INVESTIGATION OF PROTEIN PRODUCTS ENCODED BY THE HUMAN CYTOMEGALOVIRUS US22 FAMILY GENES UL23, UL24, UL43 AND US22

by

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This thesis is dedicated to John Richard Herdman (1916-2000)

SUMMARY

This thesis reports the investigation of four Human Cytomegalovirus (HCMV) genes (UL23, UL24, UL43 and US22) which belong to the twelve-member HCMV US22 gene family. US22 family genes are specific to the *Betaherpesvirus* subfamily and all *Betaherpesviruses* investigated to date code for a similar number of US22 family members, suggesting that the family plays an important role in the *Betaherpesvirus* life cycle. The family is relatively little studied and the functions provided by family members are in almost all cases unknown.

Antibodies against UL43 and US22 gene products were already available so glutathione-s-transferase (GST)-fusion proteins or synthetic peptides were used to generate antibodies to the HCMV UL23 and UL24 gene products. The anti-UL23 antibodies specifically recognised pUL23 in extracts of both pUL23 expressing bacterial cells and purified HCMV particles. The anti-UL24 antibodies specifically recognised pUL24 in extracts of both pUL24 expressing bacterial cells and in infected human foetal foreskin fibroblast (HFFF-2) cells. The anti-UL43 antibodies specifically recognised pUL43 in extracts of both pUL43 expressing bacterial cells and in infected HFFF-2 cells, while the anti-US22 antibodies specifically recognised pUS22 in infected cell extracts. The apparent molecular mass of pUL23, pUL24 and pUL43 did not differ when made in prokaryotic or eukaryotic cells and in each case was consistent with the size expected from the published DNA sequence.

The UL24 and UL43 gene products were expressed with early-late (E-L) and true-late (L) kinetics, respectively. pUL23 could not be detected in infected cell extracts and therefore the temporal classification of UL23 gene expression could not be determined. Kinetic class designations for pUL24 and pUL43 were confirmed by time course experiments which showed that pUL24 could be detected from as early as 12 h PI, reaching a plateau level at 84 h PI, while pUL43 was not detectable before 60 h PI.

In order to investigate whether pUL23, pUL24, pUL43 and pUS22 were structural or non-structural proteins, HCMV (strain AD169) particles were purified from the culture medium of infected HFFF-2 cells. Three types of particles were resolved in glycerol potassium/tartrate gradients - virions, dense bodies and non-infectious enveloped particles (NIEPs). Western immunoblotting showed that pUL24 and pUL43 were present in all particle types. pUL23 and pUS22 were present in low amounts in virions and dense bodies, and although not investigated, due to low particle numbers, are expected to be present in NIEPs. To confirm that these were virus particle components and not simply associated with co-purifying cell debris, purified virus particles were examined in the electron microscope (EM). The presence of pUL23, pUL24, pUL43 and pUS22 in virus particles was confirmed by immuno-gold tagging of the tegument region of virions and the matrix component of dense bodies. The envelope was removed from purified virus particles by treatment with detergent and pUL23, pUL24, pUL43 and pUS22 each separated with the capsid/tegument fraction, confirming their status as HCMV tegument proteins.

The intracellular location of pUL24, pUL43 and pUS22 in HCMV infected HFFF-2 cells was investigated by immunofluorescence. pUS22 was confirmed to be present in the nucleus throughout infection. In contrast, pUL24 and pUL43 were non-nuclear proteins that accumulated in the perinuclear region, concentrating in a structure juxtaposed to the nucleus. The intracellular distribution of the four proteins was further investigated at the level of cell ultra-structure by immuno-gold thin section EM. With the exception of their presence in cytoplasmic virus particles pUL23, pUL24 and pUL43 were exclusively located in large cytoplasmic protein aggregates, which correlate with the juxtanuclear structure visualised by immunofluoresence. pUS22 on the other hand appeared to be distributed throughout the cell. The protein aggregates manifested in two forms: complex structures which appeared to lack a boundary membrane and smaller, membrane-bound aggregates resembling dense bodies.

It has been suggested that the juxtanuclear structure is a site of HCMV tegument acquisition and particle maturation, and the findings reported here are in agreement with this proposition. The pUL23, pUL24 and pUL43 tegument proteins could only have been

acquired by particles interacting with the protein aggregates and indeed tegumented, but non-enveloped, particles were frequently associated with the complex-type protein aggregates.

While the HCMV UL43 gene is known to be non-essential, it was not known whether or not the UL23 and UL24 genes were required for virus replication *in vitro*. In order to isolate and propagate knockout mutant viruses in essential genes it is necessary to produce complementing cell lines. Retinal pigmented epithelial cells (RPE) and HFF cells immortalised due to expression of either human telomerase reverse transcriptase (hTERT) or the Human Papillomavirus (HPV) type 16 E6/E7 transforming genes, respectively, were employed to construct cell lines expressing pUL23, pUL24 and pUL43. Several cloned hTERT-RPE cell lines constitutively and stably expressing pUL43 were successfully obtained. However, irrespective of the cell line used, no pUL23 or pUL24 expressing line was obtained.

In order to investigate the function provided by the HCMV UL23 and UL24 genes attempts were made to generate UL23 and UL24 deletion mutants. Precise deletions in the UL23 or UL24 open reading frame (ORF) were engineered and replaced with the *E. coli* guanosine phosphoribosyl transferase (gpt) gene, to serve as a selectable marker gene. However, after four rounds of virus replication in the presence of mycophenolic acid and xanthine to enrich for gpt containing virus, followed by four rounds of plaque picking, all of the plaques yielded the wild type DNA fragment profile in Southern blots. Inability to isolate a UL23 or UL24 deletion mutant was probably due to the poor efficiency of transfection of HFFF-2 cells and/or to a low level of recombination between the HCMV plasmid mutation and the wild type virus genome during the initial stages of the experiment.

The most important finding from this investigation was the demonstration that pUL23, pUL24, pUL43 and pUS22 are tegument proteins. Three other US22 family products (pUL36, pTRS1 and pIRS1) are documented tegument components. Thus, at least seven of the twelve US22 family genes code for tegument proteins, suggesting that the products of the remaining five might be similarly located. This demonstrates for the first time a common biological feature among the US22 family members.

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I declare that this thesis consists entirely of my own work, unless specifically indicated. This thesis has not been accepted in any previous application for a degree.

ABBREVIATIONS

aa	Amino Acid
Ap-1	Activator Protein 1
bp	Base Pairs
BSA	Bovine Serum Albumin
CHX	Cycloheximide
Cos	Cosmid
CPE	Cytopathic Effect
CREB	CAMP Response Element Binding protein
DMEM	Dulbecco's Modified Eagles Medium
ELISA	Enzyme-Linked Immunosorbant Assay
EM	Electron Microscopy
ERK	Extracellular–Signal Regulated Kinase
FC	Foetal Calf Serum
FLIPs	Fas associated death domain-like interleukin 1 beta-converting enzyme (FLICE) Inhibitory Proteins
GCV	Ganciclovir
GPCMV	Guinea Pig Cytomegalovirus
gpt	Guanosine Phosphoribosyl Transferase

GST	Glutathione-S-Transferase
HAT medium	Hypoxanthine Aminopterine and Thymidine medium
HFFF-2	Human Foetal Foreskin Fibroblasts
HIV	Human Immunodeficiency Virus
HMBA	Hexamethylene Bisacetamide
HPV	Human Papillomavirus
HRP	Horse Radish Peroxidase
HSV-1	Herpes Simplex Virus – Type 1
hTERT	Human Telomerase Reverse Transcriptase
IFN	Interferon
IPTG	Isopropylthiogalactoside
IRF	Interferon Regulatory Factor
ISG	Interferon Stimulated Genes
ISRE	Interferon-Stimulated Response Element
L-glut	L-Glutamine
MAb	Monoclonal Antibody
MAPK	Mitogen-Activated Protein Kinase
MCMV	Murine Cytomegalovirus
mCP/MCP	Minor/Major Capsid Protein

MHC	Major Histocompatibility Complex
MIEP	Major Immediate-Early Promoter
m.o.i.	Multiplicity of Infection
MPA	Mycophenolic acid
NEAA	Non Essential Amino Acids
NF-kB	Nuclear Factor-kappaB
NIEPs	Non Infectious Enveloped Particles
ORF	Open Reading Frame
PAA	Phosphonoacetic Acid
PAb	Polyclonal Antibody
PCR	Polymerase Chain Reaction
Pep	Anti-Peptide Antibody
p.f.u.	Plaque Forming Units
PI	Post Infection
PMA	Phorbol 12-Myristate 13-acetate
PODs	Promyelocytic Protein Oncogenic Domains
RPE	Retinal Pigmented Epithelial
Sp1	Stimulatory Protein 1
VZV	Varicella-ZosterVirus

Chapter 1

INTRODUCTION

1.1 The clinical problem

Human cytomegalovirus (HCMV), a member of the *Betaherpesvirinae* subfamily of the *Herpesviridae* (Roizman, 1996), is an ancient virus with a global distribution. HCMV infection has been detected even in geographically isolated populations, such as the Brazilian Tiriyo Indians (Britt and Alford, 1996). While there is a relatively high (~95 %) degree of sequence similarity between the different HCMV strains that have been investigated to date, short regions of hypervariable sequence have also been identified, indicating that there may be thousands of strains of HCMV in circulation worldwide. HCMV is estimated to infect ~50 % of the North American population outside urban areas and up to ~90 % within cities. Transmission of HCMV requires direct person-to-person contact; the virus is excreted in most bodily secretions and breast-feeding is thought to be the most common source of transmitted virus (Mocarski, 1996). HCMV is of considerable medical importance. Since Rubella has been controlled by a vigorous vaccination programme HCMV has taken over as the leading cause of neonatal abnormalities, including deafness, physical abnormality and/or mental retardation (Fisher *et al.*, 2000). In the United States it is estimated that about 40,000 infants, congenitally infected with HCMV, are born each year and of these, 4,000 to 6,000 exhibit permanent neurological damage (Britt and Alford, 1996). HCMV also poses a life-threatening risk to immunosuppressed individuals, including allograft recipients and HIV infected patients and is responsible for the distressing blindness (Retinitis) that can affect some AIDs patients. However, the introduction of highly active anti-retroviral therapy (HAART) has decreased the incidence of cytomegalovirus disease in HIV-infected persons by >80 % (Salmon-Ceron, 2001). In recent years animal to human xenotransplantation has been considered a possible solution to the problem of organ supply. One of the major risks in this

procedure is zoonotic transmission of viruses. While animal cytomegaloviruses are generally considered to be species specific it has been reported that at least one animal cytomegalovirus (baboon CMV) was recoverable from a patient following baboon liver transplant in a human patient (Michaels *et al.*, 2001). Baboon CMV has also been shown to be capable of replicating in human fibroblast cells, demonstrating the potential for zoonotic transmission (Michaels *et al.*, 1997).

Resources generated to control HCMV infection in the population include HCMV vaccines and anti-HCMV drugs. The live HCMV (strain Towne) vaccine, which is now commonly used as a laboratory strain, exhibits some biological differences when compared to clinical isolates, including altered growth characteristics *in vitro* and reduced trypsin sensitivity (Britt and Alford, 1996). The Towne strain virus grows poorly in humans and although able to induce humoral and cell mediated immunity in seronegative hosts (Gonczol and Plotkin, 1990), the vaccine induced only a limited protective response when normal volunteers were subsequently challenged with wild-type virus (Plotkin *et al.*, 1989). The limited immunity and lack of understanding of the determinants of viral virulence has hindered widespread use of the vaccine. There is also an understandable reluctance to vaccinate women of child-bearing age with a potentially teratogenic virus in case they may unknowingly be pregnant at the time of vaccination (Britt and Alford, 1996). More recently, attention has focused on subunit vaccines, particularly those directed against the viral glycoprotein gB; the major antigen eliciting viral neutralising antibodies (Speckner *et al.*, 1999).

The anti-HCMV drug ganciclovir, a nucleoside analogue and a derivative of acyclovir, is licensed to treat HCMV induced retinitis in immunocompromised patients but has been associated with myelotoxicity. Foscarnet, a pyrophosphate analogue, has also been used to treat HCMV infections but is nephrotoxic and is used only in life-threatening cases or when other anti-viral drugs are no longer effective (Chan *et al.*, 2001). Clearly, there is a continued need to develop more effective and less toxic anti-HCMV therapies. The molecular genetic investigation of HCMV, in so much as it identifies new potential antiviral targets, will play an important role in facilitating new intervention strategies for HCMV. One herpesvirus characteristic that makes intervention strategies difficult to sustain is the ability of

herpesviruses to establish life-long latent or persistent infections. In the case of the *Alphaherpesvirus*, Herpes Simplex Virus-1 (HSV-1), a latent infection is established in sensory neurons. HCMV latency is associated with bone marrow CD34+ myeloid progenitor cells (Hahn *et al.*, 1998). However, it has not been conclusively established if HCMV establishes a true non-replicating latent infection in a specific cell type, as typified by HSV-1, or persists by low-level replication (Mocarski, 1996). In HSV-1 latently infected cells a specific set of viral RNAs, called Latency Associated Transcripts (LATs) are expressed (Roizman and Sears, 1996). During natural infection with HCMV a small percentage of bone marrow-derived mononuclear cells become latently infected. The latent HCMV genome is distributed at low copy number (2 to 13 genomes per cell) and is in unit-length circular form, which is a characteristic of herpesvirus latent DNA (Slobedman and Mocarski, 1999). HCMV infected granulocyte-macrophage progenitors fail to support productive viral replication but Cytomegalovirus Latency-Associated Transcripts (CLTs) are expressed from the major immediate-early gene promoter (MIEP) locus. There are sense CLTs, coded for in the same direction as productive phase transcripts but using two novel start sites in the MIEP, and anti-sense CLTs, coded for in the opposite direction and complementary to IE1 exons 2-4. The sense and anti-sense CLTs code for proteins of 94 amino acids (aa) and 152 aa, respectively (Kondo *et al.*, 1994; Kondo *et al.*, 1996). Sense CLTs are expressed during *in vitro* HCMV productive infections of dTHP-1 cells, (monocyte derived macrophages), endothelial cells and human fibroblasts (Lunetta and Wiedeman, 2000). They are also expressed during the majority of natural productive infections, and antibodies to both the 94 aa and 152 aa proteins can be detected in serum from seropositive individuals (Kondo *et al.*, 1996). The finding that HCMV CLTs are also expressed during productive infections is in accord with the finding that the HSV-1 major 2.0 kilo base pairs (kbp) LAT is also expressed during acute infection of ganglia, but at lower abundance than during latent infection (Lunetta and Wiedeman, 2000).

Due to species specificity no direct animal model exists for HCMV. Nevertheless, murine cytomegalovirus (MCMV) has been extensively used as a surrogate system, from which parallels can be inferred for HCMV in studies designed to investigate viral transmission, pathogenesis, acute infection and/or reactivation after immunosuppression, transfusion or

transplantation (Xiao *et al.*, 2000). However, since MCMV does not cross the placenta, its utility as a model for congenital HCMV infection is questionable. More recently, however, Guinea pig cytomegalovirus (GPCMV) has been reported to be a more accurate model for HCMV foetal infection, since this virus does cross the placenta and gives rise to infections that share many biological similarities with those of HCMV (McGregor and Schleiss, 2001).

1.2 Organisation of the HCMV genome and functions of viral genes

The HCMV double stranded linear DNA genome (~230 kbp) is larger than that of any other known herpesvirus. Like HSV-1, the HCMV genome has a class E organisation and so exists in the form of four genome isomers. The genome is organised as two unique regions, US and UL, which are flanked by inverted repeats sequences which permit the genome segments to invert relative to each other. One orientation was arbitrarily selected as the prototype (P) arrangement, with the others generated by inversion of the short unique sequences (IS), the long unique region (IL) and both the short and long regions (ISL). It remains to be determined whether each of the genomic inversions of HCMV is equally viable (Mocarski, 1996). Recent evidence from an HCMV (AD169) Bacterial Artificial Chromosome (BAC) produced in *E.coli* (strain DH10B), where only two of the four possible isomers were generated, showed that both isomers were infectious when transfected into HFF cells. Due to the absence of terminal fragments it was not possible to identify the two isomers that were present (Borst *et al.*, 1999).

The complete DNA sequence (229,354 bp) of HCMV strain AD169 was published by Chee *et al*, (1990), and the genome was predicted to encode at least 208 Open Reading Frames (ORFs) of more than 100 amino acids in length. ORFs were numbered sequentially and given a prefix identifying their location in the genome (e.g. TRL, UL, IRL, IRS, US or TRS) (Figure 1). In some cases, families of related ORFs appear to have been generated by gene duplication events. Nine different gene families have been recognised: RL11, US6, US22,

GCR, UL25, UL82, US1, US12 and US2. The nine gene families are represented by colour coding in Figure 1. The number of 'real genes' coded for by HCMV has been difficult to determine, but it is clear that the AD169 sequence is incomplete compared to clinical isolates. The laboratory strains of HCMV (AD169, Towne, Davis) all lack a number of genes that are present in the UL/b' region of the genome in the low passage Toledo strain and in clinical isolates. Even Toledo, however, differs from clinical isolates in that the sequences in the UL/b' region are in inverse orientation (Cha *et al.*, 1996) and because Toledo, like the other lab strains, lacks the endothelial cell tropism exhibited by fresh clinical isolates (Bolovan-Fritts and Wiedeman, 2001). There are at least two isolates of AD169 currently in circulation, the sequenced genotype (Chee *et al.*, 1990) and the more common genotype that contains an additional 929 bp sequence in the UL42/UL43 region (Dargan *et al.*, 1997).

Comparison between the published DNA sequences of HCMV and other *Betaherpesviruses* has proved a very powerful tool for evaluation of proposed ORFs, assessment of gene conservation, and identification of functional elements in particular proteins. Such comparisons provide the core data for viral evoluntionary studies (Davison, 1993). Recently, the presumed entire gene content of HCMV (i.e. strain AD169 sequence plus the Toledo UL/b' region sequence) has been re-evaluated by comparison to the chimpanzee CMV sequence (241,087 bp). The estimated number of ORFs encoding protein (i.e. 'real genes') contained in the putative wild type HCMV genome has now been rationalised to 164 to 167 genes (Dr. Andrew Davison, personal communication.). The DNA sequences of several *Betaherpesviruses* have been reported and compared with that of HCMV (strain AD169). The DNA sequence of MCMV (Smith strain) has been published and shown to consist of a 230,278 bp unique sequence with short direct repeats at either end (class F organisation). At the genetic level there are significant similarities to HCMV strain AD169, particularly in the 78 centrally located ORFs. Homologues of the HCMV UL25, UL82, US22 and GCR gene families are coded for by MCMV but there appear to be no homologues of the HCMV RL11, US1, US2, US6 or US12 gene families (Rawlinson *et al.*, 1996). The 229,896 bp DNA sequence of rat CMV (Maastricht strain) has been reported and contains all but one of the ORFs that are conserved between MCMV and HCMV (Vink *et al.*, 2000). The DNA sequence of human herpesvirus 6 (HHV-6) (strain U1102) has been published and contains

102 predicted genes, arranged co-linearly with those of HCMV (Class A organisation). The HHV-6 genome (159,321 bp) is smaller than that of HCMV, but both genomes are densely packed with minimal splicing. HCMV and HHV-6 appear to be closely related exhibiting approximately 67 % homologous proteins, however, at the ends of the genome the HHV-6 homology with HCMV diverges and the HCMV glycoprotein families RL11, US6 and US12 are not present in HHV-6 (Gompels *et al.*, 1995). The human herpesvirus 7 (HHV-7) (strain RK) genome (144,861 bp) is very closely related to that of HHV-6 and is predicted to encode 84 genes (Megaw *et al.*, 1998). Most recently, the DNA sequence of the *Tupaia* (Tree Shrew) herpesvirus, another *Betaherpesvirus*, was published (Class F organisation). Analysis of the 195,857 bp sequence predicted 158 genes, of which 76 genes had significant homology to genes in HCMV. Six gene families were recognised, although there appear to be no homologues of some US22 family genes (TRS1, IRS1, UL24 or UL43) (Bahr and Darai, 2001).

The HCMV genome contains a set of over 40 conserved herpesvirus-common 'core' genes (Figure 1 and Table 1). These are arranged in blocks that maintain relative position and orientation in the genomes of different herpesviruses and reflect evolution from a common ancestor (Davison, 1993). Few herpesvirus genes are spliced and overall the genomes have relatively few polyadenylation signals. The following HCMV genes have been predicted to be spliced: UL22A (Rawlinson and Barrell, 1993), UL36-38 (Kouzarides *et al.*, 1988), UL65 (Davis and Huang, 1985), UL83 (Ruger *et al.*, 1987), UL89 (Costa *et al.*, 1985), UL111A (Lockridge *et al.*, 2000), UL112-113 (Wright *et al.*, 1988), UL115-116, UL117-118 (Leatham *et al.*, 1991), UL122-123 (Stenberg *et al.*, 1989), US3 (Chan *et al.*, 1996) and US6 (Jones and Muzithras, 1991). The products of at least 45 HCMV ORFs (TRL4-TRL14, UL1-UL10, UL16, UL18, UL20, UL33, UL128, IRS1, US1-US13, US27; UL24; UL36; UL42 and UL43 (Mocarski, 1996; Dr. Peter Ghazal personal communication; Patterson and Shenk, 1999; Dargan *et al.*, 1997, respectively)) are dispensable for virus growth in cell culture. Overall, the products of a large number (approximately 70 %) of HCMV ORFs have not been identified. The assignment of gene function to many HCMV ORFs is based only on sequence similarity to apparently homologous genes in HSV-1. In the case of dispensable viral genes, investigation of HCMV gene function has been inhibited due to the lack of a direct HCMV

animal model system. Molecular genetic investigation of the virus is made problematic by the slow replication kinetics of the virus, the friable nature of the naked HCMV DNA and the poor transfection efficiency of HCMV permissive cell lines (i.e. Human Foetal Fibroblasts (HFF)). Very few human cell lines are fully permissive for HCMV infection and HFF cells, which give the highest yields of infectious progeny, are associated with deletion of viral sequences in the UL/b' region of the genome upon prolonged passage of the virus.

Figure 1. Schematic representation of gene arrangement on the HCMV strain AD169 genome. Broad bands represent repeat regions (TRL/IRL; IRS/TRS), thin bands represent unique long (UL) and unique short (US) regions. The nine gene families are colour-coded. Size of blocks relates to predicted size of ORFs (Chee *et al.*, 1990). Spliced genes are marked with thin line. Direction of blocks indicates direction of transcription. The core genes (red) are contained within the UL region. Diagram courtesy of Dr. Andrew Davison


0 5 10 15 20 25 30 35 40 45 50
J1L 1 2 3 4 5 6 7 8 9 10 11 12 13 TRL14 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36
50 55 60 65 70 75 80 85 90 95 100
37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69
100 105 110 115 120 125 130 135 140 145 150
70 71 72 73 74 75 76 77 78 79 80 80.5 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100 101 102 103
150 155 160 165 170 175 180 185 190 195 200
104 105 106 107 108 109 110 111 111A 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 IRL14 13 12 11 10 9 8 7 6 54 3 2 1 J1I IRS1 1 2 3 4 5 6 7 8 9 10 11
200 205 210 215 220 225
12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 TRS1 J1S
RL11 family
US22 family
UL25 family
UL82 family
US1 family
US2 family
US6 family
US12 family
US28 family
other non-core
core

Table 1. HCMV herpesvirus 'core genes' and homologous genes in HSV-1.

HCMV gene	Function, where known in HCMV	HSV-1 gene	Function, where known in HSV-1 (Umene, 1998)
	Control:		
UL69 (E-L)	*Trans*-activator (Winkler *et al.*, 2000)	UL54, ICP27 (I-E)	Multifunctional regulatory protein; stimulation of viral DNA synthesis; early-late transition; host shutoff; inhibition of host RNA splicing; nucleotidylylated; phosphorylated (essential)
	Nucleotide metabolism:		
UL45 (?)	Homologue of large subunit of ribonucleotide reductase (RR). Small subunit of RR not encoded, presumed non-functional (Lembo *et al.*, 2000)	UL39, ICP6 (I-E)	Ribonucleotide reductase large subunit (essential)
UL72 (E-L)	Predicted 388aa dUTPase, but lacks active site sequence (McGeoch, 1990).	UL50 (E)	Deoxyuridine triphosphatase, dUTPase (non essential)
UL114 (E)	Uracil-DNA-glycosylase (non-essential) (Prichard *et al.*, 1996)	UL2 (E)	Uracil-DNA glycosylase (non essential)
	DNA replication:		
UL105 (E)	Component of DNA helicase-primase complex (essential) (Pari and Anders, 1993)	UL5 (E)	Component of DNA helicase-primase complex; possesses helicase motifs (essential)
UL102 (L)	Primase associated factor (essential) (Pari and Anders, 1993)	UL8 (E)	Component of DNA helicase-primase complex (essential)
UL70 (E-L)	Component of DNA helicase-primase complex (essential) (Pari and Anders, 1993)	UL52 (E)	Component of DNA helicase-primase complex (essential)
UL57 (E)	Single-stranded DNA-binding protein	UL29, ICP8 (E)	Single-stranded DNA-binding protein (essential)
UL54 (E)	DNA polymerase. (essential) (Pari and Anders, 1993)	UL30 (E)	DNA polymerase (essential)
UL44 (E-L)	DNA polymerase processivity factor. (Ripalti *et al.*, 1995) (essential) (Pari and Anders, 1993)	UL42 (E)	DNA polymerase processivity factor (essential)
	Capsid components:		
UL86 (E-L)	Major capsid protein (MCP). HCMV pUL86 forms hexamers and pentamers of capsid shell.	UL19, VP5 (E-L)	Major capsid protein (MCP); forms hexamers and pentamers of the icosahedral shell (essential)
UL85 (E-L)	Minor capsid protein (mCP); with pUL46 forms intercapsomeric triplex	UL18, VP23 (E-L)	Minor capsid protein (mCP); with VP19c forms intercapsomeric triplexes (essential)
UL49A (?)	Small capsid protein (SCP) (Borst *et al.*, 2001)	UL35, VP26 (L)	Small capsid protein (SCP) present on outer tip of VP5 hexons but not pentons (non essential)
UL46 (E-L)	Minor capsid binding protein (mC-BP), with pUL85 forms	UL38, VP19c	Minor capsid protein with VP23 forms intercapsomeric triplexes (essential)

	intercapsomeric triplex. No aa similarity to HSV-1 VP19c (Gibson *et al.*, 1996)	(L)	
UL80 (L)	Protease, containing 5 conserved sites. Auto-proteolytic cleavage at sites 643 and 256, then at 143 for de-activation	UL26, VP24 and VP21 (L)	Serine protease cleaving itself and UL26.5; VP24 (N-portion and protease domain of UL26) and VP21 (C-portion of UL26 and involved in scaffold formation of B capsid) (essential)
UL80A (L)	Assembly (scaffolding) protein for cleavage maturation in the nucleus. Self interaction, via N-terminal sequences, important for interaction with MCP (Oien *et al.*, 1997)	UL26.5, VP22a (E-L)	Internal protein of immature capsids; assembly protein involved in scaffold formation of B capsids (non essential)
	Tegument components:		
UL99, pp28 (L)	Localised to surface of cytoplasmic capsids (Wing and Huang, 1995)	UL11 (E-L)	Myristylated tegument protein; role in envelopment and transport of virions (non essential)
UL97 (E-L)	Serine/threonine kinase. Phosphorylates ganciclovir (GCV). (essential) (Prichard *et al.*, 1999)	UL13, VP18.8 (E-L)	Protein kinase (non essential)
UL48 (L)	Structural protein	UL36, VP1/2 (L)	Very large tegument protein, ~273 kDa; required for release of viral DNA from capsids (essential)
UL77 (E)	Predicted 642aa	UL25 (E-L)	Required for DNA cleavage/packaging; influences penetration
	Envelope components:		
UL55, gB (E)	Role in cell entry, binds heparin sulphate. Binding to ligand upregulates cellular transcription factors Sp-1 and NF-kB (Yurochko *et al.*, 1997) (essential) (Britt and Mach, 1996)	UL27, gB (E)	Role in cell entry. Binds heparan sulphate; fusion; binds tegument proteins including VP16 (essential)
UL75, gH (E-L)	Mediates fusion of the virus envelope during virus entry (essential) (Mocarski and Courcelle, 2001)	UL22, gH (L)	Heterodimer; complexes with gL; role in cell entry (essential)
UL115, gL (L)	Complexes with gH, role in cell entry (probably essential)	UL1, gL (E-L)	Complexes with gH; role in cell entry. (essential)
UL100, gM (E-L)	Integral membrane protein, complexes with gN (Mach *et al.*, 2000)	UL10, gM (L)	Probable integral membrane protein. Complexes with gN (non essential)
UL73, gN (E-L)	Complexes with gM (Mach *et al.*, 2000). Highly polymorphic	UL49A, gN (L)	Possible membrane glycoprotein. Complexes with gM (non essential)
UL50 (?)	Predicted 397aa	UL34 (?)	Membrane-associated phosphoprotein (essential)
	DNA packaging and morphogenesis:		
UL56 (E)	Terminase subunit. 130 kDa protein, interacts with pUL89. (Buerger *et al.*, 2001)	UL28, ICP18.5 (E)	Required for DNA cleavage/packaging. Role in capsid maturation. (essential)

UL51 (?)	Predicted 157a/a protein	UL33 (?)	Role in capsid assembly/DNA cleavage and packaging (essential)
UL98 (E-L)	DNA, alkaline exonuclease (Sheaffer *et al.*, 1997)	UL12 (E)	DNA alkaline nuclease involved in maturation/packaging of progeny genomes (non essential)
UL104 (E)	Predicted 697aa	UL6 (E-L)	DNA cleavage/packaging; constituent of nucleocapsid but not required for capsid formation. Possibly portal protein (essential)
UL87 (?)	Predicted 941aa	UL21 (E-L)	Role in virion morphogenesis (non essential)
UL89 (E-L)	Terminase subunit (Underwood *et al.*, 1998)	UL15 (E-L)	Possible terminase? Required for DNA cleavage/packaging (essential)
	Unknown Function:		
UL47 (E-L)	Predicted 982aa	UL37 (E-L)	Possible interaction with ICP8 (UL29) (essential)
UL103 (L)	Predicted 249aa	UL7 (E-L)	Unknown (not known if essential)
UL95 (E-L)	Predicted 531aa	UL14 (E-L)	Unknown (not known if essential)
UL94 (L)	p53, plays a central role in regulation of UL94 promoter (Wing *et al.*, 1998). Function unknown	UL16 (E-L)	Unknown (non essential)
UL93 (L)	Predicted 594aa	UL17 (E-L)	Unknown (essential)
UL76 (?)	Predicted 325aa. Regulatory protein (Wang *et al.*, 2000b)	UL24 (E)	Unknown, syncytium formation (non essential)
UL53 (E)	Predicted 376aa	UL31 (L)	Unknown; nucleotidylylated phosphoprotein (essential?)
UL52 (L)	Predicted 668aa	UL32 (L)	Required for DNA cleavage/packaging (essential)
UL71 (?)	Predicted 411aa	UL51 (E-L)	Unknown (non essential)

HCMV gene function is provided (with reference where applicable) and the function of the homologous gene in HSV-1 is provided (Umene, 1998). HCMV genes are only classified as essential or non-essential where this has been specifically demonstrated. Kinetic Class is given in parentheses (Immediate-Early, I-E; Early, E; Early-Late, E-L; Late, L).

1.3 The virus replication cycle

1.3.1 Kinetic class of virus gene expression

The replication cycle of HCMV is slow compared to that of other herpesviruses, and infectious virus progeny are not detected before 48-72 h PI (Mocarski, 1996). The transcription of all herpesvirus genes occurs in a tightly regulated cascade and therefore genes can be classified into kinetic classes: immediate-early, early and late.

Immediate-Early (I-E) genes are transcribed by host cell RNA polymerase II and are the first viral gene products made in the infected cell. Four immediate-early gene loci (UL36-38, IE1/IE2, TRS1/IRS1, US3) are expressed from non-continuous regions on the HCMV genome (Mocarski, 1996). IE2, the major transactivator, operates co-operatively with IE1 and accessory proteins (e.g. UL36-38, TRS1, IRS1) to regulate early and late gene expression.

Early (E) genes are the second set of viral genes to be transcribed. Transcription requires the presence of immediate-early proteins and is not affected by inhibitors of viral DNA replication, such as phosphonoacetic acid (PAA) or ganciclovir (GCV). Early gene products generally code for enzymes and proteins involved in DNA synthesis, packaging and maturation.

Late genes are the last set of viral genes to be transcribed and generally encode structural proteins. Late genes are divided into two subsets; 'early-late' (E-L) transcripts are first detected 12-36 h PI and proteins are made only in minor amounts in the presence of viral DNA inhibitors; 'true-late' (L) protein accumulation is strictly dependent on viral DNA replication.

While this simplistic scheme provides a convenient system by which individual HCMV genes can be grouped, in truth the reality is more complex, as there is a delay between transcript production and protein synthesis in the case of some genes, indicating that translational

control mechanisms contribute to overall regulation of gene expression. For example, expression of gpUL4 is repressed at the translational level by the presence of a short upstream open reading frame (uORF2) within the UL4 transcript leader. Thus, although the UL4 mRNA is detectable at 6 h PI and reaches a plateau after 24 h PI the UL4 protein product is detectable only at 48 h PI. Mutation of uORF2 eliminates ribosomal stalling and results in abundant early expression of gpUL4 (Alderete *et al.*, 2001).

1.3.2 HCMV induced effects on intracellular signalling

Binding of HCMV particles, via envelope glycoproteins, to cell surface receptors and co-receptors results in the immediate stimulation of several cellular signalling pathways, including the mitogen-activated protein kinase (MAPK) pathway, which leads to an increase in nuclear factor NF-kB binding to DNA, and a phospholipase C (PLC) pathway, which leads to Ca^{2+} release and second messenger activity (Fortunato *et al.*, 2000). Binding of glycoprotein gB to its cell surface receptor results in activation of a subset of host cell genes normally induced by interferon (IFN)-α, a response normally associated with the cell anti-viral response (Navarro *et al.*, 1998; Boyle *et al.*, 1999; Preston *et al.*, 2001), although the virus utilizes a different signalling pathway to that normally induced by IFN-α. Indeed, the virus-induced signalling mechanism does not lead to nuclear localisation and assembly of ISGF3, but rather leads to the formation of a novel complex, that binds the interferon stimulated reponse element (ISRE) and which contains interferon-regulatory factor 3 (IRF-3) and CREB-binding protein (Fortunato *et al.*, 2000). The benefit the virus derives from induction of this subset of IFN-inducible cellular genes immediately after infection is unknown. Later in infection, the cells become refractory to IFN-α mediated stimulation and several cellular genes, including major histocompatibility complex (MHC) Class I, IRF-1, MxA and 2'5'-oligoadenylate synthetase are affected. HCMV infection is also known to repress IFN-γ signal transduction, including repression of IFN-γ induced upregulation of MHC Class II gene transcription. If cells are infected prior to exposure to IFN-γ, a dramatic reduction in the signalling factors JAK 1

(Janus Kinase 1) and p48 is responsible for the down-regulation of MHC Class II transcription (Sedmak *et al.*, 1994; Miller *et al.*, 1998). However, if cells are exposed to IFN-γ prior to, or during, infection then down-regulation of MHC Class II transcription is achieved by a different signalling mechanism, involving inhibition of transcription of Class II Transactivator (CIITA), which itself is required for the *trans*-activation of the MHC Class II gene (Le Roy *et al.*, 1999).

Infection of fibroblasts with HCMV results in stimulation of extracellular–signal regulated kinases (ERKs), due to inhibition of a cellular phosphatase. Stimulation of ERKs activity appears to be bi-phasic. At early times (within the first 24 h PI) ERK activity is transient and dependent on viral immediate-early gene expression. At late times PI, ERK2 activation may also be induced by binding of the T cell chemokine RANTES to the HCMV US28 CC-chemokine receptor expressed on the surface of infected cells, resulting in an increase in intracellular Ca^{2+}, and presumably second messenger activity (Billstrom and Worthen, 2001). Thus, the kinase activity of ERKs appears to be required at all stages of HCMV replication. ERK kinase is responsible for phosphorylation of another cellular protein, p90 ribosomal kinase (p90RSK), which operates to up-regulate cAMP-response element binding protein (CREB) and to inactivate CDC2/cyclin B regulatory kinase MYT1 and thus has a role in establishing an optimal cellular environment for virus growth (Fortunato *et al.*, 2000; Richards *et al.*, 2001). ERKs are also involved in protein/protein interactions with viral gene products, since ERK2 has been shown to phosphorylate IE2 (Harel and Alwine, 1998).

Binding of HCMV to a host cell receptor induced the immediate (maximum levels 15-30 min PI) activation of phosphatidylinositol 3-kinase (PI3-K), a protein kinase involved in intracellular signalling pathways. A second tier of PI3-K activation, which followed viral I-E protein expression was detected at 4 h PI (Johnson *et al.*, 2001). PI3-K is responsible for activation of the cellular transcription factor, NF-kB, and the protein kinases Akt and p70S6K. The phosphorylated form of Akt has been shown to be involved in inhibition of apoptosis (Kandel and Hay, 1999). In uninfected cells the NF-kB heterodimer (p65 and p50 subunits) is cytoplasmic and bound to an inhibitor molecule, IkB (Baldwin, 1996). Following stimulation of a kinase pathway, IkB is phosphorylated and degraded in an ubiquitin-mediated

manner. NF-kB then translocates to the nucleus, binds DNA and activates many genes, including several anti-apoptosis genes (Romashkova and Makarov, 1999). Johnson *et al.*, (2001) hypothesise that PI3-K, induced by HCMV infection, phosphorylates and degrades IkB, resulting in activation of NF-kB, which may then bind the HCMV MIEP to induce viral I-E protein synthesis. The importance of the early stimulation of PI3-K for HCMV replication was shown by experiments in which PI3-K activity was specifically inhibited. There was a decrease in I-E and E gene expression and DNA replication was strongly inhibited, resulting in a reduction in infectious virus progeny of ~10,000 fold (Johnson *et al.*, 2001). Thus, in some host cells at least, the mitogenic pathway involving PI3-K activity plays an important role in facilitating HCMV lytic cycle replication.

HCMV infection generally results in arrest of the cell cycle progression at the G_1/S transition point, although cells infected during the S phase can complete the cycle before arresting in at the next G_1 (Fortunato *et al.*, 2000). HCMV mediated control over cell cycle progression operates by controlling expression of several key regulatory proteins, including cyclins A, B, D and E (Salvant *et al.*, 1998) and by eliminating the function of the tumour suppressor proteins, p53 (Fortunato and Spector, 1998) and retinoblastoma protein, Rb (Poma *et al.*, 1996). In synchronised cells infected in G_0, cyclin D1 levels are decreased, while the level of the mitotic cyclin (cyclin B) is markedly increased at early times PI. The levels of cyclin E and its associated kinase are increased, and consequently the cell moves into G_1. Cyclin E activation is critical, since inhibition of its kinase, CDK2, blocks HCMV replication (Bresnahan *et al.*, 1997). The activation of cyclin E is dependent on viral early gene expression and is associated with changes in the Rb-related protein p130 (McElroy *et al.*, 2000). Infected cells are maintained at the G_1/S transition and are unable to replicate their DNA because the predominant S-phase cyclin (cyclin A) is expressed in negligible amounts and is barely detectable.

1.3.3 The major stages in the HCMV replication cycle

Following cell surface binding and membrane fusion, the de-enveloped virus enters the cytoplasm, at which stage it may shed some tegument proteins. HCMV can penetrate many cell types, including epithelial cells, fibroblasts, smooth muscle cells, endothelial cells and macrophages. Undifferentitated, transformed or anueploid cells are non-permissive for HCMV lytic replication. Most peripheral blood monocytes are also non-permissive, although a latent infection can occur, but upon their differentiation to macrophages the cells then become permissive and the virus reactivates (Sinzger and Jahn, 1996). In some cases HCMV inter-strain differences in endothelial cell tropism have been shown to be due to differences in the ability of the virus to transport the infecting capsids towards the nucleus, rather than to differences in the ability of the virus to enter cells *per se* (Sinzger *et al.*, 2000). Such inter-strain differences presumably lie in genes encoding tegument or capsid proteins. By analogy with HSV-1, it is likely that HCMV nucleocapsids are transported along microtubules to dock at a nuclear pore, where the viral DNA is released into the nucleus (Sodeik *et al.*, 1997).

The linear HCMV genome circularises upon its entry into the nucleus and becomes associated with a region in the nucleus known as a promyelocytic protein (PML)-oncogenic domain (POD) (also known as ND10). PODs are spherical structures with an average diameter of 0.3 to 0.5 μm, consisting of a central core surrounded by an electron-dense capsule. Several dozen PODs form in the nucleus of most cells and are located within inter-chromatin regions and associated with the nuclear matrix, although their function in normal uninfected cells is unknown (Wilkinson *et al.*, 1998; Drouin *et al.*, 2001). In HCMV infected cells, the periphery of PODs has been implicated as the site of initiation of immediate-early gene transcription. A nuclear compartment, where DNA replication occurs, is also associated with the periphery of PODs (Ishov *et al.*, 1997).

Compared to other herpesviruses there is a significant delay before HCMV DNA synthesis is initiated, presumably caused by the slow build up of early gene products, some of which are required for viral DNA replication. It is thought that herpesvirus DNA replicates by a 'rolling circle' mechanism, generating long concatamers, which are subsequently cleaved to unit

length for packaging. It is assumed that recombination and genome inversion also occur between concatemeric DNA molecules. Eleven HCMV genes (UL36-38, UL44, UL54, UL57, UL70, UL84, UL102, UL105, UL112-113, IE1/IE2, TRS1/IRS1) are essential for transient complementation of oriLyt-dependent DNA replication (Pari and Anders, 1993). Several of these genes are herpesvirus core genes (Table 1) and homologous to HSV-1 genes required for viral DNA replication, including the DNA polymerase (UL54), polymerase processivity factor (UL44), single stranded DNA binding protein (UL57) and the three protein components of the helicase-primase complex (UL70, UL102, UL105). The functions of the remaining HCMV genes (UL36-38, UL84, UL112-113, IE1/IE2, TRS1/IRS1) required for DNA replication in the transient replication system are not fully understood, but HCMV DNA replication may require more than the six herpesvirus-common replication functions.

Concatemeric virus DNA is cleaved to unit length by the activity of the terminase enzyme (coded for by HCMV genes UL56 and UL89) and packaged into pre-formed capsids, which are translocated to patches in the inner nuclear membranes, where they may acquire some tegument proteins before envelopment by budding through the inner nuclear membrane into the lumen of the nuclear membrane. Passage of the virus from the nucleus to the cell surface has been the topic of much research and forms part of the discussion section of this thesis.

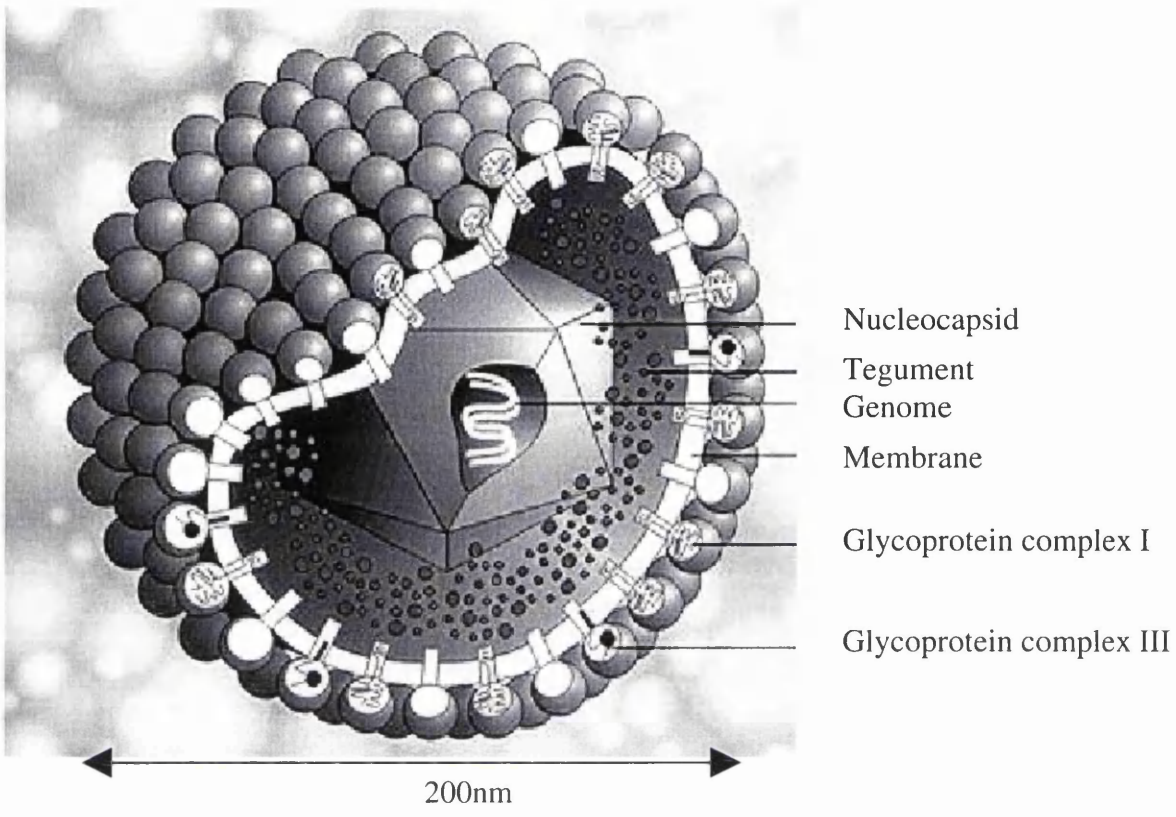


Figure 2. Cut away diagram of the HCMV virion. The envelope and tegument is cut away to reveal the iscosahedral nucleocapsid. Surface glycoprotein complexes are shown as spheres. Diagram obtained from www.biografix.de, with permission from Dr. Marko Reschke.

1.4 Structural components of HCMV particles

Three types of virus-related particles are released into the extracellular medium from HCMV infected cells in culture. These are the mature infectious virion and two non-infectious particles; the so-called non-infectious enveloped particle (NIEP) and the dense body. Dense bodies and NIEPs can be distinguished from virions and each other by morphology in the electron microscope and by biochemical analysis. Dense bodies, which are present only in the cytoplasm of infected cells, lack the nucleocapsid and DNA of the virion and are predominately composed of a single tegument protein pp65 (pUL83) (Irmiere and Gibson, 1983). Whether dense bodies are produced during *in vivo* infection or whether they represent an artefact of the *in vitro* culture systems is controversial. In support of the latter view, it has been noted that dense bodies are not produced in tissue culture cells infected with MCMV (Mocarski, 1996). It has been suggested that dense bodies may consist of surplus cellular and viral products accumulated in a storage vacuole that is subsequently voided into the extra-cellular medium (Severi *et al.*, 1992). NIEPs are similar to virions in protein composition and particle morphology. However, the capsid structure lacks the DNA genome, and instead contains an additional capsid shell scaffolding protein, which is absent in the mature infectious particle (Irmiere and Gibson, 1983). As with dense bodies, it is not clear whether NIEPs are made *in vivo* and these may also be produced as an artefact of the *in vitro* culture system.

As has been reported for HSV-1 (Roizman and Sears, 1996), several forms of HCMV nucleocapsid structures (A, B and C capsids) can be identified in infected cells. Type A capsids are empty and lack the DNA genome, whereas B capsids also lack the genome but contain the scaffolding proteins, are present in the nucleus but are not yet enveloped. Type C capsids are present in the cytoplasm of infected cells and in fully mature enveloped virions (Gibson, 1996). Herpesvirus virions (Figure 2) are composed of the double-stranded viral

DNA genome packaged inside the icosahedral-shaped capsid, which is embedded in a complex amorphous protein layer known as the tegument, and the whole then enclosed within a lipid bilayer envelope decorated with surface viral glycoprotein spikes (Roizman, 1996). To date, approximately 30 HCMV genes are reported to code for products that are contained in the virion particle. A similar number of HSV-1 genes (at least 34) have also been reported to code for particle proteins (Rixon, 1993; Steven and Spear, 1997).

1.4.1 The capsid

The icosahedral capsid composed of 162 capsomeres (150 hexons and 12 pentons) is a group characteristic of herpesviruses. However, certain features of the capsid shell can vary between members of the family. The HCMV capsid shell is larger than that of HSV-1 (diameters of 130 nm and 125 nm, respectively) and has to accommodate a genome that is some 37 % larger than HSV-1. A comparison of HCMV and HSV-1 capsid shell architecture showed differences in hexamer spacing and relative tilt, in morphology at the tips of the hexons, and in the average diameter of the scaffold (Butcher *et al.*, 1998). While the internal volume of the HCMV capsid was some 17 % larger than that of HSV-1 this was insufficient, of itself, to account for packaging of the larger HCMV genome and it was subsequently shown that the HCMV genome was more densely packed than that of HSV-1 (average inter-layer spacing of 23 Å, versus 26 Å, respectively) (Bhella *et al.*, 2000).

Assembly of the herpesvirus capsid shell occurs in the nucleus and in HSV-1 involves the products of six genes that encode seven proteins; five of which constitute the capsid structure [UL19 (VP5), UL38 (VP19c), UL18 (VP23), UL35 (VP26), UL26 (VP24)] and two scaffolding proteins that are required for capsid assembly but do not form part of the final capsid structure [UL26 (VP21) and UL26.5 (VP22a)] (Oien *et al.*, 1997). Homologous genes and proteins are coded for by HCMV and other herpesviruses (Table 1). The HCMV capsid shell contains at least four proteins: the major capsid protein (MCP) (pUL86), the minor capsid protein (mCP) (pUL85), mCP binding protein (mC-BP) (pUL46) and the small capsid

protein (SCP) (UL48/49) (Gibson, 1996). The HCMV MCP, a 1,370 amino acid protein, is the main protein constituent of the capsid, comprising the pentamers and hexamers of the capsid. The HCMV mCP and mC-BP are present in the ratio 2:1 and constitute the inter-capsomeric triplex, that links adjacent capsomeres, and are the homologues of HSV-1 VP23 and VP19c. The HSV-1 VP26 protein is not essential for the production of a stable capsid shell structure and its location at the tips of the hexon is thought to direct tegument attachment (Zhou *et al.*, 1995). The HCMV SCP is less well studied than its HSV-1 counterpart (VP26), but in contrast to VP26, the SCP has been shown to be essential for generation of infectious progeny in tissue culture cells (Borst *et al.*, 2001). The role of capsid shell proteins in interaction with tegument proteins is relatively little studied. However, it has been demonstrated that HCMV tegument proteins are attached to the pentons, hexons and triplexes of HCMV capsids (Chen *et al.*, 1999). HSV-1 tegument proteins have also been shown to be attached to the pentons (Zhou *et al.*, 1995).

The HCMV 'Assembly protein' (pUL80A), is a 36kDa scaffolding protein, derived from the carboxyl-terminal half of the HCMV UL80 85kDa gene product. It is one of four proteins that are auto-proteolytically cleaved from pUL80 by the HCMV protease activity, which is located within the amino-terminal half of pUL80. pUL80A, like its HSV-1 counterpart pUL26.5, forms a scaffold around which the capsid shell is assembled. Self-interaction, via N-terminal sequences, are required for scaffold assembly, and C-terminal sequences are required for interactions with the MCP (Oien *et al.*, 1997). Once assembled, protease cleavage of a short C-terminal region allows the scaffolding proteins to vacate the nucleocapsid, leaving space to accommodate the viral genome. As no DNA molecule is packaged in NIEPs the pUL80A remains associated with the capsid, suggesting that pUL80A may be directly involved in packaging of the genome. Since pUL80A is not a component of the mature virion this protein too must be removed at some stage during genome packaging.

1.4.2 The envelope

The envelope of HCMV particles is composed of viral proteins embedded in a host derived membrane (Mocarski, 1996). Compared to other herpesviruses, HCMV codes for a relative abundance (~50) of putative glycoprotein (gp) genes, many of which appear to have no homologues in other herpesviruses (Chee *et al.*, 1990). Most of the glycoproteins encoded by the virus have yet to be studied and the functions they provide are unknown. It is not even yet clear which are expressed on the surface of the infected cell and/or in the virion envelope.

At least eight HCMV glycoproteins with homologues in other herpesviruses are known to be involved in forming glycoprotein complexes (gcI, gcII and gcIII) in the virion envelope. HCMV gB (gpUL55) (~150 kDa) is the major envelope glycoprotein and homodimeric molecules of gB are linked by disulphide bonds to form glycoprotein complex gcI. HCMV gB binds to heparin sulphate on the cell surface and is essential for virus penetration, fusion of infected cells and cell to cell spread of infection (Boyle and Compton, 1998; Singh and Compton, 2000). The nascent HCMV gB protein is proteolytically cleaved into two components (a 446 amino acid 55 kDa C-terminal fragment and a 460 amino acid 93 kDa N-terminal fragment), which remain coupled by disulphide bonding (Mocarski, 1996).

HCMV gB is the major antigen for induction of complement-dependent neutralising antibodies and is an important candidate for generation of a subunit vaccine. Vaccine development, however, is complicated by the finding that at least four major genotypes of gB exist in the population, along with several rare variants (reviewed in Pignatelli *et al.*, 2001). Nearly all HCMV infected individuals develop anti-gB antibodies, targeted primarily to the AD-1 region (amino acids 552-635). Neutralization reaches 50 % efficiency at best, indicating that during natural infection the virus may escape the immune system by inducing production of non-neutralizing gB antibodies, or by producing viruses with variant gBs (Speckner *et al*, 1999).

Another abundant and important constituent of the HCMV envelope is the glycoprotein complex gcII, which consists of gM (gpUL100) and gN (UL73) (Mach *et al.*, 2000). It is

probable that the gcII complex contains more than a single gM/gN dimer (Hobom *et al.*, 2000). The gcII complex binds heparin and is thought to play a role in virus entry and membrane fusion. Homologues for gM and gN are known for HSV-1, where they also form a complex. However, while the gM/gN complex was essential for replication of HCMV, it was not essential for HSV-1, although the HSV-1 mutants replicated to lower titres and exhibited reduced virulence *in vivo* (MacLean *et al.*, 1993).

HCMV glycoproteins gH (gpUL75), gL (gpUL115) and gO (gpUL74) form complex gcIII, which is involved in membrane fusion and virus penetration. Homologues for gH and gL are also coded for by HSV-1 where they are essential genes with a function in virus release and entry (Desai *et al.*, 1988). gH is also a target for complement-independent anti-CMV neutralising antibodies (Mocarski, 1996). While complexes gcI, gcII and gcIII all promote membrane fusion, the mere binding of gB or gH by themselves to cell surface receptors induced signal transduction resulting in a increase in the levels of the Sp1 and NF-kB transcription factors (Yurochko *et al.*, 1997).

The envelope of HCMV particles also contains gene products that exhibit homology to G-coupled receptors (GCRs) (UL33, UL78, US27, US28) and C-C chemokine receptors (US28) (Margulies *et al.*, 1996; Zipeto *et al*, 1999; Beisser *et al*, 2001). GCRs are known to transduce extracellular signals to alter intracellular processes and the HCMV GCR genes may function similarly during the initial virus binding stage of infection. The HCMV envelope associated C-C chemokine receptor (US28) remains expressed on the surface of latently-infected THP-1 monocytes. It has been suggested that circulating infected monocytes expressing US28 *in vivo* might promote dissemination of HCMV by adhering to CX_3C expressing endothelial cells (Beisser *et al.*, 2001).

Table 2. HCMV tegument proteins

HCMV Gene (Kinetic Class)	Function of HCMV Protein	Homologues in *Betaherpesviruses*					*α-herpes*
		MCMV	HHV-6	HHV-7	Rat	Tupaia	HSV-1
UL18 (L)	Glycoprotein. Immune evasion, homologue of MHC Class I	m144			r144		
UL25 (L)	Phosphoprotein (Zini *et al.*, 1999)	M25	U14	U14	R25	T25	UL9?
UL26 (E)	Phosphoprotein (Baldick and Shenk, 1996)	M26			R26	T26	
UL32, pp150 (L)	Phosphoprotein. (Baxter and Gibson, 2001)	M32	U11	U11	R32	T32	
UL36 (I-E)	Phosphoprotein Viral inhibitor of Fas mediated apoptosis (vICA) (Skaletskaya *et al.*, 2001)	M36	U16/ U17	U16/ U17	R36	T36	
UL47 (E-L)	Phosphoprotein. Unknown. function (Baldick and Shenk, 1996).	M47	U30	U30	R47	T47	UL37
UL48 (L)	Phosphoprotein. Unknown. Function. HSV-1 homolog needed for release of DNA from capsid	M48	U31	U31	R48	T48	UL36
UL56 (E)	Terminase subunit (Giesen *et al.*, 2000a)	M56	U40	U40	R56	T56	UL28
UL65	Phosphoprotein Protein Kinase (Davis *et al.*, 1984)						
UL69 (E-L)	Phosphoprotein. *Trans*-activator of MIEP (Winkler and Stamminger, 1996)	M69	U42	U42	R69	T69	UL54
UL77 (E)	HSV-1 homolog role in capsid assembly.	M77	U50	U50	R77	T77	UL25
UL82 (pp71) (L)	Phosphoprotein *Trans*-activator of MIEP (Liu and Stinski, 1992)	M82	U54	U54	R82	T82	
UL83 (pp65) (E-L)	Phosphoprotein Protein kinase? (Schmolke *et al.*, 1995)	M83	U54	U54	R83	T82	
UL87 (?)	HSV-1 homolog nucleotidylated virion protein	M87	U58	U58	R87	T87	UL21

UL88	Phosphoprotein. Unknown. function (Baldick and Shenk, 1996)	M88	U59	U59	R88	T88	
UL94 (L)	Phosphoprotein. Unknown function	M94	U65	U65	R94	T94	UL16
UL97 (E-L)	Phosphoprotein. Serine/thronine kinase (Wagner *et al.*, 2000)	M97	U69	U69	R97	T97	UL13
UL99, pp28 (L)	Phosphoprotein. Unknown function. HSV-1 homolog required for virus envelopment	M99			R99	T99	UL11
IRS1 (I-E)	Phosphoprotein *Trans*-activator of MIEP (Romanowski *et al.*, 1997)	M143	U95	U95			
TRS1 (I-E)	Phosphoprotein *Trans*-activator of MIEP (Romanowski *et al.*, 1997)	M143	U95	U95			

Members of the US22 gene family are underlined. HCMV functional homologues in MCMV (Rawlinson *et al.*, 1996), HHV-6 (Gompels *et al.*, 1995), HHV-7 (Megaw *et al.*, 1998; Nicholas, 1996), Rat CMV (Vink *et al.*, 2000), *Tupaia* (Tree Shrew) herpesvirus (Bahr and Darai, 2001) and *Alphaherpesvirus* HSV-1 (α) (Umene, 1998) are provided.

1.4.3. The tegument

The tegument of HCMV particles, which constitutes ~40 % of the virion protein mass, contains a high proportion of phosphoproteins (Table 2) (Gibson, 1996). Most of the HCMV tegument proteins listed in table 2 have homologues in other *Betaherpesviruses* and the *Alphaherpesvirus*, HSV-1, implying an important herpesvirus conserved function/s. Several genes (e.g. UL32, UL36, UL82, UL88) appear to have no homologues in HSV-1, but have homologues in the other *Betaherpesviruses*, indicating an important *Betaherpesvirus*-specific function.

The HCMV tegument contains in excess of 20 proteins (Table 2) representing a significant genetic investment in this structure. Investigation of the role of the tegument and its importance for HCMV replication is at an early stage. It seems probable, however, that the function provided by tegument proteins will be complex and possibly inter-related and

multifunctional. Some tegument proteins may perform key architectural roles or function to facilitate virus egress. Others, delivered to cells at the time of infection, may operate prior to immediate-early protein synthesis, to induce a favourable intracellular environment for lytic replication in the infected cell. Such tegument proteins may be determinants of tissue tropism or be involved in abrogating host defences. The HCMV tegument contains several proteins, including pp71 (pUL82), pUL69 and pTRS1/pIRS1 that can *trans*-activate the virus I-E genes and/or other viral or cellular promoters. *Trans*-activation by these proteins is described in section 1.6.

The major HCMV tegument components are pp65 (pUL83 or 'lower matrix protein') and pp150 (pUL32 or 'basic phosphoprotein'), which are both highly immunogenic (Gibson, 1996; Mocarski, 1996). pp65 is an abundant virion protein (~18 % of total virion protein mass) and is the main protein constituent of dense bodies (~95 % of particle mass) (Irmiere and Gibson, 1983). Surprisingly, this abundant protein appears to be dispensable (Schmolke *et al.*, 1995). After infection with virus or dense bodies in cells treated with CHX (i.e. prior to immediate-early protein synthesis) pp65 localises to the nucleus, although its function at this time is as yet not understood (Hensel *et al.*, 1995). The UL83 gene is *trans*-activated by IE1/IE2, via a mechanism that involves an octamer sequence in the UL83 promoter, to express pp65 as an early-late protein (Depto and Stenberg, 1989). Following its translation, pp65 is present in the nucleus of infected cells throughout the replication cycle, but at late times PI is also found in the cytoplasm (Hensel *et al.*, 1995). pp65 is strongly associated with the nuclear matrix and accumulates along the nuclear periphery where it is associated with nuclear lamins (Sanchez *et al*, 1998). The UL83 product exhibits serine/threonine protein kinase activity, (although it has no homology to any other known protein kinase) and is capable of both autophosphorylation and phosphorylation of the major immediate early protein IE1. It is thought that phosphorylation of IE1 prevents cleavage of IE1 into peptides that are bound by MHC Class I and presented at the cell surface where they would be recognised by T cells (Gilbert *et al.*, 1996). pp65 also binds a cellular protein, 'Polo-like kinase activity' (PIK1), and may affect intracellular signalling by altering PIK1 activity, nucleotide preference, substrate specificity or sub cellular location (Gallina *et al.*, 1999).

The UL32 gene product (pp150) is a tegument protein that is essential (Zipeto *et al.*, 1993) for virus replication and constitutes about 20 % of the total virion mass. pp150 is post-translationally modified by O-linked glycosylation, (a modification frequently associated with proteins that localise to nuclear membranes) and is one of the most immunogenic proteins present in the virus particle. HCMV seropositive individuals have high titres of anti-pp150 antibodies (Gibson, 1996; Mocarski, 1996). pp150 could be detected in the nucleus of infected cells at early times PI (up to 48 h) where it was associated with both viral assembly compartments and the nuclear membrane, but at 72 h PI it was exclusively cytoplasmic (Hensel *et al.*, 1995). In contrast, more recent investigations have failed to detect pp150 in the nucleus at all, but have shown it to be present in the cytoplasm throughout infection, accumulating in a stable juxtanuclear structure (Sanchez *et al.*, 2000b). The function of pp150 is unknown but, like its Simian CMV (SCMV) gene homologue, HMCV pp150 is tightly bound to the capsid (Trus *et al.*, 1999; Chen *et al.*, 1999) by an interaction involving the MCP and the conserved sequence in the N-terminus of the pp150 protein (Baxter and Gibson, 2001). Given its intimate interaction with the capsid structure it is likely that pp150 provides an anchor for attachment of additional tegument proteins that subsequently build around the particle. If pp150 is acquired as a tegument protein in the nucleus (Hensel *et al.*, 1995) then it is possible that it might also play a role in the nuclear egress of particles.

The UL99 gene is expressed with true-late kinetics to yield pp28, a highly immunogenic protein that is a component of the virion tegument (Landini *et al.*, 1987). In infected cells pp28 was exclusively located in the cytoplasm in a compartment that overlaps the ER-Golgi-intermediate compartment (Sanchez *et al.*, 2000a). Thus, virus particles must acquire pp28 tegument protein from a site in the cytoplasm. The MCMV M99 gene (homologue of HCMV UL99) was also expressed with late gene kinetics producing a protein that is also a tegument component (Cranmer *et al.*, 1994). The function of pp28 is unknown, but its homologue in HSV-1, (gene UL11), produces a N-terminal myristylated tegument protein, exclusively located in the cytoplasm and has roles in virus envelopment and virion particle translocation (Loomis *et al.*, 2001).

In HCMV infected cells pp28, pp65 and pp150 and three envelope glycoproteins (gB, gH, gp65) all localised to the same cytoplasmic juxtanuclear structure. This structure only developed late in infection when production of progeny virus was increasing rapidly. Indeed, pp28 levels were not readily detectable until this structure had formed (Sanchez *et al.*, 2000b). The derivation of the juxtanuclear structure is unclear, but it was not derived from components of the ER, ER-Golgi-intermediate compartment, *cis* or medial Golgi, or lysosomes, but it did partially colocalise with the *trans*-Golgi network and appeared to lie in close proximity to the microtubule organising centre. Based on their observations, Sanchez *et al*, (2000b), proposed that this accumulation of tegument and envelope proteins in the juxtanuclear structure might represent a cytoplasmic site of HCMV particle tegumentation and maturation. In this regard it is of interest to note that the mature cleaved form of gB was found within the cytoplasmic juxtanuclear structures, suggesting retrieval of gB from a cytoplasmic compartment distal to the Golgi. This is deduced because gB cleavage is accomplished by furin, within the late secretory/endocytic vesicles (Vey *et al.*, 1995).

pUL25 is an 82 kDa tegument protein expressed with true-late kinetics and is a major target for the anti-HCMV antibody response (Baldick and Shenk, 1996; Zini *et al.*, 1999; Lazzarotto *et al.*, 2001). The MCMV M25 gene (homologous to HCMV UL25) also encodes a tegument protein. However, M25 is additionally associated with chromatin in the nucleus of infected cells and may function as a transcriptional *trans*-activator (Dallas *et al.*, 1994; Wu *et al.*, 1999). In contrast, HCMV pUL25 appeared to be present only in the cytoplasm of infected cells where it localised to large dense-body-like structures in the cytoplasm (Zini *et al.*, 1999). It seems probable that the dense-body-like structures described by Zini *et al.*, (1999), are the same cytoplasmic juxtanuclear structures described by Sanchez *et al.*, (2000b), in which the pp150, pp28 and pp65 tegument proteins have all been detected.

UL56 is a herpesvirus core gene, homologous to HSV-1 gene UL28 (Table 1). Both are essential genes that code for a subunit of the viral terminase enzyme (Buerger *et al.*, 2001). Terminase activity is required for the cleavage of concatameric DNA, an essential step in packaging of the viral genome. pUL56 contains nuclear localisation signals and is transported into the nucleus by cellular importin. pUL56 can be detected in the infected cell nucleus from

about 12 h PI where it is associated with virus replication compartments (Giesen *et al.*, 2000a; Giesen *et al.*, 2000b). pUL56 exhibits endonuclease activity *in vitro* and this is probably the function it provides in the terminase complex during HCMV DNA packaging. HCMV pUL56 binds the AT rich core sequence in *pac*1 (5'-TAAAAA) and *pac*2 (5'-TTTTAT), sequences contained within the repeat *'a'* sequences, to form a protein-DNA complex (Bogner *et al.*, 1998). Unexpectedly, pUL56 was demonstrated to be a structural protein separating with the capsid/tegument fraction when virions were de-enveloped by treatment with detergent (Bogner *et al.*, 1993). Given its function as part of an enzymatic terminase activity this is a counter-intuitive finding – more so since its partner protein, pUL89, has not been reported as a structural component. It is possible that pUL56, by itself, remains bound to the genomic termini after cleavage and is packaged along with the genome. This might explain the absence of pUL56 in dense bodies (Bogner *et al.*, 1993). However, even this seems an unlikely explanation since only two molecules of pUL56 could be packaged/virion and it seems unlikely that the sensitivity of Western immunoblotting would be sufficient to detect this. Dubious thought it might appear, the HSV-1 homologue (UL28) of HCMV UL56 has also been reported to be a virus particle component (Taus and Baines, 1998).

UL94, a herpesvirus core gene (Table 1), expresses a 36 kDa protein with true-late kinetics. The function provided by this tegument protein has yet to be determined. However, this true-late gene is notable for the novel way in which gene expression is regulated. Transient transfection assays show that UL94 contains a positive regulatory element (PRE) downstream of the UL94 mRNA start site, and a negative regulatory element (NRE) upstream of the TATA box (Wing *et al.*, 1996; Wing *et al.*, 1998). The PRE is dominant over the NRE at late times of infection whereas at early times no PRE activity can be detected. The NRE therefore has a pivotal role in controlling pUL94 levels early in infection. The NRE contains binding sites for cellular p53, which is involved in transcriptional regulation and progression of cellular DNA synthesis. Thus, p53 plays a central role in regulation of the UL94 promoter, which is not activated prior to onset of viral DNA replication.

The pUL97 tegument protein is expressed with early-late kinetics and phosphorylated within 1 hour of synthesis (van Zeijl *et al.*, 1997). pUL97 is transported to the nucleus where it

functions as a protein kinase and is responsible for the phosphorylation of pUL44 (polymerase processivity factor), which is a protein required for viral DNA replication (Prichard *et al.*, 1999). The replication of a HCMV UL97 deletion mutant is severely impaired in tissue culture, indicating that although not strictly essential, pUL97 plays an important role during replication. The pUL97 tegument protein delivered to the newly infected cell may facilitate the initial rounds of viral DNA replication. Alternatively pUL97 may have a more general role as a phosphotransferase, phosphorylating cellular and/or viral proteins early in the infectious cycle. pUL97 also activates the nucleoside analogue ganciclovir (GCV) by phosphorylation to GCV-mono-phosphate. Cellular enzymes complete the conversion of GCV-mono-phosphate to GCV-tri-phosphate, which causes chain termination when incorporated into the growing DNA strand. Viral resistance to GCV is associated with mutations in UL97 or to mutations in UL54 (DNA polymerase). Remarkably, the MCMV homologue M97 does not phosphorylate GCV. MCMV is, however, sensitive to GCV, indicating that mouse cell enzymes are capable of conversion of GCV to GCV-tri-phosphate. A recombinant MCMV expressing HCMV UL97 gene exhibited enhanced susceptibility to GCV (Wagner *et al.*, 2000).

HCMV has evolved a complex system of mechanisms to escape host immunosurveillance. An example of such a complex interlocking system is provided by the way in which the virus is able to down regulate MHC Class I and II molecule expression on the surface of infected cells (Wiertz *et al.*, 1996; Miller *et al.*, 2001). This is achieved by co-operative function of glycoproteins expressed from genes US2, US3, US6 and US11. gpUS2 and gpUS11 induce the rapid export of MHC Class I from the ER to proteosomes (Ben Arieh *et al.*, 2001; Benz *et al.*, 2001). gpUS11 dislocates newly synthesised class I molecules from the ER to the cytosol, where they are degraded by the proteosome (Wiertz *et al.*, 1996). The reticulum-lumenal domain of gpUS2 allows tight interaction with class I molecules encoded by the HLA-A locus, but not HLA-B7, HLA-B27, HLA-Cw4, HLA-E, and such differentiation between the products of distinct MHC Class I loci may explain why HCMV appears to have multiple strategies for MHC Class I down regulation (Gewurz *et al.*, 2001). Indeed, Machold *et al.*, (1997), showed that gpUS2 and gpUS11 have different specificities for MHC Class I molecules. gpUS3 forms a complex with MHC Class I preventing its transport to the cell

surface, while gpUS6 inhibits the transporter associated with antigen presentation (TAP) so that peptides are inhibited from presentation to MHC Class I in the ER (Jones *et al.*, 1996; Lehner *et al.*, 1997). To avoid natural killer cell destruction of infected cells which lack surface MHC Class I molecules at least two other HCMV genes are expressed. UL40 codes for a glycoprotein which upregulates expression of HLA-E, a non-classical MHC Class I molecule that inhibits NK cell lysis by interacting with the NK surface C-type lectin receptor CD94/NKG2A (Tomasec *et al.*, 2000; Ulbrecht *et al.*, 2000), while the UL18 gene codes for a surface glycoprotein (gpUL18) that mimics the MHC Class I molecule.

Of the genes involved in immune regulation only gpUL18 is a tegument protein (Table 2). Since gpUL18 is delivered to the cell at the time of infection it must be assumed that its function is required very early (Baldick and Shenk, 1996). gpUL18 may be required at this time because the I-E gpUS3 protein prevents expression of I-E protein derived antigens, resulting in a reduction of MHC Class I molecules on the cell surface. As one would expect, none of these immune evasion genes, including the UL18 tegument protein is required for growth in tissue culture cells (Browne *et al.*, 1992; Jones and Muzithras, 1992).

On the basis that MCMV gene m144 and HCMV UL18 both encoded MHC Class I-like products it has been considered that these genes are homologues, however, m144 does not share amino acid sequence similarity or even genome co-linearity with HCMV UL18 (Rawlinson *et al.*, 1996). Even at the level of protein folding there are differences between m144 and pUL18. While the gpUL18 molecular structure exhibits a groove, similar to that seen in MHC Class I molecules, and which could serve as a binding site for endogenous peptides, m144 has a substantial deletion within the counterpart of its alpha-2 domain and is not capable of forming a groove that could bind peptides (Chapman and Bjorkman, 1998). These results suggest that UL18 and m144 differ structurally and might provide different functions. The rat CMV gene r144 encodes a 36 kDa protein and the gene has 19 % and 30 % homology to HCMV UL18 and MCMV m144 genes, respectively. DNA sequence analysis suggests that r144 is also unlikely to be capable of binding antigenic peptides. Like the HCMV UL18 mutant an r144 null mutant has been shown to grow as well as wild type virus in a variety of cell lines (Beisser *et al.*, 2000).

The tegument of HCMV particles contains an antibody Fc receptor capable of binding all subtypes of human IgG, but not other immunoglobulin isotypes. The Fc receptor is likely to be involved in preventing antibody-mediated clearance of HCMV (Stannard and Hardie, 1991; Antonsson and Johansson, 2001). Recently, HCMV ORFs TRL11 and IRL11 have been shown to encode a glycoprotein of 34 kDa, and this has tentatively been identified as the Fc receptor (Lilley *et al.*, 2001).

Unexpectedly, the HCMV tegument has also been reported to contain a specific subset of viral transcripts, termed 'virion RNAs' originating from ORFs UL21.5, UL109, IRL4 and IRL7 (Bresnahan and Shenk, 2000a; Greijer *et al.*, 2000). The functions provided by the products of these genes are unknown. It is known, however, that pUL21.5 localises to the Golgi network and that the TRL4/IRL4 gene is the most abundantly transcribed gene at early times. Curiously, no protein product has been identified for this major early transcript in infected cells, and it is therefore possible that it operates as a functional mRNA molecule. It is assumed that delivery of virion RNAs to the cytoplasm of the host cell allows for their immediate translation, prior to transcription of the infecting genome.

Several host cell proteins, including β_2-microglobulin, annexin II, an actin-like protein and enzymes, such as capsid-associated protein phosphatase PP2A and DNA polymerase, have also been reported to be associated with particles, although the functions provided remain to be investigated (Baldick and Shenk, 1996; Gallina *et al.*, 1999; Michelson *et al.*, 1996).

In summary, the tegument contains a complex set of proteins, which are delivered to the infected cell at the time of infection; some proteins are involved directly or indirectly in initiating lytic infection and others in abrogating host defences. It is interesting to note that some of the most immunogenic proteins expressed by HCMV (pp150, pp65, pUL25 and pp28) are tegument proteins and are therefore protected from antibodies by the viral envelope (Lazarotto *et al.*, 2001). Although 3-D imaging studies of the HCMV tegument structure are in their infancy, early results indicate that tegument proteins are not haphazardly arranged but that the structure is likely to be highly ordered (Chen *et al.*, 1999). It is possible that some tegument proteins might be acquired in the nucleus and others in the cytoplasm of infected cells.

1.5 The HCMV US22 gene family

The HCMV US22 gene family has been relatively little studied and contains 12 loosely related gene members (Table 3). The function/s provided by family members is for the most part unknown. pTRS1, pIRS1 and pUL36 are known to be components of the virus tegument and pIRS1, pUL36 and pUL43 have been demonstrated to be dispensable (Romanowski *et al.*, 1997; Jones and Muzithras, 1992; Patterson and Shenk, 1999; Dargan *et al.*, 1997). There are homologues for most of the HCMV US22 gene family members in other *Betaherpesviruses;* HHV-6 (Gompels *et al.*, 1995), HHV-7 (Megaw *et al.*, 1998; Nicholas, 1996), MCMV (Rawlinson *et al*, 1996), Rat CMV (Vink *et al.*, 2000) and *Tupaia* (Tree shrew) herpesvirus (Bahr and Darai, 2001), but US22 family genes have not been identified in *Alphaherpesviruses* or *Gammaherpesviruses.* Each of the sequenced *Betaherpesvirus*es codes for a similar number of US22 famliy genes, suggesting that the family *per se* provides important functions during *Betaherpesvirus* replication.

Table 3 US22 gene family members

HCMV US22 Gene	HCMV ORF Location (size-amino acids)	HHV-6	HHV-7	MCMV	Rat	Tree Shrew
UL23	27866-28717 (342)	U2 (P), ~UL29	U2	M23 (P) / m25.1	R23	T23
UL24	28936-30009 (358)	U3	U3	M24	R24	
UL28	34757-35893 (379)	U7	U5/7spliced	M28	R28	T28
UL29	35926-37005 (360)	U8	U8			T29
UL36 EX1	49354-49863 (67)	U17	U17EX1 (IE-B)	M36EX1	R36	T36
UL36 EX2	48246-49751 (408)	U16 (P), ~US23	U17EX1 (IE-B)	M36EX2	R36	T36
UL43	54825-56093 (423)	U25 (P), ~UL24	U25	M43 (P)	R43	
IRS1	190631-193231 (846)	U95(P), ~MCMV IE2	U95 (P), ~MCMV IE2	m143 *	r143*	
US22	210803-212581 (593)	DR7	DR7 Spliced	m139 m25.2 m128Ex3 IE2	r139	T5
US23	212646-214439 (592)			m140 m143	r140	T2
US24	214520-216034 (500)			m141	r141	T3
US26	216659-218503 (603)	DR2	DR2 spliced	m142	r142	
TRS1	227044-229470 (788)	U95 (P), ~MCMV IE2	U95 (P), ~MCMV IE2 DR1spliced DR6spliced	m143 * m128Ex3 IE2	r143*	

The table lists the HCMV US22 gene homologues in HHV-6 (Gompels *et al.*, 1995), HHV-7 (Megaw *et al.*, 1998; Nicholas, 1996), MCMV (Rawlinson *et al.*, 1996) and *Tupaia* (Tree Shrew) herpesvirus (Bahr and Darai, 2001). Note that the number of such genes is also generally conserved. (P) means positional homologue with the closest (~) homologous gene by FASTA scoring provided. * refers to nearest homologue but amino acid similarity is low. HCMV UL36 is spliced.

Membership of the US22 gene family depends on the presence of one or more of four amino acid sequence motifs that are conserved among family members (Chee *et al.*, 1990). Motif I has been defined as GxxoxoxWP (o = any hydrophobic residue, x = any residue) and is

contained in products from HCMV genes UL23, UL24, UL28, UL29, UL36 and UL43 (Efstathiou *et al.*, 1992). Motif I is poorly conserved in HCMV US and TRS1/IRS members; the motif oxoxxPxxW is found in products from HCMV genes US22, US23 and US24 (Efstathiou *et al.*, 1992). Motif II has been defined as ooCCxxxLxxoG (Kouzarides *et al.*, 1988; Chee *et al.*, 1990) and is highly conserved, being found in all of the HCMV US22 family members except products from genes UL28, UL29 and TRS1/IRS. Motifs III and IV are less well defined and consist of long regions of sequence containing short runs of charged and hydrophobic residues located towards the carboxy-terminal regions of the protein (Kouzardies *et al.*, 1988; Chee *et al.*, 1990). HCMV gene products pUL23, pUL24, pUL36 and pUL43 have all four conserved amino acid motifs.

The function provided by the motifs are unknown but their existence argues for some similarity in biochemistry or biology of US22 family gene products. Interestingly, the spacing of US22 motif I and II in HCMV appears to be conserved (20-28 amino acids) among family members and this may reflect some similarity in protein folding and/or function (Table 10). The direct repeat (DR) regions of HHV-6 appear to be related to sequences located in the US region of HCMV and contain many of the US22 gene homologues (Gompels *et al.*, 1995). Likewise in HHV-7, several US22 family genes, are located at the genomic termini (Table 3) (Megaw *et al.*, 1998). Homologues of the HCMV UL36 exons 1 and 2 exist in both HHV-6 and HHV-7 and the positions of the splice donor/acceptor sequences are well conserved (Nicholas, 1996). Splicing may also be important for expression of some gene family members in HHV-7 since it has been predicted that splicing of DR1 to DR2 and DR6 and DR7 would result in proteins containing US22 motifs in their usual order (Megaw *et al.*, 1998).

Approximately twelve MCMV ORFs have homology to members of the HCMV US22 gene family (Rawlinson *et al.*, 1996). MCMV US22 gene family motif ooCCxD/E$(x)_{1\text{-}4}$oxxoG appears to be related to the HCMV US22 family motif ooCCxxxLxxoG and occurs first in the peptide sequence of all MCMV US22 family members except M43 and M128 and is only partially conserved in M25.1 (Rawlinson *et al.*, 1996). Motif GxxoxoxWP is found only in MCMV M24 and M36 (exon 1). The major immediate-early locus of MCMV is co-linear with that of HCMV and MIE splicing patterns are maintained (HCMV IE1/IE2, MCMV

IE1/IE3). However, in the case of MCMV a gene (IE2), which is a member of the US22 gene family, is expressed in the opposite orientation from the major IE products (IE1 and IE3). MCMV IE2 has no functional counterparts in HCMV, HHV-6 or HHV-7, although a homologous gene (r128) is coded for by rat CMV (Vink *et al.*, 2000). MCMV IE2 is a *trans*-activator of the MCMV MIEP, but is dispensable (Cardin *et al.*, 1995; Manning and Mocarski, 1988).

As was the case with HCMV little is known of the functions provided by the MCMV US22 family genes. MCMV M43 (UL43 homologue) has, however, been shown to be a determinant for viral growth in the salivary gland (Xiao *et al.*, 2000). M139, M140 and M141 (US22, US23 and US24 homologues, respectively) are early proteins that are distributed throughout the infected cell. Gene M139 codes for two proteins of 72 kDa and 61 kDa, while M140 and M141 each encode a single protein of 56 kDa and 52 kDa, respectively. M139 does not appear to be required for replication in macrophages, whereas deletion of M140 or M141 results in impaired replication in macrophages and spleen tissues. M140 is dominant and when deleted resulted in reduced expression of M141 protein. Thus, M140 and M141 appear to act both co-operatively and independently to regulate MCMV replication in a cell-type specific manner and are predicted to influence cell tropism and viral pathogenesis (Hanson *et al.*, 2001).

To date only one viral mutant with a defect in a HCMV US22 family gene, (UL36), has been generated (Patterson and Shenk, 1999), although a naturally occurring mutant with a partial deletion of gene UL43 has been identified (Dargan *et al.*, 1997). Few antibodies have been generated to identify US22 family gene products; those generated are against pTRS1/pIRS1 (Romanowski *et al.*, 1997), pUS22 (Mocarski *et al.*, 1988) and pUL36 (Patterson and Shenk, 1999).

The HCMV gene US22, after which the US22 gene family was named, was predicted to code for a 66.9 kDa (593 amino acid) protein (Chee *et al.*, 1990), but a protein of ~76 kDa has been detected in infected cells and also in pUS22 expressing bacterial cell extracts, using an anti-US22 antibody (Mocarski *et al.*, 1988; Dal Monte *et al.*, 1998). The discrepancy is size could, in-part, be due to a recently discovered DNA sequencing error in the published AD169

sequence (Chee *et al.*, 1990), since the corrected sequence indicates that translation commences at an upstream methionine (Parvis Akter, personal communication). The US22 gene is expressed with early gene kinetics and pUS22 can be detected from 24 h PI (Mocarski *et al.*, 1988). At both early and late times in infection pUS22 was located by immunofluroescence in the nucleus of infected cells. Confusingly, however, cell fractionation studies showed that most pUS22 separated with the cytoplasmic fraction with a proportion released from cells as a soluble protein at both early (24 h) and late (72 to 120 h) times in infection.

The UL36-38 gene locus is complex, coding a number of gene products, only one of which (pUL36) is a member of the US22 gene family, and contains all four of the US22 family amino acid sequence motifs. Proteins from the UL36-38 locus have previously been shown to be essential for transient complementation of HMCV ori-Lyt-dependent DNA replication (Pari *et al.*, 1993; Pari and Anders, 1993). One or more protein products from the UL36-38 locus are also reported to co-operate with the major immediate-early genes IE1 (72 kDa) and IE2 (86 kDa) to synergistically enhance transcriptional *trans*-activation from some cellular (heat shock protein 70 (*hsp70)* and brain creatine kinase) and HCMV gene (UL112) promoters (Colberg-Poley *et al.*, 1992). The UL36-38 gene locus encodes four products, pUL36, gpUL37, pUL37 exon 1 (pUL37x1) which are expressed with I-E gene kinetics and pUL38 which is expressed with E gene kinetics (Tenney and Colberg-Poley, 1991a; Tenney and Colberg-Poley, 1991b). A smaller spliced variant of gpUL37, called gpUL37$_M$, has also been described (Goldmacher *et al.*, 1999). The 1.65 kbp UL36 ORF is spliced and the junctions of exon 1 and 2 occur immediately prior to US22 gene family motif II (Kouzarides *et al.*, 1988). An intron of the UL36 gene contains an element (5'-TGGAAAG-3') that is identical to the SV40 enhancer core element. The UL36 mRNA is 3' co-terminal with that of 3.4 kbp UL37 mRNA and is contained entirely within the non-coding region of UL37 mRNA. UL37 mRNA is predominately transcribed at immediate-early times, but only at low abundance because expression is stringently regulated. UL37x1 mRNA, which is initiated from the same promoter, and UL36 mRNA, which is initiated from a downstream promoter, are more abundant messages. UL36 mRNA is detectable in the cytoplasm of infected cells by 4 h PI, increased by 8 h PI and remains abundant through 24 h PI (Colberg-Poley, 1996).

The extent to which transcriptional *trans*-activating activity of the UL36-38 locus resides in the UL36 gene product has yet to be investigated. However, the UL36 ORF has two regions with sequence homology to the bulky hydrophobic residues that are located in the activation domain of HSV-1 VP16, suggesting that pUL36 might function as a transcriptional *trans*-activator. In one region (UL36 exon 2, amino acids 389 to 423) homologous to VP16 activation domain, five out of six bulky hydrophobic residues and six out of ten acidic residues are conserved. In the other region (UL36 exon 1, amino acids 7 to 32) of sequence homology with VP16, bulky hydrophobic residues (two identical and two conservative substitutions) and critical acidic residues (one identical and six conservative substitutions) are also conserved (Colberg-Poley *et al.*, 1992). An alternative protein that might account for the *trans*-activating properties of the UL36-38 locus and the synergism observed in the presence of IE1/IE2 is the gpUL37 gene (Colberg-Poley *et al.*, 1998). Zhang *et al.*, (1996), have shown that gpUL37 exhibits *trans*-activating activity and that the acidic domain of pUL37x1 is dispensable for *trans*-activation of the *hsp70* promoter but is required for IE1/IE2 synergism.

gpUL37 is a type I membrane-glycoprotein, modified by *N*-linked glycosylation (Al-Barazi and Colberg-Poley, 1996). After being processed through the secretory apparatus gpUL37 is depositied in mitochondria (Colberg-Poley *et al.*, 2000). Recently, each of the spliced variants of UL37 (gpUL37, pUL37x1 and $gpUL37_{M)}$ have been shown to function as mitochondria-localised inhibitors of apoptosis (vMIA), inhibiting Fas-mediated apoptosis (Goldmacher *et al.*, 1999). Curiously, the Towne strain of CMV does not express gpUL37 or $gpUL37_{M}$, indicating that pUL37x1 alone is sufficient to block apoptosis during infection.

The HCMV UL36 gene is first expressed at I-E times, and is continuously present throughout the replication cycle. While pUL36 is a tegument protein it has been shown to be dispensable for replication and infectivity (Patterson and Shenk, 1999).

The role of the pUL36 tegument protein immediately after its delivery to the cell is almost certainly to inhibit the initiation of apoptosis by the newly infected cell. The UL36 gene product inhibits caspase-8 activated (vICA) apoptosis by binding the pro-domain of caspase-8 to prevent activation of Fas-mediated apoptosis. vICA does not share sequence homology with FLICE-inhibitory proteins (FLIPs) or other known suppressors of apoptosis and

therefore UL36, like UL37, appears to encode a new class of cell-death suppressor (Skaletskaya *et al.*, 2001).

A further two members of the US22 gene family, pIRS1 (91.0 kDa) and pTRS1 (84.0 kDa), are known to be tegument proteins (Romanowski *et al.*, 1997). The TRS1 gene is transcribed as a 2.7 kbp message that is unspliced. The presence of either pTRS1 or pIRS1 is essential for transient complementation of ori-Lyt-dependent DNA synthesis (Pari *et al.*, 1993), although pIRS1 has been shown to be dispensable for virus replication in cultured cells (Jones and Muzithras, 1992). pIRS1 and pTRS1 are present throughout the infected cells at immediate-early and early times PI, but become more abundant and accumulate in the cytoplasm at late times PI (Romanowski and Shenk, 1997). Overall, pIRS1 and pTRS1 are 55 % identical and despite divergence after amino acid 549 the C-terminus of both proteins is charged, and this may be important for protein-protein or protein-DNA interactions. The *trans*-activation properties of pIRS1 and pTRS1 are considered in section 1.6.

1.6 Transcriptional *trans*-activation in HCMV infected cells

HCMV encodes several proteins that function as transcriptional *trans*-activators of viral and cellular genes. Such *trans*-activators are thought to operate as part of a complex regulatory network that controls viral and cellular gene expression, to co-ordinate virus replication and cell biochemistry so that an optimal intracellular environment is provided for virus replication. The activity of *trans*-activators might also influence whether the virus enters the lytic or latent infection pathway. Some *trans*-activators are particle components (pIRS1, pTRS1, pUL69, pp71 (pUL82)), while others are expressed at immediate-early times PI (IE1 72, IE2 86) (Romanowski *et al.*, 1997; Winkler *et al.*, 1995; Liu and Stinski, 1992). Curiously, pIRS1 and pTRS1 are both delivered to the infected cell as particle components and also expressed at immediate-early times.

1.6.1 The major immediate-early (MIE) gene locus

The MIE locus is under the control of the MIE promoter (MIEP), which is subject to both positive and negative regulation and contains a large number of binding sites for cellular transcription factors and for some viral proteins (Mocarski, 1996). Analogous promoter-enhancers are found upstream of the predominant I-E gene locus in MCMV and SCMV and homologous enhancer elements control expression of US3 (Kim *et* al., 2002; Biegalke, 1999; Colberg-Poley, 1996). The MIEP can be separated into the enhancer region (-500 to + 1, relative to the IE1/IE2 transcription start site), specific NF-1 binding region (-800 to –500) and modulator region (-1,100 to –800). Numerous cellular transcription factors (e.g. p53, YY1, NF-kB, ATF, CREB, AP1, Sp1, C/EBP, SREB, NF1, TNF-α) are known to bind within and upstream of the enhancer region and downstream of the transcription start site (Mocarski, 1996; Prosch *et al.*, 2001; Zhang *et al.*, 2001). The enhancer contains repeated sequence elements (16-, 18-, 19- and 21- bp repeats) that are involved in binding cellular transcription factors, interspersed with unique sequences. This complex array of binding sites permits the MIEP to operate in the context of a broad range of different cell types and to respond to a changing intracellular environment. Deletion of the MIEP distal enhancer region (-300 to –582) resulted in a decrease in expression from the MIEP and the IE US3 promoters, but only during low m.o.i. infections (Meier and Pruessner, 2000). The distal enhancer is composed of at least two components, which function independently but co-operate to activate transcription from the MIEP and hence viral replication. One part, a 47 bp segment, has binding sites for CREB/ATF, Sp1 and YY1, which at low m.o.i. are thought to function to activate the MIEP and to promote virus replication (Meier *et al.*, 2002). The modulator region appears to be dispensable, since a mutant virus lacking the modulator exhibited only slightly delayed growth kinetics in human fibroblasts (Meier and Stinski, 1997). Early experiments suggested that the modulator region was involved in repression of the MIEP in non-differentiated Tera-2 and THP-1 cells (Nelson *et al.*, 1987; Shelbourn *et al.*, 1989; Kothari *et al.*, 1991; Huang *et al.*, 1996). However, when the modulator deletion mutant

was grown in undifferentiated cells IE gene expression from the MIEP remained at a minimal level, suggesting that differentiation-dependent control of the MIEP is complex and involves regulatory mechanisms other than, or additional to, that associated with the modulator region (Meier and Stinski, 1997).

The principal HCMV *trans*-activators are the nuclear phosphoproteins IE1 72 kDa (IE1 72) and IE2 86 kDa (IE2 86), which are expressed by differential splicing from the MIE gene locus and share 85 amino acids at their amino terminus (Stenberg *et al.*, 1989; Stenberg, 1996). The less abundant IE2 product IE2 55 kDa (IE2 55), produced when an intron in exon 5 is removed, is only transiently expressed reaching maximum levels at 1-6 h PI. IE2 55 is a *trans*-activator of the MIEP and is functionally antagonistic to IE2 86; IE2 55 relieves repression of the MIEP induced by IE2 86 binding to the *crs* element in the promoter. However, IE2 55 does not bind the *crs* element and appears to operate via another region in the promoter (Baracchini *et al.*, 1992). Another variant form of IE2 is IE2 40 kDa (IE2 40), an unspliced product expressed from exon 5 alone. IE2 40 is the most abundant IE2 product made late in the infection cycle and functions both a repressor of I-E gene expression and a *trans*-activator of E and L genes (Mocarski, 1996).

1.6.2 The major immediate-early protein IE1

The most abundant I-E gene product from the MIE locus is coded for by MIE exons 1, 2, 3 and 4 (gene UL123), giving rise to a 72 kDa protein (IE1 72). IE1 72 is produced throughout infection, peaking at 5-8 h PI and dipping to a low level at the start of DNA replication (Mocarski, 1996). The functions provided by IE1 72 appear to be multifarious and are not yet fully understood, although it plays an important role in initiating lytic cycle replication. In cells infected at high m.o.i. IE1 72 was dispensable but in cells infected at low m.o.i. IE1 72 was required, since the replication of a IE1 72 deletion mutants was severely impaired at low m.o.i. (Greaves and Mocarski, 1998).

IE1 72 *trans*-activates the MIEP (Table 4), indirectly via a mechanism involving binding of NF-kB to the 18 bp repeats in the MIEP promoter (Cherrington and Mocarski, 1989). It is thought that in the absence of virus particle *trans*-activators (i.e. when cells are transfected with naked infectious HCMV DNA) that NF-kB binding to the MIEP is responsible for initiating IE1 expression from the promoter (Mocarski *et al.*, 1996). By itself IE1 72 is a weak *trans*-activator of heterologous promoters (Table 4), but in transient transfection experiments the level of transcription of some target promoters was synergistically enhanced when IE1 72 and IE2 86 were co-expressed (Hagemeier *et al.*, 1992a; Klucher *et al.*, 1993). The mechanism by which IE1 72 operates to *trans*-activate gene transcription is complex, depending on the promoter structure and interactions with different proteins involved in the transcription complex (Table 4). In the case of heterologous promoters IE1 72 operates in a TATA-box independent manner (Hagemeier *et al.*, 1992b). For example, IE1 72 interacts directly with the CCAAT box-binding factor CTF1 and augments activation of the TATA-less DNA polymerase alpha promoter (Hayhurst *et al.*, 1995), while activation of the TATA-less dihydrofolate reductase (DHFR) promoter involves an interaction between IE1 72 and the E2F binding factor (E2F1) (Margolis *et al.*, 1995) (Table 4).

IE1 72 is also involved in the control of cell cycle regulation through various protein/protein interactions. For example, IE1 72 indirectly influences activity of the transcription factor E2F, which is required for activation of cellular genes in the S phase of cell growth. HCMV infection induces expression of p107 (a retinoblastoma protein) known to complex with E2F to prevent E2F-dependent gene activation. By directly binding to p107 IE1 72 overcomes the transcriptional repression on E2F-responsive promoters (Poma *et al.*, 1996) (Table 4). Another route by which IE1 72 influences the cell cycle is via IE1 72 mediated activation of activator protein-1 (AP-1) expression. AP-1 is a cellular transcription factor (largely composed of c-*fos* and c-*jun* subunits) which is important for regulation of cell growth and differentiation. IE1 72 activates AP-1 at the post transcriptional level through interactions, with a cellular protein kinase (mitogen-activated protein kinase or extracellular signal-regulated kinase kinase kinase 1 (MEKK 1) (Kim *et al.*, 1999).

Although IE1 72 does not appear to bind DNA directly, it is associated with cellular chromatin where it probably interacts with nuclear DNA-binding proteins, such as cellular transcription factors Sp1, E2F1 and CTF-1 and components of TFIID (Lukac *et al.*, 1994; Lukac *et al.*, 1997; Margolis *et al.*, 1995; Xu *et al.*, 2001). The association with chromatin requires a C-terminus acidic domain of IE1 72 (amino acids 421-486), although this domain is not required for disruption of PODs (Wilkinson *et al.*, 1998). Several cellular proteins are associated with PODs, including Sp100, NDP-55, NDP-52, PIC-1, Int-6. HAUSP, Daxx, small ubiquitin-like modifier (SUMO-1) and promyelocytic leukaemia protein (PML), a growth and tumour suppressor protein (reviewed in Wilkinson *et al.*, 1998; Xu *et al.*, 2001). IE1 72 localises to PODs within 1-2 h PI and prior to IE2 86 appearance in PODs. IE1 72 interacts with PML, by a mechanism that involves the IE1 72 ring finger motif, and which results in displacement of PML from PODs and its redistribution in a diffuse form in the nucleoplasm. IE1 72 is sumoylated at position 450 (lysine) by SUMO-1 to generate a 90 kDa IE1 isoform in both infected cells and in transiently transfected cells in which IE1 is expressed by itself (Xu *et al.*, 2001). IE2 86 is also modified by SUMO-1 by a mechanism involving the SUMO-conjugating enzyme, Ubc9, although this enzyme does not appear to be involved in sumoylation of IE1 72 (Ahn *et al.*, 2001). In the case of HSV-1, PML displaced from PODs by interaction with ICP0 results in degradation of PML in proteosomes (Parkinson and Everett, 2000). In contrast, displacement of PML from PODs by HCMV IE1 72 does not result in proteosome degradation in HCMV infected cells. In uninfected cells PML is normally involved in repression of gene transcription, but the function of both HSV-1 ICP0 and HCMV IE1 72 is to overcome this PML-mediated repression to permit viral gene expression (Xu *et al.*, 2001).

1.6.3 The major immediate-early protein IE2

HCMV IE2 86 (UL122), coded for by exons 1,2,3 and 5 is the second most abundantly expressed I-E protein. IE2 86 can bind DNA via sequences in the C-terminus of the protein (Schwartz *et al.*, 1994), binding to a 15 bp *cis*-repression signal (crs) near the MIE transcription

start site. IE2 86 binding to the crs negatively regulates MIEP activity resulting in auto-repression of IE2. The crs sequence is orientation independent and can confer IE2 responsiveness of heterologous promoters (Cherrington *et al.*, 1991; Macias and Stinski, 1993; Spector, 1996) (Table 4). IE2 86 induced repression of MIEP activity via binding to the crs is not simply due to steric hindrance, since IE2 86 appears to alter RNA polymerase II so that the transcription complex is unable to form on the promoter (Lee *et al.*, 1996).

IE2 86, unlike IE1 72, is a strong promiscuous *trans*-activator that can enhance transcription from a number of viral (E and L) and cellular promoters (Table 4), in a TATA box dependent manner (Lukac *et al.*, 1994; Haymeier *et al.*, 1992b; Sommer *et al.*, 1994; Kim *et al.*, 2000b). The ability of IE2 86 to activate transcription correlates with its ability to bind the TATA binding protein (TBP), a component of the basal transcription factor TFIID, and with its ability to bind transcription factors, such as c-*jun* and *jun*B (Scully *et al.*, 1995), which themselves bind sequence elements located upstream of the promoter (Hagemeier *et al.*, 1992b) (Table 4). IE2 86 *trans*-activation may also arise from abrogation of transcriptional repression: for example, alleviation of histone mediated repression (Klucher *et al.*, 1993), or Dr-1 mediated repression (Caswell *et al.*, 1996). The ability of IE2 86 to engage in protein-protein interactions is extremely important and facilitates the formation of multiprotein-DNA complexes on viral or cellular promoters.

IE2 86 expression levels are tightly regulated by auto-repression, but also influenced by the presence of the early virus protein pUL84. pUL84, a 75 kDa protein (He *et al.*, 1992; Spector and Tevethia, 1994) functions as a *trans*-dominant inhibitor of immediate-early mediated *trans*-activation (Gebert *et al.*, 1997). pUL84 binds pIE2 86 to promote binding of IE2 86 to the crs resulting in down regulation of the MIEP. Since high levels of IE2 expression are cytotoxic, pUL84 may fulfil an important regulatory function maintaining IE2 at non-cytotoxic levels. Another HCMV gene product, pUL76, appears to *trans*-activate the MIEP when present in low concentrations in transfected cells but, curiously, functions as a repressor of the MIEP when present at high levels. The mechanism, whether direct or indirect, remains obscure (Wang *et al.*, 2000b).

The carboxy terminal half (amino acids 290-579) of IE2 86 is required for binding to the crs, while both the amino and carboxy termini are required for IE2 86 *trans*-activation activity (Spector, 1996). Precise mapping of the domains within IE2 86 required for DNA binding has been problematic since binding seems to be promoter dependent. The domain of amino acids 86-542 appears to be important for protein/protein interactions, since this region binds a number of cellular regulatory proteins (Sp1, Tef-1, c-*jun*, *jun*B, ATF-2, NF-kB, protein kinase A-phosphorylated delta-CREB, p300, CBP, Nil-2A, CHD-1 and UBF) which are important for RNA transcription (Spector, 1996). The ability of IE2 86 to engage in protein/protein interactions is modified by its phosphorylation state. ERK2, a member of the MAPK family, is known to phosphorylate IE2 86 at several different sites (Spector, 1996; Harel and Alwine, 1998). In this regard it is of interest to note that binding of HCMV to its cellular receptors induces intracellular signalling that leads to stimulation of the MAPK pathway and to the up-regulation of ERK2 (Fortunato *et al.*, 2000).

IE2 86 is a partner is several important protein/protein interactions (Table 4). IE2 86 binds the tumour supressor retinoblastoma (Rb) protein, via interactions with the A/B pocket or the C-terminus of Rb (Hagemeier *et al.*, 1994; Sommer *et al.*, 1994; Fortunato *et al.*, 1997). Likewise, IE2 86 contains two domains (amino acids 241 to 369 and amino acids 1 to 85) that can independently affect Rb function (Fortunato *et al.*, 1997). Rb is intimately involved in the regulation of cell cycle progression. During the transition to G_1, Rb becomes progressively more phosphorylated. In its under-phosphorylated state, Rb operates to suppress growth during the G_0-G_1 phase of the cell cycle. This under-phosphorylated Rb forms a range of complexes with different transcription factors, including the cellular transcripition factor E2F, which is required for the activation of some cell genes required for S phase induction. Hence, Rb functions to control cell proliferation. The direct binding of Rb by HCMV IE2 86, results in alleviation of Rb-induced repression of promoters bearing E2F binding sites. The complex of Rb/IE2 86 results in the sequestration of both proteins and has the effect of alleviating IE2 86-mediated self repression of the MIEP and suppressing IE2 86-induced *trans*-activation of promoters (Hagemeier *et al.*, 1994). Interestingly, IE2 86 by itself has a role in cell cycle control, since it can block cell cycle progression in an Rb-independent manner (Murphy *et al.*,

2000). Based on its interaction with IE2 86, it has been suggested that Rb might control the HCMV lifecycle and account for the slow growth of HCMV in cells (Choi *et al.*, 1995).

IE2 86 also binds to cellular p53 to inhibit p53-dependent *trans*-activation. Two domains of the IE2 86 protein are involved, the N-terminal region of the protein binds p53 but has negligible effect on the ability of p53 to bind specific DNA sequences; the C-terminal sequences also bind to p53 and this interaction leads to a reduction in the *trans*-activation activity of p53 (Tsai *et al.*, 1996). p53 is an important negative regulator of transcription, cellular DNA synthesis and cell proliferation. p53 transcription is enhanced in cells under stress and the p53 protein *trans*-activates several cellular genes coding transcription factors, including p21WAF, Cip1, bax and hdm2. p21WAF and bax are involved in the induction of apoptosis, while hdm2 is a negative regulator of p53 activity. HCMV infection results in up-regulation of hdm2 and p53 is sequestered into the cytoplasm (Wang *et al.*, 2000a). Although IE2 86 was shown to bind p53, it appeared not to be responsible for sequestering p53 into the cytoplasm and binding of IE2 86 to p53 does not appear to abrogate the p53-induced G_1 checkpoint (Bonin and McDougall, 1997).

An IE2 deletion mutant virus has recently been isolated using an IE2 expressing cell line transfected with a mutated HCMV (AD169) BAC, and confirms the long held assumption that UL122 (IE2) is an essential gene. The UL122 BAC mutant never generated plaques, and although mRNA and proteins from the other I-E genes could be detected, mRNA from early genes could not be detected by RT-PCR. Therefore these experiments confirm that IE2 86 is responsible for *trans*-activation of most viral promoters and that IE2 86 is essential for switching from E to L gene expression (Marchini *et al.*, 2001).

1.6.4 Synergy between IE1 and IE2

Many transient transfection assays have shown that co-expression of IE1 72 and IE2 86 resulted in synergistic *trans*-activation of a variety of cellular and viral promoters (Lukac *et al.*,

1994). The mechanism, by which, synergy is obtained is not clear. No direct protein/protein interaction between IE1 72 and IE2 86 has been demonstrated. However, IE1 72 and IE2 86 may perform functions similar to those of TBP associated factors (TAFs) and both have been reported to interact with TAFs. IE1 72 and IE2 86 can simultaneously bind to human TAF (11)130, suggesting that this TAF may provide bridging interactions between the two proteins for transcriptional activation and synergy (Lukac *et al.*, 1997). The cellular transcription factor Sp1 can also bind TAT(11)130 and recruit the TF11D complex, and co-transfection experiments have shown that recruitment of Sp1, or TBP, to the MIEP markedly decreased the synergistic effect of IE1 on IE2-mediated *trans*-activation (Kim *et al.*, 2000a).

Table 4. Viral and cellular gene promoters *trans*-activated by IE1 and/or IE2.

	COMMENTS
Viral Promoters	**Indirect Activation by HCMV IE1 72**
MIEP	IE1 72 induces NF-kb binding to 18 bp repeats, stimulating expression from MIEP (Cherrington and Mocarski, 1989)
UL54	Promoter activation dependent on *cis*-acting sequence containing a novel 18 bp inverted repeat sequence (IR1) and an ATF binding site. The IR1 site was important for gene regulation only at early times while the ATF site was used at late times. pTRS1/pIRS1 and UL112-113 proteins *trans*-activate the UL54 promoter in association with IE1/IE2. (Kerry *et al.*, 1996; Kerry *et al.*, 1997).
UL44	IE1 required for promoter expression (Greaves and Mocarski, 1998).
UL83 (pp65)	Activation dependent on octamer sequence in promoter. Activated by IE1 or IE2 (Depto and Stenberg, 1989)
HIV LTR	10-base pair element of HIV LTR is absolutely required (Walker *et al.*, 1992).
Cellular Promoters	**Indirect Activation by HCMV IE1 72**
c-*fos* c-*jun* *hsp70*	A minimal inducible promoter element was contained 50-60 bp upstream of transcription start site, while IE2 86 alone induced expression. Synergy observed with IE1 72 and IE2 86 together. *hsp70* - Promoter constructs containing a variety of different TATA elements activated by IE1 72 or IE2 86 (Hagemeier *et al.*, 1992a)
DHFR	Dihydrofolate reductase (DHFR) transcription activated modestly by IE2 86 and IE2 55 (E2F independent). Activation by IE1 72 is E2F dependent (Margolis *et al.*, 1995).
DNA polymerase alpha	Not activated by IE2. Strongly activated by IE1. Removal of CCAAT box abolishes high level activation by IE1. IE1 interacts with CCAAT box binding protein CTF1. IE1 72 augments CTF1-mediated activation of promoter (Hayhurst *et al.*, 1995).
TNF-alpha	IE1 or IE2 up-regulate expression (Geist *et al.*, 1994)
Rb p107	IE1 interacts with p107 and relieves p107-mediated transcriptional repression of E2F-responsive promoters (Poma *et al.*, 1996)
PML	IE1 72 interacts with PML and disrupts PODs in a proteosome-independent manner. Sumoylation of IE1 is not required for this. IE1 can overcome PML induced transcriptional

	repression (Wilkinson *et al.*, 1998; Xu *et al.*, 2001)
Viral Promoters	**Direct Activation by HCMV IE2 86**
MIEP	Interacts with cis acting signal (crs) to down regulate its own transcription (Cherrington *et al.*, 1991)
UL4	UL4 promoter has upstream negative element located -168 bp to -134 bp that binds a cellular protein or IE2 86. Binding of IE2 86 lifts repression (Huang *et al.*, 1995).
	Indirect Activation by HCMV IE2 86
UL112-113	Activated by IE1 72 alone but synergistically enhanced by IE2 86 (Colberg-Poley *et al.*, 1992). Sequences between -113 and -58 required for activation by IE2 86. 3 regions of promoter (-286 to -257, -248 to -218 and -148 to -120) bind strongly to IE2 86 and show homology to crs repression sequence in MIEP (Schwartz *et al.*, 1994). CREB and CBP required for activation of the promoter in conjunction with IE2 86, possibly a multiprotein complex including CREB, p300 or CBP (Schwartz *et al.*, 1996). One product of the UL112-113 gene (pp43) responsible for IE2 dependent activation of UL54 promoter (Li *et al.*, 1999).
HIV LTR	Activation of HIV LTR by IE2 is TATA box dependent (Hagemeier *et al.*, 1992b). IE2 86 terminal region (amino acids 99 to 194) and carboxy region (amino acids 170 to 579) necessary for activation (Yeung *et al.*, 1993).
Cellular Promoters	**Indirect Activation by HCMV IE2 86**
huRNP A1	Hetergeneous ribonucleoprotein A1 (huRNP A1). IE2 86 N-terminal sequences (amino acids 1-290) and C-terminal (amino acids 291-579) required (Wang *et al.*, 1997).
HDAC-1	Histone deacetylase-1 (HDAC-1) IE2 86 region 280-380 aa required for HDAC-1 binding. IE 72 does not bind to HDAC-1. In presence of HDAC-1 IE2 86 can activate the cyclin E promoter (Murphy *et al.*, 2000).
ICAM-1	Intracellular adhesion molecule -1 (ICAM-1). Direct activation by IE proteins on promoter. Deletion of promoter revealed that 5' flanking sequence required for maximum activation (Burns *et al.*, 1999)
	Direct Activation by HCMV IE 86
c-*fos* c-*jun* *jun*B *hsp70*	IE2 86 has preference for specific TAT elements and Tef-1, Sp-1 CAAT and ATF transcription factor binding sites. Not significantly activated by IE1 72 alone but synergy in activation between IE1 72 and IE2 86. No activation of transcription factors AP1 or OCT (Lukac *et al.*, 1994). IE2 86 protein interacts with c-*jun* and *jun*B. Three independent regions of IE2 86 shown to interact *in vitro* with c-*jun*, two of which are essential for activation. IE2 86 binds directly to c-*jun* promoter through a sequence located just upstream of the AP-1 site between nucleotides -125 to -97. This discrete domain shares sequence homology with the cis-repression signal on the IE gene (Scully *et al.*, 1995)

1.6.5 The role of other HCMV proteins (pp71, pUL69, pIRS1, pTRS1) in transcriptional *trans*-activation

UL82 is expressed from a 1.9 kbp mRNA, that is 3' co-terminal with the 4 kbp UL83 transcript, to produce pp71 (Nowak *et al.*, 1984). pp71 is a tegument protein which is thought to be attached to the major capsid protein and its SCMV homologue is also thought to be

similarly located (Trus *et al.*, 1999). The pp71 tegument protein is translocated to the nucleus immediately after infection (Hensel *et al.*, 1996). Although only a minor particle component, pp71 appears to be critical for initiating lytic cycle replication, since it is a potent *trans*-activator of the MIEP (Liu and Stinski, 1992). pp71 may also function as a more general *trans*-activator, since pp71 alone, or in combination with other viral proteins (e.g. UL69), can activate promoters from different viruses (Homer *et al.*, 1999; Winkler *et al.*, 1995). pp71 appears not to interact directly with the MIEP but promotes binding of transcription factors ATF or AP1 to the MIEP (Liu and Stinski, 1992). Since, naked HCMV DNA is infectious, pp71 cannot be strictly required for infectivity. However, co-transfection of pp71 expression plasmids with naked infectious HCMV DNA enhanced the infectivity up to 80-fold (Baldick *et al.*, 1997). A pp71 deletion mutant exhibited a multiplicity dependent phenotype; at high input multiplicities (10 p.f.u./cell) pp71 was not required, but a low m.o.i. the virus mutant was severely restricted, confirming that the gene plays an important role during virus replication. During low input m.o.i. infection the immediate-early genes (IE1, IE2, UL37x1, UL38, UL106-UL109 and UL115-119) could not be detected. After a single passage of the pp71 deletion mutant virus in a complementing cell line the progeny virus recovered its ability to replicate in normal cells when infected at low m.o.i., indicating that the pp71 delivered to cells as a tegument component was critical (Bresnahan *et al.*, 2000; Bresnahan and Shenk, 2000b).

Interestingly, both the IE1 72 (Mocarski *et al.*, 1996) and pp71 virus mutants exhibit multiplicity dependent phenotypes. Since the tegument delivered pp71 functions downstream to induce IE1 72 expression the phenotype of both mutants may be due to failure to produce sufficient IE1 72 to initiate sustained activation of the MIEP.

The HCMV UL69 gene is a herpesvirus core gene (homologous to UL54 in HSV-1) (Davison, 1993) (Table 1). The literature is confused with regard to the kinetic class of UL69 gene expression. Mocarski, (1996), reported that pUL69 is expressed with immediate-early kinetics, while Winkler *et al.*, (1994), and Chambers *et al.*, (1999), report early-late kinetics. pUL69 is a tegument protein but of the three isoforms of pUL69, (105 kDa, 110 kDa, 116 kDa), only the 110 kDa isoform is incorporated into HCMV particles (Winkler and

Stamminger, 1996). The UL69 tegument protein is delivered to cells at the time of infection and is presumed to function at I-E or pre-I-E times PI. pUL69 alone or synergistically in combination with pp71 can stimulate activation of the MIEP. The pUL69/pp71 effect on the MIEP may be transitory, since IE1 72 is the major protein involved in auto-stimulation of the MIEP (Winkler *et al.*, 1995). pUL69 exhibits a broad range of *trans*-activation activity, *trans*-activating several HCMV and heterologous viral or cellular promoters. By itself pUL69 was capable of *trans*-activating expression from some early HCMV (UL112) promoters but not the UL86 late promoter (Winkler *et al.*, 1994). pUL69 localises to DNA replication compartments in the nucleus and is thought to interact with a cellular protein, hSPT6, thought to regulate chromatin structure. It has been suggested that *trans*-activation of promoters by pUL69 is due to competition between pUL69 and histones for DNA binding sites. Mutagenesis of the central conserved domain of pUL69, resulted in loss of the interaction between pUL69 and the hSPT6 protein and the *trans*-activation potential of pUL69, suggesting that the interaction is essential for the pUL69 *trans*-activation function. The carboxy terminus of hSPT6 normally interacts with histone H3 and leads to nucleosome assembly, which is thought to be repressive for transcription. pUL69 was shown to antagonise the interaction of the hSPT6 protein with histone H3, and therefore inhibit nucleosome assembly. Thus, pUL69 exhibits broad range *trans*-activating properties due to de-repression of a chromatin regulatory protein (Winkler *et al.*, 2000).

The tegument proteins pIRS1 and pTRS1, (US22 family members), *trans*-activate I-E, E and L promoters when expressed alone or gave enhanced levels when in combination with IE1/IE2 in transient transfection assays (Romanowski and Shenk, 1997). pTRS1 is absolutely required, in combination with IE1 and IE2, for activation of the late ICP36 promoter (Stasiak and Mocarski, 1992). In summary, pIRS1/pTRS1 do not appear to be strong or broad range *trans*-activators, although they can synergise with IE1 and IE2 to activate a range of heterologous promoters. The mechanism by which pIRS1 and pTRS1 operate as accessory proteins in IE1/IE12 mediated *trans*-activation is not known.

$pIRS1^{263}$ is a truncated version of pIRS1, expressed from a promoter within the IRS1 ORF and entirely encoded within the C-terminal domain and same reading frame as pIRS1.

pIRS1^{263} is entirely located in the nucleus and is part of a regulatory loop, antagonizing transcriptional *trans*-activation associated with pIRS1 and pTRS1 (Romanowski and Shenk, 1997).

The complex interactions between the various different HCMV *trans*-activators, which together control HCMV gene expression, are not fully understood. The tegument proteins pp71, pUL69, pIRS1 and pTRS1 are delivered to the cell immediately after infection and are likely immediately translocated to the nucleus. pp71 in combination with pUL69 is probably responsible for the initial *trans*-activation of the MIEP. The tegument proteins pIRS1 and pTRS1 are also capable of *trans*-activating other I-E promoters (Romanowski and Shenk, 1997). The IE1 72 produced as a result of tegument protein *trans*-activation of the MIEP then probably takes over responsibility for the sustained *trans*-activation of the MIEP. pp71 and pUL69 only modestly activate expression of the immediate-early US3 gene, although expression is up-regulated by the combination of IE1, IE2, pIRS1 and pTRS1. This synergistic activation of US3 gene expression is inhibited by products from genes UL37x1, UL38, and UL84, the latter also promoting inhibition of MIEP expression, representing a critical step in the switch from immediate-early to early gene expression (Biegalke, 1999). At late times in infection the IE2 40 product is produced and this is thought to negatively regulate the MIEP when I-E products are no longer required (Mocarski, 1996).

1.6.6. HHV-6 *trans*-activators

In the case of the *Betaherpesvirus* HHV-6, at least seven genes have been defined as transcriptional *trans*-activators on the basis of *in vitro* chloramphenicol acetyl transferase (CAT) assays and several of these are the homologues of HCMV US22 gene family members. HHV-6 proteins expressed from the U86-U89 and U16-U19 gene regions are functionally analogous to the HCMV IE1/IE2 and UL36-38 proteins, and therefore are termed IE-A and IE-B, respectively (Gompels *et al.*, 1995). U41, a herpesvirus core gene (homologous to HCMV UL69) exhibits *trans*-activation activity. Because HHV-6 has been proposed to be a

co-factor in the progression of HIV infection to AIDs, the ability of HHV-6 *trans*-activating genes to *trans*-activate the HIV-LTR promoter has been investigated (Nicholas and Martin, 1994). Kashanchi *et al.*, (1994), reported that the HHV-6 DR7 gene region (which has homology to HCMV gene US22) could specifically *trans*-activate the HIV-LTR and required only a minimal promoter element containing the TATA box. Another HHV-6 gene region (termed B701), which has weak homology with the HCMV US22 gene family, is similarly capable of *trans*-activating the HIV-LTR promoter, via the NF-kB binding site (Geng *et al.*, 1992).

Despite the identification of *trans*-activators in other *Betaherpesviruses,* little light has been shed on how all the proteins interact during an infection. To date, *trans*-activation has been studied using transient transfection assays. Such an artificial system cannot reveal the interactions that occur *in vivo*, and indeed may suggest *trans*-activating activities that do not exist *in vivo.* It is clear that the situation is complex and viral gene products may interact with cellular promoters to create the appropriate cellular environment for viral growth. It is expected that the recent introduction of gene micro-array technology will shed more light on the total cellular response to HCMV infection and provide a more complete picture of the complex pattern of transcript activation.

1.7 Aims of thesis

The US22 gene family is little studied and yet the existence of homologues to most of the family genes in other sequenced *Betaherpesviruses* indicates that the family is important. The HCMV US22 family genes UL23 and UL24 were selected as targets for investigation because they have not been previously studied and because their ORFs appeared to be relatively uncomplicated. There was no evidence of an overlapping ORF and the UL23 and UL24 ORFs were not spliced. The presence of a non-coding region and TATA boxes between the ORFs suggests that each gene might have its own promoter, although the UL23 and UL24

genes were, however, 3'-co-terminal sharing a poly(A) site (Figure 19). Since antibodies were available, the UL43 and US22 genes were included in this investigation to broaden the scope of the project, permitting a comparison between several different US22 family members. At the outset of this investigation no anti-UL23 or UL24 antibody reagents were available and therefore the aims of the investigation were to:

1) Generate antibody reagents (polyclonal and monoclonal) to specifically detect UL23 and UL24 gene products.

2) Employ anti-UL23, anti-UL24, anti-UL43 and anti-US22 antibodies to investigate gene expression and to characterise the protein products.

3) Investigate the intracellular location of UL23, UL24, UL43 and US22 gene products.

4) To produce cell lines expressing pUL23, pUL24 and pUL43 in order to complement the defective mutant viruses.

5) Generate knockout mutants of UL23 and UL24 to investigate gene function.

Chapter 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All chemicals were analytical grade and unless otherwise stated were purchased from the Sigma-Aldrich Company Ltd., UK, or BDH Laboratory Supplies, UK.

2.1.2 Radiochemicals

All radiochemicals were purchased from PerkinElmer Life Sciences, U.S.A.

2.1.3 Restriction Endonucleases

All restriction endonucleases were purchased from New England Biolabs (UK) Ltd. or Boehringer Mannheim Ltd., UK, and were supplied with their appropriate buffers.

2.1.4 Antibodies

Anti-VP22 monoclonal (SY34) Ascites fluid	Courtesy of Dr. Howard Marsden
Anti-HCV E2 monoclonal (AP33)	Courtesy of Dr. Arvind Patel
Anti-HCMV gB monoclonal	Capricorn

Anti-HCMV HWLF1 (US22) monoclonal	Advanced Biotechnologies Inc.
Anti-HCMV UL43 monoclonal	Courtesy of Dr. Derrick Dargan
Anti-GST monoclonal	Amersham Pharmacia Biotech

All HRP, FITC and TRITC conjugated secondary antibodies were purchased from SAPU, UK, or Sigma-Aldrich Company Ltd., UK.

2.1.5 Composition of Commonly Used Solutions and Buffers

Bacterial Culture:

L-broth	10 g/L NaCl, 10 g/L tryptone peptone (Becton Dickinson) 5 g/L yeast extract (Becton Dickinson), pH 7.5
L-broth Agar	L-broth medium with 4 g/L agar (Becton Dickinson)
2YT broth	5 g/L NaCl, 16 g/L tryptone peptone (Becton Dickinson), 10 g/L yeast extract (Becton Dickinson), pH 7.5

Eukaryotic Cell Culture:

PBS	170 mM NaCl, 10 mM Na_2HPO_4, 3.4 mM KCl, 1.8 mM KH_2PO_4, 6.8 mM $CaCl_2$, 4.9 mM $MgCl_2$, pH 7.5
TP broth	29.5 g/L tryptose phosphate (Becton Dickinson)
Trypsin	0.25 % trypsin in Tris saline, pH 7.7 (140 mM NaCl, 0.7 mM Na_2HPO_4, 5.6 mM dextrose (D-glucose), 24.8 mM Tris, 2.5 mM KCl solution, 1 N HCl containing 1 % w/v phenol red with 0.1 % v/v penicillin, 0.1 g/L streptomycin)

Versene	0.6 mM EDTA containing 0.002 % v/v phenol red in PBS

SDS-PAGE and Western Immunoblotting:

Resolving Gel Buffer	0.74 M Tris-HCl (pH 8.9) containing 1 % w/v SDS
Stacking Gel Buffer	0.122 M Tris-HCl (pH 6.7) containing 0.1 % w/v SDS
Gel Running Buffer	52 mM Tris, 53 mM glycine containing 0.1 % w/v SDS
Sample Buffer (2x Concentration)	100 mM Tris-HCl (pH 6.7) containing 20 % v/v glycerol, 4 % w/v SDS, 2 % v/v β-mercaptoethanol, 0.2 % v/v bromophenol blue
Gel Fix/Stain	Methanol, deionised water, acetic acid in ratio 50:43:7 containing 0.2 % w/v Coomassie brilliant blue
Gel De-stain	Methanol, deionised water, acetic acid in ratio 50:880:70.
Towbins Buffer	3.02 g/L Tris-HCl (pH 8.3), 14.4 g/L glycine containing 20 % v/v methanol

Southern Blotting:

Gel Soak 1 (5x Concentration)	0.6 M NaCl, 0.2 M NaOH
Gel Soak 2	0.6 M NaCl, 1.0 M Tris-HCl (pH 8.0)
SSC (20x Concentration)	3 M NaCl, 0.3 M tri-sodium citrate (BDH)
Hybridization Buffer	0.5 M $NaPO_4$ (pH 7.4) containing 7 % w/v SDS

TBE Buffer (10x Concentration)	1.25 M Tris, 27 mM EDTA, 0.4 M boric acid

2.1.6 Miscellaneous Reagents and Commercial Kits

Plasmid and DNA Handling:

Advantage® cDNA polymerase mix	Clontech
dNTP set ultrapure 100MM solutions kit	Amersham Pharmacia Biotech
Plasmid maxi kit (+ endofree plasmid maxi kit)	QIAgen
High prime DNA labelling kit	Roche
Hybaid DNA recovery kit	Hybaid
QIAquick nucleotide removal kit	QIAgen

DNA Transfection Reagents:

LipofectAMINE Plus™ transfection reagent	GibcoBRL
Transfast™ (Tfx™-50) transfection reagent	Promega
Top 10 One Shot™ competant *E. coli*	Invitrogen

Protein Handling:

ABTS microwell peroxidase substrate system	Kirkegaard & Perry Laboratories
DC protein assay kit	BIO-RAD
ECL detection reagent	Amersham Pharmacia Biotech
E-Zinc reversible stain kit	Pierce
Rainbow markers RPN 756 kit	Amersham Pharmacia Biotech

2.1.7 Miscellaneous Materials and Plastics

Chromatography paper 3mm	Whatman
ECL nitrocellulose Western blotting membranes	Amersham Pharmacia Biotech
Hybond-N	Amersham Pharmacia Biotech
Treff 'Microfuge' tubes 1.5ml polypropylene	Anachem
Ultra-Clear™ centrifuge tubes 50, 14x95mm	Beckman
Photographic paper multigrade IV 16.5x21.6cm	Ilford
X-Omat-S-film	Kodak Ltd
X-Omat duplicating/RA film	Kodak Ltd

2.2 Methods

2.2.1 Cells and Virus Handling Techniques

2.2.1.1 Cells and cell culture

Tissue culture plastics were obtained from Nunc, Bibby, Costar or Falcon. All cell culture media were purchased from GibcoBRL, unless otherwise stated.

Human Foetal Foreskin Fibroblast (HFFF-2) cells [Cat.# 86031405: European Collection of Cell Cultures (ECACC)] were grown in Dulbecco's Minimal Essential Medium (DMEM) supplemented with 10 % v/v foetal calf serum (FC), 1 % v/v non-essential amino acids (NEAA) and 1 % v/v L-glutamine (L-glut) (DMEM-Fc10). 100 Units/ml of penicillin/streptomycin was added when cells were used for virus propagation or titration.

Human Telomerase Reverse Transcriptase (hTERT) immortalised BJ-Human Foreskin Fibroblast (hTERT-HFF) cells, (Gift of Dr. J. Shay and Dr. W. Wright, University of Texas, Southwestern Medical Center, Courtesy of Geron) were grown in DMEM/Medium 199 (ratio 4:1) supplemented with 10 % v/v FC, 1 % v/v NEAA and 1 % v/v L-glut.

hTERT immortalised Retinal Pigmented Epithelial (hTERT-RPE) cells, (Gift of Dr. J. Shay and Dr. W. Wright, University of Texas, Southwestern Medical Center, Courtesy of Geron) were grown in DMEM/NUT MIX F-12 medium supplemented with 10 % v/v FC, 1 % v/v NEAA and 1 % v/v L-glut.

Mouse hybridoma cells were grown in DMEM-Fc10 containing 4 % v/v HAT supplement.

U373-MG Astrocytoma cells [Cat.# 89081403: ECACC] were grown in DMEM-Fc10 containing 1 % v/v sodium pyruvate.

PT67 RetroPack™ cells [Cat.# K1060-D: Clontech] were grown in DMEM-Fc10.

Baby Hamster Kidney (BHK-21, C13) cells were grown in Eagles Minimal Essential Medium (EMEM) supplemented with 10 % v/v new born calf serum and 10 % v/v tryptose phosphate broth (EMEM-C10).

2.2.1.2 Detection of Mycoplasma in cell cultures

Cell cultures were checked for *Mycoplasma* contamination at each passage. Approximately $5x10^4$ cells were transferred to a culture dish containing a sterile 16 mm coverslip and incubated at 37 °C with appropriate medium for 4-5 days. The coverslip with attached cells was removed, drained by touching the edge with a dry tissue and placed cell side up in a fresh tissue culture plate. A drop of methanol:acetone (2:1) solution was added to the coverslip and left for 3 min at RT, before draining. 50 µl Hoescht stain (Boehringer Mannheim) was added to the coverslip and left for 10 min at RT, before draining. The coverslip was carefully washed with distilled water, drained and allowed to air dry at RT. A drop of mounting fluid (Mowiol: Harlow Chemical Company Ltd.) was placed onto a glass microscope slide and the coverslip mounted cell face down. Coverslips were examined using a Nikon UV microscope fitted with a 395 nm filter.

2.2.1.3 Viruses and virus propagation and storage

The AD169, Davis and Towne strains of HCMV were obtained from American Type Culture Collection (ATCC) [Cat.# VR-538, VR-807, VR-977, respectively].

HCMV stocks were prepared by infecting HFFF-2 cell monolayers ($4x10^6$ cells) with HCMV at an m.o.i. of 0.01 p.f.u./cell, and when extensive CPE was evident, seeding the virus infected cells on to subconfluent monolayers of HFFF-2 cells in roller bottles (at ~10^5 infected cells/bottle) at 37 °C. At 24 h post-infection (PI) the culture medium was replaced and the infected cultures re-incubated at 37 °C. Cytopathic effect was usually apparent by the fifth day PI and became extensive by the ninth day. The supernatant medium containing cell-free HCMV was harvested every day between the ninth and fifteenth day PI, clarified by low speed centrifugation and stored at –196 °C in liquid nitrogen (long-term) or –70 °C (short-term). The infectivity of stored samples was then determined by titration.

2.2.1.4 Virus titration

HFFF-2 cells ($2x10^5$ cells/35 mm dish) were seeded overnight at 37 °C. Serial 10-fold dilutions of the virus stock were made in PBS containing 5 % v/v FC. The medium was removed from the plates and 100 µl of virus dilutions added to the cells and incubated (1 h at 37 °C) with occasional gentle shaking to spread the virus. The infected cell layer was then overlaid in DMEM-Fc10 containing antibiotics and incubated at 37 °C. At 5 days PI the medium was removed and replaced with fresh DMEM-Fc10. At 7 to 10 days PI the medium was replaced with DMEM-Fc10 containing 1 % v/v carboxymethylcellulose. When plaques were of sufficient size for counting, the cell layers were fixed/stained by adding 2 ml Giemsa stain (BDH) directly into the culture dish. The plates were incubated for at least 2 h at RT,

after which the stain was decanted and the plates gently rinsed with tap water and dried upside down overnight, at RT prior to counting of plaques. Only those plates containing between 1 and 500 plaques/35 mm dish were counted. The virus titre was calculated as the average of the estimations obtained from each of the virus dilutions yielding countable numbers of plaques.

2.2.1.5 Virus plaque purification

The medium was removed from the infected cell layer, which was then washed with DMEM. Plaques were picked from the damp cell layer using a micropipette tip. The harvested infected cells were pipetted into 0.5 ml DMEM-Fc10 containing 5 % v/v sterile glycerol, as cryo-protectant, and stored at –70 °C. Before plating of plaque purified virus the thawed infected cells were sonicated to release intracellular virus.

2.2.1.6 Purification of virions, dense bodies and NIEPs from the culture medium of HCMV infected cells

The supernatant culture medium of infected HFFF-2 cells was clarified by centrifugation in the Sorvall GSA rotor (8 k/0.5 h/4 °C). Virus related particles in the clarified supernatant were then pelleted using the Sorvall GSA rotor (12 k/2 h/4 °C). The pelleted particles were re-suspended in a small volume (500 µl) of EMEM lacking phenol red (EMEMpr$^-$). The virus-related particles (non-infectious enveloped particles (NIEPs), virions and dense bodies) were layered on top of a negative viscosity, positive density, glycerol/potassium tartrate gradient, prepared in TN buffer (0.05 M Tris-HCl, pH 7.4, 0.10 M NaCl) (Irmiere and Gibson, 1983). The gradient was prepared in a Beckman ultra-clear™ centrifuge tube using a standard two-chambered gradient maker, with 5 ml of 30 % (w/w) glycerol in TN buffer

containing 15 % (w/w) potassium tartrate in the feeder chamber and 4 ml of 35 % (w/w) potassium tartrate in TN buffer in the mixing chamber. The particles were then banded by centrifugation in the Sorvall TST41 rotor (40 k/15 min/4 °C) operating at slow acceleration and braking settings. Banded material was carefully removed by side-puncture of the centrifuge tube with an 18G-needle, drawing off the particles into a 5 ml syringe. The separated particle types were diluted with EMEMpr$^-$, pelleted using the Sorvall TST41 rotor (20 k/2 h/4 °C), re-suspended in 50-100 µl EMEMpr$^-$ and stored at –70 °C.

2.2.2 Plasmid cloning and DNA analysis techniques

2.2.2.1 Preparation of ampicillin/agar plates

Ampicillin (amp) was added to hand-hot (37 °C – 40 °C) molten L-broth/agar at a final concentration of 100 µg/ml and ~20 ml then aliquoted into 100 mm plates. A bunsen flame was used to eliminate surface bubbles. The plates were allowed to set at room temperature, dried at 37 °C for 1 h and stored at 4 °C for up to 4 weeks.

2.2.2.2 Small scale preparations of plasmid DNA from bacteria

A single bacterial colony, picked from an agar plate, was inoculated into 2 ml L-broth containing the appropriate antibiotic and incubated with shaking overnight at 37 °C. The

bacteria were pelleted in a microfuge (6 k/2 min/RT), then re-suspended in 100 µl of lysis buffer (50 mM EDTA, 10 mM Tris, 230 mM sucrose, containing 0.5 % v/v Triton X-100 and 1 mg/ml lysozyme). The tube was heated to 100 °C for 1.5 min and the cell debris pelleted (13 k/10 min/RT). The supernatant was transferred to a new microfuge tube containing 400 µl of 0.3 M NaAc pH 7.0 and then 500 µl isopropanol was added to precipitate the DNA. The samples were mixed by inversion and then placed at –20 °C for at least 1 hour. The DNA was pelleted (13 k/5 min/RT), allowed to air dry at RT for ~20 minutes and then re-suspended in 33 µl of distilled water.

2.2.2.3 Preparation of glycerol stocks of bacterial cultures

1 ml of bacterial culture grown overnight (~16 h) was added to 1 ml sterile glycerol, mixed, and stored at –70 °C. When recovering bacteria from the frozen stock, the vial was moved to dry ice and a scraping taken, using a sterile toothpick, which was then deposited into 2 ml of L-broth containing the appropriate antibiotic.

2.2.2.4 Restriction endonuclease digestion of DNA

Restriction enzyme digests were typically of 20 µl reaction volume and contained 10 units of enzyme and 0.2-1.0 µg DNA. RNase A was added for digests of small-scale plasmid DNA preparations. Digests were usually incubated for 2-4 h at 37 °C, depending on the temperature requirement of the enzyme used, and the reaction terminated by addition of 5 µl restriction enzyme stop buffer (10 mM Tris-HCl pH 7.5, 50 mM EDTA containing 10 % v/v Ficcoll® 400 and 0.25 % v/v bromophenol blue).

2.2.2.5 Agarose gel electrophoresis of DNA fragments

The concentration of agarose gel employed depended upon the sizes of the DNA fragments that had to be resolved. Typically a 0.8 % gel was used (0.8 g agarose dissolved in 100 ml of 1x TBE by boiling). 10 µl of a 10 mg/ml ethidium bromide solution was added to the cooled agarose solution, which was then poured into the gel mould to set at RT. Gel electrophoresis was at 60-120 mA during the day or 12 mA overnight. Gels were viewed under short wave UV (302 nm), when a photographic record was required, or long wave UV (365 nm) when DNA fragments were to be excised.

2.2.2.6 Dephosphorylation of 5' ends of DNA fragments

Typically the reaction contained 1-2 µl alkaline phosphatase (0.01 u/pmol of ends) and 10 µl Reaction Buffer (10x concentration) in a final volume of 100 µl. The reaction was incubated at 37 °C for 1 h and the dephosphorylated DNA then recovered using the 'QIAquick' Nucleotide Removal kit. The picomoles of ends of linear double-stranded DNA was calculated according to the formula:

(µg DNA/kb size of DNA) x 3.04 =pmol of ends

2.2.2.7 Ligation of DNA fragments

Typically DNA ligation reactions used Insert:Vector ratios 1:1 and 3:1 and included a linearised vector plasmid control for ligation efficiency. Transformation efficiency was also

checked using uncut plasmid as control. The ligation reaction volume was usually 10 µl and contained 200 ng vector DNA, 2 µl of 5x concentration ligase buffer and 0.5 µl high concentration T4 ligase (2,000,000 units/ml) (New England Biolabs). Ligations were conducted at 16 °C overnight. DNA concentrations were measured using a spectrophotometer operating at 260 nm and the amount of insert DNA required calculated according to the formula:

([ng of vector x kb size of insert]/kb size of vector) x molar ratio of insert:vector = ng of insert required.

2.2.2.8 Plasmid preparation for DNA sequencing

A single colony of *E. coli* cells harbouring the plasmid to be sequenced was picked from an agar plate and inoculated into 5 ml L-broth containing the appropriate antibiotic and grown overnight at 37 °C. The bacteria were pelleted (microfuge; 6 k/2 min/RT), re-suspended in 100 µl GTE buffer (50 mM D-glucose, 25 mM Tris-HCl pH 8, 10 mM EDTA containing 4 mg/ml lysozyme) and incubated for 10 min at RT. 200 µl of 200 mM NaOH containing 1 % w/v SDS was added to each tube, mixed by gentle inversion and then incubated on ice for 10 min. 150 µl of 3 M KAc pH 5.0 was added and the tube gently inverted to mix, and then incubated on ice for 10 min. The cell debris was pelleted (microfuge; 12 k/5 min/RT) and the supernatant drawn off and the DNA extracted by treatment with phenol/chlororform (1:1). The DNA was precipitated by addition of 2 volumes ethanol and the DNA pelleted (12 k/5 min/RT). The DNA pellet was air dried, re-suspended in 100 µl TE buffer (10 mM Tris pH 8.0, 1 mM EDTA) containing 100 µg/ml RNase A and incubated for 30 min at 37 °C. 120 µl of PEG/NaCl (2.5 M NaCl containing 20 % w/v polyethylene glycol (PEG) 6000) was added to each tube, which was gently inverted to mix and incubated on ice for 1 h. The DNA was pelleted as before, washed in 70 % ethanol, pelleted again and allowed to dry at

RT. The DNA pellet was re-suspended in 50 µl TE buffer (2 mM Tris-HCl pH 8, 0.2 mM EDTA). 6 µl of the plasmid preparation was mixed with 0.5 µl of 1/1000 dilution of oligonucleotide sequencing primer and sent for automated DNA sequencing.

2.2.2.9 DNA sequencing

Automated DNA sequencing was performed on an ABI Prism ™ 377 DNA Sequencer using Sequencing Analysis Software version 3.0 (ABI) and operated by Miss. Lesley Taylor. Files were transferred to UNIX, converted to SCF format (Bonfield and Staden, 1995), compiled using preGap4 1.1 and Gap4 v4.5 software (MRC, UK) and analysed using Wisconsin Package™ Version 10 software (Genetics Computer Group, USA). The FastA function was used to run a Pearson and Lipman search for similarity between the compiled sequence and the reference sequence.

2.2.2.10 Polymerase Chain Reaction (PCR)

PCR reactions were performed using a PTC-200 Peltier Thermal Cycler. The reaction volume was 50 µl, and was comprised of 1 µl 50xdNTPs, 1 µl cDNA polymerase mix, 2 µl primer mix (50 pmol of each primer made to final volume with water), 5 µl template (1 ng), 5 µl 10x buffer and 36 µl deionised water. Unless otherwise stated PCR conditions were as follows: 94 °C for first 5 min; then 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min x 29 cycles; then 72 °C for 5 min followed by cooling to 4 °C.

2.2.2.11 Preparation of competent bacterial cell cultures

10 ml cultures of *E. coli* cells (strain DH1 or BL21) in L-broth (no ampicillin), grown overnight, were used to inoculate 80 ml L-broth (no ampicillin) and the culture was grown for a further 2 hours. The bacteria were pelleted in the Sorvall GSA rotor (5 k/10 min/4 °C), re-suspended in 25 ml ice-cold 100 mM $CaCl_2$ and kept on ice for at least 1 h. The cells were then pelleted in the Sorvall SS-34 rotor (5 k/10 min/4 °C) and re-suspended in 0.5 ml of ice-cold 100 mM $CaCl_2$. Competent cells were kept on ice at all times and were used within 2 hours of preparation.

In some experiments Top 10 One shot™ competent *E.coli* (Invitrogen) were used.

2.2.2.12 Transformation of competent bacterial cell cultures

DNA for transfection was added to 50 µl of competent cell preparation and the mixture incubated on ice for at least 1 h. The cells were then subject to a heat shock (42 °C/1 min), added to 0.5 ml L-broth (no ampicillin) and incubated at 37 °C for 1.5 h. 100 µl cell suspension was plated on agar plates containing the appropriate antibiotic, and incubated overnight at 37 °C.

Electroporation of bacterial cells, an alternative transformation method, was also employed in some experiments. Gene Pulser cuvettes with 0.1 cm electrode gap (BIO-RAD) were cooled at –20 °C overnight and dried before use. DNA (0.1 – 1.0 µg) and 40 µl electro-competent *E. coli* (strains DH1 or BL21) were gently mixed, transferred to the cuvette and electroporated, using the Gene Pulser™ machine (BIO-RAD) set at a capacitance of 25 µFD, resistance of 400 ohms and 1.6 volts. Immediately after electroporation the cells were added

to 1 ml L-broth (no ampicillin) and grown for 1 h at 37 °C and then 50 µl cell suspension was plated onto agar plates containing the appropriate antibiotic and incubated overnight at 37 °C.

2.2.2.13 Transfection of mammalian cells

LipofectAMINE Plus™ transfection reagent was used according to the manufacturers instructions (transfection efficiency of 0.1 % to 4.0 % as determined by pcDNA3.1His B/lacZ reporter plasmid). Optimal conditions were determined for transfection of HFFF-2 cells ($2x10^5$ cells/35 mm plate): 0.25 µg DNA was mixed with 30 µl 'Plus' reagent in a total volume of 60 µl and the solution was incubated for 15 min at RT, while 2.5 µl LipofectAMINE reagent was mixed with 57.5 µl serum-free DMEM and this solution was also incubated for 15 min at RT. The DNA preparation and LipofectAMINE solutions were mixed and incubated for 30 min at RT. Cells were washed twice and then overlaid with 200 µl serum free DMEM and the transfection mix was then also added to the cells, which were incubated for 3 h at 37 °C. The cells were finally overlaid with DMEM-Fc10 and incubated for 48 h at 37 °C.

Transfast™ transfection reagent was used according to the manufacturers instructions (transfection efficiency of 0.005 % to 0.45 %). Optimal conditions were determined for transfection of HFFF-2 cells ($5x10^4$ cells/well): 0.1 µg DNA was mixed with Optimem 1 (GibcoBRL) and 6 µl Transfast™ reagent in a total volume of 250 µl and the mix was incubated for 10 min at RT. The medium was removed from the cells, which were then overlaid with the transfection solution for 4 h at 37 °C. The transfection solution was removed and the cells were overlaid with DMEM-Fc10 for 48 h at 37 °C.

Calcium phosphate transfection of mammalian cells, an alternative transfection method, was also employed in some experiments. 0.2-0.5 µg plasmid DNA was mixed with 2.75 µg calf

thymus DNA and water in a final volume of 57 µl and mixed with 66 µl of 2xHEBS (50 mM HEPES pH 7.1, 280 mM NaCl, 1.5 mM Na_2HPO_4) and 9 µl of 2 M $CaCl_2$. The medium was removed from the cell layer and the DNA/HEBS/$CaCl_2$ solution added drop-wise to the cells, which were then incubated for 40 min at 37 °C. 2 ml medium was added to the cells and they were incubated for a further 4 h at 37 °C.

The medium was then removed from the cells and the cell layer bathed in pre-warmed (37 °C) 10 % v/v DMSO in 1x HEBS for 2.5 min. The DMSO solution was decanted, the cell layer washed once with pre-warmed DMEM, overlaid with growth medium and the cultures returned to 37 °C.

2.2.2.14 Assessing transfection efficiency using β-gal staining reagent

Transfection efficiency was assessed by transfecting cell layers with plasmid pcDNA3.1HisB/lacZ (Invitrogen). At 48 h post-transfection the medium was removed and the cell layer fixed by treatment with 1 % gluteraldehyde in PBS for 1 h at RT. The gluteraldehyde solution was removed and the cells washed twice with PBS. The β-Gal staining reagent (100 mM potassium ferrocyanide, 100 mM potassium ferricyanide, 100 mM MgCl containing 0.1 % NP40 and 40 mg/ml x-gal in DMSO) was added and the cells incubated at 37 °C for approximately 2 to 3 h. The cells were periodically inspected and when blue coloured cells could be detected the staining solution was removed and the cells washed with de-ionised water. The percentage transfection efficiency was estimated as the percentage of blue cells compared to total cell number in the culture.

2.2.2.15 DNA extraction from virus infected mammalian cells

HCMV infected HFFF-2 cells on 35 mm tissue culture dishes were harvested when 100 % CPE was reached. The culture medium was replaced with 200 µl of cell lysis buffer (20 mM Tris-HCl pH 7.5, 1 mM EDTA) containing 0.5 % w/v SDS and 100 µg/ml proteinase K and incubated for at least 3 h at 37 °C. 15 µl of 5 M NaCl and 200 µl of phenol/chloroform (1:1) solutions were added to the DNA sample, gently mixed by inversion and the DNA recovered by centrifugation in a microfuge (13 k/2 min/RT). The upper phenol layer was transferred to a new tube containing 200 µl chloroform and the extraction repeated. The upper layer was again transferred to a new tube and the DNA precipitated by addition of 1ml ethanol. The DNA was pelleted in the microfuge (13 k/5 min/RT), washed with 0.5 ml ethanol and pelleted as before. The DNA pellet was air dried for ~20 min at RT and re-suspended in 25 µl water.

2.2.2.16 Southern blotting

Agarose gels containing resolved DNA fragments were placed in gel soak 1 for 1 h, then gel soak 2 for 45 min, and finally 20xSSC for 1 h. The treated gel was placed on top of Hybond-N membrane and dry paper towels and incubated overnight at RT. The DNA fragments that had transferred to the Hybond-N membrane were fixed to the membrane using a UV Stratalinker™ 1800 machine (Stratagene) operating on auto cross-linking setting. Membranes were stored at 4 °C in a sealed hybridisation bag until required.

The radioactive probe was made with the High Prime random priming kit (Roche) using 200 ng of template DNA and 50 µCi of α-^{32}P-dCTP. Hybridisation was conducted overnight at

65 °C. The membrane was washed extensively at 65 °C, twice with 2xSSC and then once with 1xSSC and then dried at 80 °C for 10 min, before exposing the membrane to X-ray film.

2.2.2.17 Extraction of total cellular RNA for use in Northern blotting

Pelleted cells (~$1x10^7$ cells) were re-suspended in 1 ml Trizol (Life Technologies) and incubated for 5 min at RT. 200 µl chloroform was then added and incubation at RT continued for a further 3 min, after which the sample was pelleted in a microfuge (12k/15 min/4 °C). The RNA containing aqueous (upper) phase was transferred to a new vial to which 0.5 volume isopropanol was added. Following incubation for 10 min at RT the RNA was pelleted (12 k/10 min/4 °C) and rinsed with 75 % ethanol. The RNA was allowed to air dry and re-suspended in 20 µl sterile TE at 55 °C for 10 min and immediately stored at –70 °C.

2.2.3 Protein analysis techniques

2.2.3.1 SDS-PAGE polypeptide electrophoresis

The Mini-PROTEAN II Cell apparatus (BIO-RAD) was used for preparation of SDS-PAGE gels. Typically a 10 % gel (1.5 mm thickness) was prepared. The resolving gel solution contained 5 ml resolving gel buffer, 8 ml distilled water, 7 ml 30 % Acrylamide/BIS (37.5:1), 20 µl TEMED and 80 µl of 25 % w/v ammonium persulfate. The stacking gel solution

contained 3 ml stacking gel buffer, 7 ml distilled water, 2 ml 30 % Acrylamide/BIS, 20 µl TEMED and 80 µl of 25 % w/v ammonium persulfate. Electrophoresis was at 100 V.

2.2.3.2 Staining of proteins resolved on SDS-PAGE gels with Coomassie brilliant blue dye

SDS-PAGE gels containing resolved polypeptides were immersed in gel fix/stain containing 0.2 % v/v Coomassie brilliant blue dye for 2-3 hours, on a slowly rocking platform and then transferred to de-stain solution overnight.

2.2.3.3 Recovery of proteins from gel slices by electro-elution

SDS-PAGE gels on which the GST fusion proteins were resolved were negatively stained with the E-Zinc reversible-stain kit (Pierce) and the GST fusion protein band excised. The fusion protein was electro-eluted from the gel slice using the Biotrap apparatus (Schleicher & Schuell) with electrophoresis at 120 V overnight. The concentration of the electro-eluted protein was then determined using the DC protein assay kit (BIO-RAD).

2.2.3.4 Western immunoblots

Western blotting of proteins resolved on SDS-PAGE gels was accomplished by electrophoretic transfer to ECL nitrocellulose membranes at 50 V for 2-3 h using the BIO-RAD Mini Trans-Blot apparatus. The blots were then treated with blocking buffer (PBS with

0.00005 % Tween 20 (PBS/T) containing 5 % w/v dried "Marvel" milk powder) overnight at 4 °C or for 2 h at 37 °C. The membranes were then washed (5 cycles of 3 min washes in PBS/T) and treated with the primary antibody. Polyclonal antibodies were typically diluted 1/500 in PBS/T containing 1 % w/v bovine serum albumin (BSA) and incubated with the membrane for 2 h at 37 °C. Hybridoma culture supernatants were used undiluted. The primary antibody was decanted and the membranes were again washed as before. The secondary HRP conjugated antibody was diluted 1/1000 in PBS/T containing 1 % w/v BSA and incubated with the membrane for 1 h at 37 °C. The secondary antibody was decanted and the membrane washed as before, treated with the ECL reagents and the membrane exposed to photographic film.

2.2.3.5 Isolation of bacterial inclusion bodies containing the GST-UL24 fusion protein

A 1 L culture of *E. coli* (strain BL21) containing the plasmid pGEX-6P-1/UL24 was grown in L-broth at 37 °C until a cell density of 0.6 OD_{600} units was reached. IPTG was then added (to 1 mM) to induce expression of the GST fusion protein and the culture incubated for a further 4 h at 37 °C. The bacteria were pelleted using the Sorvall GS-3 rotor (8 k/10 min/4 °C) and the pellet frozen at –20 °C.

The bacterial pellet was thawed, re-suspended in 50 ml ice cold PBS, lysozyme added (to 1 mg/ml) and the cells then incubated for 20 min at RT. The culture was sonicated at 4 °C (3 x 10 second bursts) and the insoluble material pelleted (6.5 k/10 min/4 °C). The pellet material was re-suspended in 5 ml ice-cold buffer 1 (0.1 M NaCl, 1 mM EDTA, 50 mM Tris-HCl pH 8.0, 2.4 mM sodium deoxycholate) and incubated on ice for 10 min. $MgCl_2$ and DNase I were added to 8 mM and 15 µg/ml, respectively, and the samples then incubated at 4 °C, with occasional mixing, until viscosity was eliminated (~30 min). Insoluble material was again pelleted (2 k/10 min/4 °C) and washed by re-suspension in 2.5 ml buffer 2 (50 mM Tris-HCl

(pH 8.0), 2 M Urea) followed by centrifugation, as before. The inclusion body preparation was washed by re-suspension in 2.5 ml buffer 3 (50 mM Tris-HCl (pH 8.0), 0.1 M NaCl, 1 mM EDTA). The final preparation of isolated inclusion bodies was re-suspended in 400 µl SDS-PAGE Sample Buffer for SDS-PAGE analysis and electrophoretic separation of the GST-UL24 fusion protein. The GST-UL24 fusion protein was recovered from SDS-PAGE gel slices by electro-elution, as described above.

2.2.3.6 Method for purification of GST-UL23 fusion proteins

A 3 L culture of *E.* coli (strain BL21) harbouring pGEX-6P-1/UL23 was prepared and induced with IPTG to produce the GST-UL23 fusion protein, as described above for GST-UL24. Bacterial cell pellets were re-suspended in ice-cold PBS and the cells disrupted by sonication (3 x 10 second bursts). Triton X-100 was added to 1 %, mixed gently for 30 min at RT and the insoluble material pelleted (10 k/10 min/4 °C). In order to bind soluble GST-UL23 fusion protein the supernatant fraction from lysed bacteria was mixed with glutathione sepharose beads, on a rotary mixer for 30 min at RT. The slurry mixture was transferred to a column, allowed to settle and then to drain completely. The column matrix was then washed extensively with 10x bed volumes PBS and allowed to drain. To recover the GST-UL23 fusion protein from the glutathione sepharose the column matrix was re-suspended in SDS-PAGE sample buffer, and boiled for 10 min. The GST-UL23 fusion protein was then separated by SDS-PAGE. The GST-UL23 fusion protein was finally recovered by electro-elution from gel slices, as described above.

2.2.3.7 Immunising rabbits for polyclonal antibody production

Rabbit polyclonal antibodies were raised against the GST-UL23 and GST-UL24 fusion proteins, in young adult female Dutch lop-ear rabbits. A 3 ml blood sample was taken prior to immunisation and allowed to clot for 1h at 37 °C. The clotted blood was pelleted (2 k/5 min/RT) and the pre-immune serum drawn off, aliquoted and stored at –20 °C. Rabbits were immunised by subcutaneous injection of 50 μg of fusion protein emulsified 1:1 with Freund's complete adjuvant (SIGMA) in a 400 μl innoculum. The immune response was boosted at 2-3 week intervals (total of 3 times) by additional injection of fusion protein (50 μg protein prepared in Freund's incomplete adjuvant) and test bleeds taken. ELISA reactions were used to test the immune serum for fusion protein specific antibodies.

Rabbit polyclonal antibodies were also raised against synthetic branched peptides of UL23 and UL24 in young adult female Dutch lop-ear rabbits. Pre-immune sera were collected as described above. Rabbits were immunised subcutaneously with 100 μg of synthetic peptide emulsified 1:1 with Freund's complete adjuvant in a 400 μl innoculum. The immune response was boosted further by injection of 100 μg peptide prepared in Freund's incomplete adjuvant at 2-3 week intervals. A final booster immunisation was conducted 1 week prior to the rabbits being bled out. ELISA reactions were used to determine the anti-peptide antibody potency.

2.2.3.8 Immunising mice for monoclonal antibody production

Mouse monoclonal antibodies were raised against GST-UL23 and GST-UL24 fusion proteins in Balb/C mice by subcutaneous injection of 20 μg of protein emulsified 1:1 in Freund's complete adjuvant in a 100 μl innoculum. Two further injections of protein in Freund's incomplete adjuvant were administered at two-weekly intervals. Three weeks after the last

boost, the animals were given a final intra-peritoneal injection of 100 μg protein in 100 μl PBS. The mice were sacrificed 5 days after the last injection and the spleen cells fused with Sp2/0-Ag 14 myeloma cells (ratio 10:1) using 50 % PEG1500 (Koch Light) in DMEM-Fc10. Cells were re-suspended in HAT medium (DMEM-Fc10 containing 4 % HAT supplement) containing 20 % v/v conditioned medium. Cells were seeded into 96-well microtitre plates and incubated for 12-14 days at 37 °C. Supernatants from colony-containing wells were removed and screened by ELISA against GST fusion proteins for protein-specific monoclonal antibodies. Cells from positive wells were removed and propagated.

2.2.3.9 Enzyme Linked ImmunoSorbant Assay (ELISA)

96 well flat-bottomed microtitre plates were coated with 50 μl of antigen, either GST fusion proteins or synthetic peptides, at 250 ng/well. The plate was closed and incubated overnight at RT. Excess antigen was decanted and the plate washed 5 times in wash buffer (145 mM NaCl, 7.5 mM Na_2HPO_4, 2.8 mM NaH_2PO_4 containing 0.5 % Tween 20). The plate was dried by tapping on paper towels. 150 μl of blocker solution (PBS + 2 % w/v BSA) was added to each well and incubated for 1 h at RT, after which and the plates were dried and stored. For the ELISA reactions, 50 μl of primary antibody was added to each well and the plate incubated for 1 h at 37 °C. The plate was washed 5 times in wash buffer and 50 μl/well of secondary (anti-mouse or anti-rabbit) antibody conjugated to HRP (diluted 1/1000 in PBS) was added and the plates again incubated for 1 h at 37 °C. The wells were washed 8 times in wash buffer prior to addition of 100 μl/well of ABTS peroxidase substrate. The ELISA reaction was incubated for 15-30 min at RT and the OD_{405} measured using an automated plate reader (Titertek Multiskan® Plus).

2.2.3.10 Extraction of infected cell immediate-early, early and late proteins

For preparation of virus immediate-early (I-E) proteins the medium on HFFF-2 cell layers ($2x10^5$ cells/35mm dish) was replaced with DMEM-Fc10 containing 200 μg/ml cycloheximide (CHX) for 1 h at 37 °C. The medium was then removed and the cells infected with HCMV at a m.o.i. of 10 p.f.u./cell in a 100-200 μl innoculum containing CHX at 200 μg/ml. The cells were allowed 1 h at 37 °C to absorb the virus and the cell layers were then washed 3 times and then overlaid with DMEM-Fc10 containing 200 μg/ml CHX. Actinomycin D (Act D) was added to the cultures at 18 h PI to a final concentration of 5 μg/ml for 30 min. CHX was then removed by 3 washes with DMEM-Fc10 containing 5 μg/ml Act D and the cells were overlaid with the same medium and incubated for 4 h at 37 °C. Protein extracts were prepared by decanting the medium, washing the cell layer with PBS and solubilizing the cells in 250 μl of SDS-PAGE sample buffer.

For preparation of virus early (E) proteins the cell layers were infected with HCMV at 10 p.f.u./cell, as described above except that CHX was not used. After the 1 h adsorption period the infected cells were washed 3 times and then overlaid with DMEM-Fc10 containing 300 μg/ml phosphonoacetic acid (PAA) and incubated for 48 h at 37 °C, prior to harvesting the protein extracts as previously described.

To prepare virus late (L) proteins the cell layers were infected, as described above, but no drugs were used. Infected cell protein extracts were then harvested at 72 h PI in SDS-PAGE sample buffer, as previously described.

In experiments in which the culture medium was probed for secreted virus proteins, the medium was removed from infected cell layers and clarified by low speed centrifugation. The clarified supernatant was then centrifuged in the Sorvall SS-34 rotor (20 k/2 h/4 °C) to pellet virus-related particles and the supernatant medium then mixed with SDS-PAGE sample buffer at a ratio of 1:2.

2.2.4 Light Microscopy

2.2.4.1 Immunofluroescence

HFFF-2 cells were seeded on sterile 13 mm glass coverslips in 24 well tissue culture trays at 5 x 10^4 cells/coverslip. Following infection with virus, or transfection with plasmids, the cells were incubated at 37 °C. After varying periods of time, the medium was removed, the coverslips washed twice with PBS and fixed in 0.5 ml fix solution (5 % v/v formaldehyde, 2 % w/v sucrose in PBS) for 10 min at RT. An alternative fix was methanol for 5 min at RT. The fixed cells were again washed with PBS and either permeabilised immediately or the coverslips stored at 4 °C in PBS. Permeabilisation was achieved by a 5 min at RT incubation of the cells in buffer (0.5 % v/v NP40, 10 % w/v sucrose in PBS) followed by 3 washes with PBS. Cells were treated for 1 h at RT with primary antibody (PAbs diluted 1/500, MAbs were undiluted) in a volume of 50-100 µl/coverslip. Primary antibody was removed by 3 washes in PBS and then the cells were treated for 1 h at RT with 100 µl secondary anti-rabbit or anti-mouse FITC conjugated IgG (diluted 1/100 in PBS containing 1 % calf serum), as appropriate. Secondary antibody was removed by 3 washes in PBS and once in de-ionised water after which the coverslips were allowed to air dry. The coverslips were mounted in Citifluor mounting solution on glass microscope slides with the edges of the coverslip sealed with clear nail varnish. The mounted slides were stored in the dark at 4 °C to preserve fluorescence and examined using the confocal microscope under UV illumination.

2.2.4.2 Confocal microscopy

Confocal microscopy was performed using a Zeiss LSM Confocal Microscope linked to a computer operating LSM510 software.

2.2.5 Electron Microscopy

2.2.5.1 Immuno-gold labelling of thin sections of HCMV infected cells for electron microscopy

HFFF-2 cells were infected with HCMV strain AD169 at a m.o.i. of 5 p.f.u./cell. At 96 h PI, the cells were scraped into PBS, pelleted in a BEEM capsule (TAAB laboratories) and fixed with 2.5 % v/v gluteraldehyde in PBS. The cell pellet was dehydrated through a series of increasing ethanol concentrations, up to 100 % ethanol, and then permeated with acrylic resin (unicryl) for 8 h at RT. The cell pellet was embedded in fresh resin, and polymerised by exposure to UV light for 4 days at –15 °C. Thin sections (70-80 nm) were removed from the block, using an ultratome fitted with a diamond knife and the sections placed on non-coated nickel EM grids (200 mesh). The cell sections were overlaid with PBS alone, non-HCMV control antibody AP33, anti-UL23 MAb 266, anti-UL24 MAb 116, or anti-UL43 Mab 92 overnight at RT. After extensive PBS washing of the cell sections they were overlaid with the secondary anti-mouse antibody, conjugated to 10 nm gold particles (Nanoprobes, Inc.) (diluted 1/30 in PBS) and incubated for 2 h at RT. The labelled sections were post-fixed with osmium tetroxide vapour for 2 h at RT, stained with uranyl acetate (saturated solution in 1:1

ethanol: water), counter stained with lead citrate, then examined in a Jeol 100S electron microscope operating at 80 KV.

2.2.5.2 Immuno-gold labelling of virus particle preparations for electron microscopy

Purified virus particles were adsorbed on parlodion coated nickel EM grids and then treated for 5 h at RT with PBS alone, non-HCMV control MAb AP33, anti-UL23 MAb 266, anti-UL24 MAb 116, anti-US22 Mab (HWFL1) or anti-UL43 MAb 92. The grids were then washed extensively in de-ionised water and overlaid with goat anti-mouse IgG conjugated to 5 nm or 10 nm gold particles (Nanoprobes, Inc.) (diluted 1/30 in PBS) overnight at RT. The gold-tagged particles were then negatively stained with phosphotungstic acid (potassium phosphotungstate acid (PTA) 3 % in distilled water pH 7.0) prior to examination in a Jeol 100S electron microscope operating at 80 KV.

Chapter 3

RESULTS

3.1 Cloning of the HCMV UL23 and UL24 ORFs

3.1.1 PCR Amplification

The UL23 ORF (nucleotides 28717-27864, Chee *et al.*, (1990)) was amplified by PCR from the cloned AD169 Hind III Y fragment (Oram *et al.*, 1982) using 5' and 3' specific oligonucleotide primers (Table 5). The 5'-primer (RA2) had *Eco*R I and *Sal* I linker sequences abutting the UL23 start codon, while the 3'-primer (RA1) had an *Xho* I linker sequence abutting the stop codon. Following amplification, in this and all subsequent PCR reactions, 2 µl of the final PCR reaction product was electrophoresed on a 50 ml 1 % agarose gel and examined under UV illumination to check the size and number of DNA bands. The UL23 PCR reaction produced a single band of the expected size of ~0.9 kbp.

The UL24 ORF (nucleotides 30009-28933, Chee *et al.*, (1990)) was amplified by PCR from an AD169 cosmid fragment (Cos 64, nucleotides 23495 – 66934, provided by Dr. Derrick Dargan) using 5' and 3' specific oligonucleotide primers (Table 5). The 5'-primer (RA4) had *Eco*R I and *Sal* I linker sequences abutting the UL24 start codon, while the 3'-primer (RA3) had an *Xho* I linker sequence abutting the stop codon. The PCR reaction produced a single band of the expected size of ~1.1 kbp.

Table 5. Oligonucleotide PCR primers designed for amplification of HCMV (AD169) ORFs or for use in DNA sequencing. Start (ATG) and Stop (TCA) codons are marked in bold in the oligonucleotides.

PCR PRIMER NAME	PRIMER USAGE	OLIGONUCLEOTIDE SEQUENCE
RA1	Amplify UL23 ORF	5'-ATA*CTCGAG***TCA**CGCGTCGTCAAAAAGTTGGTGGTC *Xho* I
RA2	Amplify UL23 ORF	5'-CCG*GAATTCGTCGAC***ATG**TCGGTAATCAAGGACTGTTTTCTC *Eco*R I *Sal* I
RA3	Amplify UL24 ORF	5'-ATT*CTCGAG***TCA**ACGGTGCTGACGTCCTTTGGGGCA *Xho* I
RA4	Amplify UL24 ORF	5'-ATT*GAATTCGTCGAC***ATG**GAGGAGACCCGGGCGGGACGTTAT *Eco*R I *Sal* I
RA5	DNA sequence vector pGEX-6P-1	5'-TACTTGAAATCCAGCAAGTA
RA6	DNA sequence vector pGEX-6P-1	5'-TCACCGTCATCACCGAAACG
RA7	DNA sequence UL24 ORF	5'-GCTCGCCCACTGACTCGGCG
RA8	DNA sequence UL24 ORF	5'-GGCGGATGATGAATCGCGCC
RA9	DNA sequence UL23 ORF	5'-CACAGCGTCTGGCCAGCTAC
RA10	DNA sequence UL23 ORF	5'-GCTCCAGTCGTACAGCCGAA
RApc3.1	DNA sequence vector pcDNA3.1+	5'-TAAGCAGAGCTCTCTGGCTA
RA2pc3.1	DNA sequence vector pcDNA3.1+	5'-AGTGGCACCTTCCAGGGTCA

3.1.2 Cloning the UL23 and UL24 ORFs into the GST fusion vector pGEX-6P-1

The 0.9 kbp UL23 PCR product was digested with *Eco*R I and *Xho* I, purified using the 'QIAquick' nucleotide removal kit and cloned into the *Eco*R I/*Xho* I sites in the GST fusion vector, pGEX-6P-1 (Amersham Pharmacia Biotech) (Figure 3). In this, and all subsequent

cloning experiments diagnostic restriction enzyme digests were performed to confirm that cloning had been successful. For DNA sequencing, oligonucleotide PCR primers were designed to amplify sequences from the vector DNA into the UL23 ORF (Table 5): a forward primer (RA5) at vector nucleotide 831 and a reverse primer (RA6) at vector nucleotide 1030. Oligonucleotide sequencing PCR primers were also designed to amplify sequences from the UL23 sequences out into the vector DNA: a reverse primer (RA9) at HCMV nucleotide 28551 and a forward primer (RA10) at HCMV nucleotide 28151. Oligonucleotide sequencing primers were always selected to allow for at least 200 bp of sequencing overlap. DNA sequencing confirmed that there were no errors in the amplified UL23 ORF and that it was cloned in-frame with the resident GST gene in the vector. pGEX-6P-1/UL23 DNA was transfected by electroporation into protease-deficient *E. coli* (strain BL21) cells to produce the GST-UL23 fusion protein.

The 1.1 kbp UL24 PCR product was digested with *Eco*R I and *Xho* I, purified using the 'QIAquick' nucleotide removal kit and cloned into the *Eco*R I/*Xho* I sites in the vector pGEX-6P-1 (Figure 3). For DNA sequencing, oligonucleotide PCR primers were designed to amplify sequences from the UL24 sequences out into the vector DNA: a reverse primer (RA7) at HCMV nucleotide 29861 and a forward primer (RA8) at HCMV nucleotide 29337. The RA5 and RA6 sequencing PCR primers (Table 5) were used to amplify sequences from the vector DNA into the UL24 ORF. DNA sequencing confirmed that the UL24 ORF was cloned in-frame with the resident GST gene in the vector, but also showed that there was a silent mutation in the amplified UL24 ORF at nucleotide 29623, (codon TTC changed to codon TTT, both of which code for phenylalanine). pGEX-6P-1/UL24 DNA was transfected by electroporation into protease-deficient *E. coli* (strain BL21) cells to produce the GST-UL24 fusion protein.

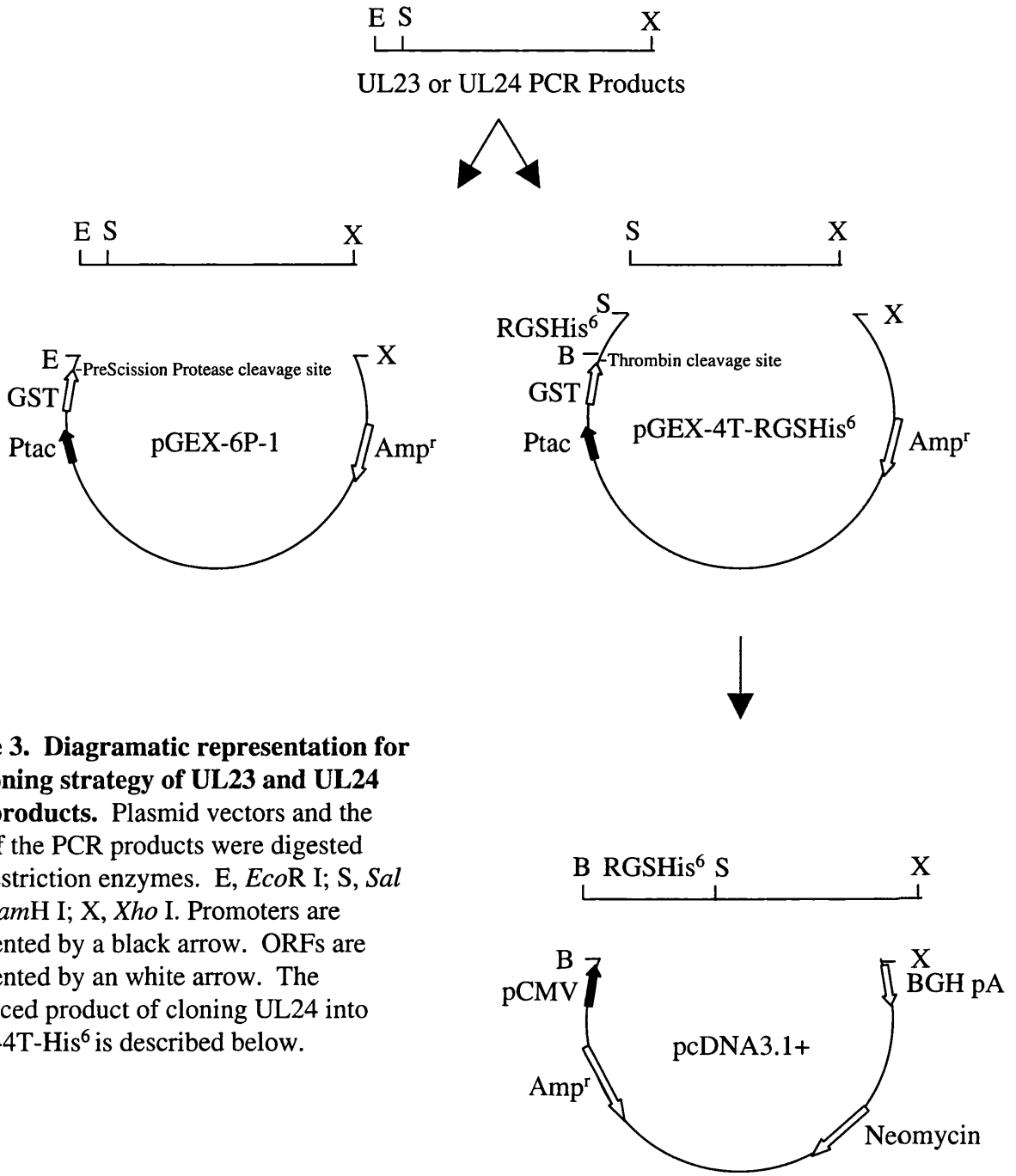


Figure 3. Diagramatic representation for the cloning strategy of UL23 and UL24 PCR products. Plasmid vectors and the ends of the PCR products were digested with restriction enzymes. E, *Eco*R I; S, *Sal* I; B, *Bam*H I; X, *Xho* I. Promoters are represented by a black arrow. ORFs are represented by an white arrow. The sequenced product of cloning UL24 into pGEX-4T-His^6 is described below.

Desired Sequence of UL24 cloned into pGEX-4T-His^6:

CTG*GGATCC* ATG AGA GGA TCT CAT CAC CAT CAC CAT CAC *GTCGAC* **ATG**
Bam H I M R G S H H H H H H *Sal* I **UL24 ORF**

Actual Sequence obtained following cloning:

CTG*GGATCC* CCG *GAATCC* *GTCGAC* **ATG**
Bam H I *Eco*R I *Sal* I **UL24 ORF in frame**

3.1.3 Cloning the UL23 and UL24 ORFs into vector pGEX-4T-3/RGSHis^6

pGEX-4T-3/RGSHis^6 is derived from the pGEX-4T-3 GST fusion vector (Amersham Pharmacia Biotech) by placement of a RGSHis^6 tag sequence after the thrombin cleavage site in the vector (generated and provided by Dr. Arvind Patel) (Figure 3). pGEX-4T-3/RGSHis^6 plasmid DNA was prepared for ligation by digesting with *Sal* I and *Xho* I and purified using the 'QIAquick' nucleotide removal kit.

The UL23 ORF was excised from the pGEX-6P-1/UL23 plasmid on a *Sal* I / *Xho* I fragment and cloned into pGEX-4T-3/RGSHis^6, placing the RGSHis^6 sequence at the 5' end of the UL23 ORF (Figure 3). DNA sequencing performed using the RA5, RA6, RA9 and RA10 PCR primers (Table 5) confirmed that there were no errors in the sequence and that the UL23 ORF was cloned in-frame with the GST gene and histidine tag sequence. pGEX-4T-3/RGSHis^6-UL23 DNA was transfected by electroportation into protease-deficient *E. coli* (strain BL21) cells to produce the GST-RGSHis^6-UL23 fusion protein.

The pGEX-6P-1/UL24 plasmid served as donor for the UL24 ORF when cloning into pGEX-4T-3/RGSHis^6. However, as the UL24 ORF contains an internal *Sal* I site at nucleotide 29134 a *Sal* I linear partial digest followed by an *Xho* I digest was required to liberate the full length UL24 ORF from the donor vector. The *Sal* I/*Xho* I fragment containing the UL24 ORF was cloned between the *Sal* I and *Xho* I sites of vector pGEX-4T-3/RGSHis^6 (Figure 3). Relatively few bacterial colonies were obtained and DNA sequencing performed using the RA5, RA6, RA7 and RA8 PCR primers (Table 5) revealed that the histidine tag sequence was lacking in the product from the ligation reaction. Nevertheless, the UL24 ORF had been cloned in frame with the GST gene in pGEX-4T-3. Although a histidine tagged version of the UL24 ORF was not produced this construct proved useful in serving as donor of the UL24 ORF when cloning into the eukaryotic expression vector pcDNA3.1+.

3.1.4 Cloning the RGSHis^6-UL23 and UL24 ORFs into eukaryotic expression vector pcDNA3.1+

The RGSHis^6-UL23 ORF was excised from the pGEX-4T-3/RGSHis^6-UL23 plasmid on a *Bam*H I/*Xho*I fragment, purified on an agarose gel and cloned into pcDNA3.1+ (Figure 3). For DNA sequencing, oligonucleotide PCR primers were designed to amplify sequences from the vector DNA into the UL23 ORF: a forward primer (RApc3.1) at vector nucleotide 808 and a reverse primer (RA2pc3.1) at vector nucleotide 1101. The RA 9 and RA10 sequencing PCR primers (Table 5) were used to amplify sequences from the UL23 sequences out into the vector DNA. Sequencing confirmed that there were no errors in the cloned UL23 ORF.

The UL24 ORF was excised from the pGEX-4T-3/UL24 plasmid on a *Bam*H I/*Xho* I fragment, purified on an agarose gel and cloned into pcDNA3.1+ (Figure 3). DNA sequencing performed using the RApc3.1, RA2pc3.1, RA7 and RA8 PCR primers (Table 5) confirmed that there were no errors in the cloned UL24 ORF, except for the silent mutation described previously.

3.2 Purification of UL23 and UL24 GST fusion proteins from *E. coli* (strain BL21) cell cultures

Small-scale cultures (10 ml) were used to optimise conditions for induction of UL23 and UL24 GST fusion proteins from the pGEX-6P-1 and pGEX-4T-3 based vectors. Variable

parameters included changes or combinations of changes to IPTG concentration, induction temperature, bacterial strain, time of induction, and growth medium (Table 6). None of the conditions employed yielded soluble GST-UL23 or GST-UL24 fusion proteins in quantities detectable by Coomassie blue stained SDS-PAGE gels or Western immunoblots, probed with anti-GST or anti-RGS antibodies (as appropriate). Although 10 ml cultures failed to yield detectable amounts of GST-UL23, small amounts of soluble GST-UL23 fusion protein were obtained from large-scale (3 L) bacterial cultures grown in 2YT broth and induced by treatment with 1 mM IPTG for 4 h at 37 °C. In contrast, soluble GST-UL24 fusion protein was never detected, even in large-scale cultures.

3.3 Purification of GST-UL23 fusion protein for antibody production

Soluble GST-UL23 fusion protein was isolated by binding to glutathione beads. The fusion protein was removed from the beads by treatment with SDS-PAGE sample buffer and the protein purified by SDS-PAGE. The fusion protein was recovered from the excised gel slices by electro-elution using the Biotrap apparatus (Schleicher and Schuell). The purity of electro-eluted fusion protein was checked by Coomassie blue dye staining of proteins resolved in SDS-PAGE gels (Figure 4A) and by Western immunoblotting, using an anti-GST antibody (Figure 4B). The GST-UL23 fusion protein appeared to run close to its predicted molecular weight of 59.0 kDa. Protein concentration assays performed on each sample batch showed that generally low levels of fusion protein (<0.1 mg/ml) were recovered. When the concentration of a sample batch was <0.1 mg/ml the protein samples were concentrated using a YM-10 Centricon® centrifugal filter device (Millipore) that retains proteins with a molecular weight > 10 kDa. The final concentration of the GST-UL23 fusion protein preparations was generally 0.3 - 1.2 mg/ml.

Table 6. Conditions for induction of GST fusion proteins.

Bacterial Strain/ Growth Media	Plasmids	Induction Temp./ Time	IPTG conc.	Soluble Fusion Protein Detected?
BL21/ 2YT	Controls: pGEX-6P-1 pGEX-4T-His^6	37 °C / 4 h	0.5, 1.0, 3.0 or 5.0 mM	GST detected under all IPTG concentrations by Western Immunoblots
***BL21/ *2YT**	**pGEX-6P-1/UL23 pGEX-6P-1/UL24**	***37 °C/ 4 h**	***1.0 mM**	**Some soluble GST-UL23 on 3 L cultures detected by Western immunoblot**
BL21/ 2YT	pGEX-6P-1/UL23 pGEX-6P-1/UL24 Control: pGEX-6P-1	37 °C / 0.5 h, 1.0 h or 4 h	1.0 mM, or 3.0 mM	No, but GST detected from control under all conditions by Western Immunoblot
BL21/ 2YT	pGEX-6P-1/UL23 pGEX-6P-1/UL24	37 °C, or 20 °C/ 4 h	0.1, 0.5, 1.0, 1.5 or 2.0 mM	No
BL21/ 2YT	pGEX-6P-1/UL23 pGEX-6P-1/UL24	37 °C, or 28 °C/ 4 h	0.1, 0.5, 1.0, 1.5 or 2.0 mM	No
BL21/ 2YT	pGEX-6P-1/UL23 pGEX-6P-1/UL24 Control: pGEX-6P-1	37 °C, or 25 °C/ 16 h	0.5, 1.0, 2.0 or 5.0 mM	No, but GST detected from control under all conditions by Western Immunoblot
BL21/ 2YT + 2 % glucose added 2 h prior to IPTG	pGEX-6P-1/UL23 pGEX-6P-1/UL24 Control: pGEX-6P-1	37 °C/ 4 h	0.0, 0.5, 1.0, 3.0 or 5.0 mM	No, but GST detected from control under all conditions by Western Immunoblot
BL21/ L-broth	pGEX-6P-1/UL23 pGEX-6P-1/UL24	37 °C/ 4 h or 6 h	1.0, 1.5 or 2.0 mM	No
DH-1/ L-broth	pGEX-6P-1/UL23 pGEX-6P-1/UL24 Control: pGEX-6P-1	37 °C, or 25 °C/ 4 h	0.5, 1.0, 2.0 or 5.0 mM	No, but GST detected from control under all conditions by Western Immunoblot
Novablue DE-3/ 2YT	pGEX-6P-1/UL23 pGEX-6P-1/UL24	37 °C/ 4 h	0.0, 0.5, 1.0, 3.0 or 5.0 mM	No
BL21/ 2YT	pGEX-4T-3/RGSHis^6-UL23 pGEX-4T-3/UL24	37 °C/ 4 h or 16 h	0.5, 1.0, 3.0 or 5.0 mM	No

*-Conditions finally chosen for growth of large-scale cultures for isolation of soluble GST-UL23 fusion protein and insoluble GST-UL24 (from inclusion bodies).

3.4 Purification of GST-UL24 fusion protein for antibody production

Analysis of IPTG induced bacterial cell extracts on Coomassie blue dye stained SDS-PAGE gels indicated that a substantial protein band of ~60 kDa, corresponding to the predicted size of the GST-UL24 fusion protein, was present in the insoluble pelleted cell debris from sonicated bacteria. Preparations of bacterial inclusion bodies were made and the GST-UL24 fusion protein separated on SDS-PAGE gels. The purity of electro-eluted fusion protein was checked by Coomassie blue staining of proteins resolved in SDS-PAGE gels (Figure 4C) and by Western immunoblotting, using an anti-GST antibody (Figure 4D).

In experiments shown in Figure 4C and 4D, the GST-UL24 fusion protein recovered from the inclusion body preparations had an apparent molecular mass of ~60 kDa in SDS-PAGE gels, which is significantly less than the 66.2 kDa predicted. The cause of this discrepancy has not been further investigated. In other experiments (e.g. Figure 7D) the GST-UL24 fusion protein resolved with the expected 66.2 kDa size.

The concentration of electro-eluted GST-UL24 fusion protein preparations were generally 0.2 – 1.0 mg/ml. An attempt to purify the GST-UL24 fusion protein from an inclusion body preparation by direct binding to glutathione beads was unsuccessful.

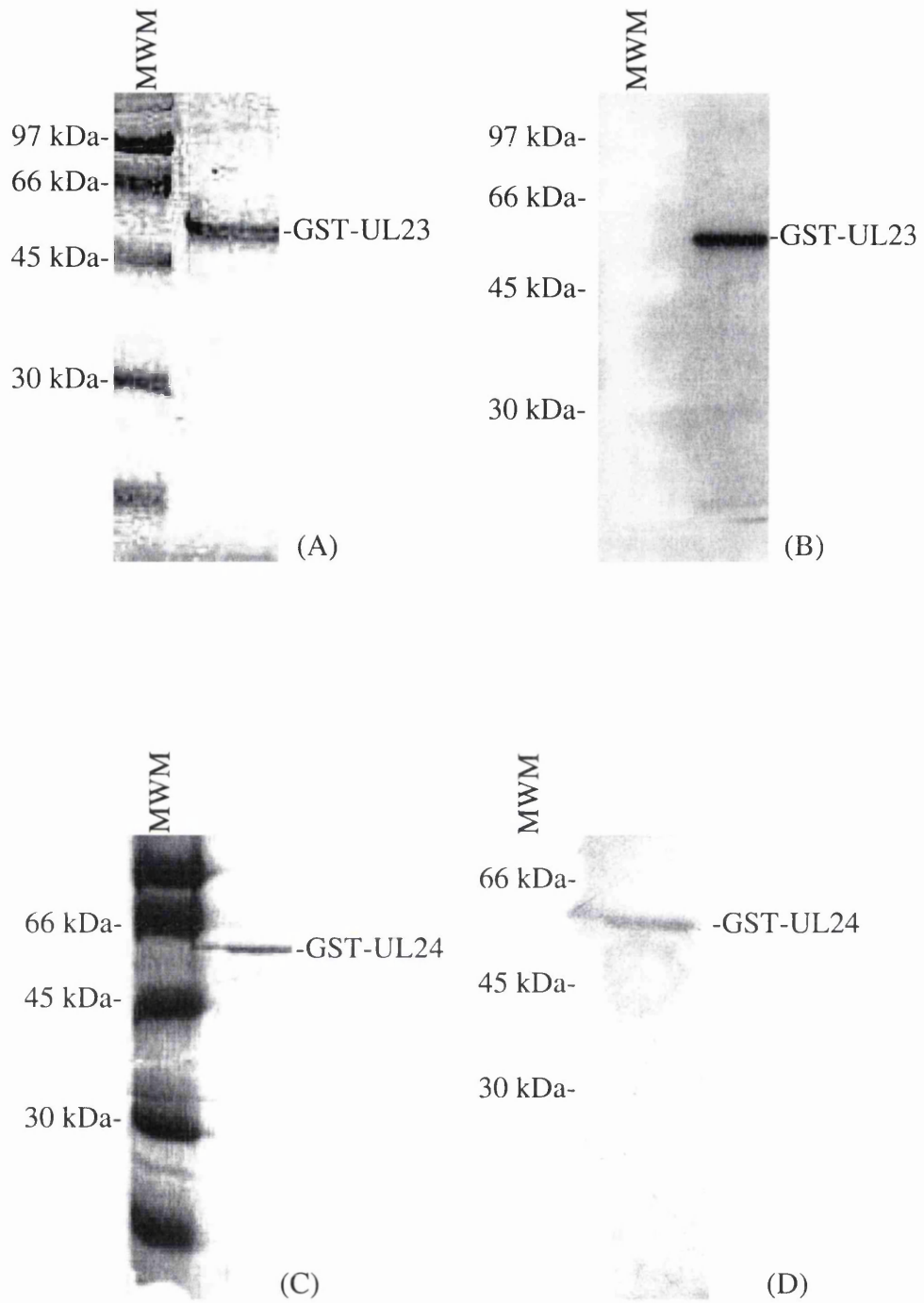


Figure 4. Isolation of UL23 and UL24 GST fusion proteins from bacterial cell extracts and investigation of the fusion protein preparations prior to immunisation of animals. (A and C) Coomassie blue stained gel showing electro-eluted GST-UL23 and GST-UL24 fusion proteins, respectively. (B and D) Western immunoblots, probed with anti-GST antibody, to confirm that the eluted proteins are GST fusion products. The predicted sizes of GST-UL23 and GST-UL24 were 59.0 kDa and 66.2 kDa, respectively. MWM, Molecular Weight Marker.

3.5 Synthesis of branched peptides corresponding to the C-terminus amino acid sequences of the UL23 and UL24 ORFs

Multiple antigen peptide systems (MAPs), originally developed by Dr. James Tam (Tam, 1996), can be used for anti-peptide antibody elicitation. MAPs are comprised of small immunogenically inert poly-lysine cores onto which peptide antigens can be synthesised. The resulting macromolecules possess a high molar ratio of peptide antigen to core molecule and are capable of eliciting a strong antibody response, often yielding significantly higher antibody titres than obtained with the monomeric counterpart attached to a carrier protein. The inert MAP core was attached to a solid phase peptide synthesis support ($Fmoc_4$-Lys_2-Lys-βAla-Wang resin) and the desired peptide antigens were synthesised directly onto the branched lysine core. Branched peptides (bold and underlined) from the C-terminus regions of UL23 (**3'-** **ADDFLQHDVGYP**CTPKNLKRMSRTGY) and UL24 (**3'-** RHQRGKPC**DPVRDYIGNHGSFR**RNEF) were prepared by Drs. T. MacDonald and H. Marsden, and generated by solid phase peptide synthesis using an Advanced ChemTec 348Ω multiple peptide-automated synthesiser using standard Fmoc chemistry. The homogeneity of the peptide product was analysed by high performance liquid chromatography (HPLC).

3.6 Raising anti-UL23 and anti-UL24 antibodies

Rabbits were injected subcutaneously with 50 μg/injection of GST fusion proteins or 100 μg/injection of branched peptides (see methods). The rabbits were sacrificed when pUL23 and pUL24 antibody titres were high, as determined by ELISA, and the serum separated to provide polyclonal antibodies. Mice were injected subcutaneously with 20 μg/injection of

GST fusion proteins. Mouse spleen cells were fused with Sp2/0-Ag 14 cells when antibody titres were high to provide hybridoma cells and the culture medium from hybridomas was the source of monoclonal antibodies (MAbs).

3.7 Testing the specificity of anti-UL23 polyclonal antibodies

The specificity of rabbit anti-UL23 polyclonal antibodies, produced against GST-UL23 fusion protein (PAb 678 and PAb 679), was investigated by Western immunoblotting using bacterially produced and thrombin cleaved GST-RGSHis^6-UL23 protein. GST-RGSHis^6-UL23 fusion protein bound to glutathione beads was cleaved with human plasma thrombin (Sigma) to yield the RGSHis^6-UL23 (33.0 kDa) and GST (26.0 kDa) protein components. PAb 678 and PAb 679 detected RGSHis^6-UL23, GST and residual uncleaved GST-RGSHis^6-UL23 proteins from the bacterial extract (data not shown). However, these experiments did not give a clean result, with many additional *E. coli* proteins also detected by the polyclonal antiserum. SDS-PAGE separation of fusion proteins results in a relatively crude preparation of GST-UL23 and rabbit antibodies will have been raised against contaminating bacterial proteins.

The specificity of rabbit anti-UL23 polyclonal antibodies, produced against branched peptides of UL23 (pep682 and pep683), was investigated by Western immunoblotting using bacterially produced RGSHis^6-UL23 protein. Pep683, anti-RGS and anti-GST antibodies all detected full-length GST-RGSHis^6-UL23 fusion protein (Figure 5A). Antibody pep683 did not detect GST but did detect the RGSHis^6-UL23 protein, which was also detected by the anti-RGS antibody. Antibody pep682 gave similar results (Figure 5B). Pep683 and anti-RGS antibodies bind the C- and N-termini of the RGSHis^6-UL23 protein, respectively, confirming that the full-length protein was expressed. Thus, the polyclonal antibodies pep682 and pep683 specifically detected pUL23. Specificity of the pep682 and pep683 antibodies was further demonstrated by the failure to detect pUL23 by Western immunoblotting following pre-adsorption of the antibodies against the UL23 peptide preparation (Figure 5B).

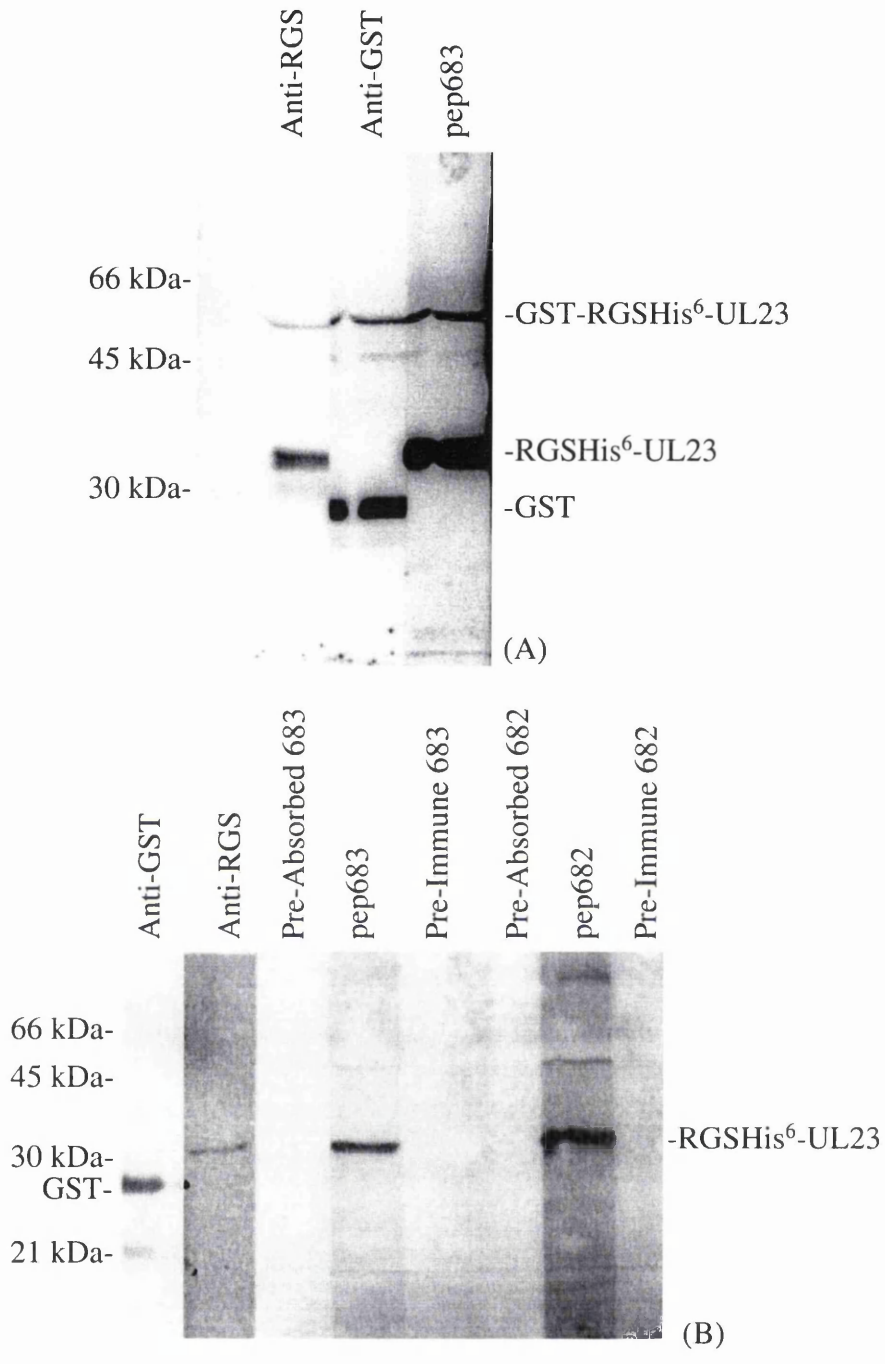


Figure 5. The anti-UL23 peptide antibodies, (pep682 and pep683), specifically identified pUL23 produced in bacterial cultures. RGSHis^6-UL23 was cleaved from GST by human plasma thrombin (50 cleavage units in 1 ml PBS) at RT overnight. (A) Western immunoblot of thrombin cleaved GST-RGSHis^6-UL23 fusion protein, using anti-RGS antibody and pep683 antibody. (B) Western immunoblot of thrombin cleaved GST-RGSHis^6-UL23 fusion protein, using antibodies pep682 and pep683 before or after pre-adsorption with the UL23 peptide used to raise the antibody. To pre-adsorb antibodies 5 µl serum was incubated with 100 µg branched peptide and 50 µl of 10 % w/v protein A sepharose (Sigma) for 1 h at 37 °C, and the sepharose bound UL23 antibodies were removed by centrifugation in a microfuge (6 k/2 min/RT).

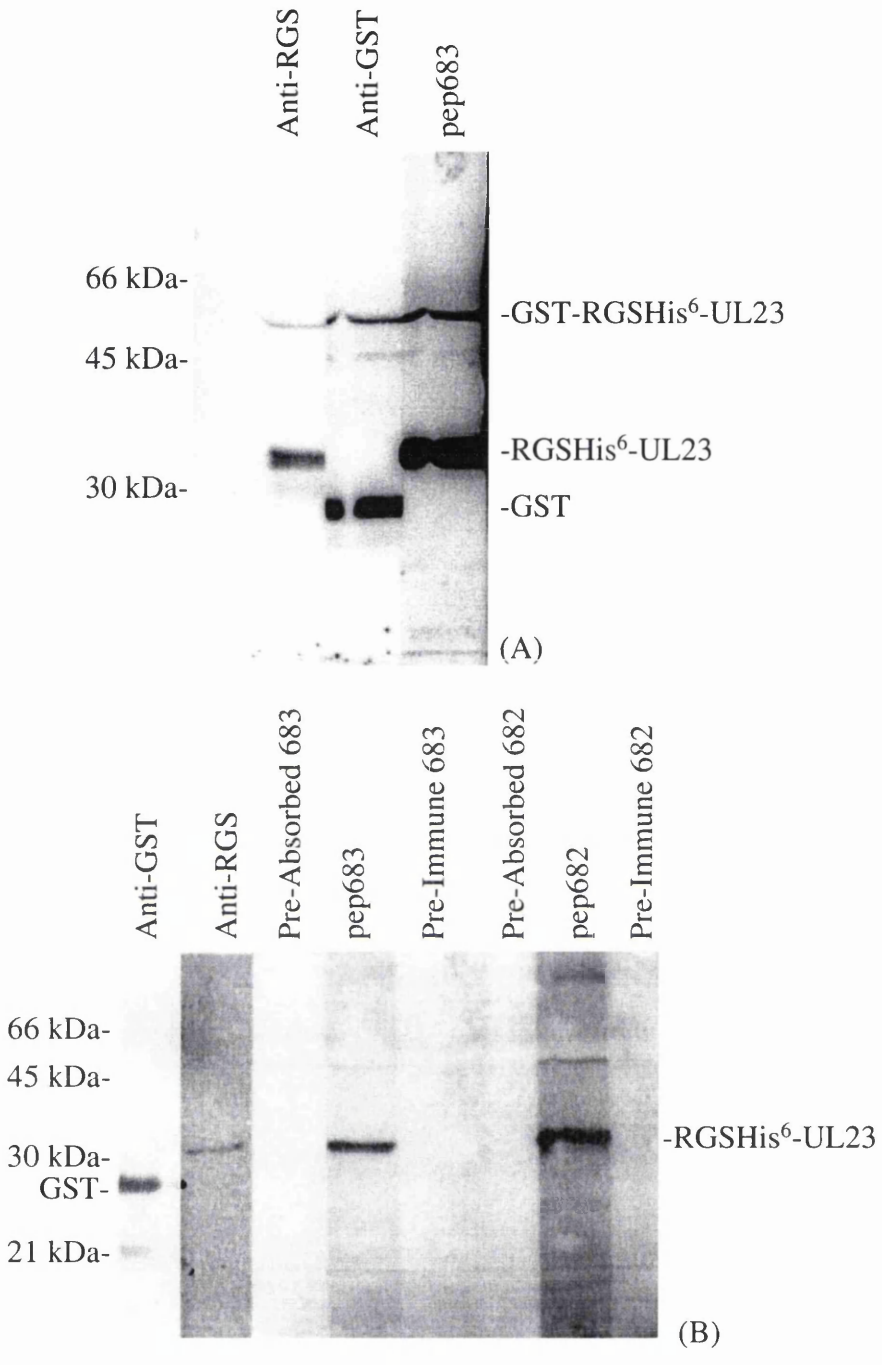


Figure 5. The anti-UL23 peptide antibodies, (pep682 and pep683), specifically identified pUL23 produced in bacterial cultures. RGSHis^6-UL23 was cleaved from GST by human plasma thrombin (50 cleavage units in 1 ml PBS) at RT overnight. (A) Western immunoblot of thrombin cleaved GST-RGSHis^6-UL23 fusion protein, using anti-RGS antibody and pep683 antibody. (B) Western immunoblot of thrombin cleaved GST-RGSHis^6-UL23 fusion protein, using antibodies pep682 and pep683 before or after pre-adsorption with the UL23 peptide used to raise the antibody. To pre-adsorb antibodies 5 µl serum was incubated with 100 µg branched peptide and 50 µl of 10 % w/v protein A sepharose (Sigma) for 1 h at 37 °C, and the sepharose bound UL23 antibodies were removed by centrifugation in a microfuge (6 k/2 min/RT).

3.8 Testing the specificity of anti-UL23 monoclonal antibodies

Monoclonal antibodies were screened by Western immunoblotting against bacterially produced and thrombin cleaved RGSHis^6-UL23 protein. Of 18 ELISA-positive hybridoma culture supernatants obtained, 13 appeared to contain UL23 specific monoclonal antibodies (MAbs). MAbs 266 and 73 were the most potent, in specifically detecting RGSHis^6-UL23 protein (Figure 6). MAb 266 has been used in subsequent experiments.

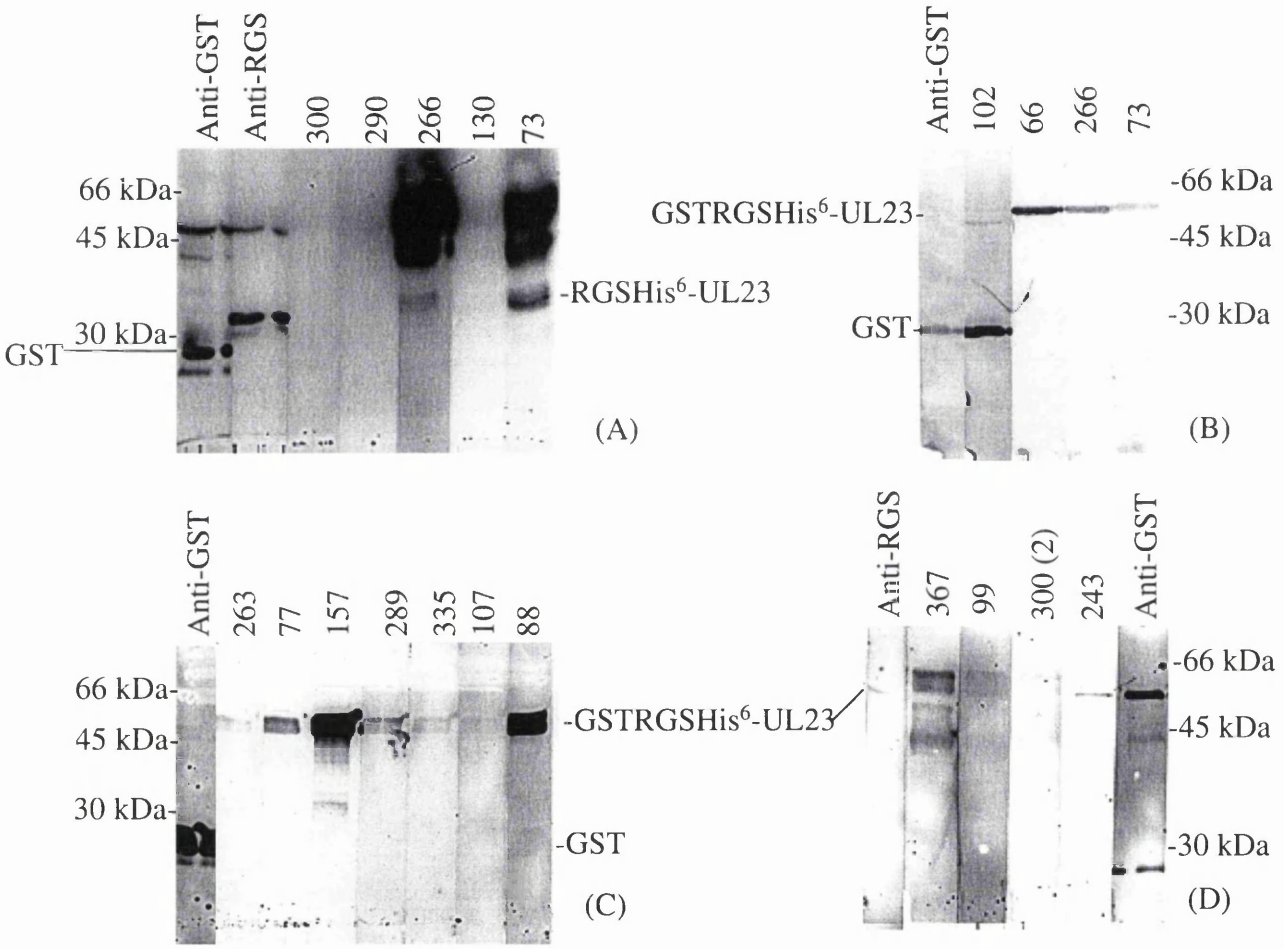


Figure 6. Screening of MAbs against RGSHis^6-UL23 fusion protein. (A) Western immunoblot against bacterially produced GST-RGSHis^6-UL23 (59.0 kDa) cleaved with thrombin to yield RGSHis^6-UL23 protein (33.0 kDa) and GST (26 kDa). (B, C, D) Western immunoblots against bacterially produced GST-RGSHis^6-UL23 eluted from the column with glutathione for 10 min at RT, and cleaved with thrombin in solution. GST-RGSHis^6-UL23 protein (59.0 kDa) and GST (26 kDa) were in detectable quantities. UL23 specific MAbs were 266, 73 (A and B), 66 (B), 263, 77, 157, 289, 335, 107 (C) and 367, 99, 300(2), 243 (D). MAbs that did not detect UL23 or were directed against GST were 300, 290, 130, 102 and 88.

3.9 Testing the specificity of anti-UL24 polyclonal antibodies

The specificity of rabbit anti-UL24 polyclonal antibodies, produced against GST-UL24 fusion protein (PAb 674 and PAb 675), was investigated by Western immunoblotting using bacterially produced UL24 (Figure 7A, B). GST-UL24 fusion protein, bound to glutathione sepharose beads, was cleaved with PreScission protease to yield pUL24. The pUL24 was then concentrated using a YM-10 Centricon® centrifugal filter device (Millipore) prior to use in Western immunoblotting. Anti-UL24 PAb 674 and PAb 675 both detected two closely spaced bands in the 40 kDa region of the gel. The lower is the major of the two bands and corresponds in size to that predicted for pUL24 (40.2 kDa). The GST protein was not detected (or was present in trace amounts) in the PreScission protease treated extract, since it remains bound to the glutathione sepharose beads. PAb 674 and PAb 675 do, however, detect purified GST (Figure 7A and B). The 40 kDa region bands were not detected by anti-GST antibody (not shown). Thus the PAb 674 and PAb 675 antibodies specifically detect pUL24.

The specificity of anti-UL24 peptide antibodies (pep684 and pep685) was investigated by Western immunoblotting using PreScission protease cleaved GST-UL24 fusion protein (Figure 7C). As expected anti-UL24 pep684 and pep685 antibodies did not detect GST. However, pep684 and pep685 detected a band of ~30 kDa thought to be related to pUL24. Since the rabbits were immunised with C-terminal peptides, the fact that full length GST-UL24 fusion protein (Figure 7C, D) is recognised by pep684 and pep685 demonstrates specificity for UL24.

PAbs 674 and 675 recognise pUL24 related products of different sizes to those recognised by pep684 and pep685. This suggests that pUL24 is either unstable *in vitro* after its cleavage from the fusion protein or that translation of the GST fusion protein terminates prematurely to yield truncated products. Since the pep684 and pep685 antibodies are directed against the C-terminal amino acid sequences of pUL24, the former appears more likely.

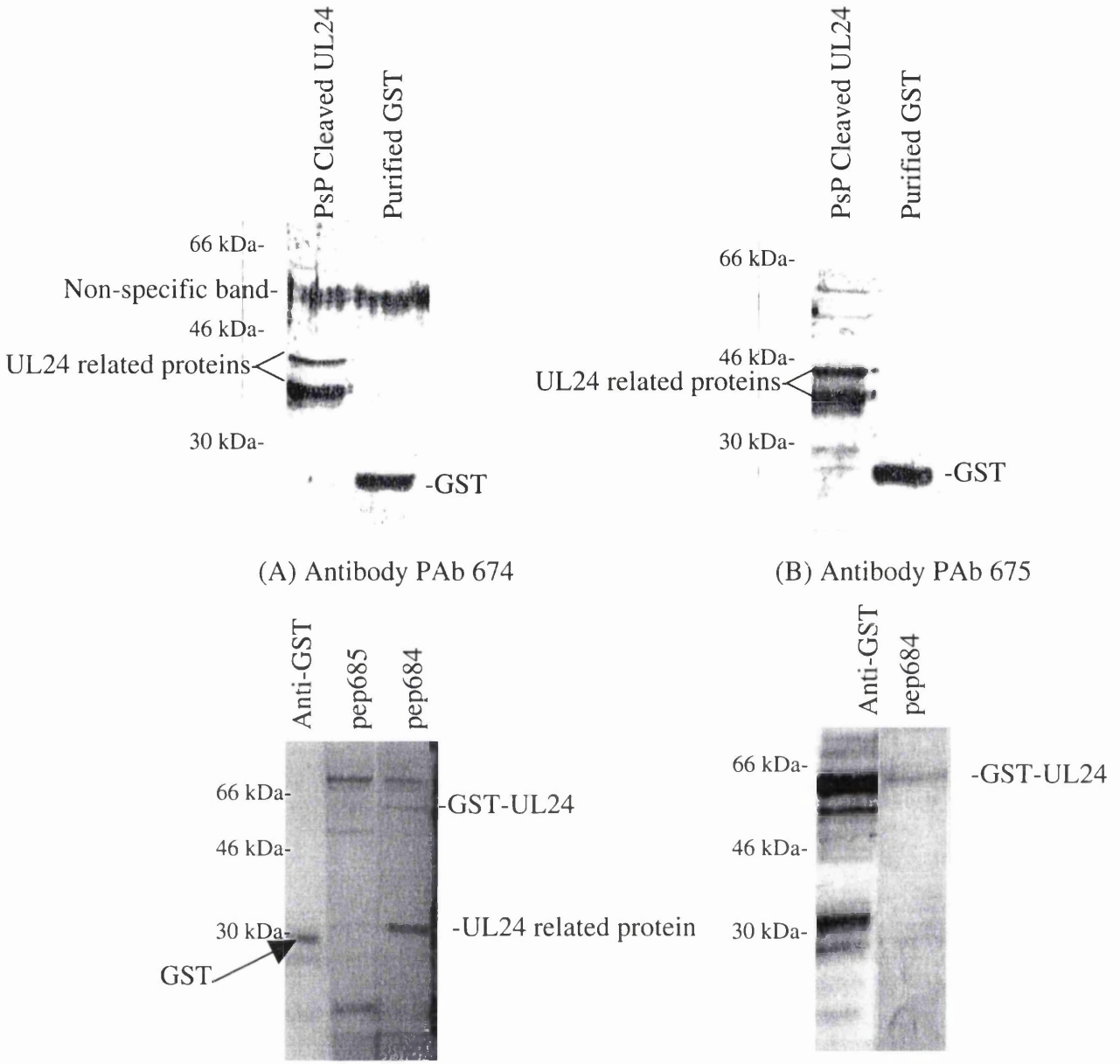


Figure 7. The anti-UL24 polyclonal antibodies specifically identified pUL24 produced in bacteria. pUL24 was cleaved from GST by PreScission Protease (PSP) (80 cleavage units in 1ml cleavage buffer [50 mM Tris-HCl pH7.0, 150 mM NaCl 1 mM EDTA, 1 mM dithiothreitol]) at 4 °C overnight. Western immunoblots of PSP cleaved UL24 and purified GST proteins, using PAb 674 (A) and PAb 675 (B). Western immunoblots of PSP cleaved UL24 (C) and an inclusion body preparation of GST-UL24 fusion protein (D), probed with pep685 and pep684 antibodies.

3.10 Testing the specificity of anti-UL24 monoclonal antibodies

Due to difficulties in obtaining sufficient pUL24 from PreScission protease cleaved fusion protein, hybridoma culture supernatants were screened by Western immunoblotting against the GST-UL24 fusion protein and purified GST. By inference, MAbs detecting the fusion protein but not the GST protein probably recognise epitopes on pUL24. Of 20 hybridomas screened all detected both the full-length GST-UL24 fusion protein and purified GST, indicating that they were directed against the GST portion of the fusion protein. However, MAb 116 reacted strongly with the complete fusion protein and only weakly with GST (data not shown). This suggests that MAb 116 might recognise an epitope that spans the GST and UL24 protein junction.

In conclusion, several antibodies (polyclonal, monoclonal or anti-peptide) were generated which specifically recognised epitopes on pUL23 and pUL24. These antibodies were used to investigate UL23 and UL24 gene expression, presence of the proteins in the virus particle and the intracellular location of the proteins. In order to gain further insight into the function of the US22 gene family proteins *per se,* antibodies directed against HCMV UL43 (provided by Dr. Derrick Dargan) and US22 (MAb HWLF1; Advanced Biotechnologies Inc.) were also employed.

3.11 Temporal class of pUL23, pUL24 and pUL43 expression

Herpesvirus gene expression is temporally regulated, occurring in three successive phases. The first genes expressed are those of the immediate-early (I-E) class, which are transcribed in

the absence of *de novo* viral protein synthesis, by cellular RNA polymerase II. The early genes (E) are defined as requiring prior IE protein synthesis, but not viral DNA replication for their expression. Early gene products are therefore made in the presence of inhibitors of virus DNA synthesis such as phosphonoacetic acid (PAA). Late genes may be separated into E-L and L subsets, which are transcribed, mainly (E-L) or entirely (L), from progeny genomes. An indication of likely gene function can be deduced from this classification of gene expression. In general, I-E genes encode regulatory functions; E genes encode enzymes, proteins required for viral DNA replication, some virus structural proteins, or proteins with a role in viral pathogenesis, while L genes generally encode structural proteins.

Unexpectedly, the polyclonal antibodies raised against the GST-UL24 fusion protein or the UL24 C-terminal peptides failed to detect pUL24 in infected cell extracts by Western immunoblotting, although UL24 related proteins of various sizes were detected in bacterial cell extracts following IPTG induction. However, a monoclonal antibody (MAb 116) raised against the GST-UL24 fusion protein recognised a protein band of ~40 kDa in Western immunoblots of infected cell extracts (Figure 8). Close approximation in size to the predicted UL24 product (40.2 kDa) indicates that MAb 116 specifically recognises pUL24 made in infected cells. To investigate the temporal class of UL24 gene expression, HFFF-2 or U373 MG cells were infected with HCMV (strains AD169, Towne or Davis) at an m.o.i. of 5 to 10 p.f.u./cell and grown under conditions for expression of I-E, E and L genes. Western immunoblots probed with MAb 116 detected a band of ~40 kDa (pUL24) in infected, but not mock infected, HFFF-2 and U373 MG cells (Figure 8A, B and C). Irrespective of the HCMV strain used the pUL24 band detected was always the same size and was always detected in cell extracts in which late (L) proteins were made. In some experiments (Figure 8A), pUL24 could also be detected, albeit weakly, in cells treated with 300 μg/ml PAA (i.e. expressing E genes) (Figure 8A). It is concluded therefore that pUL24 is expressed with kinetics of an early-late (E-L) gene.

pUL24 has proved difficult to detect in infected cell extracts and is almost certainly present in relatively small amounts. Differences in antibody potency might explain why pUL24 was not detected by the PAbs and anti-peptide antibodies.

Monoclonal (MAb 92) and polyclonal antibodies (PAb 616) raised against a GST-UL43 fusion protein (provided by Dr. Derrick Dargan) were used to investigate the temporal kinetics of UL43 gene expression. Infected HFFF-2 cell extracts were prepared as described above. PAb 616 recognised a polypeptide band of ~48 kDa (similar to the predicted product of the UL43 ORF (47.7 kDa)) in cell extracts in which late proteins were made (Figure 8D). pUL43 could not be detected in cell extracts treated with PAA, even upon prolonged exposure of the gel. Thus, gene UL43 is expressed with the kinetics of a true-late (L) gene.

pUL23 could not be detected in either HFFF-2 or U373 MG cells infected at high m.o.i. (≥ 10 p.f.u./cell) with HCMV strains AD169, Towne or Davis, or in the supernatant culture medium and therefore the temporal classification of UL23 gene expression could not be determined by these experiments. Attempts were made to enhance UL23 gene expression by incubating infected HFFF-2 cells at different temperatures (33 °C, 39.2 °C or 38.5 °C) or by overlaying cells with medium containing 1-5 mM HMBA (N,N'-Hexamethylene-bis-acetamide) (Sigma) or 1-5 mM sodium butyrate (Sigma) for the first 24 h PI. HMBA is known to affect the pattern of transcription in cells infected with HSV-1 (Preston and McFarlane, 1998; McFarlane *et al.*, 1992). Sodium butyrate has been shown to enhance HCMV yield in TPC-1 cells (Tanaka *et al.*, 1991), enhance EBV-specific DNA synthesis (Saemundsen *et al.*, 1980) and induce HCMV permissiveness in cultured endothelial cells (Radsak *et al.*, 1989). Protein extracts were harvested at various time points up to 96 h PI but no pUL23 specific bands were ever detected by Western immunoblotting from any of these experiments. Nor were pUL23 specific bands detected from experiments in which infected cells were fractionated into nuclear and cytoplasmic extracts.

While the polyclonal and monoclonal antibodies raised against the GST-UL23 fusion protein and the polyclonal antibodies raised against the UL23 synthetic peptide all recognised pUL23 made in bacteria none could detect pUL23 in infected cell extracts. This suggested either that pUL23 levels were below that which were detectable by the antibodies used, or that the reactive epitopes were unavailable, possibly due to some post-translational modification of the protein.

To investigate pUL24 and pUL43 expression throughout the virus replication cycle, HFFF-2 cell layers were either mock infected or infected with AD169 at a m.o.i. of 10 p.f.u./cell. Protein extracts were harvested every 12 h up to 120 h and examined by Western immunoblotting, using MAb 116 (anti-pUL24) or MAb 92 (anti-pUL43) as probe. Neither antibody reacted with polypeptides from the mock-infected control cell extracts (not shown). pUL24 was first detected at 12 h PI, at very low abundance, and increased in amount thereafter (Figure 9A). pUL43 was first detected at 60 h PI and increased in amount thereafter (Figure 9B). These findings are in keeping with the designation of pUL24 as the product of an early-late (E-L) gene and pUL43 as the product of a true-late (L) gene. None of the proteins, pUL23, pUL24 or pUL43 could be detected as secreted proteins in the culture medium from infected cells.

Despite numerous attempts to optimise conditions, using several protocols, none of the several anti-UL23, UL24 or UL43 antibodies detected their target protein in immunoprecipitation reactions. This has hindered attempts to investigate possible post-translational modification of the proteins.

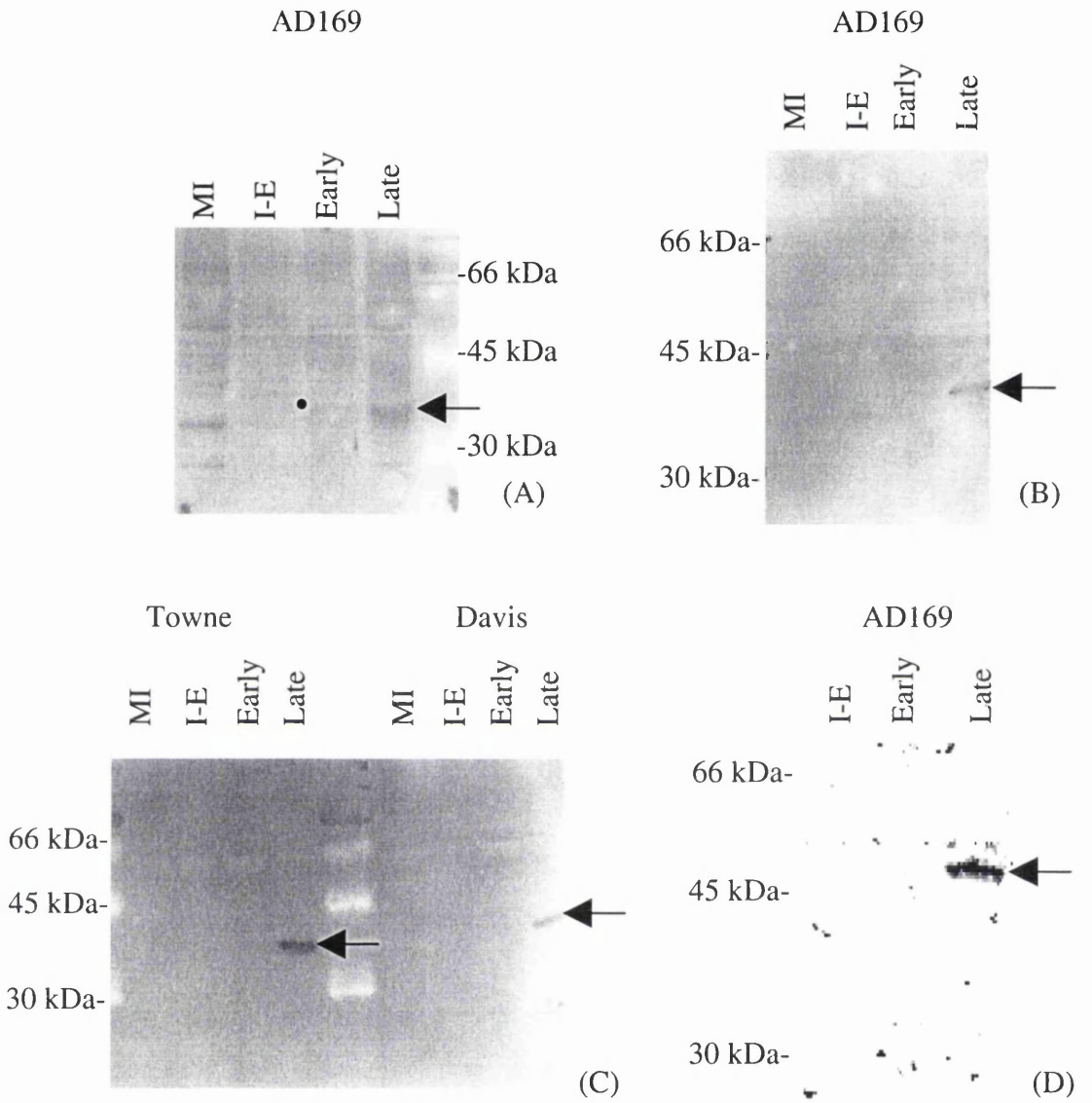


Figure 8. Temporal class of gene expression. Western immunoblots, probed with anti-UL24 MAb 116, against mock infected (MI) cell extracts or HCMV infected cell extracts made under conditions for expression of I-E, E or L proteins. (A) AD169 infected HFFF-2 cells extracts. (B) AD169 infected U373 MG cell extracts. (C) Towne and Davis strain infected HFFF-2 cell extracts. (D) Western immunoblot, probed with anti-UL43 PAb 616, against HCMV infected cell extracts made under conditions for expression of I-E, E and L proteins.

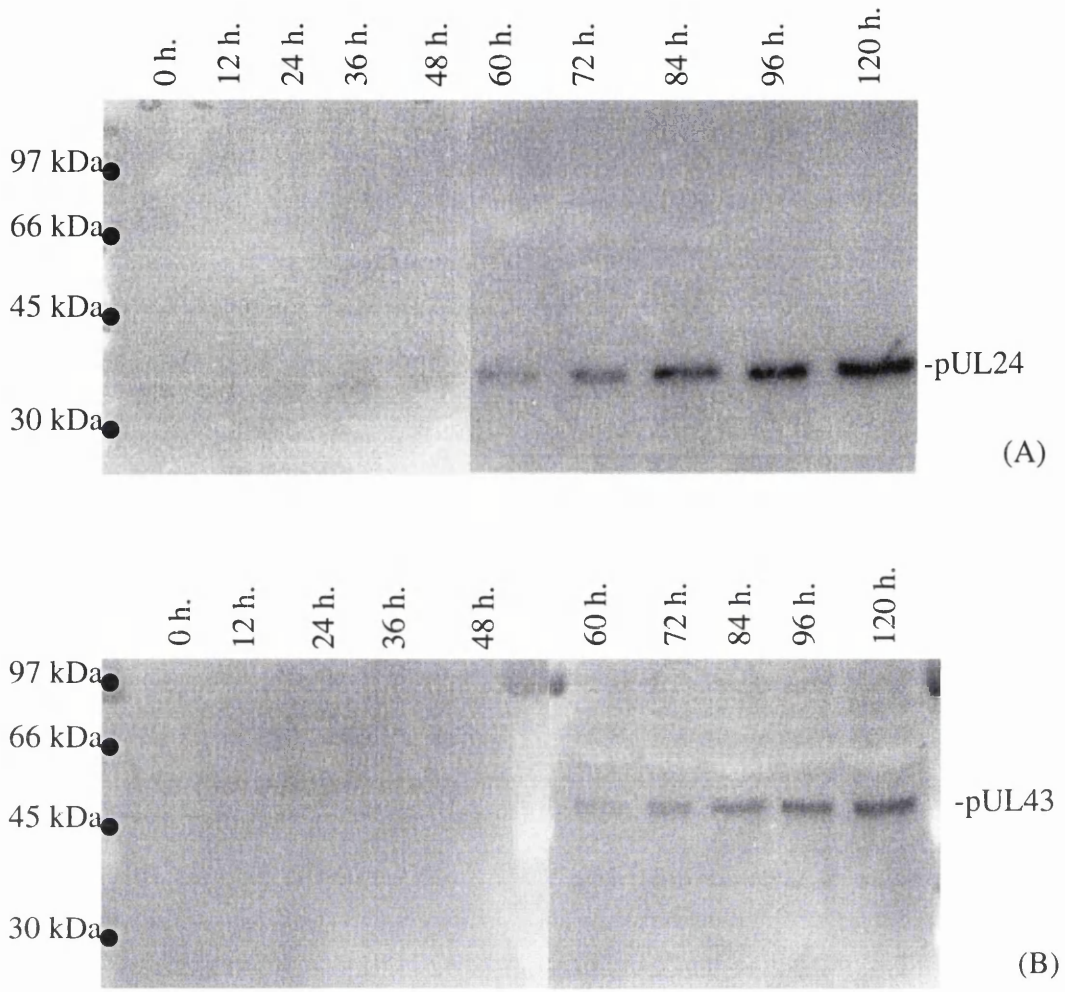


Figure 9. Synthesis of pUL24 and pUL43 during the virus replication cycle. Western immunoblots of AD169 infected HFFF-2 cell protein extracts, probed with anti-UL24 MAb 116 (A) and anti-UL43 MAb 92 (B).

3.12 pUL23, pUL24, pUL43 and pUS22 are components of the virion tegument

To investigate whether pUL23, pUL24, pUL43 and pUS22 were virus particle components, cell-released virus-related particles were pelleted in the Sorval GSA rotor (12 k/2 h/4 °C) and the particles purified by banding in negative viscosity, positive density (glycerol/potassium tartrate) gradients (Irmiere and Gibson, 1983). Three bands were obtained after gradient centrifugation, each containing different HCMV particle types. From the top of the gradient, the sequence of banded particles was; NIEPs, virions and dense bodies, as determined by SDS-PAGE analysis of the protein content of each band (Figure 10). The identification of NIEPs was confirmed by the presence of a unique virus protein band of 36 kDa (UL80A). All the structural proteins of the particle were present in the banded virion preparation, while the banded dense bodies were largely composed of the pp65 matrix protein, with the UL25 product also over-represented. These designations were subsequently confirmed by electron microscopy.

Purified virions, dense bodies and NIEPs were investigated for the presence of pUL23, pUL24, pUL43 and pUS22 by Western immunoblotting. pUL24 and pUL43 were contained in the banded virions, dense bodies and NIEPs preparations (Figure 11A and B). Curiously, both the anti-UL24 and anti-UL43 antibodies detected an additional NIEPs specific band running below pUL24 and pUL43. The significance of this observation is not clear and has not been investigated further. The anti-US22 (HWLF1 MAb) detected a protein of ~76 kDa, slightly higher than the predicted size of pUS22 (66.9 kDa), in both purified virion and dense body preparations, suggesting post-translational processing of pUS22 (Figure 11C). pUS22 was poorly detected unless the gel track was loaded with 10 fold (i.e. 10^{10} particle equivalents) normal levels of protein extract. Unfortunately, there was insufficient NIEP protein extract to allow for the 10 fold greater loading, though it is highly likely that pUS22 is also a component of NIEPs. Similarly, pUL23 was present in low abundance in purified virions (Figure 13) and could only be clearly identified in experiments where 10 fold normal concentrations of protein extract were used. Thus all of the US22 gene family proteins investigated were associated with gradient purified virus particles.

In order to establish whether pUL23, pUL24, pUL43 and pUS22 were indeed particle proteins, and not simply associated with co-purifying cell debris, the purified virions were investigated by immuno-gold negative stain electron microscopy. Rabbit polyclonal antibodies were not used during these experiments because the HCMV tegument contains an Fc receptor (Stannard and Hardie, 1991) that binds rabbit antibodies with high affinity (Antonsson and Johansson, 2001). To eliminate the possibility that our results were due to non-specific Fc binding of MAbs, a non-HCMV MAb (AP33) having the same IgG1 isotype as anti-UL23 MAb 266, anti-UL24 Mab 116 and anti-UL43 MAb 92 (antibody isotyping kit; Sigma) was used as a control. Particles treated with the non-HCMV MAb AP33 did not label with gold (Figure 12A). pUL23, pUL24, pUL43 and pUS22 were detected in tegument material adhering to capsids (Figure 12B, C, D and E respectively), or in matrix material issuing from dense bodies (not shown), where the envelope was broken. Particles with an intact membrane were not labelled with gold. Thus, pUL23, pUL24, pUL43 and pUS22 are confirmed as protein components of the virus particle and are likely contained in the tegument structure. To confirm that pUL23, pUL24, pUL43 and pUS22 were tegument components, the envelope was removed from purified virions by treatment with detergent. After centrifugation, the pelleted capsid/tegument and soluble envelope fractions were probed for pUL23, pUL24, pUL43 and pUS22 by Western immunoblot (Figure 13A, B, C, D, respectively). As expected, pUL23, pUL24, pUL43 and pUS22 were each present in the intact virions (track 1) and in the capsid/tegument fraction (track 2), but not in the envelope fraction (track 3). To confirm efficient de-envelopment of the particles, the blots were routinely stripped and sequentially re-probed with control antibodies directed against a known tegument protein, pp65 (Figure 13E) and a known envelope protein, gB (Figure 13F). pUS22 and pUL23 were present in low abundance in virions and in the capsid/tegument fraction and could only be detected in experiments where $> 10^{10}$ particle equivalents were loaded in each gel track. Since the proteins comprising the capsid structure are well known (Chee *et al.*, 1990) and do not include pUL23, pUL24, pUL43 and pUS22, they all were deduced to be tegument proteins.

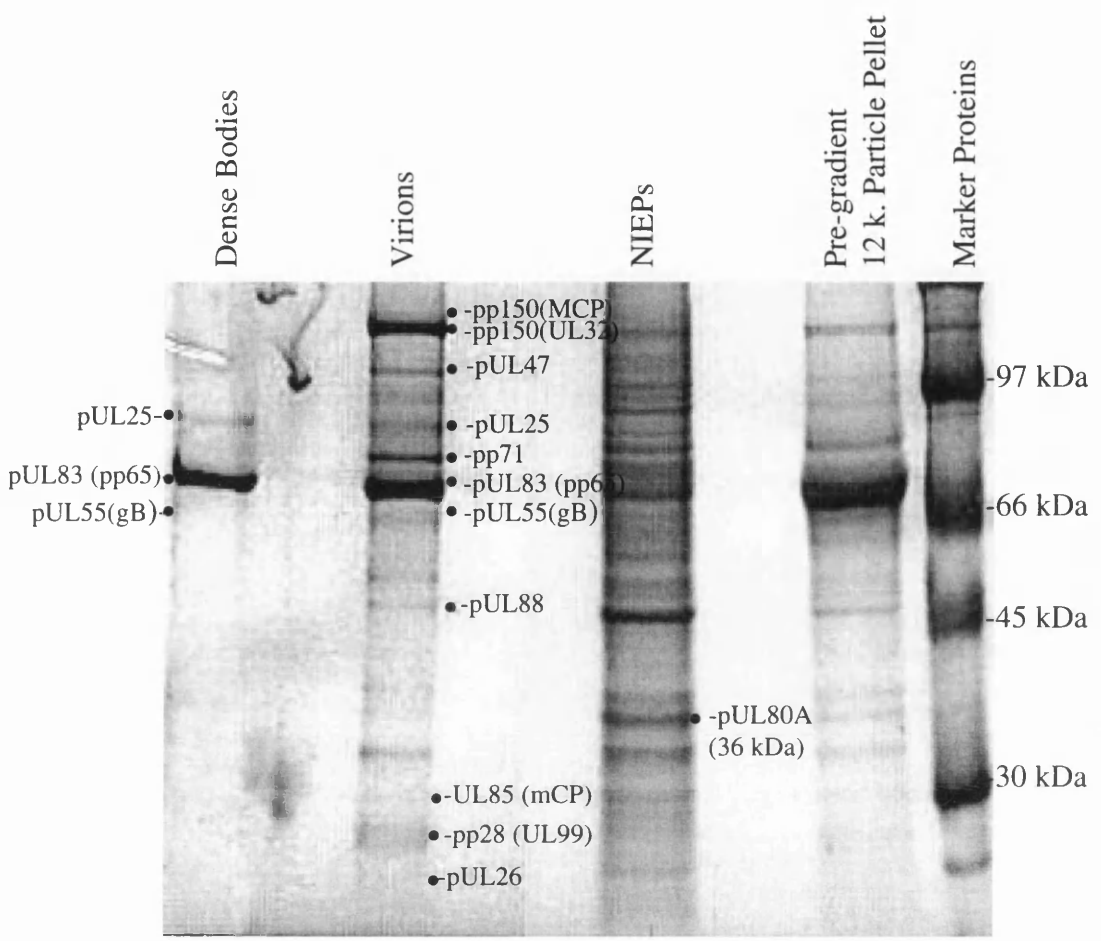


Figure 10. Polypeptide content of banded HCMV particle types. Coomassie blue stained SDS-PAGE gel showing polypeptides associated with purified HCMV dense bodies, virions and NIEPs. The '12 k particle pellet' lane represents the initial pre-gradient particle preparation. The identity of individual viral protein bands are indicated. MCP, Major Capsid Protein; mCP, Minor Capsid Protein.

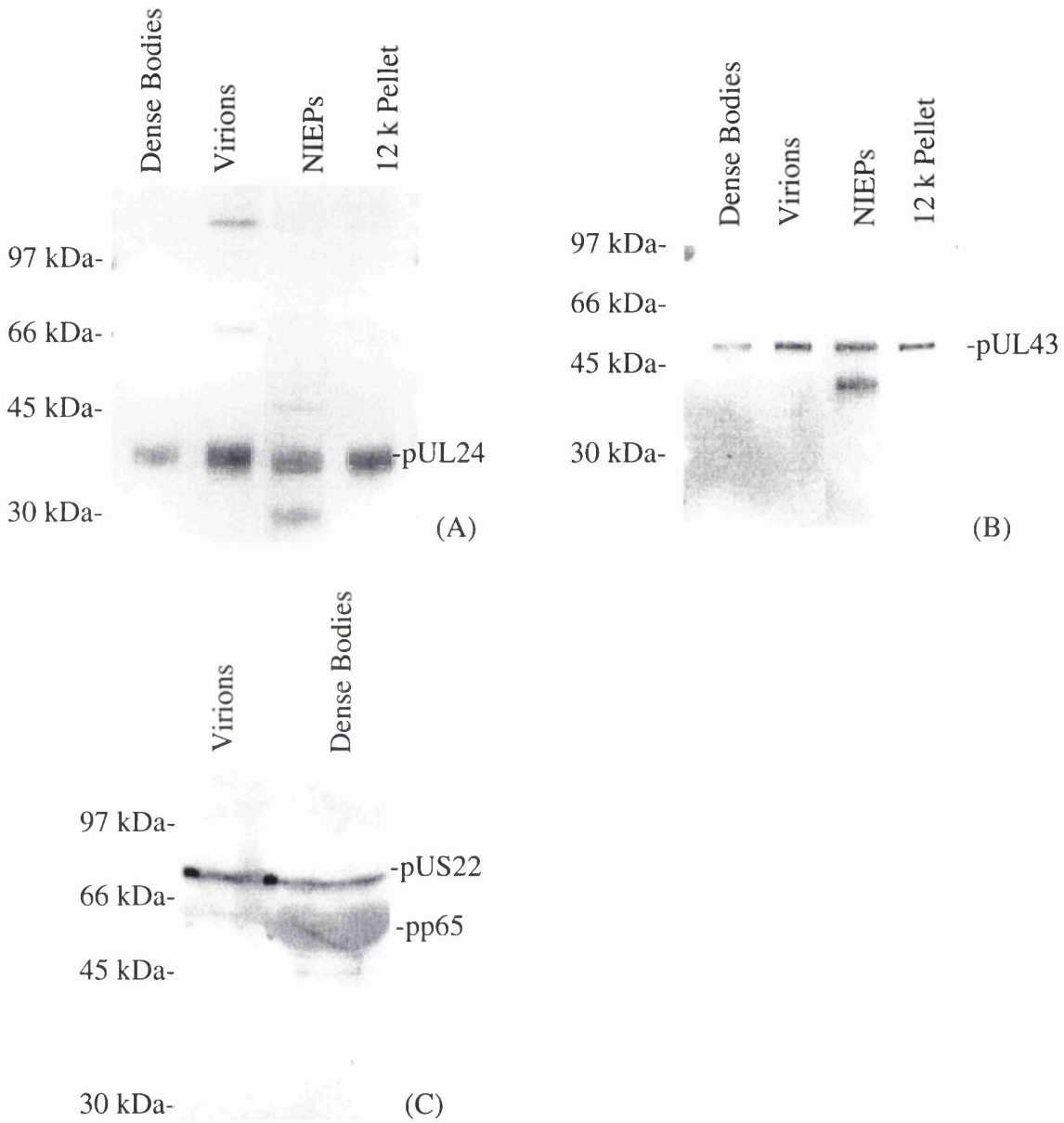
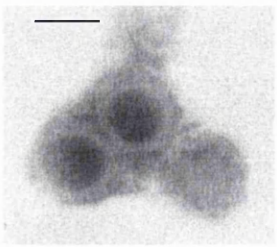
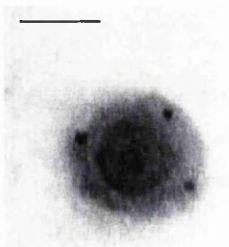


Figure 11. pUL24, pUL43 and pUS22 are contained in purified NIEPs, virions and dense bodies. Western Immunoblots, probed with anti-UL24 MAb 116 (A), stripped and re-probed with anti-UL43 Mab 92 (B). (C) Western immunblot, probed with anti-US22 MAb, showing detection of US22 in purified virions and dense bodies (NIEPs not shown). The broad band of pp65 protein in dense bodies can be seen.



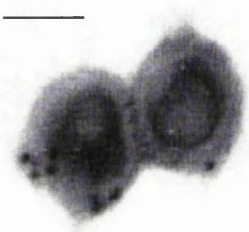
(A)

Non-HCMV MAb AP33



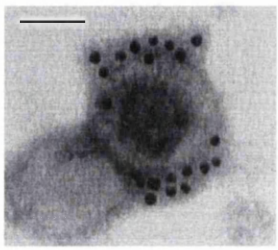
(B)

Anti-UL23 MAb 266



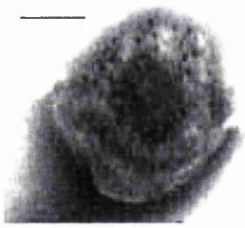
(C)

Anti-UL24 MAb 116



(D)

Anti-UL43 MAb 92



(E)

Anti-US22 MAb HWLF1

Figure 12. pUL23, pUL24, pUL43 and pUS22 are virion components. Virions were probed with non-HCMV control MAb AP33 (A), anti-UL23 MAb 266 (B), anti-UL24 MAb 116 (C), anti-UL43 MAb 92 (D) or anti-US22 MAb HWLF1 (E) for 5 h at RT, and subsequently treated with an anti-mouse gold-conjugated second antibody (1/30 dilution) overnight at RT. Virions were then negatively stained with phosphotungstic acid. Bar represents 100nm.

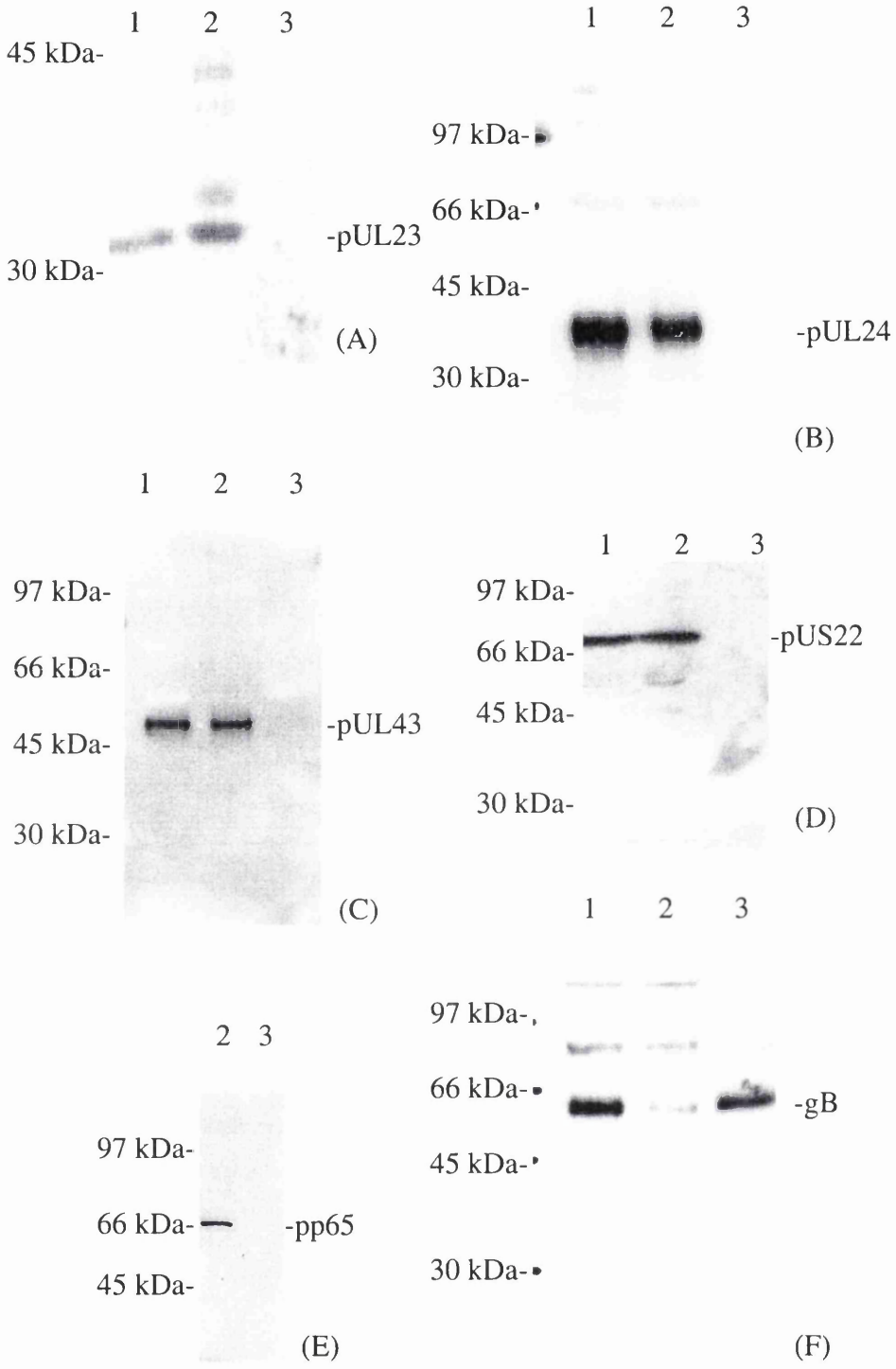


Figure 13. US22 gene family members are virion tegument proteins. Western immunoblots of intact purified virions (1), capsid/tegument (2) or envelope (3) fractions, probed with various antibodies against US22 family proteins. (A) pooled anti-UL23 PAb 679 and pep683, (B) anti-UL24 MAb 116, (C) anti-UL43 MAb 92, (D) anti-US22 MAb (HWLF1), (E) anti-pp65 MAb control, (F) anti-gB MAb control. The tracks are loaded with $5x10^9$ (B, C, E, F) or $1x10^{10}$ (A, D) virions. Particles were de-enveloped by treatment with detergent (1.0 % NP40 in TNE buffer (10 mM Tris-HCl pH 7.4, 100 mM NaCl, 1 mM EDTA)), for 30 min at 4 °C.

3.13 Intracellular location of pUL24 and pUL43

To investigate the intracellular location of pUL24 and pUL43, HFFF-2 cells grown on glass coverslips were either mock infected or infected with AD169 at a m.o.i. of 1 p.f.u./cell and processed for UV immunofluorescent microscopy at 72 h PI, using anti-UL24 MAb 116, anti-UL43 MAb 92 and anti-US22 MAb HWLF1 as probes. Rabbit polyclonal antibodies were not used in immunofluorescence due to non-specific binding of rabbit antibodies to the HCMV encoded Fc receptor. Mock infected or virus-infected HFFF-2 cells treated with the non-HCMV control antibody (MAb AP33) (Figure 14A), or mock-infected cells treated with MAb 116 (Figure 14B) or MAb 92, gave only background levels of fluorescence. In infected cells most of the pUL24 (Figure 14C) and pUL43 (Figure 14D) was concentrated in an intensely staining region or structure, juxtaposed to the nuclear membrane, though punctate staining of the perinuclear region, and to a lesser extent the cytoplasm was also observed. pUL24 and pUL43 were not detected in infected cell nuclei. Immunofluorescence studies using anti-UL23 MAb 266 did not show a clear or consistent fluorescence over background level that would permit the location of pUL23 to be determined (data not shown). As previously reported (Mocarski *et al.*, 1988) pUS22 was detected by MAb HWLF1 only in the nucleus of infected cells (Figure 14 E).

The pUL24 and pUL43 containing juxtanuclear structures in infected HFFF-2 cells were further investigated by thin section immuno-gold transmission electron microscopy. Cells infected at a m.o.i. of 5 p.f.u./cell were harvested and processed for electron microscopy at 96 h PI. Cell sections were treated with primary antibody (anti-UL23 MAb 266, anti-UL24 MAb 116 or anti-UL43 MAb 92) then subsequently with secondary anti-mouse antibody conjugated to 5 or 10 nm gold particles, as available. Cell sections treated with the non-

HCMV control MAb AP33 did not label with gold. pUL23, pUL24 and pUL43 were each located in cytoplasmic protein aggregates that manifested in two morphological forms. The first, were large complex structures, which ranged in size from 0.3-2.0 μm in diameter, appeared to lack a limiting membrane, and were located close to the nuclear membrane (Figure 15A, B, D, E). The second, which resembled dense bodies, were smaller (with diameters up to about <0.2 μm) and appeared to be bounded by a limiting membrane (Figure 15C). Evidently, the fluorescent juxtanuclear structure and the immuno-gold-tagged large complex type protein aggregates are the same structure. Virus particles, at various stages of maturation (B-capsids or DNA-containing capsids; tegumented or non-tegumented and non-enveloped) were associated with the complex type protein aggregates. Large numbers of spherical microvesicle structures (53 nm average diameter) with a concentric double ring appearance were embedded within the complex aggregates. The small dense-body like membrane-bound aggregates were not associated with virus particles, and were devoid of microvesicles and appeared to originate from the complex aggregates by envelopment of portions of the matrix (Figure 15D).

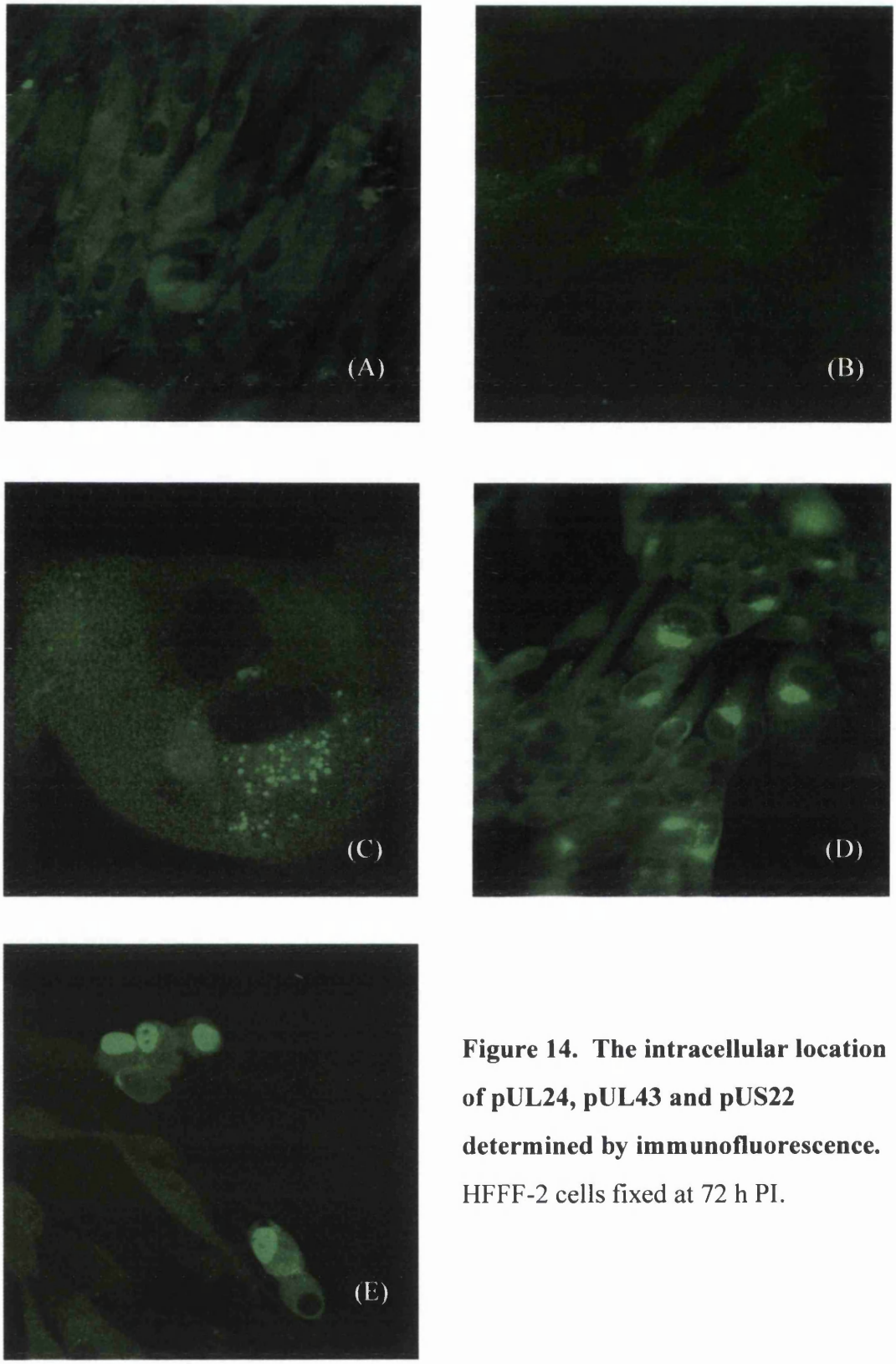
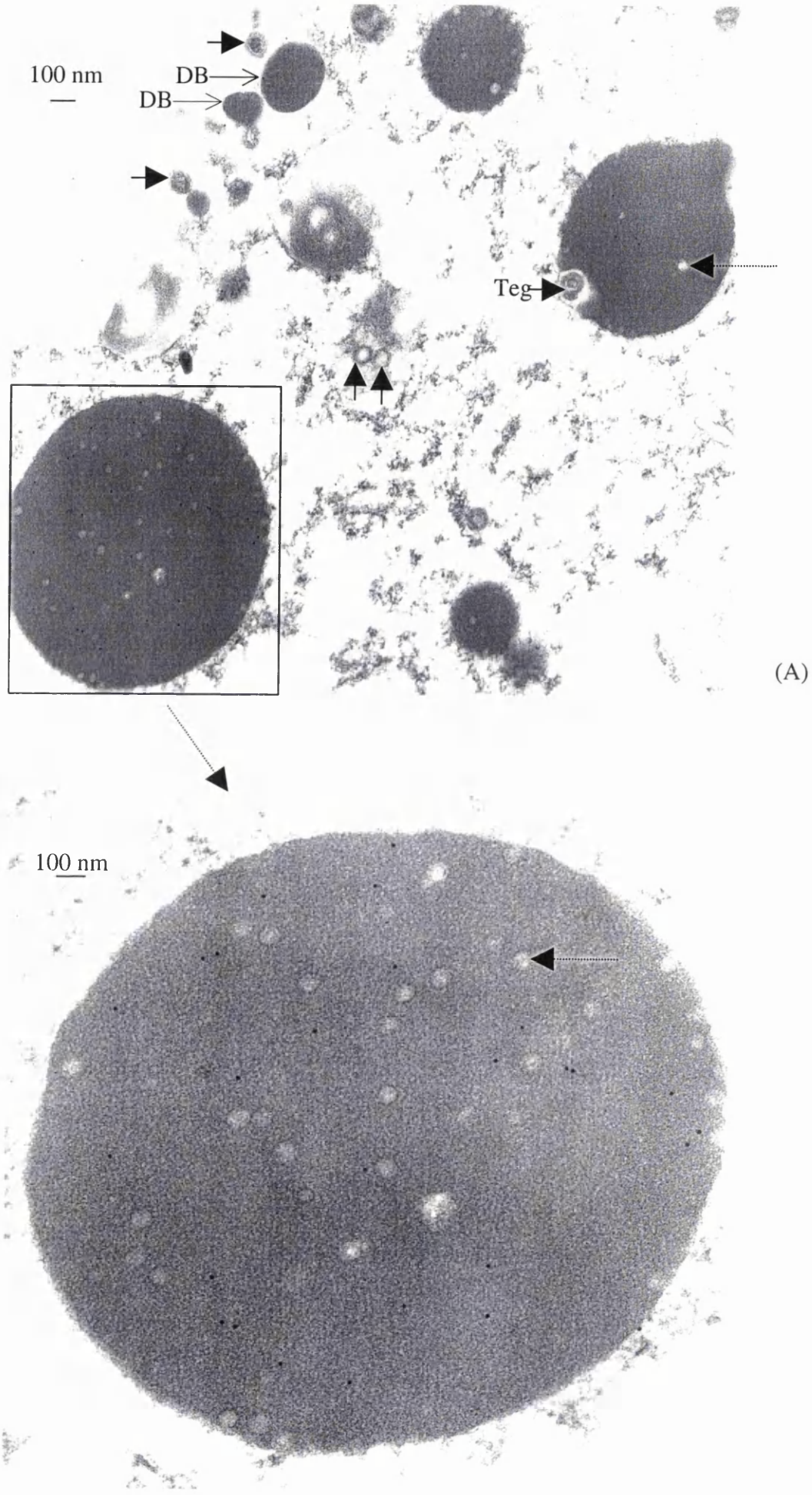
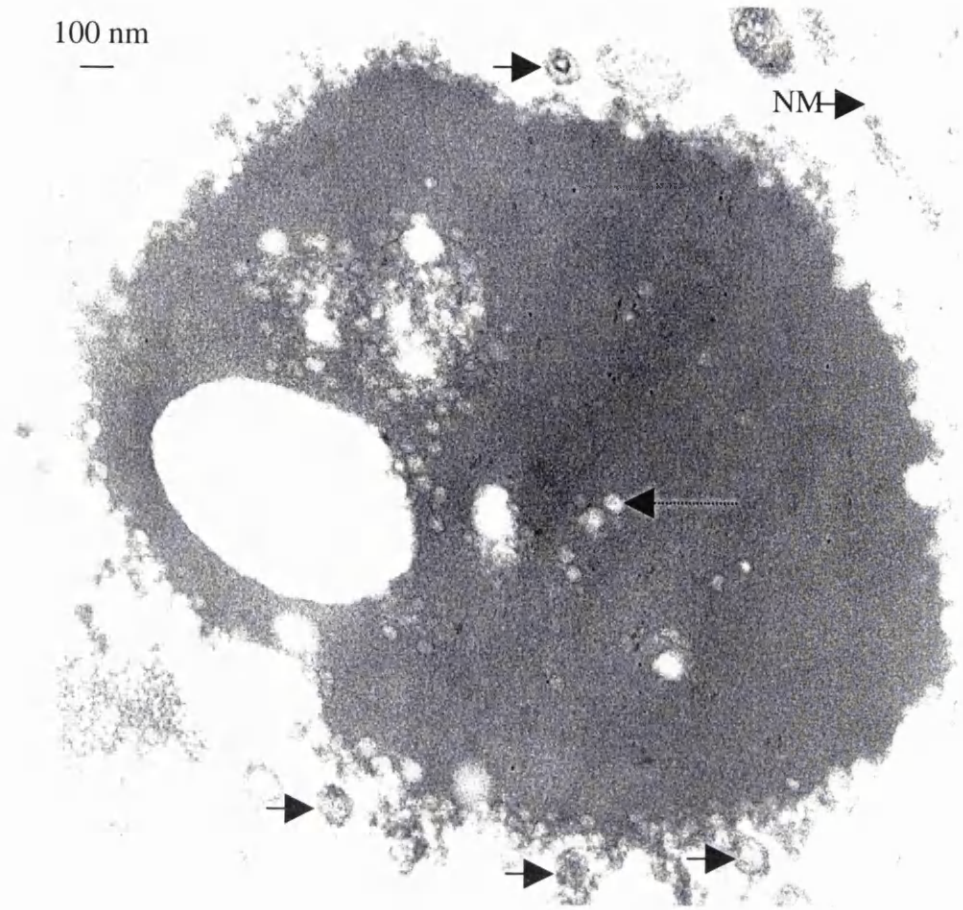


Figure 14. The intracellular location of pUL24, pUL43 and pUS22 determined by immunofluorescence. HFFF-2 cells fixed at 72 h PI.

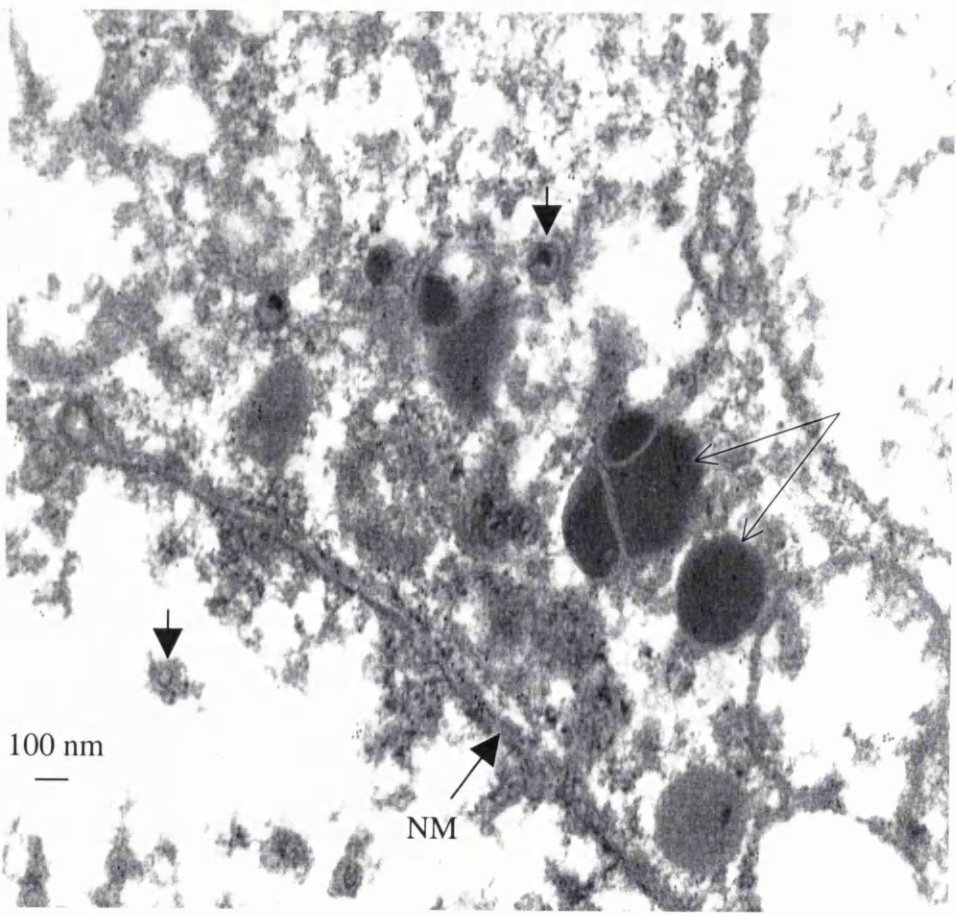
(A) Infected cells probed with non-HCMV MAb AP33 (Mag x 20)
(B) Mock infected cells probed with anti-UL24 MAb 116 (Mag x 20)
(C) Infected cells probed with anti-UL24 MAb 116 (Mag x 60)
(D) Infected cells probed with anti-UL43 MAb 92 (Mag x 20)
(E) Infected cells probed with anti-US22 MAb HWLF1 (Mag x20)

Figure 15. Gold-tagged cytoplasmic protein aggregate. Electron micrographs of HCMV infected HFFF-2 cells at 96 h PI. Cell sections were probed with (A) anti-UL24 MAb 116, or (B) anti-UL23 MAb 266, or (C, D, E) anti-UL43 MAb 92 and subsequently labelled with anti-mouse conjugated to 5 nm (A and B) or 10 nm (C,D and E) gold particles. (A) Large complex-type protein aggregate containing gold-tagged pUL24. Virus particles are arrowed; DB, dense-bodies; Teg, tegumented particle; microvesicles, broken arrow. (B) Large complex-type protein aggregate, juxtaposed to the nucleus and containing gold-tagged pUL23. Virus particles are arrowed; NM, nuclear membrane; microvesicles, broken arrow. (C) Membrane bound small protein aggregate containing gold-tagged pUL43 (double arrow). These structures, resembling HCMV dense bodies, varied greatly in size. Virus particles are arrowed; NM, nuclear membrane. (D) Virions and NIEPs associated with reduplicated membrane are embedded in this large complex-type protein aggregate (arrowed). The ground texture of the membrane-enclosed small protein aggregates (thin arrows) is similar to that of the large aggregate-though microvesicles appear to be excluded-suggesting that these are derived from the larger structure. (E) Non-enveloped, empty and DNA-containing virus particles (arrows) are associated with the peripheral surface of this large, gold-tagged pUL43-containing complex-type protein aggregate. The tegumented dense-cored virus particle appears to be detaching from the aggregate (fine arrow). Several gold-tagged virus particles associated with the aggregate are evident.

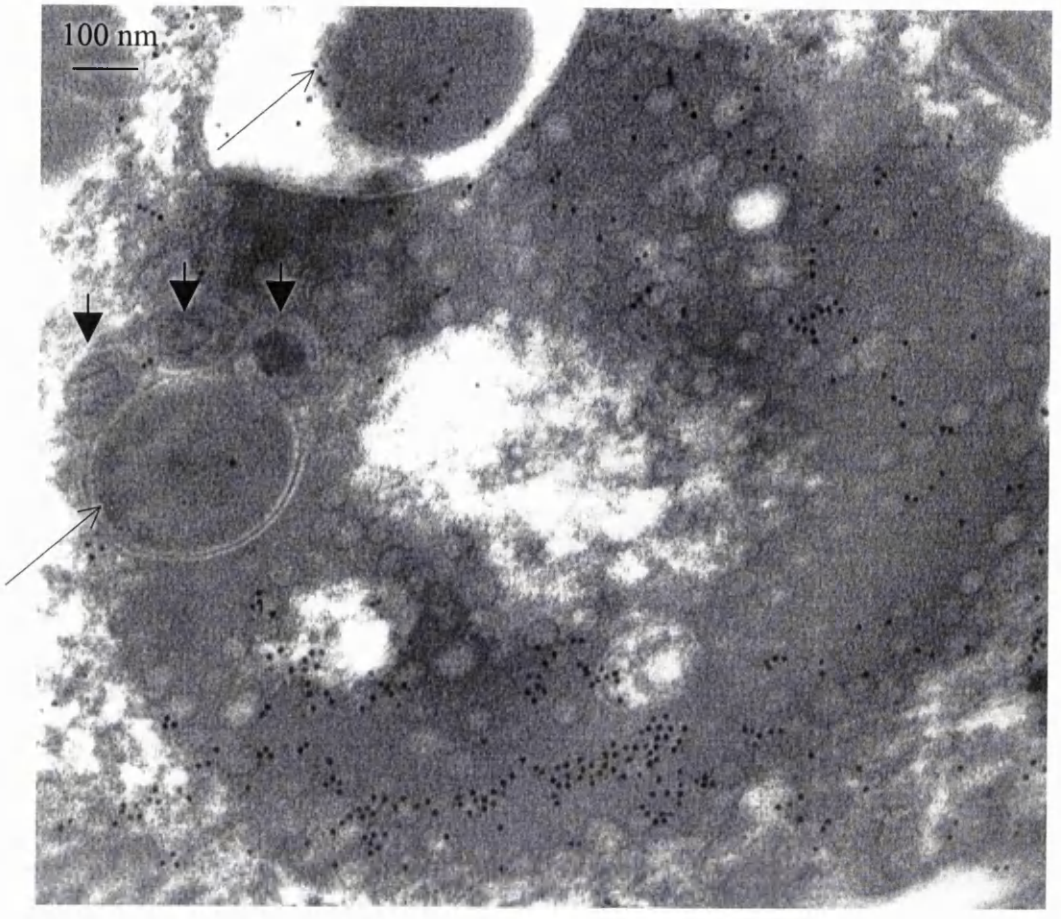

100 nm
DB
DB
Teg
(A)
100 nm



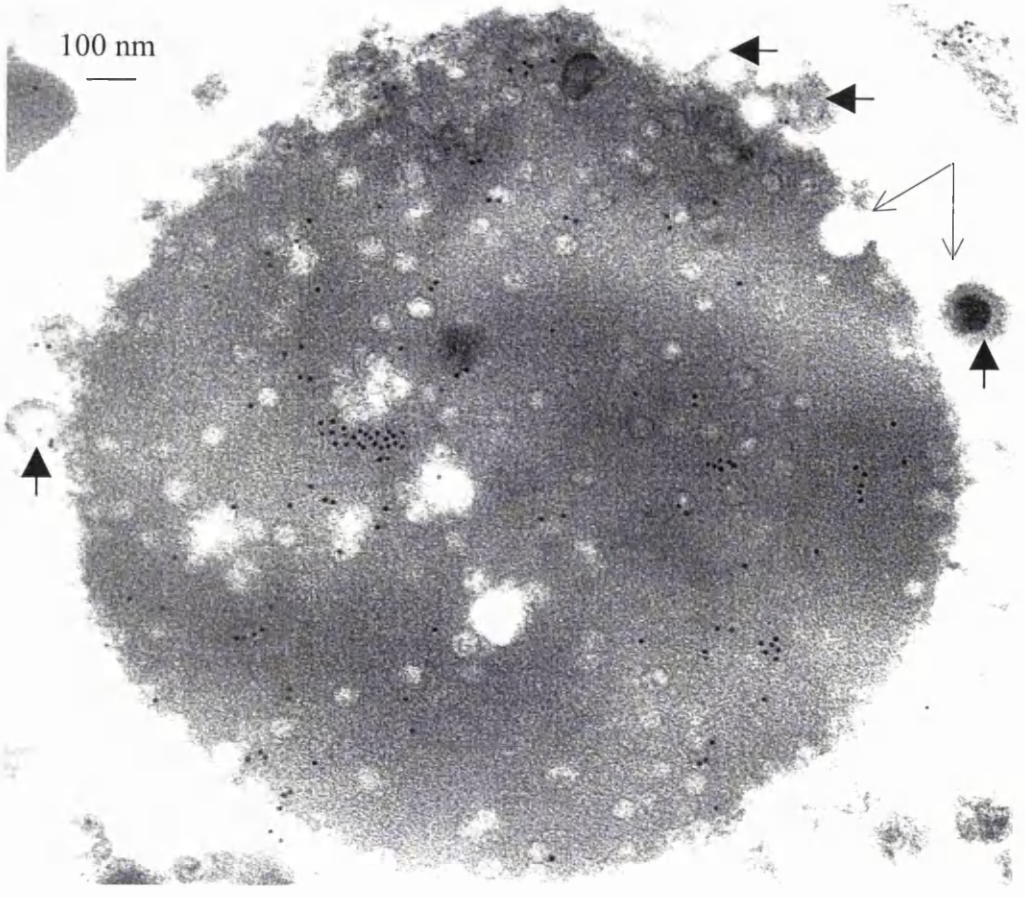
(B)



(C)



(D)



(E)

Chapter 4

RESULTS

4.1 Preparation of expressing/complementing cell lines

The existence of homologues for the HCMV UL23, UL24 and UL43 genes in the other sequenced *Betaherpesviruses* suggests that US22 family members provide an important function. Consequently, it was considered that US22 gene products might be essential for virus growth *in vitro*. If so, complementing cell lines are needed for the isolation and propagation of mutant viruses. Because they are permissive and give the highest yield of infectious progeny, human foetal fibroblast cells are the most appropriate cell type for construction of complementing cell lines. However, poor transfection efficiency and a short life span have impeded progress in this area until recently. With regard to the latter, two experimental approaches have been used to overcome senescence in fibroblast cells; firstly expression of the human papilloma virus (HPV) type 16 E6 and E7 transforming genes and secondly expression of the human telomerase reverse transcriptase gene (hTERT). Both of these systems were used in an attempt to construct cell lines expressing pUL23, pUL24 or pUL43.

4.2 Complementing cell lines based on hTERT-immortalised human retinal pigmented epithelial (RPE) cells

Before making complementing cell lines it was necessary to determine whether RPE cells were permissive for HCMV. RPE cell layers were infected with HCMV at an m.o.i. of 1 p.f.u./cell and infected cell cultures harvested at various times during the period 0-120 h PI.

The one-step growth curves obtained (Figure 16) confirmed that hTERT-RPE cells are permissive for HCMV growth, although the virus replication kinetics were delayed compared to HFFF-2 cells and lower final tires were obtained.

Transient transfection assays using a reporter plasmid (pcDNA3.1His B/lacZ (Invitrogen)) to assess transfection efficiency showed that hTERT BJ (human foreskin fibroblasts) were virtually non-transfectable by electroporation, $CaPO_4$ or various lipofection techniques (transfection efficiencies were never > 0.01 % and were usually ~0.001 %). Transfection of hTERT-RPE cells was much more efficient (~10 %). Consequently hTERT-RPE cells were used to generate expressing cell lines.

In order to generate expressing cell lines, hTERT-RPE cells in 24 well tissue culture trays were seeded at $5x10^4$ cells/well overnight at 37 °C and then transfected with 0.1 μg plasmid DNA using the LipofectAMINE Plus™ or Transfast™ lipofection reagents. The cells were transfected with the empty pcDNA3.1+ vector plasmid, pcDNA3.1+/RGSHis^6-UL23, pcDNA3.1+/UL24 or pcDNA3.1+/ RGSHis^6-UL43 (the latter provided by Dr. Derrick. Dargan). At 48 h post transfection the cells from each well were trypsinized and re-seeded into the 24 wells of a new tissue culture tray. 24 h later the medium was changed for medium containing 300 μg/ml G418 to select for cells expressing the plasmid marker gene. Drug resistant cell colonies were obtained for each of the transfected plasmids after ~2 weeks of G418 selection. Generally, only a few of the 24 wells on a tissue culture tray had colonies and usually there was only a single colony present in a positive well. When wells containing drug resistant cells were nearly confluent, the cells were trypsinized and re-seeded into a small tissue culture flask. Cells transfected with pcDNA3.1+/UL43 yielded eleven drug resistant colonies, of which six expressed pUL43 protein as determined by Western immunoblotting (Figure 17). Each of the six cell lines has continued to stably express pUL43 after recovery from –70 °C storage and following at least twenty cell passages.

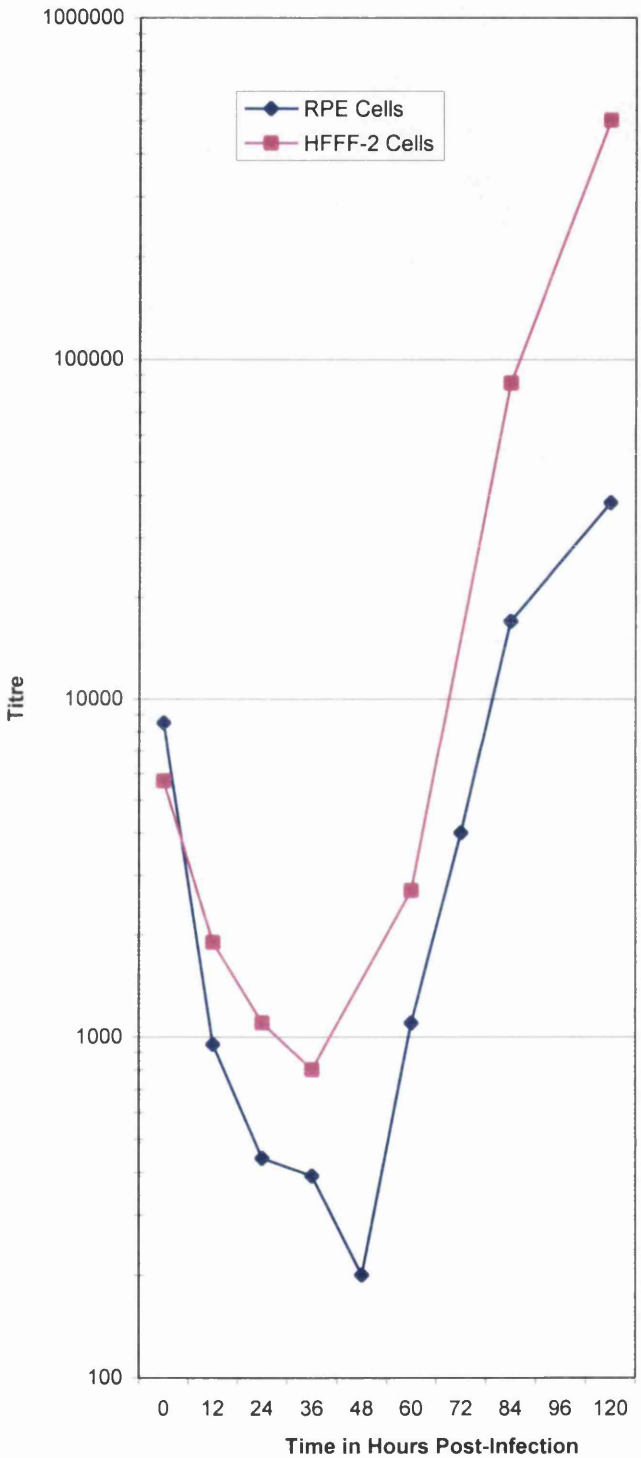
Cells transfected with pcDNA3.1+/UL24 and pcDNA3.1+/RGSHis^6-UL23 yielded twenty one and thirteen drug resistant colonies in the first experiment, respectively. However, none of these colonies appeared to express the pUL23 or pUL24 proteins when cell extracts were

probed in Western immunoblots using the full range of available anti-UL23 and anti-UL24 antibodies. Repeated transfection experiments yielded further drug resistant colonies, so that a total of thirty (pUL24) and twenty six (pUL23) colonies were finally obtained, however, without a single cell line expressing detectable amounts of pUL23 or pUL24 being obtained.

To eliminate the possibility that the pcDNA3.1+ plasmid constructs were defective HFFF-2 cells were transiently transfected with the pcDNA3.1+/RGSHis^6-UL23 plasmid and prepared for immunofluorescence at 48 h post transfection. The transfected cells were probed with mouse monoclonal antibody directed against the RGSHis^6 tag and with the rabbit anti-UL23 pep682 antibody. Confocal images (Figure 18) show that pUL23 is expressed from the pcDNA3.1+/RGSHis^6-UL23 plasmid, at least in HFFF-2 cells, and that the same protein, distributed in a region close by the nuclear membrane, is recognised by the two antibodies (Figure 18A, B, C). Cells transfected with the pcDNA3.1+/ RGSHis^6-UL43 plasmid and co-labelled with anti-RGS MAb and anti-UL43 PAb 616 show that pUL43, like pUL23, is expressed and is also located in a region close by the nuclear membrane (Figure 18D, E, F).

To check if UL23 or UL24 transcripts were being produced in the G418 resistant RPE cell lines, mRNA was extracted from a representative drug resistant cell line for each of the UL23 and UL24 plasmids. Northern blot experiments in which total mRNA was probed with UL23 and UL24 sequences (previously shown to detect UL23 and UL24 transcripts in Northern blots) failed to detect UL23 or UL24 mRNAs (Parvis Akter, personal communication).

Figure 16. One-Step Growth Curves of HCMV (AD169) Growth in RPE cells and HFFF-2 cells (1 p.f.u./cell)



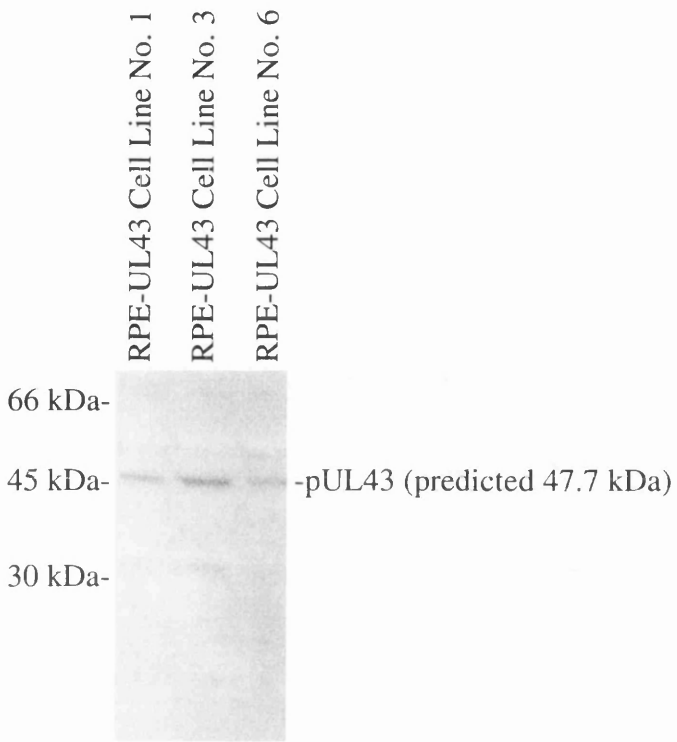


Figure 17. pUL43 is stably expressed by hTERT-RPE cells. Western immunoblot, probed with anti-UL43 MAb 92, against cell protein extracts from G418 resistant hTERT-RPE cells at cell passage number 6. pUL43 runs according to its predicted molecular weight of 47.7 kDa.

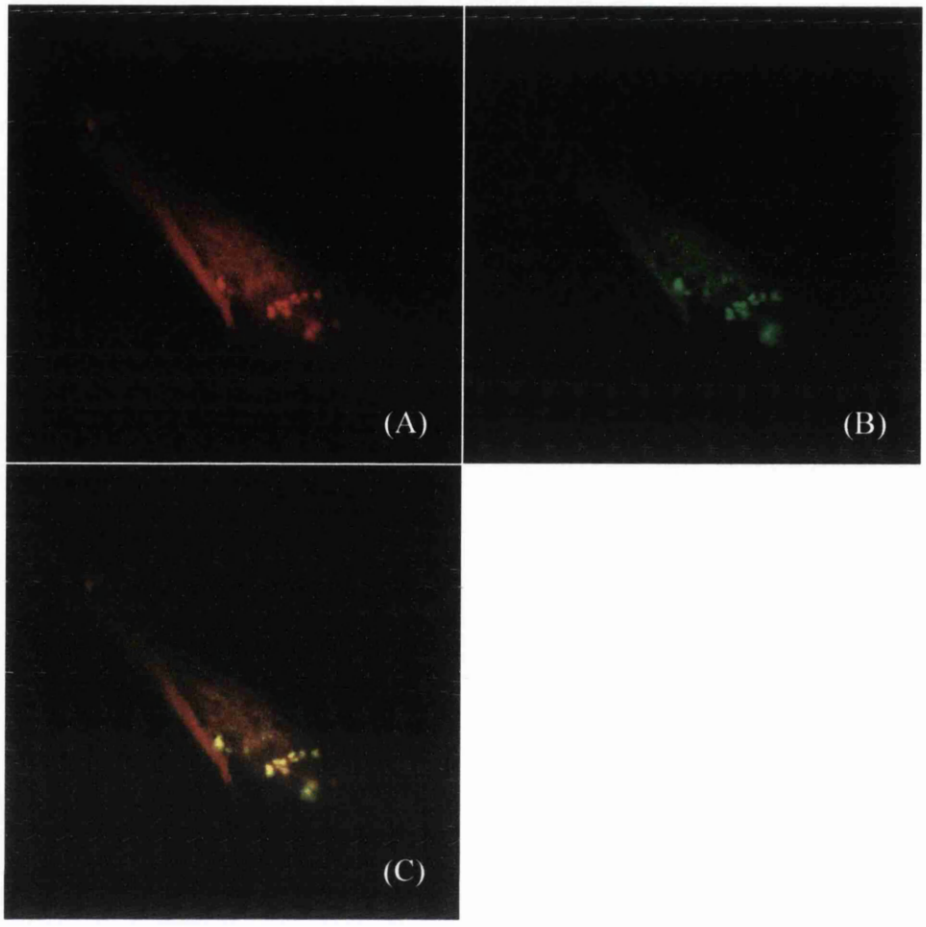


Figure 18. pUL23 and pUL43 are expressed in mammalian cells. (A, B, C) Confocal images of pUL23 in HFFF-2 cells transiently transfected with the eukaryotic expression vector plasmid pcDNA3.1+/RGSHis^{6}-UL23. The cell was probed with anti-RGS antibody (A) and anti-UL23 pep682 antibody (B). Merged image (C) shows co-localisation (yellow), indicating the primary antibodies were detecting the same protein. (D, E, F) Next Page

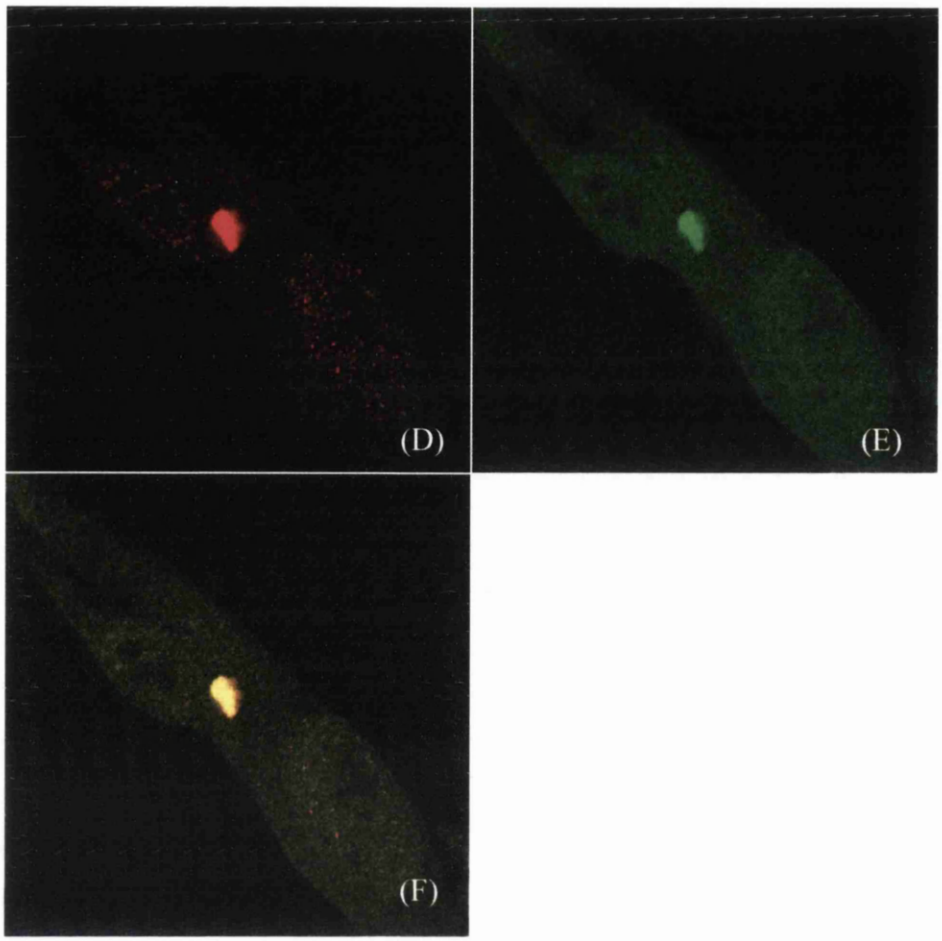


Figure 18. pUL23 and pUL43 are expressed in mammalian cells. (D, E, F) Confocal images of pUL43 in HFFF-2 cells transiently transfected with the eukaryotic expression vector plasmid pcDNA3.1+/RGSHis^{6}-UL43. The cell was probed with anti-RGS antibody (D) and anti-UL43 PAb 616 antibody (E). Merged image (F) shows co-localisation (yellow), indicating the primary antibodies were detecting the same protein.

4.3 Complementing cell lines based on hTERT-HFF (BJ) cells

Since pUL23 was expressed from the pcDNA3.1+ vector in transiently transfected human foreskin fibroblasts, it seemed appropriate to attempt to make expressing cell lines in the hTERT-HFF (BJ) cell line. hTERT-HFF cells were seeded ($5x10^4$ cells/well) overnight at 37 °C and then transfected with 1.0 μg plasmid DNA (pcDNA3.1/RGSHis^6-UL23 or pcDNA3.1+/UL24) using the LipofectAMINE Plus™ or Transfast™ lipofection reagents. The medium was changed 48 h after transfection for medium containing 300 μg/ml G418. After approximately two weeks a small number of drug resistant colonies were obtained from cells transfected with either plasmid, but these never expanded beyond a few hundred cells, despite attempts to stimulate cell growth by various methods, including removal of cells from drug selection, use of conditioned medium, overnight change in incubation temperature or addition of an HFFF-2 feeder cell layer. Such drug resistant colonies survived for at least a four-month period and bromophenol-blue dye exclusion viability tests confirmed that the cells were still viable, though in a quiescent non-replicating state.

4.4 Cloning the RGSHis^6-UL23 and UL24 ORFs into the LXSN16E6E7 amphotropic retrovirus vector

Since UL23 and UL24 complementing cell lines were not obtained using the hTERT cell systems it was decided to try to make the expressing cell lines using the pLXSN16E6E7 amphotropic retrovirus vector (kindly provided by Dr. Denise Galloway). This vector carries the HPV-16 E6 and E7 transforming genes for cell immortalisation and a G418 resistance

gene for cell line selection. The UL23 and UL24 ORF were cloned, under the control of the HCMV I-E promoter, into the vector at a site between the E6/E7 and G418 genes.

The RGSHis^6-UL23 ORF was excised from pGEX-4T-3/RGSHis^6-UL23 plasmid on a *BamH* I/*Xho* I fragment, purified on an agarose gel and cloned into LXSN16E6E7. DNA sequencing performed using the RApc3.1, RA2pc3.1, RA9 and RA10 PCR primers (Table 5) confirmed that there were no errors in the sequence of LXSN16E6E7/RGSHis^6-UL23.

Similarly, the UL24 ORF was excised from pGEX-4T-3/UL24 plasmid on a *BamH* I/*Xho* I fragment, purified on an agarose gel and cloned into LXSN16E6E7. DNA sequencing performed using the RApc3.1, RA2pc3.1, RA7 and RA8 PCR primers (Table 5) confirmed that there were no errors in the sequence of LXSN16E6E7/UL24.

4.5 Complementing cell lines based on the LXSN16E6E7 retrovirus vector

The LXSN16E6E7/RGSHis^6-UL23 or UL24 plasmid was transfected (see methods) into PT67 Retropack packaging cells, which provide all the proteins (gag, pol, amphotropic env) required for replication and packaging of the vector into virus particles.

The amphotropic retrovirus packaging cell line, PT67, was seeded (2x10^5 cells/35 mm dish) overnight at 37 °C and then transfected with 1 μg plasmid (LXSN16E6E7/RGSHis^6-UL23 or LXSN16E6E7/UL24) DNA using the Transfast™ reagent. 48 h post transfection the medium containing the released vector retrovirus was removed from cells, clarified by low speed centrifugation and filtered (0.45 μm). The virus innoculum was mixed 1:1 with DMEM-Fc10 containing 4 μg/ml polybrene and used to infect HFFF-2 cells for 2 h at 37

°C. The innoculum was decanted and replaced with DMEM-Fc10 containing 4 μg/ml polybrene and the cells were incubated for 5 h at 37 °C. The medium was then replaced with DMEM-Fc10 and the cells incubated at 37 °C. At 48 h PI the cells were trypsinized, re-seeded in to 24 well tissue culture plates, and 24 h later the medium was changed for DMEM-Fc10 containing 300 μg/ml G418.

Several experiments produced in total three UL24 colonies and one UL23 colony. These colonies expanded in size to a few hundred cells (by 2 weeks PI) but then ceased to propagate. As was the case with hTERT-HFF (BJ) cells, removal of G418 from the medium had no effect on HFFF-2 colony expansion. At approximately ten weeks PI only one (UL24) colony had expanded further, in the absence of G418, and the cells were seeded onto a 35 mm plate. However, pUL24 could not be detected by MAb 116 in Western immunoblots of protein extracts from this expanded cell line.

Clearly the retrovirus vector system was functional in these experiments since G418 resistance was acquired by the target HFFF-2 cells. The low number of drug resistant colonies obtained may reflect lower efficiency of the retrovirus vector system compared with the hTERT system.

In summary, three different cell lines using two experimental approaches have been used to attempt to produce a complementing/expressing cell line for pUL23 and pUL24. Each system has been shown to be functional since drug resistant colonies were obtained, and in the case of hTERT-RPE cells the system successfully yielded pUL43 expressing cell lines. The inability to produce an expressing cell line for pUL23 or pUL24 could be due to the proteins having a toxic effect on cells. The possibility that pUL23 and pUL24 may be cytotoxic is in keeping with the observation that they are expressed at low abundance in infected cells.

An attempt was made to demonstrate the putative toxic effect of pUL23 or pUL24 by directly transferring VP22-UL23 or VP22-UL24 fusion protein to HFFF-2 cells using the 'Voyager' system (Invitrogen). The RGSHis^6-UL23, UL24 and RGSHis^6-UL43 ORFs were

cloned into the plasmid VP22/myc-His (Invitrogen) to produce VP22-RGSHis^6-UL23, VP22-UL24 and VP22-RGSHis^6-UL43 fusion proteins, respectively. Numerous immunofluorescence experiments were conducted, probed with anti-VP22 MAb SY34 (kindly provided by Dr. Howard Marsden). BHK-21 cells transfected with the plasmids showed cytoplasmic fluorescence and when selected in G418 yielded drug resistant cells exhibiting large nuclear fluorescent aggregates, indicating that the VP22 proteins were present in the cell nucleus without killing the cells. HFFF-2 cells overlaid with filtered (0.45 μm) medium containing the VP22-UL23 or VP22-UL24 fusion protein and fixed 3-4 h later exhibited nuclear fluorescence (data not shown). Thus the VP22-UL23 and VP22-UL24 fusion proteins did not appear to be cytotoxic. These experiments, however, do not rule out the possibility that pUL23 or pUL24 expressed in their native state are cytotoxic proteins.

Chapter 5

RESULTS

5.1 Strategy for the generation of UL23 and UL24 gene knockout mutants

In order to investigate the functions provided by genes UL23 and UL24 it is necessary to make knockout mutants for each gene. Since UL23 and UL24 are located adjacently in the genome it is possible to also produce a double knockout mutant. An deletion-insertion mutagenesis strategy was employed; replacing a DNA fragment that coded for the conserved US22 motifs I and II in the UL23 and UL24 ORFs with the drug-selectable E.*coli* guanosine phosphoribosyl transferase (gpt) gene. The gpt gene product provides the capacity to salvage xanthine when cellular *de novo* guanine synthesis is blocked in the presence of the toxic compound mycophenolic acid (MPA). The use of gpt as a selectable marker for mutagenesis of MCMV (Vieira *et al.*, 1994) and HCMV (Greaves *et al.*, 1995) has been described previously. Drug selection for gpt expression is not absolute and wild type virus breakthrough is common. However, growth of recombinant viruses carrying the gpt gene is reported to be 1000 fold higher than that of the wild type virus, when grown in the presence of MPA and xanthine. Recombinant viruses expressing gpt are therefore isolated after several rounds of enrichment and plaque picking in the presence of MPA and xanthine.

A diagrammatic representation of the cloning strategy employed for the generation of plamids with UL23, UL24 and the double UL23/UL24 knockout mutations is shown in Figure 19.

5.2 PCR amplification of DNA fragments required for generation of UL23 and UL24 plasmid mutants

All the DNA fragments needed for constructing the mutants were amplified by PCR, cloned sequentially into plasmid pUC18 (Amersham Pharmacia Biotech) and checked by DNA sequencing. The UL23 ORF and flanking DNA fragments (Fragments 1 and 2, see Figure 19) were amplified from the cloned AD169 Hind III Y fragment (Oram *et al*, 1982) by PCR using oligonucleotide primers (Primers Rich1, 2, 3 and 4, see Table 7 and Figure 19). The UL24 ORF and flanking DNA fragments (Fragments 3 and 4, see Figure 19) were amplified from an AD169 cosmid fragment (Cos 64, described previously) by PCR using oligonucleotide primers (Primer Rich 5, 6, 7 and 8, see Table 8 and Figure 19). Each primer was abutted by restriction enzyme linker sequences to facilitate cloning (Tables 7 and 8). The UL23 and UL24 amplified DNA fragments were designed to have ~ 1 kbp of DNA sequence upstream and downstream of the UL23 or UL24 ORFs, respectively, to facilitate homologous recombination between plasmid contained HCMV sequences and wild type virus DNA. The gpt ORF and its associated HSV-1 Tk promoter (300-1187) were amplified by PCR from the pON1101 plasmid (kindly provided by Dr. Richard Greaves) using oligonucleotide primers (Primers Richgpt and Richgpt2, see Table 7). The SV40 poly(A) tail of gpt was not amplified.

Table 7. Oligonucleotide PCR primers for amplification of UL23 or gpt DNA fragments or for use in DNA sequencing.

Primer Name / Position	Primer Usage / Forward or Reverse primer	Oligonucleotide sequence
Rich1 / HCMV 26855	Amplify UL23 3'-end (fragment 1) / Forward	5'-AAT*GGATCC*ATGAGAGAGGCGCCGCACGCTGCATGG *Bam*H I
Rich2 / HCMV 28128	Amplify UL23 3'-end (fragment 1) / Reverse	5'-AATGGATCC*TCTAGA*TGGTCGGCAGTAACCACGTGGAACCTT *Xba* I
Rich3 / HCMV 28640	Amplify UL23 5'-end (fragment 2) / Forward	5'-AAT*CTGCAG*GGATCCGGGTTTCCACGGTCGCGACGTCTTAGG *Pst* I
Rich4 / HCMV 29706	Amplify UL23 5'-end (fragment 2) / Reverse	5'-GAC*AAGCTT*GAGATCGAGACGGACGAGGACTTCAAA *Hind* III
Richgpt / pON1101 300	Amplify gpt ORF / Forward	5'-GGAAGATCT*CTGCAG*GAGCTTCAGGGAGTGGCGCAGCTGCTT *Pst* I
Richgpt2 / pON1101 1187	Amplify gpt ORF / Reverse	5'-GCC*TCTAGA*TTAAAGCGGGGTTTGAACAGGGTTTCG *Xba* I
RAPUC1/ pUC18 126	DNA sequence vector pUC18 / Forward	5'-ATTAGGCACCCCAGGCTTTA
RAPUC2/ pUC18 361	DNA sequence vector pUC18 / Reverse	5'-TGTGCTGCAAGGCGATTAAG
23Del1 / HCMV 27201	DNA sequence UL23 fragment 1 / Forward	5'-GAATCTGGGGAATTCAACAC
23Del2 / HCMV 27606	DNA sequence UL23 fragment 1 / Forward	5'-TGTATTCGGCACGCGAAACA
gptseq1 / pON1101 718	DNA sequence gpt ORF / Forward	5'-GTGCGTTACTGGCGCGTGAA
gptseq2 / pON1101 760	DNA sequence gpt ORF / Reverse	5'-GTATCGACATGACGAATACC
34Seq1 / HCMV 29139	DNA sequence UL23 fragment 2 / Reverse	5'-GTCGACCTTATCCCCATCGT
34Seq2 / HCMV 29038	DNA sequence UL23 fragment 2 / Forward	5'-GAACGTGTTGAGATTATCTC

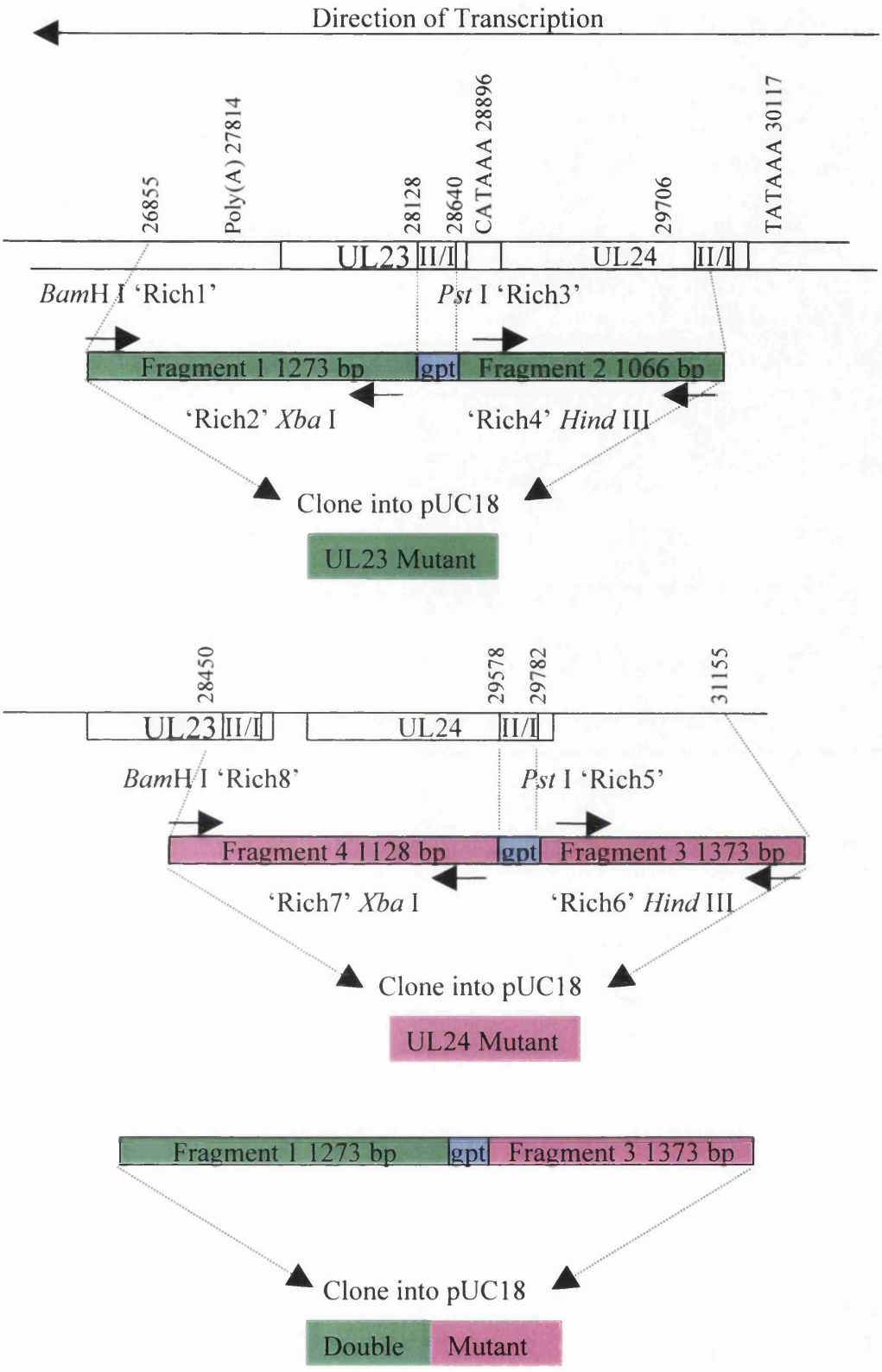


Figure 19. Diagramatic representation of the cloning strategy to produce UL23, UL24 and double knockout plasmid mutants. The arrows indicate direction of PCR primer amplification and restriction enzyme linkers used. II/I, conserved US22 motifs II and I in the UL23 and UL24 ORFs which are replaced by gpt in the recombinant viruses.

5.3 Cloning to produce a HCMV UL23 knockout plasmid mutant

The ~1.3 kbp UL23 3'-end (fragment 1) PCR product was digested with *Bam*H I/*Xba* I, purified on an agarose gel and cloned into pUC18. DNA sequencing performed using the RAPUC1, RAPUC2, 23Del1 and 23 Del2 PCR primers (Table 7) revealed that there were 3 PCR related errors in the DNA sequence of fragment 1; a silent mutation at position 28085 in ORF UL23 (codon AGC changed to codon AGT, both coding for Serine), and two point mutations at positions 27616 and 27468 in the UL22 and UL23 inter-genic region.

The ~0.9 kbp gpt PCR product was digested with *Pst* I/*Xba* I, purified on an agarose gel and cloned into pUC18 upstream of UL23 fragment 1. DNA sequencing performed using the RAPUC2, gptseq1 and gptseq2 PCR primers (Table 7) revealed that the sequence contained a silent mutation at pON1101 position 773 (codon TCC changed to codon TCT, both coding for Serine).

The ~1.1 kbp UL23 5'-end (fragment 2) PCR product was digested with *Hind* III/*Pst* I, purified on an agarose gel and cloned into pUC18 upstream of fragment 1 and gpt. DNA sequencing performed using the RAPUC2, 34Seq1 and 34Seq 2 (Table 7) PCR primers revealed that there were 2 PCR amplification errors in fragment 2, comprising of a silent mutation at position 28984 in ORF UL24 (codon AAC changed to codon AAT, both coding for Asparagine) and a point mutation at position 29347 in ORF UL24 (codon TTC, coding for Phenylalanine, changed to codon TTA, coding for Leucine).

Table 8. Oligonucleotide PCR primers for amplification of UL24 DNA fragments or for use in DNA sequencing.

PCR Primer Name / Position	Primer Usage / Forward or Reverse primer	Oligonucleotide sequence
Rich5 / HCMV 29782	Amplify UL24 5'-end (fragment 3) / Forward	5'-AAT*CTGCAG*CAGACAGCCCAGGCCGAACTCGGCCGT *Pst* I
Rich6 / HCMV 31155	Amplify UL24 5'-end (fragment 3) / Reverse	5'-GCC*AAGCTT*TATAGAAGAGCACGTTGTAATCGCGTA *Hind* III
Rich7 / HCMV 29758	Amplify UL24 3'-end (fragment 4) / Reverse	5'-CTA*TCTAGA*GAAGAAGACCGCTGTGTGCCTCATCTC *Xba* I
Rich8 / HCMV 28450	Amplify UL24 3'-end (fragment 4) / Forward	5'-CTA*GGATCC*GCACGTTGTGATCGCCCTTGTGTTTAA *Bam*H I
56Seq1 / HCMV 29801	DNA sequence UL24 fragment 3 / Forward	5'- TCGGCCGTGAGCGCCAGGCT
56Seq2 / HCMV 30499	DNA sequence UL24 fragment 3 / Reverse	5'- CTGCTACCGCCGCTGCTGCT
56Seq3 / HCMV 30442	DNA sequence UL24 fragment 3 / Forward	5'- TGCCCGAGAACGGCGCCGGC
56Seq4 / HCMV 30797	DNA sequence UL24 fragment 3 / Reverse	5'- TAAGACGGGGTCGACGAGGC
56Seq5 / HCMV 30742	DNA sequence UL24 fragment 3 / Forward	5'- ACGAATGCATACCCATGCCG
56Seq6 / HCMV 31149	DNA sequence UL24 fragement 3 / Reverse	5'- AGAGCACGTTGTAATCGCGT

5.4 Cloning to produce a HCMV UL23/UL24 double knockout plasmid mutant

The HCMV UL23/UL24 double knockout mutant cloning strategy was to clone the UL24 5'-end (fragment 3) upstream of gpt and UL23 fragment 1. The ~1.4 kbp UL24 5'-end (Fragment 3, see Figure 19) PCR product was digested with *Hind* III/*Pst* I, purified on an agarose gel and cloned into the pUC18 plasmid containing gpt and UL23 fragment 1. DNA sequencing performed using the RA7 (Table 5), RAPUC2 (Table 7), 56Seq1, 56Seq2, 56Seq3, 56Seq4, 56Seq5 and 56Seq6 (Table 8) PCR primers revealed that UL24 fragment 3 contained no sequence errors.

5.5 Cloning to produce a HCMV UL24 knockout plasmid mutant

To produce the HCMV UL24 knockout mutant, the UL23 fragment 1 was excised by *Bam*H I/*Xba* I digestion of the UL23/UL24 double mutant plasmid construct and replaced with the UL24 3'-end (fragment 4) using the same restriction enzyme linker sites (Figure 19).

The ~1.1 kbp UL24 3'-end (Fragment 4, see Figure 19) PCR product was digested with *Bam*H I/*Xba* I, purified on an agarose gel and cloned into the pUC18 plasmid downstream of gpt and fragment 3. DNA sequencing performed using the RA8, RA9 (Table 5), RAPUC1, 34Seq1 and 34Seq2 (Table 7) PCR primers revealed that there were 2 PCR amplification errors in fragment 4, comprising of a point mutation at position 28727 in the UL23-UL24 inter-genic region and a deletion mutation (consisting of a single adenosine base pair) also in the UL23-UL24 inter-genic region at position 28762.

5.6 Enriching for HCMV UL23 and UL24 gene knockout mutants

The protocol used for enrichment of recombinant viruses containing the gpt gene was as described by Greaves *et al*, (1995). HFFF-2 cells ($2x10^5$ cells/35 mm plate) were transfected with 1-8μg plasmid DNA (for each mutant plasmid construct) by the calcium phosphate method and infected 24 h later with HCMV at a m.o.i. of 3 to 5 p.f.u./cell. Alternatively, the cells were infected with HCMV at a m.o.i. of 3-5 p.f.u./cell and then transfected with mutant plasmid constructs by lipofection (Transfast™) immediately after the 1 h virus adsorption period. In either case, progeny virus was harvested 5 days after infection and the infectivity titrated. For the first and subsequent rounds of enrichment for gpt expressing viruses plates of HFFF-2 cells were infected at ~0.1 p.f.u./cell. After virus adsorption for 1 h at 37 °C, the medium on cells was replaced with DMEM-Fc10 supplemented with antibiotics and containing 10 μg/ml MPA (GibcoBRL) and 250 μg/ml xanthine (Sigma). Virus was harvested 5 days after the cultures exhibited 100 % CPE. After four rounds of enrichment for gpt expressing viruses, the virus was cloned by four rounds of plaque purification. Plaques were picked from those plates that yielded well-defined and separated plaques. Picked plaques were sonicated and immediately plated on HFFF-2 cell layers, which were then overlaid with DMEM-Fc10 containing 10 μg/ml mycophenolic acid and 250 μg/ml xanthine. After the four rounds of plaque purification the infected cells were trypsinized, re-suspended in DMEM-Fc10 and used to infect a small flask of HFFF-2 cells, to provide a virus stock. Plaque purified recombinant virus stocks were used to infect a 35 mm plate of HFFF-2 cells, which was harvested to provide infected cell DNA extracts for Southern blotting when 100 % CPE was reached. The DNA pellet was re-suspended in 25 μl sterile distilled water and digested with *Hind* III overnight at 37 °C. To screen for the presence of the gpt gene 5-10 μl of the *Hind* III digested DNA was further digested with *Pst* I/*Xba* I for ~4 h at 37 °C and the fragments separated by electrophoresis on an agarose gel, which was then processed for Southern blotting. The UL24 mutant plasmid construct was used as probe throughout, since it hybridized well to wild type virus DNA and should hybridize to bands of unique sizes for each of the recombinant viruses (Table 9).

Table 9. Expected DNA band sizes in Southern blots probed with the UL24 mutant plasmid construct, following *Hind* III then *Pst* I/*Xba* I digestion of picked plaque infected cell DNA extracts.

Type of viral DNA	Expected DNA band sizes in bp
Wild type virus	64, 1432, 3790
UL23 knockout virus	64, 888, 1071, 1432
UL23/UL24 double knockout virus	888, 1425
UL24 knockout virus	888, 1425, 3657

Conclusion

No recombinant knockout viruses were isolated. Of the 79, 57 and 97 plaque purified "recombinant" virus infected cell extracts obtained for the UL23, UL24 and double UL23/UL24 mutant plasmid constructs, respectively, all gave the band pattern expected for wild type virus in Southern blotting experiments. A representative Southern blot with accompanying agraose gel is shown (Figure 20). The possible reasons for failure to isolate a recombinant virus are outlined in the discussion section of this thesis, section 6.6.

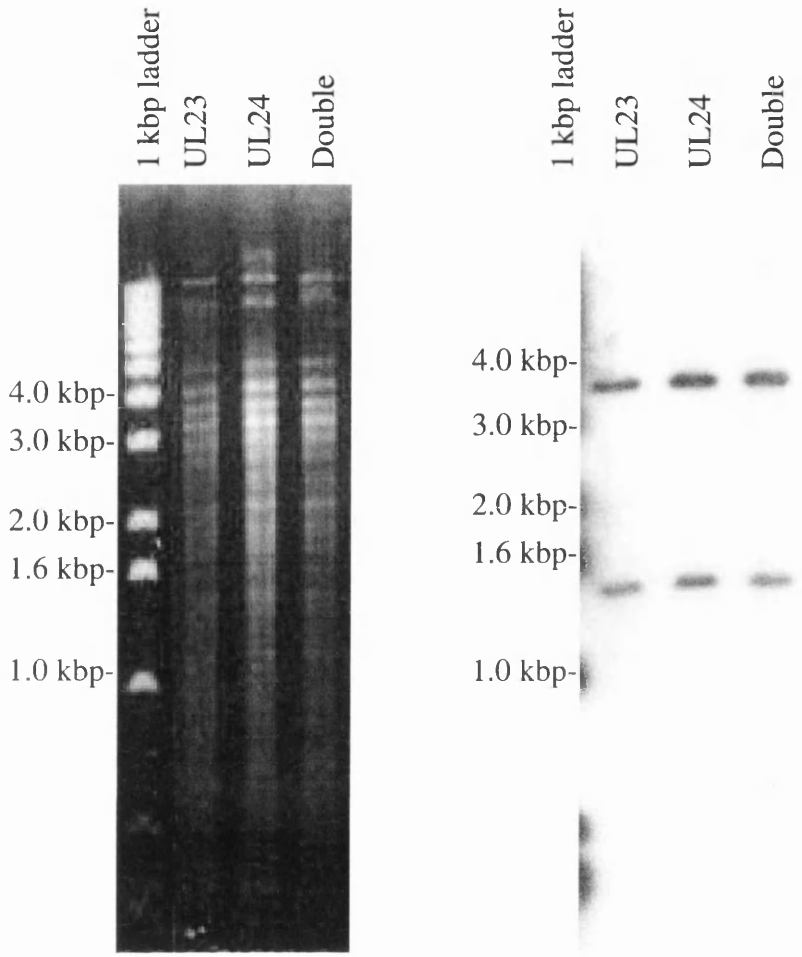


Figure 20. Screening for recombinant viruses by Southern blotting.
Representative agarose gel (A) showing total cellular and viral DNA extracted from 35mm plates of infected HFFF-2 cells at 100% CPE, and digested with *Hind* III and then with *Pst* I/*Xba* I. Track UL23 is a putative UL23 knockout mutant; UL24 is a putative UL24 knockout mutant and double is a putative knockout mutant in both UL23 and UL24. The accompanying Southern blot (B) for the gel, probed with the plasmid constructed for UL24 knockout mutant, shows that all 3 samples gave the wild-type virus profile. The expected band sizes for wild-type virus were 3790 bp and 1432 bp.

Chapter 6

DISCUSSION

Antibodies were generated to identify the HCMV UL23 and UL24 gene products. Polyclonal and monoclonal antibodies were raised against GST fusion proteins and polyclonal antibodies were also raised in rabbits against branched peptides of pUL23 and pUL24. The anti-UL24 antibodies specifically recognized a 40 kDa protein in extracts prepared from pUL24 expressing bacterial cells and from HCMV infected HFFF-2 cells. The anti-UL23 antibodies specifically recognized a 33 kDa protein in purified HCMV particle and in pUL23 expressing bacterial cell extracts. Antibodies previously raised against pUL43 specifically recognized a 48 kDa protein both in pUL43 expressing bacterial cell and HCMV infected HFFF-2 cell extracts. The anti-US22 MAb specifically recognized pUS22 (76 kDa) from HCMV infected HFFF-2 cell extracts (Mocarski *et al.*, 1988; Chee *et al.*, 1990).

The molecular masses of pUL23, pUL24 and pUL43 did not differ significantly when made in prokaryotic and eukaryotic cells and were close to the size expected from the published DNA sequence (Chee *et al.*, 1990), suggesting that none of the proteins were extensively modified by post-translational processing. Nevertheless, it is possible that some post-translational modification of pUL24 occurs, since pUL24 in infected cell or in virus particle extracts resolved as a broad fuzzy band or occasionally as a doublet of closely spaced bands in SDS-PAGE gels. The HHV-6 U3 gene (UL24 homologue) may also be post-translationally modified, since the pU3 made in eukaryotic cells has a higher molecular weight than that made in bacterial cells (Mori *et al.*, 1998). Other members of the US22 family are also thought to be post-translationally modified. For example, pUL36 consistently migrated as a doublet in SDS-PAGE gels, although the nature of the modification has yet to be established (Patterson and Shenk, 1999). Furthermore, pUS22 migrated in SDS-PAGE gels at an apparent molecular resolution of 76 kDa, which is significantly higher that the 67 kDa protein predicted from the DNA sequence. In part the difference in size is due to a mistake in the published AD169 US22 sequence (Chee *et al.*, 1990), that shortened the predicted US22 ORF,

but this cannot, by itself, account for the entire difference in the observed and predicted sizes – suggesting that pUS22 may also be post-translationally modified. Unfortunately, immunoprecipitation experiments using the anti-UL23, anti-UL24 or anti-UL43 antibodies failed to work, possibly because in each case the proteins are present in protein aggregates in infected cells and so may not have been solubilized, and this has limited the investigation of post-translational processing of these gene products.

6.1 Kinetic class of gene expression

Western immunoblots revealed that pUL24 was expressed with early-late (E-L) gene kinetics, while pUL43 was expressed with true-late (L) gene kinetics. Time course experiments showed that pUL24 could be detected from as early as 12 h PI, while pUL43 was not detectable before 60 h PI, confirming the kinetic class designations for pUL24 and pUL43 synthesis. Due to low levels of expression the 33.0 kDa UL23 protein could not be detected at all in infected cell extracts and consequently the kinetics of UL23 protein synthesis could not be determined. pUS22 has been reported to be expressed with early (E) gene kinetics (Mocarski *et al.*, 1988).

The kinetic class of transcript expression for most HCMV genes has been investigated using a HCMV gene array system, which confirmed that the US22 and UL43 genes were expressed with early and late kinetics, respectively, but failed to detect mRNA transcripts for genes UL23 or UL24, or indeed for some other US22 family genes (UL28, IRS1 or TRS1) (Chambers *et al.*, 1999). More recently, however, UL23 and UL24 mRNA transcripts have been detected, using the highly sensitive techniques of SMART-RACE PCR and Northern blotting. The results indicate that the UL23 and UL24 genes are both expressed with early-late (E-L) gene kinetics (Parvis Akter, personnal communication). Interestingly, the UL23 transcript was even less abundant than the UL24 transcript, which itself was a rare mRNA. Thus, the relative transcript levels appear to correlate with the relative levels of pUL23 and

pUL24 protein expression as detected by Western immunoblotting. The apparent paucity of UL23 and UL24 transcripts probably explains failure of transcript detection by the gene array system, as Chambers *et al.*, (1999), arbitrarily set sensitivity levels to detect more abundant messages. By analogy with UL23 and UL24, the gene array data could also indicate that the UL28, IRS1 and TRS1 transcripts will also be rare. Interestingly, the HHV-6 gene U3 transcript, detected by Northern blotting, was also rare. Like HCMV UL23, the HHV-6 U3 protein product was not detected in infected cell extracts by Western immunoblot, although it was detected in infected cells by immunofluorescence. The authors concluded that the U3 gene, like its HCMV UL24 counterpart, was expressed with early-late (E-L) gene kinetics (Mori *et al.*, 1998).

US22 family gene members are represented in each of the temporal classes of gene expression; UL36, TRS1 and IRS1 are I-E genes (Tenney and Colberg-Poley, 1991a); Romanowski and Shenk, 1997; Chambers *et al.*, 1999); US22, US23, US24 and US26 are E genes (Mocarski *et al.*, 1998, Chambers *et al.*, 1999), while UL23, UL24, UL29 and UL43 are L genes (Chambers *et al.*, 1999; this thesis). Thus the different expression kinetics among family members allows for a US22 gene family role at each phase of HCMV gene expression.

6.2 US22 family gene products are tegument components

pUS22, pUL23, pUL24 and pUL43 were detected in protein extracts prepared from purified virions and dense bodies and pUL24 and pUL43 were also contained in protein extracts from NIEPS. pUL23 and to a lesser extent pUS22 appeared to be in low abundance in virions and dense body preparations, and were only detected unambiguously when the gel was loaded with 10-fold normal protein concentration (10^{10} particle equivalents/gel track). Since insufficient NIEPs were obtained in these experiments to allow for this increased loading, it was not possible to demonstrate the presence of pUL23 and pUS22 in NIEPs, though it seems highly probable that they would be present. In order to corroborate that pUS22,

pUL23, pUL24 and pUL43 were indeed particle components and not simply associated with co-purifying cell debris, the purified virions were investigated by immuno-gold negative stain electron microscopy. As expected, pUS22, pUL23, pUL24 and pUL43 were particle components as evidenced by gold-tagging of tegument material adhering to capsids, or in matrix material issuing from dense bodies, where the envelope was broken. Final confirmation that pUS22, pUL23, pUL24 and pUL43 were indeed tegument proteins was obtained by demonstrating that these proteins were retained with the pelleted capsid/tegument structure after removal of the virus envelope by detergent treatment. Although others have suggested that pUS22 (HWLF1) is a non-structural protein (Mocarski *et al.*, 1988; Dal Monte *et al.*, 1998) the data reported here clearly show that pUS22 is a structural protein and a component of the tegument.

6.3 Intracellular location of pUS22, pUL23, pUL24 and pUL43 and models for particle maturation

The intracellular locations of several US22 family proteins have been investigated by immunofluorescence. pIRS1 and pTRS1 are present throughout the infected cell at immediate-early and early times PI, but are predominately cytoplasmic at late times (Romanowski and Shenk, 1997), although neither has been reported to be located in the juxtanuclear structure reported by Sanchez *et al.*, (2000b). Another protein, pIRS1^{263}, entirely encoded within the C-terminal region of IRS1, is predominately nuclear throughout infection. This is consistent with the proposed function of pIRS1^{263}, which is thought to antagonize pIRS1 and pTRS1-induced transcriptional *trans*-activation of pIRS1/pTRS1 responsive gene promoters. Another US22 family gene product, pUL36, made at IE times, is present throughout the virus replication cycle in AD169 infected cells. Like pIRS1 and pTRS1, pUL36 was distributed all through the infected cell but occasional intense spots of fluorescence were present in the cytoplasm (Patterson and Shenk, 1999). The pattern of

pUL36 intracellular fluorescence differed between AD169 and Towne or Toledo infected cells. In the latter case there was little nuclear fluoresence but the cytoplasm contained intensely fluorescent spots and/or 'worm-like' structures of unknown origin. Immunofluorescence experiments using the HWLF1 (anti-US22) antibody and AD169 infected HFFF-2 cells (not shown) confirmed earlier reports that pUS22, detected by antibody CH41, was located in the nucleus at both early and late times post-infection (Mocarski *et al.*, 1988). Confusingly, however, Mocarski *et al.*, (1988), also reported that in cell fractionation studies pUS22 was cytoplasmic with a proportion released from cells as a soluble protein at both early (24 h) and late (72 to 120 h) times in infection. The pUS22 amino acid sequence does indeed exhibit characteristics consistent with a secretory protein, including putative N-linked glycosylation sites and the lack of a hydrophobic transmembrane region. In EM immuno-gold thin section experiments pUS22 could not be located unambiguously, since gold was scattered all over the sectioned cells, including the nucleus, cytoplasm and protein aggregates (data not shown) and could not reliably be differentiated from the pattern obtained with antibodies giving a non-specific reaction.

In immunofluorescence experiments pUL24 and pUL43 were non-nuclear proteins located in a cytoplasmic juxtanuclear region or structure, resembling that reported by Sanchez *et al*, (2000b), which was located close to, or involving, the microtubule organising centre and associated with the post-Golgi network. The fluorescent juxtanuclear structures were further investigated by thin section immuno-gold transmission electron microscopy. These investigations correlated the fluorescent juxtanuclear structure with large protein aggregates with a complex structure. Smaller membrane-bound protein aggregates reminiscent of HCMV dense bodies were also present. The complex-type protein aggregates did not appear to be bound by a limiting membrane, but contained numerous spherical microvesicles with an average diameter of approximately 53 nm. pUL23, pUL24 and pUL43 were each located both in the complex-type protein aggregates and the smaller membrane-bound aggregates. Cytoplasmic protein aggregates are well documented in HCMV infected cells (Severi *et al.*, 1992). They have been reported to contain other viral tegument proteins pp150 (UL32), pp65 (UL83) and pp28 (UL99) and to be associated with the virus envelope glycoproteins gB (UL55), gH (UL75) and gp65 (Landini *et al.*, 1987; Hensel *et al.*, 1995; Sanchez *et al.*, 2000b).

It has been suggested that the juxtanuclear structure is a major site of HCMV tegumentation (Sanchez *et al.*, 2000b).

The site, or sites, at which tegument proteins are incorporated into the herpesvirus particle has been the topic of much research. To understand the processes by which HCMV tegumentation occurs it is necessary to consider what is known regarding the maturation of other herpesvirus particles. Most studies have used the *Alphaherpesvirus* HSV-1. Two models for HSV-1 envelopment have been reported. The first, and earliest, holds that HSV-1 particles acquire their envelope from the inner nuclear membrane. Such enveloped particles are said to be present in the lumen of the nuclear membrane and to travel along it to a junction with the ER. From the ER the enveloped particles are thought to pass into the Golgi apparatus, where final processing of the envelope glycoproteins occurs. The particles are then thought to exit the cell via the post-Golgi network and to be excreted into the medium by reverse pinocytosis. The second and more recent model holds that the HSV-1 particle, enveloped at the inner nuclear membrane, is de-enveloped at the outer nuclear membrane and gains its final envelope by budding into a cytoplasmic vacuole from which it is released at the cell surface by reverse pinocytosis. These two models have implications with regard to the site of particle tegumentation. If the first model were correct then all tegument proteins must be acquired in the nucleus, before the particle is enveloped at the inner nuclear membrane. If the second model is correct then tegumentation might be a multi-step process or even occur exclusively in the cytoplasm.

Evidence is building in support of the second model (Skepper *et al.*, 2001). It is possible that HSV-1 acquires a particular subset of tegument proteins that are predominately located in the nucleus (e.g. the UL25 gene product). On leaving the nucleus this partially tegumented particle acquires a primary envelope from the inner nuclear membrane. However, investigation of the lipid content of extracellular virions reveals that the final envelope is acquired from a Golgi-related source and so the envelope acquired at the inner nuclear membrane must be lost (Van Genderen *et al.*, 1994). Additional envelope glycoproteins, gE/I, which are known to facilitate cell-to-cell spread of infection, accumulate in the *trans*-Golgi network (TGN) early in HSV-1 infection prior to re-distribution to cell junctions

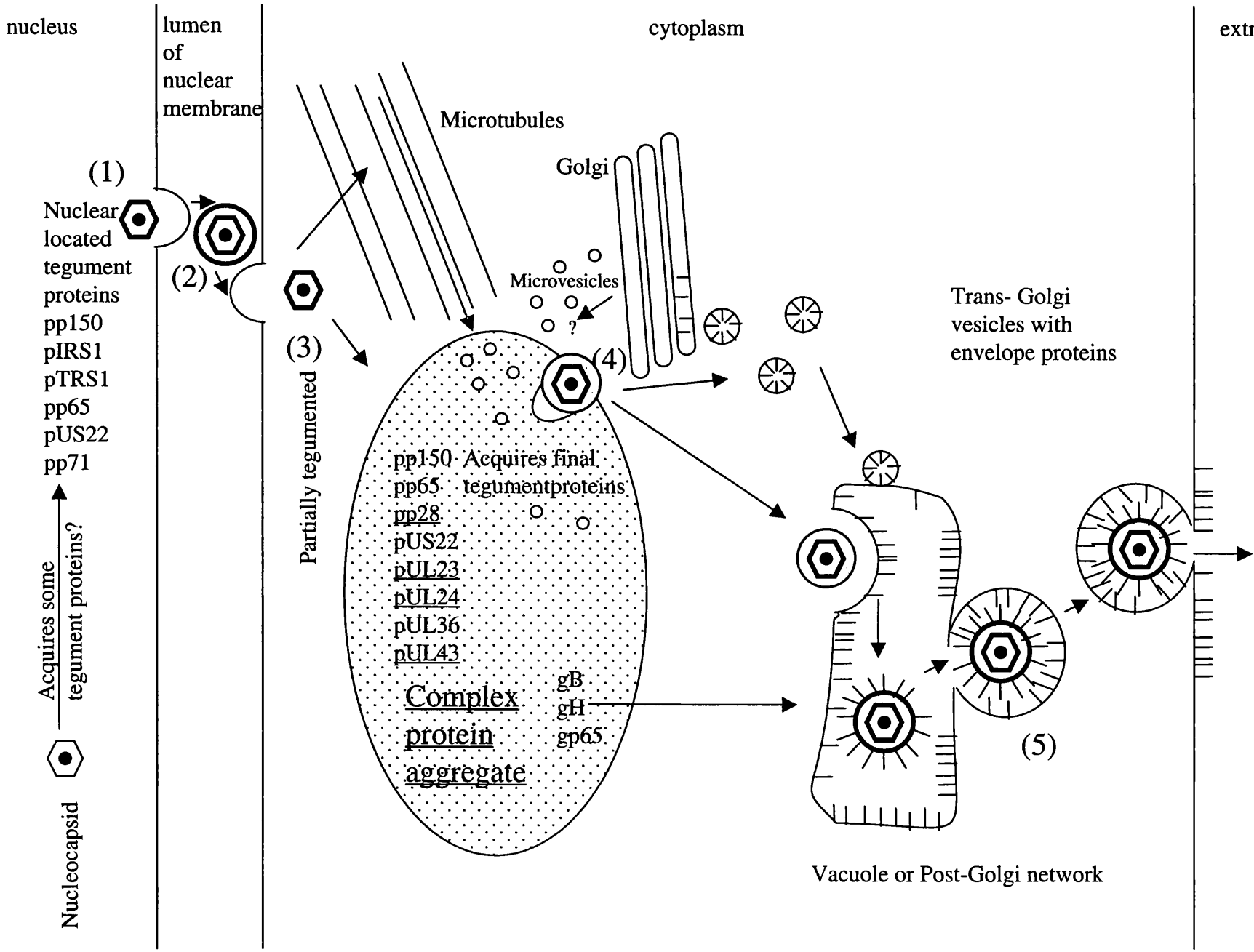
(McMillan and Johnson, 2001). Further support for final envelopment of particles in the cytoplasm has been obtained from Pseudorabies virus (an *Alphaherpesvirus)*, since simultaneous deletion of gM and gE/I leads to formation of numerous large intracytoplasmic inclusions, containing tegumented but non-enveloped capsids (Brack *et al.*, 2000). Thus, the primary inner nuclear membrane is lost when the HSV-1 particle buds from the outer nuclear membrane. The de-enveloped partially tegumented particle then acquires an additional set of tegument proteins that are present only in the cytoplasm of infected cells. The precise location/s at which this second tegumentation step occurs has not been identified but probably involves vacuoles derived from the post-Golgi network. Since HSV-1 acquires its final envelope by budding into such a vacuole it is likely that the vacuole is coated with tegument proteins. The fully mature tegumented and enveloped particle is then transported to the plasma membrane and released into the extracellular medium when the vacuole reaches the cell surface and the vacuole and plasma membranes fuse (reverse pinocytosis).

That the HSV-1 cytoplasmic envelopment model also applies to *Betaherpesviruses* was suggested by studies with HHV-6. The HHV-6 tegument is electron-dense and readily visualized in the EM. HHV-6 capsids present in the lumen of the nuclear membrane have no discernible attached tegument structure, but those present in the cytoplasm are evidently tegumented (Roffman *et al.*, 1990). More conclusive, however, is the data presented here for HCMV pUL23, pUL24 and pUL43. While pUL23, pUL24 and pUL43 are tegument proteins they were not detected in the infected cell nucleus or in any other cytoplasmic structure other than the cytoplasmic protein aggregates described. These tegument proteins must therefore have been acquired by particles maturing in the cytoplasm at the protein aggregates. Others have reported similar findings for pp28 (UL99) and pp150 (UL32) (Sanchez *et al.*, 2000a; Hensel *et al.*, 1995). Severi *et al*, (1992), reported that electron dense structures in the cytoplasm of HCMV infected cells contain DNA, RNA and viral proteins. They called these structures 'cytoplasmic dense bodies', but they appear morphologically similar to the protein aggregates described here. They suggested that cytoplasmic dense bodies represent sites where surplus cellular and viral molecules are stored prior to elimination (aggresomes). The data reported here dispute this interpretation for the complex-type aggregate for the reasons stated above. Moreover, Sanchez *et al*, (2000b), investigated the juxtanuclear structures and showed that

they were stable, did not reorganise cellular vimentin and were not resistant to nocadazole, indicating that they are probably not aggresomes.

In support of the cytoplasmic envelopment model for HCMV, we have observed that in infected HFFF-2 cells (at times > 72 h PI), tegumented, but non-enveloped particles were frequently associated with the large complex protein aggregates. Although the complex aggregates appeared to lack an enclosing membrane, reduplicated membranes and enveloped virus particles embedded within the aggregate were occasionally observed. Spherical structures with the concentric double ring appearance of micro-vesicles (Dalton, 1975) were embedded within the matrix of large juxtanuclear protein aggregates and these may be derived from elements of the post-Golgi network. Interestingly, cryo-electron microscopy has revealed that spherical membrane structures, albeit larger at ~75 nm, were located at the centre of most HSV-1 L particles (Szilágyi and Berriman, 1994); a non-infectious virus-related structure that is analogous to HCMV dense bodies. The small, membrane bound protein aggregates (dense bodies), observed in HCMV infected cells appeared to be generated by membrane envelopment of a portion of the large juxtanuclear aggregates.

Figure 21. Model for tegumentation of *beta*-herpesvirus HCMV. Nucleocapsids (thin lined hexagon) acquire some tegument proteins (thick lined hexagon) (1) and are enveloped (dark circle) at the inner nuclear membrane (2) and de-enveloped by budding through the outer nuclear membrane into the cytoplasm (3). Partially tegumented nucleocapsids are transported to complex cytoplasmic aggregates either by free cytoplasmic streaming or by transport along microtubules. Further tegument proteins (light grey) are acquired at the complex aggregate (4) and final envelopment may occur in the vicinity of the protein agrregates in a compartment derived from the trans-Golgi network or in vacuoles (5). Mature virions exit the cell by reverse pinocytosis. Tegument proteins only found within the complex cytoplasmic protein aggregates are underlined.


nucleus
lumen of nuclear membrane
cytoplasm
extracellular
Microtubules
Golgi
(1)
Nuclear located tegument proteins
pp150
pIRS1
pTRS1
pp65
pUS22
pp71
(2)
(3)
Partially tegumented
Microvesicles
?
(4)
pp150
pp65
pp28
pUS22
pUL23
pUL24
pUL36
pUL43
Complex protein aggregate
Acquires final tegumentproteins
gB
gH
gp65
Trans- Golgi vesicles with envelope proteins
(5)
Vacuole or Post-Golgi network
Acquires some tegument proteins?
Nucleocapsid

A schematic representation of a proposed HCMV maturation pathway is presented in Figure 21. Particles in the nucleus do not appear to possess an electron-dense coat of tegument proteins. However, it is possible that HCMV nucleocapsids may acquire a restricted subset of tegument proteins, including pp65 (pUL83), pp71 (pUL82) pUS22 and pp150 (pUL32), which are present in the nucleus (Figure 21, step 1). The SCMV homologue of pp71 is thought to directly bind to the major capsid protein (Trus *et al.*, 1999) and it may be that the HCMV protein behaves similarly. Likewise, pp150 is tightly bound to the HCMV capsid, by an interaction of a conserved sequence in the amino one-third of the pp150 protein with the major capsid protein (Baxter and Gibson, 2001). Since pp71 and pp150 are probably directly bound to the capsid shell they must be among the first tegument proteins acquired. The partially tegumented HCMV particle then enters the cytoplasm after envelopment and de-envelopment at the inner and outer nuclear membranes as described for HSV-1 (Figure 21, step 2 and 3). The non-enveloped particle is then translocated to the vicinity of the large complex protein aggregate either by transport of the particle along microtubules to the microtubule organising centre or by cytoplasmic flow. If the microtubule network is used, clearly either a capsid protein or a nuclear located tegument protein must be involved in binding to motor proteins on the microtubule. It is likely that nuclear acquired tegument proteins form a framework for attachment of additional tegument proteins, which are acquired later in the cytoplasm. Non-enveloped particles then acquire an electron-dense, EM visible, tegument structure from the peripheral surface of the complex aggregates (Figure 21, step 4). The tegumented particles then acquire an envelope from cell membranes originating from the Golgi apparatus, possibly by budding into a vacuole. The virus is then released from the cell by reverse pinocytosis (Figure 21, step 5). An association of the post-Golgi network and the fluorescent juxtanuclear structure has been reported (Sanchez *et al.*, 2000b) and occasionally enveloped virus particles and reduplicated membrane embedded in the complex type aggregates were seen. Dense bodies may also be generated from the complex aggregate by envelopment of the matrix material. It is also possible that virus particles may acquire an envelope by a similar mechanism.

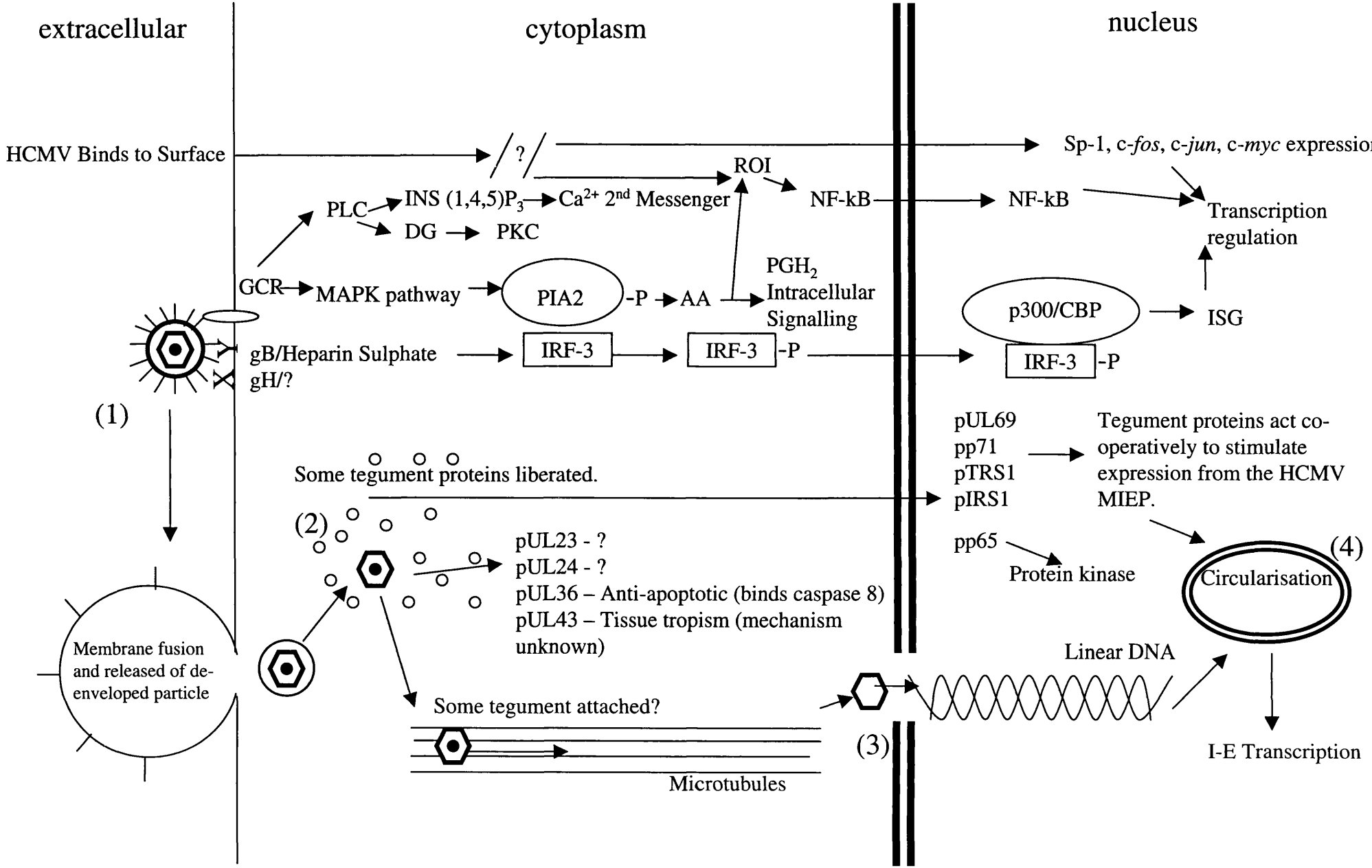
6.4 Pre-Immediate-Early functions provided by tegument proteins

The finding that pUS22, pUL23, pUL24 and pUL43 are tegument proteins suggests that they operate during the initial stages of virus infection. In order to understand the possible range of functions performed by HCMV tegument proteins it is valuable to consider the events that occur during the period between virus binding to its cellular receptors and initiation of immediate-early gene expression. Much of this has been determined using the *Alphaherpesvirus* HSV-1. HSV-1 virions bind to the receptors on the cell surface (including heparan sulphate and chondroitan sulfate proteoglycans) through specific interactions with viral envelope glycoproteins (primarily gB and gC) and interaction of gD with one of several cellular co-receptors (Whitbeck *et al.*, 2001). Herpesvirus entry mediator (HVEM or HveA), a member of the tumor necrosis family, has been identified as the cell surface protein mediating HSV-1 penetration into human T cells, although other cellular receptors, such as HveB (Nectin-2), HveC (Nectin-1), 3-*O*-sulfotransferase-3-modified heparan sulphate, may be needed for entry into other cell types (Steven and Spear, 1997; Martinez and Spear, 2001). Following binding of gD, the cytoplasmic domain of HveA interacts with a subset of adapter proteins in the TRAF family, which in turn leads to activation of two pathways: NF-kB and JNK/AP-1 (Whitbeck *et al.*, 2001). The contact initiates fusion of the viral envelope with the plasma membrane mediated by viral membrane glycoproteins gD, gB and gH/gL. After membrane fusion, the de-enveloped virus enters the cytoplasm leaving the envelope integrated into the host cell plasma membrane. Some tegument proteins may be shed from the virus particle immediately after entry while others appear to remain associated with the capsid. The viral nucleocapsid becomes attached to the microtubule network and is transported to a nuclear pore, where the capsid shell is opened to release the DNA genome, which then enters the nucleus (Batterson *et al.*, 1983). Certain tegument proteins, such as VP16 (UL48), activate transcription of the viral genome and so enter the nucleus, while others, like virion host shutoff factor (Vhs) (UL41) are retained and operate in the cytoplasm. Upon entry into the nucleus the linear DNA molecule circularises. Interaction of VP16 with host transcription

components Octamer-binding protein (Oct-I) and host cell factor (Hcf) results in immediate-early gene transcription and initiation of the virus replication cycle (Flint *et al.*, 2000).

Although the individual steps occurring between binding of HCMV particles to their cell receptor and expression of I-E genes have been less well characterised, it can be assumed that HCMV follows the main features determined for HSV-1.

Figure 22. Model for initiation of lytic infection of *beta*-herpesvirus HCMV. HCMV binds surface heparin sulphate and co-receptors (1). Glycoprotein complexes are involved in fusion of the viral envelope with the cell membrane. Some tegument proteins are liberated upon de-envelopment and entry of the particle into the cytoplasm (2). The partially tegumented nucleocapsid is assumed to be transported along microtubules to dock at the nuclear pore (3). Some tegument proteins are transported to the cell nucleus and act co-operatively to stimulate HCMV I-E gene expression (4). Binding of HCMV initiates several cell signalling pathways. IRF-3, interferon response factor; CBP, Creb binding protein; ISG, interferon-stimulated genes; GCR, G-coupled receptor; MAPK, mitogen activated protein kinase; PIA2, phospholipase A2; AA, arachidonic acid; PGH_2, Prostaglandin; ROI, reactive oxygen intermediates; NF-kB, nuclear factor-kB; PLC, phospholipase C; $INS(1,4,5)P_3$, inositol(1,4,5)-triphosphate; DG, diacylglycerol; PKC, protein kinase C; Sp1, stimulatory protein 1


extracellular
cytoplasm
nucleus
HCMV Binds to Surface
?
Sp-1, c-fos, c-jun, c-myc expression
ROI
PLC
INS (1,4,5)P3
Ca2+ 2nd Messenger
NF-kB
NF-kB
Transcription regulation
DG
PKC
GCR
MAPK pathway
PIA2
-P
AA
PGH2
Intracellular Signalling
p300/CBP
ISG
gB/Heparin Sulphate
gH/?
IRF-3
IRF-3 -P
IRF-3 -P
(1)
pUL69
pp71
pTRS1
pIRS1
Tegument proteins act co-operatively to stimulate expression from the HCMV MIEP.
Some tegument proteins liberated.
(2)
pUL23 - ?
pUL24 - ?
pUL36 – Anti-apoptotic (binds caspase 8)
pUL43 – Tissue tropism (mechanism unknown)
pp65
Protein kinase
(4)
Circularisation
Membrane fusion and released of de-enveloped particle
Linear DNA
Some tegument attached?
(3)
I-E Transcription
Microtubules

A schematic representation of the recognised steps involved in the initiation of HCMV infection of cells in shown (Figure 22). The mechanism by which HCMV enters host cells has not been completely elucidated, but like HSV-1, it is a complex process involving viral/glycoprotein-host cell receptor interactions. HCMV initially binds heparan sulphate proteoglycans expressed on the cell surface, but this is not sufficient of itself to permit virus adsorption, co-receptor proteins are required, as with HSV-1. Possible receptors include aminopeptidase N (CD13) or the 34 kDa protein annexin II (Pietropaolo and Compton, 1997) (Figure 22. step 1). Annexin II specifically binds HCMV virions, is present on the envelope of purified virions and has been shown to directly interact with HCMV gB (UL55). The mechanism of membrane fusion and liberation of the nucleocapsid into the cytoplasm is likely to be analogous to HSV-1. A glycoprotein complex, gcIII, containing HCMV gH, gL and gO (UL75, UL115 and UL74) is involved in fusion of the viral envelope with the cell membrane. Another glycoprotein complex, gcII, containing HCMV gM (UL100) and gN (UL73), appears to bind heparin and may be involved in cell entry and/or cell/cell spread of infection (Mach *et al.*, 2000). Some HCMV tegument proteins may be liberated upon de-envelopment and entry of the particle into the cytoplasm (Figure 22, step 2). Some of the tegument proteins (e.g. pp65, pp71, pUL69, pTRS1, pIRS1) are transported to the nucleus of the infected cell, where some exhibit *trans*-activating activity. Not all tegument proteins appear to be imported into the nucleus of the cell. pUL36 may act at this stage to inhibit apoptosis of the newly infected cell (Skaletskaya *et al.*, 2001). By analogy with HSV-1, it is assumed that the HCMV nucleocapsid is transported along microtubules (Figure 22, step 3) to dock at the nuclear pore, where the viral DNA is released into the nucleus (Figure 22, step 4).

The functions provided by most HCMV tegument proteins are unknown, however, some tegument proteins have protein kinase activity (e.g. pp65) or may activate cell or other tegument proteins by phosphorylation. Some tegument proteins might function to modulate the intracellular signalling events induced by virus particle binding to cell surface receptors, or to induce additional cell signalling pathways. Others may function directly or indirectly as *trans*-activators of certain cellular (e.g. transcription factors) or viral (I-E) genes. Some tegument proteins may operate to abrogate cellular anti-viral defence mechanisms (e.g.

pUL36), and others to participate in translocation of the infecting virus particle or in release of the infecting genome from the capsid.

The HCMV tegument component contains transcriptional *trans*-activators including pTRS1, pIRS1, pUL69, pUL36 and pUL82 (pp71), which promote immediate-early gene expression and the initiation of lytic cycle replication (Romanowski *et al.*, 1997; Winkler *et al.*, 1994; Patterson and Shenk, 1999; Baldick *et al.*, 1997). Some of the HCMV US22 gene family products are reported to exhibit *trans*-activating activity in transient transfection assays when expressed with the major I-E products IE1 and IE2 (Colberg-Poley *et al*, 1992; Romanowski and Shenk, 1997). Several more HHV-6 US22 gene family members have been shown to *trans*-activate the HIV LTR promoter in transient transfection assays, suggesting that at least some members of the US22 gene family may have a role in regulation of gene expression (Nicholas and Martin, 1994; Geng *et al.*, 1992).

In addition to pUL23, pUL24, pUL43 and pUS22, three other HCMV US22 family gene products (pUL36, pTRS1 and pIRS1) are documented virion tegument components (Patterson and Shenk, 1999; Romanowski *et al*, 1997). Thus seven of the twelve US22 family genes code for tegument proteins, raising the expectation that the remaining five, as yet unstudied, US22 family genes will encode products that are similarly located. pUL24, pUL36, pUL43 and pIRS1 are non-essential and cannot be required for virus particle architecture or stability (Dr. Peter Ghazal, personal communication; Patterson and Shenk, 1999; Dargan *et al.*, 1997; Jones and Muzithras, 1992;). Rather the presence of the US22 family proteins in the virus tegument argues for a role during the initial stages of infection. Curiously, pIRS1, pTRS1 and UL36 are delivered as particle components, but also expressed as immediate-early proteins, which argues that the proteins may be multi-functional and/or required at different stages of infection. For example, pTRS1 cooperates with pUL69 tegument protein to *trans*-activate the HCMV I-E promoters at the initial stages of infection (Romanowski *et al.*, 1997) but is also required in combination with IE1 and IE2 to *trans*-activate the L gene product ICP36 (UL44) (Stasiak and Mocarski, 1992).

The functions supplied by pUL23, pUL24, pUL43 and pUS22 tegument proteins are not known, but clearly the processes that occur in the period between virus binding and virus

gene expression are very complex and likely affect the ability of the virus to initiate lytic cycle replication. Given the wide range of host cells infected by HCMV there is ample scope for involvement of US22 family tegument proteins in influencing cell tropism. Infection of cells with HCMV results in activation of a range of cellular signalling events (Figure 22), including components of the MAPK, interferon reponse and phopsholipase C (PLC) pathways. It is possible that US22 family tegument proteins play a role in modulating these induced cell responses or in initiating or inhibiting other, as yet, unidentified cell responses to promote virus replication.

Surprisingly pUL23, pUL24 and pUL43 were not detected in the infected cell nuclei, although the possibility cannot be ruled out that the levels were simply below those that were detectable by the techniques employed. Alternatively, pUL23, pUL24 and pUL43 may only enter the nucleus when delivered to cells at the time of infection and not at the late times investigated here. Clearly, failure to detect pUL23, pUL24 and pUL43 in the nucleus argues against a direct involvement in gene regulation. However, at least one other HCMV *trans*-activator, gpUL37, is confined to the cytoplasm (Al Barazi and Colberg-Poley, 1996; Zhang *et al.*, 1996). Several mechanisms can be invoked to account for an indirect effect of cytoplasmic located US22 gene family tegument proteins on host or viral gene regulation. They might interact directly with host cell or viral proteins in the cytoplasm that are themselves directly or indirectly involved in gene regulation, particularly of the MIEP, or that operate as part of an intracellular signaling pathway, or they might influence transcript stability or translation. Alternatively, US22 family proteins might operate by binding to, or otherwise inhibiting, the function of cellular proteins that are involved in pathways leading to a cellular antiviral defense mechanism. The reported functions provided by the few HCMV US22 tegument proteins that have been studied to date are in keeping with a role during the initial stages of infection.

Recently, a MCMV virus mutant with a transposon insertion in the M43 (HCMV UL43 homologue) coding sequence has been reported. The mutant grows as well as wild type virus in all mice tissues studied, with the exception of the salivary gland where the virus yield was 100 to 1000 fold lower (Xiao *et al.*, 2000). In contrast, Brown *et al*, (1995), have reported that

AD169 (UK) (identical to the ΔUL42/UL43 deletion mutant (Dargan *et al.*, 1997)) replicated to a level 100 to 1000 fold greater than the AD169; ATCC stock (wild type; coding for UL43) in human thymus/liver implants in SCID mice. Taken together, these data support the view that the HCMV UL43 gene and its MCMV homologue are involved in viral tropism (Xiao *et al.*, 2000). Thus pUL43, delivered to cells at the time of infection, must have a role in the control processes that result in lytic infection, at least in some cell types.

pUL36 has also recently been reported to play a role in initiation of productive infection. The pUL36 gene product functions to inhibit apoptosis in infected cells (Skaletskaya *et al.*, 2001). The UL36 viral inhibitor of caspase-8 activation (vICA) inhibits Fas-mediated apoptosis by binding the pro-domain of caspase-8 and preventing its activation. vICA does not share sequence homology with FLICE-inhibitory proteins (FLIPs) or other known suppressors of apoptosis and therefore UL36 appears to encode a new class of cell-death suppressor. Since UL36 is dispensable for virus growth in cell culture and laboratory strains can acquire inactivating mutations in the UL36 gene, it has been suggested that the there is little selective pressure to maintain vICA function. This may be because there is redundancy in the anti-apoptotic functions provided by vICA and a viral mitochondria-localized inhibitor of apoptosis (vMIA) encoded by UL37, which has been shown to be highly conserved in clinical isolates and laboratory strains (Hayajneh *et al.*, 2001a; Hayajneh *et al.*, 2001b). The broad host range of cell types infected by HCMV *in vivo* may account for why HCMV encodes at least two anti-apoptotic proteins and so the UL36 gene may also function as a determinant of cell tropism.

Transient transfection assays have been used to investigate the function of HCMV US22 gene products pIRS1 and pTRS1, and have shown that they promote little transcriptional activation when present alone, but act synergistically with IE1 and IE2 to enhance expression from a variety of viral promoters (Romanowski and Shenk, 1997). One of the two proteins is required for transient complementation of *ori*Lyt-dependent viral DNA synthesis (Pari and Anders, 1993). Thus, the function of the tegument pIRS1 and pTRS1 proteins might be to facilitate I-E gene expression at very early stages when the IE1 and IE2 gene products are just beginning to be made.

The function of pUS22 is not known. Curiously, this secreted protein is highly immunogenic and stimulates a good IgG response. Indeed, anti-US22 IgG immunoglobulins are detected in ~40 % of CMV positive sera, even in patients with relatively low levels of anti-CMV IgGs (Dal Monte *et al.*, 1998). Since it is a secreted protein of high immunogenicity it can be speculated that it has a role as an immune decoy, allowing infected cells to avoid immune clearance by cytotoxic T lymphocytes and natural killer cells. Alternatively, it might bind a receptor/s on non-infected cells and initiate an intracellular signaling response that predisposes the cell to infection by HCMV and thus facilitates spread of infection in the infected individual.

It is possible that US22 gene family proteins might be multi-functional, providing different functions at different stages of the virus replication cycle, as implied by the different kinetics of gene expression among family members. Indeed, pUL36 has motifs that are homologous with the HSV-1 *trans*-activating protein VP16, and so may function both as a *trans*-activator and as an inhibitor of apoptosis. The disparate range of functions either reported for TRS1/IRS1, UL36 or suggested for UL43 lends some support to a recently proposed suggestion, based on computer (*in silico*) comparisons of US22 protein sequences with a data base of protein three-dimensional structures, that US22 family genes do not necessarily encode proteins of similar function (Novotny *et al.*, 2001). Nevertheless, conservation of US22 family amino acid motifs and gene homologues for most HCMV US22 genes in other sequenced *Betaherpesviruses* provides strong evidence that at least some functional element of US22 family proteins are maintained and are presumably important for protein function (Table 10).

Table 10. Spacing between US22 family motifs I and II

HCMV US22 Gene	HCMV DNA sequence with US22 motif I and II underlined.	Motif Spacing in HCMV	Motif Spacing in other *Beta Herpesviruses*
UL23	GQRVALVWPKDRCLVIRRRWRLVRDEGRDAQRLASYLCCPEPLRFVG	26	CCMV UL23 = 26 HHV-6 U2 = 26 HHV-7 U2 = 26 *Tupaia* T23 = 28
UL24	GQVLPVVWPPGWNLVLQEIETDEDFKPEDVKAWSHYLCCQTRLAFVG	26	CCMV UL24 = 26 HHV-6 U3 = 26 MCMV M24 = 26 RatCMV R24 = 26
UL28	GRWLPLCWP.... No Motif II (possible spliced gene)	N/A	
UL29	GSCVSLGWP.... No Motif II (possible spliced gene)	N/A	
UL36	GTRLHVAWPERCFIQLRSRSALGPFVGKMGTVCSQGAYVCCQEYLHPFG Spliced Gene	28	CCMV UL36 = 28 HHV-7 U17 = 29 MCMV M36 = 28 RatCMV R36 = 28
UL43	GTVLRLSWPNGWFFTYCDLLRVGYFGHLNIKGLEKTFLCCDKFLLPVG	27	CCMV UL43 = 27 HHV-6 U25 = 27 HHV-7 U25 = 27 RatCMV R43 = 27
US22	VALRNPANWFLVMREQAAIPQIYARSLAADYLCCDDTLEAVG	21	CCMV US22 = 21 *Tupaia* T5 = 21
US23	LSLGIPHNWFLQVRPGSTMPELRDQLLDDVICCPERLIVLG	20	CCMV US23 = 20 *Tupaia* T2 = 23
US24	LSLGPPKGWHVMLRTEDGIITAAKQAASKLICCREPLTPLG	20	CCMV US24 = 20 *Tupaia* T3 = 19
US26	LPISAPPGWRLDFVEFEDIFGSAAVTDGPETPEGQLICCEESLESLG	26	CCMV US26 = 26
TRS1	No Motif I or II	N/A	
IRS1	No Motif I or II	N/A	

The HCMV US22 motifs I and II are underlined with conserved amino acid residues in bold. The right hand column gives the spacing -in amino acids - between the motifs. Spacing of Motifs I and II is provided for other sequenced *Betaherpesvirus* genes.

Scrutiny of US22 family gene sequences reveals that the spacing between motif I and motif II is well conserved (20-28 aa) among the HCMV members of the family (Table 10). More importantly, the spacing of motif I and motif II is identical for HCMV and CCMV homologues and well conserved in the HHV-6, HHV-7, MCMV, RatCMV and *Tupaia* (Tree Shrew) US22 gene homologues. Conservation of spacing between the US22 family motifs I and II argues for at least local conservation in protein folding, to generate an important

functional element common to most US22 family members. The fold in the protein induced by the motifs might be involved in protein/protein interactions with a cellular protein or with different members of a conserved family of cell proteins. Alternatively, though less likely, the fold might be involved in US22 family protein interactions with DNA or RNA.

Since most, if not all, US22 family genes encode tegument proteins it may be that one or more of the US22 family amino acid motifs are required to target the protein to protein aggregates or for integration of the protein into the complex tegument structure.

The data present in this thesis have demonstrated for the first time a common biological feature of US22 family proteins that has implications for the role performed by the family *per se* and focuses future research on early events in the virus replication cycle.

6.5 pUL23 and pUL24 may be cytotoxic proteins

In setting out to study the functions provided by genes UL23 and UL24 it was considered that pUL23 and pUL24 might be essential for virus growth *in vitro*, and that complementing cell lines would be required in order to isolate virus knockout mutants. The short life-span of human foetal fibroblasts, and their poor transfection efficiency, has until recently hindered the production of complementing cell lines for HCMV. Two systems for the generation of complementing cell lines were investigated.

The first depended on immortalization of HFF cells due to expression of the HPV type 16 E6 and E7 transforming genes. E6 and E7 immortalized HFF cells have been shown to be permissive for HCMV infection (Compton, 1993) and have served as the basis for an HCMV IE1 expressing cell line that was capable of complementing an IE1 null mutant virus (Greaves and Mocarski, 1998). E6 functions by binding to, and causing unbiquitination and degradation, of cellular p53, which is a nuclear transcriptional regulator that normally induces

G_1 arrest or apoptosis in response to DNA damage or cellular stress (Flint *et al.*, 2000). E7 exhibits transforming activity by binding to cellular retinoblastoma tumour suppressor protein, Rb, preventing its ability to bind substrates such as E2F. The loss of the tumour suppressors p53 and Rb imparted by binding to E6 and E7, respectively, results in immortalisation of the cell.

The second system depends on the immortalisation of human fibroblast cell lines (HFF-R2, MRC-5, HCA2) by expression of human telomerase reverse transcriptase (hTERT) (McSharry *et al.*, 2001; Bresnahan *et al.*, 2000). hTERT fibroblasts have been shown to be fully permissive for HCMV. hTERT operates to inhibit senescence by preventing the shortening of chromosomal telomeres.

Retinal pigmented epithelial (RPE) cells and HFF cells with an extended life-span due to expression of hTERT and HPV type 16 E6/E7 transforming genes, respectively, were employed in an attempt to construct cell lines expressing pUL23, pUL24 and pUL43. hTERT-RPE cells were transfected with the eukaryotic expression vector pcDNA3.1+ carrying the UL23, UL24 or UL43 genes and grown in the presence of G418. However, no pUL23 or pUL24 expressing cell lines were obtained. In contrast, hTERT-RPE cells constitutively and stably expressing pUL43 were obtained, indicating that the hTERT RPE system was capable of generating cell lines expressing foreign genes. Failure to generate hTERT-RPE cell lines expressing pUL23 or pUL24 was not due to failure to transfect the cultures since G418 resistant colonies were obtained. It was considered that either the pUL23 and pUL24 gene products were expressed at levels that were not detectable by Western immunoblotting or that the genes were not expressed at all. Attempts to increase expression of UL23 and UL24 drug resistant colonies by growth for in the presence of compounds known to alter the transcription pattern in cells (Phorbol 12-myristate 13-acetate (PMA), or PMA in combination with Forskolin, or Forskolin alone, or 5 HMBA alone or sodium butyrate alone) were all unsuccessful. Failure to detect protein may be due to impairment of transcription or translation. Northern blot investigations failed to detect UL23 or UL24 transcripts from total RNA extracts of hTERT-RPE UL23 or UL24 G418 resistant cell lines. Whether the transcripts were not made or whether they were unstable has not been

investigated. It is possible that the UL23 or UL24 gene products were cytotoxic and so the genes were silenced in the surviving drug resistant cell lines. A trivial and less likely explanation might be that the UL23 or UL24 ORFs were deleted or disrupted as a consequence of transfection of the plasmid into cells.

Similar attempts were also made to produce a complementing cell line for pUL23 and pUL24 in human foreskin fibroblast cells (hTERT-HFF BJ), again without success. G418 resistant colonies were obtained as described above for the hTERT-RPE cell line, indicating that this part of the plasmid construct at least was expressed. Attempts were also made to generate UL23 and UL24 complementing cell lines in HFFF-2 cells using the pLXSN16E6E7 amphotropic retrovirus vector system, but again without success, although again G418 resistant cell lines were obtained. It was noted that the G418 resistant colonies of hTERT-HFF UL23 or UL24 cells lines and the pLXSN16E6E7 UL23 and UL24 cell lines all failed to propagate beyond a few hundred cells. Intriguingly, the colonies remained viable for at least 4 months in the continous presence of G418, suggesting a possible effect of pUL23 and pUL24 on cell cycle regulation.

Protein toxicity was cited as the most likely reason for the inability to produce cell lines expressing the HHV-6 U3 (pUL24 homologue) gene product (Mori *et al.*, 1998). In an attempt to investigate the presumed toxic effects of pUL23 and pUL24, the ORFs were cloned into the Voyager vector system to produce VP22-UL23 and VP22-UL24 fusion proteins, which can be directly imported (translocated) into cells from the culture medium via the VP22 moiety. Immunofluorescence experiments confirmed the presence of the VP22-UL23 and VP22-UL24 fusion proteins in the nuclei of translocated HFFF-2 cells. No toxic effects of the fusion proteins were discernable. However, it is possible that the VP22 fusion proteins do not have the same characteristics as the native UL23 and UL24 proteins.

6.6 Site directed mutagenesis of genes UL23 and UL24

In order to investigate the functions provided by genes UL23 and UL24 attempts were made to produce virus mutants with knockout mutations in either gene. The strategy was to engineer a precise deletion in the UL23 or UL24 ORFs and clone in the E. *coli* gpt gene, to serve as a selectable marker gene. After four rounds of replication to enrich for gpt containing viruses and four rounds of plaque picking in the presence of MPA and xanthine all of the plaque purified viruses yielded the wild type virus restriction endonuclease DNA band pattern in Southern blots.

That no recombinants were isolated was unexpected, since care was taken to avoid operator selectivity, by picking all of the plaques on a culture dish irrespective of size. Greaves *et al.*, (1995) have successfully used gpt as a selectable marker in HCMV mutagenesis, but also reported that the wild type virus can breakthrough the MPA/xanthine selection to give infectious virus yield at approximately 1000 fold lower levels than recombinant viruses expressing the gpt gene. In addition to wild type virus breakthrough there are several other possible explanations as to why no recombinants were isolated. Transfection efficiency is notoriously low (<1 %) in the case of HFFF-2 cells, and may have resulted in little or no uptake of mutant viral plasmids. To maximise transfection efficiency, circular plasmid molecules were used for transfections, although this may have significantly reduced the efficiency of recombination between plasmid and viral genome. Choice of HCMV strain used may be another key factor that influenced the inability to isolate a mutant virus. Greaves *et al.*, (1995), used the Towne strain of HCMV, rather than AD169, and since the former replicates faster and to higher titres the efficiency of the Towne system might be greater than that of AD169.

It is possible that UL23 and UL24 are essential for virus growth in culture and in that case recombinants cannot be isolated, in the absence of a complementing cell line. However, this explanation was apparently ruled out for UL24 when a clinical isolate with a naturally occurring deletion of the UL24 ORF was identified, although this has not yet been fully

characterised (Dr. Peter Ghazal, personnal communication). Finally, one trivial explanation might be that an insufficient number of plaques were screened. However, time constraints allowed for only a single attempt at these experiments.

Isolation of HCMV mutants using standard molecular biology techniques is a difficult and time-consuming task, primarily because of poor transfectability of HCMV permissive cell lines and the slow viral replication kinetics. Therefore, relatively few HCMV mutants have been documented to date. Overlapping cosmid fragments have been used to generate mutants in herpesvirus genomes (Cunningham and Davison, 1993; Cohen and Seidel, 1993; Kemble *et al.*, 1996). While this approach eliminates the technical problem of isolating a minor population of recombinants from within a mixed pool of wild type virus, it relies on transfecting several DNA fragments (up to 8 in the case of HCMV) into cells simultaneously and therefore is inefficient. Transposon insertional mutagenesis and bacterial artificial chromosomes (BACs) have been used to generate large banks of random mutants in HCMV and MCMV, and several reports have used these approaches to study MCMV mutants *in vivo* (Zhan *et al.*, 2000a; Zhan *et al.*, 2000b; Xiao *et al.*, 2000). The genomes of several herpesviruses have been cloned as BACs, including MCMV; EBV; PRV; HSV-1; HCMV and GPCMV (Messerle *et al.*, 1997; Delecluse *et al.*, 1998; Smith and Enquist, 2000; Saeki *et al.*, 1998; Borst *et al.*, 1999 and McGregor and Schleiss, 2001). Borst *et al.*, (1999) described the construction and stable passage of an infectious clone of HCMV maintained as a BAC and reported a technique for site-specific mutagenesis, by which they were able to produce a UL37 deletion mutant virus. More recently, the BAC technology was used to create an IE2 knockout mutant (Marchini *et al.*, 2001). The BAC technology represents a major advance in the study of herpesvirus genomes, although complementing cell lines are still required for analysis of essential viral genes. Future attempts to create UL23 and UL24 knockout mutants may be more easily performed using the BAC system.

6.7 Future work on the US22 gene family

This is the first investigation of HCMV genes UL23, UL24 and UL43, which belong to the HCMV US22 gene family and the first time that a common biological feature has been demonstrated among family members. The main finding that pUL23, pUL24, pUL43 and pUS22 (along with other US22 family members UL36, TRS1 and IRS1), are all tegument components has important implications for the overall role of the family *per se.* Seven of the twelve US22 family genes encode tegument proteins, raising the expectation that the products of the remaining five genes will be similarly located. This important finding implicates the US22 family in events that occur during the early stages of infection and this will be the focus of future research on the US22 gene family.

Using yeast two-hybrid systems with cellular and/or viral cDNA library it should be possible to investigate protein/protein interactions between pUL23, pUL24, pUL43 and pUS22 proteins and host cell factors or other viral proteins. The pUL43 expressing cell line might be used in gene array experiments to investigate possible effects of pUL43 on cell gene regulation. It should be possible to construct the UL23, UL24 and UL43 knockout mutants using the HCMV BAC system, though it is possible that the genes will be non-essential for growth in tissue culture and have no recognisable phenotype – at least in HFFF-2 cells.

These studies also contribute to the debate regarding the intracellular site at which tegument proteins are acquired. It will be important to investigate whether the large protein aggregates described here are also present in other cell types infected with HCMV and whether they are also present in cells infected with other *Betaherpesviruses.*

Chapter 7

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