. The protein content of each fraction was then quantitated using a standard protein assay (section 2.6.4).

2.9.2 Preparation and purification of recombinant UL54

2.9.2.1 Infection of Sf cells with baculovirus AcNPV-UL54

Typically, 12 large flasks of *Sf* cells at approximately 70% confluency (3.5×10^7 cells/ flask) were infected with recombinant baculovirus AcNPV-UL54 at a MOI of 5-10 in 2 ml medium. Virus was allowed to adsorb for 1 hour, then 20 ml medium was added to flasks which were then incubated for 3 days at 28°C. Cells were harvested 3 days post infection by shaking into the medium. The contents of each flask were then transferred to a roller bottle of *Sf* cells at a confluency of 1×10^6 cells/ml. Cells were incubated at 28°C for 3 days.

2.9.2.2 Harvesting of infected sf cells and extraction of protein

Cells infected with AcNPV-UL54 were treated to extract the soluble protein content exactly as described in section 2.9.1.2, with the exception that 20 ml of NSC buffer was used to resuspend the cells prior to homogenisation. After the final centrifugation, the supernatant containing UL54 was dialysed extensively overnight at 4°C in 4 litres of POL dialysis buffer.

2.9.2.3 Purification of UL54

Recombinant UL54 was purified from dialysed insect cell lysate by a two-step chromatographic process. UL54 was initially captured from the lysate onto a column consisting of the phosphocellulose beads. Phosphocellulose acts as a cationic exchanger, which binds positively charged UL54 via negatively charged phosphate groups on the beads. UL54 is eluted from this column by applying a NaCl gradient. The second stage of purification utilises a ss-DNA cellulose column. DNA cellulose acts as an affinity column which consists of ss-DNA attached to cellulose beads to

which DNA-binding proteins adhere. UL54 binds to this column and is eluted using a NaCl gradient. All chromatography was carried out using a Pharmacia Biotech 'AKTA *purifier*'.

Phosphocellulose column purification of Sf cell extract containing UL54
Column preparation: Whatman P11 cellulose phosphate was prepared according to the manufacturers instructions and then packed to a final volume of 15 ml inside a Pharmacia glass chromatography column (diameter 1.6 cm). The column was then equilibriated using a salt gradient of 50 mM-0.85M NaCl overnight at a flow rate of 1.0 ml/minute.

Sample loading and run details: Dialysed extract containing UL54 was loaded onto the phosphocellulose column using low salt buffer at a flow rate of 1.0 ml/minute then a salt gradient of 50 mM-0.85M NaCl was applied over a volume of 250 ml. UL54 was eluted at approximately 0.35 M NaCl. Fractions of 4.5 ml volume were collected throughout. Small samples of 50 µl volume were taken from each fraction to be used in subsequent analysis. The remainder of each fraction was stored at -70°C. Fraction samples were split between 2 gels and analysed using SDS-PAGE followed by Coomassie blue staining and western blotting analysis (section 2.6.3), using a UL54-specific antiserum to allow unambiguous identification of the peak fractions containing UL54.

DNA cellulose Column Purification of UL54 from phosphocellulose fractions
Column preparation: Sigma ss DNA cellulose was swollen in Buffer A then packed to a volume of 9.6 ml in a Pharmacia glass chromatography column (diameter 1.6 cm) and then equilibriated using a 50 mM-0.65 M NaCl gradient using a flow rate of 1ml/minute.

Sample loading and run details; Peak UL54-containing fractions eluted from the phosphocellulose column were pooled, transferred to dialysis membrane which was sealed at either end and then dialysed overnight in 3 litres of low-salt DNA cellulose column buffer. The pooled fractions were then loaded onto the ss DNA cellulose column in low-salt buffer using a flow rate of 1.0 ml/minute. A NaCl gradient of 50 mM-0.65 M was then applied to the column, causing elution of bound UL54 protein at approximately 0.3 M NaCl. Fractions of 1.5 ml were collected throughout the salt gradient, with 50 µl samples removed from each for analysis. Fractions were stored at -70°C. The purity of the UL54 preparation was determined by SDS-PAGE analysis of the peak fractions followed by Coomassie blue staining and western blot analysis. The

protein content of each fraction was then quantitated using a Bio-Rad protein assay (section 2.6.4).

Chapter 3

Generation and characterisation of UL102-specific monoclonal antibodies

3.1 Introduction

Antibodies are an essential tool in the study of proteins and their function. Monoclonal antibodies (MAbs) are especially useful as their highly specific and unique binding properties can be exploited for many purposes. A further advantage is that they can be produced repeatedly and in limitless quantities. Since the aim of this project was to characterise UL102, it was necessary first to raise UL102-reactive antibodies. Producing MAbs was the primary objective as this would likely give rise to a range of antibodies each with unique individual specificities, but collectively, with reactivity to a variety of epitopes on the UL102 protein. The production of a panel of MAbs is also more likely to result in a selection of antibodies which, collectively, are suitable for a wider range of applications. This is an important consideration, as polyclonal antiserum does not always suit every application. Also, once epitope-mapping of a monoclonal antibody has been carried out, its unique specificity to a defined region can be employed for working with truncated forms of the protein which contain that epitope. This characteristic was particularly desirable for the purpose of dissecting putative interactions between UL102 and other replication proteins.

Having isolated MAbs, it is necessary to characterise their properties to determine their affinities and the applications in which they are useful. This is an important objective as the hybridoma cell lines used in this study were selected and isolated on the basis of screening against UL102 in ELISA only. For the purposes of this project, the three most appropriate immunochemical techniques in which to test the reactivities to UL102 of each monoclonal were; Western blotting, immunofluorescence and immunoprecipitation. The reactivities of the MAbs in each of these techniques was tested.

Another aspect of antibody characterisation which is useful for their application in protein analysis is epitope-mapping. Having UL102-specific antibodies that mapped to defined regions would be potentially useful for investigating the regions of UL102 involved in binding to other replication proteins, and also for assigning functions to different domains of the protein. Fine epitope mapping of the UL102-specific MAbs was outwith the constraints of this project. However the epitopes recognised by the UL102-specific MAbs in Western blotting were approximately located by testing their reactivities to truncated UL102 GST-fusion proteins which are described in Chapter 4. In addition, each MAb was tested in ELISA for reactivity to peptides corresponding to C-terminal and C-proximal regions of UL102, allowing a finer mapping of the epitopes

recognised by C-terminal reactive MAbs. MAbs whose reactivity mapped to the C-terminus of UL102 were potentially useful for the purpose of investigating the putative interaction between UL102 and UL54, as the homologous interaction in HSV-1 between the UL8 and UL30 proteins is mediated by the C-terminal region of UL8 (Marsden *et al.*, 1997). Figure 3.1 depicts the reagents used for locating epitopes recognised by the UL102 MAbs.

Principles of monoclonal antibody production

MAbs are secreted from single clones of hybridoma cells, these cells being created following the fusion of myeloma cells and antibody-producing immune lymphocytes. Polyethylene glycol (PEG) is the agent used to promote membrane fusion between the cells. This process was first described by Kohler & Milstein, (1975). Hybridoma cells therefore inherit both immortality from the myeloma cells and antibody-producing capability from the B lymphocytes. Following fusion, the hybrid cells are selected from the mixture of spleen cells, myeloma cells and hybrids by the addition of HAT (hypoxanthine, aminopterin, thymidine) to the culture medium. Aminopterin is an antibiotic which inhibits *de novo* nucleic acid synthesis by blocking purine and pyrimidine synthesis. However in normal cells, this pathway can be bypassed by using the salvage pathway, which requires the substrates hypoxanthine and thymidine for purine and pyrimidine synthesis, respectively. Since myeloma cells are deficient in the salvage pathway, they do not survive. Neither do the unfused spleen cells due to their limited life span in culture. Hence the only cells which survive are hybrid cells which have inherited the ability to utilise the salvage pathway from the spleen cells and long-term viability from the myeloma cells. Some of these hybrid cells will also have the antibody producing capacity of the splenic lymphocytes. The culture supernatant from single colonies of the hybridoma cells is then tested for the presence of the desired antibody. Cell lines positive for the secretion of antibody are propagated and frozen down in liquid N_2 vapour for long-term storage.

Inbred Balb/c mice were immunised with purified UL102 mixed with adjuvant. After a good antibody response had been achieved, the spleens were removed and the extracted spleen cells were fused with Sp2/0-Ag14 mouse myeloma cells (Shulman *et al.*, 1978). Hybridoma cell colonies were tested for the secretion of UL102-specific antibody by enzyme-linked immunosorbent assay (ELISA).

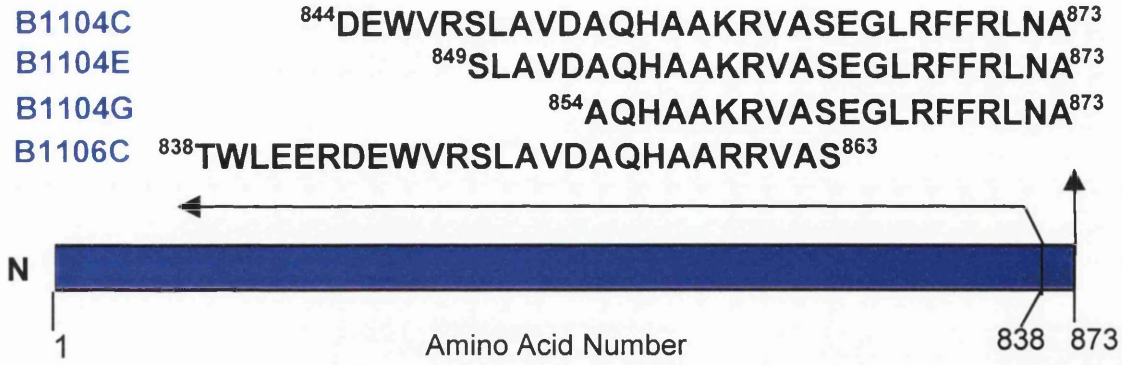


Figure 3.1A Biotinylated peptides used to map epitopes recognised by UL102 MAbs

Schematic diagram indicating regions of the C-terminus of UL102 to which N-terminally biotinylated peptides B1104C, B1104E, B1104G and B1106C correspond. UL102 is represented as a solid bar with the N and C-termini indicated. The amino acid numbers of UL102 represented by each peptide are also indicated.

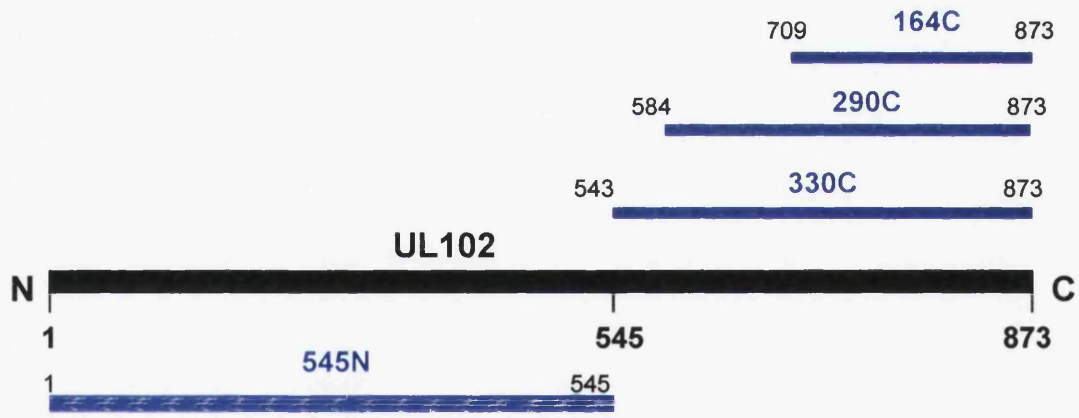


Figure 3.1B Truncated UL102 proteins used to map reactivites of UL102 MAbs

Schematic diagram showing regions of the UL102 protein expressed in bacteria and used for screening reactivity of UL102 MAbs in Western blotting. UL102 is represented as a solid bar with its N- and C-termini indicated. The regions of UL102 cloned and expressed are also depicted by solid bars with the amino acid numbers of UL102 which each region spans indicated. The nomenclature of the truncated UL102 proteins is given in blue text.

3.2 Chapter-specific methods

3.2.1 Generation of UL102-specific MAbs

3.2.1.1 Preparation of immunogen

UL102 was purified from insect cell lysate by two-column chromatography as described in section 2.9.1. Figure 3.2 shows Coomassie blue stained gels of UL102-containing fractions from both stages of the purification process, analysed by SDS-PAGE. UL102 of at least 95% purity was used for immunisation.

3.2.1.2 Immunisation schedule

Female Balb/c mice were immunised subcutaneously initially using 5μg of soluble recombinant UL102 protein emulsified in Freund's complete adjuvant. This was followed by three booster injections of 60μg recombinant UL102 protein emulsified in Freund's incomplete adjuvant at two week intervals. Sera from test bleeds was titrated against UL102 protein in ELISA to ascertain which animals exhibited the best antibody response. In preparation for the fusion, the 2 best-responding mice were given final intra-peritoneal boosts of protein as follows;

Mouse 1; 200μg of UL102 five days prior to the fusion.

Mouse 2; 150μg of UL102 four days prior to the fusion.

3.2.1.3 Preparation of spleen cells for fusion protocol

Mice were killed by cervical dislocation and the spleens removed immediately and placed in sterile DMEM medium on ice. Spleen cells were isolated by puncturing the spleen surface several times with a 26-G needle and injecting sterile medium into the spleen using another 26-G needle and syringe, forcing cells out through the perforations. The extracted cells were pelleted at 1400rpm (Sorvall RTH-250 rotor) for 10 minutes at 4°C then the supernatant was removed and 10ml of sterile DMEM was used to resuspend the cells, which were then counted.

3.2.1.4 Preparation of Myeloma Cells

Confluent Sp2/0-Ag14 cells were harvested by shaking into their existing medium and then pelleted at 1400 rpm (Sorvall RTH-250 rotor) for 10 minutes at 4°C. The cell pellet was stored on ice. Some of the supernatant was retained for use in the conditioned medium to be added subsequently to the cells following fusion.

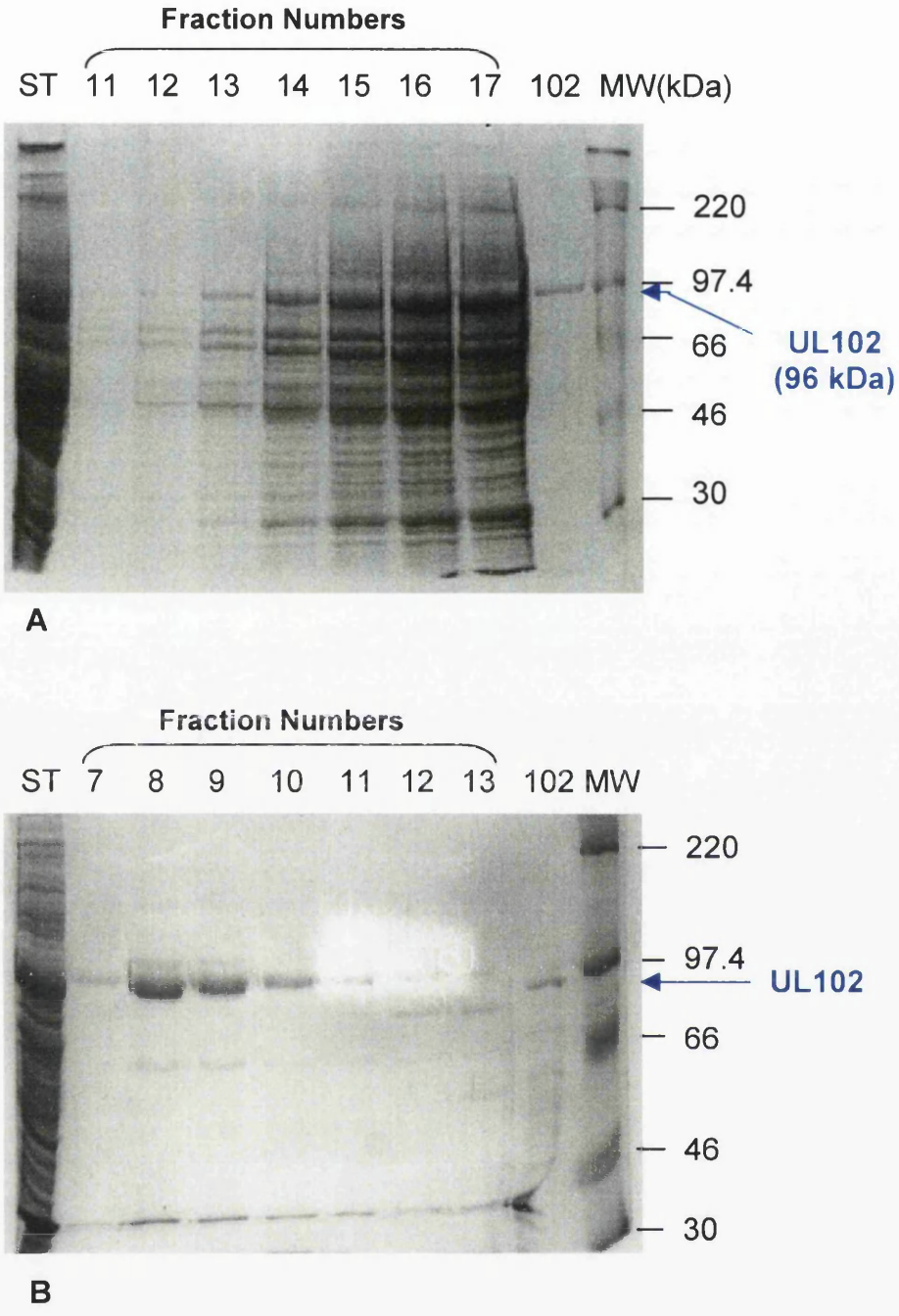


Figure 3.2 Purification of UL102 protein
Coomassie blue stained gels showing SDS-PAGE analysis of fractions collected from **A** DEAE-Sepharose column chromatographic separation of crude insect cell lysate containing UL102, and **B** Hydroxyapatite column purification of UL102 from pooled DEAE-Sepharose fractions. Lanes labelled 'ST' contains the start material injected onto each column. Fractions numbers are indicated. Lanes labelled '102' contain an earlier preparation of purified UL102 to act as a marker. Lanes labelled 'Mw' contain molecular weight marker proteins. Sizes in kDa are shown. The position of UL102 on each gel is indicated.

3.2.1.5 Fusion protocol

1×10^7 myeloma cells and 1×10^8 spleen cells, were mixed in a 50 ml tube, then pelleted at 1400 rpm (Sorvall RTH-250 rotor) for 5 minutes. The supernatant was removed and the cell pellet tapped loose. Fusion of the splenocytes and myeloma cells was achieved by adding 1ml of 50% PEG (1ml of PEG mixed with 1ml of DMEM at 37°C) to the cells and mixing gently. After 1 minute, the PEG was diluted 1:2 using 1ml of DMEM. Dilution of the PEG was repeated by adding a further 4, 8 and 16 ml DMEM at 2, 3 and 4 minutes respectively. The cells were then centrifuged at 1400 rpm (Sorvall RTH-250 rotor) for 15 minutes and re-suspended in conditioned HAT medium, comprising 75% fresh HAT medium and 25% pre-conditioned Sp2/0-Ag14 cell medium (medium in which Sp-2 cells had previously been grown), to a final concentration of 10^7 Sp2/0-Ag14 cells per 100 ml. The cell suspension was distributed into 96 well microtitre plates, by adding 150 µl per well and incubated at 37°C in a humidified incubator with 5% CO_2.

3.2.1.6 HAT selection and maintenance of fused myeloma/spleen cells

The cells were checked after 3 days to ensure the HAT medium was killing the Sp2/0-Ag14 cells. At 7-10 days following the fusion, the wells were monitored for the appearance of large colonies of hybrid myeloma/spleen cells. The supernatant from wells containing single colonies of diameter a third of the well was screened for reactivity against recombinant UL102 protein by ELISA, as described below.

3.2.1.7 ELISA screening of hybridoma cell supernatant

Immulon 1 microtitre plates were coated with 200 ng/well of purified recombinant UL102 protein diluted in PBS at 37°C overnight, then blocked using 2% BSA in PBS (100 µl/well) for 1 hour at 37°C. Hybridoma cell supernatant (50 µl) was added to the wells and incubated at 37°C for one hour then plates were washed 6x in PBS + 0.005% Tween 20. The plates were tapped dry before adding 50 µl/well of anti-mouse-HRP conjugate secondary antibody and incubating at room temperature for 1 hour. Unbound conjugate was removed by washing 6x using PBS/Tween-20 and plates tapped dry. 100µl per well of ABTS-peroxidase substrate was added and the colour change in each well after twenty minutes was measured by reading the optical density at 405 nm. UL102-reactive supernatant was designated as that producing a colour change greater than that produced by control supernatant from Sp-2 cells.

3.2.1.8 Propagation of positive-secreting hybridoma cell lines

Cells secreting positive antibody were transferred to small flasks and topped up with fresh HAT medium. Once cells reached confluency in the small flasks, the supernatant was tested again for reactivity against UL102 to ensure they were still secreting UL102-reactive antibodies. If positive for anti-UL102 antibody, cells were harvested and transferred to medium flasks and, ultimately, large flasks. Once cells were confluent in large flasks, the supernatant was collected and frozen in aliquots at -20°C. The hybridoma cells were aliquoted in HAT medium containing 10% DMSO then frozen down for long-term storage in liquid N_2.

3.2.2 Characterisation of UL102-specific MAbs

3.2.2.1 Testing reactivity of MAbs against UL102 in Western blotting

Semi-purified recombinant UL102 was used to screen the UL102 MAbs for reactivity in western blotting. Peak fractions eluted from DEAE-sepharose column purification of *Spodoptera frugiperda (Sf)* cell extracts containing UL102 were subjected to SDS-PAGE and transferred to nitrocellulose as described in sections 2.6.1 and 2.6.2. The nitrocellulose membranes were cut into 0.5 mm strips and incubated in blocking buffer overnight at 4°C. Individual strips were incubated with 1 ml of a single hybridoma cell supernatant at room temperature for 1 hour. The remainder of the Western blotting protocol was as described in section 2.6.3.

3.2.2.2 Testing reactivity of MAbs to UL102 in immunofluorescence

Sf cells were seeded at $1x10^5$cells/ well in 24-well plates containing 13mm coverslips. The next day, existing medium was removed and the cells were infected using 1 ml per well of recombinant baculovirus AcNPV-UL102 at a MOI of 10 (diluted using complete *Sf* cell medium) then replaced at 28°C overnight. After 24 hours, the cells were washed 3 times using PBS-Tween. They were then fixed by adding 200µl/well of -20°C acetone/methanol (1:2) solution and incubating at -20°C for 20 minutes. After a further 3 washes with PBS-Tween, 200µl/well of blocking solution (1% FCS in PBS-Tween) was added and left at 37°C for 1 hour. This solution was removed then 400µl of undiluted supernatant medium from monoclonal hybridoma cells was added to each well. After 1 hour at 37°C, cells were washed 3 times using PBS-Tween (1%). A 1:200 dilution of α-mouse-FITC conjugate was added to the cells (150µl/well) and incubated at RT for 1 hour then the cells were again washed 3 times using PBS-Tween. Coverslips were mounted onto a small drop of Citifluor solution (UKC) on microscope slides. Cells were viewed under x 400 magnification using a Nikon Microphot SA

microscope and a FITC filter.

3.2.2.3 Screening MAbs for reactivity against UL102 by immunoprecipitation

3.2.2.3.1 Expression of radiolabelled UL102 protein

24-well dishes were seeded with $1x10^6$ *Sf* cells/well in 1ml medium. At approximately 70% confluency, they were infected with 100µl of concentrated recombinant baculovirus AcNPV-UL102 or wild-type Pac6 baculovirus at a MOI of 5-10. Virus was adsorbed for 1 hour at 28°C, then fresh medium was added and the cells were incubated at 28°C overnight. The next day, the medium was replaced with 500µl of methionine-free *Sf* cell medium containing 30µCi per well of ^{35}S-methionine. Cells were incubated at 28°C overnight. The medium was removed and the cells were harvested in 500µl of cold TBS and then pelleted by centrifugation at 6000 rpm (microfuge). The supernatant was discarded and the cells were washed twice more in 500µl TBS. The cells were centrifuged again at low speed, the supernatant was removed and the pellet frozen at -70°C. Proteins were extracted by resuspending the cell pellet in 150µl of cold AE buffer and incubating on ice for 20 minutes. Extracts were then centrifuged at 35000 rpm for 30 minutes at 4°C in a Beckman TLA-100.2 rotor (Beckman TLA-100 benchtop ultracentrifuge). The supernatant containing extracted proteins from AcNPV-UL102-and Pac6-infected cells was then analysed by SDS-PAGE to check that UL102 had been expressed. The gels were dried and exposed to photographic film overnight.

3.2.2.3.2 Immunoprecipitation of UL102 from insect cell extracts

Proteins were extracted in cold AE buffer as described above. Cell extracts were mixed with 100µl of MAb (neat hybridoma cell supernatant) for 2.5 hours at 4°C. 50µl of a 50% suspension of Protein A-sepharose beads in buffer AE was then added and extracts were mixed for a further 1.5 hours at 4°C. Samples were then centrifuged at 6000 rpm (microfuge) for 2 minutes to pellet the protein A sepharose beads and the supernatant was discarded. The beads were then washed to remove any proteins not specifically bound. The beads were re-suspended in 500µl of cold AE buffer and then centrifuged at 6000 rpm (microfuge) for 2 minutes. The supernatant was discarded and the beads were washed twice. After the final wash, the pelleted beads were mixed with 50µl of SDS-PAGE sample buffer and boiled for 5 minutes to dissociate the bound proteins. The beads were briefly centrifuged again at 6000 rpm (microfuge) and the supernatant was analysed by SDS-PAGE, together with a whole insect cell lysate sample to allow identification of the UL102 protein band. Following electrophoresis, gels were vacuum dried at 80°C for 1 hour, exposed to X-ray photographic film

overnight and the resulting autoradiograph was developed.

3.2.2.4 Testing MAbs for recognition of UL102 C-terminal/C-proximal peptides in ELISA

UL102 C-terminal and C-proximal peptides, B1104C, B1104E, B1104G and B1106C, that were specifically biotinylated at their N-terminus, were diluted (200ng/well in 50µl PBS) and bound to streptavidin coated microtitre wells by incubation at 37°C overnight. Wells were blocked for 1 hour using 5% dried milk in PBS-Tween, washed using PBS-Tween (1%) and then 50µl of MAb (undiluted supernatant medium from hybridoma cells) was added to individual wells and incubated at 37°C for 1 hour. The remainder of the ELISA protocol was as described in section 3.2.1.7.

3.2.2.5 Testing reactivities of MAbs against UL102 GST-fusion proteins in Western blotting

Full-length and truncated UL102 GST-fusion proteins (UL102-GST, 544N-GST, 330C-GST, 290C and 164C) were inducibly expresed in *E. coli.* After centrifugation, bacterial cell pellets were mixed with SDS-PAGE sample buffer and boiled for 10 minutes. Samples were then resolved by SDS-PAGE and transferred to nitrocellulose as described in section 2.6.2. Blots were then incubated with individual MAbs (2 ml of undiluted hybridoma cell medium supernatant) for 1 hour. Blots were washed and then incubated with 5 ml of a 1:1000 dilution of anti-mouse HRP conjugated antibody for 1 hour. After further washing, blots were treated with ECL reagents and exposed to photographic film.

3.3 Results

3.3.1 Generation of UL102 MAbs

51 hybridoma cell lines secreting UL102-specific antibodies were isolated following the fusion of Sp2/0-Ag14 myeloma cells and spleen cells from mice immunised with purified recombinant UL102.

3.3.2 Characterisation of UL102 MAbs

3.3.2.1 Reactivity of MAbs in Western blotting

Over half (29/51) of the UL102-specific MAbs recognised denatured UL102 protein bound to nitrocellulose blots. Figure 3.3 shows the reactivity of a subset of the UL102 MAbs. The protein band recognised by some of these MAbs was confirmed as UL102 as it co-migrated with the protein band recognised by the polyclonal UL102 antisera 373 and was of the correct molecular weight. MAb numbers 658, 626, 546, 549, 683, 707 and 778 are positive. MAb 555 does not react with full-length UL102 but reacts with a protein band of smaller molecular weight. Western blot positive MAbs are indicated by '+' in summary Table 3.3.

3.3.2.2 Reactivity of MAbs in immunofluorescence

All MAbs were screened for their ability to recognise UL102 by immune-fluoresence. Each MAb was incubated with fixed *Sf* cells that had been infected with UL102-expressing recombinant baculovirus AcNPV-UL102 or wild type Pac6 baculovirus. Twenty eight MAbs produced a fluorescent signal in the UL102-expressing cells but no significant background fluorescence in the Pac6-infected cells. These MAbs were scored as '+' for IF reactivity, with scores of '++' and '+++' indicating increasing strength of fluorescence in Table 3.3. Figure 3.4 shows IF images of UL102- and pac6-infected cells treated with positive or negative staining MAbs. MAbs 70 and 546 were judged to react whilst MAbs 532 and 807 were non-reactive.

3.3.2.3 Reactivity of MAbs in immunoprecipitation experiments

Twelve MAbs were capable of immune-precipitating UL102 from extracts of *Sf* cells infected with recombinant baculovirus AcNPV UL102 whilst no proteins were precipitated from mock-infected *Sf* cell extracts. Figure 3.5 shows the results from a typical screening experiment. MAb numbers 657, 658 and 707 clearly immunoprecipitate UL102 whereas MAbs numbers 559, 672 and 683 do not.

3.3.2.4 Reactivity of MAbs to UL102 C-terminal/C-proximal peptides

MAbs were tested for their ability to recognise four peptides (Figure 3.1A)

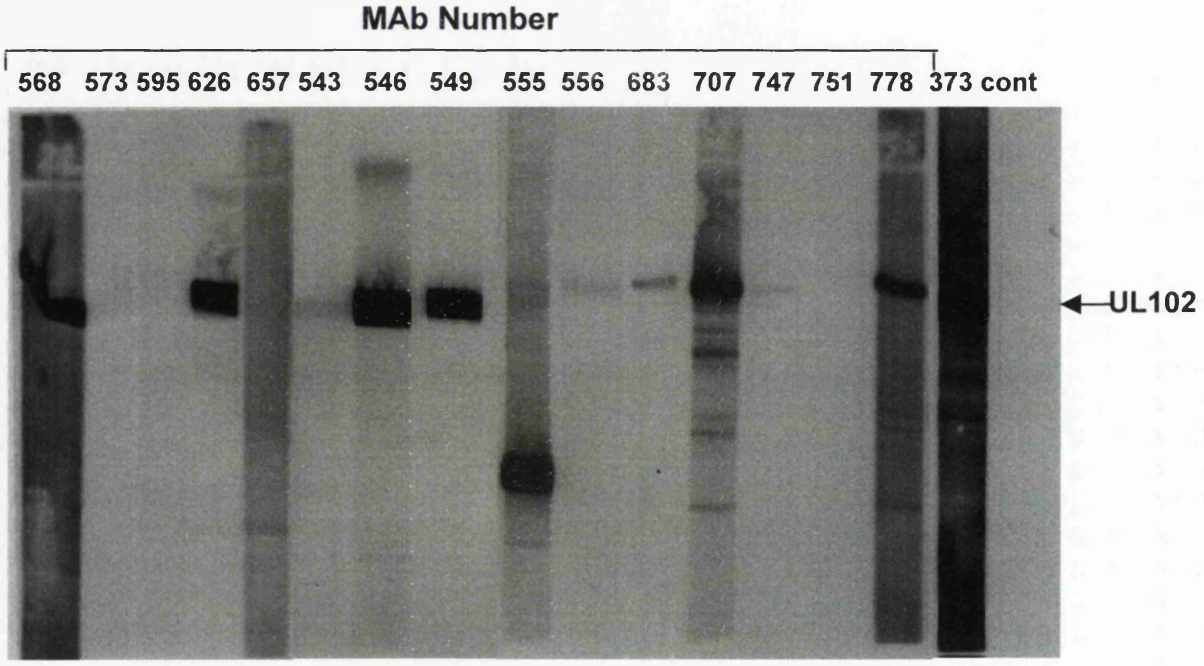


Figure 3.3 Reactivity of MAbs to UL102 in western blotting
Semi-purified *sf* cell extract containing UL102 was resolved by SDS-PAGE and electroblotted onto nitrocellulose. Nitocellulose strips were incubated with 1ml Mabs (neat supernatant from individual hybridoma cell lines), polyclonal UL102 antisera 373 (1:200) or control hybridoma cell supernatant (labelled cont), as indicated. Strips were then washed and incubated with either anti-mouse HRP- or anti-rabbit HRP-conjugated antibody, as appropriate. After further washing, strips were treated with ECL reagents and exposed to photographic film which was subsequently developed. The position of UL102 on the strips is indicated.

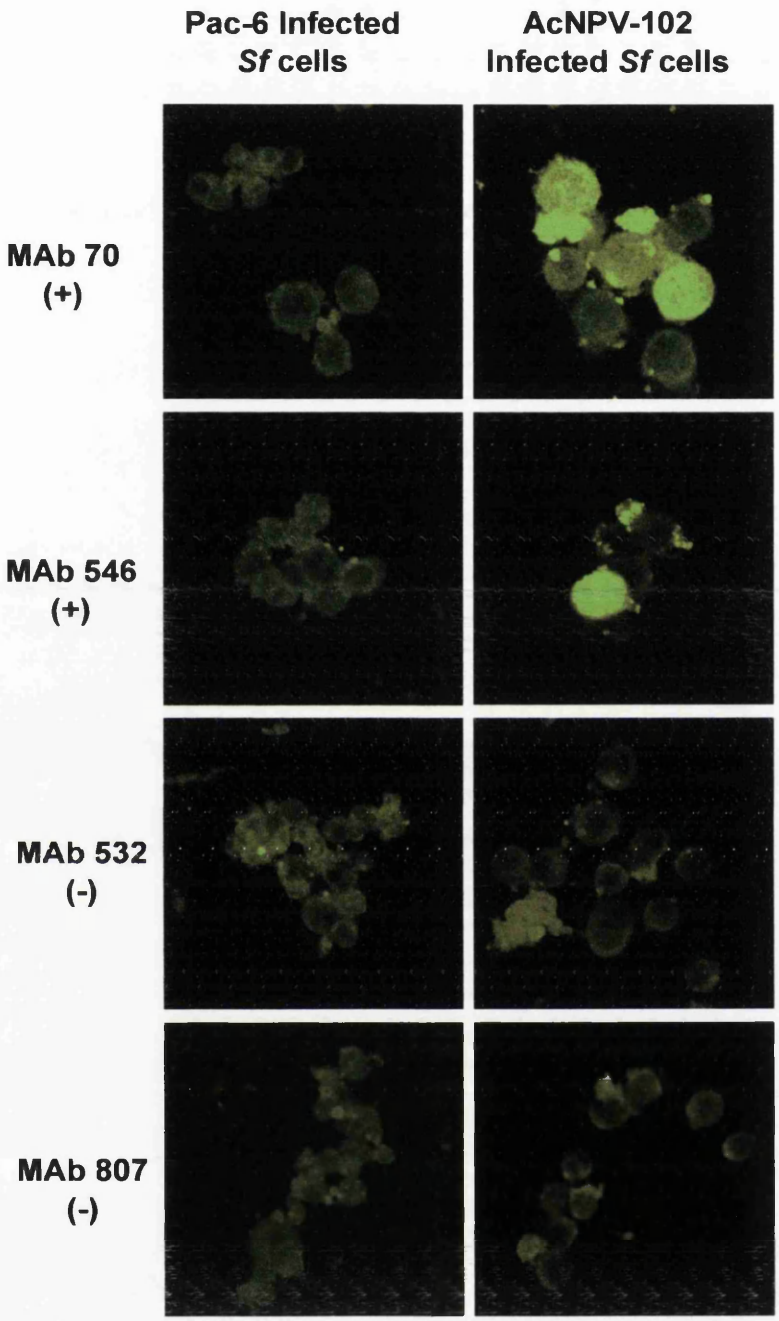


Figure 3.4 Reactivity of Mabs to UL102 in immunefluorescence assays
Sf cells were infected at a MOI of 5-10 with recombinant baculovirus AcNPV-UL102 or wild-type baculovirus Pac6 as indicated. Following a 24 hour infection, cells were fixed and incubated with Mabs for 1 hour then washed and incubated with a 1:100 dilution of anti-mouse FITC conjugated antibody for a further hour. Cells were washed again and, after drying, placed face down in citifluor mounting agent on microscope slides. The cells shown were viewed under x40 magnification.

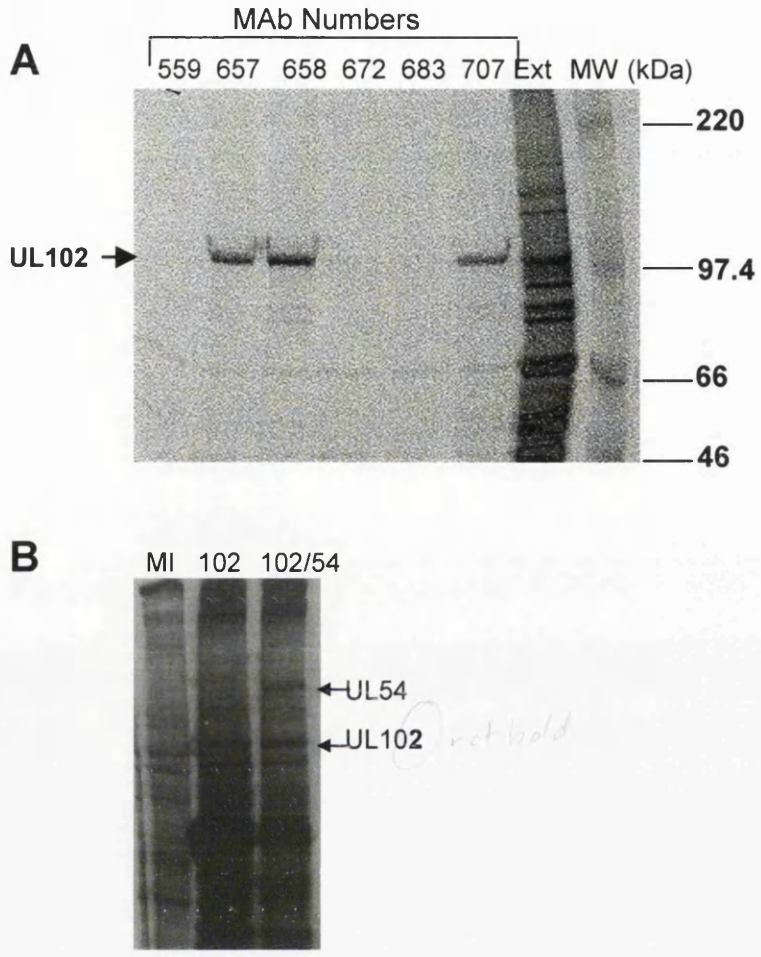


Figure 3.5 Reactivity of MAbs to UL102 in immunoprecipitation experiments

Sf cells were infected with recombinant baculovirus AcNPV-UL102 at an MOI of 5-10. After 24 hours, the medium was replaced with methionine-free *Sf* medium containing ^{35}S-methionine and incubated for a further 24 hours. Cells were harvested and washed 3 x in TBS. Proteins were extracted in cold AE buffer and mixed with individual MAbs for 1 hour at 4°C, then a suspension of protein A-sepharose was added. After mixing for a further hour, protein A-sepharose beads were pelleted by centrifugation, washed 3 x in AE buffer then boiled in SDS-PAGE buffer. Beads were pelleted by centrifugation and the supernatant was analysed by SDS-PAGE. Gels were dried and exposed to photographic film overnight, which was then developed. **Figure 3.5A** shows the results obtained for a subset of the UL102 Mabs. MAb numbers are indicated, along with the position of UL102. 'Ext'- clarified whole cell extract, 'MW'- Molecular weight markers. **Figure 3.5B** shows SDS-PAGE analysis of radiolabelled proteins extracted from *Sf* cells either mock infected (MI), infected with AcNPV-UL102 (102) or doubly-infected with AcNPV-UL102 and AcNPV-UL54 (102/54).

corresponding to UL102 C-terminal/proximal regions in ELISA. Over a quarter of the MAbs bound to at least one of these peptides. A positive result was attributed where sufficient binding of the MAb and then HRP-conjugated secondary antibody had produced a colour change, upon addition of a chromatogenic substrate, of greater than 0.5 OD units. The 13 MAbs which recognised at least one of the 4 peptides screened in this assay are listed in Table 3.1 below.

MAb No	B1104C	B1104E	B1104G	B1106C
59	+	+		
70	+	+		
123	+	+		
154	+	+		
175	+	+	+	
188	+	+	+	
278	+	+		
532	+	+	+	
546				+
549	+	+		
568	+	+	+	
778	+	+		
782	+	+		

Table 3.1 UL102 MAbs which recognise UL102 C-terminal/C-proximal peptides
A '+' indicates binding of MAbs to individual peptides as labelled.

OD measurements obtained for each of the 13 reactive MAbs tested against all 4 peptides are shown in Figure 3.6. Generally, B1104G was bound relatively weakly as compared to B1104C and B1104E, producing significantly lower OD measurements. The majority of the MAbs bound both peptides B1104C and B1104E but only one MAb (546) bound peptide B1106C.

3.3.2.5 Testing Western blot-positive MAbs for ability to recognise full-length and truncated UL102 GST-fusion proteins

MAbs capable of recognising UL102 in Western blotting were further tested for their ability to recognise a range of truncated UL102 GST-fusion proteins expressed in bacteria in Western blotting. One C-terminally and three N-terminally truncated UL102 proteins, depicted in Figure 3.1, were used in this experiment. A full-length UL102 GST-fusion construct and GST protein alone were also included as positive and negative binding controls, respectively. The reactivity of each WB-positive MAb to UL102 GST-fusion proteins is summarised in Table 3.2 below. Figure 3.7 shows the Western blotting results obtained for two of the MAbs screened against the UL102

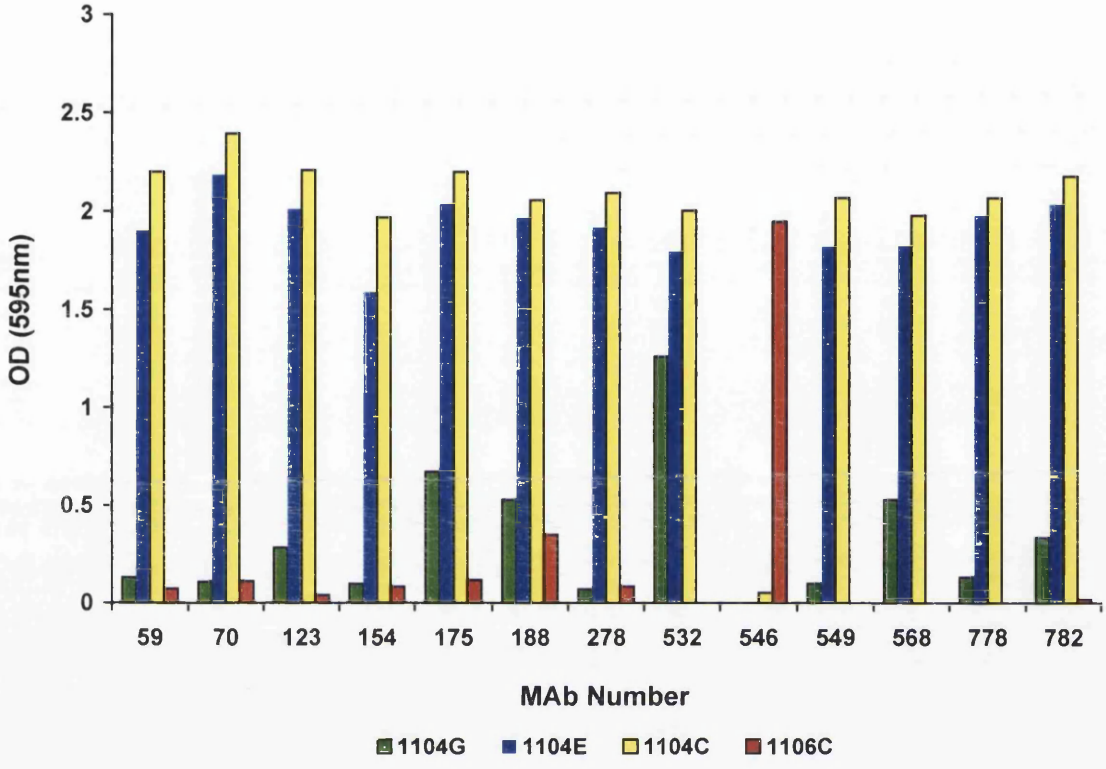
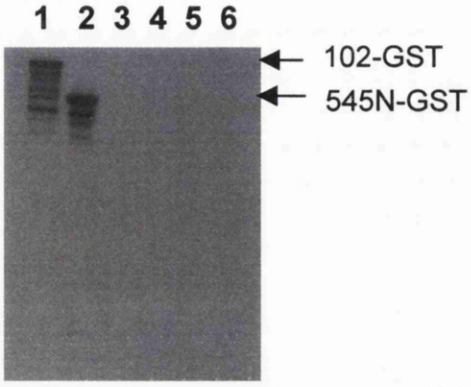
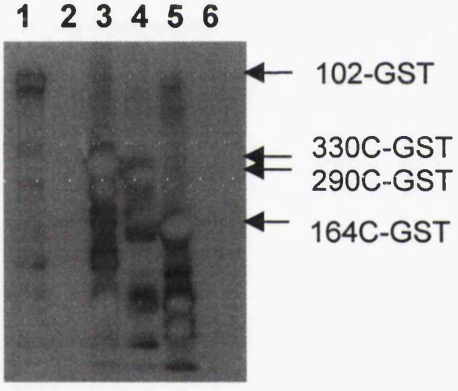


Figure 3.6 Reactivity of MAbs to UL102 C-terminal and C-proximal peptides

MAbs were incubated with biotinylated peptides B1104C, B1104E, B1104G and B1106C bound to streptavidin-coated microtitre wells. After washing to remove any unbound MAb, anti-mouse HRP-conjugated antibody was added. Unbound secondary antibody was removed by further washes, then chromogenic ABTS-peroxidase substrate was added to each well. The optical density (595nm) in each well was measured after 30 minutes using a plate reader. There are four readings shown for every MAb, each bar corresponding to one of the 4 peptides, as indicated in the legend.



MAb145:
N-terminally reactive



MAb70:
C-terminally reactive

Figure 3.7 Reactivity of MAbs to UL102 GST-fusion proteins in Western blots

Whole bacterial cells expressing UL102 GST-fusion proteins were boiled in SDS-PAGE buffer and then resolved by SDS-PAGE. Proteins were transferred to nitrocellulose membranes. After blocking with milk protein, membranes were incubated with individual MAbs for 1 hour then washed and incubated with anti mouse HRP-conjugated antibody for a further hour. Membranes were washed again, treated with ECL Western blotting reagents and exposed to photographic film. Labels 1-6 refer to whole bacterial cells expressing proteins as follows: 1= UL102-GST, 2= 544N-GST, 3=330C-GST, 4=290C-GST, 5=164C-GST and 6=GST only.

GST-fusion proteins. MAb 145 reacts with full-length UL102 and the 544N truncated UL102 protein. In contrast, MAb 70 reacts with full-length UL102 and all three C-terminal UL102 proteins. In both cases, there are multiple bands due to degradation products of the expressed proteins. The bands corresponding to the 330C, 290C and 164C proteins appear bubble-like. The intensity of signal (light emission following addition of ECL substrates) from the middle of these protein bands is diminished. This 'quenching' phenomenon was observed several times when heavily loaded gels were treated with ECL reagents.

	UL102 GST-fusion protein Reactivity					
MAb No	**UL102**	**544N**	**330C**	**290C**	**164C**	**GSTonly**
59	Y	Y	Y	Y	Y	N
70	Y	N	Y	Y	Y	N
81	Y	Y	N	N	N	N
123	Y	N	Y	Y	Y	N
145	Y	Y	N	N	N	N
154	Y	Y	Y	Y	Y	N
157	Y	Y	N	N	N	N
175	Y	Y	Y	Y	Y	N
185	Y	Y	N	N	N	N
188	Y	N	Y	Y	Y	N
266	NON-REACTIVE					
278	Y	N	Y	Y	Y	N
508	NON-REACTIVE					
532	Y	N	Y	Y	Y	N
537	NON-REACTIVE					
539	NON-REACTIVE					
543	NON-REACTIVE					
546	Y	N	Y	Y	Y	N
549	Y	N	Y	Y	Y	N
555	Y	N	Y	Y	Y	N
556	NON-REACTIVE					
568	Y	N	Y	Y	Y	N
626	Y	Y	N	N	N	N
658	Y	Y	N	N	N	N
683	NON-REACTIVE					
707	Y	Y	N	N	N	N
747	NON-REACTIVE					
778	Y	N	Y	Y	Y	N
782	Y	N	Y	Y	Y	N

Table 3.2 Reactivities of MAbs to UL102 GST-fusion proteins in Western blotting
Binding of MAbs to UL102 GST-fusion proteins is indicated by a 'Y'. 'N' means no binding between the protein and MAb was detected.

As expected, no MAbs reacted with GST protein alone and most MAbs displayed reactivity to either N-terminal or C-terminal regions of UL102. However, MAbs 59, 154

and 175 were found to be reactive to both regions of the protein and this observation is discussed further in section 3.4.2.

3.3.2.6 Defining epitope-containing regions of UL102 for each MAb

The combination of the C-terminal peptide reactivity data with the results from the screening against truncated UL102 proteins made it possible to determine regions of UL102 containing the epitopes recognised by each of the MAbs. Five epitope-containing regions of UL102 were defined, as depicted in Figure 3.8. The epitopes recognised by MAbs which did not recognise any of the truncated UL102 proteins or any of the C-terminal peptides could not be defined to any particular region of UL102, Hence the epitope-containing region to which they have been assigned (region 1) consists of full-length UL102. MAbs mapping to region 2 (residues 1-545) were those which did not bind any of the UL102 C-terminal peptides but recognised the UL102 GST fusion protein 545N in WB. Therefore the epitopes recognised by this subset of MAbs lie within the first 545 residues of UL102. Region 3 was recognised by one MAb only (number 555), which reacts with all the C-terminal UL102 GST fusion proteins, collectively spanning residues 546-873, but did not react with any of the C-terminal UL102 peptides (residues 838-873). Therefore the UL102 region to which this MAb reacts lies between residues 546-838. Region 4 (residues 838-863), corresponding to peptide B1106C, is recognised by MAb 546 only. Although this peptide contains six unique residues (838-843), it cannot be excluded that downstream residues also contribute to epitope formation, hence the precise location of the epitope within the peptide cannot be determined. Region 5 (aa's 844-873) corresponds to the UL102 residues spanned by peptides B1104C, B1104E and B1104G. Hence, MAbs which recognised any of these peptides could be epitope-mapped to this region. The region to which each MAb maps is indicated in Table 3.3.

3.4 Discussion

3.4.1 Locating epitopes recognised by UL102-specific MAbs; Use of C-terminal UL102 peptides

Interactions between several of the HSV-1 replication proteins are known to be mediated by the C-terminal regions of one or both of these proteins. Examples include the interactions between proteins UL8/UL30 (Marsden *et al.*, 1997) and UL30/UL42 (Tenney *et al.*, 1993; Digard *et al.*, 1993; Stow, 1993; Marsden *et al.*, 1994; Digard *et al.*, 1995). As one of the aims of this project was to investigate the putative interaction between UL102 and another HCMV replication fork protein, UL54, antibodies which

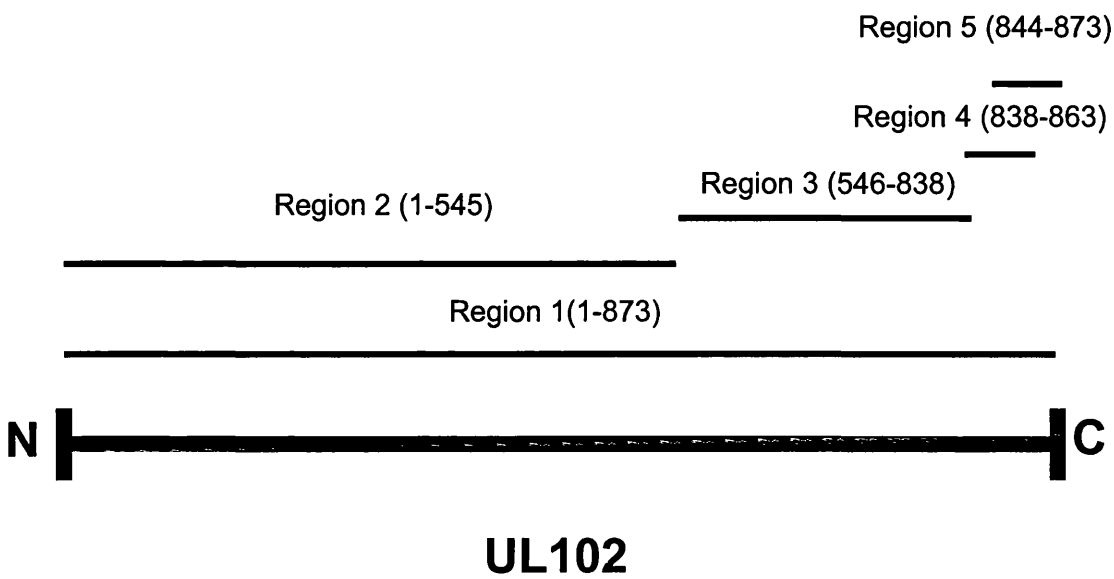


Figure 3.8 Summary of epitope-containing regions of UL102
UL102 is represented as a solid bar with its N- and C-termini indicated. Above it are marked 4 epitope-containing regions, with the amino acid numbers of UL102 which they represent written in brackets.

	Reactivities					Reactivities					Reactivities			
MAb No	WB	IF	IP	Epitope Region	MAb No	WB	IF	IP	Epitope Region	MAb No	WB	IF	IP	Epitope Region
24	-	+++	-	1	454	-	+	-	1	555	+	+++	-	3
59	+	-	-	5	508*	+	-	-	1	556*	+	-	-	1
70	+	+++	-	5	511	-	-	-	1	568	+	+++	-	5
81	+	+	-	2	519	-	+	+	1	573	-	-	-	1
123	+	-	-	5	532	+	-	+	5	595	-	-	-	1
145	+	-	-	2	535	-	++	-	1	626	+	++	-	2
154	+	+	-	5	536	-	+	-	1	657	-	+	+	1
157	+	-	-	2	537*	+	++	-	1	658	+	++++	+	2
162	-	+	-	1	539*	+	+	-	1	672	-	-	-	1
175	+		+	5	540	-	+	-	1	683*	+	-	-	1
185	+	+	+	2	543*	+	+	-	1	707	+	+++	+	2
188	+	+	+	5	546	+	++++	-	4	747*	+	-	-	1
232	-	+	-	1	547	-	-	-	1	751	-	-	-	1
266*	+	+	-	1	548	-	-	-	1	778	+	++++	-	5
278	+	-	+	5	549	+	+++	-	5	782	+	+++	-	5
407	-	+	+	1	551	-	-	+	1	807	-	-	-	1
414	-	+++	-	1	553	-	-	-	1	809	-	-	+	1

Table 3.3 Summary of the reactivities of UL102-specific MAbs

The table summarises the findings for each MAb after testing for reactivity against UL102 in three immunoassays; Western blotting (WB), immune-fluorescence (IF) and immune-precipitation (IP). Antibodies that recognise UL102 in a given assay are marked '+' in the appropriate column, whereas '-' denotes no reactivity in that assay. Data for the reactivity of each MAb in IF has been graded with '+' indicating weak fluorescence and '++', '+++' and '++++' indicating increasing strengths of fluorescence. Also listed for each MAb is the region of UL102 containing the epitope to which it binds. MAbs have been allocated a number corresponding to a region of UL102 as described in Figure 3.8. MAbs marked with a '*' are those which recognised insect cell expressed UL102 protein in WB, but not UL102 protein expressed in bacteria.

bind the C-terminal region of UL102 were potentially of use in determining whether any demonstrated interactions are also mediated by this region, as was found for the HSV-1 counterparts. Hence, each monoclonal was screened for reactivity against peptides corresponding to C-terminal and C-proximal regions of UL102 in ELISA. Four overlapping peptides spanning this region of UL102 were used for this purpose, to allow the identification of short amino acid sequences containing or contributing to the epitopes in this region to which any MAbs bound.

Of the 51 MAbs, 13 recognised at least one of the 4 peptides (Figure 3.1A) spanning the C-terminal region (Table 3.1). Furthermore, 8 of these bound to both peptides B1104C and B1104E, but neither peptide individually, indicating that these MAbs recognise an epitope common to both peptides. By comparing the sequences of B1104C and B1104E to B1104G (Figure 3.1A), to which these MAbs do not bind, it can be concluded that the amino acid sequence SLAVD (aa's 849 to 853) must be involved in the formation of this epitope. However, this sequence does not appear to be the sole sequence involved in formation of the epitope, because it is also present in peptide B1106C, and none of the 8 MAbs bound to this peptide. B1106C differs from B1104C and B1104E as it lacks the C-terminal 10 residues of UL102 (EGLRFFRLNA), indicating that this sequence may also contribute to the formation of the epitope which is recognised by these MAbs.

As 2 distal regions within the C-terminal 25 amino acid residues of UL102 are required for recognition by these 8 MAbs, this suggests that they may together form a conformational epitope. For these regions to be in close enough proximity to allow binding of the antibody, the extreme C-terminus of UL102 may form a loop structure. Structural information on UL102 obtained using the "predict-protein" programme (Rost, 1996) predicts that the extreme C-terminal residues of UL102 are either looped or are not predicted to form any recognised structures (Figure 8.1). Considering that a large proportion of the C-terminally reactive MAbs recognise the epitope(s) formed by these regions, it seems that this is an immunodominant region of the protein. An additional three MAbs; 175, 188 and 568, show limited reactivity to peptide B1104G as well as B1104C and B1104E. It is possible that these MAbs bind to an alternative epitope common to all 3 sequences or perhaps more likely that they have reduced reactivity to 1104G as its N-terminally truncated sequence does not allow complete formation of the dominant epitope present in B1104C and B1104E. MAb 546 shows unique reactivity to peptide 1106C and is the only MAb to bind to this sequence. As it does not recognise any of the other peptides, it can be concluded that the residues unique to this peptide

must contribute to formation of the epitope to which it binds. However, it is possible that other residues of the peptide may also contribute to epitope formation.

The C-terminus of UL102, specifically the 36 terminal amino acids represented by the four peptides used in this epitope mapping experiment, seems to be strongly immunogenic as a quarter (13/51) of the UL102 MAbs recognise epitopes residing in this region of the protein. This is quite a high proportion considering that the protein is 873 amino acids in length and hence this region represents only 4% of the total protein. This observation provides an insight into the possible native conformation of UL102, suggesting that the C-terminal region of UL102 occupies an exposed, exterior position in the UL102 molecule and is therefore available for recognition by B-lymphocytes which would therefore produce antibodies reactive to this region.

3.4.2 Locating epitopes recognised by UL102-specific MAbs; Use of truncated UL102 proteins

MAbs which recognised full-length insect cell-expressed UL102 by Western blotting were also screened for their ability to recognise bacterially-expressed truncated UL102 proteins by Western blot also. One C-terminally (545N) and three N-terminally truncated (330C, 290C and 164C) forms of UL102 were used in these experiments. This allowed the epitopes recognised by these MAbs to be mapped to broad regions of UL102, and also identified which MAbs were suitable for use in conjunction with these truncated forms of the protein in subsequent experiments.

MAbs were found to recognise either the C-terminally truncated 545N protein or all 3 N-terminally truncated proteins; 330C, 290C and 164C. None of the MAbs recognised any of the C-terminal UL102 GST-fusion proteins individually, suggesting that they all recognise epitopes residing in the terminal 164 residues of UL102. All MAbs, with the exception of MAb 555, which recognised C-terminal UL102 GST-fusion proteins also recognised at least one of the 4 UL102 C-terminal peptides, so the region of UL102 containing the epitopes recognised by these MAbs can be further defined to residues 838-873, which are the UL102 aa's represented by these peptides.

Three MAbs (59, 154 and 175) showed reactivity to both N-terminal and C-terminal UL102 GST-fusion proteins. Clearly it is not possible for a MAb to do this as they should, by definition, bind to one specific epitope only. It was concluded that these were antibodies derived from more than one clone. These hybridoma cell lines were subcloned by Dr Susan Graham, who found that all 3 contained dominant clones

whose specificity mapped to the C-terminal region of UL102 and no subclones reactive with the N-terminal fragment could be recovered. The dominant clones were propagated and adopted to solely represent each cell line. Hence any information referring to locations of epitopes for MAb numbers 59, 154 or 175 was elucidated from these dominant hybridoma cell lines.

Of the 29 western positive MAbs, 8 were found to be non-reactive to any of the bacterially-expressed full-length or truncated UL102 proteins (MAb numbers 266, 508, 537, 539, 543, 556, 683 and 747). One possible explanation for this lack of reactivity is that the formation of the epitopes recognised by these MAbs is dependent on post-translational modification of UL102, such as glycosylation or phosphorylation, which is not carried out in bacterial cells. An alternative explanation may be that the GST-fusion may interfere with the folding of the expressed truncated protein, resulting in the epitope(s) to which these MAbs react being masked. All the MAbs which recognised insect cell-expressed UL102 but none of the bacterially expressed UL102 proteins recognise epitopes within region 1 (Figure 3.8). Hence, mapping of the regions to which these MAbs react may provide useful information regarding sites of modifications made to UL102.

3.4.3 Reactivities of UL102-specific MAbs in immunochemical assays: Western blotting, immunoprecipitation and immunofluorescence

A significant proportion (58%) of the MAbs were reactive to UL102 in Western blotting. There was variability in the strengths of signals and also specificities of binding amongst these MAbs. As can be seen in Figure 3.3, several MAbs also bind smaller molecular weight protein bands, which probably correspond to degradation products of full-length UL102 containing the epitopes recognised by these particular MAbs. The epitopes recognised by the Western blot-positive MAbs are unlikely to be conformational (ie formed as a consequence of the tertiary fold of UL102) since they retain the ability to bind their cognate MAbs following extensive denaturation by SDS-PAGE. Many of these MAbs also recognise UL102 in IF, IP or both, hence these linear epitopes must also be available when UL102 is in a more native form. An interesting anomaly is apparent when the Western blot results and the epitope mapping data is considered together. The eight MAbs which appeared to recognise conformational epitopes on the basis of the C-terminal peptide data are all capable, however, of binding to UL102 in Western blotting. It is possible that the loop structure may withstand the denaturing process or reform after blotting onto the nitrocellulose membrane.

Many of the MAbs (29/51) also recognise UL102 in immune-fluorescence assays. Eighteen of these are also positive for Western blotting, indicating that the epitopes which they recognise are probably linear and are likely to be present in the same form when UL102 is expressed in insect cells, presumably in a more native form. The other 11 IF-positive MAbs must recognise conformational epitopes that are disrupted when the protein is denatured by SDS-PAGE. None of these MAbs recognise epitopes located in the extreme C-terminal region of UL102.

A far smaller proportion (12/51) of the MAbs were capable of immune-precipitating UL102 from extracts of cells infected with AcNPV-UL102 baculovirus. This perhaps reflects the fact that UL102 epitopes are presented in this assay amongst a complex, dynamic mixture of insect cell proteins and hence this is a more stringent test of the MAb binding affinities. Five of the IP-positive MAbs do not react with UL102 in Western blotting, probably due to denaturation of conformational epitopes. Again, none of these five MAbs map to the C-terminus of UL102. A subset of 9 MAbs do not recognise UL102 in any of the immunoassays tested, making them useful only for detection of the protein in ELISA.

The panel of 51 UL102-specific MAbs described in this chapter represent a diverse and potentially powerful resource for the study of UL102. The spectrum of MAb reactivities available enables the analysis of full-length and truncated forms of UL102 in a variety of assays. The reactivities of all the UL102 MAbs are summarised in Table 3.3. These antibodies were used to investigate the putative interaction between UL102 and UL54 and also the intracellular localisation of full-length and truncated UL102 proteins by immunofluorescence, described in Chapters 5 and 7, respectively.

Chapter 4

Generation of UL102-expressing constructs

4.1 Introduction

The UL102 characterisation studies undertaken in this project required that the UL102 gene be cloned into several different vectors for expression in a variety of cell systems, including bacterial and mammalian cells, in addition to the insect-cell system already established for the expression and purification of the UL102 protein. At the outset of the study the main characterisation studies intended included;

A) Investigation of the putative interaction between UL102 and UL54 proteins

As it was anticipated that an interaction between UL102 and UL54 would be demonstrated, a primary objective was to clone several regions of the UL102 gene in order to express truncated UL102 proteins, with which the regions involved in interaction with UL54 could be investigated. A bacterial system was selected to express the truncated proteins, because of the relative ease of generation and expression of constructs, as compared to recombinant baculovirus production. The Pharmacia GST Gene Fusion System was chosen, in which the cloned protein is expressed as an N-terminal fusion with the 26 kDa Glutathione-S-transferase (GST) protein from *Schistosoma japonicum.* This confers the advantage of simple and quick purification of the fusion protein (provided it is soluble) by affinity chromatography using Glutathione Sepharose 4B and the GST tag can then be cleaved. The system uses the pGEX series of vectors (Figure 4.1). The UL102 gene fragments were cloned into either pGEX-2T, pGEX-4T2 or pGEX-4T3, dependent on the availability of suitable in-frame restiction sites.

B) UL102 Immunofluorescence studies

A second aim was to perform immunofluorescence studies on UL102, to determine its intracellular localisation when expressed alone, or in combination with other HCMV replication proteins. For this purpose, the mammalian expression vector pCMV10 was chosen. pCMV10 is a pUC-based vector which contains a multiple cloning site downstream of the HCMV major IE promoter sequence (Stow *et al., 1993;* Figure 4.2). It can produce high levels of expression of cloned genes in mammalian cells. The full-length UL102 gene, as well as a UL102 gene fragment encoding a C-terminally truncated UL102 protein were cloned into pCMV10. In addition, a UL102 gene fragment encoding an N-terminally truncated protein was cloned into mammalian expression vector pCMV-Tag 2B (Stratagene), which is described further in section 7.1.

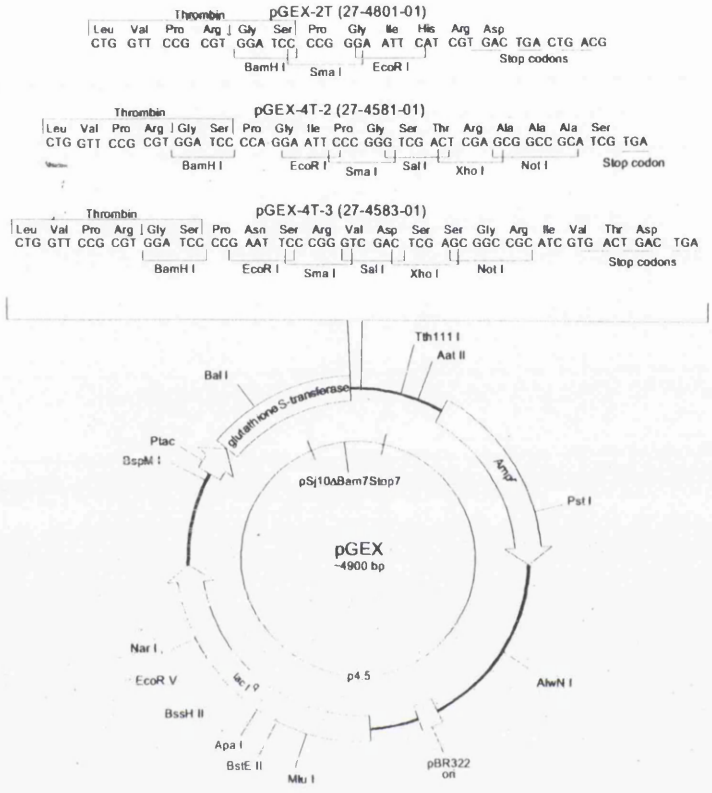


Figure 4.1 pGEX Vector Map

A schematic diagram of the pGEX series of vectors is shown with the position and orientation of the fusion sequence and the multiple cloning site (MCS) region indicated. Above the vector map are the MCS regions from the three pGEX vectors into which regions of the UL102 gene were cloned: pGEX-2T, pGEX-4T2 and pGEX-4T3, with the positions of each restriction site indicated. The map was reproduced from the Pharmacia catalogue.

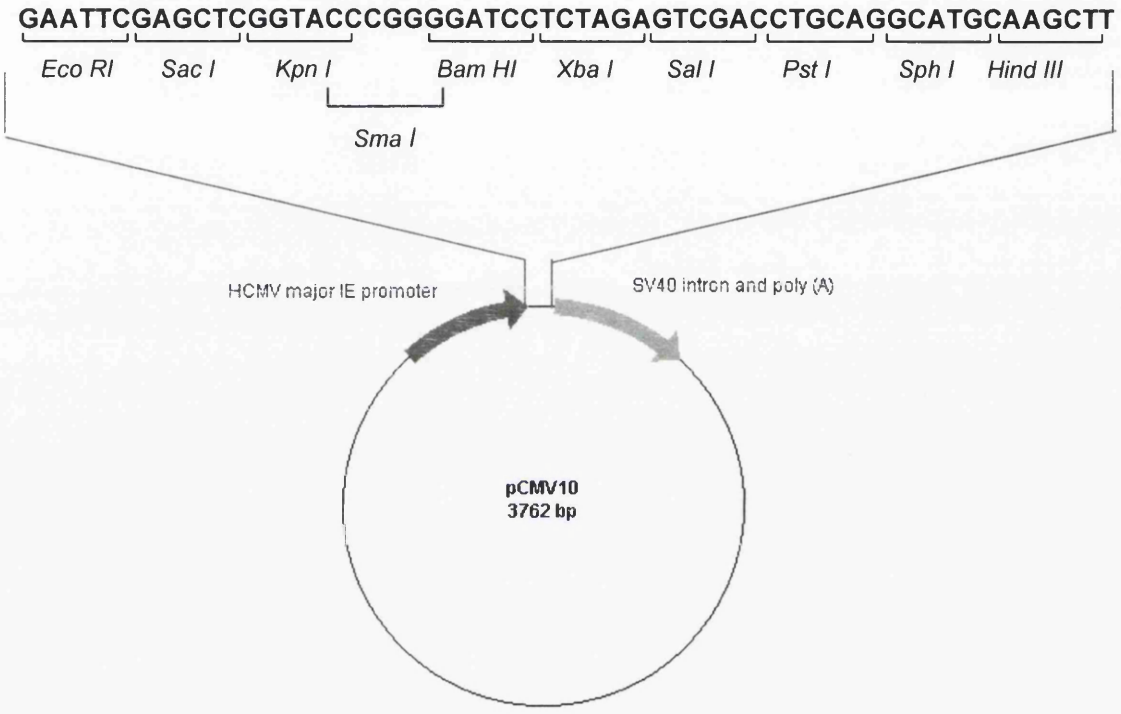


Figure 4.2 Map of mammalian expression vector pCMV10
The pCMV10 vector is represented in schematic form, with the positions and orientations of the HCMV IE promoter and SV40 intron and poly (A) sequences indicated by large arrows. The position of the multiple cloning site (MCS) is also shown. An enlarged diagram of the MCS indicates the restriction sites included and their relative positions.

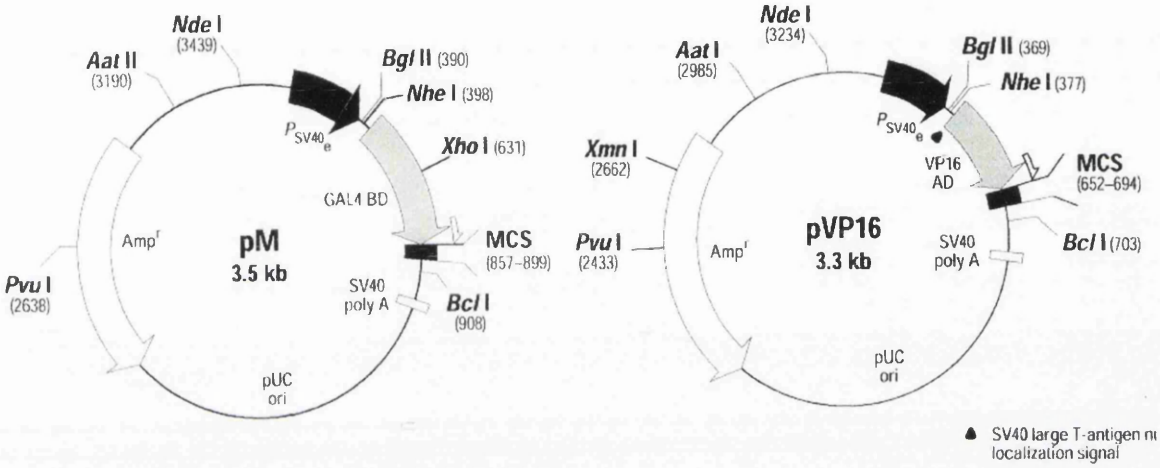
C) **Investigation of the putative UL102-UL54 interaction using the mammalian-2-hybrid system.** The final system in which the UL102 gene was expressed was the Clontech mammalian-2-hybrid system, which was used to investigate the putative interaction between the UL102 and UL54 proteins, when expressed in mammalian cells. This two-hybrid system consists of two vectors; pVP16, which expresses the first protein as a fusion with the activation domain (AD) of the HSV VP16 transcriptional activator and pM, which expresses the second cloned protein as a fusion with the DNA-binding domain (DNA-BD) from the yeast GAL-4 protein. Both UL102 and UL54 proteins were cloned into each of the vectors so that each protein could be expressed as a fusion with either the AD or BD (Figure 4.3).

4.1.1 Truncated UL102 proteins

Cloning regions of the UL102 gene in order to express truncated UL102 proteins was necessary to investigate which regions are involved in mediating putative functions, such as interactions with other replication proteins or binding to nucleic acids. As the main focus of the project was investigating the putative interaction with UL54, the C-terminal half of UL102 was of particular interest, as the multiple interactions between HSV replication proteins characterised to date are predominantly mediated by C-terminal regions within one of one of the respective proteins. Three portions of the UL102 gene encoding C-terminal regions and one portion encoding an N-terminal region of the UL102 protein were cloned. These regions are shown in Figure 4.4. The nucleotide numbers, relative to the A of the ATG start codon, and the amino acids of UL102 to which each of these regions corresponds is listed in Table 4.1 below.

UL102 Fragment Name	UL102 nt Numbers (inclusive)	UL102 aa Numbers	Total Number of aa's	Nomenclature of encoded protein	Predicted Mw of GST-Fusion (kDa)
1635 bp	1-1635	1-545	545	545N	86
993 bp	1630-2622*	543-873	330	330C	62.3
873 bp	1750-2622*	583-873	290	290C	58.9
495 bp	2128-2622*	709-873	164	164C	44

Table 4.1 Summary of the UL102 gene fragments cloned for expression as GST-fusion proteins (These numbers include the UL102 stop codon nucleotides, which do not encode an amino acid residue) nt=nucleotides.



pM/pVP16 MCS

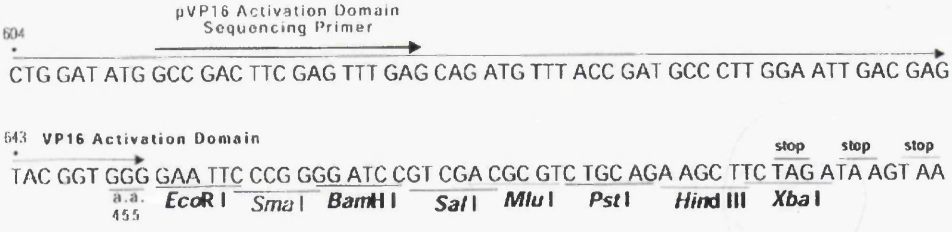


Figure 4.3 Maps of Clontech Mammalian-2-Hybrid system vectors; pM and pVP16

Schematic diagrams of the pVP16 and pM vectors from the Clontech mammalian-2-hybrid system are shown. The positions and orientations of the promoter, SV40 poly (A), and fusion protein sequences are indicated. Beneath the vector map, the sequence of the MCS which is common to both vectors, is given. The locations of all the available restriction sites are given. Vector maps were reproduced from the Clontech catalogue.

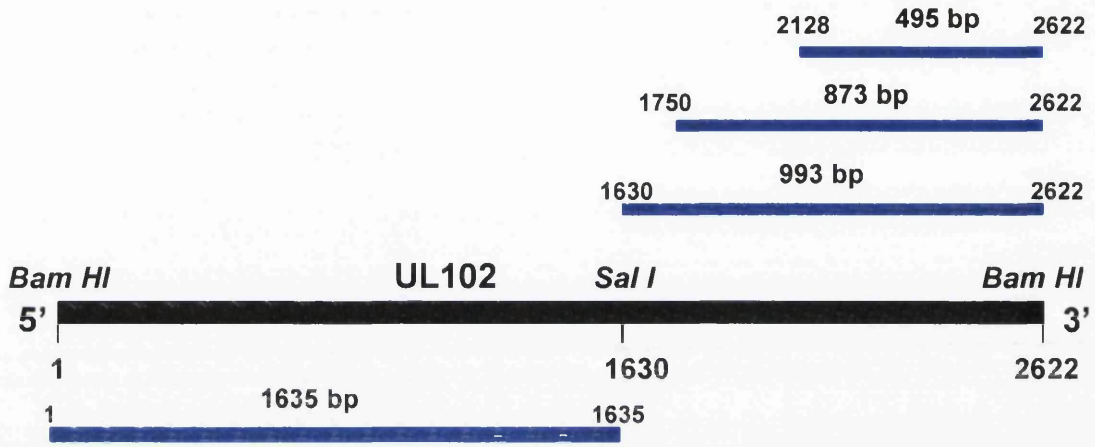


Figure 4.4 Regions of the UL102 gene cloned for expression of truncated UL102 proteins

Schematic diagram showing regions of the UL102 gene which were sub cloned in order to express truncated versions of the UL102 protein. The UL102 gene is represented as a solid bar with the 5' and 3' termini indicated. The regions of the gene which were sub-cloned are also depicted by solid bars, with the UL102 nucleotide numbers which each region spans also indicated. The *Bam* HI sites, introduced by PCR and used originally to clone the UL102 gene are indicated, as is the unique *Sal* I site at nucleotide position 1630, which was used to create the 1635 bp and 993 bp fragments.

The C-terminal regions that were cloned were chosen after consulting the 'predict-protein' analysis of UL102 (Appendix 1). This analysis predicts that an α-helical domain is contained within the C-terminal 110 aa's, and this region is preceded by a stretch of over 60 aa's which are predicted not to form a distinct structural domain. It was decided to express this predicted α-helical domain in its entirety to increase the likelihood that it would retain a more native secondary structure and form a functional domain. The final 495 nucleotides (nts) of UL102 (2128-2622 inclusive), encoding the C-terminal 164 aa's which include this predicted α-helical domain, were amplified by PCR and cloned. The 873 bp region (nts 1750-2622 inclusive), encoding UL102 aa's 583-873 and which incorporates the predicted α-helical domain and a further 126 upstream aa's, were also amplified by PCR and cloned. The 1635 and 993 bp fragments were cloned using the unique *Sal* I site at nt position 1630, which divides UL102 into two large N- and C- terminal regions. As both fragments were subsequently cloned into vectors using this *Sal* I site at the 3' or the 5' ends of the 1635 bp and 993 bp fragments, respectively, both encode the aa's specified by the *Sal* I site, and these aa's have been included in the total number of aa's encoded by each fragment. Hence, the nomenclature used to refer to these gene fragments throughout the text (1635 bp and 993 bp) also includes the nucleotides spanned by the *Sal* I site. The PCR primers and restriction sites used to clone the four UL102 gene fragments are illustrated in Figure 4.5.

Initially the UL102 fragments were cloned into pGEX vectors to express as GST-fusion proteins in bacteria, which could then be used to investigate the putative UL102-UL54 interaction by ELISA. The UL102 gene fragments could subsequently be cloned into the other expression vectors to investigate the roles of these regions in other aspects of UL102 function. The 1635 bp and 993 bp UL102 fragments were cloned into expression vectors pCMV10 and pCMV-Tag2B, respectively, for use in immuno-fluorescence studies.

The sources of the UL102 and UL54 genes for sub-cloning into different vectors were plasmid pTZ18u-102, which consists of the UL102 gene amplified by PCR using primers which specify *Bam* HI sites at the 5' and 3' ends of the gene, and plasmid PY-POL, which consists of cloned copy of the UL54 gene amplified by PCR using primers specifying Xba I sites at the 5' and 3' ends of the gene.

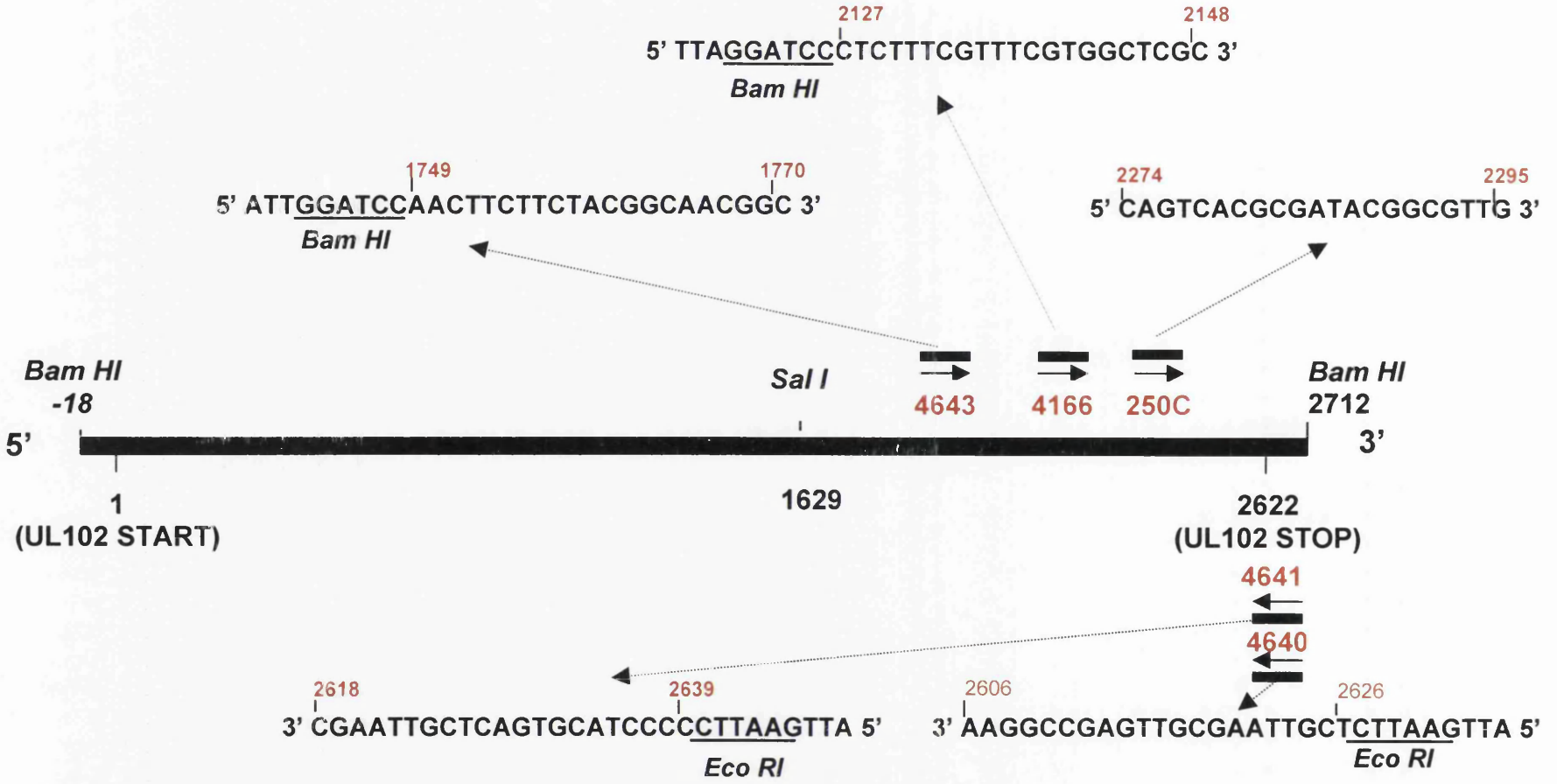


Figure 4.5 Schematic diagram showing primers used to amplify by PCR and sequence regions of the UL102 gene
Schematic diagram showing the position and orientation of primers used to generate regions of the UL102 gene cloned into pGEX vectors for the expression of truncated UL102 GST-fusion proteins in bacteria. The UL102 gene is represented by a solid bar with the start and end nucleotide numbers indicated. The position and orientation of the primers used to amplify regions of the UL102 gene and sequence the cloned fragments are indicated by solid bars either above or below the UL102 gene. The primer names are given in red text, as are the UL102 numbers which each primer spans. Where the primers incorporate restriction sites, these are underlined and named beneath the sequence.

4.2 Chapter specific methods

4.2.1 General cloning procedures

In all cases described below, the cloning of inserts into vectors was carried out using standard cloning techniques for ligation, transformation and isolation of plasmid DNA as described in sections 2.5.1.5, 2.5.1.7 and 2.5.1.8. Recombinant plasmid DNA isolated was analysed by restriction digestion and electrophoresis on a 1% agarose gel as described in sections 2.5.1.1 and 2.5.1.2.

4.2.2 Cloning of full-length UL102 and truncated UL102 gene fragments into pGEX vectors for expression as GST-fusion proteins in *E. coli*

4.2.2.1 PCR amplification of the UL102 495 bp and 873 bp fragments

The partial UL102 sequences spanning nucleotides 2128-2622 inclusive (495 bp) and 1750-2622 inclusive (873 bp) were amplified by PCR from a cloned copy of the *Hind* III restriction fragment of HCMV strain AD169. The amplification was carried out using the Clontech Advantage cDNA PCR kit, according to the manufacturer's instructions. The primers used were (Figure 4.5);

For amplification of the 495 bp fragment;

4166: 5' TTAGGATCCCTCTTTCGTTTCGTGGCTCGC 3' and

4640: 5' ATTGAATTCTCGTTAAGCGTTGAGCCGGAA 3',

For amplification of the 873 bp fragment;

4643: 5' ATTGGATCCAACTTCTTCTACGGCAACGGC 3' and

4641: 5' ATTGAATTCCCCCTACGTGACTCGTTAAGC 3'

These primers incorporate *Bam* HI and *Eco* RI sites (underlined) at the 5' and 3' ends of each fragment, respectively. Reaction mixtures were subjected to an initial denaturation step of 94°C for 1 minute, then allowed to cycle 30 times through the following sequence of temperatures; 1) template denaturation at 94°C for 30 seconds, 2) primer annealing at 50°C for 40 seconds, 3) DNA polymerisation for 4 minutes at 68°C. A final elongation step at 68°C for 3 minutes was also performed. Products were analysed on a 1% TAE EtBr agarose gel alongside DNA markers and products of the correct size were excised from the gel and purified using the BIO 101 Geneclean kit, following the manufacturer's instructions.

4.2.2.2 Cloning of the amplified 495 bp and 873 bp fragments into pUC 119

Purified 495 bp and 873 bp PCR products and pUC 119 vector DNA were cloned into the pUC 119 vector MCS as *Bam* HI-*Eco* RI inserts.

4.2.2.3 Sequencing of cloned UL102 495 bp and 873 bp fragments

Single pUC 119-495 bp and pUC 119-873 bp clones were chosen for sequencing analysis, to ensure the fidelity of each of the amplified fragments to the original UL102 clone sequence. Both the 495 bp and 873 bp inserts were sequenced using;

A) Standard pUC universal and reverse sequencing primers (NEB), which bind to regions upstream and downstream of the pUC MCS to give sequencing data across the MCS in both sense and anti-sense directions.
B) An internal primer, 250C, corresponding to UL102 nts 2293-2313 (Figure 4.5).
C) For the larger 809 bp insert, an additional internal primer corresponding to UL102 nts 2153-2173.

Sequencing reactions consisted of 3μg of mini-prep plasmid DNA, 10 pmoles of primer and 4μl of ABI sequencing reaction buffer in a total volume of 10μl and were carried out in triplicate (Sequencing facility in the Institute of Virology). An ABI automated sequencer was used for double stranded sequencing of the DNA, using the dideoxy method of Sanger, (1977). Triplicate sequences obtained using each primer were compared to generate a consensus sequence. Consensus sequences derived from each of the primers were then compared with that of the original UL102 clone using the FASTA programme (Wisconsin package version 9.1, Genetic Computer Group (GCG) software, Madison, Wisc.).

4.2.2.4 Sub-cloning of the 495 bp and 873 bp fragments into the pGEX-4T2 vector

Following confirmation that both the cloned 495 bp and 873 bp fragments contained no nucleotide changes from the original UL102 sequence, they were excised as *Bam* HI-*Eco* RI fragments from the pUC 119-495 bp and pUC 119-873 bp plasmids and cloned into the MCS of vector pGEX-4T2 as *Bam* HI-*Eco* RI inserts.

4.2.2.5 Cloning of the UL102 1635 bp and 993 bp fragments into vectors pGEX-4T2 and pGEX-4T3, respectively

The 1635 bp UL102 fragment was excised from plasmid pTZ18u-102 by digesting with *Bam* HI and *Sal* I, which cuts the *Bam* HI site upstream of the UL102 start codon and the *Sal* I site at UL102 nt position 1630, and cloned into the MCS of vector pGEX-4T2 as a *Bam* HI/ *Sal* I insert. The 993 bp UL102 fragment was excised from the MCS of plasmid pTZ18u-102 by digesting with *Sal* I, which cuts UL102 at nt position 1630 and the MCS of pTZ18u-102 downstream of the UL102 stop codon, and cloned into the MCS of vector pGEX-4T3.

4.2.2.6 Cloning of the complete UL102 gene into the pGEX-2T vector

The whole UL102 gene was excised from plasmid pTZ18u-UL102 by digestion using *Bam* HI and cloned into the *Bam* HI site of the pGEX-2T vector.

4.2.3 Expression of UL102 GST-fuslon proteins in *E. coli*

Colonies of *E. coli DH5*α cells, containing the plasmid clones of full-length and truncated UL102 GST-fusion proteins; pGEX 1635, pGEX 993, pGEX 873 and pGEX 495, were picked and grown overnight in 5 ml 2YT medium containing ampicillin (2YTA). The following day, 100 μl of the overnight culture was transferred to 10 ml fresh 2YTA medium and grown until the absorbance at 600nm (A_{600}) of each culture reached between 0.6 and 0.8 units. Expression of the GST-fusion proteins was induced by adding IPTG to a final concentration of 1mM to each culture and growing for a further 1-2 hours at 37°C, with shaking. The final A_{600} reading of each culture at 600nm was taken, then 1.5 ml of each culture was transferred to a microfuge tube and centrifuged for 10 seconds at 12000 rpm (microfuge) and the supernatant discarded. Cell pellets were resuspended in a volume of PBS equal to the A_{600} value multiplied by ten. Resuspended cells were lysed by sonication on ice until the suspension became clear and then centrifuged at 12000 rpm (microfuge) to pellet insoluble material. 50% glutathione sepharose-B beads (20μl/sample) were added to the supernatant and mixed for 10 minutes. After centrifugation to pellet the beads, the supernatant was removed and the beads were washed three times in 100μl of PBS. After the final wash, the pelleted beads were resuspended in 10μl of Glutathione elution buffer and mixed for 10 minutes. The beads were centrifuged at 12000 rpm briefly and the supernatant, containing eluted proteins, was retained for SDS-PAGE analysis.

4.2.3.1 Experiments to increase the solubility of the UL102 GST-fusion proteins

UL102 GST-fusion proteins were expressed and purified as described in section 4.2.3, with the following variations in order to investigate the effect of these parameters on the solubility of the expressed proteins;

A) Temperature at which cultures were grown; 25°C or 37°C.
B) Concentration of IPTG used to induce expression: Final concentrations of either 0.5mM or 0.1mM were used.
C) Composition of the bacterial cell lysis buffer. Four buffers were compared: Buffer G (10% glycerol), PBS-Tween (1%), PBS-Tween (2%) and PBS/Triton-X-100 (1%).

4.2.4 Constructs used in immunofluorescence studies

4.2.4.1 Cloning of the UL102 gene into expression vector pCMV10

The entire UL102 gene was excised from plasmid pTZ18u-UL102 as a *Bam* HI fragment and cloned into the *Bam* HI site of the pCMV10 vector MCS.

4.2.4.2 Cloning of the UL102 1635 bp gene fragment into vector pCMV10

The UL102 1635 bp gene fragment was excised from plasmid pGEX-2T2-1635 by digesting with *Bam* HI and *Sal* I restriction enzymes and cloned into vector pCMV10 as a *Bam* HI/*Sal* I insert.

4.2.4.3 Cloning of the UL102 993 bp gene fragment into vector pCMV-Tag2B

The UL102 993 bp gene fragment was excised from plasmid pGEX-4T3-993 by digesting with *Sal* I and cloned into MCS of vector pCMV-Tag 2B as a *Sal* I insert.

4.2.5 Constructs used for mammalian-2-hybrid studies

4.2.5.1 Cloning of the UL102 gene into the pVP16 and pM vectors

The UL102 gene was excised from plasmid pTZ18u-UL102 using the *Eco* RI site in the MCS upstream of the UL102 start codon and the *Hind* III site in the MCS downstream of the UL102 stop codon. It was cloned into vectors pVP16 and pM as a *Eco* RI / *Hind* III insert.

4.2.5.2 Cloning of the UL54 gene into the pM and pVP16 vectors

Plasmid PY-POL was digested with *Xba* I to excise the UL54 gene. The UL54 gene was then cloned into *Xba* I digested and de-phosphorylated pM and pVP16 vectors as an *Xba* I insert.

4.3 Results

4.3.1 Cloning of UL102 fragments for expression in bacteria

4.3.1.1 Amplification of UL102 495 bp and 873 bp fragments by PCR

Figure 4.6A shows the products from the PCR reactions described in sections 4.2.2.1, following electrophoresis on a 1% agarose gel. These reactions resulted in the generation of products of 495 and 873 bp respectively. Lanes A and B contain 5μl samples (from 50μl total volume) of duplicate reactions containing the 495 bp product, whilst lanes C and D contain 5μl samples of duplicate 873 bp reaction mixtures.

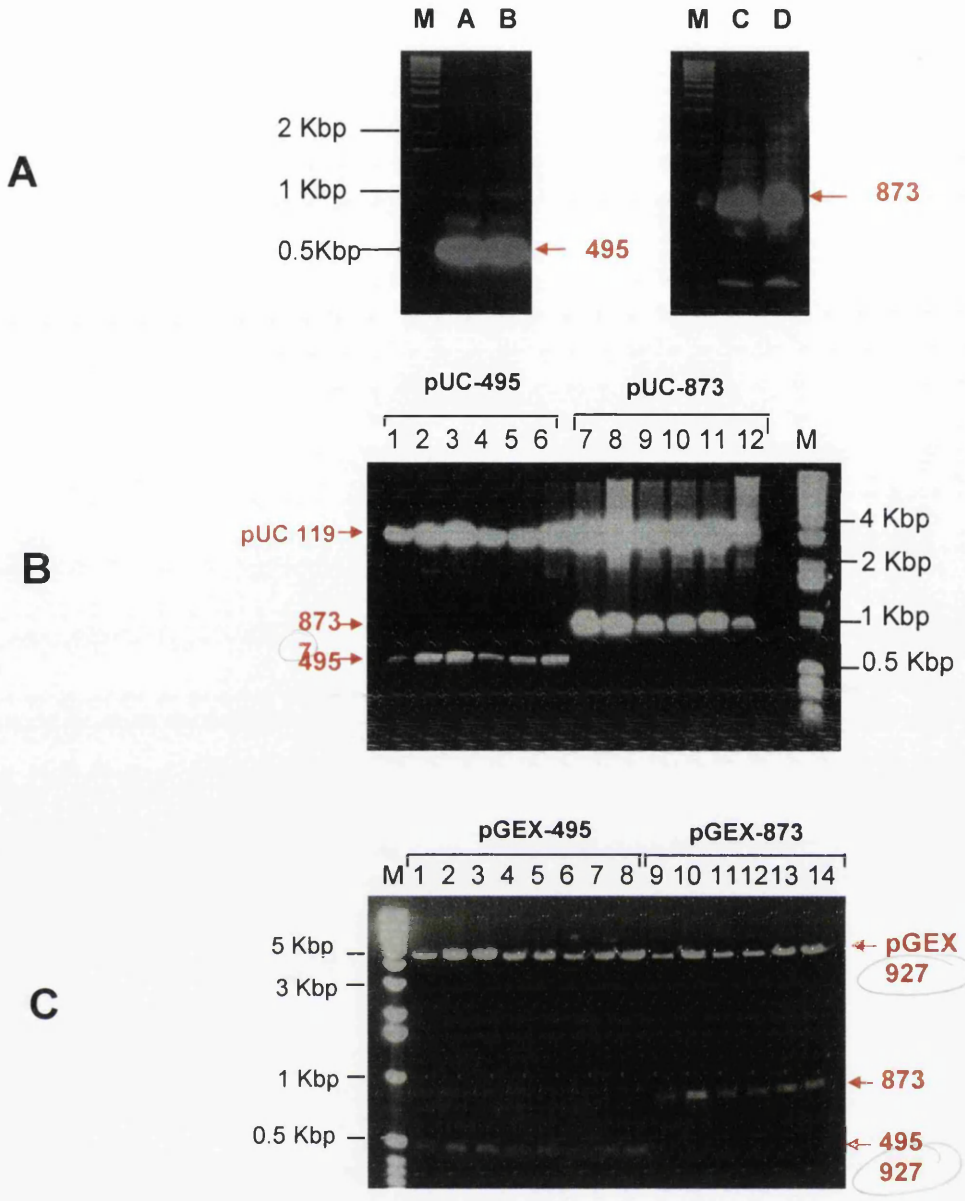


Figure 4.6 PCR amplification and cloning of UL102 495 bp and 873 bp fragments

DNA products were electrophoresed on 1% TAE agarose gels containing EtBr. 5μl of mini-prep DNA was digested in a total volume of 10μl, then each digest was mixed with 2.5μl of 5X DNA-loading buffer prior to electrophoresis:

A: Products obtained from PCR amplification of fragments of the UL102 gene corresponding to the final 495 and 873 nts using UL102 sequence-specific primers. 5μl from the total PCR reaction mixture of 50μl was mixed with 2μl of 5X DNA-loading buffer in a 10μl volume then loaded onto the gel.

B: Products formed following *Bam HI/Eco RI* restriction digestion of recombinant plasmid DNA isolated following cloning of the UL102 495 bp and 873 bp PCR products into pUC119 vector DNA.

C: Products formed following *Bam HI/Eco RI* restriction digestion of recombinant plasmid DNA isolated following cloning of the UL102 495 bp and 873 bp PCR products into pGEX-4T2 vector DNA.

Lanes marked M contain DNA molecular size markers.

4.3.1.2 Cloning of UL102 495 and 873 bp PCR products into pUC 119

Recombinant plasmid DNA isolated following the cloning of the 495 bp and 873 bp PCR products into pUC 119 was digested with *Bam* HI and *Eco* RI to confirm that the inserts were present. Both the 495 bp and 873 bp PCR products had been successfully cloned into the pUC 119 vector, as only bands corresponding to the linearised pUC 119 vector and either the 495 (Figure 4.6B, lanes 1-6) or 873 bp products (Figure 4.6B, lanes 7-12).were observed.

4.3.1.3 Sequencing of the UL102 495 bp and 873 bp cloned PCR products

Sequencing analysis of the cloned 495 bp and 873 bp UL102 fragments in plasmid pUC 119 revealed that there were no nt differences between either PCR product and the original UL102 sequence. Hence, further sub-cloning into appropriate pGEX vectors could proceed.

4.3.1.4 Cloning of UL102 495 bp and 873 bp fragments into pGEX-4T2

Recombinant plasmid DNA isolated was digested with *Bam* HI and *Eco* RI, to verify the presence of the 495 bp and 873 bp inserts and a vector band of the right size for pGEX-4T2 (4.9 kbp). Figure 4.6C shows that all 8 pGEX-495 clones contain vector and insert bands of the appropriate size, (lanes 1-8) as do all 6 pGEX-873 clones (lanes 9-14).

4.3.1.5 Cloning of UL102 1635 bp and 993 bp fragments into pGEX vectors

To verify that the 1635 and 993 bp UL102 fragments had been cloned successfully into the pGEX-4T2 and pGEX-4T3 vectors, respectively, the recombinant plasmid DNA isolated was digested with the appropriate restriction enzymes. The pGEX-1635 clones were digested with *Bam* HI and *Sal* I then resolved on a 1% agarose gel. All 12 recombinant plasmids picked contain the pGEX vector and the 1635 bp DNA bands (Figure 4.7A). The presence and orientation of the 993 bp insert was determined by digesting the pGEX-993 clones with *Xho* I. When the insert is in the correct orientation, restriction digest with *Xho* I cuts at UL102 nt position 2518, 196 bp's from the 3' end of the insert and also in the pGEX MCS, downstream of the *Sal* I site used to clone the fragment, generating two DNA bands of sizes 196 bp (plus a few additional bp corresponding to the vector sequence between the *Sal* I and *Xho* I sites and the sequence between the UL102 stop codon and the *Sal* I site) and 5 kbp (5 kbp band corresponds to the linearised pGEX vector and the remainder of the 993 bp insert). As can be seen in Figure 4.7B, clones 1 and 6 consisted of vector and insert bands of the appropriate sizes. Clones 2, 6, 10 and 11 consist of DNA bands of the correct size, but

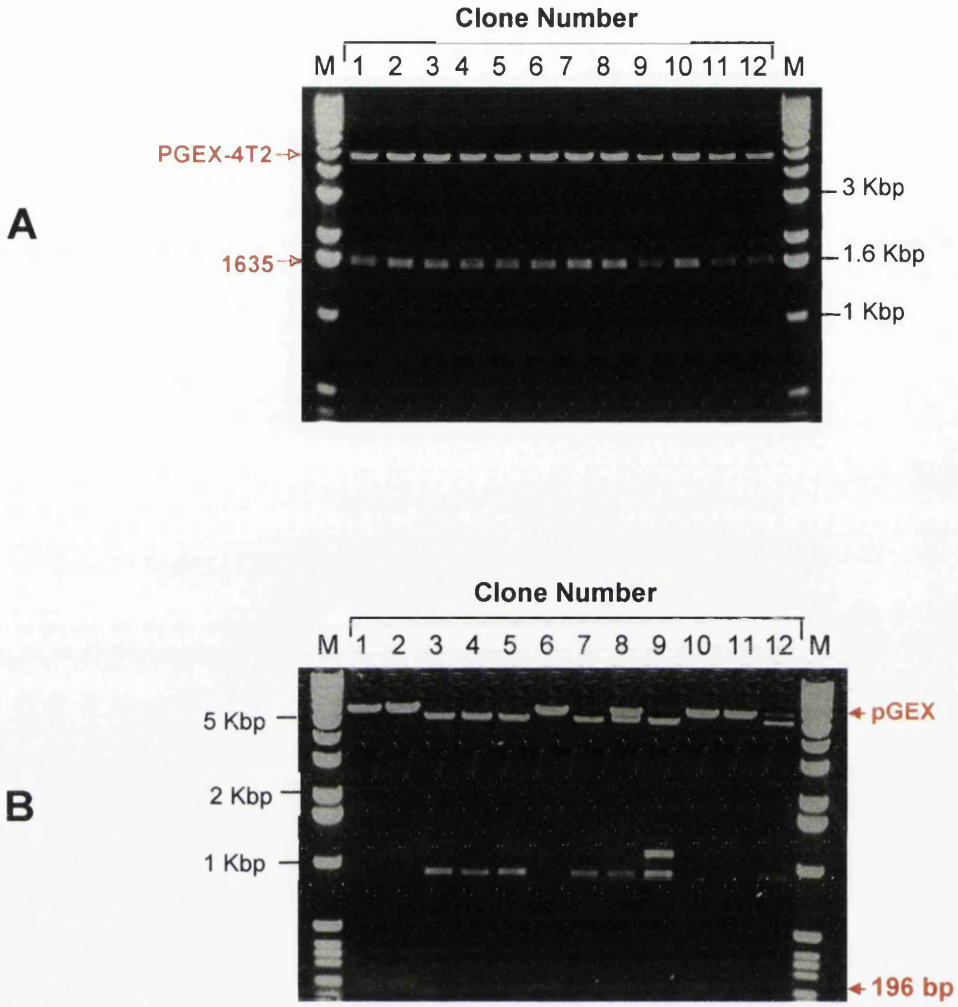


Figure 4.7 Cloning of 1635 bp and 993 bp UL102 gene fragments into pGEX vectors

The following DNA products were electrophoresed on 1% TAE agarose gels containing EtBr. 5µl of mini-prep DNA was digested in a total volume of 10µl, then each digest was mixed with 2.5µl of 5X DNA-loading buffer prior to electrophoresis.

A: Products formed following *Bam HI/Sal I* restriction digestion of recombinant plasmid DNA isolated following cloning of the UL102 1635 bp fragment into pGEX-4T2 vector DNA.

B: Products formed following *Xho I* restriction digestion of recombinant plasmid DNA isolated following cloning of the UL102 993 bp fragment into pGEX-4T3 vector DNA.

Lanes marked M contain DNA molecular size markers.

also contain an additional band (approximately 3 kbp), which is difficult to see on the reproduced gel.

4.3.1.6 Cloning of the complete UL102 gene into the pGEX-2T vector

Recombinant plasmid DNA isolated was digested with *Sal* I and *Eco*RI then electrophoresed on a 1% agarose gel, to confirm the presence and orientation of the UL102 insert. These restriction enzymes were chosen as they allow distinction of clones containing the UL102 insert in either orientation. *Sal* I cuts UL102 internally at nt position 1630 and *Eco*RI cuts pGEX-2T downstream of the *Bam* HI site in the MCS. If UL102 is in the correct orientation in pGEX-2T, digestion with *Sal* I and *Eco*RI produces two DNA fragments; one of approximately 1 Kb corresponding to the UL102 3'-terminal 993 nts and the other of 5 Kbp, corresponding to the pGEX-2T vector and the remainder of the UL102 gene. Figure 4.8 shows that all the clones isolated, with the exception of clones 1, 8, 9, and 16, generate fragments corresponding to these products.

4.3.2 Expression of UL102 GST fusion-proteins in bacteria

SDS-PAGE analysis of *E. coli* extracts containing the various UL102 GST-fusion proteins was carried out to check for expression of each protein. Samples of clarified cell lysates were taken before and after the addition of IPTG to each culture. Proteins were resolved on a 7.5% polyacrylamide gel along with a sample of the final eluent from each culture following purification using Glutathione sepharose-4B (GS-4B) (Figure 4.9). For each of the UL102 GST-fusion constructs, there is a protein band in the post-induction sample which is either absent or significantly increased from the pre-induction sample. In all cases this band is of the predicted molecular weight of the expressed UL102 GST-fusion protein and the 29 kDa GST tag. Although all the UL102-GST fusion proteins clearly express in the DH5α cells, very little or none of these proteins were subsequently purified from the bacterial cell lysate using Glutathione sepharose-4B (lane 3), with the exception of the 290C-GST truncated UL102 fusion protein, where small amounts were recovered from the GS-4B purification. Further analysis of samples taken from various stages of the expression and purification process for each of the UL102 GST-fusion proteins revealed that the majority of the expressed protein was found in the insoluble fraction of the cell lysate. SDS-PAGE analysis of samples taken from expression and purification of the 330C-GST and 290C-GST fusion protein is shown in Figure 4.10. These show that the expressed protein was largely found in the insoluble fraction although a small amount of 290C-GST was found in the soluble fraction (lane 6).

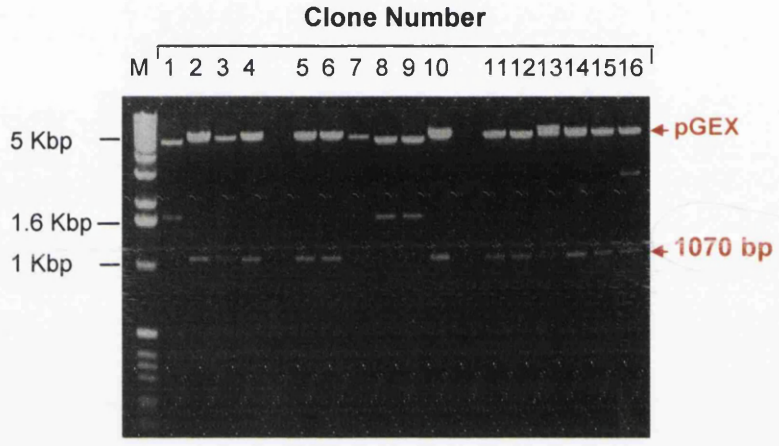


Figure 4.8 Cloning of the UL102 gene into vector pGEX-2T

Recombinant plasmid DNA isolated following cloning of the UL102 gene into pGEX-2T vector DNA was digested with *Sal* I/*Eco* RI restriction enzymes. 5μl of mini-prep DNA was digested in a total volume of 20μl, then each digest was mixed with 5μl of 5X DNA-loading buffer prior to electrophoresis on a 1% TAE agarose gel containing EtBr. Lanes marked M contains DNA molecular size markers.

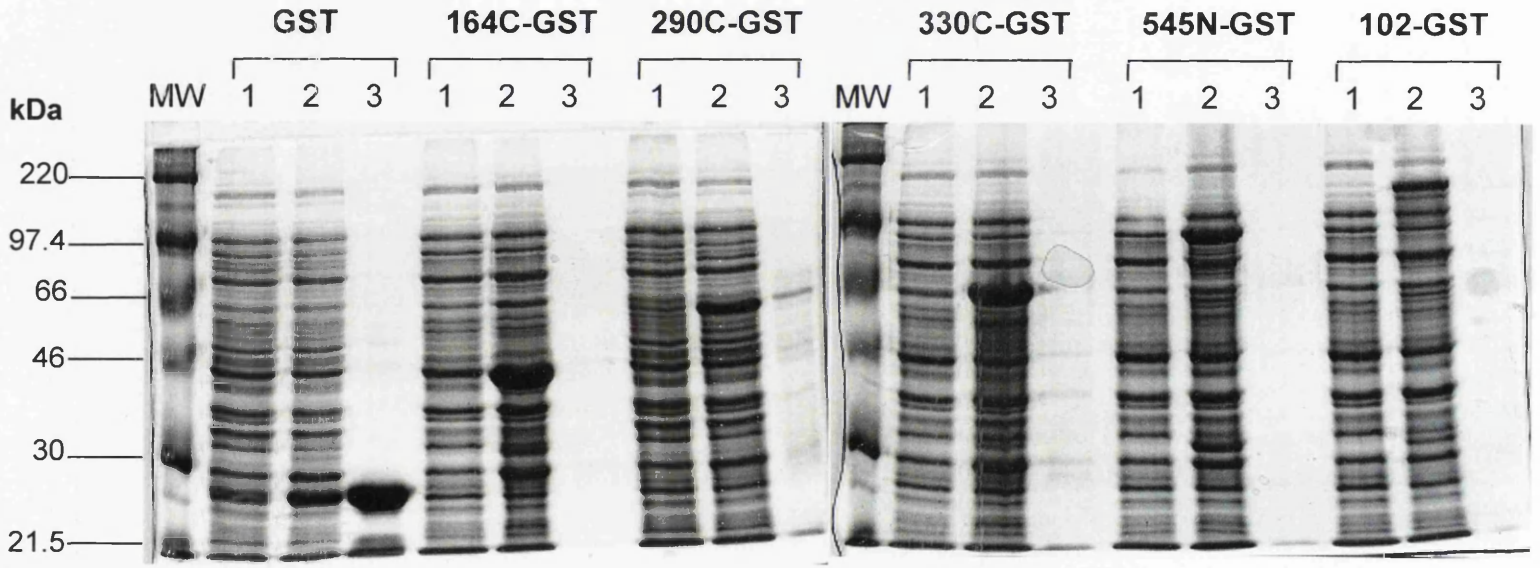


Figure 4.9 Expression of full-length and truncated UL102 GST-fusion proteins in DH5α bacteria

Bacterial cells transformed with recombinant UL102-pGEX plasmids were grown in 2YTA at 37°C, with shaking, until the A_{600} reached between 0.6 and 0.8. Samples of each culture were removed then 0.1mM IPTG was added and growth was continued for a further 1.5 hours. After removal of post-induction culture samples, the bacterial cells were lysed and the clarified cell lysates were mixed with 50% GS-4B. The GS-4B beads were washed 3 times to remove non-specifically bound proteins and then mixed with glutathione elution buffer to elute bound GST-fusion proteins. The beads were centrifuged briefly to pellet and the supernatant retained for analysis. The following samples taken for each UL102 GST-construct (as indicated) were analysed by SDS-PAGE on a 7.5% polyacrylamide gel;

1= Pre-IPTG induction bacterial cell lysate, 2= Post-IPTG induction bacterial cell lysate, 3= Eluate from GS-4B beads

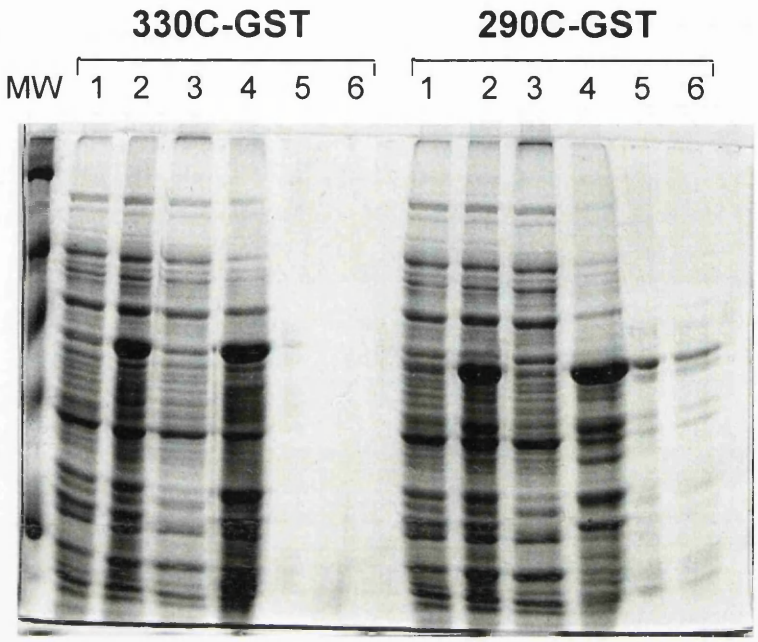


Figure 4.10 SDS-PAGE analysis of samples taken from stages in the expression and purification of UL102 330C-GST and 290C-GST fusion proteins

Bacterial cells transformed with recombinant UL102-pGEX plasmids expressing the 330C-GST and 290C-GST UL102 fusion proteins were grown in LBA at 37°C, with shaking, until the A_{600} reached between 0.6 and 0.8. Samples of each culture were removed then 0.1mM IPTG was added and growth was continued for a further 1.5 hours. After removal of post-induction culture samples, the bacterial cells were lysed by sonication and centrifuged to pellet insoluble material, which was retained for analysis. The clarified cell lysates were mixed with 50% GS-4B. The GS-4B beads were washed 3 times to remove non-specifically bound proteins and then mixed with glutathione elution buffer to elute bound GST-fusion proteins. The beads were centrifuged briefly to pellet and the supernatant and beads were retained for analysis. The following samples taken were taken for each UL102 GST-construct (as indicated), analysed by SDS-PAGE on a 7.5% polyacrylamide gel and then stained using Coomassie blue;

1= Pre-induction whole bacterial cells, 2= post-induction whole bacterial cells, 3= lysate following incubation with GS-4B, 4= Insoluble material following lysis, 5= Sample of GS-4B beads following elution of GST-fusion proteins, 6= Final eluate from GS-4B.

4.3.2.1 Varying expression and purification conditions to increase solubility of the UL102 GST-fusion proteins

To overcome the solubility problems associated with the expression of the majority of the UL102 GST-fusion proteins, various expression and purification conditions were utilised to determine their effect on the soluble yield of each of these proteins. The following parameters were investigated:

Effect of culture growth temperature: Growing the bacterial cultures at 25°C or 37°C had no effect on the solubility of the expressed proteins, as no increase in the final amount of protein eluted following purification using GS-4B was seen (Data not shown).

Effect of concentration of IPTG used to induce expression of proteins: Similarly, the final concentration of IPTG used to induce expression of the proteins made no detectable difference to the amount of purified protein isolated (Data not shown).

Effect of buffer composition in which bacterial cells were lysed: Four different buffer types were compared for their effect on the solubility of the UL102 GST-fusion proteins. Pelleted bacterial cells were resuspended, prior to lysis, in either: Buffer G, PBS-Triton-X-100 (1%), PBS-Tween (1%) or PBS-Tween (2%). Figures 4.11 and 4.12 show samples recovered from various stages of expression and purification of each fusion proteins, analysed by SDS-PAGE on 7.5% polyacrylamide gels and stained with Coomassie blue. Lanes numbered 6 in each gel contain the final eluted proteins from GS-4B. For each UL102 GST-fusion protein there are four lane 6 samples corresponding to each of the 4 lysis buffers used.

For the full-length UL102 GST and the 330C GST-fusion proteins, none of the buffer conditions used resulted in the recovery of any protein from the GS-4B. In the case of the 545N GST-fusion protein, all the buffer conditions used resulted in a small amount of purified protein being recovered. There was little difference in the amount of protein recovered between the different buffers used and in all cases this amount was small compared to the total amount of protein expressed. A very small amount of protein was present in the eluate from the 290C GST-fusion protein samples. As was found for the 545N GST-fusion, the lysis buffer used made no difference to the amount of protein recovered and this amount was a very small proportion of the total amount expressed (lane 2).

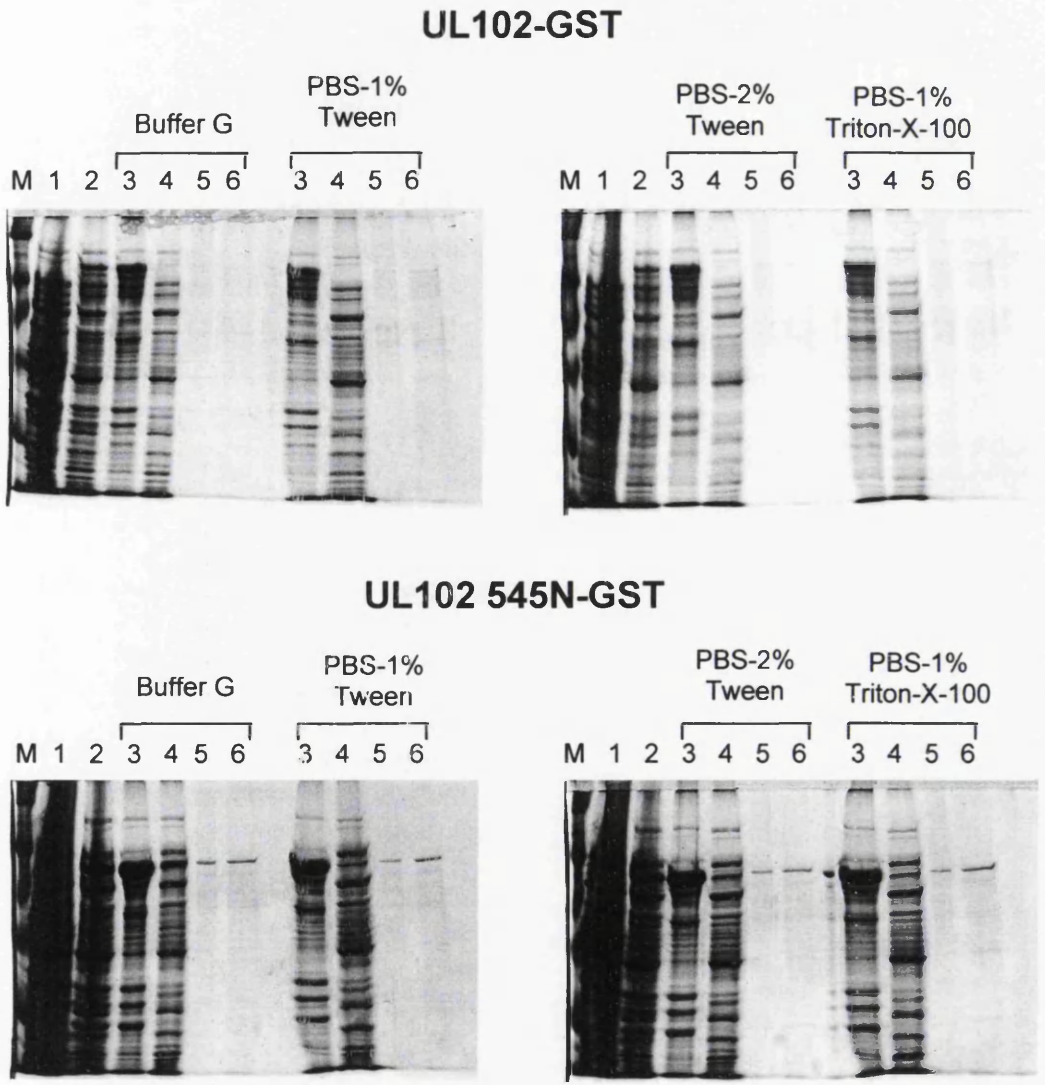


Figure 4.11 Effect of composition of lysis buffers on the solubility of full-length UL102 GST and 545N-GST fusion proteins

UL102-GST fusions proteins were expressed in DH5α bacterial cells. Whole cell samples were taken before and after the addition of 0.1mM IPTG to induce expression of the proteins. Cells were harvested by centrifugation then re-suspended in one of the following buffers;

- Buffer G
- PBS-Tween (1%)
- PBS-Tween (2%)
- PBS-Triton X 100 (1%)

Cells were then lysed by sonication on ice until the suspension cleared. Lysates were centrifuged to pellet the insoluble material. Cell lysates were then mixed with GS-4B for 10 minutes to capture the expressed GST-fusion proteins. The GS-4B beads were pelleted by brief centrifugation and washed 3x in the appropriate buffer. Bound GST-fusion proteins were eluted by addition of GEB and mixing for 10 minutes. GS-4B beads were pelleted by centrifugation and the supernatant was retained. The following samples were electrophoresed on a 7.5% polyacrylamide gel and stained using Coomassie blue: 1= Pre-induction whole bacterial cells, 2= Post-induction whole bacterial cells, 3= Insoluble pellet material, 4= supernatant following binding GS-B, 5=GS-4B beads sample, 6= Final eluate from GS-4B.

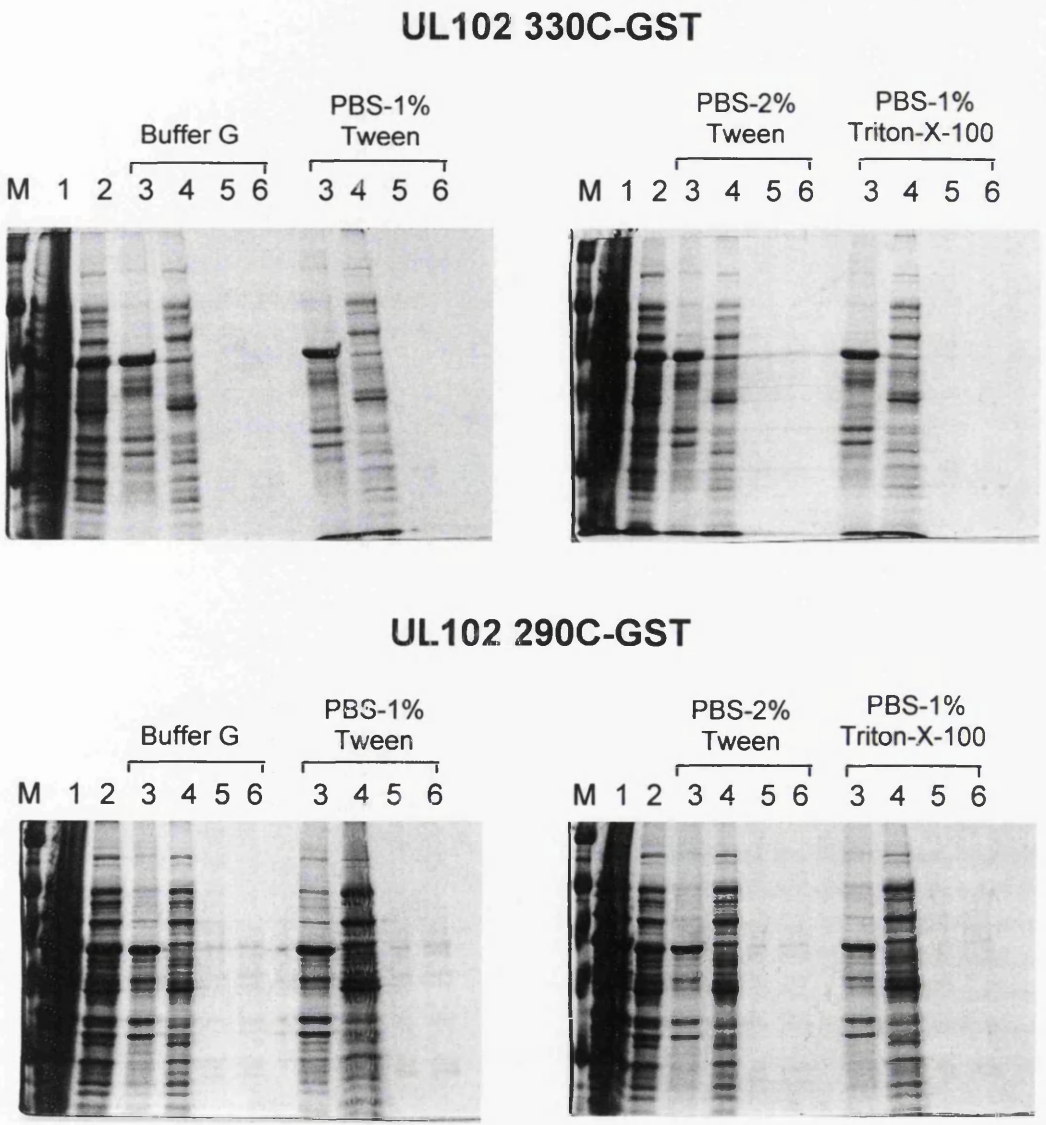


Figure 4.12 Effect of composition of lysis buffers on the solubility of UL102 330C-GST and 290C-GST fusion proteins

UL102-GST fusions proteins were expressed in DH5α bacterial cells. Whole cell samples were taken before and after the addition of 0.1mM IPTG to induce expression of the proteins. Cells were harvested by centrifugation then re-suspended in one of the following buffers;

- Buffer G
- PBS-Tween (1%)
- PBS-Tween (2%)
- PBS-Triton X 100 (1%)

Cells were then lysed by sonication on ice until the suspension cleared. Lysates were centrifuged to pellet the insoluble material. Cell lysates were then mixed with GS-4B for 10 minutes to capture the expressed GST-fusion proteins. The GS-4B beads were pelleted by brief centrifugation and washed 3x in the appropriate buffer. Bound GST-fusion proteins were eluted by addition of GEB and mixing for 10 minutes. GS-4B beads were pelleted by centrifugation and the supernatant was retained. The following samples were electrophoresed on a 7.5% polyacrylamide gel and stained using Coomassie blue: 1= Pre-induction whole bacterial cells, 2= Post-induction whole bacterial cells, 3= Insoluble pellet material, 4= supernatant following binding GS-B, 5=GS-4B beads sample, 6= Final eluate from GS-4B.

4.3.3 Constructs used in immunofluorescence

4.3.3.1 UL102 in vector pCMV10

Recombinant plasmid DNA isolated was digested with *Bam* HI to verify the presence of the UL102 insert in pCMV10. All six clones isolated generate vector and UL102 insert bands of the correct sizes (Figure 4.13A). The orientation of the UL102 insert in each plasmid was determined by digesting with *Sal* I, which cuts the UL102 gene uniquely at nt position 1630 and also the pCMV10 MCS, downstream of the UL102 stop codon. When UL102 is cloned in the correct orientation, *Sal* I digestion yields a DNA fragment of 993 bp, corresponding to the 3'-terminal 993 bp nt's of UL102, and also a 5.4 kbp fragment, consisting of the pCMV10 vector and the remainder of the UL102 gene. Clones 2, 3, 5 and 6 contain DNA bands of the correct sizes, confirming these clones consist of the UL102 gene cloned into pCMV10 in the correct orientation (Figure 4.13B).

4.3.3.2 Cloning of the UL102 1635 bp gene fragment into pCMV10

To verify the presence of the 1635 bp insert in vector pCMV10, recombinant plasmid DNA isolated was digested with *Bam* HI and *Sal* I. This should generate vector and insert DNA fragments of 3.8 kbp and 1635 bp, respectively. The results of these digests are shown in Figure 4.14A. All the clones isolated (numbers 1-10) generate vector and insert bands of the correct sizes.

4.3.3.3 Cloning of the UL102 993 bp gene fragment into pCMV-Tag 2B

To verify the presence and orientation of the 993 bp insert in the pCMV-Tag 2B vector, recombinant plasmid DNA was digested with *Bam* HI and *Eco*RV. *Bam* HI cuts the pCMV-Tag MCS upstream of the 993 bp insert and *Eco*RV cuts the 993 bp insert at nt position 679. If the insert is in the correct orientation, this yields DNA fragments of 4.3 kbp, 679 bp and 400 bp (although only 314 bp of this fragment encodes UL102, the remainder is insert sequence beyond the stop codon). Figure 4.14B shows that clone numbers 1, 4, 5, 6, 8, 9 and 12 produce DNA fragments of the correct size.

4.3.4 Constructs used in mammalian-2-hybrid studies

4.3.4.1 Cloning of UL102 into the pVP16 and pM vectors

The presence of the UL102 gene in the recombinant pVP16 and pM plasmids isolated was verified by triply digesting the DNA using *Eco* RI , *Hind* III and *Sal* I restriction enzymes. This excises the UL102 gene from the pVP16/pM vectors and cuts it at the unique UL102 *Sal* I site at nucleotide position 1630, resulting in vector DNA fragments

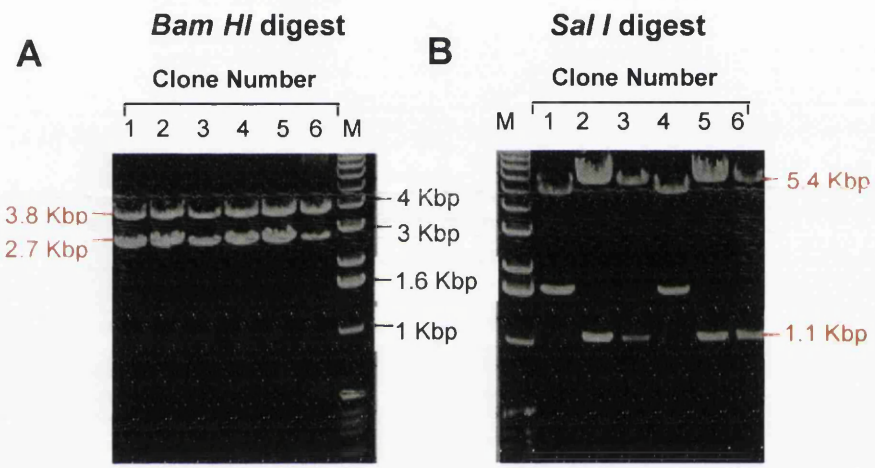


Figure 4.13 Cloning of the UL102 gene into vector pCMV10
Products formed following *Bam HI* **(A)** or *Sal I* **(B)** restriction digestion of recombinant plasmid DNA isolated following cloning of the UL102 gene into pCMV10 vector DNA. 5µl of mini-prep DNA was digested in a total volume of 10µl, then each digest was mixed with 2.5µl of 5X DNA-loading buffer prior to electrophoresis on a 1% TAE agarose gel containing EtBr. Lanes marked M contain DNA molecular size markers.

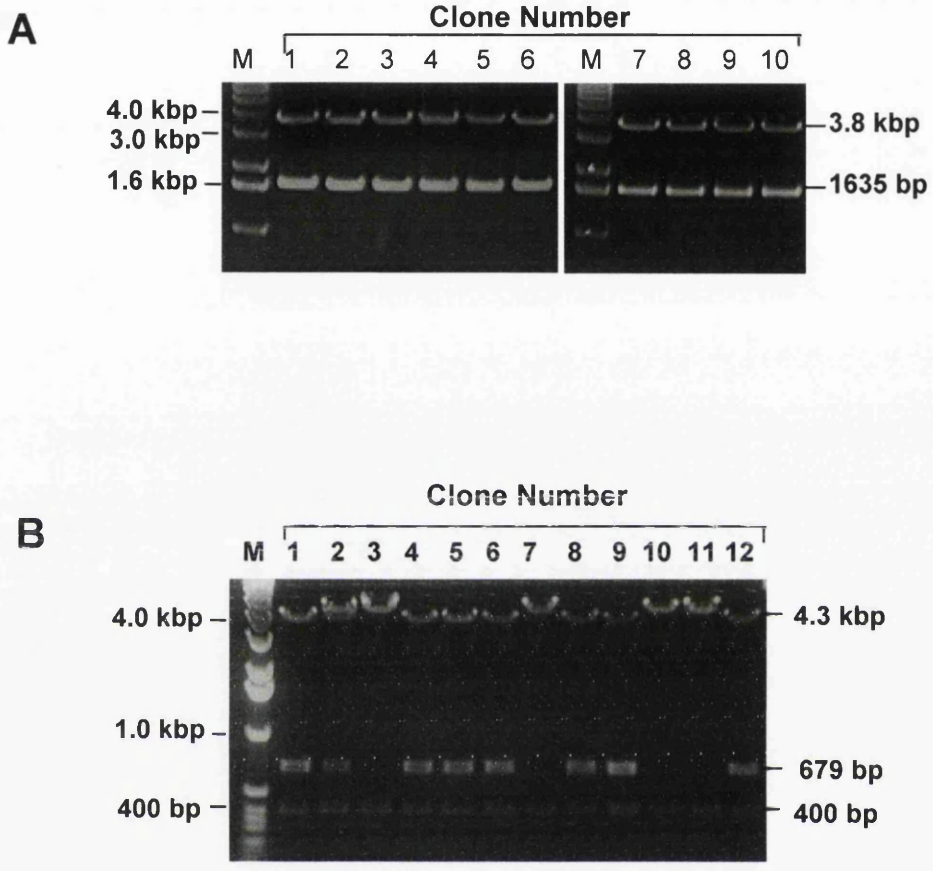


Figure 4.14 Cloning of the UL102 1635 bp fragment into vector pCMV10 and the 993 bp fragment into vector pCMV-Tag2B.
The following DNA products were electrophoresed on 1% TAE agarose gels containing EtBr. 5µl of mini-prep DNA was digested in a total volume of 10µl, then each digest was mixed with 2.5µl of 5X DNA-loading buffer prior to electrophoresis;
A: Products formed following *Bam* HI/*Sal* I restriction digestion of recombinant plasmid DNA isolated following cloning of the UL102 1635 bp fragment into vector pCMV10.
B:Products formed following *Bam* HI/*Eco* RV restriction digestion of recombinant plasmid DNA isolated following cloning of the UL102 993 bp fragment into pCMV-Tag2B vector.
Lanes marked M contain DNA molecular size markers.

of 3.3 kb (pVP16) or 3.5 kb (pM) and UL102 DNA bands of 1630 bp and 992 bp (which appear slightly larger in the gel due to the presence of vector sequences from pTZ18u-102 which flank the UL102 start and stop codons). Clones 4, 6 and 11 contain DNA bands of the correct molecular weight for UL102 cloned in the correct orientation in pVP16 vector (Fig 4.15A). Fig 4.15B shows that clones 1, 3, 4, 5 and 7 contain bands of the right size for UL102 cloned into pM in the correct orientation (also shown are the *Eco* RI/*Hind* III digests for the pM-UL102 recombinant plasmids which show the presence of the UL102 insert).

4.3.4.2 Cloning of the UL54 into the pM and pVP16 vectors

To verify the presence and orientation of the UL54 gene in the isolated recombinant plasmid DNA, it was digested using *Sal* I restriction enzyme. *Sal* I cuts the UL54 gene at a unique site at nt position 813 and also cuts the pM/pVP16 MCS upstream of the UL54 insert. If the UL54 gene is cloned in the correct orientation, this results in the generation of DNA fragments of 850 bp and 6.4 Kbp for the pM-UL102 recombinant plasmids or 850 bp and 6.2 Kbp for UL54 cloned into the pVP16 vector. Figure 4.16 shows that clones 2, 3, 4, 6, 10 and 11 contain bands of the correct molecular weight for UL54 cloned into the pM vector (Fig 4.16A), whereas clones 4, 6, 7, 9 and 12 contain the correct size bands for UL54 cloned in the correct orientation into pVP16 (Fig 4.16B) (also shown are the *Xba* I digests for the pVP16-UL54 recombinant plasmids which show the presence of the UL54 insert).

4.4 Discussion

The full-length UL102 gene, as well as two UL102 gene fragments, were successfully cloned into mammalian expression vectors pCMV10 or pCMV-Tag, allowing an investigation of the intracellular localisation of these proteins in transfected cells (Chapter 7). The successful cloning of UL102 and UL54 into pM and pVP16 vectors enabled the putative interaction between these proteins to be investigated using the mammalian-2 hybrid system (M-2-H) (see Chapter 5). If an interaction was demonstrated using this system, the 5' and 3'-truncated UL102 gene fragments could then be cloned into the M-2-H vectors to determine which regions of UL102 were involved in binding to UL54. The truncated UL102 proteins could also be expressed in the M-2-H system to determine regions of interaction with other HCMV replication proteins, such as the UL70 and UL105 proteins, in future UL102 characterisation studies. In addition, the entire UL102 gene and 5' and 3' truncated UL102 gene fragments were successfully cloned into pGEX vectors, which allowed the expression

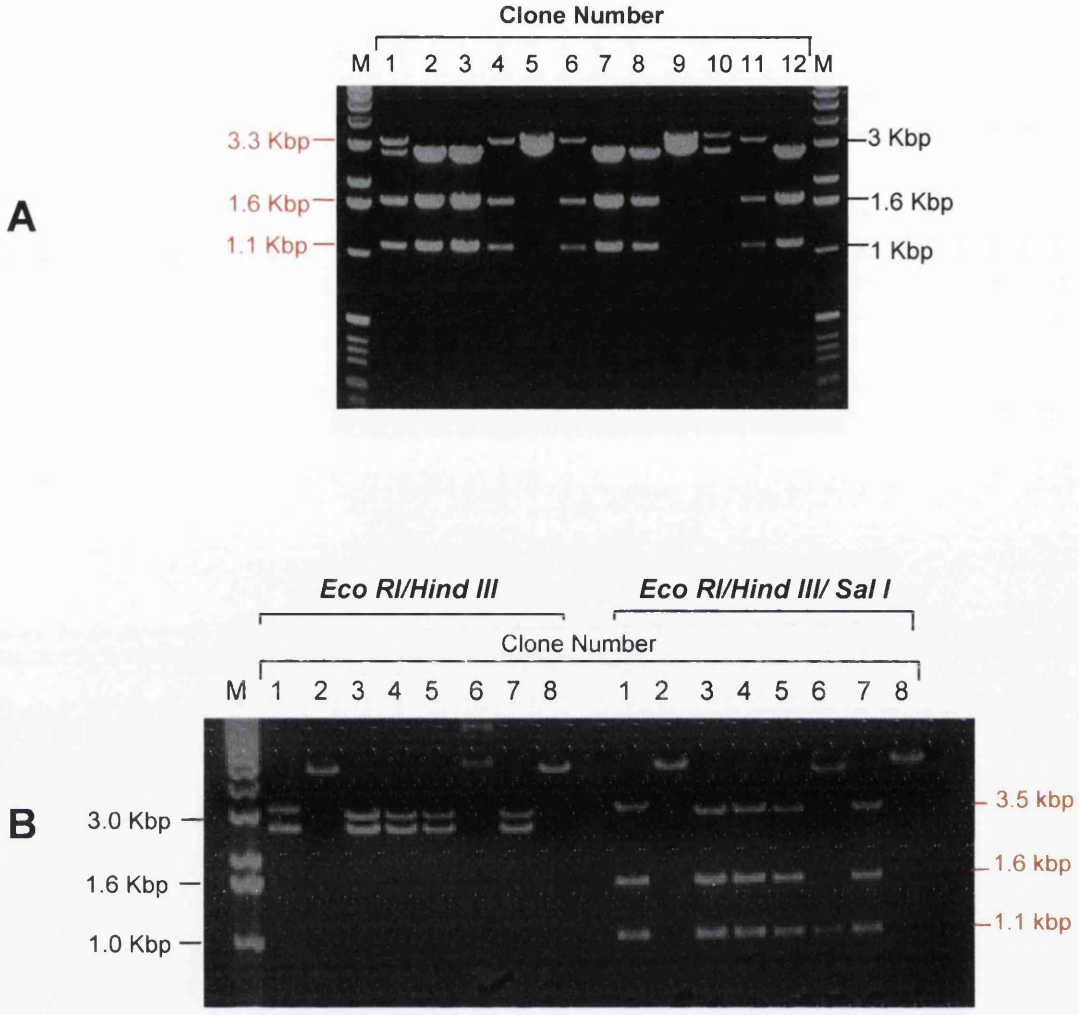


Figure 4.15 Cloning of the UL102 gene into mammalian-2-hybrid vectors
The following DNA products were electrophoresed on a 1% TAE agarose gel containing EtBr. 5µl of mini-prep DNA was digested in a total volume of 20µl. 10µl of each digest was mixed with 2.5µl of 5X DNA-loading buffer prior to electrophoresis.;
A: Products formed following *Eco* RI/*Hind* III/*Sal* I triple restriction digestion of recombinant plasmid DNA isolated following cloning of the UL102 gene into pVP16 vector DNA.
B: Products formed following either *Eco* RI/*Hind* III or *Eco* RI/*Hind* III/*Sal* I restriction digestion of recombinant plasmid DNA isolated following cloning of the UL102 gene into pM vector DNA (as indicated).
Lanes marked M contain DNA molecular size markers.

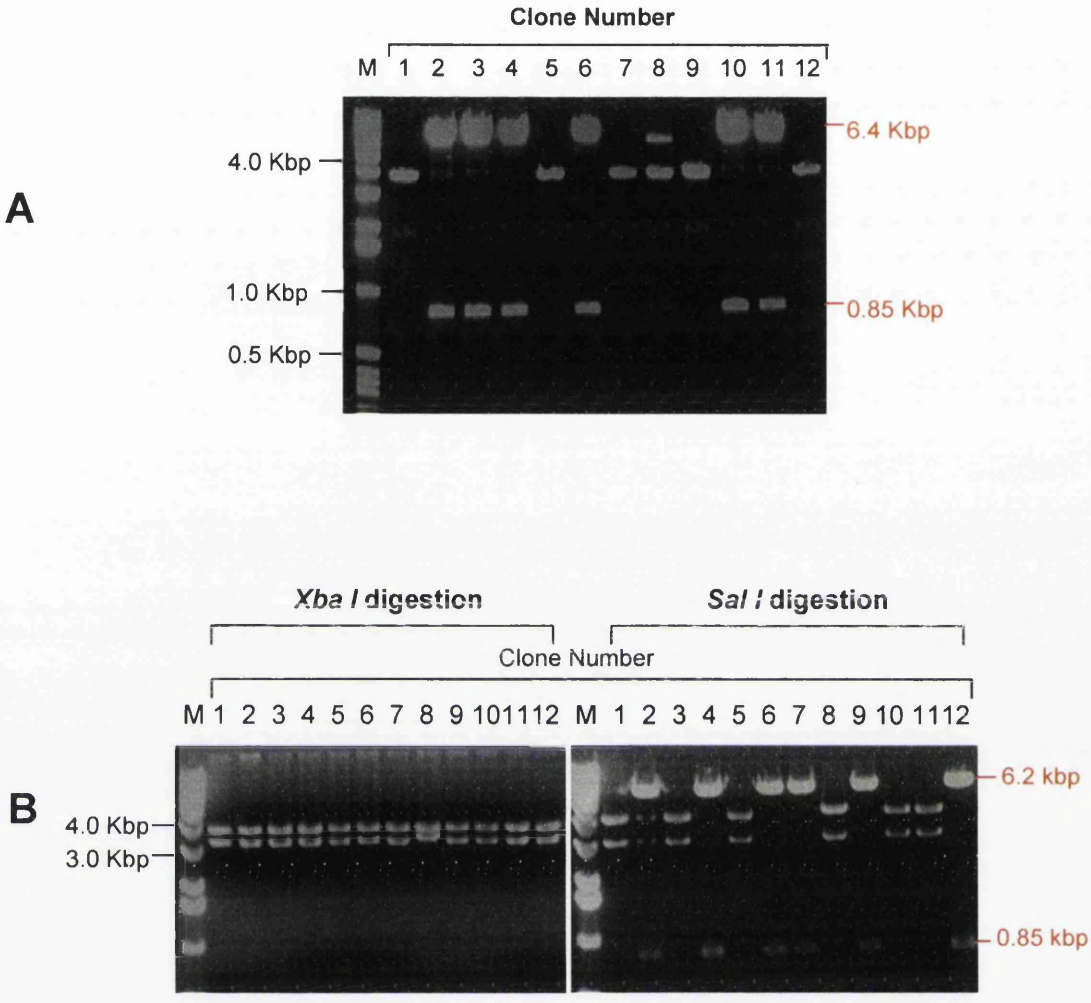


Figure 4.16 Cloning of the UL54 gene into mammalian-2-hybrid vectors
The following DNA products were electrophoresed on a 1% TAE agarose gel containing EtBr. 5μl of mini-prep DNA was digested in a total volume of 10μl then each digest was mixed with 2.5μl of 5X DNA-loading buffer prior to electrophoresis.
A:. Products formed following *Sal I* digestion of recombinant plasmid DNA isolated following cloning of the UL54 gene into pM vector DNA.
B: Products formed following *Xba I* or *Sal I* digestion of recombinant plasmid DNA isolated following cloning of the UL54 gene into pVP16 vector DNA (as indicated).
Lanes marked M contain DNA molecular size markers.

of both full-length and four truncated forms of the UL102 protein as GST-fusions in bacteria. Although these proteins were largely insoluble, they were utilised for defining regions of UL102 which are recognised by UL102 MAbs (section 3.3.2.5) and could, in principle, be used to perform UL102 structure-function studies if they were expressed in alternative systems.

Purification of the UL102 GST-fusion proteins was hindered by their lack of solubility. As only the 290C-GST protein was sufficiently soluble to allow purification using GS-4B, various aspects of the expression and purification protocol were altered to try and increase the solubility of the UL102-GST fusion proteins. The parameters investigated included the bacterial culture growth temperature and the concentration of IPTG used to induce expression. By using either lower growth temperatures or lower concentrations of IPTG, the rate of expression of the fusion proteins may be reduced so that proper folding may occur and the protein may be less likely to gather in inclusion bodies. However, neither the growth temperature or concentration of IPTG were found to have any effect on the solubility of the expressed proteins. The final parameter altered to try and increase the solubility of the fusion proteins was the composition of the buffer in which the bacterial cells were lysed. Four different buffers were compared; Buffer G, PBS-1% Tween, PBS-2% Tween and PBS-1% Triton X-100. Buffer G has been used to extract baculovirus-expressed UL102 and also HSV-1 UL8 protein from insect cells (Parry *et al.*, 1993; Marsden *et al.*, 1998). PBS formed the basis for the other buffers used, with either Tween-20 or Triton-X-100 detergents added. The presence of detergents in the lysis buffer can increase recovery of the insoluble protein by reducing the intermolecular interactions which lead to aggregation, or at higher concentrations, detergents cause denaturing of the protein, allowing it to be solubilised from inclusion bodies. For the full-length UL102 and 330C-GST fusion proteins, none of the buffers used increased the solubility of the proteins sufficiently to allow purification. However, in the case of the 545N-GST and 290C-GST proteins, all four lysis buffers increased the solubility of the proteins sufficiently such that small amounts could be recovered using GS-4B. The presence of detergents in the lysis buffers likely decreased aggregation of insoluble protein, making it accessible to the GS-4B. The amount of 545N-GST and 290C-GST proteins recovered from the GS-4B in all cases was small compared to the total amount expressed. However, if the volumes of cultures were to be increased, then this would allow purification of small amounts of protein.

It is possible that altering other conditions during expression and purification of the full-

length UL102 and 330C-GST fusion proteins might increase their solubility so that they could be purified. Other parameters that could be investigated include the timing of induction, the period of induction or aeration levels of the cultures. Isolation of the protein from inclusion bodies could be carried out by denaturation of the aggregated protein in common denaturants, such as 4-8 M urea or 4-8 M guanidium hydrochloride, followed by attempted refolding by the removal of the denaturant by dilution or dialysis. However, extensive denaturation caused by such denaturants would require that the proteins would need to be properly refolded to regain function.

A lack of solubility of recombinant expressed HCMV replication proteins, including UL102, has been reported by other workers (McCue & Anders, 1998). Owing to the poor solubility of the HCMV helicase-primase proteins when expressed using recombinant baculovirus, they have subsequently utilised the Semliki Forest virus expression system to express these proteins and have reported that their solubility is improved using this system.

Sufficient constructs were obtained from the work described in this chapter to allow most of the objectives of my project to be pursued.

Chapter 5

Investigation of the putative interaction between UL102 and UL54

5.1 Introduction

In HSV-1, the UL8 protein interacts specifically with UL30, the DNA polymerase catalytic subunit (Marsden *et al.*, 1997). An interaction has also been reported between BBLF2/3 and BALF5, the EBV counterparts of HSV-1 UL8 and UL30 (Fujii *et al.,* 2000). These findings suggest that this interaction may be conserved amongst the herpesviruses. The aim was to establish whether a specific interaction also occurs between the homologous proteins in HCMV, UL102 and UL54 and if so, to establish which regions of UL102 were involved in the interaction. Three different methods were used to investigate the putative UL102-UL54 interaction:

A: ELISA interaction assay

An ELISA-based assay was developed to investigate the UL102-UL54 interaction. This method has been used previously to demonstrate interactions between the HSV replication proteins UL8 and UL30 (Marsden *et al.*, 1997) and UL30 and UL42 (Marsden *et al.*, 1994). The assay utilised purified UL102 and UL54 proteins and either UL102- or UL54-specific MAbs. UL54 was immobilised onto microtitre plate wells. Following a blocking step, UL102 was added to the wells and incubated at 37°C. Binding between UL102 and UL54 was detected by adding a UL102-specific MAb. Formation of antigen-antibody complexes was visualised using a peroxidase-coupled α-mouse antibody, which catalyses a colour-change reaction when a chromogenic substrate is added. The colour change of the added substrate in each well was then measured using a microtitre plate reader. Extensive washing to remove unbound proteins and components was carried out in between each stage of the protocol. Hence, a signal is only produced where binding between the two proteins and all the subsequently added components has occurred (Figure 5.1). All 51 UL102-specific MAbs, described in Chapter 3, were screened in this assay for their ability to detect an interaction between UL54 and UL102. In addition, 8 UL54-specific MAbs were tested for their ability to detect UL54 bound to UL102 by performing the assay with the proteins added in the reverse order.

B: Co-immunoprecipitation experiments

Co-immunoprecipitation is a widely used technique for demonstrating protein-protein interactions. Extracts from insect cells containing radiolabelled UL102 and UL54 were mixed with either UL102- or UL54-specific MAbs. The MAb-antigen complexes formed were captured on Protein-A-sepharose, analysed by SDS-PAGE and visualised by autoradiography. Twelve UL102 IP-positive MAbs (section 3.3.2.3) were screened for their ability to co-precipitate UL54 along with their cognate antigen, UL102. In addition,

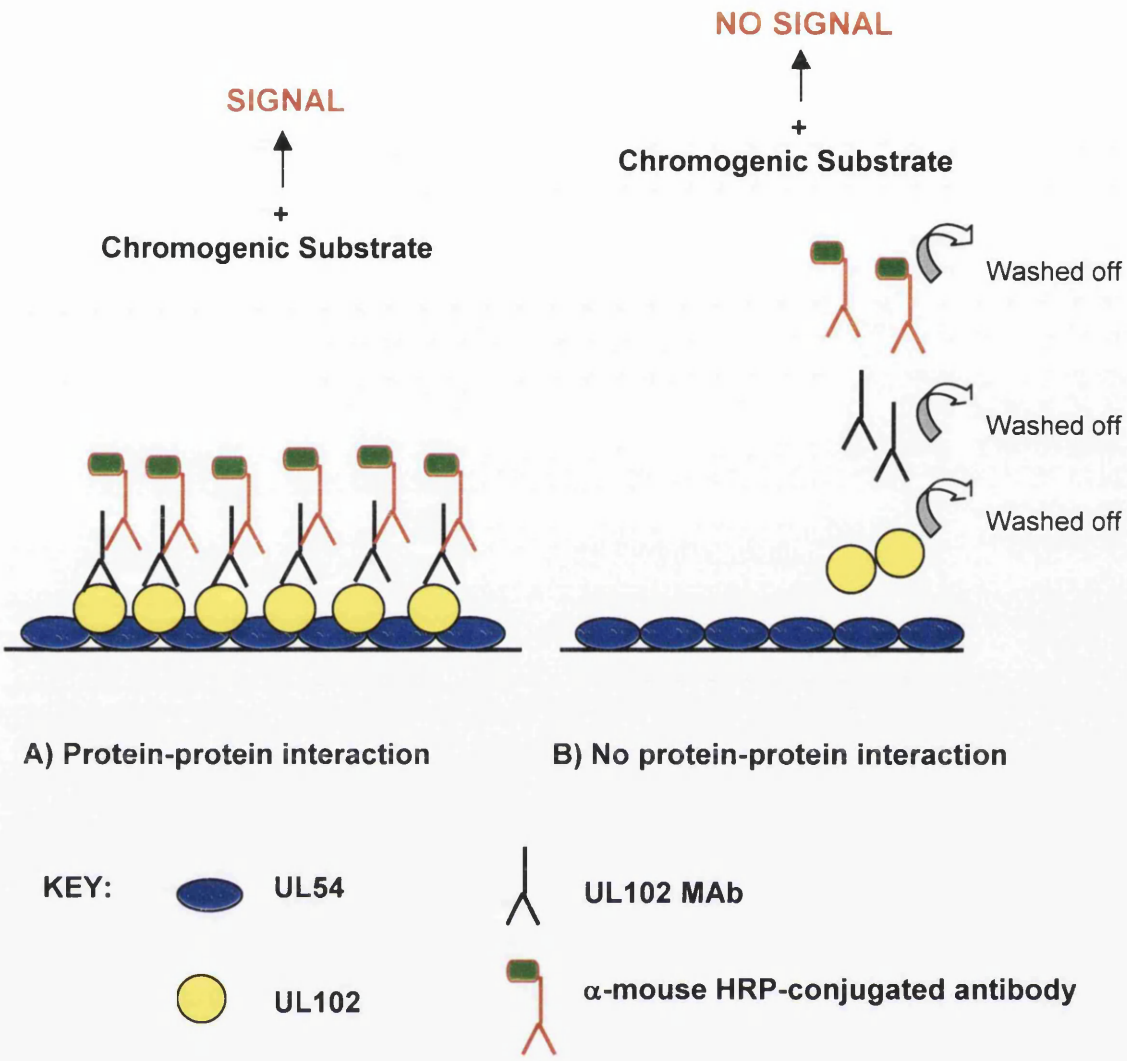


Figure 5.1 UL102-UL54 Interaction ELISA
Schematic diagram illustrating the UL54-UL102 interaction ELISA. **Fig 1A** depicts the complexes formed if the proteins bind to each other. If UL102 interacts with UL54, it will be subsequently be bound by UL102-specific MAb added to the wells. Binding of the UL102-specific MAb is then detected by addition of an HRP-conjugated α-mouse antibody followed by a chromogenic substrate (ABTS-peroxidase). The colour change of the substrate in each well is then measured by reading the OD (595nm) using an ELISA plate reader. The detection of the colour-change signal is therefore dependent on the binding of all the added components following immobilisation of UL54 to the wells. **Fig 1B:** If UL102 does not bind to UL54, it is lost from the wells during the washes following the incubation period. Hence, there is no binding of the subsequently added UL102-specific MAb or HRP-conjugated α-mouse antibody and these components are lost during washing of the wells. No signal can therefore be produced upon addition of the chromogenic substrate.

a panel of UL54-specific MAbs was screened to determine whether any could specifically co-precipitate UL102 as well as UL54 from cell extracts containing both proteins.

C: Mammalian-2-hybrid system

The Clontech mammalian-2-hybrid (M-2-H) system was utilised to determine whether UL102 and UL54 interact *in vivo* in mammalian cells. It is based on the same principle as yeast-2-hybrid systems, in which the two proteins of interest are expressed as fusions with the DNA-binding domain (DNA-BD) and the activation domain (AD) from two different transcription factors, respectively. If the proteins interact, the DNA-BD and AD will be brought in close enough proximity to activate transcription of the Chloramphenicol transferase (CAT) reporter gene, which is co-transfected into the cells along with the plasmids expressing the fusion proteins. Cell extracts are then assayed for CAT activity to determine whether the proteins have interacted. The system is outlined in Figure 5.2.

The pM vector is used to express one of the proteins of interest as a N-terminal fusion to the DNA-BD from the yeast GAL4 protein. The pVP16 vector creates a fusion between the N-terminus of the second protein and the AD derived from the HSV VP16 protein. The CAT gene is situated downstream of five consensus GAL4 binding sites and a promoter derived from the adenovirus E1b gene on the pG5CAT reporter vector. Vector pM3-VP16, which expresses a fusion of the GAL4 DNA-BD and the VP16 AD, was also used as a positive control plasmid.

Recombinant plasmid combinations of either pVP16-UL102 and pM-UL54 or pM-UL102 and pVP16-UL54 were transfected into Hela cells along with the pG5CAT reporter plasmid. The transfected cell extracts were then assayed for CAT activity using a conventional radioactive CAT assay.

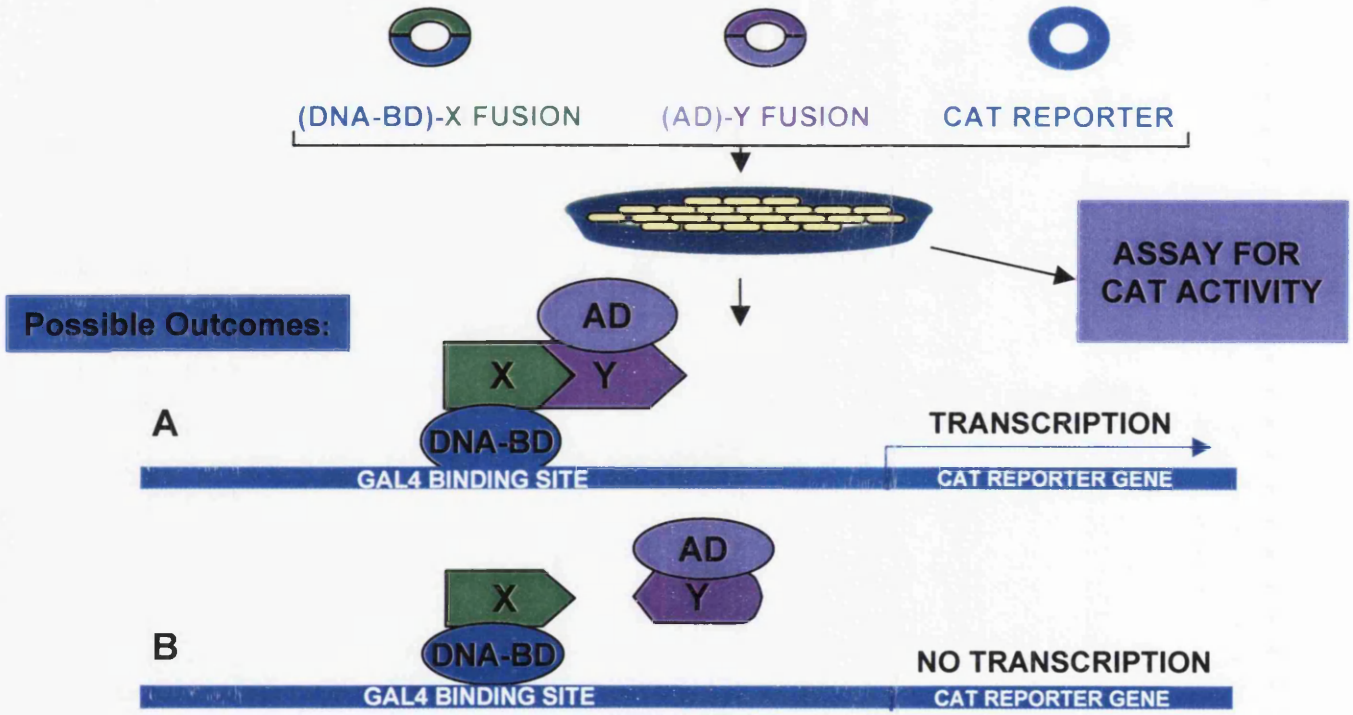


Figure 5.2 Principles of the Mammalian-2-hybrid system
Mammalian cells are transfected with plasmids expressing protein X as a fusion with the DNA-binding domain (BD) derived from the GAL4 transcription factor and protein Y as a fusion with the activation domain (AD) from the HSV VP16 transcriptional activator . A reporter plasmid consisting of the CAT reporter gene downstream of GAL4 binding sites is also transfected into the cells. If proteins X and Y interact, the AD and BD are brought sufficiently close to enable transcription of the CAT reporter gene. 48 hours post-transfection, the transfected cells are harvested, lysed and the resulting extracts are assayed for CAT activity. Detection of CAT activity therefore indicates that the two proteins have interacted **(A)**, whereas if no interaction has occurred, then no CAT activity is detected **(B)**.

5.2 Chapter Specific Methods

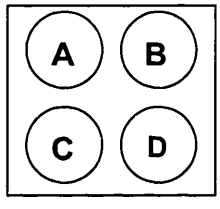
5.2.1 Investigation of the UL102-UL54 interaction by ELISA

5.2.1.1 Purification of UL102 and UL54 proteins

Recombinant UL102 and UL54 proteins were expressed in *Sf* insect cells and purified as described in sections 2.9.1 and 2.9.2 respectively. The purity of each protein was determined by SDS-PAGE analysis followed by Coomassie blue staining and then quantified using a Bio-Rad protein assay, according to the manufacturer's instructions.

5.2.1.2 UL102-UL54 interaction ELISA protocol

Groups of four wells in 96-well Immulon 1 microtitre plates were coated with protein, diluted in PBS, as indicated below using 50µl/well by incubating overnight at 37°C. The concentrations used are indicated in the figure legends.



A: UL54
B: UL54
C: PBS only
D: PBS only

Microtitre plates were washed 8 times in ELISA wash buffer (EWB) to remove any unbound protein. Any parts of the well surface not coated with the proteins indicated were then blocked using 2% BSA in PBS by incubation for 1 hour at 37°C. Wells were washed in EWB to remove the BSA/PBS. UL102 protein was added (50µl/well) to wells A and C in each group of 4 wells at the concentrations indicated in the figure legends and then incubated for 1 hour at 37°C. Wells were washed 8 times in EWB to remove unbound UL102. A UL102 MAb (50µl/well of neat hybridoma cell supernatant) was added to each group of 4 wells and incubated for 1 hour at 37°C and then washed 8 times in EWB. A 1:1000 dilution of α-mouse HRP-conjugated antibody in PBS was added to each well (50µl/well) and plates were incubated at room temperature for 1 hour. Unbound secondary antibody was removed by washing plates 8 times in EWB. Wells were tapped dry before adding 100µl/well of ABTS-peroxidase substrate. The colour change of the substrate was detected by measuring the OD (595nm) in each well after 30 minutes using a Titertek plate reader. All 51 UL102 MAbs were tested for their ability to detect UL102 bound to UL54 using this interaction ELISA. In addition, a panel of UL54-specific MAbs were tested for their ability to detect the UL102-UL54 interaction. In this case, wells were coated as described with UL102 initially and UL54 was subsequently added following the blocking step.

5.2.1.3 Control protein interaction ELISA

Immulon 1 microtitre plate wells were coated with either UL54 or control proteins alcohol dehydrogenase (AD), carbonic anhydrase (CA) or BSA diluted in PBS at the concentrations indicated in the results figures. Following coating of the microtitre wells with protein, the ELISA protocol was performed exactly as described in section 5.2.1.2. UL102 protein was added to all of the above wells at a concentration of 100ng/well in a volume of 50µl unless otherwise indicated in the results figures. UL102 MAb number 532 was used to detect bound UL102.

5.2.1.4 Modifying ELISA conditions to minimise non-specific interactions

The effect of varying ELISA protocol conditions on the detection of non-specific interactions between UL102 and control proteins was investigated. The protocol used was exactly as described in section 5.2.1.3 with the exception of varying the following two parameters;

A: Secondary protein diluent: Two types of diluent, PBS (pH 7.2) and Tris-buffer (pH 8) were compared initially and then the Tris buffer diluent was used at four different pH values (7.5, 8.0, 8.5, 9.0) for comparison.

B: Wash buffer: Two different types of wash buffer, EWB (145 mM NaCl, 7.5 mM Na_2HPO_4, 2.8 mM NaH_2PO_4, 0.05% Tween-20) and IP wash (100 mM Tris (pH 8), 10 mM NaCl, 2 mM EDTA) were compared. Subsequently, the effect of detergent (0.5% deoxycholate, 1% NP40) and salt concentration (50-500 mM) in the IP wash buffer was also investigated.

5.2.2 Investigation of the UL102-UL54 interaction using co-immunoprecipitation experiments

UL102 and UL54 proteins were expressed from recombinant baculoviruses AcNPV-UL102 and AcNPV-UL54, radiolabelled using ^{35}S-methionine and extracted from *Sf* insect cells as described in section 3.2.2.3. Extracts from doubly-infected cells or extracts from cells singly-infected with either AcNPV-UL102 or AcNPV-UL54, which had been mixed together, were then incubated with either UL102 or UL54 MAbs (neat hybridoma cell supernatant), using 100µl/extract for 2.5 hours at 4°C. The remainder of the immunoprecipitation protocol was as described in section 3.2.2.3.

5.2.3 Investigating the putative UL102-UL54 interaction using the M-2-H system

5.2.3.1 Cloning of UL102 and UL54 genes into M-2-H vectors

The UL102 and UL54 genes were cloned into each of the M-2-H vectors, pM and pVP16, as described in sections 4.2.4.1. and 4.2.4.2.

5.2.3.2 Transfection of Hela cells with M-2-H vectors

35mm dishes were seeded with 4 x 10^5 cells/plate in 2 ml medium. Combinations of M-2-H vectors were mixed with cationic liposomes and serum-free Optimem medium (Gibco-BRL) as described in section 2.8.2 to create transfection mixtures of 500μl final volume. The existing medium was removed from each plate and the cells were washed twice in PBS. Transfection mixtures were added to the plates and incubated for 3 hours at 37°C. The transfection mixture was then removed from each plate and the cells were washed once in PBS. Cells were replenished with 2mls fresh medium and incubated at 37°C for 48 hours. After 48 hours, the medium was removed and cells were washed once in PBS. Cells were scraped from the plate surface into 1 ml PBS and kept on ice. Cells were pelleted by brief centrifugation in a microfuge, the supernatant was removed and the cells washed once more in 1 ml PBS. Following another brief centrifugation the final cell pellet, representing all the cells harvested from one plate, was stored at -20°C.

5.2.3.3 Determination of CAT activity in cell lysates by radioactive CAT assay

Cell pellets were thawed and resuspended in 75 μl of 250 mM Tris buffer (pH 7.5) by vortexing. Cells were then lysed by 3 rounds of freeze-thaw treatment (5 minutes on dry ice followed by 5 minutes in a 37°C water bath). Lysed cells were centrifuged for 5 minutes at 12000 rpm (microfuge) to pellet the insoluble material and each supernatant was transferred to a fresh tube. The supernatant was then mixed with 15μl of CAT assay mix, consisting of 1μl 50 mM acetyl-co-A, 1μl of ^{14}C-chloramphenicol and 13μl of 250 mM Tris (pH 7.5), and incubated at 37°C for 2 hours. Samples were centrifuged briefly before adding 250μl of ethyl acetate and vortexing for 20 seconds. Samples were centrifuged for 5 minutes at 13000 rpm and the upper phase was transferred to a fresh tube. The liquid was evaporated under vacuum for 20 minutes in a speedvac. Pellets were resupended by vortexing in 25 μl of ethyl acetate and then spotted onto silica gel-coated TLC plates and developed for 20 minutes in a 95:5 (vol:vol) mixture of chloroform:methanol. Plates were air-dried and then exposed to Kodak X-Omat film overnight.

5.3 Results

5.3.1 Investigating the putative UL102-UL54 interaction by ELISA

5.3.1.1 UL102-UL54 interaction assay

Figure 5.3 shows the results of an interaction ELISA in which UL102 MAbs were used to detect binding between UL102 and immobilised UL54. All 51 MAbs were used in this assay, but only a selection of the results are shown. MAb numbers 59, 70, 123, 154, 185 and 707 produced a signal in the presence of both proteins, whereas no significant signal was produced in the presence of either protein individually or in the absence of any protein, as expected. MAb 547 produced a signal only marginally higher than background. The detection of a signal indicates that a UL102 protein-UL102 MAb complex has formed and hence binding between UL102 and UL54 had occurred. These findings were substantiated by the results obtained from ELISAs in which UL102 and UL54 had been added in the reverse order and where UL54 MAbs had been used to detect the binding of UL54 to immobilised UL102. The results obtained for 8 UL54-specific MAbs are given in Figure 5.4. UL54 MAb numbers 243 and 355 produce a strong signal in the presence of both proteins, although there is also a less intense signal produced in wells containing UL54 only. No significant signal is detected in wells containing either UL102 only or no protein. The generation of a signal from wells containing both proteins indicates an interaction between UL102 and UL54. MAb 43 produces a slightly stronger signal in the presence of both proteins, but not appreciably stronger than background. Those UL102- or UL54-specific MAbs that did not produce a signal from wells containing both proteins may bind to regions of UL102 and UL54 which are masked as a result of the interaction.

5.3.1.2 Control protein ELISA

To investigate whether the UL102-UL54 binding observed using the interaction ELISA was specific, the experiments were repeated as before but with the inclusion of control proteins alcohol dehydrogenase (AD), carbonic anhydrase (CA) and BSA as well as UL54. Wells were coated with serial 3-fold dilutions of UL54 or control proteins and 100ng/well of UL102 was subsequently added to each well. The results of one such ELISA are shown in Figure 5.5. A strong signal was detected from wells containing UL102 and UL54, indicating binding between the two proteins had occurred, as previously demonstrated (Figures 5.3 and 5.4). However, signals significantly stronger than background were also detected from wells containing UL102 and the control proteins AD or CA. This finding was unexpected, and although the UL102-AD and UL102-CA interactions did not result in as strong a signal as that produced by the

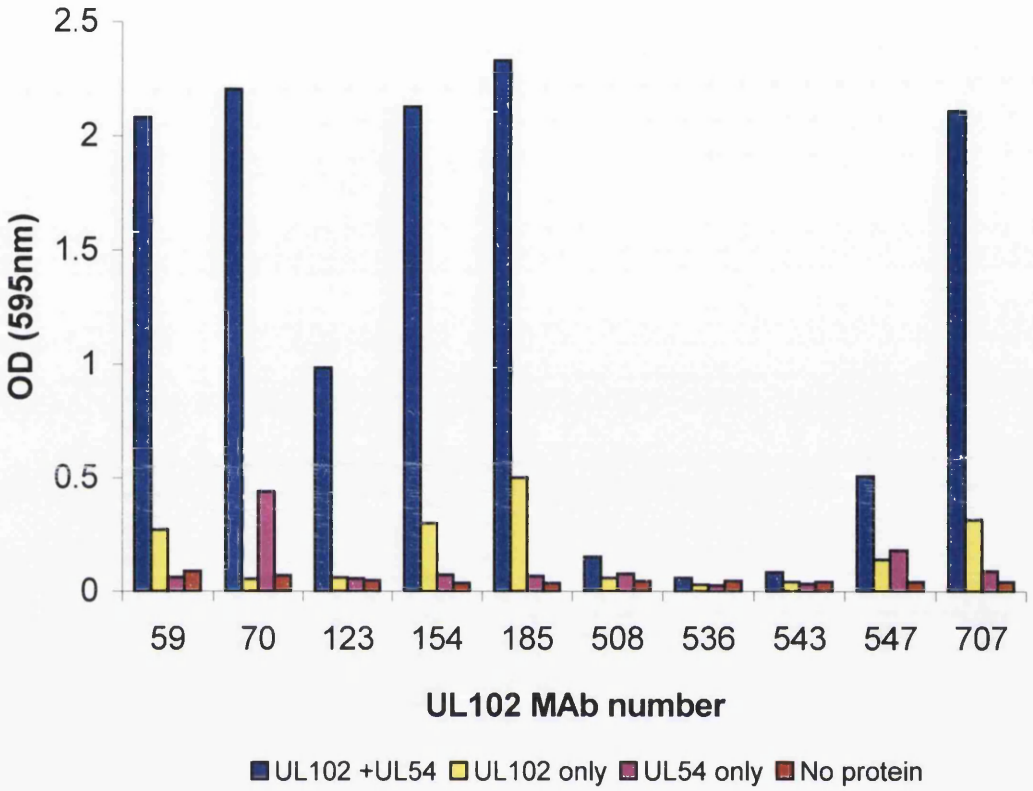


Figure 5.3 UL102-UL54 interaction ELISA using UL102-specific MAbs
Microtitre wells were coated with either 400 ng UL54 in PBS, or PBS only, then blocked for 1 hour using 2% BSA in PBS. 100 ng/well of UL102 was added to each well and incubated for 1 hour at 37°C. After washing, 50µl/well of UL102 MAbs were added and incubated at 37°C for 1 hour. Unbound MAbs were washed off and 50µl/well of a 1:1000 dilution of α-mouse-HRP was added to each well and incubated for 1 hour at room temperature. Unbound secondary antibody was washed off and 100µl of ABTS-peroxidase substrate was added to each well. The OD (595nm) of each well was measured after half an hour. For each MAb, there are four data bars, corresponding to wells containing either UL102 and UL54, UL102 only, UL54 only or no protein, as indicated in the key.

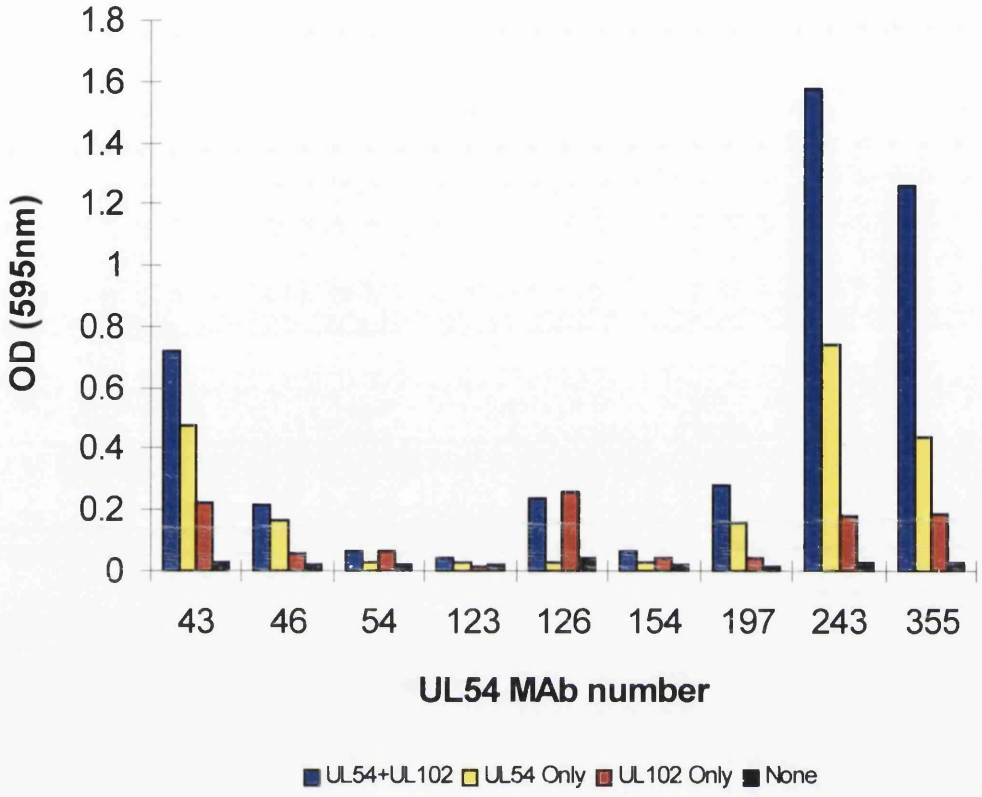


Figure 5.4 UL102-UL54 interaction ELISA using UL54-specific MAbs
Microtitre wells were coated with either 200 ng UL102, or PBS only then blocked for 1 hour using 2% BSA in PBS. 200 ng/well of UL54 was added to each well and incubated for 1 hour at 37°C. After washing, 50μl/well of UL54 MAbs were added and incubated at 37°C for 1 hour. Unbound MAbs were washed off and 50μl/well of a 1:1000 dilution of α-mouse-HRP was added to each well and incubated for 1 hour at room temperature. Unbound secondary antibody was washed off and 100μl of ABTS-peroxidase substrate was added to each well. The OD (595nm) of each well was measured after half an hour. For each MAb, there are four data bars, corresponding to wells containing either UL102 and UL54, UL54 only, UL102 only or no protein, as indicated in the key.

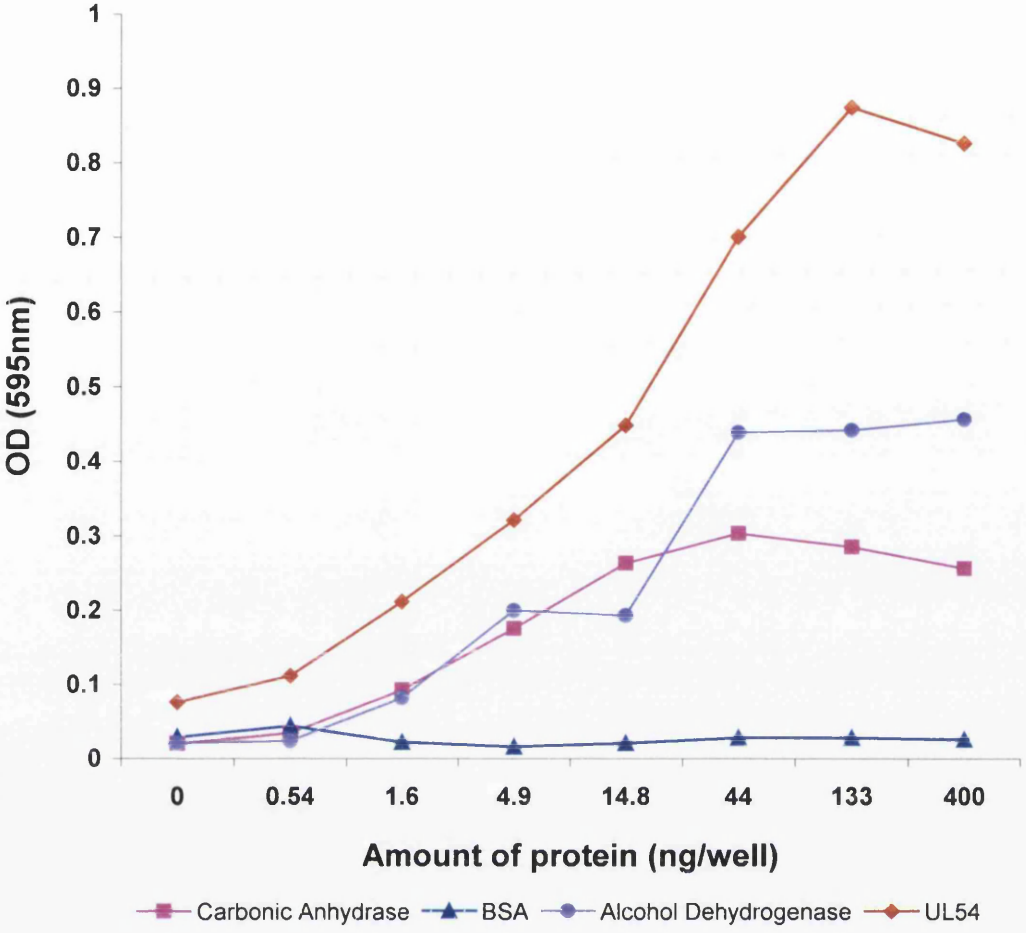


Figure 5.5 Interaction of UL102 with control proteins in ELISA
Microtitre wells were coated with either UL54, carbonic anhydrase, BSA or alcohol dehydrogenase at the indicated concentrations. After blocking any remaining binding sites with 2% BSA in PBS for 1 hour at 37°C, 100ng/well of UL102 was added and plates were incubated for 1 hour at 37°C. Unbound UL102 was washed off using EWB. UL102 MAb 532 (50µ/well) was added to each well and incubated at 37°C for 1 hour. Plates were washed again using EWB and then 50µl of α-mouse HRP-conjugated antibody was added to each well and incubated for 1 hour at room temperature. Unbound secondary antibody was washed off and plates were tapped dry. 100µl of ABTS-peroxidase substrate was added to each well. After 30 minutes, the OD (595nm) in each well was measured.

UL102-UL54 interaction, they were sufficiently strong to suggest that UL102 is capable of interacting with control proteins in a non-specific manner under the conditions used. As expected, no signal was detected from wells containing BSA and UL102, indicating no binding between these proteins had occurred.

5.3.1.3 Varying interaction ELISA parameters

Several ELISA protocol parameters were varied in order to determine their effect on detection of non-specific interactions between UL102 and control proteins. The first of these was the effect of the type of protein diluent and wash buffer used. Protein diluents PBS and Tris buffer were compared initially. The effects of the different diluents are shown in Figure 5.6. Use of PBS diluent, in this experiment, did not result in any distinction between the UL102-UL54 interaction and the UL102-AD and UL102-CA interactions. Use of Tris buffer to dilute the proteins resulted in a slight distinction between the UL102-UL54 and the UL102-AD and UL102-CA interactions, but this was no greater than the difference in strength of signals between UL102-UL54 and UL102-AD and UL102-CA originally observed (Fig 5.5).

The second parameter investigated in this experiment was the effect of the composition of the buffer used to wash the ELISA plates. The original ELISA wash buffer (EWB) was compared to IP wash buffer, which contained NP40 and deoxycholate detergents, to determine the effect of detergents on the formation of non-specific interactions (Figure 5.6). When PBS diluent was used in conjunction with IP wash, there was little difference in the strengths of signal detected from the UL102-UL54 interaction and the UL102-AD and UL102-CA control protein interactions, although the overall strength of each signal was increased. The combination of IP wash and Tris diluent led to a reduction in the overall strength of signals detected and there was a slight decrease in the relative strengths of the signals generated from the UL102-AD and UL102-CA interactions compared to that from the UL102-UL54 interaction. However, the UL102-AD and UL102-CA signals were still significantly higher than background.

The effect of changing the pH value of the Tris buffer used to dilute the proteins was also investigated. In these experiments, IP wash was used throughout. Four Tris buffer diluent pH values were compared; 7.5, 8.0, 8.5 and 9.0. At pH 7.5, the signal detected from the UL102-UL54 interaction was slightly greater than those from the UL102-AD and UL120-CA interactions, in agreement with previous findings. At pH values of 8.5 and above, all interactions, including that between UL102 and UL54, were almost completely inhibited, with the signals detected from each interaction reduced to

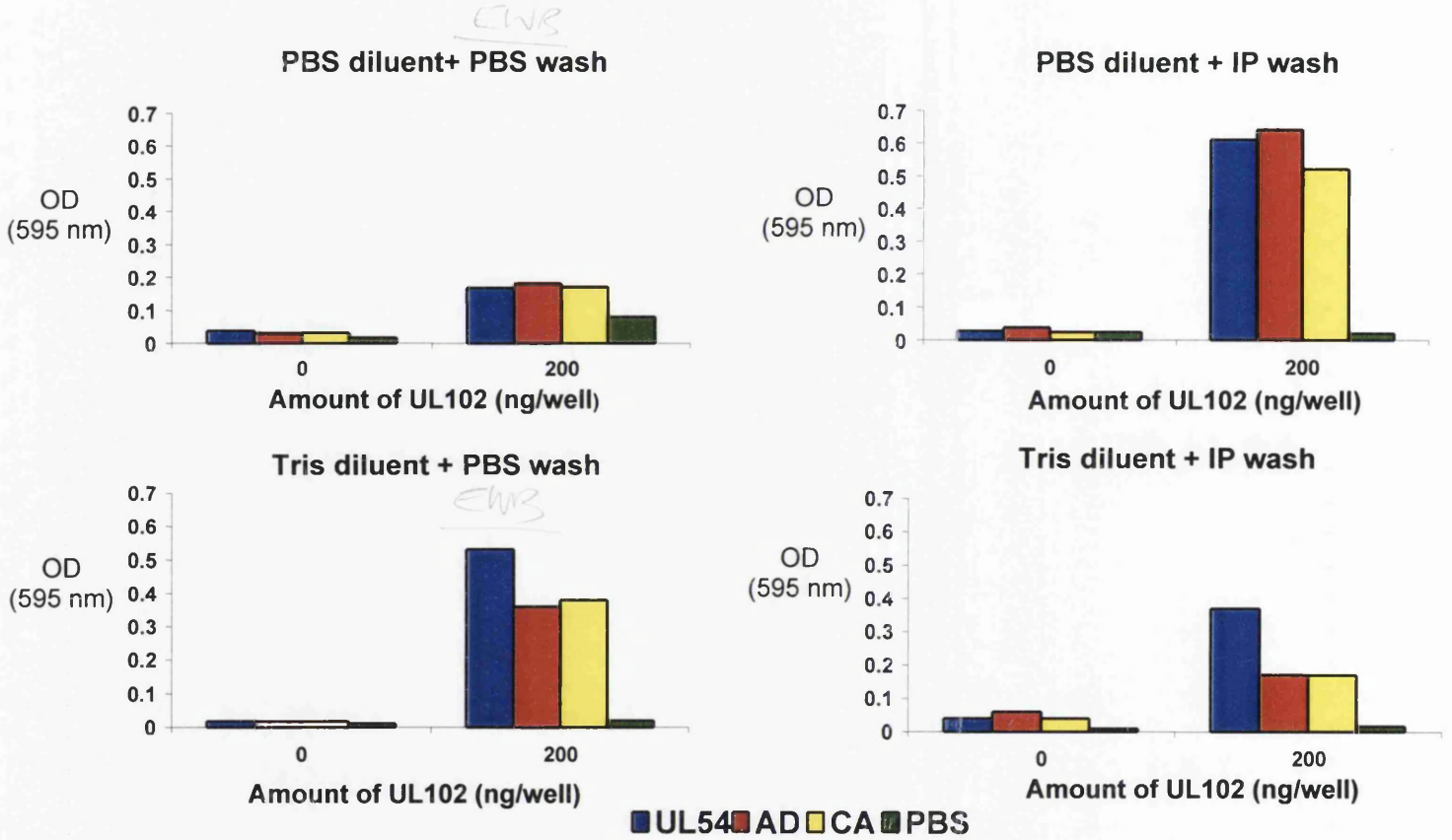


Figure 5.6 Effect of varying parameters of the UL102-UL54 interaction ELISA
Microtitre wells were coated with 200 ng of either UL54, alcohol dehydrogenase (AD), carbonic anhydrase (AD) or BSA diluted in either PBS or Tris. After a blocking step, either protein diluent only or 200 ng/well of UL102 was added and incubated for 1 hour at 37 °C. After washing in either PBS wash or IP wash, 50 µl of UL102-specific MAb 532 was added to each well and incubated at 37 °C for 1 hour. After washing, 50 µl/well of 1:1000 α-mouse-HRP conjugated antibody was added and incubated for 1 hour at room temperature. After a final wash in buffers as indicated, 100 µl/well of ABTS-peroxidase substrate was added to each well. The OD (595 nm) in each well was measured after 30 minutes using a plate reader.

background levels (data not shown). The final parameter investigated was the effect of the salt concentration of the wash buffer used during the ELISA. Four salt concentrations were compared; 50, 100, 200 and 500 mM NaCl. Proteins were diluted in Tris throughout. The wash buffer salt concentration was found to have no effect on either the overall magnitude of the signals generated or the relative strengths of the signals generated from the UL102-AD and UL102-CA interactions compared to that generated by the UL102-UL54 interaction (data not shown).

5.3.2 Co-immunoprecipitation experiments

Twelve UL102 IP-positive MAbs identified in section 3.3.2.3 were used in immune-precipitation experiments to determine whether UL54 could be specifically co-precipitated along with UL102 from insect cell extracts containing UL102 and UL54. Figure 5.7 shows SDS-PAGE analysis of the immune complexes formed after mixing the UL102 IP-positive MAbs 809 and 658 with insect cell extracts containing UL102, UL102 and UL54 or mock-infected cell extracts. Two IP-negative UL102 MAbs (numbers 70 and 546) were also included in the experiment for comparison. As expected, MAb numbers 658 and 809 did not form complexes with any proteins from the mock-infected cell extract and both precipitated UL102 from UL102-containing extracts. Both MAbs also precipitated very small amounts of UL102 from the doubly-infected cell extract, as expected. However, neither MAb co-precipitated UL54 from the cell extract containing both UL102 and UL54. IP-negative UL102 MAbs 70 and 546 did not precipitate proteins from either mock-infected, UL102-containing or UL102/UL54-containing cell extracts, as expected.

UL54 MAbs were also utilised in the immunoprecipitation experiments. The immune complexes formed when UL54 MAbs were mixed with cell extracts containing UL102, UL54, UL102 and UL54 or mock-infected extracts were analysed by SDS-PAGE. Representative results for one UL54 IP-positive and one UL54 IP-negative MAb are shown in Figure 5.8. MAb 20016 precipitated UL54 from the extracts of cells expressing UL54 only and from extracts of cells expressing UL102 and UL54 individually, which had been mixed together, as expected. No UL54 was precipitated from the extract from cells expressing both UL102 and UL54, however this can be explained by the small amount of UL54 in the cell extract initially (see 'cell extract' lane labelled 102/54). In the cell extracts containing both UL102 and UL54, only UL54 was precipitated using MAb 20016. None of the other UL54-specific MAbs tested in these immunoprecipitation experiments co-precipitated UL102 together with UL54 (data not shown from experiments carried out by Mary Murphy). UL54-specific MAb MM355 was

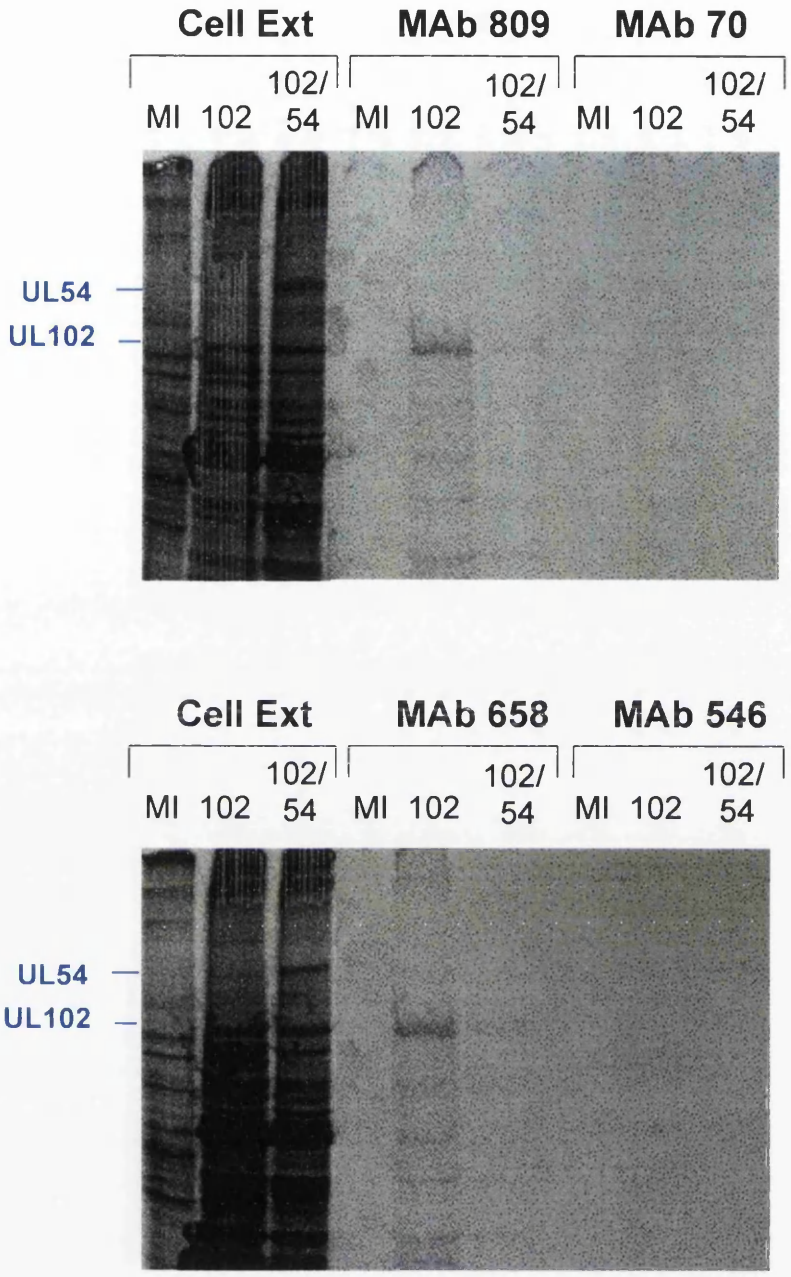


Figure 5.7 Co-immunoprecipitation experiments using UL102 MAbs
Sf cells extracts from mock-infected cells (MI) or cells expressing either UL102 only (102) or both UL102 and UL54 (102/54) were incubated with UL102 MAbs as indicated for 2.5 hours at 4°C, before adding a suspension of protein A-sepharose. After mixing for a further hour, protein A-sepharose beads were pelleted by centrifugation, washed 3 x in AE buffer then boiled in SDS-PAGE buffer. Beads were pelleted by centrifugation and the supernatant was analysed by SDS-PAGE. Gels were dried and exposed to photographic film overnight. Lanes labelled cell extract are crude insect cell extract from either mock-infected cells (MI) or cells infected with recombinant baculoviruses expressing either UL102 (102), or UL102 and UL54 (102/54).

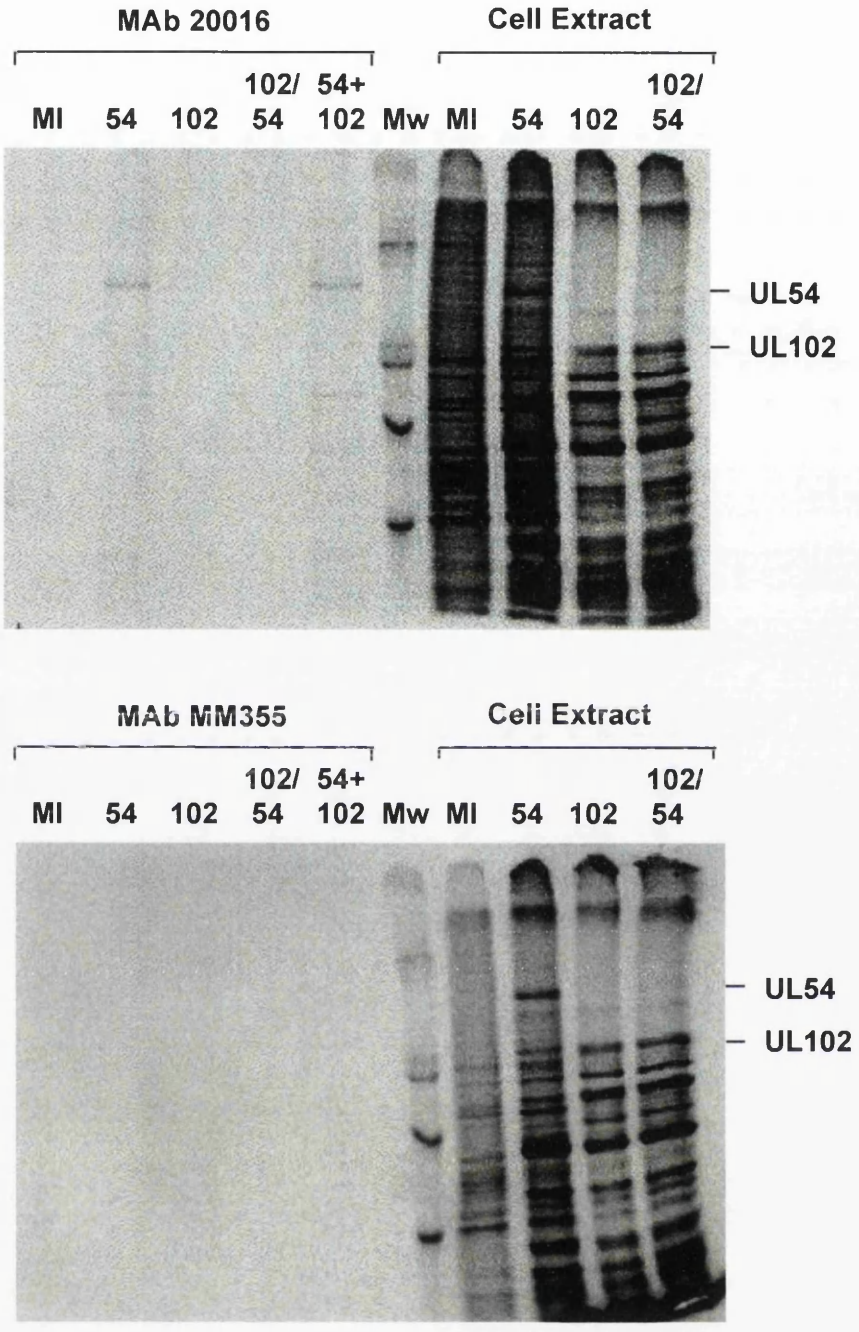


Figure 5.8 Co-immunoprecipitation experiments using UL54-specific MAbs
Sf cells extracts from cells expressing either UL102 and UL54 individually, both proteins (102/54) or extracts from cells singly expressing UL102 and UL54, which had been mixed together (102 + 54), were incubated with UL54 MAbs as indicated for 2.5 hours at 4°C, then a suspension of protein A-sepharose was added. After mixing for a further hour, protein A-sepharose beads were pelleted by centrifugation, washed 3 x in AE buffer then boiled in SDS-PAGE buffer. Beads were pelleted by centrifugation and the supernatant was analysed by SDS-PAGE. Gels were dried and exposed to photographic film overnight. The first 5 lanes on each gel show the immune complexes captured when UL54 MAbs were mixed with the cell extracts indicated. MI refers to mock-infected cell extracts. Lanes marked MW contain ^{14}C-labelled molecular weight marker. Lanes labelled cell extract are crude insect cell extract infected with recombinant baculoviruses expressing either UL102, UL54 or both proteins, as indicated.

used as a negative control MAb and did not precipitate any proteins from any of the cell extracts, as expected.

Considering that UL102 and UL54 were present in similar amounts in the mixed extracts used and that the conditions used allowed both proteins to be precipitated by their cognate MAbs, this suggests that under these conditions, UL102 and UL54 did not interact when co-expressed in insect cells, or when in solution through mixing extracts from cells expressing UL102 and UL54 individually.

5.3.3 Mammalian-2-hybrid experiments

Initial experiments were performed to ensure that the system was working in the Hela cells which were chosen to perform the mammalian-2-hybrid experiments. This involved transfecting the cells with combinations of control plasmids then performing CAT assays on the cell extracts to ensure CAT activity was only present in the appropriate cell extracts. Figure 5.9 shows the results of the CAT assay performed on cell extracts following transfection with M-2-H control plasmids. CAT mediates the transfer of acetyl onto one or both of the hydroxyl groups on the chloramphenicol molecule, resulting in either mono- or di-acetylated forms which are then resolved by thin layer chromatography. CAT activity was only detected, as expected, in extracts from cells transfected with varying amounts of the positive control plasmid, pM3-VP16 and pG5CAT (lanes 3, 4, 5, 6 and 7). No CAT activity was detected in untransfected cells or negative control extracts (lanes 1, 2 and 8), as expected. Similarly, no CAT activity was detected in cells transfected with plasmids pM-UL54, pVP16 and pG5CAT, (lanes 9 and 10), indicating that in this experiment, no interaction occurred between UL102 and UL54 when co-expressed in Hela cells.

Consistent with this result, experiments performed with varying amounts of expression vectors pM-UL54 and pVP16-UL102 also resulted in a lack of detectable CAT, indicating an inability of UL102 to interact with UL54 in this system (Figure 5.10). Lanes 1-4 are extracts from cells acting as negative CAT controls and as expected, no CAT activity was detected in these extracts. CAT activity was detected in extract from cells transfected with the positive control plasmid pM3-VP16, indicating that both the transfection and CAT assay had been successful (lane 5). Lanes 6-10 contain extracts from cells transfected with vectors pVP16-UL102, pM-UL54 and pG5CAT in varying amounts and ratios. No CAT activity was detected in any of these cell extracts. Up to 10μg of each of the plasmids had been transfected into the cells, hence it seems unlikely that the lack of activity was a consequence of low intracellular amounts of

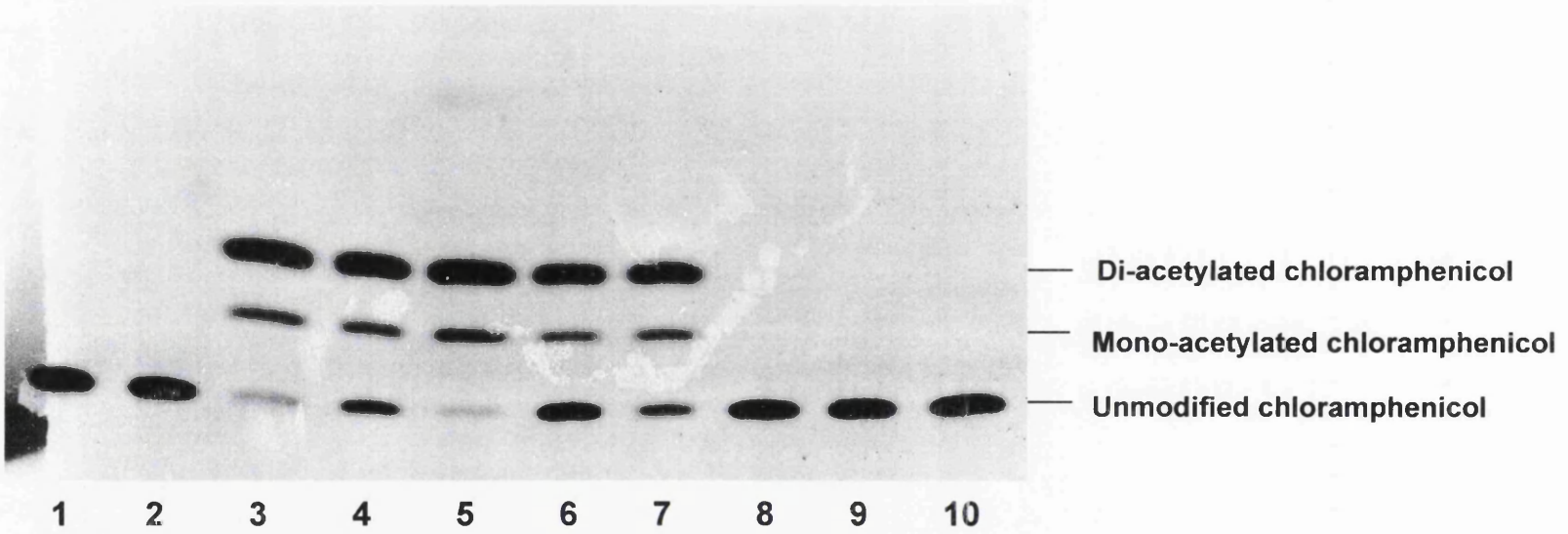


Figure 5.9 Mammalian-2-hybrid assay optimisation

Hela cells were transfected with M-2-H plasmids as indicated below. 48 hours post-transfection, cells were harvested, lysed and the clarified cell extracts were subjected to a radioactive CAT assay protocol as described in section 5.2.3.3. CAT assay samples were spotted onto silica gel-coated TLC plates and developed in a 95:5 mixture of chloroform/methanol for 20 minutes. The plate was air-dried and exposed to kodak x-omat film overnight at -70°C. Multiple bands for any given sample in the resulting autoradiograph confirm the presence of modified chloramphenicol, either in a mono- or di-acetylated form, indicating CAT enzyme activity in that sample. The contents of each lane are as follows;

1= Untransfected cells, **2**= Basal control (pM + pVP16 + pG5CAT), **3**= pM3-VP16 + pG5CAT (1μg :1μg), **4**= pM3-VP16 + pG5CAT (2 μg :1μg), **5**= pM3-VP16 + pG5CAT (2 μg :2 μg), **6**= pM3-VP16 + pG5CAT (4 μg : 2 μg) **7**= pM3-VP16 + pG5CAT (3 μg : 1μg), **8**= pM3-VP16 only (1μg), **9**= pVP16-102 + pM-54 + pG5CAT (1μg : 1μg : 1μg), **10**= pVP16-102 + pM-54 + pG5CAT (2μg : 2μg : 2μg)

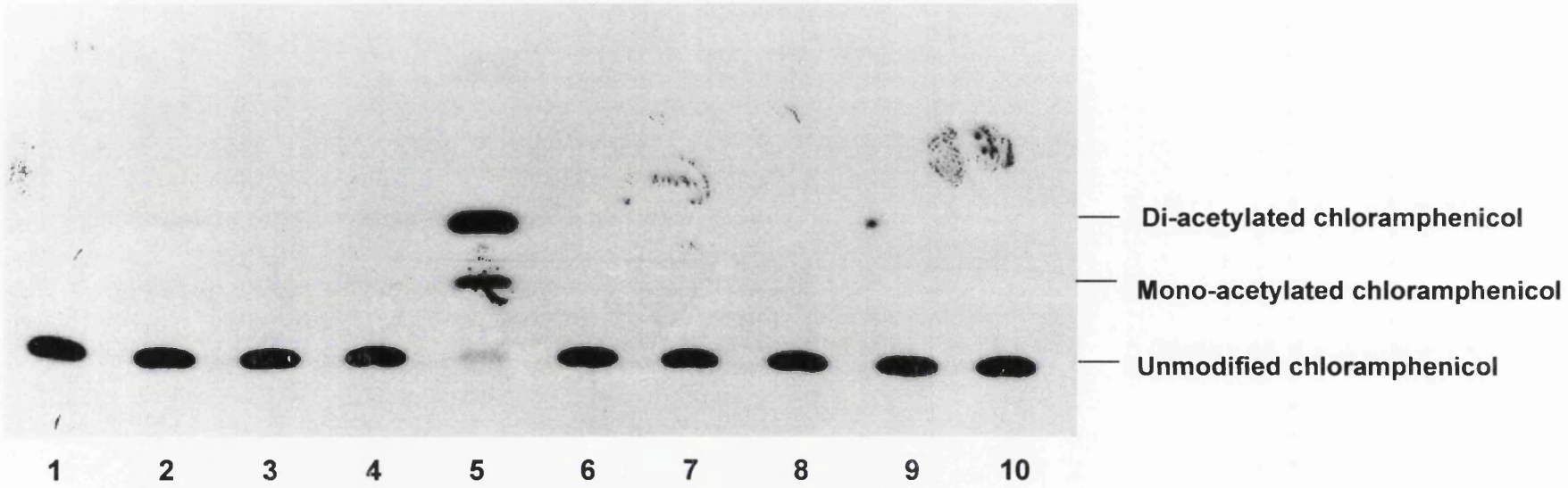


Figure 5.10 Investigation of the putative UL102-UL54 interaction using the M-2-H system (1)
Hela cells were transfected with M-2-H plasmids as indicated below. 48 hours post-transfection, cells were harvested, lysed and the clarified cell extracts were subjected to a radioactive CAT assay protocol as described in section 5.2.3.3. CAT assay samples were spotted onto silica gel-coated TLC plates and developed in a 95:5 mixture of chloroform/methanol for 20 minutes. The plate was air dried and exposed to kodak x-omat film overnight at -70°C. Multiple bands for any given sample in the resulting autoradiograph confirm the presence of modified chloramphenicol, either in a mono- or di-acetylated form, indicating CAT enzyme activity in that sample. The contents of each lane are as follows;
1= Untransfected cells, **2**= pM + pVP16 + pG5CAT (1μg : 1μg : 1μg), **3**= pM-54 + pVP16 + pG5CAT (1μg : 1μg : 1μg),
4= pM + pVP16-102 + pG5CAT (1μg : 1μg : 1μg), **5**= pM3-VP16 + pG5CAT (1μg : 1μg), **6**= pVP16-102 + pM-54 + pG5CAT (2μg : 2 μg : 2 μg), **7**= = pVP16-102 + pM-54 + pG5CAT (5μg : 5 μg : 5 μg), **8**= = pVP16-102 + pM-54 + pG5CAT (10μg: 10 μg : 10 μg) **9**= pVP16-102 + pM-54 + pG5CAT (5μg : 5 μg : 1 μg), **10**= = pVP16-102 + pM-54 + pG5CAT (2μg : 2 μg : 1 μg).

UL102 and UL54 protein. In contrast, 1μg of the positive control vector pM3-VP16 was sufficient to produce significant levels of CAT activity. These findings suggest that under the conditions used, UL102 and UL54 do not interact in Hela cells when expressed as fusion proteins with VP16 AD and GAL4 DNA-BD, respectively.

In order to examine the possibility that potential conformational changes in UL102 and UL54 when expressed as fusion proteins with either VP16 or GAL4 prevented an interaction occurring between them, the experiments were repeated using vectors pM-UL102 and pVP16-UL54. These vectors express UL102 and UL54 as fusions with the GAL4 and VP16 proteins, respectively. Figure 5.11 shows the results of CAT assays performed on cell extracts from this experiment. No CAT activity was detected in the negative control samples (lanes 1, 2 and 5), whereas activity was detected in the extracts from positive control samples (lanes 3 and 4), as expected. Lane numbers 6-12 contain extracts from cells transfected with UL102- and UL54-expressing vectors and reporter plasmid pG5CAT. Lanes 6-8 contain extracts from cells transfected with pVP16-UL102 and pM-UL54, the combination of vectors which had been used previously (Figure 5.10). No CAT activity was detected in these cell extracts, in agreement with previous results. Neither was any CAT activity detected in cell extracts transfected with the other combination of UL102- and UL54-expressing vectors, pM-UL102 and pVP16 (lanes 9-12) and pG5CAT. Four different amounts and ratios of vectors were used, but even when 10μg of each plasmid was used, no CAT activity was detected in any of the samples.

UL102 and UL54 were expressed in two combinations as fusion proteins in Hela cells, but even when the amounts of DNA transfected were 10 times greater than that which resulted in expression of CAT using the positive control vector pM3-VP16, no CAT activity was detected in cells transfected with either combination of UL102- and UL54-expressing vectors, indicating that UL102 and UL54 do not interact in Hela cells under the conditions used.

5.4 Discussion

The results from initial interaction ELISAs using both UL102 and UL54 MAbs demonstrated that UL102 and UL54 could interact, although the specificity of the interaction could not be established from these experiments alone. Hence, several control proteins were included in the ELISA and the ability of UL102 to bind to these proteins was investigated. Unexpectedly, these experiments showed that UL102 was capable of binding in a non-specific manner to the control proteins AD and CA. It is

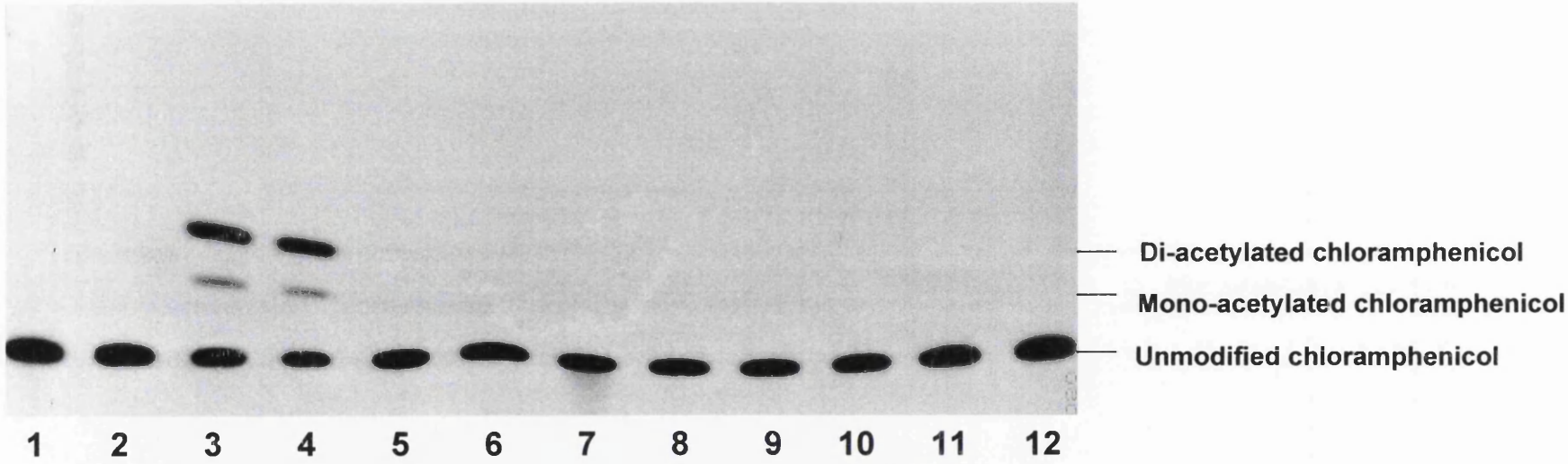


Figure 5.11 Investigation of the putative UL102-UL54 interaction using the M-2-H system (2)
Hela cells were transfected with M-2-H plasmids as indicated below. 48 hours post-transfection, cells were harvested, lysed and the clarified cell extracts were subjected to a radioactive CAT assay protocol as described in section 5.2.3.3. CAT assay samples were spotted onto silica gel-coated TLC plates and developed in a 95:5 mixture of chloroform/methanol for 20 minutes. The plate was air dried and exposed to kodak x-omat film overnight at -70°C. Multiple bands for any given sample in the resulting autoradiograph confirm the presence of modified chloramphenicol, either in a mono- or di-acetylated form, indicating CAT enzyme activity in that sample. The contents of each lane are as follows;
1= Untransfected cells, **2**= pM + pVP16 + pG5CAT (1μg:1μg:1μg), **3**= pM3-VP16 + pG5CAT (1μg:1μg), **4**= pM3-VP16 + pG5CAT (2μg :2μg) **5**= pM3-Vp16 only (1μg), **6**= pVP16-102 + pM-54 + pG5CAT (1μg:1μg:1μg), **7**= pVP16-102 + pM-54 + pG5CAT (2μg:2μg:2μg), **8**= pVP16-102 + pM-54 + pG5CAT (5μg:5μg:1μg), **9**= pM-102 + pVP16-54 + pG5CAT (1μg:1μg:1μg), **10**= pM-102 + pVP16-54 + pG5CAT (2μg:2μg:2μg), **11**= pM-102 + pVP16-54 + pG5CAT (5μg:5μg:1μg), **12**= pM-102 + pVP16-54 + pG5CAT (10μg:10μg:2μg).

perhaps not surprising that two purified proteins brought into contact in microtitre wells might interact to some degree, however the signals detected from wells containing UL102 and control proteins were considerably higher than background. This led me to investigate the effect of varying several parameters of the ELISA protocol on the formation of non-specific interactions between UL102 and control proteins.

The first parameter which was investigated was the effect of the buffer in which the proteins were diluted and added to the wells. PBS buffer and Tris buffer were compared, but neither significantly decreased the detection of non-specific interactions between UL102 and the control proteins AD and CA compared to UL102-UL54 interaction. The effect of the composition of the buffer used to wash the wells was also investigated. The original PBS wash buffer was compared with IP wash buffer, which contained deoxycholate and NP-40 detergents, to increase the stringency of the wash conditions. The inclusion of detergents was found to have little effect on the relative strengths of the signals detected as a result of non-specific interactions (UL102-AD and UL102-CA) compared to the signal detected from the UL102-UL54 interaction. When Tris diluent was used in conjunction with IP wash, the strengths of signals detected from the UL102-AD and UL102-CA interactions were slightly decreased but still significantly higher than background. Overall, the strengths of the signals resulting from all the interactions was decreased using this combination of diluent and wash buffer. Two other parameters were also investigated; the effect of the pH value of the protein diluent and the salt concentration of the wash buffer. The data resulting from these experiments is not shown, but neither was found to influence the relative strengths of the signals detected from the non-specific interaction as compared to the UL102-UL54 interaction.

An interaction ELISA was also developed in the laboratory to study the HSV-1 UL8-UL30 protein interaction. When control proteins were incorporated into the UL8-UL30 ELISA, there was also a degree of non-specific binding between the UL8 or UL30 and the control proteins. However in this case, increasing the stringency of the ELISA wash conditions by introducing detergents was sufficient to decrease the non-specific interactions to background level, whilst detection of the UL8-UL30 interaction was unaffected. Similar results were seen with the HSV-1 UL8-UL9 interaction ELISA (data not shown, Mary Murphy, personal communication).

Although an interaction between UL102 and UL54 was consistently demonstrated using the interaction ELISA, the failure to establish ELISA conditions under which non-

specific interactions between UL102-AD and UL102-CA were minimised and which allowed the distinction between specific and non-specific interactions, resulted in concluding that this assay was unsuitable for investigating the putative UL102-UL54 interaction.

Co-immunoprecipitation experiments were also used to investigate the UL102-UL54 interaction. This commonly used method for demonstrating protein-protein interactions is more stringent than the interaction ELISA method. This is because the proteins of interest are presented in a complex mixture of other proteins, derived from the insect cells in which the two proteins were expressed. When UL102-specific or UL54-specific MAbs were individually mixed with insect cell extracts containing both UL102 and UL54, none co-precipitated either UL54 or UL102 along with their cognate proteins. In further experiments, the stringency of the IP wash buffer used was decreased by omitting the detergents so that if the interaction was weak, then it would be less likely to be disrupted. However, even in the absence of detergents, no UL102-UL54 complexes were precipitated using either UL102- or UL54-specific MAbs from insect cell extracts containing both proteins (Mary Murphy, personal communication). The results from the immunoprecipitation experiments provided no evidence that UL102 and UL54 interact in insect cell extracts containing both proteins under the conditions used. Further work to investigate the UL102-UL54 interaction could involve performing similar co-immunoprecipitation experiments on HCMV-infected cell extracts. This would provide a more meaningful indication of whether the proteins interact in the context of viral infection. Yokohama *et al.*, (1999) showed the co-immunoprecipitation of the three EBV helicase-primase complex subunits from cells in which lytic EBV replication had been induced.

The third approach adopted to investigate the UL102-UL54 interaction was a mammalian-2-hybrid system, this method being the closest to simulating the conditions that UL102 and UL54 would encounter *in vivo* during infection. UL102 and UL54 were both expressed as fusions with either the HSV VP16 AD or the GAL4 BD utilised by the M-2-H system. Various amounts and ratios of plasmid DNA expressing the fusion proteins and the CAT reporter plasmid were used, however no CAT activity was detected in extracts from cells transfected with UL102- and UL54-expressing plasmids, in either combination. Since CAT activity was detected in extracts from cells transfected with the positive control vector, the lack of activity could not be attributed to ineffective transfection or CAT assay protocols. Hence, these results indicate that UL102 and UL54 do not interact in Hela cells when expressed as fusion proteins with

either the GAL4 DNA-BD or the VP16-AD under the conditions used. However, the possibility that any interaction between UL102 and UL54 may be disrupted due to the presence of the N-terminal fusion domains on each protein cannot be excluded. The fusion domains may interfere with folding and the native conformation of each protein thus inhibiting the interaction. By analogy with the known characteristics of the HSV-1 UL8-UL30 interaction, the region of UL102 which may interact with UL54 is the C-terminal region. Hence, the presence of a N-terminal fusion may not have a profound effect on the conformation of this region of the protein and therefore, on its ability to interact with UL54. The region of UL30 with which UL8 interacts in HSV-1 has not been defined, and hence no prediction can be made with regard to possible UL102-interacting regions of UL54. However, as the C-terminus of UL30 is known to mediate its interaction with UL42 (Tenney *et al.*, 1993; Digard *et al.*, 1993; Stow, 1993), it is highly unlikely that this region would be involved in binding to UL8 also.

To investigate the putative interaction using the mammalian-2-hybrid more fully, the truncated UL102 proteins described in Chapter 2 could be cloned into the M-2-H vectors and then screened against UL54. This would investigate whether specific domains of UL102 can interact with UL54. Other studies in which 2-hybrid systems have been used to investigate interactions between herpesvirus replication proteins have been described. Constantin and Dodson, (1999) used the yeast-2-hybrid system to map regions of interaction between the UL52 and UL8 subunits of the HSV helicase-primase complex. Interestingly, they found that the interaction between a N-terminally truncated form of the UL52 protein and UL8 gave a stronger signal using this system then the interaction between full-length UL52 and UL8. Another consideration is the cell type used to perform the M-2-H assays. Hela cells were chosen in this study for their ease of growth and transfection, however it is noted that they are a highly transformed cell line. Hence it would also be of interest to perform the M-2-H assays in a cell line permissive for HCMV infection, if possible.

Using three distinct methods of investigation, I found no evidence that UL102 and UL54 specifically interact *in vitro* or *in vivo*. The methods used are not exhaustive and hence it is possible that the interaction occurs but is below the threshold of detection of the three methods used. The interaction may be weak or transient, making detection difficult outwith the context of viral replication in HCMV-permissive cells. Another consideration is that an interaction between UL102 and UL54 may be dependent on the presence of other HCMV replication proteins. Both UL102 and UL54 exist as part of viral enzyme complexes in infected cells. UL102 closely associates with UL105 and

UL70 to form the helicase-primase complex and UL54 forms a polymerase holoenzyme with UL44. The behaviour of either protein may altered when it is complexed with the other components of each enzyme complex. Hence, it would be worthwhile investigating the interaction using the whole viral enzyme complexes. Work is currently underway in the laboratory to express the HCMV helicase-primase complex from recombinant baculoviruses in insect cells. Once this is achieved, then the purified complex could be used in interaction ELISA experiments to investigate the putative UL54 interaction or extracts containing the whole complex could be used in immune-precipitation experiments.

The lack of evidence to support an interaction between UL102 and UL54 does raise the possibility that the proteins do not interact. This would distinguish UL102 and UL54 from their counterparts in both HSV (UL8 and UL30) and EBV (BBLF2/3 and BALF5) which have been shown to specifically interact with each other (Marsden *et al.*, 1997; Fujii *et al.*, 2000). The significance of these interactions in the process of DNA replication *in vivo* has not been demonstrated. However, the interaction between the helicase-primase associated protein and the polymerase catalytic sub-unit could facilitate recruitment of the polymerase complex to the site of newly unwound and primed DNA at the replication fork. Neither the HSV UL8-UL30 or the EBV BBLF2/3-BALF5 interaction have been shown to be essential for viral DNA replication. However, Barnard *et al.*, (1997) have shown that a truncated UL8 protein, lacking the C-terminal 33 residues is unable to support viral DNA synthesis in a transient replication assay. Considering that this region is required for the interaction with UL30 (Marsden *et al.*, 1997), this suggests that the interaction is necessary for viral DNA synthesis. The model for recruitment of POL during herpesvirus DNA replication (section 1.4.6) would not apply to HCMV if UL102 and UL54 do not specifically interact. This would point to an alternative mode of recruitment for POL and also indicate that UL102 does not fulfill one of the functions predicted by analogy with HSV UL8.

In HSV, the UL8-UL30 interaction is suggested as an explanation for the stimulatory effect of UL8 upon the extension of primers by the POL holoenzyme during lagging strand synthesis (Sherman *et al.*, 1992). In this model the UL8-UL30 interaction serves to increase the efficiency with which POL locates primers on the DNA template. Hence, it would be useful to determine whether UL102 has a similar stimulatory effect on the extension of RNA primers by the HCMV polymerase holoenzyme in simulated lagging strand synthesis as this may provide alternative evidence as to whether UL102

and UL54 interact with each other and address whether UL102 fulfills one of the roles predicted by analogy with UL8.

Chapter 6

Investigation of the DNA-binding properties of UL102

6.1 Introduction

A specific enzymatic function for UL102 has not been established, although its HSV counterpart UL8 appears to augment the enzymatic activities of, and interact with, several other HSV replication proteins. UL102 may have a similar role in HCMV DNA replication and be involved in recruiting and mediating interactions between the HCMV replication fork proteins. The mechanism of recognition of the HCMV origin of replication is not yet understood, but it is likely that the helicase-primase complex is one of the first viral protein complexes to arrive, being required to unwind the duplex DNA. Hence it is possible that UL102 interacts with DNA either at this stage or at later stages of DNA replication. Establishing whether UL102 is capable of interacting with DNA would therefore be of use in determining its function at the replication fork. The homologous HSV replication protein, UL8, does not bind DNA under conditions which allowed binding between other HSV replication proteins and DNA oligonucleotides (Parry *et al.*, 1993). The aim of the research presented in this chapter was to determine whether UL102 can bind DNA and hence establish whether it behaves like its HSV counterpart, UL8, in this respect. Similar DNA-protein binding assays as those carried out by Parry *et al.*, (1993) were used to investigate the DNA-binding ability of UL102.

At the replication fork, various forms of DNA are present: double-stranded DNA (ds DNA), single-stranded DNA (ss DNA), and also DNA-RNA hybrids, formed as a result of RNA priming of unwound ss DNA to enable elongation by the viral DNA polymerase. UL102 potentially has the opportunity to interact with any of these forms of DNA at the replication fork. It was therefore appropriate to test the ability of UL102 to bind synthetic oligonucleotide templates representing either ss DNA, ds DNA or DNA-RNA hybrids. The template sequences used in this study were derived from bacteriophage ϕX174, which was chosen as it has been previously utilised in studies of DNA replication, including studies on the HSV helicase-primase proteins (Tenney *et al.*, 1994). The sequence of the oligonucleotides used in the binding assays corresponded to the preferred priming site for the HSV helicase-primase complex on ϕX174 DNA (Tenney *et al.*, 1995). A 50-mer oligonucleotide was used as the ss DNA template and another complementary 50-mer oligonucleotide was annealed to this to make a ds DNA template. In addition, two 10-mer RNA oligonucleotides complementary to two non-contiguous regions of the 50-mer DNA molecule were synthesised. These RNA oligonucleotides were annealed to the 50-mer DNA oligonucleotide either individually or together to create hybrid DNA-RNA templates. The sequences of the templates used to test the DNA-binding ability of UL102 are shown in Figure 6.1.

ss DNA Template

5′C T T C T T C G G T T C C G A C T A C C C T C C C C G A C T G C C T A T G A T G T T T A T C C T T T G3′ ϕX174A

ds DNA Template

5′C T T C T T C G G T T C C G A C T A C C C T C C C C G A C T G C C T A T G A T G T T T A T C C T T T G3′ ϕX174A

3′G A A G A A G C C A A G G C T G A T G G G A G G G C T G A C G G A T A C T A C A A A T A G G A A A C5′ ϕX174B

DNA/RNA Hybrid Template 1

5′C T T C T T C G G T T C C G A C T A C C C T C C C C G A C T G C C T A T G A T G T T T A T C C T T T G3′ ϕX174A

3′U G A C G G A U A C5′ RNA 1

DNA/RNA Hybrid Template 2

5′C T T C T T C G G T T C C G A C T A C C C T C C C C G A C T G C C T A T G A T G T T T A T C C T T T G3′ ϕX174A

3′U G A C G G A U A C5′ 3′A A U A G G A A A C5′ RNA1/RNA2

Figure 6.1 Oligonucleotide templates used in UL102 DNA-binding assays
The nucleotide sequences of each of the templates is given. The nomenclature for each of the oligonucleotides is indicated in blue text.

The ability of UL102 to bind the oligonucleotide templates was investigated by mixing purified UL102 protein with each of the templates in binding buffer either in the presence or absence of 50 mM NaCl. Salt is known to affect the strength of binding between a protein and DNA. Increasing salt concentrations will distinguish between weak and strong protein-DNA interaction, with only tightly binding proteins surviving high salt conditions. The binding reaction were carried out using buffer containing either no NaCl or 50 mM NaCl to provide information as to the effect of salt on the putative protein-DNA interactions.

A DNA band-shift assay was used to determined whether binding had occurred. Various control reactions were also carried out to ensure the conditions allowed other well characterised DNA-protein interactions to occur. Purified HSV UL29 protein was used as a ss DNA-binding protein control, as it is known to preferentially bind ss DNA in a non-sequence specific manner (Ruyechan & Weir, 1984). Similarly, HSV UL42 protein was chosen as a ds DNA-binding protein control for its ability to preferentially bind ds DNA (Bayliss *et al.,* 1975; Gallo *et al.*, 1988). Lastly, HSV UL8 was included in the binding assays to act as a negative DNA oligonucleotide-binding control, and to confirm it behaved as previously described (Parry *et al.*, 1993).

6.2 Chapter specific Methods

6.2.1 Preparation of purified UL102

UL102 was expressed and purified from *Sf* insect cells as described in section 2.9.1. The purity of the protein was determined by SDS-PAGE followed by Coomassie blue staining. Protein concentration was quantitated using a Bio-Rad protein assay, according to the manufacturers instructions.

6.2.2 5' radiolabelling of oligonucleotides

50 pmoles of single-stranded DNA oligonucleotide ϕX174A and 50 pmoles each of single-stranded RNA oligonucleotides 1 and 2 (Figure 6.1) were 5' labelled using 20 units of bacteriophage T4 polynucleotide kinase (PNK) and 75μCi of [γ-^{32}P]-ATP in 1x PNK buffer (NEB) for 30 minutes at 37°C. The reaction was stopped by the addition of 2μl 0.5M EDTA and the DNA was purified by phenol/chloroform extraction followed by 3 rounds of ethanol precipitation. DNA/RNA pellets were finally resuspended in 50μl of TE buffer (pH 7.5) and stored at 4°C.

6.2.3 Annealing reactions

Equivalent molarities (12 pmoles) of each of the following sets of oligonucleotides were mixed and annealed by heating to 95°C for 5 minutes then allowing to cool gradually to room temperature. Annealed oligonucleotides were then stored at 4°C.

1) ϕX174A and ϕX174B (unlabelled).
2) ϕX174A and RNA 1.
3) ϕX174A and RNA 1/RNA 2.

6.2.4 DNA-protein binding assay

Oligonucleotide templates (5ng/reaction) were mixed with either 50 or 500ng of purified protein in a total volume of 20μl of binding buffer (with or without 50 mM NaCl) and incubated for 30 minutes at 37°C. Reactions were stopped by adding 5μl of 5x DNA loading buffer and analysed by non-denaturing electrophoresis in a 6% polyacrylamide gel made with 1x TAE for 1.5 hours at 100mA. Gels were dried at 80°C for 2 hours then exposed to X-OMAT UV film overnight at -70°C and the resulting autoradiograph was developed.

6.3 Results

6.3.1 Ability of UL102 to bind oligonucleotide templates in salt-containing buffer

Under binding conditions in which 50 mM NaCl was present, no shift in the mobility of the oligonucleotides incubated with UL102 was observed, hence it was concluded that UL102 did not bind to any of the templates (Figure 6.2, lanes 1-8). However, under the same conditions, as expected, binding did occur between the HSV UL29 protein and the ssDNA template (lane 10) and also between the HSV UL42 protein and the dsDNA template, as expected (lane 12). The binding between UL29 and UL42 and the ss and ds DNA templates respectively appeared to be protein concentration-dependent, as incubation with 50ng of UL29 or UL42 failed to cause a shift in mobility of the templates, whereas incubation with 500ng resulted in a clear shift. As expected, no binding between the HSV UL8 protein and either ss DNA or ds DNA templates was observed (lanes 13-16).

6.3.2 Ability of UL102 to bind oligonucleotide templates in salt-free buffer

In the absence of salt from the binding buffer, incubation with UL102 still did not cause any shift in mobility of the oligonucleotide templates (Figure 6.3, lanes 1-8). Binding between the control proteins UL29 and UL42 and the ssDNA and dsDNA templates, respectively, was still observed (lanes 10 and 12). Similarily, the absence of salt made

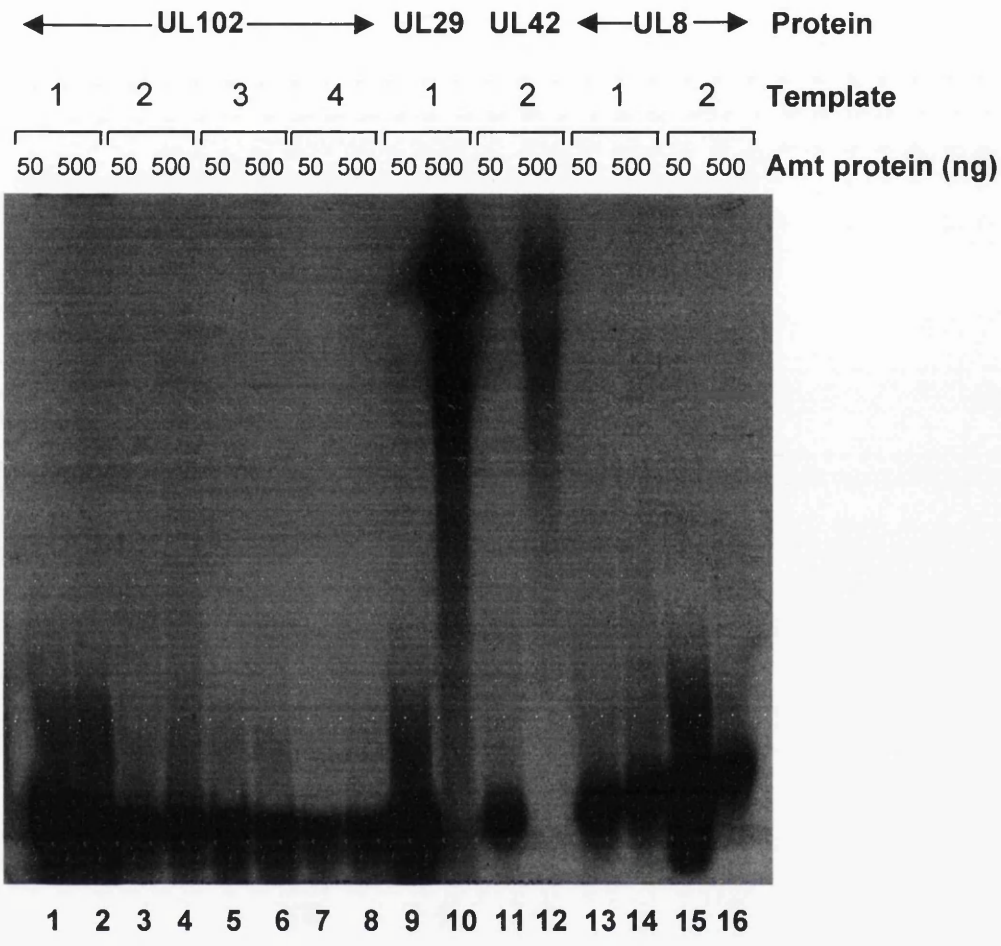


Figure 6.2 Ability of UL102 to bind DNA in buffer containing salt

Proteins were mixed with [γ^{32}P]-ATP 5'-labelled oligonucleotide templates in binding buffer containing 50 mM NaCl and incubated for 30 minutes at 37°C. Reactions were stopped by the addition of DNA-loading buffer and then electrophoresed in a 6% non-denaturing polyacrylamide gel (containing TAE). The gel was dried and then exposed to X-OMAT UV film overnight at -70°C. The resulting autoradiograph was developed using an X-omat developer. Templates used are as follows; 1= ss DNA, 2= ds DNA, 3= DNA/RNA Hybrid 1 and 4= DNA/RNA Hybrid 2.

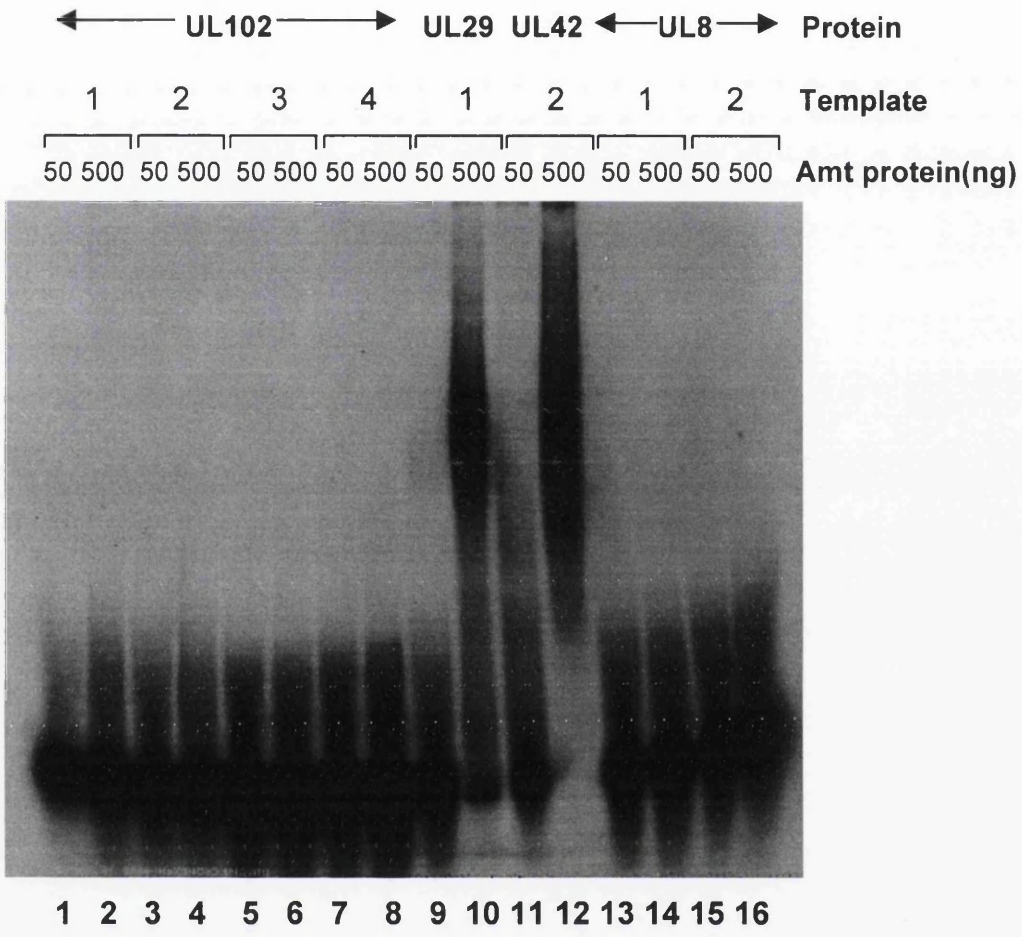


Figure 6.3 Ability of UL102 to bind DNA in buffer lacking salt

Proteins were mixed with [γ^{32}P]-ATP 5'-labelled oligonucleotide templates in binding buffer without NaCl and incubated for 30 minutes at 37°C. Reactions were stopped by the addition of DNA-loading buffer and then electrophoresed in a 6% non-denaturing polyacrylamide gel (containing TAE). The gel was dried and then exposed to X-OMAT UV film overnight at -70°C. The resulting autoradiograph was developed using an X-omat developer. Templates used are as follows; 1= ss DNA, 2= ds DNA, 3= DNA/RNA Hybrid 1 and 4= DNA/RNA hybrid 2.

no difference to the UL8 reactions, as no binding to either ss or dsDNA occurred under these conditions either (lanes 13-16).

6.4 Discussion

Under the assay conditions used, no binding between UL102 and four different DNA oligonucleotides was observed. However, binding between the HSV replication protein UL29 and ss DNA oligonucleotides and between UL42 and ds DNA oligonucleotides, was observed in the same assay. Considering that the assay conditions were suitable to allow DNA-binding by other herpesvirus replication proteins, these findings suggest that UL102 does not bind to either ss or ds DNA, or DNA/RNA hybrids. The data also confirms the findings of Parry *et al.*, (1993), who found that UL8 did not bind to any form of DNA in a similar band shift assay.

It cannot be excluded, however, that the sequence of the nucleotide templates chosen for this assay may influence the DNA-binding ability of UL102. The sequences chosen were derived from preferred priming sites by the HSV helicase-primase complex on bacteriophage ϕX174 DNA, and were intended as non-sequence specific templates to investigate DNA binding by UL102. It is possible that UL102 is capable of binding DNA, but in a sequence specific manner. In order to exclude this possibility, assays utilising templates corresponding to sequences of the HCMV genome, perhaps including the replication origin, could be performed. It seems unlikely, though, that the nucleotide sequence would have a profound difference on the ability of UL102 to bind to DNA. Most sequence-specific DNA-binding proteins bind non-specifically initially, then track along the DNA until they reach their specific recognition sequence (Kornberg & Baker, 1992). Some degree of non-specific DNA binding is therefore exhibited by sequence-specific DNA-binding proteins. Such binding was not demonstrated for UL102 in this set of experiments.

An important point to note, also, is that UL102 was tested in a purified form for its ability to bind DNA. In the context of viral DNA replication, UL102 exists as part of the closely associated helicase-primase complex, and hence its behaviour may be different when expressed alone. It is possible that the DNA-binding ability of UL102 may change when it is complexed with UL70 and UL105. An investigation of whether UL102 can bind DNA when associated with the other helicase-primase subunits would be complicated owing to the intrinsic DNA-binding ability of UL70 and UL105. However, a UV cross-linking experiment may indicate whether UL102, in addition to UL70 and UL105, binds to DNA. Radiolabelled DNA would be allowed to interact with the purified

helicase-primase complex and then irradiated with UV light to cross-link the protein to the DNA. The complex would then be digested with proteases, resolved by non-denaturing electrophoresis and transferred to nitrocellulose. The identity of the protein fragments could be determined by probing with antibodies reactive to each subunit of the complex in turn. Following immunoblotting to identify the protein fragments, the nitrocellulose membrane could be exposed to film and the resulting autoradiograph compared with the Western blot data. Cross-referencing of the protein fragments of known identity and the radioactive bands would indicate which protein had interacted with the DNA.

Although the possibility that UL102 binds DNA cannot be excluded, the results of the DNA band-shift assay presented here would suggest that this is not the case. This has implications when considering possible functions of UL102 at the replication fork. A DNA replication protein which does not bind DNA seems unusual, however, the finding that HSV UL8 does not appear to bind nucleic acids either, adds credence to this proposal. In other eukaryotic systems, proteins involved in DNA replication which do not bind DNA have been reported, such as proliferating cell nuclear antigen (PCNA) which is a DNA polymerase auxiliary protein (Tsurimoto and Stillman, 1991).
The observation that UL102 does not bind DNA excludes a number of putative functions at the replication fork, for which binding DNA directly is necessary. Such functions include locating the helicase-primase complex to the origin of replication and stabilising the association between newly synthesised primers and ss DNA templates. This is a possible role which has been inferred by analogy to HSV UL8, which is known to increase the efficiency of extension of primers by the HSV DNA polymerase (Sherman *et al.,* 1992). UL102 could still have a role in locating the helicase-primase complex to the origin by association with another protein which binds the origin directly. This situation would be analogous to that which is believed to occur during assembly of the HSV DNA replication complex, in which UL8 interacts with the viral origin binding protein UL9.

Chapter 7

Intracellular localisations of the components of the HCMV helicase-primase complex in transfected and HCMV-infected cells

7.1 Introduction

Localisation of HCMV helicase-primase proteins

In HSV-1, the UL8 protein has been shown to be essential for efficient nuclear localisation of the heterotrimeric helicase-primase complex. In the absence of UL8, the UL5 and UL52 proteins remain predominantly cytoplasmic when expressed individually or together in Vero and BHK cells (Barnard *et al*, 1997). The aim in this part of the study was to establish whether UL102 is required for nuclear localisation of the other components of the HCMV helicase-primase complex, like its HSV-1 counterpart, UL8. The intracellular localisations of the UL102, UL105 and UL70 proteins when expressed individually or in various combinations in mammalian cells was investigated.

Vector pCMV10, described in section 4.1, was used to express the UL102 protein and UL102 MAbs were then used to detect the intracellular location of the protein by indirect immunofluorescence microscopy. Owing to the unavailability of UL105- and UL70-specific antibodies, these proteins were expressed as fusion proteins with either the FLAG (DYKDDDDK) or c-myc (EQKLISEEDL) epitope tags using Stratagene pCMV-Tag mammalian expression vectors. The intracellular locations of UL105 and UL70 were then detected using an anti-FLAG MAb. The pCMV-tag series of vectors allows the expression of the protein of interest as an N-terminal or C-terminal fusion with either the FLAG or c-myc epitope tags under the control of the CMV immediate early promoter (Figure 7.1). Both UL105 and UL70 were cloned into pCMV-Tag 2B. UL70 was also cloned into pCMV-Tag 3B for expression as a c-myc fusion protein so that it could be distinguished from FLAG-tagged UL105 when both proteins were expressed together.

Initially, the cellular localisations of the UL102, UL105 and UL70 proteins when expressed individually in Vero and/or BHK cells was determined. Plasmids expressing UL102 and either UL105 or UL70 were then co-transfected to determine whether co-expression with UL102 influenced the localisation of UL105 and UL70. Finally, the localisation of the UL102 and UL105 proteins when co-transfected with a UL70-expressing plasmid was examined.

Localisation of truncated UL102 proteins

Preliminary UL102 localisation studies indicated that it was capable of localising to the nucleus in the absence of any other HCMV replication proteins. Analysis of the UL102 amino acid sequence revealed several putative nuclear localisation signals (NLS), which typically comprise a short peptide sequence of basic amino acids (Garcia-Bustos

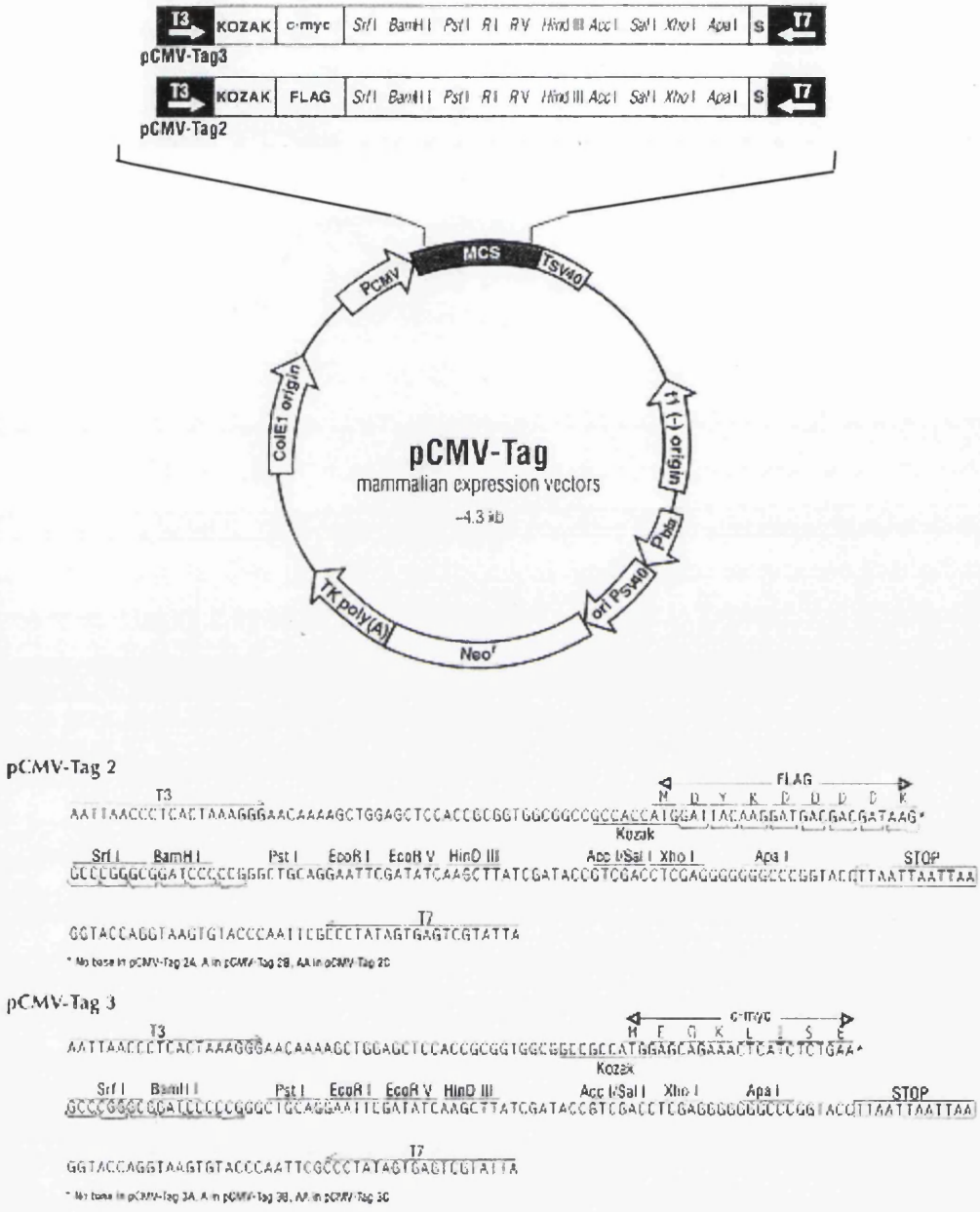


Figure 7.1 pCMV-Tag Vector Map
A schematic representation of the pCMV-Tag series of vectors is shown, with the positions and orientations of the CMV major immediate-early promoter and the multiple cloning site indicated. Further details of the sequence of the MCS in both pCMV-Tag2/Tag3 vectors are given below. The locations of the fusion epitope tags and the restriction sites are shown. The map was reproduced from the Stratagene catalogue.

et al., 1991), although less typical sequences can act as NLS (see discussion). Two UL102 sequences that resemble typical NLS were identified in the N-terminal region of the protein and a further two sequences which are less typical but could still potentially function as NLS were identified in the C-terminal region. The putative NLS and the residues of UL102 to which they correspond are shown below;

UL102 Residue Numbers	**Sequence**
189-194	HKKLRR
481-486	RFRSRR
692-697	RFVARR
847-853	RFFR

UL102 truncated genes spanning nucleotides 1-1635 and 1630-2622, which express the N-terminal 545 and the C-terminal 330 amino acids respectively, were cloned into mammalian expression vectors pCMV10 and pCMV-Tag2B, respectively (sections 4.3.3.2 and 4.3.3.3). The pCMV10 vector could not be used to express the 993 bp fragment as it lacks a suitable ATG start codon required to express the 5' deleted fragment. The localisation of the C-terminally and N-terminally truncated UL102 proteins was then examined by immunofluorescence microscopy to investigate which region of UL102 confers nuclear localisation.

Detection of UL102 in HCMV-infected HFFF cells

In addition to immune-fluorescence studies on UL102 in transfected cells, I carried out experiments to detect UL102 expression during the course of HCMV infection in HFFF cells. This would establish the ability of the UL102 MAbs raised against protein produced in insect cells to react with UL102 made in mammalian cells and the ability to detect the small amounts of UL102 which are expressed during HCMV infection. The experiments would also establish the kinetics of UL102 expression during the course of natural infection. HFFF cells were infected with HCMV strain AD169 and then harvested at various time points between 16 and 138 hours post-infection. UL102 was then detected by double immunolabelling with UL102-specific MAb number 658 and α-mouse-FITC conjugated antibody followed by fluorescence microscopy. UL102-specific MAb 658 was chosen because it was one of the strongest reacting MAbs against UL102 expressed in insect cells in immunofluorescence screening experiments (Section 3.3.2.2).

7.2 Chapter specific Methods

7.2.1 Cloning of UL105 and UL70 proteins into expression vectors

7.2.1.1 Cloning of the HCMV UL105 gene into the pCMV-Tag 2A vector

The source of the UL105 gene was plasmid pacCL29.105 which consisted of the pacCL29 baculovirus transfer vector containing the UL105 gene in the MCS. This clone of UL105 had been amplified by PCR with the introduction of *Bam* HI and *Eco* RI sites at the 5' and 3' ends of the gene, respectively. The UL105 gene was excised from pacCL29.105 in two stages. In the first, the 3' terminal 720 bp of the UL105 gene was excised using the *Bam* HI site at nucleotide position 2148 and the *Eco* RI site downstream of the stop codon which had been introduced by PCR. This fragment was cloned into vector pCMV-Tag 2A as a *Bam* HI-*Eco* RI insert using methods described in sections 2.5.1.5 and 2.5.1.7. In the second stage of cloning the first 2147 nucleotides of UL105 were excised from pacCL29.105 as a *Bam* HI fragment using the *Bam* HI site upstream of the start codon and the internal *Bam* HI site at nucleotide position 2148. pCMV-Tag 2A vector containing the initial UL105 *Bam* HI-*Eco* RI insert was digested with *Bam* HI and the 2148 bp fragment was ligated into the vector as a *Bam* HI insert.

To verify the presence of the entire UL105 gene insert, recombinant plasmid DNA was digested with *Bam* HI and *Eco* RI, which yields a vector DNA fragment of 4.3 Kb and two insert DNA fragments of sizes 2148 bp and 720 bp. The orientation of the 2148 bp UL105 *Bam* HI insert was determined by digesting the recombinant plasmid DNA with *Xba* I and *Xho* I enzymes. *Xba* I cuts the UL105 gene internally at nucleotide position 408 whilst *Xho* I cuts twice at nucleotide positions 2416 and 2668. If the *Bam* HI insert is present in the correct orientation in pCMV-Tag 2A, then *Xba* I-*Xho* I digestion yields DNA fragments of sizes approximately 4.6 kbp, 2.1Kbp, 0.25 kbp and 0.17 Kbp. Restriction digestion products were analysed on a 1% agarose gel.

7.2.1.2 Cloning of the HCMV UL70 gene into pCMV-Tag 2A/3A vectors

The source of the UL70 gene was plasmid pacCL29.70 which consisted of the pacCL29 baculovirus transfer vector with the UL70 gene (amplified by PCR with *Bam* HI and *Eco* RV restriction sites inserted at the 5' and 3' termini, respectively) cloned into the MCS. UL70 was excised from plasmid pacCL29.70 by digesting with *Bam* HI and *Eco* RV enzymes and then ligated into the pCMV-Tag 2A and pCMV-Tag 3A vectors as a *Bam* HI-*Eco* RV insert using methods described in sections 2.5.1.5 and 2.5.1.7. The presence of the UL70 gene insert in vectors pCMV-Tag 2A/3A was determined by digesting recombinant plasmid DNA isolated with *Bam* HI and *Eco* RV.

This should yield vector and insert DNA fragments of 4.3 and 2.8 Kbp. respectively. In addition, recombinant clone number 3 from the ligation of UL70 into pCMV-Tag 2A vector was triply digested with *Bam* HI, *Eco* RI and *Eco* RV enzymes, which yield DNA fragments of 4.3 Kbp, 2.5 Kbp and 0.35 Kbp. Restriction digestion products were analysed on a 1% agarose gel.

7.2.2 Transfection and antibody staining protocols

7.2.2.1 Transfection of BHK and Vero cells using cationic liposomes

13 mm circular glass coverslips were placed into wells in 24-well plastic plates. The wells were then seeded with either 0.5 x 10^5 Vero cells or BHK cells in 1ml of the appropriate medium. The following day, provided cells were approximately 70% confluent, they were transfected using cationic liposomes prepared as described in section 2.8.1. Plasmid DNA was mixed with liposomes and serum-free Optimem medium (Gibco-BRL) to prepare transfection mixtures of total volume 500 μl/well which were then added to each well as described in section 2.8.2. 24 hours post-transfection, the cell medium was removed, cells were washed once in PBS and then fixed by adding 500 μl of fix solution and incubating at room temperature for 15 minutes. After removing the fix solution, cells were washed twice using PBS.

7.2.2.2 Antibody staining of transfected BHK and Vero cells

Cells were incubated with 500 μl of permeabilisation solution for 10 minutes at room temperature then washed twice using PBS/1% FCS. Coverslips were then placed cell-side down onto 20 μl of primary antibody in a linbro dish lid and incubated for 1 hour at room temperature. Coverslips were washed three times rapidly in situ using PBS/1% FCS and then turned over and subjected to a further three 5 minute washes, with gentle agitation. Fluorescent secondary antibody, either α-mouse-FITC conjugate (1:100), or α-rabbit-Cy5 conjugate (1:200) was diluted in PBS/1% FCS placed onto a fresh linbro lid (20 μl/coverslip). Coverslips were then placed cell side down onto the secondary antibody and incubated for 1 hour at room temperature in the dark. Coverslips were then washed again as described above. After the final wash, the coverslips were dipped into de-ionised water and air-dried in the dark. Citifluor mounting agent (UKC) at room temperature was used to mount the cells on microscope slides. Cells were then viewed under x 400 magnification lens using a Zeiss LSM 510 confocal microscope. Cells were scanned with lasers emitting light of wavelengths 488 or 633 nm, to detect either FITC or Cy5-conjugated secondary antibodies, as appropriate.

7.2.3 Detection of UL102 in HCMV-infected cells

7.2.3.1 Infection of HFFF cells with HCMV strain AD169

Linbro wells containing 13 mm glass coverslips were seeded with 5 x 10^4 HFFF cells in 1 ml medium. The following day, the medium was removed and cells were infected with HCMV strain AD169 at a MOI of 10 diluted in a total volume of 200 μl HFFF medium. Virus was adsorbed for 1 hour at 37°C then 1ml of fresh medium was added. An equal number of wells were treated with HFFF medium only to act as mock-infected controls. Cells were incubated at 37°C. At the following time points post-infection; 15, 23, 40, 63, 70, 88, 114 and 138 hours, coverslips of HCMV- or mock-infected cells were removed and fixed as described in section 7.2.3.2.

7.2.3.2 Antibody staining of HCMV- or mock-infected HFFF cells

Cells were permeabilised and stained with UL102-specific MAb 658 and α-mouse-FITC conjugated antibody as described in section 7.2.2.2 and examined using a Nikon Microphot SA microscope and a FITC filter.

7.3 Results

7.3.1 Cloning of UL105 and UL70 proteins into pCMV-Tag2A/3A vectors.

7.3.3.1 Cloning of the UL105 gene into vector pCMV-Tag2A

Recombinant plasmid DNA was subjected to restriction enzyme analysis as described in section 7.2.1.1. Figure 7.2A shows that all clones isolated (1-12) contain bands of the correct sizes (*Bam* HI/*Eco* RI digest). The orientation of the *Bam* HI UL105 insert was determined by additionally digesting the clones using enzymes *Xba* I and *Xho* I, which generates DNA fragments of 4.6 kbp, 2.1 kbp, 0.25 kbp and 0.17 kbp if the insert is in the correct orientation. The products of the digestion are shown in Figure 7.2B. Clone numbers 1, 3, 5, 7, 8, 9, 11 and 12 produce fragments of the correct sizes.

7.3.1.2 Cloning of the UL70 gene into vectors pCMV-Tag2A/3A.

Recombinant DNA isolated was subjected to restriction enzyme analysis as described in section 7.2.1.2. Figure 7.3A shows that all 12 clones produce DNA fragments of the correct sizes. Clone number 3 was additionally digested with *Bam* HI, *Eco* RI and *Eco* RV enzymes, to further verify the presence of the UL70 insert. Figure 7.3A shows this clone generates DNA fragment of the correct sizes: 4.3 kbp, 2.5 kbp and 0.35 kbp, which are produced if the UL70 insert is present in pCMV-Tag2A. Figure 7.3B show the products of the digestion of the pCMV-Tag3A recombinant clones. Clone numbers 2-12 produce vector and insert DNA bands of the correct sizes.

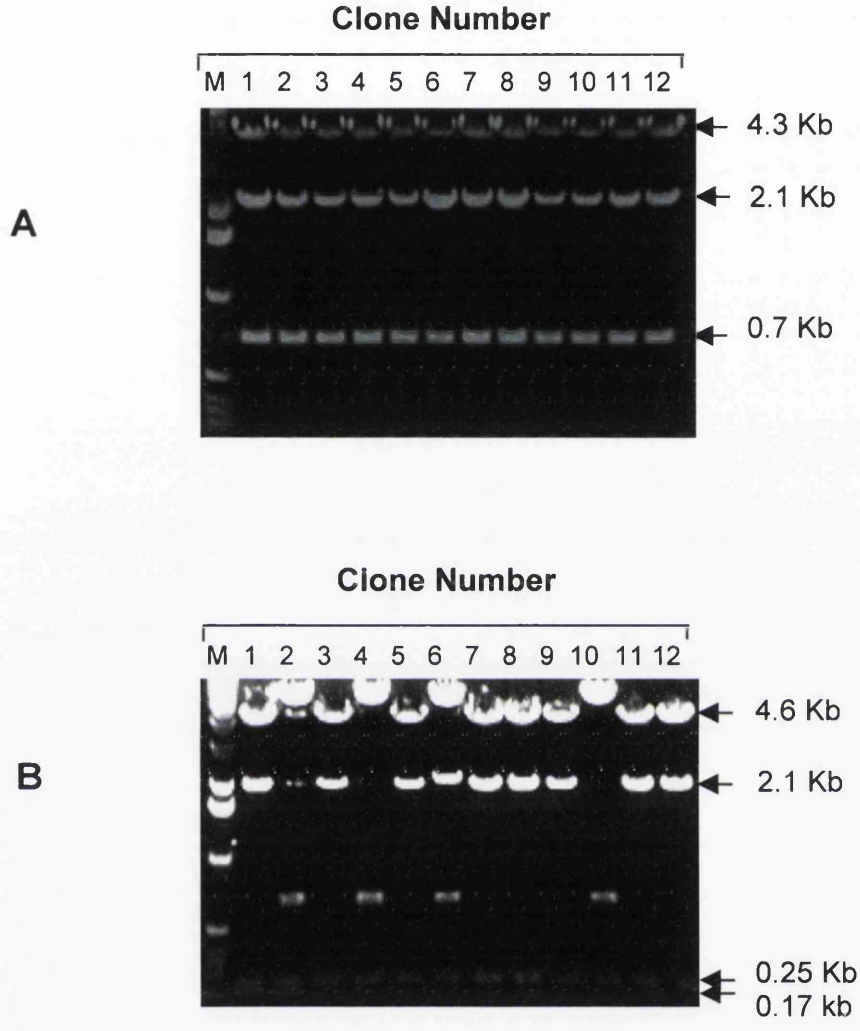


Figure 7.2 Cloning of the UL105 gene into vector pCMV-Tag2A
The following DNA products were electrophoresed on 1% TAE agarose gels containing EtBr;
A: Products following Bam HI/Eco RI digestion of recombinant plasmid DNA isolated following cloning of UL105 into vector pCMV-Tag2A. 5µl of mini-prep DNA was digested in a total volume of 10µl, then 2.5 µl of DNA-loading buffer was added prior to electrophoresis.
B: Products following Xba I/Xho I digestion of recombinant plasmid DNA isolated following cloning of UL105 into vector pCMV-Tag2A. 5µl of mini-prep DNA was digested in a total volume of 10µl, then 2.5 µl of DNA-loading buffer was added prior to electrophoresis. Lanes marked M contain DNA molecular size markers.

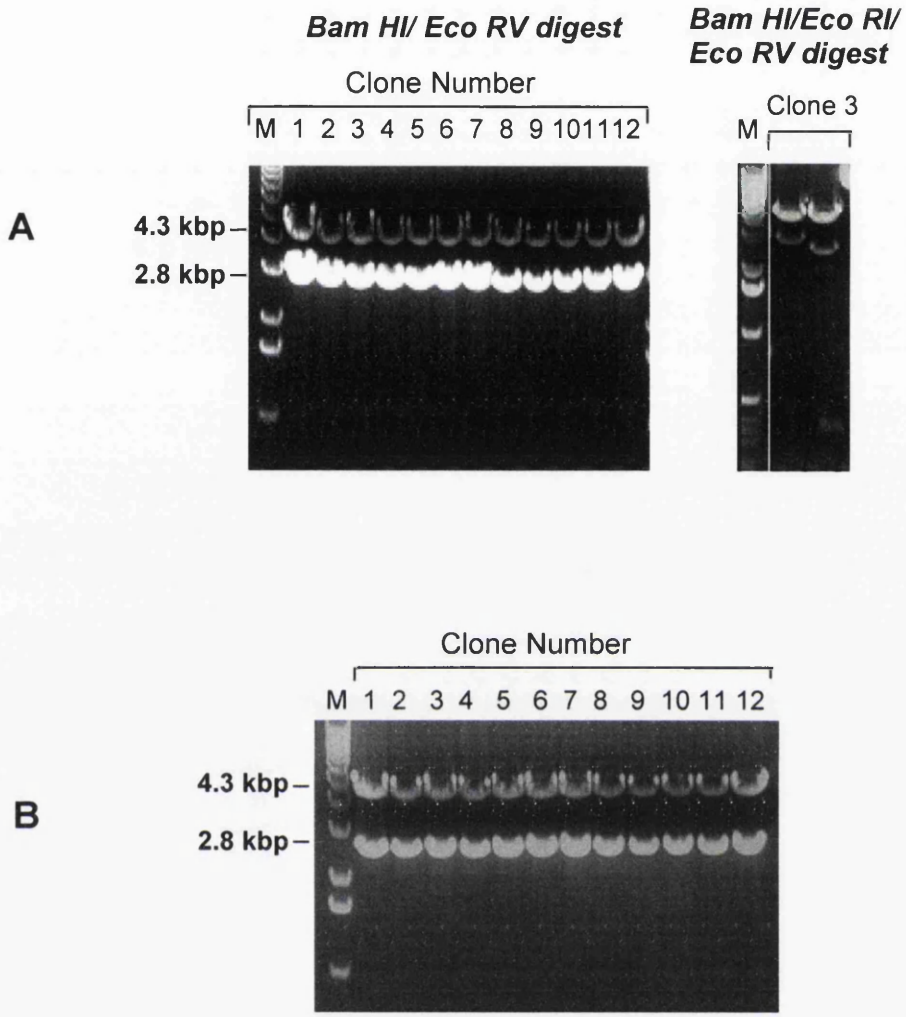


Figure 7.3 Cloning of the UL70 gene into vectors pCMV-Tag2A/3A
The following DNA products were electrophoresed on 1% TAE agarose gels containing EtBr;
A: Products following Bam HI/Eco RV or Bam HI/Eco RI/ Eco RV digestion of recombinant plasmid DNA isolated following cloning of UL70 into vector pCMV-Tag2A, as indicated. 5μl of mini-prep DNA was digested in a total volume of 10μl, then 2.5 μl of DNA-loading buffer was added prior to electrophoresis.
B: Products following Bam HI/Eco RV digestion of recombinant plasmid DNA isolated following cloning of UL70 into vector pCMV-Tag3A. 5μl of mini-prep DNA was digested in a total volume of 10μl, then 2.5 μl of DNA-loading buffer was added prior to electrophoresis. Lanes marked M contain DNA molecular size markers.

7.3.2 Intracellular localisation of UL102 expressed alone in mammalian cells

Recombinant mammalian expression vector pCMV10-UL102 was transfected into both BHK and Vero cells in order to perform intracellular localisation studies on transiently-expressed UL102. Figure 7.4 shows typical immune-fluorescence images of BHK cells expressing UL102 and stained using MAb 658 (panels A, B and C). UL102 appears to be present in all parts of the cell, except the nucleoli, but the nucleus is particularly densely stained. In order to establish whether the distribution of UL102 was truly nuclear or just perinuclear, a series of scans at different depths throughout the section of the cell was taken. This process is known as a Z-stack. Hence, one of the mid-point scans will show a horizontal section right through the nucleus. If UL102 was perinuclear in localisation, then no fluorescence would be detected in the nucleus at this point. The Z-stack clearly shows nuclear fluorescence in all sections through the cell, confirming that UL102 has a true nuclear localisation (panel D).

A similar staining pattern is observed in Vero cells (Figure 7.5, panels A, B and C). Fluorescence, indicating the localisation of UL102, can be seen in both the cytoplasm and nucleus, but the nuclear staining is much more intense. Again, to confirm that the UL102 distribution was throughout the nucleus and not just perinuclear, a Z-stack scan was performed. Nuclear fluorescence was observed in all sections throughout the cell, indicating a true nuclear localisation of UL102 in Vero cells also (panel D). The results presented are representative of experiments which were performed at least three times.

7.3.2 Localisation of truncated UL102 proteins

BHK cells were transfected with plasmids expressing the truncated UL102 proteins 545N or 330C and then stained using the N-terminally reactive UL102 MAb 707 or C-terminally reactive UL102 MAb 778, respectively. Images of BHK cells expressing the 545N or 330C UL102 proteins are shown in Figure 7.6. The 545N protein clearly localises uniformly to the cytoplasm (panels A, B and C). In contrast, the 330C truncated UL102 protein localises to the nucleus of BHK cells (panels D, E and F), although fluorescence is also observed less densely in the cytoplasm also. These results are unexpected as the sequences which most closely resembled established NLS were all located in the N-terminal half of the UL102. The results presented are representative of experiments which were performed at least three times.

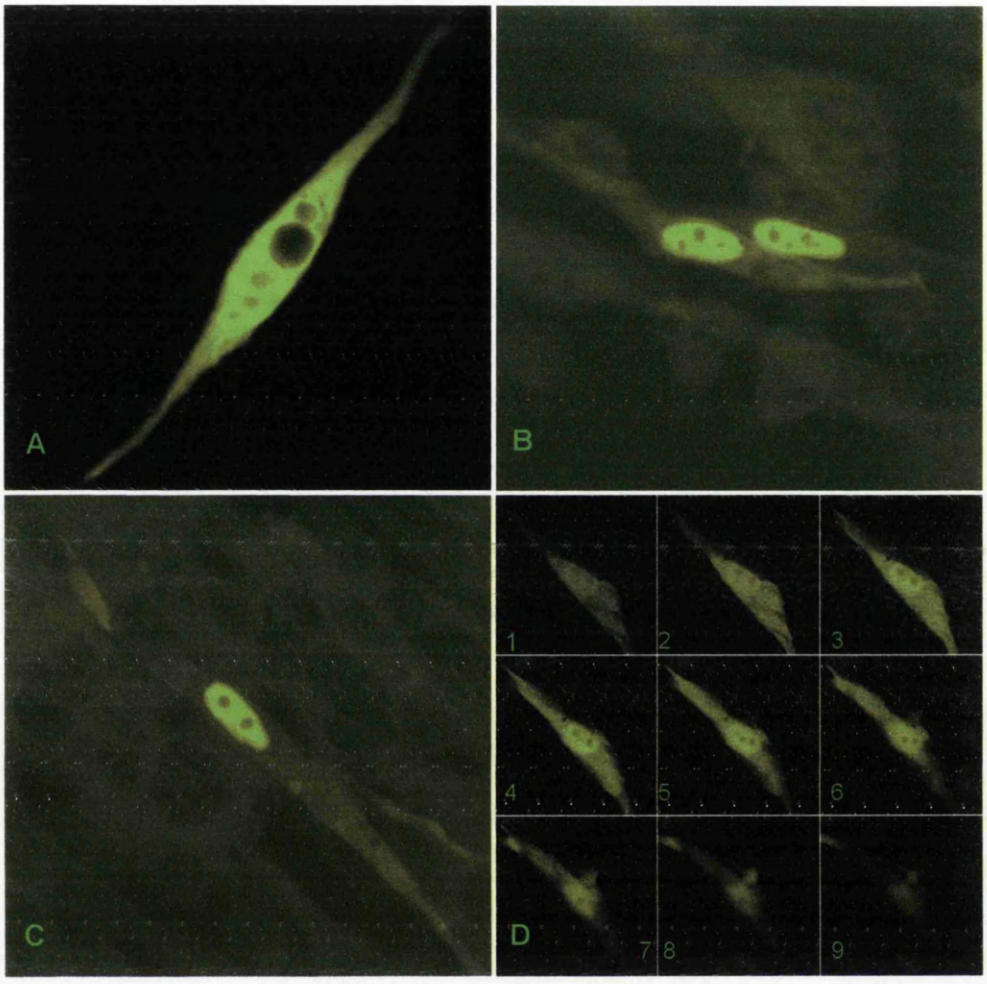


Figure 7.4 Localisation of UL102 in transfected BHK cells
BHK cells were transfected with plasmid pCMV10-102, which expresses UL102 protein. Twenty-four hours post-transfection, the cells were fixed and permeabilised before staining using UL102-specific MAb 546. After washing, the cells were further incubated with α-mouse-FITC conjugated secondary antibody. The cells were mounted in citifluor on microscope slides and then visualised under x 400 magnification using a Zeiss LSM 510 confocal microscope and scanned using a 488 nm wavelength laser. Three different groups of cells expressing UL102 are shown (panels A, B and C). Panel D (1-9) shows a Z-stack scan through a UL102-expressing cell.

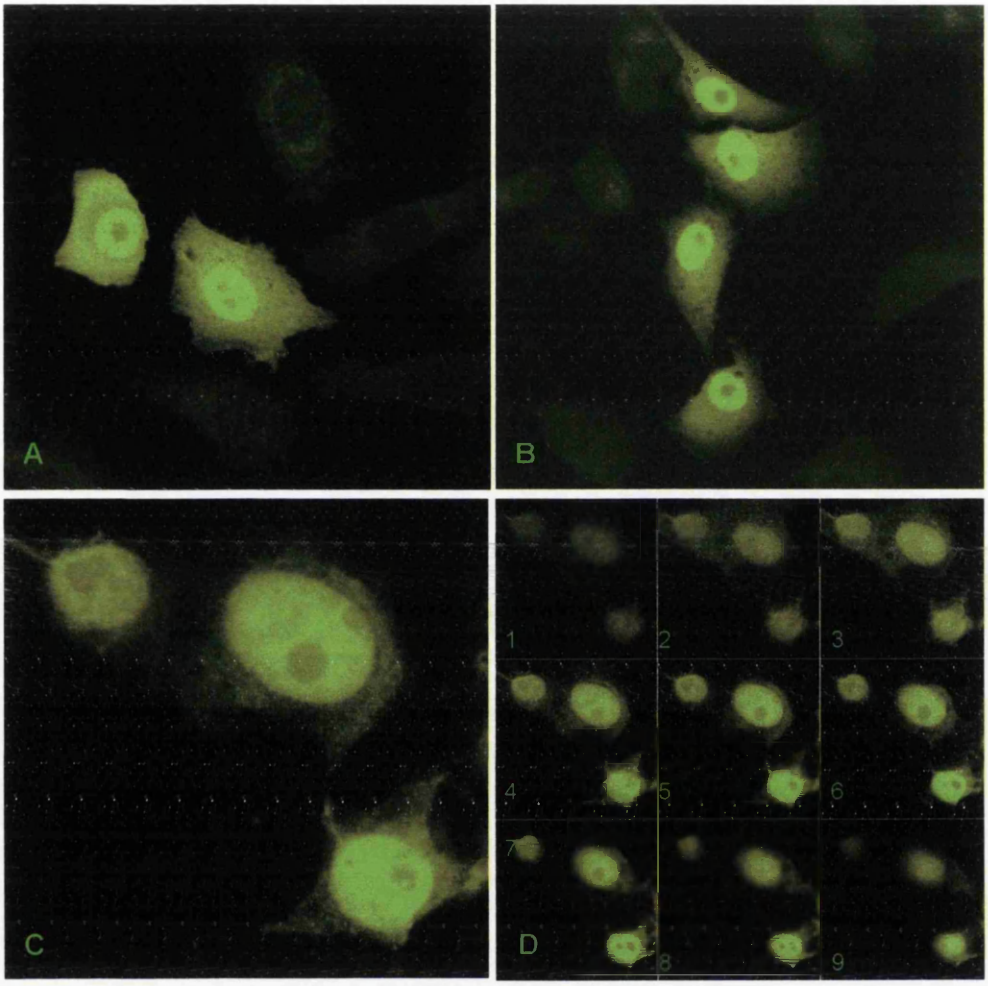


Figure 7.5 Localisation of UL102 in transfected Vero cells
Vero cells were transfected with plasmid pCMV10-102, which expresses UL102 protein. Twenty-four hours post-transfection, the cells were fixed and permeabilised before staining using UL102-specific MAb 546. After washing, the cells were further incubated with α-mouse-FITC conjugated secondary antibody. The cells were mounted in citifluor on microscope slides and then visualised under x 400 magnification using a Zeiss confocal microscope and scanned using a 488nm wavelength laser. Three different groups of cells are shown (panels A, B and C). Panel D (1-9) shows a Z-stack scan of the same group of cells as shown in panel C.

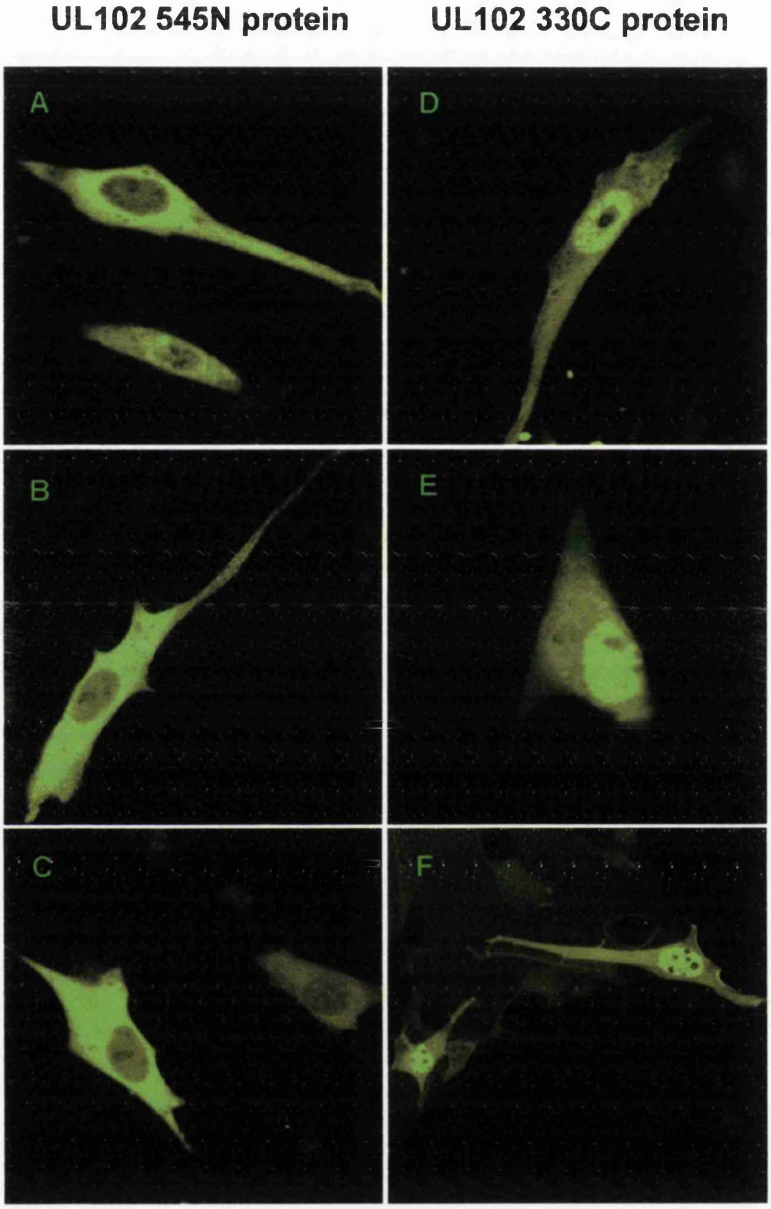


Figure 7.6 Intracellular localisation of UL102 truncated proteins 545N and 330C

BHK cells were transfected with plasmids expressing either UL102 545N or UL102 330C protein. Cells were fixed and permeabilised 24 hours following transfection. Cells transfected with the 545N-expressing plasmid were incubated with UL102-specific MAb 707 whilst those transfected with the 330C-expressing plasmid were incubated with UL102-specific MAb 778. After washing, cells were further incubated with α-mouse-FITC conjugated antibody then mounted in citifluor solution. Cells were viewed under x 400 magnification on a Zeiss confocal microscope and scanned using a laser at 488 nm wavelength. Panels A, B and C show BHK cells expressing the UL102 545N protein. Panels D, E and F show BHK cells expressing the UL102 330C protein.

7.3.3 Localisation of HCMV helicase-primase components

7.3.3.1 Localisation of UL105 and UL70 expressed individually in BHK cells

BHK cells were transfected with individual plasmids pCMV-Tag/UL105 or pCMV-Tag/UL70 expressing UL105-FLAG and UL70-FLAG fusion proteins, respectively. After staining with FLAG-specific MAbs and FITC-conjugated secondary antibody, cells were visualised using a laser scanning microscope. Typical images of BHK cells expressing UL70 are shown in Figure 7.7 (panels A, B and C). UL70 localises exclusively to the cytoplasm in these cells. UL105 also displays a cytoplasmic localisation when expressed on its own, similar to that of UL70 (Figure 7.7, panels D, E and F). The results presented are representative of experiments which were performed at least three times.

7.3.3.2 Localisation of UL105 and UL70 when co-expressed with UL102

BHK cells were then doubly transfected with plasmids expressing either UL105 and UL102 or UL70 and UL102, to ascertain whether co-expression of UL102 influences the cellular localisation of either UL105 or UL70. Images of two different cells, A and B, co-expressing UL105 and UL102 are shown in Figure 7.8. There are three images of each cell: 1) cell stained for UL105 (green), 2) cell stained for UL102 (red) and 3) merged image of 1 and 2. In both the cells shown, UL105 displays a cytoplasmic localisation, whereas UL102 is predominantly nuclear and no co-localisation of the two proteins is seen. When co-expressed, the localisations of UL105 and UL102 do not vary from their localisations when expressed individually, indicating that UL102 does not interact with UL105 to alter its intracellular localisation in the absence of any other HCMV replication proteins.

Like UL105, the intracellular localisation of UL70 does not alter when it is co-expressed with UL102 in BHK cells (Figure 7.9). Two images of BHK cells co-expressing UL70 and UL102 are shown (A and B). In both, UL70 (green) is localised in the cytoplasm and UL102 (red) displays a nuclear localisation. Hence, co-expression of UL70 and UL102 does not result in a change in localisation of either protein compared to when expressed on their own. The results presented are typical of those obtained from experiments which were performed at least twice.

7.3.3.3 Localisation of UL105 and UL102 when co-expressed with UL70

Finally, the localisation of the UL102 and UL105 proteins when co-expressed with UL70 in BHK cells was investigated. Cells were transfected with plasmids pCMV10-102, pCMV-Tag2B/UL105 and pCMV-Tag3B/UL70, which express UL102, FLAG-

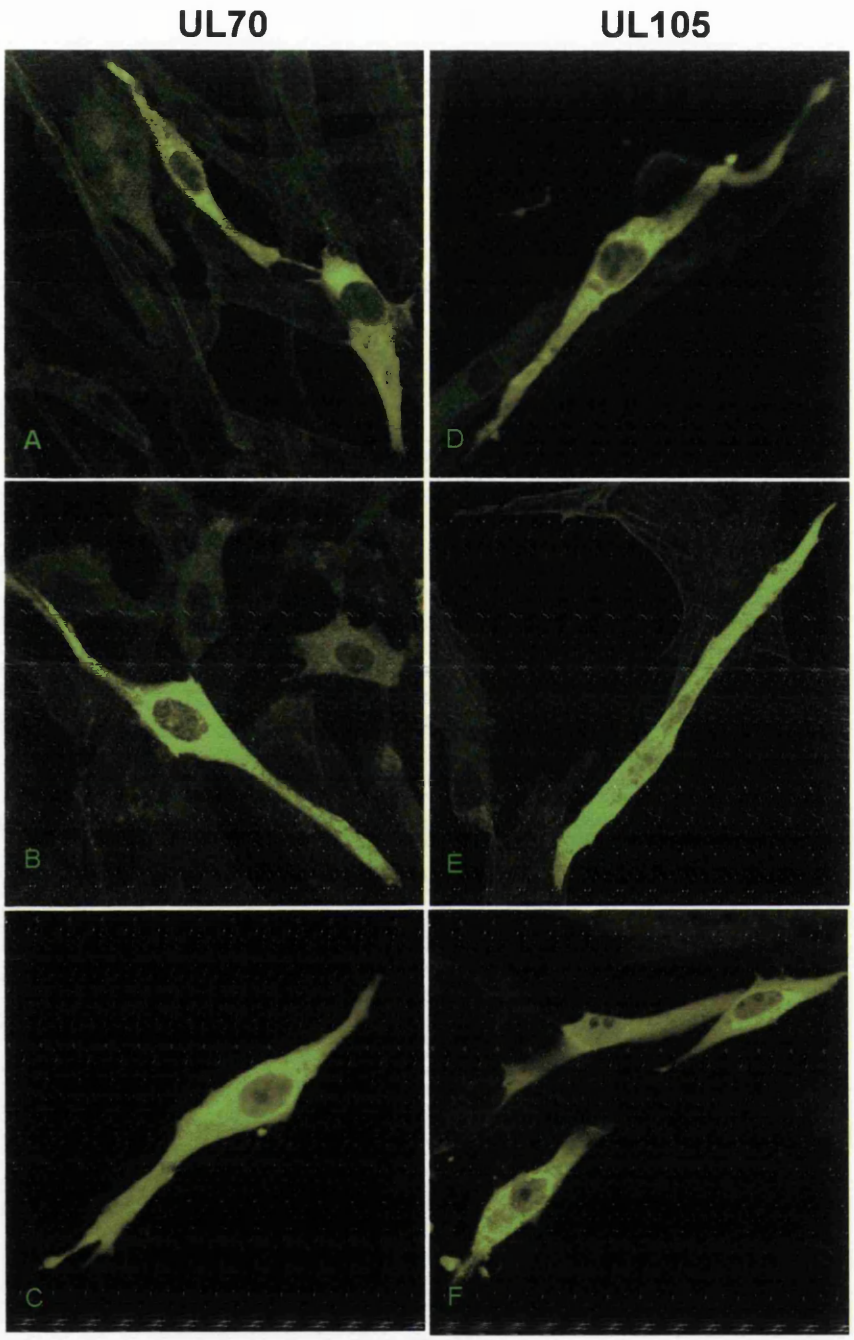


Figure 7.7 Intracellular localisations of UL70 and UL105 in transfected BHK cells

BHK cells were transfected with plasmids expressing either FLAG-tagged UL105 or FLAG-tagged UL70. Cells were fixed and permeabilised 24 hours following transfection. Cells were then incubated with a FLAG-specific MAb followed by α-mouse-FITC conjugated secondary antibody as described in section 7.2.2.2. After washing, cells were mounted in citifluor solution and viewed under x 400 magnification on a Zeiss LSM 510 confocal microscope. Cells were scanned using a laser at 488 nm wavelength. Panels A, B and C show cells expressing UL70 and panels D, E and F show cells expressing UL105.

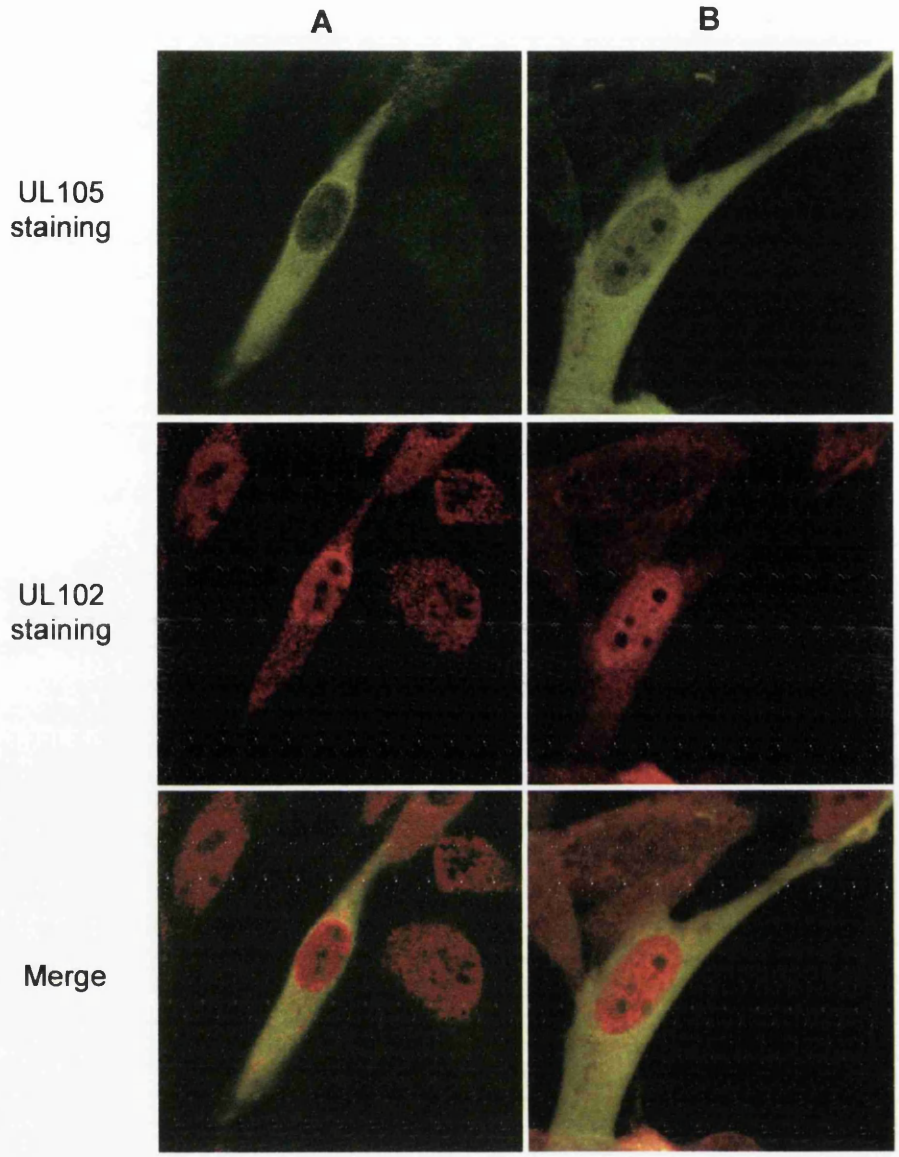


Figure 7.8 Localisation of UL105 and UL102 co-expressed in BHK cells
BHK cells were transfected with plasmids expressing UL102 and FLAG-tagged UL105. Cells were fixed and permeabilised 24 hours following transfection. Cells were then incubated with a mixture of UL102 polyclonal antisera 724 and a FLAG-specific MAb, followed by a mixture of α-mouse-FITC and α-rabbit-Cy5 conjugated secondary antibodies. After washing, cells were mounted in citifluor solution and viewed under x 400 magnification on a Zeiss LSM 510 confocal microscope and scanned using 488 nm and 633 nm laser wavelengths simultaneously. Two different groups of cells are shown; A and B. For each group of cells there are three images corresponding to 1) visualisation of UL70 (green), 2) visualisation of UL102 (red) and 3) images 1 and 2 merged (co-localisation of proteins is indicated by yellow).

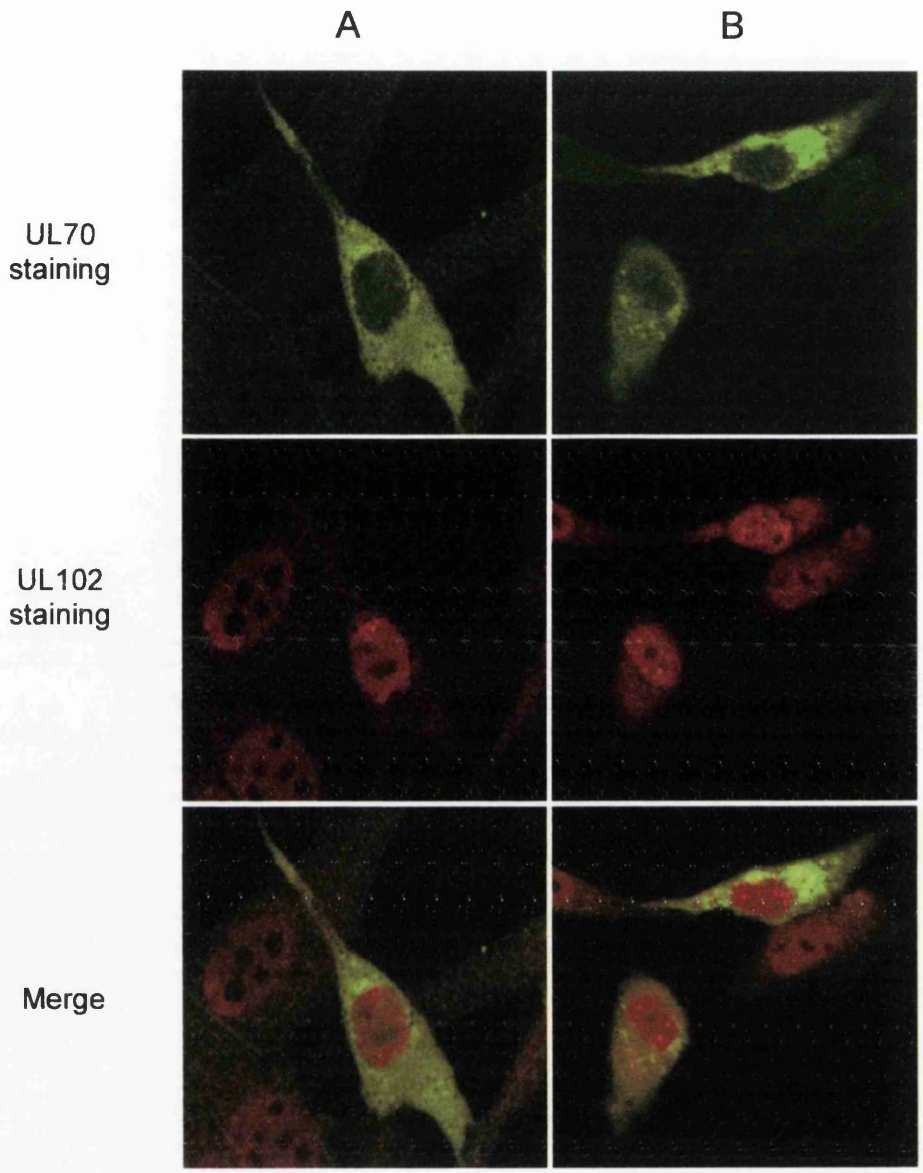


Figure 7.9 Localisation of UL70 and UL102 co-expressed in BHK cells
BHK cells were transfected with plasmids expressing UL102 and FLAG-tagged UL70. Cells were fixed and permeabilised 24 hours following transfection. Cells were then incubated with a mixture of UL102 polyclonal antisera 724 and a FLAG-specific MAb, followed by a mixture of α-mouse-FITC and α-rabbit-Cy5 conjugated secondary antibodies. After washing cells were mounted in citifluor solution and viewed under x 400 magnification on a Zeiss LSM 510 confocal microscope and scanned using 488 nm and 633 nm laser wavelengths simultaneously. Two different groups of cells are shown; A and B. For each group of cells there are three images corresponding to 1) visualisation of UL70 (green), 2) visualisation of UL102 (red) and 3) images 1 and 2 merged (co-localisation of proteins is indicated by yellow).

tagged UL105 and C-myc-tagged UL70, respectively. Cells were fixed and doubly-stained using polyclonal UL102 antibody 724 and an anti-FLAG MAb, to detect UL105. Two different groups of cells stained for UL102 and UL105 are shown in Figure 7.10 (A and B). Three images of each group of cells are shown, corresponding to 1) UL105 localisation (green), 2) UL102 localisation (red) and 3) images 1 and 2 merged. In cell group A, the cell labelled '1' (panel B), UL102 is visible in the nucleus. In the same cell stained for UL105, (panel A) UL105 is also visible in the nucleus, in contrast to previous findings on its localisation when expressed alone or together with UL102. When the images in panels A and B are superimposed, UL102 and UL105 are co-localised within the nucleus of cell number 1 (panel C). Similar results are found in the cell labelled 1 (Group B), in which UL102 and UL105 are again both localised to the nucleus (panels G and H). These results indicate that in cells transfected with plasmids expressing all three HCMV helicase-primase proteins, the localisation of UL105 is altered from cytoplasmic to nuclear.

It should be pointed out that this experiment was only performed once and therefore needs to be repeated in order to confirm these preliminary findings and in addition, the necessary control of demonstrating that the pCMV-Tag3A-70 construct expresses c-myc-tagged UL70 was absent from this experiment. However, a definite change in the localisation of UL105 from cytoplasmic to nuclear was observed when this construct was included in the transfection of BHK cells together with plasmids expressing UL105 and UL102. Therefore the most likely conclusion of these experiments is that, in the absence of either UL102 or UL70, UL105 is cytoplasmic but that when co-expressed, all three components of the helicase-primase complex localise to the nucleus.

7.3.3.4 Antibody controls for immune-fluorescence experiments

Transfection and antibody staining controls were carried out to exclude the possibility that any of the fluorescence observed in the above described transient transfection experiments was an artefact of either the vectors or the antibodies used. Two vectors were used to express the UL102, UL105 and UL70 proteins; pCMV10 and pCMV-Tag 2B/3B. Cells were transfected with either empty pCMV10 or pCMV-Tag vectors and then labelled with the same antibodies as those used to label cells transfected with protein-expressing vectors. Images of cells transfected with either pCMV10 or pCMV-Tag vectors and stained with antibodies are shown in Fig 7.11. As expected, no fluorescence was observed in any of the control cells, confirming that the fluorescence corresponded to proteins expressed from either pCMV10 or pCMV-Tag.

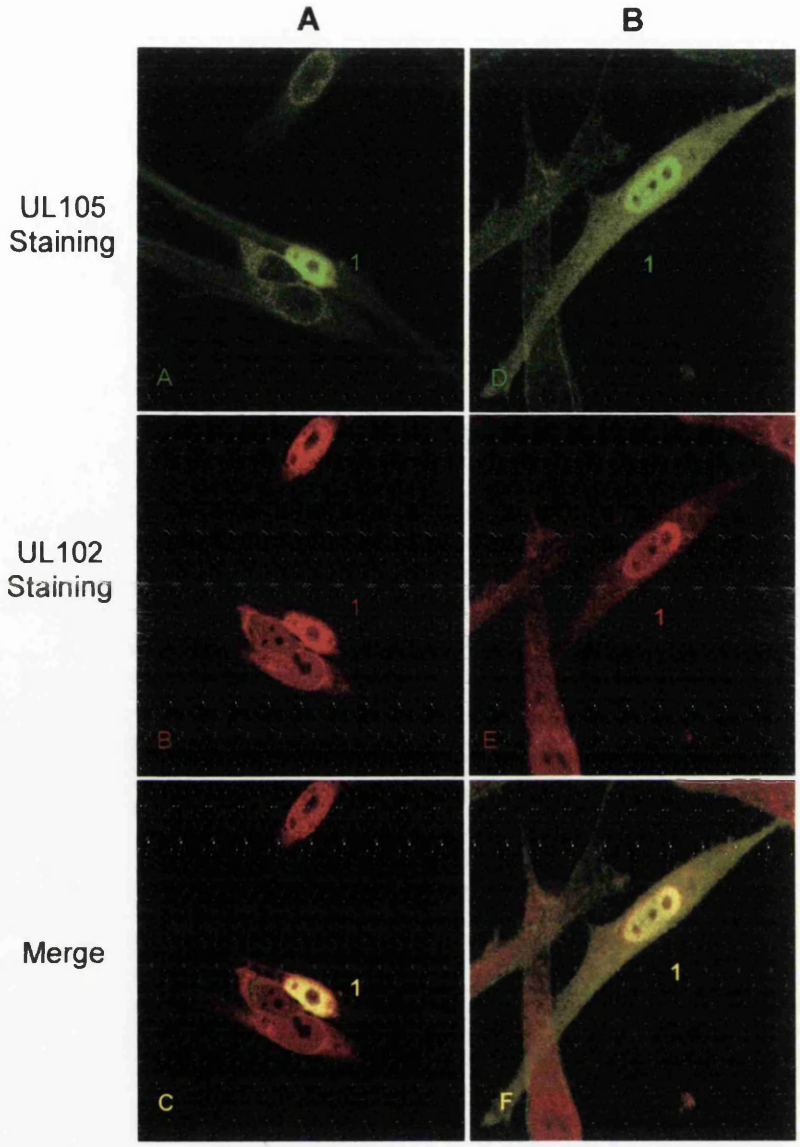


Figure 7.10 Localisation of UL105 and UL102 in triply-transfected BHK cells
BHK cells were transfected with plasmids expressing UL102, a UL105-FLAG fusion protein and c-myc-tagged UL70. Cells were fixed and permeabilised 24 hours following transfection. Cells were then incubated with a mixture of UL102 polyclonal antisera 724 and a FLAG-specific MAb, followed by a mixture of α-mouse-FITC and α-rabbit-Cy5 conjugated secondary antibodies. After washing cells were mounted in citifluor solution and viewed under x 400 magnification on a Zeiss LSM 510 confocal microscope using 488 nm and 633 nm wavelengths simultaneously. Two different groups of cells are shown: A and B. For each group of cells there are three images corresponding to 1) visualisation of UL105 (green), 2) visualisation of UL102 (red) and 3) images 1 and 2 merged (co-localisation of proteins is indicated by yellow). Individual cells are labelled with a number for reference in the text.

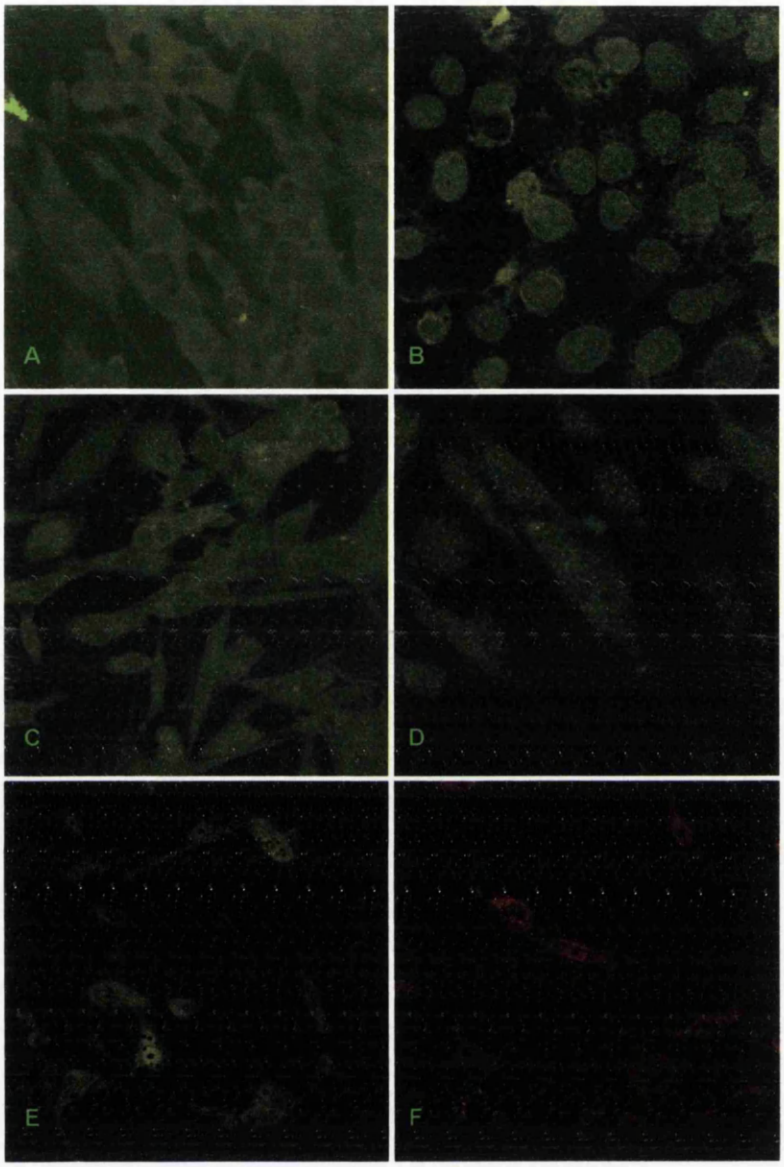


Figure 7.11 Controls for immune-fluorescence experiments
Cells were transfected with the plasmids indicated using methods described in section 7.2.2.1 and then stained with either monoclonal or polyclonal antibodies as indicated below:
Panel A: BHK cells transfected with plasmid pCMV10 and stained using UL102-specific MAb 546 (control for pCMV10-102 transfections in BHK cells). **Panel B**: Vero cells transfected with plasmid pCMV10 and stained using UL102-specific MAb 546 (control for pCMV10-102 transfections in Vero cells). **Panel C**: BHK cells transfected with plasmid pCMV10 and stained with UL102-specific MAb 707 (control for expression of UL102 545N protein using plasmid pCMV10-1635 in BHK cells). **Panel D**: BHK cells transfected with plasmid pCMV-Tag2B and stained using UL102-specific MAb 778 (control for expression of 330C UL102 protein using plasmid pCMV-Tag2B-993 in BHK cells). **Panel E:** BHK cells transfected with plasmid pCMV-Tag2B and stained with FLAG-specific MAb (control for expression of UL105 and UL70 proteins using vectors pCMV-Tag2B-105 and pCMV-Tag2B-70, respectively in BHK cells). **Panel F:** BHK cells transfected with plasmid pCMV10 and stained with UL102 polyclonal antisera 724 (control for expression of UL102 using plasmid pCMV10-102 in BHK cells)

7.3.4 Temporal expression of UL102 in HCMV-infected cells

HFFF cells were infected with HCMV strain AD169 and then fixed at various time points up to 138 hours post-infection. They were then stained with UL102 MAb number 658 followed by an α-mouse-FITC conjugated antibody and then examined by fluorescence microscopy. Images of HCMV-infected cells from each time point are shown in Figure 7.12. At 16 hpi (panel A), only a few individual cells display faint nuclear fluorescence and these cells are in isolation (1-2/13mm coverslip). Neighbouring cells may be beginning to express UL102 but at a level below the threshold of detection for the antibodies used. By 23 hpi (panel B), several small groups of cells (2-3 per coverslip) display faint nuclear fluorescence, indicating that infection has spread from individual infected cells to neighbouring cells, which are also expressing UL102. At 40 hpi (panel C), increasing numbers of cell clusters are expressing UL102 and the intensity of nuclear fluorescence has increased. By 70 hpi (panel D), whole sections of the cell monolayer are expressing UL102. The nature of the nuclear staining is also more specific, with distinct areas of the nucleus containing more intense fluorescence. The numbers of cells detectably expressing UL102 increases between 88 and 114 hpi and by 138 hpi almost the entire cell monolayer is expressing UL102, (panels E, F and G). The distinct nuclear staining is more obvious at these stages in the infection also. Images of enlarged individual cells exhibiting this distribution of UL102 are inset in panels F and G, showing cells at 114 and 138 hpi . Within these cells, up to four large globular intranuclear compartments densely stained for UL102 are visible.

Mock-infected cells were also harvested at the same time points and stained with UL102 MAb 658. In contrast to the HCMV-infected cells, no fluorescence was observed in cells fixed at any of the time points, indicating that the fluorescence observed was as a consequence of HCMV infection and correlated to expression of UL102 (cells fixed and stained between 16 and 114 hpi, data not shown). Panel H shows mock-infected cells at 138 hpi, in which no fluorescence is observed after double-labelling with UL102-specific MAb and α-mouse-FITC conjugated antibody.

7.4 Discussion

7.4.1 Intracellular localisation of full-length and truncated UL102 proteins

When expressed on its own in both BHK and Vero cells, UL102 displayed a diffuse nuclear localisation. In this respect, UL102 is distinct from its HSV-1 counterpart, UL8,

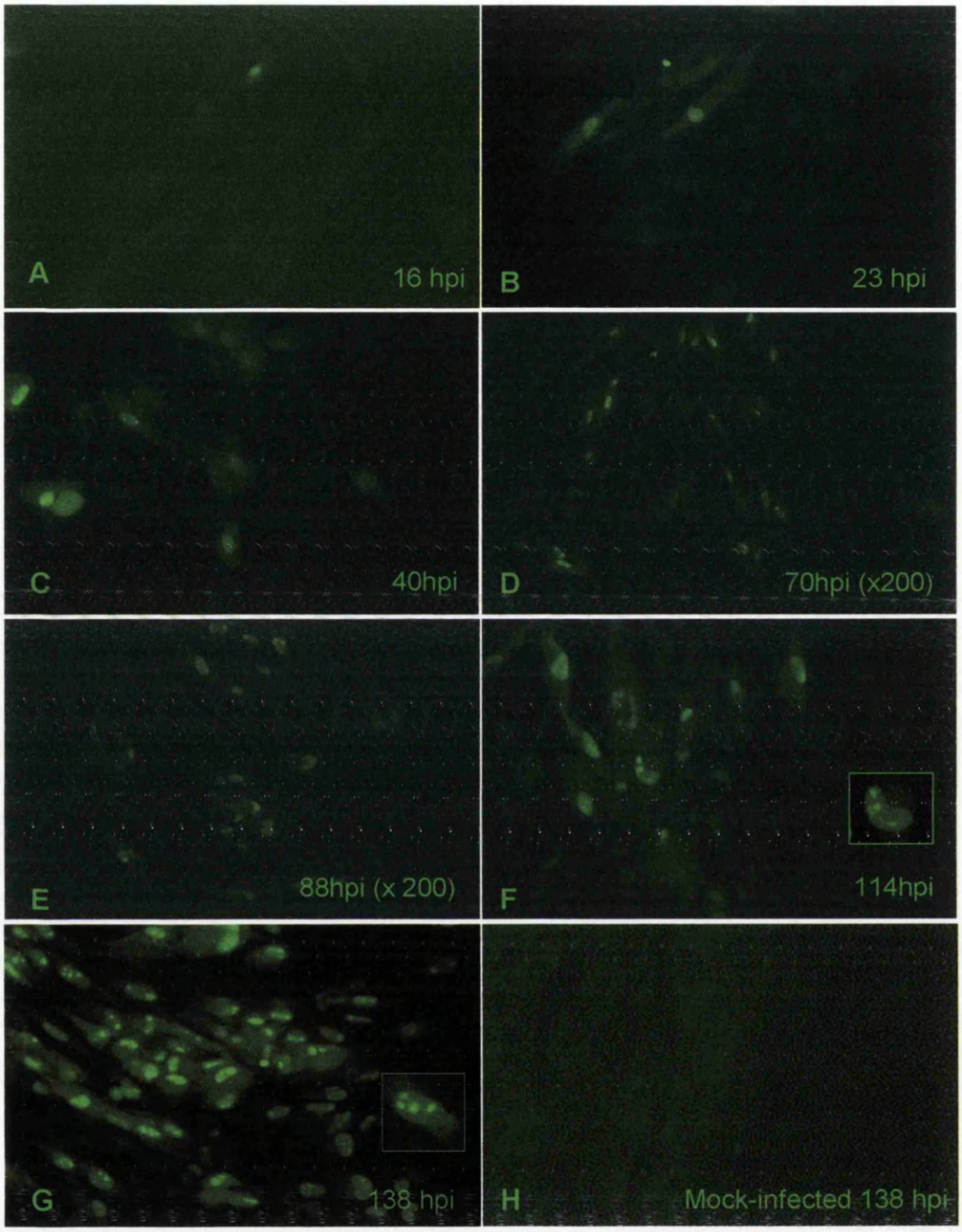


Figure 7.12 Detection of UL102 in HCMV-infected HFFF cells by fluorescence microscopy

HFFF cells were infected with HCMV strain AD169 and then fixed at the hours post-infection (hpi) indicated. Cells were stained with UL102-specific Mab 658 followed by α-mouse FITC conjugated secondary antibody as described in Section 7.2.3.2. Cells were viewed using a Nikon Microphot-SA microscope using a FITC filter at x 400 magnification unless indicated otherwise. Panels A-G are images of HCMV-infected cells fixed at the time points indicated. Panel H is an image of mock-infected HFFF cells at 138 hpi, for comparison with panel G (HCMV-infected cells at 138 hpi). Comparison of panels G and H demonstrates that the fluorescence observed is as a consequence of HCMV infection and expression of UL102.

which localises to the cytoplasm (Calder *et al.*, 1992). This result suggests that UL102 contains an endogenous nuclear localisation signal (NLS). Two putative NLS, whose sequences closely resembled those of typical established NLSs (Garcia-Bustos *et al.*, 1991), were readily identified within the N-terminal 486 residues of UL102 as well as two less typical potential NLSs in the C-terminal region of the protein (see section 7.1).

In order to investigate the location of the UL102 NLS, the truncated UL102 proteins 545N and 330C, which span the 545 N-terminal and the 330 C-terminal residues of UL102, respectively, were expressed in BHK cells to determine their intracellular localisations. As the 545N UL102 protein contained both typical putative NLS, it was anticipated that it would localise to the nucleus. Unexpectedly, it displayed an exclusively cytoplasmic localisation. In contrast, the 330C, which contains less typical candidate NLS, displayed a nuclear localisation in BHK cells.

Whilst the identification of a sequence, or sequences which resemble the deduced NLS from a known nuclear protein may offer a preliminary indication that a protein may localise to the nucleus, it is clear from studies on NLS function that the presence of such a sequence is not necessarily sufficient to confer uptake into the nucleus (reviewed by Garcia-Bustos *et al.*, 1991). One of the first difficulties in identifying putative NLSs within a protein primary sequence is that there is no good consensus sequence for NLSs. The prototypical NLS is considered to be that from the SV40 large T-antigen (PKKKRK), which satisfies the classical criteria of a stretch of generally less than 10 amino acids which contains a high proportion of the basic residues lysine (K) and arginine (R). More recently, alternative forms of NLS have been defined, such as that identified in the nucleoplasmin protein, which consist of two short stretches of basic amino acids separated by a spacer region of 10 amino acids which are not necessarily basic and can be mutated without affecting the function of the NLS. This type of NLS motif, which has been termed as a bipartite nuclear targeting sequence, has been identified in roughly half of nuclear proteins within the Swissprot data base, whereas only 5% of non-nuclear proteins contain such a sequence (Robbins *et al.*, 1991). These findings suggest that bipartite motifs may be a frequently occurring form of NLS. In addition, work carried out on the influenza virus nucleoprotein (NP) has shown that the sequences of the motifs which interact with cellular transport factors and confer nuclear localisation in this protein correspond to SxGTKRSYxxM and TKRSxxxM (Wang *et al.*, 1997). Neither of these motifs bears any obvious homology with either the prototypic SV40 T-Ag or bipartite forms of NLS. Hence, it is clear that

there is a variety of amino acid motifs which can be utilised for nuclear localisation. Another factor which determines the function of a putative NLS is the context and position of the signal. Studies investigating the effect of the NLS positioning within a protein sequence have indicated that the signal needs to be present on an exposed surface of the protein (Roberts *et al.*, 1987) so whilst there may be a typical NLS within the primary sequence of a protein, if it is located within a buried hydrophobic domain then it will be masked and inactivated.

It follows that it is perhaps not surprising that the predicted localisations of the truncated UL102 proteins were not borne out, considering the variety of motifs that may confer nuclear localisation and the factors which influence their functioning. The putative NLSs identified in the 545N protein may actually be hidden in the native protein, alternatively they may possibly function in conjunction with another putative NLS within the C-terminal region of UL102 to confer nuclear localisation, but are inactivated as a result of misfolding of the truncated protein. The putative signals located within the C-terminal 330 residues of UL102, although less akin to classical NLS may act synergistically to mediate entry of this part of the protein. It is known that the effects of several weak NLS can be additive. Establishing which, if any, of the identified putative NLS within UL102 is responsible for transport into the nucleus could be achieved through mutagenesis of each in turn and assessing the intracellular localisation of the expressed mutant proteins. It is possible that other sequences within UL102, without obvious homology to SV40 T-Ag NLS, may be involved in conferring nuclear localisation to UL102. Therefore future work on resolving the localisation of the UL102 NLS should involve re-examining the aa sequence of UL102 to identify sequences with homology to bipartite NLS. If any homologous sequences were identified then mutational analysis could be performed to assess their role in translocation of UL102 to the nucleus.

7.4.2 Localisation of UL102, UL105 and UL70 proteins expressed in BHK cells

The localisations of all three HCMV helicase-primase proteins when expressed individually was investigated. As described in section 7.4.1, UL102 was found to localise to the nucleus when expressed alone. However, both UL70 and UL105 displayed a cytoplasmic localisation when expressed on their own in BHK cells, in common with their HSV-1 counterparts, UL52 and UL5 (Calder *et al.*, 1992). As the helicase-primase proteins ultimately localise to replication compartments within the nucleus, this raises the possibility that UL102 has a role in the nuclear translocation of the other helicase-primase proteins. Hence the effect of co-expression of UL102 with

either the UL105 or UL70 proteins was investigated. The localisation of both UL105 or UL70 was not influenced by co-expression with UL102, as both remained cytoplasmic and displayed no co-localisation with UL102 in the nucleus. Similar results were observed in experiments investigating the effect of co-expression of HSV-1 UL8 on the localisation of the UL5 and UL52 proteins, in which neither protein was found to efficiently co-localise to the nucleus when expressed with UL8 alone (Calder *et al.*, 1992).

Finally, the effect of co-transfecting plasmids expressing UL102, UL105 and UL70 upon the localisation of UL105 and UL102 in BHK cells was examined. As expected, the nuclear localisation of UL102 was unchanged, however the localisation of UL105 changed from cytoplasmic to nuclear, co-localising with UL102. Whilst UL102 is capable of translocating to the nucleus when expressed on its own, both UL105 and UL70 proteins displayed strictly cytoplasmic localisation when expressed individually or when co-expressed with UL102. Only when cells are transfected with plasmids expressing all three proteins does UL105 localise to the nucleus. This result, although preliminary, indicates that for nuclear localisation of the HCMV helicase-primase proteins to occur, all three subunits need to be present. This finding is analogous to the situation in HSV-1, where efficient nuclear localisation of the helicase-primase proteins only occurs when all three subunits are present (Calder *et al.*, 1992). The same finding was reported also for the EBV helicase-primase proteins, BBLF2/3, BSLF1 and BBLF4. Whilst the counterpart of HCMV UL102, BBLF2/3, displays a mixed nuclear/cytoplasmic distribution on its own, the other two subunits, BSLF1 and BBLF4 localise to the cytoplasm when expressed individually. Only when all three proteins are co-expressed do all the sub-units localise to the nucleus (Gao *et al.,* 1998).

Although the expression of UL70 in these cells has not been directly demonstrated in this experiment, the results indicate that the inclusion of the UL70-expressing construct changes the localisation of UL105 from cytoplasmic to nuclear when UL102 is also present. The most likely explanation for the observed change in UL105 localisation is that UL70 is expressed, enabling the formation of the HCMV helicase-primase complex and its subsequent translocation to the nucleus. The dependence of UL105 (and possibly UL70) on the presence of the other sub-units of the complex is consistent with the model proposed for HSV-1, in which the helicase-primase proteins are transported to the nucleus as a complex (Calder *et al.,* 1992). It is noted that the effect of co-expression of UL105 with UL70 on the cellular localisation of UL105 has not been

examined so far, hence it is possible that UL70 may on its own be sufficient to confer nuclear localisation to UL105. However, UL105 was not observed in the nucleus of any cells in the absence of UL102 in this experiment.

If the helicase-primase proteins are transported to the nucleus as a complex and the ability of UL102 to enter the nucleus using a putative NLS is exploited for this purpose, it might be expected that the pair-wise expression of UL102 with UL70 and UL102 with UL105 would result in nuclear localisation of UL70 and UL105, as co-immunoprecipitation studies have demonstrated that the HCMV helicase-primase subunits can form pair-wise associations with each other (McMahon *et* al., 1999). This, however was not observed in these studies. It is possible that the recognition of the putative UL102 NLS, or the context in which it is presented, may be altered when UL102 is complexed with UL105 and UL70, in such a way that confers transport into the nucleus. Another possibility is that the putative UL102 NLS is not responsible for transporting the complex into the nucleus and that a different signal, which is only present or is unmasked when the sub-units are associated is utilised, or possibly that nuclear entry relies on the cumulative effects of several NLSs which are present within the complexed proteins.

In order to establish whether the putative NLSs identified in UL102 are required for nuclear uptake of the complex, mutational analysis of the each putative NLS in could be performed in turn and the ability of the mutated UL102 proteins to facilitate nuclear localisation of the UL105 and UL70 subunits determined by fluorescence microscopy. Where mutation resulted in an inability of either protein to enter the nucleus, the appropriate NLS would have to be transferred to a non-nuclear protein and its intracellular localisation assessed to determine whether the NLS conferred transport into the nucleus and hence demonstrate proof of function.

7.4.3 UL102 in HCMV-infected cells

HCMV- and mock-infected cells were fixed and stained using UL102-specific MAb 658 at various time points up to 138 hpi. UL102 was detected in the nuclei of cells infected with HCMV strain AD169 but not in mock-infected cells, as expected. Initially, the UL102 signal was weak and detectable in only a few cells, which represent the foci of infection (16 hpi). As expected, the number of cells expressing UL102 and the intensity of the UL102 signal increased progressively throughout subsequent time

points as the infection spread throughout the cell monolayer. The pattern of UL102 localisation within the nucleus also altered as the infection proceeded. Whereas at early stages of infection, UL102 appeared to display a diffuse nuclear localisation, by 88 hpi, UL102 localised to distinct compartments within the nucleus. These structures are particularly apparent in the cells stained at 114 and 138 hpi (Figure 7.12, panels G and H), corresponding to times during infection at which active viral DNA synthesis occurs. Within these cells, up to four large globular intranuclear compartments containing UL102 are visible. These compartments appear very similar to the viral replication compartments in HCMV infected HF cells described by Sarisky & Hayward (1996a). These compartments are typical of those associated with active DNA replication in HCMV, as well as other herpesviruses, which contain the viral replication machinery and progeny DNA. Sarisky & Hayward (1996a) visualised these compartments in infected cells by staining for UL44, the HCMV polymerase accessory protein. The nuclear distribution of UL44 which they described at late times in infection matches very closely my findings for UL102 distribution in HCMV-infected cells, as expected.

To confirm that the structures to which UL102 localises are truly viral replication compartments representing sites of active DNA synthesis, the experiment could be repeated, but incorporating a BUdR pulse prior to fixing and immunofluoresence analysis of cells. BUdR is a thymidine analog which is incorporated into growing DNA chains. Hence, if followed by double-immunolabelling of the infected cell monolayer using UL102-specific antisera and anti-BUdR antibody, this approach would determine whether active DNA symthesis occurs in the intranuclear compartments to which UL102 localises.

In contrast to the findings on the localisation of UL44 by Sarisky & Hayward (1996a), I did not observe the localisation of UL102 to smaller intranuclear pre-replicative structures at early times in infection, prior to onset of viral DNA synthesis (16-40 hpi, see Figure 7.12, panels A, B and C). Rather, UL102 displayed a diffuse nuclear distribution. As no other studies on the localisation of UL102 during the course of HCMV infection have been published, no comparisons can be made. However, it is possible that these results indicate that UL102 does not localise to pre-replicative nuclear structures, unlike the other HCMV core replication proteins UL44 and UL57 and the auxiliary protein UL112-113 (Sarisky & Hayward, 1996a; Penfold & Mocarski, 1997).

Smith and Pari, (1995a) performed Northern analysis on HCMV-infected total cell RNA to investigate the time course of UL102 expression. They detected a specific UL102 transcript at 24 hours post-infection but could not detect it at earlier times in infection. My findings suggest that UL102 is transcribed and expressed at earlier stages of infection (16 hpi) in a small number of cells in an infected monolayer. This discrepancy may be due to variations in the sensitivity of the methods used. However, Penfold & Mocarski, (1997) examined the formation of HCMV viral replication compartments by staining infected cells for UL57 and UL44, which are both also early essential replication proteins, like UL102. They detected both proteins at 12 hours post-infection, a time-scale for expression which is more consistent with my findings on UL102.

Chapter 8

Discussion

8.1 Discussion

The set of replication proteins required for origin-dependent HCMV DNA replication include six core replication fork proteins which are conserved amongst the herpesvirus family. So far, the HCMV core replication proteins have not been well characterised, hence their proposed functions and characteristics have been predicted by analogy with their more extensively studied HSV-1 counterparts. The aim of this project was to investigate the properties of UL102, one of the essential HCMV DNA replication proteins, in order to determine whether it shares common properties with its HSV-1 counterpart, UL8, and assess whether it may play a similar role to UL8 during DNA synthesis. The following specific properties of UL102 were investigated:

A) The ability to interact with the HCMV DNA polymerase catalytic subunit, UL54.
B) The DNA-binding properties of UL102.
C) The intracellular localisation of UL102 when expressed alone in mammalian cells and the effect of co-expression with UL102 on the intracellular localisations of the other two HCMV helicase-primase subunits, UL70 and UL105.

In summary, the UL102 characterisation studies presented in this thesis revealed that; i) a specific physical interaction between UL102 and UL54 could not be demonstrated, raising the possibility that the interaction between the helicase-primase complex and the DNA polymerase catalytic subunit may not be common to all herpesviruses, ii) UL102 does not bind DNA or DNA/RNA hybrid oligonucleotide templates which represent the forms of nucleic acid present at the replication fork, iii) unlike HSV-1 UL8, UL102 displays a nuclear localisation when expressed alone in mammalian cells and therefore likely contains an endogeneous nuclear localisation signal (NLS), iv) UL102 does not influence the intracellular cytoplasmic localisations of either UL70 or UL105 when it is co-expressed with either protein in BHK cells, but when it is expressed with both UL70 and UL105 it results in UL105 (and likely also UL70) being transported to the nucleus and v) UL102 localises in HCMV-infected HFFF cells to sub-nuclear regions that might likely correspond to viral replication compartments.

With respect to its DNA-binding ability and the ability to influence the intracellular localisation of the other helicase-primase subunits, UL105 and UL70, when it is co-expressed with either protein in mammalian cells, UL102 was found to behave like its HSV-1 counterpart, UL8. Taken together with the results obtained by McCollum *et al.*, (1999) who demonstrated that UL102, like UL8, is dispensable for the enzymatic activities of the HCMV helicase-primase complex, it is clear that UL102 shares several

characteristics in common with HSV-1 UL8. However, in other respects, most notably the inability to demonstrate a specific interaction between UL102 and the polymerase catalytic subunit UL54, and also the ability to translocate to the nucleus when expressed on its own, UL102 displays characteristics different to UL8. In the light of these findings, UL102 may not perform the identical functions as HSV-1 UL8 during viral DNA replication. As it possesses some characteristics in common with but others that are different from those of UL8, a reasonable prediction is that UL102 performs some but perhaps not all of the roles which have been so far attributed to UL8.

A comprehensive comparison of UL102 and UL8 is not yet possible as many of the biochemical properties of UL102 have still to be investigated and compared to the known properties of UL8. *In vitro* studies have demonstrated that UL8 is necessary for efficient primer utilisation by the HSV-1 polymerase, stimulates primer synthesis and mediates an interaction between the helicase-primase complex and the ss DNA-binding protein UL29, which optimises the utilisation of UL29-coated DNA templates (Sherman *et al.*, 1992; Tenney *et al.*, 1994; Tanguy Le Gac *et al.*, 1996). A thorough study is required to determine whether UL102 modulates the functions of the UL70-UL105 sub-assembly of the HCMV helicase-primase complex and/or influences primer utilisation by the HCMV DNA polymerase. In HSV-1 the UL8-UL30 interaction possibly facilitates the UL8-mediated increased efficiency of primer utilisation by the DNA polymerase, as it would serve to recruit polymerase directly to the site of unwound and newly-primed template DNA. Hence, as a specific physical association between UL102 and UL54 could not be demonstrated in this study, it would be especially pertinent to determine whether UL102 increases primer utilisation by the UL54-UL44 polymerase complex.

The inability to demonstrate an interaction between UL102 and UL54 in the *in vitro* studies presented in Chapter 5 raises the possibility that they do not, in fact, physically interact. This would distinguish UL102 and UL54 from their counterparts in both HSV-1 (UL8 and UL30, respectively) and EBV (BBLF2/3 and BALF5, respectively), which have been shown to interact with each other (Marsden *et al.*, 1997; Fujii *et al.*, 2000),. The demonstration of a homologous interaction in both HSV-1, an alphaherpesvirus and EBV, a gammaherpesvirus, makes it tempting to speculate that it is common to all herpesviruses. Whilst the findings of Fujii *et al.*, (2000) confirmed the interaction between the BBLF2/3 and BALF5 proteins predicted by analogy to HSV-1, it is to be noted that the methodology used was not as stringent as that adopted by Marsden *et al.*, (1997) in demonstrating the HSV-1 UL8-UL30 interaction. An approach involving

immunoprecipitation followed by western blotting was used by Fujii *et al.,* (2000) to demonstrate the BBLF2/3- BALF5 interaction. They detected BALF5 which had co-precipitated with BBLF2/3 by resolving and transferring the captured antibody-protein complexes onto nitrocellulose and probing with an anti-BALF5 antibody. This clearly demonstrates co-precipitation of BALF5 with BBLF2/3 but does not however, illustrate the specificity of the interaction as it gives no indication of the presence of other proteins which may have also co-precipitated with BBLF2/3.

The possibility that UL102 does not physically interact with UL54 is curious considering the predicted structural similarities in the C-terminal regions of HCMV UL102 and HSV-1 UL8. As it has been demonstrated using peptide inhibition studies that the C-terminal 33 residues of UL8 are involved in the interaction with UL30 (Marsden *et al.*, 1997), the similar C-terminal structure predicted for the same region in UL102 is consistent with its putative interaction with UL54. The "predict-protein" programme (Rost, 1996) suggests that the C-terminal regions of UL102 and UL8 both comprise an α-helical region, flanked by residues which are either predicted to form loops or have no predicted structure, summarised in Figure 8.1 (compiled using the sub-set of predicted secondary structures with an expected average accuracy of greater than 82%). Ordering the residues within the predicted helical region onto an α-helical wheel reveals that the helices are amphipathic in nature, meaning that when the α-helical region is viewed from above, one half of the helix is comprised predominantly of hydrophillic residues whilst the other half is comprised predominantly of hydrophobic residues (Nigel Stow, personal communication). The conservation of this specific structure in the same regions of UL8 and UL102 suggests that it may serve a common function and hence it would be of interest to determine whether this region mediates any of the other known functions of UL8 in addition to the interaction with UL30. If so, then this may indicate a possible function for this region of UL102 also.

The predicted UL102 C-terminal structural data is consistent with the epitope mapping of UL102-specific MAbs (section 3.3.2.4), which revealed that a high proportion of the MAbs recognise epitope(s) in the UL102 C-terminal region. Specifically, the C-terminal 25 residues (represented by peptide B1104E, Figure 3.1A) were found to contain the epitope(s) recognised by 12 out of 51 of the UL102-specific MAbs generated, indicating that this region occupies an exposed, exterior position in native folded UL102 and is therefore available for recognition by B-lymphocytes.
As described in Chapter 7, UL102 was found to be distinct from HSV-1 UL8 in its ability

HCMV UL102 (residues 823-873)

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G T A T V S P P S C S S S S A T W L E E R D E W R V R S L A V D A Q H A A R R V A S E G L R F F R L N A
L         L L L L L       H H H H H H H H H H H H H H H H H H H H H H H H H H H H H H H H H H H H   L
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HSV-1 UL8 (residues 700-750)

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R P L V L P P V D C A H H L R E I L R E I E L V F T G V L A G V W G E G G K F V Y P F D D K M S F L F A
L L L L L L L     H H H H H H H H H H H H H H H H H H H H H H H H H     L L L         L L           L
```

Figure 8.1 Summary of predicted secondary structures for the C-terminal regions of the HSV-1 UL8 and HCMV UL102 proteins

The sequence of the C-terminal 52 amino acid residues of both HSV-1 UL8 and HCMV UL102 is given, with the predicted secondary structure of each residue indicated below in blue text. H=helix, L=looped, no symbol underneath indicates that no prediction is made for that particular residue. Predictions were obtained using the 'predict-protein' programme (Rost, 1996).

to translocate efficiently to the nucleus independently of the other helicase-primase subunits, indicating that it contains an endogeneous NLS. As, in contrast, UL105 and UL70 display a cytoplasmic localisation when expressed alone, it is tempting to speculate that UL102 may therefore mediate, by virtue of association, transport of the other helicase-primase subunits into the nucleus. However, although it has been demonstrated that UL102 can form pair-wise associations with both UL105 and UL70 (McMahon *et al.*, 1999), it was not found to alter the localisation of either UL105 and UL70 when co-expresed with either protein as might be expected.

Preliminary results presented in section 7.3.3.3 suggest that UL105 only localises to the nucleus in cells transfected with UL102-, UL70- and UL105-expressing constructs, in a situation analogous to that found with the HSV-1 helicase-primase proteins in which efficient localisation of the helicase-primase proteins only occurs when all three subunits are present (Calder *et al.*, 1992; Barnard *et al.*, 1997). These results indicate that the HCMV helicase-primase proteins may enter the nucleus as a complex, possibly utilising an NLS(s) which is manifested only upon association of the three subunits and raise the possibility that UL102 is perhaps not responsible for nuclear uptake of the helicase-primase complex.

Why then, would UL102 contain a NLS enabling it to translocate to the nucleus independently of the other helicase-primase proteins? One possibility is that UL102 performs another function which requires that it be translocated to the nucleus on its own. This is a very speculative proposition, however evidence that another HCMV replication fork protein, UL44, performs a completely unrelated function in addition to its role as the DNA polymerase accessory subunit during DNA synthesis has recently been presented by Loh et al., (2000). In this study, it was demonstrated that UL44 contains an Arg-Gly-Asp or RGD sequence, a motif which is recognised by cell surface adhesion molecules called integrins, and further that recombinant UL44 could mediate cell adhesion. Although it is not yet known whether this property of UL44 has any functional significance during the course of HCMV infection, the demonstration that an individual protein can potentially perform two extremely diverse roles is nonetheless extremely interesting. It is also of interest to note that Loh *et al.*, (2000) have identified an RGD motif in the sequence of UL102 (residues 58-60).

It was also observed in immunofluorescence studies on UL102 expression in HCMV-infected cells that UL102 does not appear to localise to distinct pre-replicative nuclear structures like other HCMV replication proteins, such as UL44, UL57 and UL112-113

(Sarisky & Hayward, 1996a; Penfold & Mocarski, 1997). It would be interesting to establish whether UL102 does or does not co-localise to pre-replicative structures along with other HCMV replication proteins. This could be achieved by performing immunofluorescence analysis of HCMV-infected cells labelled with antibodies specific for UL102 and another replication protein known to localise to pre-replicative structures, such as UL44. If my preliminary results were corroborated, it would be tempting to speculate that UL102 does not localise to replication compartments until such times in infection when viral DNA synthesis commences (indicated by the formation of large intranuclear viral replication compartments) because it is performing another function at a different cellular location.

The research presented in this thesis demonstrates that several UL102 characteristics are distinct from those of its HSV-1 counterpart, UL8 and furthermore that knowledge regarding the HSV-1 replication fork proteins cannot necessarily be extrapolated to other herpesviruses. It is well established that HCMV is distinct from HSV-1 in several aspects of its molecular biology which are relevant to DNA replication, such as the size and complexity of its origin of replication, orilyt, the apparent lack of a virally encoded origin-binding protein and the fact that it stimulates host cellular protein synthesis when HSV-1, in contrast, induces the opposite effect. It is therefore perhaps not surprising that it may also differ from HSV-1 and other herpesviruses in its precise mode of DNA replication.

The differences in the initiation of DNA replication of HSV-1 and HCMV pose an interesting question as to the sequence and mechanism of recruitment of the essential HCMV replication proteins/complexes to the origin of replication. Following the initial and as yet undefined events at the origin of replication, the helicase-primase complex is most likely the next viral enzyme complex to arrive, being required for essential DNA unwinding and RNA priming activities. In HSV-1, it is likely that the helicase-primase complex is recruited by virtue of the interaction between UL8 and the origin-binding protein, UL9. As no equivalent of HSV-1 UL9 has so far been identified in HCMV, the mechanism of recruitment of the viral helicase-primase remains enigmatic. It is possible that a cellular protein may be involved in the initiation of replication, as HCMV orilyt contains multiple cellular transcription factor binding sites (Anders et al., 1992) and conceivably, motifs which act as binding sites for other, unidentified cellular proteins. In HSV-1, both ori_s and ori_L contain binding sites for a cellular protein known as OF-1, and mutational analysis indicates that binding of OF-1 is required for maximum origin function (Dabrowski et al., 1994; Hardwicke & Schaffer, 1995). The

binding of cellular transcription factor Sp1 to consensus binding sites within ori_S has also been reported to enhance the efficiency of ori_S-dependent replication (Nguyen-Huynh & Schaffer, 1998). Likewise in EBV, Sp1 and another cellular transcription factor, ZBP-89, bind EBV orilyt and stimulate its replication (Baumann et al., 1999). The authors also demonstrated that both proteins interact with the viral DNA polymerase complex (BALF5-BMRF1), and suggest that the interaction may facilitate the formation of the origin-bound viral replication complex. Hence a pertinent experiment may be to perform a two-hybrid screen, using a library produced from an HCMV permissive cell line, such as HFFF with UL102 acting as the 'bait' protein. This may lead to the identification of cellular proteins involved in initiation of replication at orilyt which interact with the helicase-primase complex.

In addition to carrying out two-hybrid screens against a library of proteins derived from a host cellular source, it would also be potentially informative to use a two-hybrid system to investigate putative interactions between UL102, or other helicase-primase subunits, and the HCMV auxiliary replication proteins. There are conflicting reports on the requirements for the auxiliary proteins in origin-dependent HCMV DNA replication (Sarisky & Hayward, 1996a; Ellsmore, 2000), but if any supplies an origin-specific function, then it is reasonable to anticipate it might interact with a component of the helicase-primase complex to facilitate the recruitment of the complex to the origin.

The rationale for this type of investigation is supported by the results of studies involving the EBV helicase-primase complex and auxiliary replication proteins. In similarity with HCMV, maximum efficiency of EBV origin-dependent DNA replication in a transient replication assay is dependent not only on the presence of the six core replication fork proteins but also upon two additional auxiliary proteins, Zta and Rta (Fixman et al., 1992). Rta is not absolutely required but significantly increases replication efficiency. Zta, however is a transactivator which also performs an EBV origin-specific function and is absolutely essential for DNA replication (Fixman et al., 1995). Gao et al., (1998) have shown that Zta interacts with all components of the helicase-primase (BBLF2/3, BSLF1 and BBLF4) and converts the intracellular localisations of myc-tagged BSLF1 and BBLF4 from cytoplasmic to nuclear in a transient transfection assay. Further, they demonstrated that the helicase-primase proteins interact with EBV origin-bound Zta, indicating that the interaction may serve to stabilise an origin-bound replication complex.

An interaction between HSV-1 UL8 and the HSV-1 ss DNA-binding protein, UL29 is

strongly suggested by the work of Falkenberg et al., (1997) and Tanguy Le Gac et al., (1996). Hence by analogy with HSV-1, UL102 may interact with UL57, the HCMV ss DNA-binding protein. This could also be investigated by means of a two-hybrid system, or other means for detection of protein-protein interactions, such as those utilised for the investigation of the putative UL102-UL54 interaction (Chapter 5). If an interaction between UL102 and any other viral or cellular protein was demonstrated, then structure-function analysis of the interaction could be performed using the UL102 reagents described in Chapters 3 and 4, including a panel of UL102-specific MAbs and constructs expressing truncated forms of the UL102 gene.

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Appendix 1

'Predict-protein' analysis of HCMV UL102

PHD results (normal)

```
                ....,....1....,....2....,....3....,....4....,....5....,....6
AA              MTAQPPLHHRHHPYALFGTSCHLSWYGLLEASVPIVQCLFLDLGGGRAEPRLHTFVVRGD
PHD_sec                        E  EE EEE  EEE    EEEEEEEE          EEEEEEE
Rel_sec         99997876778877532111111111211111241157777548887653225887744 6
SUB_sec         LLLLLLLLLLLLLLL.....................EEEEEE.LLLLLL...EEEEE..L

P_3_acc         eeeebeb beebbbbbbebbb bbb bbbebbbebbbbbbbebeee bebeb bbbb ee
Rel_acc         31010100010020522022306340142342205939774112212210120254510 1
SUB_acc         ..............b.......b.b..b..b...bb.bbbb.............bbb...

                ....,....7....,....8....,....9....,....10...,....11...,....12
AA              RLPPAEVRAVHRATYAALASAVTTDADERRRGLEQRSAVLARVLLEGSALIRVLARTFTP
PHD_sec            HHHHHHHHHHHHHHHHHHH  HHHHHHHHHHHHHHHHHHHHH HHHHHHHHHH
Rel_sec         99826687899999999998752559989874577899999999763799999999728 7
SUB_sec         LLL.HHHHHHHHHHHHHHHHH.LHHHHHHH.HHHHHHHHHHHHHH.HHHHHHHHHH.LL

P_3_acc         eb ebeb bbbebbbbbbbbbbbeebeee ee ee bbbbbebbbebbbbbebbbeebbe
Rel_acc         20120251732273137650342020331131051022549074432043907571010 0
SUB_acc         .....b.b...b...bbb..b..........e....bbb.bbb...b.b.bbb.....

                ....,....13...,....14...,....15...,....16...,....17...,....18
AA              VQIQTDASGVEILEAAPALGVETAALSNALSLFHVAKLVVIGSYPEVHESRVVTHAAERV
PHD_sec          EEEE    HHHHHHHHHH   HHHHHHHHHHHHHHHEEEEEE          EEE  HHH
Rel_sec         43663556324675513323215998988898743245788416655663313312135 3
SUB_sec         ..EE.LLL...HHHH.......HHHHHHHHHHH....EEEE..LLLLLL.........H.

P_3_acc         b bebe ebbebbebbeebbbebbbbbebbbbbbbbebbbbbb beb ee bbebebeeb
Rel_acc         20610200132953351021324633015464208728685221022023145000332 3
SUB_acc         ..b........bb..b......bb....bbbb..bb.bbbb..........bb.......

                ....,....19...,....20...,....21...,....22...,....23...,....24
AA              SEEYGTHAHKKLRRGYYAYDLAMSFRVGTHKYVLERDDEAVLARLFEVREVCFLRTCLRL
PHD_sec         HHH  HHHHHHHHHHHHHHHHHHEEE    EEEE   HHHHHHHHHHHHHHHEEHHHHHE
Rel_sec         46314579988652113343111113145213641331899999976332112544312 3
SUB_sec         .H...HHHHHHHH..............L...E.....HHHHHHHH.......H.....

P_3_acc         beebbeebbeeb ebbbbbbbbbbb bbbbe bbeeeeebbbbebbeb ebbbb bbbeb
Rel_acc         35303114024213100820464251121020443012445651312500575402531 3
SUB_acc         .e.....b..e......b..bbb.b.......bb....ebbbb....b..bbbb..b...

                ....,....25...,....26...,....27...,....28...,....29...,....30
AA              VTPVGFVAVAVTDEQCCLLLQSAWTHLYDVLFRGFAGQPPLRDYLGPDLFETGAARSFFF
PHD_sec         E     EEEEEE  HHHHHHHHHHHHHHHHHHHHHH                    EE
Rel_sec         21433479981572345666789999999999722796131466974222222111116
SUB_sec         ......EEEE.LL...HHHHHHHHHHHHHHHHHH..LLL....LLLL............L

P_3_acc         bbbbbbbbbbbbeebbbbbbebbbbbbbebbbebbbe eebee bbeebbeebbb bbbb
Rel_acc         31023478854123186364115200610557110011111131201223200120333 1
SUB_acc         .....bbbbbb....bb.bb..b...b..bbb.........................

                ....,....31...,....32...,....33...,....34...,....35...,....36
AA              PGFPPVPVYAVHGLHTLMRETALDAAAEVLSWCGLPDIVGSAGKLEVEPCALSLGVPEDE
PHD_sec               EEEE  HHHHHHHHHHHHHHHHHHHH      EE    EEE
Rel_sec         89999845774241467799999999999998447621122578143223145448984 2
SUB_sec         LLLLLL.EEE.....HHHHHHHHHHHHHHHH..LL.....LLL........L..LLL...

P_3_acc         eebeebbbbbbbbbbbbbeebbbebbbebbbbbbbeebbebbbebebebbbbbbebeeee
Rel_acc         13101225495244111412261245437651303114500203114122530110121 1
SUB_acc         .......bbbb.bb...b...b..bbb.bbb......bb.......b...b.........

                ....,....37...,....38...,....39...,....40...,....41...,....42
AA              WQVFGTEAGGGAVRLNATAFRERPAGSDRRWLLPRCRVTTATVKTTSWKSARSTGGAHPP
PHD_sec          EEEEEE    EEEEE                                  EEE
Rel_sec         11423315884255522222358888775213799987789998992551447899999 9
SUB_sec         .......LLL..EEE......LLLLLLLL...LLLLLLLLLLLLLL.EE...LLLLLLLL

P_3_acc         bebbbbebeeebb bbbebbee eeeee  bbbeeeeeeeeeeeeeeeeeeeeee ee
Rel_acc         31424111011461506132131112420014116053536726533271151022211 1
SUB_acc         ..b.b......bb.b.b.........e....b..e.e.e.ee.ee...e..e........
```

```
                ....,....43...,....44...,....45...,....46...,....47...,....48
AA              SDDATFTVHVRDATLHRVLIVDLVERVLAKCVRARDFNPYVRYSHRLHTYAVCEKFIENL
PHD_sec             EEEEEEEE    EEEHHHHHHHHHHHHHHHH      EEE E    HHHHHHHHHHH
Rel_sec         98753899996135424422699999999986522579612321123432899999999 8
SUB_sec         LLLL.EEEEEE..L......HHHHHHHHHHHHHH..LLLL..........HHHHHHHHHH

P_3_acc         eeebebbb b ebbbbbbbbbebbeebbbebbeb ebee b bbbebbb bbbeebbebb
Rel_acc         22320605080150110589416711665443161200005023112130468124430 5
SUB_acc         .....b.b.b..b....bbbb.bb..bbbeb..b......b.........bbb..bb..b


                ....,....49...,....50...,....51...,....52...,....53...,....54
AA              RFRSRRAFWQIQGLLGYISEHVTSACASAGLLWVLSRGHREFYVCDGYSGHGPVSAEVCV
PHD_sec         HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH   EEEEEE      E EEEEE
Rel_sec         76489999999999756899989999987766655751353799983656889622289 88
SUB_sec         HH.HHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHHH..L.EEEEE.LLLLLLL...EEEE

P_3_acc          beb ebbbebbebbb bbebbbbbbbbbbbbbbbee eebbbbebbeee ebbbebbb
Rel_acc         20121154207004300643072256423146484411011826001111101126267 5
SUB_acc         ...-..bb..b..b...bb..b..bbb...bbbbbb.....b.b...........b.bbb


                ....,....55...,....56...,....57...,....58...,....59...,....60
AA              RTVVDCYWRKLFGGDDPGPTCRVQESAPGVLLVWGDERLVGPFNFFYGNGGAGGSPLHGV
PHD_sec         EE E HHHHHHH         EEEEE    EEEEE         EEEEE           EE
Rel_sec         72332253587357899981355545896699861522234333554178889766211 3
SUB_sec         E.....H.HHH.LLLLLLL..EEE.LLLLEEEEE.L........EE..LLLLLLLL....

P_3_acc          bbbebbbeebbeeeeeeeebebeeebeebbbbbbee bbebbbbbbbeeeeeebeb bb
Rel_acc         04761415121010123101315221020576501121120031340101002000104 3
SUB_acc         .bbb.b.b.............b......bbbb...........b...........b.


                ....,....61...,....62...,....63...,....64...,....65...,....66
AA              VGGFAAGHCGGACCAGCVVTHRHSGGGGGSGVGDADHASGGGLDAAAGSGHNGGSDRVSP
PHD_sec         E                EEEEEEEE
Rel_sec         23322446566532317898762479875542434133347311224678799876525 7
SUB_sec         .......LLLLL....EEEEEE..LLLLLL.........L......LLLLLLLLLL.LL

P_3_acc         bbbbbbb bbbbbbbbbbbbbe eeeeeeebbbebe beeeebebbbbee eeeeeebbe
Rel_acc         55327610411555636583310044221102004114012022552112011011120 2
SUB_acc         bb..bb..b..bbbb.bbb.....ee........b..b......bb.............


                ....,....67...,....68...,....69...,....70...,....71...,....72
AA              STPPAALGGCCCAAGGDWLSAVGHVLGRLPALLRERVSVSELEAVYREILFRFVARRNDV
PHD_sec                      HHHHHHHHHHHHHHHHHH    HHHHHHHHHHHHHHHHH
Rel_sec         99922357720145531222355677666999863466118999999999999873226 44
SUB_sec         LLL...LLL....LL......HHHHHHHHHHHHH..LL..HHHHHHHHHHHHHH...L..

P_3_acc         e  ebbbbbbbbbbeeebbbbbbbbbb bbbbbeeebbbbebebbbeebbb bbb eeeb
Rel_acc         41011210177732003252754063113010423041211613231176615351001 2
SUB_acc         e........bbb......b.bbb.b.......b...b....b......bbb.b.b.....


                ....,....73...,....74...,....75...,....76...,....77...,....78
AA              DFWLLRFQPGENEVRPHAGVIDCAPFHGVWAEQGQIIVQSRDTALAADIGYGVYVDKAFA
PHD_sec            EEE           E        HHH   EEEEE  HHHH         HHHHH
Rel_sec         32013434785633575653113554431132244476403335641228741102699 9
SUB_sec         ........LLLL..LLLLL....LL...........EE.....HH....LL.....HHHH

P_3_acc         ebbbb b eeeeeb eebbbbbbbbb bbbbeebebbbeb eebbbbebbbbbbbbebbb
Rel_acc         05555101311114100514506600022442031565051205284121034070174 5
SUB_acc         .bbbb........b...b.bb.bb.....bb....bbb.b...b.bb.....b.b..bbb


                ....,....79...,....80...,....81...,....82...,....83...,....84
AA              MLTACVEVWARELLSSSTASTTACSSSSVLSSALPSVTSSSSGTATVSPPSCSSSSATWL
PHD_sec         HHHHHHHHHHHHHHHHH     EE EE   E      EEE     EEE         HHHH
Rel_sec         99999999999999962456422132133112457741333766221328988843215 78
SUB_sec         HHHHHHHHHHHHHHHH..LL.............LLL.....LLL.....LLLLL....HHH

P_3_acc         bbbbbbebbbeebbeeeebbeebbbbbebbbebbeebbbeeeeebebeeeebeeebbebb
Rel_acc         45175714392232120120012300004531210220100200411012120000411 2
SUB_acc         bb.bbb.b.b.................bb.............b...........b...
```

```
                ....,....85...,....86...,....87...,....88
AA              EERDEWVRSLAVDAQHAARRVASEGLRFFRLNA
PHD_sec         HHHHHHHHHHHHHHHHHHHHHHHHH   EEEE
Rel_sec         888999999875699999999875212142439
SUB_sec         HHHHHHHHHHHHHHHHHHHHHHHHH........L

P_3_acc         ee eebb bbbbebe bbeebbeebb bb bee
Rel_acc         331240602562072033423604131251005
SUB_acc         ....e.b..bb..b....e..b.e....b...e
```

Appendix 2

'Predict-protein' analysis of HSV-1 UL8

PHD results (normal)

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                ....,....1....,....2....,....3....,....4....,....5....,....6
AA              MDTADIVWVEESVSAITLYAVWLPPRAREYFHALVYFVCRNAAGEGRARFAEVSVTATEL
PHD_sec              EEEEE  EEEEHHHHHH    HHHHHHHHHHHHHHH    EEEEEEEEEE HHHH
Rel_sec         99984354211133421234114894399999998755443366522257999752465 6
SUB_sec         LLLL..E..............LL..HHHHHHHHHHH....LLL...EEEEEEE..HHH

P_3_acc         eebeebb beebbbbbbbbbbbeeeeeebbebbbbbb eeeeebebebbebbbebeeb
Rel_acc         11110500510565784835330122012171799398901011401526718060231 3
SUB_acc         .....b..b..bbbbbbb.b.........b.bbb.bbb.....e..b.bb.b.b.....


                ....,....7....,....8....,....9....,....10...,....11...,....12
AA              RDFYGSADVSVQAVVAAARAATTPAASPLEPLENPTLWRALYACVLAALERQTGPVALFA
PHD_sec         HHHH      HHHHHHHHHHHH              HHHHHHHHHHHHHHHHHH     EEE
Rel_sec         77416874325799999999516786877779954788999999999999999184325 4
SUB_sec         HH..LLL...HHHHHHHHHHH.LLLLLLLLLLL.HHHHHHHHHHHHHHHHH.LL...E.

P_3_acc         eebeebeebbbbebbbbbebbbebebbbb bbee ebbebbbbbbbbbbe eebe bbbb
Rel_acc         13111001433115125715600003024002010025057254858541010310662 0
SUB_acc         .......b....b..bb.bb.......b........b.bb.bbbbbbb.......bb..


                ....,....13...,....14...,....15...,....16...,....17...,....18
AA              PLRIGSDPRTGLVVKVERASWGPPAAPRAALLVAEANIDIDPMALAARVAEHPDARLAWA
PHD_sec         E EE      EEEEEEE           HHHHHHH      HHHHHHHHHH   HHHHHH
Rel_sec         20113498662687973388999985213778541332356468788774248668899 9
SUB_sec         ......LLLL.EEEEE..LLLLLLLL...HHHH......LL.HHHHHHH...LLHHHHHH

P_3_acc         bbebeee ebbbbbebeeeeeeeeeebb bbbbebebebebeebbbebbbbeeeeeebbbb
Rel_acc         21121210124557221101101301215755131424060005508026311001491 9
SUB_acc         ..........bbbb............bbbb...b.b.b...bb.b..b......bb.b


                ....,....19...,....20...,....21...,....22...,....23...,....24
AA              RLAAIRDTPQCASAASLTVNITTGTALFAREYQTLAFPPIKKEGAFGDLVEVCEVGLRPR
PHD_sec         HHHHHH            EEEEE  HHHHHHHHHHH              HHHHHHHHEE
Rel_sec         99999538841457987489747334543577655389975577732377764541214 5
SUB_sec         HHHHHH.LL...LLLLL.EEE.L...H..HHHHHH.LLLLLLLLL...HHHH.H.....L

P_3_acc         bbbbb eeeeeeeeeebebebbbeebeb bebeebee eeeeebebbbbbebbeeebeee
Rel_acc         19374131231121012081623226150012110010012231112055150001410 4
SUB_acc         .b.bb............b.b....b.b.................bb.b....b..e


                ....,....25...,....26...,....27...,....28...,....29...,....30
AA              GHPQRVTARVLLPRDYDYFVSAGEKFSAPALVALFRQWHTTVHAAPGALAPVFAFLGPEF
PHD_sec             EEEEEEE        EE        HHHHHHHHHHHHHHHHH      HHHHHHE
Rel_sec         99732699998378632133246431259999999999999985388531454431298 65
SUB_sec         LLL..EEEEEE.LLL.......L....HHHHHHHHHHHHHHHHH.LLL...H.....LLLL

P_3_acc         e eeebbbbbbbbeebbbbbbebeebbbbbbbbbbebbbbbb eeeebbbbbbbbbbbeb
Rel_acc         01121439187621050475000210411775776015244300001043299845301 4
SUB_acc         .....b.b.bbb...b.bbb......b..bbbbbb..b.bb.......b..bbbbb...b


                ....,....31...,....32...,....33...,....34...,....35...,....36
AA              EVRGGPVPYFAVLGFPGWPTFTVPATAESARDLVRGAAAAYAALLGAWPAVGARVVLPPR
PHD_sec              EEEEEE              HHHHHHHHHHHHHHHHH          EEE
Rel_sec         77999863699881589104445887314689999999999999928974323215234 78
SUB_sec         LLLLLLL.EEEEE.LLL.....LLLL...HHHHHHHHHHHHHHHH.LLL......E...LL

P_3_acc          eebee bbbbbbbbbb bbbebebebeebeebb ebbbbbbbbebbbbbbbbebbbbbe
Rel_acc         11301200265563301010521051020411380053031101111433263073000 0
SUB_acc         .........bbbb.......b...b....b...b..b..........b...b..b.....


                ....,....37...,....38...,....39...,....40...,....41...,....42
AA              AWPGVASAAAGCLLPAVREAVARWHPATKIIQLLDPPAAVGPVWTARFCFPGLRAQLLAA
PHD_sec                 HHHHHHHHHHH     EEEEEEE          EEEE   HHHHHHHHHH
Rel_sec         88733212147725799999999169992887873544368742563272589999999 9
SUB_sec         LLL.......LL.HHHHHHHHHH.LLLL.EEEEE.L...LLL..EE..L.HHHHHHHHHH

P_3_acc         bbeeebeebbeeb eebeebbbebeeeeebbebbeeebebeeebbb b bbeb bebbbb
Rel_acc         01110011011100215216221113102330642002112110071505006050822 4
SUB_acc         ..............b..b...........bb..........b.b.b..b.b.b..b
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                ....,....43...,....44...,....45...,....46...,....47...,....48
AA              LADLGGSGLADPHGRTGLARLDALVVAAPSEPWAGAVLERLVPDTCNACPALRQLLGGVM
PHD_sec         HHHHHH           HHHHHHHHHH          HHHHHHH    HHHHHHHHHHHHHH
Rel_sec         99998524524689613899988875299981118999874247624699999999853
SUB_sec         HHHHHH..L..LLLL..HHHHHHHHH.LLLL...HHHHHH...LL..HHHHHHHHHHHH.

P_3_acc         bbebbeeb beeebeebbb bebbbbbbbbee bebbbeeb eebeebbebb ebbebbb
Rel_acc         10240201011122111301610364672121051036104011002611480173205 4
SUB_acc         ...b.............b...bbbb.....b...b..b......b..bb..b...bb


                ....,....49...,....50...,....51...,....52...,....53...,....54
AA              AAVCLQIEETASSVKFAVCGGDGGAFWGVFNVDPQDADAASGVIEDARRAIETAVGAVLR
PHD_sec         HHHEEEHHHHH     EEEEEEE              HHHHHHHHHHHHHHHHHHHHHHHHH
Rel_sec         54423212542025216873114431002269963188999999999999999999999
SUB_sec         H.......H....L..EEE...........LLLL..HHHHHHHHHHHHHHHHHHHHHHHH

P_3_acc         bbbbbbbeebbeeeebbbbbbbeebbbbbbeeeeeeeebbbbbbeebeebbeebbebbbe
Rel_acc         32966080103221079972001026452301012100180031123217322241528 0
SUB_acc         ..bbb.b........bbbb......bbb..........b.........b....b.b.b.


                ....,....55...,....56...,....57...,....58...,....59...,....60
AA              ANAVRLRHPLCLALEGVYTHAVAWSQAGVWFWNSRDNTDHLGGFPLRGPAYTTAAGVVRD
PHD_sec         H          EEEEE EEEEE       EEEE                HHHHHHHHHHHH
Rel_sec         81577799860455321023341212201134176787653438897446999999999 9
SUB_sec         H.LLLLLLLL..EE..................LLLLLLL...LLLL..HHHHHHHHHHH

P_3_acc         bebbebeeebeb bebbbbbbbbbbeebbbbbb eeeeeebbbbbb eebeeebeeebee
Rel_acc         10102010111504046433863430001362001210205132211021010803072 2
SUB_acc         ..........b.b.bbb..bb.b......b.........b...........b...b..


                ....,....61...,....62...,....63...,....64...,....65...,....66
AA              TLRRVLGLTTACVPEEDALTARGLMEDACDRLILDAFNKRLDAEYWSVRVSPFEASDPLP
PHD_sec         HHHHHHHHH         HHHHHHHHHHHHHHHHHHHHHH       E
Rel_sec         99999999467899752136899999999999999973388621013478865657996
SUB_sec         HHHHHHHH.LLLLLLL...HHHHHHHHHHHHHHHHHHHH..LLL......LLLLLLLLLL

P_3_acc         bbeebbeebbee eeeeeeebeebbebbbeebbbebbee  ebebbbbeeeeeeeeeebb
Rel_acc         08114511111013121001510352094216700651100101354011111200322 2
SUB_acc         .b..bb............b...b..bb..bb..bb........bb.............


                ....,....67...,....68...,....69...,....70...,....71...,....72
AA              PTAFRGGALLDAEHYWRRVVRVCPGGGESVGVPVDLYPRPLVLPPVDCAHHLREILREIE
PHD_sec                          EEEEEE                          HHHHHHHHHHHHH
Rel_sec         41115764133546995799984699898897624889987789842199999999999
SUB_sec         ....LLL....L.LLLLEEEEE.LLLLLLLLLL..LLLLLLLLLL...HHHHHHHHHHHH

P_3_acc         ebbbeebebbbeeeee ebbbbbeeeeeeeeebbbebbbebbbbbebbbbbbbeebbeebb
Rel_acc         10211030410021210048250222200012111211011233152921182256216 2
SUB_acc         ........b.........bb.b.......................b.b...b..bb..b.


                ....,....73...,....74...,....75
AA              LVFTGVLAGVWGEGGKFVYPFDDKMSFLFA
PHD_sec         HHHHHHHHHHH         E
Rel_sec         99999998663249984101476434331 9
SUB_sec         HHHHHHHHHH...LLL.....LL......L

P_3_acc         ebbbbbbbbbeeeee e e ebeeebebbbe
Rel_acc         05600460300111020011011111643 1
SUB_acc         .bb..bb..................bb..
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