Characterisation of selected members of the Alphaherpesvirus ICP0 family of proteins.

by

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Abstract.

The HSV-1 immediate early protein ICP0 is a well-characterised regulator of gene expression, which enhances expression of all classes of genes throughout the viral genome. ICP0 plays an important role in reactivation of HSV-1 from quiescent infection, and for efficient initiation of the lytic cycle. One way ICP0 may favour the lytic cycle is by altering the intracellular environment by targeting cellular proteins for degradation through its RING finger mediated E3 ubiquitin ligase activity. Amongst the proteins that have been found to be degraded in cells expressing ICP0 are certain components of cellular nuclear sub-structures known as ND10 or PML nuclear bodies. These proteins may be part of a cellular repression mechanism that inhibits viral gene expression and leads to the assembly of viral genomes into a quiescent chromatin structure. Alternatively, disruption of ND10 could release transcription factors that stimulate viral gene expression. All of the aforementioned activities have been shown to be dependent of ICP0 having a functional RING finger domain, a cysteine-rich sequence that is situated near the N terminus of the protein and is structurally coordinated by the presence of two zinc atoms. ICP0 and its isolated RING finger domain have been shown to act as E3 ubiquitin ligases *in vitro* and cause substrate independent polyubiquitin chain formation in the presence of the E2 ubiquitin conjugating enzymes UbcH5a and UbcH6.

The proteins of interest to this study are the homologues of ICP0 that are expressed by other members of the alphaherpesvirus sub-family, including BICP0 of BHV-1, Eg63 of EHV-1, EP0 of PRV and Vg61 of VZV. The homology between these proteins is limited to the RING finger domain and indeed mainly to the zinc coordinating and certain other structurally important residues therein. Previously, it was shown that in a similar manner to that of ICP0, these related proteins to varying degrees cause the disruption of ND10 by affecting certain cellular proteins, and they induce the formation of colocalising conjugated ubiquitin. Together, this evidence suggested that these members of the ICP0 family of proteins also act as E3 ubiquitin ligase enzymes.

Using the techniques of protein expression, purification and *in vitro* ubiquitin conjugation assays, the work described in this thesis demonstrates that the isolated RING finger domains of the ICP0 related proteins, to varying degrees, act as E3 ubiquitin ligase enzymes. In addition, they simulate the same E2 ubiquitin conjugating

enzymes UbcH5a and UbcH6, in a manner similar to that previously shown for ICP0. It was also shown that the isolated RING finger domains of the ICP0 family of proteins could form stable interactions with specific E2 partners *in vitro*. Transfection assays additionally demonstrated that the full-length versions of the ICP0 related proteins formed foci that co-localised with certain E2 proteins *in vivo*. In addition, studies using truncated versions of the RING finger domains of Eg63 and BICP0 identified differences between their activities in partnership with the E2 enzymes UbcH5a and UbcH6 that depended on residues on the C-terminal side of their core RING finger domains. Finally, using a transfection assay, it was shown that even though the homology of the ICP0 family of protein is largely limited to their RING finger domains, to varying degrees they are able to complement ICP0-null mutant HSV-1.

Abbreviations.

A.

A.A. Amino acid.

Ac. *Autographa californica.*

Amp. Ampicillin.

Amp^r. Ampicillin resistance.

ATP. Adenosine triphosphate.

B.

Bac. Baculovirus.

BAC. Bacterial artificial chromosome.

b.p. Base pair.

BICP0. The BHV-1 homologue of ICP0.

BHK. Baby hamster kidney

BHV-1. Bovine herpes virus type 1.

BM. Boiling mix.

BSA. Bovine serum albumin.

C.

C- Carboxy.

°C. Degrees Celsius.

CHV. Canine herpes virus.

Ci. Curie.

CIP. Calf intestinal phosphatase.

Cm. Chloramphenicol.

Cm^r. Chloramphenicol resistance.

CPE. Cytopathic effect.

CsCl. Caesium chloride.

D.

Da. Dalton.

dH_20. Distilled water.

DNA. Deoxyribonucleic acid.

DMSO. Dimethyl sulphoxide.

DTT Dithiothreitol.

E.

E. Early.

EBV. Epstein Barr Virus.

E.coli. Escherichia coli.

EDR. Envelopment, deenvelopment, reenvelopment pathway.

Eg63. The EHV-1 homologue of ICP0.

EHV-1. Equine herpes virus type 1.

EM. Electron Microscope.

ELISA. Enzyme linked immunoabsorbent assay.

EP0. The PRV homologue of ICP0.

EtBr Ethidium bromide.

ER. Endoplasmic Reticulum,

F.

FdA. Fold activation.

FHV. Feline herpes virus.

FITC Fluoroscein thiocynate.

FCS. Foetal calf serum

G.

GAGS. Glycosaminoglycans.

Gen. Gentamycin.

Gen^r. Gentamycin resistance.

GFP. Green fluorescent protein.

GHV. Gallid herpesvirus.

H.

HCMV. Human cytomegalovirus.

6xHis. 6 histidine residues

hr. Hour.

HCF. Host cell factor.
HVB. Herpes B virus.
HHV. Human herpesvirus
HSV. Herpes simplex virus.
Hve. Herpesvirus mediator.

I.

IE. Immediate-Early.
ICP. Infected cell protein
IE. Immediate early
Ig. Immunoglobulin
IRL. Internal repeat long
IRS. Internal repeat short
IPTG Isopropylthio-β-D-galactoside

K.

k. Kilo (10^3)
Kan. Kanamycin.
Kan^r. Kanamycin resistance.
kbp Kilobase pair.
KSHV. Kaposi's Sarcoma-Associated Herpesvirus

L.

L. Late.
l Litre.
LAT. Latency associated transcript.
LB. Luria Broth.

M.

μ Micro (10^{-6}).
μg Microgram.
μl Microlitre.
M Molar.
m Milli (10^{-3}).
mA milliamps.
mAb Monoclonal antibody.
Mdm2. Mouse double mutant 2.
Min. Minute.
M.O.I. Multiplicity of infection.
Min. Minute.
mg. Milligrams.
ml. Millilitres.
mM. Millimolar.
mRNA. Messenger ribonucleic acid.
MW. Molecular weight.

N.

n Nano (10^{-9}).
N- Amino.
NaCl Sodium chloride.
NA. Not applicable.
NP40 nonidet P40.

O.

OBP. Origin binding protein.
OD. Optical density.
o/n Overnight.
ORF. Open reading frame.
Ori. Origin of replication.

P.

PAGE. Polyacrylamide gel electrophoresis.
PBS-T. Phosphate buffered saline supplemented with 0.1% Tween-20.
p.f.u. plaque-forming unit.
PCR. Polymerase chain reaction.
Plq. Plaque

PRV. Pseudorabies virus.

PS. Penicillin/streptomycin.

R.

RGB. Resolving gel buffer.

RNA. Ribonucleic acid.

RnaseA. Ribonuclease A.

r.p.m. Revolutions per minute

RT. Room temperature.

S.

Sec. Second.

^{35}[S] Sulphur-35-radioisotope.

SDS. Sodium dodecyl sulphate.

SF. *Spodoptera frugiperda.*

SGB. Stacking gel buffer.

SV40. Simian virus 40.

T.

TCA. Trichloroacetic acid.

TEMED.N,N,N',N',-tetramethylethylene diamine.

tk. Thymidine kinase.

TNF. Tumour necrosis factor.

Tris. Tris (hydroxymethyl) aminoethane.

ts. Temperature sensitive.

U.

U.V. Ultra-violet light.

Ub. Ubiquitin.

UL. Unique long.

US. Unique short.

UV. Ultra violet.

V.

V. Volts.

v/v. Volume to volume ratio.

v/w. Volume to weight ratio.

VZV. Varicella zoster virus.

Vg61. The VZV encoded ICP0 homologue.

W

WT Wild type.

X.

X-gal. 5-bromo-4-chloro-3-indolyl-β-D-galactosidase.

Y.

YTB. Yeast tryptone broth.

Amino acid abbreviations

Amino acid	Three letter code	One letter code	Properties
Alanine	Ala	A	Non-polar (hydrophobic)
Arginine	Arg	R	Basic
Asparagine	Asn	N	Polar, uncharged
Aspartic acid	Asp	D	Acidic
Cysteine	Cys	C	Polar, uncharged
Glutamine	Gln	Q	Polar, uncharged
Glutamic acid	Glu	E	Acidic
Glycine	Gly	G	Polar, uncharged
Histidine	His	H	Basic
Isoleucine	Ile	I	Non-polar (hydrophobic)
Leucine	Leu	L	Non-polar (hydrophobic)
Lysine	Lys	K	Basic
Methionine	Met	M	Non-polar (hydrophobic)
Phenylalanine	Phe	F	Non-polar (hydrophobic)
Proline	Pro	P	Non-polar (hydrophobic)
Serine	Ser	S	Polar, uncharged
Threonine	Thr	T	Polar, uncharged
Tryptophan	Trp	W	Non-polar (hydrophobic)
Tyrosine	Tyr	Y	Polar, uncharged
Valine	Val	V	Non-polar (hydrophobic)

DNA Abbreviations.

Base	One letter code
Adenine (Purine)	A
Cytosine (Pyrimidine)	C
Guanine (Purine)	G
Thymidine (Pyrimidine)	T
Uracil (Pyrimidine)	U

Chapter 1 – Introduction - Part I – Herpesviridae.

1.0. Herpesviruses.

Herpesviruses are members of the *Herpesviridae*, a large family of viruses containing over 120 known species (Roizman & Sears, 1996), infecting a wide spectrum of vertebrates and a least one invertebrate. Generally, at least one herpesvirus has been described in most animal species including fish, amphibians, reptiles, birds and especially mammals, including cattle, pigs, and man (Roizman, 2003). The occurrence of host specific herpesviruses is an indication they have evolved with their hosts over long periods of time and are exquisitely well adapted to them (Davison, 2002). Herpesviruses differ widely in their pathogenic potential, but share the ability, after primary infection, to remain latent throughout the lifetime of the host. To date eight distinct human herpesviruses have been identified causing various disease states; an overview of these viruses is shown in figure 1.0A.

1.0.1. Classification of the herpesviruses.

Herpesviruses are amongst the largest and most complex of viruses and their classification is primarily based on the four conserved features of the virion (Roizman & Sears, 1996).

i) An electron opaque core that contains a single linear double stranded DNA molecule, typically 120-230 Kbp in length, with a varying G+C content of between 31 and 75%.

ii) An icosahedral capsid that is approximately 125 nm in diameter, which surrounds the nucleic acid.

iii) An amorphous tegument, a matrix that surrounds the capsid.

iv) The envelope, a lipid bilayer containing glycoprotein spikes.

common name	subfamily	G + C (%)	DNA size (Kbp)	common disease / symptoms
HSV-1	α	68.3	152	cold sores + other occasional symptoms
HSV-2	α	69	152	genital lesions
VZV	α	46	125	chicken pox / shingles
EBV	γ	60	172	infectious mononucleosis (glandular fever), Burkitt's lymphoma + other cancers
HCMV	β	57	229	Cytomegalia
HHV-6	β	42	162	exanthum subitum or roseola infantum
HHV-7	β	45	145	none known
KSHV	γ	53	140.5	Kaposi's sarcoma, PEL, MCD

Figure 1.0A. *The human herpesviruses.*

Overview of the eight herpesviruses that have so far been identified to infect humans.

Abbreviations: Herpes Simplex Virus (HSV), Varicella-Zoster Virus (VZV), Epstein-Barr virus (EBV), Human Cytomegalovirus (HCMV), Human Herpesvirus (HHV), Kaposi's Sarcoma-Associated Herpesvirus (KSHV), Primary Effusion Lymphoma (PEL), Multicentric Castleman's Disease (MCD).

Herpesviruses have been classified into three groups based upon tissue tropism, pathogenicity and behaviour under laboratory conditions and all share four significant properties:

i) All herpesviruses specify enzymes and other factors involved in DNA synthesis, e.g. DNA polymerase, helicase, primase, origin binding protein, and DNA metabolism e.g. thymidine kinase, thymidylate synthetase, dUTPase, ribonucleotide reductase. Herpesviruses specify at least one protease and a variable number of protein kinases.

ii) The synthesis of viral DNA and assembly of capsids occurs in the nucleus, with initial envelopment of the capsid happening as it migrates through the nuclear envelope.

iii) The production of infectious progeny virus is generally accompanied by the destruction of the infected cell.

iv) Herpesviruses have the ability to remain latent in their natural hosts.

Various additional criteria, such as antigenic properties, have been used for classifying herpesviruses into lower taxa of subfamily, genus and species. These criteria have been superseded by relationships based on comparisons of viral DNA or amino acid sequences (Davison, 2002). This is discussed in a later section.

1.0.2. Subfamily classification of herpesviruses

The subfamilies of the *herpesviridae* are classified into three groups *Alpha-, Beta-,* and *Gamma-herpesvirinae* (Roizman *et al.*, 1981), the division being based upon the biological properties of the viruses. Historically, subfamily classification was based on biological characteristics, such as host range, duration of reproductive cycle, cytopathology and characteristics of latent infection, these are summarised below:

Alphaherpesvirinae.

Herpesviruses within this subfamily demonstrate a variable host range *in vitro*, a relatively short reproductive cycle (<24 hours), rapid spread within tissue culture that results in the efficient destruction of infected cells, and a capacity to establish a latent state of infection, predominantly within sensory ganglia. The subfamily of *Alphaherpesvirinae* has been further divided into two genera; *Simplexviruses*, which include herpes simplex virus type-1 and type-2 (HSV-1 and HSV-2), and *Varicelloviruses*, which includes varicella-zoster virus (VZV) (Roizman & Sears, 1996).

Betaherpesvirinae.

Herpesviruses within this subfamily demonstrate a restricted host range, a long reproductive cycle and slow growth in tissue culture. Infected cells frequently become enlarged in appearance (cytomegalia) and latency occurs in secretary glands, lymphoreticular cells, kidney and other tissues (Roizman & Sears, 1996). The subfamily of *Betaherpesvirinae* has been further divided into three genera; *Cytomegalovirus*, which includes the human cytomegalovirus (HCMV-1), *Muromegalovirus*, which includes murine cytomegalovirus, and *Roseolovirus*, which includes the human herpesvirus type-6 and type-7 (HHV-6 and HHV-7) (Roizman & Sears, 1996).

Gammaherpesvirinae.

Members of this subfamily typically demonstrate an *in vitro* host range restricted to the species of the natural host. *In vitro,* all members of this subfamily replicate in lymphoblastoid cells and some also cause lytic infection within epithelioid and fibroblastic cells. Within the natural host, viruses tend to be specific for either T or B-lymphocytes. Viruses within this subfamily frequently maintain a latent state of infection within lymphoid tissue. The subfamily of *Gammaherpesvirinae* has been further divided into two genera, *Lymphocryptovirus*, which includes the Epstein-Barr virus (EBV) and *Rhadinovirus*, which includes human herpes virus type-8 (HHV-8).

1.0.3. Sequence-based classification of the herpesviruses.

The traditional approach to classification of herpesviruses was based on the biological properties of the viruses. However, due to the advent of sequencing technology the classification was reanalysed based upon DNA sequence homology and genome organisation (Davison, 2002). Currently, more sequence information is available for herpesviruses than any other large DNA virus family; they are highly diverse, ranging in size from 125 to 230 kbp, with around 70 to 200 genes and they exhibit widely differing base composition and patterns of repeated sequences (McGeoch, 1989; Roizman *et al.*, 1992). Extensive sequencing of the genomes of several mammalian herpesviruses from all three subfamilies indicates they share around 40 genes, by the criteria of their genomic position and amino acid sequence (McGeoch *et al.*, 1995). The available data has helped reassess the subfamily classification, taking into consideration factors such as the arrangement of terminal sequences required for DNA packaging, the presence of nucleotides subject to methylation, conservation and positioning of genes and gene clusters. The classification of members of the herpesvirus family based on sequence data caused only a few alterations to the original classification system (Davison, 2002). In particular, HHV-6, HHV-7 and GHV-1 were originally classified as *Gammaherpesvirinae* as they exhibited lymphotropism. Following sequence analysis HHV-6, HHV-7 were reassigned to the *Betaherpesvirinae* and GHV-1 to *Alphaherpesvirinae* as their genomes more closely resembled other members of their 'new' respective families (Buckmaster *et al.*, 1988; Roizman *et al.*, 1992). Furthermore, comparison of the vast array of sequence data has enabled the identification of a common ancestral origin of the herpesvirus family. It was estimated that the three subfamilies arose approximately 180 to 220 million years ago and the subsequent divisions within these families arose 80 to 60 million years ago (see figure 1.0.3A) (McGeoch et al, 1995).

1.1.0 HSV biology

The herpes simplex viruses consist of two serotypes HSV-1 and HSV-2, and were the first of the human herpesviruses to be discovered. Due to their biological properties and the relative facility with which they can be cultured in the laboratory, they are among the most intensely investigated of the herpesviruses (reviewed by Roizman & Sears,

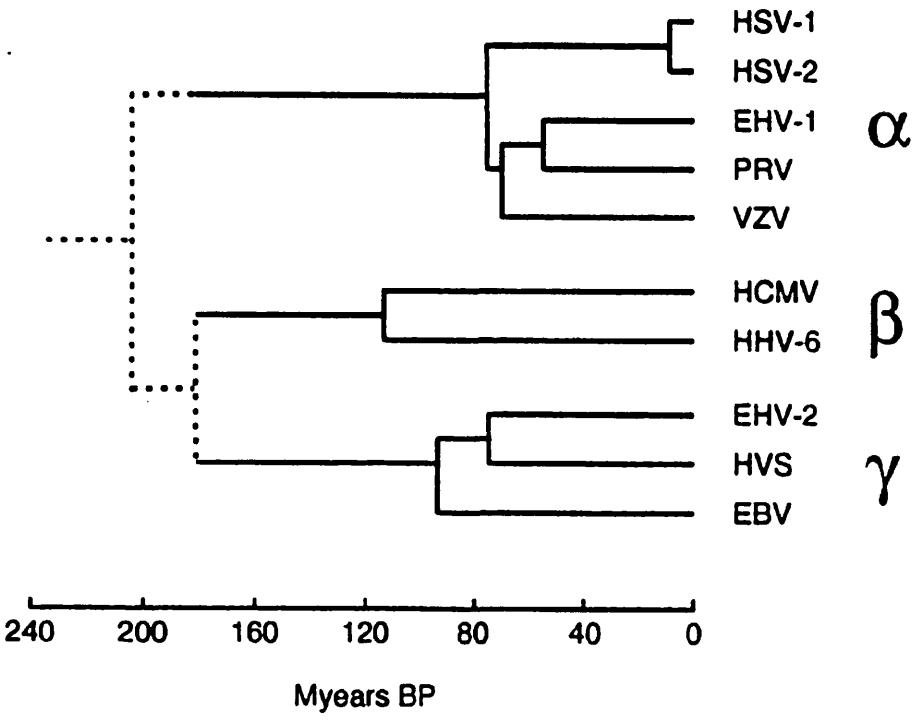


Figure. 1.0.3A. *Evolutionary divergence of the Herpesvirus family.*

Horizontal lines of branches are proportional to their respective time of divergence. Subfamily groups (only key members are shown) are indicated by the presence of their appropriate Greek letter. The oldest parts of the tree are indicated by broken lines to represent the low confidence in the exact time scale of divergence. BP = Before present time. This figure was adapted from McGeoch et al., 1995.

1996). Furthermore, like other members of the herpesvirus family, the herpes simplex viruses are highly evolved and capable of establishing both persistent lytic and latent infection for the lifetime of the host, thus ensuring their ubiquity is maintained amongst the human population. HSV-1 is normally associated with orofacial infections and rare cases of encephalitis, whereas HSV-2 usually causes genital infections. However, both forms of HSV can enter the body at either site of infection owing to the presence of cell surface target receptor heparan sulphate proteoglycans (Whitley & Roizman, 2001).

1.1.1. Ubiquity and transmission in the population.

Although HSV is highly infectious, it is not transmitted casually from person to person. The enveloped virions are relatively unstable in atmospheric conditions and close interpersonal contact is usually required for their transmission. HSV is transmitted easily through body fluids and the direct contact of infected to uninfected mucous membranes. Transmission of the virus is enhanced if the uninfected membrane that is in contact with the infected membrane is damaged. Symptomatic infection by HSV is usually noted by the presence of a lesion, which occurs in conjunction with the period of highest viral shedding (Stanberry, 1986). Obvious measures can be taken to limit the spread of the virus from person to person or from one part of the body to another by simply limiting contact with the symptomatic area. These precautions are not fully effective when the ubiquity of the virus amongst the population is considered. It is likely that an individual may be unaware that asymptomatic shedding is occurring, as they appear to be symptom-free. Patients with recurrent genital disease have been documented to shed HSV-2 as often as 10% of the time that they appear to be lesion-free (Kohl, 1997). In fact, the majority of individuals throughout their life are unaware of encountering the virus or of becoming infected (Connelly & Stanberry, 1995). Symptomatic and asymptomatic shedding ensures the continued spread of HSV-1 and HSV-2, as it exploits its host's need for social contact. Transmission and ubiquity in the population is reviewed in more detail elsewhere (Bernstein, 1991; Stanberry, 2000; Whitley, 2002; Whitley & Roizman, 2001).

1.1.2. Definition and serotypes of the herpes simplex viruses.

The genome of HSV-1 and HSV-2 share 47-50% base sequence homology, and their genetic maps are largely co-linear (Roizman & Sears, 1996). They differ in restriction

endonuclease (RE) cleavage sites and apparent size of viral proteins. Antigenic differences can be detected (Roizman *et al.*, 1984) and distinctive biological markers include virus titres, plaque size and growth in cultured cells. Due to occasional base substitutions/deletions resulting in changes in restriction endonuclease sites or a.a. sequence, and because of the variability of certain repeated sequences, epidemiologically unrelated isolates of the same HSV serotype do not contain identical DNA sequences (Chou & Roizman, 1990).

1.1.3. HSV-1 virion structure.

HSV-1, like other members of the alphaherpesvirus family, has four distinct components that make up its particle: the DNA core, nucleocapsid, tegument, and envelope (Wildy *et al.*, 1960). A generalised structure is shown in figure 1.0.1A, while a more detailed electron micrograph is shown in figure 1.1.3A.

The HSV-1 virion consists of electron-opaque core, containing the viral genome, which is surrounded by an icosahedral capsid and an outer lipid envelope. The icosahedral capsid consists of 162 capsomers, of which 150 are hexameric and 12 are pentameric. The hexamers are located on the faces and edges of the capsid and the pentamers are on the vertices (Booy *et al.*, 1991). More recent studies have used electron cryomicroscopy to produce a highly detailed structure of the HSV-1 capsid at a resolution of 8.5 angstroms, providing a wealth information regarding the secondary folding of the capsid proteins (Zhou *et al.*, 2000).

The amorphous tegument layer contains over 20 distinct structural proteins which are thought to influence the process of infection (Rixon, 1993). The outer surface of the tegument is enclosed by the envelope – a trilaminar lipid membrane of host origin, containing a number of glycoproteins (named gB to gM), which are involved in attachment and penetration of the virus into the host cell (Schrag *et al.*, 1989). The specific roles of the virion proteins will be discussed in more detail later.

1.1.4 The viral genome.

The HSV-1 genome consists of a large single double stranded DNA molecule, encoding at least 84 different polypeptides (Ward & Roizman, 1994). It is at least 152 kbp in size

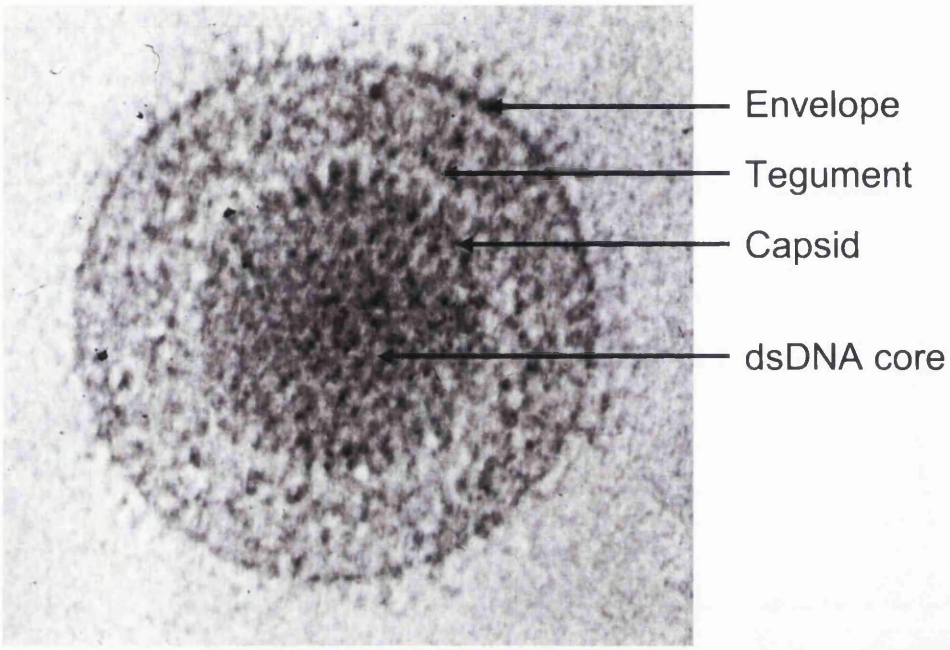


Figure. 1.1.3A *Electron micrograph of a frozen hydrated HSV-1 virion.*

The major structural features of the virion, including envelope, tegument, capsid, and DNA core are indicate by the appropriate arrows. The diameter of capsid is approximately 125 nm and the whole virion is approximately 200 nm. This figure was reproduced with permission from Dr. Frazer Rixon, MRC Virology Unit, Glasgow.

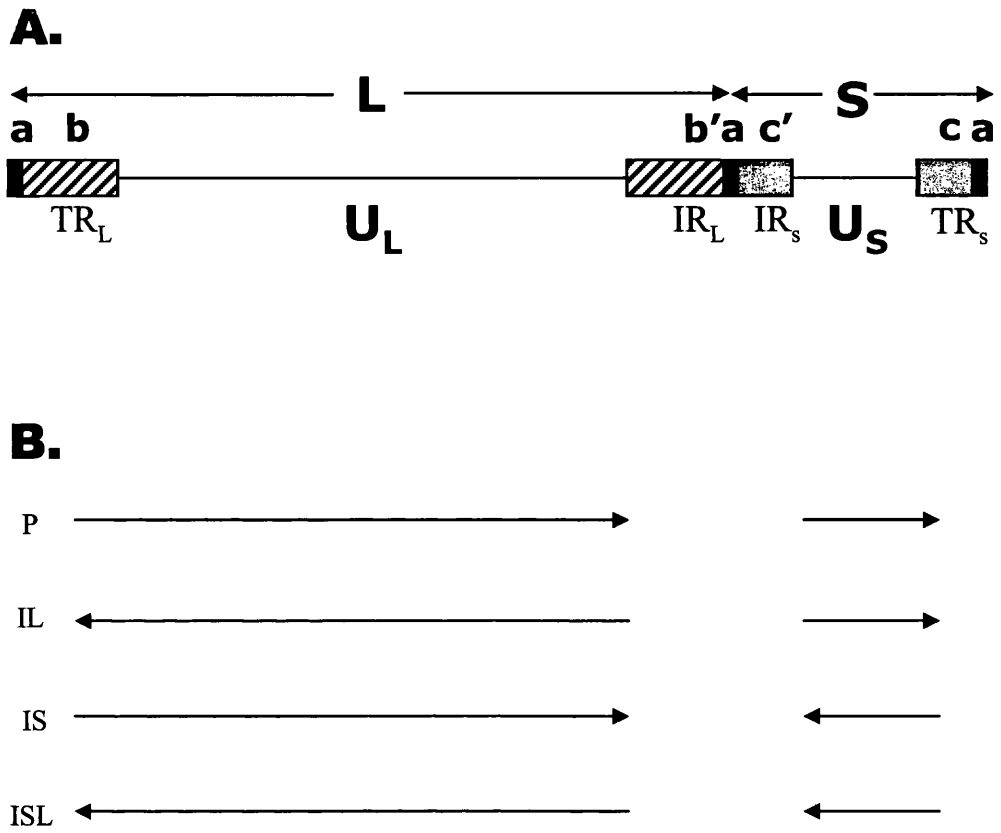


Figure. 1.1.4A. *Schematic illustration of the structure of the HSV-1 genome.*

The HSV-1 genome contains unique long (U_L) and unique short (U_S) sequences, the *"a"* sequences, the terminal repeats (TR_L and TR_S) and internal repeats (IR_L and IR_S) are shown. (B) Schematic illustration of the four possible isomers of the U_L and U_S sequences: P (prototype), IL (inversion of L), IS (inversion of S), and ISL (inversion of both S and L).

with a high G + C content of around 68% (McGeoch *et al.*, 1988) and consists of two covalently linked segments designated L (Long) and S (Short). Primarily, the variation in the size of the genome is due to the number of reiterations of a 400 bp repeat unit termed the 'a' sequence (Hayward *et al.*, 1975; Wadsworth *et al.*, 1976; Wadsworth *et al.*, 1975), which is located at the termini of the genome and at the junction between the L and S segments (Whitley *et al.*, 1998). Each of the long and short components is composed of unique sequences (U_L or U_S respectively). The U_L region is 107.9 kbp and the unique short U_S region is 13 kbp. The inverted repeat sequences flanking U_L are known as *b*, whereas those flanking U_s are known as *c*. Thus as shown in figure 1.1.4A, U_L is flanked by the sequence a'b and its inversion b'a' around 9.2 Kbp each, whereas U_S is flanked by sequences a'c and its inversion c'a, each of 6.6 Kbp (McGeoch *et al.*, 1988). The two unique segments can invert relative to each other via the repeat sequences, such that a population of HSV DNA molecules consists of four equimolar isomers (see figure 1.1.4A).

More than 84 different genes are distributed throughout the genome (Ward & Roizman, 1994), sixty-five mapping in U_L, fourteen in Us and two copies of four genes (including the IE-1 gene that encodes ICP0) flanking U_L. In general, each gene has its own promoter to direct transcription, although some transcripts share 3' ends. Amongst the various naming terminologies that have been used, one is the naming of a viral gene according to the relative position within the genome e.g. U_L 1-56 and U_S 1-11.

1.2.0. Stages of productive infection of HSV-1.

Productive infection of a cell by HSV-1 involves the following steps (figure 1.2.0A): entry (viral attachment and adsorption), gene expression, DNA synthesis and assembly/egress of progeny virions (Roizman & Sears, 1996). From the initial infection of fully permissive tissue culture cells, to the final viral release takes approximately 18-20 hr.

1.2.1. Viral attachment to cell surface receptors.

Entry of HSV-1 into the host cell is mediated by the glycoproteins situated within the viral envelope and proceeds in a sequential manner. In HSV-1 and the rest of the alphaherpesvirus family 12 glycoproteins have been identified so far: glycoprotein B (gB), gC, gD, gE, gG, gH, gI, gJ, gK, gL, gM and gN (predicted) (Spear, 2004). However, only gB, gD, gH, and gL play an indispensable role in cell fusion and entry (Spear, 2004). Initially, the virus binds to heparin sulphate moieties on the host cell surface, via gB and gC (Herold *et al.*, 1994). This initial step enhances infection, however gC (but not gB) has been shown to be dispensable for viral growth and replication *in vitro*, although infectivity of gC-negative virus is reduced by as much as 10-fold (Herold *et al.*, 1991). However, it is thought that the degree of reduction in HSV infectivity as a result of gC deletion may be dependent on virus serotype and cell type (Cheshenko & Herold, 2002). Furthermore, the glycosaminoglycan (GAG) heparin sulphate is thought to be the primary receptor for HSV-1, as cell lines devoid of GAG have been shown to be 85% resistant to infection. Consequently, as cells remain partially susceptible to infection it indicates that heparin sulphate is the primary, but perhaps not the sole initial receptor for HSV-1 (Banfield *et al.*, 1995a; Gruenheid *et al.*, 1993; Shieh *et al.*, 1992). Proposed candidates for secondary or co-receptors have included the fibroblast growth factor receptor (FGFR) (Baird *et al.*, 1990) and chondroitin sulphate (Banfield *et al.*, 1995b).

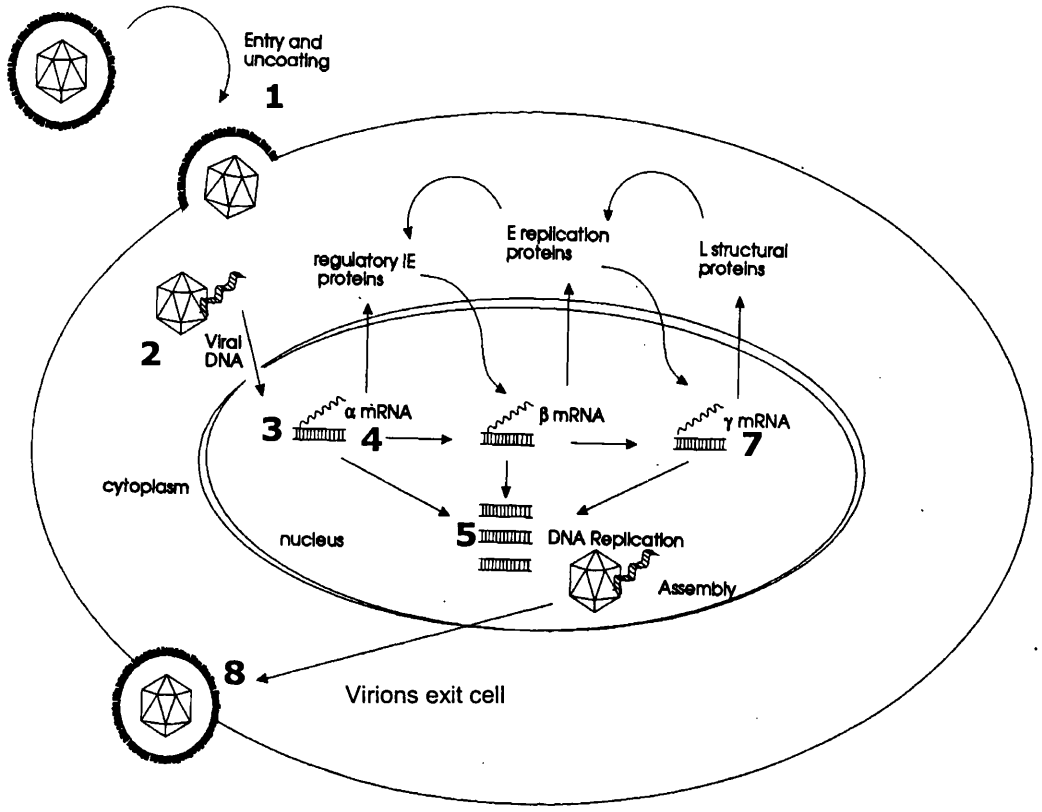


Figure 1.2.0A. *Life cycle of HSV-1.*

The stages of HSV-1 infection are: 1) Receptor binding and membrane fusion. 2) Release of the viral nucleocapsid and tegument into the cell cytoplasm and transport of the nucleocapsid to the nuclear pore. 3) Release of viral DNA into the nucleus. 4) Transcription and translation of the viral immediate early and early genes. 5) Viral DNA synthesis. 6) Transcription and translation of the viral late genes 7) Capsid assembly and DNA packaging. 8) Egress of the progeny virus. α, β and γ represent IE, Early and Late mRNA, respectively. Diagram adapted from Ackermann, *et al.* 1998.

1.2.2. Viral absorption into the cell.

The interaction of gB and gC with heparan sulphate alone is not sufficient for viral entry into the cell. Glycoprotein D is required to interact with one of several cellular receptors, termed herpes virus entry proteins (Hve) (Spear *et al.*, 2000), which trigger the fusion of the viral envelope with the cell membrane. The Hve proteins are composed of members of three families of receptor, the tumour necrosis factor (TNF) receptor family, including HveA (Montgomery *et al.*, 1996; Whitbeck *et al.*, 1997) and the immunoglobulin super family, including nectin-1 (HveB) (Warner *et al.*, 1998), nectin-2 (HveC) (Geraghty *et al.*, 1998), and heparan sulphate receptors which have been modified by 3-*O*-sulphatotransferases (Shukla *et al.*, 1999). An overview of the binding and fusion process of the HSV-1 virion is shown in figure 1.2.2A. The fusion process also requires the action of other viral glycoproteins, gB and a heterodimer of gH-gL. The role of all the virus glycoproteins is reviewed in further detail elsewhere (Spear, 2004; Spear & Longnecker, 2003).

Following viral penetration and internalisation into the cell, several of the tegument proteins play a crucial role. These include the virion host shut off protein (VHS), which degrades mRNA and causes an early shut down of host protein synthesis (Kwong & Frenkel, 1987). Virus capsids are transported to the nuclear membrane by a process mediated by microtubules, where the viral DNA and at least some tegument proteins (VP16, VP1 and VP2) enter the nucleus via a nuclear pore by an unknown mechanism (Sodeik *et al.*, 1997). The viral DNA in the nucleus forms an episome and it is at this point, where the course of infection depends on whether the virus undergoes lytic or latent infection.

1.2.3. Viral gene expression.

Upon internalisation into the nucleus, HSV-1 exploits the host cell machinery to ensure expression of viral proteins necessary for replication and cell-to-cell spread. The viral DNA encodes proteins that are essential for viral DNA replication and structural proteins, which make up the virion envelope and are involved in viral DNA packaging. Like other members of the herpesvirus family, HSV-1 gene expression involves the sequential cascade of three sets of genes that are named immediate early (IE), early (E) and late (L), and which are classified according to their time of expression during the

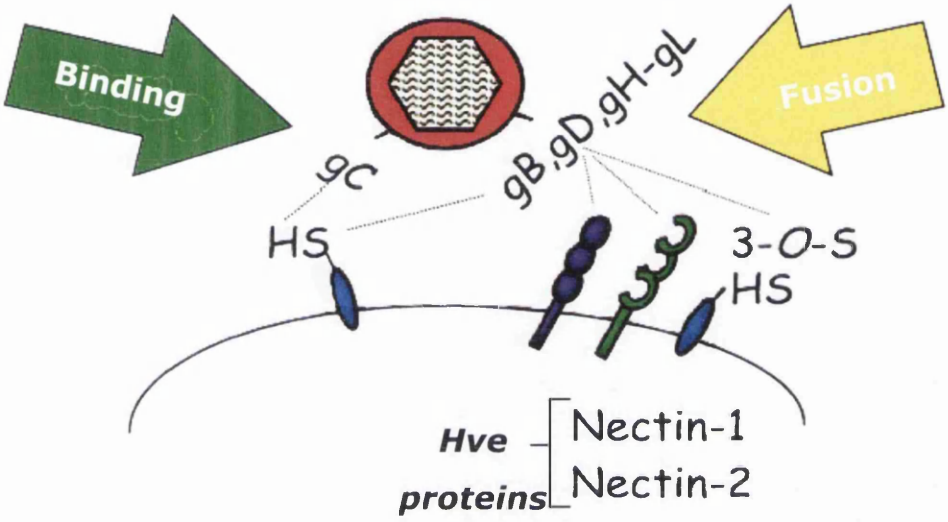


Figure 1.2.2A. *Cell surface receptors and viral ligands that participate in HSV entry.*

Only five of the HSV-1 glycoproteins gB, gC, gD, gH and gL have been shown to participate in viral entry. Binding of virus to cells can be mediated by the binding of gB or gC to heparan sulphate (HS) chains on cell surface proteoglycans, which facilitates the binding of gD to one of its cell surface receptors. These include the herpesvirus entry proteins (Hve) nectin-1 and nectin-2, and specific sites in heparin sulphate generated by certain 3-O-sulphate transferases. Binding of gD to any one of these receptors triggers fusion of the viral envelope with the cell membrane. This membrane fusion requires the action of gB and gH-gL heterodimers as well as gD and a gD receptor. Diagram adapted from Spear, 2004.

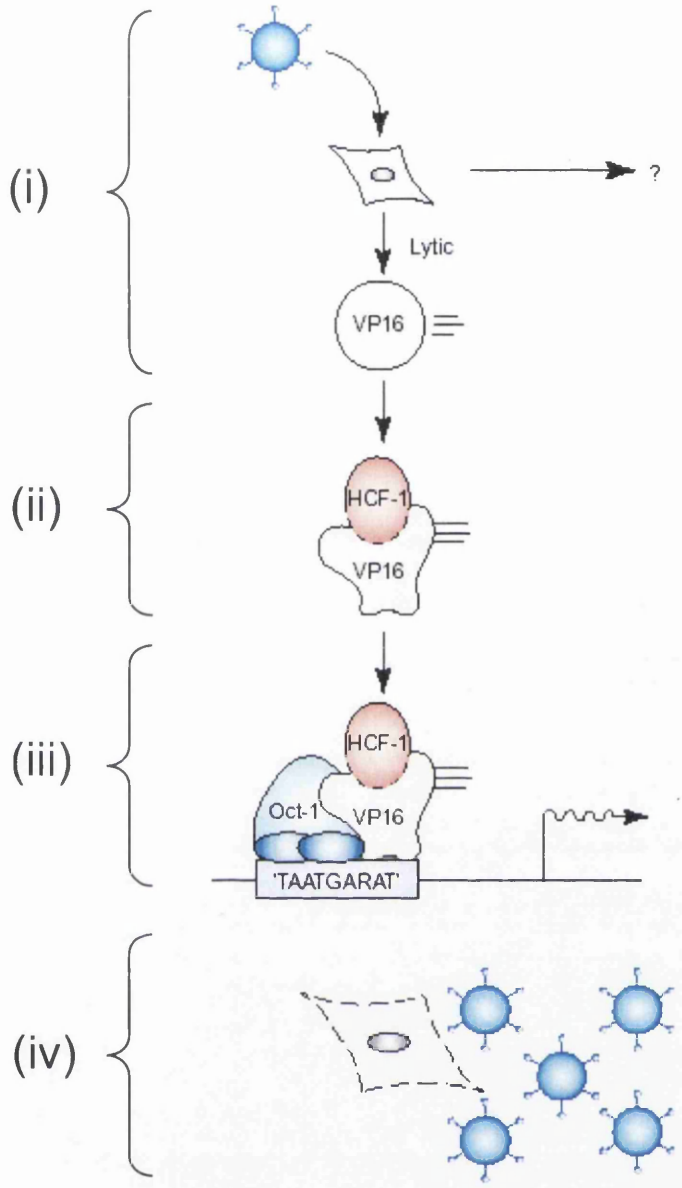


Figure 1.2.3A. ***The role of VP16-induced complex in the initiation of viral gene expression.***

Upon HSV infection the virus can enter a lytic or latent phase **(i).** In the lytic mode, VP16 is delivered to the host cell. **(ii)** VP16 then associates with HCF-1. **(iii)** This leads to VP16-induced complex assembly on HSV immediate-early (IE) promoters with Oct-1., which initiates a cascade of gene expression. **(iv)** Resulting in virus production and lysis of the host cell The diagram is adapted from Wysocka & Herr, 2003.

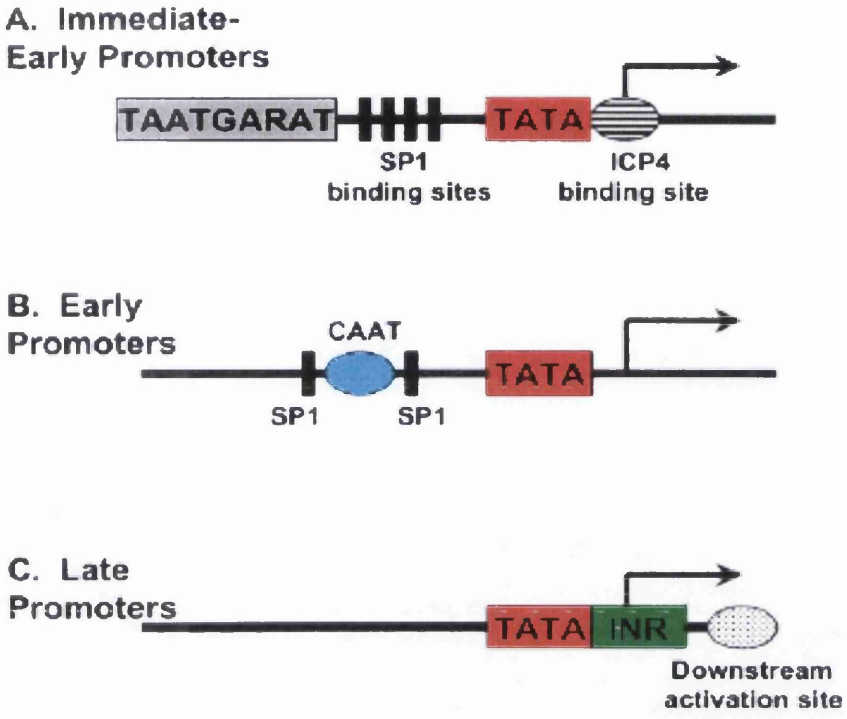


Figure 1.2.3B. *Schematic representation of IE, E, and L HSV-1 promoters.*

The general arrangement and composition of cis-acting regulatory elements for generic IE, early and late promoters is shown. The actual number and arrangement of elements varies among promoters of each class.

(A) In addition to the TATA element, IE promoters have TAATGARAT elements in the upstream region through which the viral VP16 protein mediates transcriptional activation. Binding sites for eukaryotic transcription factors such as SP1 are also present upstream from the TATA element and in at least some IE promoters, binding sites for ICP4.

(B) Early promoters have binding sites for eukaryotic transcription factors upstream of the TATA element but no identified cis-acting regulatory elements further downstream.

(C) Late promoters have an initiator element (Inr) at the start of transcription, and in at least some late promoters, a downstream activation site. Diagram adapted from Weir, 2001.

viral replication cycle (Clements *et al.*, 1977; Honess & Roizman, 1974). Transcription of the viral DNA within the nucleus of host cells is carried out by host DNA-dependent RNA polymerase II, which is responsible for the synthesis of all viral mRNA (Alwine *et al.*, 1974; Costanzo *et al.*, 1977).

Figure 1.2.3A shows an overview of the steps that initiate gene expression in the host cell nucleus. Following internalisation into the cell and the induction of the lytic infection cycle, the tegument protein VP16 (a key activator of the lytic infection), forms a trimeric association with the cellular transcription factor Oct-1 (octamer DNA-binding protein) and the cell proliferation factor HCF-1. The interaction of HCF and VP16, promotes stable interaction with Oct-1 and the association of VP16 with Oct-1 enables the former to bind to DNA (Hughes *et al.*, 1999). The sub-cellular distribution of HCF has been shown to be altered during HSV-1 infection and particularly during the early stages of infection, and it is thought to act as a nuclear import factor for VP16 (La Boissiere *et al.*, 1999; La Boissiere & O'Hare, 2000). Furthermore, during latency it has been shown that HCF remains in the cytoplasm of sensory neurons; however under experimental conditions that induce the initiation of the lytic infection, it is rapidly relocated into the nucleus. This indicates a possible role for HCF in the switch between latency and productive infection (Kristie *et al.*, 1999)

The Oct-1-VP16-HCF-1 complex initiates immediate early gene expression by binding to the upstream consensus IE promoter element 'TAATGARAT' which is present in at least one copy in all HSV IE promoters (Gaffney *et al.*, 1985). An overview of the composition of the different promoter types within the HSV-1 genome is shown in figure 1.2.3B. Oct-1 binds directly to the TAAT region of the motif and the recruitment of VP16 is dependent on the presence of the GARAT half of the sequence (where R is a purine) (O'Hare *et al.*, 1988). The availability of Oct-1 can influence the efficiency of initiation of the HSV lytic cycle as recently it was shown that at low M.O.I. the initiation of the lytic cycle is greatly impaired in Oct-1 deficient cells. However, Oct-1 deficiency could be overcome at high M.O.I., suggesting that induction of transcription by VP16 can occur through other IE regulatory elements (Nogueira *et al.*, 2004).

The IE genes are defined as those expressed in the absence of viral protein synthesis and are ICP0, ICP4, ICP22, ICP27 and ICP47, which will discussed in more detail in section 1.2.4. IE protein expression serves to transactivate early gene expression, which in turn

produces proteins that are generally required for DNA replication, such as DNA polymerase, single-stranded DNA-binding protein (SSB or ICP8), the DNA helicase-primase complex, and the origin binding protein. Early genes also include enzymes that are required for nucleotide metabolism and DNA repair (Roizman & Sears, 1996).

The synthesis of E genes can be detected as early as 2 hours post infection and reaches a peak at 5 - 7 hours. Viral DNA synthesis commences shortly after the onset of early gene expression and continues up to 15 hours post-infection, with a peak between 7 - 10 hours post-infection (Roizman & Sears, 1996). The regulated cascade of gene expression ends with the expression of L genes that generally encode structural proteins of the virion. Late genes can be subdivided into leaky late, such as VP5 encoded by UL19, and true late, such as VP19c encoded by UL38. Expression of leaky late genes can occur in the absence of DNA synthesis, although their expression is enhanced during DNA replication, the true late genes are only expressed after the onset of DNA replication. Late gene expression peaks at 8-10 hours post-infection and persists for the remainder of the lytic cycle (Harris-Hamilton & Bachenheimer, 1985). Further detailed review of HSV-1 gene expression can be found elsewhere (Preston, 2000; Roizman & Sears, 1996).

1.2.4. Proteins encoded by the immediate early genes of HSV-1.

The IE gene products play an essential role in the regulation of the viral gene expression (see figure 1.2.4A) and they control the host cell to create an environment conducive for viral replication. ICP4 and ICP27 are essential regulatory proteins in all experimental systems (*in vitro* and *in vivo*); ICP0 and ICP22 are dispensable in at least some systems, but the evidence indicates that each plays an important regulatory role in viral gene regulation. Furthermore, collectively the immediate early proteins have been implicated in being essential for the induction of apoptosis during the early stages of viral infection (Sanfilippo *et al.*, 2004). Below is a brief review of the immediate early gene products, highlighting their key biological functions. As it is not possible to go into depth with all of the immediate early gene products, appropriate references are included in each section. However, a detailed review of ICP0 can be found in section 1.4.0.

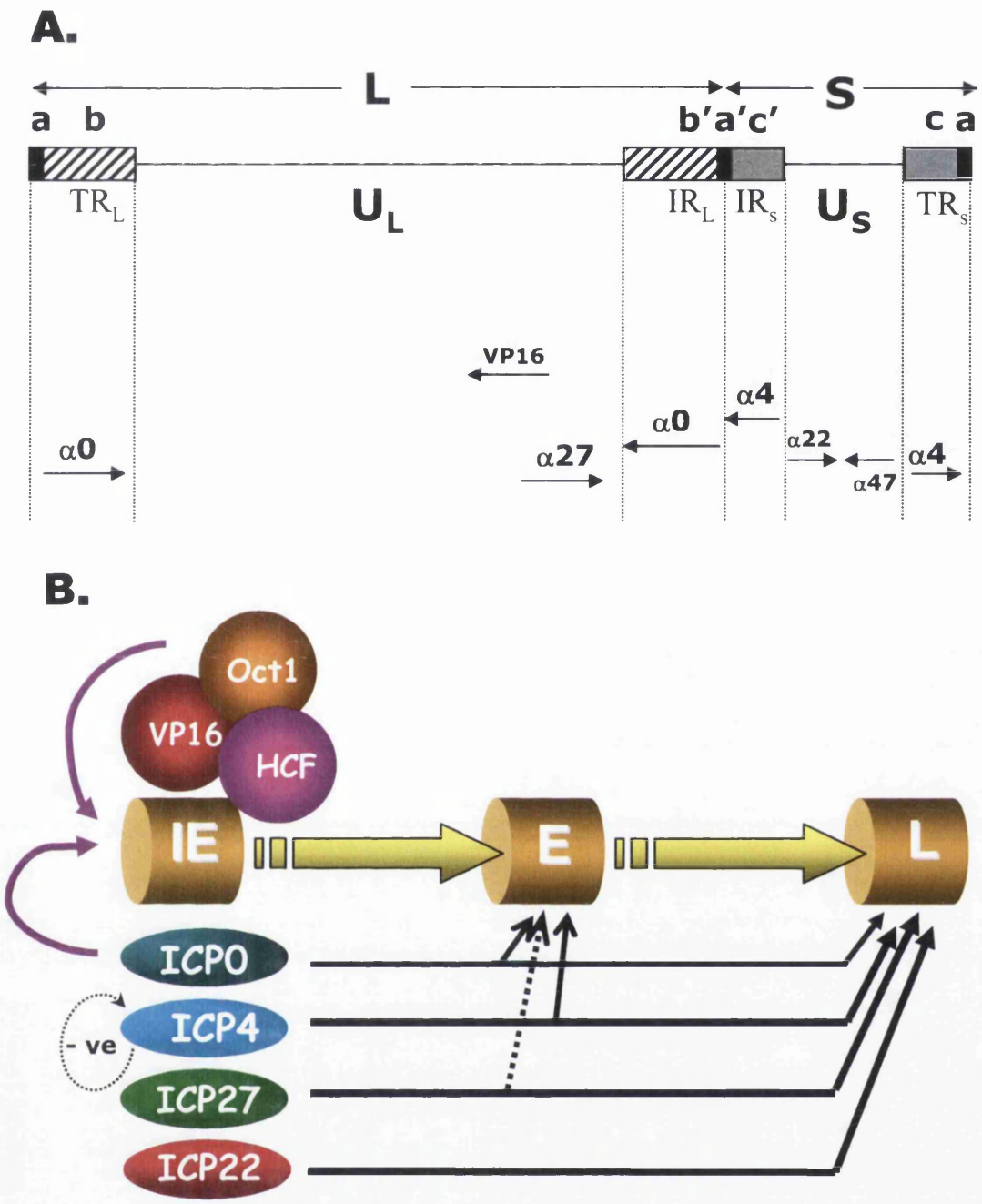


Figure 1.2.4A. *Schematic diagram showing the location of IE genes within the genome of HSV-1 and the regulation HSV-1 gene expression by the IE proteins.*

The immediate early gene are ICP0 (IE1 or α0), ICP27 (IE2 or α27), ICP4 (IE3 or α4), ICP22 (IE4 or α22) and ICP47 (IE5 or α47). ICP0 can transactivate all classes of genes.) is essential for the activation of transcription of the early and late genes. It can also regulate expression of IE proteins including itself and ICP0. ICP27 is essential for the expression of late genes and several early genes. ICP22 also contributes to the efficient expression of L gene products. Further details can be found within the text. Diagram B, adapted from Everett, 2000.

The role of ICP27.

ICP27 is an essential regulatory IE gene encoding a 63 KDa nuclear phosphoprotein, being required for the expression of certain early genes and most late genes. ICP27 functions at least in part at the transcriptional level (McCarthy *et al.*, 1989; McLauchlan *et al.*, 1989). Furthermore, the protein associates with RNA polymerase II holoenzyme and the viral transcription factor ICP4, which is thought to reflect its role in stimulating early and late gene expression and/or its role in inhibiting host transcription (Zhou & Knipe, 2002). Furthermore, ICP27 can also function to repress expression from certain HSV-1 encoded promoters in the presence of ICP4 or ICP0 (Sekulovich *et al.*, 1988). Through stimulating the expression of the early genes that encode essential replication proteins ICP27 promotes viral DNA replication (McCarthy *et al.*, 1989).

Despite evidence that ICP27 functions at the transcriptional level, recent evidence indicates that its major roles are at the post-transcriptional level. For example, ICP27 stimulates the usage of certain polyadenylation signals and inhibits host gene expression by preventing splicing, which contributes to the shut-off of host protein synthesis seen during productive infection (Bryant *et al.*, 2000; Hardwicke & Sandri-Goldin, 1994; Hardy & Sandri-Goldin, 1994). Furthermore, ICP27 has been shown to shuttle between the nucleus and cytoplasm, and additionally can cross link with viral RNA in the nucleus and cytoplasm, suggesting a role for it in the nuclear export of viral transcripts (Phelan *et al.*, 1997; Sandri-Goldin, 1998; Soliman *et al.*, 1997). More recent studies have shown ICP27's ability to export RNA is specific for intron-less viral mRNAs. Furthermore, ICP27 *in vitro* and in virally infected cells has been shown to bind to REF and TAP, factors involved in cellular mRNA export (Chen *et al.*, 2002a; Koffa *et al.*, 2001). An ICP27 mutant that does not interact with REF was inactive in viral mRNA export (Koffa *et al.*, 2001). It was proposed that ICP27 associates with viral mRNAs and recruits TAP via its interaction with REF proteins, therefore enabling otherwise inefficiently exported viral mRNAs to access the TAP-mediated export pathway (Koffa *et al.*, 2001). Research has supported the role of TAP in ICP27-mediated export of viral mRNA (Chen *et al.*, 2002a) and additionally showed that over-expression of a TAP mutant that cannot bind to nucleoporins inhibits ICP27 translocation (Chen *et al.*, 2002a). The ability of ICP27 to bind to RNA and shuttle between the nucleus and cytoplasm has been shown to be required for efficient cytoplasmic localisation of the long UL24 transcript (Pearson *et al.*, 2004), which encodes nuclear associated proteins

that have a supposed role in inhibiting cell to cell fusion during infection (Sanders *et al.*, 1982; Tognon *et al.*, 1991). Finally, in certain human cells, ICP27 prevents apoptotic cell death, which is otherwise induced by early events in viral infection (Aubert *et al.*, 1999).

The role of ICP4.

ICP4 is a 175-kDa protein encoded by both copies of the RS1 or IE3 gene within the repeats flanking the S segment of the viral genome. ICP4 acts as a transactivator of early and late gene expression and is essential for lytic infection to progress past expression of the immediate early genes alone (Preston, 1979; Watson & Clements, 1980). Furthermore, ICP4 acts to down regulate its own expression and that of other IE proteins such as ICP0 (DeLuca *et al.*, 1985; Roberts *et al.*, 1988). As a transactivator, ICP4 serves to increase transcription by increasing the assembly of transcription complexes within promoter sequences (Grondin & DeLuca, 2000). ICP4 contains a DNA binding region that is essential for its transactivation function (DeLuca & Schaffer, 1988; Michael *et al.*, 1988; Paterson & Everett, 1988), however no specific ICP4 binding sites have yet been identified on more than a minority of the early and late promoters that ICP4 activates (Everett, 1984; Imbalzano *et al.*, 1990; Smiley *et al.*, 1992). Mutational studies have shown that complete deletion of ICP4 severely impairs expression of early and late genes (DeLuca *et al.*, 1985).

Several lines of evidence have proposed that ICP4 transactivation occurs through interaction with TATA-binding protein (TBP) and general transcription factor TFIIB (Gu *et al.*, 1995a; Kuddus *et al.*, 1995) and that these interactions are facilitated by the cellular high mobility group factor 1 (HMG1) protein and DNA (Carrozza & DeLuca, 1998). Most recently *in vitro* studies have shown using the structurally distinct promoters, the early tk and the late gC promoter, that TFIIA was necessary for ICP4-mediated activation of the latter. This suggests a mechanism of action by which ICP4 regulates the early and late promoters (Zabierowski & DeLuca, 2004).

The role of ICP47.

ICP47 is encoded by the US12 gene and is non-essential for replication and doesn't act as a regulatory protein in the same manner as ICP0, ICP4 and ICP27 (Mavromara-

Nazos *et al.*, 1986). ICP47 is able to bind to the cytosolic face of TAP, a transporter which is associated with antigen processing (Tomazin *et al.*, 1996), this process inhibits the translocation of peptides across the ER (Fruh *et al.*, 1995; Hill *et al.*, 1995), which is thought to have an important function in inhibiting antigen presentation to CD8 T lymphocytes. Processing of viral proteins for recognition by the cytotoxic T lymphocytes normally involves degradation of the proteins in the cytosol of an infected cell followed by transport of the resulting peptides into the endoplasmic reticulum (ER) by TAP. Due to the lack of translocated peptides inside the ER, MHC class I molecules fail to assemble and therefore MHC peptide complexes do not reach the cell surface for immune recognition. Thus ICP47 may provide a mechanism by which HSV-1 escapes immune surveillance by the host (York & Rock, 1996)

The role of ICP22.

ICP22 is a 68 kDa protein expressed from the IE4 gene and is involved in gene regulation although its role is yet to be defined. ICP22 null mutants grow as well as wild type HSV-1 in many cell types (Post & Roizman, 1981), however they exhibit a reduced level of ICP0 expression and a subset of late genes (Sears *et al.*, 1985). The carboxyl terminus of ICP22 is also encoded by a second overlapping transcript that has been designated as US1.5 (Carter & Roizman, 1996). Analysis of the ICP22 and US1.5 shared domain has shown that it is necessary for optimal expression of several L genes, and HSV-1 deletion mutants lacking these domains are avirulent in experimental animal systems (Ogle & Roizman, 1999).

ICP22 has also been implicated in the production of an aberrantly phosphorylated form of cellular RNA polymerase II, however the significance of this remains unclear (Rice *et al.*, 1995). Interestingly, the role of ICP22/U_S1.5 is tied to that of the UL13 protein kinase (Purves *et al.*, 1987). The UL13 kinase, along with the US3 kinase, phosphorylates ICP22/US1.5, and HSV mutants lacking functional UL13 have similar phenotypes to those of ICP22 null mutants (Rice *et al.*, 1995). Studies that are more recent have indicated that ICP22 and UL13 have an important relationship with cell cycle regulated protein cdc2, which is activated in HSV-1 infected cells. Specific inhibition of this cellular kinase results in inhibition of expression of the same set of late genes that are inhibited by inactivation of ICP22 or UL13 (Advani *et al.*, 2000). How

cdc2 actually regulates the expression of this subset of late genes remains to be elucidated.

1.2.5. The role of HSV-1 early proteins.

The E gene products encode proteins involved in viral DNA synthesis, DNA binding proteins, thymidine kinase and other enzymes. The temporal regulation of the early class of genes is mediated by cis-acting elements in E promoters and by ICP4 and ICP0, which work synergistically with one another to stimulate expression of these genes (and late genes).

Studies to develop the understanding of temporal regulation of early genes in HSV-1 were originally carried out using the gene encoding thymidine kinase (UL23). The studies showed the tk, like other early promoters contain regulatory elements similar to that of other eukaryote promoters, such as a TATA element linked to two upstream binding sites for the transcription factor Sp1 and a CAAT element. Both elements were required for efficient tk gene transcription during HSV infection (Everett, 1983, 1984). No additional *cis*-acting elements were necessary for expression during infection (Coen, 1986). Besides these elements, analysis of the gD gene expression revealed the cap-site region is necessary for E promoter activation; deletion of this region moderately decreased gD gene expression (Everett, 1983, 1984). An overview of the early promoter structure can be found in figure 1.2.3B.

These results were intriguing as a viral gene such as tk, which apparently contains all of the cis-acting regulatory elements that are sufficient for expression in the eukaryotic cell, is not expressed when present in the viral genome until HSV-1 IE proteins are synthesised (Coen *et al.*, 1986). Subsequent studies showed that ICP4 is necessary for expression of the tk gene in the viral genome, that transactivation is mediated through the TATA element of the tk promoter, and that expression is enhanced by the presence of the CAAT motif and Sp1 binding sites. Furthermore, the tk TATA element alone can direct modest transcription in the absence of the upstream regulatory elements, but only in the presence of functional ICP4 (Imbalzano *et al.*, 1991). It is thought that the TATA element appears to influence the strength of expression rather than the timing (Cook *et al.*, 1995).

From the detailed analysis of the UL37 and UL50 early promoters, it has been shown that individual HSV early promoters contain a variety of cis-acting regulatory elements, commonly found in eukaryotic genes transcribed by RNA polymerase II (Pande *et al.*, 1998). In conjunction with earlier studies carried out on the tk gene, the results demonstrate the composition and arrangement of these elements differ considerably among HSV early promoters, but all are found upstream from an essential TATA element (Weir, 2001). Therefore, unlike the IE promoters, the E promoters do not contain regulatory elements that are specific to viral genes, and instead the E promoters rely on cis-acting sequences that are in common with those of typical cellular genes (Everett, 1984). This is supported by research that showed the HSV-1 gD promoter transactivation does not require elements other than those involved in promoter function in the absence of the IE proteins (Everett, 1983, 1984). Indeed, HSV-1 is unable to differentiate between viral and cellular promoters when the latter are placed in the context of the viral genome (Smiley *et al.*, 1987).

1.2.6. The role of the late gene products.

Expression of the L genes is activated by the IE genes, but only after viral DNA synthesis has occurred. Again, ICP0 and ICP4 work synergistically with one another to stimulate their expression. In addition, ICP27 can further enhance the expression of certain L genes, beyond the levels achieved by the combination of ICP4 and ICP0 (Everett, 1986; Sacks *et al.*, 1985). Furthermore, ICP22 has been implicated in stimulating the expression of L genes in a cell type specific manner (Sears *et al.*, 1985).

Concurrently with DNA replication, viral late gene expression is initiated as early gene expression begins to decline. These processes are linked, since inhibition of DNA replication also inhibits late gene expression and prolongs the expression of early genes (Weir, 2001). The L genes encode the structural proteins of the capsid, tegument and envelope. Following the expression of the L genes, the viral DNA concatamers are cleaved into genome length units and packaged into the capsids. The cleavage and packaging signal derives from 'a' sequences that lie between the repeat regions of the DNA concatamer (see figure 1.1.4A) (Roizman & Sears, 1996).

A detailed analysis has been carried out on the composition of late gene promoters (see figure 1.2.3b). Like IE and E promoters, the L promoters require a TATA element and

appear to be identical to other viral or eukaryotic TATA elements that account for temporal expression (Johnson *et al.*, 1986). As mentioned previously, ICP4 is necessary for the activation of L gene expression and mediates its action through the TATA element, the same mechanism as used for early promoters. Late promoters are functionally silent before DNA replication even at a time when ICP4 is present. The IE and early promoters differ from the true late promoters, as the latter do not possess cis-acting regulatory elements upstream from the TATA element. Instead, L promoters contain other elements downstream from the TATA including an initiator element at the start of transcription (Guzowski & Wagner, 1993; Kibler *et al.*, 1991; Mavromara-Nazos & Roizman, 1989). The regulatory elements so far identified as necessary for efficient late promoter activity also appear to be identical to the eukaryotic regulatory elements. The key feature that distinguishes E and L promoters from one another is the arrangement and composition of these regulatory elements suggesting a potential mechanism to how the transcriptional machinery distinguishes between the E and L promoter during the course of infection. (Weir, 2001)

There are two sets of L genes referred to as leaky-late or $\gamma 1$ genes and the true HSV-1 late genes referred to as γ_2 genes that require DNA replication for any appreciable accumulation of their mRNAs (Weir, 2001). The leaky-late genes are expressed to a degree in the absence of DNA replication, but require DNA synthesis for maximal expression (Weir, 2001). Expression is classed as leaky due to cis-acting regulatory sequences upstream of the TATA element similar to early promoters, as well as an initiator element at the start of transcription similar to strict late promoters (Huang *et al.*, 1993; Lieu & Wagner, 2000)

Further studies have proposed that ICP8 is required for late gene expression (Chen & Knipe, 1996; Gao & Knipe, 1991; McNamee *et al.*, 2000). ICP8 mutants exhibit reduced levels of gene expression, possibly due to the reduced levels of DNA replication that results from defective ICP8. However, certain mutants of ICP8 inhibit late gene expression to a far greater extent than they inhibit viral DNA replication, suggesting a more direct role in late gene expression (Chen & Knipe, 1996; Gao & Knipe, 1991). Furthermore, it has been shown that the viral regulatory proteins ICP4 and ICP22 co-localise with RNA polymerase II and viral DNA replication proteins late in infection (Leopardi *et al.*, 1997). More recently, a study to further elucidate the role of ICP8 have shown that it coprecipitates with a vast array of proteins including proteins

involved in DNA replication (Taylor & Knipe, 2004). Possibly further analysis of these proteins will give new insight into what part ICP8 plays in HSV-1 replication.

1.2.7 The role of VHS in HSV host cell interactions and viral gene expression.

The uncoating of the virion following fusion of the viral envelope with the cell membrane results in the release of the tegument proteins into the cytoplasm of the host cell. Some of these proteins serve to stall host protein synthesis and accelerate the activation of viral gene transcription (reviewed by Roizman and Sears, 1996). The primary mechanism that initiates the shut-off of host protein synthesis during the initial stages of infection is regulated by the virion associated host shut-off (VHS) protein; classed as early (primary or virion associated) shut off. VHS instigates the shut-off of host cell protein production by globally increasing the rate of mRNA degradation in the cytoplasm (Kwong & Frenkel, 1987), resulting in a decrease in the levels of the corresponding proteins (Koppers-Lalic *et al.*, 2001; Strom & Frenkel, 1987). Viral mRNA is also degraded, however its rate of synthesis is greater than that of VHS-induced degradation (Elgadi *et al.*, 1999; Karr & Read, 1999; Krikorian & Read, 1991; Lam *et al.*, 1996). The effect of VHS is magnified by virus-induced suppression of host mRNA synthesis, mediated through repression of primary transcription (Spencer *et al.*, 1997) and pre-mRNA splicing (Hardy & Sandri-Goldin, 1994). The affect of VHS is enhanced by additive effects of ICP27, so that the two functions lead to a reduction in the abundance of host mRNA during infection (Hardwicke & Sandri-Goldin, 1994; Song *et al.*, 2001). The available data suggest that VHS functions as an RNase or as a subunit of an RNase. Its amino acid sequence contains similarity to a family of cellular nucleases that are involved in DNA replication and repair (Doherty *et al.*, 1996; Everly *et al.*, 2002; Everly & Read, 1997), in particular to FEN-1, an endo/exonuclease that helps remove RNA primers from Okazaki fragments during DNA replication in eukaryotes (Lieber, 1997).

The process by which VHS-dependent RNase mediated degradation selectively targets mRNAs as opposed to other cellular RNAs remains unclear, as it cannot discriminate the 5' cap or 3' poly(A) tail of mRNA (Smiley, 2004). However, some evidence suggests that VHS is preferentially targeted to regions of translation initiation on mRNAs, as the 5' end of HSV thymidine kinase mRNA is degraded before the 3' end *in vivo* (Karr & Read, 1999). It was proposed that VHS is targeted to mRNAs through

interactions with one or more components of the cellular translation initiation machinery. This is illustrated by the fact that VHS has been shown to directly bind the cellular translation initiation factor eIF4H (Feng *et al.*, 2001).

VHS is present in all neurotropic alphaherpesviruses and therefore is likely to play a fundamental role during infections. However, the phenotype of a HSV-1 VHS null mutant in cell culture, unlike in animal models, is still relatively similar to wild type HSV-1 (Read & Frenkel, 1983; Read *et al.*, 1993; Smibert *et al.*, 1994). The distinct difference in the virulence of the virus *in vitro* to that of *in vivo* indicated that VHS plays a crucial role as a HSV-1 virulence factor (Strelow *et al.*, 1997; Strelow & Leib, 1995, 1996). VHS has been shown to affect host immunity in a number of ways, in particular mRNA degradation induced by VHS and the effects of ICP47 contribute to the loss of major histocompatibility complex (MHC) class I and reduce the levels of MHC class II at the cell surface (Hinkley *et al.*, 2000; Koppers-Lalic *et al.*, 2001; Tigges *et al.*, 1996). This renders infected cells resistant to lysis by cytotoxic T lymphocytes, an effect that is predicted to impair antigen presentation and lessen both cellular and humoral responses (Trgovcich *et al.*, 2002). The biological affects of VHS are reviewed in further detail elsewhere (Smiley, 2004).

1.2.8. DNA replication.

The viral DNA reaches the nucleus as soon as 30 minutes post-infection where it forms a circular episome (Uprichard & Knipe, 1996). HSV DNA replication occurs in specialised structures formed in the nucleus of infected cells, called replication compartments (Quinlan *et al.*, 1984), which are located adjacent to subnuclear structures called ND10 (Maul *et al.*, 1996). Within the replication compartments are protein products of the early genes that are essential for viral DNA replication.

Several HSV-1 proteins are required for ori (origin of replication) dependent DNA replication. These are components of the helicase primase complex (U_L5, U_L8, U_L52), an origin binding protein (U_L9), a single stranded DNA binding protein (U_L29 or ICP8), DNA polymerase (U_L30) and its processivity factor (U_L42). All the genes encoding these proteins are located in the U_L segment of the genome (Challberg, 1986; Crute *et al.*, 1989; Wu *et al.*, 1988).

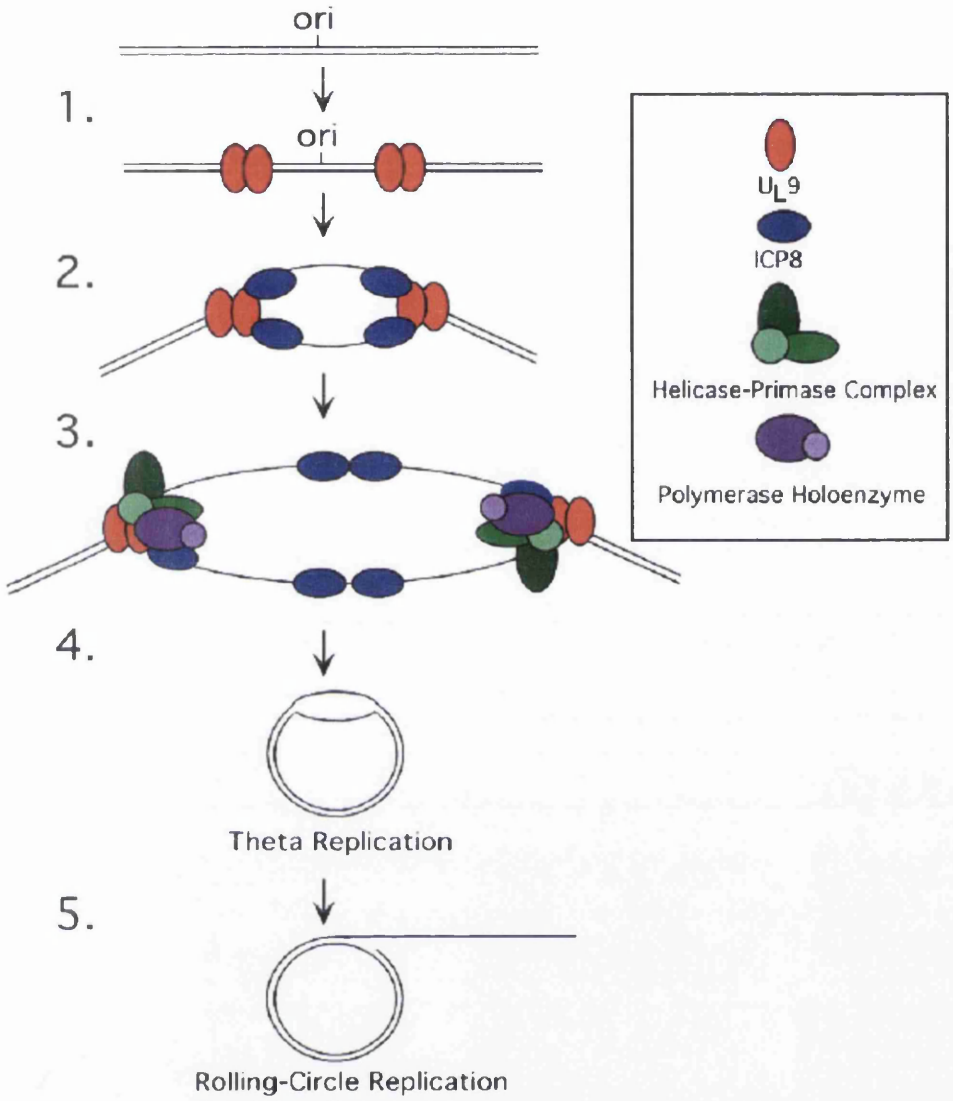


Figure 1.2.8A. *Schematic representation of HSV-1 DNA Replication.*

1), UL9, the origin binding protein, binds to specific sites at an origin of DNA replication (either *ori*L or *ori*S) and starts to unwind the DNA. 2) ICP8, the single-stranded DNA binding protein, is recruited to the unwound DNA. 3) UL9 and ICP8 recruit the helicase-primase complex and the polymerase holoenzyme replication proteins to the replication forks. 4) DNA synthesis initially proceeds via a theta replication mechanism, but then switches to a rolling-circle replication mechanism. (Diagram adapted from Taylor *et al*, 2001).

DNA replication is thought to be initiated by the theta mode of replication (Roizman & Sears, 1996) and occurs at one of the three cis-acting elements within the HSV-1 genome that function as origins of replication (ori) (see figure 1.2.8A). The origin binding protein (UL9) forms a homodimer and binds via its N-terminal region to specific sequences within the ori sequence (Elias *et al.*, 1992), in association with the single stranded DNA binding protein ICP8 (UL29). Helicase activity associated with UL9 is stimulated by the presence of ICP8, which binds to the C–terminal segment of UL9 and enables the separation of the two DNA strands permitting entry of the host DNA polymerase complex (a heterodimer of UL30/UL40) (Boehmer *et al.*, 1994; Boehmer & Lehman, 1997). Theta replication begins before switching to the rolling circle mode and the addition of the DNA helicase-primase (primosome) complex comprising of a 1:1:1 ratio of the UL5/UL8/UL52 gene products (Boehmer & Nimonkar, 2003). DNA synthesis is continuous along one strand and discontinuous along the other strand. The growth of the DNA fork is maintained by ICP8 and results in accumulations of large concatemeric DNA molecules at specific sites within the nucleus (de Bruyn Kops & Knipe, 1988; Quinlan *et al.*, 1984).

Other HSV-1 viral proteins important for DNA replication include those involved in nucleic acid metabolism, such as thymidine kinase (UL23), ribonucleotide reductase (UL39 and UL40), dUTPase (UL50), uracil DNA glcosylase (UL2) and alkaline exonuclease (UL12). The functions of these proteins have been reviewed recently elsewhere (Boehmer & Nimonkar, 2003).

The hypothesis of circularization of the HSV-1 genome upon its entry into the nucleus has recently been challenged. Instead it has been suggested that replication initially occurs from linear genome templates and that the genome only circularises in the absence of ICP0 (Jackson & DeLuca, 2003); this is discussed in more detail in section 1.4.11.

1.2.9. Viral capsid assembly.

Expression of the late proteins drives capsid assembly and occurs within the nucleus. However, the capsid proteins VP5, VP26, VP23 lack nuclear localisation signals and consequently form complexes with NLS-containing proteins VP19C or pre-VP22a in the cytoplasm for transport into the nucleus (Rixon *et al.*, 1996). A mature HSV-1

capsid is comprised of an outer shell containing penton-shaped subunits of the major capsid protein, VP5 and hexons of VP5 and VP26 (Newcomb *et al.*, 1996; Newcomb *et al.*, 1999). These subunits are connected by triplex structures formed by the two minor capsid proteins, VP19C (UL38) in one copy and VP23 (UL18) in two copies. Two genes encoding multiple non-structural proteins, UL26 (VP21 and VP24) and UL26.5 (pre-VP22a, VP22a), are also necessary for efficient capsid formation. VP21, pre-VP22a, and VP22a are scaffolding proteins (Thomsen *et al.*, 1994). VP24 is a serine protease required for capsid maturation (Taylor *et al.*, 2002). Cellular factors are not required for capsid assembly since the process can be completed *in vitro* using purified viral protein components (Newcomb *et al.*, 1999). Capsid shells containing the scaffold proteins are then loaded with viral DNA, with concomitant loss of the scaffold. A schematic representation of capsid assembly is shown in figure 1.2.9A.

1.2.10. Packaging of progeny DNA

The UL12 and UL12.5 genes encode alkaline nucleases, which aid the processing of branched DNA molecules prior to cleavage, although these proteins are not essential for cleavage or packaging of viral DNA. Mutants that fail to express an alkaline nuclease (AN) exhibit a reduced viral yield by 100- to 1000-fold, which is thought to be from the cumulative effects of relatively small reductions in viral DNA synthesis, DNA packaging, capsid egress from the nucleus and the ability of progeny particles to initiate new cycles of infection (Bronstein *et al.*, 1997; Martinez *et al.*, 1996; Porter & Stow, 2004b). DNA replication results in the production of large, branched, head-to-tail concatamers of viral DNA (Zhang *et al.*, 1994). However, in the absence of AN gene the DNA replicative intermediates have a more complex structure with an increased frequency of branches (Martinez *et al.*, 1996) and appear to be more prone to recombination events (Porter & Stow, 2004a). Furthermore, structural abnormalities have also been detected in the genomes of progeny virions (Porter & Stow, 2004b)

The replicated DNA is packaged into the capsid and cleaved into unit length monomers at the novel a-a junction. This results in a single copy of the genome being packaged per capsid, with an "a" sequence being present at each terminus of the DNA molecule (Mocarski & Roizman, 1982). The "a" sequence is thought to act as a marker in order to determine when a full genome length has been packaged and should be cleaved. The cleavage of DNA is also dependent upon capsid formation as mutants that fail to express

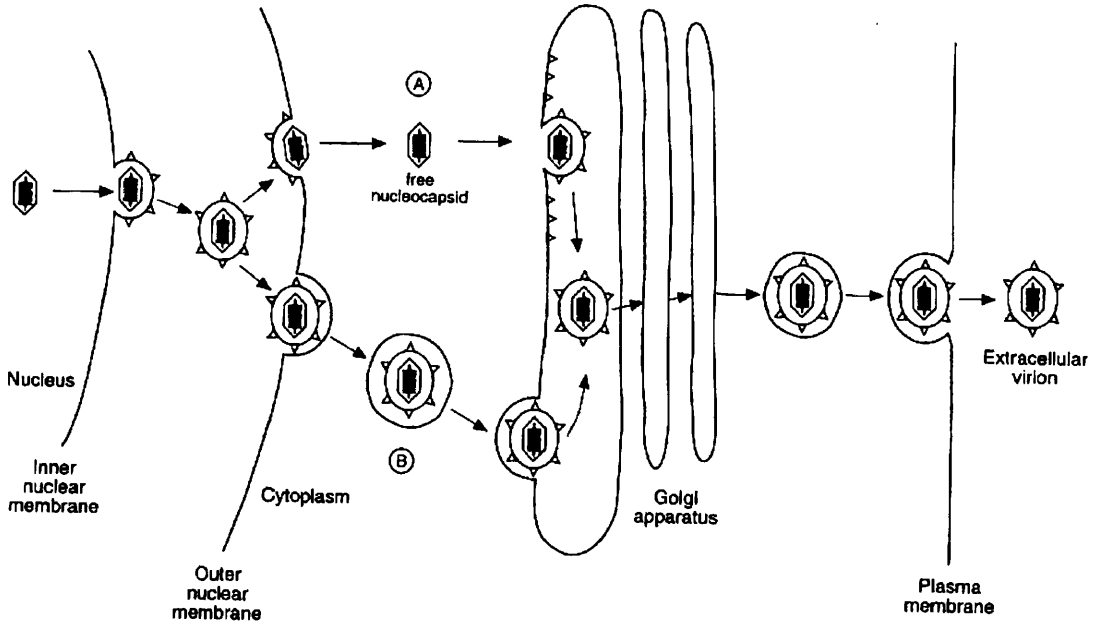


Figure 1.2.10A. *Schematic illustration of the hypothesised routes of virion egress from the cell nucleus.*

Pathway A - envelopment, de-envelopment, and re- envelopment (EDR) pathway. This involves the enveloped particle in the inter-membrane space of the nuclear envelope becoming de-enveloped by fusing with the outer nuclear membrane, causing release of the free nuclear capsid into the cytoplasm. This nucleocapsid becomes enveloped by fusion to the Golgi apparatus. **Pathway B**- The lumenal pathway. This involves the enveloped particle in the inter-membrane space of the nuclear envelope budding into a cytosolic vesicle. The vesicle moves or is transported to the Golgi apparatus where it fuses with the Golgi membrane, releasing an enveloped particle into the lumen of the Golgi apparatus. At this point the two pathways converge. Pathway A predicts that the virion envelope derives from the Golgi apparatus membrane while pathway B predicts that the virion envelope derives from the inner nuclear membrane. The diagram was adapted from Knipe, et al. 1996.

either the VP5 or VP23 capsid proteins appear to be unable to cleave concatemeric DNA (Desai *et al.*, 1993). In addition to the major components of the procapsid, several DNA packaging proteins (UL6, UL15, UL17, UL25, UL28, UL32 and UL38) are necessary for cleavage and packaging of concatemeric HSV-1 DNA. Studies have shown that UL6 protein is localized at a single vertex of the capsid and purified UL6 product forms a dodecameric ring and probably acts as portal protein, which acts as a docking site for the other components of the DNA-packaging machinery and the viral genome (Newcomb *et al.*, 2001). This is further supported by research that showed UL6 interacts with UL15 and UL28, proteins that are thought to also play a role in establishing a functional DNA packaging complex (White *et al.*, 2003). Studies have also suggested UL25 plays an important role in generation of capsids containing DNA (Addison 1984). It is thought to occur during the later stages of infection when the DNA is added to the capsid, prior to their release into the cytoplasm (Stow, 2001), with UL25 acting to ensure the DNA is retained in the capsids (McNab *et al.*, 1998). Additionally UL25 has been shown to interact with specific capsid proteins and viral DNA, thereby anchoring the viral DNA to the capsid (Ogasawara *et al.*, 2001). The exact mechanism by which DNA enters the capsid has yet to be identified. However, the structure of the DNA within virions would suggest that the DNA is packaged by a spooling mechanism forming coiled layers (Zhou et al., 1999). During infection the DNA is thought to be released from the capsid in a reverse process, whereby the DNA uncoils from the centre outwards (Zhou *et al.*, 1999).

1.2.11. Tegument acquisition and virion egress.

Following DNA packaging, the HSV-1 nucleocapsid acquires an envelope by budding through the inner nuclear membrane, but it is uncertain whether this envelope is retained during virus maturation and egress or whether mature progeny virions are derived by de-envelopment at the outer nuclear membrane followed by re-envelopment in a cytoplasmic compartment (Skepper *et al.*, 2001). Although the exact mode of tegument and envelope acquisition is not known, there are currently two popular models to describe this stage of the reproductive cycle. An overview of both pathways is shown in figure 1.2.10A. One model, the luminal pathway, proposes that capsids acquire an envelope at the inner nuclear membrane, with subsequent modifications to the tegument and envelope glycoproteins occurring as the virion exits the cell through the Golgi apparatus by the exocytic pathway. Research supporting this hypothesis includes a

comparative ultra-structural study showing that after intranuclear assembly, progeny nucleocapsids exit by budding at the inner leaflet of the nuclear membrane into the perinuclear space, resulting in acquisition of a primary envelope (Granzow *et al.*, 2001).

The second model, the envelopment, de-envelopment, and re-envelopment (EDR) pathway, proposes that capsids exiting the nucleus acquire an envelope by budding through the inner nuclear membrane into the perinuclear space. These membrane bound capsids are then de-enveloped at the outer nuclear membrane and naked capsids are released into the cytoplasm. Capsids acquire a tegument on route to the Golgi apparatus where they are re-enveloped and acquire membrane-associated proteins before exiting the cell via an exocytic pathway. Research to support this model includes a study using immunogold labelling to track the distribution of gD that was expressed in conjunction with an endoplasmic reticulum retention signal. Most ER-tagged gD was found between the inner and outer nuclear membranes, whereas very little of the protein was present on the extracellular virus particles. This implies that the primary envelope acquired during egress through the nuclear membrane had been lost and the mature virion must have obtained an envelope from a post-ER cytoplasmic compartment (Skepper *et al.*, 2001).

To add further confusion to which is the correct model of virion egress, electron microscopy studies carried out previously to the ones mentioned above supported both models, as enveloped capsids were identified within cytoplasmic vesicles and naked capsids were observed in the perinuclear space (Campadelli-Fiume *et al.*, 1991; Steven & Spear, 1997). More detailed reviews of virion maturation and egress can be found elsewhere (Roizman & Sears, 1996).

1.3.0. Latent infection.

A key characteristic of HSV-1 is that it has two states of infection, lytic and latent. The virus can establish a latent infection in the ganglion tissue of sensory neurons that lasts for the lifetime of the host. All persons seropositive for HSV-1 harbour a latent infection.

Unlike a persistent lytic infection, during latency, no viral progeny are produced and only very limited amounts of gene transcription can be detected. Following primary infection at an oral or genital mucosal surface, the virus travels along the innervating neuronal axon to the neuronal cell body (see figure 1.3.0A). Once within the neuron, the virus enters a quiescent state in which the lytic gene products are not produced. In a subset of seropositive individuals, clinically apparent re-activation events occur periodically, and virus, or at least viral genetic information, is thought to pass intra-atonally to neuroepithelial junctions where infection of epithelial cells results in productive infection and, ultimately, lesions typical of a reactivated infection (Preston, 2000) (see figure 1.3.0A).

The establishment of latent herpesvirus infections can be essentially viewed as a passive phenomenon i.e. it requires no viral gene expression or gene products, as replication-defective virus can establish latent infections (Garber *et al.*, 1997). Virus resides in latently infected ganglia in a non-replicating state, with expression of the lytic genes being extremely low or undetectable (Wagner *et al.*, 1995). This is in keeping with the fact that latent HSV genomes are harboured within the nucleus of a non-dividing sensory neurone and do not need to replicate; indeed the challenge arises from the need for the virus to reactivate from a transcriptionally quiescent, non-replicating cell (Wagner *et al.*, 1995). This transcriptional silence may allow the virus to remain hidden in the cell, thus avoiding immunodetection.

Certain stimuli can reactivate the virus from the latent state. Following these stimuli, newly synthesised virus follows axons back to the peripheral site and replication proceeds at the skin or mucous membranes (Mellerick & Fraser, 1987; Rock & Fraser, 1985). The signals and mechanisms involved in this process are poorly understood, but it appears that certain physical stresses, such as illness or exposure to ultraviolet light,

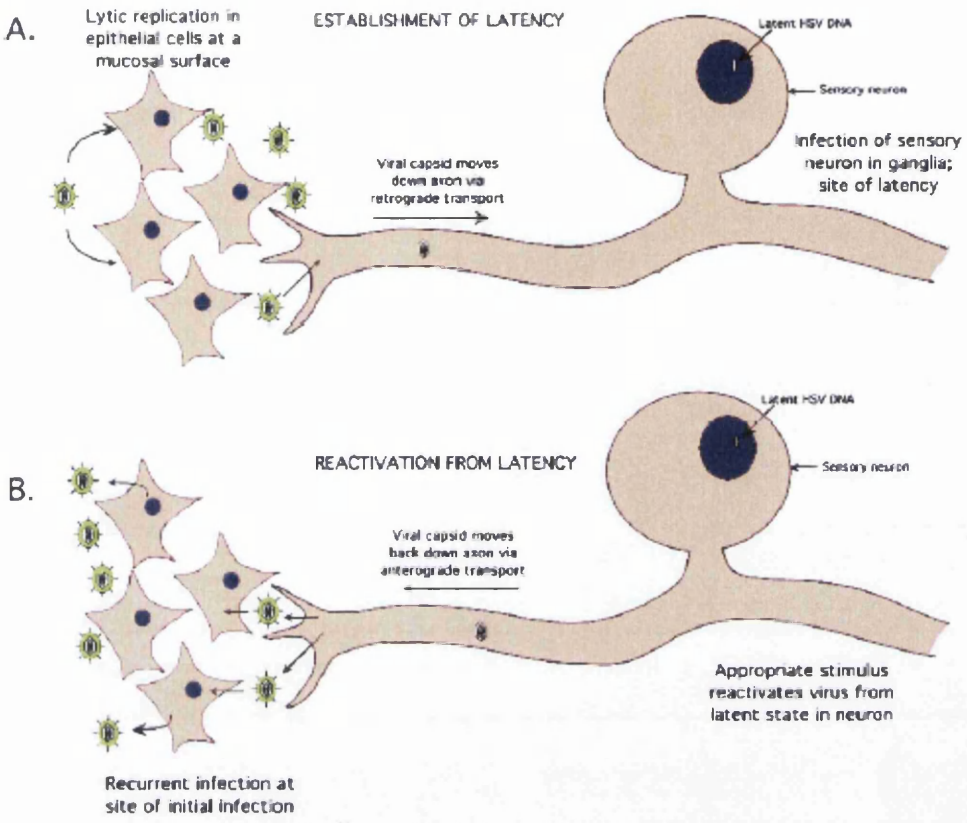


Figure 1.3.0A. *Schematic illustration of the establishment and reactivation of HSV-1 latent infection.*

(A). HSV-1 replicates initially in epithelial cells at the genital or oral mucosal surface. The virus enters innervating sensory neurons where it travels up the axon to the neuronal cell body. Once in the neuron, the virus establishes a latent infection that may last the lifetime of the host.

(B). Upon the proper stimulus, the virus can reactivate, travel back down the axon, and establish another round of lytic replication.

Diagram adapted from Taylor, *et al.* 2001.

increase the chance of reactivation. Spontaneous reactivation occurs in spite of specific humoral and cellular immunity in the host. However, the body's immune system limits local virus replication so that recurrent infections are less extensive and severe, furthermore reactivation can be of a symptomatic or an asymptomatic nature (Wagner *et al.*, 1995)

1.3.1. The role of latency associated transcripts (LAT).

Within the latently infected cell, the alphaherpesvirus genome is maintained as a nucleosomal, circular episome. Low levels of genome replication might occur or be necessary for the establishment or maintenance of a latent infection from which virus can be efficiently reactivated (Mellerick & Fraser, 1987; Rock & Fraser, 1985). Latent infection with a member of the alphaherpesvirus family can be viewed as having three separable phases: establishment, maintenance and reactivation. In the establishment phase, the virus must enter a sensory neurone, and following entry, there must be a profound restriction of viral gene expression so that the cytopathic results of productive infection do not occur (Preston, 2000).

Thus, productive cycle genes are transcriptionally and functionally quiescent and only a 8.3 Kbp latency associated transcript (LAT) is expressed and accumulates to low levels in latently infected neurons. However, the 2.0 and 1.5 Kbp introns derived from the 8.5 Kbp are abundant and very stable (Krause *et al.*, 1988; Rock *et al.*, 1987; Stevens *et al.*, 1987). The expression of LAT is controlled by the latency specific promoter, which has a number of regulatory elements important in neuronal expression over extended periods. Currently, no LAT-encoded protein has been conclusively demonstrated to exist, therefore it is thought that LAT transcripts may have other functions or activities. It has been shown that LAT negative viruses have increased immediate early gene expression in neurons, suggesting that LATs may limit viral gene expression (Garber *et al.*, 1997) and promote a latent state. An alternate view is that LATs are an antisense mechanism, because most of the 8.3-kb LAT transcript is transcribed from the long terminal repeat region of the viral genome and its transcription continues into the short terminal repeat region. On the opposite DNA strand to that of the LATs is the gene that encodes the immediate early regulatory protein ICP0. The LAT transcript extends across the ICP0 coding region and continues to just downstream from the termination of the ICP4 coding region (see figure 1.3.1A) (Chen *et al.*, 1997). It has been proposed that

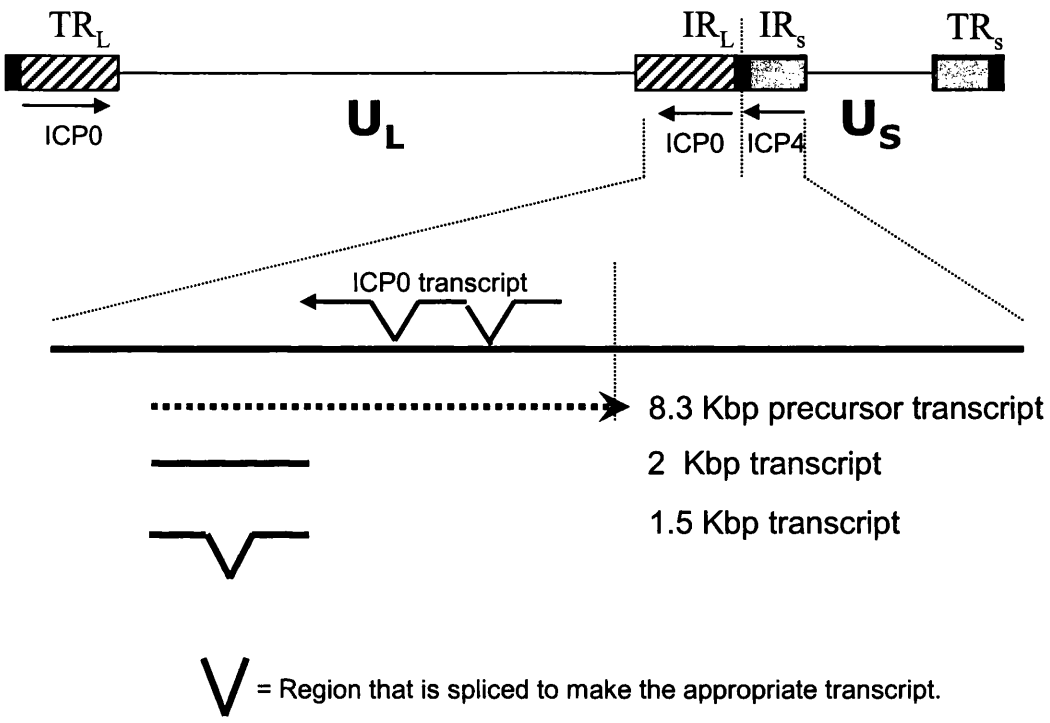


Figure 1.3.1A. *Schematic illustration of the establishment of HSV-1 latent infection.*

Location of transcripts in the long repeat region of HSV-1 DNA. The internal repeat of HSV-1 is depicted, although the same gene arrangement exists in the terminal repeat. The horizontal line below represents the relative position of sequences within the ICP0/LAT region. The various LAT transcripts are shown. See text for further details.

LATs prevent the induction of the lytic cycle by acting as anti-sense RNA to block the expression of ICP0 (Stevens *et al.*, 1987). This theory is supported by transient transfection assays, which suggested that LATs inhibit the transactivating ability of ICP0 (Farrell *et al.*, 1991). Although studies carried out using a LAT deletion mutant were unable to detect an increase in levels of the ICP0 transcript in infected cells (Chen *et al.*, 2002b). However, it has recently been suggested that LAT may play a role in controlling the expression of ICP0 at the post-transcriptional level during HSV-1 reactivation from latency (Thompson *et al.*, 2003).

The view that LAT are in some way involved in establishing latent infection and preserving the primary nervous system is supported by reports that have suggested in rabbit trigeminal ganglia and certain animal models that LAT negative viruses more efficiently initiate lytic infection than LAT positive viruses, (Perng *et al.*, 2000a; Perng *et al.*, 2000b). Interestingly, it has been recently shown that no difference in the induction of apoptosis could be observed between a LAT exon 1 deletion virus and wild type. However, when the deletion was extended to include the 5' end of 2.0 Kbp LAT, the number of infected cells undergoing apoptosis increased (Ahmed et al., 2002). The potential anti-apoptotic role of LAT is further supported by research that showed that following infection of a HSV-1 mutant, where the LAT promoter and part of the 5' end of the LAT primary transcript had been deleted, there was an increase in neuronal cell death during the acute stage of infection of mice (Perng *et al.*, 2000b). In contrast, using the same mutant virus when compared to wild-type, it was reported that there was not a significant increase in the level of neuronal death, in fact only a very few cells were undergoing apoptosis (Thompson & Sawtell, 2000, 2001). Therefore, the role of LAT in protecting neurones from apoptosis remains a controversial issue.

The molecular basis of reactivation is not known. Stimuli that are effective in inducing reactivation in human subjects include axonal injury, fever, physical or emotional stress and exposure to ultraviolet light. Expression of only a 350 bp region of LAT near its 5' end is both necessary and sufficient to facilitate reactivation in several animal models (Davido & Leib, 1996). Thus, viruses with deletions in the transcription unit do not re-activate as efficiently as the wild type following the induction process (Chen *et al.*, 1997).

It has been observed from the analysis of infected mice trigeminal ganglia that a small number of cells could be detected that were expressing productive cycle genes during latent infection (Feldman *et al.*, 2002). It was proposed that this may represent a population of cells where reactivation can occur to allow low levels of gene expression, but these are insufficient to initiate a recurrent infection (Feldman *et al.*, 2002). The significance of this observation remains unclear. The role of LATs has been reviewed in more detail elsewhere (Kent *et al.*, 2003; Preston, 2000).

Chapter 1- Part II – A review of the biological properties of the immediate early protein ICP0.

1.4.0. Introduction to ICP0.

ICP0 is a member of the HSV-1 immediate early class proteins and its expression is initiated by the trimeric association of VP16, Oct-1 and the cellular protein HCF (see figure 1.2.4A). It has a multi-functional role during both the lytic and latent stages of HSV-1 infection. Key features of ICP0 and its various biological functions are outlined below.

1.4.1. The IE-1 gene.

The gene encoding ICP0 (IE-1 or α0) is diploid and maps to the inverted repeats, which flank the unique sequences of U_L, IR_L and TR_L (see figure 1.2.4A). The IE-1 gene encodes a protein of 775 amino acids in size and the primary IE-1 transcript is only one of four to be transcribed from the HSV-1 genome that includes introns and becomes spliced (Perry *et al.*, 1986). The two introns are between codons 19 and 20, and 241 and 242 in the ICP0 open reading frame (Perry *et al.*, 1986). The intron 1 RNA is 767 bp in size (Perry *et al.*, 1986) and has been shown to accumulate in the cytoplasm and contain elements that may regulate the expression of the gene (Gu *et al.*, 1995b; Poon *et al.*, 2002). Both introns contain in-frame stop codons, therefore if alternate or incomplete splicing of the primary transcript occurs it is possible that multiple ICP0 related protein products could be generated. If the first or second intron of ICP0 was incompletely spliced this would cause the premature termination of translation, thus generating a product of either 72 or 262 residues in size. Studies were carried out using a mutant strain of HSV-1, which only contained intron 2. It was shown the 262 residue product of an unspliced transcript that includes intron 2 could be detected at low levels in BHK cells infected with wild type virus. Additionally, low level accumulation of the 262 residue product is cell type dependent and does not occur following infections with viruses that have lost intron 1 and 2 or intron 2 alone (Everett *et al.*, 1993b).

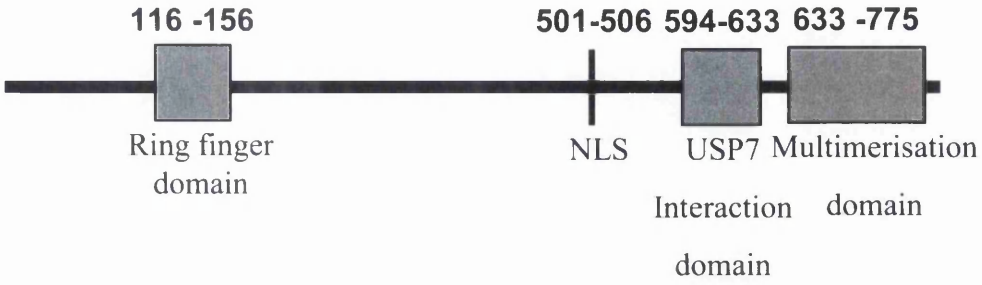
The ICP0 262 residue protein is also known as ICP0R and is thought to function as a promiscuous repressor of transcriptional activation by ICP0 and heterologous transcriptional activators (Weber *et al.*, 1992), potentially by titrating cellular factor(s) away from ICP0 (Spatz *et al.*, 1996). Furthermore, ICP0R has been shown to be modified by ubiquitination (Weber *et al.*, 1999), although its mechanism of action is not yet fully understood.

1.4.2. The structure of the ICP0 protein.

The ICP0 protein contains 775 a.a. residues and has a predicted molecular weight of 78 KDa, although its electrophoretic mobility in denaturing gels indicates that its size is approximately 110 kDa (Heine *et al.*, 1974). The variation between the predicted and actual size is thought to be due to phosphorylation of a serine rich region between residues 554-591 (Ackermann *et al.*, 1984), but it is also due to the nature of the intrinsic amino acid composition and sequence of the protein. Phosphorylation is carried by a number of proteins including the viral protein kinase UL13 (Ogle *et al.*, 1997) and the cell cycle kinase cdc2 (Advani *et al.*, 2001; Advani *et al.*, 2000). Throughout the infection cycle ICP0 becomes sequentially phosphorylated and it has been proposed that phosphorylation status correlates with the cellular localisation of the protein (Advani *et al.*, 2001). Phosphorylation is also thought to play an important role in determining the activity of the protein, such as in gene expression assays (Davido *et al.*, 2002). Other post-translational modifications of ICP0 include nucleotidylation (Blaho *et al.*, 1993) and ubiquitination (Canning *et al.*, 2004). Post-translational modification of ICP0 will be discussed in further detail later in this section.

ICP0 carries out various functions within the cell, a result of containing multiple functional domains (see figure 1.4.2A). Mutational studies have shown that ICP0 includes a basic a.a. cluster that acts as a nuclear localization signal (residues 501 to 506) (Everett, 1988a); an USP7 interaction domain (Everett *et al.*, 1997; Meredith *et al.*, 1995; Meredith *et al.*, 1994); a multimerisation domain (residues 633 to 775) (Ciufo *et al.*, 1994; Everett *et al.*, 1991) and a C_3HC_4 zinc-binding RING finger motif (between residues 116 and 156) which is crucial for the majority of ICP0 biological functions (Barlow *et al.*, 1994). The cysteine-histidine motif classifies the protein as a member of the RING finger family and this motif is conserved within the ICP0 family of proteins throughout the alphaherpesvirus family. Furthermore, ICP0 also shares homology with

A.



B.

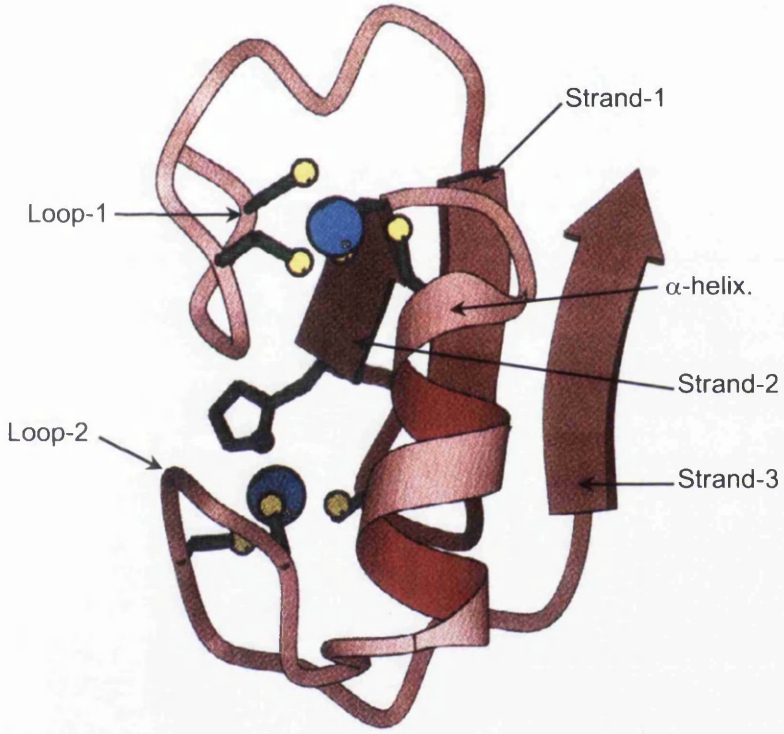


Figure 1.4.2A. *Schematic illustration of the functional domains within ICP0 and the structure of the RING finger domain of Eg63.*

A) The relative position of the functional domains of ICP0 are illustrated. B) The structure of the RING finger domain Eg63 was determined by NMR. The coordinating zinc residues are shown in blue and the cysteine and histidine residues in yellow. The secondary structure features of the RING finger domain are also shown.

RING finger domains in proteins expressed by a wide range of other organisms (Freemont, 1993). Recently, downstream of the ICP0 RING finger domain and before the nuclear localisation signal, three segments have been mapped to contain dominant phosphorylation sites (Davido and Schaffer, in press). The various domains of ICP0 are discussed in more detail in the following section.

1.4.3. The structure of the RING finger domain of ICP0.

The presence of the conserved cysteine – histidine zinc chelating domain attributes ICP0 to the RING finger family of proteins. The term RING is an acronym for 'really interesting new gene' as the domain was initially identified in the protein encoded by the human gene, RING1 (Lovering *et al.*, 1993). The consensus sequence for RING finger motifs is $C\text{-}X_2\text{-}C\text{-}X_{(9\text{-}39)}\text{-}C\text{-}X_{(1\text{-}3)}\text{-}H\text{-}X_{(2\text{-}3)}\text{-}C\text{-}X_2\text{-}C\text{-}X_{(4\text{-}48)}\text{-}C\text{-}X_2\text{-}C$, where X denotes any amino acid (Freemont, 1993). The alphaherpesvirus EHV-1 ICP0 homologue, Eg63, was used to resolve the structure of the RING finger domain (see figure 1.4.0A). The NMR analysis of the secondary structure of the homologue showed, starting from the N-terminus, that this motif consists of an irregular loop, two strands of β-sheet, two turns of an α-helix (two zinc atoms are present, one at either end) and a second irregular loop and the third strand of β-sheet (Barlow *et al.*, 1994; Everett *et al.*, 1993a).

1.4.4. The RING finger protein family.

RING finger domains can be found in a large number of proteins expressed by different species ranging from yeast to mammals and including double-stranded DNA viruses (Freemont, 2000). In many cases so far analysed, RING finger proteins play a crucial role in ubiquitin conjugation pathway which is thought to reflect their absence in prokaryotes as they do not utilise this biochemical pathway (Freemont, 2000). RING finger proteins are also involved in a range of cellular processes including development, oncogenesis, apoptosis, and viral replication (Borden & Freemont, 1996). However, the first protein to implicate RING finger domains as E3 ubiquitin ligase enzymes and thus play a crucial role in the ubiquitin conjugation pathway was c-Cbl, which was shown to undergo a protein-protein interaction with components of the ubiquitination conjugation pathway (Joazeiro *et al.*, 1999). From the initial identification, a growing number of RING finger proteins with similar functions have been identified.

1.4.5 The role of the RING finger domain of ICP0 in HSV-1 infection.

The various biological properties of ICP0 that have been observed over the years have largely been shown to be RING finger dependent. In cell culture, an ICP0 RING finger negative virus (FXE) has been shown to have greatly reduced growth characteristics causing a 1000-fold reduction in the yield of progeny virus (Everett, 1989). Furthermore, FXE is unable to reactivate HSV-2 latent genomes *in vitro*, thus highlighting the important role the RING finger domain plays in reactivation (Harris *et al.*, 1989). Also using an *in vitro* neuronal model it was shown the ability of FXE to establish a latent infection was impaired by approximately 10-fold compared to wild-type virus, as measured by cellular expression of the LATs and also the amount of viral DNA in neuronal culture during latency (Wilcox *et al.*, 1997). The role of ICP0 in HSV-1 infection is discussed in detail later in this section.

1.4.6. The RING finger domain is crucial for the functions of ICP0.

ICP0's ability to act as a transactivator of HSV-1 gene expression is dependent on the presence of its RING finger domain (Cai & Schaffer, 1989; Everett, 1987, 1988a). Furthermore, studies have shown that mutation of lysine 144, glutamine 148 and asparagine 151 within the RING finger domain reduces the transactivation ability of ICP0 (Everett *et al.*, 1995a). The mutation of lysine residue 144 had the most deleterious affect, causing a reduction in the transactivation ability of the protein to levels similar to the RING finger mutant FXE. It was concluded the alpha helix region of the RING finger domain must be crucial for ICP0's transactivation ability (Everett *et al.*, 1995a). The role of ICP0 as a transactivator of gene expression will be discussed in further detail later in this section.

1.4.7. The ICP0 RING finger domain and its role in the degradation of cellular proteins.

During the early stages of HSV-1 infection ICP0 localises to and disrupts cellular nuclear sub-structures called PML nuclear bodies or ND10 (Maul *et al.*, 1993). The ability of ICP0 to disrupt ND10 was shown to be RING finger dependent, although

localisation of ICP0 to ND10 was not (Maul & Everett, 1994). Disruption of ND10 by ICP0 is caused by the RING finger dependent degradation of cellular proteins PML and Sp100. Centromere proteins CENP-C and CENP-A are also degraded in response to ICP0, resulting in the disruption of centromeres (Everett *et al.*, 1999a; Everett *et al.*, 1999b; Lomonte *et al.*, 2001; Parkinson *et al.*, 1999). The effect of ICP0 on cellular proteins is described in more detail in section 1.5.11.

1.4.8. ICP0 nuclear localisation signal.

The nuclear localisation signal (NLS) of ICP0 is a highly basic region encoded by residues 501-506 and is similar to the SV40 large T antigen NLS. Its activity was deduced from deletion studies, which showed that its removal caused the retention of ICP0 in the cytoplasm (Everett, 1988a). Further studies showed that insertion of a short oligopeptide (VRPRKRR) at amino acid residue 500 in an ICP0 NLS deletion mutant causes restoration of wild type characteristics (Mullen *et al.*, 1994). However, residues flanking the nuclear localisation signal on its C–terminal side were also shown to be crucial for NLS activity (Everett, 1988a). Additionally, ICP0's ability to act as a transactivater of gene expression in the presence of ICP4 was also reduced by removal of the NLS (Everett, 1988a). Studies using a virus that expressed a mutant form of ICP0 containing a NLS deletion exhibited a reduction in synthesis of viral proteins, formation of plaques (in tissue culture) and the level of progeny virus (Mullen *et al.*, 1994).

1.4.9. Multimerisation domain of ICP0.

Glycerol gradient studies have shown purified ICP0 exists as a dimer or higher order oligomer in solution (Everett *et al.*, 1991). The region 617-712 of ICP0 was shown to be crucial, while adjacent residues enhanced multimerisation activity (Ciufo *et al.*, 1994). Studies carried out elsewhere mapped the multimerisation domain to a similar region, between residues 633-755 (Meredith *et al.*, 1995). Deletion of residues 633-680 from ICP0 when expressed from a HSV-1 mutant, caused a diffuse nuclear staining pattern of the protein instead of the normal punctuate foci (Maul & Everett, 1994). The C-terminal deletion of the multimerisation domain of ICP0 reduced its ability to act as a transactivator of gene expression (Everett, 1988a). Furthermore, a series of mutant HSV-1 viruses containing deletions of the multimerisation domain exhibited reduced

growth efficiency compared to wild type. It was concluded the C-terminal region of ICP0 is important for viral gene expression and growth (Everett, 1989).

1.4.10. The USP7 interaction domain of ICP0.

Following HSV-1infection of multiple cell types, ICP0 has been shown to bind to a 135 KDa cellular protein (Meredith *et al.*, 1994), via residues 594-633 in the C-terminal region of ICP0 (Meredith *et al.*, 1995). The cellular protein was later identified to be ubiquitin specific protease 7 (USP7), a member of a large family of enzymes that cleave ubiquitin from either alpha- or isopeptide linked chains (Everett *et al.*, 1997),

Studies using HSV-1 strains that contained mutations within this domain exhibited impaired growth compared to wild-type HSV-1 in cell culture (Meredith *et al.*, 1995). Transfection studies showed mutations of the USP7 binding motif reduced ICP0's ability to act as a promiscuous transactivator of gene expression, when compared to the wild-type protein (Everett, 1988a; Everett *et al.*, 1999d). Mutation of the USP7 binding domain, in particular of either of two lysine residues at positions 620 and 624 of ICP0 severely impairs ICP0 binding efficiency to USP7 and growth of virus carrying these lesions in cell culture, suggesting that the interaction between ICP0 and USP7 is biologically relevant (Everett *et al.*, 1999d).

Interestingly, even though the USP7 domain of ICP0 contributes to the efficiency of viral growth, it is only conserved in HSV-1, HSV-2 and HVB, may be a reflection of the viruses being closely related. No other member of the alphaherpesvirus ICP0 family of proteins where detailed sequence information exists has been so far identified to contain this domain. The biological significance of the relationship between ICP0 and USP7 is discussed in further detail in section 1.5.7.

1.5.0. The role of ICP0 in HSV-1 infection.

As our understanding of ICP0 has developed, it has become apparent that it is a multifunctional protein. It has been implicated to have crucial roles in viral growth, gene expression, reactivation from latent or quiescent infection and interaction with an ever-growing list of cellular proteins. The aim of this section is to review the diverse cellular roles of ICP0.

1.5.1. ICP0 is not essential for HSV-1 growth following high multiplicity infection.

To understand the biological function of ICP0 it is necessary to characterise the phenotype of an ICP0-null virus. Various studies have attempted to characterise the phenotype of an ICP0-null virus, the aim of this section is to outline these findings.

ICP0 is required for low multiplicity infections.

One of the first studies carried out used the HSV-1 mutant dl1403, which contains a 2 Kbp deletion in both the TR_L and IR_L copies of the ICP0 gene. The mutant virus was shown to infect BHK cells, however the apparent titre of the progeny virus was 20 to 100-fold lower than that of wild type HSV-1 (Stow & Stow, 1986). However, following high M.O.I. infection (5 p.f.u./cell) with dl1403 it was shown that the efficiency of viral protein production was quantitatively similar to that of wild type HSV-1, as was replication and encapsidation of the virus (Stow & Stow, 1986). The difference in phenotype of dl1403 to wild-type HSV-1 was most noticeable following infection carried out at a low multiplicity and in certain cell types, as at a high M.O.I. the phenotype of the mutant was similar to that of wild type HSV-1 (Stow & Stow, 1986). Following low M.O.I., it was shown that dl1403 displays reduced plaque forming efficiency, in particular in human foetal lung cells (HFL) and less so in BHK cells and Vero cells (Stow & Stow, 1986). The conclusions from these studies were supported by similar findings from research that was carried out elsewhere (Sacks & Schaffer, 1987). The work carried out by both research groups implies firstly, that ICP0 has a cell type dependent affect, being required in different cells to varying degrees. Second, a multiplicity dependent affect, being essential for progression of infection at low M.O.I., but not at a high M.O.I. Importantly, these studies identified for the first time that ICP0-

null viruses exhibit a high particle to plaque forming unit ratio (Sacks & Schaffer, 1987; Stow & Stow, 1986).

Later studies examined the growth characteristics of mutants with defined lesions in the ICP0 gene. The mutant viruses were studied in single-step growth curve experiments, by assaying for plaques in a variety of cell types and by analysis of viral polypeptide synthesis during productive infection at high and low multiplicities (Everett, 1989). At low multiplicity, mutations in ICP0 reduced viral gene expression in certain cell types, in particular HFL cells. Mutations within the RING finger domain had the most dramatic affect on the growth characteristics. However, at a high M.O.I. the mutant viruses exhibited a phenotype similar to that of wild type virus. It was suggested that following low multiplicity infections of cells in culture the inactivity of ICP0 leads to failure to progress into lytic infection in certain cell types (Everett, 1989).

The requirement for ICP0, following low multiplicity infections was further supported elsewhere. It was shown in Vero cells transfected with infectious ICP0-null mutant viral DNA, virus production was delayed for 2 days and the levels of de novo synthesis of viral proteins were reduced. Furthermore, at low M.O.I. (0.004 p.f.u./cell) it was shown that 90% or more of the coding sequence of ICP0 was required for efficient replication of HSV-1 (Cai & Schaffer, 1989).

In conclusion, the aforementioned studies highlight that ICP0 is essential for low multiplicity infection, however this defect can be overcome at high multiplicity of infection. Furthermore, once an ICP0-null virus has entered lytic infection, viral gene expression and yield of progeny particles are similar to those of wild-type HSV-1 (Cai & Schaffer, 1992; Everett, 1989; Stow & Stow, 1986). This suggests that a high dose of ICP0-null viral genomes can overcome any potential cellular repression mechanism that would normally inhibit the same infection at a low multiplicity, thereby not able to prevent the cell committing to the lytic cycle.

ICP0-null virus replicates as efficiently as wild-type HSV-1 in U20S cells.

Following low multiplicity infection of the osteosarcoma cell line U2OS, an ICP0-null mutant virus exhibits growth characteristics similar to that of a wild type virus (Yao & Schaffer, 1995). Compared with Vero cells, U2OS cells significantly enhanced the

plaque-forming efficiency of an ICP0-null mutant by over 100-fold. In contrast, the plaque-forming efficiencies of wild-type virus in both cell types remained similar (Yao & Schaffer, 1995). Further analysis showed the IE and E promoters, (but not the L promoters) were responsive to a cellular activity in U2OS cells, as they exhibited increased basal levels of expression (Yao & Schaffer, 1995). These results suggested that there is cellular activity in U2OS cells that can substitute for the function of ICP0 at low M.O.I. Alternatively, there may be cellular repression mechanisms, which are normally targeted by ICP0 in other cell types, e.g. HFL cells that are not present to inhibit the progression of infection in U2OS cells. Furthermore, these studies highlight that an ICP0-null virus is intrinsically as infectious as wild-type virus. However, in restrictive cell lines such as HFL or Vero cells, the probability that a potentially infectious ICP0-null mutant virus particle will initiate plaque formation is greatly reduced. Consequently, the majority of cells harbour the viral genome in a quiescent state; however, the addition of ICP0 can cause their reactivation.

1.5.2. The concept of threshold in ICP0-null mutant HSV-1 infections.

A recent study has identified the importance of threshold when characterising the phenotype of an ICP0-null virus (Everett *et al.*, 2004). Initially the titres of an ICP0-null mutant virus and wild-type HSV-1 were determined on U2OS cells, as the absence of ICP0 from the mutant virus does not impair its growth in this cell line; therefore, an equivalent titre on the basis of potentially infectious virus particles for the two viruses can be obtained (Everett *et al.*, 2004).

The particle to p.f.u. ratio of an ICP0-null virus was shown to be about 500 times greater in HFFF-2 human fibroblast cells than in U2OS cells. This reflects a much reduced probability that a potentially infectious mutant virus particle will initiate a productive infection in the restrictive cell type (Everett *et al.*, 2004). However, when HFFF-2 cells were infected with wild-type HSV-1 and ICP0-null virus at multiplicity of 10 p.f.u. per cell (based on titres in U2OS cells), equivalent levels of progeny virus and gene expression could be detected. This was despite the fact that 10 p.f.u. per cell of the wild type and mutant viruses equates to about 0.5 and only 0.01 p.f.u. per cell, respectively, if the titrations had been conducted in HFFF-2 cells (Everett *et al.*, 2004). However, if the infections were performed at 1 p.f.u. per cell (based on titres in U2OS cells), viral gene expression and the production of viral progeny were shown to proceed

much more efficiently in a wild-type HSV-1 than in the ICP0-null mutant virus infection. Therefore, the 500-fold defect in plaque forming efficiency of an ICP0-null mutant virus does not mean that 500-fold more virus is required to initiate a productive infection, as viral expression and progeny virus production of the ICP0-null virus occurs at a similar efficiency to that of the wild type virus, if the multiplicity is above a certain threshold, which in the case of HFFF-2 cells is about 10 U2OS cell p.f.u. per cell. Below this threshold the phenotype of the ICP0-null mutant virus is increasingly exemplified (Everett *et al.*, 2004). Overall it was concluded that the multiplicity-dependent defect of ICP0-null mutant HSV-1 is not linear and that there is a broad threshold of input virus above which infection becomes ICP0 independent (Everett *et al.*, 2004). This threshold was shown to vary between cell types. When an ICP0-null mutant virus is used to infect cells below threshold, the true consequences of an ICP0-dependent infection can be studied. However, above the threshold, the infection becomes ICP0-independent and the rate of the developing infection is proportional to the viral input copy number.

The phenotype of an ICP0-null virus.

It was originally shown the number of Vero cells that were infected by an ICP0-null mutant viruses, as judged by expression of ICP4, far exceeded the number expected on the basis of the input p.f.u. (based on titrations in Vero cells) (Cai & Schaffer, 1992). Similarly, from examining the fate of individually infected cells, it was shown that even in the most restrictive cell type, HFFF-2, the number of cells expressing viral proteins following a low M.O.I. infection with ICP0-null mutant HSV-1 dl1403 far exceeds the number of cells that enter productive infection (Everett *et al.*, 2004). Further analysis of the non-productively infected cells revealed viral proteins could be detected for extended periods, in particular, ICP4 levels were similar to those in productively infected cells (Everett *et al.*, 2004). The non-productively infected cells were assigned to four classes: quiescent, with no detectable viral protein expression; stalled and expressing an apparently incomplete set of IE proteins; stalled at the IE stage; or stalled at a stage at which expression of some early proteins has occurred but DNA replication has not been initiated (Everett *et al.*, 2004). This study highlights that cells infected with an ICP0-null virus can undergo a multitude of fates. Therefore single-cell assays at a low MOI may provide a more realistic description of the phenotype of an ICP0-null virus than methods that utilise whole cell population approaches (Everett *et al.*, 2004).

1.5.3. ICP0 is a transactivator of gene expression.

The first significant discovery in understanding the role of ICP0 in HSV-1 infection, was the identification that it acts as a potent transactivator of gene expression in transfection assays (Everett, 1984). These assays were important in identifying the synergistic relationship between ICP4 and ICP0. Alone ICP4 could activate the gD early promoter, however in the presence of ICP0 the level of activation was increased 20-fold. Besides these studies, ICP0 was shown to able to independently transactivate all three classes of HSV-1 genes, including the thymidine kinase promoter (Chen *et al.*, 1991; Everett, 1984; Gelman & Silverstein, 1985; O'Hare & Hayward, 1985; Quinlan & Knipe, 1985).

Later studies showed that ICP0 is capable of being a promiscuous transactivator of gene expression. A variety of promoters other than those of HSV-1 were reported to be transactivated by ICP0, these include the SV40 early promoter, HSV-2 28K promoter, the HIV long terminal repeat and the long terminal repeat of human endogenous retrovirus K (Everett, 1988b; Kwun *et al.*, 2002; Mosca *et al.*, 1987; O'Hare & Hayward, 1985). Interestingly, the cellular rabbit ϵ-globin and human β-globin promoters have also been reported to be transactivated by ICP0 (Everett, 1985). The analysis of the diverse promoter types transactivated by ICP0 has not led to the identification of a consensus motif that is required for ICP0 to activate gene expression, but instead any reporter cassette with a promoter containing elements recognised by the basal RNA polymerase II transcription machinery appears to be responsive to ICP0. This implies that ICP0 does not act by binding directly to the DNA, this is further supported by research that showed that ICP0 does not form a stable complex with DNA in solution (Everett *et al.*, 1991). Later studies analysed the level by which ICP0 activates viral gene expression by comparing the activation level of specific promoters from all three classes of HSV genes. Using CAT and RNase protection assays, it was shown that ICP0 activates gene expression by increasing the amount of mRNA synthesis, but not translation (Jordan & Schaffer, 1997). These results indicated ICP0 must target a shared mechanism or acts prior to the transcription factors commencing transcriptional stimulation.

Studies that are more recent have implicated cyclin dependent kinases (cdks) to have a role in the mechanism by which ICP0 acts as a transactivator. Cdks are intrinsically associated with the cell cycle and are thought to regulate certain cellular proteins that are involved in many viral pathways or processes necessary for their replication. It was shown that in the presence of the cdk inhibitor roscovitine, ICP0's transactivating ability was inhibited. It was hypothesised that certain cdks regulate the function of cellular enzymes which modify ICP0, and are required for its transactivating ability (Davido *et al.*, 2002).

1.5.4. The role of ICP0 in the reactivation of HSV-1 from quiescent infections.

It has been shown that ICP0 is required for reactivation of HSV-1 from quiescence in cultured cells. The model involved wild-type HSV-1, HSV-2 or ICP0 negative virus, infection of HFL cells, which were subsequently grown at 42°C, a temperature non-permissive for viral replication, thus preventing the onset of the lytic infection. The wild-type virus and the ICP0-null virus established latent infection equally as efficiently as one another, indicating that ICP0 is not required for the establishment of latency *in vitro* (Russell *et al.*, 1987). Further supporting this premise were results that showed that cultured cells quiescently infected with HSV-2 could be induced to reactivate when super-infected with HSV-1, but not with an ICP0-null virus.

The ICP0-null virus used in the aforementioned study was dl1403, which contains a deletion that includes the 3' end of the ICP0 gene, which is complementary to the 5' end of the LAT. It was therefore proposed that the phenotype observed could be a result of a loss of LAT function (Harris *et al.*, 1989). Therefore, follow on studies involved constructing HSV-1 mutants with more defined lesions in the ICP0 gene. Using the same *in vitro* latency system as used previously (Russell *et al.*, 1987) it was shown that adenovirus recombinants expressing ICP0 could reactivate HSV-2 from latent infection. Furthermore, it was identified the RING finger domain of ICP0 was essential for the reactivation of latent genomes, while the 3' region of the ICP0 gene that overlaps with LAT or the sequence involved in self-multimerisation and nuclear localisation was not (Harris *et al.*, 1989).

Studies carried out using integrated quiescent adeno-associated virus (AAV) genomes have shown that expression of the AAV replication proteins can be induced by infection

of HSV-1 or by transfection of plasmids expressing ICP0. Interestingly it was shown that this process was RING finger dependent, but in addition the presence of the USP7 binding motif in the ICP0 mutants was also required (Geoffroy *et al.*, 2004).

1.5.5. The role of ICP0 in the reactivation of HSV-1 from latent infections.

The role of ICP0 in latent infection has been most extensively studied using the mouse model. It was shown using the mouse ocular model that ICP0-null HSV-1 viruses are able to replicate in ganglia with reduced efficiency. These results suggested that ICP0 might play an important role in establishment and/or reactivation from latency (Leib *et al.*, 1989). Interestingly, contradictory findings were published elsewhere, using the mouse footpad model it was shown that ICP0 is dispensable for the establishment, maintenance and reactivation from latency in infected mouse ganglia. However, the ICP0-null virus exhibited a reduced virulence, as the time taken to reactivate was greater than that of wild-type HSV-1 (Clements & Stow, 1989). Variations in the results between the two research groups may be a reflection of the mouse model used, multiplicity of infection or the strain from which the ICP0-null virus was derived.

Later studies supported previous findings that have suggested ICP0 does not play a role in the establishment of latent infection. The virulence of a series of ICP0 mutants with C-terminal truncations, but retaining the region that partially encodes the LAT, was assessed using the mouse ocular model. The results indicated that all mutants enter and reactivate from latent infection, albeit less efficiently than wild type HSV-1 (Cai *et al.*, 1993). Interestingly, it was also shown that an ICP0 LAT double mutant's ability to reactivate from latency could be restored by the addition of one copy of the ICP0 gene into the viral genome. These results allowed a distinct difference to be identified in the role that ICP0 and LATs play in the establishment and reactivation of HSV-1 from latency (Cai *et al.*, 1993).

Studies that are more recent have indicated that an ICP0-null virus displays a phenotype similar to wild-type HSV-1 *in vivo*. Using the mouse ocular model transiently immuno-suppressed mice were inoculated with an ICP0-null virus, which was able to reach wild type levels of viral load in trigeminal ganglia of latently infected mice (Halford & Schaffer, 2000). Additionally it was shown that infection of an ICP0-null virus at high multiplicities was detrimental for the establishment of latency in mice (Halford &

Schaffer, 2000). Subsequent studies showed that following infection with an ICP0-null virus and wild-type HSV-1 with the same latent viral loads that ICP0 is essential for efficient reactivation from trigeminal ganglia (Halford & Schaffer, 2001). Furthermore, latent ICP0-null HSV-1 could be 100% reactivated from trigeminal ganglia by super-infection with an ICP4-null mutant that expresses ICP0. These results support the theory that ICP0 is required for efficient reactivation of HSV-1 from latency (Halford & Schaffer, 2001).

Additional studies from the same group showed that latent HSV-1 could be reactivated from latently infected primary trigeminal ganglia cultures by super-infection with adenoviruses vectors expressing ICP0, ICP4 or VP16. By using an adenovirus expressing GFP it was shown that super-infection with the parent adenovirus vector alone was not sufficient to induce reactivation (Halford *et al.*, 2001).

On the assumption ICP0 is responsible for reactivation of HSV-1 from latency, research has been carried out to determine what induces ICP0's expression. It has been shown that factors in the trigeminal ganglia of transgenic mice can differentially regulate the IE-1 and the IE-4 promoters, in the absence of all other viral proteins (Loiacono *et al.*, 2002). Reporter transgenic mice were exposed to UV irradiation or hyperthermia to test whether stimuli known to reactivate HSV-1 could activate viral IE promoters. By measuring the beta-galactosidase levels, it was shown that ICP0 promoter activity was significantly increased in the trigeminal ganglia of mice, which have undergone UV irradiation or been subjected to hyperthermia. Interestingly, parallel studies showed that the IE-3, IE-2 and gC gene promoters driving the reporter transgene failed to be activated. It was subsequently hypothesized that ICP0's promoter is a target for activation by host transcription factors in sensory neurons that have undergone damage (Loiacono *et al.*, 2003).

1.5.6. The effects of ICP0 on cellular proteins and cells.

ICP0 is a multifunctional protein, which is a reflection of the fact that it has been implicated in interacting with a diverse range of cellular factors. In the following section, the different cellular proteins that ICP0 has been reported to interact with and the possible implications of these interactions on HSV-1 replication will be reviewed.

1.5.7. ICP0 interacts with a member of the ubiquitin specific protease family of proteins.

ICP0 interacts via a motif located at in its C-terminal third with ubiquitin specific protease 7 (USP7), also known as herpes associated ubiquitin specific protease (HAUSP). It was shown using indirect immunofluorescence that USP7 is predominantly a nuclear protein and that in some cell types it can form punctate structures that are associated with ND10 (Everett *et al.*, 1997). Mutation or deletion of the USP7 binding domain causes ICP0 function in virus growth and gene expression assays to be reduced. (Everett *et al.*, 1999d). These studies indicated that the ICP0-USP7 interaction is biologically relevant.

However, until recently the exact function of USP7 in relation to ICP0 was unclear. Normally USP enzymes have two functions, firstly to cleave ubiquitin chains from substrate proteins, thereby preventing proteasome mediated degradation of substrate proteins and increasing the pool of free cellular ubiquitin. Secondly, USP enzymes cleave ubiquitin precursor molecules to generate free ubiquitin for use in the ubiquitin conjugation pathway. As both USP7 and ICP0 associate at ND10, it was conceivable that USP7 might be involved in cleaving SUMO-1 from isoforms of PML, however this was shown not to be the case (Everett *et al.*, 1998b).

It was not until recently that the real breakthrough in understanding the significance of the ICP0-USP7 relationship occurred. It was found that ICP0 efficiently ubiquitinates itself *in vitro* through its E3 ubiquitin ligase activity, and that USP7 protected ICP0 from this activity be removing the polyubiquitin chains. Furthermore, the instability of ICP0 in infected cells was greatly increased if it was unable to bind to USP7, or if siRNA treatment was used to reduce the cellular levels of USP7 (Canning *et al.*, 2004). This effect was later shown to be particularly pronounced during early times of low multiplicity infections (Boutell et al., submitted). It was also shown that, following infection with a mutant of HSV-1 expressing a form of ICP0 that is unable to bind to USP7, the mutant ICP0 proteins accumulated to lower levels than the wt protein, causing a reduction in the level of expression of other viral proteins in a cell type dependent manner (Boutell et al., submitted). These results may be due to an increased level of ICP0 degradation in the cell, a result of the failure to bind to USP7. Consequently, if less ICP0 were present, it would be predicted that a reduction in the

level of viral protein expression would occur. In very low multiplicity infections, this effect would cause reductions in the number of viral genomes entering the lytic infection and the level of plaque formation. These effects were indeed observed (Boutell et al., submitted).

1.5.8. Reciprocal activities of USP7 and ICP0.

Following the studies that showed that the interaction with USP7 is biologically relevant for optimum function of ICP0, it was shown that USP7 is ubiquitinated *in vitro* and degraded during HSV-1 infection. ICP0 was shown to degrade USP7 in a RING finger dependent manner, which was reliant on the two proteins being able to interact (Boutell et al., submitted). It was shown that stabilisation of ICP0 during the early stages of infection was particularly important following low M.O.I., but not at a high M.O.I. It was proposed that the latter was observed because the increased levels of synthesis of ICP0 could compensate for instability caused by increased levels of USP7 degradation (Boutell et al., submitted).

1.5.9. The role of ICP0 in cell cycle regulation.

It has been shown the phenotype of an ICP0-null virus (impaired growth and synthesis of viral proteins) was partially restored to that of wild-type HSV-1 when Vero cells at the G0/G1 stage of the cell cycle were used as hosts (Cai & Schaffer, 1991; Ralph *et al.*, 1994). It was suggested that putative cellular functions are expressed at the transition of G0 into G1 phase of the cell cycle that can functionally substitute for ICP0 transactivation. Conversely, this also suggested that ICP0 might be able to mimic or promote cellular conditions that are present during specific stages of the cell cycle.

It was shown by yeast two-hybrid assays and glutathione S-transferase pull down assays that ICP0 is able to form complexes with cyclin D3 *in vitro* (Kawaguchi *et al.*, 1997b). Furthermore, when ICP0 and cyclin D3 were expressed together from a recombinant HSV-1 mutant, both proteins were shown to localise to ND10. Using the same mutant virus, it was shown that substitution mutation D199A caused ICP0 to remain at ND10 and cyclin D3 was prevented from colocalising with ICP0 (Van Sant *et al.*, 1999). Studies using an ICP0-null or D199A ICP0 mutant strain of HSV-1 indicated that cyclin D1 and D3 were degraded more rapidly than in wild type infections, suggesting that

ICP0 plays a role in promoting their stability (Van Sant *et al.*, 1999; Van Sant *et al.*, 2001b).

Based on the previous studies, the same group carried out research to elucidate the mechanism by which ICP0 promotes D-type cyclin stability. It was shown that ICP0 acts as an E3 ubiquitin ligase (Boutell *et al.*, 2002; Hagglund *et al.*, 2002) and promotes the autoubiquitination of cdc34 (Hagglund *et al.*, 2002). It was interpreted that the ICP0-mediated autoubiquitination and subsequent degradation of cdc34 must promote cyclin D1 and D3 stability (Van Sant *et al.*, 2001b), as in other systems cdc34 has been implicated in their degradation (Jackson & Eldridge, 2002). However, research carried out elsewhere has conclusively proved that ICP0 does not promote the stability of D-type cyclins. A variety of cell types was infected with an ICP0-null and wild-type HSV-1 at equivalent multiplicities that ensured that both infections were proceeding at equivalent rates (see section 1.5.1. for further details). When carefully controlled in this manner, it was found that the D-type cyclins were equally unstable during wt and ICP0-mutant HSV-1 infections (Everett, 2004). The author was unable to find evidence to support the theory that ICP0 promoted the stability of either cyclin D1 or cyclin D3, by preventing their degradation. Furthermore, following varying M.O.I. and even when high levels of ICP0 were expressed, no affect on cdc34 could be observed throughout HSV-1 infection (Everett, 2004).

Research carried out elsewhere has studied different aspects of how ICP0 affects the cell cycle. It was reported that infection of a mutant virus that expresses ICP0 as the only IE protein causes cell cycle arrest at G1/S and G2/M. However, a HSV-1 mutant variant expressing no IE proteins failed to induce this phenotype (Hobbs & DeLuca, 1999). Supporting the previous findings, it was shown that by transfecting plasmids expressing ICP0, the cell cycle could be stalled at the G1/S boundary. Furthermore, by using wt and ICP0-null mutant HSV-1 to infect synchronised cells it was found that ICP0 resulted in the cell cycle becoming stalled at the pseudo-prometaphase stage (Lomonte & Everett, 1999). The mitotic arrest was shown to be caused by ICP0-induced proteasome-mediated degradation of centromere proteins CENP-C and CENP-A (Everett *et al.*, 1999a; Lomonte *et al.*, 2001), both of which are associated with the inner kinetochore plate, which has an essential role in cell division.

1.5.10 ICP0 interacts with and regulates components of transcription and translation.

ICP0 has been shown to target various cellular factors involved in the regulation of gene expression. Studies have shown that ICP0 degrades the catalytic subunit of DNA-dependent protein kinase (DNA-PKs) during HSV-1 infection (Lees-Miller *et al.*, 1996) in a RING finger dependent manner (Parkinson *et al.*, 1999). Furthermore, it was shown the ICP0-induced degradation of DNA-PKs appeared to be beneficial for HSV-1 infection, as the virus replicates slightly more efficiently in cell lines that do not express DNA-PKs, especially at low multiplicities of infection (Parkinson *et al.*, 1999).

Other studies have shown using the yeast two hybrid system that ICP0 interacts with the cytoplasmic translation regulation protein elongation factor 1δ (EF-1δ), via the C-terminal region of ICP0 (Kawaguchi *et al.*, 1997a). The interaction between ICP0 and EF-1δ is thought to occur during the later stages of HSV-1 infection, when ICP0 migrates to the cytoplasm. The HSV-1 protein, UL13 is thought to be involved in the hyperphosphorylation of EF-1δ (Kawaguchi *et al.*, 1998), an effect that is associated with efficient translation. However, the implications of EF-1δ interaction with ICP0 are not fully understood.

Using the yeast two hybrid system it was shown that ICP0 interacts with transcription factor BMAL1, via residues located between amino acids 95 and 450 (Kawaguchi *et al.*, 2001). Furthermore, ICP0 and BMAL1 when coexpressed together significantly increased transcription of a luciferase reporter construct compared to ICP0 or BMAL1 alone. It was suggested this activity was due to ICP0 forming a transcriptionally active complex with BMAL1 in cell culture. It was also inferred that ICP0 acts to stabilize BMAL1, as it was more prone to degradation in cells that had been previously infected by an ICP0-null virus. The biological significance of these observations is not clear, as binding sites for BMAL1 in the HSV-1 genome have not been characterised and, as discussed previously, no specific transcription factor binding sites are required for regulation of gene expression by ICP0.

Using transfection assays it was shown that ICP0 recruits and colocalises with class I and II histone deacetylase (HDAC) enzymes (Lomonte *et al.*, 2004). Furthermore, type II HDAC enzymes were shown to interact with ICP0 *in vitro*, via a domain associated with regulation of MEF-2, a transcription factor involved in muscle development and

neuronal survival (Lomonte *et al.*, 2004). Using transfection reporter assays it was shown that ICP0 can overcome HDAC5 mediated repression of MEF-2. It was concluded this mechanism could be involved in the relief from repression of viral chromatin that is widely thought to be a consequence of ICP0 activity (Lomonte *et al.*, 2004).

1.5.11. The affect of ICP0 on ND10 and centromeric proteins.

During HSV-1 infection, disruption of ND10 occurs via a mechanism that is dependent on ICP0 and its RING finger domain (Maul *et al.*, 1993) (Everett & Maul, 1994; Maul & Everett, 1994). This disruption of ND10 is brought about by ICP0 inducing the proteasome-dependent degradation of its major constituents PML and Sp100, particularly their SUMO-1 modified forms (Chelbi-Alix & de The, 1999; Everett *et al.*, 1998a; Parkinson & Everett, 2000). The dependence on the proteasome degradation pathway was proved by research that showed the integrity of ND10 could be preserved in the presence of the proteasome inhibitor MG132 (Everett *et al.*, 1998a). Furthermore, the ability of ICP0 to disrupt ND10 correlates very well with its ability to initiate lytic infection and induce reactivation, as these function were also inhibited by MG132 (Everett *et al.*, 1998a). Centromeric proteins CENP-C and CENP-A are also degraded by the proteasome pathway in response to ICP0, leading to the disruption of centromere structure and concomitant mitotic delay and failure (Everett *et al.*, 1999a; Lomonte & Everett, 1999; Lomonte *et al.*, 2001). Consistent with these observations it was later shown that ICP0 can cause the formation of colocalising, conjugated ubiquitin in both transfected and infected cells, at ND10 and centromeres (Everett, 2000). It has also been shown that ICP0 recruits USP7 following transfection of ICP0 or during HSV-1 infection of cultured cells. The binding of USP7 by ICP0 was shown to contribute to, but not be essential for, ICP0-mediated disruption of ND10 (Everett *et al.*, 1999d; Everett *et al.*, 1997; Parkinson & Everett, 2001).

1.5.12. Why does ICP0 disrupt ND10 bodies?

The ability to disrupt ND10 correlates very well with the biological activities of ICP0. Why this should be the case remains a matter of debate. One possibility is that ND10 are disrupted in order to release factors that are essential for the continued progression of HSV-1 infection (Negorev & Maul, 2001). However, it has also been proposed that

ND10 structures play an essential role as part of a cellular repression mechanism, which prevents or stalls the progression of viral infection. It has been shown that ND10 proteins become deposited at sites juxtaposed to parental HSV-1 genomes early during infection, whereupon viral transcription and the formation of replication compartments occurs (Everett & Zafiropoulos, 2004; Ishov & Maul, 1996; Maul *et al.*, 1996). The role of ND10 proteins being part of a potential anti-viral mechanism is supported by research that has shown the ND10 components Sp100 and PML are interferon-induced and have been implicated in the repression of gene expression. Additionally, various studies have linked ICP0 with resistance to the effects of interferon (Eidson *et al.*, 2002; Harle *et al.*, 2002; Mossman *et al.*, 2000; Mossman & Smiley, 2002; Nicholl *et al.*, 2000; Preston *et al.*, 2001; Taylor *et al.*, 1998). In contrast, high-level PML expression does not appear to inhibit HSV-1 infection (Chelbi-Alix & de The, 1999; Lopez *et al.*, 2002). Furthermore, ICP0-null mutant HSV-1 does not appear to replicate more efficiently in mouse fibroblasts that do not express PML (Chee *et al.*, 2003). It is possible that no affect was observed after PML over-expression because ND10 proteins do not have a role in viral gene expression, or perhaps because PML may not be the major component of the suggested repression mechanism. Degradation of PML may be a side affect of an ICP0-mediated activity towards other cellular components.

1.5.13. The disruption of ND10 by other viruses.

As mentioned in the previous section, ND10 are disrupted by many viruses, including other members of the *alphaherpesvirinae*. This section will briefly review the affect of other DNA viruses on ND10 structures.

HCMV and its immediate early protein IE1.

The beta herpesvirus HCMV disrupts ND10 early during infection by the action of IE regulatory protein IE1 (Ahn & Hayward, 2000; Korioth *et al.*, 1996; Wilkinson *et al.*, 1998). The properties of an ICP0-null HSV-1 mutant are similar to those of an HCMV IE1 negative virus; both mutant viruses enter the lytic cycle inefficiently, but the defect can be overcome by using a high multiplicity of infection (Mocarski & Courcelle, 1996). The similarities are extended to the fact that HCMV genomes are found at the periphery of ND10, whereupon replication compartments were formed (Ahn *et al.*, 1998; Ishov & Maul, 1996). The potential for ND10 to act as a repressor of HCMV

replication is suggested by the fact that cell lines expressing high levels of PML are poorly infected by HCMV (Ahn & Hayward, 2000), although this has been shown not to be the case for HSV-1 (Chelbi-Alix & de The, 1999; Lopez *et al.*, 2002). Furthermore, using transfection assays it was shown that expression of the IE1 protein inhibits the transcriptional repression function of PML (Lee *et al.*, 2004).

EBV and HHV-8 and their regulatory proteins.

The gamma herpesvirus Epstein Barr Virus (EBV) and Human Herpes Virus-8 are lymphotrophic viruses and replicate persistently to a greater extent than other members of the alpha or betaherpesviruses. The key biological differences that exist may reflect the differing relationships of the viruses with ND10. Upon infection the EBV protein, EBNA-5 becomes associated with ND10, although it does not affect their stability (Szekely *et al.*, 1993). However, using transfection assays, it was shown that high level expression of the BZLF1 protein causes ND10 disruption (Adamson & Kenney, 2001; Bell *et al.*, 2000).

During EBV lytic infection several ND10 components have been shown to be disrupted including Sp100, hDaxx and NDP55, which are lost rapidly, while PML is dispersed more slowly (Bell et al., 2000). A more recent study has suggested that EBV nuclear protein SM induces the expression of Sp110b (a member of the Sp100 family of proteins), and recruits it to replication compartments. SM utilises Sp110 to increase the stability of lytic EBV transcripts (Nicewonger *et al.*, 2004).

Key differences occur that distinguish the relationship of EBV genomes with ND10 during lytic and latent infection. It was shown that EBV genomes do not associate with ND10 components during latency, and instead they become attached to interphase chromosomes, ensuring the passage of their genome into daughter cells (Bell *et al.*, 2000; Wu *et al.*, 2000). When the lytic cycle is resumed, as with HSV-1 and HCMV, replication compartments juxtaposed to ND10 are formed. In a similar manner to that of EBV, HHV-8 has been shown to form structures that resemble replication compartments juxtaposed to ND10 and that contain the latency associated nuclear antigen, LANA (Wu *et al.*, 2001).

Papovaviruses.

Papovaviruses undergo episomal replication and rely on the presence of various cellular host proteins for viral DNA synthesis. During SV40 replication, foci containing the viral genome are formed and these are juxtaposed to ND10. This process is reliant on the SV40 large T antigen and an intact SV40 origin of DNA replication.

The human papilloma virus genome also associates with ND10, and this is thought to be a result of the E1 and E2 proteins that are involved in DNA replication. The HPV L2 protein was also shown to accumulate at ND10, which then recruits the L1 protein. L2 induces the dispersal of ND10 component Sp100 and the recruitment of Daxx (Swindle *et al.*, 1999). Interestingly it has recently been shown that using a PML negative cell line, L2 can still recruit Daxx in the absence of functional PML proteins (Becker *et al.*, 2004).

Studies on the HPV-1 E4 protein have shown that it also induces ND10 reorganisation *in vitro* and *in vivo*, relocating PML from ND10 bodies to inclusion bodies during the lytic cycle. These studies indicate that, as with other DNA viruses, ND10 reorganisation is part of the HPV replication cycle (Roberts *et al.*, 2003). Again, similar to HHV-8 and EBV, HPV genomes associate with chromosomes during mitosis to ensure their passage into daughter cells.

Adenoviruses.

The adenovirus type 5 (Ad5) open reading frame 3 (orf3) product disrupts ND10 and causes the redistribution of PML to 'fibrous-like' structures during the early phase of infection (Carvalho *et al.*, 1995). During the late phase of infection the protein pIX is thought to participate in the potential neutralisation of ND10, by participating in the relocation of PML to clear-amorphous (c.a.) inclusions bodies located in the nucleus (Rosa-Calatrava *et al.*, 2003). It was suggested that role of pIX was essential for efficient viral infection as prevention of this process caused a reduction in viral yield (Rosa-Calatrava *et al.*, 2003). Interestingly other ND10 constituents, including the interferon induced Sp100, have been found at c.a. inclusion bodies (Souquere-Besse *et al.*, 2002).

1.5.14 Introduction to the SUMO modification pathway.

Both PML and Sp100, which are major components of ND10, are major substrates for SUMO-modification, and during HSV-1 infection the action of ICP0 leads to preferential loss of the SUMO-modified species of these proteins (Everett et al., 1998). The aim of this section is briefly to outline the cellular role of SUMO and the purpose it plays in viral infection with particular reference to that of ICP0.

The best-characterised members of the SUMO (Small Ubiquitin-like Modifier) family are SUMO-1, SUMO-2, and SUMO-3. The enzymatic process is biochemically analogous to, but functionally distinct from ubiquitination conjugation (Schwartz & Hochstrasser, 2003). As with ubiquitination, there is an E1 enzyme that activates SUMO-1 by a forming a thiolester linkage in an analogous manner to that of the ubiquitin E1 enzyme. Unlike in the ubiquitination pathway, there is only a single E2 enzyme (UbcH9) and only a handful of SUMO E3 enzymes have been defined. A further difference between the two pathways is that, unlike in ubiquitination, SUMO is frequently joined to target proteins via a lysine residue situated in a specific amino acid sequence motif (Rodriguez *et al.*, 2001). However, unlike ubiquitin modification of a target substrate, the fate of the SUMO-modified protein does not normally result in degradation, in fact, it can have multiple and quite diverse affects on the protein. These include enhancing the stability of a protein (Bies & Wolff, 1997; Bresnahan *et al.*, 1996), and modulation of the transactivation activity and/or subcellular localisation of a protein (Schwartz & Hochstrasser, 2003). Interestingly, enhanced stability of a SUMO-modified substrate can be a direct consequence of competing with the ubiquitin conjugation pathway. If SUMO-modification takes place on the same lysine residue that is a preferential target for ubiquitination, this protects the lysine residue from ubiquitination and this may prevent or delay the initiation of proteasome mediated degradation of the protein (Pickart, 2001a).

PML is a major constituent of ND10 and its expression is sharply up-regulated by interferon (Chee *et al.*, 2003; Regad & Chelbi-Alix, 2001). SUMO-modification is a crucial part of PML biological function, because mutation of the major SUMO-modification sites of PML affects its ability to form foci with ND10 and for it to recruit specific proteasome regulatory components (Lallemand-Breitenbach *et al.*, 2001).

Multiple viruses utilise the SUMO-modification pathway to modify their regulatory proteins. It has been shown that bovine papillomavirus protein E1 relies on SUMO-1 modification for its import into the nucleus (Rangasamy & Wilson, 2000; Rangasamy *et al.*, 2000) and adenovirus E1B-55KDa utilises it for nuclear accumulation and for its cellular transforming functions (Endter *et al.*, 2001). It has been shown that the HCMV immediate early-1 protein (IE1) is modified by SUMO-1 and immediate early-2 protein (IE2) is modified by SUMO-1, SUMO-2 and SUMO-3 (Ahn *et al.*, 2001; Hofmann *et al.*, 2000; Muller & Dejean, 1999; Spengler *et al.*, 2002; Xu *et al.*, 2001). SUMO-modification of IE2 is thought to enhance its transactivation capacity (Ahn *et al.*, 2001; Hofmann *et al.*, 2000). Interestingly, recent studies from mutating the lysine residue of the SUMO-modification motif of IE1 showed that viruses containing this mutation exhibited reduced rates of growth compared to wild type, suggesting that SUMO-modification is required for the full activity of IE1 (Nevels *et al.*, 2004). However, this mutation did not affect the ability of IE1 to localise to and disrupt ND10 and to bind to chromatin (Nevels *et al.*, 2004). However, most interestingly, the failure to modify IE1 with SUMO-1 resulted in reduced levels of expression of IE2, indicating that IE1 SUMO modification is essential for IE2 accumulation and the progression of efficient HCMV replication (Nevels *et al.*, 2004).

1.5.15 ICP0 and SUMO-modification.

Unlike some of the other viral regulatory proteins mentioned previously, ICP0 does not contain a SUMO modification motif. ICP0 induces the proteasome-mediated degradation of PML and Sp100 and in particular their SUMO-1 modified isoforms (Everett *et al.*, 1999c; Parkinson & Everett, 2000). Recently, it was shown that ICP0 increases the level of ubiquitinated PML in transfected cells and mutation of a SUMO-modification site in PML reduced the level of its degradation by ICP0 (Boutell *et al.*, 2003). Furthermore, ICP0 mutants that don't locate to ND10 efficiently have a reduced ability to induce PML degradation (Everett *et al.*, 1998a; Parkinson *et al.*, 1999).

The available data indicate that it is important for PML and ICP0 to locate to and accumulate in the same nuclear substructure, however as degradation still occurs at a reduced level when this process is inhibited, this suggests there may be additional factors that play a part. It has been shown that isopeptidase SENP-1, an enzyme capable of deconjugating SUMO-modified isoforms of PML, can be recruited to and colocalise

with ICP0 at defined foci during the early stages of infection, although it remains to be determined if this plays a role in ICP0 induced loss of PML-SUMO-1 conjugates (Bailey & O'Hare, 2002).

Chapter 1- Part III – The biology of ubiquitination.

1.6.0. Introduction to ubiquitination.

Ubiquitin is a small 76-residue polypeptide that plays an essential role in eukaryotes through its covalent conjugation to other intracellular proteins. Substrate proteins become ubiquitinated via the ubiquitin conjugation pathway, which generally involves three defined sets of enzyme, an E1 (ubiquitin activating enzyme), an E2 (ubiquitin conjugating enzyme) and an E3 (ubiquitin ligase), which determines substrate specificity. The fate of a substrate protein arises primarily at the stage of ubiquitination, which is mediated by a specific E2-E3 interaction occurring, as this determines how many ubiquitin molecules and to which lysine residue the ubiquitin is added to the substrate (see figure 1.6.0A). When more than one ubiquitin molecule is added to a substrate, it becomes marked with a polymer of ubiquitin peptides, a polyubiquitin chain, which is an intracellular signal to target the protein for proteasome-mediated degradation by a multi-subunit ATP-dependent protease known as the 26S proteasome. However, when a substrate is only marked with a single or a few ubiquitin molecules, this can cause proteolysis in the lysosome. Furthermore, the lysine residues targeted for ubiquitination play a crucial role in determining the fate of the protein. If polyubiquitin chains are linked via lysine 48 of ubiquitin, the substrate will be targeted for proteasome mediated degradation, but if linked via lysine 63 the substrate may become involved in non-proteolytic signalling (Pickart, 2001a).

The ubiquitin conjugation pathway is associated with the regulation of a diverse number of critical cellular functions including the progression of the cell cycle and antigen presentation (Pickart, 2001a). It is thought to act as a regulator in these various cellular processes by mediating the selective proteasome-mediated degradation of regulatory proteins involved in these diverse cellular processes (Pickart, 2001a). Non-proteolytic fates of a ubiquitinated proteins include ribosomal function (Spence *et al.*, 2000), post-replication DNA repair (Hofmann & Pickart, 1999; Spence *et al.*, 2000), the initiation of the inflammatory response (Deng *et al.*, 2000), and the function of certain transcription factors (Kaiser *et al.*, 2000).

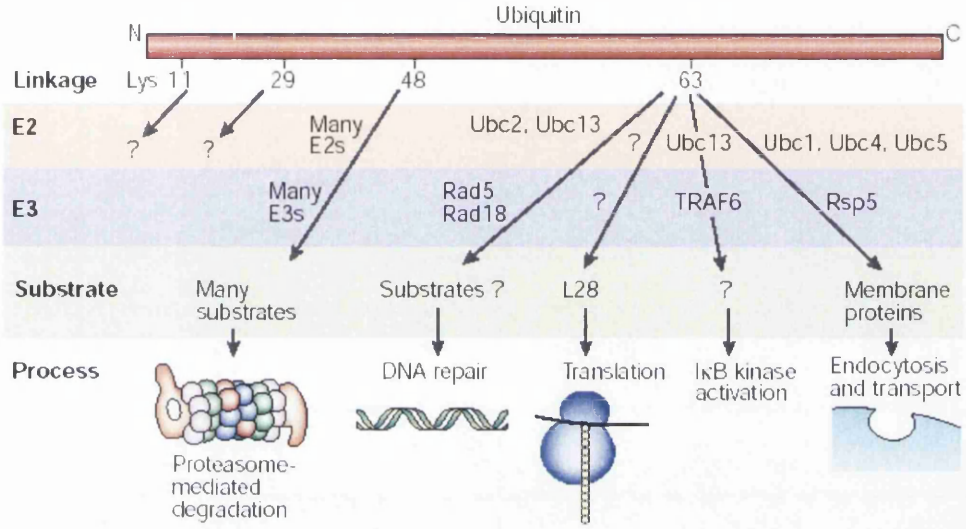


Figure 1.6.0A *The fate of a substrate protein following ubiquitination.*

The initially the fate of a protein is partially determined by which lysine (Lys) residue the ubiquitin is linked to the substrate protein, which is mediate by the specific E2-E3 interaction. The functions of Lys11 and Lys29-linked chains are unknown. Lys 48-linked chains target proteins to proteasome-mediated degradation and Lys 63-linked chains have a range of fates. The diagram was adapted from Weissman, 2001.

As ubiquitin-dependent proteolysis plays an important role in many diverse cellular functions, when it becomes deregulated it can have dire affects on the host organism. Previously it has been implicated as a causative factor in certain cancers and several genetic diseases (including cystic fibrosis, Angelman's syndrome, and Liddle syndrome). Reviews of these issues can be found elsewhere (Ciechanover & Brundin, 2003; Liu, 2004; Schwartz & Ciechanover, 1999).

1.6.1 The machinery of ubiquitination.

Ubiquitination of a substrate protein occurs as follows (see figure 1.6.1A): The first enzyme in the ubiquitination pathway, the E1, via an ATP-dependent reaction forms a thiolester bond between its active site cysteine and the carboxyl-terminal glycine of an ubiquitin molecule. The activated ubiquitin on the E1 is subsequently transferred to the active site cysteine of any number of E2 enzymes. Then a generalised view to what happens next is that the E3 ubiquitin ligase binds to both the ubiquitin-charged E2 and the substrate, then the E3 facilitates formation of an isopeptide linkage between the carboxyl-terminal glycine residue of ubiquitin and the ϵ-amino group of an internal lysine residue on the substrate, or in an ubiquitin molecule already attached to the substrate (Hershko & Ciechanover, 1998; Pickart, 2001a).

The final enzyme involved in the enzymatic cascade that leads to the ubiquitination of a target substrate is the E3 ubiquitin ligase. These enzymes can be classified into two families of proteins, those containing HECT (Homologous to E6-AP Carboxyl Terminus) domains (Huibregtse *et al.*, 1995), and the members of the RING finger related protein family (Freemont, 2000; Joazeiro & Weissman, 2000). Both families of E3 ubiquitin ligases have a distinct amino acid motif that has led to their identification in various eukaryotic organisms and viruses. HECT and RING finger domain proteins serve the same purpose of targeting substrates for ubiquitination, despite using different mechanisms of action.

1.6.2 An overview of the enzymes involved in the ubiquitin conjugation pathway.

The organisation of the ubiquitin conjugation enzymes is hierarchical (see figure 1.6.2A). In both humans and yeast, there is only one E1 enzyme that activates ubiquitin for the entire array of downstream E2 and E3 enzymes (McGrath *et al.*, 1991;

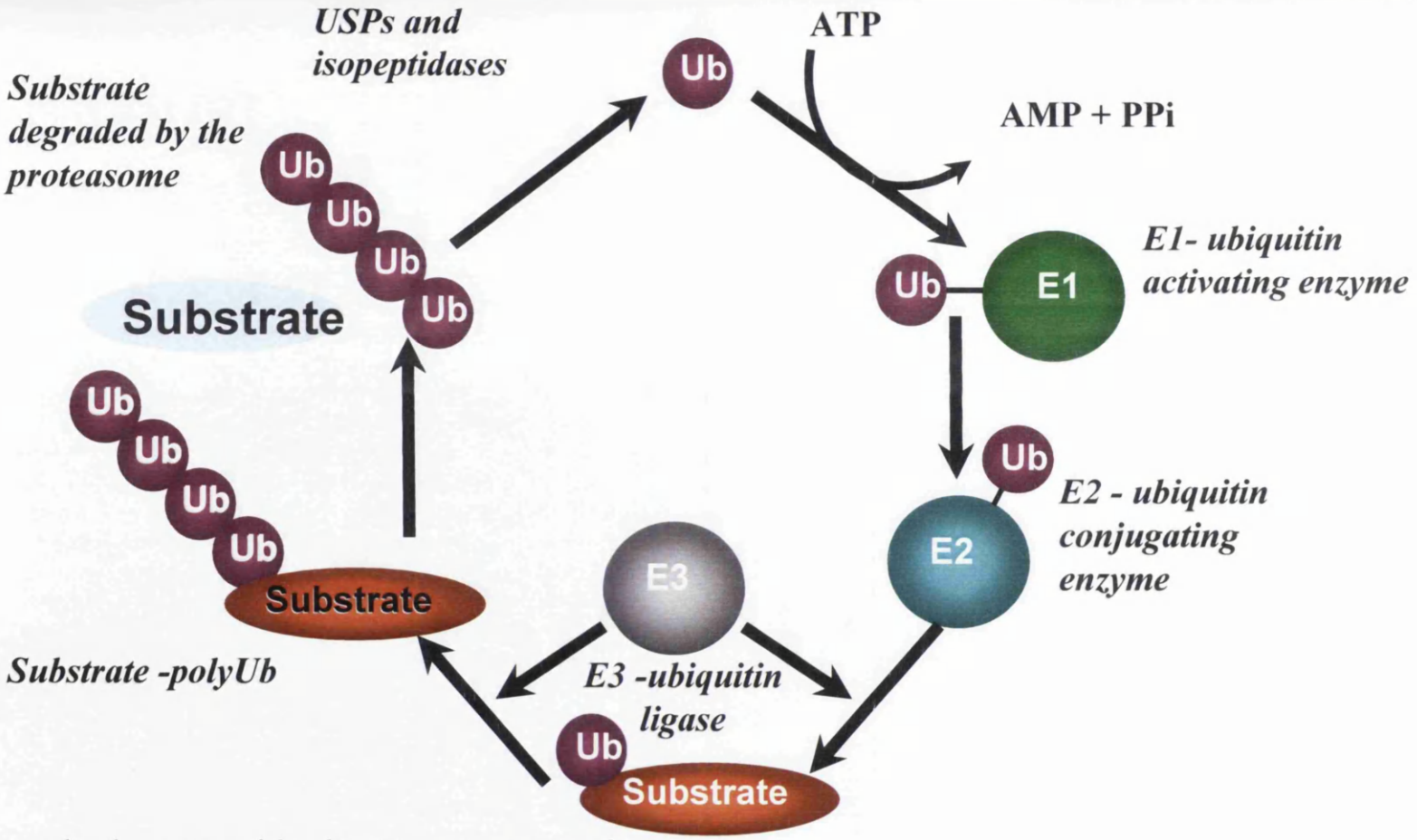


Figure 1.6.1A. *A generalised overview of the ubiquitin conjugation cycle.*

Initially an ubiquitin becomes activated by an E1 enzyme, which involves the formation of a thiolester linkage from the ubiquitin to the active site cysteine of the E1, in an ATP-dependent reaction. Whereupon, the E2 enzyme forms a thiolester bond between the sulphydryl group at its active site cysteine and the carboxyl group of the C-terminal glycine residue of the activated ubiquitin molecule. The E3 ligase then facilitates the transfer of the ubiquitin from the active site of the E2 to a substrate protein. The ubiquitin is linked via an isopeptide bond from its C-terminal carboxyl group to the amino group at the end of the side chain of a lysine residue in the substrate, or if the substrate is already ubiquitinated to a lysine side chain on another ubiquitin molecule. The poly-ubiquitin chain is broken back into single ubiquitin proteins by isopeptidases and ubiquitin specific proteases

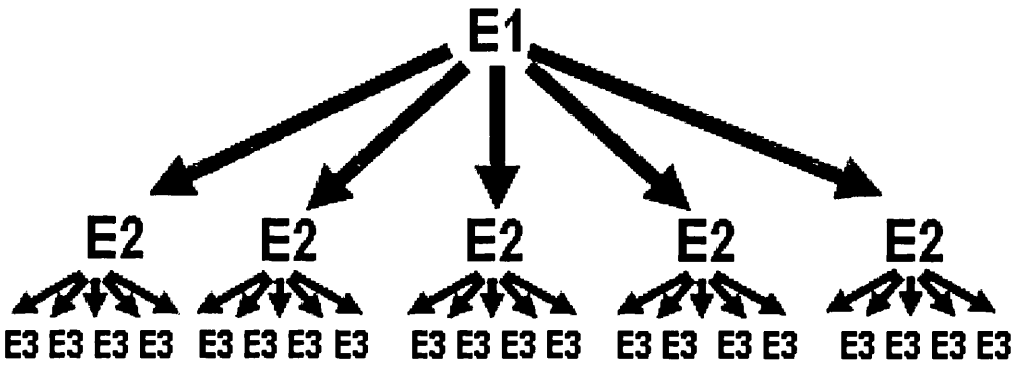


Figure 1.6.2A. *Hierarchical relationship between the enzymes inolved in ubiquitin conjugation.*

Many organisms encode within their genomes only a single E1 (ubiquitin activating enzyme), and a few E2 (ubiquitin conjugating enzymes). However, the number of E3s (ubiquitin ligase) enzymes that they encode, depends on the organism and can range into hundreds.

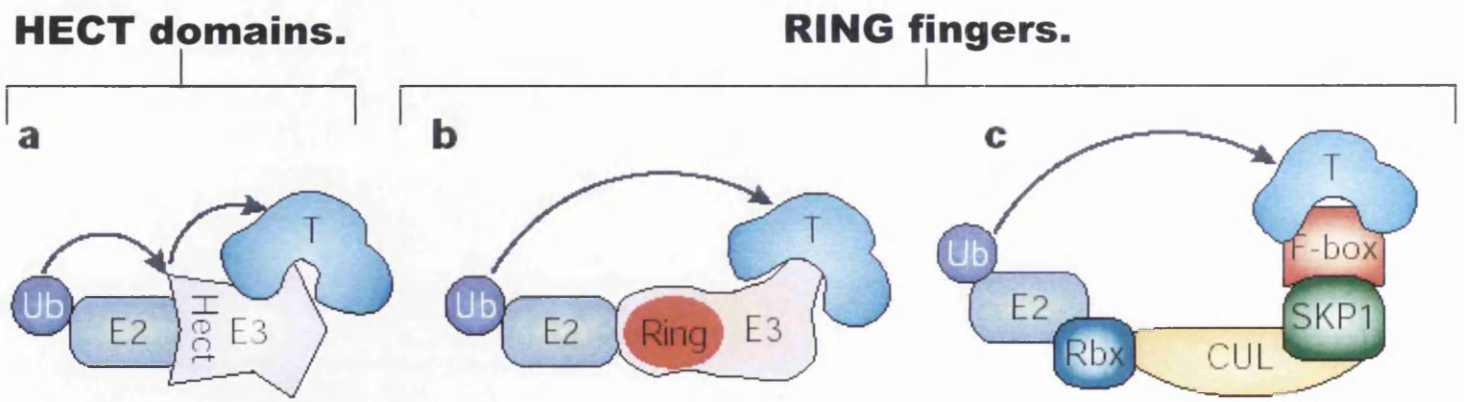


Figure 1.6.4A. *Mechanisms of substrate ubiquitination by HECT and RING finger domains.*

A) The HECT domain forms an intermediate thiolester linkage with ubiquitin before transferring the ubiquitin to the lysine residue in the target protein.

B) Unlike HECT domains, RING finger protein E3 ubiquitin ligase enzymes do not participate directly in the transfer of ubiquitin to the target and instead they act only as a mediator ensuring the correct interaction between the E2 the and the target protein occurs. The E2 protein forms a thiolester bond with ubiquitin, where upon it is transferred directly to the target substrate.

C) A relative of the RING finger domain E3 ligases are the cullin-based multi-subunit E3 enzymes such as SCF. The components of SCF are four polypeptides SKP1, Cullin, an F-box and the Rbx1 protein. The F box protein provides substrate specificity, while the Rbx1 is the RING finger domain, which provides E2 specificity. Diagram adapted from Sullivan, *et al.* 2003

Zacksenhaus & Sheinin, 1990). There are greater numbers of E2 enzymes, with around 30 predicted to be encoded by the human genome. Each E2 is thought to serve several E3 enzymes. Finally there is a large, ever growing number of E3 ubiquitin ligase enzymes (Pickart, 2001a), which recognise a set of substrates that share one or more ubiquitination signals (Hershko & Ciechanover, 1998; Hochstrasser, 1996).

1.6.3. The E1 ubiquitin activating enzyme.

Cells expressing a temperature-sensitive E1 first led to the discovery that ubiquitination is essential for cell cycle progression (Mayer *et al.*, 1989). It has been shown the E1 has a poor affinity for ubiquitin prior to the binding of ATP (Haas *et al.*, 1982; Hershko, 1983), whereupon it can bind two molecules of ubiquitin, one as a thiolester and the other as an adenylate. The activated ubiquitin is then transferred to the active site cysteine in the E2 via an ATP-dependent reaction. The carboxyl-terminal glycine residue of ubiquitin is essential for its activation by E1. The chemistry of E1 activation is reviewed in more detail elsewhere (Haas *et al.*, 1982).

1.6.3 The E2 ubiquitin conjugating enzyme.

The E2 family of enzymes share a highly conserved core domain consisting of 150 amino acids (Pickart, 2001a). The members of the UbcH5 family of E2 enzymes (UbcH5a, b and c) share over 90% sequence homology to one another and over 70% similarity with UbcH6. Some E2 enzymes have substantial amino- or carboxyl-terminal extensions or insertions within their core domain (Pickart, 2001a), which are thought to determine E3 specificity. However, substrate specificity is determined by the E3, therefore one E2 can have diverse biological functions depending on which E3 it interacts with. For example, the E2 UbcH1 is upregulated in wasting muscle and implicated in spermatogenesis (Pickart, 2001a).

1.6.4. The E3 ubiquitin ligase enzymes.

Eukaryotic genomes encode a vast number of E3 ubiquitin ligase enzymes, which are classified as either HECT or RING finger domain proteins on the basis of their subunit composition (Pickart, 2001a). Both the HECT and RING finger domains serve the same purpose of targeting substrate proteins for ubiquitination, although they achieve this

through different mechanisms (see figure 1.6.4A). In this section, the key characteristics of HECT and RING finger domains are discussed.

HECT Domains.

Studies into Human Papilloma Virus (HPV) E6-dependent ubiquitination of p53 led to the identification of the first HECT E3 ligase, E6-AP (E6-Associated Protein) (Scheffner, 1998). It was initially shown that a domain of about 350 residues at the carboxyl-terminus of E6-AP contained large degree of homology to domains that exist in otherwise unrelated proteins (Huibregtse *et al.*, 1995). Henceforth the name HECT domain was used, as these regions were shown to be Homologous to E6-AP Carboxyl Terminus.

It has been shown that the active site of a HECT domain contains a conserved lysine residue situated 35 amino acids upstream of the carboxyl-terminus, which is essential for its function. The N-terminal portion of the HECT domain is involved in substrate recognition, E2 specificity and it also regulates subcellular localisation (Weissman, 2001). During the ubiquitination process, the HECT domain interacts with a specific E2 partner, then mediates the transfer of a ubiquitin molecule from the E2 to the conserved lysine residue within its active site and then finally to a lysine reside within the substrate protein (Pickart, 2001a). Compared to the large number of RING finger related proteins, only around 50 HECT domain E3 ubiquitin ligase enzymes have been identified in mammals, and they are also less numerous in other organisms such as yeast (Sullivan *et al.*, 2003).

The zinc-binding RING finger domain E3 ubiquitin ligases.

The members of the largest family of E3 ubiquitin ligase enzymes are characterised by the presence of a RING finger domain (see figure 1.6.4A), a conserved motif of generally seven cysteine and one histidine residues, CX2CX(9–39)CX(1–3)HX(2–3)C/HX2CX(4–48)CX2C (X is any amino acid) that coordinate two zinc atoms in a cross-braced fashion (Borden & Freemont, 1996). Unlike HECT domains, RING fingers do not participate directly in the transfer of ubiquitin, instead act as mediators to ensure the correct interaction of the E2 and target substrate.

The are several types of RING finger domain proteins, which include the multi sub-unit based E3 complexes such as SCF (Skip1-Cullin-F box) (Jackson & Eldridge, 2002). The cullin and Skip-1 proteins provide a core scaffold, where the Rbx1 acts as a mediator to ensure ubiquitination of the target substrate. However, the substrate specificity is determined by the F-box protein, which contains the appropriate protein-interaction domain (Craig & Tyers, 1999; Willems *et al.*, 1999).

Two further types of domain that are related to the RING finger are the U-box and PHD domains, several of which have been found in proteins expressed by viruses and mammals. Interestingly transcription factor NF-X1 has been shown to contain overlapping RING and PHD finger consensus sequences, however only the PHD domain is required for its *in vitro* ubiquitination activity (Fang *et al.*, 2003). The U-box is distantly related to the RING finger in sequence and functions in a similar fashion (Hatakeyama *et al.*, 2001), but it does not require chelated zinc atoms, instead it uses intra-molecular interactions to maintain structural integrity (Ohi *et al.*, 2003). Furthermore, unlike the other RING finger proteins, many of which contain a conserved tryptophan residue, U-box domains contain a cysteine residue in its place. The number of RING finger domain proteins found in diverse organisms, including viruses, is rapidly growing. Detailed reviews can be found elsewhere (Fang & Weissmann, 2004; Freemont, 2000; Pickart, 2001b; Weissman, 2001).

Single and multi-subunit E3 ubiquitin ligase enzymes.

E3 ubiquitin ligase enzymes can be generally classified as either single or multi-subunit. Essentially, what determines a single subunit E3 is its ability to interact with both the appropriate E2 and the substrate. An example of such a E3 is Mdm2, a RING finger E3 that has an amino-terminal p53 binding domain and a carboxyl-terminal RING finger that together with an E2 enzyme leads to the ubiquitination p53 (Fang *et al.*, 2000; Honda *et al.*, 1997). Other proteins, which are predicted to function in a similar fashion, include c-Cbl and perhaps ICP0. Importantly, single subunit E3 enzymes also include HECT domain proteins such as Nedd4 (Fang & Weissmann, 2004).

Multi-subunit E3 ubiquitin ligases, such SCF, require additional factors other than the substrate and the E2 to provide the correct interactions and regulatory apparatus. The benefit of a multi-subunit E3 is that their structural organisation that enables the

ubiquitin ligase associated with it to target multiple substrates for degradation (Jackson & Eldridge, 2002; Kamura *et al.*, 2002). For example, with the SCF complex, substrate specificity is provided by the F-box protein, of which there are at least 46 in humans (Sullivan *et al.*, 2003).

1.6.5. Deubiquitinating enzymes.

The final component of ubiquitin cycle is provided by the deubiquitinating enzymes (DUBs). Figure 1.6.5A gives an overview of their function. The mammalian genome is thought to encode over 65 deubiquitinating enzymes (Wing, 2003), which comprise of two classes of thiol proteases - the ubiquitin carboxy-terminal hydrolases (UCH) and the ubiquitin processing proteases, also referred to as ubiquitin specific proteases (USPs). Even though two classes of enzymes have been defined, key functional differences have not yet been defined (Wing, 2003).

The roles of the DUBs include cleavage of multi-ubiquitin chains from residual peptides (Papa & Hochstrasser, 1993), shortening of protein-bound multi-ubiquitin chains by sequentially removing the terminal ubiquitin group (Lam *et al.*, 1997), and removal of complete polyubiquitin chains from an intact substrate molecule (Canning et al., 2004). The role of UCHs is to catalyse the removal of carboxy-terminal extensions from ubiquitin precursors, as ubiquitin is initially expressed either fused to itself or to a ribosomal subunit (Pickart, 2001a). USPs are generally thought to be involved in removing ubiquitin from larger proteins and the disassembly of multi-ubiquitin chains (Chung & Baek, 1999).

DUBs also play a role in preventing the accumulation of residual multi-ubiquitin chains at the proteasome, thus ensuring the normal movement of ubiquitinated proteins to and through the proteasome (Weissman, 2001). In addition, DUBs have been shown to be constitutively active in the removal of ubiquitin from substrates, as inhibition of proteasome function causes the accumulation of mostly non-ubiquitinated proteins (Wing, 2003). An overview of the roles of the DUBs is shown in figure 1.6.4A.

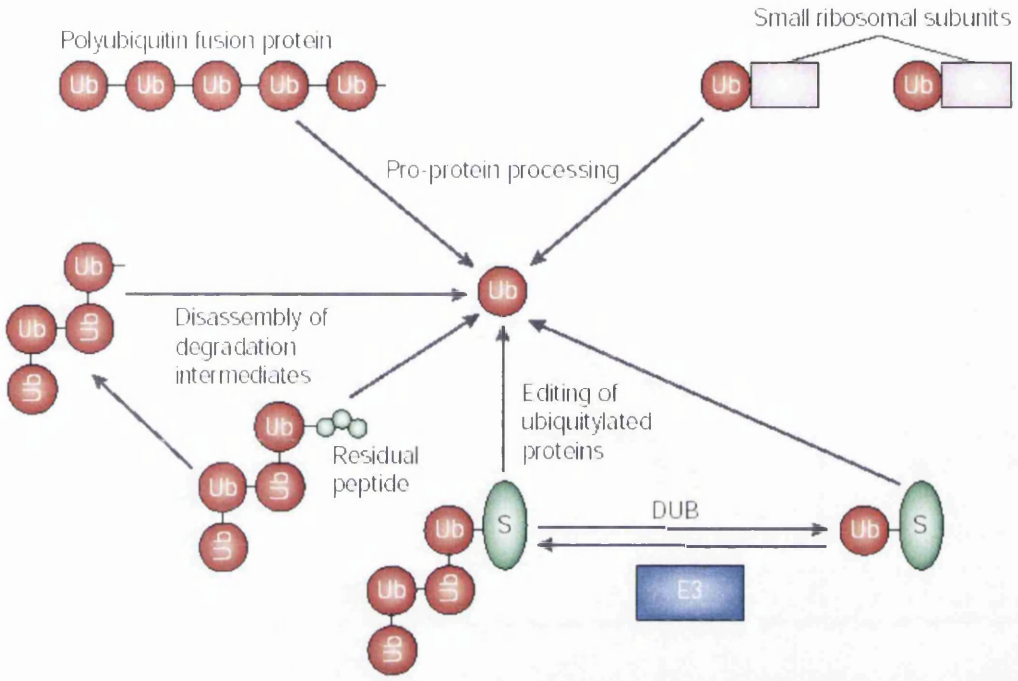


Figure 1.6.5A. *The many functions of deubiquitinating enzymes.*

The roles of deubiquitinating (DUB) enzymes is as follows: Initially the ubiquitin protein is synthesized fused to a additional ubiquitin protein (polyubiquitin) or with small ribosomal subunits, which are then processed by cleavage at the carboxy-terminal glycine by the DUB to free the ubiquitin. Once the substrate protein has been degraded, ubiquitin must be freed from residual peptides and disassembled. DUB enzymes also reverse the activity of E3 enzymes, by removing the poly-ubiquitin chains from substrates (S). This may occur in specific cellular locations where ubiquitination is occurring or at the proteasome. Adapted from Weissman, 2001.

Chapter 1- Part IV – ICP0 functions as an E3 ubiquitin ligase.

1.7.0. Introduction.

Previous research has shown that ICP0 disrupts ND10 and degrades its major constituents Sp100 and PML (Chelbi-Alix & de The, 1999; Everett *et al.*, 1998a; Everett *et al.*, 1999c). ICP0 also causes the cell cycle to stall at the pseudo-prometaphase stage by inducing the degradation of the centromere proteins CENP-C and CENP-A (Everett *et al.*, 1999a; Everett *et al.*, 1999b; Lomonte & Everett, 1999; Lomonte *et al.*, 2001). All of the aforementioned activities were shown to occur in a proteasome-dependent manner, as the presence of MG132 (a proteasome inhibitor) preserved the integrity of cellular structures targeted by ICP0. Furthermore, the RING finger was shown to be crucial, as mutation of this region abolished the ability of ICP0 to disrupt cellular structures. Shortly afterwards it was shown that ICP0 induces and colocalises with conjugated ubiquitin at centromeres and ND10 structures (Everett, 2000; Parkinson & Everett, 2001). Taken together, these data provided compelling evidence that ICP0 acts as an ubiquitin E3 ligase. This was conclusively proven when ICP0 and its isolated RING finger domain were shown to catalyse the formation of polyubiquitin chains *in vitro* (Boutell *et al.*, 2002; Hagglund *et al.*, 2002). The aim of this section will be to review the work carried out to characterise the E3 ubiquitin ligase activity of ICP0.

1.7.1. The E3 ubiquitin ligase activity of ICP0.

It is possible to assess the activity of a potential E3 ligase by using a simple *in vitro* assay, since substrate-independent polyubiquitin chain formation can occur in a buffer containing ATP, E1, the appropriate member of the E2 family of proteins and the E3 ubiquitin ligase itself. Polyubiquitin chain formation can be easily detected via SDS-PAGE analysis and Western blotting (Lorick *et al.*, 1999). The approach was undertaken to assess if ICP0 acts as an E3 ligase *in vitro*, using a truncated version of ICP0 (ICP0-262), which was expressed as a GST fusion protein in bacteria. ICP0-262 contains the residues of ICP0 exons 1 and 2, plus 21 residues derived from intron sequences, fused to GST. Purified ICP0-262 was incubated with a range of human E2

enzymes and was shown to stimulate UbcH5a and UbcH6 and cause the formation of variable length polyubiquitin chains (Boutell *et al.*, 2002). The studies also showed that the isolated residues of exons 1 and 2 of ICP0 [ICP0(241)], and full length ICP0 had identical E3 ligase activity to that of ICP0-262 (Boutell *et al.*, 2002). It should be noted that another study also identified that the RING finger domain of ICP0 stimulates UbcH5a and UbcH6 to form polyubiquitin chains *in vitro* (Hagglund *et al.*, 2002).

Transfection studies showed full-length ICP0 in a RING finger dependent manner clearly sequesters UbcH5a and UbcH6 in HEp-2 cells (Boutell *et al.*, 2002). Furthermore, studies carried out elsewhere have provided a link between E2 specificity and degradation of constituents of ND10. It was shown that mutation of the cysteine residue within the active site of UbcH5a delayed or blocked the degradation of PML and Sp100 and the subsequent dispersal of ND10 (Gu & Roizman, 2003). However, the same mutation in UbcH6 had no affect on the integrity of ND10 or indeed its constituents. Therefore it was concluded that functional UbcH5a is essential for the E3 activities of ICP0 that are required for the degradation of PML and Sp100 and the dispersal of ND10 (Gu & Roizman, 2003).

The RING finger domain of an E3 ligase plays a central role in mediating interaction with the required E2 ubiquitin-conjugating enzyme and the transfer of ubiquitin to substrate proteins (Freemont, 2000; Joazeiro & Weissman, 2000). As the RING finger domain is the hub of an E3 ligase's activity, mutation within this region has generally been shown to be deleterious, such as preventing the formation of polyubiquitin chains *in vitro* in the presence of active E2 partners. (Pickart, 2001a). Therefore, mutational analysis was carried out on the RING finger domain of ICP0 (241), to deduce the role of specific amino acids in polyubiquitin chain formation. It was shown that only deletion of the RING finger domain and insertion of 4 residues at ICP0 codons 150 and 162 abrogated ICP0's ability to act as an E3 ligase in the presence of UbcH5a (Boutell *et al.*, 2002). Furthermore, point mutations within the RING finger domain of ICP0-241 (K144E, W146A, Q148E and N151D) and some insertion mutants (*ins*188 and *ins*197) had little deleterious affect (Boutell *et al.*, 2002). Even mutation of the conserved tryptophan residue which is present in the majority of E3 ligase enzymes and is thought to be particularly important for activity (Freemont, 2000; Joazeiro & Weissman, 2000; Pickart, 2001a) had no effect on the E3 ligase activity of ICP0 (241) (Boutell *et al.*, 2002). However, in contrast to having little affect on the E3 ligase activity of ICP0

(241) *in vitro*, the majority of the aforementioned mutations had deleterious affects on ICP0's ability to simulate gene expression and disrupt ND10 (Everett *et al.*, 1995a; Everett, 1987; O'Rourke *et al.*, 1998). The fact that ICP0 appeared to retain E3 ligase activity *in vitro* despite the presence of certain mutations in the RING finger domain was probably due to the ubiquitin E3 ligase assay being insufficiently sensitive to detect subtle variations in activity (Boutell *et al.*, 2002).

The interaction between an E2 and an E3 protein is an integral part of the ubiquitination process and this can normally be detected via such means as yeast-two hybrid, GST-pull down and immunoprecipitation assays. However, no stable interaction between ICP0 or its RING finger domain with UbcH5a or UbcH6 was detected (Boutell *et al.*, 2002; Hagglund & Roizman, 2002). However, these studies conclusively prove that ICP0 acts in a RING finger dependent manner to utilise the ubiquitin conjugation pathway, which is essential for its major functions, such as gene expression during the lytic cycle and reactivation from quiescence (Boutell *et al.*, 2002; Everett *et al.*, 1998a; Hagglund *et al.*, 2002).

1.7.2. The E2 partners of ICP0.

ICP0 causes polyubiquitin chain formation in the presence of UbcH5a and UbcH6 which, as noted above, are highly related (Boutell *et al.*, 2002; Hagglund *et al.*, 2002). It is probably the conserved sequence similarity between UbcH5a and UbcH6 that enables ICP0 to stimulate polyubiquitin chain formation in their presence (Boutell *et al.*, 2002).

UbcH5a has also been implemented as a cognate E2 partner of a growing number of E3 ligases (Hakli *et al.*, 2004; Itoh *et al.*, 2003; Lorick *et al.*, 1999; Spencer *et al.*, 1999; Vandenberg *et al.*, 2003; Yang & Du, 2004; You & Pickart, 2001), as has UbcH6 (Anan *et al.*, 1998; Nuber *et al.*, 1996; Yang & Du, 2004). HPV E6 has also been shown to require members of the UbcH5 family for the ubiquitination of p53 (Jensen *et al.*, 1995; Rolfe *et al.*, 1995; Scheffner *et al.*, 1994)

Apart from the fact that many RING finger E3 ubiquitin ligases use these same two E2 enzymes, it is unclear why ICP0 should use UbcH5a and UbcH6 in preference to other E2 enzymes, as one E2 can serve several different E3 ligases. It is possible that ICP0 has additional E2 partners, as within the relatively small genome of *S.cerevisiae* there

are 13 genes that encode E2 enzymes and more are likely to be found in higher eukaryotes (Hochstrasser, 1996). Therefore as the number of characterised E2 enzymes increases so may the number of ICP0's E2 partners.

Other studies have suggested that there may be a third E2 partner of ICP0, namely cdc34 (Hagglund & Roizman, 2002). This conclusion was originated from data that showed cdc34 could be immunoprecipitated with proteasomes from HSV-1-infected cells (Van Sant *et al.*, 2001a). It was shown that the ICP0 RING finger domain could not stimulate cdc34 *in vitro* (Boutell *et al.*, 2002; Hagglund & Roizman, 2002), but despite this an interaction was identified between the two proteins (Hagglund & Roizman, 2002; Hagglund *et al.*, 2002). It was reported that ICP0 residues 543 to 768 stimulated autoubiquitination of cdc34, despite the lack of an interaction between cdc34 and this segment of ICP0 (Hagglund & Roizman, 2002; Hagglund *et al.*, 2002). Despite this, it was proposed that ICP0 must interact with cdc34 via its RING finger domain and stimulate cdc34 by residues located near its C-terminus (Hagglund & Roizman, 2002; Hagglund *et al.*, 2002; Van Sant *et al.*, 2001a). Contrary to this, results published elsewhere showed that full-length ICP0 neither stimulated cdc34 activity *in vitro* nor recruited cdc34 to ICP0 foci in transfected cells (Boutell *et al.*, 2002). The region of ICP0 between residues 543 and 768 does not contain any homology to a motif from either the HECT domain or RING finger family of proteins. However, a possible explanation of the cdc34 stimulation is the amount of the GST-fusion component used. In optimum ubiquitin ligase assay conditions, amounts of an E3 ligase as low as 3.5 ng can stimulate polyubiquitin formation. However, Hagglund and Roizman (2002) used 5 μg of the GST fusion protein containing ICP0 residues 543 to 768 in their study. In such high amounts, GST fusion proteins that lack authentic E3 ligase activity have been shown to induce cdc34 stimulation *in vitro* (Deffenbaugh *et al.*, 2003).

1.7.3. Substrates of ICP0 mediated ubiquitination.

After initial studies identified that ICP0 has E3 ligase activity, the next step was to identify the substrates of ICP0. On the basis that ICP0 induces the degradation of PML during HSV-1 infection, PML was investigated as a potential substrate of ICP0 *in vitro*. Initial studies using purified E1, E2, ICP0 and SUMO-1 modified or unmodified PML failed to detect ubiquitination of PML *in vitro*. However, it was found that ICP0 increased the levels of ubiquitinated PML in transfected cells (Boutell *et al.*, 2003). It

was concluded that ICP0-mediated degradation of PML *in vivo* is either indirect or requires additional factors that are yet to be identified (Boutell *et al.*, 2003).

The same study went on further to characterise the affect SUMO-modification had on the integrity of PML in the presence of ICP0 (Boutell *et al.*, 2003). Co-expression studies showed that isoforms of PML that contained a mutation at lysine 160, which normally becomes SUMO-modified, were degraded by ICP0 to a lesser degree than wild-type PML. It was therefore implied that SUMO-modification plays an important role in the efficiency of ICP0-induced degradation of PML (Boutell *et al.*, 2003).

Because it had been reported that p53 is protected from mdm2-induced degradation via binding to USP7 (Li et al., 2002), and because USP7 interaction contributes to ICP0 activity (Canning *et al.*, 2004; Everett *et al.*, 1998b, 1999d), it was investigated whether ICP0 had any affect on p53. It was shown that in a RING finger dependent manner and in the presence of UbcH5a or UbcH6, ICP0 ubiquitinates p53 *in vitro*. Furthermore, ICP0-induced ubiquitination of p53 could also be detected *in vivo*, albeit to a much lesser degree than that induced by mdm2 (Boutell & Everett, 2003b). Further studies demonstrated that p53 metabolism during HSV-1 infection is a complicated issue. It was found that p53 becomes stabilized during HSV-1 infection by a mechanism that is dominant over any potential ICP0-mediated ubiquitination of p53 (Boutell & Everett, 2004). The purpose of the relationship between ICP0 and p53 currently remains unclear. However, it should be noted that several viruses affect the stability of p53 by targeting it for ubiquitination and subsequent proteasome degradation, such as E6-AP in conjunction the E6 protein of HPV 16 and 18 and the adenovirus E1B-55K/E4-orf6 complex.

1.7.4. Other viruses and the proteasome degradation pathway.

A number of viruses across a wide spectrum of host and tissue tropisms utilise the ubiquitin-proteasome pathway for a range of different functions (see figure 1.7.4A) such as host cell entry, transcription and reactivation, avoidance of apoptosis and the immune response, and virus release and budding (Banks *et al.*, 2003). How the pathway is manipulated varies between viruses, for instance HPV encodes a protein that redirects a cellular E3 ligase to ubiquitinate substrates that would not normally be targeted in a non-infected cell. However, EBV encodes proteins that manipulate the proteasome

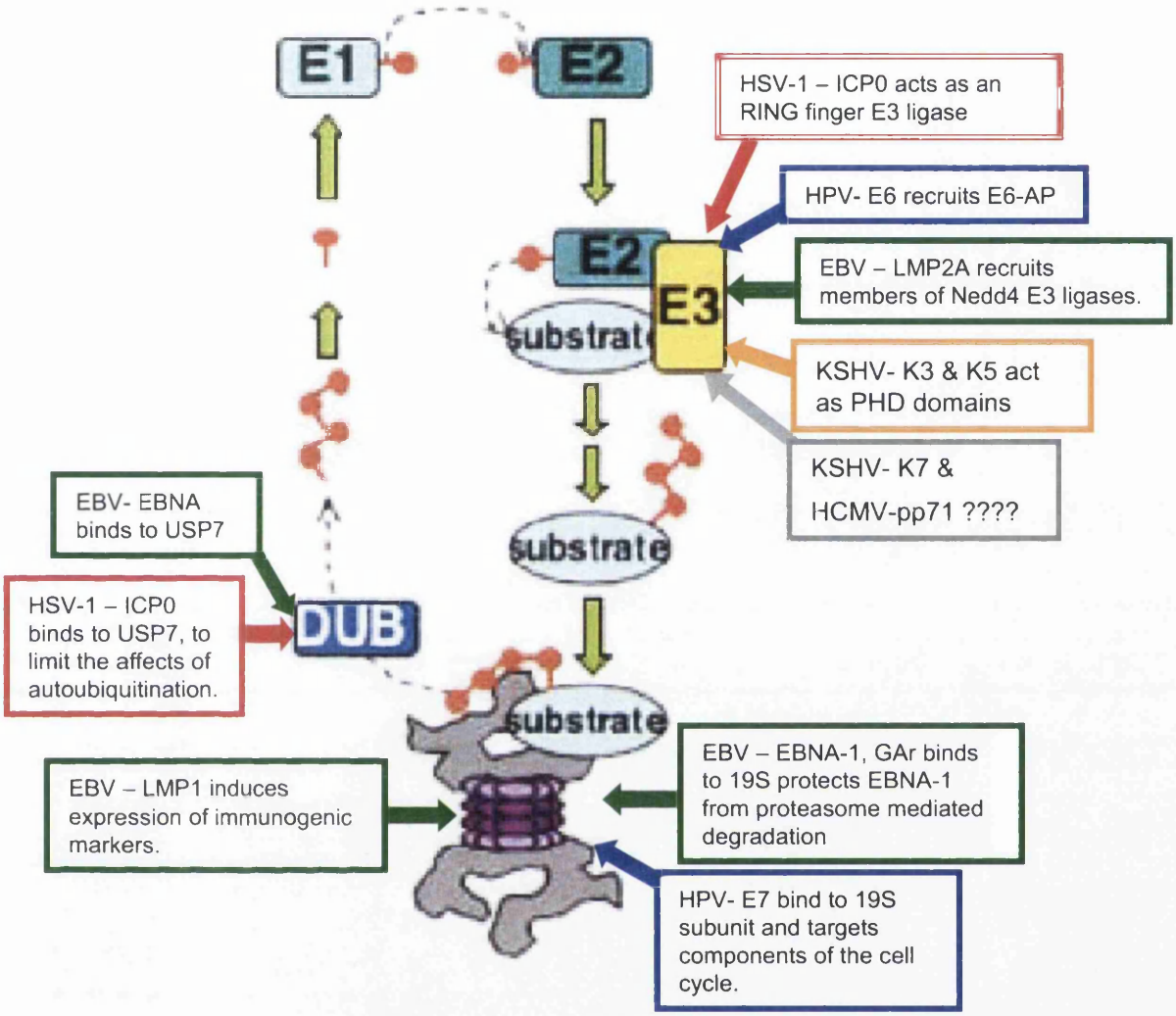


Figure 1.7.4A. *Overview of how specific DNA viruses manipulate the proteasome degradation pathway.*

The substrate becomes ubiquitinated via the sequential activity of a ubiquitin activating enzyme (E1), a ubiquitin conjugating enzyme (E2) and a ubiquitin ligase (E3). The ubiquitinated protein can under go numerous fates including endocytois and proteasome mediated degradation. Ubiquitin is recycled through the activity of deubiquitinating enzymes (DUB). Different viruses utilises the pathway at different points and for different outcomes. This is discussed in further detail in the text. Based on a diagram from Maccuri, *et al.*, 2004.

degradation pathway at multiple points, such as mediating ubiquitination of target substrates and also preventing specific substrates from becoming ubiquitinated. The aim of this section will be to outline specific examples of viruses other than HSV-1 that utilise the proteasome pathway, with particular reference to the herpesvirus family.

Human Papilloma Virus.

HPV provided perhaps the earliest characterised example of a virus that utilises the proteasome degradation pathway. The HPV E6 protein mediates the ubiquitination of target substrates by associating with the cellular HECT domain E6-accessory protein (E6-AP) to target p53 for degradation (Thomas *et al.*, 1999). E6 binds to residues 391 and 408 of E6-AP, which are flanked by hydrophobic residues that are essential for binding (Elston *et al.*, 1998). E6 additionally binds to p53 and an ubiquitin conjugating enzyme and mediates the transfer of ubiquitin from the E2, via a thiolester intermediate, to p53. It has been shown that E6-AP utilises a number of E2 enzymes including UbcH5 and UbcH6 (Jensen *et al.*, 1995; Rolfe *et al.*, 1995; Scheffner *et al.*, 1994), and also UbcH7 and UbcH8. The E6-AP protein is thought to determine E2 specificity in conjunction with the E6 protein, which additionally provides substrate specificity (Kumar *et al.*, 1997). This is supported by research that has shown that unless E6 binding occurs, E6-AP does not normally target p53 (Scheffner & Whitaker, 2003). Furthermore, during specific stages during the HPV lifecycle, p53 becomes phosphorylated and only then is it susceptible to E6/E6-AP mediated degradation, therefore indicating a precise mechanism of p53 regulation (Bech-Otschir *et al.*, 2001). The ability of HPV to degrade p53 is crucial for ensuring the progress of the viral life cycle as otherwise p53 would induce apoptosis.

Other proteins have been shown to be subject to indirect ubiquitination by E6, including the apoptosis-promoting Bak protein. It was shown that Bak is targeted by E6-AP in the absence of E6, but in its presence this activity is enhanced (Thomas & Banks, 1998). Furthermore, similar to ICP0, E6-AP has been shown to autoubiquitinate itself, which is thought to represent a feedback control mechanism (Kao *et al.*, 2000).

The prevention of apoptosis via the E6/E6-AP interaction is not the only function for which HPV uses the proteasome degradation pathway. It has been shown that the E7 protein mediates the degradation of a number of proteins associated with the cell cycle

via binding to the 19S proteasome subunit (Munger *et al.*, 2001). This causes the cell to enter the S phase of the cell cycle, creating an environment ideal for viral DNA replication (Munger *et al.*, 2001). More detailed reviews of the significance of the E6/E6-AP interaction and the function of E7 can be found elsewhere (Banks *et al.*, 2003; Scheffner & Whitaker, 2003).

Human Cytomegalovirus.

The HCMV UL82 gene product pp71 stimulates quiescent cells to enter the cell cycle. It has been shown to target hyper-phosphorylated forms of the retinoblastoma (Rb) family of proteins for proteasome-mediated degradation (Kalejta *et al.*, 2003; Kalejta & Shenk, 2003). It is not currently clear by what mechanism pp71 achieves this activity.

Epstein Barr Virus.

Epstein Barr Virus (EBV) is probably more intrinsically involved in the ubiquitin conjugation pathway than any other virus so far characterised, manipulating the pathway at different points depending on the stage of its infection cycle.

EBV establishes a persistent infection within the B cell population of the host, therefore latency and reactivation must be tightly regulated since virus production is associated with cell death. A number of virally encoded proteins are associated with maintaining the balance between lytic and latent infection, such as the latency membrane protein 2A (LMP-2A). The stimulation of the B-cell receptor (BCR) on infected B cells is thought to initiate a cell signalling cascade, which triggers productive infection (Masucci, 2004). It has been suggested LMP-2A regulates this process through its ability to inhibit BCR signalling by interfering with the activity of BCR-associated tyrosine kinases, Lyn and Syk (Miller *et al.*, 1994). LMP-2A binds to a member of the HECT Nedd4 ubiquitin E3 ligase family and via its amino terminal sequences to Lyn or Syk, which results in their eventual degradation (Winberg *et al.*, 2000). LMP-2A also undergoes ubiquitination by members of Nedd4 family of E3 ligases and this is thought to be important in the modulation of BCR signalling (Masucci, 2004). By preventing exit from latency, it is thought that LMP-2A may rescue antigen-triggered EBV-carrying memory B cells and allow their homing to lymphoid follicles where other EBV proteins play a crucial role in preventing apoptosis (Winberg *et al.*, 2000).

EBNA-1 is an essential protein expressed throughout the EBV life cycle and it interacts with different components of the ubiquitin pathway. The N-terminal half of EBNA-1 contains a long repetitive sequence exclusively composed of Gly and Ala residues (GAr) that varies in length between different EBV isolates (Falk *et al.*, 1995). The GAr region is thought to play an important role in preventing immunodetection of infected B cells. Interestingly one of its mechanisms of action is to inhibit ubiquitin-dependent proteolysis, as when linked to other viral or cellular proteasome substrates their degradation was severely inhibited (Levitskaya *et al.*, 1997). The GAr sequences do not require any of the enzymes involved in the ubiquitin conjugation pathway for their affect. It is thought to inhibit proteasome-mediated degradation by interfering with the interaction of the ubiquitinated substrate with the 19S proteasome subunit thereby promoting the premature release of the substrate (Masucci, 2004). Like ICP0, it has recently been shown that EBNA-1 interacts with USP7 (Holowaty *et al.*, 2003b). Although the significance of this interaction remains unclear, it was proposed that USP7 is involved in regulating EBNA-1 during transcription and translation of the viral genome (Holowaty *et al.*, 2003b). Additional studies from the same group showed that EBNA-1 competes with p53 for USP7, thus affecting the function of p53 *in vivo* (Holowaty *et al.*, 2003a). It is possible that EBNA-1 is increasing the degradation of p53 by reducing the cellular pool of available USP7 and preventing the deubiquitination of p53, thus inhibiting the induction of apoptosis.

EBV-infected B cells can become oncogenic, resulting in uncontrolled proliferation. To contain this hazard, the viral proteins initiate a plethora of B-cell activation markers, rendering the infected cells highly immunogenic and therefore easily attacked by T-cell-mediated immune responses (Masucci, 2004). LMP-1 is crucial for regulating this mechanism by various methods, including being subjected to proteasome-mediated degradation. This process causes the generation of peptides involved in signalling cascade that in turn affects the immunogenicity of the infected cell (Dukers *et al.*, 2000).

Kaposi's Sarcoma-Associated Herpes Virus.

Kaposi's Sarcoma-Associated Herpes Virus (KSHV) encodes two membrane-bound proteins, K3 and K5, which modulate immune recognition of infected cells (Coscoy *et al.*, 2001). K3 and K5 both include N-terminal PHD domains that are related to the

RING finger domain family (Coscoy & Ganem, 2003). K5 was shown to cause the formation of substrate-independent polyubiquitin chains in the presence of UbcH5a (Coscoy *et al.*, 2001). Both K3 and K5 promote evasion of the immune system by ubiquitinating the major histocompatibility complex class I chains, which leads not to proteasomal, but to endosomal degradation (Coscoy & Ganem, 2003; Coscoy *et al.*, 2001). Furthermore, it has been recently reported that K7 targets a regulator of the ubiquitin conjugation pathway to ubiquitinate p53, which is thought to prevent the induction of cellular apoptosis in KSHV-infected cells (Feng *et al.*, 2004).

1.8.0. The ICP0 Family of Proteins.

1.8.1. Sequence comparisons of the members of the ICP0 family of proteins.

All the alphaherpesviruses for which appropriate DNA sequences are available encode a protein related to ICP0 by virtue of their possession of a RING finger domain: BICP0 in bovine herpes virus (BHV) -1 (Wirth *et al.*, 1992) and its homologue in BHV-5 (Delhon *et al.*, 2003); the product of gene 63, Eg63, in EHV-1 (Telford *et al.*, 1992); the product of gene 61, Vg61, in VZV (Davison & Scott, 1986); EP0 in PRV (Cheung, 1991); CICP0 in CHV (Miyoshi *et al.*, 2000); FICP0 in FHV-1 (Sussman & Maes, 1997); and the product of the RL2 gene in HVB (Perelygina *et al.*, 2003). All of these proteins are expressed from genomes that are composed of unique long and unique short regions, and gene expression is divided into three classes in a similar manner to that of HSV-1. However, only ICP0, Eg63, Vg61 are IE products, while CICP0 and EP0 have been shown to be transcribed during the early phase of gene expression (Cheung, 1991; Honess & Roizman, 1974; Miyoshi *et al.*, 2000; Nagpal & Ostrove, 1991). BICP0 is classed as an immediate early gene even though it can be translated from immediate early and early mRNA transcripts (Koppel *et al.*, 1997; Wirth *et al.*, 1992).

By comparison of the amino acid sequences of these related proteins using the ClustalW alignment program (Thompson *et al.*, 1994) it is possible to identify the RING finger motif (Figure 1.8.1A). Apart from core residues within the RING finger motif, the only readily apparent homology shared amongst the ICP0 related proteins is that of the nuclear localisation signal. It is striking that the majority of the members of the ICP0 protein family do not contain a sequence similar to that of the USP7 binding region or the multimerisation domain; these sequences are limited to the proteins encoded by HSV-1, HSV-2 and HVB (Everett *et al.*, 1998a; Parkinson & Everett, 2000, 2001). This may be a reflection of the fact that HVB is more closely related to HSV-1 than any other member of the alphaherpesvirus family. Examination of the RING finger sequences revealed that even within this conserved motif, with the exception of the zinc-coordinating and a few other structurally important residues, the sequence similarity amongst the ICP0 related proteins is limited. Because of this extensive sequence divergence, it was unclear whether all the proteins in the ICP0 family shared the same biological functions (Everett *et al.*, 1995b).

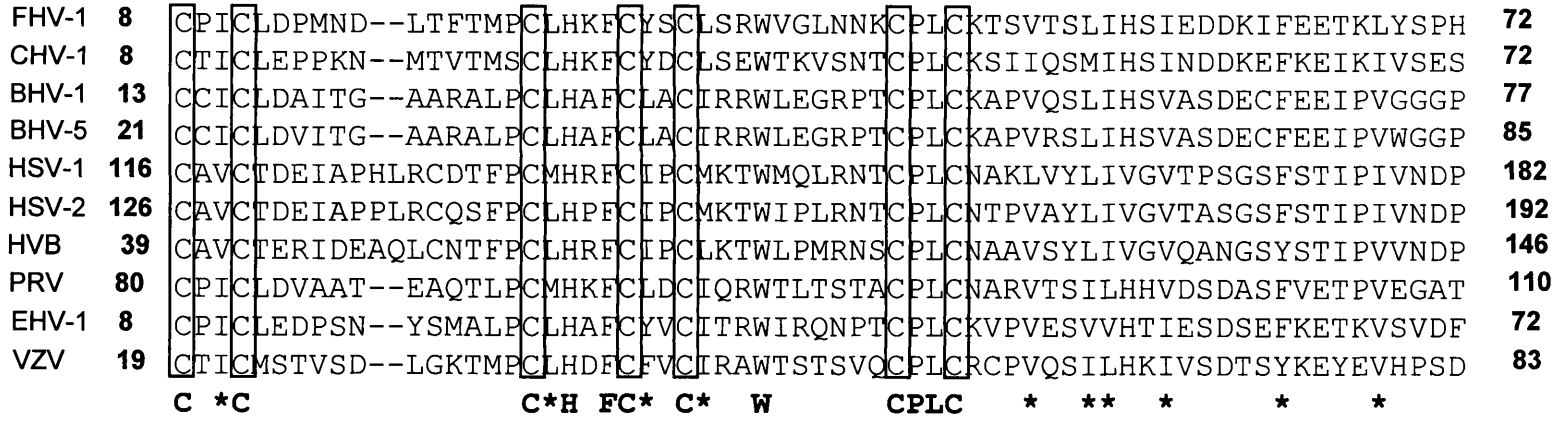


Figure 1.8.1A. *Comparison of the deduced amino acid sequences of the RING finger domains of the alpha-herpesvirus ICP0 family of proteins.*

The alignment was carried out using ClustalW (Thompson *et al*, 1994.). The coordinates of the amino acid residues relating to the initiating methionine are indicated. Gaps are introduced into the sequence in dashes for best alignment. Boxed amino acids residues indicate the structurally coordinating residues of the RING finger domain. A consensus sequence of the conserved residues and positions of similar hydrophobic residues (*) are shown underneath. FHV = Feline Herpes Virus; CHV = Canine Herpes Virus; BHV = Bovine Herpes Virus; HSV = Herpes Simplex Virus, HVB = Herpes B Virus; PRV = Pseudorabies Virus; Equine Herpes Virus; VZV = Varicella Zoster Virus.

1.8.2 The biological functions of the alphaherpesvirus ICP0 related proteins.

Of all of the members of the ICP0 family so far identified, the most extensively studied proteins are BICP0, Eg63, EP0 and Vg61. This section will review their known biological functions.

1.8.3. The role of Vg61.

Vg61 is encoded by ORF61 of the VZV genome. There are conflicting reports about the ability of this protein to activate or repress gene expression. In transient expression assays it was shown that ORF61 acts as a transactivator of gene expression, and acts as a repressor of the transactivating ability of ORF62 (the VZV equivalent of HSV-1 ICP4) and ORF4 (Moriuchi *et al.*, 1992; Moriuchi *et al.*, 1993). This is unlike ICP0, which does not have a transrepressing function and can function in synergy with the HSV-1 transactivator ICP4.

However despite these differing results, it has been reported that cell lines expressing ORF61 complement the growth of HSV-1 mutants which fail to express functional ICP0 or VP16 (Moriuchi *et al.*, 1993). However, strains of HSV-1 that had mutations within the ICP4 and ICP27 genes were unable to grow in these cells. It was therefore concluded that despite the differences in their sequences and effects on the cognate promoters in transient expression assays, VZV ORF61 is the functional homologue of ICP0 (Moriuchi *et al.*, 1992). To analyse how the cysteine and histidine residues contribute to the function of the Vg61 RING finger domain, each of the residues were individually mutated. None of the mutants retained the ability to act as a transactivator of gene expression (Moriuchi *et al.*, 1994). Furthermore, it was shown that a functional chimera protein could be made by exchanging the N-terminal portion (which contains the RING finger domain) of the Vg61 protein with that of ICP0. The fusion protein retained its transregulatory function (Moriuchi *et al.*, 1994). This probably reflects the fact that the Vg61 RING finger domain, like the corresponding domain in ICP0, is crucial for the majority, if not all of its biological functions.

1.8.4. The role of Eg63.

The Eg63 protein of EHV-1 has been shown partially to complement an HSV-1 ICP0 deficient virus. A recombinant HSV-1 virus was constructed that expressed the Eg63 protein in place of ICP0. Comparison of the growth properties to that of wild type indicated that Eg63 is able to fulfil partially, but not completely, the roles of ICP0 during virus growth in tissue culture (Everett *et al.*, 1995b). Similar to ICP0, Eg63 is able to act as a transactivator of gene expression of all three classes of viral promoters in the EHV-1 genome; up-regulating the expression of the immediate-early promoter (driving expression of the EHV-1 homologue of ICP4), the early thymidine kinase promoter, the late IR5 promoter and the late glycoprotein K promoter (Bowles *et al.*, 1997; Bowles *et al.*, 2000). Furthermore, Eg63 also acts as promiscuous transactivator of gene expression in transfection assays, up regulating gene expression from the ICP6 promoter of HSV-1 (Bowles *et al.*, 1997; Everett *et al.*, 1995b). Using transfection assays it was shown that individually the Eg63 and EHV-1 ICP4 homologue proteins are powerful regulators of gene expression. However, when expressed together EHV-1 ICP4 inhibited the ability of the Eg63 protein to transactivate the IE and late promoters (Kim *et al.*, 1999). However, in the presence of the EHV-1 ICP27 homologue, ICP4 acts as synergistic transactivator to regulate the expression of the early and late promoters (Bowles *et al.*, 2000). The mechanism of the antagonistic relationship between Eg63 and the ICP4 homologue may be due to their interactions with components of the cellular transcriptional machinery. The transcription factors TFIID and TFIIB are components of the pre-initiation complex (PIC), which is essential for gene expression. These transcription factors indirectly mediated the binding to specific promoter complexes, interacting with TATA binding protein (TBP). It was shown that regions of Eg63 interact directly with TFIIB and TBP and may contribute to the transactivation properties of Eg63 (Kim *et al.*, 2003). However, regions of the EHV-1 ICP4 homologue has also been shown to bind to TBP and the same region of TFIIB, and this interaction is necessary for its full transactivation properties (Albrecht *et al.*, 2003; Jang *et al.*, 2001). Furthermore, it was shown that Eg63 and the EHV-1 ICP4 homologue also interact with one another. It was proposed that this binding of the ICP4 homologue in some way sequesters Eg63 and prevents it interacting with the aforementioned transcription factors or they compete for binding to the transcription factors and mediate the initiation of gene expression (Kim *et al.*, 2003). The EHV-1 ICP4 homologue is only able to activate the E promoters, however Eg63 is able to activate all promoters types,

therefore it was proposed that the antagonistic relationship between Eg63 and the ICP4 homologue may prevent Eg63 inappropriately activating L promoters during early times of infection (Kim *et al.*, 2003). If the assumption is made that this hypothesis is correct and we extend it to other members of the alphaherpesvirus family, it becomes confusing. HSV-1 ICP4 has also been shown to interact with TFIIB and TBP (Smith *et al.*, 1993), and furthermore it has been suggested to interact with ICP0 (Yao & Schaffer, 1994) and synergistically they promote gene expression of early and late promoters (Everett, 1986). Furthermore, the ability of the EHV-1 ICP4 homologue to act as a transrepressor of late gene expression is unique, as no other alphaherpesvirus ICP4 homologue have shown to have this function (Bowles *et al.*, 2000). It is likely that the confusion that arises from these results is a consequence of the limitations of the plasmid reporter transfection assays and *in vitro* protein-protein interaction methods that have been employed.

It has been shown, as with the other ICP0 homologue that Eg63's ability to act as a transactivator is dependent on a functional zinc RING finger domain, as a series of deletion mutants spanning this region were unable to activate the E and L promoters of EHV-1 genome (Bowles *et al.*, 2000). Like ICP0, the Eg63 protein is phosphorylated during infection and deletion of a serine rich region (residues 210 to 217) reduced by over 70% the ability of Eg63 protein to activate gene expression (Bowles *et al.*, 2000).

Studies carried out recently have used an EHV-1 Eg63-null mutant virus. The initial studies have shown that transfection of rabbit kidney cells with a BAC encoding an EHV-1 Eg63 null genome causes the production of infectious progeny. This indicates that Eg63 is not essential for EHV-1 replication (Yao *et al.*, 2003). Experiments to assess the effect of the Eg63 deletion on EHV-1 gene expression revealed that mRNA expression of the all three major classes was reduced when compared to that of wild type virus. It has also been shown that the absence of ICP0 causes a reduction in mRNA synthesis during HSV-1 infection (Jordan & Schaffer, 1997). Furthermore, plaque number and plaque size of Eg63-null virus were less than that of wild type EHV-1. However, the temporal cascade from early to late viral gene expression in the EHV-1 genome was not prevented or delayed by the absence of Eg63. The authors suggested that Eg63 gene did not disrupt the temporal aspects of EHV-1 gene regulation (Yao *et al.*, 2003). However, for future research it will be important to consider how the M.O.I. and cell type affect the phenotype of an Eg63-null virus.

1.8.5. The role of EP0.

A PRV EP0 knock out virus was shown to replicate less efficiently in tissue culture and swine. Furthermore, it was shown to have a similar growth cycle to wild type PRV *in vivo*, however in cell culture, plaques were smaller and the titre of the progeny virus was distinctly lower than that of the wild type. It was concluded that EP0 is non-essential for PRV replication; however, viral growth is greatly enhanced in its presence (Cheung *et al.*, 1994). The restricted growth characteristics were thought to be analogous to those of ICP0-deficient HSV-1 viruses (Cheung, 1996). It was also demonstrated that, as in the case of ICP0, the RING finger of EP0 is essential for its ability to transactivate gene expression of the thymidine kinase (TK) and glycoprotein X (gX) promoters (Watanabe *et al.*, 1995). More recently it was suggested that EP0 could also act as a transrepressor and inhibit the activity of glycoprotein E gene (Chang *et al.*, 2002) and VHS (virion host shutoff protein) promoters (Chang *et al.*, 2004).

Furthermore, it has been shown in transfection assays using an EP0-deficient PRV virus that growth could be complemented in cells expressing Eg63 or ICP0 (Moriuchi *et al.*, 1995). EP0 was also shown, using transient expression assays, to be able to increase expression from both HSV-1 and VZV promoter reporter cassettes, indicating its functional homology to Eg63 and ICP0 (Moriuchi *et al.*, 1995). It has also been shown that EP0 acts synergistically with the PRV ICP4 homologue IE180 to enhance the transcription of early and late genes in a manner similar to that of ICP0 and ICP4 (Ono *et al.*, 1998).

1.8.6 The role of BICP0.

The BHV-1 homologue BICP0 has also been shown to be an IE, zinc-binding nuclear protein that functions in transient transfection assays as either a transactivator or transrepressor of all classes of BHV-1 promoters (Fraefel *et al.*, 1994) and also of certain heterologous promoters (Zhang & Jones, 2001) including the HSV-1 TK promoter (Inman *et al.*, 2001).

Functional requirement for zinc ions has not yet been shown for any of the aforementioned ICP0 homologues; it has only been shown that the RING finger

domains of Eg63, ICP0 and Vg61 bind zinc (Everett *et al.*, 1993a). However, in BICP0 it was demonstrated that its ability to act as a transactivator of gene expression is dependent on the presence of zinc ions (Fraefel *et al.*, 1994). This conclusion may reflect the fact that the zinc atoms are structurally important for the integrity of the RING finger domain.

It has been shown that BICP0 can induce aggregation of chromatin structures in transfected cells in a RING finger dependent manner (Inman *et al.*, 2001). This activity may be related to BICP0's ability to interact with class I histone deacetylase (HDAC), which plays a role in many important cellular functions including chromatin assembly (Zhang & Jones, 2001). ICP0 has also been shown to colocalise and reorganise type I and type II HDAC enzymes, and to interact with specific members of the HDAC type II family (Lomonte *et al.*, 2004). BICP0 and ICP0 may target HDAC family members as the may be part of a cellular repression mechanism. It has been shown that ICP0 negative virus can be reactivated from quiescence by expression of ICP0 from an external source or, albeit to a lesser degree by the addition of trichostatin A, an HDAC inhibitor (Everett *et al.*, 1998c; Harris *et al.*, 1989; Hobbs & DeLuca, 1999; Preston & Nicholl, 1997; Samaniego *et al.*, 1998).

It has also been shown that deletion of amino acids spanning residues 356-677 at the C-terminus of BICP0 prevents the translocation of the protein to the nucleus (Inman *et al.*, 2001). This phenotype would suggest that BICP0 contains a nuclear localisation signal (NLS) within its C-terminal region.

Interestingly, the function of BICP0 was shown to be blocked by prostaglandin D2. Prostaglandins are a class of naturally occurring carbon fatty acids that are produced in response to various external stimuli, such as cell injury and inflammation. They have also been implicated in regulating the replication of several DNA and RNA viruses, including HSV-1 in cultured cells (Santoro, 1997). It was shown that wild-type BHV-1 infection, increased PGD(2) levels in cells in culture and this affect was BICP0-dependent, as it was not observed with a BICP0 negative virus. Furthermore, PGD(2) added exogenously repressed BHV-1 replication in cultured cells. It was therefore suggested that PGD(2) impairs the ability of BICP0 to act as a transactivator of gene expression, by an as yet uncharacterised mechanism (Saydam *et al.*, 2004).

From outlining what is known about the activities of the members of the ICP0 family of proteins it appears that they have many functional similarities. However, there are also differences in their activities that may be explained by their limited homology outside or indeed even within their RING finger domains.

1.8.7. The effect of the ICP0 homologues on cellular structures.

It is likely that ICP0 carries out its role in activation of transcription and reactivation from latency by interacting with various cellular components (as reviewed in section 1.5.6), such as its strong binding to USP7, and the degradation of specific constituents of ND10. Studies have been carried out to assess whether BICP0, Eg63, EP0 and Vg61 have the same effects as ICP0 on protein stability and cellular sub-structures. A summary of these results is shown in figure 1.8.8A. No obvious sequence homology to the USP7 binding domain of ICP0 has been found in the aforementioned homologues, but it was investigated whether the related proteins still affect USP7 by interacting via an uncharacterised binding domains. The following conclusions were made: BICP0, Eg63, EP0 and Vg61 all cause changes to ND10 structures in transfected cells. It was shown that Sp100 was affected more readily than PML, and even more so than CENP-C in centromeres. Using a mutant HSV-1 virus that expressed Eg63 in place of ICP0 it was shown that PML, Sp100 and CENP-C were disrupted in a fashion similar to that of ICP0, therefore it was proposed that Eg63 might also be dependent on proteasome degradation pathway for disruption of cellular components. It was proposed that the difference in specificity of the ICP0 homologues on components of ND10 suggests that there may be additional components that are more preferentially targeted. This facet may reflect the differences in hosts and cell types that are infected by the viruses (Parkinson & Everett, 2000).

Despite the variations between the properties of these particular members of the ICP0 family of proteins, the data indicate that the ICP0 homologues may function via similar biochemical pathways. Indeed, all these proteins induced the formation of co-localising conjugated ubiquitin. It was therefore suggested that like ICP0, the ICP0 family of proteins function via a related mechanism involving the E3 ubiquitin ligase pathway (Parkinson & Everett, 2001).

Properties	ICP0	BICP0	Eg63	EP0	Vg61
Dispersal of PML	+++	+++	++	+	*No*
Dispersal of Sp100	+++	+++	+++	++	+
Dispersal of CENP-C	+++	+++	++	++	+
Redistribution of USP7	+++	*No*	*No*	*No*	*No*
Induce loss of SUMO modified PML	+++	*No*	*No*	*No*	No
Induce colocalisation of conjugated ubiquitin	+++	+++	+++	++	+

Figure 1.8.7A. *Summation of the biological properties of the ICP0 homologues.*

A series of studies were carried out (Parkinson & Everett, 2000, 2001) to characterise the affect of selected members of the ICP0 family of proteins on constituents of ND10 domains and centromeres. The activity of ICP0 is shown as a comparison.

1.8.8. Conclusion of the comparison of the biological properties of the ICP0 homologues.

The previous sections have shown that there are a distinct number of similarities shared amongst the ICP0 related proteins. However, there are differences between the intrinsic activities of the ICP0 homologues that may be a reflection of the sequence divergence within the RING finger domains and their flanking sequences. It is clear from the studies by Parkinson & Everett (2000 & 2001) that the members of the ICP0 family of proteins are all likely to function through the ubiquitin- proteasome pathway; however it remained to be established that this was indeed the case at the biochemical level.

Chapter 2.0 Materials and Methods

2.1 Materials.

2.1A Plasmids.

The following plasmids were provided by the acknowledged authors:

pGEX2TNMCR (Everett *et al.*, 1997; Meredith *et al.*, 1994). This vector is designed for inducible, high-level bacterial expression of proteins or protein fragments as fusions with *Schistosoma japonicum* glutathione S-transferase (GST). Expression of GST fusion proteins is under the control of the IPTG-inducible tac promoter and an internal *lac* I^q gene, which ensures that expression is inducible when, transformed into certain strains of *E.coli.* Plasmid *pGEX2TNMCR was* derived from pGEX2T, which is commercially available from Pharmacia.

pGEX241 (Boutell *et al.*, 2002). A derivative of pGEX2TNMCR in which the GST open reading frame is fused to the N-terminal 241 codons of ICP0 (encoded by the gene IE1 exons 1 and 2).

pGEX211 (Boutell *et al.*, 2002). A derivative of pGEX241 from which the ICP0 coding sequence beyond the *Kpn*I site in codon 212 has been deleted.

pGEX241K144E, pGEX241W146A, pGEX241Q148E, pGEX241N151D, pGEX241*ins*150, pGEX241*ins*162, pGEX241ins188, pGEX241*ins*197 (Boutell *et al.*, 2002). A series of plasmids derived from pGEX241 containing point and insertion mutations in the core RING finger domain coding region of ICP0. The mutants and their derivatives were constructed by inserting exon 2 fragments from previously characterised mutant plasmids (Everett *et al.*, 1995a; O'Rourke *et al.*, 1998) in the place of the wild type fragment.

pGEXENX (R.D. Everett, unpublished). A derivative of pGEX2TNMCR containing the *NcoI-DraI* fragment encoding the first 63 residues of the EHV-1 gene 63 protein (Eg63) linked to the GST open reading frame. The plasmid was derived from the previously characterised p110ENX (Everett *et al.*, 1995b).

pCIrtagBICP0, pCIrtagEg63, pCIrtagEP0, pCIrtagVg61 (Parkinson & Everett, 2001). A series of plasmids expressing N- terminal tagged proteins of the ICP0 family expressed by BHV-1, EHV-1, PRV and VZV respectively. The tag sequence encodes an epitope from the C-terminal peptide of the HSV-1 UL30 protein that is recognised by rabbit polyclonal serum r113 (Marsden *et al.*, 1994). The vector is pCIneo (Promega), in which open reading frames of choice can be inserted downstream of the HCMV IE promoter to allow expression in mammalian cells.

pPP65BICP0, pPP65Eg63, pPP65EP0, pPP65Vg61. (Parkinson & Everett, 2000). A series of plasmids expressing the ICP0 family of proteins with an N-terminal tag derived from the HCMV pp65 protein. Expression is driven by the HCMV IE promoter.

pET24aUbcH5a, pET24aUbcH6, pET24aUbcH7, pET24aUbc10, pET24acdc34 (Boutell *et al.*, 2002). A series of plasmids allowing high-level expression of polyhistidine tagged E2 enzymes in bacteria. Expression of the coding sequence of the E2s is under control of IPTG-inducible T7 promoter. The vector plasmid is pET24a (Novagen).

pFastBac-HTa. This vector contains a pUC9 origin of replication for propagation in *E. coli*. It also contains mini Tn7 elements that permit site-specific transposition of the gene of interest into a bacmid containing the entire baculovirus genome (Luckow *et al.*, 1993), propagated in DH10Bac bacteria (see section 2.1F). Cloning of an open reading frame into the multi-cloning site introduces a polyhistidine tag onto the N-terminus of the expressed protein, allowing purification by metal chelate affinity chromatography. Upstream of the inserted open reading frame site lies the baculovirus polyhedron promoter to allow high-level expression within insect cells. The plasmid was purchased from Invitrogen.

pCMV2UbcH6 (Boutell *et al.*, 2002), pCMV2cdc34 (R.D. Everett, unpublished). These plasmids express FLAG-tagged E2 enzymes UbcH6 and cdc34 in transfected mammalian cells by virtue of the HCMV IE promoter.

2.1B Enzymes.

Restriction enzymes were obtained from Boehringer Mannheim or New England Biolabs. DNase, RNase A and lysozyme were purchased from Sigma and T4 polynucleotide kinase, T4 DNA ligase, calf intestine phosphatase, *E.coli* DNA polymerase I Klenow fragment and proteinase K were purchased from Boehringer Mannheim. PCR was carried out using *Thermus aquaticus* T4 DNA polymerase (Taq polymerase) obtained from Boehringer Mannheim.

2.2C Synthetic oligonucleotides.

Oligonucleotides for PCR amplification and sequencing were synthesised and purified by MWG Biotech and Sigma-Genosys.

2.3D Cell lines.

All cell media and supplements were obtained from Gibco-BRL. The following cell lines were used in experimental work and were obtained from the cytology department of the Institute of Virology. The formulations of the media are described below, and in future sections of the text the supplemented media used for cell growth are referred to as complete media.

HEp-2. An epithelial cell line which was grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 100 units/ml penicillin and 100 μg/ml streptomycin.

Spodoptera frugiperda 21 (Sf21). An insect cell line derived from larval ovarian tissue grown in complete TC-100 medium, supplemented with 10% FCS and 100 units/ml penicillin and 100 μg/ml streptomycin.

Vero. A cell line derived from African green monkey kidney cells; grown in DMEM supplemented with 10% FCS and 100 units/ml penicillin and 100 μg/ml streptomycin.

2.1E Bacterial strains.

The following strains of *E. coli* were used:

E. coli DH5α *(*F'/*end*A1 *hsd*R17 (r_k^-mk+) *sup*E44 *thi*l *rec*Al *gyr*A (Nal^r) relAlΔ (*lac*ZYA-*arg*F) U169 (Φ80*dlac*Δ(lacZ)M15) was purchased from Invitrogen and was used for maintenance and propagation of plasmid DNA.

E. coli BL21 (DE3) pLysS (F$^-$ *ompT* $r_B^-m_B^-$) was used for bacterial expression of proteins. The bacterial strain contains an integrated lysogenic λ bacteriophage DE3 that has an IPTG-inducible promoter from which T7 RNA polymerase is expressed and the plasmid pLysS that encodes T7 lysozyme. The bacteria were purchased from Novagen.

E. coli DH10 Bac (F- *mcr*A.(*mrr-hsd*RMS-*mcr*BC) ö80*lac*Z.M15 .lacX74 *deo*R *rec*A1 *end*A1 *ara*D139 .(*ara*, *leu*)7697 *gal*U *gal*K ë- *rps*L *nup*G/bMON14272/pMON7124) contains a baculovirus shuttle vector (bacmid) and a helper plasmid that provides the Tn7 transposition function (Barry, 1988). The bacteria were purchased from Invitrogen.

2.1F Bacterial culture media.

The DH5α *E. coli* strain was grown in LB medium (10 g NaCl, 10 g Bactopeptone, 5 g yeast extract in 1 L water, pH 7.5) and all other strains in 2YT broth (5 g NaCl, 16 g Bactotryptone, 10 g yeast extract in 1 L water). Agar plates were made with 1.5% (w/v) agar in LB. Where necessary, media and agar plates were supplemented with antibiotics at the following concentrations: 70 μg/ml Ampicillin, 50 μg/ml of Kanamycin, 10 μg/ml Tetracycline, 7 μg/ml Gentamycin. The antibiotics used depended on the resistance being conferred by the plasmid being harboured by the bacteria. When blue/white screening was used to detect positive colonies, agar plates were supplemented with the following reagents: 100 μg/ml Bluo-gal (Novagen), 40 μg/ml IPTG.

2.1G Solutions.

Recipes of general solutions have been listed below, the details of all other solutions have been included either in the text or at the end of the relevant section.

Formamide dye mix:	10 mM EDTA, 1 μg/ml xylene cyanol FF, 1 μg/ml bromophenol blue in formamide.
10x Loading buffer:	(for agarose gels and non-denaturing polyacrylamide gels): 1x TBE, 1% SDS, 50% glycerol, 1 μg/ml bromophenol blue.
PBS (A):	170 mM NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2P04, pH 7.2.
STET:	8% sucrose, 5% Triton-X 100, 50 mM EDTA, 50 mM Tris-HCl (pH 8).
20x TBE:	2.5 M Tris, 0.8 M boric acid, 54 mM EDTA.
1x TE:	10 mM Tris-HCl, 1 mM EDTA (pH 8).
Transfer buffer:	40mM Tris, 48mM glycine, 20% methanol, 0.4% SDS
EP0 265 buffer:	100 mM Tris-HCl, 150 mM NaCl, 10% Glycerol, 0.1% NP40, 20 mM β-mercaptoethanol.

2.1H Radiochemicals.

Chemical	Stock concentration	Supplier
[^{35}S]-L-methionine	(10μCi/μl)	Amersham

2.1I Chemicals.

All chemicals and reagents were purchased from BDH Chemicals UK or Sigma Chemical Company, unless otherwise stated below or in relevant sections:

Amersham Life Science:	Rainbow protein markers.
Beecham Research:	Ampicillin.
Bio-Rad:	Ammonium perisulphate (APS), N,N,N',N'-tetramethylethylenediamine (TEMED), coomassie brilliant blue.
Boehringer Mannheim:	Protease inhibitors.
Difco:	Agar.
Fisons:	Ammonium hydroxide, acetone.
Fluka:	Formaldehyde
Invitrogen:	IPTG, Lipofectamine PLUS liposomal transfection reagent.

Marvel:	Dried skimmed milk
Melford Laboratories Ltd:	Caesium chloride
National Diagnostics:	30% acrylamide (2.5% cross-linker)
Prolabo:	Boric acid, butanol, chloroform, ethanol, glacial acetic acid, glycerol, hydrochloric acid, isopropanol, methanol
Scotlab:	N, N'-methylene-bis-acrylamide 19:1
UKC Chemical Laboratories:	Citifluor

2.1J Antibodies.

Penta-HIS:	Commercially available (Novagen) mouse monoclonal antibody used against polyhistidine epitope tagged proteins.
Anti-FLAG (M2):	A mouse monoclonal antibody commercially available from Sigma that recognises the FLAG epitope tag.
Anti-UL30 (r113):	A rabbit polyclonal antibody raised against an epitope from the C-terminal peptide of HSV-1 UL30 gene (Marsden *et al.*, 1994).
Anti-c-myc (9E10):	A mouse monoclonal antibody commercially available from Santa Cruz Biotechnology.
Anti-Ubiquitin (FK2):	A mouse monoclonal antibody that targets polyubiquitinated or monoubquitinated proteins (Fujimuro *et al.*, 1994). The antibody recognises the isopeptide link between ubiquitin and a protein to which it is conjugated. It is commercially available from Affiniti.
Anti-Ubiquitin(P4D1)	A mouse monoclonal antibody that recognises all forms of ubiquitin. It is commercially available from Santa Cruz Biotechnology.

2.1K Commonly used proteins.

The ubiquitin activating enzyme (E1) and the ubiquitin conjugating enzymes (E2) used in the studies described herein were sourced from communal lab stocks. A detailed overview of the methods used to express and purify the E1 and E2 enzymes is described elsewhere (Boutell *et al.*, 2002).

2.2. Methods.

The Use of Centrifuges.

In the following section, specific centrifuges are used frequently and are referred to as follows: A 'microfuge' is a MSE Micro Centaur. A 'bench top centrifuge' is a Sorvall RT7. All other types of centrifuges are described fully in the text where used.

2.2 Nucleic Acid Manipulation

2.2A Nucleic acid restriction enzyme digestion.

Restriction enzyme digestion was carried out according to manufacturer's instructions. Normally, 0.5 μg of plasmid DNA was used in diagnostic restriction digestion of cloned DNA. 10 μg - 20 μg of DNA was digested to generate DNA fragments for cloning purposes.

2.2B Blunt-end formation at DNA 5' overhangs.

Blunt-ending of 5' DNA overhangs was used to enable the ligation of DNA fragments with incompatible cohesive ends. The restriction digestion of the DNA was allowed to proceed as normal. Upon completion, the mixture was incubated in the presence of 50 μM of all four dNTPs and 2 units of DNA polymerase I Klenow fragments for 30 min to 1 hr.

2.2C Dephosphorylation of DNA fragments.

The 5' and 3' ends of vector DNA were dephosphorylated to prevent re-circularisation of vector fragments during ligation reactions. Restriction digestion of the vector was allowed to proceed as normal (see section 2.2A) in the presence of 1 unit of calf intestinal phosphatase, added at the start of the restriction digestion reaction, and a second unit was added halfway through the reaction.

2.2D Ligation of DNA fragments.

DNA ligation reactions were carried out with a vector/insert ratio of usually 3:1, in the presence of 1x ligase buffer and 1 unit of T4 DNA polymerase in a total volume of 20 µl. The reaction was incubated at RT for 30 min to overnight.

2.2E Annealing and purification of oligonucleotide linkers.

To make a double-stranded oligonucleotide linker, the two complementary oligonucleotides were annealed in the following reaction mix: 2 µl of 5' phosphorylated synthetic oligonucleotides (100 pM/µl), 3 µl of 500 µM ATP, 2 µl 10x T4 Phosphate Nucleotide Kinase (T4 PNK) buffer and 2 units of T4 PNK in a total volume of 20 µl. The reaction was incubated at 37°C for 30 min, then at 95°C for 5 min and then left to cool to RT. The samples were supplemented with 1x loading buffer then subjected to electrophoresis on a non-denaturing acrylamide gel (see section 2.4C). The gel was stained in ethidium bromide at a concentration 1 µg/ml and visualised on a long wave U.V. transilluminator. The annealed oligonucleotide band was excised and eluted as follows: Initially the gel fragment containing the band was crushed to a pulp, 400 µl of 1x TE was added, and the mixture was placed into a 1.5 ml reaction tube. The tube was placed in a shaking incubator and incubated overnight. The eluted DNA was separated from the gel pulp by filtering the mix through siliconised glass wool and the annealed oligonucleotide was concentrated and recovered by ethanol precipitation (see section 2.2I).

2.2F PCR of DNA fragments.

PCR was performed using *P.f.u.* Turbo DNA polymerase (Stratagene). In each case the reaction was carried out in buffers supplied and under conditions recommended by the manufacturer. Reactions were performed in thin-wall 0.5 ml reaction tubes, overlaid with mineral oil and placed in a Techne thermal cycler. A typical reaction protocol with *P.f.u.* Turbo polymerase is shown in Table 2.2F

dH_2O	11.5µl
10x P.f.u. buffer	2.5µl
10 mM dNTPs	5µl
DMSO (2% final)	1µl
DNA (20 ng/µl final)	5µl
Forward primer. 10 pmol/µl	1µl
Reverse primer. 10 pmol/µl	1µl

Table 2.2F. Reaction components of a typical PCR reaction.

The cycling conditions were as follows: 1 x 94°C for 30 sec, 35 x (94°C for 30 sec + 57°C for 1 min + 72 °C 1 min), and a final extension step of 72°C for 10 min.

2.4G DNA purification from agarose gels

Agarose gel electrophoresis (see section 2.4A) was initially used to resolve digested DNA fragments. The DNA fragment of interest was excised from gels under long wave U.V. using a U.V. Products Inc transilluminator and the DNA was recovered using Qiagen Qiaquick gel extraction kit.

2.2H Phenol/chloroform extraction of DNA.

This procedure was usually carried out during multiple restriction digestions, where the enzymes used shared incompatible buffers. Consequently, it was necessary to remove the incompatible buffer before proceeding to the next digestion. Initially the volume of the DNA was increased to 200 µl by the addition of dH_2O. An equal volume of phenol/chloroform (1:1) was added to the DNA solution and briefly vortexed before being centrifuged at 13,000 r.p.m. for 5 min at RT in a microfuge and then the upper aqueous phase was removed; this step was repeated a further 2-3 times. Upon completion, to remove traces of the phenol/chloroform the DNA was precipitated with ethanol (see section 2.2I).

2.2I Ethanol precipitation.

The sample to be precipitated was increased in volume to 500 µl with dH_20. The DNA was precipitated by the addition of 0.1 volumes of 3 M sodium acetate and 2 volumes of 100% ethanol. The mixture was vortexed and incubated for 20 min on dry ice. The precipitated DNA was pelleted by centrifugation at 13,000 r.p.m. for 10 min at RT in a microfuge. The supernatant was discarded and approximately 500 µl of 70%-80% ethanol was added to remove excess salts. The DNA was pelleted again by centrifugation at 13,000 r.p.m. for 5 min at RT. The DNA pellet was air dried for approximately 5 - 10 min before being resuspended in the appropriate volume of dH_2O. The DNA was stored at -20°C until required.

2.3 Plasmid preparation.

2.3A Small scale preparation of plasmid DNA.

The small scale DNA preparation method was generally used for diagnostic purposes. A single bacterial colony from a selective plate was inoculated into 10 ml of YTB (supplemented with the appropriate antibiotic) and incubated at 37°C overnight. The following day 1.5 ml of the bacterial culture was dispensed into a 1.5 ml reaction tube and centrifuged for 1 min at 13,000 r.p.m. in a microfuge. The supernatant was discarded and 200 µl of STET and 10 µl of 10 mg/ml lysozyme was added and the samples were vortexed and incubated at RT for 1 min. The samples were then boiled for 1 min and then centrifuged for 15 min at 13,000 r.p.m. The supernatant was transferred to a fresh 1.5 ml reaction tube, and an equal volume of isopropanol was added, and the tubes were inverted several times and centrifuged at 13,000 r.p.m. for 10 min. The supernatant was discarded and 400 µl of 80% ethanol was added and the samples were centrifuged for 5 min at 10,000 r.p.m. The ethanol was removed and the pellets were air dried for approximately 2 min. The pellets were resuspended in 30-40 µl of dH_20 or 1x TE and 1 µl of 1 mg/ml RNase was added. The preparations were stored at -20°C.

2.3B Large scale preparation of plasmid DNA

Large scale plasmid preparations were usually carried out on cultures of *E. coli* DH5α bacteria derived from colonies containing plasmids the identity of which had been initially confirmed by the miniprep method (see section 2.3A). 300 ml of LB (supplemented with relevant antibiotics) was inoculated with a single bacterial colony and incubated overnight. The culture was transferred to a GSA centrifuge tube and centrifuged at 10,000 r.p.m. for 10 min (Beckman GPR centrifuge). The supernatant was discarded and the pellet was resuspended in 20 ml of STET. The resuspended culture was transferred to a 50 ml Falcon tube and 2.5 ml of 10 mg/ml of lysozyme was added and incubated at RT for 1 min. The mixture was brought to the boil and placed in a boiling water bath for 1 min, then transferred to a Beckman SS34 rotor tube and centrifuged at 18,000 r.p.m. for 45 min at 4°C in a Beckman GPR centrifuge. The supernatant was transferred to a 50 ml Falcon tube and 0.9 volumes of isopropanol were added and centrifuged at 3,000 r.p.m. for 5 min for 4°C (Bench top centrifuge). The supernatant was then discarded and the pellet was resuspended in 5 ml of 1x TE and brought up to 6.3 ml in volume with 1x TE. 7 g of CsCl was added and swirled gently to dissolve and 0.2 ml 10 mg/ml ethidium bromide was added and the tube was placed on ice for 10 - 15 min. The sample was centrifuged at 3,000 r.p.m. for 10 min at 4°C (Bench top centrifuge). Using a 10 ml syringe the sample was transferred to a 6 ml 65V13 tube that was then filled to the neck. The tubes were sealed, balanced and centrifuged overnight or for 16 hrs at 45,000 r.p.m. at 15°C (Beckman ultra centrifuge). The plasmid DNA band was removed from the tube by inserting a needle into the neck of the tube and a second needle with syringe just below the DNA band. The solution containing the band was removed then mixed thoroughly with an equivalent volume of butanol saturated with 1x TE. The layers were separated by centrifuged at 3,000 r.p.m. at 4°C for 10 min (bench top centrifuge), then the upper layer containing butanol with extracted ethidium bromide was removed. This procedure was repeated 2-3 times to remove all of the ethidium bromide. The sample was placed in dialysis tubing (Pierce) and dialysed against 1x TE for 2 hr, then transferred to a Starsdedt tube and treated with RNase A at 100 μg/ml at 65°C for 1 hr. The samples were cooled and treated with Proteinase K at 100 μg/ml in the presence of 0.1% SDS at 37°C for 1 hr. Subsequently, an equal volume of phenol saturated with 1x TE was added, and the tube was inverted several times and centrifuged at 3,000 r.p.m. for 10 min (Bench top centrifuge). The upper aqueous phase was added to a new Starstedt tube and an equal volume of

chloroform was added, inverted several times and centrifuged at 3,000 r.p.m. for 10 min at 4°C. The upper aqueous phase was removed to a 15 ml Falcon tube and ethanol precipitated (see section 2.2I). The DNA was pelleted by centrifuging at 3,000 r.p.m. for 10 min at 4°C (Bench top centrifuge.). The precipitated DNA was resuspended in 400 µl of 1x TE containing 0.25M NaCl, and again ethanol precipitated (see section 2.2I). The pellets were washed with 400 µl of 80% ethanol and the samples centrifuged for 2 min at 13,000 r.p.m. (Microfuge). The pellets were resuspended in 100 µl - 300 µl of 1x TE.

2.4 DNA analysis.

2.4A Non-denaturing agarose gels.

DNA fragments produced by PCR or restriction digestion were resolved by non-denaturing gel electrophoresis. Initially between 0.5% and 2% agarose mix was made in either 1x TBE or 1x TAE, the latter was used when gel purifying DNA fragments. After boiling to dissolve the agarose, the mixture was cooled, poured into a horizontal slab gel apparatus with a suitable well-forming comb, and allowed to set. DNA samples were supplemented with the appropriate amount of agarose gel loading buffer and always loaded on the gel in conjunction with a sample of a marker DNA ladder (1 Kbp, New England Biolabs). The gel was run at 12 V/cm in either 1x TBE or 1x TAE buffer depending on the corresponding gel type. 1x TBE gels were generally used for diagnostic purposes to resolve cloned DNA that had been restriction digested. 1x TAE gels were used for the resolution of DNA fragments that would be used for cloning purposes. Following electrophoresis the gel was stained in ethidium bromide solution (1 µg/ml) for 2-3 min and then rinsed in water. The DNA was visualised under U.V. light (normally short wave, but long wave for preparative gels). Photography was carried out using The Imager (Appligene).

2.4B Non-denaturing polyacrylamide gels.

Vertical non-denaturing 12% polyacrylamide gels (acrylamide: N, N'-methylene-bis-acrylamide 19:1) gels prepared in 1x TBE were used to purify annealed synthetic oligonucleotides. Polymerisation was initiated by adding 0.01 volumes 10% ammonium persulphate and 0.001 volumes TEMED, then the mixture was applied to a Bio-Rad Mini Protein II apparatus with a suitable comb. After electrophoresis, the DNA bands were visualised by ethidium bromide staining and eluted by the method described above (Section 2.2E).

2.4C Sequencing of DNA.

Sequencing of DNA was carried out as a service using DNA sequence analysis machines either in house, or at the Molecular Biology Sequencing Unit, Institute of Biomedical Sciences, University of Glasgow.

2.5 Manipulation of Competent Bacteria.

2.5A Preparation of electro-competent bacteria.

Initially a single *E.coli* DH10 Bac colony was inoculated into 10 ml of YTB (supplemented with the appropriate antibiotics when necessary) and incubated at 37°C overnight. The culture was then used to inoculate 1 litre of LB broth, again supplemented with the appropriate antibiotics when necessary. The culture was grown to mid-log phase and transferred to the appropriate number of 350 ml Falcon tubes and left on ice for 30 min, then centrifuged for 15 min at 3,500 r.p.m. (Bench top centrifuge). The supernatant was decanted and the pellets were resuspended in a total of 160 ml of sterile dH_20 and combined. The bacteria were pelleted again by centrifugation at 3,500 r.p.m. for 15 min at 4°C (Bench top centrifuge). The supernatant was discarded and the pellet was resuspended in a total volume of 500 ml of sterile dH_20 in a GSA tube and centrifuged at 3,500 r.p.m. for 15 min at 4°C (Beckman GPR centrifuge). The supernatant was again discarded and the pellet was resuspended in 40 ml of dH_20 and transferred to a pre-chilled 50 ml (SS34) tube and centrifuged for 6,000 r.p.m. for 15 min at 0°C (Beckman GPR centrifuge). The supernatant was discarded and the pellet

was resuspended in 2 ml dH_20 + 10 ml glycerol. The resuspended bacteria were aliquoted into 1.5 ml reaction tubes that were placed in a dry ice/methanol bath to snap freeze. The frozen bacteria were stored at -70°C

2.5B Transformation of electro-competent bacteria.

Typically 1-3 µl of DNA was mixed with approximately 80 µl of electro-competent bacteria and electroporated using a Hybond cell shock electroporator (following the manufacturer's guidelines) in a 0.1 cm Gene Pulser® cuvette (Bio-Rad). Following electroporation the bacteria were resuspended in 1 ml YTB, and incubated at 37°C for 45 min in an orbital shaker. Variable quantities of up to 200 µl of the culture were plated out onto an L-Broth agar plate containing the appropriate antibiotics and incubated overnight at 37°C.

2.5C Heat shock transformation of competent bacteria.

Calcium chloride prepared competent bacteria were purchased from commercial sources. Initially 50 µl of *E. coli* DH5α (Invitrogen) or 15 µl of *E. coli* BL21 (pLysS) (Novagen) were thawed on ice. A volume of plasmid DNA or ligation reaction containing 20 - 30 ng of DNA was added to the thawed competent bacteria, mixed gently. The mixture was incubated on ice for 20 min, then heat shocked at 37°C for 20 sec and returned to ice for a further 2-4 min. A 500 µl volume of YTB was promptly added to the bacteria, and then the mixture was incubated at 37°C for 30-60 min. A 100-200 µl aliquot of the bacterial suspension was plated onto LB Agar plates supplemented with the appropriate antibiotics and incubated at 37°C overnight.

2.6 Tissue Culture.

2.6A Growth of mammalian cells in culture.

Mammalian derived cells were passaged in sterile, 175 cm^2 plastic flasks (Nunc) in the appropriate media (see section 2.3D), and incubated at 37°C in a humidified incubator under 5% CO_2. Confluent monolayers were harvested by washing first with versene (0.6 mM EDTA in PBS(A), 0.2% phenol red) and then trypsin/versene (1:2) (supplied by the

Institute of Virology Media Services) and then resuspended in 10 ml of the appropriate medium. For continual passage, HEp-2 and Vero cells were split in a 1:10 ratio every 3-4 days.

2.6B Growth of insect cells in culture.

Insect derived Sf21 cells were passaged in sterile, 175 cm^2 plastic flasks (Nunc) in complete TC-100 medium (see section 2.3D), and incubated at 28°C without venting. Confluent monolayers were harvested by removing the growth media and tapping sharply on the back of the flask multiple times, until the cells became detached from the flask surface. The cells were resuspended in 10 ml of appropriate growth media and split 1:10 to seed new flasks. Cells were also grown in suspension cultures in burrler roller bottles (Nunc). All cells were grown to a confluencey of 70%-80% before splitting.

2.6C Recovering Sf21 cells from liquid nitrogen.

Cryovials containing frozen cell suspensions were removed form liquid nitrogen storage and the cells were immediately thawed in a 37°C water bath. The thawed cells were transferred to a 15 ml Falcon tube and resuspended in 4 ml of complete TC-100 and centrifuged (Bench top centrifuge) at 1000 r.p.m. for 5 min, the supernatant was discarded and this step was repeated. The cell pellet was resuspended in 4 ml of growth medium and the suspension was added to a 125 cm^2 flask (Nunc), then an additional 15 ml of medium was added and the cells were incubated at 28°C until 70% confluent and then passaged.

2.7 Immunofluorescence and Transfection of Cells in Culture.

2.7A Transfection of plasmid DNA

Cultured cells were transfected by the Lipofectamine PLUS method, as recommended by the supplier (Gibco-BRL). HEp-2 cells were seeded at a density of 7.5 x 10^4 the day prior to transfection into 24-well Linbro well units (Nunc) containing a sterile coverslip. The cells were supplemented with the appropriate medium and incubated overnight at

37°C/5% CO_2. The following method states the amount of transfection reagent for a single well, but normally the PLUS and lipofectamine mixes were made up in sufficient quantity for all the samples being transfected. Normally between 100 ng - 500 ng of plasmid DNA was dispensed into 15 ml Falcon tubes, then 1 µl of PLUS reagent was added to 25 µl of serum-free DMEM and this mixture was added to the DNA, mixed and then incubated at RT for 15 min. During the incubation 1 µl of Lipofectamine reagent was mixed with a further 25 µl of serum-free DMEM, then this mixture was added to the DNA-PLUS mix solution and incubation was continued for a further 15 min at RT. During the incubation, the cells were washed with serum-free DMEM, first taking off and retaining at 37°C the 'conditioned' medium in which the cells had been growing. The Falcon tubes containing the DNA/Lipofectamine/PLUS reagent mix were supplemented with 200 µl of serum-free medium, then the whole 250 µl mix was added to the Linbro wells containing the washed cells, which were then incubated at 37°C/5% CO_2 for 3 hr. Subsequently, 750 µl of the retained conditioned medium was added to each of the Linbro wells containing the transfected cells and incubation was continued for 16 hr.

2.7B Large scale transformation of cells in culture.

The method mentioned in section 2.7A was modified for the transfection of 35 mm plates, as follows. The cells were seeded the day prior at 4 x 10^5 cells per plate. The volumes of the PLUS reagent and Lipofectamine were increased as follows: 4 µl PLUS reagent in 100 µl serum-free medium, and 4 µl Lipofectamine reagent in 100 µl serum-free medium per plate. At the time of addition of the mixtures to the cells, an extra 800 µl serum free medium was included to ensure the cells were completely covered with medium. After the 3 hr incubation 1.5 ml of conditioned medium was added to the cells, and incubation was continued accordingly.

2.7C Complementation assay.

Separately 1 µg of infectious viral *dl*1403 DNA (Stow and Stow, 1986) and 100 ng of plasmid DNA expressing one of the ICP0 family of proteins were mixed and made up to a total of 2 µg DNA with pUC9. The DNA was transfected into the cells as described in section 2.7B, except that at the end of the procedure 2 ml of medium supplemented with

1% human serum was added. At 16 hr post transfection, the medium was removed and fresh medium containing 1% human serum was added. At 72 hr post transfection, the medium was removed and 1 ml of Giemsa stain was added to each of the wells for approximately 30 sec and removed by washing thoroughly in a large sink containing water. The plates were inverted and left to dry before the plaques were counted.

2.7D Fixation of cell for immunostaining.

Cells were grown on glass coverslips and transfected as described in preceding sections. Initially the growth medium was removed from the cells, which were then washed twice with 1 ml of PBS before the addition of 0.5 ml of formaldehyde fix solution (19 ml PBS(A), 1 ml formaldehyde and 0.4 g of sucrose). The cells were then incubated at room temperature for 10 min, the fix solution was discarded, the cells were washed twice with 1 ml of PBS(A), then 0.5 ml of permeablisation solution (19 ml PBS, 1 ml 10% NP40, 2 g sucrose) was added to the cells which in turn were incubated at RT for 10 min. Upon completion of this incubation the cells were washed twice in 1 ml PBS supplemented with 1% calf serum.

2.7E Indirect immunofluorescence.

Dilutions of antibodies (see section 2.1I) were made in PBS supplemented with 1% calf serum (PBS/1% FCS). A 15-20 µl aliquot of the antibody dilution was placed in the centre of one of the raised circles in an up-turned lid of a 24-well Linbro dish. The coverslip was placed fixed cell side down onto the antibody and incubated at RT for 30-60 min. Following the antibody incubation, 15-20 ml of PBS/1% FCS was gently pipetted onto the lid, immersing the coverslips. The first wash was immediately removed, and then the wash was repeated a further two times. The coverslips were then turned cell side up and washed 3 more times over a 15 min period. The secondary antibody dilution was made in PBS(A)/1% FCS (see appropriate section for details). Specific concentrations of antibody combinations are stated where used. A 15 - 20 µl volume of secondary antibody dilution mixture was placed in the centre of a well on a fresh upturned Linbro lid. The coverslip were placed cell side down on the antibody dilution and incubated for 30-60 min in the dark at RT. The coverslips were then washed as previously mentioned. Finally coverslips were washed once in water, air dried and mounted cell side down on a glass slide with 3 µl-5 µl of an anti-fade

mounting agent in PBS/glycerol (Citifluor). The coverslips were fixed to the slide by applying clear nail varnish around their perimeters, and then stored a 4°C in the dark until required. Immunofluorescence analysis was carried out on a Zeiss LSM 510 confocal microscope and the images were edited using PhotoShop 6.0 software.

2.8 Production of recombinant baculoviruses.

A synopsis of the methodology and theory behind creating a recombinant baculovirus is outlined in section 3.0.

2.8A Transposition of expression cassettes to bacmid plasmids within E. coli DH10 Bac bacteria.

E. coli DH10α were electroporated according to the method stated in section 2.5B, with the exception of the bacteria having an extended final incubation of 3 hr at 37°C. The prolonged recovery period was to allow the transposition of the recombinant gene within the Tn7 elements of the pFastBacHTa plasmid, to the integration site in the bacmid containing the entire baculovirus genome within the *E. coli* DH10 Bac bacteria. To select for the desired recombinants, the bacteria were plated onto LB agar plates supplemented with 50 μg/ml kanamycin, 7 μg/ml gentamycin, 10 μg/ml tetracycline, 100 μg/ml Bluo-gal, 40 μg/ml IPTG and incubated at 37°C for 24 hrs. The antibiotics select for the bacmid, the pFastBac plasmid, and the plasmid expressing the proteins necessary for the site-specific recombination. A recombination event occurring at the correct integration site results in failure to express functional β-galactosidase, therefore producing a white instead of a blue colony in the presence of IPTG and Bluo-Gal. White colonies were rare within a high background of blue ones. Normally, 5 white positive colonies were picked and purified on the selective plates to eliminate false positives and isolate clonally pure colonies.

2.8B Isolation of recombinant bacmid DNA.

Single positive colonies containing recombinant bacmid DNA were used to inoculate 5 ml of YTB supplemented with 50 μg/ml kanamycin, 7 μg/ml gentamycin and 10 μg/ml tetracycline, and then the cultures were incubated overnight. An aliquot of the culture

was transferred to a 1.5 ml reaction tube and centrifuged at 3,000 r.p.m. for 5 min. The supernatant was removed and resuspended by gentle vortexing in 300 µl of solution I (15 mM Tris-HCl [pH 8.0], 10 mM EDTA, 100 µg/ml RNase A). This was followed by 03 ml of solution II (0.2 N NaOH, 1% SDS), then the mixture was vortexed gently and incubated at RT for 5 min. Finally, 300 µl of 3 M potassium acetate (pH 5.5) was added and the mixture was placed on ice for 5 to 10 min. The sample was then centrifuged for 1! min at 14,000 x g in a bench top centrifuge. The supernatant was transferred to a 1.5 ml reaction tube containing 0.8 ml isopropanol, the contents were mixed by inversion several times to mix, then the tube was placed on ice for 20 min or -20°C overnight. The sample was centrifuged for 15 min at 14,000 x g in a bench top centrifuge. The supernatant was removed and 0.5 ml 70% ethanol was added, followed by the inversion of the tube to wash the pellet. Again the sample was centrifuged for 5 min at 14,000 r.p.m. in a bench top centrifuge. The supernatant was removed and the DNA pellet was left to air dry for 5 to 10 min. The pellet was resuspended in 40 µl of 1x TE or distilled water, and stored at -20°C.

2.8C Transfection of Sf21 cells with recombinant bacmid DNA.

Sf21 cells were seeded 1 x 10^6 cells per 35 mm dish on the day prior to transfection in 2 ml of complete TC-100 medium. Normally 3-5 µg of recombinant bacmid DNA was used for the transfection of Sf21 cells. The transfection protocol is described in section 2.7A. Cells were left for 5-6 days or until signs of infection could be observed, whichever was the sooner. Following transfection a 10 ml syringe bung was used to scrape the cells from the dish. The cell supernatant mix was removed to a 15 ml Falcon tube and centrifuged at 3,000 r.p.m. for 10 min (Bench top centrifuge.). The supernatant was placed in a cryovial and left at 4°C, protected from light.

2.8D Amplification and harvesting of recombinant baculoviruses.

Amplification of Baculovirus stage I.

Sf21 cells were seeded 1 x 10^6 cells per 35 mm dish on the day prior to infection, in 2 ml of complete TC-100 medium. The supernatant was removed from the cells and discarded, then 200 µl of supernatant from the initial supernatant harvest (section 2.8C) was added. The cells were rocked at RT for 1 hr, to aid viral adsorption, then 2 ml of

complete TC-100 medium was added and the cells were incubated for 5-6 days at 28°C or until cellular morphology was indicative of infection. The supernatant was harvested as previously described (section 2.8C), however cell pellets were retained for recombinant protein expression analysis and the supernatant was stored at 4°C. Cell pellets were resuspended in 200 µl of PBS and 100 µl of 3x SDS-PAGE boiling mix and placed in a boiling bath for approximately 5 min. 15 µl of the resuspended cell pellet was analysed by SDS-PAGE and Western blot analysis (sections 2.11A-2.11D). Expression of the recombinant proteins was detected by using anti-6HIS or anti-myc antibodies as relevant (section 2.1I).

Amplification of Baculovirus stage II.

Initially 125 cm^3 flasks (Nunc) were seeded with Sf21 cells at 1.0 x 10^6 the day prior to infection with supernatant virus from amplification stage I stocks that had been shown to express the desired protein by Western blot analysis. The medium was removed from the flasks, then 300 µl of virus stock supernatant from amplification stage I was added to the cells in combination with 1 ml of complete TC-100 medium and the cells were rocked at RT for 1 hr, to aid viral adsorption. An additional 4 ml of complete TC-100 medium was added to the cells, which were incubated for 3-5 days at 28°C or until a cytopathic effect due to virus infection was observed, whichever was sooner. The flasks were shaken firmly to remove the remaining cells from the surface. The cell suspension was transferred to a 15 ml Falcon tube which was centrifuged at 3,000 r.p.m. for 10 min (Bench top centrifuge). The supernatant was removed to a new 15 ml Falcon tube and stored at 4°C.

Amplification of Baculovirus stage III.

Burrler roller bottles (Nunc) were seeded at 4.5 x 10^8 Sf21 cells in a total volume of 300 ml complete TC-100 medium, then 1 ml of amplification stage II supernatant from an isolate positive for expression of the desired protein was added. The culture was incubated at 28°C for 4-5 days. The cell suspension was centrifuged at 5,000 r.p.m. for 10 min in a sterile centrifuge bottle (GSA rotor, Beckman GPR centrifuge), then the supernatant was transferred to further sterile centrifuge tubes and centrifuged at 10,000 r.p.m. for 3-4 hr at 4°C (Beckman GSA rotor; Beckman GPR centrifuge). The

supernatant was discarded and the viral pellet was resuspended in 4 ml complete TC-100 medium. The viral suspension was stored in 1 ml aliquots in cryovials, and kept at 4°C if for immediate use or at -70°C for long-term storage.

2.8E Titration of baculovirus stocks.

Sf21 cells were seeded at 1 x 10^6 cells in 2 ml complete TC-100 medium onto 35 mm plates the day prior to infection, to attain 60%-70% confluence. Serial 10-fold dilutions of the original virus stock were prepared in complete TC-100 medium from neat to 10^{-8} in a total volume of 300 µl. The media from the 35 mm dishes containing the Sf21 cells was removed and 100 µl from each of the dilution series was added per 35 mm dish, in duplicate. An additional 100 µl of complete TC-100 medium was added per plate and the plates were rocked at RT for one hr. During virus absorption, 25 ml of complete TC-100 medium was heated to 42°C in a water bath, then 25 ml of sterile 5% (w/v) sea-plaque agarose was melted and equilibrated at 42°C. After virus adsorption, the supernatant was removed from the cells and the equilibrated complete TC-100 medium and sea-plaque agarose were mixed and 1.5 ml aliquots were added immediately to the cells. The plates were then left for 10-15 min at RT, then 1.5 ml of complete TC-100 medium was added to the plates which were then incubated at 28°C for 5-6 days. The surface medium was removed and replaced with complete TC-100 medium supplemented with Neutral Red solution (2% v/v of stock), then incubation of the plates at 28°C was continued overnight. The stain solution was then removed and the plates were inverted on tissues to dry before the plaques were counted and the viral titre was calculated.

2.8F Labelling of proteins in baculovirus infections with ^{35}S methionine.

35 mm dishes were seeded with 1 x 10^6 Sf21 cells the day prior to infection. The medium was removed and the virus was added at an m.o.i. of 5 p.f.u./cell in a total volume of 200 µl. The cells were rocked for 1 hr at RT to aid viral adsorption to the cells, whereupon 1.5 ml of complete TC-100 medium was added and the cells incubated at 28°C. At 24-30 hrs p.i., the medium was discarded and replaced with 1.5 ml of complete TC-100 medium formulated without added methionine. After 1 hr, the medium was removed and 1.5 ml of methionine-free complete TC-100 medium mixed

with normal medium at a ratio of 4:1 and containing 30 μCi/ml of [^{35}S]-methionine was added. The cells were incubated for 18 h. A 10 ml syringe bung was used to scrape the cells from the dish. The cell supernatant mix was transferred to a 1.5 ml reaction vial and pelleted at 6,500 r.p.m. in a microfuge for 3 min. The supernatant was discarded as radiochemical waste and the pellet was resuspended in 1 ml PBS-A, and centrifuged at 6,500 r.p.m., this step was repeated and the pellet was retained. The cell pellet was resuspended in 100-150 μl 3x boiling mix and the lysates were stored at -20^{o}C prior to SDS-PAGE analysis (see 2.11A). After electrophoresis, the gels were mounted on card and dried under vacuum, and exposed to Kodak X-OMAT film.

2.8G Purification of baculovirus expressed recombinant proteins.

Sf21 cells were grown in burrlers (Nunc) to a density of 3 x 10^6 in 300 ml, and were infected at an M.O.I. of 3 and incubated at 28°C for 72 hr. Upon completion, the cell culture was transferred to a Beckman GSA tube and centrifuged at 3,000 r.p.m. for 5 min (Beckman GPR centrifuge). The supernatant was removed and the cell pellet was resuspended in 5 ml of PBS-A and transferred to a 15 ml Falcon tube and centrifuged at 3,000 r.p.m. (Bench top centrifuge) for 5 min, this step was repeated. The retained cell pellet at this point could be stored at -70°C. The pellet was resuspended in 1 ml of buffer A (100 mM Tris-HCl [pH 8], 500 mM NaCl, 10% Glycerol, 1% NP40, 20 mM β-mercaptoethanol, 4% Complete^{TM} (EDTA-free) protease inhibitors [Boehringer Mannheim]). The pellet was resuspended through a needle (26G $^3/_8$) 15-20 times, to produce a homogenous mix. The volume of the extract was brought up to 7 ml in a Falcon tube with buffer A, and placed horizontally in soni-bath (Kerry) and sonicated for approximately 30 sec. The extract was placed on ice for 30 min, accompanied by intermittent vortexing. The supernatant was then transferred to a snap cap tube (Nunc) and centrifuged at 13,000 r.p.m. for 10 min at 4°C (Beckman GPR centrifuge). The supernatant was transferred to a 15 ml Falcon tube. The volume was increased to 14 ml by adding 100 mM Tris-HCl (pH 8) + 4% Protease inhibitors. Initially the buffer was added 1 ml at a time for the first 3 ml and the remainder was then added. Then 200 μl of Ni-NTA coupled to Sepharose® CL-6B (Qiagen) was washed in 3 x 1 ml in Buffer A/50% 100 mM Tris-HCl pH 8. Each wash step was accompanied by a centrifugation step to pellet the beads and discard the wash buffer (13,000 r.p.m. for 30 sec, microfuge). The washed beads were added to the protein extract, which was in turn

incubated at RT for 1 - 2 hr accompanied by intermittent vortexing. The beads were then pelleted by centrifugation for 5 min at 1,000 r.p.m. (Bench top centrifuge). The supernatant was discarded and the beads were washed as follows; 4 x 1 ml, Buffer A/50% 100 mM Tris-HCl pH 8, and 4 x1 ml in Buffer A/50% 100 mM Tris-HCl + 20 mM imidazole pH 8. Each wash step was followed by centrifugation (10 sec at 13,000 r.p.m., microfuge.) to pellet the beads and discard the wash buffer. The proteins were eluted for 5 min at RT in 300 µl of Buffer A/50% 100 mM Tris-HCl pH 8, supplemented with 250 mM imidazole. The beads were collected as previously mentioned and the eluate was retained. The elution step was carried out a further 3 times The eluates were placed in dialysis tubes (Pierce), and dialysed in 100 mM Tris-HCl (pH 8), 150 mM NaCl, 0.1 % NP40, 5 % Glycerol, 10 mM β-mercaptoethanol, for 2 hr at 4°C. Protein expression was analysed by SDS-PAGE analysis and/or Western blotting.

2.8H Infection of mammalian cells with baculoviruses.

Mammalian cells were seeded on 13 mm glass cover slips in 24 well Limbro plates (Nunc) at 0.75 x 10^5 in 1 ml of medium and incubated overnight. The medium was removed and baculoviruses expressing members of the ICP0 family of proteins from the HCMV promoter were used to infect cells at an M.O.I. of 100-500 insect cell p.f.u. per cell. The viral dilutions were made up to 150 µl in volume with the appropriate cell growth medium before being added to the cells. The cells were incubated at 37°C for 1 hr with regular mixing, whereupon an additional 750 µl of cell growth medium was added and the incubation was continued for a further 24 hrs.

2.9 Biochemical assays.

The biochemical assays stated in this section are a generalisation of the experimental procedures used. Variations are stated clearly in the appropriate sections.

2.9A In vitro thiolester assay.

The *in vitro* thiolester assay was carried out in 1 x reaction buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM ATP in 1.5 ml reaction tubes. The individual assay components were as follows: 500 nM E1, 5 μg of ubiquitin and 3-5 μg E2 (The E2s used are stated in the results section) in a total volume of 10 μl. The E1 and E2 enzymes were sourced from communal lab stocks (see section 2.1K). To attain working concentrations, where needed, assay components were diluted with 1x reaction buffer. The reaction was repeated twice for each E2. The mixtures were incubated at 28°C for 20 min, one set of reactions was immediately stopped with 3 x gel loading buffer containing 500 mM DTT, the remaining set of reactions was stopped in 3 x gel loading buffer containing no reducing agent. The reactions were subjected to electrophoresis on 10% BIS-Tris Nupage gels (Invitrogen). The gels were run according to the manufacturer's instructions and in buffers also supplied by the manufacturer and then coomassie stained (see section 2.11B).

2.9B In vitro ubiquitination assay.

The reaction was carried out in 1.5 ml reaction tubes, in a total volume of 10 μl and its components were: 50 nM E1 (see section 2.1K), 500 ng of ubiquitin, 50-250 nM E2 (see section 2.1K) and 10-100 ng E3. The 10 x reaction buffer contained 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 5 mM ATP. To attain working concentrations, where needed assay components were diluted with 1 x reaction buffer. The reaction mix was incubated at 37°C for 1 hr and stopped by the addition of 3x gel loading buffer. The samples were resolved by SDS-PAGE analysis and the ubiquitin products were detected by Western blotting using either FK2 (Affiniti) or P4D1 (Santa Cruz Biotechnology) anti-ubiquitin antibodies.

2.9C GST-pull down assays.

GST pull down assays (Smith & Johnson, 1988) were used to detect if the GST fusion protein interacted with specific target proteins. Initially soluble extracts were made of the GST fusion and target proteins (see section 2.10A). Glutathione agarose beads were rehydrated in PBS-A/1% NP40 at RT for 1-2 hr to make a 1:1 (v/v) slurry. Samples (200 µl) of bacterial extracts expressing the GST fusion and the target proteins were mixed separately in 1.5 ml reaction tubes with 50 µl of glutathione agarose bead slurry and mixed end-over-end for 1 hr at 4°C. The reaction tubes were centrifuged at 13,000 r.p.m. for 30 sec (Microfuge.). The supernatants containing the target proteins were retained and the beads were discarded; this procedure 'pre-cleared' the extract of any insoluble or aggregated target proteins that could give artefactual positive results. The beads from the tube with the bacterial extract expressing GST-tagged proteins were retained and the supernatant was discarded. These beads were then washed 3 times with PBS-0.1% NP40, each time recovering the beads by centrifugation in a microfuge (13,000 r.p.m. for 30 sec). The GST beads were then mixed with the clarified soluble extract of the target proteins in a fresh 1.5 ml reaction tube. The reaction tube was mixed end-over-end at 4°C for 1 hr. The beads were recovered by centrifugation at 13,000 r.p.m. for 30 sec (Microfuge) and the supernatant was discarded. The beads were washed 3 times in 750 µl of buffer A (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5% Glycerol, 0.05% NP40). Each wash step was followed by centrifugation for 30 sec at 13,000 r.p.m. (Microfuge), with the beads being retained and the supernatant discarded. The proteins were eluted from the glutathione beads by the addition of 20 µl of buffer B (250 mM Tris pH 7.5, 0.1% NaCl, 0.1% NP40, 50 mM reduced glutathione) and then incubated at RT for 15 min. The beads were recovered as previously mentioned and the eluate was retained. The elution step was repeated. Finally, 3x SDS-PAGE loading buffer was added to the eluates and the samples were either stored at -20°C or resolved by SDS-PAGE analysis. The presence of a particular protein was detected by Western blot analysis.

2.10 Purification of Recombinant Proteins.

2.10A Production of soluble extracts from E.coli BL21 expressing recombinant proteins.

Plasmids of the pGEX or pET24a series were transfected into BL21 (DE3) pLysS bacteria (see 2.5B) and the resultant colonies were grown overnight on agar plates supplemented with the appropriate antibiotics. From 5 to 10 fresh colonies were picked and inoculated into 100 ml of YTB, supplemented with the appropriate antibiotics and grown at 37°C, to mid-log phase. IPTG was added to a final concentration of 0.1 mM and incubation was continued for 4 hrs at 28°C. Bacteria were harvested in a bench top centrifuge at 5,000 r.p.m. for 10 min. The bacterial pellets were resuspended in EP0 265 buffer and lysed using a Branson sonifier 450 soni-probe, giving a number of 20 second bursts set at the 50% duty cycle and an output of 5, and monitoring to avoid frothing of the sample. When the mixture showed clear signs of clarification, debris was removed by centrifugation for 20 min at 8,000 r.p.m. in a Beckman GPR centrifuge, and the soluble extracts were stored at -20°C.

2.10B Purification of GST-tagged proteins from bacteria.

Bacterial lysates containing the GST-fusion proteins (see section 2.10A) were purified by mixing 300 µl of soluble protein extracts with 100 µl of pre-swollen glutathione agarose beads (50% [v/v] with PBS) in a 1.5 ml reaction tube, with regular mixing over a period of 30 min on ice. The mixture was then centrifuged in a microfuge at 13,000 r.p.m. for 30 sec, the supernatant was then discarded and the beads were then washed 3 times in 150 µl of EP0 265 buffer. Each wash step was followed by centrifugation at 13,000 r.p.m. for 30 sec to collect the beads. A 100 µl volume of EP0 265 buffer supplemented with 50 mM glutathione was used to elute the protein from the beads. The elution was carried out on ice, accompanied by intermittent resuspension, for 20 min. The eluate was clarified by microfuge centrifugation at 13,000 r.p.m. for 30 sec and then dialysed immediately in a dialysis tube (Pierce) against 50 mM Tris-HCl pH 7.5, 50 mM NaCl, 2.5 mM β-mercaptoethanol for 2 hr and stored at -70°C.

2.11 Analysis of Proteins.

2.11A SDS polyacrylamide gel electrophoresis.

Proteins were resolved by electrophoresis through SDS polyacrylamide mini gels (Laemmli, 1970) using the Bio-Rad miniprotein gel apparatus. The resolving gels were usually made at an acrylamide concentration of 10% (Table 2.11A). The gel was set using a Bio-Rad Mini-Protean II gel former, the mix being poured between two glass plates and over laid with butanol. Once set the butanol was flushed away with water. The gel was then over laid with stacking gel (Table 2.11A) and a gel comb inserted. Once the stacking gel had set the comb was removed and the wells were flushed with water. The gel cassette was placed in a Bio-Rad Mini Protean II gel apparatus and the gel tanks were filled with SDS-PAGE running buffer. Protein samples supplemented with 3x gel loading buffer were placed in a boiling bath for 2-5 min and loaded into the wells. The gels were subjected to electrophoresis at 150 V for approximately 75 min.

Solution	7.5% Resolving Gel	Stacking Gel
30% Acrylamide 2.5% cross linker	2.5 ml	0.4 ml
RGB	2.5 ml	NA
SGB	NA	0.6 ml
dH_20	5 ml	1.4 ml

Table 2.11A. Solutions used to make SDS-polyacrylamide gels. Amounts of 30% acrylamide and water were adjusted accordingly for preparations of 6%, 10% or 12.5% resolving mixes. To each resolving mix 80 µl 10% ammonium persulphate and 8 µl of TEMED were added and 20µl 10% ammonium persulphate and 3µl TEMED were added to stacking gel mixes, in order to catalyse setting of the gel.

2.11B Coomassie brilliant blue staining of SDS-PAGE gels.

Protein gels were stained with Coomassie Blue (0.2% Coomassie Brilliant Blue in 50:50:7 ratio of methanol:water:acetic acid) for 5 min and destained in protein gel destain solution (5% methanol, 7% acetic acid in water).

2.11C Transfer of proteins from SDS-PAGE gels to nitrocellulose filters.

Resolved proteins in SDS gels were detected by immunoblotting (Western blotting). After electrophoresis, the gel was placed on a piece of nitrocellulose membrane, and both were sandwiched between pieces of Whatman 3mm filter paper; which in turn were sandwiched between two sponges (Bio-Rad). The whole procedure was carried out with the items submerged in transfer buffer and care was taken to eliminate any air bubbles in the sandwich. A Bio-Rad Mini Protean II transblot cell was used to transfer the proteins to a nitrocellulose filter (Hybond) by electrophoresis at 250 mA for a minimum of 2 hrs (Towbin *et al.*, 1979).

2.11D Immunodetection of proteins.

Following the transfer of proteins to the nitrocellulose membrane, the membranes were placed in PBS-T containing 5% dried milk (Marvel) and incubated at RT for 1 hr or overnight at 4°C. The filter was then incubated in 10 - 20 ml PBS-T / 5% dried milk containing the primary antibody (made up to the appropriate dilution) at RT for 3 hr or overnight at 4°C, with constant mixing. The blot was then washed five times in PBS-T for 15 min at RT per wash. Then secondary antibody in PBS-T / 2% dried milk was added to the filter and incubated at RT for 1 hr, again with constant shaking. The secondary antibodies were used at the following concentration: anti-mouse IgG whole molecule peroxides conjugates were used at 1/1,000 dilution and the rabbit anti-goat peroxidase were used at 1/80,000. Following incubation the blots were washed again as previously described. The bound antibodies were detected using the NEN enhanced chemiluminescence (ECL) system. Usually 1 ml of each of the two reagents provided in the kit were mixed, placed on the nitrocellulose filter and vigorously mixed. The filters were mounted on glass plates, covered with sheets of cling film and exposed to Kodak X-OMAT-S film for variable lengths of time.

2.11E Detection of radiolabelled proteins

Proteins were resolved by SDS-PAGE analysis and then the gels were vacuum dried. The dried gel was then exposed to Kodak X-OMAT S film.

2.11F Stripping and reprobing nitrocellulose membranes.

In order to reprobe membranes with a second antibody it was first necessary to strip the membranes of any previous antibodies. The filter membrane was submerged in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl (pH 6.7) and incubated at 55°C for 1 hr with intermittent agitation. The membrane was washed thoroughly in PBS-T and then blocked and reprobed as previously described.

Chapter 3 – Part I - Biochemical characterisation of the RING finger domains of the ICP0 family of proteins.

3.0.0 General Introduction.

The aim of this series of experiments was to characterise the *in vitro* biochemical activity of the RING finger domains of the ICP0 related proteins. The RING finger domain is a cysteine/histidine-rich zinc binding motif that is present in a wide variety of proteins expressed by many different organisms, and which frequently has been associated with E3 ubiquitin ligase activity (Freemont, 1993; Joazeiro & Weissman, 2000; Pickart, 2001a). In the case of ICP0 and the members of the ICP0 family, the RING finger motif is situated towards the N-termini of the proteins (Barlow *et al.*, 1994). Its location is conserved throughout the family of ICP0 related proteins, including in members not used in these studies. The presence of the RING finger is the main characteristic that links the ICP0 family of proteins, since there is limited sequence homology elsewhere between the different members of the protein family. Even within the RING finger itself there is considerable diversity amongst the family members, with only the zinc-coordinating cysteine and histidine, and a limited number of others residues being conserved (Miyoshi *et al.*, 2000; Parkinson & Everett, 2000, 2001). The RING finger domains of the ICP0 related proteins were initially selected for investigation (rather than their full-length counterparts); previous research carried out on ICP0 has shown this region is largely responsible for its multifunctional activities. Mutational studies of ICP0 RING finger domain have shown that it is essential for the activation of HSV-1 gene expression (Cai & Schaffer, 1989; Chen *et al.*, 1991; Everett, 1987, 1988a), the initiation of the lytic cycle (Everett, 1989) and for the E3 ubiquitin ligase activity of ICP0 (Boutell *et al.*, 2002). A comprehensive review of the properties of the RING finger domain of ICP0 can be found in part II of the Introduction.

The small size of the RING finger domains of the ICP0 related proteins (the largest being approximately 90 a.a.) allows their expression in bacteria. Use of a bacterial system such as *E.coli* BL21 has many advantages over other protein expression systems,

such as rapid growth of the bacteria to a high density with potentially large accumulations of the recombinant gene product. Furthermore, there was already an established method for the expression and purification of the ICP0 RING finger domain in *E.coli* BL21 (Boutell *et al.*, 2002).

Initially, the RING finger domain coding regions were separately cloned into the vector pGEX-2TNMCR (Everett *et al.*, 1997; Meredith *et al.*, 1994), enabling a GST tag to become fused to the N-terminus of the expressed recombinant protein. The pGEX-2TNMCR series of vectors containing the ICP0 related RING finger domains were expressed in *E.coli* BL21, from which lysates were made. The GST-fusion proteins were then affinity-purified by adsorption to glutathione agarose beads (Smith & Johnson, 1988), washed, eluted and used in *in vitro* biochemical assays.

The following chapter is divided into the following sections, the first of which (Part I) details the methodology used to create the GST fusion proteins containing the ICP0 related RING finger domains used to determine their E3 ligase activities. Part II assesses the E3 ligase activity of truncated versions of the BICP0 and Eg63 RING finger domains. Part III analyses the auto-ubiquitination carried out by the ICP0 related proteins *in vitro*. Part IV determines if the ICP0 related RING finger domains form stable interactions with the E2-conjugating enzymes that they stimulate.

3.0.1 Construction of the ICP0 related RING finger domain expression plasmids.

The vector pGEX-2TNMCR (Everett *et al.*, 1997; Meredith *et al.*, 1994) (see figure 3.0.1A) was used to introduce an N-terminal GST tag to the isolated ICP0 related protein RING finger domains (see figure 3.0.1B). Initially the DNA fragments encoding the domains were isolated from the pFastBac HTa series of plasmids, which were originally used to express the full-length ICP0 related proteins (see chapter 4). The restriction sites used to excise the 3' end of the DNA fragments encoding the ICP0 related RING domains were chosen because they were approximately 100 - 200 bp after the last cysteine residues of the RING finger domains. The amino acid sequence within each of the ICP0 related protein RING finger domains is shown in section 3.0.1B and an overview of the cloning procedure is given below and in figure 3.0.1C.

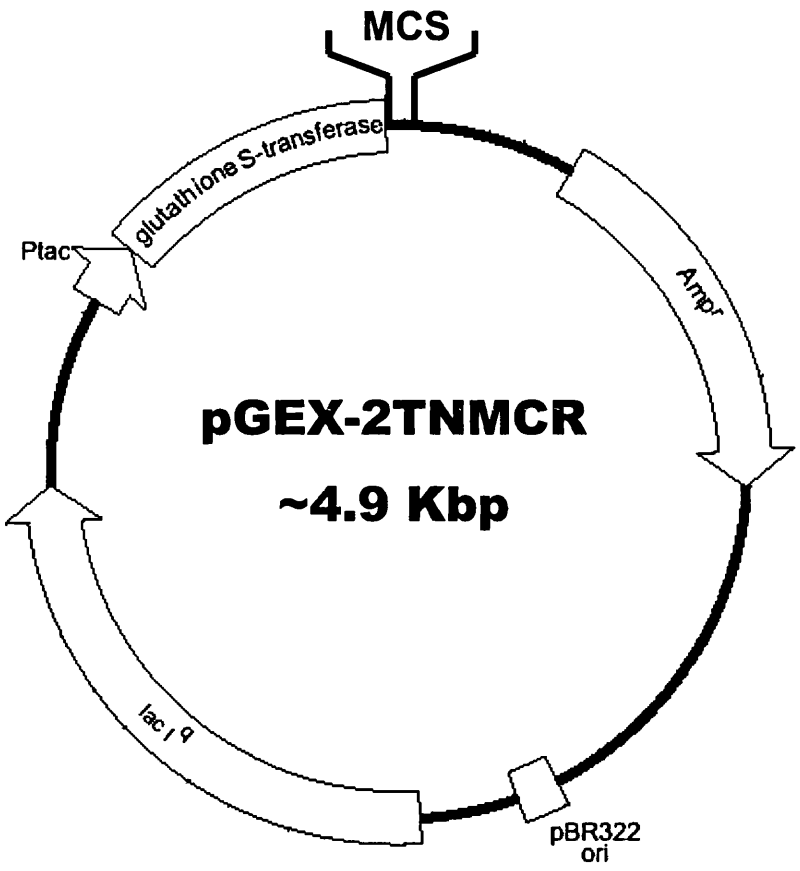


Figure 3.0.1A *Schematic overview of the salient features of the expression vector pGEX-2TNMCR.*

MCS= multiple cloning sites; Amp^r = ampicillin resistance gene; pBR322 ori = plasmid origin of replication; Ptac = promoter; Lac I^q = repressor gene. (Adapted from the pGEX-2TNMCR plasmid map, *Amersham.*).

BHV-1, BICP0 RING finger domain and flanking sequence (109 a.a.).

NcoI
MAPPAAAPELGSCCICLDAITGAARALPCLHAFCLACIRRWLEGRPTCPL 50

CKAPVQSLIHSVASDECFEEIPVGGGPGADGALEPDAAVIWGEDYDAGPI 100

AvaI
DLTAADGEAS

EHV-1, Eg63 RING finger domain and flanking sequence (105 a. a.).

NcoI
MATVAERCPICLEDPSNYSMALPCLHAFCYVCITRWIRQNPTCPLCKVPV 50

ESVVHTIESDSEFKETKVSVDFDYDSEEDEDSFEGQFLAVDSGDAPANIS 100

NcoI
AWNGPM

PRV, EP0 RING finger domain and flanking sequence (265 a.a.).

NcoI
MGCTVSRRRTTTAEASSAWGIFGFYRPRSPSPPPQRLSLPLTVMDCPICL 50

DVAATEAQTLPCMHKFCLDCIQRWTLTSTACPLCNARVTSILHHVDSDAS 100

FVETPVEGATDVDGEEDEPVGGGFAVIWGEDYTEEVRHEEAEGQGSGSGS 150

RARPRVPVFNWLYGQVSTVIESDPIREAVVDNIVEIIQEHGMNRQRVTEA 200

MLPMFGANTHALVDTLFDISAQWMRRMQRRAPMSHQGVNYIDTSESEAHS 250

XhoI
DSEVSSPDEEDSGASS

VZV, Vg61 RING finger domain and flanking sequence (229 a.a.).

NcoI
MDTILAGGSGTSDASDNTCTICMSTVSDLGKTMPCLHDFCFVCIRAWTST 50

SVQCPLCRCPVQSILHKIVSDTSYKEYEVHPSDDDGFSEPSFEDSIDILP 100

GDVIDLLPPSPGPSRESIQQPTSRSSREPIQSPNPGPLQSSAREPTAESP 150

SDSQQDSIQPPTRDSSPGVTKTCSTASFLRKVFFKDQPAVRSATPVVYGS 200

NcoI
IESAQQPRTGGQDYRDRPVSVGINQDPRTM

Figure 3.0.1B. *The amino acid sequence of the RING finger domains of the ICP0-related proteins.*

The amino acid sequence of the RING finger domains and flanking sequences of the ICP0 related proteins are shown. The positions of the 5' and 3' restriction enzyme sites used to excise the regions from the parental plasmids are shown on the amino acid sequences. The parental vectors were the pFastBac HTa series of plasmids used to express the full length versions of the ICP0 related proteins (see chapter 4). The RING finger domains of the ICP0 related proteins are indicated in red, with the first and last cysteine denoted (*). Flanking sequences that are not part of the RING finger domains are denoted in black.

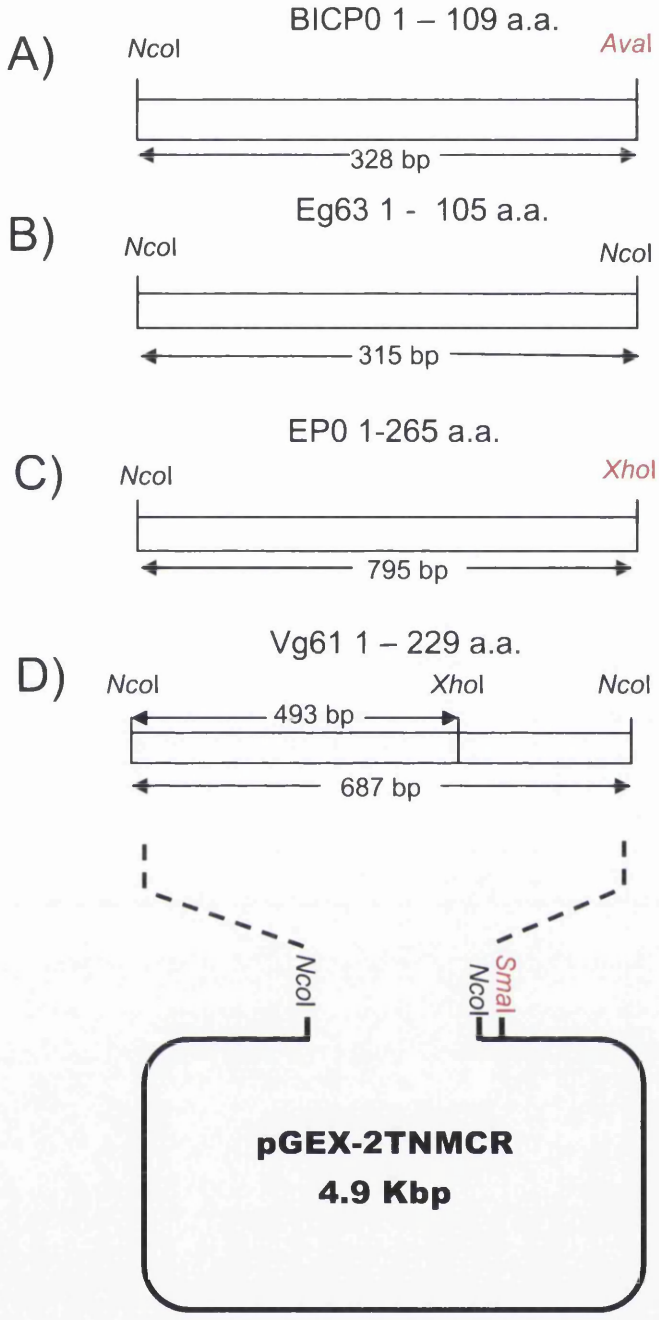


Figure 3.0.1C *Schematic representation of the construction of the pGEX plasmids expressing the RING finger domains of the ICP0-related proteins*

A) The 328 bp DNA fragment of the RING finger domain of BHV-1 BICP0 was removed by *Nco*I and *Ava*I restriction digestion of the parental vector pFastBac HTa BICP0. The 3' end of the DNA fragment excised by *Ava*I restriction digestion was filled in by blunt ending reaction (see materials and methods for further details). **B**) The 315 bp DNA fragment encoding the RING finger domain of EHV-1 Eg63 was removed by an *Nco*I restriction digestion of the parental vector pFastBac HTa Eg63. **C**) The 795 bp DNA fragment encoding the RING finger domain of PRV EP0 was removed by separate *Xho*I and *Nco*I restriction digestion of the parental vector pFastBac HTa EP0. The 3' end of the DNA fragment excised by *Xho*I restriction digestion was filled in by blunt ending reaction. **D**) The 687 bp DNA fragment encoding the RING finger domain of VZV Vg61 was removed by an *Nco*I restriction of the parental vector pFastBac HTa Vg61. To accommodate the DNA fragments A-D pGEX-2TNMCR was digested as follows: DNA Fragments B and D, with the restriction enzyme *Nco*I; DNA fragments A and C, with the restriction enzymes *Nco*I and *Sma*I.

The vector pFastBac HTa BICP0 was digested with *Ava*I at the 3' end of the DNA fragment encoding the RING finger domain and then blunt-ended (see section 2.2B). After ethanol precipitation to remove the reagents used in the blunt-ending reaction, *Nco*I was used to cut at the 5' end of the DNA fragment encoding the BICP0 RING finger domain, which was then isolated using gel electrophoresis and purified accordingly (see methods section).

The vector pFastBac HTa EP0 was digested with *Xho*I at the 3' end of the DNA fragment encoding the RING finger domain and then blunt-ended and purified. *Nco*I was then used to cut at the 5' end of the DNA fragment encoding the EP0 RING finger domain, which was then isolated and purified using gel electrophoresis.

Plasmid pFastBac HTa Eg63 was digested with *Nco*I, which cuts at the 5' and 3' ends of the DNA fragment encoding the Eg63 RING finger domain. The DNA fragment was then isolated and purified using gel electrophoresis.

The DNA fragment encoding Vg61 was isolated from pFastBac HTa Vg61. The method was the same as that used to isolate the DNA fragment encoding the Eg63 RING finger domain.

The recipient vector pGEX-2TNMCR was digested with *Nco*I, allowing insertion of the isolated DNA fragments encoding the RING finger domains of Vg61 or Eg63. For insertion of the DNA fragments encoding the BICP0 and EP0 RING finger domains, pGEX-2TNMCR was cut with *Nco*I and *Sma*I. The DNA fragments encoding the ICP0 related protein RING finger domains and the appropriately digested vector were then ligated and transformed into *E.coli* DH5α. The resulting colonies were screened by diagnostic restriction enzyme digestions of small-scale plasmid preparations. The identities of plasmids harbouring the desired restriction fragments were further confirmed by expression analysis and DNA sequencing. Expression analysis simply involved transfecting the pGEX vectors encoding the RING domains into *E.coli* BL21 and examining the subsequent lysates by SDS-PAGE and Coomassie staining. This method was important for the clones containing the coding sequences of Vg61 or Eg63 RING finger domains, as the *Nco*I restriction fragments could have been inserted into the recipient vector in either orientation. Only isolates positive for the correct

recombinant protein expression were further analysed by DNA sequencing. The individual pGEX series of plasmids expressing the ICP0 related RING finger domains will henceforth be referred to as pGEX-BICP0, pGEX-Eg63, pGEX-EP0 and pGEX-Vg61 and the expressed proteins collectively will be referred to as the GST-ICP0 related proteins or individually as GST-BICP0, GST-Eg63, GST-EP0 and GST-Vg61.

3.0.2 Expression and purification of the GST-ICP0 related proteins.

The pGEX series of plasmids encoding the GST-ICP0 related proteins were transformed into *E.coli* BL21. Several (5 to 10) bacterial colonies were pooled and cultured in YTB (supplemented with ampicillin) until mid-log phase, whereupon IPTG was added to induce expression of the recombinant protein. Incubation was continued for a further 3-5 hours at 28°C. The bacteria were then pelleted and resuspended in lysis buffer and sonicated (see section 2.10 for further details). After initial trials, the lysis buffer EP0 265 (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% NP40, 20 mM β-mercaptoethanol) was found to maximise the solubility of the GST-ICP0 related proteins. An example of a typical protein purification profile is shown in figure 3.0.2A (see section 2.10B for further details on the protein purification and dialysis methods). Figure 3.0.2B shows samples of the purified GST-ICP0 related proteins that have been resolved by SDS-PAGE analysis. From this data it is possible to identify several breakdown or misread proteins in each of the samples. Furthermore, RING finger domain proteins frequently display aberrant gel mobilities, so from the data it is not possible to be certain which of the bands represents the primary translation product. Despite these factors, in most of the resolved purified protein samples there is a dominant band that has a mobility close to that of the predicted size of the intact protein (marked on the relevant figures).

3.0.3. Quantification of the purified GST-ICP0 related proteins.

To ensure that equal amounts of the purified GST-fusion proteins were used in repeated biochemical assays, densitometric analysis was used to determine the concentrations of each preparation. Samples of the proteins to be quantified and aliquots of BSA standards of known concentrations were resolved by SDS-PAGE. The gel was stained with

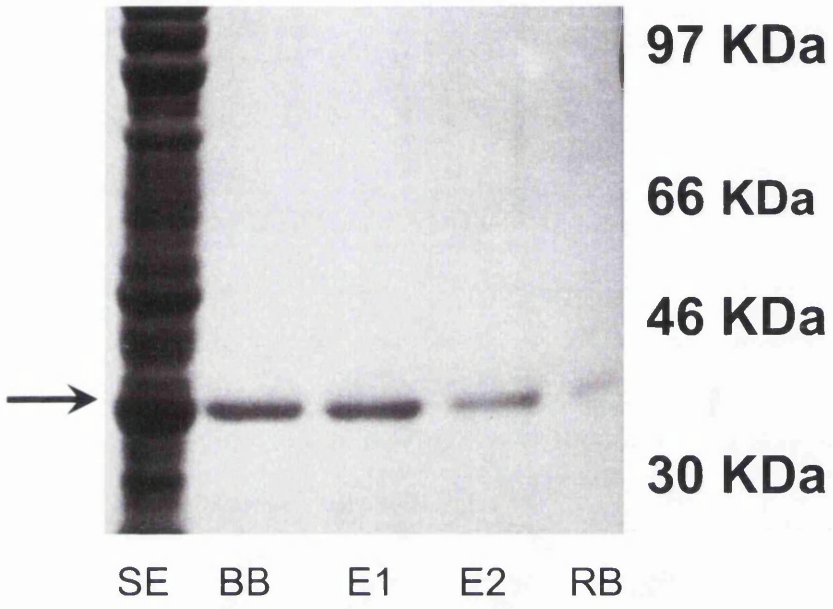
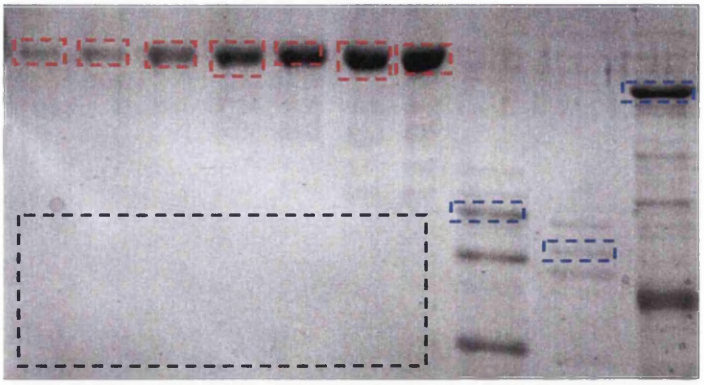


Figure 3.0.2A. *Purification profile of the BICP0 RING finger domain.*

Samples of GST-BICP0 from a typical expression and purification experiment were resolved by SDS-PAGE and visualised by Coomassie staining. The arrow marks the BICP0 protein band that corresponds with its predicted size. The abbreviations of each extract and the quantities resolved by SDS-PAGE are as follows: SE = soluble extract (5 µl); BB = bound beads (10 µl); E1 = elution 1 (20 µl); E2 = elution 2 (20 µl); RB = remaining beads (10 µl).

A)



B)

Lane Number	Name	Adjusted Volume (Intensity x mm²).	Amount.
1	BSA std. 0.1 µg	26.22	0.1 µg
2	BSA std. 0.3 µg	52.41	0.3 µg
3	BSA std. 0.5 µg	124.22	0.5 µg
4	BSA std. 0.7 µg	199.49	0.7 µg
5	BSA std. 0.9 µg	261.88	0.9 µg
6	BSA std. 1.1 µg	305.15	1.1 µg
7	BSA std. 1.3 µg	351.99	1.3 µg
8	Eg63	27.98	0.15 µg
9	BICP0	97.74	0.39 µg
10	EP0	196.65	0.72 µg
	Background	0	N/A

C)

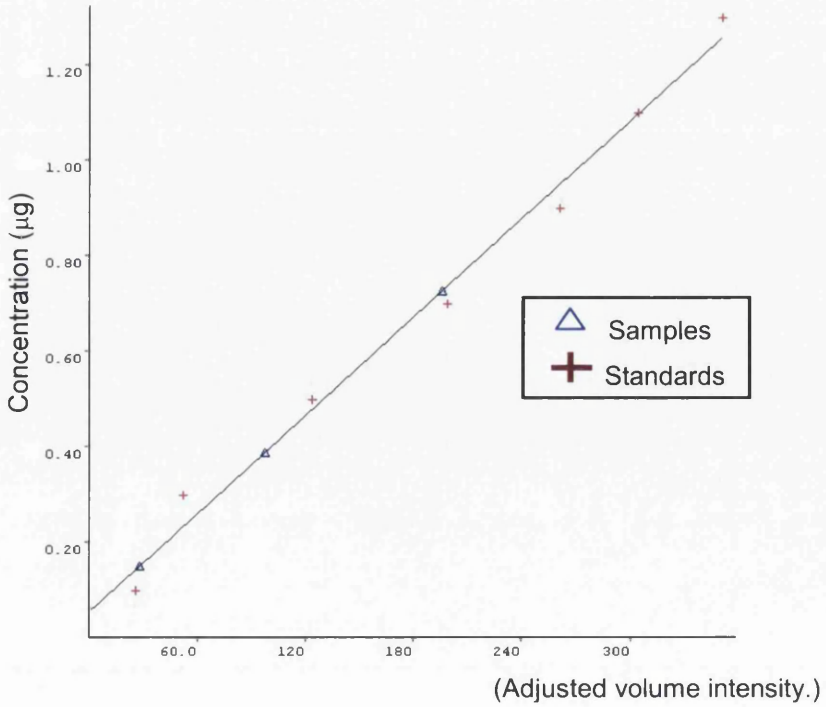


Figure 3.0.3A. *Quantification of the purified ICP0 GST-RING finger domains.*

A) 5 µl of purified GST-BICP0, GST-Eg63 and GST-EP0 (lanes 8-10) were resolved by SDS-PAGE, in conjunction with BSA standards (lanes 1-7), that ranged in concentration from 0.1 µg to 1.3 µg. The resolved proteins were visualised by Coomassie staining and then thoroughly destained. The gel image was captured under white light using The Imager (Applegene). The amounts of the RING finger domains was determined from the captured gel image using 'BioRad Quality One software v4.22'. The amount of proteins were determined by initially highlighting the amounts standards, lanes 1-7 (shown in red), then selecting the background (shown in black), finally selection of the unknown samples i.e. the GST-ICP0 related proteins (lanes 8-10).

B) The volumes of the selected samples, relative to background (lanes 1-10) were automatically calculated, using the BioRad Quality One Software. Volume is the total intensity data inside a defined boundary (i.e. the selected area). It is calculated as follows: Volume = [The intensities of the pixels inside the volume boundary] x [the area of a single pixel]. From the volume the adjusted volume is calculated as follows: Adjusted Volume = [volume of the background] – [volume of the sample or standard]. The adjusted volumes of the BSA standard and RING finger protein domains are shown in the table.

C) The adjusted volumes of the BSA standards were plotted against their relative amounts, with a corresponding line of best fit. The adjusted volumes of the RING finger domains are plotted against the line of best fit to determine their amounts relative to the BSA standards. The amounts of the ICP0 related proteins in the 5 µl sample volume are shown in the table.

Coomassie blue, destained, and then a digital image was captured, which was then subjected to volume analysis using the BioRad Quantity One Software (Version 4.22). An example of this process is shown in figures 3.0.3A and B. The concentrations of the GST-fusion proteins were determined every time a new stock of protein was purified from bacterial lysates. When the activity of more than one of the GST-ICP0 related proteins was to be compared, the proteins were quantified on the same SDS-PAGE gel. This was important as the quantification method employed would only give an indication of concentration relative to that of the BSA standards and furthermore concentration of protein samples varied slightly when re-analysed, due to experimental variables.

3.1.0. Introduction to the E3 ubiquitin ligase and thiolester assays.

The ability to disrupt ND10 correlates very well with the biological activities of ICP0. It is thought they are targeted to release factors essential for the continued progression of HSV-1 infection (Negorev & Maul, 2001). However, it also been proposed that ND10 structures play an essential role as part of a cellular repression mechanism, which prevents or stalls the progression of viral infection. This is supported by the fact that components of ND10 are interferon induced and have been implicated in the repression of gene expression (this is discussed in further detail in the Introduction). Additionally, various studies have linked ICP0 with resistance to the effects of interferon (Eidson *et al.*, 2002; Harle *et al.*, 2002; Mossman *et al.*, 2000; Mossman & Smiley, 2002; Nicholl *et al.*, 2000; Preston *et al.*, 2001; Taylor *et al.*, 1998). Therefore, the absence of ICP0 from the viral genome can have various deleterious affect on the progression of viral infection. It was recently shown that infection of HFL cells with an ICP0-null virus at low MOI can lead to multiple fates, including stalling of viral gene expression and the host cell undergoing apoptosis (Everett *et al.*, 2004).

ICP0 has been shown to disrupt ND10 in a proteasome-dependent manner, as in the presence of the proteasome inhibitor MG132, it is unable to stimulate HSV-1 lytic infection or reactivation from quiescence. (Everett *et al.*, 1998c). Consistent with these observations it was later shown that ICP0 can cause the formation of colocalising, conjugated ubiquitin in both transfected and infected cells, at ND10 and at centromeres (Everett, 2000). Furthermore, in a RING finger dependent manner ICP0 can act as an potent E3 ligase *in vitro*, inducing the formation of polyubiquitin chains in the presence of UbcH5a and UbcH6, (Boutell *et al.*, 2002) and targeting p53 for ubiquitination (Boutell & Everett, 2003a). It was also shown that E2 enzyme UbcH5a plays a role in inducing the degradation of ND10 components during HSV-1 infection (Gu & Roizman, 2003).

The ICP0 related proteins have also been implicated as E3 ubiquitin ligases, as they have been shown to colocalise with conjugated ubiquitin and disrupt components of ND10 structures to varying degrees (Parkinson & Everett, 2000, 2001). However, the ICP0 related proteins, unlike ICP0, do not affect the conjugation of SUMO-1 to PML in

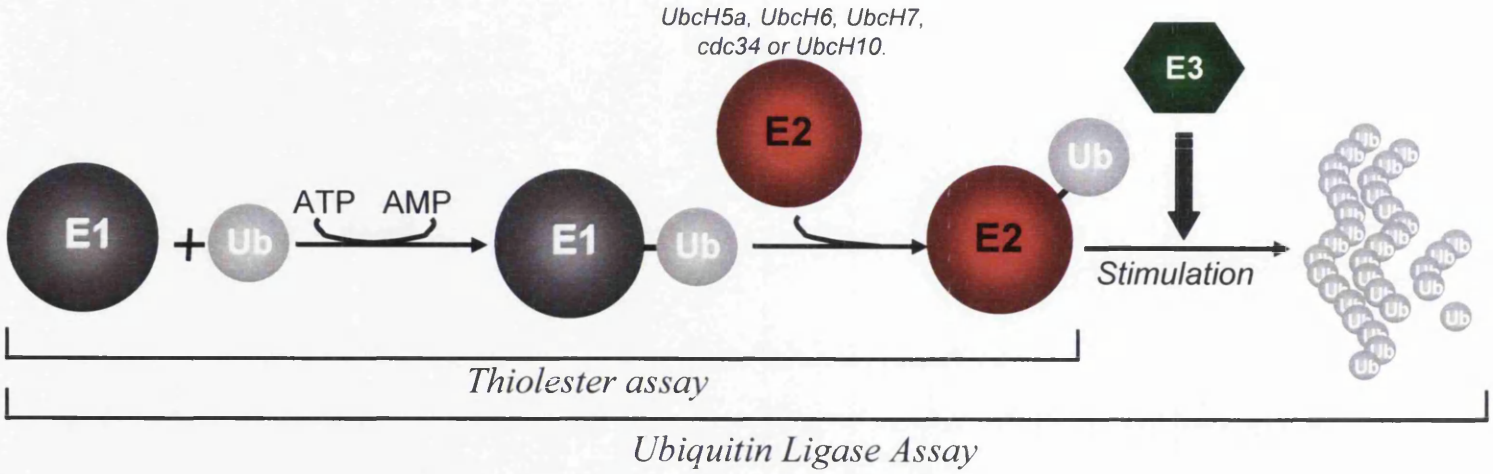


Figure 3.1.0A. *Thiolester and ubiquitin ligase assay overview.*

Thiolester assay = Initially an ubiquitin becomes activated by an E1 enzyme, which involves the formation of a thiolester linkage, from the ubiquitin to the active site cysteine of the E1, in an ATP-dependent reaction. Whereupon, the E2 enzyme forms a thiolester bond between the sulphydryl group at its active site cysteine and the carboxyl group of the C-terminal glycine residue of the activated ubiquitin molecule.

Ubiquitin ligase assay = The E3 ligase then facilitates the transfer of the ubiquitin from the active site of the E2 to a substrate protein. The ubiquitin is linked via an isopeptide bond from its C-terminal carboxyl group to the amino group at the end of the side chain of a lysine residue in the substrate, or if the substrate is already ubiquitinated to a lysine side chain on another ubiquitin molecule.

co-transfection assays (Parkinson and Everett, 2002). The differences in the biological properties of the ICP0 related proteins to that of ICP0 might be a reflection of the divergence of sequence within or outside of their conserved RING finger domains. Furthermore, the variation in sequence may have an implication on the specific E3 ubiquitin ligase activities of the ICP0 related proteins.

The GST-ICP0 related proteins (described in section 3.0.2) were used in assays with a panel of E2 ubiquitin conjugating enzymes to identify and characterise their potential E3 ubiquitin ligase activities. The panel of E2 enzymes included UbcH5a and UbcH6, which ICP0 has been shown to stimulate and cause the formation of polyubiquitin chains *in vitro* (Boutell *et al.*, 2002). Studies have also suggested there could be a third E2 partner of ICP0, namely cdc34, since this E2 was reported to become autoubiquitinated in the presence of a GST-fusion protein containing ICP0 residues 543 to 768, thus proposing a second ubiquitin E3 ligase site on ICP0 (Hagglund *et al.*, 2002; Van Sant *et al.*, 2001a). Even though the residues reported to be associated with cdc34 stimulation are located towards the C-terminus of ICP0, and appear not to be highly conserved or are absent from the other members of the ICP0 family, cdc34 was included in the panel of E2 enzymes used in these studies for completeness. The initial E3 ubiquitin ligase experiments involved screening the activities of the GST-ICP0 related proteins against a panel of E2 enzymes that included UbcH5a, UbcH6 and cdc34, to determine which of these enzymes could be stimulated to form polyubiquitin chains *in vitro*.

Before assessing the E3 ubiquitin ligase activities of the ICP0 related proteins, a thiolester assay was carried out to determine if the preparations of the E2 enzymes were functional. A thiolester assay is similar to the E3 ubiquitin ligase assay, except no E3 ligase is present (see figure 3.1.0A). Initially, an ubiquitin molecule becomes activated by the E1 ubiquitin activating enzyme, which involves the formation of a thiolester linkage between the ubiquitin and the active site cysteine of the E1, in an ATP-dependent reaction. Next, in a transesterification reaction the E2 enzyme forms a thiolester bond between the sulphydryl group at its active site cysteine and the carboxyl group of the C-terminal glycine residue of the E1-linked ubiquitin molecule, thus transferring the ubiquitin from the E1 to the E2. If this was an ubiquitin ligase assay and an E3 ubiquitin ligase was present, the E3 would then facilitate the transfer of the

ubiquitin from the active site of the E2 to a lysine residue in the substrate protein via an isopeptide bond between the C-terminal carboxyl group of the ubiquitin to the amino group at the end of the lysine side chain. If the substrate had already been ubiquitinated, further ubiquitin moieties can be added to a lysine side chain on the ubiquitin molecule at the end of the growing polyubiquitin chain.

Unlike isopeptide bonds, thiolester linked conjugates are not stable in the presence of reducing agents such as β-mercaptoethanol or DTT. Therefore, following the thiolester assay to determine if the preparations of the E2 enzymes were functional and to preserve the thiolester bond, the samples were resuspended in SDS-PAGE loading buffer that did not contain reducing agents.

3.1.1. Thiolester assay to determine the activity of E2 enzyme preparations.

A thiolester assay was used to determine if preparations of purified E2 ubiquitin conjugating enzymes UbcH5a, UbcH6, UbcH7, UbcH10 and cdc34 were active (see figure 3.1.1A) The assay was carried out according to section 2.9A. The E1 and E2 components of the thiolester assay were made in-house and according to methods stated previously (Boutell *et al.*, 2002) and purified ubiquitin was supplied by Sigma. Figure 3.1.1A shows that the E2 enzymes UbcH5a, UbcH6, UbcH7, UbcH10 and cdc34 were all functional, as in a non-reducing environment (-DTT) they formed a thiolester with ubiquitin. The mono-ubiquitinated form of each of the E2 enzymes is indicated by the presence of a second band of lower electrophoretic mobility in each lane. This band is not present in the equivalent lanes containing products from the same reactions that had subsequently been incubated with DTT. The results indicated the preparations of the E2 enzymes were functional and they could be used in ubiquitin E3 ligase assays with GST-ICP0 related proteins.

3.1.2. Optimisation of the E3 ubiquitin ligase assay.

The protocols used to purify the GST-ICP0 related proteins, and the ubiquitin E3 ligase assay conditions, were based on those optimised for ICP0 (Boutell *et al.*, 2002). Therefore, to ensure the conditions were optimal and the ICP0 related proteins were active, an initial ubiquitin E3 ligase assay was carried out.

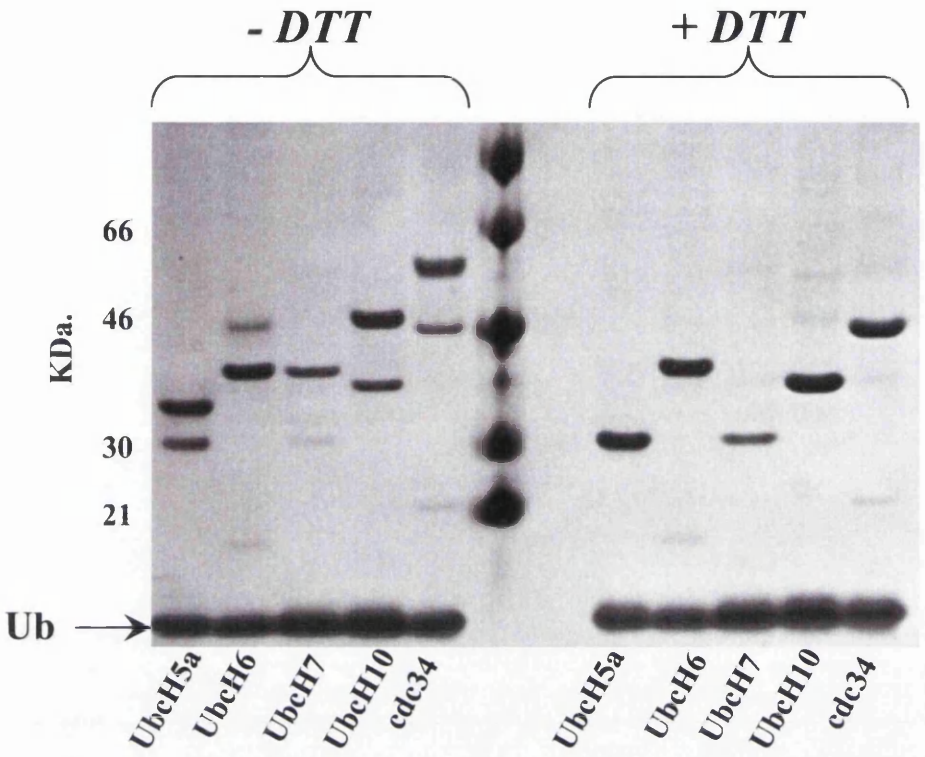
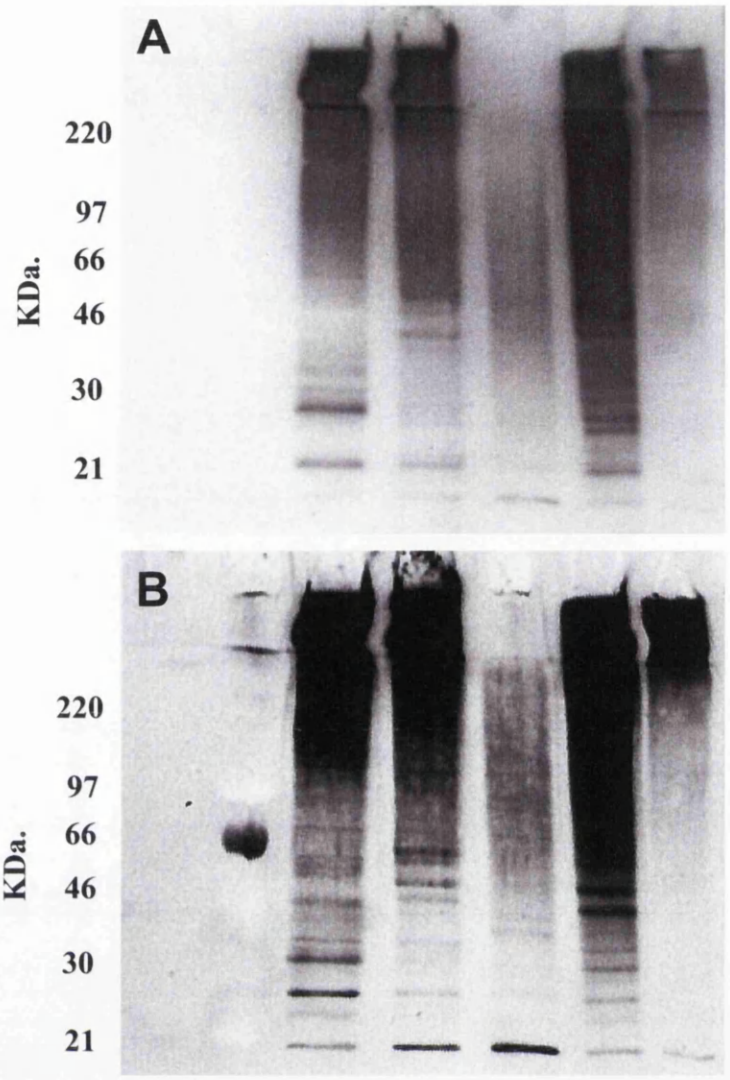


Figure 3.1.1A *In vitro thiolester assay to determine E2 functionality.*

In vitro thiolester assay was carried out using the following assay components: 500 nM E1, 5 μg of ubiquitin and 3–5μg of each E2, (UbcH5a, UbcH6 UbcH7 UbcH10 and cdc 34). The assay was carried out in duplicate and incubated at 28°C for 15 min. One set of reactions was stopped by the addition of boiling mix containing DTT and the second set with boiling mix not containing DTT. The reactions were resolved by SDS PAGE analysis on a 5-12% BIS-Tris gel (Invitrogen), according to manufacturer's instructions and visualised by Coomassie staining. Below each lane, it is denoted which E2 was resolved.

Ub= Excess Ubiquitin.



	1	*MW*	*2*	*3*	*4*	*5*	*6*
E1	+		+	+	+	+	+
UbcH5a	+		+	+	+	+	+
241	-		+	-	-	-	-
BICP0	-		-	+	-	-	-
Eg63	-		-	-	+	-	-
Vg61	-		-	-	-	+	-
EP0	-		-	-	-	-	+
Ub	+		+	+	+	+	+

Figure 3.1.2A. *Optimisation* of the *E3 ligase assay.*

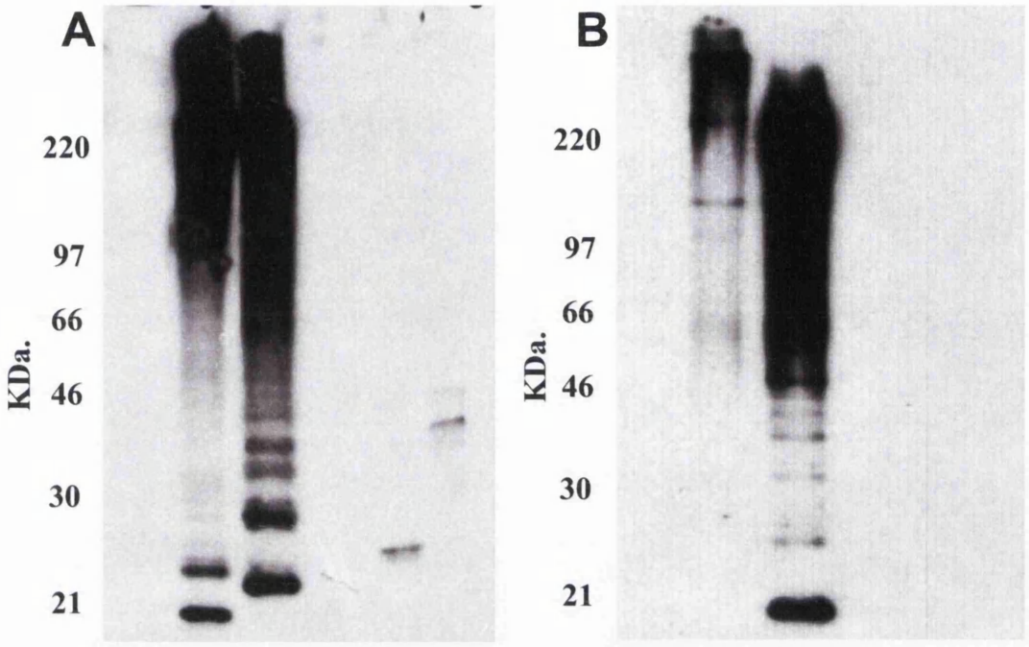
In vitro ubiquitin E3 ligase assay was carried out as described in section 2.9B. The components of each reaction are denoted in the table. The E3 ligase reactions were resolved by SDS-PAGE analysis and visualised by Western blotting. In lanes 2-6 each of the purified RING finger domains were assessed for potential E3 ligase assay in the presence of UbcH5a. MW = Molecular weight markers. 1 = Control (No E2 present). Blot A, was probed using anti-ubiquitin antibody P4D1 (1/1000) and blot B with FK2 (1/1000), both antibodies were detected by secondary hrp-conjugated anti-mouse antibody (1/1000).

The basic ubiquitin E3 ligase assay method and conditions are outlined in section 2.9B, using the GST-ICP0 related proteins and the E2 enzyme UbcH5a (see figure 3.1.2A). All of the GST-ICP0 related proteins stimulated UbcH5a to form high molecular weight polyubiquitin chains (Figure 3.1.3A panel A lanes 3 – 6). Furthermore, the activities of the GST-ICP0 related proteins were similar to that of GST-241 of ICP0 (lane 2). However, there are differences in the amount and molecular weight of the polyubiquitin formed; this will be discussed in more detail later in this chapter. The results indicated that all of the ICP0 related RING finger fusion proteins were successfully purified and active in the ubiquitin E3 ligase assay, using conditions optimised previously for ICP0 (Boutell *et al.*, 2002).

The Western blot in figure 3.1.2A was stripped of the anti-ubiquitin (P4D1) monoclonal antibody and reprobed with FK2 monoclonal antibody, to determine if there was any difference in the results obtained. Both antibodies identify ubiquitin, however FK2 targets the isopeptide bond that links the ubiquitin proteins and P4D1 targets the ubiquitin protein itself. From comparing panels A and B, it can be seen that there were no significant differences in the results obtained using either antibody. This was an important prerequisite for further experiments as the antibodies were used interchangeably to determine if polyubiquitin formation had occurred.

3.1.3. E2 library screen to characterise the E3 ligase activities of the GST-ICP0 related proteins.

It was shown in section 3.1.2. that the GST-ICP0 related proteins function as ubiquitin E3 ligase enzymes and stimulate UbcH5a to form polyubiquitin chains *in vitro*. However, it is possible that the GST-ICP0 related proteins also have other E2 partners. Using the same assay conditions as in section 3.1.2, the GST-fusion proteins were screened against a panel of E2 ubiquitin conjugating enzymes. To ensure consistency in the assay conditions, components that were used in all reactions were made into a 'master mix', which was then aliquoted into the required number of reaction tubes and the individual E2 enzymes were added. The tubes were incubated at 37°C for 1 hour and the reactions were stopped by adding 3-times concentrated SDS-PAGE loading buffer. Further experimental details can be found in section 2.9B.

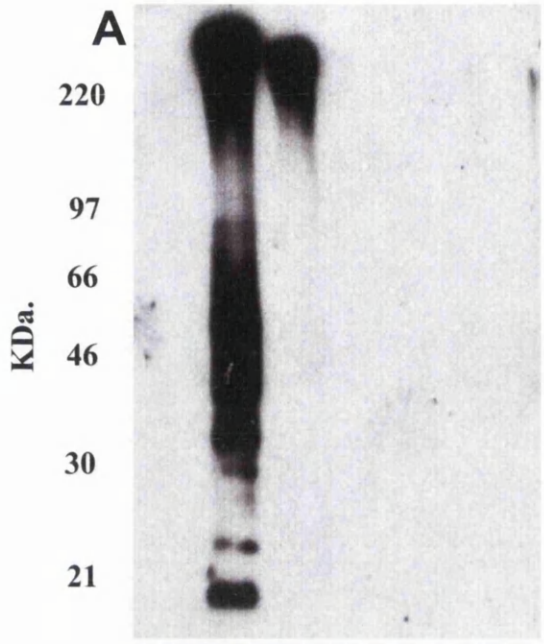


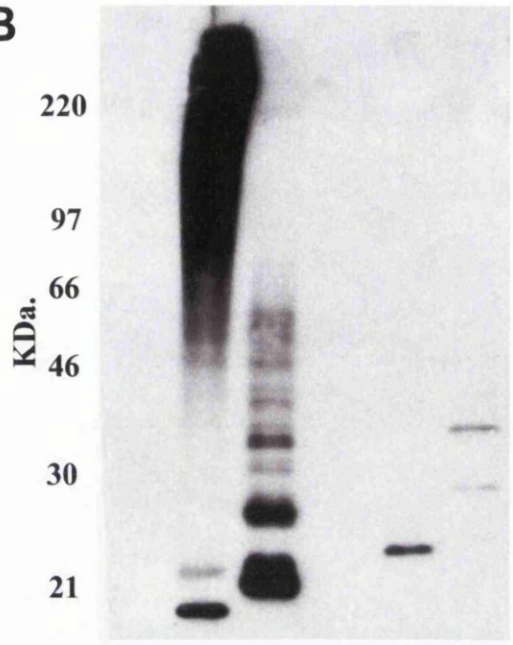
	1	*2*	*3*	*4*	*5*	*6*
E1	+	+	+	+	+	+
UbcH5a	-	+	-	-	-	-
UbcH6	-	-	+	-	-	-
UbcH7	-	-	-	+	-	-
UbcH10	-	-	-	-	+	-
CDC34	-	-	-	-	-	+
BICP0	+	+	+	+	+	+
Eg63	-	-	-	-	-	-
Ub	+	+	+	+	+	+

1	*2*	*3*	*4*	*5*	*6*
+	+	+	+	+	+
-	+	-	-	-	-
-	-	+	-	-	-
-	-	-	+	-	-
-	-	-	-	+	-
-	-	-	-	-	+
-	-	-	-	-	-
+	+	+	+	+	+
+	+	+	+	+	+

Figure 3.1.3A. *E3 ligase assays to determine the E2 specificity of the RING finger domains of GST-BICP0 (A) and GST-Eg63 (B).*

In vitro ubiquitin ligase assay was carried out as described in section 2.9B. A panel of E2 enzymes were screened for activity in the presence of the RING finger domains of GST-BICP0 and GST-Eg63. The RING finger domains of GST-BICP0 and GST-Eg63 were assessed for potential E3 ligase in the presence of varying E2 enzymes. The components of each reaction (including the E2 used) are denoted in the table. The E3 ligase reactions were resolved by SDS-PAGE analysis and visualised by Western blotting. **Blot A & B**, Lane 1 = Control (No E2 present); **Blot A**, Lane 2-6 = The RING finger domain of BICP0 only stimulated UbcH5a and UbcH6 to cause polyubiquitin formation; **Blot B,** Lane 2-6 = The RING finger domain of GST-Eg63 only stimulate UbcH5a and UbcH6 to cause polyubiquitin formation. Blot A & B were probed using anti-ubiquitin antibody P4D1 (1/1000) followed by secondary hrp-conjugated anti-mouse antibody (1/1000).





	1	*2*	*3*	*4*	*5*	*6*
E1	+	+	+	+	+	+
Control	-	-	-	-	-	-
UbcH5a	-	+	-	-	-	-
UbcH6	-	-	+	-	-	-
UbcH7	-	-	-	+	-	-
UbcH10	-	-	-	-	+	-
CDC34	-	-	-	-	-	+
EP0	+	+	+	+	+	+
Vg61	-	-	-	-	-	-
Ub	+	+	+	+	+	+

1	*2*	*3*	*4*	*5*	*6*
+	+	+	+	+	+
-	-	-	-	-	-
-	+	-	-	-	-
-	-	+	-	-	-
-	-	-	+	-	-
-	-	-	-	+	-
-	-	-	-	-	+
-	-	-	-	-	-
+	+	+	+	+	+
+	+	+	+	+	+

Figure 3.1.3B. *E3 ligase assays to determine the E2 specificity of the RING finger domains of GST-EP0 (A) and GST-Vg61 (B).*

In vitro ubiquitin ligase assay was carried out as described in section 2.9B. A panel of E2 enzymes were screened for activity in the presence of the RING finger domains of GST-EP0 and GST-Vg61. The RING finger domains of BICP0 and GST-Eg63 were assessed for potential E3 ligase in the presence of varying E2s. The components of each reaction (including the E2 used) are denoted in the table. The E3 ligase reactions were resolved by SDS-PAGE analysis and visualised by Western blotting. **Blot A & B**, Lane 1 = Control (No E2 present); **Blot A**, Lane 2-6 = The RING finger domain of GST-EP0 only stimulated UbcH5a and UbcH6 to cause polyubiquitin formation; **Blot B,** Lane 2-6 = The RING finger domain of GST-Vg61 only stimulated UbcH5a and UbcH6 to cause polyubiquitin formation. Blot A & B were probed using anti-ubiquitin antibody P4D1 (1/1000) followed by secondary hrp-conjugated anti-mouse antibody (1/1000).

The results were as follows: Figure 3.1.3A, Panel A lane 1 indicates that E1 and GST-BICP0 cannot form polyubiquitin chains alone. However, when E1, ubiquitin and GST-BICP0 were incubated in the presence of UbcH5a (lane 2) and UbcH6 (lane 3), the E2 enzymes were able to form variable length polyubiquitin chains, but the E2 enzymes UbcH7, UbcH10 and cdc34 were not. Similarly, figure 3.1.3B shows that GST-Eg63 also has E3 ligase activity and could stimulate UbcH5a (lane 2) and UbcH6 (lane 3), but not the other E2 enzymes. GST- EP0 and GST-Vg61 also acted as ubiquitin E3 ligases in a similar manner, again stimulating only UbcH5a and UbcH6 (figure 3.1.3B, panels A and B).

3.1.4. Conclusions.

The results from screening the GST-ICP0 related proteins against a panel of E2 proteins indicate that they act as E3 ubiquitin ligases and their specificity is limited to UbcH5a and UbcH6. Furthermore, the GST-ICP0 related proteins do not contain a second E3 ligase domain associated with cdc34 stimulation. Collectively, these results show the GST-ICP0 related proteins are E3 ligases and the intrinsic activity is similar to that previously shown for ICP0. The similar results of the ICP0 related proteins to that of ICP0 are likely to be a consequence of the conserved sequence within their RING finger domains. Indeed, many families of cellular RING finger proteins show a similar pattern of E2 enzyme stimulation, so it is likely that some characteristic of RING finger domains in general determines their E2 specificity. A large degree of homology is also shared between the E2 enzymes of the UbcH5 family and UbcH6. The UbcH5 family members (UbcH5a, UbcH5b and UbcH5c) share over 90% sequence similarity to one another, with UbcH5a sharing 75% homology to UbcH6 (Jensen *et al.*, 1995; Nuber *et al.*, 1996). Therefore, it is probably the conserved sequence or structural similarity between UbcH5a and UbcH6 that enables the ICP0 related proteins to stimulate both of the E2 enzymes. UbcH5a has also been implicated as the E2 partner of a growing number of E3 ubiquitin ligases (Hakli *et al.*, 2004; Itoh *et al.*, 2003; Lorick *et al.*, 1999; Spencer *et al.*, 1999; Vandenberg *et al.*, 2003; Yang & Du, 2004; You & Pickart, 2001) as has UbcH6 (Anan *et al.*, 1998; Nuber *et al.*, 1996; Yang & Du, 2004), many of them members of the RING finger class.

In contrast to the ICP0 related proteins of the alphaherpesviruses that stimulate both UbcH5a and UbcH6, Human Papilloma Virus (HPV) E6 protein has been shown to utilize only members of the UbcH5 family for the ubiquitination of target substrate p53. However, unlike ICP0, HPV is not a RING finger protein and instead recruits the cellular E3 ligase HECT domain protein H6-AP to ubiquitinate p53 (Jensen *et al.*, 1995; Rolfe *et al.*, 1995; Scheffner *et al.*, 1994). In Drosophila, the Inhibitory Apoptosis Proteins (IAP), c-IAP1 and c-IAP2, have been shown to act as ubiquitin E3 ligases and are able to stimulate both UbcH5a and UbcH6 to form polyubiquitin chains *in vitro* (Yang & Du, 2004). These results are remarkably similar to those in this chapter and those obtained previously with ICP0. The studies carried out in this chapter were sufficient to determine that the ICP0 related proteins have the same E2 partners as ICP0 *in vitro*, although it possible that other partners may exist. In the relatively small genome of *S. cerevisiae,* 13 genes exist that encode E2 enzymes (Hochstrasser, 1996), and more are likely to be found in higher eukaryotes. Therefore, as the number of defined E2 proteins increases, so may the number of potential E2 partners of ICP0 and its related proteins.

3.2.0. Characterisation of the ubiquitin E3 ligase activities of the ICP0 related RING finger domains.

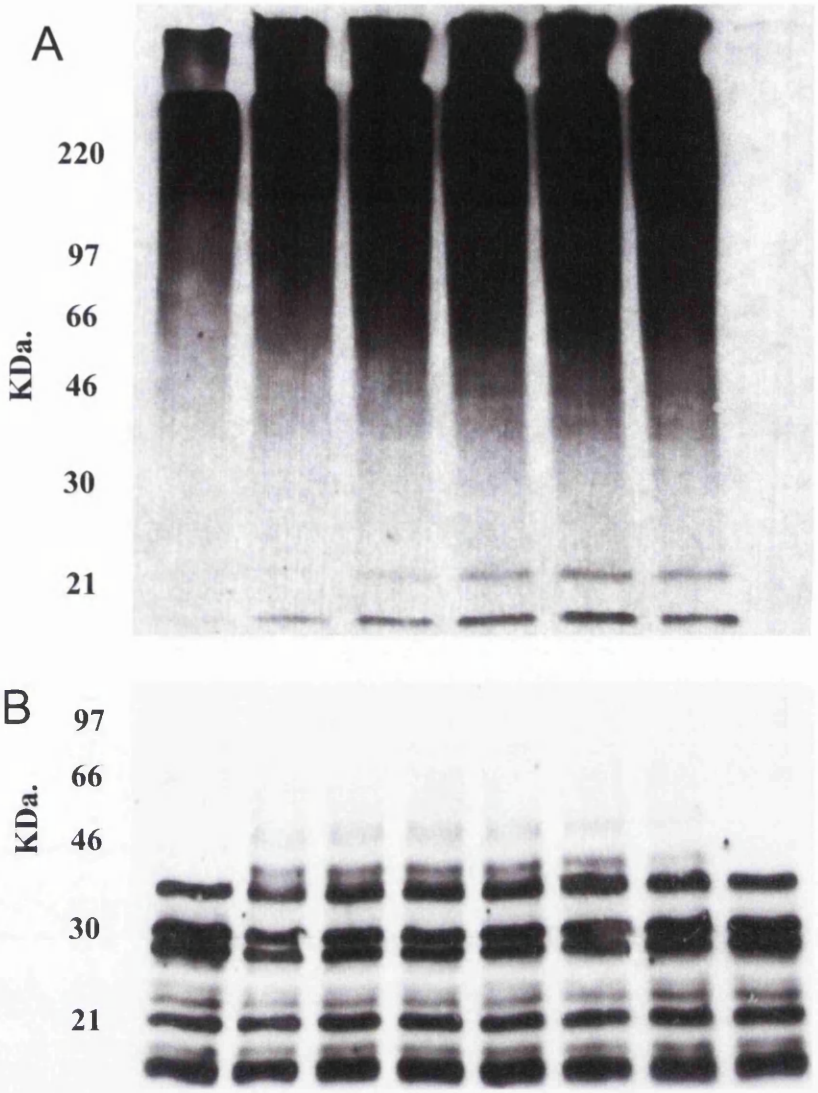
The following sections describe a series of experiments that characterise the *in vitro* biochemical activities of the RING finger domains of the selected members of the ICP0 related proteins.

3.2.1. Rate of polyubiquitin formation by GST-Eg63.

This ubiquitin E3 ligase assay was carried out to determine the rate of polyubiquitin formation by GST-Eg63 in the presence of the E2 enzymes UbcH5a (figure 3.2.1A) and UbcH6 (figure 3.2.1B). Sixteen sets of reactions were set up (8 for UbcH5a and 8 for UbcH6) which were incubated (see materials and methods section for further details) and stopped at a series of time points. Figure 3.2.1A lane 1 indicates that GST-Eg63 stimulation of UbcH5a causes rapid polyubiquitin formation, within 1 minute of the initiation of the E3 ligase reaction. Polyubiquitin continued to accumulate during the first 60 minutes of the incubation (lane 1 to lane 7). In lane 7 (120 minutes), the amount of polyubiquitin detected was similar to that in lane 6 (60 minutes). This may be due to one of the components of the assay becoming inactive or the amount of free ubiquitin becoming limiting, or the maximum level of polyubiquitin that was possible to detect using Western blotting was reached. Lane 8 is the control reaction, where all assay components were present except UbcH5a. Figure 3.1.5B shows the rate of polyubiquitin formation in the presence of UbcH6 and GST-Eg63. Polyubiquitin chains were detectable at 15 minutes and continued to accumulate up to the final time point, 120 minutes. Therefore the kinetics of the E3 ligase reactions with UbcH5a appear to more efficient than UbcH6.

3.2.2. Conclusions from the series of experiments to determine the rates of polyubiquitin formation.

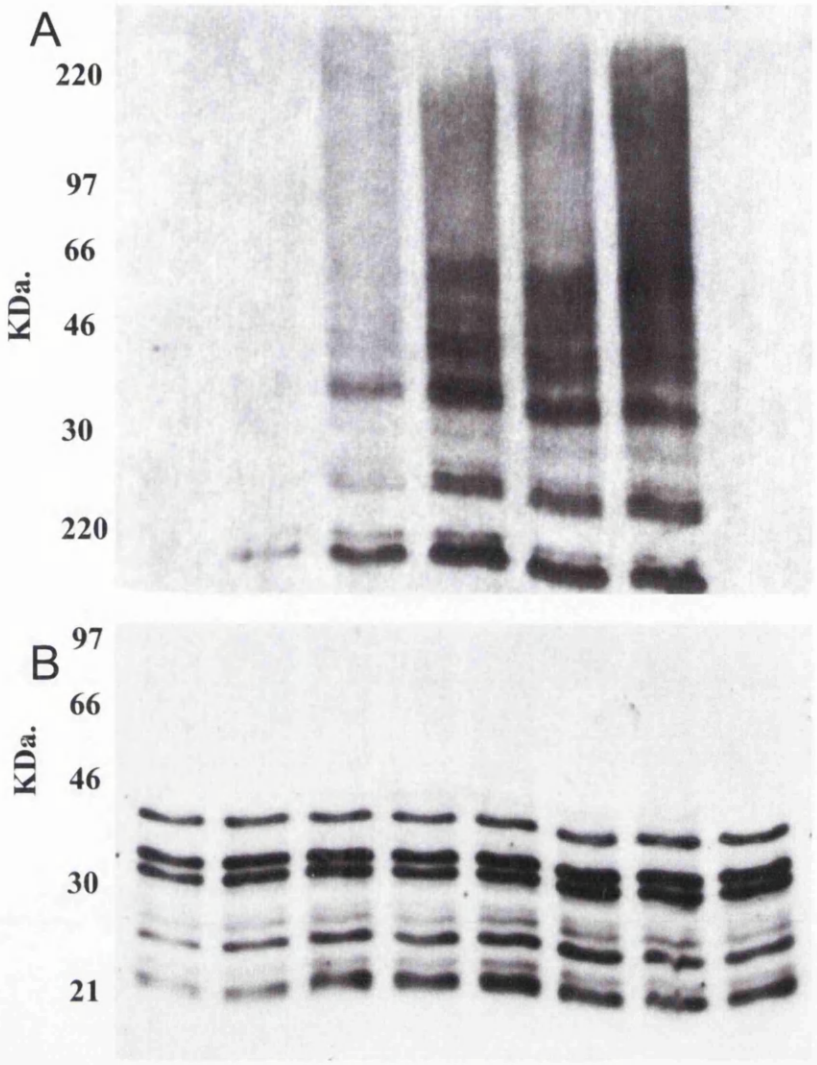
The results indicated there were differences in the kinetics of the reaction of GST-Eg63 with UbcH5a and UbcH6. This may be down to some distinct difference in the intrinsic biological activity of GST-Eg63 with UbcH6, although it is more likely explained by the UbcH6 preparation being intrinsically less active than that of UbcH5a. When GST-Eg63



	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
E1	+	+	+	+	+	+	+	+
Eg63	+	+	+	+	+	+	+	+
UbcH5a	+	+	+	+	+	+	+	-
Ub	+	+	+	+	+	+	+	+
Time Reaction Stopped (min)	**1**	**5**	**15**	**30**	**45**	**60**	**120**	**120**

Figure 3.2.1A. *Characterisation of the E3 ligase activity of GST-Eg63 in the presence of UbcH5a, over time.*

In vitro ubiquination assay was carried out as described in section 2.9B. In lanes 1-7 replicate E3 ligase reactions were carried out and terminated at different time points (see table) to assess the rate of poly-ubiquitin formation of GST-Eg63 in the presence of UbcH5a. Lane 8 = control (no E2 present). The E3 ligase reactions were resolved by SDS-PAGE analysis and visualised by Western blotting. Blot A was probed with the primary antibody α-Ub (P4D1) at 1/1000 followed by the secondary antibody hrp-conjugated anti-mouse at 1/1000. Blot B was probed with anti-GST (1/1000), followed by the secondary antibody hrp-conjugated anti-rabbit (1/1000).



	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
E1	+	+	+	+	+	+	+	+
Eg63	+	+	+	+	+	+	+	+
UbcH6	+	+	+	+	+	+	+	-
Ub	+	+	+	+	+	+	+	+
Time Reaction Stopped (min)	**0**	**5**	**15**	**30**	**45**	**60**	**120**	**120**

Figure 3.2.1B. *Characterisation of the E3 ligase activity of GST-Eg63 in the presence of UbcH6, over time.*

In lanes 1-7 replicate E3 ligase reactions were carried out and terminated at different time points (see table), to assess the rate of poly-ubiquitin formation of GST-Eg63 in the presence of UbcH6. Lane 8 = control (no E2 present). The ubiquitin ligase reaction was carried out according to conditions outlined in the previous figure.

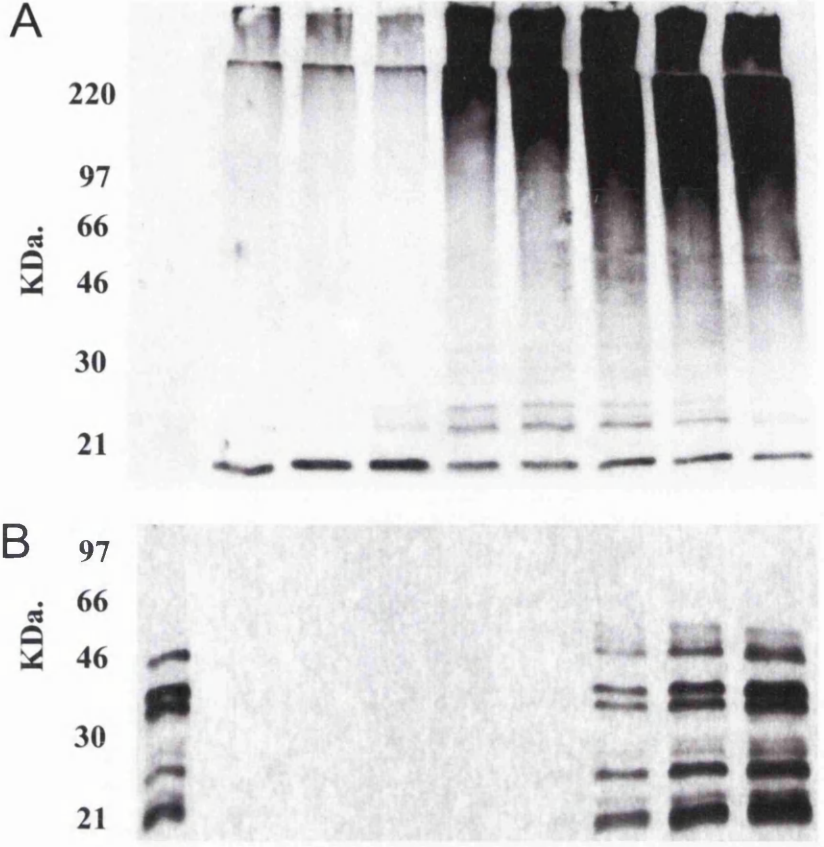
stimulates UbcH5a the rapid formation of polyubiquitin chains occurs, within the first minute of initiation of the E3 ligase reaction. As the reaction progresses the shorter polyubiquitin chains appear to accumulate, up to approximately the 60th minute. However, when the same assay was carried out in the presence of UbcH6, no polyubiquitin chains formation was detectable until 15 minutes. Thereafter, instead of polyubiquitin chain length being increased over time as in the case with UbcH5a, the chains appear to be uniform in length and increases in amount as the reaction progresses. This indicates an apparent difference in the biochemical activities of GST-Eg63/UbcH5a and GST-Eg63/UbcH6. It is likely that the variations observed are probably due to the biochemical differences between the E2 enzymes. Indeed, similar differences between the UbcH5a and UbcH6 reactions were observed with the other GST-ICP0 related proteins.

3.2.3. Concentration dependence of the polyubiquitination activity.

In the following series of experiments the ubiquitin E3 ligase activities of the GST-ICP0 related proteins at different concentrations were compared. The GST-ICP0 related proteins were quantified as described above, however a limitation that should be borne in mind is that some of the truncated species of the RING finger constructs that are present after purification (see figure 3.0.2A) may have enzymatic activity. Therefore the following data can provide only an approximation of the relative activities of the different RING fingers.

In figure 3.2.3A GST-Eg63 was titrated from 1.25 ng to 120 ng, while the other ubiquitin E3 ligase assay components were used at the amounts stated in materials and methods. The results indicate (Figure 3.2.3A, panel A) that 7.5 ng of GST-Eg63 was sufficient to stimulate polyubiquitin chain formation by UbcH5a. The results indicate that as the amount of GST-Eg63 increased the amount of polyubiquitin formation also increased.

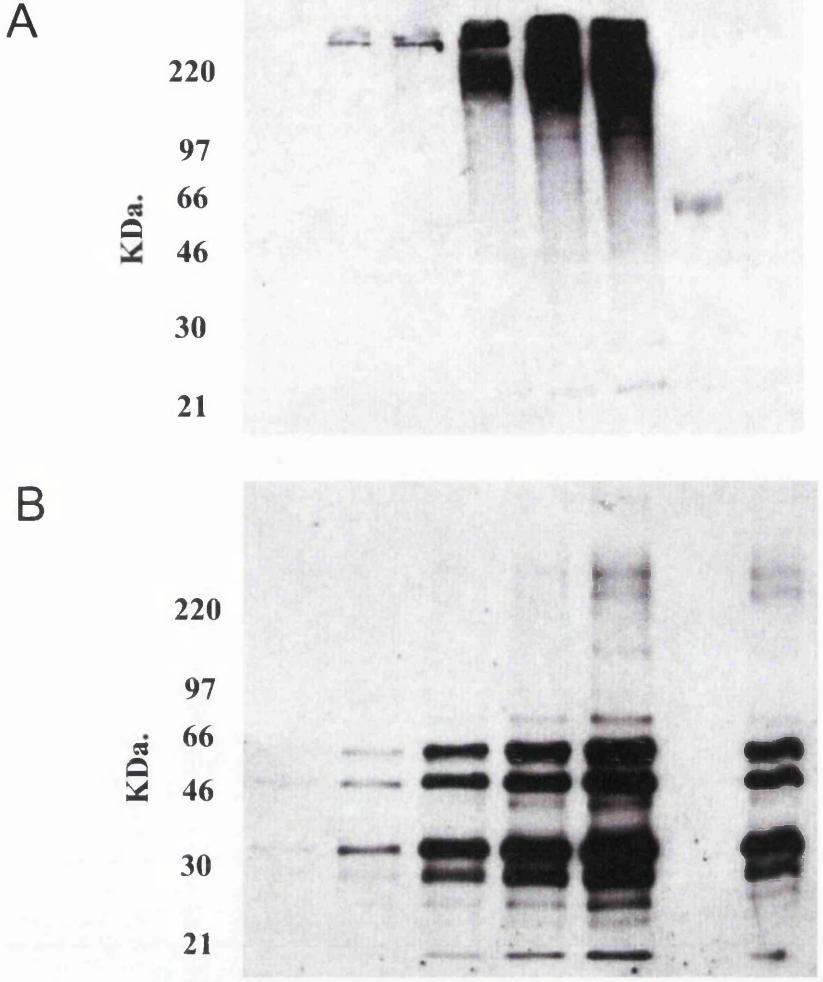
In figure 3.2.3B, the E3 ubiquitin ligase assay was carried out using GST-BICP0, which was titrated from 7.5 ng to 120 ng, the other components were used at the concentrations stated in materials and methods. The results indicated (Figure 3.2.3B, panel A) that 30 ng of BICP0 was required for detectable polyubiquitin chain formation in the presence of UbcH6. These results suggest the intrinsic activity of GST-BICP0 is



	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
E1	+	+	+	+	+	+	+	+	+
UbcH5a	-	+	+	+	+	+	+	+	+
Ub	+	+	+	+	+	+	+	+	+
Eg63 (ng)	**60**	**1.25**	**2.5**	**5**	**7.5**	**15**	**30**	**60**	**120**

Figure 3.2.3A. *Characterisation of the E3 ligase activity of the titrated RING finger domain of GST-Eg63 in the presence of UbcH5a.*

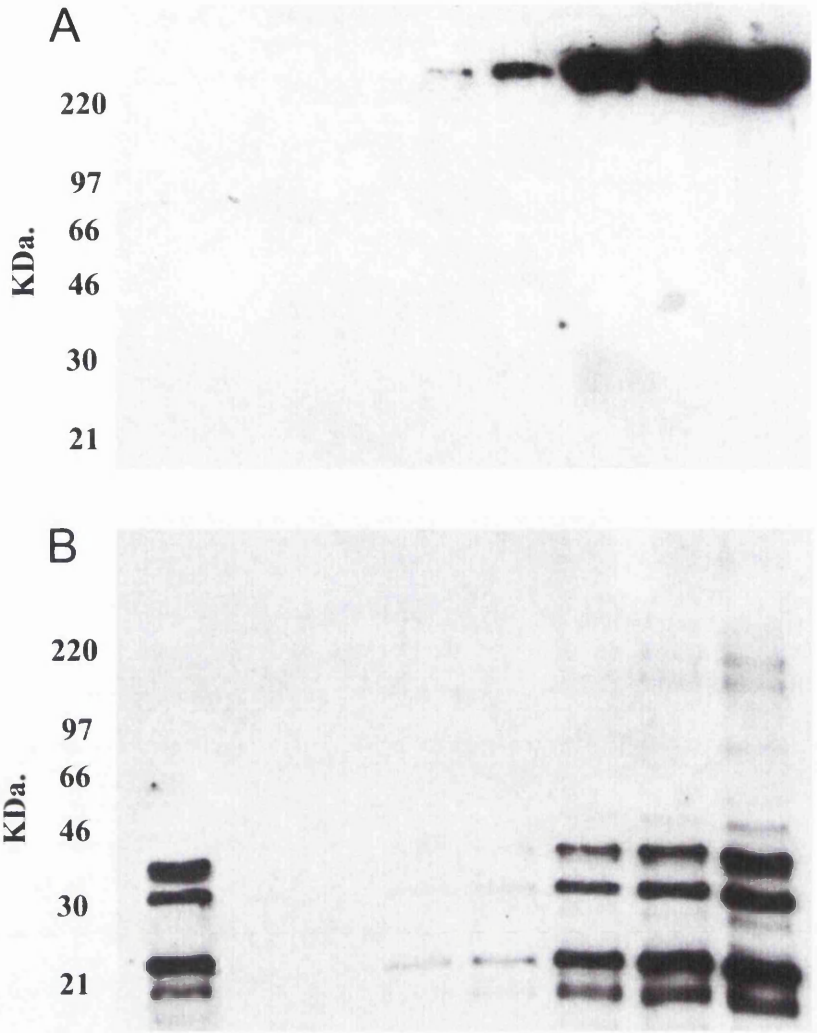
The RING finger domain of Eg63 was titrated, to assess its E3 ligase activity in the presence of UbcH5a. In lanes 2 to 9 the E3 ligase assay was carried out in the presence of UbcH5a. Lanes 1 = Control (no UbcH5a was present). With the exception of the E3 ligase concentration the reactions were carried out according to the method described in section 2.9B.



	1	*2*	*3*	*4*	*5*	*MW*	*6*
E1	+	+	+	+	+		+
UbcH6	+	+	+	+	+		-
Ub	+	+	+	+	+		+
GST-BICP0 (ng)	**7.5**	**15**	**30**	**60**	**120**		**60**

Figure 3.2.3B. *Characterisation of the E3 ligase activity of the titrated RING finger domain of BICP0 in the presence of UbcH6.*

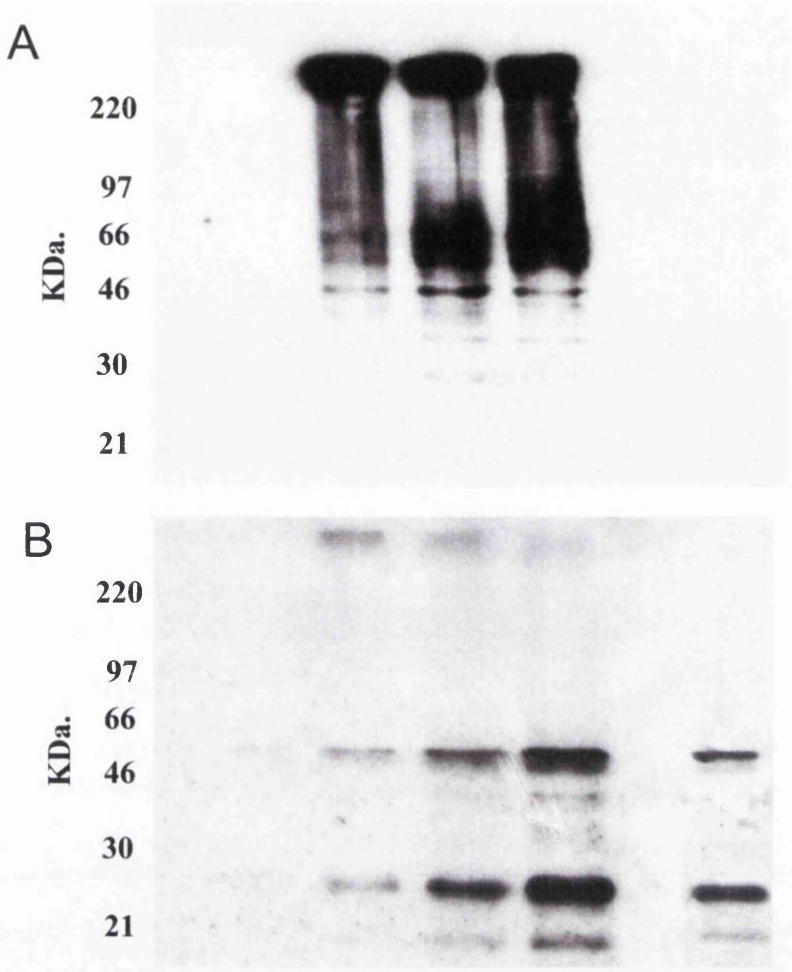
The RING finger domain of BICP0 was titrated, to assess its E3 ligase activity in the presence of UbcH6. In lanes1 to 5 the E3 ligase assay was carried out in the presence of UbcH5a. MW = Molecular weight markers. Lanes 6 = Control (no UbcH6 was present). The ubiquitin ligase reactions were carried out according to the method outlined in the previous figure.



	1		*MW*	*2*	*3*	*4*	*5*	*6*
E1	+			+	+	+	+	+
UbcH5a				+	+	+	+	-
Ub	+			+	+	+	+	+
BICP0 (ng)	**60**			**7.5**	**15**	**30**	**60**	**120**

Figure 3.2.3C. *Characterisation of the E3 ligase activity of the titrated RING finger domain of BICP0 in the presence of UbcH5a.*

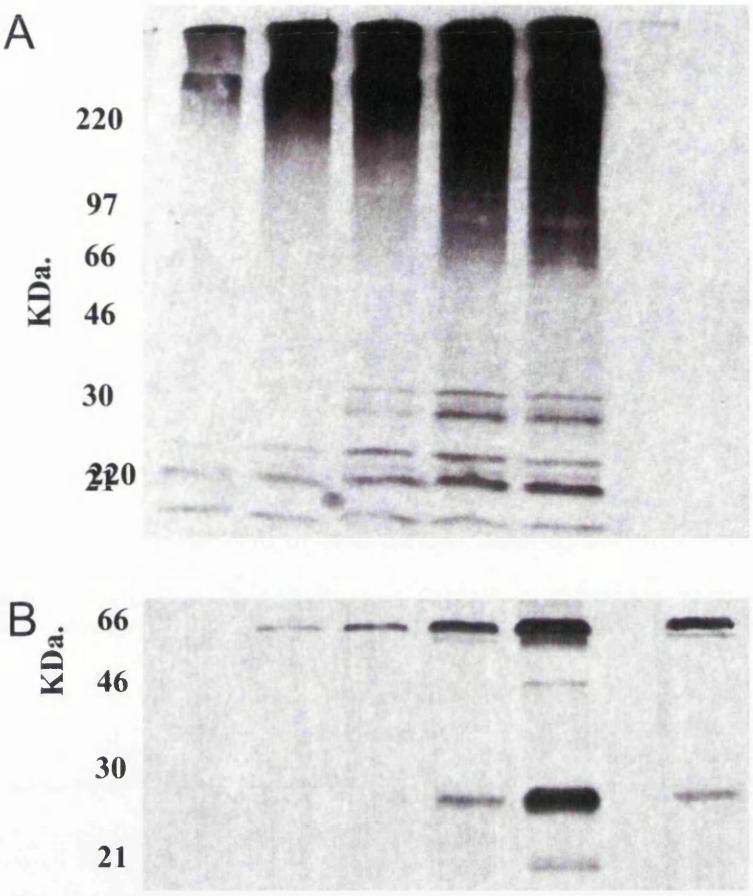
The RING finger domain of BICP0 was titrated, to assess its E3 ligase activity in the presence of UbcH5a. In lanes 2 to 6 the E3 ligase assay was carried out in the presence of UbcH5a. MW = Molecular weight markers. Lanes 1 = Control (no UbcH5a was present). The ubiquitin E3 ligase reactions were carried out according to the method outlined in the previous figure.



	1	*2*	*3*	*4*	*5*	*MW*	*6*
E1	+	+	+	+	+		+
UbcH6	+	+	+	+	+		-
Ub	+	+	+	+	+		+
GST-EP0 (ng)	**7.5**	**15**	**30**	**60**	**120**		**60**

Figure 3.2.3D. *Characterisation of the E3 ligase activity of the titrated RING finger domain of GST-EP0 in the presence of UbcH6.*

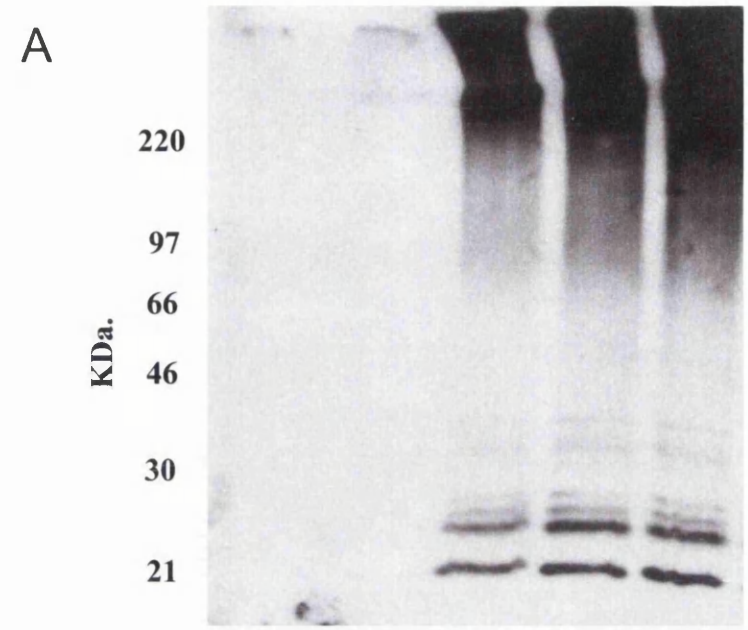
The RING finger domain of GST-EP0 was titrated, to assess its E3 ligase activity in the presence of UbcH6. In lanes 1 to 5 the E3 ligase assay was carried out in the presence of UbcH6. Lanes 6 = Control (no UbcH5a was present). MW = Molecular weight markers. The ubiquitin ligase reactions were carried out according to the method outlined in the previous figure.

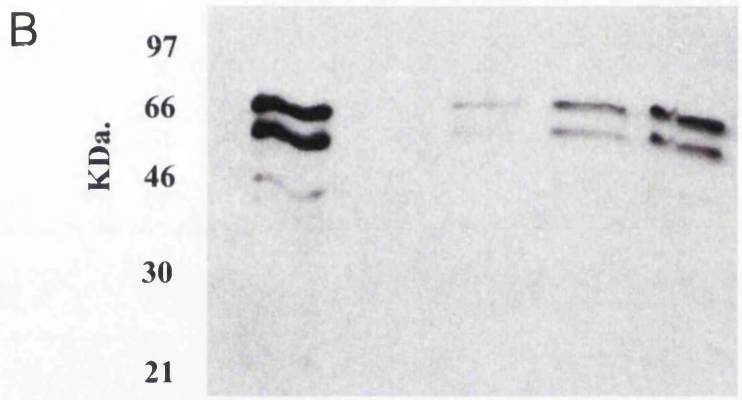


	1	*2*	*3*	*4*	*5*	*MW*	*6*
E1	+	+	+	+	+		+
UbcH5a	+	+	+	+	+		-
Ub	+	+	+	+	+		+
GST-EP0 (ng)	**7.5**	**15**	**30**	**60**	**120**		**60**

Figure 3.2.3E. *Characterisation of the E3 ligase activity of the titrated RING finger domain of GST-EP0 in the presence of UbcH5a.*

The RING finger domain of GST-EP0 was titrated, to assess its E3 ligase activity in the presence of UbcH5a. In lanes 1 to 5 the E3 ligase assay was carried out in the presence of UbcH5a. Lanes 6 = Control (no UbcH6 was present). MW = Molecular weight markers. The ubiquitin ligase reactions were carried out according to the method outlined in the previous figure.





	1	*2*	*3*	*4*	*5*
E1	+	+	+	+	+
UbcH5a	-	+	+	+	+
Ub	+	+	+	+	+
GST-Vg61 (ng)	**60**	**7.5**	**15**	**30**	**45**

Figure 3.2.3F. *Characterisation of the E3 ligase activity of the titrated RING finger domain of Vg61 in the presence of UbcH5a.*

The RING finger domain of GST-Vg61 was titrated, to assess its E3 ligase activity in the presence of UbcH5a. In lanes 2 to 5 the E3 ligase assay was carried out in the presence of UbcH5a. Lanes 6 = Control (no UbcH5a was present). The ubiquitin ligase reactions were carried out according to the method outlined in the previous figure.

ess than GST-Eg63; the possible reasons behind this will be discussed at the end of this ection.

n figure 3.2.3C, GST-BICP0 was titrated from 7.5 ng to 120 ng in the presence of UbcH5a. In this experiment, the polyubiquitin chains formed were of such high nolecular weight that they have remained in the upper portion of the gel. Consequently, i is only possible to conclude that 15 ng of GST-BICP0 is sufficient to stimulate UbcH5a activity in the reaction conditions used.

n figure 3.2.3D, GST-EP0 was titrated from 7.5 ng to 120 ng and the remaining ubiquitin E3 ligase assay components were used at the concentrations stated in materials and methods. The results indicated (Figure 3.1.6D, panel A) that 30 ng of GST-EP0 was required for detectable polyubiquitin chain formation in the presence of UbcH6. However, under the same ubiquitin ligase assay conditions in figure 3.2.3E, only 7.5 ng of GST-EP0 was sufficient to stimulate UbcH5a activity.

In figure 3.2.3F GST-Vg61 was titrated from 3.75 ng to 30 ng and the remaining ubiquitin E3 ligase assay components were used at the concentrations stated in materials and methods. The results indicated (Figure 3.1.6D, panel A) that 7.5 ng of GST-Vg61 was required for detectable polyubiquitin chain formation in the presence of UbcH5a.

3.2.4. Conclusions.

The results indicate that approximately 7.5 ng of GST-ICP0 related proteins was needed to simulate UbcH5a and approximately 30 ng was needed to simulate UbcH6 and cause the formation of polyubiquitin. As mentioned previously the difference in the intrinsic activity of the reactions was probably a reflection of the specific activity of each E2. However, the data indicated, but by no means conclusively that the same approximate amounts of each of the ICP0 related proteins were needed to stimulate UbcH5a or UbcH6. From the current data, it was not possible to determine which of the GST-ICP0 related proteins was intrinsically more active than others. It became apparent when carrying out these experiments that some of the GST-ICP0 related proteins and E2 enzymes were unstable, quickly losing activity after a few freeze-thaw cycles.

Chapter 3 – Part II - Biochemical characterisation of the minimal functional domains of the ICP0 related proteins, BICP0 and Eg63.

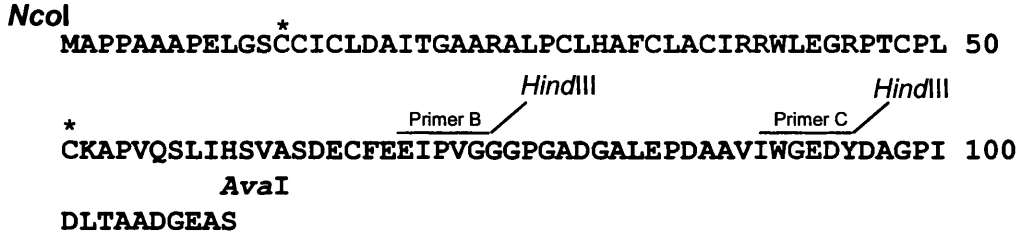
3.3.0. Introduction.

The aim of the experiments described in this section was to further characterise the *in vitro* E3 ligase activities of GST-BICP0 and GST-Eg63. PCR mutagenesis was used to truncate the 3' end of the DNA fragment encoding GST-BICP0. A truncation of GST-Eg63, previously termed ENX, was already available in-house and was originally used to determine the NMR structure of the RING finger domain of the ICP0 related proteins (Barlow *et al.*, 1994; Everett *et al.*, 1993a). The methods used to express and purify the GST-BICP0 and GST-Eg63 truncations were the same as those used in section 3.0.0.

3.3.1 Construction of the minimal functional domains of GST-BICP0.

PCR mutagenesis was used to truncate the 3' end of the DNA fragment encoding GST-BICP0. Two truncated versions were constructed: BICP0 1_90 260 bp (90 a.a.) in size and BICP0 1_75, 225 bp (75 a.a.) in size (see figure 3.3.1A). The PCR method employed for the construction of BICP0 1_75 used the vector pGEX GST-BICP0 as a template; BICP0/GST/FW (specific for a DNA sequence within the GST tag), BICP0/75/REV, (specific for a DNA sequence around the 225 bp of the BICP0 RING finger domain) were the forward and reverse primers respectively. BICP0 1_90 was constructed using the same template vector and forward primer, while the reverse primer BICP0/90/REV (specific for a sequence around 270 bp of the DNA fragment encoding the BICP0 RING finger domain) was used in place BICP0/75/REV. Each of the reverse primers encoded a *Hind*III site at their 5' ends, to enable the introduction of a *Hind*III into the 3' end of the PCR amplified DNA product. The PCR reaction components, cyclic conditions, the amino acid sequence of the region amplified and the DNA sequence of the primers, are all shown in figure 3.1.1A.

Construction of the BICP0 RING finger domain truncations.



Primer A = BICP0/GST/FW (5'-ATC GAC CAT GGC GCC-3')

Primer B = BICP0/75/Rev (5'-GAT CCA AGC TTA CCC CAC AGG GAT CTC-3')

Primer C = BICP0/90/Rev (5'-GAT CCA AGC TTA GTC CTC GCC CCA GAT-3')

Figure 3.3.1A. O*verview of the PCR procedure used to construction BICP0 truncations.*

The amino acid sequence of the BICP0 RING finger domain is marked in red, with the first and last cysteine residues marked (*). The BICP0 RING finger domain truncations were produced by PCR mutagenesis, using primers A, B and C (nucleotide sequences shown below) and the template vector pGEX GST-BICP0. Primer A (forward) binds upstream of the *Nco*I site within the template vector (not shown) and was used in conjunction with primer B or C in the PCR reactions. Primer B (reverse primer), binds to a region specific for a.a. codon 75-80 of the template vector. Primer C (reverse primer), to a region specific for a.a. codon 90-95 of the template vector. Primers B & C were designed to introduce a a *Hind*III restriction enzyme site into the 3' of PCR product. The primers were used in the following combinations A + C and A + B and the PCR reaction components (20 ng pGEX GST-BICP0, dNTPs 10 mM, reaction buffer 10 x, 1 unit p.f.u. turbo, 2 % DMSO, H_2O.) and cyclic conditions were the same for both reactions (1 x 94 °C for 5 min, 35 x [94 °C for 30 seconds, 57 °C for 1 min, 72 °C for 1 min], 1 x 72 °C for 10 min).

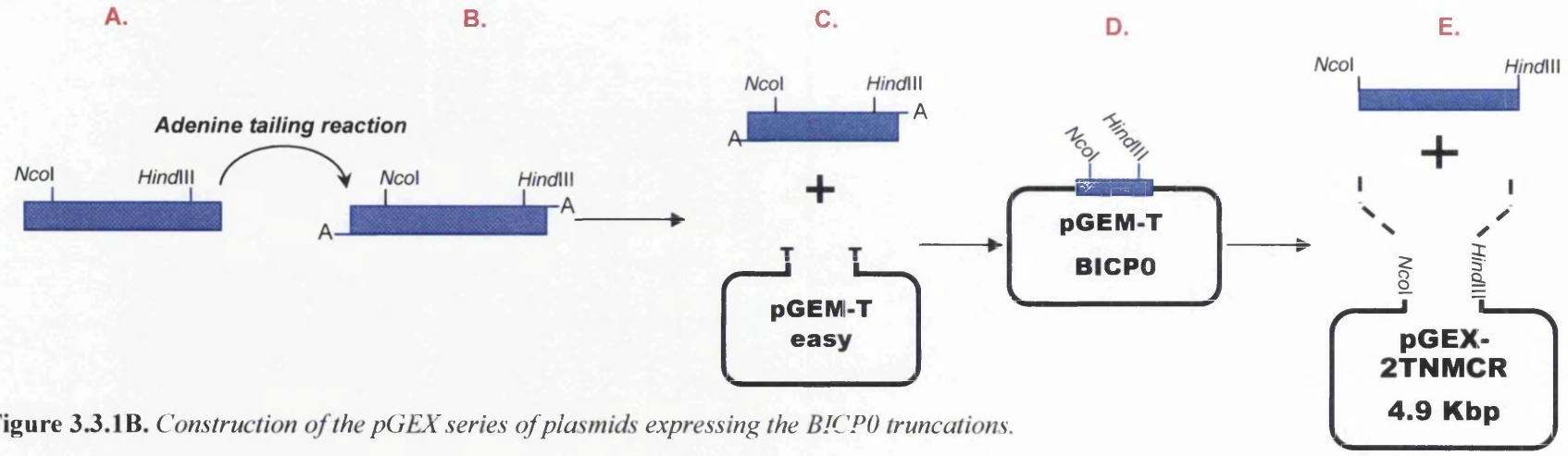


Figure 3.3.1B. *Construction of the pGEX series of plasmids expressing the BICP0 truncations.*

A) The blue box represents the PCR product BICP0 1_75 or BICP0 1_90, which were cloned using the same strategy. The relative positions of the *Nco*I and *Hind*III restriction sites within the PCR product are shown. It was not possible to cut the isolated PCR product with the restriction enzymes *Nco*I or *Hind*III, therefore it was necessary to sub-clone the PCR product into the vector pGEM-T easy. B) Initially, it was necessary to carry out an deoxyadenosine (A)-tailing reaction (see methods section for details), as the proof reading DNA polymerases, Pfu Turbo, used in these reaction does not add A residues to the 3' end of each strand of the PCR product. C) The PCR product was then ethanol-precipitated, ligated in to pGEM-T easy, then transformed into *E.coli* DH5α. D) A selection of the resultant colonies were prepared using the mini-prep method and plasmid DNA was isolated, the identity of which was confirmed using restriction analysis. E) *Nco*I and *Hind*III were used to re-isolate the PCR product from pGEM-T BICP0. The PCR product was gel purified, ethanol precipitated and ligated into *Nco*I-*Hind*III digested pGEX-2TNMCR.

EHV-1 – GST-Eg63 RING finger truncation.

NcoI

MATVAER*CPICLEDPSNYSMALPCLHAFCYVCITRWIRQNPTCPL*CKVPV 50

ESVVHTIESDSEF

Figure 3.3.1C. *Sequence of the GST-Eg63 RING finger truncation.*

The amino acid sequence of the DNA fragment encoding the RING finger domain truncations of GST-Eg63 (originally 106 a. a. in length) as described previously (Everett *et al.,* 1995). The RING finger domains of GST-Eg63 is marked in red, with the first and last cysteine residues of the domain marked (*).

The PCR amplified DNA products encoding BICP0 1_75 and BICP0 1_90 were cloned into pGEM-T easy (Promega), according to manufacturer's instructions (see figure 3.3.1B). The DNA fragments encoding the GST-BICP0 products and the vector were ligated and transformed into *E.coli* DH5α. Subsequently, colonies were screened by diagnostic restriction enzyme digestions of small-scale plasmid preparations. The resulting plasmids were termed pGEM-BICP0 1_75 and pGEM-BICP0 1_90. Once the identities of the pGEM series of plasmids had been confirmed the DNA fragments encoding BICP0 1_75 and BICP0 1_90 were subcloned, as follows:

The vector pGEM BICP0 1_75 was digested with *Nco*I which cuts at the 5' end and *Hind*III which cuts at the 3' end of the DNA fragment encoding BICP0 1_75, which was then isolated by gel electrophoresis and purified accordingly. The recipient vector pGEX-2TNMCR was digested with *Nco*I and *Hind*III, to allow insertion of the DNA fragment encoding BICP0 1_75. The DNA fragment encoding BICP0 1_90 was also subcloned from pGEM-1_90 into the recipient vector pGEX-2TNMCR, following the same procedure as before.

The DNA fragments encoding the BICP0 truncations and the appropriately digested vector pGEX-2TNMCR were ligated and transformed into *E.coli* DH5α. The resultant colonies were screened by making small-scale plasmid preparations and diagnostic restriction enzyme digestions. The identities of the plasmids harbouring the desired restriction fragments were further confirmed by expression analysis and DNA sequencing. Expression analysis simply involved transfecting the pGEX vectors encoding either of the BICP0 mutants into *E.coli* BL21 and examining the subsequent lysates by SDS-PAGE and Coomassie staining (data not shown). Only isolates positive for the correct recombinant protein expression were further analysed by DNA sequencing.

The Eg63 truncation, Eg63 1_63 had been previously cloned into pGEX-2TNMCR, and its expression verified (Everett *et al.*, 1995b; Everett *et al.*, 1993a). The solution structure of this segment of Eg63 has been determined by NMR, to provide the first characterised structure of a RING finger domain (Barlow *et al.*, 1994). The amino acid sequence of the Eg63 1_63 truncation is shown in figure 3.3.1C.

The individual pGEX series of plasmid expressing the BICP0 and Eg63 truncations will henceforth be referred to as pGEX BICP0 1_75, pGEX BICP0 1_90, pGEX Eg63 1_63 and the expressed proteins as GST-BICP0 1_75, GST-BICP0 1_90 and GST-Eg63 1_63.

3.3.2. Expression and purification of the truncations of GST-BICP0 and GST-Eg63.

The pGEX series of plasmids encoding the GST-BICP0 truncation proteins and GST-Eg63 1_63 were transformed into *E.coli* BL21 and subsequently purified, using the same method as stated in section 3.0.2. However, using this procedure, it was not possible to purify GST-BICP0 1_75, this may have been due to the protein folding incorrectly or being insoluble in the lysis buffer used. Only GST-BICP0 1_90 and GST-Eg63 1_63 were used for further experiments in this section.

Figure 3.3.2A shows SDS-PAGE analysis of purified samples of GST-BICP0 1_90, GST-Eg63 1_63, GST-BICP0 and GST-Eg63. This shows the PCR mutagenesis procedure was successful in reducing the size of the GST-BICP0 and GST-Eg63, as the electrophoretic mobilities of GST-BICP0 1_75 and GST-Eg63 1_63 were comparatively lower. In the resolved protein samples there are several breakdown products. However, there are dominant bands that have an electrophoretic mobilities close to those of the predicted sizes of the intact GST-ICP0 related proteins; these are marked on the figure.

3.3.3. Initial E3 ubiquitin ligase assay to determine the activity of GST-BICP0 1_90 and GST-Eg63 1_63.

The ubiquitin E3 ligase assay was carried out to determine the activity of GST-BICP0 1_90 and GST-Eg63 1_63, using the method detailed in section 2.9B. This was an important prerequisite to future experiments, since truncation of GST-BICP0 and GST-Eg63 may have caused the proteins to fold incorrectly and become inactive. Furthermore, the established E3 ubiquitin ligase assay conditions may not have been optimal for these proteins. The initial ubiquitin E3 ligase assays were carried out using the GST-Eg63 1_63 and GST-BICP0 1_90 in the presence of UbcH5a (see figure 3.1.2A) using conditions optimised for ICP0 (Boutell *et al.*, 2002).

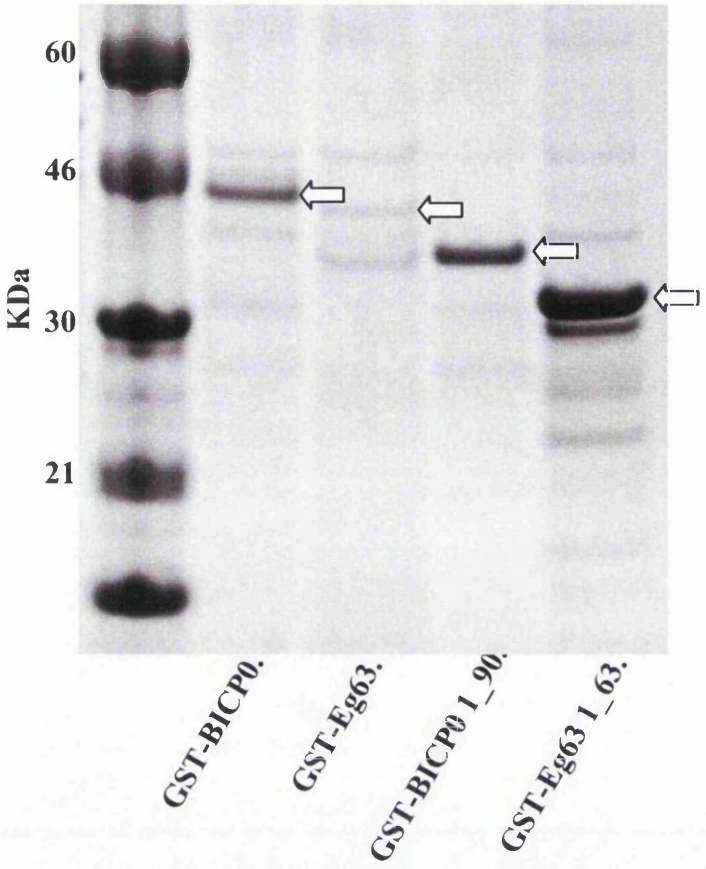
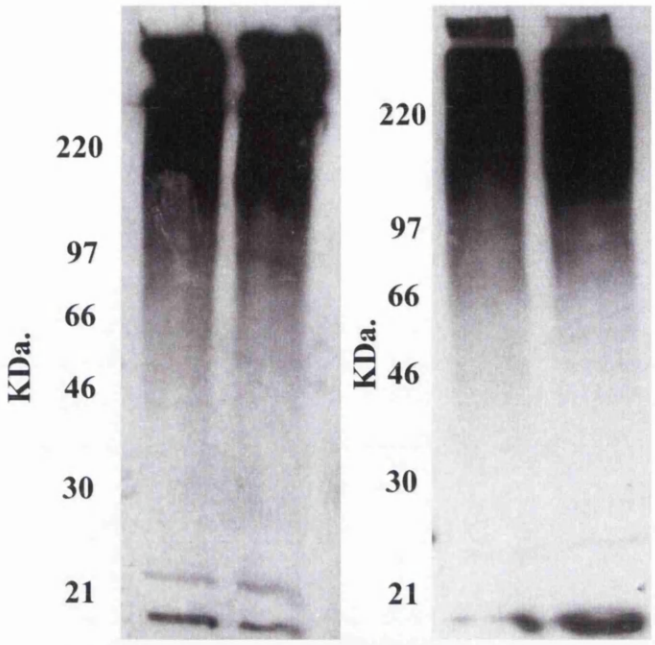


Figure 3.3.2A. *Profile of extracts of the purified RING finger domains GST-BICP0 and GST-Eg63 and their truncated forms.*

Ten microlitres of extract containing GST-RING finger fusion proteins were resolved by SDS-PAGE and visualised by Coomassie staining. Below each lane, it is denoted which extract of purified RING finger domain was resolved. Due to the nature of the purification method, partially degraded or truncated proteins were additionally purified; the arrow in each lane identifies the band that corresponds to the predicted electromobilities of the protein.



	1	*2*		*3*	*4*
E1	+	+		+	+
UbcH5a	+	+		+	+
Ub	+	+		+	+
GST- BICP0	**1 µl**	-		-	-
GST- BICP0 1_90	-	**1 µl**		-	-
GST-Eg63	-	-		**1 µl**	-
GST-Eg63 1_63	-	-		-	**1 µl**

Figure 3.3.3A. *E3 ligase activity of GST-BICP0 1_90 and GST-Eg63 1_63.*

The in vitro ubiquitin ligase assay was carried out as described in section 2.9B. The components of each reaction are denoted in the table. The products of the E3 ligase reactions were resolved by SDS-PAGE and visualised by Western blotting. GST-BICP0 1_90 (Lane 2) and GST-Eg63 1_63 (Lane 4) were assessed for potential E3 ligase activity in the presence of UbcH5a. As GST-BICP0 (lane 1) and GST-Eg63 (lane 3) had been previously shown to stimulate UbcH5a, they were used as positive controls, The blot was probed using anti-ubiquitin antibody P4D1 (1/1000) followed by the secondary hrp-conjugated anti-mouse antibody (1/1000).

Figure 3.3.3A shows that GST-BICP0 (Lane 1) and GST-BICP0 1_90 (Lane 2) both produced polyubiquitin chains in the presence of UbcH5a. Similar results were obtained with GST-Eg63 (Lane 3) and GST-Eg63 1_63 (Lane 4). The results indicated that GST-Eg63 1_63 and GST-BICP0 1_90 were successfully purified and active in the ubiquitin E3 ligase assay. Furthermore, it appears from the Western blots that the levels of polyubiquitin formation by GST-BICP0 and GST-Eg63 were similar to those induced by their respective truncation proteins.

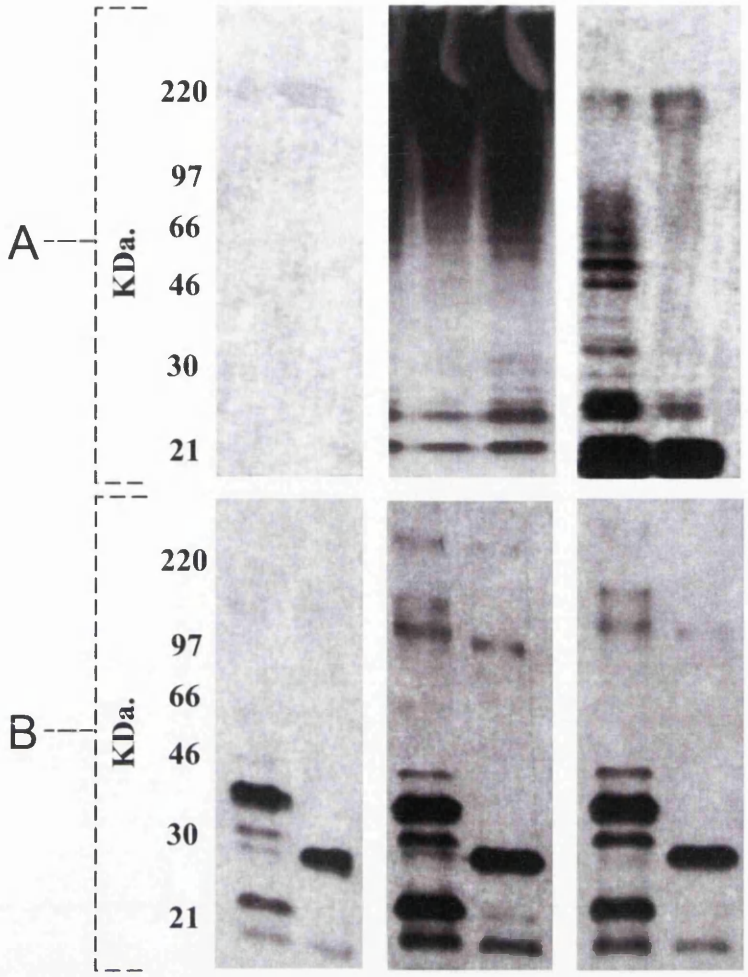
3.3.4. Quantification of GST-BICP0 1_90 and GST-Eg63 1_63.

To ensure equal amounts of the purified GST-BICP0 1_90, GST-Eg63 1_63, GST-BICP0 and GST-Eg63 were used in subsequent E3 ubiquitin ligase assays, densitometric analysis was used to determine the concentration of each preparation (data not shown). The method employed was the same as previously used in section 3.0.3.

3.3.5. Comparison of the E3 ubiquitin ligase activities of GST-BICP0 and GST-Eg63 and their truncated forms.

The E3 ligase assay was carried out as previously described in section 2.9B. The E3 ligase activities of GST-BICP0 1_90, GST-BICP0, GST-Eg63 and GST-Eg63 1_63 were compared in the presence of UbcH5a and UbcH6 (see figure 3.3.5A).

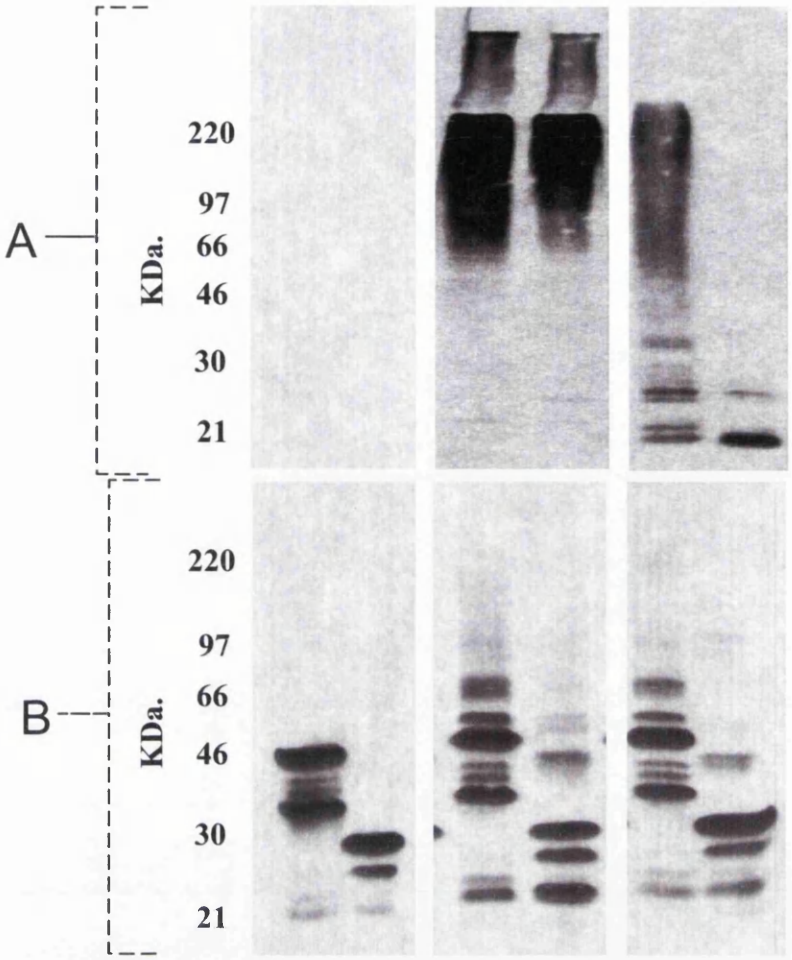
The results from these assays were as follows: Figure 3.3.5A panel A, lane 1 indicates that E1 with GST-BICP0 (Lane 1) or GST-BICP0 1_90 (Lanes 2) does not lead to the formation of polyubiquitin chains alone. However, when E1, ubiquitin and GST-BICP0 (Lanes 3) or GST-BICP0 1_90 (Lanes 4) were incubated in the presence of UbcH5a they facilitated the formation of variable length polyubiquitin chains *in vitro*. Similarly, incubation of E1, ubiquitin and GST-BICP0 (Lanes 5) or GST-BICP0 1_90 (Lanes 6) in the presence of UbcH6, facilitated the formation of variable length polyubiquitin chains *in vitro*. The data also shows (Figure 3.3.5A, panel B) that when the assay was carried out using equivalent amounts of GST-BICP0 (Lane 3) and GST-BICP0 1_90 (Lanes 4) both proteins possessed similar intrinsic E3 ligase activities in the presence of UbcH5a (Figure 3.3.5A, panel A). Furthermore, GST-BICP0 (Lane 5) and GST-BICP0 1_90



	1	*2*	*3*	*4*	*5*	*6*
E1	+	+	+	+	+	+
UbcH5a	-	-	+	+	-	-
UbcH6	-	-	-	-	+	+
Ub	+	+	+	+	+	+
BICP0	+	-	+	-	+	-
BICP0 1_90	-	+	-	+	-	+

Figure 3.3.5A. *Comparison of the E3 ligase activities of the GST-BICP0 and GST-BICP0 1_90 RING finger domains, in the presence of UbcH5a and UbcH6.*

The in vitro ubiquination assay was carried out as described in section 2.9B. The components of each reaction are denoted in the table. The products of E3 ligase reactions were resolved by SDS-PAGE and visualised by Western blotting. **Blot A**: the RING finger domain proteins GST-BICP0 and GST-BICP0 1_90 were added in equal amounts into the E3 ligase assay, to compare their ability to cause the formation of polyubiquitin chains in the presence of UbcH5a (lanes 3 and 4) and UbcH6 (lanes 5 and 6). Lane 1 and 2 = Control, no E2 was present in these reactions. **Blot B** : Lanes 1 – 6, shows that comparable amounts of the RING finger domain proteins GST-BICP0 and GST-BICP0 1_90 were used in the E3 ligase reactions. Blot A was probed with α-Ub at 1/1000 followed by the secondary antibody hrp-conjugated anti-mouse antibody 1/1000. Blot B was probed with anti-GST (1/1000) followed by secondary hrp-conjugated anti-rabbit (1/1000) antibody.



	1	2	3	4	5	6
E1	+	+	+	+	+	+
UbcH5a	-	-	+	+	-	-
UbcH6	-	-	-	-	+	+
Ub	+	+	+	+	+	+
Eg63	+	-	+	-	+	-
Eg63 1_63	-	+	-	+	-	+

Figure 3.3.5B. *Comparison of the E3 ligase activities of the RING finger domain GST-Eg63 and GST-Eg63 1_63, in the presence of UbcH5a and UbcH6.*

Blot A: the RING finger domain proteins GST-Eg63 and GST-Eg63 1_90 were added in equal amounts into the E3 ligase assay, to compare there ability to cause the formation of polyubiquitin chains in the presence of UbcH5a (lanes 3 and 4) and UbcH6 (lanes 5 and 6). Lane 1 and 2 = Control, no E2 was present in these reactions. **Blot B** : Lanes 1 – 6, show that comparable amounts of the RING finger domain proteins GST-Eg63 and GST-Eg63 1_90 were used in the E3 ligase reactions. This set of experiments was carried out using identical conditions to those outlined in the previous figure.

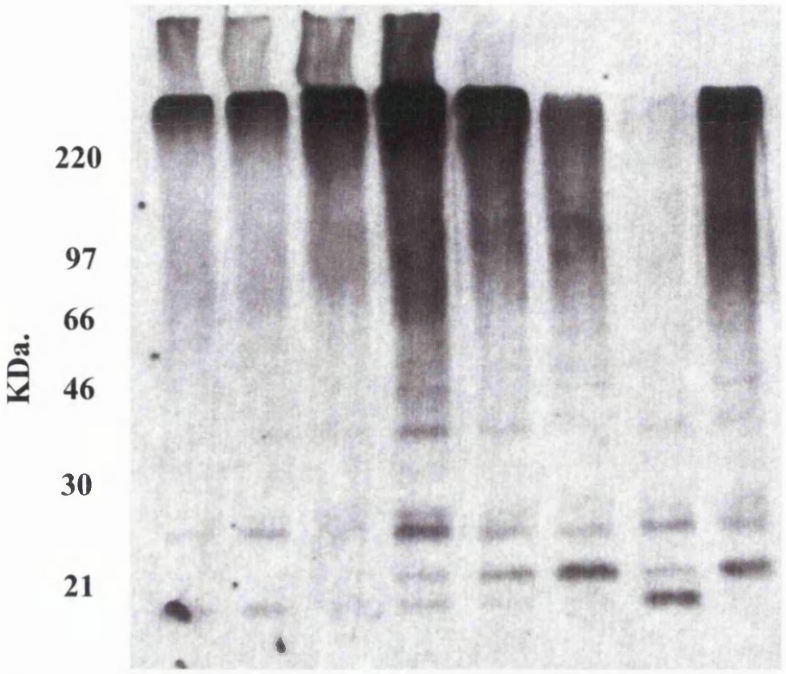
(Lane 6) both cause the formation of polyubiquitin chains in the presence of UbcH6, but significantly the intrinsic activity of GST-BICP0 1_90 appears be less than that of GST-BICP0.

The same sets of experiments were carried out with GST-Eg63 and GST-Eg63 1_63 (Figure 3.3.5B), the results were as follows: Figure 3.3.5B panel A, lane 1 indicates that incubation of E1 with GST-Eg63 (Lane 1) and Eg63 1_63 (Lanes 2) did not lead to the formation of polyubiquitin chains. However, when E1 ubiquitin and GST-Eg63 (Lane 3) and GST- Eg63 1_63 (Lane 4) were incubated in the presence of UbcH5a, formation of similar amounts of polyubiquitin chains *in vitro* occurred. When E1, ubiquitin and GST-Eg63 (Lane 5) were incubated in the presence of UbcH6, GST-Eg63 facilitated the formation of variable length polyubiquitin chains *in vitro*, however, under the same assay conditions Eg63 1_63 (Lane 6) could not. The data shows when GST-Eg63 (Lane 3) and GST-Eg63 1_63 (Lane 4) were used at equivalent concentrations (Figure 3.3.5B panel B) they exhibit similar intrinsic E3 ligase activity in the presence of UbcH5a. However, under the same assay conditions GST-Eg63 1_63 (Lane 6), unlike GST-Eg63 (Lane 5) has little or no ability to cause the formation of polyubiquitin chains in the presence of UbcH6.

The data suggests the removal of 43 residues from the C terminus of GST-Eg63 to create GST-Eg63 1_63 caused the near abrogation of the truncated protein's ability to stimulate UbcH6 and prevented the formation of polyubiquitin chains *in vitro.* However, both proteins retained the ability to cause similar levels of polyubiquitin formation in the presence of UbcH5a. Similarly, the removal of 20 a.a. from the C terminus of GST-BICP0 to create GST-BICP0 1_90 caused a significant reduction in the protein's ability to facilitate the formation of polyubiquitin chains in the presence of UbcH6. Collectively the data suggests that residues that flank the C terminal sides of the RING finger domains of BICP0 and Eg63 are essential for the stimulation of UbcH6, but not UbcH5a.

3.3.6 Inhibition assay to characterise the loss of the E3 activity of GST-Eg63.

The inability of Eg63 1_63 to stimulate UbcH6 may have been due to its failure to bind to the E2; in other words, residues on the C-terminal side of the core RING domain may



	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*
E1	+	+	+	+	+	+	+	+
Ub	+	+	+	+	+	+	+	+
UbcH6	+	+	+	+	+	+	+	+
Eg63 (ng)	**30**	**30**	**30**	**30**	**30**	**30**	**-**	**60**
Eg63 1_63 (ng)	**15**	**30**	**60**	**-**	**-**	**-**	**60**	**-**
Eg63 (ng)	**-**	**-**	**-**	**15**	**30**	**60**	**-**	**-**

Figure 3.3.6A. *Characterisation of the E3 ligase activity of the titrated RING finger domains of GST-Eg63 and GST-Eg63 1_63 in the presence UbcH6.*

The in vitro ubiquination assay was carried out as described in section 2.9B. The GST-Eg63 and GST-Eg63 1_63 RING finger domains were titrated into each reaction. The table denotes the combination and amount of each protein used. In lanes 1-7, 30 ng of the RING finger domain of GST-Eg63 were present per reaction and in lanes 1-3 GST-Eg63 1_63 was present from 15 – 60 ng. In lane 4 – 6 additional RING finger domain GST-Eg63 was present, from 15 – 60 ng. Lane 7 and 8 = Control reactions only GST-Eg63 1_63 and GST-Eg63 were present. The E3 ligase reactions were resolved by SDS-PAGE and visualised by Western blotting. The blot was probed with α-Ub at 1/1000 followed by secondary hrp-conjugated anti-mouse antibody 1/1000.

play an essential part in its interaction with UbcH6. Alternatively, the two proteins might interact but in a non-productive manner. In the latter case, Eg63 1_63 might act as a competitive inhibitor of the reaction in the presence of longer versions of the Eg63 RING domain by sequestering UbcH6 protein. To address this possibility, the ubiquitin E3 ligase assay was performed with UbcH6 and varying amounts of GST-Eg63 and GST-Eg63 1_63. Thus, if GST-Eg63 1_63 sequestration of the E2 is occurring, as its concentration increases the formation of polyubiquitin induced by GST-Eg63 should decrease. The same conditions were used as those previously outlined in the materials and methods, section 2.9B.

The results of the Eg63 1_63 inhibition assay are shown in figure 3.3.6A. In the samples analysed in lanes 1-6 and 8, 30 ng of GST-Eg63 was present. In lanes 1 – 3, GST-Eg63 1_63 was titrated into the reaction in amounts increasing from 15 – 60 ng. The results indicate that increasing the amount of GST-Eg63 1_63 did not decrease the level of polyubiquitin formed; in fact the levels remain relatively uniform. In lanes 4 – 6 as a control, additional GST-Eg63 was titrated into the reaction and caused an increase in polyubiquitin formation, compared to lanes 1 - 3, in the presence of UbcH5a. A slight decrease in the levels of polyubiquitin in lanes 5 and 6 was observed, most likely due to the presence of an inhibitory substance in this sample of purified protein. Lane 7 and 8 were control reactions containing only GST-Eg63 1_63 and GST-Eg63, respectively.

3.3.7. Conclusions.

The results from this section have shown that deletion of regions located on the C-terminal sides of the core RING finger domains of GST-BICP0 and GST-Eg63 affect their ability to stimulate the formation of polyubiquitin chains in the presence of UbcH6, but not UbcH5a. In the presence of UbcH6, GST-BICP0 and GST-Eg63 caused a greater degree of polyubiquitin formation than GST-BICP0 1_90, which in turn had a greater affect than GST-Eg63 1_63. This pattern of decreasing activity would suggest that larger the C-terminal deletion, the greater the abrogation of the RING finger domain proteins' ability to stimulate UbcH6. Collectively, this data suggests that within the deleted 40 a.a. of GST-Eg63 and the 20 a.a. of GST-BICP0, there are regions essential for the formation of polyubiquitin in the presence of UbcH6 (see figure 3.3.7A).

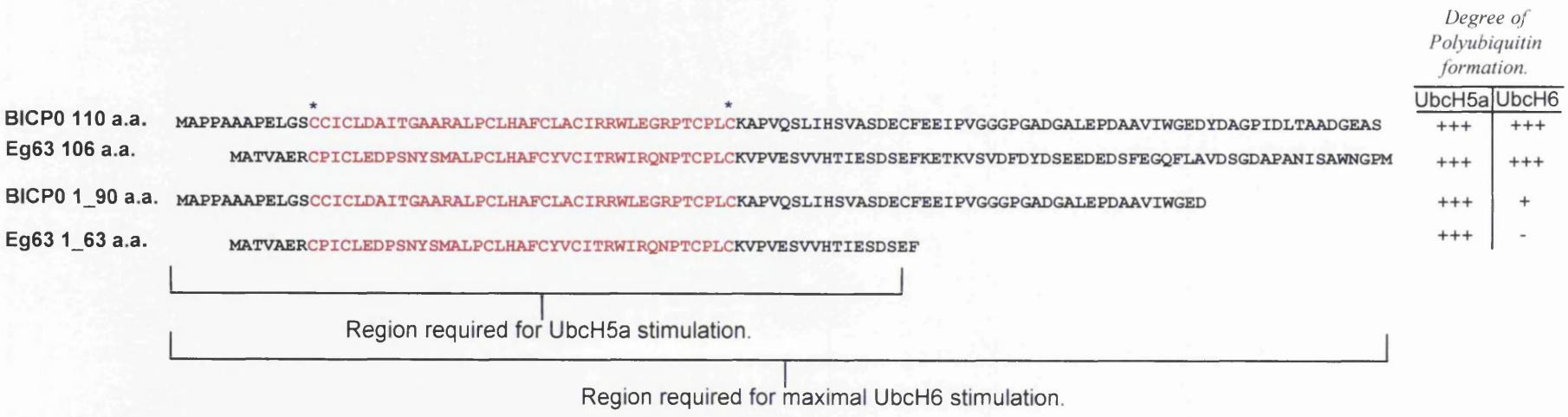


Figure 3.3.7A. *Regions of the ICP0-related proteins essential for the stimulation of UbcH5a and UbcH6.*

The ICP0-related proteins are aligned according to the relative position of their RING finger domains, the first and last cysteine are indicated (*). The table indicates the relative level of polyubiquitin formation of the ICP0-related proteins in the presence of the E2 enzymes UbcH5a and UbcH6a under identical conditions.

Furthermore, these results suggest that these same sequences of GST-Eg63 and GST-BICP0 are not essential for the formation of polyubiquitin in the presence of UbcH5a.

The RING finger domains of E3 ubiquitin ligase enzymes have previously been shown to be the hub of their activity and their disruption causes reduction or abrogation of the proteins' ability to function. This is discussed in detail in the Introduction and also reviewed in detail elsewhere (Freemont, 2000; Joazeiro & Weissman, 2000; Pickart, 2001a). Furthermore, mutations between the boundary of the first and last cysteine residues of the RING finger domain of ICP0 have been shown to reduce or abrogate its ability to facilitate the formation of polyubiquitin in the presence of UbcH5a (Boutell *et al.*, 2002). Previously, it was proposed the ability of ICP0 to stimulate both UbcH5a and UbcH6 was a result of its interaction with conserved residues or an area of structural homology between the two E2 proteins (Boutell *et al.*, 2002). This hypothesis is still valid, but despite the structural homology of UbcH5a and UbcH6, the results presented here indicate that more than one region of the ICP0 related proteins are required for stimulating UbcH5a and UbcH6.

Research carried out on other E3 ubiquitin ligase enzymes has shown that the RING finger domain is largely responsible for facilitating the formation of polyubiquitin in the presence of specific E2 partners. Therefore, it is likely that the RING finger domains of GST-Eg63 and GST-BICP0 were responsible for specific E3 ligase activity in the presence of UbcH5a and UbcH6. Furthermore, the truncated RING finger domain of GST-Eg63 1_63 is sufficient for E3 ligase activity in the presence of UbcH5a. The study has also shown that regions located on the C-terminal sides of the core RING finger domains of GST-BICP0 and GST-Eg63 are additionally required for the stimulation of UbcH6. However, from the available data it is not possible to conclude why these regions are essential. There is little sequence homology outside of the RING finger domain of the ICP0 related proteins, therefore it is not possible to conclude if the loss of activity was due to deletion of a conserved motif or residues. It is most likely that C-terminal deletions of GST-BICP0 and GST-Eg63 1_63 may have caused the proteins to adopt structural configurations, which prevent efficient stimulation of UbcH6, but not UbcH5a. In conclusion, the data from this study shows that GST-BICP0 and GST-Eg63 can facilitate the formation of polyubiquitin chains *in vitro*, but the mechanism used to stimulate UbcH5a differs in detail from that with UbcH6.

Chapter 3 – Part III - Autoubiquitination of the ICP0 related proteins.

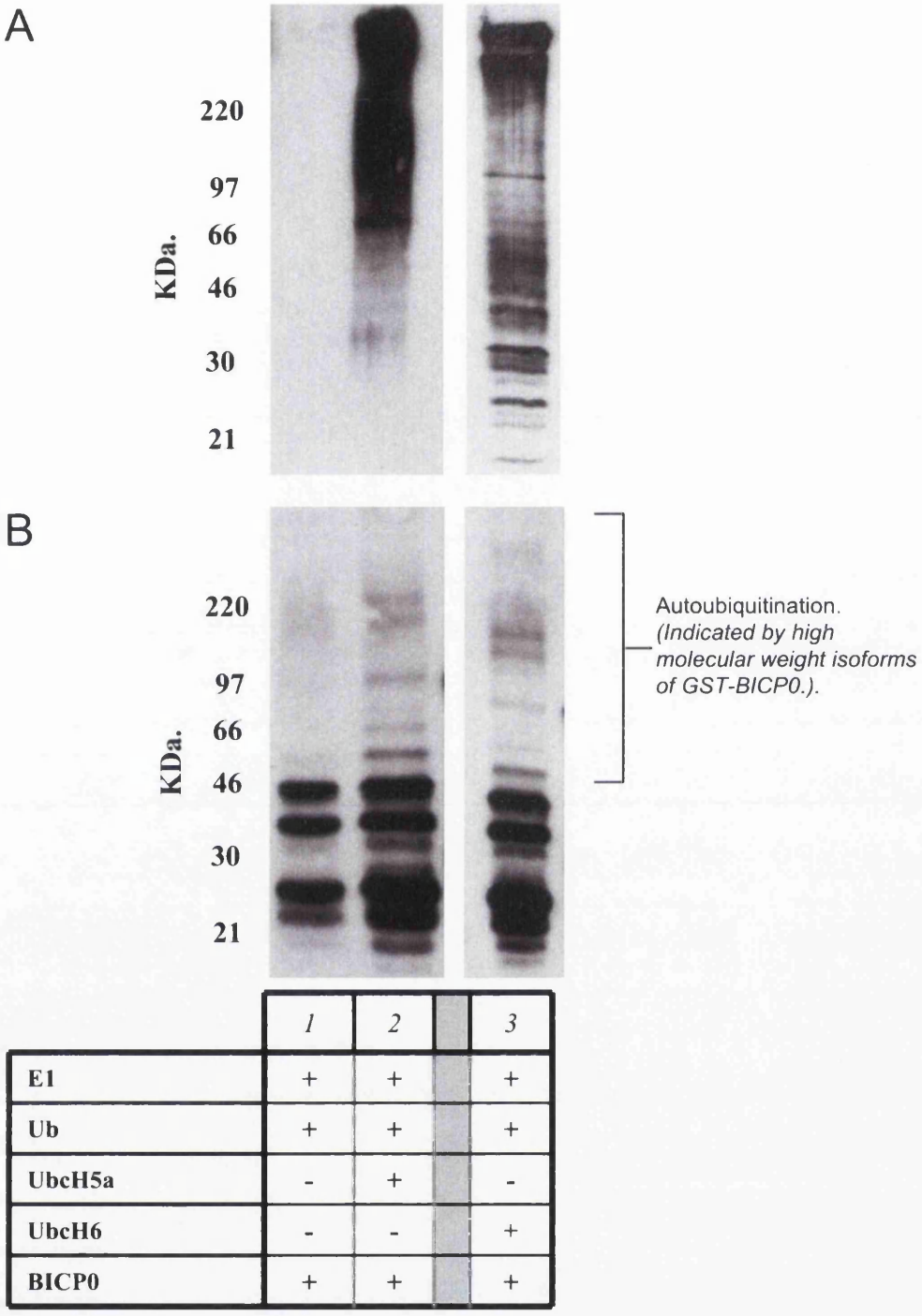
3.4.1. Introduction.

Experiments described in this section were performed in order to highlight the ability of the ICP0 related proteins to autoubiquitinate themselves *in vitro*. Autoubiquitination is a common occurrence amongst RING finger E3 ligases, where the RING finger or the fusion partner acts as a proxy substrate. ICP0 has been shown to autoubiquitinate *in vitro* (Boutell *et al.*, 2002) and *in vivo* (Canning *et al.*, 2004). In the latter case, it is targeted for proteasome-mediated degradation, promoting its own turnover. In contrast, ICP0 also promotes its own stability by interacting with USP7 (ubiquitin-specific protease 7), a member of a family of cellular enzymes that cleave ubiquitin from either alpha or isopeptide-linked chains (Everett *et al.*, 1997). The ICP0-USP7 interaction was shown to be essential in preventing autoubiquitination and the subsequent proteolysis of ICP0 *in vivo* (Canning *et al.*, 2004). Mutational studies have shown that if the ICP0-USP7 interaction is prevented, the ability of ICP0 to function in gene expression and viral growth assays is greatly reduced (Everett *et al.*, 1999d).

The ICP0 related proteins used in this study do not contain a USP7 binding motif, despite the importance of ICP0 interaction with USP7. However, the GST-ICP0 related proteins do autoubiquitinate themselves or their fusion partner *in vitro*. The following section refers to figures therein and elsewhere in this chapter, highlighting examples of autoubiquitination of the GST-ICP0 related proteins in the E3 ubiquitin ligase assays.

3.4.2. Autoubiquitination of the GST-ICP0 related proteins.

The ubiquitin E3 ligase assay was carried out using GST-BICP0 in the presence of UbcH5a and UbcH6, according to the method described previously (see section 2.9B). GST-BICP0 was used here since the autoubiquitinated isoforms of BICP0 were the most easily identifiable of the GST-ICP0 related proteins.



	1	2		3
E1	+	+		+
Ub	+	+		+
UbcH5a	-	+		-
UbcH6	-	-		+
BICP0	+	+		+

Figure 3.4.2A. *Autoubiquitination of GST-BICP0 1_110 during the E3 ligase activity.*

In vitro ubiquination assay were carried out as described in section 2.9B. The components of each reaction are denoted in the table. The products of the E3 ligase reactions were resolved by SDS-PAGE analysis and visualised by Western blotting. The RING finger domain protein of GST-BICP0 was titrated into the assay to assess levels of autoubiquitination resulting from the formation of polyubiquitin in the presence of UbcH5a and UbcH6. **Blot A:** In lanes 2 and 3, the RING finger domain of GST-BICP0 stimulate UbcH5a and UbcH6, respectively. **Blot B:** The reprobed membrane shows higher molecular weight autoubiquitinated isoforms of the RING finger domain of GST-BICP0 are present when polyubiquitin formation occurs in the presence of UbcH5a (lane 2) and UbcH6 (Lane 3). When no E2 was present in the reaction mix the ubiquitinated isoforms of the RING finger domain of GST-BICP0 are not present. Blot A was probed with α-Ub at 1/1000 followed by the secondary antibody hrp-conjugated anti-mouse antibody 1/1000. Blot B was probed with anti-GST (1/1000) and secondary hrp-conjugated anti-rabbit (1/1000).

Similar to previous findings, the results from these assays were as follows: figure 3.4.2A (panel A), lane 1 indicates that E1 and GST-BICP0 cannot form polyubiquitin chains alone. However, when E1, ubiquitin and GST-BICP0 were incubated in the presence of UbcH5a (lane 2) and UbcH6 (lane 3), it stimulated the E2 enzymes to form variable length polyubiquitin chains *in vitro*. In figure 3.4.2A (panel B) it is possible to see that GST-BICP0 causes polyubiquitin chain formation in the presence of UbcH5a (lane 2) and UbcH6 (lane 3), where upon it becomes autoubiquitinated. It is possible to identify autoubiquitination due to the presence of multiple high molecular weight isoforms of GST-BICP0, present in lanes 2 and 3, but not in lane 1 (marked on figure 3.4.2A). Notably in the E3 ligase assay the pattern of autoubiquitinated GST-BICP0 is markedly similar in presence of UbcH5a and UbcH6, despite different E2s being involved in the reaction.

Similar results can be seen in figure 3.3.5A (panel B) (see relevant section for specific experimental details). GST-BICP0 and GST-BICP0 1_90 both undergo autoubiquitination in the presence of UbcH5a (Lanes 3 and 4) and UbcH6 (Lanes 5 and 6). Figure 3.3.5B (panel B) also shows that GST-Eg63 and GST-Eg63 1_63 both undergo autoubiquitination in the presence of UbcH5a (Lanes 3 and 4) and UbcH6 (Lanes 5 and 6). However, the patterns of high molecular weight autoubiquitinated species of GST-Eg63 1_63 are different in the presence of UbcH6 (Lane 5) and UbcH5a (Lane 6). The decrease in high molecular weight species of autoubiquitinated GST-Eg63 1_63 may be a reflection of the protein's decreased E3 ligase activity in the presence of UbcH6.

3.4.3. Conclusions.

The proteins GST-BICP0, GST-BICP0 1_90, GST-Eg63, GST-Eg63 1_63 all under go varying degrees of autoubiquitination when either UbcH5a or UbcH6 are present in the E3 ligase assay. Single or multiple lysine residues within the RING finger domains or that of the GST moiety may be targeted for ubiquitination. The results in Figure 3.3.5B suggest that GST-Eg63 1_63 undergoes a lower level of autoubiquitination in the presence of UbcH6, than that of UbcH5a; probably a consequence of the protein's abrogated ability to form polyubiquitinated chains in the presence of UbcH6.

If similar to ICP0, autoubiquitination of the ICP0 related proteins has an important *in vivo* significance. These proteins would therefore make interesting targets for future research. Unlike ICP0, the ICP0 related proteins do not contain a USP7 binding site, and therefore may use a different ubiquitin specific protease or mechanisms of self-regulation to prevent their proteolysis *in vivo*. Furthermore, the majority of the ICP0 related proteins contain one conserved lysine residue within their RING finger domains that is not shared with ICP0. It has been shown that ICP0 RING finger domain contains two lysines near to one another and it is thought that either or both of these residues are targeted for autoubiquitination (Canning *et al.*, 2004). Therefore, mutation of the conserved lysine residue of the RING finger domains of ICP0 related proteins could help further elucidate the significance of autoubiquitination in the ICP0 family of proteins.

Chapter 3 – Part IV - Physical interactions between the GST-ICP0 related proteins and E2 enzymes.

3.5.0. Introduction.

The aim of the experiments described in this section was to determine if the ICP0 related RING finger domain proteins form stable interactions with UbcH5a and UbcH6 *in vitro*. In general it is widely accepted that the interaction of the E2 ubiquitin-conjugating enzymes with the RING finger domain plays a central role in mediating the transfer of ubiquitin to substrate proteins (Freemont, 2000; Joazeiro & Weissman, 2000). In agreement, the majority of characterised E3 ligases have been shown to form stable interactions via their RING finger domains with their E2 partners, for example c-Cbl and the E2, UbcH7 (Zheng *et al.*, 2000); AO7 and the E2, UbcH5 (Lorick *et al.*, 1999); HHARI and the E2s, UbcH7 and UbcH8 (Moynihan *et al.*, 1999). However, occasionally E2-E3 complex interactions can also occur with sequences outside of the RING finger domain, such as in the case of the Ubc2p-Ubr1p interaction. It was shown that the RING domain of the E3 Ubr1p was necessary for polyubiquitination, but not for interaction with the E2 Ubc2p (Xie & Varshavsky, 1999). However, research carried out previously showed that despite clear data indicating that ICP0 stimulates UbcH5a, and UbcH6 it was not possible identify a stable interaction between the proteins (Boutell *et al.*, 2002).

It was decided to use GST-pull down assays in an attempt to identify an interaction between the ICP0 RING finger domain proteins and UbcH5a and UbcH6. Bacterial extracts containing either HIS linked UbcH5a, UbcH6 or cdc34 were mixed with glutathione beads that had been previously charged with one of the GST-ICP0 related proteins (see Chapter 3, section I for further details) and incubated at 37°C for a short period of time. The beads were then isolated and the coprecipitated proteins were eluted and analysed by SDS-PAGE and Western blotting (specific experimental details can be found in section 2.9C). The E2 enzyme cdc34 was additionally used in the assays, as it has been reported that it is stimulated to form autoubiquitinated products by residues

within the C-terminal third of ICP0 *in vitro* (Van Sant *et al.*, 2001a). Furthermore, it was also suggested that cdc34 forms a stable interaction with the RING finger domain of ICP0 (Van Sant *et al.*, 2001a). However, a report published elsewhere has shown that neither full-length ICP0 nor its isolated RING finger domain was able to induce the formation of polyubiquitin chains in the presence of cdc34 (Boutell *et al.*, 2002). The series of experiments described in this section will also analyse if GST-ICP0 (241) or any of the GST-ICP0 related proteins form stable interactions via their RING finger domains with cdc34.

3.5.1. The GST-pull down assay.

Initially, bacterial extracts were made containing the GST-ICP0 related proteins or HIS tagged UbcH5a, UbcH6 and cdc34, as follows:

The pGEX series of plasmids encoding the GST-ICP0 related proteins and the pET24a series of plasmid encoding UbcH5a, UbcH6, or cdc34 (Boutell *et al.*, 2002) were transformed into *E.coli* BL21. Several (5 to 10) bacterial colonies were pooled and cultured in YTB (supplemented with ampicillin or kanamycin, as appropriate) until mid-log phase, whereupon IPTG was added to induce expression of the recombinant protein. Incubation was then continued for a further 3-5 hours at 28°C. The bacteria were then pelleted and resuspended in lysis buffer and subjected to sonication. The lysis buffer EP0 265 (100 mM Tris-HCl pH 7.5, 150 mM NaCl, 10% Glycerol, 0.1% NP40, and 20 mM β-mercaptoethanol) was again used. The bacterial extract was clarified by high-speed centrifugation and stored appropriately (see section 2.10 for further details).

The soluble extracts containing bacterially expressed members of the GST-ICP0 related proteins were mixed with glutathione-agarose beads, then the beads with bound fusion proteins were harvested by centrifugation and washed extensively to remove unbound proteins. Beads charged with GST alone were prepared to pre-clear extracts containing the E2 enzymes and to serve as a control. Bacterial extracts containing expressed HIS-UbcH5a, HIS-UbcH6 or HIS-cdc34 were then pre-treated with beads linked to GST to deplete from the extract proteins that might bind non-specifically to the beads, then the supernatants from these incubations were mixed with beads charged with the GST-

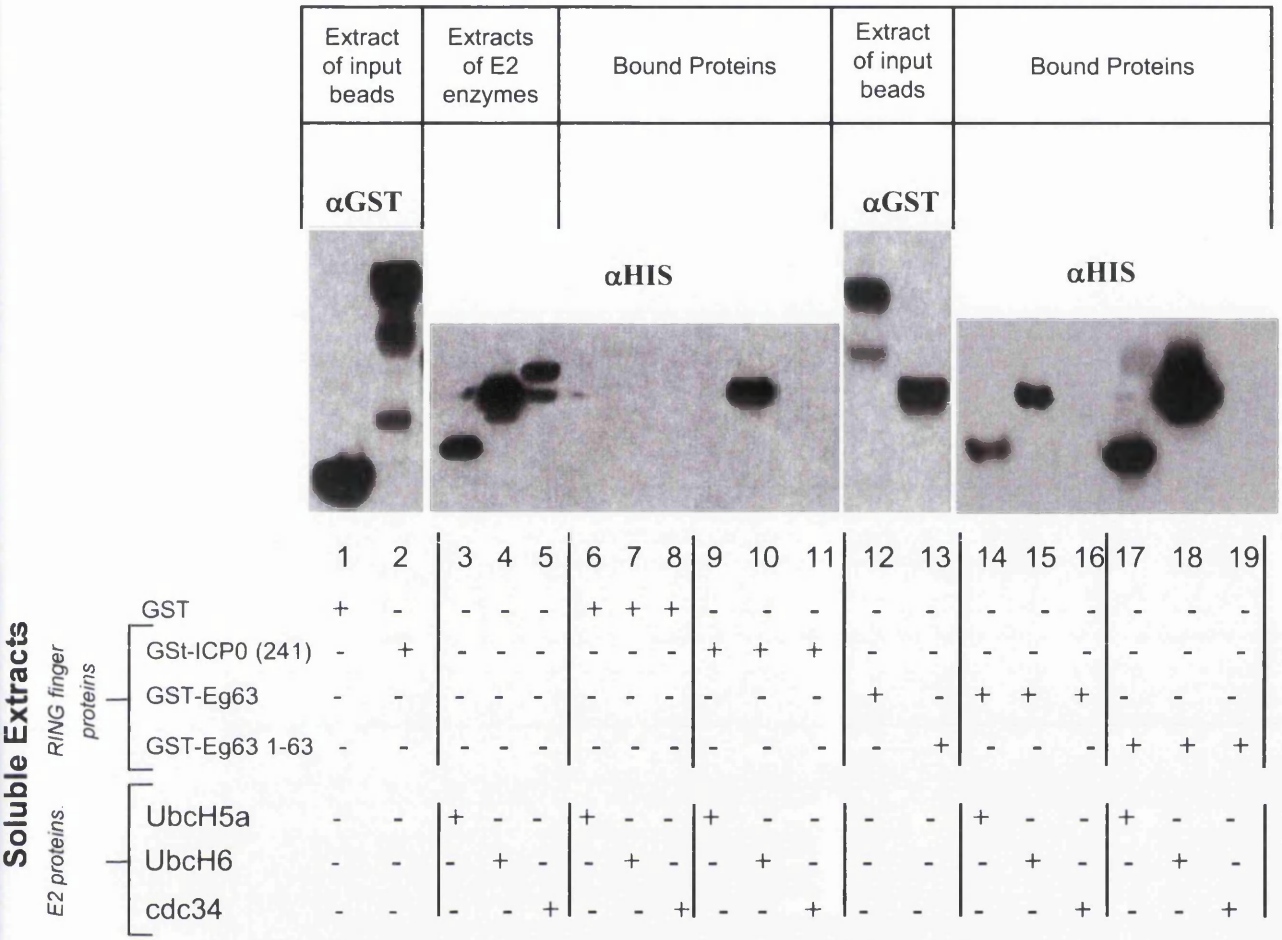


Figure 3.5.1A. *GST-pull down assay to determine if the RING finger domain proteins form stable complexes with selected E2 enzymes* in vitro.

The GST-pull down assays were carried out according to the method stated in section 2.9C. Pre-cleared extracts containing either HIS-UbcH5a, HIS-UbcH6 and HIS-cdc34, were incubated with glutathione beads, charged with GST, GST-ICP0 (241), GST-Eg63 or GST-Eg63 1_63. The combination of proteins that were incubated together is shown in the table. The mixtures were washed and the co-precipitated proteins were eluted and then analyzed by SDS-PAGE followed by Western Blotting. The antibodies used to identify the proteins is stated above each of the blots and were used at the following concentrations: anti-HIS used at 1/750 followed by the secondary antibody hrp-conjugated anti-mouse 1/1000 anti-GST at 1/1000; followed by the secondary antibody hrp-conjugated anti-rabbit at 1/1000.

RING finger fusion proteins. The mixtures were subsequently analyzed for co-precipitated proteins by SDS-PAGE and Western blotting. Specific experimental details can be found in section 2.9C. To ensure accuracy in the results obtained the interaction conditions used were highly stringent, in particular, the bound proteins were eluted from the beads with reduced glutathione before analysis, rather than simply analysing the proteins present in the bead pellets. The results were as follows:

The results of GST-pull down assays to determine if GST, GST-241, GST-Eg63 and GST-Eg63 1_63 formed stable interactions with UbcH5a, UbcH6 or cdc34 are shown in figure 3.5.1A. Lanes 1 and 2 show samples of the GST and GST-ICP0 (241) bead preparations used in the subsequent stages of the assay. Lanes 3 - 5 are positive controls, showing UbcH5a, UbcH6 and cdc34, respectively in the bacterial extracts used for the interaction assays. Lanes 12 and 13 show samples of the GST-Eg63 and GST-Eg63 1_63 beads. Lanes 6 - 11 contain eluted proteins from the interaction assays using GST and GST-ICP0 (241) beads. The results indicate that GST does not form a stable interaction with UbcH5a, UbcH6 or cdc34 (lanes 6 – 8). However, from lanes 9 - 11 it is possible to observe that GST-ICP0 (241) forms a stable interaction with UbcH6, but not UbcH5a or cdc34 under the conditions employed. Additionally it can be observed that GST-Eg63 (lanes 14-16) and GST-Eg63 1_63 (lanes 17-19) form stable interactions with UbcH5a and UbcH6, but not cdc34.

Figure 3.5.1B is a continuation from the previous set of results. Lanes 1, 2, 9 and 10 show samples of the GST-BICP0 1_90, GST-BICP0, GST-EP0, and GST-Vg61 beads used in the pull-down experiments, respectively. Lanes 3 - 5 indicate that BICP0 1_90 forms a stable interaction with UbcH6, but not UbcH5a or cdc34. Identical results were also observed with GST-BICP0 (Lane 7), GST-EP0 (Lane 12) and GST-Vg61 (Lane 15).

3.5.2. Conclusions from the interaction assays.

The data shows that all of the GST-ICP0 related proteins form stable interactions with either or both of their E2 partners, UbcH5a and UbcH6. However, GST-ICP0 (241) and none of the GST-ICP0 related proteins formed stable interactions with cdc34. This

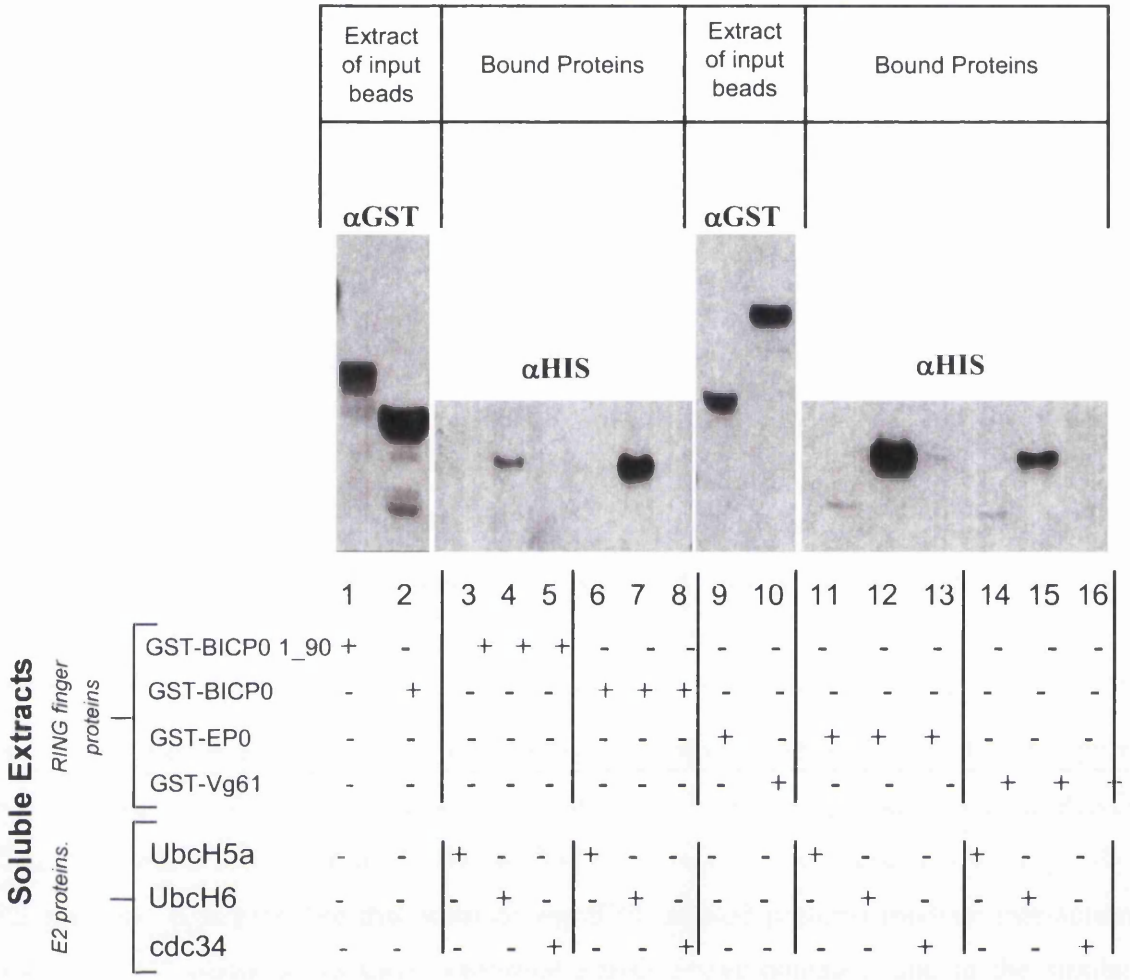


Figure 3.5.1B. *GST-pull down assay to determine if the RING finger domain proteins form stable complexes with selected E2 enzymes in vitro.*

The GST-pull down assays were carried out according to the method stated in section 2.9C. Pre-cleared extracts containing either HIS-UbcH5a, HIS-UbcH6 and HIS-cdc34, were incubated with glutathione beads, charged GST-BICP0 1_90, GST-BICP0, GST-EP0 or GST-Vg61. The reactions were carried out according to the method described in the previous figure.

contradicts data that what was previously published, which suggested the RING finger domain of ICP0 forms a stable interaction with cdc34 (Van Sant *et al.*, 2001a).

The levels of interaction with UbcH6 varied between the GST-ICP0 related proteins, from using a basic pull down assay it is not possible to interpret if this was significant or not, as it may be down to minor experimental variables. Out of all of the GST-ICP0 related proteins only GST-Eg63 1_63 and GST-Eg63 formed strong stable interactions with UbcH5a, this may have again been due to minor experimental variables. However, it is probable that all of the GST-ICP0 related proteins do form stable interactions with UbcH5a and in future by further optimization of the assay conditions it may be possible to detect these.

Additionally the data showed that GST-Eg63 1_63 and BICP0 1_90 formed stable interactions with UbcH6, despite their reduced ubiquitin ligase activity in its presence. Furthermore, as GST-Eg63 1_63 contains only 20 a.a. from the last cysteine in its RING finger domain to the C-terminal end of the expressed protein fragment (see figure 3.3.1C), this would indicate that like most other E3 ubiquitin ligase enzymes the RING finger domain is more than likely responsible for mediating the interaction with specific E2 partners. It is probable that most of the ICP0 related proteins mediate interaction with their E2 partners via their conserved RING finger domains, due to the similar intrinsic E3 ligase activity and biological properties they share. This has been shown to be the case for other E3 ligases, for example the IAP family of proteins all share a conserved RING finger domain and have all been shown to stimulate and interact with UbcH5a in a similar fashion (Yang & Du, 2004).

Chapter 3 – Part V - Interaction assays between mutant versions of GST-ICP0 (241) and E2 ubiquitin conjugating enzymes.

3.6.1. Introduction.

The RING finger domain plays a central role in mediating interaction between E3 ubiquitin ligase enzymes with the required E2 ubiquitin-conjugating enzyme, and in mediating the transfer of ubiquitin to substrate proteins (Freemont, 2000; Joazeiro & Weissman, 2000). As the RING finger domain is the hub of an E3 ligases activity, mutation within this region has generally been shown to be very deleterious for the ability of varied E3 enzymes to interact with their E2 partners and to stimulate the formation of polyubiquitin chains in *vitro* (Pickart, 2001a). In the case of ICP0-241 it was shown that insertion of 4 residues at a.a. 150 and 162, deletion of the RING finger domain or sequence 162-188, abrogated ICP0's ability to act as an E3 ligase in the presence of UbcH5a (Boutell *et al.*, 2002). However, point mutations within the RING finger domain of ICP0-241 (K144E, W146A, Q148E and N151D) and some insertional mutants (*ins*188 and *ins*197) had little or no deleterious affect (Boutell *et al.*, 2002). Even mutation of the conserved tryptophan which is present in the majority of RING finger E3 ligase enzymes and has been shown when mutated to be exceptionally deleterious in other related proteins (Freemont, 2000; Joazeiro & Weissman, 2000; Pickart, 2001a), had little affect on the E3 ligase activity of ICP0-241 (Boutell *et al.*, 2002), or the *in vivo* activity of ICP0 (O'Rourke *et al.*, 1998). However, in contrast to having little affect on the E3 ligase activity of ICP0 (241) the majority of the aforementioned mutations had an extremely deleterious affect on ICP0 ability to simulate gene expression and disrupt ND10 in transfection assays (Everett *et al.*, 1995a; Everett, 1987). The following series of experiments were designed to determine if the ICP0-241 series of mutations (see figure 3.6.1A) were able to interact with UbcH5a or UbcH6.

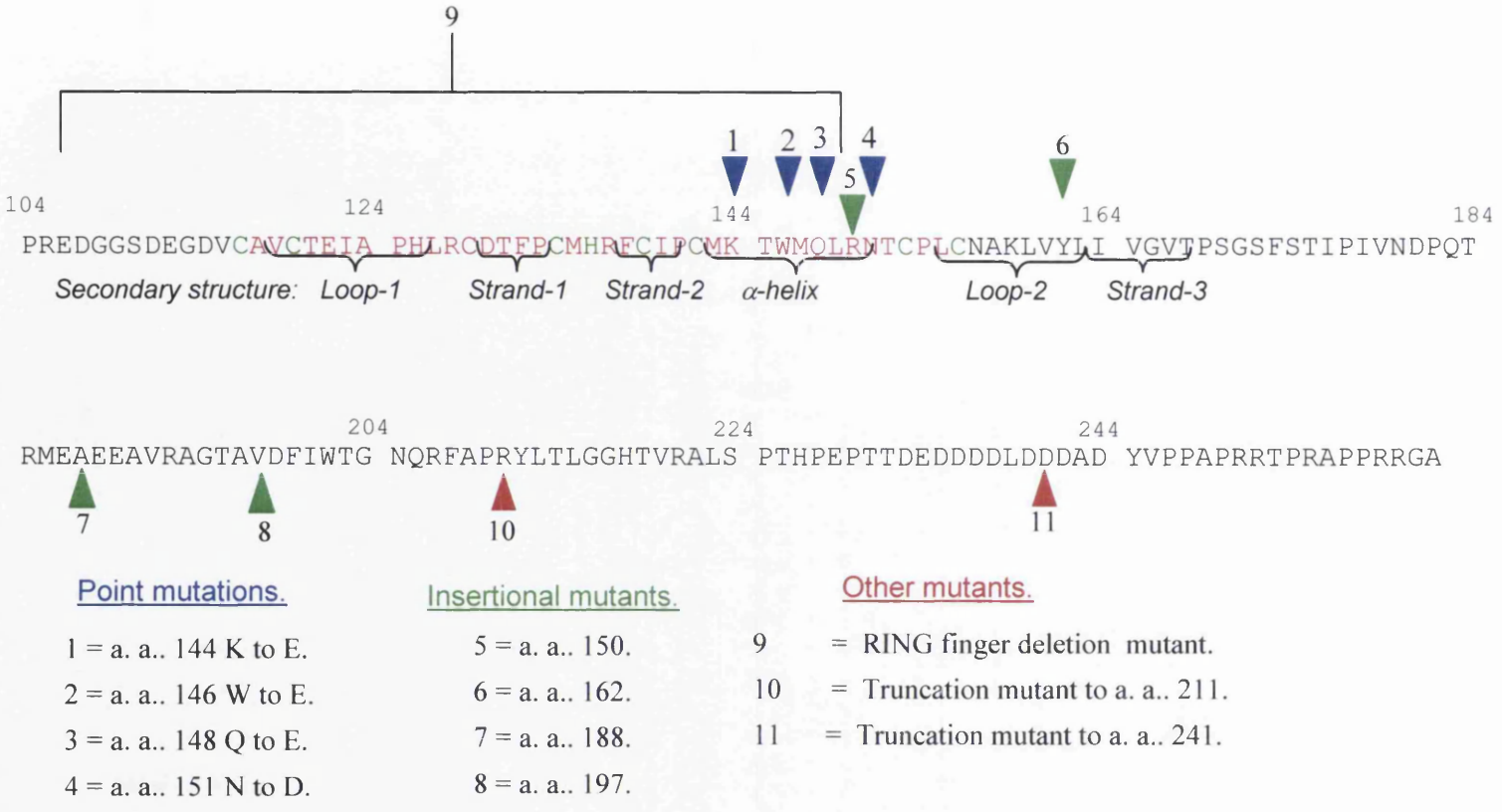


Figure 3.6.1A. *An overview of the ICP0 241 series of mutants.*

The amino acid sequence of the ICP0 truncation mutant 262 is shown (from a. a. 104). The specific mutations shown were made in the back ground of ICP0 (241), their relative positions are marked on the sequence. The key secondary structure features of the RING finger domain of ICP0 241 are shown in italics as determined by NMR analyses of the RING finger domain of the ICP0 homologue Eg63 (Barlow *et al.*, 1994), see figure 1.4.2A for the assignment of the secondary structure features. The zinc coordinating residues are marked green in the sequence.

3.6.2. Interaction assays using the GST-ICP0 (241) series of mutants.

Initially soluble bacterial extracts were made containing the GST-ICP0-241 series of mutant proteins and HIS tagged UbcH5a and UbcH6, as follows:

The pGEX series of plasmids encoding the GST-ICP0-241 mutants and the pET24a series of plasmids encoding UbcH5a and UbcH6 (Boutell *et al.*, 2002) were transformed into *E.coli* BL21. Soluble extracts of the bacteria containing the expressed proteins was made according to the method stated in section 3.5.1. The GST-pull down assays were carried out using soluble extracts of the GST-ICP0-241 mutants, which were mixed separately with bacterially expressed HIS-UbcH5a and HIS-UbcH6. Again, the assay was carried out according to the method stated previously in section 3.5.1.

GST-pull down assays were used to determine if GST, GST-262 (FXE), GST-241 and GST-211 (extracts of which are shown in lanes 1-4, respectively) form stable interactions with UbcH5a and UbcH6, the results of which are shown in figure 3.6.2A. Lanes 5 and 6 are positive controls showing UbcH5a and UbcH6, respectively. Lanes 7 and 8 are negative controls and indicate that GST does not form a stable interaction with UbcH5a or UbcH6. Lanes 9 and 10 indicate GST-262 FXE the RING finger deletion mutant, does not form a stable interaction with either UbcH5a or UbcH6. However, from lanes 11 and 12 it is possible to observe that GST-241 forms a stable interaction with UbcH6 but not UbcH5a under the conditions employed in this experiment. Replicate results were observed with GST-211 (lanes 13 and 14).

GST-pull down assays were carried out to determine if GST-241ins150, GST-241ins162, GST-241ins188 and GST-241ins192 (extracts of which are shown in lanes 1-4, respectively) form a stable interaction with UbcH5a or UbcH6, the results of which are shown in figure 3.6.2B. Lanes 6 and 7 are positive controls, containing UbcH5a and UbcH6, respectively. Lanes 8 and 9 are negative controls, indicating that GST does not form a stable interaction with either UbcH5a or UbcH6. Lanes 10 and 11 indicate that GST-241ins150 forms a stable interaction with UbcH5a and UbcH6, as does GST-241ins162 (lanes 12 and 13) and GST-241ins192 (lanes 16 and 17). However, the results indicate that under the current experimental conditions GST-241ins188 (lanes 14 and 15) could only form a stable interaction with UbcH6.

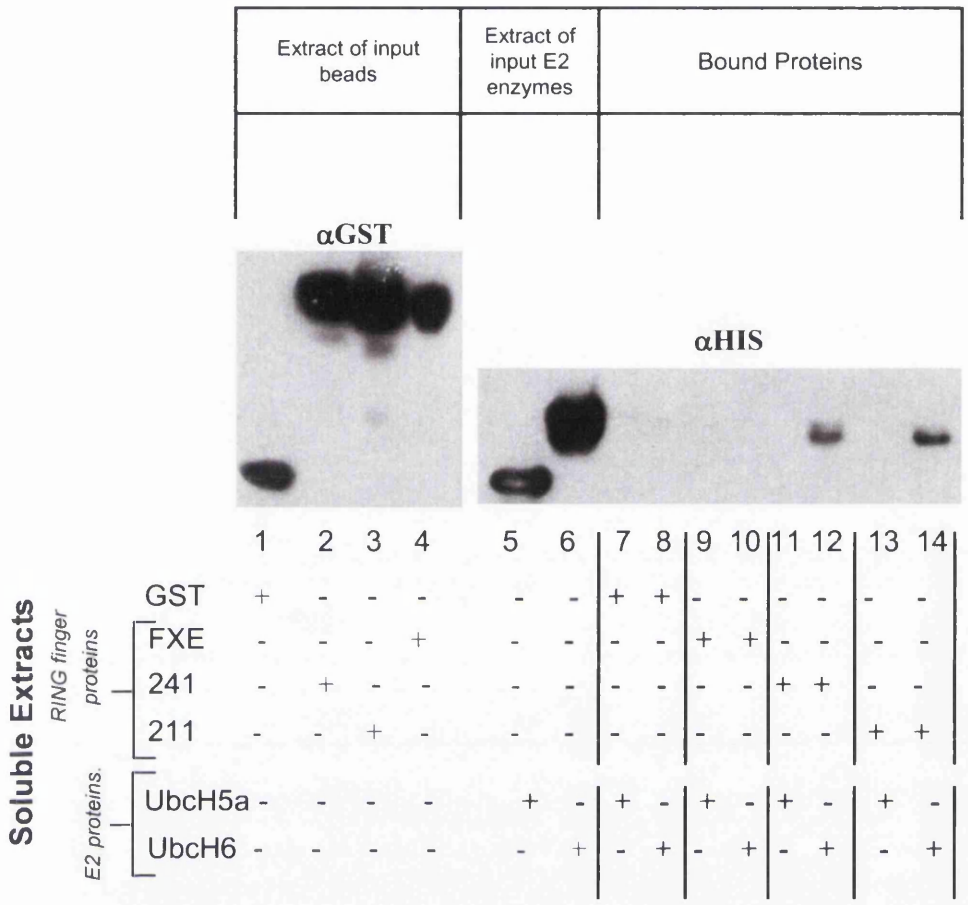


Figure 3.6.2A *GST-pull down assay to determine if the ICP0 RING finger domain mutants form stable complexes with selected E2 enzymes in vitro.*

The GST-pull down assays were carried out according to the method stated in section 2.9C. Pre-cleared extracts containing either HIS-UbcH5a or HIS-UbcH6, were incubated with glutathione beads, charged with GST, GST-ICP0 (241), GST-211 and GST-FXE. The combination of proteins that were incubated together is shown in the table. The mixtures were washed and the co-precipitated proteins were eluted and then analyzed by SDS-PAGE followed by Western Blotting. The antibody used to visualise the proteins was as follows anti-HIS used at 1/750 followed by the secondary antibody hrp-conjugated anti-mouse at 1/1000.

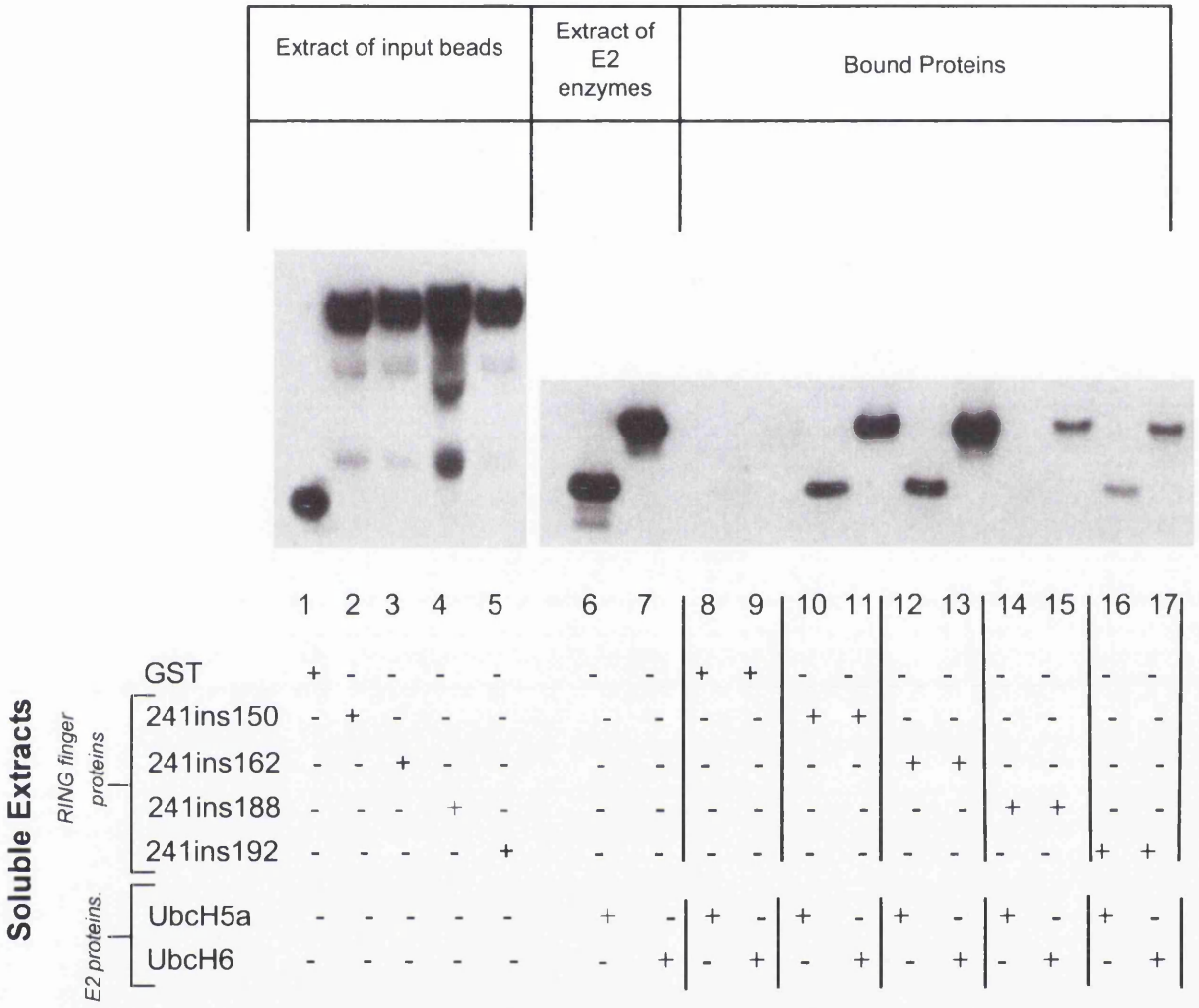


Figure 3.6.2B. *GST-pull down assay to determine if the ICP0 RING finger domain mutants form stable complexes with selected E2 enzymes in vitro.*

Pre-cleared extracts containing either HIS-UbcH5a or HIS-UbcH6 were incubated with glutathione beads, charged with RING finger domain mutants of ICP0, GST-241ins150, GST-241ins162, GST-241ins188 or GST-241ins192. The combinations of proteins that were incubated together are shown in the table. The assay was carried according to the method out lined in the previous figure.

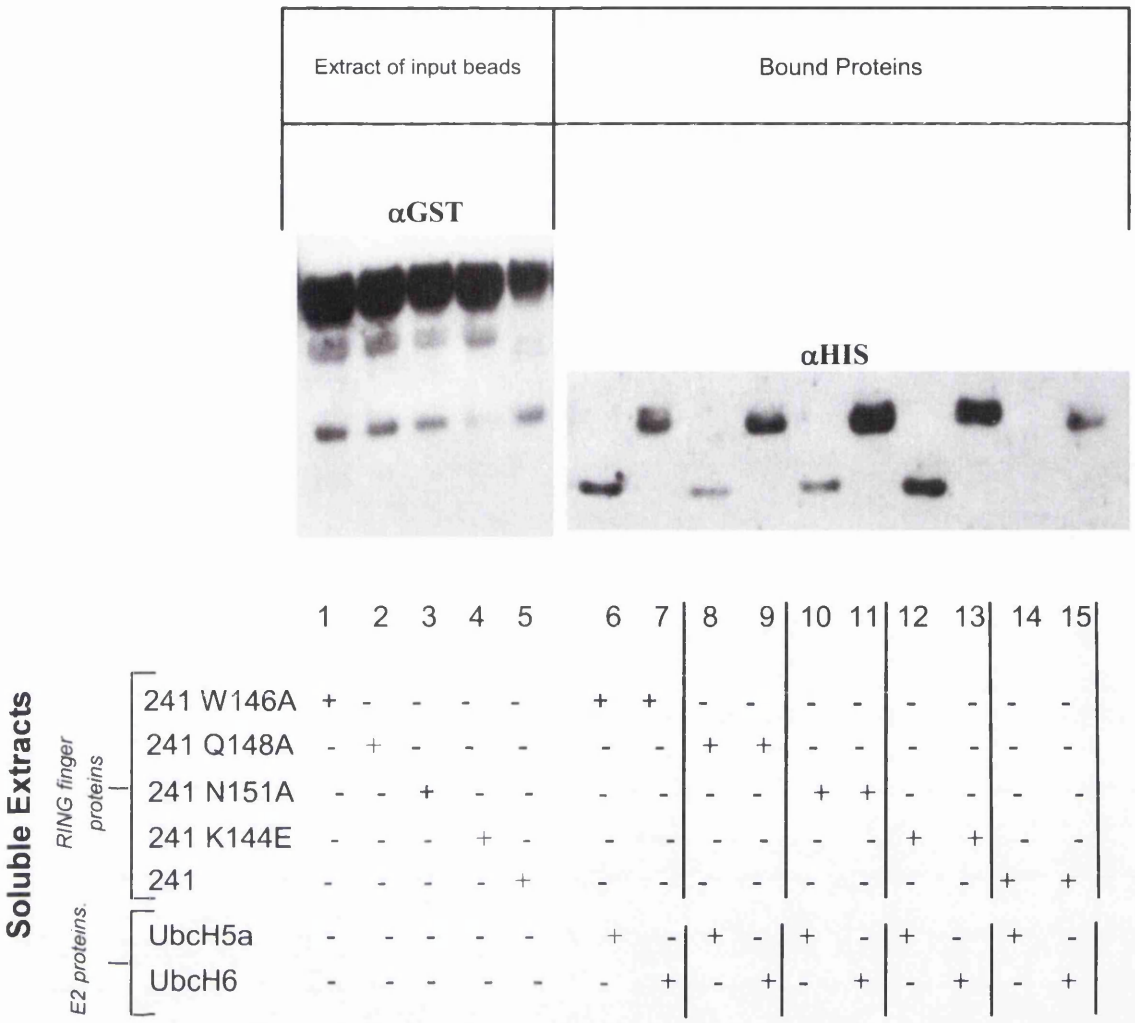


Figure 3.6.2C *GST-pull down assay to determine if the ICP0 RING finger domain mutants form stable complexes with selected E2 enzymes in vitro.*

The GST-pull down assays were carried out according to the method stated in section 2.9C. Pre-cleared extracts containing either HIS-UbcH5a or HIS-UbcH6, were incubated with glutathione beads, charged with RING finger domain mutants of ICP0, 241 W146A, 241 Q148A, 241 N151A or 241 K144E. The combinations of proteins that were incubated together are shown in the table. The assay was carried according to the method outlined in the previous figure.

Figure 3.6.2C is a continuation from the previous set of results. The results determine whether GST-241W146A, GST-241Q148A, GST-241N151A, GST-241K144E and GST-241 (extracts of which are shown in lanes 1-5, respectively) form stable interactions with UbcH5a and UbcH6. Lanes 6 and 7, indicate that GST-241W146A is capable of forming a stable interaction with UbcH5a and UbcH6 respectively, as can GST-241Q148A (Lanes 8 and 9), GST-241N151A (Lanes 10 and 11) and GST-241K144E (Lanes 12 and 13). However, the results indicate under the current experimental conditions GST-241 (lanes 14 and 15) could only form a stable interaction with UbcH6.

3.6.3. Conclusions from the interaction assays.

The results showed that subtle mutation of the RING finger or its flanking sequence failed to abrogate ICP0 ability to form a stable interaction with UbcH6. However, deletion of the RING finger domain of ICP0 (262-FXE) managed to prevent an interaction with UbcH6.

Significantly, the data also indicated that most of the ICP0-241 mutants (see figure 3.6.1A) appeared to form a stable interaction with UbcH5a unlike ICP0-241, which was previously shown to interact with UbcH6 alone. It is possible this is down to minor experimental variables and further optimization of the assay would probably enable an interaction between ICP0 (241) and UbcH5a to be detected. However, it is equally possible that mutations, with exception of the RING finger deletion mutant caused a change in conformation of the protein, which enabled a more stable interaction between ICP0 (241) and UbcH5a to be detected. All of the point mutants used in this study were within the a-helix region of ICP0 (241) (see figure 3.6.1A), which has been predicted to be critical for the function of the protein (Barlow *et al.*, 1994). However, the insertional and point mutants used in this study had been shown not to affect the transactivation ability of ICP0 (241), therefore they were predicted not to cause any major alterations in the protein fold or charge of the a-helix region (Barlow *et al.*, 1994). In addition, later research showed that the same point and insertional mutants had no or only slightly effected ICP0 (241) ability to stimulate UbcH5a and cause the formation of polyubiquitin chains in vitro (Boutell *et al.*, 2002). In light of the studies described herein it is possible that subtle mutation of the RING finger domain and flanking

sequence is insufficient to cause significant changes in the biological properties of the protein (Barlow *et al.*, 1994; Boutell *et al.*, 2002), but is sufficient to cause a change in conformation of the protein that allows a stable interaction between ICP0 (241) and UbcH5a to be detected in vitro.

Chapter 4 - Characterisation of members of the ICP0 family of proteins by expression of their full-length versions.

4.0. Expression of members of the ICP0 family of proteins using recombinant baculoviruses.

4.0.1 Introduction.

The aim of this project was to create a series of recombinant baculoviruses (*Autographa californica* nuclear polyhedrosis virus [AcNPV]) for expression of full-length members of the ICP0 family of proteins in Sf21 insect cells (see section 2.3D) and in HEp-2 cells. Baculoviruses have been used as vectors for the efficient expression of recombinant proteins in insect cells since the early 1980s, and more recently they have been adapted as gene delivery vectors into cultured mammalian cells, in particular hepatic cell lines (Hofmann *et al.*, 1995; Kost & Condreay, 1999, 2002; Luckow *et al.*, 1993).

Two sets of baculovirus were constructed to allow expression in mammalian and insect cell lines. For expression of recombinant genes encoding the ICP0 related proteins in Sf21 insect cells, use is made of the strong polyhedron promoter of AcNPV, already located within the pFastBac HTa vector (see figure 4.0.1A). However, for the expression of the full length proteins in mammalian cells it was necessary to insert the HCMV IE promoter/enhancer upstream of the recombinant gene, as the polyhedron promoter is unable to drive expression in mammalian cells (Kost & Condreay, 2002). The insertion of a mammalian active promoter allows expression only of the recombinant gene from the viral genome in infected mammalian cells, as it has previously been shown that mammalian cells are non-permissive for baculovirus replication, and expression of any of the insect virus proteins, even from its IE genes, is undetectable (R.D. Everett personal communication.).

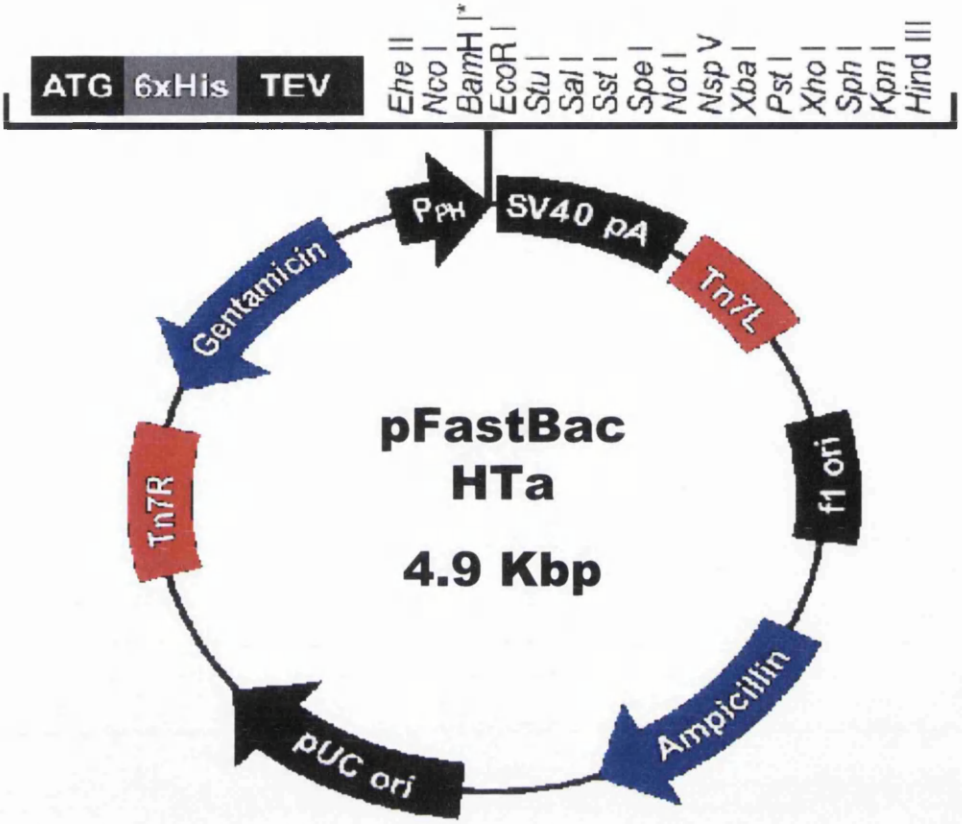


Figure 4.0.1A. *Schematic overview of the salient features of the plasmid pFastBac HTa.*

Further details are stated in the text. pFastBac HTa key features are as follows. ATG: Initiation Codon. 6 x HIS: Poly-histidine tag, pUC ori: Origin of replication, F1 ori: phage F1 origin of replication. Ampicillin and gentamycin: Genes encoding antibiotic resistance. P_{PH}: Polyhedron promoter. SV40 pA: Polyadenylation signal. Tn7L and Tn7R: Mini-Transposable elements. Diagram adapted from the Invitrogen 'Bac-to-Bac Baculovirus Expression System' laboratory manual.

Figure 4.0.1B. *Overview of the construction of a recombinant baculovirus.*

A) The foreign gene, i.e. the DNA fragment containing the ORF encoding one of the ICP0 family of proteins, was ligated into the MCS of the pFastBac HTa vector, then the resultant transfer plasmid was transformed into *E.coli* DH10 Bac. **B**) Site specific transposition occurs between the pFastBac HTa containing the recombinant gene and the bacmid. Antibiotic selection and blue/white screening was used to identify *E.coli* DH10 Bac expressing recombinants. The white colonies were restreaked onto the same selective media to produce clonally pure isolates. **C**) The positive colonies were cultured in media supplemented with the appropriate antibiotics and the high molecular weight bacmid DNA was isolated by alkaline lysis. **D**) The bacmid DNA was transfected into Sf21 cells. **E**) Low titre baculovirus was isolated from the cell culture after 5-7 days. **F**) The low titre baculovirus stock was used to infect further Sf21 cells. Repeated infection of Sf21 cells and harvesting of viral supernatant was carried out, the volume of cells in culture being increased at each stage of the amplification process. **G**) The cell pellets that were collected during the amplification process were analysed by Western blotting for recombinant protein expression. High titre stocks were harvested from one baculovirus positive for expressing the heterologous protein of the correct size. **H**) The recombinant protein could be easily analysed by immunodetection, using either an anti-HIS or anti-Myc antibodies. **I**) Plaque assays were performed to determine the titre of the baculovirus stocks. **J**) Sf21 cells were infected with recombinant baculoviruses. **K**) The expressed recombinant protein was isolated by a metal chelate affinity chromatography. **L**) Baculoviruses containing a heterologous gene downstream from HCMV promoter were used to infect mammalian cells. Protein expression was identified by immunofluorescence in cell culture.

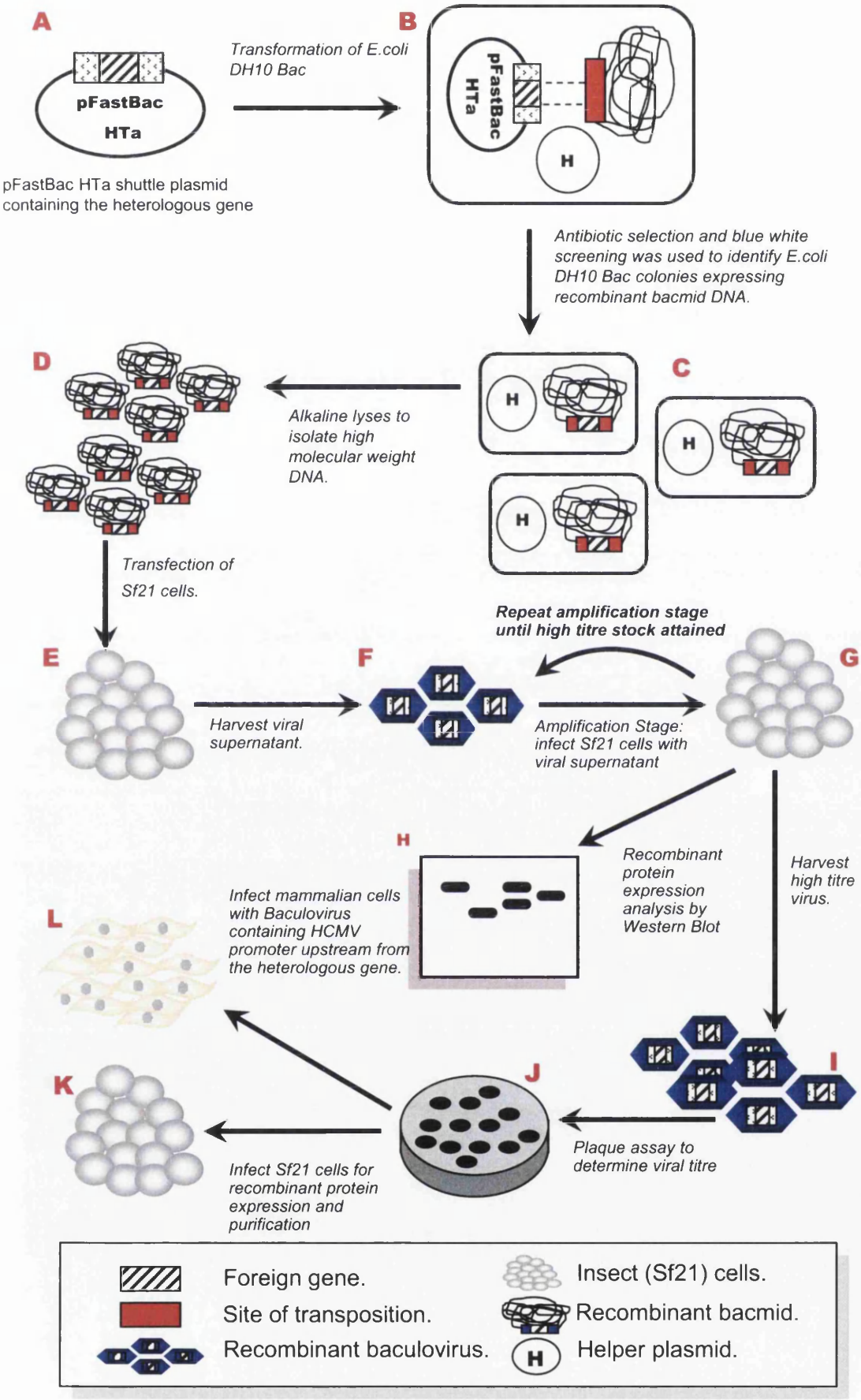


Figure 4.0.1B. *Overview of the construction of a recombinant baculovirus.*

The Bac-to-Bac system (Invitrogen) was employed to create the recombinant baculoviruses. The main features of baculovirus construction are as follows (an overview is shown in figure 4.01B). Initially the heterologous gene was cloned into the multiple cloning sites (MCS) of the vector pFastBac HTa (see figure 4.0.1A). Insertion of the foreign gene into the MCS in the correct frame introduces a polyhistidine (6 x HIS) tag at the N-terminus of the recombinant protein, enabling purification by metal chelate affinity chromatography. The tag also allows immunodetection of the protein. The MCS are positioned within a mini-Tn7 region, a transposing element that allows site-specific transposition of the heterologous gene from the vector to receptor attTn7 sites that are present within the baculovirus genome, which is contained within a bacmid. The bacmid is propagated in the bacteria *E. coli* DH10 Bac, in conjunction with a helper plasmid (pMON7124) which provides the Tn7 transposition function by encoding a transposase enzyme (Luckow *et al.*, 1993). After introduction of the pFastBac HTa recombinant plasmid into *E. coli* DH10 Bac (see section 2.1E), transposition occurs at low frequency, but clones in which transposition has occurred can be selected for by multiple antibiotics and blue/white screening. Transposition of the mini-Tn7 element to the attTn7 element of the bacmid causes the disruption of the LacZα peptide, so colonies expressing the recombinant gene are white in a background of blue colonies that contain the unaltered bacmid.

The following chapter is divided into two parts. Part I will describe the construction of the baculoviruses for recombinant protein expression within Sf21 cells, and the subsequent results. Part II will describe the construction of the baculoviruses expressing the HCMV driven ICP0-family members in mammalian cells and the subsequent results.

Chapter 4 - Part I - *In vitro* analysis of the ICP0 family of proteins.

4.0.2 Cloning of the genes encoding members of the ICP0 protein family into pFastBac HTa.

A schematic representation of the cloning strategy employed is shown in figure 4.0.2A. Initially the fragments encoding the ICP0 family genes were isolated from the pCI-rtag series of plasmids (Parkinson & Everett, 2001), taking advantage of the *Nco*I sites that occur at the initiating ATG codon in each case, as follows:

The vector pCIrtagBICP0 was initially digested with the restriction enzyme *Xho*I at the 3' end of the DNA fragment encoding BICP0. A partial restriction digestion using the enzyme *Nco*I was used to generate multiple DNA fragments of approximately 2.2 Kbp, 1.8 Kbp and 0.4 Kbp. The 2.2 Kbp fragment containing the entire BICP0 coding region was isolated for the remaining parts of the cloning procedure.

The vector pCIrtagEg63 was initially digested with the restriction enzyme *Xho*I at the 3' end of the DNA fragment encoding Eg63. A partial restriction digestion using the enzyme *Nco*I was used to generate multiple species of DNA fragments of approximately 1.8 Kbp, 1.5 Kbp, and 300 bp. The 1.8 Kbp fragment was isolated for the remaining parts of the cloning procedure.

The vector pCIrtagVg61 was initially digested with the restriction enzyme *Not*I at the 3' end of the DNA fragment encoding Vg61. A partial restriction digestion using the enzyme *Nco*I was used to generate multiple DNA fragments of approximately 1.8 Kbp, 1.1 Kbp, and 700 bp. The 1.8 Kbp fragment was isolated for the remaining parts of the cloning procedure.

The multiple restriction digestion was carried out on the vector pCIrtagEP0. The restriction enzymes *Nco*I was used to cut at the 5' and *Hind*III at the 3' end of the DNA fragment encoding the EHV-1 ICP0 homologue EP0. The 1.6 Kbp fragment was isolated for the remaining parts of the cloning procedure.

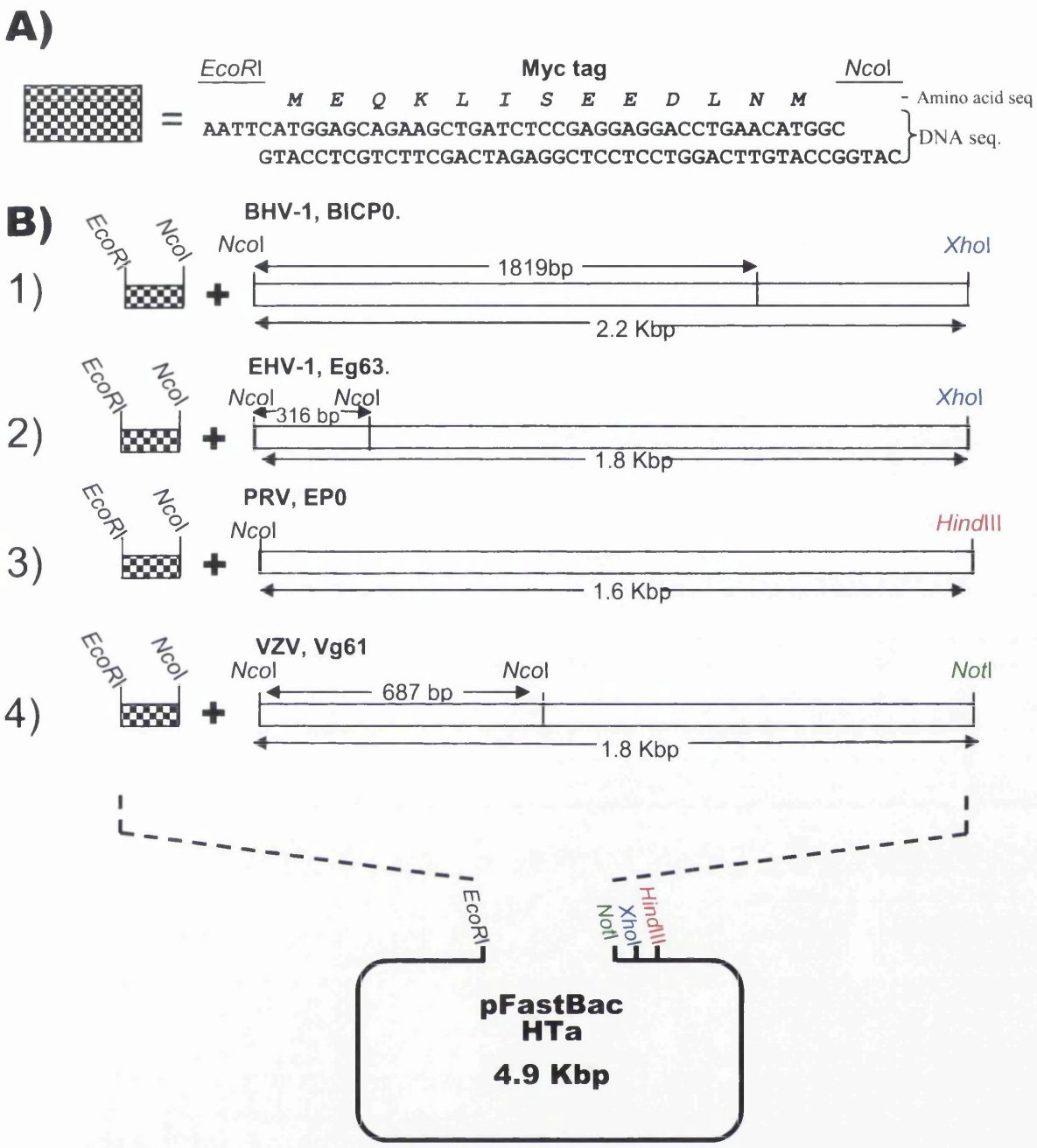


Figure 4.0.2A *Schematic representation of the construction of the pFastBac HTa plasmids expressing the ICP0-related proteins.*

A) The chequered box represents an oligonucleotide linker that encodes the myc epitope tag. The DNA sequence and the corresponding amino acid sequence is shown. The linker contained a 3 prime site that is compatible with the 5' *Nco*I restriction site of the ORF encoding ICP0 homologues (1-4). The 5' restriction site of the oligo is compatible with the 5' *EcoR*I site of the recipient vector pFastBacHTa. **B)** 1) The 2.2 Kbp DNA fragment containing the BICP0 ORF of BHV-1 was removed by an *Nco*I and *Xho*I restriction digestion of the parental plasmid pCIrtagBICP0. The vector pFastBac HTa was digested with *EcoR*I and *Xho*I (shown in blue) to accommodate the fragment and linker (A). 2) The 1.8 Kb DNA fragment encoding the Eg63 ORF of EHV-1 was derived from pCIrtagEg63 removed by an *Nco*I and *Xho*I restriction digestion. The vector pFastBac HTa was digested with *EcoR*I and *Xho*I (shown in blue) to accommodate the fragment and linker (A). 3) The 1.6 Kbp DNA fragment encoding EP0 of PRV was removed by an *Nco*I and *Xho*I restriction digestion of the parental plasmid pCIrtagEP0. The vector pFastBac HTa was digested with *Nco*I and *Hind*III (shown in red) to accommodate the fragment. 4) The 1.8 Kbp DNA fragment encoding Vg61 of VZV was removed by an *Nco*I and *Not*I restriction digestion of the parental plasmid pCIrtagVg61. The vector pFastBac HTa was restriction digested with *Nco*I and *Not*I (shown in green) to accommodate the fragment.

There are no efficient antibodies available for all of the ICP0 related family of proteins; it was therefore necessary to include a method for the immunodetection of the proteins. As previously mentioned, insertion of the ICP0 related protein coding sequence into the MCS of pFastBac HTa enables the fusion of a 6 x HIS tag to the N-terminus of the expressed protein. Furthermore, an N-terminal myc epitope tag was also used. The tag was introduced by ligating an oligonucleotide linker encoding the myc epitope in conjunction with the isolated DNA fragments encoding the ICP0 related proteins into suitably restriction-digested pFastBac HTa (see figure 4.0.2A). The ligated DNA fragments were transformed into *E. coli* DH5α. The resultant colonies were screened by making small-scale plasmid preparations and diagnostic restriction enzyme digestions. Clones harbouring the desired plasmids were identified and then large-scale plasmid preparations were made. The identities of all the constructs were confirmed by DNA sequencing using primers that generated sequences covering both the 5' and 3' cloning junctions.

4.0.3 Construction of recombinant baculoviruses.

An overview of the procedures required in the construction of a recombinant baculovirus is shown in figure 4.01B. The pFastBac HTa clones expressing the members of the ICP0 family of proteins (BICP0, Eg63, EP0 and Vg61) were individually transformed into *E.coli* DH10 Bac (see section 2.1E). Colonies expressing the heterologous gene were identified by drug selection and blue/white screening (see section 4.0.1); the bacmid DNA was then isolated from purified clones. The bacmid DNA was transfected into Sf21 cells and a low titre recombinant baculovirus stock was produced after 5-7 days. The viral titre was amplified by repeated infection of increasingly large Sf21 cell cultures. High titre baculovirus stocks were stored at -70°C and used for recombinant protein expression.

4.0.4 Analysis of Sf21 insect cell extracts for the expression of the ICP0 related proteins after low titre baculovirus infection.

During the amplification of the recombinant baculoviruses, infected cell extracts were probed for recombinant protein expression by Western blot analysis. It was necessary to ensure that the recombinant protein expressed was of the correct size, as it was possible that there may have been a mutation introduced during the cloning or the amplification process. During the amplification process of the virus, Sf21 cells in 35 mm dishes were repeatedly infected (2-3 times) to increase the viral titre. Cell pellets from the final 35 mm plate amplification stage were retained and analysed for expression of the ICP0 related proteins (see figure 4.0.4A).

The Western blot analysis shown in figure 4.0.4A indicates that all the baculoviruses expressed recombinant proteins of approximately the correct size. From comparing the profile of lanes 1-4 to that of lane 5 it is possible to see that the anti-myc antibody is highly specific for the tagged recombinant proteins as no background binding can be observed in the control (lane 5). In lane 1 the protein expressed by Bac BICP0 is of the predicted size of approximately 97 KDa. In lane 2 the protein expressed by Bac Eg63 has mobility consistent with its predicted size of 94 KDa. In lane 3 and 4 the proteins expressed by Bac EP0 and Bac Vg61 are again consistent with their predicted sizes of 70 KDa and 67 KDa respectively. However, in all the samples there were signs of degraded, cleaved or incomplete recombinant proteins; these are the bands lower than the predicted size of the recombinant protein that are detected by the antibody. Therefore, the Western blot analysis was sufficient to determine that the recombinant baculoviruses were expressing the desired members of the ICP0 family of proteins at this stage of the viral titre amplification procedure.

4.0.5 Analysis of Sf21 cell extracts for expression of the ICP0 related proteins after high titre baculovirus infection.

High titre stocks of the baculoviruses expressing the ICP0 related proteins were harvested, and a plaque assay was carried out on each to determine their titre (see section 2.8E for further details).

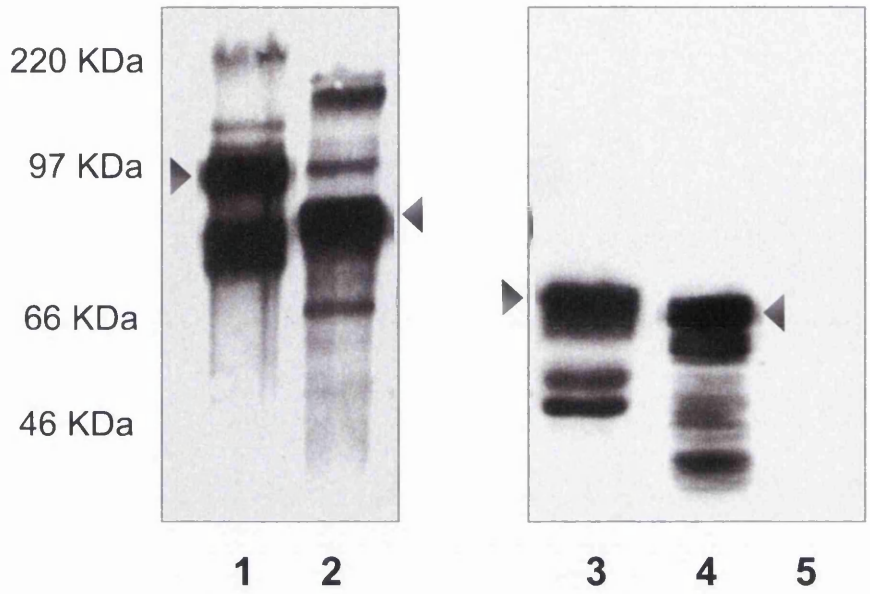


Figure 4.0.4A *Western blot analysis of Sf21 cell pellet extracts for recombinant protein expression following infection with baculoviruses expressing the ICP0 related proteins.*

200 µl of supernatant containing low titre recombinant baculoviruses expressing either BICP0, Eg63, EP0 and Vg61 was used to infect Sf21 cells seeded on 35 mm dishes (see methods section for further details). The cell pellets were harvested 72 hours post infection and resuspended in 200 µl of PBS and 100 µl of 3 x boiling mix. 15 µl of each of the samples was resolved by SDS-PAGE and the proteins were transferred to a nitrocellulose membrane. The nitrocellulose membrane was probed with the primary antibody anti-myc (9E10) at a concentration of 1/1000. This was followed by anti-mouse secondary at a concentration of 1/1000. The Sf21 cells extracts analysed are those following infection with: 1= Bac BICP0, 2= Bac Eg63, 3= Bac EP0, 4= Bac Vg61 5= Mock extract (no infection). The arrows indicate the bands of the sizes expected of the full length ICP0-related proteins.

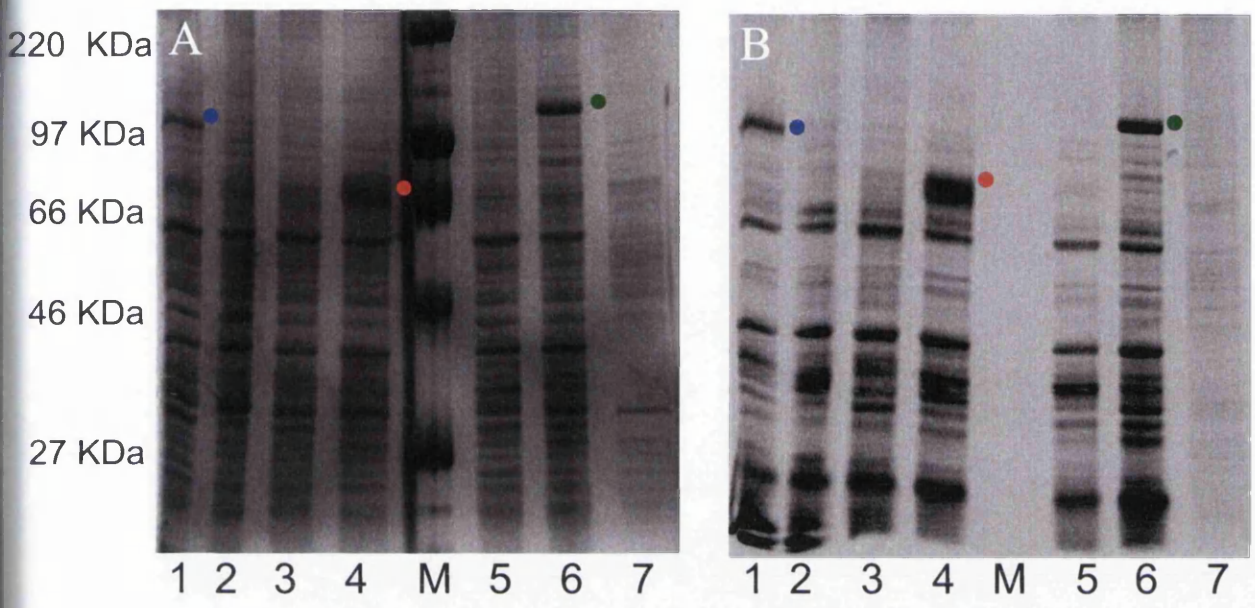


Figure 4.0.5A. *Coomassie staining and ^{35}S-methionine labelling of baculovirus infected Sf21 cell extracts.*

Sf21 cell extracts from high titre baculovirus infections were harvested and resolved by SDS-PAGE. Protein expression was detected by either Coomassie staining (A) or ^{35}S-methionine labelling of cell extracts (B). The lanes contain extracts from the following infections, 1= Bac BICP0, 2= Bac Eg63, 3= Bac EP0, 4= Bac Vg61, M= Marker, 5= Bac HTA, 6= Bac ICP0, 7= Mock (no infection). The recombinant proteins expressed by the baculoviruses are indicated by (•) BICP0, (•) Vg61 (•) ICP0.

Sf21 cells were infected at an MOI of 5 p.f.u./cell with the titred recombinant baculoviruses Bac BICP0, Bac Eg63, Bac EP0, Bac Vg61 and the control virus Bac HTa that does not have a recombinant gene insert. During infection, the cells were radiolabelled with ^{35}S-methionine as described in section 2.8F. The infected cells were harvested, lysed in SDS-gel boiling mix and the proteins were resolved by SDS-PAGE and analysed for radiolabelled recombinant protein expression (see section 2.8F). The results are shown in figure 4.0.5A.

TC-100$_{-met}$ complete media supplemented with ^{35}S-methionine was added approximately 24 hrs post infection. Consequently, any protein made within the cell after this time point, including those encoded by the baculoviruses, would incorporate the ^{35}S-methionine. From the radiolabelled cell extracts (panel B), the following interpretations could be made: comparison of lane 7 to lanes 1-5 indicates there is a low abundance of radiolabelled proteins 24 hrs post infection expressed by the uninfected cells. To aid identification of the novel proteins encoded by the recombinant baculoviruses expressing the ICP0 related proteins, lane 5 contains Sf21 cell extracts that had been infected with Bac HTa, a baculovirus that does not express a recombinant protein. The distinctive ladder of proteins encoded by Bac HTa is similar in lanes 1-4 and 6. This indicates that none of the matching radiolabelled protein bands are the ICP0 related protein's encoded by the recombinant baculoviruses (lanes 1-4). In lane 1 a band can be observed running above the 97 KDa marker (indicated on figure 4.0.5A), indicative of the expression of BICP0 from Bac BICP0, as this correlates with the proteins predicted size. Lane 4 indicates the VZV homologue of ICP0, Vg61 is being expressed by Bac Vg61, as the protein migrates at the predicted size of around 70 KDa. Furthermore, there appears to be multiple isoforms of the protein that could be due to either post-translational modification such as phosphorylation, or partial degradation of the protein, causing a slight smear. This characteristic of Vg61 was also observed when this protein was expressed from a plasmid-based system in HEp-2 cells (Parkinson & Everett, 2000). Lane 6 shows the extract of cells infected with Bac ICP0; the recombinant protein is running at its predicted size of around 110 KDa (Boutell *et al.*, 2002). Therefore, the baculoviruses encoding ICP0, BICP0 and Vg61 are expressing the desired proteins. In contrast, expression of neither Eg63 nor EP0 could be detected by either radiolabelling (panel B) or Coomassie staining (panel A) of proteins in extracts of cells that had been infected with Bac Eg63 and Bac EP0. The predicted sizes of the

proteins if expressed would be approximately 97 KDa for Eg63 of EHV-1 and 70 KDa for EP0 expressed by PRV.

The infections using Bac Eg63 and Bac EP0 were repeated and the Sf21 insect cell extracts were analysed by Western blotting (data not shown), but it was not possible to detect expression of the recombinant proteins from either Bac EP0 or Bac Eg63 (data not shown). Furthermore, the construction of Bac Eg63 and Bac EP0 was repeated from the initial transformation of *E.coli* DH10Bac with pFastBac HTa containing the Eg63 or EP0 ORFs. As before, using the final high-titre baculoviruses it was still not possible to detect expression of proteins Eg63 or EP0.

4.0.6 Limitations of the use of baculoviruses for recombinant protein expression.

From using the final high titre stocks of the recombinant baculoviruses Bac Eg63 and Bac EP0 to infect Sf21 cells, it was not possible to attain high-level recombinant protein expression. This data contradicts the analysis of cell extracts from low titre viral infection (Figure 4.0.4A), which indicated that all of the recombinant baculoviruses could express heterologous proteins of the correct size. Detection of expression of the desired proteins during the amplification of the baculoviruses was an important prerequisite before moving onto the next stage of the baculovirus method. Normally cell extracts of 5 independent recombinant baculoviruses expressing the same protein would be analyzed for the expression of proteins of the correct size. Only then would one baculovirus positive for recombinant protein expression be selected for continued amplification until a high titre stock was obtained. The results indicate that the expression of the recombinant proteins from Bac Eg63 and Bac EP0 was unstable after passaging of the virus. The amplification of these viruses was repeated from low titre stocks, positive for recombinant protein expression, and again the results indicated that recombinant protein expression was unstable. The instability was further confirmed when the whole baculovirus construction was repeated from the initial transformation of *E.coli* DH10 Bac with pFastBac HTa expressing the recombinant genes. Again recombinant protein expression was detected in Sf21 cell extracts previously infected with recombinant baculoviruses from the initial rounds of viral amplification, but not from the final high titre stocks.

There are various possibilities as to why recombinant protein expression from Bac Eg63 and Bac EP0 was unstable. The instability of protein expression from the recombinant baculoviruses may be due to viruses that have lost the ability to express the recombinant transgene (non-expressing viruses) or defective interfering (DI) viruses that accumulate upon amplification of the viral titre; it is important to distinguish between the two types. It has been widely reported that defective interfering baculoviruses accumulate upon serial passage. This has shown to be a major obstacle in the large scale production of recombinant viruses and proteins in cell culture systems, this has been described as the passage effect (Kool *et al.*, 1991; Pijlman *et al.*, 2002; Pijlman *et al.*, 2001; van Lier *et al.*, 1992). However, defective interfering recombinant baculoviruses are dependent on a 'helper virus' for replication as they contain major genomic deletions of up to 43%, including the heterologous gene (Carstens, 1982; Lee & Krell, 1992; van Lier *et al.*, 1994). Consequently, as the recombinant baculoviruses are serial passaged, the viral titre decreases rapidly as the DI viruses have a replicative advantage over that of the intact recombinant baculovirus (Pijlman *et al.*, 2001) and thus become the predominant strain. This feature indicates that Bac Eg63 and Bac EP0 are not contaminated with DI viruses as they had a viral titre (approximately 1 x 10^9) similar to of recombinant baculoviruses Bac BICP0 and Bac Vg61, both of which had a high level of recombinant protein expression.

This conclusion is further supported by ^{35}S-methionine labelling of Bac Eg63 and Bac EP0. The results indicated that both baculoviruses had low recombinant protein expression compared to that of Bac BICP0 and Bac Vg61; however other shared viral encoded proteins were at similar levels. This was because all of the viruses had similar titres (as determined by the plaque assays) and the infections were carried out at the same MOI. Thus if the Bac Eg63 and Bac EP0 stocks were contaminated with DI particles their titre would be much lower than that of Bac BICP0 and Bac Vg61 and it would be necessary to use a higher MOI to attain the comparative results shown (see figure 4.0.5A). Consequently, it is possible to conclude that the high titre viral stocks were not contaminated with defective inferring virus, but as the viral titre remained comparable to that of other recombinant baculoviruses, they were contaminated by replication competent or interfering baculoviruses that fail to express the desired proteins.

The results indicate that upon amplification of the baculovirus titre, the non-expressing viruses become predominant as recombinant protein expression decreases and viral titre remains high. Therefore, the non-expressing viruses must have a replicative advantage over the baculoviruses expressing the recombinant proteins Eg63 or EP0. The non-expressing viruses could be generated because of baculovirus genomic instability in insect cells. Recently a study was carried out (Pijlman *et al.*, 2003), that looked at the stability of heterologous gene expression in baculoviruses. The technique used to make the virus was the Bac-to-Bac system (Invitrogen) as employed in this study. Their results indicated that recombinant protein expression was only just detectable by passage 10. By using restriction mapping to analyse the viral genome, it was determined that this was due to instability of the sequences expressing the transgene, as the non-essential expression cassette (mini F replicon, antibiotic resistance genes and the heterologous gene) were deleted from the viral genome upon passage in insect cells. Therefore, the baculovirus mutants carrying the deletion become the predominant species and thus cause a drop in recombinant protein production. It is likely that this is analogous to what happened with Bac Eg63 and Bac EP0. It may be that genetic rearrangement of the baculovirus genome occurs due to selective pressure from the host cell, perhaps caused by the toxicity of the ICP0 homologues Eg63 and EP0, leading to the generation of a non-expressing virus that has replicative advantage, and becomes amplified upon serial passaging.

Another possibility for the drop in recombinant protein production was that a contaminating virus was introduced either due to a mixed population of bacmids being transfected into Sf21 insect cells during the first stage of viral amplification or a contaminating virus arose in the initial amplification stages. It has been shown that plaque purifying recombinant baculoviruses made using the Bac-to-Bac system can improve the level of expression of a heterologous protein that was initially expressed at low level from high titre virus stock, due to the presence of a contaminating virus (R.D. Everett, unpublished results). However, as the construction of Bac Eg63 and Bac EP0 was repeated from a transfection with the original pFastBac HTa encoding Eg63 and EP0, and the same loss of recombinant protein expression at high titre occurred, it is therefore unlikely that the same experimental error introduced a contaminating virus a second time. The following question arises, why would one set of recombinant baculoviruses (Bac Eg63 and Bac EP0) be more susceptible to repeated loss in

heterologous protein production, when this characteristic has not been observed with other recombinant baculoviruses made in the same way? However, what is clear is that it is not possible to conclude from the current experimental data whether the loss of recombinant gene expression is due to a contaminating virus or to a genetic rearrangement of the baculovirus genome.

In future, to increase the likelihood of heterologous protein expression from Bac Eg63 and Bac EP0, it may be necessary to take the recombination approach to the construction of recombinant baculoviruses. This involves the infection of insect cells with a baculovirus in conjunction with transfection of a plasmid carrying the heterologous gene alone. Recombination occurs between homologues regions of the viral genome and the plasmid, followed by several rounds of plaque purification to isolate the virus and thus reduce the chance of interfering viruses. A possible advantage of the recombination approach to baculovirus construction is the reduced size of the expression cassette used, as it only contains a coding sequence for the heterologous gene and homologous flanking sequence to that of the baculovirus genome, which enables recombination to occur. Previously, it has been shown that baculovirus genome stability can be improved by reducing the amount of superfluous coding sequence of the expression cassette that is present in the Bac-to-Bac system, i.e. removal of antibiotic resistance gene, etc (Pijlman *et al.*, 2003). Reduction in the size of the cassette is thought to reduce the selective pressure on the viral genome for genetic rearrangement. Although it has been reported that a baculovirus genome can take insertion of foreign sequences of up to 25 Kbp in length and remain genetically stable (Vlak *et al.*, 1988). However, it could be that the selective pressure for genetic rearrangement of the bacmid could be due to toxicity of the encoded protein, rather than the size of the corresponding sequence. The traditional approach to recombinant baculovirus construction would obviously take longer, but if it resulted in a more stable homogeneous population of recombinant viruses, it would no doubt make up for the time advantages of the Bac-to-Bac system.

4.0.7 Purification of baculovirus expressed recombinant proteins.

It was only possible to obtain expression of the desired proteins from the high titre viral stocks of Bac BICP0 and Bac Vg61, as indicated in figure 4.0.5A. Consequently, only

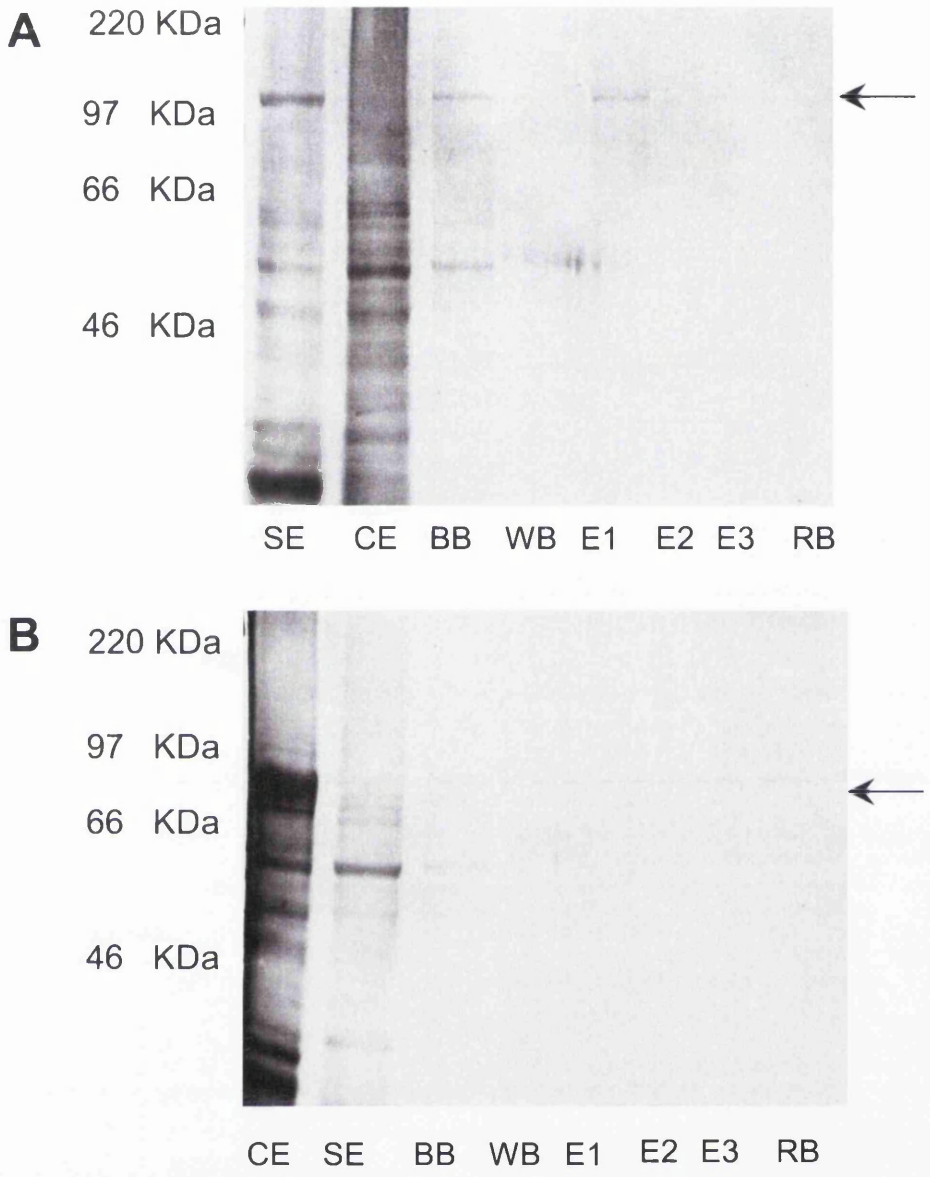


Figure 4.0.7A. *Purification profile of BICP0(A) and Vg61(B), from Sf21 insect cells.*

5 µl of each extract was resolved by SDS-PAGE and visualised by Coomassie staining. The abbreviations are as follows CE = Crude extract, SE= Soluble extract, BB= Bound beads, WB= Washed Beads, E1= Elution 1, E2= Elution 2, E3= Elution 3, RB = Remaining beads.

these viruses were used to infect Sf21 cells for recombinant protein expression and purification (see section 2.8G). Sf21 cells were seeded at a cell density of 1 x 10^6/ml in 300 ml of medium and infected at an MOI of 5 with Bac BICP0 or Bac Vg61 and incubated for 3 days at 28°C. Extracts were made from the cells and subjected to metal chelate affinity chromatography, samples were retained at each stage of the protein purification. Each sample was analysed by SDS-PAGE, as shown in figure 4.0.7A. As BICP0 and Vg61 were expected to have similar physical properties to those of ICP0, the buffer employed was the same as that used to purify ICP0 (Boutell *et al.*, 2002).

The purification profile of BICP0 is shown in figure 4.0.7A panel A. It was not possible to identify clearly the expressed recombinant protein by Coomassie staining of the whole cell extract. However, the 97 KDa BICP0 proteins was visible in the soluble extract lane, indicating that at least some of the baculovirus-expressed BICP0 was soluble. Furthermore, the expressed BICP0 protein could be bound to the nickel beads by virtue of it's HIS tag, and could be eluted from the beads in the first imidazole wash (Elution 1). For future use, the eluted sample of BICP0 was dialysed against HIS dialysis buffer (see section 2.8G) and stored frozen at -70°C.

The purification profile of Vg61 is shown in 3.0.7A panel B. From analysis of the crude extract it is possible to see that Vg61 (approximately 70 KDa in size) was expressed at a greater level than that of BICP0. However, the protein was largely insoluble as the Vg61 protein band was depleted in the soluble extract, although there were minor bands visible of mobility similar to that of Vg61 in the soluble extract (between approximately 66 KDa and 85 KDa). However, at best there were only trace amounts of these bands bound to the nickel beads, and none were detectable in the imidazole elution fractions.

4.0.8 Introduction to ubiquitin ligase assay using BICP0.

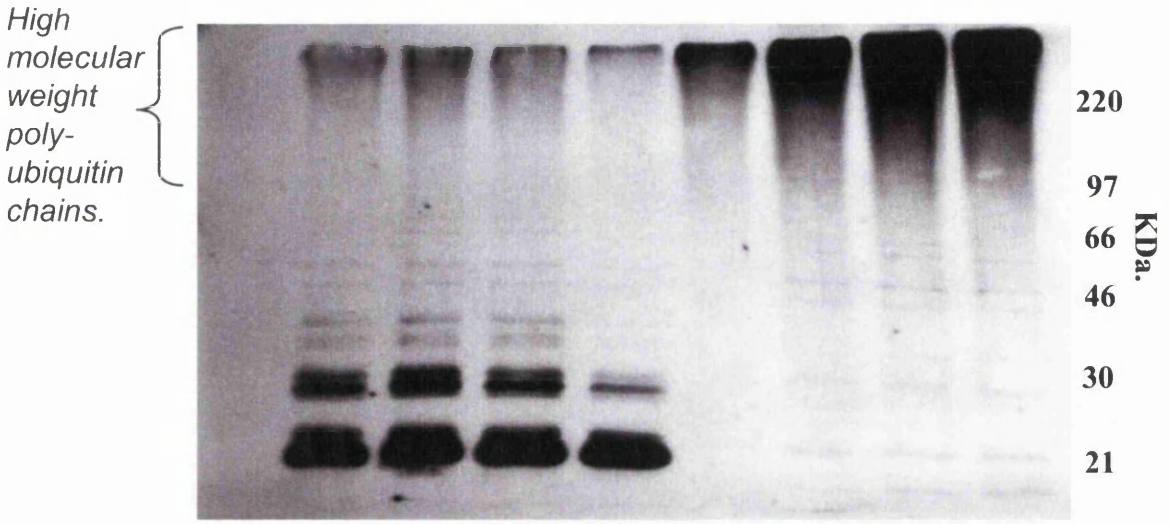
The aim of this assay was to determine if BICP0 had ubiquitin E3 ligase activity similar to that of its truncated forms GST-BICP0 and GST-BICP0 1_90 as shown Chapter 3. The method used was identical to the one used to assess the ubiquitin ligase activity of the GST tagged ICP0 related proteins. The ubiquitin ligase assay was carried out in the presence of UbcH5a and UbcH6, as these proteins had already been shown in Chapter 3 to be the E2 partners of the GST-ICP0 related proteins.

4.0.9 Ubiquitin ligase assay using purified BICP0.

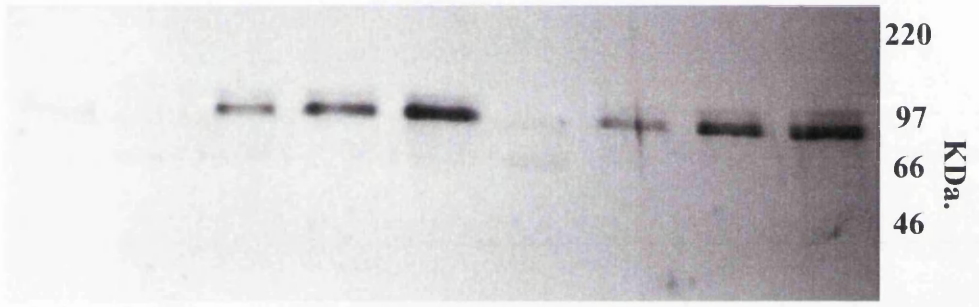
A sample of purified BICP0 was used in an ubiquitin ligase assay to determine if it had E3 ligase activity. The assay was carried out as stated in section 2.9B, with the exception that BICP0 was titrated into the reaction (1 – 4 μl of the eluted protein). The data indicates (figure 4.0.9 panel A) that BICP0 stimulates UbcH5a (Lanes 6 – 9) and UbcH6 (Lanes 2 – 5) and causes the substrate-independent formation of unanchored variable length high molecular weight poly-ubiquitin chains, in a fashion similar to that of ICP0 (Boutell *et al.*, 2002). Lane 5 (figure 4.0.8 panel A) contains a reduced amount of poly-ubiquitin compared to that in lane 4, even though more BICP0 was present (figure 4.0.8 panel B). This effect may be due to an inhibitory agent that remained in sample, even after dialysis. Figure 4.0.8 Panel B shows the same blot re-probed with the anti-myc tag antibody.

The results clearly indicate that full length BICP0 acts as an E3 ligase in a fashion similar to that of its truncated forms (see Chapter 3). The similarities between full length BICP0 and its GST-RING finger domain proteins also extend to the molecular weight of the poly-ubiquitin that is formed depending on if UbcH5a or UbcH6 was in the ubiquitin ligase reaction mix. When UbcH5a is stimulated by full length or truncated GST-BICP0, mainly high molecular weight polyubiquitin is formed, and when UbcH6 is stimulated the polyubiquitin formed ranges from low to high molecular weight causing a uniform distribution down the gel. The characteristic pattern of polyubiquitin is also similar to that observed with the GST-ICP0 related proteins. The significance of the polyubiquitin chain length is discussed in further detail in Chapter 3.

A



B



	1	2	3	4	5	6	7	8	9
E1	+	+	+	+	+	+	+	+	+
UbcH5a	-	-	-	-	-	+	+	+	+
UbcH6	-	+	+	+	+	-	-	-	-
Ub	+	+	+	+	+	+	+	+	+
BICP0	1.5 µl	1 µl	2 µl	3 µl	4 µl	1 µl	2 µl	3 µl	4 µl

Figure 4.0.9A *E3 ligase activity of BICP0.*

In vitro ubiquination assay was carried out as described in section 2.9B, with the exception of the concentration of the full length protein BICP0. The protein BICP0 (20 ng/µl) was titrated into the assay to assess its potential activity as an E3 ligase. In lanes 2 to 5 the E3 ligase assay was carried out in the presence of UbcH6, in lanes 6 to 9 in the presence of UbcH5a. Lane 1 = Control (No E2 present.). Blot A was probed with α-Ub at 1/1000 and blot B α-myc 1/1000, followed by the secondary antibody hrp-conjugated anti-mouse antibody 1/1000 .

Chapter 4 - Part II - *In vivo* analysis of ICP0 related proteins.

4.1.0 Introduction to studies analysing the in vivo biological properties of the ICP0 related proteins.

The results presented in the previous section and in Chapter 3 indicate that full length BICP0 and the other ICP0 related proteins *in vitro* are able to stimulate UbcH5a and UbcH6 in a similar fashion to that of ICP0, as described previously (Boutell *et al.*, 2002). As an alternative approach to assess the biological significance of the *in vitro* ubiquitin ligase activity, the distribution of the UbcH5a, UbcH6 and cdc34 in the presence of the ICP0 related proteins could be investigated in transfected cells. Previously it had been demonstrated that the E3 activity of ICP0 itself was not an *in vitro* artefact and could be replicated *in vivo*, as colocalisation between ICP0, UbcH5a and UbcH6 could be observed, but not between ICP0 and cdc34 (Boutell *et al.*, 2002). However, it has been reported that cdc34 and ICP0 do have a relationship *in vivo* and *in vitro*. ICP0 was reported to interact with cdc34 and mediate its relocation to ND10 structures within the nucleus (Kawaguchi *et al.*, 1997b; Van Sant *et al.*, 2001b). Furthermore, a fragment of ICP0 unrelated to the RING finger was reported to cause limited auto-ubiquitination of cdc34 (Van Sant *et al.*, 2001a), causing it to be degraded in a proteasome-dependent manner (Hagglund *et al.*, 2002). If cdc34 is targeted in this manner by ICP0, it could explain why ICP0 might bring about the reported stabilization of both cyclin D3 and D1 during HSV-1 infection (Van Sant *et al.*, 2001b). The relationship of ICP0 and cdc34 is discussed in more detail in the Introduction, but as described there, it is likely that most or all the observations concerning ICP0 and cdc34 are artefacts. Indeed, an E3 ligase activity of ICP0 with cdc34 can not be reproduced (Boutell *et al.*, 2002), and more recent analysis has proved that ICP0 has no effect on cdc34, cyclin D1 and cyclin D3 during HSV-1 infection (Everett, 2004). Nonetheless, it was important to examine if the ICP0 related proteins' relationships with cdc34 differ from that of ICP0. The homology between ICP0 and the ICP0 related proteins is limited to that of their RING finger domains, thus it is feasible that the ICP0 related proteins may have different biological properties with cdc34 *in vivo*, even though they do not stimulate cdc34 and facilitate the formation of polyubiquitin *in vitro*. Therefore, the aim

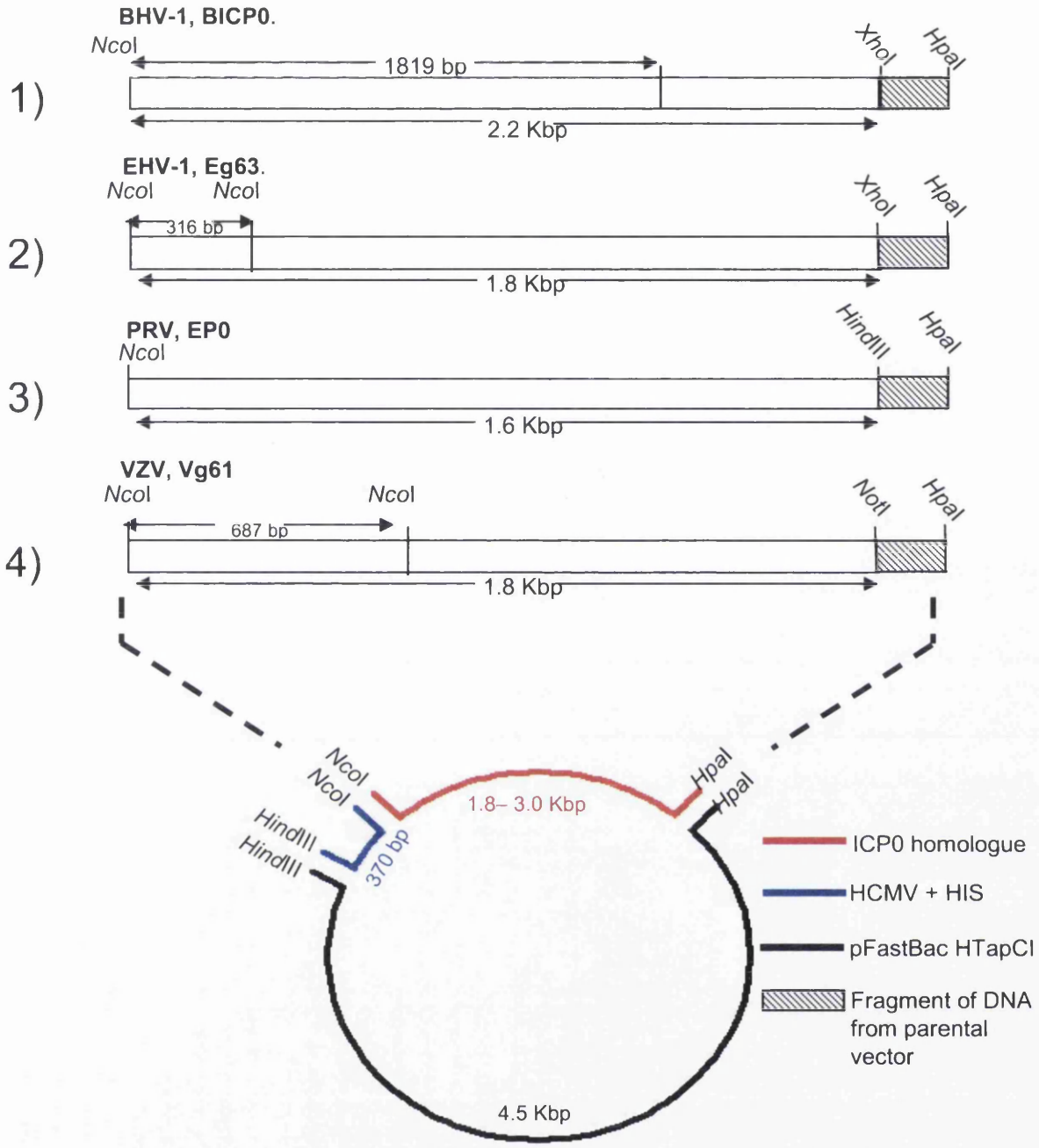


Figure 4.1.2A *Schematic representation of the construction of pFastBac HTa CMV plasmids expressing ICP0 related proteins.*

The plasmids were constructed as follows: 1) A fragment encoding BICP0 was removed from pFastBac HTa BICP0 by using the restriction enzymes *Nco*I and *Hpa*I. 2) A fragment encoding Eg63 was removed from pFastBac HTa Eg63 by using the restriction enzymes *Nco*I and *Hpa*I. 3) A fragment encoding EP0 was removed from pFastBac HTa EP0 by using the restriction enzymes *Nco*I and *Hpa*I. 4) A fragment encoding Vg61 was removed from pFastBac HTa Vg61 by using the restriction enzymes *Nco*I and *Hpa*I. The ICP0 related proteins ORFs (1 – 4) were ligated into pFastBac HTapCI (*Hind*III-*Hpa*I) in conjunction with the DNA fragment HCMV + HIS (*Hind*III – *Nco*I). The HCMV + HIS fragment (shown in blue) encodes the HCMV IE promoter upstream from an in frame 6 x HIS tag. It was isolated from pFastBac HTa CMV.HIS. ICP0 by an in frame *Nco*I – *Hind*III digestion. Insertion of this DNA fragment in to the recipient vector pFastBac HTapCI would allow from the resulting baculoviruses in frame expression of 6 x HIS tagged ICP0 encoding ORFs.

promoter situated upstream from a sequence encoding a 6 x HIS tag, and was excised from pFastBacHta-CMV.HisICP0 by digestion with *Hind*III and *Nco*I at the 5' and 3' ends of the desired fragment, respectively. The three fragments were ligated as follows: The recipient vector pFastBacHTa-pCI was digested with *Hind*III (5') and *Hpa*I (3'). This enabled in-frame ligation of the DNA fragment containing the HIS tag and HCMV IE promoter upstream of the fragment encoding the ICP0 homologue (see figure 4.1.1A.). The ligated plasmid was transformed into *E.coli* DH5α. The cloned DNA was isolated by the mini-prep method and screened by restriction analysis and sequencing to confirm the identity of positive clones.

Once the pFastBacHTa-pCI series of plasmids expressing the HCMV IE promoter driven ICP0 homologues had been constructed and their identities had been confirmed by DNA sequencing (see section 2.4D), the method used to create the recombinant baculoviruses was identical to that stated previously in section 3.0.3. The resultant baculoviruses expressing the HCMV driven ICP0 related proteins were named Bac CMV BICP0, Bac CMV Eg63, Bac CMV EP0 and Bac CMV Vg61. Stocks of these viruses were generated and titrated following the same methods described previously for baculoviruses used for recombinant protein expression in insect cells.

4.1.3. Confirmation of the identity of recombinant baculoviruses expressing HCMV driven ICP0 related proteins.

HEp-2 cells were infected with the baculoviruses encoding the CMV driven ICP0 related proteins to confirm that they were expressed in mammalian cells, and that the proteins were of the correct size. Cell extracts were analysed for recombinant protein expression by Western blot analysis (See figure 4.1.3A). The expressed recombinant proteins from Bac CMV BICP0 (Lane 2) and Bac CMV Vg61 (Lane 5) were of the correct size, BICP0 approximately 97 KDa and Vg61 66 KDa. Low-level expression was detected from Bac CMV EP0 (Lane 4), and the expressed protein was again of the expected size. However, Bac CMV Eg63 expressed only low levels of an apparent recombinant protein, but this was not of the expected size of approximately 97 KDa. The generation of Bac CMV Eg63 was repeated and the same problems of low expression of the protein and incorrect size occurred. Expression levels from this group of baculoviruses as detected by Western blot analysis were variable; therefore

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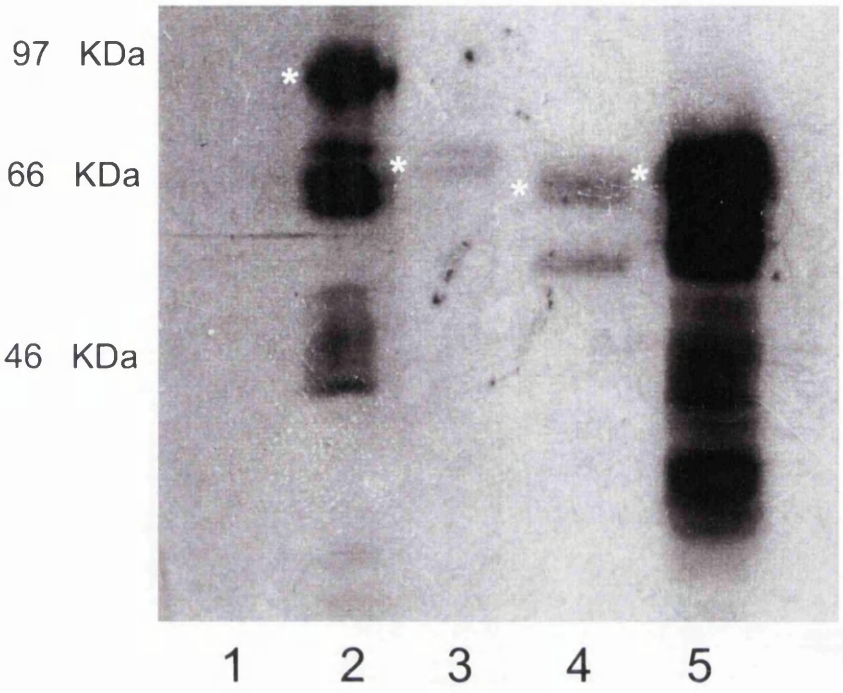

Figure 4.1.3A. *Analysis of HEp-2 cell extracts for recombinant protein expression.*

Bac CMV BICP0, Bac CMV Eg63, Bac CMV EP0 and Bac CMV Vg61 were used to infect HEp-2 cells at an MOI of 100 and incubated at 32°C for approximately 24 hours. The cell pellets were analysed for recombinant protein expression by SDS-PAGE analysis and Western blotting. The blot was probed with primary anti-6 x HIS antibody at a concentration of 1/750 and secondary hrp-conjugated anti-mouse antibody at 1/1000. The lanes are extracts of HEp-2 cells infected with the following: 1 = Mock extract (no infection), 2 = Bac CMV BICP0, 3 = Bac CMV Eg63, 4 = Bac CMV EP0, Bac CMV Vg61. The expressed recombinant proteins are indicated (*) See text for analysis.

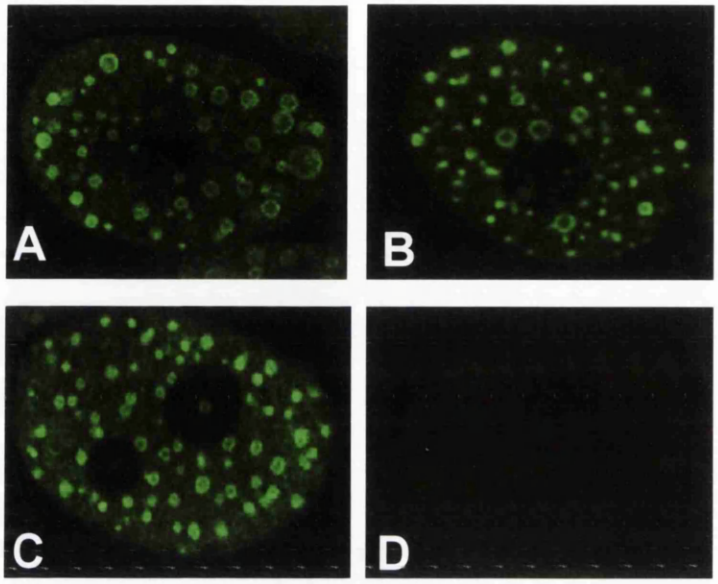

Figure 4.1.4A. *Immunostaining of HEp-2 cells following infection with Bac CMV BICP0, Bac CMV Vg61 and Bac CMV ICP0.*

All baculoviruses were used at an MOI of 200 and cells were fixed 24 hours post transfection. A) A typical cell infected with Bac CMV BICP0. B) A typical cell infected with Bac CMV ICP0. C) A typical cell infected with Bac CMV Vg61 D) Negative (No infection). The negative cell was isolated from the same field of view as the Bac CMV Vg61 cell. This was to provide an indication of background fluorescence. The cells were stained with the primary mouse monoclonal antibody anti-HIS at a concentration 1/750. The secondary antibody used was FITC-conjugated goat anti-mouse IgG (Amersham) at 1/100.

immunostaining was used to determine if the baculoviruses expressed detectable levels of the recombinant proteins in individual HEp-2 cells.

4.1.4. Immunostaining of HEp-2 cells following baculovirus infection.

HEp-2 cells were infected with Bac CMV BICP0, Bac CMV Eg63, Bac CMV EP0 and Bac CMV Vg61 as stated in section 2.8H. The infections were repeated and reproducible results were attained for Bac CMV BICP0 and Bac CMV Vg61, by infecting HEp-2 cells at an MOI of 200 based on the titres of these viruses in Sf21 insect cells (see figure 4.1.4A). The expressed BICP0 produced ring-like structures in the nucleus. The larger rings were incomplete and arcs of stained material frequently appeared. The foci of the expressed Vg61 tended to be quite small and uniform in size, but not in shape. Unlike BICP0, Vg61 was uniformly distributed around the foci. The morphology of the foci of BICP0 and Vg61 was consistent, and very similar to that previously described using a plasmid transfection approach (Parkinson & Everett, 2000, 2001), indicating that the baculovirus method was successful in expressing the recombinant proteins in cultured cells.

The baculovirus approach was an efficient method to observe the biological properties of BICP0 and Vg61 *in vivo*. By using Bac BICP0 and Bac Vg61 at an MOI of 200 the majority of cells seeded at 1 x 10^5 were consistently infected during repeated experiments, a higher ratio than the transfection approach. Using recombinant baculovirus to transduce the mammalian cells eliminated the variability in the number of positive cells that occurs when using the transfection approach to study the expression of recombinant proteins. The ability of the baculoviruses to transduce a large number of cells means they will be highly useful reagents to study the biological properties of BICP0 and Vg61 in a large number of cell types.

4.1.5. Plasmid based expression of the full-length ICP0 related proteins in cultured cells.

The original aim of this series of experiments was to carry out the analysis of the members of the ICP0 family of proteins using the baculovirus expression system. However, as mentioned in sections 4.1.2 and 4.1.3, it was not possible to express Eg63 or EP0 using the baculovirus clones that had been isolated. Therefore, to study the members of the ICP0 family of proteins and to maintain consistency a plasmid based transfection system was employed as used previously (Parkinson & Everett, 2000, 2001). The ICP0 family members were expressed using the pCIrtag plasmid system, which is an adaptation of the plasmid pCIneo (Promega). A schematic overview of this system is shown in figure 4.1.5A. The ICP0 related proteins were tagged at their N-terminal ends with an epitope derived from the C-terminal end of HSV-1 protein UL30, which is referred to in the remainder of the text as 'rtag'. The plasmids coding the ICP0 related proteins were co-transfected with plasmid encoding FLAG tagged UbcH6 and cdc34. Ideally, a plasmid expressing FLAG-tagged UbcH5a would also be used for these cotransfection experiments, but in this series of experiments the levels of expression of FLAG-tagged UbcH5a were insufficient to be readily detected by immunofluorescence (data not shown). The sections below outline the methodology taken to analyse the biological properties of the ICP0 related proteins with UbcH6 and cdc34.

4.1.6. Immunostaining of HEp-2 cells cotransfected with plasmids expressing ICP0 related proteins and UbcH6 or cdc34.

The pCIrtag plasmid based system was used to express the members of the ICP0 family of proteins that are the subject of this study, while FLAG-tagged UbcH6 and cdc34 were expressed from plasmids based on the pCMV2 vector. Plasmids expressing an ICP0 related protein with an N-terminal rtag were co-transfected with a plasmid expressing FLAG-tagged UbcH6 or cdc34. One-day post transfection, the cells were fixed, permeabilised, then stained with rabbit serum r113 to detect the rtag, and monoclonal antibody M2 to detect the FLAG tag. The primary antibodies were detected using FITC or Cy5 labelled secondary antibodies, then the samples were examined by confocal microscopy. When expressed by themselves, the E2 ubiquitin conjugating

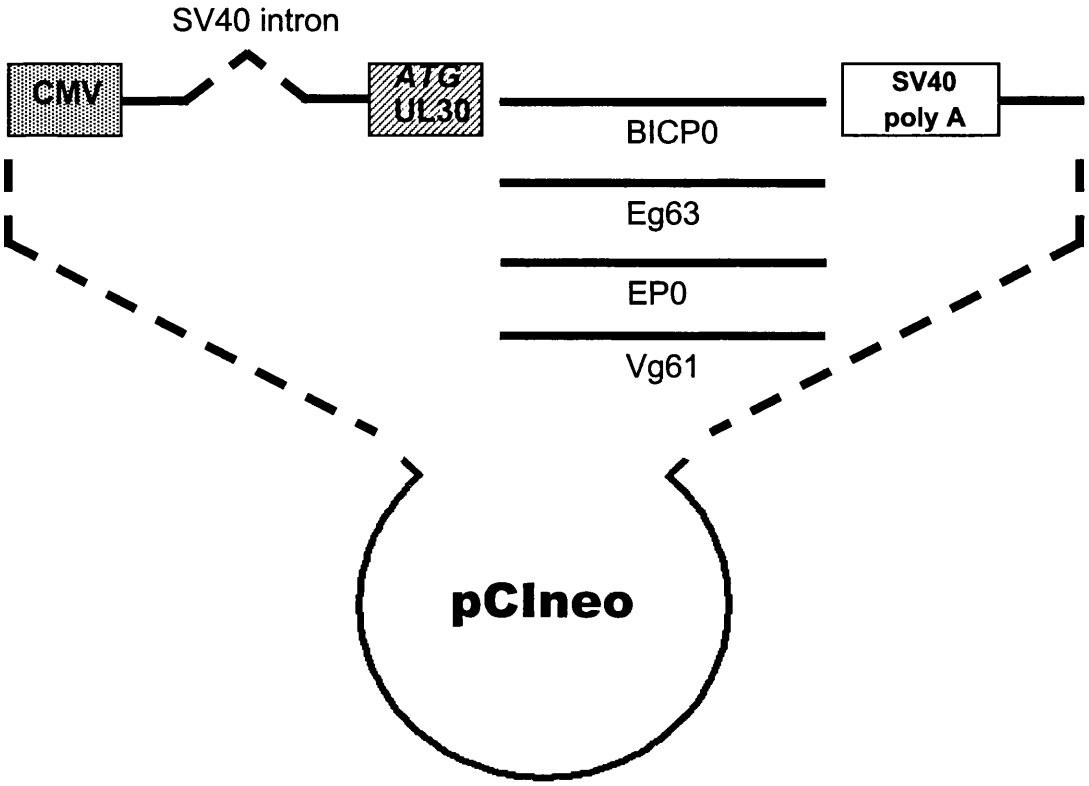

Figure 4.1.5A *Schematic diagram of the pCI-rtag series of plasmids expressing the ICP0 related proteins.*

The pCI-rtag plasmid is derived from pCIneo. The plasmid contains a HCMV promoter to enable expression of the ICP0-derived family of proteins in mammalian cells post transfection. Down stream from the HCMV promoter is a SV40 intron followed by the C terminal peptide of HSV-1 UL30. The UL30 coding sequence is upstream from the ICP0-derived family coding sequence, consequently when expression occurs from the HCMV IE promoter it is introduced as a N terminal fusion tag.

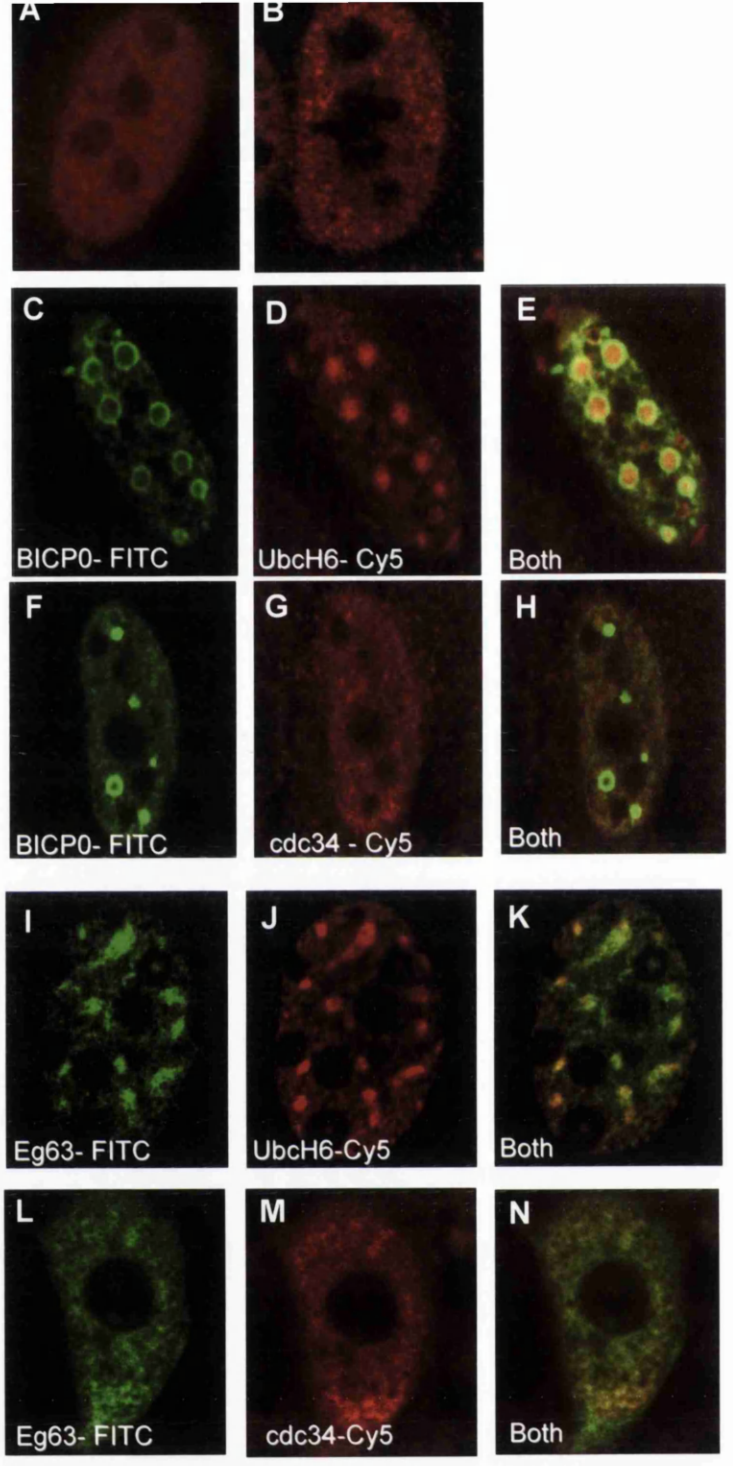

Figure 4.1.6A. *Immunofluorescence of BICP0 and Eg63 with UbcH6 or CDC34 in vivo.*

Figure 4.1.6B. *Immunofluorescence of EP0 and Vg61 with UbcH6 or CDC34 in vivo.*

HEp-2 cells were transfected with plasmids expressing tagged (FLAG) E2 enzymes, UbcH6 or cdc34 and the tagged (rtag) ICP0 homologues, EP0 and Vg61. The antibodies were used at the following concentrations, anti-FLAG tag monoclonal M2 (Sigma) was used at 1/300, with the secondary antibody FITC goat anti-mouse (IgM) (Sigma 1/100); the primary antibody anti-r113 1/5000, followed by the secondary Cy5-conjugated goat anti-rabbit IgG (Amersham; 1/500). A) The Cy5 labelled UbcH6 control. B) The Cy5 labelled cdc34 control. (C to E) The single and merged channels for cells coexpressing EP0 and UbcH6. (F to H) The single and merged channels for cells coexpressing EP0 and cdc34. (I to K) The single and merged channels for cells coexpressing Vg61 and UbcH6. (L to N). The single and merged channels for cells coexpressing Vg61 and cdc34.

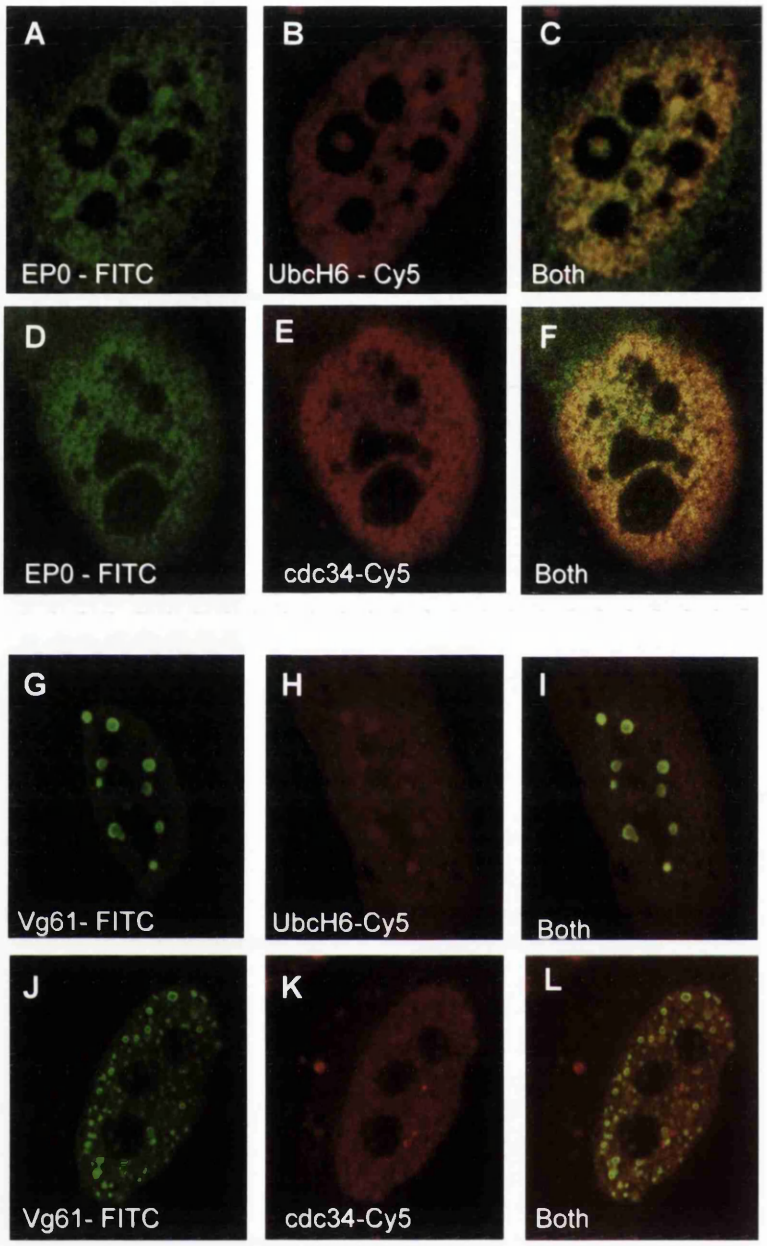

Figure 4.1.6B. *Immunofluorescence of EP0 and Vg61 with UbcH6 or CDC34 in vivo.*

enzymes UbcH6 and cdc34 were diffusely distributed, mostly in the nucleus of the cell (figure 4.1.6A, panel A and B). In the presence of a co-expressed member of the ICP0 family, UbcH6 was partially sequestered in co-localising foci, particularly in the presence of BICP0 (figure 4.1.6A, panel C to E), Vg61 (figure 4.1.6B, panel G to I), Eg63 (figure 4.1.6A, panel I to K.), and to a lesser extent with EP0 (figure 4.1.6, panel A to C). However, it was clear that none of the ICP0 related proteins, BICP0 (figure 4.1.6B, panel F to H), Eg63 (figure 4.1.6A, panel L to N), EP0 (figure 4.1.6B, panel D to F), or Vg61 (figure 4.1.6b, panel J to L), sequestered cdc34 into colocalising foci. These results are very reminiscent of those obtained with ICP0 itself (Boutell *et al.*, 2002).

4.1.7 Conclusion of the immunofluorescence using the ICP0 related proteins.

The results shown in figure 4.1.6A and 4.1.6B are typical of the immunofluorescence observations seen with the members of the ICP0 family of proteins and the E2 enzymes UbcH6 and cdc34. From repeated experiments, the following interesting consistencies were observed: BICP0 tended to form large arcs or rings (figure 4.1.6A, panel C) while sequestered UbcH6 (figure 4.1.6A, panel D) were localised within the BICP0 ring-like structures. Consequently, when the channels are merged (figure 4.1.6A panel E), BICP0 is distributed around the periphery of the foci and UbcH6 is in the middle. From 4.1.6B panel F and G it can be seen that formation of BICP0 foci occurs in the presence of cdc34 although cdc34 is not recruited into the BICP0 foci. EP0 (figure 4.1.6B, panel A to C) also sequestered UbcH6 (figure 4.1.6A, panel A), although foci formation by this member of the ICP0 related proteins was rare, normally limited to only a few cells in the sample and frequently the foci were located only in the nucleolus. When either Eg63 (figure 4.1.6A, panel L to N) or EP0 (figure 4.1.6B, panel D to F) proteins were expressed in the presence of cdc34 it was not possible to observe foci formation in cells, even though immunostaining was indicative of co-expression. This may be due to expression levels from the transfected plasmids encoding Eg63 and EP0, although this series of experiments was repeated with varying concentrations of plasmid to eliminate this possibility. Finally, Vg61 (figure 4.1.6B, panel J to L) formed foci in the presence of co-expressed cdc34 (although there was no co-localisation), however these foci were distinctly smaller than the foci observed when transfected with UbcH6 (figure 4.1.6B, panel G to I). From the immunofluorescence it could be seen that each of the ICP0

homologues formed foci that were characteristic of that specific protein and these results replicate observations made previously using the same expression system (Parkinson & Everett, 2000, 2001).

Chapter 4 - Part III – Complementation of an ICP0 negative virus with the ICP0 related proteins.

4.2.0. Introduction to the complementation assay.

The HSV-1 strain *dl*1403 (Stow & Stow, 1986) is an ICP0 negative virus that initiates lytic infection inefficiently, especially in low MOI infections of certain cell lines (Everett *et al.*, 2004). This phenotype is particularly apparent in limited-passage human fibroblast cells and to a lesser degree in BHK and Vero cells. However, in U20S cells ICP0 is not required for efficient HSV-1 infection and *dl*1403 replicates as efficiently as a wild type virus (Yao & Schaffer, 1995). In cells where the mutation does limit growth, the phenotype is multiplicity dependent and can be overcome at high MOI (Sacks & Schaffer, 1987). However, at a low MOI the ICP0 negative virus becomes quiescent, although the lytic cycle can be initiated by introducing ICP0 into the cells (Harris *et al.*, 1989; Preston, 2000; Stow & Stow, 1989). This series of experiments analyses whether the ICP0 related proteins, like ICP0, can overcome the cellular repression mechanisms that prevent expression from the quiescent *dl*1403 genomes, thus enabling the lytic infection to be resumed.

4.2.1. Optimisation of the complementation assay of dl1403 by the ICP0 related proteins.

Initially to deduce the optimal amount of plasmid encoding the ICP0 related proteins that was needed to complement *dl*1403 and to act as a basis for future experiments, Vero cells were seeded on 35 mm plates and cotransfected with 100 ng - 300 ng of pCIrtag plasmids encoding ICP0, BICP0, Eg63 or pCIrtag alone (negative control) and 2 μg of *dl*1403 DNA (section 2.7C). Following transfection, the cells were incubated for 72 hours, where upon the plaques were counted (see table 4.2.1). The tabulated data was also plotted for easier comparison (see figure 4.2.1A) of the degree of complementation that occurred (inferred from the number of plaques). The results show that it is possible to complement *dl*1403 with a plasmid encoding HCMV IE driven ICP0 or an ICP0 related protein using the transfection method. Furthermore, the results indicate that complementation was most efficient using 200 ng of plasmid encoding ICP0 or the

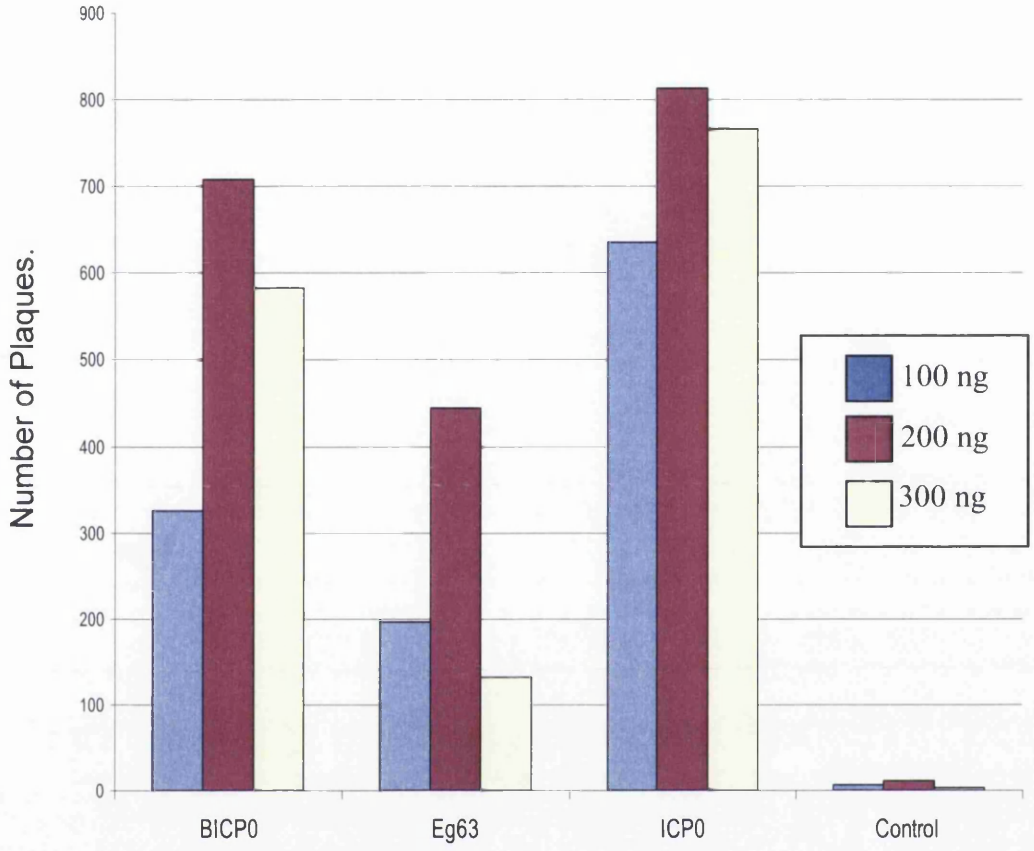

Figure 4.2.1A. *Optimisation of the complementation assay of the ICP0 negative virus dl1403.*

The members of the ICP0 are shown along the X axis and the number of plaques are along the Y axis. 35 mm dishes seeded with Vero cells were transfected with 2 μg of *dl*1403 DNA and 100 – 300 ng of plasmids encoding ICP0 or the ICP0 related proteins. At 72 hours post transfection the plaques were counted following Giemsa staining (see methods section for further details). pUC9 was used as a negative control. The legend indicates the amount of plasmid in ng encoding ICP0 or the ICP0 related protein or the negative control.

ICP0 related protein, and 2 μg of *dl*1403 DNA. Following these initial positive results all of the plasmid encoded ICP0 related proteins were tested in this assay.

	Number of plaques			
Amount of plasmid	BICP0	Eg63	ICP0	Negative control
100 ng	326	196	636	7
200 ng	508	445	813	12
300 ng	703	132	767	3

Table 4.2.1. Optimisation of the complementation assay of *dl*1403.

4.2.2. Complementation assay of dl1403 using full-length members of the ICP0 family of proteins.

The aim of this experiment was to test whether all of the ICP0 family were able to complement *dl*1403. The method used was identical to that used in section 4.2.1. The experimental data was variable between different experiments due to varying transfection efficiencies, therefore to make the data more comparable between replicate experiments the fold activation was calculated (see table 4.2.2). The mean fold

	BICP0		Eg63		EP0		Vg61		ICP0		Control	
	Plq#	FdA#	Plq#	FdA#	Plq#	FdA#	Plq#	FdA#	Plq#	FdA#	Plq#	FdA#
1	328	109	98	33	101	34	25	8	438	146	3	-
2	172	86	80	40	50	25	10	5	210	105	2	-
3	420	140	186	62	129	43	45	15	531	177	3	-
Mean	-	112	-	45	-	34	-	9	-	143		

Table 4.2.2. Complementation of *dl*1403 with pCI-rtag plasmids expressing members of the ICP0 family of proteins.

Plq# = Number of plaques. FdA# = Fold activation. The fold activation was calculated by number of plaques for each homologue per experiment divided by that of the negative control. The mean was taken of the fold activation and was plotted in figure 4.2.2A.

activation and standard error of the mean are shown in figure 4.3.1A. The complementation assays demonstrate how efficiently the members of the ICP0 family complement *dl*1403. ICP0 gave the highest fold activation and thus complements *dl*1403 most efficiently. From the results with the other members of the ICP0 family it can be seen that BICP0 has the greatest activity, whereas Eg63 and EP0 are significantly

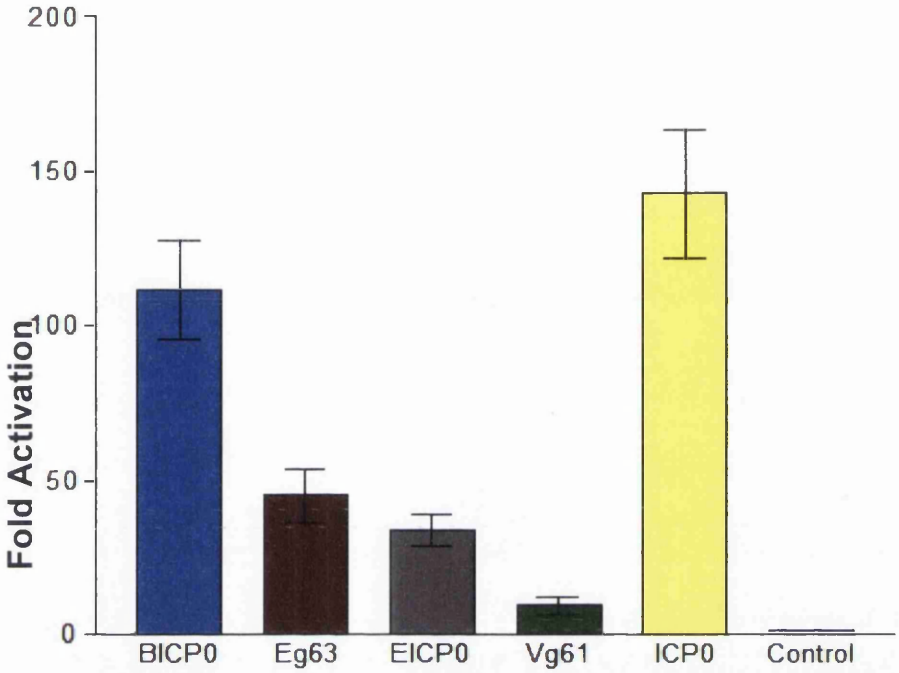

Figure 4.2.2A. *Complementation of the ICP0 negative virus dl1403.*

The ICP0 homologues are shown along the X–axis and fold activation is shown along the Y-axis. pUC9 was used as a negative control. 35 mm dishes seeded with Vero cells were transfected with 1 μg of *dl*1403 DNA and 200 ng of plasmid encoding ICP0 or the ICP0 related proteins. At 72 hours post transfection the plaques were counted following Giemsa staining (see methods section for further details) and the fold activation was calculated. The fold activation is plotted in conjunction with the standard error of the mean, shown by the error bars.

less efficient, with both having a similar effect. Finally, Vg61 gave the lowest level of complementation. The control indicates that in Vero cells without the presence of a co-transfected plasmid expressing a member of the ICP0 family, the recovery of virus from transfected *dl*1403 DNA was very low under the conditions employed. These data indicate that the members of the ICP0 family of proteins can complement an ICP0 negative virus and relieve cellular repression mechanisms so that the lytic cycle can commence.

4.2.3. The significance of the complementation assay of the dl1403 by the ICP0 related proteins.

The results in section 4.2 confirm what is already known and expand upon previous studies of cross-complementation of the ICP0 related proteins of BHV-1, EHV-1, PRV and VZV. It has been previously shown that the ICP0 related proteins encoded by ORF 61 of VZV and ICP0 of HSV-1 can complement a PRV EP0 deletion mutant in cell culture (Moriuchi et al., 1995) and reactivate it from latency in animal models (Smith & Cheung, 1998). Furthermore, cell lines expressing ORF61 partially complement the growth of HSV-1 ICP0 negative mutants which fail to express functional ICP0 (Moriuchi *et al.*, 1992). The Eg63 protein of EHV-1 was also shown to partially complement a HSV-1 ICP0 deficient virus (Everett *et al.*, 1995b). The results with Eg63 and Vg61 support the previously published data (Everett *et al.*, 1995c; Moriuchi *et al.*, 1992). The data in section 4.2 indicate that BICP0 of BHV-1 and EP0 of EHV-1 are also able to complement an HSV-1 ICP0 deletion mutant. Significantly, the degree of complementation (inferred from the level of fold activation) varies amongst the ICP0 related proteins; BICP0 was the most efficient at complementing an ICP0 negative virus, with Eg63, EP0 and Vg61 only being able to carry out this to a lesser degree. However, when making this conclusion it is important to take in to account the relative transfection efficiencies of the different plasmids, and probably the degree of expression of the individual ICP0 family members. From the immunofluorescence work carried using the plasmids to express the ICP0 related proteins shown earlier in this chapter and published previously (Parkinson & Everett, 2000, 2001), it is possible to conclude that using the same amounts of plasmids, BICP0 and Vg61 were expressed more efficiently than Eg63 and EP0. This is an important factor to take into account when deducing which ICP0 related protein most efficiently complements *dl*1403. If Eg63 and EP0 could be expressed at levels similar to those of the other ICP0 related proteins, the

degree of complementation may have been higher, perhaps closer to that of BICP0. However, despite Vg61 being expressed well in transfected cells, it had the lowest activity in the complementation assay. Therefore, out of the all of the ICP0 related proteins tested, Vg61 was the least efficient at complementing the ICP0-null virus *dl*1403.

Chapter 5 - General Discussion.

5.0.1. The alphaherpesvirus homologues of ICP0 act as ubiquitin E3 ligases.

The role of ICP0 as an ubiquitin E3 ligase is becoming increasingly well characterised, from initial studies that showed ICP0 binds to USP7 (Everett *et al.*, 1997) and disrupts constituents of ND10 (reviewed in Everett, 2001) to the most recent that have characterised the ICP0-mediated ubiquitination of p53 *in vitro* (Boutell & Everett, 2003b, 2004). However, unlike ICP0, the functions of its homologues in other members of the alphaherpesvirus family were relatively unclear. It had been previously shown BICP0, Eg63, EP0 and Vg61 to varying degrees cause the formation of colocalising conjugated ubiquitin and disrupt the constituents of ND10 and in some cases centromeres (Parkinson & Everett, 2000, 2001). Furthermore, Eg63 had been shown to disrupt ND10 in a proteasome-dependent manner (Parkinson & Everett, 2000). Collectively, this research provided strong evidence that the ICP0 family of related protein functions via an active ubiquitin-proteasome degradation pathway. However, due to the variability in the result observed by Parkinson & Everett (2000 & 2001) and the fact the homology of the ICP0 related proteins is largely limited to the structurally coordinating residues of their N-terminal RING finger domains, it was not conclusive that the ICP0 related proteins all worked via the same mechanism to disrupt ND10.

This study shows that despite the difference in the intrinsic biological properties and their limited homology, the members of ICP0 family of proteins all function as ubiquitin E3 ligases, in a manner similar to one another and to that of ICP0. By using the isolated RING finger domains, it was shown that the ICP0 related proteins stimulate the same E2 partners as ICP0. Furthermore, in the case of BICP0 the E3 ligase activity of the full-length protein appeared to be similar to that of its isolated RING finger domain (GST-BICP0). Despite the fact that the E3 ligase activity of all of the full-length ICP0 homologues was not characterised, the data suggests the isolated RING finger domain provides an accurate indication of E3 ligase activity and E2 specificity. This is supported by previous research that showed full-length ICP0 has similar E3 ligase activity to its isolated RING finger domain (ICP0-241) (Boutell *et al.*, 2002). Transfection assays further illustrated the fact that the ICP0 family of proteins have E3

ligase activity similar to that of ICP0, and they were shown to colocalise with exogenous UbcH6 in transfected cells.

Previous research has generally shown the ICP0 homologues share similar biological properties. In particular, they are all strong transactivators of heterologous and homologous promoters in gene expression assays (see section 1.8.0. and references therein). Furthermore, this study has shown that to varying degrees the ICP0 related proteins can complement an ICP0-null virus. In addition, the ICP0 related proteins were shown to function as E3 ligases in a similar manner, *in vitro.* Consequently, one of the most significant variations in the biological properties of the ICP0 family of proteins is their ability to disrupt constituents of ND10 (Parkinson & Everett, 2000, 2001).

It has been proposed that ND10 play an essential role in repressing viral gene expression and disruption of a characterised or as yet uncharacterised constituent by ICP0 may be crucial in allowing HSV-1 replication to resume. Based on this hypothesis, the differing ability to disrupt ND10 may contribute to the variation in the biological properties amongst the alphaherpesviruses. It is possible that the alphaherpesviruses that more readily disrupt ND10 or its constituents will be more likely to reactivate from latency. For instance, VZV is associated with one or two reactivativation events throughout the lifetime of the infected host, and Vg61 least readily disrupts certain components of ND10 (see figure 1.8.6A), thus preventing the removal of a potential ND10 viral repression mechanism. However, it should be considered that the constituents of ND10, which VZV may more readily target are yet to be identified, or that this could possibly be a deliberate function of the virus to ensure that it remains latent. The latter has been shown to be the case for other herpesviruses such as EBV, which tightly controls the balance between lytic/latent infections via a number of proteins. One such protein is LMP-2A that has a role in interfering with the signalling cascade that would lead to the induction of the lytic cycle in infected B cells (Masucci, 2004).

An additional mechanism, which could determine frequency of viral reactivation amongst the alphaherpesviruses, is substrate interaction and specificity. Previously, ICP0 has been shown to interact with p53 via residues within its C terminus (Boutell & Everett, 2003b). It is therefore possible as sequence homology amongst the ICP0 family of proteins is largely limited to the RING finger domain and the divergent sequence out side of it may determine which cellular or ND10 components and indeed to what degree

become degraded. The varying RING finger flanking sequence amongst the ICP0 family of proteins may therefore be determining specificity and affinity for substrate proteins that could be part of a ND10-mediated viral repression mechanism. For example, ICP0 readily disrupts ND10 substructures unlike Vg61, consequently this may partially explain why VZV reactivates less frequently than HSV from latency.

5.0.2. Characterisation of the E3 ligase activity of Eg63.

The E3 ubiquitin ligase activity of the ICP0 homologue Eg63 was extensively characterised within these studies. Initially, it was shown that the isolated RING finger domain of Eg63 (GST-Eg63) has E3 ligase activity similar to that of the other GST-ICP0 related proteins. However, upon deletion of approximately 40 amino acids from the C-terminus of GST-Eg63, to create GST-Eg63 1_63, caused nearly a complete abrogation of the protein's ability to stimulate UbcH6, but not UbcH5a, in the ubiquitin ligase assay. It was possible the inability of GST-Eg63 1_63 to stimulate UbcH6 may have been due to its failure to bind to the E2, suggesting that the deleted residues from the C-terminal side of the core RING domain may play an essential part in their interaction. However, it was also possible the deletion caused the two proteins to interact in a non-productive manner, whereby Eg63 1_63 sequesters UbcH6. The interaction assay showed that despite deletion of residues from the C-terminal side of the core RING domain, a stable interaction still occurred between GST-Eg63 1_63 and UbcH6. The inhibition assay using an optimised amount of GST-Eg63 showed that at the amount used GST-Eg63 1_63 did not sequester UbcH6. However, this possibility cannot be ruled out, the results observed may have been due to an insufficient amount of GST-Eg63 1_63 being present in the reaction to sequester UbcH6, therefore at a higher amount a decrease in poly-ubiquitin formation by GST-Eg63 may have been observed. Conversely, an excess of UbcH6 may have been present in the reaction, therefore by using a lower amount a decrease in the activity of GST-Eg63 may have been detected.

5.0.3. Autoubiquitination of the ICP0 homologues in vitro.

ICP0 has been shown to autoubiquitinate itself *in vitro* (Boutell *et al.*, 2002) and *in vivo* (Canning *et al.*, 2004). In the latter case, it is targeted for proteasome-mediated degradation, promoting its own turnover. To promote its own stability ICP0 interacts with USP7 (ubiquitin-specific protease 7), a member of a family of cellular enzymes

that cleave ubiquitin from either alpha or isopeptide-linked chains, via residues located within its C-terminus (Everett *et al.*, 1997). Research has shown that if the ICP0-USP7 interaction is in some way inhibited, the ability of ICP0 to function in gene expression and viral growth assays is greatly reduced (Everett *et al.*, 1999d), as the ICP0-USP7 interaction has been shown to promote the stability of ICP0 *in vivo* (Canning *et al.*, 2004).

The results shown in this study indicate the ICP0 homologues, like ICP0, undergo autoubiquitination *in vitro*. However, unlike ICP0, its homologues used in this study do not bind to USP7, nor indeed contain a USP7 binding domain. On the face of it, this would suggest the ICP0 homologues are more susceptible to autoubiquitination and subsequent proteolysis than ICP0. However, this is on the assumption that the ICP0 homologues are equally as susceptible to autoubiquitination. From table 5.0.3A, it can be seen that BICP0, Eg63 and EP0 contain fewer lysine residues than ICP0. Furthermore, as determined by the Poisson distribution the number of lysine residues present in each of the ICP0 related proteins is far less than would be expected. The ICP0 homologues of HSV-1, HSV-2 and HVB contain more lysine residues than BICP0, Eg63 and EP0, but additionally they contain a USP7 binding site. Therefore, it is possible that two mechanisms have evolved to protect the stability of the ICP0 homologues *in vivo*. Firstly, BICP0, Eg63 and EP0 do not contain a USP7 binding site, however to compensate they only contain a few lysine residues; therefore by reducing the number of residues, this would decrease the level of autoubiquitination and subsequent chance of proteolysis. Furthermore, the folding of the protein may limit the

ICP0 Homologue	USP-7 binding site.	K	Length (a.a.)	Exp K
EP0	*No*	1	410	21.3
BICP0	*No*	2	676	35.2
Eg63 (ICP0) HSV-1	*No*	3	532	27.7
ICP0 (HVB)	*Yes*	8	775	40.3
ICP0 (HSV-2)	*Yes*	6	699	36
Vg61	*No*	6	825	42.9
		24	467	24.2

Table 5.0.3A *The number of lysine residues within the ICP0 family of proteins. The workshown was carried out by D. Gatherer.*

The table shows the actual number and expected (Exp) number of K residues as determined by the Poisson distribution (calculations not shown) within each of the ICP0 related proteins.

number of lysine residues susceptible to autoubiquitination and the level of viral expression of BICP0, Eg63 or EP0 may be sufficient to compensate for the level of proteolysis that occurs from autoubiquitination. Second, ICP0 and its homologues in HSV-2 and HVB contain a larger number of lysine residues, which is still significantly lower than the number expected (see table 5.0.3). Therefore, it is equally possible these proteins have evolved to contain a low number of lysine residues to limit proteolysis in the cell. However, the USP7 binding domain may provide an additional safeguard to limit proteolysis due to the increased number of lysine residues compared to BICP0, Eg63 and EP0. This theory is supported by the fact that BICP0 is most similar in size to ICP0, however it does not contain a USP7 binding domain, but may compensate to limit autoubiquitination by containing only two lysine residues to that of ICP0's eight. The potential increased stability of BICP0 may partially explain why it has been shown to have a phenotype most similar to that of ICP0, when it comes to disrupting ND10 and its various constituents (see Parkinson & Everett, 2000 and figure 1.8.6A) and complementing an ICP0-null virus. It is also important to consider that the human genome encodes at least 90 potential deubiquitinating enzymes (Chung & Baek, 1999) many of these deubiquitinating enzymes are thought act as a "proof-reader" targeting a specific subset of ubiquitinated proteins (Chung & Baek, 1999). However, BICP0 or any other of the ICP0 homologues that cannot bind to USP7 may maintain their stability *in vivo* by binding to a yet uncharacterised deubiquitinating enzyme. Furthermore, as the aforementioned homologues contain only a low number of lysine residues and subsequent autoubiquitination may be relatively low, the non-specific activity of certain deubiquitinating enzymes may be sufficient in helping to promote their stability.

Despite the majority of the ICP0 homologues containing a low number of lysine residues, Vg61 contains 24, which is equal to a protein of its predicted size. This may mean Vg61 undergoes a significantly higher amount of autoubiquitination and the protein is intrinsically less stable than the other ICP0 related proteins *in vivo*. Furthermore, this may explain why out of all of the ICP0 homologues it has a phenotype least similar to ICP0, as assessed by its ability to complement an ICP0-null virus and disrupt ND10 and its various constituents. The phenotype of Vg61 is most similar to ICP0 variants that contain mutations within their USP7 binding domain as they degrade PML and Sp100 with reduced efficiency (Parkinson & Everett, 2000) and their activity in gene expression and viral growth assays was also reduced (Everett *et al.*, 1999d). The phenotype of an ICP0-USP7 binding domain mutant was thought to be partially

explained by a decrease in the stability of ICP0 due to an inability to prevent autoubiquitination and subsequent proteolysis (Canning *et al.*, 2004). Therefore, the large number of lysine residues in Vg61 and its potentially increased susceptibility to autoubquitination and subsequent proteolysis may explain why its phenotype is more similar to that of an ICP0-USP7 mutant, than wild-type ICP0. The may also contribute to the reduced levels of reactivation VZV undergoes compared to that of HSV-1, due to inability of Vg61 to relieve the potential repression mechanism of viral gene expression imposed by ND10, this is discussed in more detail in section 5.0.1A.

5.0.4. The phenotype of the ICP0 (241) RING finger domain mutants.

The RING finger mutants of ICP0 (241) used in this study (see figure 3.6.1A), were originally derived from full-length ICP0 mutants, which have been extensively characterised elsewhere. It had been shown that the majority of the mutations had an extremely deleterious affect on full length ICP0's ability to simulate gene expression, disrupt ND10 in transfection assays and colocalise with conjugated ubiquitin, *in vivo* (Everett *et al.*, 1995a; Everett, 1987, 2000). The most notable exception was the point mutation W146A, as it had little affect on the biological properties of ICP0. Theses results were surprising as the tryptophan residue is conserved in the majority of RING finger E3 ligase enzymes and has generally been shown to be exceptionally deleterious affect to the function of a protein (Freemont, 2000; Joazeiro & Weissman, 2000; Pickart, 2001a).

The ICP0 (241) mutants were also used to characterise the E3 ligase activity of the RING finger domain of ICP0 *in vitro* (Boutell *et al.*, 2002). It was shown that point mutations within the RING finger domain of ICP0 (241) (K144E, W146A, Q148E and N151D) and the insertional mutant *ins*197, had little or no deleterious affect on the E3 ligase activity of the protein (Boutell *et al.*, 2002). However, *in vivo* the mutations K144E or N151D were previously shown to be deleterious on full-length ICP0's ability to colocalise with conjugated ubiquitin (Everett, 2000). Collectively, this suggests the K144E an the N151D mutations of the RING finger domains inhibit ICP0 function as an E3 ligase *in vivo*, but have a limited affect on this activity *in vitro*. The insertional mutants of ICP0-241 (ins150, ins162 or ins188) had an exceptionally deleterious affect on ICP0's ability to facilitate the formation of poly-ubiquitin chains *in vitro* (Boutell *et al.*, 2002), and colocalise with conjugated ubiquitin *in vivo* (Everett, 2000). However,

overall the *in vitro* data characterising the E3 ligase activity of the ICP0-241 mutants, correlated well with its ability to colocalise with conjugated ubiquitin.

This study shows that despite the varying affects the mutations had on the biological properties of ICP0, generally all of the mutants appeared to form a stable interaction with UbcH5a and UbcH6. In fact, compared to ICP0 (241) the ability of ICP0 (241) mutants to form stable interactions with UbcH5a and UbcH6 was enhanced, as ICP0 (241) was unable to form a stable interaction with UbcH5a. As mentioned previously, the inability of ICP0-241 and certain other members of the ICP0 family to form a stable interaction with UbcH5a may be due to assay conditions or the quality of the reaction components. However, it could be down to the kinetics of the E2/E3 interaction. Traditionally, based on the structural characterisation of various RING finger E3s and their E2 partners, it is thought that the two proteins form a rigid stable interaction. The role of the E3 ubiquitin ligase is to position the E2 and substrate for catalysis (Pickart, 2001a; Weissman, 2001). However, what this model does not consider is that when a substrate becomes poly-ubiquitinated, it grows at 8 KDa per reaction cycle, therefore how does this rigid structure accommodate the increasing size of the ubiquitinated substrate? Deffenbaugh et al., (2003) used the multi-subunit RING E3 ligase SCF (see section 1.6.4A) and the E2 enzyme cdc34, to propose the 'hit and run' model of substrate ubiquitination. The kinetics of the reaction between the E2 and E3 was calculated and indicated that instead of forming a rigid structure, the E3 complex is in dynamic association/dissociation equilibrium with ubiquitin-charged cdc34 (Deffenbaugh *et al.*, 2003). The substrate only becomes ubiquitinated when it binds to the E3 complex whereupon the ubiquitin charged E2 is released prior to substrate ubiquitination. Therefore, the E3/E2 interaction under normal conditions may only occur in a transient manner.

It is possible using the 'hit and run model' that UbcH5a only forms a transient interaction with ICP0 (241). However, when the RING finger domain of ICP0-241 is mutated, structural changes prevent a transient interaction occurring, thus a stable interaction could now be detected using the GST-pull down method, as shown. The following question then arises, if the 'hit and run model' is correct why does non-mutated ICP0 (241) form a stable interaction with UbcH6? It is possible that during the GST-pull down assay UbcH6 is not in a conformation that would allow a transient interaction to occur.

5.0.5. Further work to characterise the ICP0 family of proteins.

Characterising the E3 ligase activity of the ICP0 family of proteins.

An initial area of future research would be to optimise the expression and purification of the full-length ICP0 homologues. As mentioned previously the recombinant baculoviruses that expressed Eg63 or EP0 were unstable, therefore repeating the baculovirus method, but introducing a plaque purification stage may lead to the generation of more stable recombinants. In addition, it would be necessary to develop a purification strategy for Vg61 as it was insoluble using the method previously optimised for ICP0. Once a full set of functional recombinant baculoviruses had been gained, they could initially be used to confirm the E3 ligase activity of the full-length ICP0 homologues was similar to their isolated RING finger domains. Furthermore, the baculovirus containing the CMV promoter could be used as an effective tool to determine if the ICP0 homologues undergo novel protein-protein interactions or interact with UbcH5a or UbcH6 and indeed any other E2 ubiquitin conjugating enzymes *in vitro*.

Additional studies on the GST-ICP0 family of proteins would include, using the ICP0 (241) bank of mutants to determine if they have E3 ligase activity with UbcH6. This would be an important area for further work as deletion of C terminal residues from GST-Eg63 and GST-BICP0 had a deleterious affect on their E3 ligase activity in the presence of UbcH6. Therefore, using more 'subtle' mutations may provide insight into which residues or areas within or outside of the RING finger domain are essential for UbcH6 stimulation. Furthermore, it would be interesting to repeat the inhibition assay, but use GST-Eg63 1_63 titrated to a higher concentration, to determine if UbcH6 becomes sequestered and the E3 ligase activity of GST-Eg63 was inhibited.

Characterisation of Vg61.

As mentioned previously, Vg61 has 24 lysine residues, therefore in theory should make it particularly susceptible to autoubiquitination and proteasome mediated degradation. A recombinant baculovirus or plasmid based approach could be used to determine if Vg61 or indeed any other member of the ICP0 homologues undergo autoubiquitination *in*

vivo. Furthermore, if Vg61 is subjected to a larger degree of degradation *in vivo* compared to the other ICP0 homologous, it would interesting to see if larger concentrations of Vg61 in transfection assays caused the disruption of ND10 and other cellular components in a fashion more similar to ICP0. Furthermore, MG132 could be used to determine if inhibition of the proteasome improves the stability of Vg61 *in vivo*, furthermore it could be used to aid the identification of autoubiquitinated isoforms of Vg61. As Vg61 does not have a USP-7 binding domain it would be interesting to characterise how it protects its stability *in vivo*, therefore would make it a particularly interesting target for protein-protein interaction studies. Other areas of work could look at a complementation assay to determine how efficiently a Vg61-null virus is reactivated from latency by ICP0 compared to that of Vg61.

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