

**Studies of the ubiquitin conjugating (UBCv) enzyme
encoded by African swine fever virus**

by

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To Joyce,
Mum and Dad

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Abstract

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Ubiquitin conjugating (UBC) enzymes play a key role in eukaryotes during the post-translational modification of proteins by covalent attachment of ubiquitin. A gene was identified in the double stranded DNA genome of African swine fever virus (ASFV) which was predicted to encode a protein with high homology to eukaryotic UBC enzymes. This ASFV encoded enzyme (UBCv) was expressed in *E. coli* and was shown to have ubiquitin conjugating activity *in vitro*. Antisera against recombinant UBCv were used to detect UBCv in ASFV infected cells. UBCv was shown to be a cytosolic protein present throughout the early and late stages of ASFV replication and was packaged in ASFV virions. Attempts to inhibit UBCv activity during ASFV infection using antisense oligonucleotides were unsuccessful, and a recombinant ASFV mutant with the UBCv gene disrupted by the luciferase reporter could not be isolated. However, ASFV replication was impaired late in infection in TS20 cells at a temperature which inhibits the ubiquitin conjugating pathway.

No novel ubiquitinated proteins could be detected in ASFV infected cells by immunoblotting, although an unspecific increase of cellular ubiquitin conjugation was observed in early infection. However, virus factories were intensely stained late in ASFV infection by immunofluorescence using anti-ubiquitin antisera. In addition, several ubiquitinated structural proteins were detected in purified ASFV extracellular particles by both immunoblotting and immunogold electron microscopy. An 18 kDa ubiquitinated structural protein, probably localized in the virion periphery, was purified to homogeneity and the sequence of its N-terminal 10 amino acids was determined. The N-terminal sequence of this protein matched exactly the predicted product of a gene of unknown function encoded by the ASFV genome.

Acknowledgements

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Abbreviations

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| | |
|------|--|
| AMP | adenosine 5' monophosphate |
| AraC | cytosine arabinoside |
| ASF | African swine fever |
| ASFV | African swine fever virus |
| ATP | adenosine 5' triphosphate |
| bp | base pair |
| CIP | Calf intestinal phosphatase |
| CPE | cytopathic effect |
| cpm | counts per minute |
| DMEM | Dulbeccos modified Eagles medium |
| DNA | deoxyribonucleic acid |
| dNTP | 2'-deoxy- 5' triphosphate nucleotides |
| ds | double stranded |
| DTT | dithiothreitol |
| dUTP | 2'deoxyuridine 5' triphosphate |
| E1 | ubiquitin activating enzyme |
| E2 | ubiquitin conjugating enzyme |
| E3 | ubiquitin ligase |
| ECL | enhanced chemiluminescence |
| EDTA | ethylenediamine tetra-acetic acid |
| ER | endoplasmic reticulum |
| ETF | early transcription factor |
| FCS | foetal calf serum |
| FITC | fluorescein isothiocyanate |
| gpt | guanine phosphoribosyltransferase |
| GST | glutathione-S-transferase |
| HRP | horse radish peroxidase |
| IEF | isoelectric focusing |
| IgG | immunoglobulin G |
| IPTG | isopropyl-1-thio- β -D-galactoside |
| kb | kilobase pairs |
| kDa | kiloDaltons |
| LTF | late transcription factor |
| luc | luciferase |
| MGF | multigene family |
| MHC | major histocompatibility complex |
| MOI | multiplicity of infection |

| | |
|-------|---|
| mRNA | messenger RNA |
| Mwt | molecular weight |
| NP40 | Nonidet P40 |
| OD | optical density |
| OG | <i>n</i> -octyl- β -D-glucopyranoside |
| ORF | open reading frame |
| PAGE | polyacrylamide gel electrophoresis |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PEG | polyethylene glycol |
| Ppi | pyrophosphate |
| PVDF | polyvinyldifluoride |
| RNA | ribonucleic acid |
| SDS | sodium dodecyl sulfate |
| TEM | transmission electron-microscopy |
| TEMED | N,N,N',N'-tetraethylenediamine |
| TIR | terminal inverted repeats |
| Tris | tris(hydroxymethyl)amino ethane |
| Ub | ubiquitin |
| UBC | ubiquitin conjugating enzyme |
| UBCv | UBC encoded by ASFV |
| VV | Vaccinia virus |

Chapter 1

Introduction

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With the advent of international trade, the once remote and little known African swine fever (ASF) disease has become an important economic threat to the pig industry worldwide. The lack of prophylaxis for ASF and its potential devastating effect has spurred considerable interest in its causative agent, African swine fever virus (ASFV). The combined efforts of many laboratories since the 1960s have revealed many ASFV characteristics which indicate, much to the frustration of taxonomists, that ASFV is a resolutely unique virus. Molecular biology has recently opened several new insights into the understanding of the distinctive replication cycle of ASFV, one of which is the finding that ASFV encodes a ubiquitin conjugating enzyme. Since its discovery in eukaryotes a decade ago, the ubiquitin conjugation pathway has been found to play key roles in a growing number of apparently unrelated cellular processes. In addition, a disparate set of recent observations has led to the realization that ubiquitin conjugation is probably also relevant to virus infections. This view is supported by the particular case of ASFV which is the subject of this study.

1.1 African swine fever

1.1.1 Geographical distribution and control

The African swine fever (ASF) disease was first described in Kenya early this century (Montgomery, 1921) and was subsequently reported in many countries of sub-Saharan Africa (reviewed by Wardley *et al.*, 1983; Vinuela, 1985a; Wilkinson, 1989). The disease was first introduced outside Africa in 1957 when infected waste food was fed to pigs near Lisbon airport, Portugal. Ingestion of infected pig meat by pigs is also the suggested cause of many of the ASF outbreaks in Europe, the Caribbean and South America. Most of these outbreaks were successfully eradicated (France, Belgium, the Netherlands, the Caribbean and Brazil) but ASF is currently enzootic in sub-Saharan Africa, Portugal, Sardinia and Spain.

Since there is neither a vaccine nor a cure for ASF, the only effective measures for disease control are mass slaughter and strict import policies (Mackenzie, 1993). These rudimentary measures are not only difficult to enforce but are also very expensive, as illustrated by the \$45 million cost in 1978 of the Malta ASF eradication program. Similar ASF eradication programs, involving the destruction and re-stocking of the entire pig population, were also successfully carried out in the Dominican Republic, Cuba and Haiti. These eradication programmes were simplified by the insular nature of these countries. Despite colossal efforts, other countries such as Sardinia, Spain and Portugal have not managed to eradicate ASF, which underlines the need for new and effective methods of disease control.

1.1.2 Causative agent, host range and transmission

The difficulties encountered in the fight against the spread of ASF are partly due to the complex host range of ASFV. Domestic and wild pigs (*Sus scrofa*) are the only animals in which ASFV infection produces disease. However, the virus also replicates in warthogs (*Phacochoerus aethiopicus*), bush pigs (*Potamochoerus porcus*) and soft ticks (*Ornithodoros* genus) but produces no apparent disease (Detray, 1963; Plowright *et al.*, 1968, 1969). These apparently healthy carriers act as reservoirs of ASFV and

infected soft ticks are also an important disease vector since they can transmit ASFV to the pigs on which they feed (Wilkinson, 1981). Once ASFV is introduced into a pig population it may be transmitted in the absence of the tick vector by simple contact with infected pigs.

The disease caused by ASFV in pigs varies from peracute to subacute or even chronic depending on the ASFV isolate. The primary route of ASFV infection in pigs is the upper respiratory tract. The virus replicates in the tonsils and rapidly spreads to the lymph nodes of the head. Virus propagation from the lymphatic system to the blood follows shortly and generalized infection may occur as soon as 48 hours after contact exposure. Cells of the lymphoreticular system are the main targets for ASFV replication. The first sign of disease is fever which is followed by internal haemorrhages caused by impairment of the endothelial cell functions. Death usually occurs within seven days of the onset of clinical signs although subacute disease may be more prolonged.

While many isolates from Africa are highly virulent (100% mortality), other ASFV isolates such as in recent outbreaks in Spain and Portugal are less virulent (under 50% mortality) although they still produce high morbidity (Mebus and Daidiri, 1980). The pigs which recover from ASFV infection are thought to play an important role in the spread of ASFV since they may be persistently infected for periods of six months or more. Although recovered pigs are resistant to challenge with the homologous ASFV isolate, pigs challenged with heterologous isolates are usually not protected (Hess, 1971; Thomson *et al.*, 1979). Pigs which die from ASFV infection do so before an effective immune response is mounted, but surprisingly pigs that have recovered from the disease do not appear to produce neutralizing antibodies against ASFV (DeBoer, 1967; DeBoer *et al.*, 1969). However, resistance to ASFV infection can sometimes be induced by passive immunization with immunoglobulins from pigs that have recovered from a homologous ASFV infection (Hess, 1971; Wardley *et al.*, 1985), which indicates that other antibody dependent immune mechanisms are involved in protection. One recent report claims that the infectivity of virulent ASFV isolates is neutralized *in vitro* with convalescent swine serum (Zsak *et al.*, 1993), whereas previous findings have suggested the presence of antibodies which reduce but do not neutralize virus infectivity (DeBoer, 1967; Parker and Plowright, 1968; DeBoer *et al.*, 1969; Ruiz Gonzalvo *et al.*, 1986). Further understanding of the

unusual and somewhat controversial immune response to ASFV will be necessary if an effective vaccine against ASFV is to be produced.

1.2 African swine fever virus

1.2.1 Particle structure

The large icosahedral ASFV particles (figure 1.1) are similar to vertebrate iridovirus particles such as frog virus 3 (Breese and DeBoer, 1966; Pan *et al.*, 1970; Carrascosa *et al.*, 1984). The intracellular ASFV particles are approximately 190 nm across and are composed of three main concentric structures. The spherical nucleoprotein core is approximately 80 nm in diameter and is surrounded by an internal lipid membrane which is closely associated with the external protein capsid. In addition, extracellular ASFV particles are enveloped with a loose external membrane similar in structure to the cell unit membrane. The icosahedral structure of ASFV particles is conferred by the periodical arrangements of 13 nm hexagonal capsomers which form the virus capsid.

Extracellular ASFV particles contain at least 54 structural proteins with molecular weights ranging from 10 to 150 kDa (Tabares *et al.*, 1980a; Carrascosa *et al.*, 1985; Esteves *et al.*, 1986). The term 'structural protein' refers to a protein present in virions, but does not necessarily imply that it participates in the virion structure. Approximately one quarter of the ASFV structural proteins are suspected to consist of cellular proteins, such as actin and tubulins, that are packaged into virions or are spuriously co-purified with virions (Tabares *et al.*, 1980a; Carrascosa *et al.*, 1985; Esteves *et al.*, 1986). The location in the virion of certain major ASFV structural proteins was identified by electron-microscopy of immuno-gold labelled ASFV particles (Carrascosa *et al.*, 1986, 1993). The largest ASFV structural protein VP150 is situated in the virion nucleo-protein core and at the vertices of the capsid (one of these locations might be due to cross-reactivity with another virion protein), structural proteins VP37 and VP17 are found close to the internal lipid membrane, structural proteins VP72 is located in the virus capsid whereas VP12 is located outside the capsid, probably in the virus envelope. Extracellular ASFV particles do

not appear to contain any glycoproteins (Tabares *et al.*, 1983; Del Val *et al.*, 1986; Del Val and Vinuela, 1987) but two myristylated (28 and 13 kDa) and two phosphorylated (35 and 17 kDa) proteins are detected in virus particles (Salas, M.L. *et al.*, 1988; Aguado *et al.*, 1991). Finally in common with vaccinia virus (the prototype *orthopoxvirus*), several enzymatic activities are associated with ASFV particles (table 1.1), most of which are involved in the synthesis and processing of messenger RNA (discussed in §1.2.3).

| Reference | Enzyme Activity |
|---|---------------------------------|
| Kuznar <i>et al.</i> , 1980 | DNA dependent RNA polymerase |
| Salas <i>et al.</i> , 1981 | Poly A polymerase |
| Salas <i>et al.</i> , 1981 Neilan <i>et al.</i> , 1993 | RNA guanylyl transferase |
| Salas <i>et al.</i> , 1981 | RNA guanine-7-methyltransferase |
| Salas <i>et al.</i> , 1981 | RNA 2'-O-methyl-transferase |
| Vinuela, 1985a | Deoxyribonuclease |
| Salas <i>et al.</i> , 1983 | DNA topoisomerase |
| Kuznar <i>et al.</i> , 1981 | Nucleoside triphosphohydrolases |
| Polatnick <i>et al.</i> , 1974 | Protein kinase |
| Baylis <i>et al.</i> , 1993a | |

Table 1.1 Enzyme activities present in ASFV particles.

The enzyme activities detected in ASFV particles are indicated together with the relevant bibliographic references.

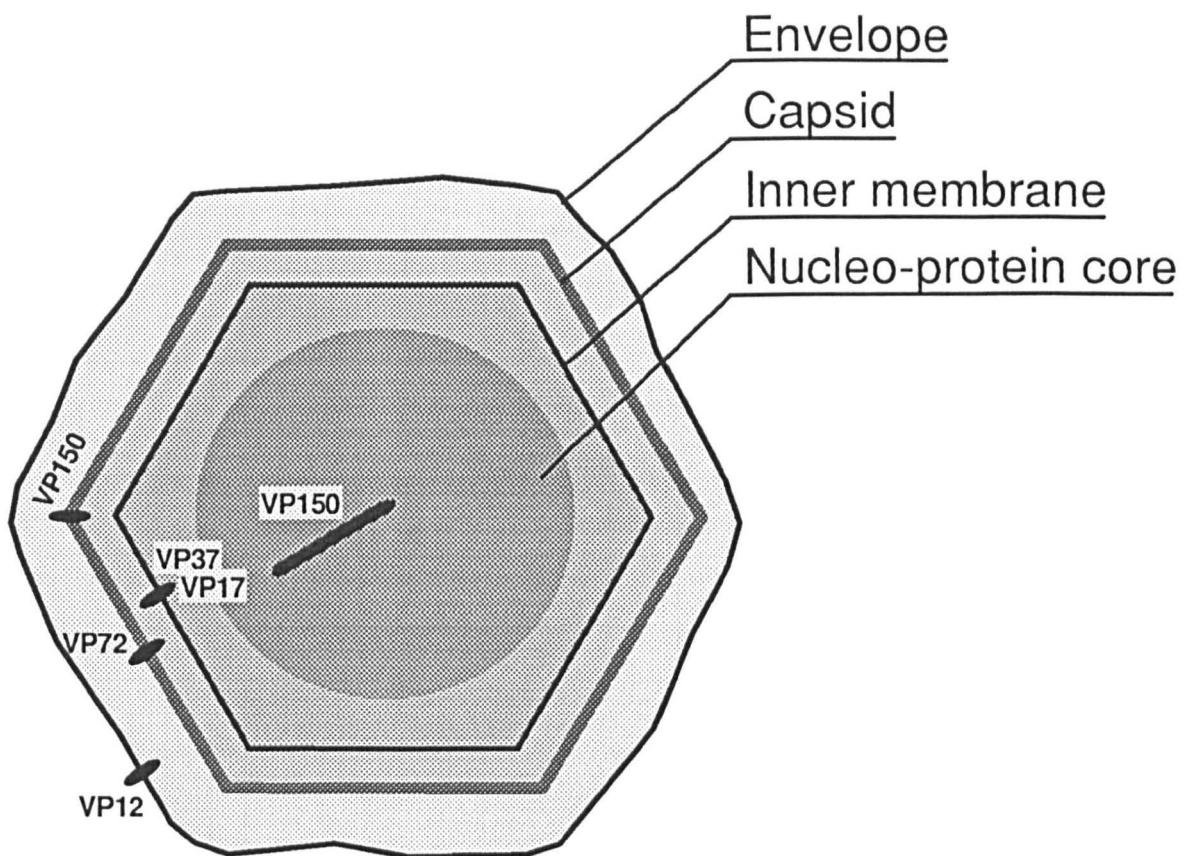


Figure 1.1 Structure of ASFV particles

A schematic representation of ASFV extracellular particles is indicated together with the location of major ASFV structural proteins (see §1.2.1).

1.2.2 Genome structure

The ASFV genome is composed of a linear double stranded DNA molecule (figure 1.2) with an A+T content of approximately 60% (Enjuanes *et al.*, 1976b; Vinuela, 1985a, b). The genome is between 170 and 190 kb in length and both its ends are cross linked by terminal hairpin loops into a single polynucleotide chain (Ortin *et al.*, 1979; Gonzalez *et al.*, 1986; Blasco *et al.*, 1989). The terminal hairpin loops consist of two inverted and partially paired 17 bp sequences that are almost entirely composed of A and T bases. The hairpin loops are followed by terminal inverted repeats (identical but oppositely oriented sequences at the two ends of the genome) which contain several sets of tandem direct repeats of unknown function (Sogo *et al.*, 1984; Vinuela, 1985a; Dixon *et al.*, 1993). Although additional arrays of tandem repeats are found in other parts of the genome, the sequences of the repeat units between the various arrays are unrelated. It is thought that these tandem repeats might act as recombination 'hot spots' or transcription regulatory signals.

Restriction endonuclease mapping of the genomes of several ASFV isolates reveals frequent insertions and deletions of up to 8 kb in defined regions of the genome (Wesley and Pan, 1982; Dixon and Wilkinson, 1988; Blasco *et al.*, 1989; Dixon *et al.*, 1990; Sumption *et al.*, 1990). With the exception of a single locus 90 kb from the left end of the ASFV genome, the central 100 kb portion of the genome is conserved in length whereas the regions near the ends of the genome (right hand 22 kb and left hand 48 kb) are more variable. Many of these variations are probably due to differences in the number of repeats in the arrays of direct tandem repeats. However, sequencing of the terminal genome regions indicates that many ASFV genome variations arise from deletions, duplications and translocations of redundant genes belonging to three different multigene families (MGF) (Almendral *et al.*, 1990; Gonzalez *et al.*, 1990; Vydelingum *et al.*, 1993). Members of the MGF 360 (so called because the genes of this family contain an average of 360 codons) are present at one or both ends of all the ASFV isolates sequenced. Members of MGF 110 are present in the majority of ASFV isolates although two virus clones were isolated which did not contain any MGF 110 members (Aguero *et al.*, 1990). Loss of MGF 110 did not reduce virulence of these virus clones in domestic pigs. Members of MGF 100 are located at the right end of the genome of the Malawi LIL20/1 ASFV

isolate but sequence information for this MGF is not yet available from other isolates (Vydelingum *et al.*, 1993). The function of these MGFs is unknown but their presence in many different isolates and the large part of the virus genome that they constitute (5 kb or more) suggests that they must be important for virus replication or transmission. Since the homologous genes of a MGF are all slightly different from each other, it is possible that these MGFs are a source of genetic diversity which allows ASFV to adapt to its various hosts and environments. The view that the ends of the ASFV genome are important for determining cellular tropism and host specificity but not for virus replication is supported by the observation that major deletions (up to 17 kb) occur in the genome termini upon adaptation of ASFV field isolates to tissue culture cells (Tabares *et al.*, 1987).

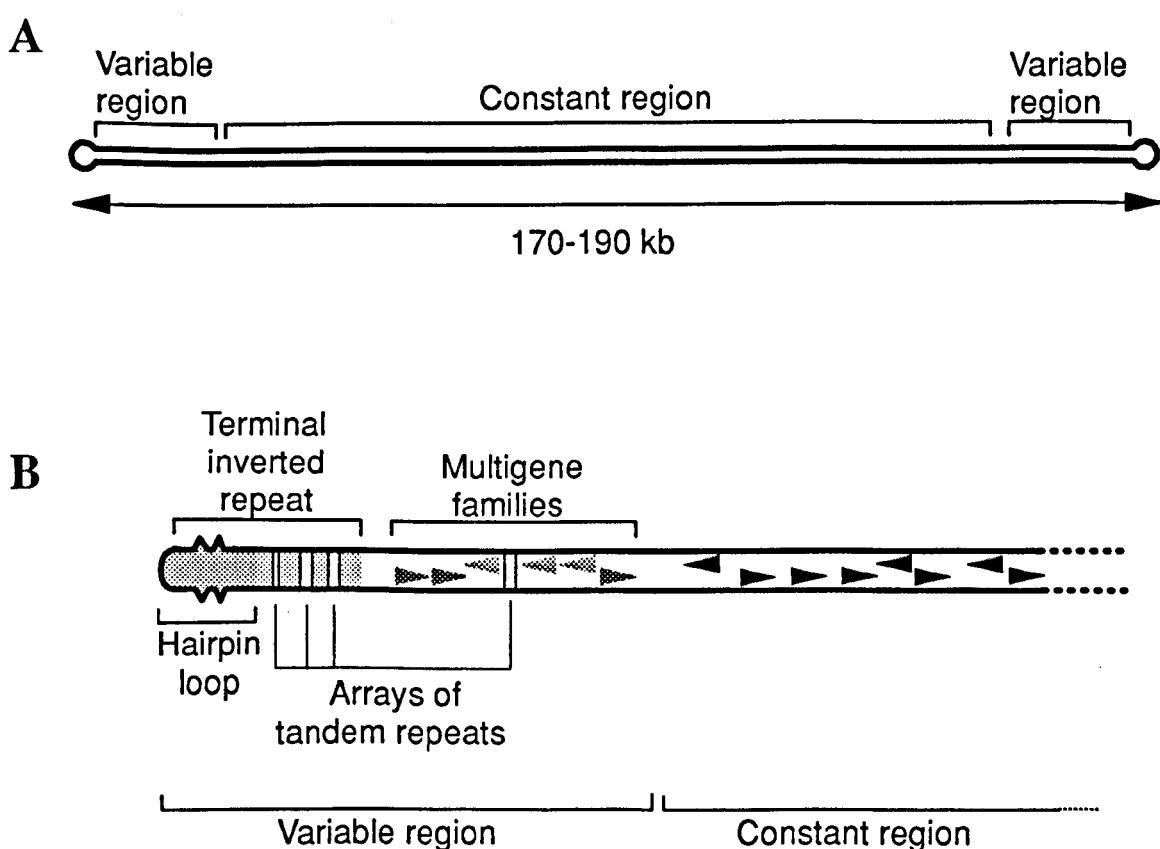


Figure 1.2 Structure of the ASFV genome

Panel A: Each strand of the ASFV DNA is represented by a single line. Panel B: detail (not to scale) of one of the genome termini shows the organization of terminal inverted repeats (TIRs), tandem arrays of direct repeats, cross linked terminal hairpin loops and multigene families (see §1.2.2)

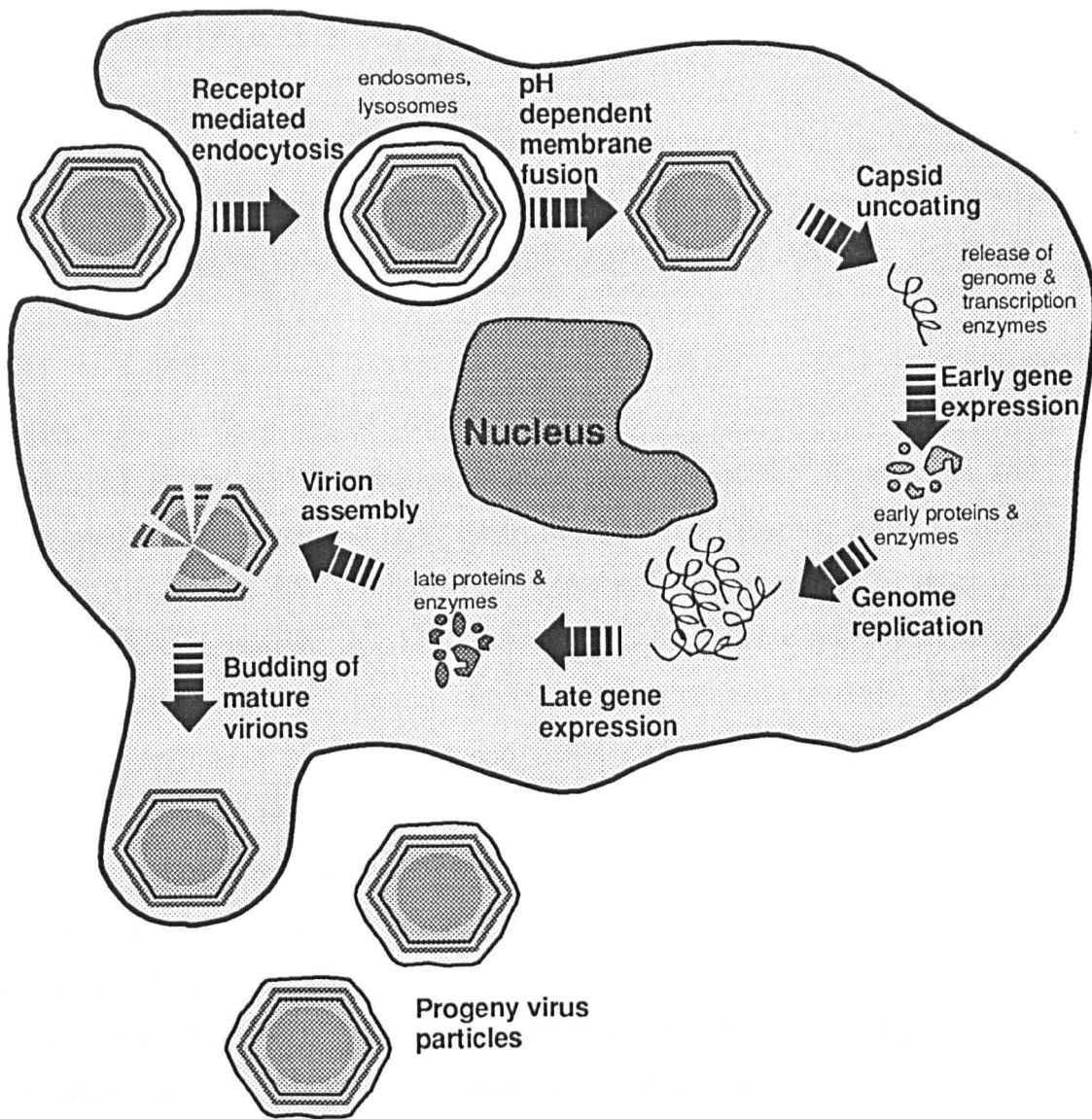


Figure 1.3 The replication cycle of ASFV in the host cell

The diagram shows the various events (virus entry, early gene expression, DNA replication, late gene expression, virus morphogenesis and virus release) that take place during the replication of ASFV in a permissive host cell (see §1.2.3).

With the exception of the presence of extensive MGFs and internal arrays of repeated sequences, the overall genome structure of ASFV described above is very similar to that reported for poxviruses (reviewed by Moss, 1990a, b).

1.2.3 Replication cycle

The replication cycle of ASFV in its host cell (summarized in figure 1.3) is remarkably similar to the replication of poxviruses such as vaccinia virus (Moss, 1990a). The sequence of events involved in the replication of ASFV is described below.

Virus entry

The natural target for ASFV infection in pigs are cells of the macrophage and monocyte lineage (Malmquist and Hay, 1960; Moulton and Coggins, 1968; Wardley *et al.*, 1979; Pan, 1987). ASFV binds to the target cell via a saturable receptor that is present on cells permissive to ASFV infection, such as swine macrophages and certain tissue culture cell lines, but not on cells that are resistant to ASFV infection such as rabbit or human macrophages and tissue culture L-cells (Alcami *et al.*, 1989b, 1990). Although this ASFV cell receptor has not yet been identified, it was shown *in vitro* that opsonization of ASFV particles with anti-ASFV immunoglobulins class G (IgGs) did not affect virus infectivity, even when the macrophage immunoglobulin Fc receptors (FcRs) were pre-saturated with non-immune antibodies (Alcami and Vinuela, 1991). These experiments indicate that the macrophage FcR does not participate *in vitro* in virus attachment or in antibody-dependent enhancement (ADE) of infectivity.

The virus attachment protein has been identified as a 12 kDa protein (VP12) which is present as a 17 kDa dimer in virus particles (Carrascosa *et al.*, 1991). The sequence of the ASFV gene encoding the VP12 attachment protein indicates that a 22 amino acid transmembrane domain is present in its 61 residues, suggesting that it is anchored in the ASFV envelope (Alcami *et al.*, 1992; Carrascosa *et al.*, 1993). The sequence of the gene encoding VP12 is highly conserved amongst tissue culture adapted and field isolates of ASFV (Angulo *et al.*, 1992) and anti-VP12 antibodies are readily detected in ASFV infected pigs (Angulo *et al.*, 1993). However, although

ASFV attachment to target cells is strongly inhibited *in vitro* by the addition of soluble recombinant VP12, none of the antisera directed against VP12 were shown to affect the binding of ASFV to the host cell or to neutralize virus infectivity (Angulo *et al.*, 1993). This suggests that the VP12 determinants responsible for the recognition of the cellular receptor are not accessible to antibodies or that an alternative virus envelope protein is also involved in virus attachment (Carrascosa *et al.*, 1993).

Following interaction with the cellular receptor, ASFV enters the cell by energy-dependent adsorptive endocytosis in coated pits and is later detected in endosomes and finally in lysosomes (Geraldes and Valdeira, 1985; Valdeira and Geraldes, 1985; Alcami *et al.*, 1989a). Non-enveloped virus is then detected in the cytosol and all these steps are completed in approximately 15 minutes. The release of virus particles from secondary lysosomes into the cytosol is inhibited by lysosomotropic agents such as chloroquine which indicates that a low pH trigger is necessary for fusion of the virus envelope with the lysosomal limiting membrane (Geraldes and Valdeira, 1985; Valdeira and Geraldes, 1985; Alcami *et al.*, 1989a).

Early gene expression

Once in the cytosol, the virus capsid is uncoated which leads to the solubilization of the nucleo-protein core. In addition to the virus genome, ASFV particles contain the enzymes and factors necessary for the synthesis of functional early messenger RNAs (see §1.2.1). The resistance of ASFV transcription to α -amanitin, which inhibits the activity of the cellular RNA polymerase-II, demonstrates that ASFV gene expression is independent of the cellular transcription machinery contained in the host cell nucleus (Salas, J. *et al.*, 1988). Indeed, the replication of ASFV takes place in discrete cytoplasmic foci designated virosomes or virus factories. Each infectious ASFV particle is thought to initiate a small cytoplasmic virus factory which migrates towards the nucleus where it fuses with other ASFV factories to form prominent perinuclear inclusions (Carvalho *et al.*, 1988). The virus factories are rich in membranous material (thought to be derived from the endoplasmic reticulum or Golgi apparatus, Breese and DeBoer, 1966; Moura Nunes *et al.*, 1975) and are closely associated with microtubules and intermediate filaments (Carvalho *et al.*, 1988). ASFV infection also induces a major re-organisation of the cell cytoskeleton

illustrated by the decondensation of microtubules, the concentration close to the cell membrane of actin containing microfilaments and the retraction of intermediate filaments from the cell periphery to around the cell nucleus (Carvalho *et al.*, 1988). These perturbations of the cytoskeleton by ASFV infection show similarities to those induced during infection by the iridovirus frog virus 3 (Murti and Goorha, 1983; Murti *et al.*, 1988a), whereas alterations induced by poxviruses infection only affect the cell's microfilaments (Hiller *et al.*, 1979, 1981).

The solubilization of the ASFV nucleo-protein core in the cytosol allows the viral RNA polymerase contained within it to initiate the transcription of a subset of ASFV genes, designated early genes, which represent approximately 50% of the coding capacity of the ASFV genome (Salas *et al.*, 1986). The early ASFV RNA transcripts are capped, methylated (m^7GpppN^m cap structure) and polyadenylated by enzymes contained in the nucleo-protein core (Salas *et al.*, 1981) to yield fully functional mRNAs which are then translated by the host cell ribosomes into early proteins. Included amongst the 40 to 50 early proteins encoded by ASFV are virus structural components as well as enzymes necessary for replication of the virus DNA genome (Tabares *et al.*, 1980b; Esteves *et al.*, 1986; Santaren and Vinuela, 1986; Escribano and Tabares, 1987; Urzainqui *et al.*, 1987).

DNA replication

Early synthesis of ASFV encoded nucleotide precursor synthesis enzymes, such as a ribonucleotide reductase, thymidine kinase and thymidylate kinase (Polatnick and Hess, 1979; Blasco *et al.*, 1990; Boursnell *et al.*, 1991; Yanez *et al.*, 1993b) and a DNA polymerase (Polatnick and Hess, 1972) is followed by replication of the virus DNA genome which begins approximately 4 to 8 hours after infection (Tabares and Sanchez Botija, 1979; Costa, 1990). Although replication of the ASFV genome takes place mainly in virus factories, the presence of a functional host cell nucleus is essential for synthesis of virus genomic DNA (Ortin and Vinuela, 1977). The presence early in infection (4 hours post-infection) of ASFV DNA in the nuclei of infected macrophages suggests that an early nuclear stage might be necessary for replication of the ASFV genome (Garcia-Beato *et al.*, 1992b). In contrast, replication of the genome of poxviruses is strictly extranuclear although some factors (including the large subunit of the RNA polymerase II) may be recruited from the host cell

nucleus (Moss, 1990b).

The analysis of ASFV genomes undergoing replication suggests the presence of head to head and tail to tail concatemeric DNA molecules (Ortin *et al.*, 1979; Almendral *et al.*, 1984; Gonzalez *et al.*, 1986; Caeiro *et al.*, 1990). During replication of poxvirus genomes, similar DNA concatemers are present and are probably intermediates formed during the replication process (Moyer and Graves, 1981). The replication of poxvirus DNA is thought to be initiated after a specific single strand nick close to one or both of the terminal cross links. The extension of the free 3' end thus generated allows self-priming of newly-synthesized DNA and duplication of the whole genome into a linear mirror image concatemer. Monomeric genomes are excised from the intermediate concatemeric DNA forms by specific single strand nicking close to the genome termini, followed by ligation of the terminal hairpin loops to yield mature genomes. The structural similarities between the genomes of poxviruses and ASFV (see §1.2.2) together with the presence of identical replicative intermediates suggest that ASFV DNA may be replicated by a mechanism analogous to that proposed for poxviruses.

Late gene expression

The onset of DNA replication marks a turning point in the pattern of ASFV gene expression (Tabares *et al.*, 1980b; Esteves *et al.*, 1986; Santaren and Vinuela, 1986; Escribano and Tabares, 1987). Many genes that were expressed immediately upon virus entry are shut off following the replication of ASFV DNA ('transient early' genes) while other previously silent genes only begin expression after DNA replication ('late' genes). Expression of a subset of early genes continues after DNA replication and are referred to as 'persistent early' genes. The early to late switch in gene expression does not proceed in the presence of inhibitors of DNA replication such as cytosine arabinoside which demonstrates that replication of the ASFV genome is necessary for the expression of late ASFV genes. The mechanism regulating the temporal switch in gene expression is unknown although it is expected that a succession of transcription factors regulate gene expression in a manner similar to that demonstrated for vaccinia virus (VV) (reviewed by Keck *et al.*, 1990; Moss, 1990b; Moss *et al.*, 1991). The VV cascade model of gene regulation is based on three temporal gene classes designated early, intermediate and late. The expression of VV

early genes is dependent on an early transcription factor (ETF) which is present in virus particles. Early gene expression results in replication of the VV DNA genome which provides a naked template necessary for the expression of at least three intermediate genes. The products of these three intermediate genes are late transcription factors (LTF) which activate expression of late genes. One of the late genes encodes for the ETF which is packaged in progeny VV particles, ready to activate early gene expression during the next round of infection.

In contrast to early ASFV transcripts, which only map to specific regions covering half the genome, the synthesis of late mRNA occurs throughout the genome (Salas, *et al.*, 1986). Unlike poxviruses the expression of cellular proteins is not shut off in ASFV infected cells late post-infection, although cellular expression is slightly reduced at very late times in infection (Esteves *et al.*, 1986). Approximately 70 virus proteins are expressed late in ASFV infection, many of which consist of structural components (such as VP150 and VP72) that are integrated in progeny virus particles (Esteves *et al.*, 1986; Santaren and Vinuela, 1986; Escribano and Tabares, 1987; Urzainqui *et al.*, 1987).

Virus morphogenesis and release

The first completed progeny virus particles are detected in cytoplasmic virus factories approximately 8 to 10 hours after infection with ASFV (Breese and DeBoer, 1966; Moura Nunes *et al.*, 1975). The details of the morphogenesis of ASFV virions are not well understood, essentially because of the lack of clear intermediate virus structures. Apart from apparently mature hexagonal virus particles which usually contain a nucleo-protein core, the virus factories only contain unidentified small spherical membrane vesicles presumed to represent early stages of virion formation. The hexagonal virions present in virus factories are often seen associated with arrays of ribosome-like particles, probably engaged in the synthesis of virus structural proteins (Breese and DeBoer, 1966). Apart from structural protein components, the progeny virus particles also encapsidate the virus DNA genome and ASFV encoded enzymes including those necessary for transcription of early mRNA in the next round of infection. At least 14 cellular proteins (including actin and tubulins) are also incorporated into mature virions, although it is not known whether these are necessary for infectivity or whether they are accidentally packaged into virus particles or are

contaminating cellular proteins which are not eliminated during virion purification (Tabares *et al.*, 1980a; Carrascosa *et al.*, 1985; Esteves *et al.*, 1986).

Approximately 12 hours after ASFV infection, mature virus particles start to migrate from the viral factories towards the cell membrane (Breese and DeBoer, 1966; Moura Nunes *et al.*, 1975). The migration of virions is inhibited by colchicine (Arzuza *et al.*, 1992) which suggests that the cellular microtubules network might be responsible for the translocation of mature ASFV particles. The mature virus particles are released from infected cells either by budding through the cell membrane, thus acquiring an external envelope, or by cell lysis giving rise to naked extracellular particles (Breese and DeBoer, 1966; Moura Nunes *et al.*, 1975). The role of the external envelope in virus infectivity is still not clear since naked intracellular virus particles were found to be infectious (Moura Nunes *et al.*, 1975). Furthermore, the origin of the external envelope in extracellular virus particles has recently been put in question by two independent observations. Firstly, detailed observation of intracellular virus particles has suggested that ASFV might acquire a close-fitting external envelope inside the cytoplasm (Arzuza *et al.*, 1992), as has been shown in other enveloped viruses including poxviruses (Moss, 1990a). By budding through the cell membrane, the extracellular ASFV particles would then gain an additional loose envelope which is not important for infectivity and is often subsequently lost. The second argument comes from the study of the intracellular localization of the ASFV attachment protein VP12 (Angulo *et al.*, 1993) which is detected in virus factories and is associated with mature virus particles migrating to the cell membrane, but is not detected in the cell plasma membrane. Since the VP12 attachment protein is thought to be located in the external envelope of mature ASFV particles, this observation supports the hypothesis that the virus external envelope is incorporated intracellularly before ASFV buds from the infected cell. The apparent intracellular assembly of ASFV membranes is similar to that observed during the morphogenesis of vaccinia virus particles (Moss, 1990a). Although VV particles do not contain a protein capsid, intracellular VV particles are enwrapped by a double membrane layer which is thought to be derived from cisternal membranes of the intermediate compartment between the ER and the Golgi complex (Sodeik *et al.*, 1993). A small proportion of VV particles then migrate to the cell plasma membrane and are enveloped by two additional membrane layers of unknown origin, one of which is lost

during VV release by fusion with the plasma membrane. Further examination of ASFV morphogenesis will be required to characterize the external virion layers and proteins which are likely play an important role in infection and in eliciting the host immune response.

1.2.4 Post-translational modification of ASFV proteins

In the course of ASFV replication at least eight virus induced proteins are phosphorylated in infected cells (Tabares *et al.*, 1983; Salas, M.L. *et al.*, 1988). Five of these phosphoproteins are packaged into virus particles, three of which are located in the external layers of the virion. It is not known whether these are phosphorylated by the protein kinase detected in purified ASFV particles, although this is suggested for two other small virus structural proteins which were found to be good substrates *in vitro* for the protein kinase packaged in virions (Polatnick *et al.*, 1974; Salas, M.L. *et al.*, 1988). One non-structural phosphoprotein of approximately 32 kDa (VP32) varies in apparent molecular weight amongst ASFV isolates, and is one of the most highly antigenic ASFV proteins (Alfonso *et al.*, 1992; Prados *et al.*, 1993). The VP32 phosphoprotein is expressed abundantly early in infection and forms a large homo-oligomeric complex of 220 kDa (Andres *et al.*, 1993). The location of VP32 in ASFV infected cells is still unclear since it has been proposed to be either membrane anchored (Santaren and Vinuela, 1986; Alcaraz *et al.*, 1992a, b; Alfonso *et al.*, 1992), intracellular (Prados *et al.*, 1993) or even secreted (Alfonso *et al.*, 1992). The possible membrane location of VP32, as well as its high antigenicity and antigenic variation suggest that it may participate in the host immune response against ASFV.

At least five of the proteins induced early in ASFV infected cells are glycosylated and infectious virus production is greatly reduced by glycosylation inhibitors such as glucosamine or tuncamycin (Tabares *et al.*, 1983; Del Val *et al.*, 1986; Del Val and Vinuela, 1987). Although two glycolipids of cellular origin are present in purified extracellular ASFV particles (see §1.2.1), no glycoproteins are detected in the virions which is an unusual property for an enveloped virus. Since viral glycoproteins are often surface proteins involved in cell recognition and in inducing a host protective immune response, it is possible that the lack of

glycosylated structural proteins plays a role in the evasion of ASFV from the immune system. A total of ten late and one early virus induced proteins in infected cells were shown to be fatty acid acylated with myristic acid but none were modified with palmitic acid (Aguado *et al.*, 1991). Although two of these myristylated ASFV proteins (28 and 13 kDa) are packaged into virus particles, their function and location in the virus is not yet determined.

Polyprotein processing is the last post-translational modification which has been demonstrated in ASFV infected cells (Lopez-Otin *et al.*, 1989; Simon-Mateo *et al.*, 1993). This mechanism of protein expression is usually associated with retroviruses (Oroszlan and Luftig, 1990) and some positive-strand RNA viruses such as picornaviruses which express a single polyprotein from a monocistronic RNA transcript (Belsham, 1993). The ASFV VP220 polyprotein is translated late in infection from a single ASFV gene and is subsequently myristylated and processed, through an ordered cascade of proteolytic cleavages, into four individual proteins (VP150, VP37, VP34 and VP14). All the cleavage sites recognized in the VP220 polyprotein have a Gly-Gly-X consensus sequence similar to that thought to be recognized in the maturation of adenovirus structural proteins and in the cellular polyubiquitin precursors (Lopez-Otin *et al.*, 1989). The protease responsible for the processing of the ASFV polyprotein has however not been identified. The four ASFV proteins processed from the VP220 polyprotein are all located in virus factories and also represent major structural proteins in ASFV particles. This suggests that the ASFV polyprotein might be important for some step of virus morphogenesis, perhaps allowing the four major structural proteins to be present in proximity and/or in equimolar amounts.

1.2.5 Partial sequence of the ASFV genome

The sequence of a 55 kb region from the right end of the genome of a virulent ASFV isolate is now available (Dixon *et al.*, 1993). This data shows that the ASFV genes are coded equally on both strands of the genome, in contrast to the poxvirus genome organisation in which genes close to the terminal regions of the genome are preferentially read towards the genome termini (Goebel *et al.*, 1990; Smith *et al.*, 1991). The ASFV genes are generally closely spaced and rarely appear to overlap.

Apart from being rich in A and T bases, the upstream regulatory sequences do not appear to conform to any evident consensus sequence (Hammond, 1992; Dixon *et al.*, 1993). In contrast, the sequence alignment of poxviruses promoters has revealed consensus sequences the regulatory role of which has been confirmed by mutational analysis (Moss, 1990b; Moss *et al.*, 1991). Vaccinia virus promoters for all three classes of VV genes (early, intermediate and late) consist of short (20-30 bp) upstream sequences, rich in A and T residues, which contain two critical regulatory elements (core and initiator) separated by a fixed length spacer (Davison and Moss, 1989a, b; Baldick *et al.*, 1992). Although there is no evident sequence correlation between VV and ASFV promoters, some ASFV genes were shown to be expressed by the vaccinia transcription machinery in vaccinia virus infected cells transfected with ASFV DNA (Hammond and Dixon, 1991).

At least 65 open reading frames (ORFs) are present in the 55 kb right hand region of the genome and the predicted amino acid sequences of 15 of these ORFs have homologies to proteins of known function (table 1.2). As expected from the cytoplasmic site of ASFV replication, a number of ORFs encode proteins which have homologies with enzymes such as DNA topoisomerase type II and DNA ligase that are involved in DNA replication (Baylis *et al.*, 1992; Garcia-Beato *et al.*, 1992a; Hammond *et al.*, 1992; Yanez and Vinuela, 1993), or with enzymes such as RNA polymerase subunits, transcription elongation factor TFS II and capping enzyme involved in mRNA synthesis and processing (Dixon *et al.*, 1993; Lu *et al.*, 1993; Neilan *et al.*, 1993; Yanez *et al.*, 1993a). Two ASFV ORFs encode predicted proteins which have homology with known virulence factors encoded by other viruses, including the ICP34.5 neurovirulence associated protein of herpes simplex virus (Chou and Roizman, 1990), and the protease inhibitor which in cowpox virus inhibits the interleukin-1 β converting enzyme (Ray *et al.*, 1992).

Finally two ORFs encode predicted proteins which are homologous to enzymes involved in the post-translational modification of proteins. One predicted protein is similar to a serine protein kinase. This protein has been expressed in *E. coli* and shown to have the predicted enzyme activity (Baylis *et al.*, 1993a). The second ORF has considerable homology to a family of ubiquitin conjugating enzymes (described in detail in §1.3 below) and represents the first such enzyme identified encoded on a virus genome (Hingamp *et al.*, 1992; Rodriguez *et al.*, 1992b).

| ORF | Homology | Identity |
|------|-----------------------------------|--|
| g1L | RNA polymerase (large subunit) | VV: 21% Yeast: 27% |
| g3L | DNA ligase | VV: 20% Yeast: 21% T4: 23% |
| g4R | mRNA capping (large subunit) | VV: 23% |
| g10L | Helicase | VV (NTPase I): 18% j10L: 24% |
| i2R | RNA polymerase subunit | Yeast (RPB5): 21% Human (23 kDa): 28% |
| i8R | DNA topoisomerase type II | Yeast: 22% Human: 24% |
| j1L | RNA polymerase subunit | Yeast (RPB3): 23% |
| j8L | Protein kinase | VV (B1R): 21% Human (pim-1): 24% |
| j9L | Helicase | VV (ETFI): 21% VV (NTPase I): 28% |
| j10L | Helicase | VV (A18R): 20% |
| j11R | Nif S like | Yeast: 25% |
| k1R | dUTPase | E. coli: 24% VV: 24% |
| k9L | RNA polymerase subunit | VV (rpo30): 25% Mouse (TFSII): 23% |
| k12L | Ubiquitin conjugating enzyme | Yeast (UBC1-10): 31-45% |
| l14L | Neurovirulence factor | HSV (ICP34.5): 51% |

Table 1.2 Homology between the predicted protein products of ASFV open reading frames and proteins of known function.

The table shows the protein sequence homologies of the predicted products of the open reading frames (ORFs) present in the right hand 55 kb of the Malawi LIL20/1 ASFV isolate (Dixon *et al.*, 1993). The percentage of identical amino acids are also indicated. The percentages were calculated using the complete protein sequence apart from the HSV ICP34.5 protein in which the identity was calculated from a conserved 40 amino acid carboxyl terminal domain. VV: vaccinia virus; Yeast: *Saccharomyces cerevisiae*; T4: bacteriophage T4; HSV: herpes simplex virus.

1.2.6 Taxonomic classification of ASFV

The early observations of ASFV such as its particle structure, cytoplasmic site of replication and large DNA genome led to its classification in the *Iridoviridae* family (Matthews, 1982). However, subsequent analysis has revealed that many characteristics of ASFV replication are similar to viruses of the *Poxviridae* family. Indeed the ASFV genome, which has terminal cross links and inverted terminal repeats is similar in structure to that of poxviruses, whereas the genomes of iridoviruses are circularly permuted. Moreover, in common with poxviruses, ASFV transcription is not dependent on host cell RNA polymerase II and the virions contain all the enzymes and factors necessary for early mRNA synthesis. Finally, expression of ASFV genes is temporally regulated in a manner similar to poxviruses since late gene expression is dependent on virus DNA replication.

The sequencing of the ASFV genome has, however, shown that the genome organisation of ASFV genes is not co-linear with that of the orthopoxviruses (Goebel *et al.*, 1990; Johnson *et al.*, 1993). Furthermore, the presence of ASFV DNA but not poxvirus DNA in the host cell nucleus indicates that there are important differences in the replication strategy of ASFV and poxviruses. On the basis of its distinctive features, ASFV has been removed from the family Iridoviridae and is now classified in a separate unnamed family of which ASFV is the only member (Brown, 1986).

1.3 The ubiquitin conjugation system

1.3.1 The ubiquitin polypeptide

Ubiquitin is a small polypeptide found universally in eukaryotic cells, either free or covalently attached to cellular proteins (reviewed by Rechsteiner, 1988; Jentsch *et al.*, 1990; Hershko, 1991a; Hershko and Ciechanover, 1992; Jentsch, 1992). The amino acid sequence of ubiquitin is highly conserved in evolution, with at most 4% divergence between yeast, plants and animals. Amongst the 76 amino acids of the ubiquitin polypeptide, 11 residues are acidic and 11 residues are basic giving the protein a neutral isoelectric point of 6.7. The molecular weight of ubiquitin is 8.5

KDa as determined by gel filtration, but its unusually high electrophoretic mobility in SDS-PAGE can lead to an apparent molecular weight as low as 5.5 KDa (Ciechanover *et al.*, 1978). This is probably due to the incomplete denaturation of the protein which is consistent with the first described characteristic of ubiquitin: its unusually high stability. After treatment with urea, alcohol or thermal denaturation (up to 85°C), ubiquitin can easily refold into its native conformation (Rechsteiner, 1988).

The crystal structure of ubiquitin (Vijaykumar *et al.*, 1987a, b) shows that it has a packed globular structure, with 90% of its residues involved in hydrogen-bonded secondary structure which might account for the pronounced stability of the protein. The surface of ubiquitin can be divided into three physico-chemical regions: a basic face and an acidic face which are on opposite sides of the molecule and flank a hydrophobic region. The four C-terminal residues protrude from the core of the protein making the Gly⁷⁶ residue available for conjugation to other proteins.

1.3.2 The ubiquitin conjugation pathway

All known ubiquitin-related functions are mediated through conjugation of ubiquitin to proteins. The mechanism of ubiquitin conjugation, which is now regarded as a central and universal eukaryotic post-translational modification, was originally determined in reticulocytes (Ciechanover *et al.*, 1980; Hershko *et al.*, 1980, 1983; Hershko, 1988). These studies show that the ligation of ubiquitin to cellular proteins is a multi-step process (figure 1.4).

The first step is the ATP dependent activation of ubiquitin by a large (105 kDa) E1 enzyme also known as the ubiquitin activating enzyme. Free ubiquitin, which is one of the most abundant small proteins in the cell, is available for activation from a large intracellular pool. The activation of ubiquitin consists of thiol ester bond formation between its free C-terminal Gly⁷⁶ with an internal cysteine residue of E1. The reaction involves the formation of a ubiquitin-AMP adenylate intermediate which accounts for the ATP requirement of ubiquitin conjugation. In the second step, activated ubiquitin is transferred from the E1 enzyme to one of a large family of ubiquitin carrier isozymes (E2s), also known as ubiquitin conjugating (UBC) enzymes. Again, a thiol ester bond covalently links ubiquitin by its C-terminal Gly⁷⁶.

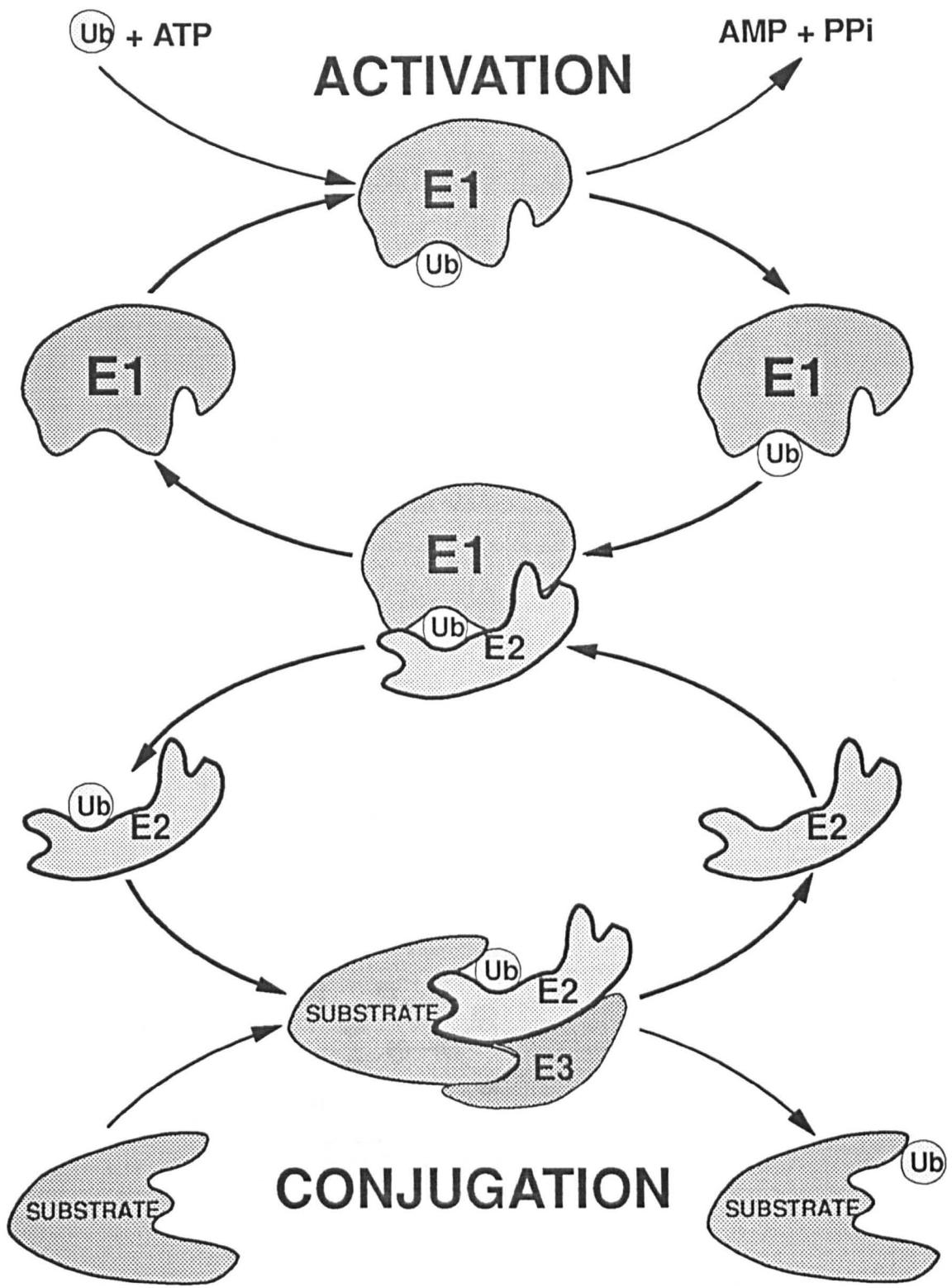


Figure 1.4 The ubiquitin conjugation pathway.

The diagram shows the steps and enzymes responsible for the conjugation of ubiquitin to cellular proteins. Ub: ubiquitin, E1: ubiquitin conjugating enzyme, E2: ubiquitin conjugating enzyme (UBC), E3: ubiquitin ligase.

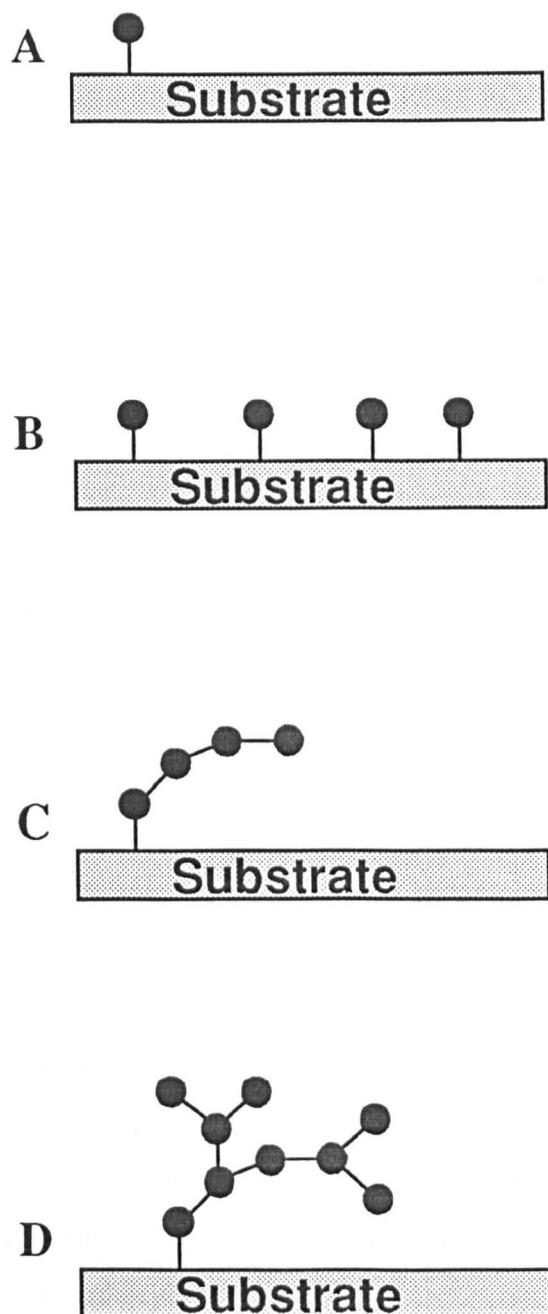


Figure 1.5 Structure of various types of ubiquitin conjugates.

The lollipops represent ubiquitin molecules. Panel A: mono-ubiquitinated conjugate; Panel B: multi-ubiquitinated conjugate; Panel C: linear poly-ubiquitinated conjugate; Panel D: branched poly-ubiquitinated conjugate.

to a conserved internal cysteine residue of the UBC enzymes.

The final conjugation step consists of the transfer of ubiquitin from the UBC enzyme to a specific protein substrate. The resulting ubiquitinated protein is known as a ubiquitin conjugate, in which ubiquitin is attached to the substrate protein via an amide bond between the C-terminal Gly⁷⁶ of ubiquitin and the ϵ -amino group of an internal lysine residue of the substrate. Many protein substrates have been shown to be multi-ubiquitinated (figure 1.5) (Hershko *et al.*, 1980; Hochstrasser, 1992; Jentsch, 1992). This phenomenon is the result of several ubiquitin molecules being conjugated either to different lysine residues of the substrate, or to the internal Lys⁴⁸ and Lys⁶³ residues of previously conjugated ubiquitin (Chau *et al.*, 1989; Chen and Pickart, 1990; Gregori *et al.*, 1990; Cook *et al.*, 1992a; Banerjee *et al.*, 1993). The latter type of poly-ubiquitination leads to the formation of linear and branched poly-ubiquitin chains anchored to a single lysine of the protein substrate.

The specificity for the protein substrate is usually dictated by the UBC enzyme, although in some instances a third enzyme (E3 or ubiquitin ligase) is also necessary for substrate recognition (Elias and Ciechanover, 1990; Sharon *et al.*, 1991; Sung *et al.*, 1991a; Madura *et al.*, 1993). By binding to specific UBC enzymes, the ubiquitin ligases are thought to complement or even modify the specificity of the UBC enzyme for protein substrates. An additional class of enzymes, the ubiquitin hydrolases, are responsible for the release of ubiquitin molecules from ubiquitin conjugates by hydrolysis of the Gly⁷⁶- ϵ NH₂ isopeptide bonds (Wilkinson *et al.*, 1989; Tobias and Varshavsky, 1991; Baker *et al.*, 1992; Hadari *et al.*, 1992; Eytan *et al.*, 1993). Conjugated ubiquitin is therefore in equilibrium with the free ubiquitin pool, which suggests that ubiquitin conjugation is a dynamic process analogous to protein phosphorylation.

1.3.3 Functions of the ubiquitin conjugation pathway in yeast

Although the mechanisms of ubiquitin conjugation were originally determined in rabbit reticulocytes, the yeast *Saccharomyces cerevisiae* is now the model for the functional and genetic analysis of the ubiquitin pathway in eukaryotes (reviewed by Jentsch *et al.*, 1991; Jentsch, 1992). The yeast genes encoding many of the enzymes of the pathway have been identified (table 1.3), and in many instances homologous

| PROTEIN | GENE | FUNCTION |
|-----------|--|---|
| E1 | <u>UBA1</u> | Ubiquitin activating enzyme (105 kDa). |
| E2 | <u>UBC1*</u> | Ubiquitin conjugating (UBC) enzymes -Bulk proteolysis; Essential for early growth after germination. |
| | UBC2 | -DNA repair; Sporulation; Cell division control S→G2 transition ; N-end rule with UBR1 ubiquitin ligase (ex RAD6). |
| | <u>UBC3</u> | -Cell division control, G1→S transition (ex CDC34). |
| | <u>UBC4*</u> | -Bulk proteolysis of misfolded & abnormal proteins; Stress response (ex HSP16). |
| | <u>UBC5*</u> | -Similar function to UBC4. |
| | UBC6 | -Integral ER membrane enzyme; Selective proteolysis (ex DOA2). |
| | UBC7 | -Confers cadmium resistance; Selective proteolysis. |
| | UBC8 | -Unknown. |
| | <u>UBC9</u> | -Cell division control at G2/M stage. |
| | UBC10 | -Peroxisome biogenesis (ex PAS2). |
| | Others | -Other UBC enzymes probably remain be discovered. |
| E3 | UBR1 | -Ubiquitin ligase; Recognition of protein substrates by the N-end rule of proteolysis (mediated by UBC2). |
| | Others | -Might be responsible for recognition of other degrons. |
| Hydrolase | YUH1 UBP1 UBP2 UBP3 | -Ubiquitin hydrolases; Specifically cleave and recycle ubiquitin from ubiquitin conjugates or from ubiquitin fusion precursors. |
| Ubiquitin | <u>UBI1</u> <u>UBI2</u> <u>UBI3</u> <u>UBI4</u> | -UBI1 to UBI3: fusions of ubiquitin with ribosomal subunits; 'Chaperone' like function of ubiquitin in ribosome assembly. -Poly-ubiquitin precursor; Stress inducible. |
| Protease | <u>PRE1</u> <u>PRE2</u> <u>PRE3</u> <u>PRE4</u> Others | -26S protease (multicatalytic complex); Numerous subunits, including the yscE protease complex (20S proteasome); Degrades poly-ubiquitinated proteins and has ubiquitin hydrolase activity. |

Table 1.3 The ubiquitin conjugation pathway in yeast

The various yeast proteins involved in the ubiquitin pathway are indicated together with their encoding genes (the alternative names of genes encoding UBC enzymes are indicated in brackets with the prefix 'ex'). Genes that are underlined are essential for yeast survival. An asterisk (*) marks a group of UBC genes of overlapping function where individual genes are not essential if at least one other gene in the group is functional.

genes have been found in animals and plants. The following outline of the major components of the ubiquitin pathway in yeast illustrates its wide and fundamental role in the eukaryotic cell.

Ubiquitin is expressed either as a polyubiquitin precursor or as a fusion protein with various ribosomal proteins (Dworkinrastl *et al.*, 1984; Ozkaynak *et al.*, 1987; Warner, 1989). The ubiquitin moiety in the ribosomal fusion protein has been shown to enhance the efficiency of incorporation of the ribosomal proteins into mature ribosomes, suggesting a co-translational 'chaperone' role for ubiquitin (Finley *et al.*, 1989). The yeast UBI4 polyubiquitin gene, which has five ubiquitin genes arranged head to tail, is strongly induced during stress and is essential for yeast survival under these conditions (Finley *et al.*, 1987). In both types of ubiquitin fusions, the precursors are rapidly processed by ubiquitin hydrolases into single ubiquitin molecules which then join the free ubiquitin pool. The yeast ubiquitin activating enzyme E1 is encoded by the single UBA1 gene (McGrath *et al.*, 1991). Deletion of the UBA1 gene is lethal and this highlights the essential role played by the ubiquitin pathway in eukaryotic metabolism (Ciechanover *et al.*, 1984; Finley *et al.*, 1984; Gropper *et al.*, 1991; Mitchell *et al.*, 1991; Leyser *et al.*, 1993; Mori, M. *et al.*, 1993).

The key enzymes in the ubiquitin conjugation pathway which determine substrate specificity are the UBC (or E2) enzymes, 10 of which have been identified in yeast. Three UBC enzymes (UBC1, UBC4 and UBC5) have overlapping functions and are involved in bulk intracellular protein degradation (Seufert and Jentsch, 1990; Seufert *et al.*, 1990; Treier *et al.*, 1992; Girod and Vierstra, 1993; Girod *et al.*, 1993; Zhen *et al.*, 1993). The ubiquitin conjugates generated by these UBC enzymes are poly-ubiquitinated which targets them for degradation by a ubiquitin-specific protease complex (see §1.3.5). Together, these three UBC enzymes mediate the intracellular turnover of normally occurring short lived proteins as well as misfolded and abnormal proteins. This latter role is especially important in cells under stress conditions. In addition, the yeast cadmium inducible UBC7 enzyme participates in the elimination of abnormal proteins generated during cadmium exposure (Vassal *et al.*, 1992; Jungmann *et al.*, 1993). The yeast UBC6 enzyme is the only integral membrane UBC enzyme identified to date (Sommer and Jentsch, 1993). UBC6 is located in the endoplasmic reticulum (ER) with its catalytic domain facing the cytosol

where it is thought to mediate the selective degradation of ER membrane proteins.

In contrast, the three yeast UBC2, UBC3 and UBC9 enzymes are all involved in sensitive regulatory functions. The UBC2 gene, previously identified as the DNA repair gene RAD6, encodes an enzyme with very pleiotropic functions (Jentsch *et al.*, 1987; Morrison *et al.*, 1988; Sullivan and Vierstra, 1989; Reynolds *et al.*, 1990; Schneider *et al.*, 1990; Koken *et al.*, 1991a, b; Woffendin *et al.*, 1991). The phenotypes of yeast mutants with an inactive UBC2 gene include sensitivity to mutagens, defects in meiotic recombination and sporulation, slow growth as well as partial blocking of cell cycle progression (Siede, 1988; Picologlou *et al.*, 1990; Ellison *et al.*, 1991). Single mutations in the UBC2 active site cysteine have demonstrated that all the roles attributed to UBC2 are dependent on its ubiquitin conjugating activity (Sung *et al.*, 1990, 1991b). It is possible that UBC2 has a number of distinct regulatory substrates or that its diverse functions are mediated by a single fundamental role in DNA metabolism. An additional role for UBC2, in cooperation with the E3 enzyme UBR1, is to mediate the degradation of intracellular proteins according to the N-end rule (see §1.3.4). The cellular importance of N-end rule-mediated proteolysis is not known since yeast mutants lacking this pathway have no significant phenotype (Bartel *et al.*, 1990). The yeast UBC3 and UBC9 enzymes are located in the nucleus and are essential for the progression of cell division at two stages of the cell cycle (Goebl *et al.*, 1988; Jentsch, 1992; Chen *et al.*, 1993). The UBC3 gene (previously identified as CDC34) is necessary for the G1 to S transition of the cell cycle, whereas yeast mutants in the UBC9 gene are arrested in the G2/M phase of the cell cycle. The latest UBC enzyme to be identified in yeast is UBC10 which is essential for the biogenesis of peroxisomes, although its precise role is unknown (Wiebel and Kunau, 1992).

The diversity of the functions attributed to UBC enzymes is apparently difficult to reconcile with their single ubiquitin conjugating catalytic activity. It is possible, however, that the apparently non-catabolic roles of several UBC enzymes, such as UBC2 and UBC3 which are responsible for DNA repair and cell cycle control respectively, are indirectly mediated by degradative processes. The targets of these UBC enzymes might be regulatory proteins, such as histones or cyclins, which are targeted for degradation by ubiquitination. A unifying proteolysis theory for the function of ubiquitin conjugation will, however, only be validated when specific

substrates for individual UBC enzymes are identified and the consequences of their ubiquitination are fully understood.

1.3.4 The recognition of substrate proteins by UBC enzymes

Although a variety of physiological substrates for ubiquitin dependent degradation are known (see §1.3.5), the UBC enzymes responsible for their specific ubiquitination are mostly unidentified. Consequently, the mechanism of substrate recognition by the UBC and E3 enzymes is still poorly defined (reviewed by Ciechanover and Schwartz, 1989; Rechsteiner, 1991; Hershko and Ciechanover, 1992; Jentsch, 1992). To date, the only physiological substrate of UBC enzymes that has been studied in detail is the yeast MAT α 2 transcriptional regulator. The naturally short-lived MAT α 2 repressor is poly-ubiquitinated *in vivo* which targets it for subsequent proteolysis (Hochstrasser *et al.*, 1991). Two distinct ubiquitination pathways involving no less than four UBC enzymes (UBC4, UBC5, UBC6 and UBC7) are responsible for the ubiquitination of MAT α 2 (Chen *et al.*, 1993). Furthermore, the UBC6 and UBC7 enzymes associate in a heterodimeric complex allowing them to specifically recognize the Deg1 ubiquitination signal of MAT α 2 which consists of the N-terminal 67 amino acids. This finding suggests that a network of interacting UBC enzymes may determine the substrate specificity of ubiquitin conjugation. The association of different combinations of UBC enzymes in distinct complexes would greatly expand the repertoire of substrates that could be specifically recognized and ubiquitinated.

Comparison of the protein sequences of the UBC enzymes characterized show that all UBC enzymes have a conserved catalytic core domain of approximately 16 kDa, which contains approximately 150 amino acids (Jentsch, 1992). The core domain has at least 35% amino acid identity between all UBC enzymes, including a centrally located cysteine residue which is required for the formation of a thiolester bond with ubiquitin. In addition to the conserved core domain, certain UBC enzymes such as UBC2 and UBC3 have a C-terminal extension or tail. The sequences of the C-terminal tails of UBC enzymes are not related to each other and might contribute to the enzyme's substrate specificity, acting in *cis* rather than in *trans* as is the case with E3 ubiquitin ligases. This hypothesis has recently been substantiated by the study of chimeric UBC2/UBC3 constructs in yeast (Kolman *et al.*, 1992; Silver *et al.*,

1992). A chimeric UBC enzyme consisting of the catalytic core of UBC2 and the C-terminal tail of UBC3 was capable of restoring the full UBC3 cell cycle function in mutant yeast cells carrying an inactivated UBC3 gene. However, this chimeric UBC enzyme retained at least some of the functions specific to UBC2, which indicates that the C-terminal tail expands rather than supplants the specificity of the catalytic core.

Another approach for determining the interaction between UBC enzymes and their substrates involves detailed analysis of proteins known to be ubiquitinated. The study of the ubiquitin-dependent degradation of cyclins has identified a hypothetical 'destruction box' of sequence RxxLxxIxN which is necessary for cyclin degradation and is located upstream of the ubiquitinated lysine site (Glotzer *et al.*, 1991). It is not known whether cyclins are the substrates for the UBC enzymes known to be involved in cell division control. Other substrates for ubiquitin dependent degradation have been shown to contain stretches of sequence enriched in proline, glutamic acid, serine and threonine residues, referred to as PEST sequences (Rogers *et al.*, 1986). A third ubiquitination signal is illustrated by the N-end rule of ubiquitin-dependent proteolysis which predicts the half life of a protein according to the identity of its N-terminal amino acids. This signal is known as the N-degron (Varshavsky *et al.*, 1987; Varshavsky, 1992). In yeast this pathway is catalysed by the UBC2 enzyme in conjunction with the E3 ubiquitin ligase UBR1 (Bartel *et al.*, 1990; Dohmen *et al.*, 1991; Sharon *et al.*, 1991; Sung *et al.*, 1991a). No physiological substrates for this pathway are known in yeast, but in *Xenopus* oocytes the c-mos proto-oncogene product (Mos) is rapidly ubiquitinated upon fertilization according to the N-end rule pathway and is subsequently degraded, following which the zygote enters mitosis (Nishizawa *et al.*, 1992, 1993).

1.3.5 The fate of ubiquitinated proteins

It is becoming increasingly evident that the most common outcome of ubiquitination is proteolysis (reviewed by Hershko, 1988, 1991a, b; Rechsteiner, 1991; Hershko and Ciechanover, 1992; Hochstrasser, 1992; Varshavsky, 1992). Not only is the ubiquitin / ATP-dependent pathway responsible for the bulk of non-lysosomal intracellular proteolysis, but recent observations also tentatively suggest that the ubiquitin and lysosomal degradation systems might be functionally interrelated (Doherty *et al.*,

1989; Ciechanover *et al.*, 1991b; Gropper *et al.*, 1991; Mayer *et al.*, 1991, 1992; Lenk *et al.*, 1992; Simeon *et al.*, 1992). The exact nature and extent of this putative link are, however, still unclear.

One well defined pathway for the degradation of ubiquitin conjugates involves a large protease complex which is specific for poly-ubiquitinated proteins (Chau *et al.*, 1989; Gregori *et al.*, 1990). This ubiquitin-dependent protease is present in the cytosol and the nucleus and has been identified as a 26S (or 1500 kDa) multi-catalytic protease. The 26S protease consists of three subunits CF1, CF2 and CF3 which only associate into the 26S complex in the presence of ATP (Waxman *et al.*, 1987; Driscoll and Goldberg, 1990; Seufert and Jentsch, 1992). The CF3 subunit has been identified as the 20S proteasome, which is a large cytosolic and nuclear protease (comprising some 12-15 related polypeptides) with three distinct endopeptidase activities (Kanayama *et al.*, 1992). The eukaryotic 20S proteasome polypeptides are related to each other and to the two types of proteasomal polypeptides found in Archaeabacteria. It is not known whether the eukaryotic 20S proteasome can function alone or if it is only active as part of the larger 26S protease. The CF1 and CF2 subunits of the 26S protease appear to be activators and inhibitors respectively of the 20S proteasome, which suggests that substrate degradation is intricately regulated (Chu-Ping *et al.*, 1992a, b; Li and Etlinger, 1992). The poly-ubiquitinated proteins recognized by the 26S protease are broken down into small oligopeptides, and a specific ubiquitin hydrolase activity associated with the 26S protease recycles intact ubiquitin molecules for further rounds of conjugation (Eytan *et al.*, 1993; Papa and Hochstrasser, 1993).

The presence of metabolically stable intracellular ubiquitin conjugates suggests that some functions of the ubiquitin pathway may be independent of proteolysis. In chromatin of higher eukaryotes for instance, up to 15% of histone H2A is ubiquitinated, predominantly in the mono-ubiquitinated form, with no apparent subsequent degradation (Goldknopf and Bush, 1978; Matsui *et al.*, 1979; Wu *et al.*, 1981). Variations in the levels of ubiquitinated histones are synchronous with cell division, which suggests a role for histone ubiquitination in modulating chromosomal DNA structure or accessibility (Hacques and Marion, 1989; Mori, M. *et al.*, 1993). A growing number of transmembrane proteins have been shown to be ubiquitinated but it is not known if these are subsequently degraded. These include the growth

hormone receptor (Leung *et al.*, 1987), the lymphocyte homing receptor (St. John *et al.*, 1986; Siegelman *et al.*, 1986), the high-affinity IgE receptors (Paolini and Kinet, 1993), the T cell antigen receptor (Cenciarelli *et al.*, 1992) and the platelet-derived growth factor (PDGF) receptor (Yarden *et al.*, 1986; Mori *et al.*, 1992). Ubiquitination of the PDGF, IgE and T cell antigen receptors was shown to be induced by binding of the receptor ligand, suggesting a role for ubiquitination in transmembrane signal transduction. In the case of the PDGF receptor, ubiquitination has been shown to play a negative regulatory role in its mitogenic signalling, possibly by promoting the degradation of the ligand-activated receptor or by altering the signal transducing properties of the modified receptor (Mori, S. *et al.*, 1993).

1.4 The role the ubiquitin pathway in virus infections

1.4.1 Involvement of the cellular ubiquitin system in virus infections

Considering the numerous ramifications of the ubiquitin system in cellular metabolism, it seemed inevitable that the replication of viruses would be shown to involve the ubiquitin pathway.

The simplest involvement is the specific packaging of free ubiquitin in avian leukosis virus (ALV) particles (Putterman *et al.*, 1990). The adventitious packaging of ubiquitin is improbable since other small cellular peptides are excluded from virions, and ubiquitin is present in virions at a concentration fivefold higher than in the cytosol. Although the function of the free ubiquitin in ALV particles is not known, it is conceivable that it associates non-covalently with virus proteins and plays a role in virion structure. In contrast, the structural proteins of several plant viruses are covalently ubiquitinated (Dunigan *et al.*, 1988; Hazelwood and Zaitlin, 1990). These viruses might utilize a chaperone-like activity of ubiquitin for virus morphogenesis. Alternatively, these virus structural protein-ubiquitin conjugates could be the result of a host cyto-protective reaction which attempts to prevent infection by targeting virus proteins for proteolysis. The ubiquitin-dependent degradation system is, in fact, implicated in plant defence mechanisms against viral infections (Becker *et al.*, 1993). In tobacco plants that over-express a ubiquitin analog which does not

support ubiquitin-dependent proteolysis because the Lys⁴⁸ site of polyubiquitin branch formation is substituted for Arg, tobacco mosaic virus (TMV) replication is reduced and the host systemic acquired resistance system is activated, as indicated by the induction of pathogenesis-related proteins. The mechanism by which the ubiquitin pathway affects the plant defense mechanism is unclear, although it is speculated that a specific defence activator protein, normally inactivated by ubiquitin-dependent proteolysis, is stabilized in cells under stress or exposed to viral infection.

Several lines of evidence also suggest that the ubiquitin pathway plays an important part in the animal host immune response to viral infections (figure 1.6) (reviewed by Driscoll and Finley, 1992; Goldberg and Rock, 1992; Howard and Seelig, 1993). This hypothesis stems from the observation that at least two major histocompatibility (MHC) class I gene products associate with the 20S proteasome (the major component of the 26S ubiquitin specific protease) to form the low molecular-weight polypeptide (LMP) particle. The LMP particle is suspected to be the cytosolic protease involved in class I antigen processing (Parham, 1990), by generating antigenic viral peptides that are transported into the lumen of the ER where they associate with nascent class I MHC molecules and are presented on the cell surface to cytotoxic T cells. Furthermore, the MHC gene products, which are associated with the proteasome, appear to cause subtle changes in the peptidase activities of the 20S and 26S proteasomes, and are induced by γ -interferon which is a property shared by many other components of the MHC presentation machinery (Yang *et al.*, 1992; Driscoll *et al.*, 1993; Gaczynska *et al.*, 1993). In addition, an amino terminal modification of a virus protein that increases ubiquitin-dependent degradation by the N-end rule also enhances its presentation with class I molecules (Townsend *et al.*, 1988). Finally, a direct link between the ubiquitin conjugation system and class I presentation was demonstrated in an antigen-presenting cell line with a thermolabile E1 activating enzyme (Michalek *et al.*, 1993). Although growth of mutant cells at the non-permissive temperature did not inhibit class I-restricted presentation of an ovalbumin peptide neo-synthesized from a minigene, processing for class I presentation of full length ovalbumin introduced into the cytosol was inhibited. These results implicate the ubiquitin-dependent proteolytic pathway in the production of antigenic peptides.

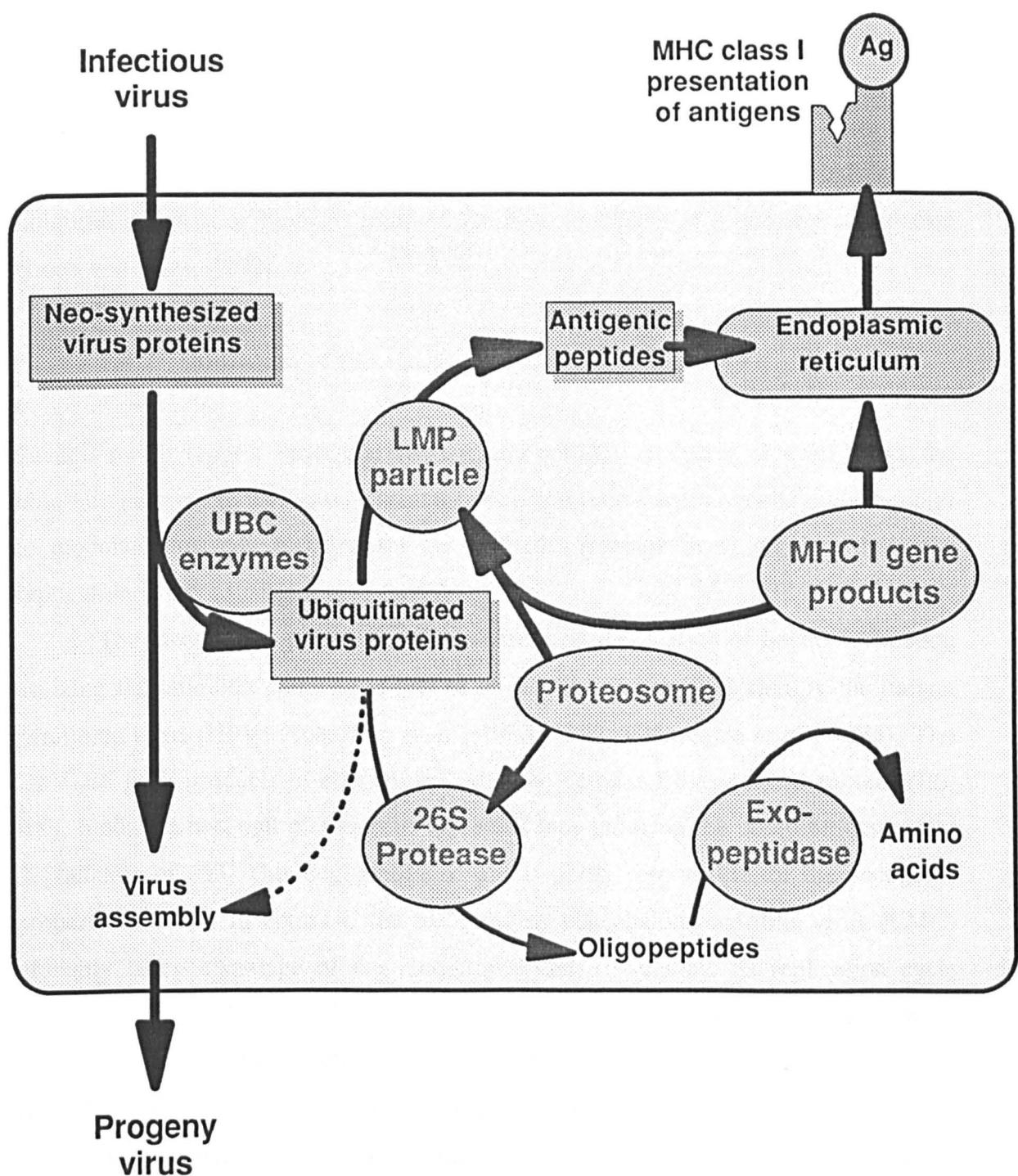


Figure 1.6 The class I-restricted processing of antigenic peptides by the ubiquitin-dependent degradation pathway

The diagram summarizes the current hypothesis concerning the ubiquitin-dependent processing and presentation of class I antigens.

Another interferon induced response in virus infected cells leads to the rapid expression of a ubiquitin cross-reactive protein (UCRP) (Ahrens *et al.*, 1987, 1990; Haas *et al.*, 1987). The 15 kDa UCRP, which is composed of a tandem di-ubiquitin like sequence, is induced by α - and β -interferons in all cells responsive to these cytokines and is also slowly secreted from these cells. The intracellular form of the protein, which can be conjugated to other cellular proteins, might modulate the ubiquitin pathway or mediate some of the diverse effects of interferon stimulation (Loeb and Haas, 1992).

1.4.2 Utilization of the ubiquitin system by viruses

Several recent reports show that viruses may actively modulate or even hijack the ubiquitin pathway to their own advantage. The ubiquitin system may be utilized either to modulate host cell function for the viruses advantage or to regulate the virus replication cycle.

The most striking example of virus induced modulation of host cell function utilizing the ubiquitin pathway is that of an E3 like enzyme encoded by the human papilloma virus (HPV) (Scheffner *et al.*, 1990, 1992; Huibregtse *et al.*, 1993). The HPV E6 gene product, in cooperation with the cellular E6-associated protein (E6-AP), binds the host cell p53 tumour repressor thus inducing the ubiquitin-dependent degradation of p53. This degradation of p53 is partly responsible for the oncogenic property of HPV. In contrast, the picornavirus encephalomyocarditis virus (EMC) probably takes advantage of the ubiquitin system to regulate its replication cycle (Oberst *et al.*, 1993). The EMC encoded protease 3C is indeed rapidly degraded by an ATP-dependent proteolytic system present in reticulocyte lysates, a property typical of the ubiquitin-dependent degradation pathway.

Another example of a virus encoded component of the ubiquitin pathway is illustrated by the togavirus bovine viral diarrhoea virus (BVDV) (Meyers *et al.*, 1989, 1991). The cytopathogenic phenotype of two strains of BVDV were correlated with a genomic insertion of sequences 97% identical to ubiquitin. These BVDV ubiquitin insertions are thought to be present to provide a cleavage site in a virus polyprotein precursor (Meyers *et al.*, 1992). The baculovirus *Autographa californica* nuclear polyhedrosis virus (AcMNPV) similarly encodes a protein with 76% identity to

ubiquitin which is essential for virus replication (Guarino, 1990). This viral ubiquitin homolog (v-ubi) can be attached to substrate proteins by the ubiquitin conjugating system. These v-ubi conjugates are, however, not as effectively targeted for degradation as ubiquitin conjugates and high molecular weight v-ubi conjugates are detected in virus nucleocapsids (Guarino, 1993). The v-ubi protein might therefore act as a ubiquitin analog which could interfere with the ubiquitin system, perhaps by protecting virus proteins from proteolysis. In addition, a phospholipid modified ubiquitin molecule is a major component of AcMNPV particles, where it is anchored inside the virus envelope facing the virus capsid. The purpose of this novel membrane anchored ubiquitin is not known, although roles in virion assembly, virus stability and in early infection events have been proposed (Guarino, 1993).

The UBC enzyme encoded by African swine fever virus is a novel example of a virus encoded key component of the ubiquitin system and is the subject of this study (Hingamp *et al.*, 1992; Rodriguez *et al.*, 1992b).

1.5 Project aims

The overall objective of this project is to determine the function of the African swine fever virus (ASFV) encoded ubiquitin conjugating enzyme (UBCv). The eukaryotic ubiquitin conjugating enzymes are so versatile that *a priori* speculations on the role of UBCv cover most of the stages of ASFV replication, as well as potential roles in manipulating either the host cell to facilitate virus replication or the host immune response. The most likely potential roles for UBCv include the uncoating of the virus capsid, the early to late switch in gene expression, the assembly of virus particles, the control of host cell gene expression and/or state of differentiation and interference with the host immune response. The study of the UBCv enzyme might further our understanding both of virus-host interactions, as well as the involvement of the ubiquitin system in virus infections. In addition, if the substrates for the UBCv enzyme can be identified, then ASFV will provide the first comprehensive model for ubiquitin conjugation.

The project will involve three complementary approaches. The first will deal with the *in vitro* analysis of the UBCv enzyme activity. The second will examine

UBCv in ASFV infected cells, in order to obtain data on the expression and localisation of the enzyme. The construction of an ASFV mutant with an inactivated UBCv gene will also be part of this study. The third approach will concentrate on the identification of physiological substrates for UBCv. If successful, the genes for these protein substrates might in turn be characterized and sequenced.

Chapter 2

UBCv gene sequence and *in vitro* activity

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The complete sequence of the right hand 55 kb region of the ASFV genome (Malawi LIL20/1 isolate) contains nearly 70 open reading frames (ORFs), 15 of which encode putative homologues of cellular enzymes (Dixon *et al.*, 1993). This study focused on the UBCv gene which was predicted to encode a protein with a convincing, albeit unexpected, homology to eukaryotic ubiquitin conjugating (UBC) enzymes. To verify that this homology was not coincidental, the ubiquitin conjugating activity of recombinant UBCv was assayed *in vitro*.

2.1 Structure of the UBCv gene

2.1.1 Nucleic acid sequence of UBCv from ASFV Malawi LIL20/1

Malawi LIL20/1 is a highly virulent ASFV field isolate collected from ticks in East Africa (Dixon and Wilkinson, 1988). The sequencing of the right hand 55 kb of the genome of Malawi LIL20/1 (Dixon *et al.*, 1993) located the UBCv gene approximately 12 kb from the right hand terminus (figure 2.1). It is the thirteenth open reading frame on *SalI* fragment k and is read on the complementary strand of the genome (leftwards) and has therefore been named k13L.

The 642 bp long k13L open reading frame (figure 2.2) has a 62% A+T content which is close to that of 61% calculated for the right hand 55 kb portion of the ASFV genome. This high A+T ratio confers a relatively low melting temperature to the dsDNA which might be important for ASFV replication in the cells of the soft

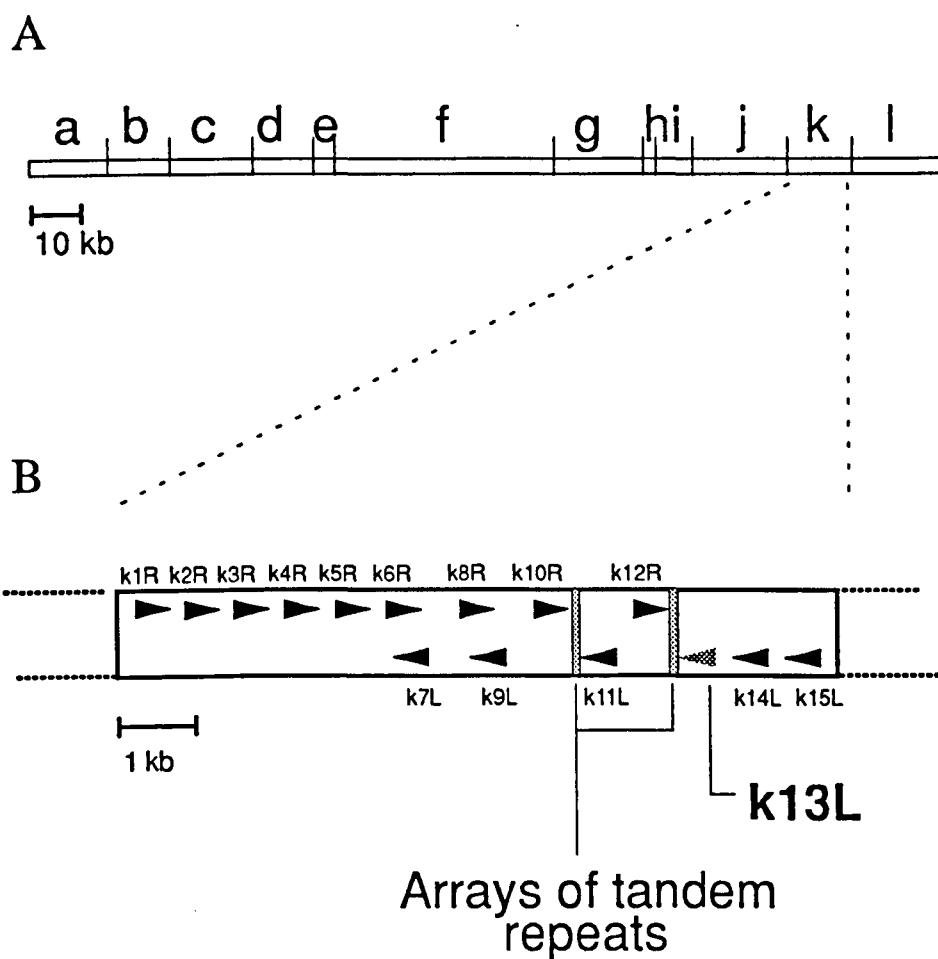


Figure 2.1 Location of UBCv on the ASFV genome.

Panel A: *Sall* map of the genome of the Malawi LIL20/1 isolate of ASFV (Dixon, 1988). Panel B: Detail of the gene arrangement on *Sall* fragment k showing the UBCv gene (k13L) encoded on the complementary DNA strand.

tick vector since these have a lower body temperature than mammals. The coding sequences contained in the right hand 55 kb region of the Malawi LIL20/1 genome were used to generate a standard codon preference table for ASFV genes (Devereux *et al.*, 1984). When compared to this table, the codon usage throughout k13L does not present any bias, towards either typical coding or non-coding regions (figure 2.3, A). Only one rare ASFV codon, with an occurrence frequency of 9%, is present in the whole k13L ORF. In contrast, over 50 rare codons are distributed throughout the k13L ORF when compared to the codon preference of *Escherichia coli* (figure 2.3, B).

The 30 bp sequence upstream of the k13L start codon is especially rich in A and T bases (43% and 30% respectively) which is similar to observations made for vaccinia virus early promoters (Davison and Moss, 1989a) and certain ASFV genes (Almazan *et al.*, 1992; Hammond, 1992). The motif TAAAT which occurs upstream of late and some early vaccinia virus genes occurs at a position 9 bp upstream of the k13L start codon and has also been reported in some early ASFV genes (Hammond, 1992). Transcriptional initiation sites of several ASFV genes have been mapped to positions a short distance (8 to 70 bp) upstream of AUG codons (Almazan *et al.*, 1992, 1993; Yanez *et al.*, 1993b), but promoters have not otherwise been defined and no consensus sequences are apparent by comparison of ASFV promoter sequences.

A signal for the termination of ASFV gene transcription genes consists of at least seven consecutive Ts (Almazan *et al.*, 1993). A 9T signal is first found 404 bp downstream from the stop codon of the k13L ORF. This suggests that the k13L messenger RNA is unduly long (1.1 kb), or that some alternative termination signal is recognized. Seventy percent of the ASFV genes identified so far do not have a 7T signal downstream from their ORF (Dixon *et al.*, 1993). However, the analysis of ASFV mRNA by Northern blot hybridization with a UBCv specific probe has shown that the UBCv transcript is approximately 1.1 kb long (Rodriguez *et al.*, 1992b), which suggests that the 9T sequence downstream from k13L might be the termination signal for UBCv transcription.

| | | |
|------|--|------|
| -40 | AAGGAATTAAACCAAGGAATTAAATTCATATT <u>AAAATAGCCATGGTTCCA</u> | 9 |
| | k13L -> M V S S | |
| 10 | GTTTTTACTGGCAGAGTACAAAAACCTAATAGTGAATCCCTCTGAGCAT | 59 |
| | F L L A E Y K N L I V N P S E H | |
| 60 | TTCAAAATCTCAGTGAATGAAGATAATTGACTGAATGGGATGTCACTT | 109 |
| | F K I S V N E D N L T E W D V I L | |
| 110 | AAAAGGCCACCTGACACTCTTATGAGGGAGGCTTATTCAAAGCAAAGA | 159 |
| | K G P P D T L Y E G G L F K A K I | |
| 160 | TTGTCTTCCTCCAAAATACCCATATGAACCACCCAGATTAACATTCA | 209 |
| | V F P P K Y P Y E P P R L T F T | |
| 210 | TCTGAAATGTGGCATCCCAATATCTACTCTGATGGAAATTATGTATT | 259 |
| | S E M W H P N I Y S D G K L C I S | |
| 260 | TATCTGCATGGAGACAATGCTGAAGAACAGGGATGACTTGGTCTCCGG | 309 |
| | I L H G D N A E E Q G M T W S P A | |
| 310 | CTCAAAAGATTGATACCGTACTTCTTAGTGTAAATTCTCTGCTCAATGAG | 359 |
| | Q K I D T V L L S V I S L L N E | |
| 360 | CCAAATCCAGATTCTCCAGCAAATGTAGATGCAGCTAAAGCTACCGTAA | 409 |
| | P N P D S P A N V D A A K S Y R K | |
| 410 | ATATCTATATAAAGAGGATTAGAATCATACCCCATGGAAGTTAAAAGA | 459 |
| | Y L Y K E D L E S Y P M E V K K T | |
| 460 | CTGTCAAAAATCATGGATGAGTGTTCAGCGGAAGACATAGAATATTT | 509 |
| | V K K S L D E C S A E D I E Y F | |
| 510 | AAAAATGTTCCAGTGAATGTTCTACCAAGTACCCAGTGATGATTATGAAGA | 559 |
| | K N V P V N V L P V P S D D D Y E D | |
| 560 | TGAAGAAATGGAGGATGGCACCTATATCTTAACCTATGATGATGAGGATG | 609 |
| | E E M E D G T Y I L T Y D D E D E | |
| 610 | AAGAAGAGGATGAAGAGATGGATGATGAGTAGTGCTGATTAAATGCATA | 659 |
| | E E D E E M D D E * | |
| 660 | ACATATTAGTTACTTACACTT <u>AGTGCTTAGATTTAGTGTTAAACTTT</u> | 709 |
| | <u>a</u> <u>b</u> <u>c</u> | |
| 710 | <u>d</u> <u>e</u> <u>f</u> <u>g</u> <u>h</u> <u>i</u> | 759 |
| 760 | <u>d</u> <u>e</u> <u>c</u> <u>d</u> | 809 |
| 810 | <u>e</u> <u>c</u> <u>d</u> <u>f</u> | 859 |
| 860 | <u>c</u> <u>g</u> <u>f</u> | 909 |
| 910 | <u>c</u> <u>h</u> <u>i</u> | 959 |
| 960 | <u>TTTGGCTGTTAGCGAAAAAAAAATCAGAAAAATTATAATCTGTATA</u> | 1009 |
| 1010 | TAATTCTGCTGATGTATATATT <u>TTTTTTGTTAAATGAAATT</u> term. | 1059 |

Figure 2.2 Nucleic acid sequence of the UBCv gene.

Complementary DNA sequence of a 1.1 kb region of *Sall* fragment k of the Malawi LIL20/1 genome comprising the k13L ORF (UBCv gene). The protein translation of k13L is given on the second row. The downstream tandem repeats (a through i), the TAAAT upstream motif and the 9T putative termination signal are underlined.

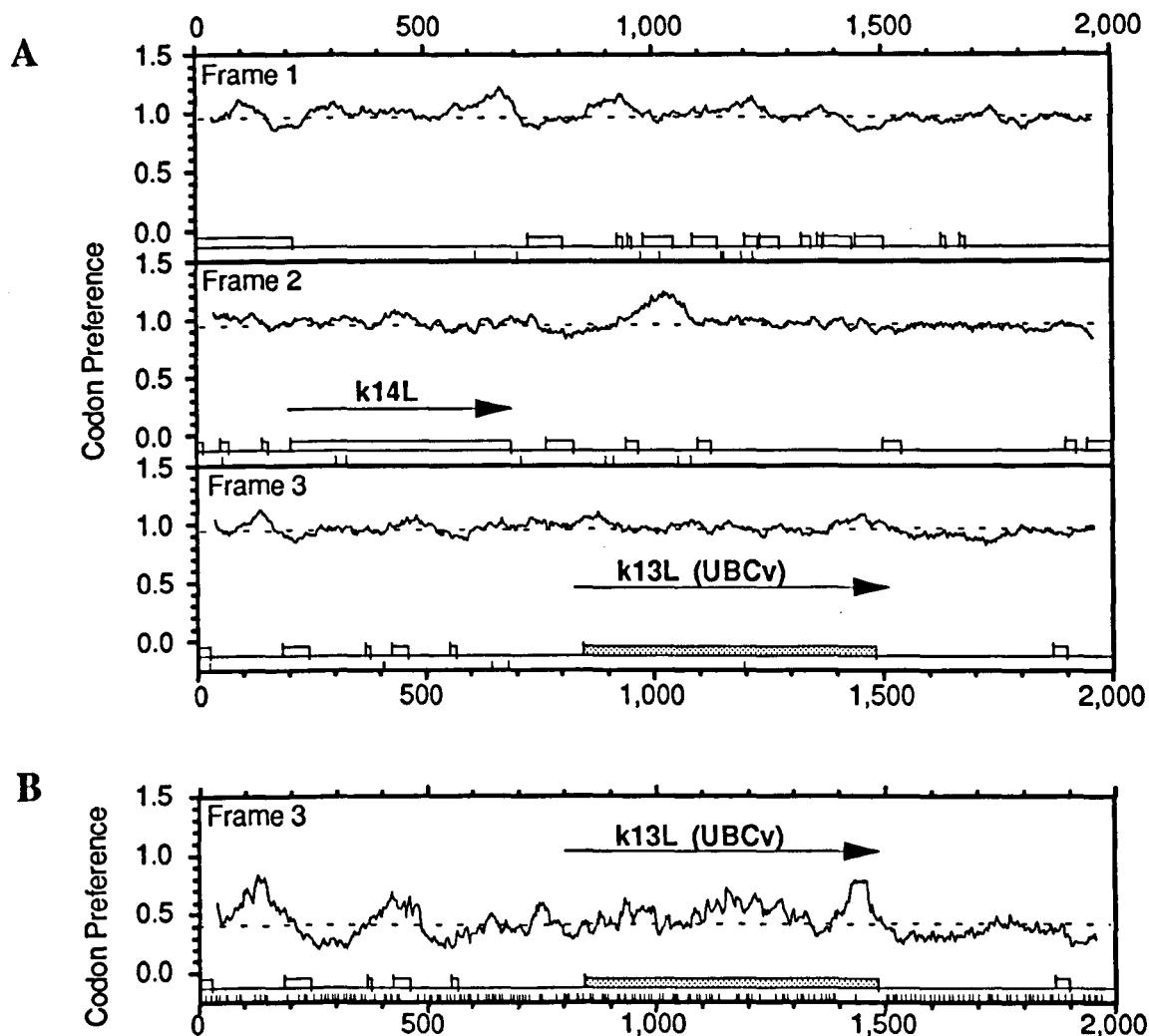


Figure 2.3 Statistical analysis of the codon usage in k13L.

A synonymous codon 'preference' table for ASFV was calculated using 63 ORFs spanning the 55 kb right hand region of the Malawi LIL20/1 genome. In panel (A), the codon usage along a 2 kb region surrounding k13L was compared to the codon preference of ASFV. Values lower than 1.0 indicate that less preferred synonymous codons are used in the sequence analyzed. ORFs are represented by open boxes and rare codons (typical occurrence frequency of less than 10%) are indicated by ticks under the ORF boxes. The position of the k13L ORF is indicated. In panel (B), the codon usage was compared to the codon preference table of *E.coli* ECOHIGH.COD. Output is from the CODONPREFERENCE program of the University of Wisconsin GCG software package (Devereux *et al.*, 1984).

An array of tandem repeats is located in the intergenic region between k13L and k12L. This array consists of 20 repeated units of closely related 14 bp sequences. Although other intergenic arrays of tandem repeats exist elsewhere in the genome, their sequences are not related to each other and the function of these repeats is unknown.

The nucleic acid sequence for the UBCv gene has also been reported for the attenuated BA71V isolate of ASFV (Rodriguez *et al.*, 1992b). The UBCv gene in BA71V has similar features to the ones described above for Malawi LIL20/1 and the nucleic acid coding sequences are 91% identical.

2.1.2 Detection of UBCv in other ASFV isolates by PCR

To assess whether the UBCv gene is present in other ASFV isolates and may therefore have an important role in virus replication, the UBCv gene was amplified by polymerase chain reaction (PCR) from genomic DNA of 20 ASFV isolates which were obtained from infected domestic pigs or ticks in Africa and Europe. The DNA was extracted from virus purified from infected pig blood (Payne and Wilkinson, personal communication). The sequences of the oligonucleotides used as primers in the PCR are complementary to the 5' and 3' ends of the UBCv gene, either from Malawi LIL20/1 (Mw1 set) or from BA71V (Ba1 set) (Table 2.1).

PCR using the Ba1 set of primers amplified a DNA fragment of the expected size (approximately 650 bp) from the nine ASFV genomes tested of European origin (figure 2.4, A). In contrast, PCR with Ba1 primers failed to amplify any sequences from the genomic DNA of seven African ASFV isolates, including Malawi LIL20/1 (figure 2.4, B). However when using the Mw1 set of primers, the PCR amplified a DNA fragment of the expected size (650 bp) from eleven ASFV genomes of African origin (figure 2.4, C). This difference between the European and African ASFV isolates confirmed the previous groupings observed both by restriction enzyme site mapping of virus genomes and by PCR amplification of other ASFV sequences (Williams, Payne and Wilkinson, personal communication).

| Set | No. | Posi-tion | Restric-tion site | Oligonucleotide sequence (5' to 3') |
|-----|-----|-----------|-------------------|---|
| Mw1 | UB3 | 5'end | <i>PstI</i> | ACCGGTCTGCAGGG <u>ATGGTT</u> TCCAGTTTTTACT |
| | UB4 | 3'end | <i>HindIII</i> | GTGTGAAAG <u>CCTCTACTCATC</u> ATCCATCTCTT |
| Mw2 | UB5 | 5'end | <i>BamHI</i> | ACCGGTGG <u>ATCCATGGTTCC</u> AGTTTTTACT |
| | UB6 | 3'end | <i>BamHI</i> | GTGTGAGG <u>ATCCCTACTCATC</u> ATCCATCTCTT |
| Ba1 | UB7 | 3'end | none | TTACTCATCATCCATCTCTTC A |
| | UB8 | 5'end | none | TAGCAGAGTACAGACACCTG A |

Table 2.1 Sequence of the oligonucleotides used as primers for the amplification by PCR of the k13L ORF.

Start and stop codons are underlined. The nonsense nucleotide hexamers at the 5' ends of the oligonucleotides are intended to help binding of restriction enzymes during digestion. *PstI*, *HindIII* and *BamHI* sites (in bold in the nucleotide sequence) were added to facilitate cloning into plasmid vectors (see §2.3.1 and §3.1.1).

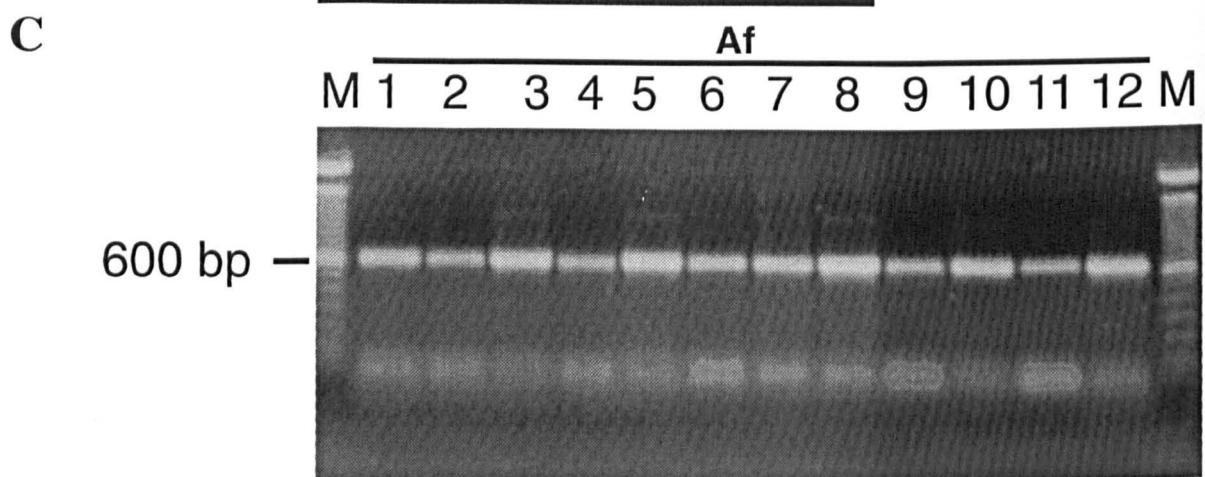
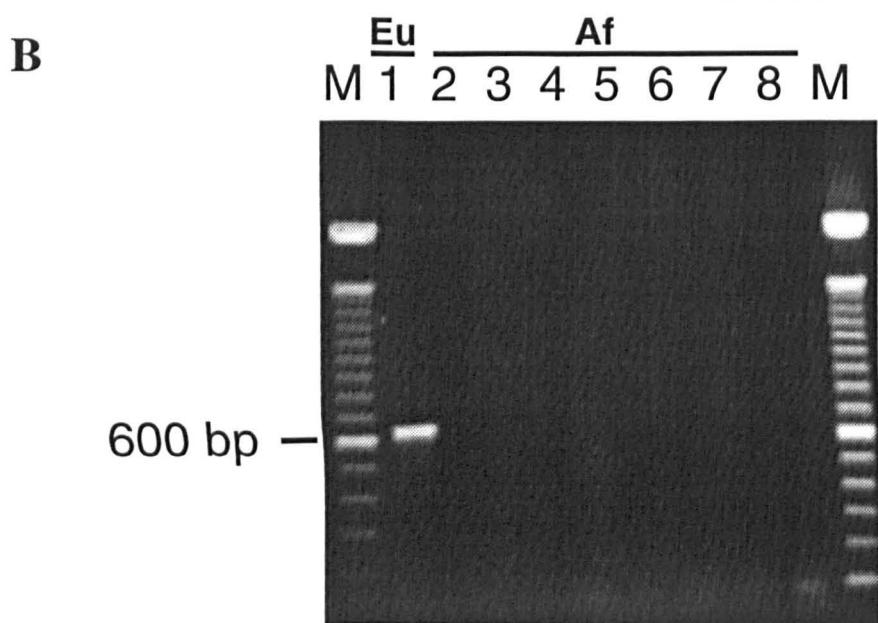
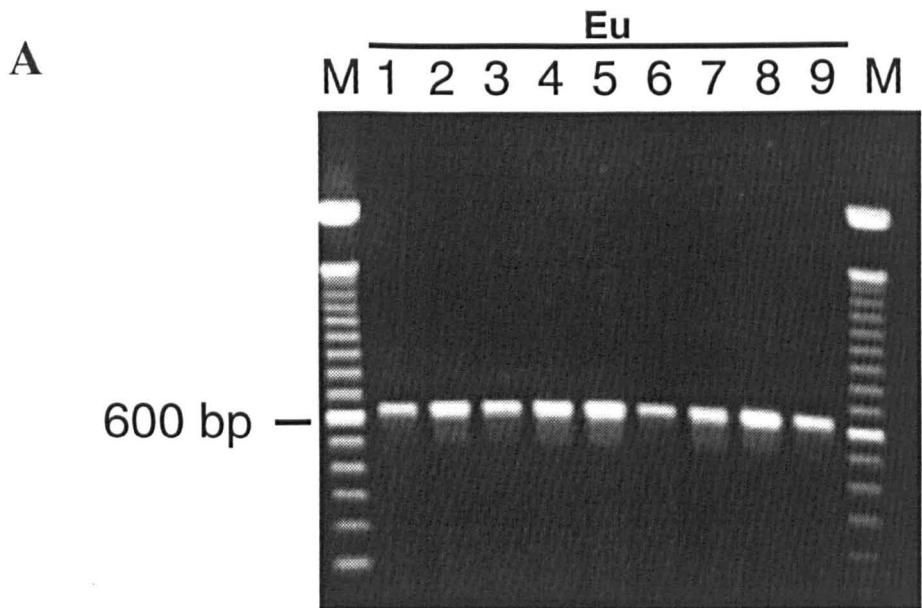


Figure 2.4 PCR amplification of the UBCv gene from African and European isolates of ASFV.

Figure 2.4 PCR amplification of the UBCv gene from African and European isolates of ASFV (facing page).

A library of genomic DNA from ASFV isolates originating from Europe (Eu) or from Africa (Af) was screened for the presence of the UBCv gene by PCR. The PCR primers used were complementary to the 5' and 3' ends of the UBCv gene sequence, derived either from the BA71V isolate (panels A and B) or from the Malawi LIL20/1 isolate (panel C). PCR products were separated by 0.6% agarose gel electrophoresis. Side lanes (M): 100 bp DNA ladder, the position of the 600 bp marker (double intensity) is indicated. The ASFV isolates were for panel A lanes 1-9: ANG70, LIS57, LIS60, MAL78, SAR82, ITA83, BEL85, TCN86, TCN87; panel B lanes 1-8: RSA85/1, TAN87, MOZ60, BUR84/1, BON83, CHA86, LIL20/1, DED86; panel C lanes 1-12: CHA86, NAM82/2, BUR84/1, MOZ60, HIN54, KAT67, KIM1, BAR2, BEL92/1, LIL20/1, LIV13/33, TAN87.

2.2 Amino acid sequence of UBCv

2.2.1 Predicted polypeptide structure of UBCv

The k13L ORF encodes a putative 213 amino acid UBCv protein with a predicted molecular weight of 24.7 kDa and an isoelectric point of 4.0. Computer predictions of the hydrophilicity of UBCv (figure 2.5, A) showed that it contained no extensive hydrophobic regions, indicating that UBCv was probably not membrane associated. Amongst the secondary structure features predicted for UBCv (figure 2.5, B), three α -helices and two β -sheets were located in similar positions to those observed in the crystal structure of the Ath1 UBC enzyme from *Arabidopsis thaliana* (Cook *et al.*, 1992b). Two further β -sheets and one α -helix present in Ath1 were not predicted in the relevant positions in UBCv.

Scanning the UBCv sequence against a protein motif library (Prosite, 06/93 version, Bairoch, 1992) suggested that the UBCv sequence contained two potential post-translation modification sites. The first was an asparagine glycosylation site ($NxTx$; x is any residue apart from a proline) at position 29, and the second was a tyrosine phosphorylation site ($KxxxDxxY$) at position 38. Although these two sites were also detected in the UBCv sequence from BA71V, they were not present in 20 other UBC enzyme sequences analyzed. The final motif detected spanned residues 74 to 88 of UBCv and corresponded to the universal signature of the active sites of UBC isozymes.

2.2.2 Comparison of UBCv amino acid sequence with other UBC enzymes

The amino acid sequences of UBCv and nine yeast UBC isozymes were aligned and compared. A core structure which consists of approximately 150 amino acids at the N-terminus is conserved in all UBC enzymes and contains the catalytic site (Jentsch, 1992). The sequence surrounding the active site cysteine is extremely well conserved (figure 2.6, A), except for a 10 amino acid insertion which is present in UBCv and the yeast UBC3, UBC6 and UBC7 enzymes.

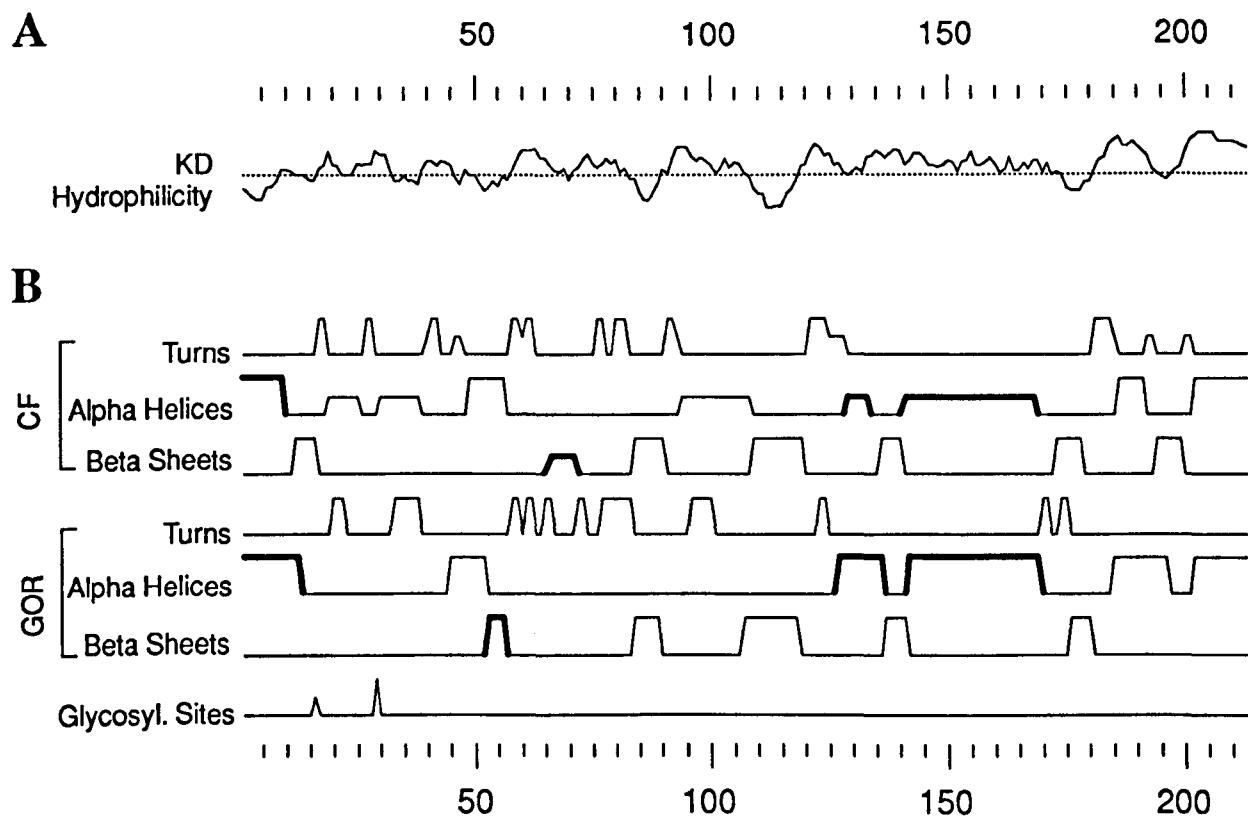


Figure 2.5 Predicted hydrophilicity and secondary structure of UBCv
 Panel A: Hydrophilicity according to Kyte-Doolittle (KD, Kyte and Doolittle, 1982) across the peptide sequence of UBCv. Panel B: Secondary structure according to either Garnier-Osguthorpe-Robson (GOR) or Chou-Fasman (CF) (Devereux *et al.*, 1984). Putative glycosylation sites are shown on the bottom row. Conserved β -sheets and α -helices (compared to the structure of Ath1, Cook *et al.*, 1992b) are indicated in bold. Outputs are from the PEPTIDESTRUCTURE program of the University of Wisconsin GCG software package.

A carboxyl-terminal tail of variable length extends from the core region. The sequences of these tails are unrelated although they share a large proportion of acidic residues (figure 2.6, B). The 56 amino acid C-terminal tail of UBCv contains 26 aspartic and glutamic acid residues (46%), including an uninterrupted track of 10 of these residues.

The percentages of amino acid identity and similarity between UBCv and yeast UBCs are given in table 2.2. The identity of UBCv over the N-terminal conserved domain ranged from 25% compared to UBC9 to 41% compared to UBC7. When the acidic tails were included, the percentage similarity of UBCv was lowest compared to UBC6 and highest compared to UBC3 (23% and 51% respectively).

| Yeast UBC enzyme | Percent identity (core) | Percent similarity (core + tail) |
|------------------|----------------------------|-------------------------------------|
| UBC1 | 27% | 25% |
| UBC2 | 32% | 43% |
| UBC3 | 38% | 51% |
| UBC4 | 36% | 40% |
| UBC5 | 36% | 41% |
| UBC6 | 28% | 23% |
| UBC7 | 41% | 46% |
| UBC8 | 26% | 29% |
| UBC9 | 25% | 27% |

Table 2.2

Percentage of amino acid identity and similarity between UBCv and yeast UBC1 to UBC9 enzymes.

The percentage of identical amino acids between the UBCs was determined using the conserved N-terminal regions (core). The percentage of similar amino acids over the whole length (core + tail) of the UBC polypeptides was determined using the PAM250 matrix (Dayhoff *et al.*, 1983). Calculations were made using the DISTANCES program of the University of Wisconsin GCG package (Devereux *et al.*, 1984).

B



Figure 2.6 Alignment of the protein sequences of UBC enzymes from ASFV and yeast.

Panel A (facing page): Alignment of the conserved catalytic core of UBC enzymes. Black boxes indicate residues that agree with the consensus (defined by a residue present in more than 5 sequences). Decreasing shades of grey represent decreasing degrees of conservation in residue substitutions. The star (*) marks the position of the active site cysteine. The insertion downstream from the active site is underlined. Panel B: Alignment of the carboxyl-terminal tail of UBC enzymes. Grey boxes indicate acidic amino acids (Asp and Glu). The alignment was produced by the GCLUSTALV program of the University of Wisconsin GCG software package. Y1 to Y9 : UBC1 to UBC9 from yeast (protein sequences were translated from the nucleotide sequences obtained from the EMBL and GBONLY databases). Mw and Ba: sequence of UBCv from Malawi LIL20/1 and BA71V ASFV isolates respectively.

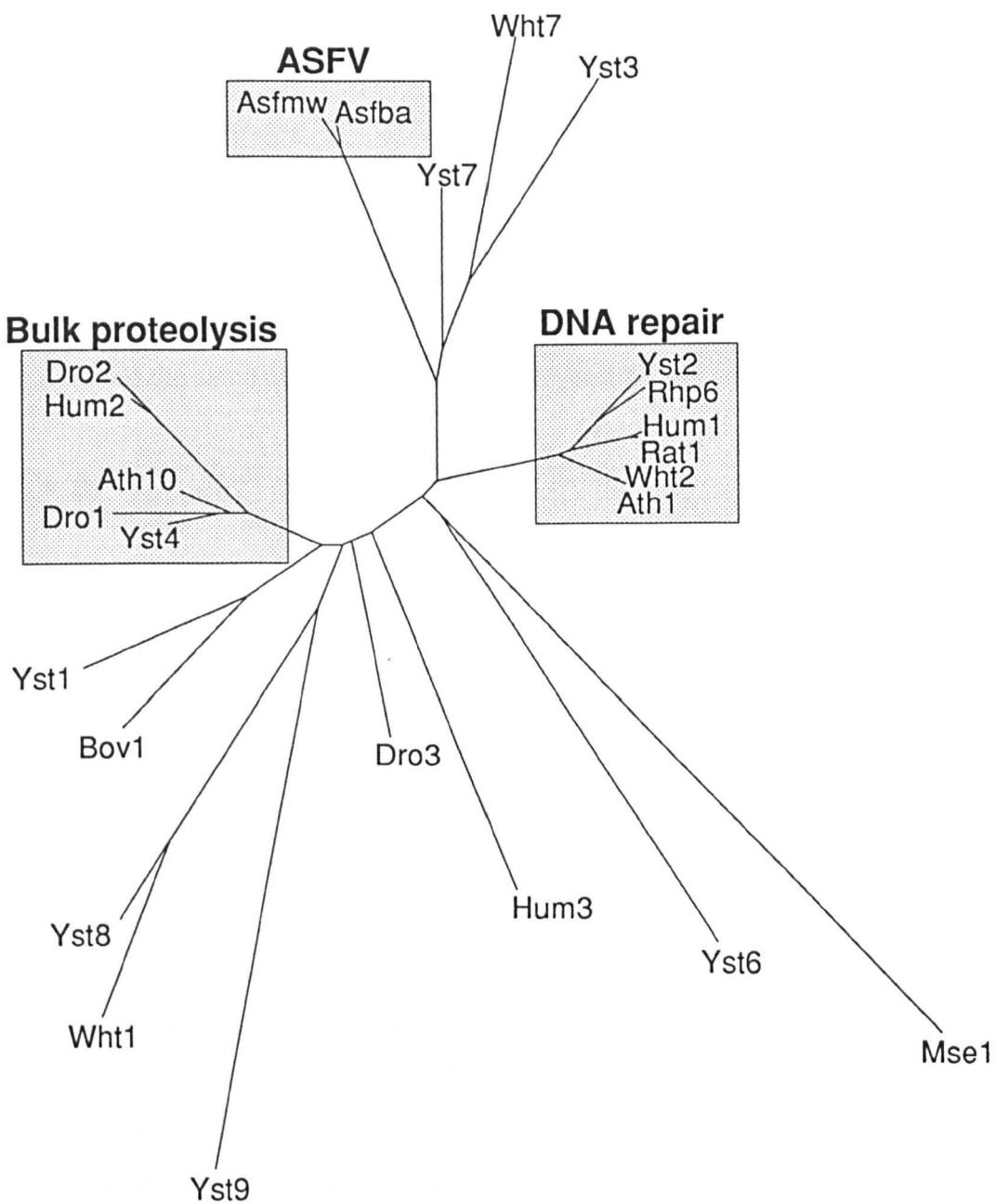


Figure 2.7 Dendrogram of sequence similarity between various UBC enzymes. The length of the lines to the nodes joining the various UBC enzymes are proportional to their sequence divergence. Clustering of distinct groups of enzymes are boxed. The protein sequences of the UBC enzymes used to calculate the dendrogram were translated from nucleotide sequences in the EMBL and GBONLY databases. Yst: yeast (*Sc*), Wht: wheat, Dro: drosophila, Mse: mouse, Hum: human, Ath: *Arabidopsis thaliana*, Bov: bovine, Rhp: *Saccharomyces pombe*, Rat: rat, Asfmw: ASFV (Malawi LIL20/1), Asfba: ASFV (BA71V). Output is from the University of Washington PHYLIP software package. The tree was calculated according to the neighbour joining method using the percentage of similarity (PAM250 matrix, Dayhoff *et al.*, 1983) in the UBC's core amino acid sequences.

The percentage similarity between 25 UBC enzyme sequences present in the database was used to plot a homology dendrogram (figure 2.7). This diagram suggested the existence of distinct UBC subgroups between which the sequence divergence was lower than 25%. The clusterings partly reflected known similarities in UBC function such as enzymes involved in DNA repair or in bulk protein degradation. Although the UBCv enzyme did not appear to belong to any of these subgroups it shared the same phylogenetic branch as yeast UBC3 and UBC7 enzymes.

2.3 UBCv *in vitro* enzyme activity

2.3.1 Production of recombinant UBCv

Many UBC enzymes have been expressed in *E. coli* and shown to retain their activity *in vitro* (Jentsch *et al.*, 1987; Goebel *et al.*, 1988; Sullivan and Vierstra, 1989; Seufert *et al.*, 1990; Haas *et al.*, 1991; Qin *et al.*, 1991). Moreover, prokaryotic vectors are the preferred expression system for the study of UBC enzymes since they are devoid of contaminating endogenous enzymes of the ubiquitin pathway.

The k13L ORF was amplified by PCR using as template DNA from bacteriophage Lambda clone LMw22 which contains the ASFV genome fragment *Sal*I k (Malawi LIL/20 isolate, Dixon, 1988). The UB3 and UB4 oligonucleotide primers (Table 2.1) used in the reaction contained sequences from the 5' and 3' ends of the k13L ORF and added *Pst*I and *Hind*III restriction sites. The PCR reaction yielded a unique product of approximately 670 bp (expected length was 668 bp) (figure 2.8, A).

The PCR product was digested with *Pst*I and *Hind*III and ligated to *Pst*I/*Hind*III digested plasmid vector pKK 233-2 to yield plasmid PH1 (figure 2.9). The cloning process introduced four additional codons at the 5' end of the original k13L ORF (which translates into four non-authentic N-terminal residues Met¹-Ala²-Ala³-Gly⁴). The expression vector pKK 233-2 is derived from plasmid pBR 322 and has both ampicillin and tetracyclin resistance genes (Amann and Brosius, 1985). A P_{trc} promoter (hybrid P_{trp}-lac promoter) and T1/T2 terminators are present on each side of a *Nco*I/*Pst*I/*Hind*III polylinker. Expression of the gene cloned downstream

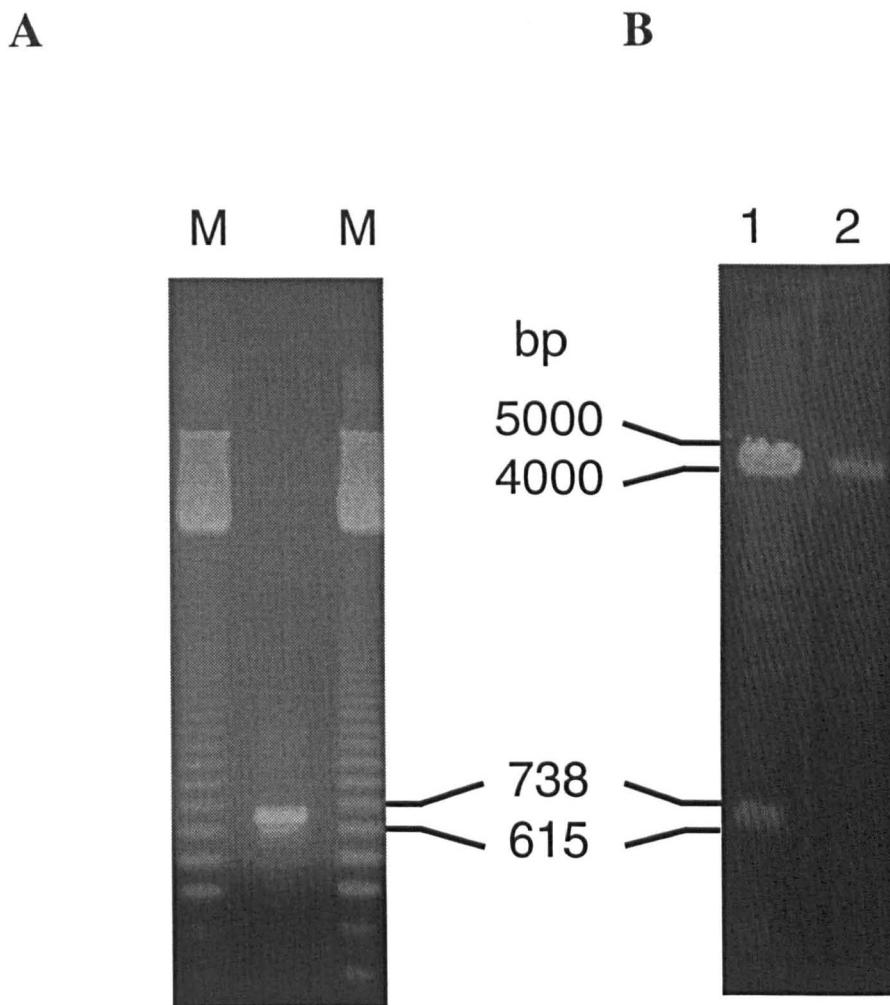


Figure 2.8 Amplification of UBCv by PCR and cloning in pKK 233-2.

Primers UB3 and UB4 (see table 2.1) were used to amplify UBCv (k13L ORF) by PCR from Lambda clone LMw22 containing ASFV fragment *Sal*I k. Panel (A) shows a 1% agarose gel electrophoresis of the purified PCR product. Side lanes (M): 123 bp DNA ladder. The purified PCR product was cloned in the *Pst*I / *Hind*III site of plasmid pKK 233-2 to yield plasmid PH1. Panel (B) shows a 0.6% agarose gel electrophoresis of the *Pst*I / *Hind*III digests of plasmid DNA from an *E. coli* colony transformed with PH1 (lane 1) or with plasmid pKK 233-2 alone (lane 2).

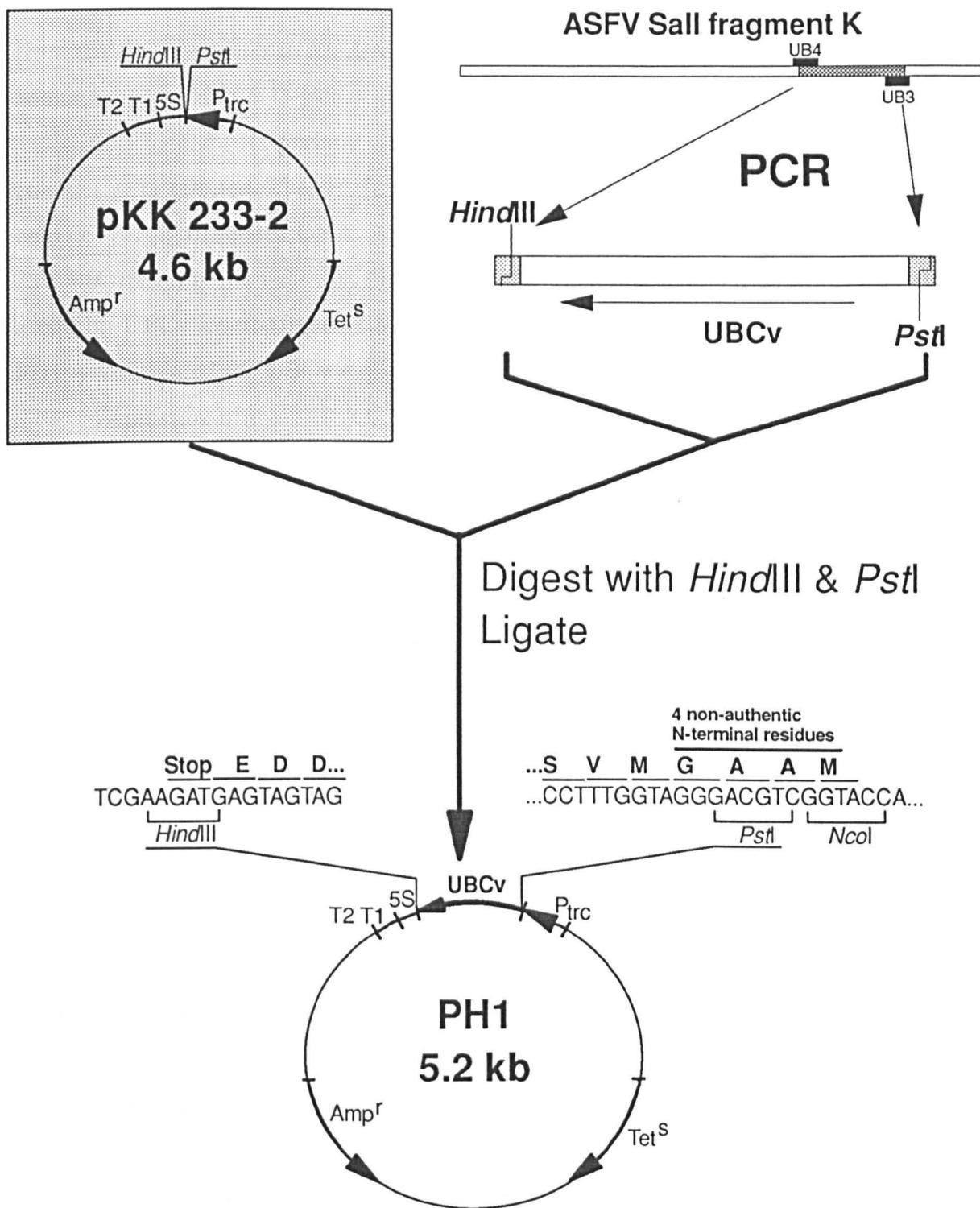


Figure 2.9 Diagram showing the subcloning of the UBCv gene in expression vector pKK 233-2.

The UBCv gene was amplified by PCR using as template DNA from bacteriophage Lambda clone LMw22 which contains *Sall* fragment k of the Malawi LIL20/1 ASFV genome). The amplified UBCv gene was inserted into plasmid pKK 233-2 by double digestion of both the vector and the PCR product with *Pst*I and *Hind*III restriction enzymes followed by ligation, yielding recombinant plasmid PH1.

from the P_{trc} promoter is regulated by the lac operator and is therefore induced by adding isopropylthio- β -D-galactoside (IPTG) to the cell culture.

The ligated PH1 plasmid was transformed into *E. coli* and ampicillin resistant colonies were selected. The efficiency of transformation was 4.10^4 ampicillin resistant colonies per μg of ligated DNA. Analysis of plasmid DNA from resistant colonies by *PstI* / *HindIII* digestion showed that 50% of the ampicillin resistant clones contained a 650 bp UBCv insert (figure 2.8, B). Clones harbouring the recombinant plasmid PH1 were isolated for UBCv expression.

Expression of the k13L ORF was induced in *E. coli* containing PH1 by adding IPTG to cultures. Proteins in the resulting cell lysates were then analyzed by SDS-polyacrylamide gel electrophoresis (SDS/PAGE). Coomassie blue staining of proteins was not sufficiently sensitive to detect the expressed protein, consequently *E. coli* proteins were labelled with [³⁵S]methionine at various times after induction with IPTG. Upon induction, 50% of the clones containing the PH1 plasmid expressed a novel protein with an apparent molecular weight of 30 Kda (figure 2.10). This protein was not detected in induced cells containing the vector pKK233-2 alone (data not shown).

Expression of this 30 kDa protein was detected 10 minutes after induction and continued at a constant level for at least another 60 minutes (data not shown). This novel protein was likely to be UBCv despite the discrepancy between the apparent and predicted molecular weights of UBCv (30 kDa versus 24.4 kDa), since aberrant migration in SDS/PAGE was also observed with other UBC enzymes and might have been due to the freely extending acidic tail (Goebel *et al.*, 1988; Morrison *et al.*, 1988; Sullivan and Vierstra, 1989).

2.3.2 Purification and assay of a ubiquitin activating enzyme E1

A ubiquitin activating (E1) enzyme is an essential component for the *in vitro* assays of the UBCv enzyme. A homogenous preparation of E1 enzyme was purified from pig brain tissue by affinity chromatography on a ubiquitin-sepharose column as described in figure 2.11 (Ciechanover *et al.*, 1982).

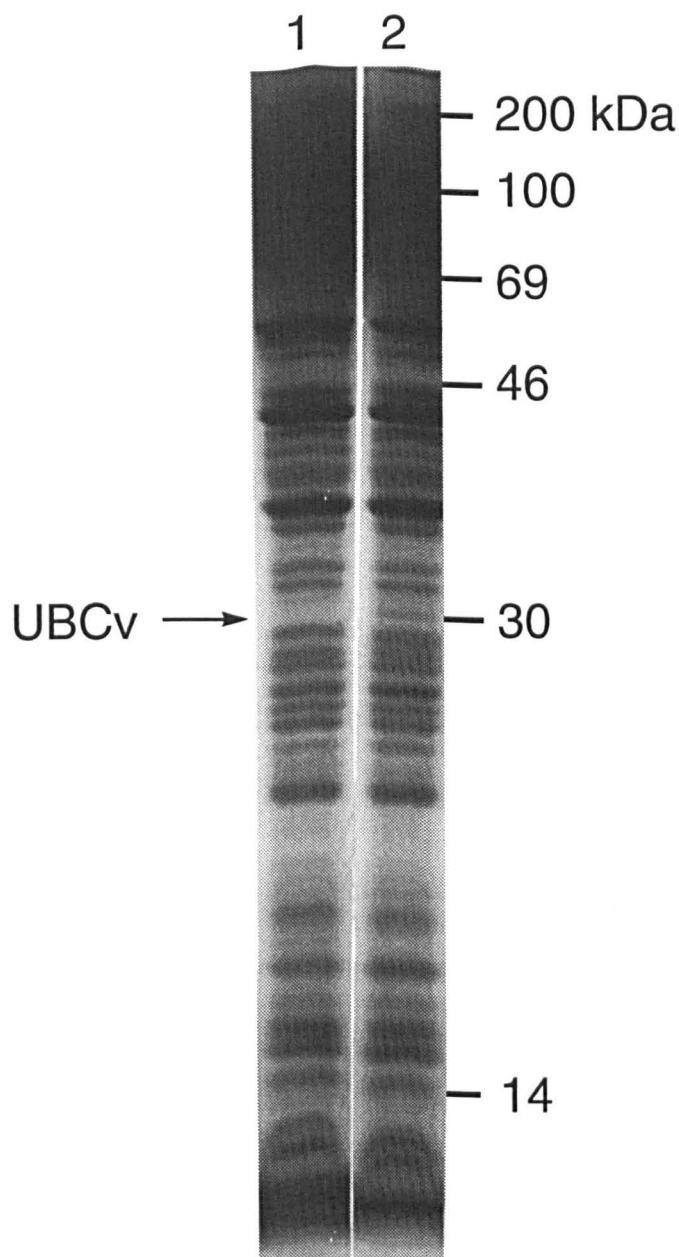


Figure 2.10 Expression of the UBCv protein in *E. coli* harbouring plasmid PH1. IPTG was added to an *E. coli* cell culture containing PH1 in early exponential phase to induce expression of UBCv. The culture was incubated for 30 minutes before a pulse of [35 S]methionine was added for 10 minutes. Cells were pelleted and lysed by adding sample buffer. Proteins were separated by 20% SDS/PAGE and detected by fluorography of the dried gel. Lane 1: cell lysate before induction; Lane 2: cell lysate 30 min after induction. The arrow head shows the position of the novel UBCv protein. Molecular weights in kDa are indicated on the right.

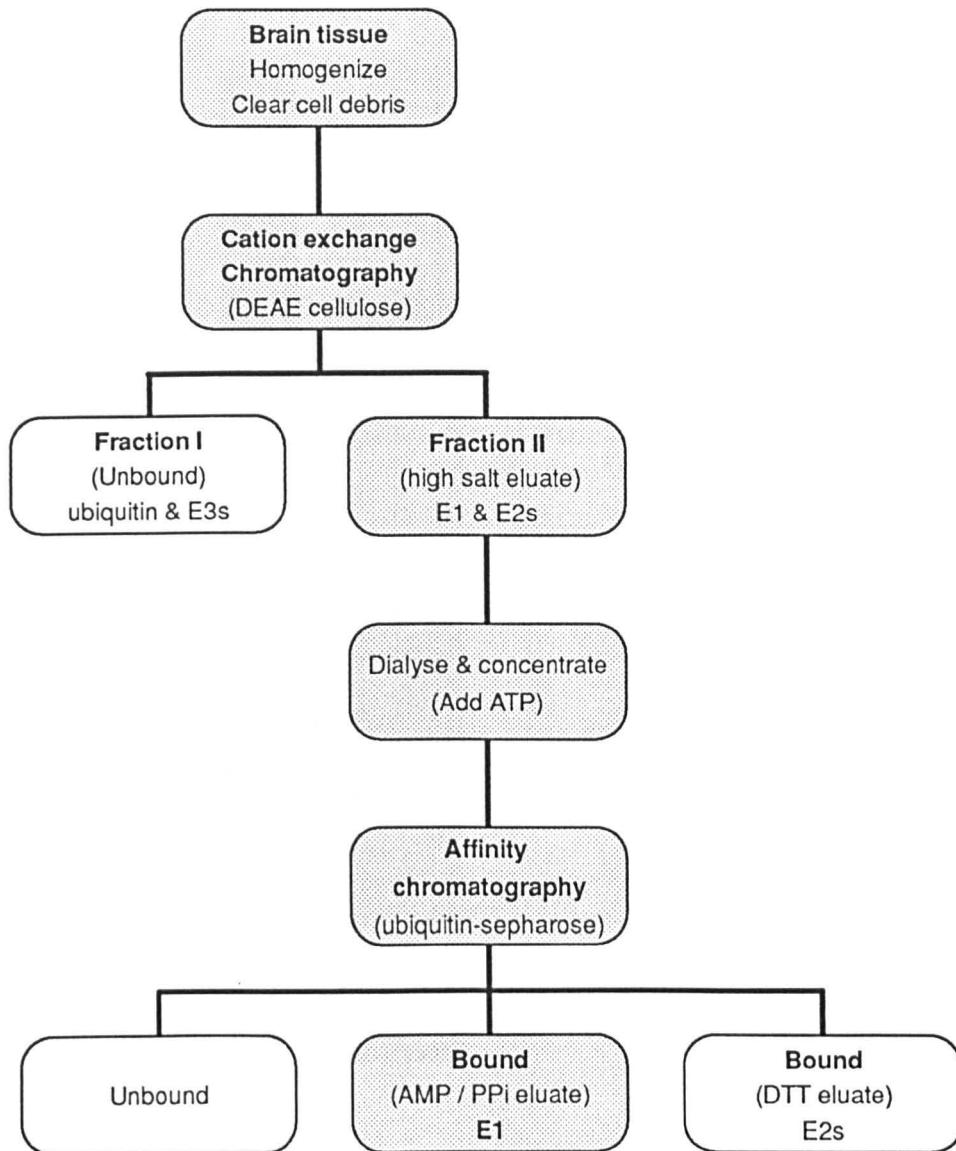


Figure 2.11 Diagram describing the purification of an E1 enzyme from pig brain.

A soluble extract from a fresh piglet brain was depleted of endogenous ubiquitin by DEAE cellulose ion-exchange chromatography. The bound proteins, including E1, were eluted by a high salt buffer and applied to a ubiquitin-sepharose affinity column. In the presence of ATP, the E1 enzyme covalently bound to the immobilized ubiquitin through a thiol ester link, whereas other protein contaminants were washed through the column. The E1 enzyme then was eluted off the column with an AMP and PPi buffer, a mixture that specifically reverses the binding reaction of E1 with ubiquitin.

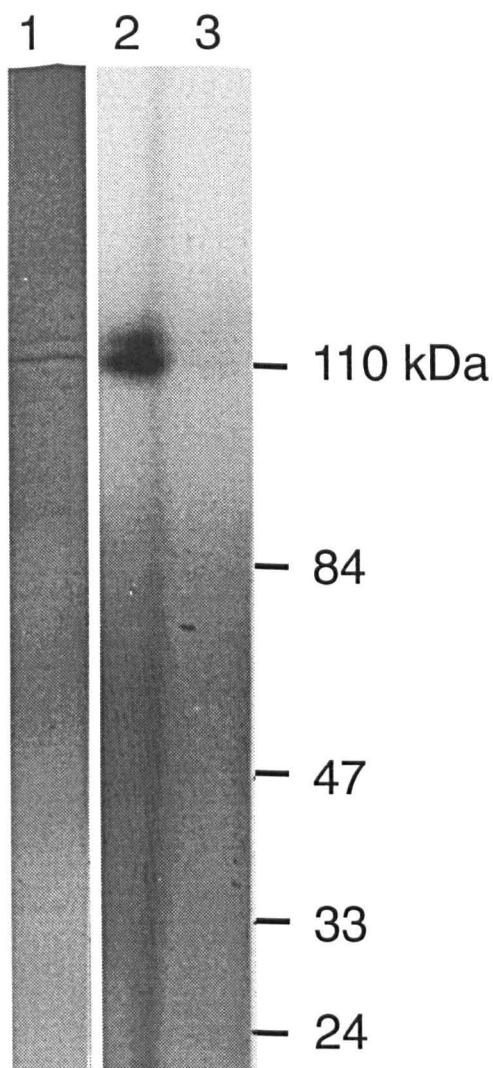


Figure 2.12 Purity and *in vitro* activity of a ubiquitin activating enzyme E1 from pig brain.

Lane 1: Coomassie staining of 7-20% SDS/PAGE of proteins eluted off a ubiquitin affinity column loaded with piglet brain extract; lane 2: 10 minutes incubation of affinity purified E1 with [¹²⁵I]Ubiquitin and ATP (autoradiography of 7-20% SDS/PAGE); lane 3: same reaction as in lane 2 but without ATP. Molecular weights in kDa are indicated to the right.

SDS/PAGE analysis of the affinity purified material followed by Coomassie blue staining (figure 2.12, lane 1) showed that the eluted material consisted of a protein doublet (Mwts 100 kDa and 107 kDa). These molecular weights are those expected for the two isoforms of the mammalian E1 enzyme (Cook and Chock, 1992).

A sample of the affinity purified preparation was tested for E1 activity by incubating it for 10 minutes with [¹²⁵I]Ubiquitin in the presence of ATP. The products of the reaction were analyzed by non-reducing SDS/PAGE followed by autoradiography (figure 2.12, lane 2). In these conditions two radioactive ubiquitin adducts of 108 and 115 kDa were formed. These adducts were not detected when the reaction mixture was either submitted to reducing conditions (data not shown) or if ATP was omitted (figure 2.12, lane 3).

These observations indicated that the affinity purified proteins were E1 enzyme isoforms capable of forming an ATP dependent thiolester bond with ubiquitin. Furthermore the E1 preparation was free of UBC enzyme contaminants and was suitable for use in UBCv activity assays.

2.3.3 *In vitro* assay of UBCv activity

Recombinant UBCv was prepared from the crude soluble extract of an induced *E. coli* culture containing plasmid PH1. A sample of recombinant UBCv was incubated with [¹²⁵I]Ubiquitin, purified pig brain E1 and ATP. After 15 minutes, the products of the reaction were analyzed by SDS/PAGE under non-reducing conditions (figure 2.13, lane 1).

A single 37 kDa radioactive ubiquitin adduct was formed. This ubiquitin adduct was sensitive to reducing conditions (figure 2.13, lane 4) which indicated that the ubiquitin adduct was the result of a thiolester bond. Formation of the 37 kDa ubiquitin adduct was also both ATP and E1 dependent (figure 2.13, lanes 2 & 3). No E1 ~ ubiquitin adduct was visible, suggesting that ubiquitin activation was the rate limiting step. When recombinant UBCv was substituted for a protein extract from an *E. coli* culture containing the pKK 233-2 vector alone, no ubiquitin adduct was

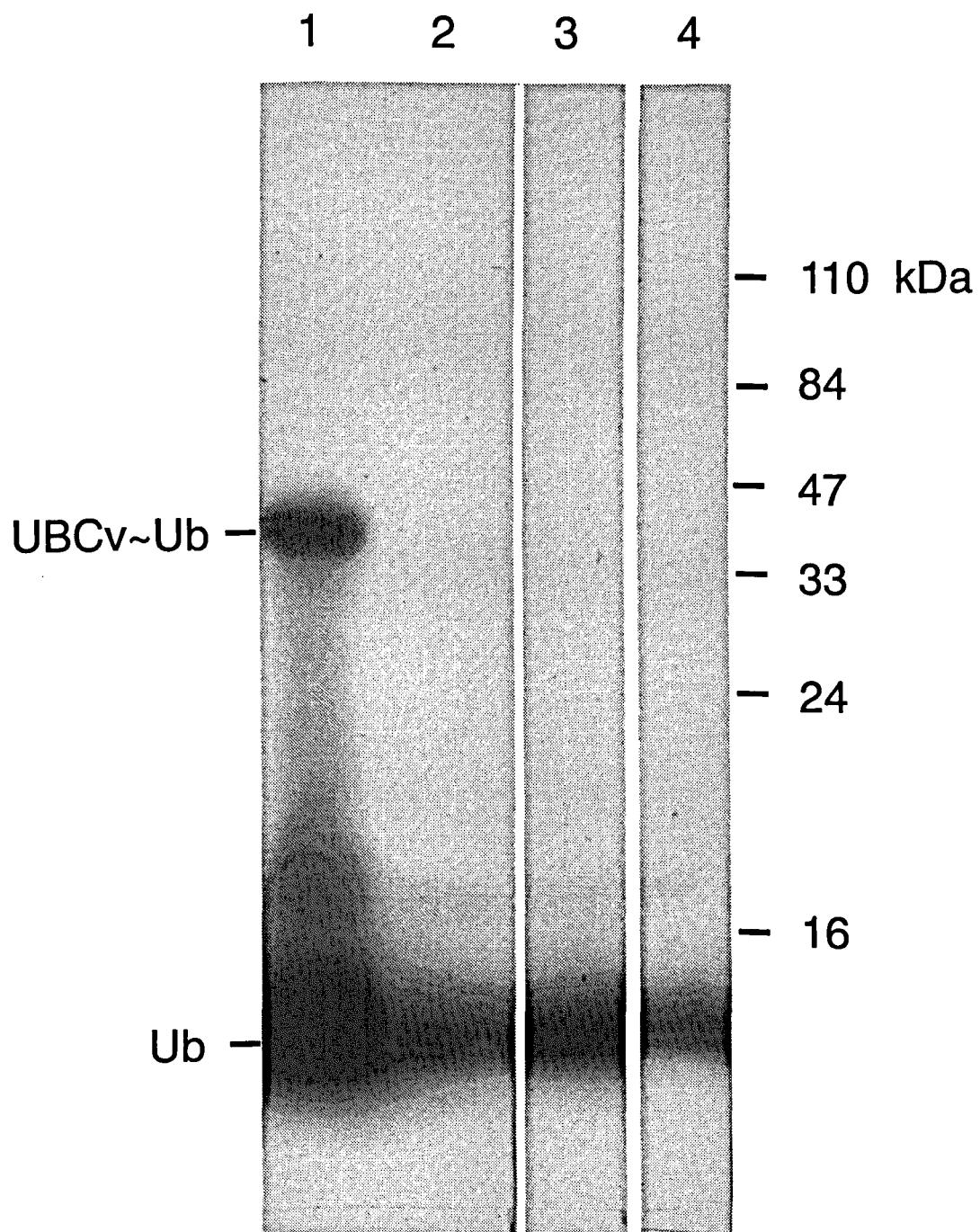


Figure 2.13 Formation of a thioester bond between UBCv and ubiquitin.

Reaction mixtures were incubated for 15 minutes and contain in lanes 1 and 4: recombinant UBCv, [125 I]ubiquitin, affinity purified pig brain E1 and ATP; lane 2: as lane 1 without E1; lane 3: as lane 1 without ATP. The reactions were stopped by adding sample buffer with (lane 4) or without (lanes 1-3) β -mercaptoethanol. Reaction products were separated by 7-20% SDS/PAGE and detected by autoradiography of dried gels. The positions of ubiquitin (Ub) and of the UBCv-ubiquitin thioester complex (UBCv-Ub) are indicated. Molecular weights in kDa are shown on the right.

observed (data not shown) confirming that the 37 kDa thiolester adduct consisted of UBCv~[¹²⁵I]ubiquitin.

In vitro studies have shown that UBC enzymes with an acidic C-terminal tail can ubiquitinate histones (Jentsch *et al.*, 1987; Goebel *et al.*, 1988; Sullivan and Vierstra, 1989; Haas *et al.*, 1991). In these experiments, ubiquitin-H2A conjugate formation occurred in a reaction that was not dependent on added E3 enzymes. To determine whether such a ubiquitin-histone conjugate could be formed by UBCv in an E3 independent reaction, histone H2A was added to the same reaction mixture as for the thiolester assay (recombinant UBCv, pig brain E1, [¹²⁵I]Ubiquitin and ATP). The incubation time was extended to 60 minutes and the products of the reaction were analyzed by reducing SDS/PAGE. The reducing conditions ensured that only isopeptide links rather than thiolester bonds were detected (figure 2.14).

A complex mixture of radioactive adducts was formed that corresponded to three distinct classes of ubiquitin conjugates. These could be more easily identified by comparison with the control assay containing no added histones (figure 2.14, A). Surprisingly, when no protein substrate was present six ubiquitin conjugates were formed with apparent molecular weights of 17, 36, 37, 42, 46 and 54 kDa.

A number of other UBC enzymes (including the yeast UBC3 enzyme) have given rise to similar *in vitro* patterns of conjugation (Sung *et al.*, 1988; Chau *et al.*, 1989; Arnold and Gevers, 1990; Braatz *et al.*, 1992). These patterns were assigned to the UBC enzyme's ability to transfer ubiquitin to themselves, as well as to free or conjugated ubiquitin. These activities are known as auto- and poly-ubiquitination. The results obtained with UBCv suggest that it has such activities since the ubiquitin conjugates that are formed could correspond to Ub₂ (17 kDa ubiquitin dimer), to two species of UBCv-Ub where ubiquitin is linked to different lysine residues of UBCv (36 and 37 kDa), and to poly-ubiquitinated forms of UBCv: UBCv-Ub₂ (42 and 46 kDa) and UBCv-Ub₃ (54 kDa). Western blotting analysis using monospecific anti-UBCv antiserum confirmed that the high molecular weight adducts consisted of UBCv-ubiquitin conjugates (S. Twigger, personal communication).

The addition of histone H2A to the reaction resulted in the formation of two novel ubiquitin conjugates (figure 2.14, B, lane 1) which could correspond to mono- and di-ubiquitin forms of H2A: H2A-Ub (26 kDa) and H2A-Ub2 (32 kDa).

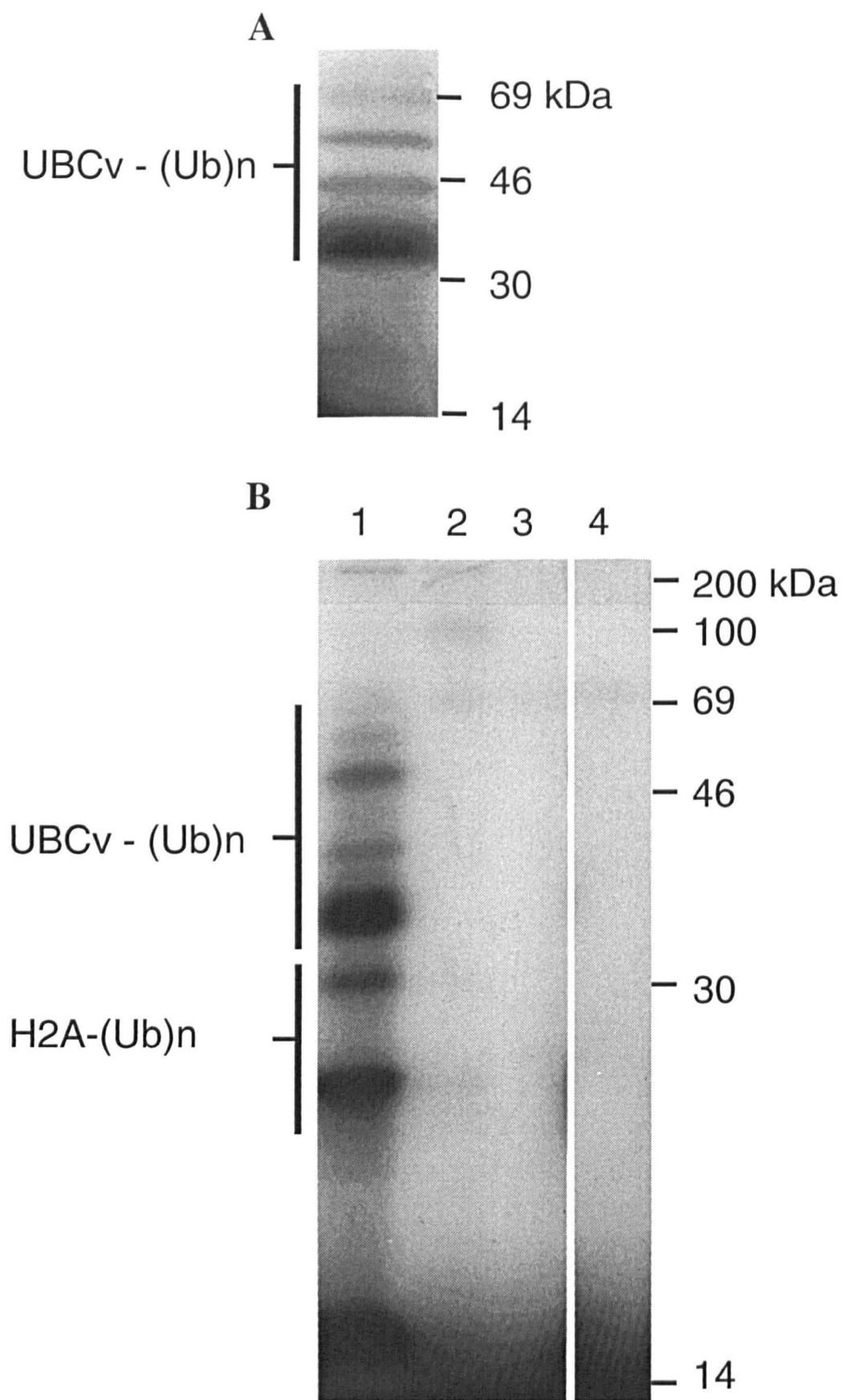


Figure 2.14 Ubiquitin conjugate formation in the presence of UBCv.

Figure 2.14 Ubiquitin conjugate formation in the presence of UBCv (facing page).

Reaction mixtures were incubated for 60 minutes and contained in panel A: recombinant UBCv, [125 I]Ubiquitin, pig brain E1 and ATP. Panel B, lane 1: as panel A with added histone H2A; lane 2: as lane 1 without E1; lane 3: as lane 1 without ATP; lane 4: as lane 1 with UBCv replaced by extracts from *E. coli* containing vector pKK 233-2 alone. Reactions were stopped by boiling in sample buffer containing β -mercaptoethanol. Reaction products were separated by 7-20% SDS/PAGE and detected by autoradiography of dried gels. The positions of ubiquitin (Ub), ubiquitin_n polymers (Ub_n), histone H2A-ubiquitin conjugates (H2A-Ub) and UBCv-ubiquitin conjugates (ASFV UBC-Ub) are indicated. Molecular weights in kDa are shown on the right.

The 17 kDa ubiquitin dimer and most of the UBCv auto-ubiquitination ladder were still visible in reactions in which H2A was added. Formation of all these conjugates was shown to be E1, ATP and UBCv dependent (figure 2.14, B, lanes 2,3 & 4).

The three classes of substrates for the UBCv enzymes were therefore UBCv itself, ubiquitin and histones. Conjugation assays with histone H2B and lysozyme showed that these substrates were also ubiquitinated by UBCv (data not shown).

1.4 Discussion

The analysis of the k13L ORF encoding UBCv revealed a structure typical of ASFV genes, indicating that UBCv was likely to be a functional gene. Observations such as the codon usage and the high A+T content of k13L suggested that the UBCv gene was not a particularly recent acquisition of the ASFV genome. This view was supported by the PCR screening which suggested that the UBCv gene was a universal feature of African and European ASFV isolates. Moreover the tissue culture adapted BA71V isolate (Tabares *et al.*, 1987), which has undergone several genome rearrangements during tissue culture adaptation, also contained an intact UBCv gene (Rodriguez *et al.*, 1992b). The ubiquitous presence of UBCv in ASFV, in spite of its location in the more variable region of the genome, supported the hypothesis that UBCv is potentially important for ASFV replication.

The protein sequence deduced from the UBCv gene was homologous to eukaryotic UBC isozymes. Amino acid identity between UBCv and cellular UBC enzymes reached 40% which is higher than usually observed when comparing ASFV proteins to their cellular homologues (typically around 20%) as would be predicted since UBC enzymes are a very conserved protein family. The conserved N-terminal 150 residues of UBC enzymes were also present in UBCv as was the active site cysteine. The C-terminal region of UBCv was similar to that of yeast UBC2, UBC3 and UBC8 since all contain a large number of acidic residues. Overall however, UBCv did not appear to be closely related to any particular subtype of UBC enzymes, suggesting that UBCv might have evolved a novel function.

The *in vitro* experiments demonstrated that recombinant UBCv could, in an E1 and ATP dependent reaction, form an adduct with ubiquitin. The UBCv enzyme

could also covalently transfer ubiquitin to protein substrates. This evidence indicated that UBCv had the general *in vitro* characteristics of UBC enzymes. Furthermore the formation of ubiquitin conjugates catalyzed by UBCv was highly specific since UBCv ubiquitinated itself, ubiquitin and histones but none of the numerous *E. coli* proteins present in the assays.

UBCv shares its histone ubiquitination property with the yeast UBC2 and UBC3 enzymes (Pickart and Rose, 1985; Jentsch *et al.*, 1987; Goebel *et al.*, 1988; Sung *et al.*, 1988; Prakash, 1989; Sullivan and Vierstra, 1989; Haas *et al.*, 1991). Since all three enzymes have an acidic C-terminal extension, it is possible that ionic interactions with basic proteins such as histones might be involved in substrate recognition. Further evidence for the importance of the C-terminal tail in determining substrate specificity comes from experiments in which the C-terminal tail of UBC3 was transferred to the N-terminal core of other UBC enzymes (Kolman *et al.*, 1992; Silver *et al.*, 1992). Since this portable determinant probably conferred substrate specificity, it is likely that the C-terminal tail of UBCv is important for the viral enzyme's substrate specificity and function. The lack of apparent homology between the tails of UBCv and other UBC enzymes suggested that UBCv had a distinct physiological target. This was also suggested from complementation experiments carried out with UBCv in yeast UBC mutants (Jentsch, personal communication). In a yeast UBC2 null mutant expressing UBCv, none of the wild type UBC2 phenotypes (DNA repair or sporulation) were restored although growth of the yeast mutant was slightly restored. The N-terminal core of the yeast UBC2 enzyme retained some of its functions in the absence of a C-terminal tail (Morrison *et al.*, 1988; Sung *et al.*, 1988; Prakash, 1989), partly through interactions with an E3 enzyme (Dohmen *et al.*, 1991; Sharon *et al.*, 1991; Sung *et al.*, 1991a; Madura *et al.*, 1993). Thus the possibility that the function (or one of the functions) of UBCv is mediated by an E3 enzyme (either cellular or virus encoded) can not be completely ruled out.

The UBCv enzyme also shared an *in vitro* poly-ubiquitination property with UBC2 and UBC3 (Sung *et al.*, 1988; Haas *et al.*, 1991; Banerjee *et al.*, 1993). From the results presented here, the exact nature of the poly-ubiquitinated conjugates formed by UBCv could not be resolved. They might have been the result either of successive ubiquitination at distinct lysine sites within the substrate, or of processive

ubiquitination of previously conjugated ubiquitin (poly-ubiquitin chains). The apparent capacity of UBCv to synthesize dimers of ubiquitin is similar to that of yeast UBC8 (Chen and Pickart, 1990; Qin *et al.*, 1991). Since in this reaction ubiquitin acted as a substrate, it is tempting to speculate that UBCv may also be capable of forming poly-ubiquitin chains. This hypothesis is supported by the prediction that the short insertion downstream from the active site of UBCv might be responsible for the catalysis of poly-ubiquitin chains (Haas *et al.*, 1991; Van Nocker and Vierstra, 1991; Cook *et al.*, 1992b).

The issue of poly-ubiquitin conjugate formation is important since it is thought to be responsible for targeting substrates for degradation and would therefore suggest that UBCv can target proteins for proteolysis (Chau *et al.*, 1989; Gregori *et al.*, 1990). Although the ability to target substrates for degradation would support a UBCv role in virion uncoating, it would argue against a chaperone-like function for UBCv which might be required for certain functions such as virion morphogenesis. The ability of UBCv to poly-ubiquitinate itself might target UBCv for proteolysis, providing a feed back mechanism for control of UBCv activity in ASFV infected cells, a role similar to that proposed for the yeast UBC3 auto-ubiquitination property (Banerjee *et al.*, 1993).

It must be stressed that *in vitro* assays may produce misleading artifacts for several reasons. Firstly, bacterial recombinant UBCv might not be properly folded or modified and might therefore not have the same activity as *in vivo*. Secondly, concentrations of the protagonists in the assay were entirely arbitrary and potential cofactors (such as E3 enzymes) might have been absent. Further analysis of the function of the UBCv enzyme was investigated by studies of UBCv during ASFV infection of cells.

Chapter 3

Analysis of UBCv in ASFV infected cells

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The structure of the UBCv gene and the *in vitro* activity of recombinant UBCv both suggested that the ASFV encoded UBC enzyme was functional. This hypothesis was investigated further by looking at the expression of UBCv during ASFV infection of cells. Antibodies specific for UBCv were produced in rabbits and used to detect UBCv's presence in ASFV infected cells and ASFV particles. In addition, attempts were made to inhibit the UBCv enzyme activity during ASFV infection, the effects of which might help elucidate the role of UBCv during ASFV replication.

3.1 UBCv expression in ASFV infected cells

3.1.1 Preparation of antiserum against UBCv

Immunological methods for the detection of proteins require high affinity, monospecific antibodies. Antisera were raised against UBCv in rabbits using antigens which consisted of either synthetic peptides or whole recombinant UBCv (table 3.1). The sequence of the synthetic peptides covered either UBCv's highly conserved active site (with a high probability of antibody accessibility), or its N- and C-termini which were specific for UBCv and less likely to cross-react with the host's UBC isozymes.

Two rabbits were immunized with whole recombinant UBCv purified from an *E. coli* culture transformed with plasmid PH2. The PH2 plasmid was constructed (figure 3.1) by inserting the UBCv gene in frame with the glutathione-S-transferase (GST) gene of the expression vector pGEX-2T (Promega). The expression of the

| Antigen | | | | | α -serum |
|---------|---------|----------------------|-------|-------------|-----------------|
| Name | Pos. | UBCv region | + Cys | Amount | Name |
| P9 | 75-94 | Active site | no | 150 μ g | LD1 |
| P11 | 116-134 | Downstream act. site | yes | 150 μ g | LD3 |
| P17 | 1-19 | N-terminus | yes | 150 μ g | LD4/LD5 |
| P31 | 195-213 | C-terminus | yes | 150 μ g | LD6/LD7 |
| rUBCv | 1-213 | Recombinant UBCv | N/A | 15 μ g | LD8 |
| rUBCv | 1-213 | Recombinant UBCv | N/A | 100 μ g | LD9 |

Table 3.1 Antigens used in the production of anti-UBCv antisera.

The antigens used in the rabbit immunizations consisted either of synthetic peptides (Px) or of pure recombinant UBCv (rUBCv). The sequence of the antigen is referred to by its position in the UBCv polypeptide sequence (Malawi LIL20/1). When a cysteine residue was not present in the synthetic peptide sequence, a cysteine residue was added to its N-terminus (+Cys) in order to provide a site for linking to sepharose or a carrier protein. The amounts of antigen used for each inoculation are indicated.

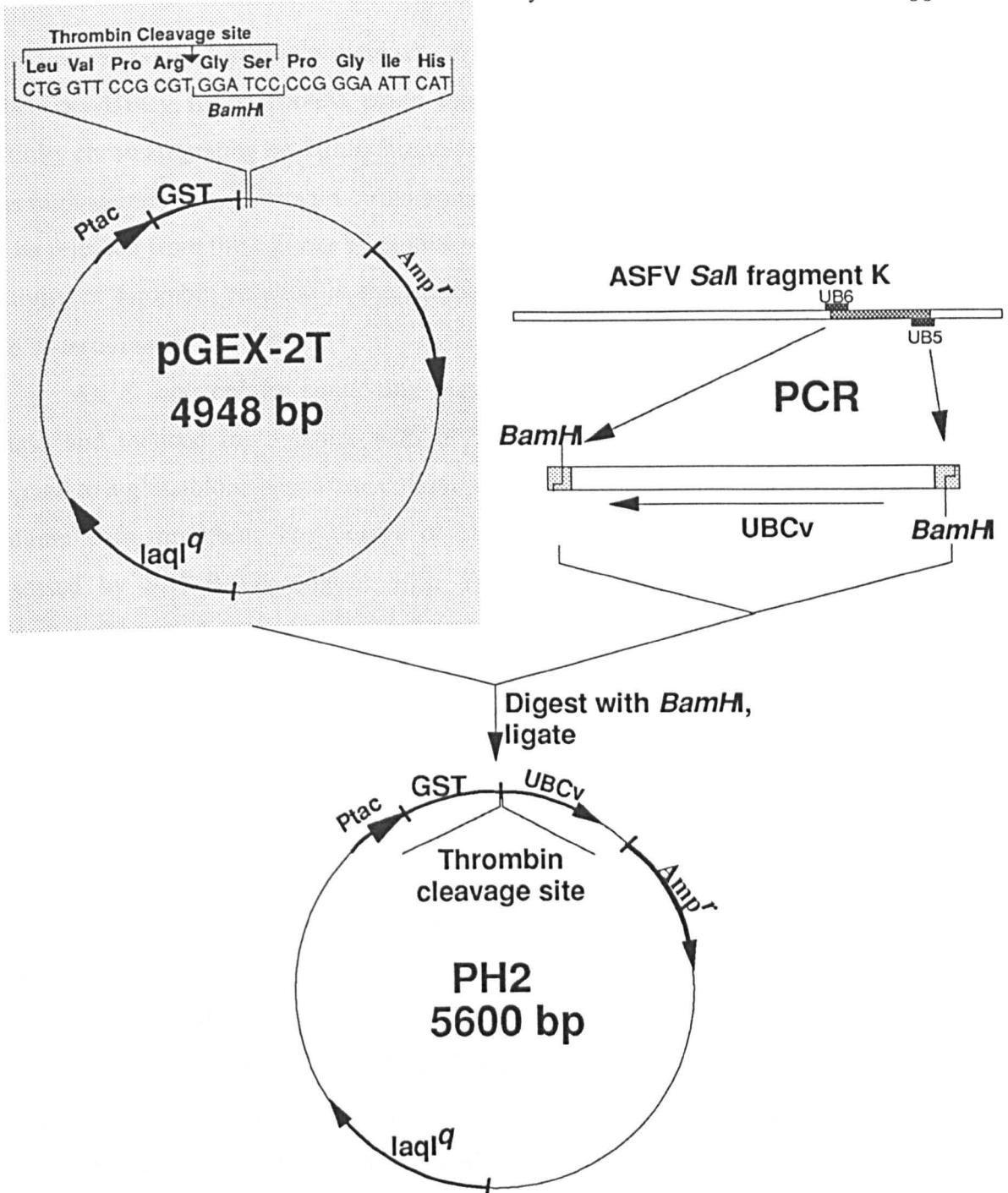


Figure 3.1 Cloning of the UBCv gene in expression vector pGEX-2T

The UBCv gene was amplified by PCR with the Mw2 set of primers (table 2.1) using as template DNA from bacteriophage lambda clone LMw22 (Dixon, 1988). The unique 650 bp fragment produced in the reaction was digested with BamHI. The pGEX-2T plasmid (Promega corporation) was digested with BamHI and dephosphorylated with calf intestine phosphatase (CIP). The digested UBCv insert and pGEX-2T vector were then ligated and transformed in *E. coli*. Clones expressing the GST-UBCv fusion protein were identified in *E. coli* cell lysates by Western blot analysis using UBCv anti-peptide antiserum.

resulting GST-UBCv fusion protein was driven by the IPTG inducible Ptac promoter. The GST-UBCv fusion protein could then be purified from the *E. coli* lysate by affinity chromatography on a glutathione-sepharose column for which the GST moiety has high affinity. The thrombin cleavage site between GST and UBCv allowed UBCv to be released from the column by elution with the thrombin protease. The proteolytic activity of thrombin resulted in the addition of a non-authentic Gly-Ser dipeptide at the N-terminus of UBCv.

An *E. coli* culture containing plasmid PH2 was grown to mid-exponential phase and induced for 3 hours with IPTG. The resulting soluble cell lysate was applied to a glutathione-sepharose column and UBCv was released by cleavage on the column with thrombin. The degree of UBCv purity attained by this method was assessed by SDS/PAGE (figure 3.2). The preparation consisted mainly of two proteins (30 and 31 kDa) which were not present in an *E. coli* culture containing the pGEX-2T vector alone. The two proteins are likely to correspond to two forms of UBCv since they both cross reacted with the UBCv anti-peptide antiserum (figure 3.3). The smaller of the two UBCv proteins might have been an early termination product since the UBCv coding sequence contained many codons which rarely occur in *E. coli* (see § 2.1.1). The other minor and faster migrating proteins were probably additional early termination or degradation products of UBCv which would not interfere with the production of anti-UBCv antisera.

Approximately 1 mg of over 90% pure UBCv was recovered from a 1 litre *E. coli* culture containing plasmid PH2. The relative low yield of UBCv recovery (the yield of recombinant proteins using pGEX-2T normally ranges from 5 to 10 mg per litre) was probably due to the ASFV codon usage which was not optimal for high expression in *E. coli*. The UBCv eluate from the glutathione-sepharose column was used with no further treatment for the rabbit immunizations.

The nine anti-UBCv antisera were tested for their ability to detect recombinant UBCv in Western blots (figure 3.3). All the antisera detected the UBCv doublet and at least one of the smaller fragments. Nonetheless, at an equal dilution, the LD8 and LD9 antisera (figure 3.3, lanes 8 & 9) raised against recombinant UBCv were more sensitive than the anti-peptide antisera (figure 3.3, lanes 1-7). The LD9 antiserum was therefore used hereafter in all UBCv detection experiments.

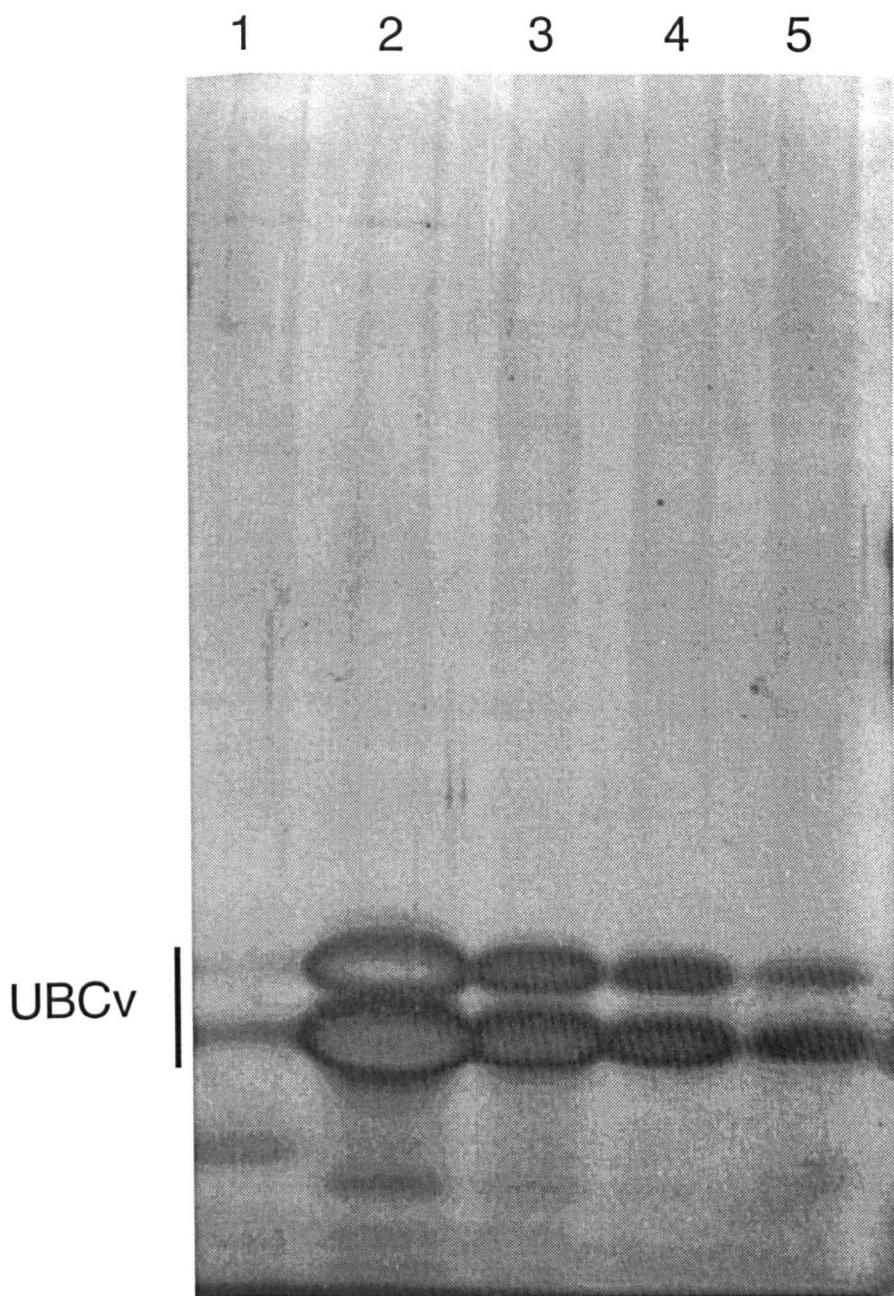


Figure 3.2 Analysis of UBCv purified from an *E. coli* culture harbouring plasmid PH2.

The GST-UBCv fusion protein was affinity purified on a glutathione-sepharose column from lysates of *E. coli* cells containing plasmid PH2. The four successive fractions eluted with thrombin were subjected to 10% SDS-PAGE and proteins were detected by silver staining (lanes 2-5). In lane 1, the *E. coli* culture contained the pGEX-2T vector alone. The position of the UBCv doublet is indicated.

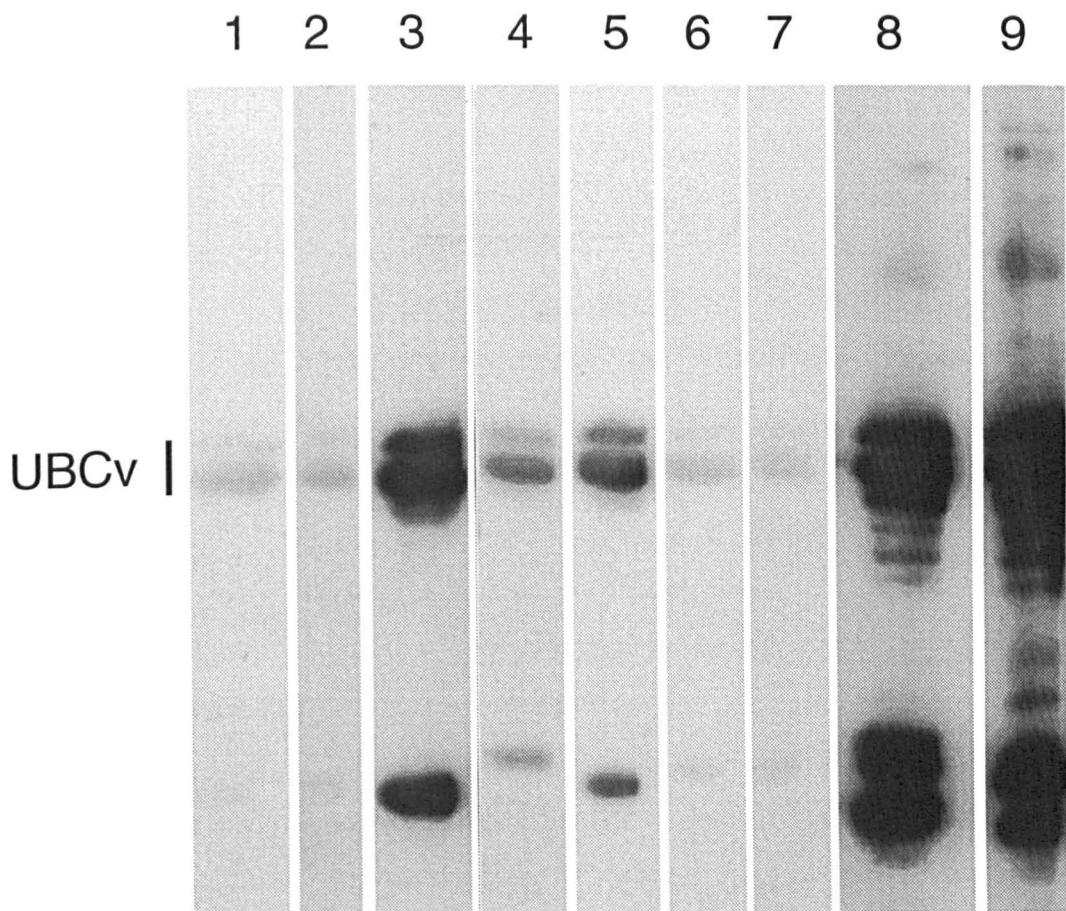


Figure 3.3 Detection of recombinant UBCv using various anti-UBCv antisera. Recombinant UBCv was affinity purified from *E. coli* cell lysates containing plasmid PH2. Purified recombinant UBCv was subjected to 12% SDS-PAGE followed by Western blotting. The blotted proteins were probed with nine antisera (at the same dilution) raised in rabbits (see table 3.1) against either synthetic peptides (LD1 to LD7) or whole recombinant UBCv (LD8 and LD9) (lanes 1 to 9 respectively). Enhanced chemo-luminescence (ECL) was used to detect bound antibodies. The position of the UBCv doublet is indicated.

3.1.2 Expression of UBCv in ASFV infected cells

Pig monocytes were infected with the Malawi LIL20/1 ASFV isolate at a high multiplicity of infection and harvested at various times post-infection. Infected cell extracts were analyzed by SDS-PAGE followed by Western blotting. When the blots were probed with anti-UBCv antiserum (figure 3.4, B), a 29 kDa immunoreactive protein was detected in all the infected cell extracts but not in mock-infected monocytes. This protein was not detected with the pre-immune serum (figure 3.4, A) which provided further evidence that this 29 kDa protein was UBCv and demonstrated that UBCv was present in ASFV infected cells from at least 3 to 24 hours post-infection. As estimated from the intensity of the bands, the amount of UBCv was approximately constant throughout the ASFV replication cycle. Since the number of adherent cells severely decreased after 24 hours of infection, the total amount of protein loaded on the gel for this time point was considerably lower than in early infection. This suggests that the relative concentration of UBCv in infected cells actually increased at 24 hours post-infection.

ASFV infected pig monocytes were also pulse labelled with [³⁵S]methionine and labelled proteins were immunoprecipitated with anti-UBCv antiserum. A 29 kDa protein was immunoprecipitated (figure 3.5) indicating that UBCv was synthesized in infected cells from at least 3 to 24 hours post-infection. Although UBCv was actively expressed throughout the ASFV replication cycle, its expression was particularly high early in infection (3 hours post-infection).

A time course of infection was also carried out for the Uganda-A ASFV isolate which was adapted to grow in IBRS2 tissue culture cells. Infected cell extracts were analyzed by SDS/PAGE followed by Western blotting. The expression of ASFV proteins was monitored by probing the blots with pig hyperimmune anti-ASFV antiserum (figure 3.6, A). The early to late switch in protein expression was clearly visible between 10 and 24 hours post-infection, with the expression of 10 novel late proteins (L1 to L10). When the blots were probed with anti-UBCv antiserum (figure 3.6, B), an immunoreactive protein of 29 kDa was detected from 4 to 24 hours post-infection. In this experiment the total amount of protein loaded on the gel at each time point was the same and the results show that the concentration of UBCv in infected

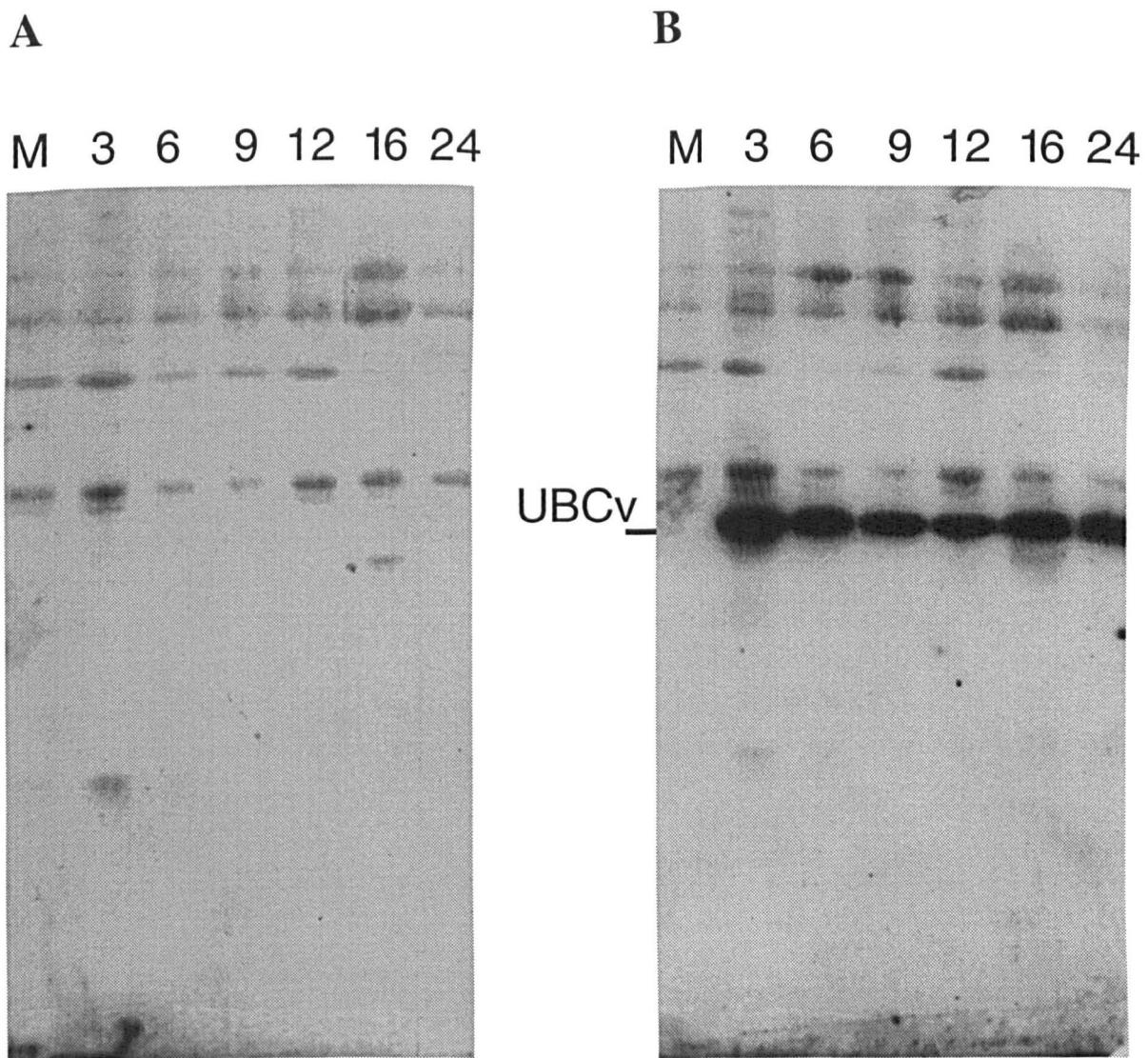


Figure 3.4 Steady state levels of UBCv in ASFV infected pig monocytes.

Pig peripheral blood monocytes were infected with the Malawi LIL20/1 ASFV isolate at a high multiplicity of infection (MOI > 10). At 3, 6, 9, 12, 16 and 24 hours post-infection, the proteins in infected cells were analyzed by 15% SDS/PAGE followed by Western blotting. The blots were probed either with anti-UBCv LD9 antiserum (panel B) or with the LD9 pre-immune serum (panel A). Detection of bound antibodies was performed by ECL. In lane M, monocytes were mock infected. The position of UBCv and molecular weight markers (in kDa) are indicated.

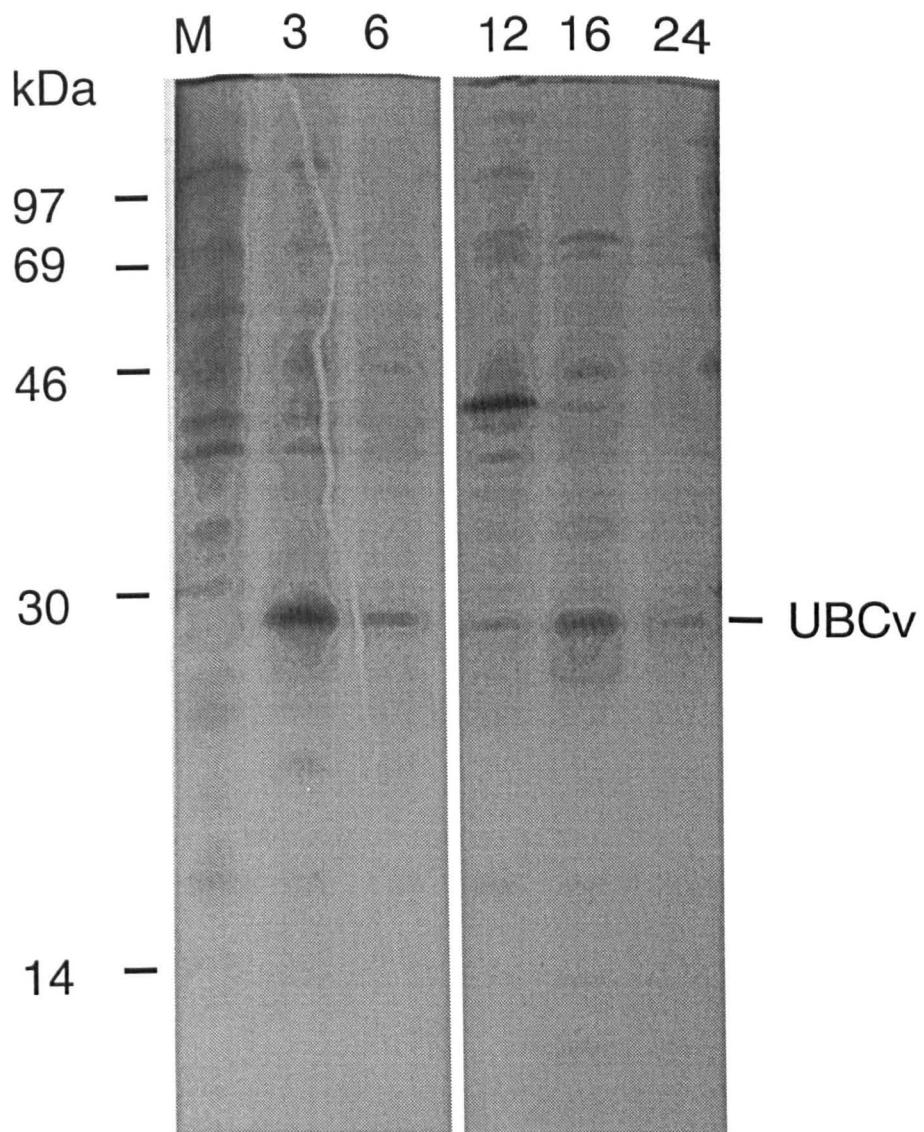


Figure 3.5 Expression of UBCv in ASFV infected pig monocytes.

Pig peripheral blood monocytes were infected with the Malawi LIL20/1 ASFV isolate. At 3, 6, 12, 16 and 24 hours post-infection, the cells were incubated for one hour with [35 S]methionine before being lysed. Soluble proteins were successively incubated with anti-UBCv LD9 antiserum and protein A-sepharose. Immunoprecipitated proteins were separated by 15% SDS/PAGE and detected by fluorography. In lane M, cells were mock infected. The position of UBCv and molecular weight markers (in kDa) are indicated.

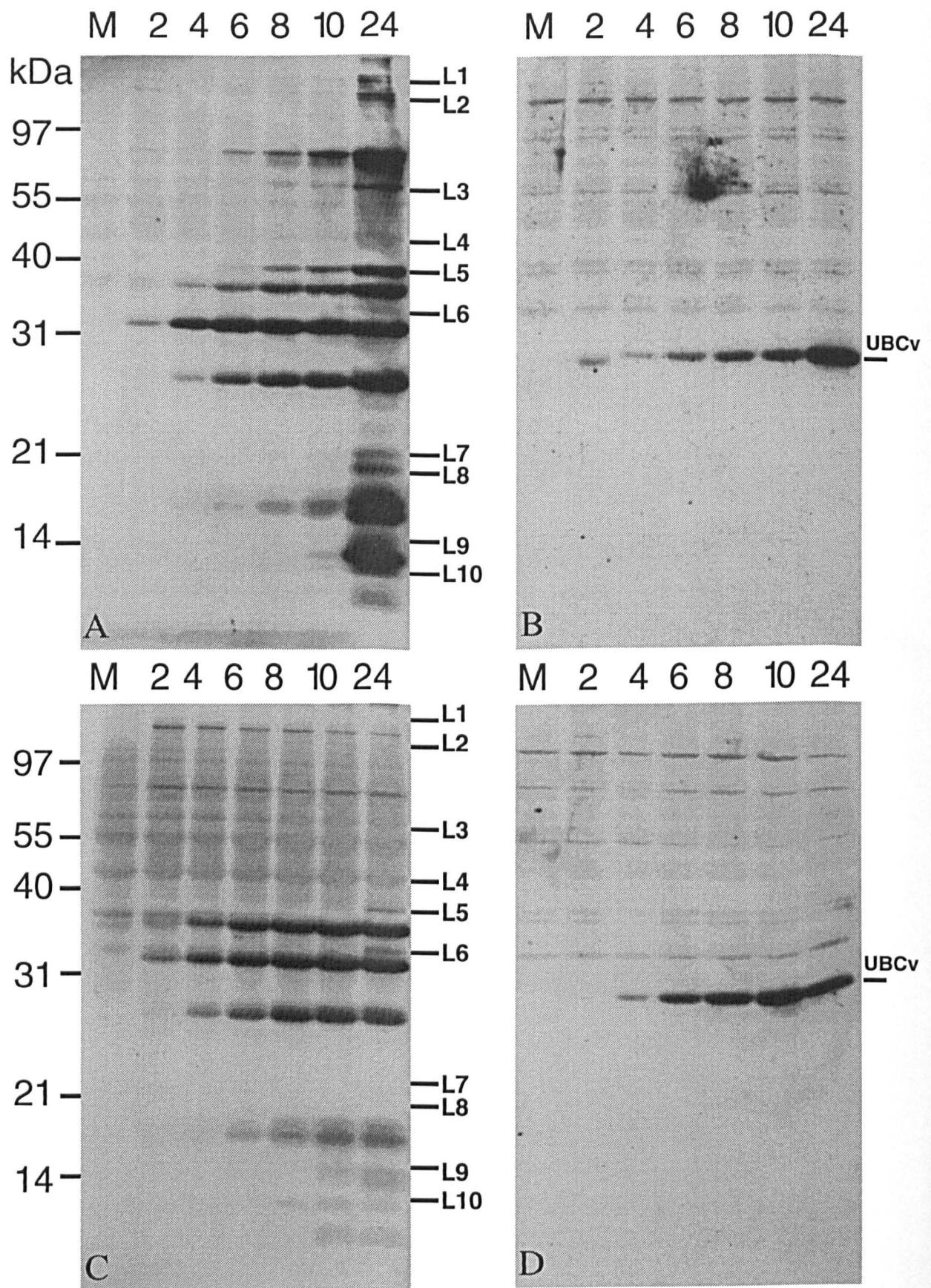


Figure 3.6 Effect of an inhibitor of DNA synthesis (AraC) on the level of UBCv in ASFV infected cells.

Figure 3.6 Effect of an inhibitor of DNA synthesis (AraC) on the level of UBCv in ASFV infected cells (facing page).

Tissue culture IBRS2 cells were infected with the Uganda-A ASFV isolate in normal media (panels A and B) or in the presence of 50 $\mu\text{g.ml}^{-1}$ of AraC (panels C and D). At 2, 4, 6, 8, 10 and 24 hours post-infection infected cell extracts were subjected to 12.5% SDS/PAGE followed by Western blotting. The blots were probed either with pig hyperimmune anti-ASFV antiserum (panels A and C) or with anti-UBCv LD9 antiserum (panels B and D). Bound antibodies were detected by ECL. The position of UBCv, late proteins (L1 to L10) and molecular weight markers (in kDa) are indicated.

cells increased steadily during ASFV replication. These results suggest that UBCv is stable since its relative synthesis is greatest early in infection but it accumulates to higher levels late in infection.

Late ASFV gene expression depends on the replication of the viral genome and can be suppressed by adding an inhibitor of DNA synthesis such as cytosine-arabinoside (AraC) to ASFV infected cells (Esteves *et al.*, 1986; Santaren and Vinuela, 1986; Escribano and Tabares, 1987). To confirm that UBCv was an early ASFV protein, IBRS2 cells were infected with the Uganda-A ASFV isolate in the presence of AraC. The infected cell extracts were again analyzed at various times post-infection by SDS/PAGE followed by Western blotting. Probing the blots with anti-ASFV antiserum confirmed that AraC strongly inhibited the expression of ASFV late proteins since only low levels of two out of ten late proteins (L5 and L6) were detected (figure 3.6, C). When the blots were probed with anti-UBCv antiserum (figure 3.6, D), the UBCv enzyme was still detected from 4 to 24 hours post-infection in the presence of AraC although a slight decrease in UBCv immunoreactivity at 24 hours post-infection was observed. The UBCv enzyme was therefore present early until late in ASFV infected cells although its expression was not dependent on the replication of the viral genome. This indicated that the UBCv enzyme was part of the persistent early category of ASFV proteins.

3.2 Subcellular localization of UBCv

3.2.1 UBCv localisation in ASFV infected cells

Indirect immunofluorescence using anti-UBCv antiserum was carried out on fixed and permeabilized tissue culture IBRS2 cells infected with the Uganda-A ASFV isolate. Although no staining was observed in mock infected cells (figure 3.7, A), the specific UBCv signal in infected cells only rose slightly above background late in infection (figure 3.7, B). The staining by anti-UBCv antiserum appeared diffuse but confined to the cytoplasm, with no detectable staining of the plasma membrane, nucleus or viral factories. In a parallel experiment the ASFV viral factories were readily stained in infected cells using anti-DNA monoclonal antibodies (figure 3.8).

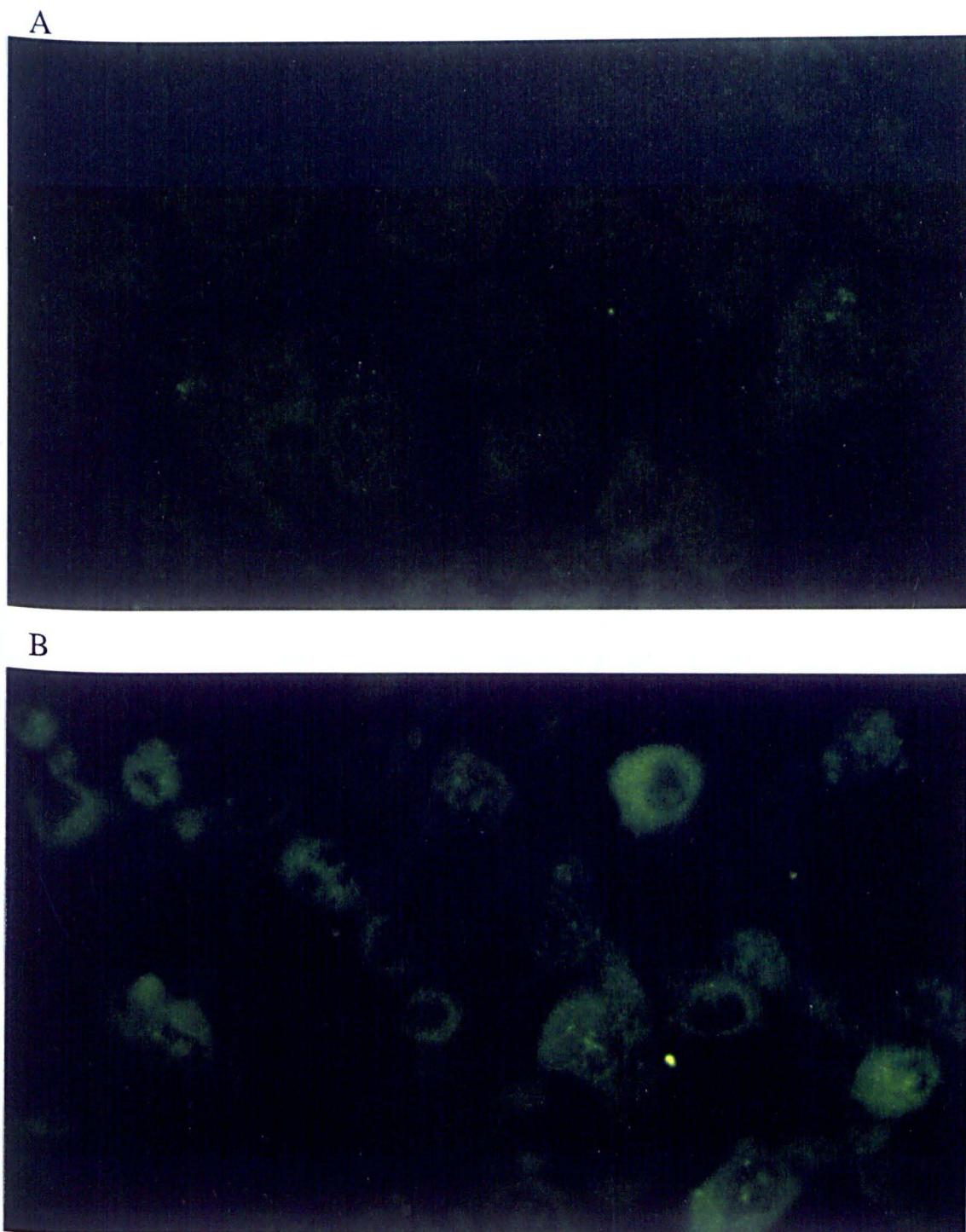
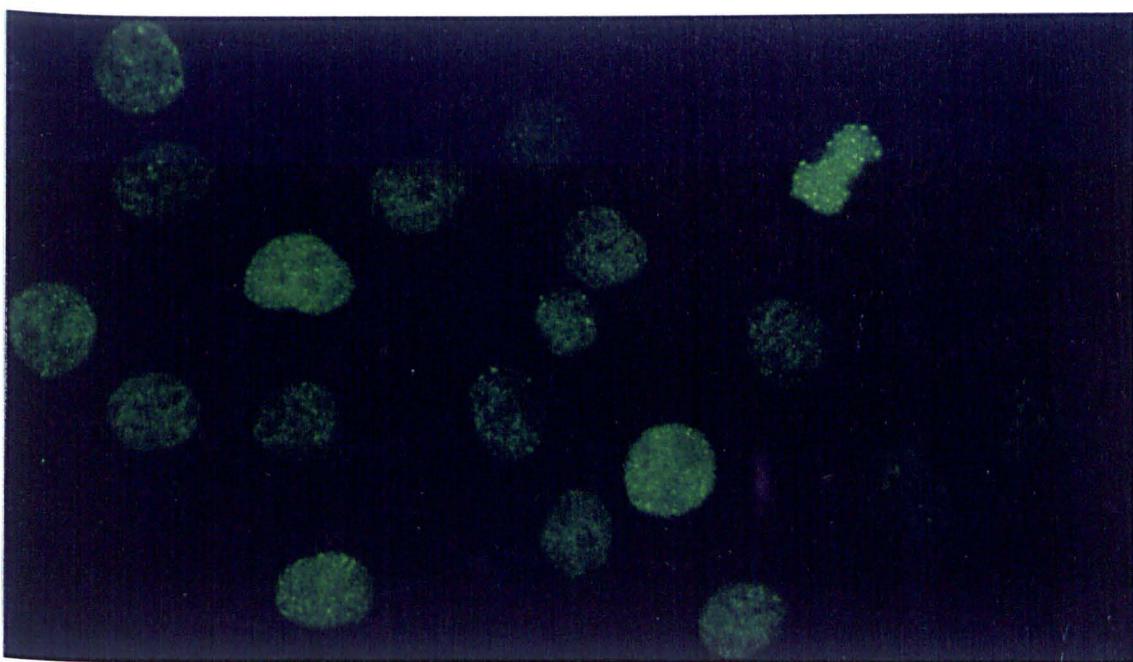


Figure 3.7 Indirect anti-UBCv immunofluorescence of ASFV infected cells.
Tissue culture IBRS2 cells were either mock infected (panel A) or infected with the Uganda-A ASFV isolate (panel B). At 16 hours post-infection, the cells were fixed and permeabilized in ice cold acetone/methanol. The fixed cells were probed with anti-UBCv LD9 antiserum followed by FITC conjugated secondary antibody. The photographs were taken with a Vickers incidence fluorescence microscope (x400 magnification).

A



B

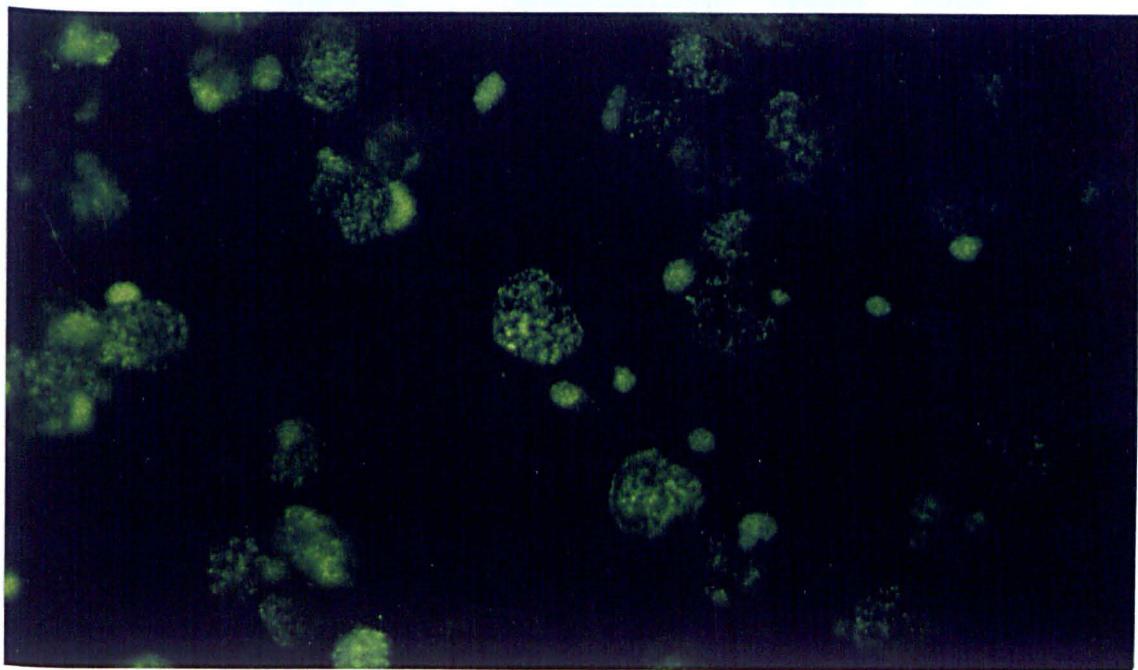


Figure 3.8 Indirect anti-DNA immunofluorescence of ASFV infected cells.

Tissue culture IBRS2 cells were either mock infected (panel A) or infected with the Uganda-A ASFV isolate (panel B). At 16 hours post-infection, the cells were fixed and permeabilized in ice cold acetone/methanol. The fixed cells were probed with anti-DNA monoclonal antibodies (Boehringer Mannheim) followed by FITC conjugated secondary antibody. The photographs were taken with a Vickers incidence fluorescence microscope (x400 magnification).

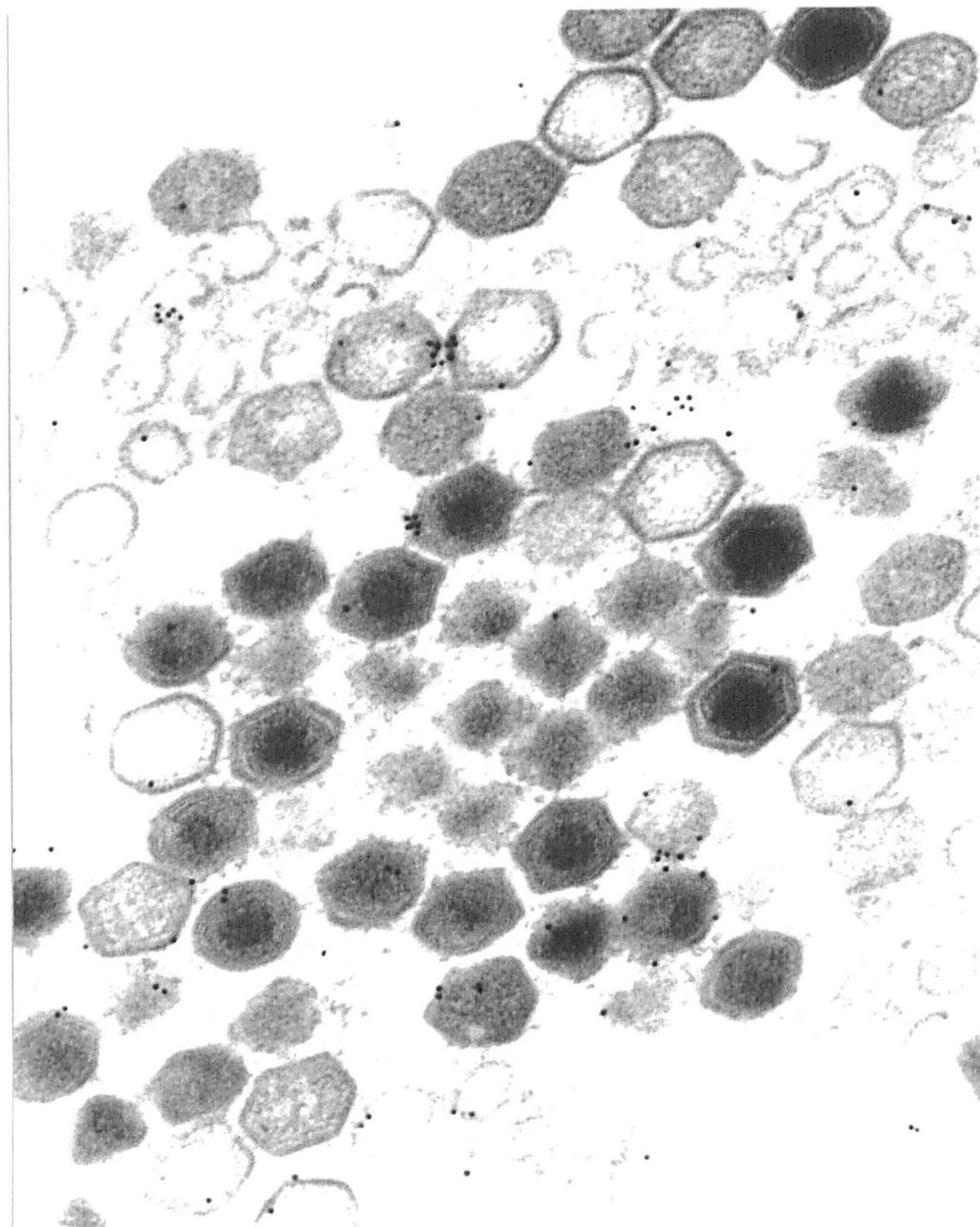


Figure 3.9 Anti-UBCv immunogold labelling of ASFV infected cells, detail of a virus factory.

Tissue culture IBRS2 cells were infected with the Uganda-A ASFV isolate. At 16 hours post-infection, the cells were fixed and embedded in araldite. Thin sections were probed with anti-UBCv antiserum followed by gold labelled secondary antibodies. The photograph was taken with a Philips EM410 electron microscope (x26000 magnification).

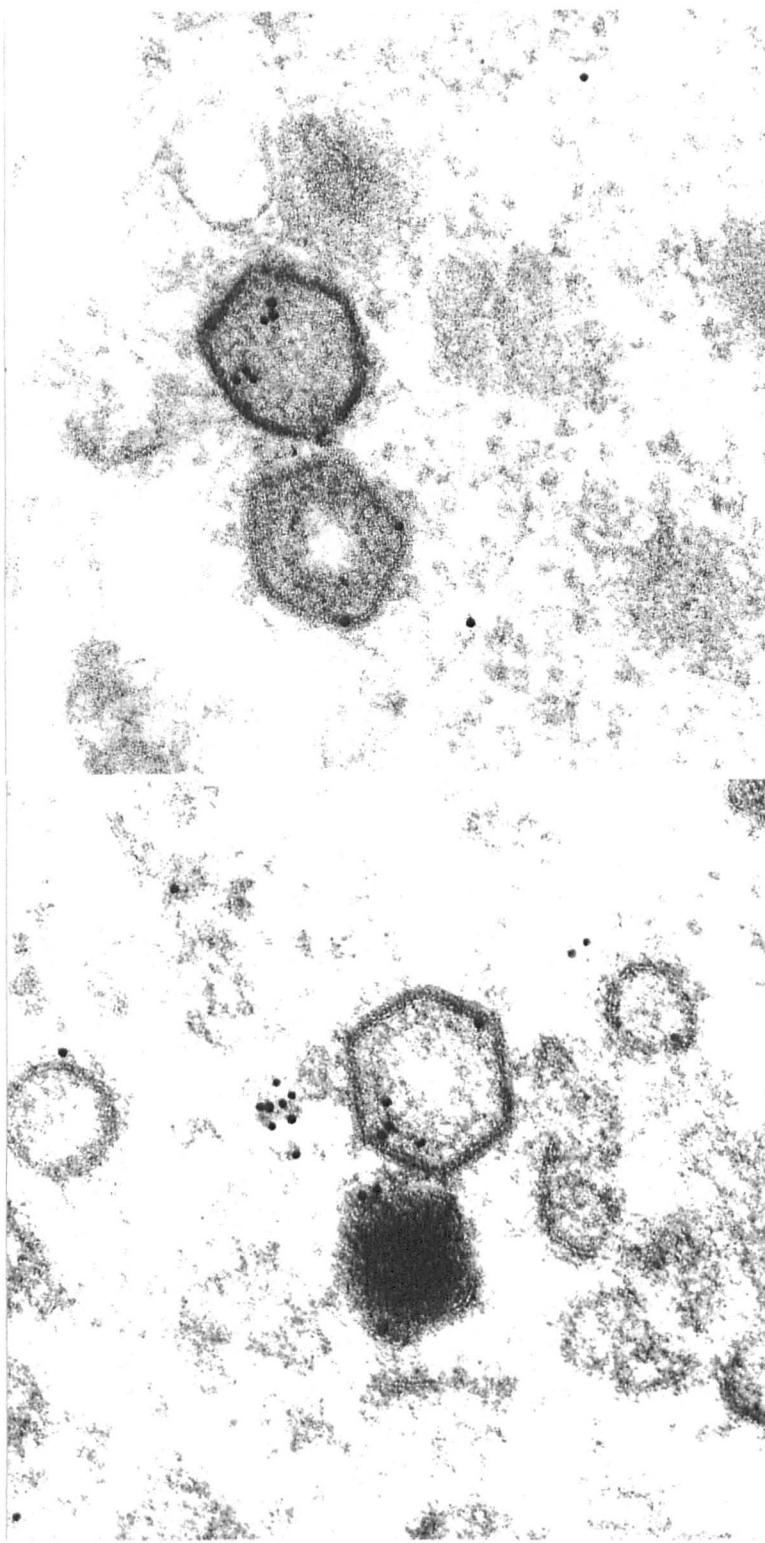


Figure 3.10 Anti-UBCv immunogold labelling of ASFV infected cells, details of virus particles.

Tissue culture IBRS2 cells were infected with the Uganda-A ASFV isolate. At 16 hours post-infection, the cells were fixed and embedded in araldite. Thin sections were probed with anti-UBCv antiserum followed by gold labelled secondary antibodies. The photographs were taken with a Philips EM410 electron microscope (x60000 magnification).

The tissue culture cells infected with ASFV were also analyzed by electron microscopy. The cells were fixed and thin sections were immunogold labelled with anti-UBCv antiserum. The gold labelled anti-UBCv antibodies were equally distributed between the cytoplasm and the viral factories without appearing to decorate any specific intracellular structure, although gold particles, sometimes in clusters, were occasionally seen associated with virus capsids (figure 3.9). At a higher magnification, gold labelled anti-UBCv antibodies could also be seen inside 'empty' and electron dense ASFV virions (figure 3.10).

3.2.2 Detection of UBCv in purified ASFV particles

To investigate the possibility that UBCv was present in ASFV virions, extracellular Uganda-A ASFV virus particles were purified by equilibrium centrifugation in Percoll density gradients (Carrascosa *et al.*, 1985). The protein content of the purified virus preparation was assessed by two dimensional gel electrophoresis (figure 3.11). Approximately 50 proteins were detected including the two major ASFV structural proteins, VP72 and VP150, which is consistent with previous observations made with ASFV virion proteins (Esteves *et al.*, 1986; Urzainqui *et al.*, 1987). Furthermore the virus preparation was free of vesicle contaminants since no proteins of above 150 kDa molecular weight could be detected (Carrascosa, AL *et al.*, 1985).

The purified ASFV preparation was fixed and thin sections were immunogold labelled using anti-UBCv antiserum (figure 3.12). Gold particles were clearly seen associated with approximately 50% of the ASFV extracellular particles. The purified ASFV proteins were also separated by SDS/PAGE and Western blotted. Probing the blot with anti-UBCv antiserum revealed a single immunoreactive 29 kDa protein (figure 3.13, lane 2). This confirmed that UBCv was present in ASFV particles, albeit in low amounts since no major corresponding 29 kDa protein could be detected by silver staining (figure 3.13, lane 1).

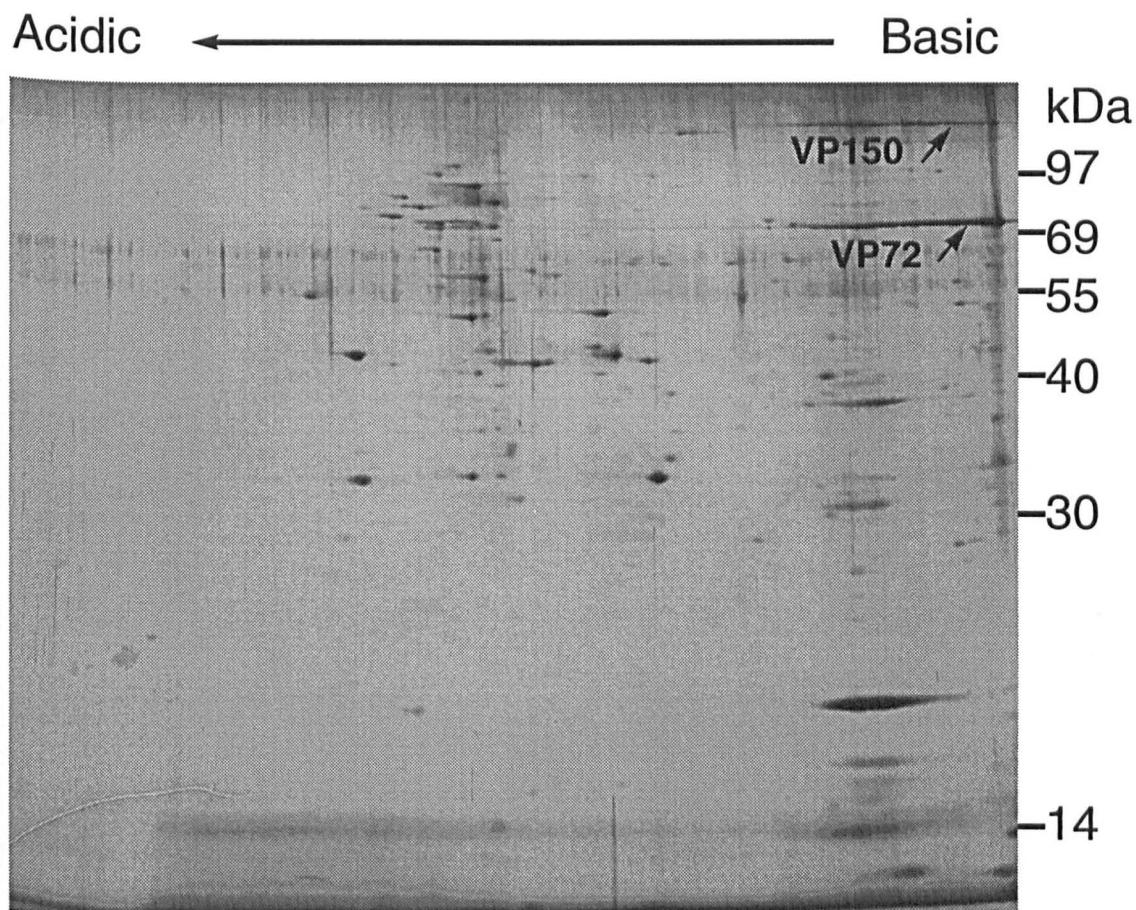


Figure 3.11 Two-dimensional analysis of proteins in purified extracellular ASFV particles.

The Uganda-A ASFV isolate was grown in IBRS2 cells and purified by equilibrium centrifugation in Percoll density gradients (Carrascosa *et al.*, 1985). The purified virus was subjected to isoelectric focusing (IEF) in the first dimension followed by 12.5% SDS/PAGE in the second dimension. The separated proteins were detected by silver staining. The positions of molecular weight markers (in kDa) and of the two main ASFV structural proteins VP72 and VP150 are indicated.

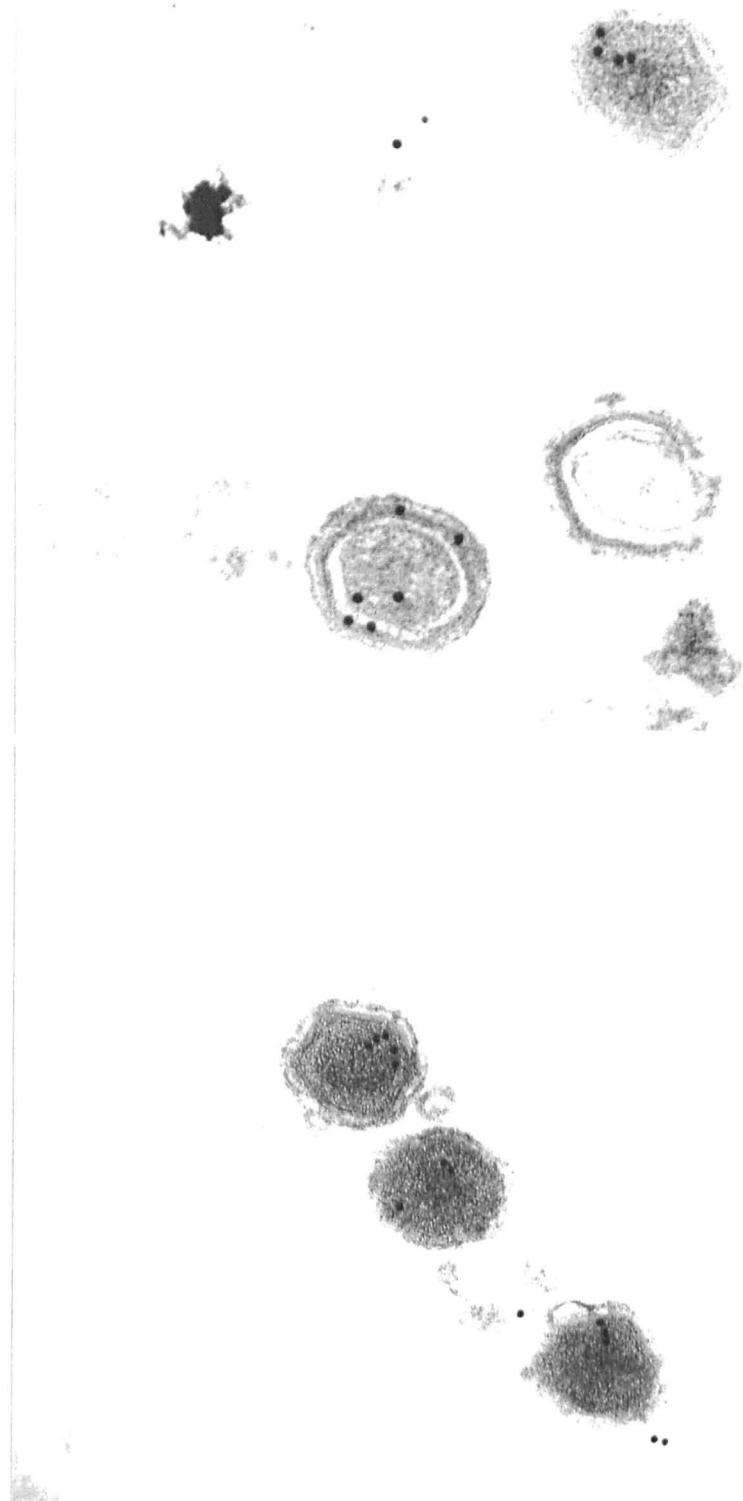


Figure 3.12 Anti-UBCv immunogold labelling of purified extracellular ASFV particles.

Percoll purified ASFV particles (Uganda-A isolate) were fixed and embedded in araldite. Thin sections of ASFV particles were probed with anti-UBCv antiserum followed by gold labelled secondary antibodies. The photographs were taken with a Philips EM410 electron microscope (x120000 magnification).

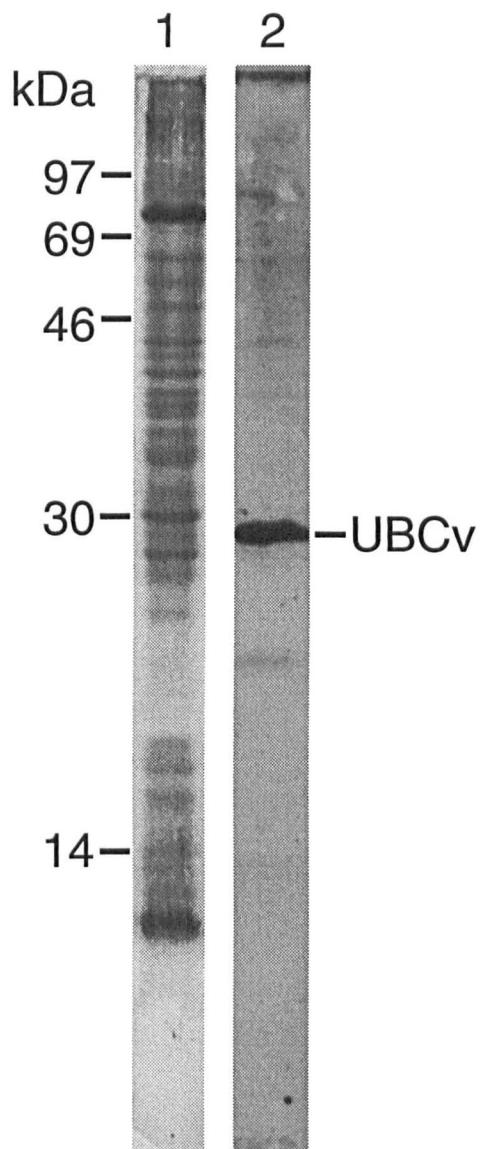


Figure 3.13 Presence of UBCv amongst the structural proteins of purified extracellular ASFV particles.

Proteins from Percoll purified ASFV particles (Uganda-A isolate) were separated by 15% SDS/PAGE and either silver stained (lane 1) or Western blotted (lane 2). The blot was probed with anti-UBCv antiserum and bound antibodies were detected by ECL. The positions of molecular weight markers (in kDa) and UBCv are indicated.

3.3 Inhibition of UBCv activity during ASFV replication

3.3.1 Effect of antisense UBCv oligonucleotides on UBCv expression

The successful inhibition of virus gene expression by synthetic antisense oligonucleotides has been demonstrated with RNA and DNA viruses alike (Agrawal, 1992; Bishofberger and Wagner, 1992) although no experiments involving ASFV have been reported. The expression of the targeted virus genes was shown to be severely reduced by incubating infected cells with antisense oligonucleotides complementary to the gene's 5' end (Matsukura *et al.*, 1989; Leiter *et al.*, 1990).

Vero tissue culture cells infected with the BA71V ASFV isolate were chosen for the UBCv antisense experiments because the efficiency of oligonucleotide uptake by pig macrophages was undefined. The two synthetic oligonucleotides tested were either complementary to the coding strand of the 5' end of the UBCv gene (UBCv⁽⁺⁾) or of random sequence (dN) (table 3.2).

Vero cells were infected with the BA71V ASFV isolate in the presence or absence of the UBCv⁽⁺⁾ and dN oligonucleotides. The infected cells were harvested at various times post-infection and analyzed by immunoblotting using anti-ASFV antiserum (figure 3.14, A). Both early and late ASFV proteins were expressed in the presence of either of the two oligonucleotides. At 8 hours post-infection however, the expression of late ASFV proteins appeared to be retarded in the presence of the

| Name | Target | Sequence (5' to 3') |
|---------------------|-----------------|-----------------------------------|
| UBCv ⁽⁺⁾ | UBCv, 5'end | CTGCTATTAAAAACCTGGAA <u>ACCAT</u> |
| dN | Random sequence | d(AGCT) ₂₅ |

Table 3.2 Sequence of the antisense oligonucleotides.

The 24- and 25-mer oligonucleotides were phosphorothioate analogues (sulphur backbone) as it was shown that their resistance to nucleases increased their intra- and extra-cellular half-lives (Hoke *et al.*, 1991; Agrawal, 1992; Thierry and Dritschilo, 1992). The UBCv⁽⁺⁾ oligonucleotide was complementary to the first 8 codons of the UBCv gene from the BA71V ASFV isolate (Rodriguez *et al.*, 1992b). The start codon is underlined.

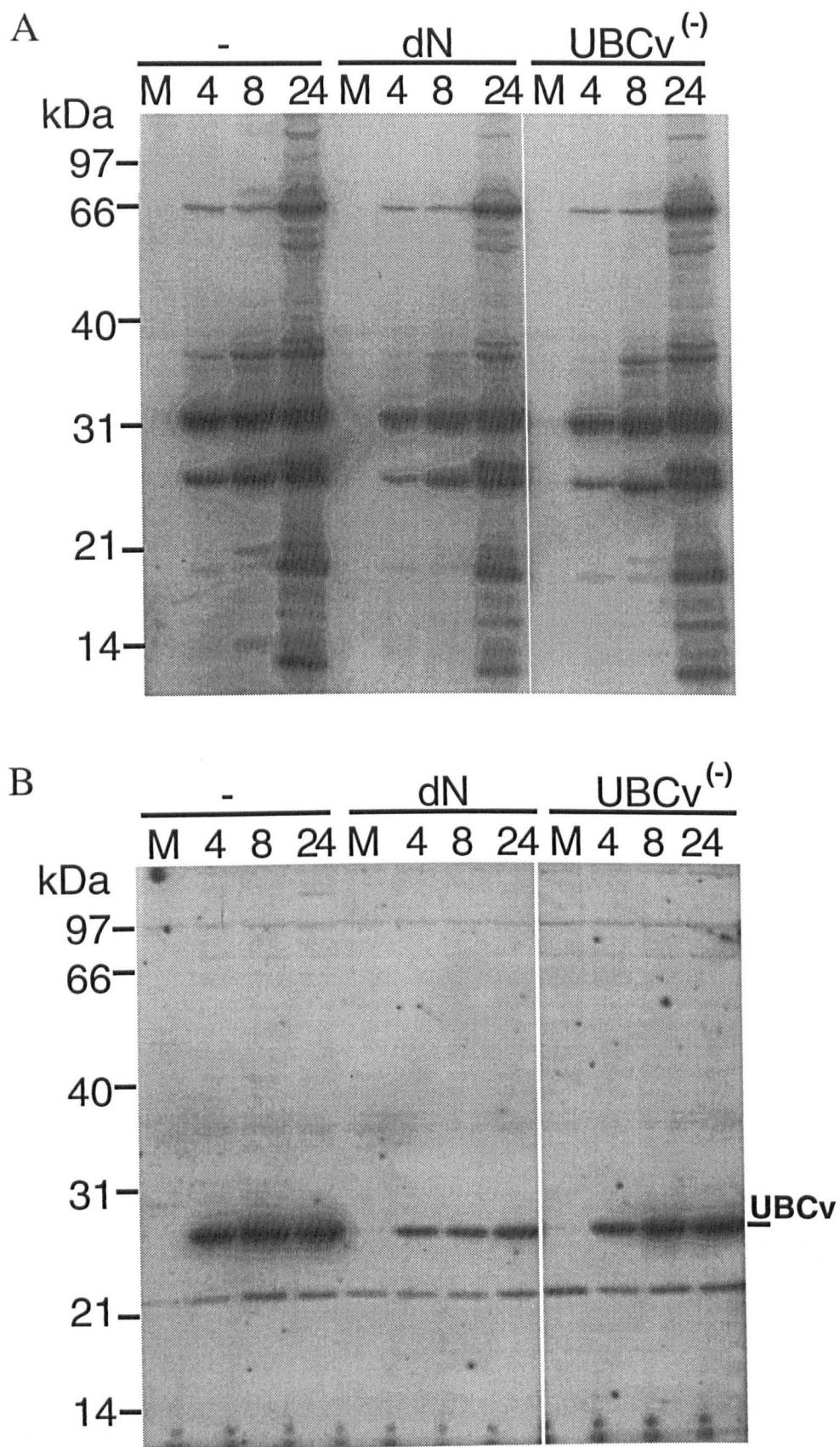


Figure 3.14 Effect of an antisense UBCv oligonucleotide on ASFV replication and on UBCv expression.

Figure 3.14 Effect of an antisense UBCv oligonucleotide on ASFV replication and UBCv expression (facing page).

Vero tissue culture cells were pre-incubated either with no oligonucleotide (-), the UBCv⁽⁺⁾ antisense oligonucleotide (25 μ M) or the dN random oligonucleotide (25 μ M) for 16 hours before infection with the BA71V ASFV isolate. At 4, 8 or 24 hours post-infection, the infected cells were harvested and analyzed by SDS/PAGE followed by Western blotting. The blots were either probed with hyperimmune pig anti-ASFV antiserum (panel A) or with anti-UBCv LD9 antiserum (panel B). Bound antibodies were detected by ECL. The positions of UBCv and molecular weight markers (in kDa) are indicated.

UBCv⁽⁺⁾ and dN oligonucleotides. Probing the blots with anti-UBCv antiserum (figure 3.14, B) showed that UBCv was expressed from 4 to 24 hours post-infection even in the presence of the UBCv⁽⁺⁾ antisense oligonucleotide. Although a slight decrease in UBCv immunoreactivity was observed at all time points in the presence of the UBCv⁽⁺⁾ antisense oligonucleotide, an even greater decrease was observed in the presence of the dN random sequence oligonucleotide.

Taken together these results indicated that the synthetic UBCv⁽⁺⁾ antisense oligonucleotide did not specifically affect UBCv expression and was therefore not suitable for inhibiting UBCv activity in ASFV infected cells.

3.3.2 ASFV replication in cells impaired in the ubiquitin pathway

The TS20 cells are temperature sensitive cell cycle mutants of the Chinese hamster E36 cell line (Kulka *et al.*, 1988; Lenk *et al.*, 1992). The defect in the mutant TS20 cells is due to their thermolabile ubiquitin activating (E1) enzyme. Since UBC enzymes are incapable of using ubiquitin without an E1 enzyme, the TS20 cells are unable to support ubiquitin conjugation at the non-permissive temperature.

The TS20 and E36 wild type cells were infected for various durations with the Uganda-A ASFV isolate at the permissive or non-permissive temperature. The infected cell extracts were analyzed by immunoblotting using anti-ASFV antiserum. At the permissive temperature, the pattern of viral protein expression in both the TS20 and E36 cells was typical of ASFV infection in tissue culture cells (figure 3.15, A and C). Early ASFV proteins were detected from 2 to 8 hours post-infection and 10 additional late ASFV proteins (L1 to L10) were detected at 24 hours post-infection.

At the non-permissive temperature the infection appeared to proceed normally from 2 to 8 hours post-infection in the TS20 and E36 cells (figure 3.15, B and D). At 24 hours post-infection however late ASFV protein expression was altered in both cell types at the non-permissive temperature. In the E36 cells 4 out of the 10 late proteins were detected (L3, L5, L7 and L8), whereas in the TS20 cells only two late proteins could be detected (L4 and L5). Furthermore, in TS20 cells one late protein (L1) was prematurely expressed from 4 hours post-infection onwards and two

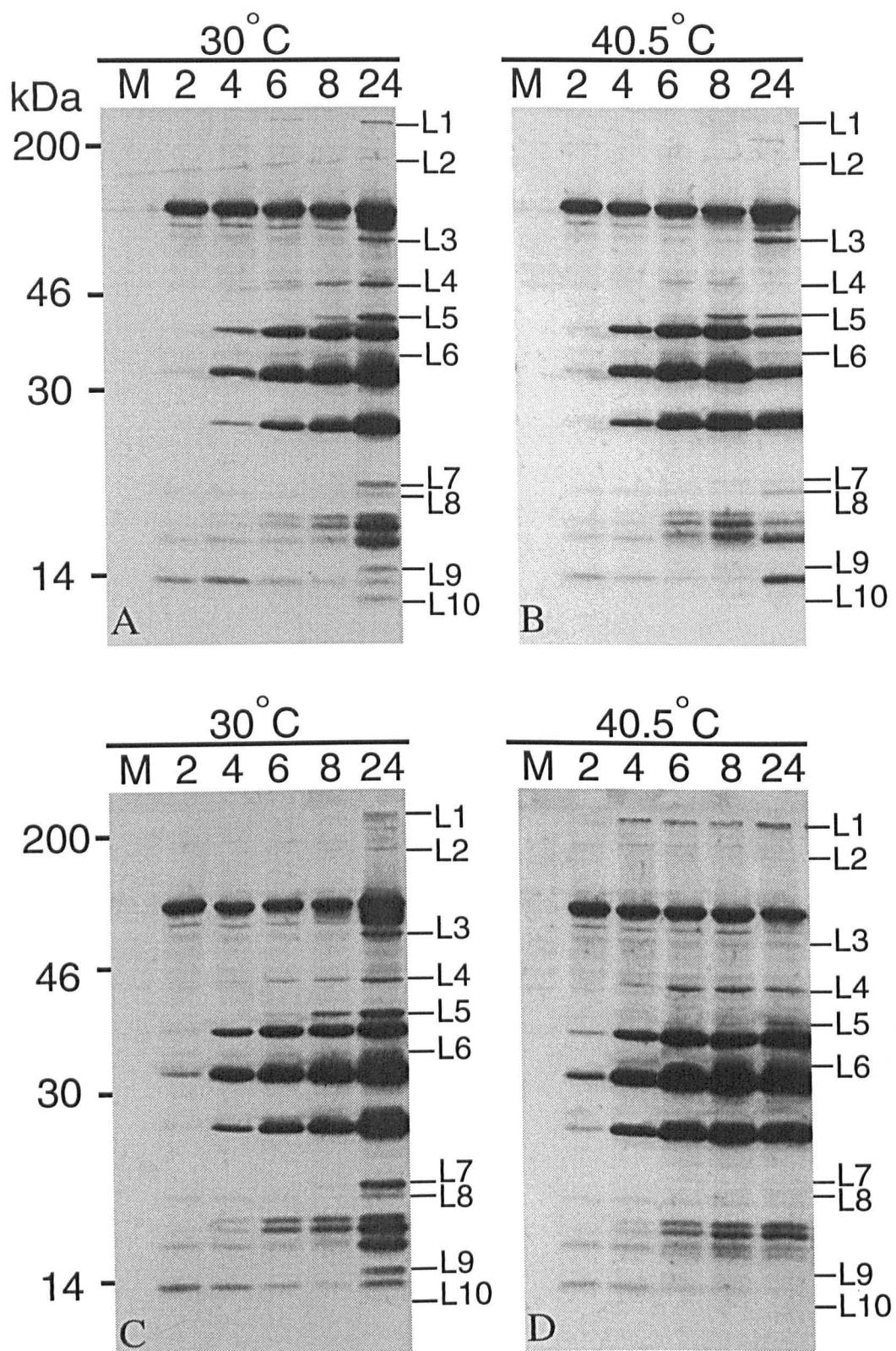
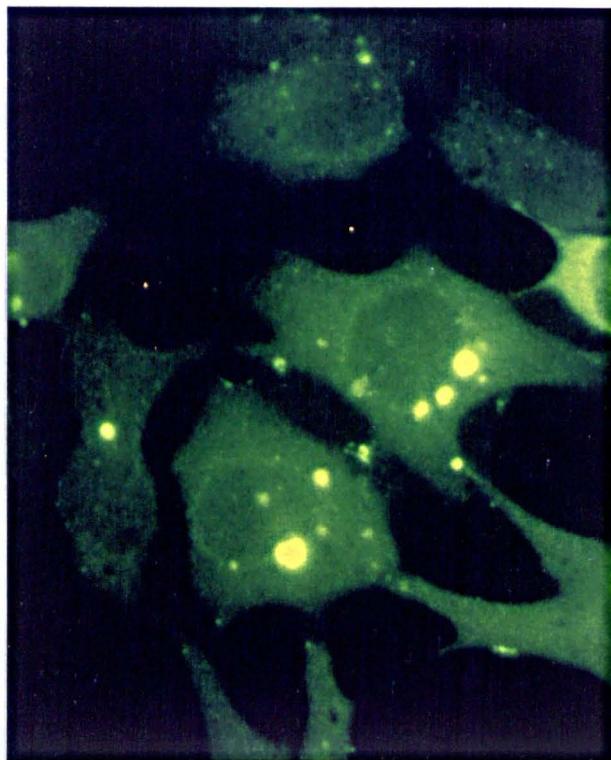


Figure 3.15 ASFV protein expression in E36 and TS20 cells at the permissive and non-permissive temperatures.

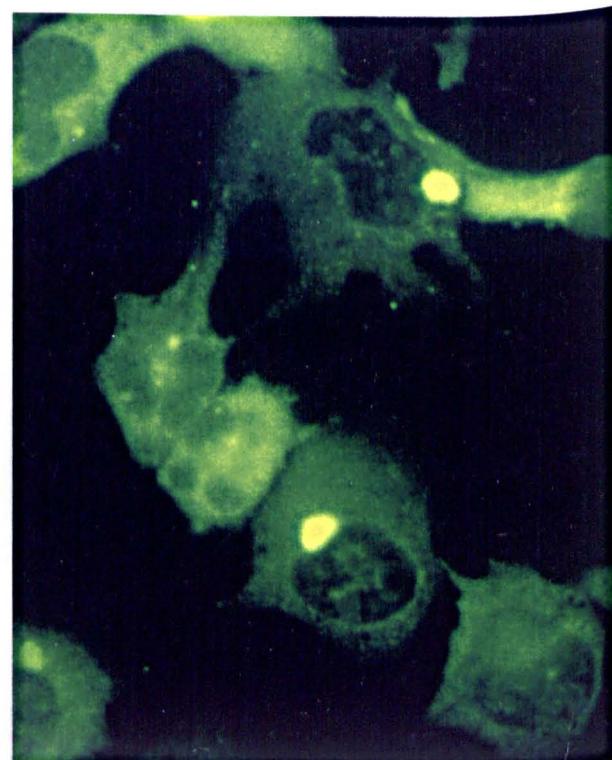
Figure 3.15 ASFV protein expression in E36 and TS20 cells at the permissive and non-permissive temperatures (facing page).

E36 wild type cells (panels A and B) or TS20 mutant cells (panels C and D) were infected with the Uganda-A ASFV isolate. From 2 hours before infection onwards the cells were incubated either at the 30°C permissive temperature (panels A and C) or at the 40.5°C non-permissive temperature (panels B and D). At 2, 4, 6, 8, 10 and 24 hours post-infection the cells were lysed and the extracts analyzed by 12.5% SDS/PAGE followed by Western blotting. The blots were probed with anti-ASFV hyperimmune pig antiserum and bound antibodies were detected by ECL. In lane M the cells were mock infected. The position of molecular weight markers (in kDa) and of late proteins (L1 to L10) are indicated.



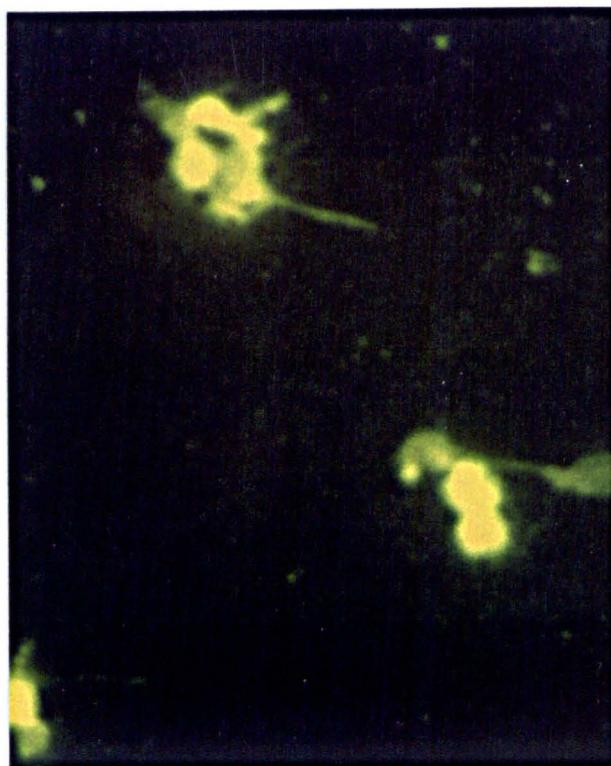
A

E36

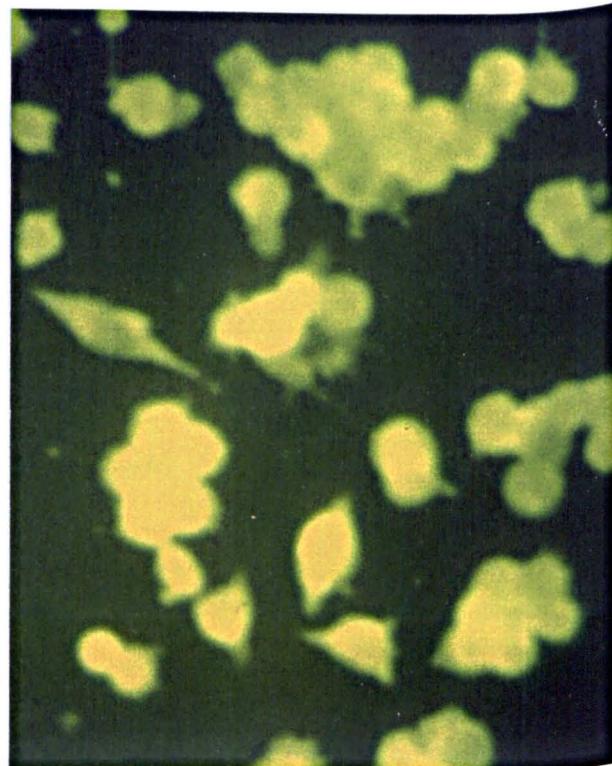


B

TS20



C



D

Figure 3.16 Indirect anti-ASFV immunofluorescence of E36 and TS20 cells infected with ASFV at the non-permissive temperature

Figure 3.16 Indirect anti-ASFV immunofluorescence of E36 and TS20 cells infected with ASFV at the non-permissive temperature (facing page).

E36 wild type cells (panels A and C) and TS20 mutant cells (panels B and D) were infected with the Uganda-A ASFV isolate for either 3 hours (panels A and B) or 24 hours (panels C and D). The cells were incubated from 2 hours before infection onwards at the 40.5°C non-permissive temperature. The infected cells were fixed in acetone/methanol and probed with anti-ASFV hyperimmune pig antiserum followed by FITC-conjugated secondary antibodies. The photographs were taken with a Vickers incidence fluorescence microscope (magnification: panels A and B x400, panels C and D x200).

persistent early proteins (14 and 16 kDa) were absent late in infection at the non-permissive temperature.

The ASFV infected TS20 and E36 cells were also observed at the non-permissive temperature by immunofluorescence using anti-ASFV antiserum. The TS20 and E36 cells showed similar patterns of immunofluorescence at 3 hours post-infection (figure 3.16, A & B). The cytosol of E36 and TS20 cells was diffusely stained and perinuclear viral factories were clearly visible. At 24 hours post-infection both TS20 and E36 cells were intensely stained indicating that high levels of viral proteins were present (figure 3.16, C & D). The number of remaining TS20 infected cells at 24 hours post-infection was however significantly higher (over three times) than for the infected E36 wild type cells, possibly indicating that cell lysis was delayed or inhibited. This phenomenon was not observed at the permissive temperature (data not shown).

These results suggested that in cells defective in the ubiquitin conjugation pathway, the replication of ASFV proceeded normally through the early phase but the late phase of infection was partially impaired or delayed. Since in wild type cells the expression of late ASFV proteins was also altered at the non-permissive temperature, albeit not as much as in the mutant TS20 cells, it is likely that part of the ASFV late phase impairment in TS20 cells was due to heat shock. Because of the interference of heat shock stress with ASFV replication during the long incubation periods necessary for ASFV infection (over 36 hours), this system is not suited for further investigations into the nature of the ASFV late phase impairment in TS20 cells.

3.3.3 Construction of an ASFV UBCv null mutant

The role of several poxvirus genes has been elucidated by constructing mutant 'knock-out' viruses (Blasco *et al.*, 1991; Alcami and Smith, 1992; Johnson *et al.*, 1993). In these experiments the targeted genes were inactivated by disruption with a reporter gene (figure 3.17). This general approach has also been shown to be applicable to ASFV (Rodriguez *et al.*, 1992a). Efficient isolation of these mutant viruses can be achieved in tissue culture by using dominant selection reporters such as the neomycin resistance or guanine phosphoribosyltransferase (gpt) genes (Franke *et al.*, 1985; Falkner and Moss, 1988).

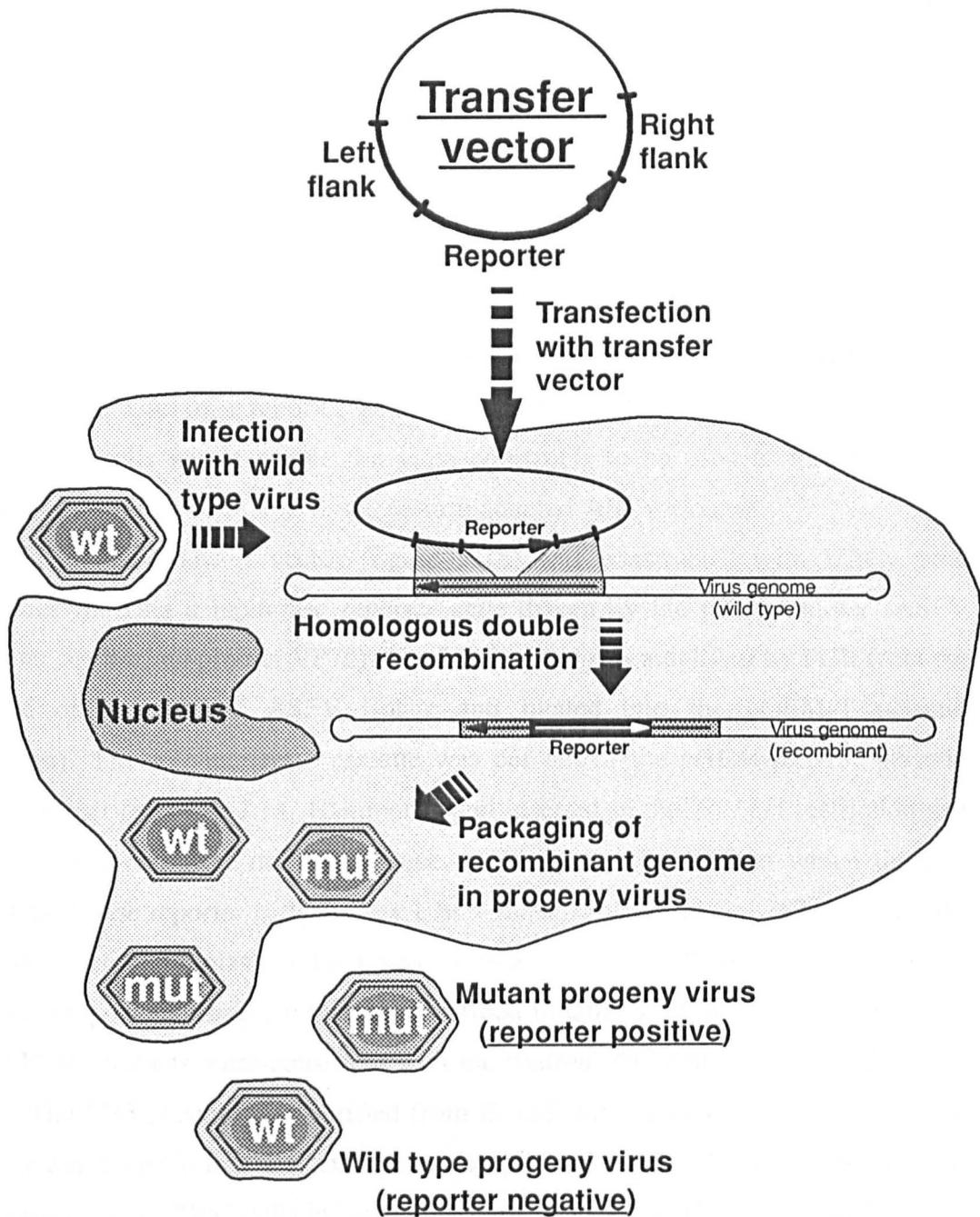


Figure 3.17 Schematic representation of the generation of 'knock-out' mutants by homologous double DNA recombination.

Cells infected with the virus are transfected with a DNA vector which contains a reporter gene flanked by sequences homologous to the viral gene to be disrupted. During the replication of the virus, the exogenous DNA is integrated in the viral genome by homologous double recombination. The progeny mutant viruses are then selected with the help of the reporter gene.

Cells of the macrophage lineage are the only cells in which virulent ASFV isolates replicate. Growth of these cells is not inhibited in the media used for these selection markers (Vydelingum, personal communication). These selectable markers cannot therefore be used to isolate recombinant viruses expressing these marker genes in macrophages. The firefly luciferase gene was therefore chosen as the reporter in ASFV 'knock-outs' since although it confers no selection advantage, it is a very sensitive marker which can be used in all cell types since there is no background luciferase activity (Rodriguez *et al.*, 1988; Kovacs and Mettenleiter, 1991; Rodriguez *et al.*, 1992). Use of a reporter gene that can be used both in tissue culture and macrophage cells would enable the same constructs to be used to manipulate the genomes of both virulent and tissue culture adapted ASFV isolates.

The PH3 transfer vector (figure 3.18) was constructed with UBCv gene sequences flanking a luciferase reporter gene driven by the p72 promoter (ASFV promoter for the late protein VP72). The UBCv gene was amplified by PCR from the genome of the BA71V ASFV isolate and ligated into the pGEM-T plasmid (Promega). The p72-luciferase cassette was cut out of the pGEM-p72/luc plasmid (generous gift from Dr. J.M. Hammond) and inserted in the *Tth11I* restriction site located in the centre of the UBCv gene, yielding plasmid PH3 in which the p72 driven luciferase reporter is flanked by UBCv sequences 347 bp and 282 bp in length. The construction of plasmid PH3 was verified by the PCR amplifications of its various components using the primers described in table 3.3 (figure 3.19). The sizes of the PCR products were consistent with the desired PH3 construct (table 3.4).

The PH3 plasmid was purified from *E. coli* and the activity of the luciferase reporter was tested in transient expression assays. PH3 plasmid DNA was transfected into tissue culture IBRS2 cells infected with the Uganda-A ASFV isolate. At 24 hours post-infection the infected cells were lysed and the extracts were mixed with a buffer containing coenzyme A and beetle luciferin. The luminescent reaction between the luciferase enzyme and its luciferin substrate was measured with a liquid scintillation counter. The luminescence of the infected cell extracts transfected with PH3 was over 20.10^6 counts per minute (cpm). In contrast, the background luminescence of both non-infected and non-transfected cell controls remained below 50 cpm.

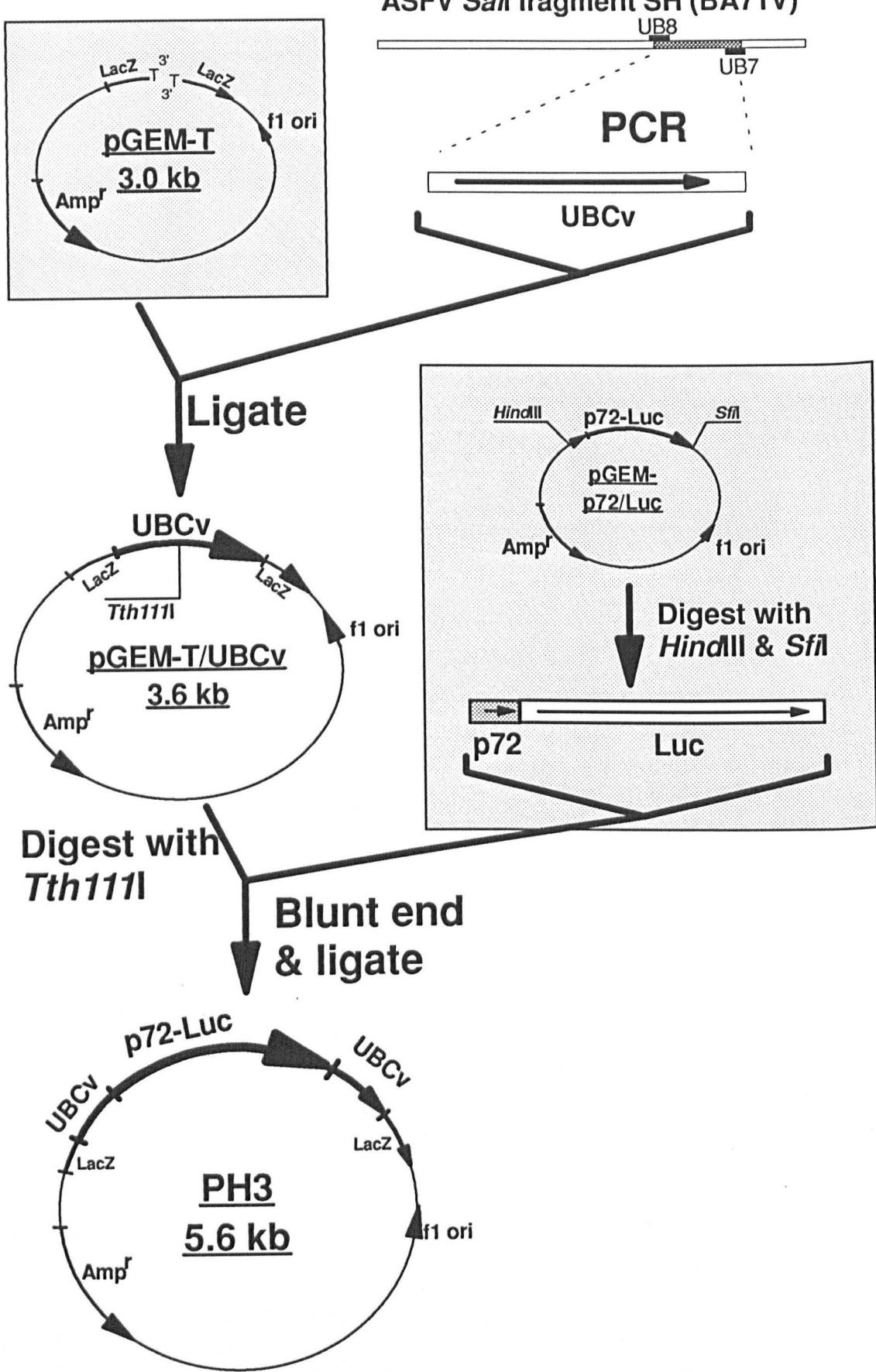


Figure 3.18 Construction of the PH3 transfer vector

Figure 3.18 Construction of the PH3 transfer vector (facing page).

The UBCv gene was amplified by PCR with primers UB7 and UB8 (table 3.3) using as template DNA from a plasmid clone (SH) containing the *SaII* fragment H of the BA71V ASFV isolate (Almendral *et al.*, 1984). The unique 650 bp PCR product was purified using a Magic PCR column (Promega) and ligated in plasmid pGEM-T (Promega) yielding plasmid pGEM-T/UBCv. The pGEM-T/UBCv plasmid was digested with the *Tth11I* restriction enzyme and the DNA ends generated were end filled using the Klenow fragment of DNA polymerase and dNTPs, then dephosphorylated with calf intestinal phosphatase (CIP). The p72-luc cassette was constructed by Dr. J. Hammond. Briefly, the p72 promoter was amplified by PCR with the p72-1 and p72-2 primers (table 3.3) from the genome of the Malawi LIL20/1 ASFV isolate and the unique 250 bp PCR product was cloned in the *BamHI* site of the pGEM-luc plasmid (Promega). The p72-luc cassette was then cut out of the pGEM-p72/luc plasmid with the *HindIII* and *SfiI* restriction enzymes and the DNA ends generated were end filled using the Klenow fragment of DNA polymerase and dNTPs. The 2 kb p72-luc fragment was purified from an agarose gel using the Geneclean kit (Bio101) and ligated with the *Tth11I* cut pGEM-T/UBCv vector. The ligation mixture was transformed in *E. coli* JM101 cells and an ampicillin resistant colony containing the PH3 plasmid was grown in 1 litre of LB and 370 µg of PH3 plasmid DNA was purified using a Magic Maxiprep column (Promega).

| Name | Position | Sequence (5' to 3') |
|-------|--------------------|------------------------------|
| UB7 | UBCv, 3' end | TTACTCATCATCCATCTCTTCA |
| UB8 | UBCv, 5' end | TAGCAGAGTACAGACACCTG |
| UB9 | UBCv, I/S 3' end | CATAGTCTTCCATCAGGGTAA |
| UB10 | UBCv, I/S 5' end | ACTTGGTCTCCGGCTCAAA |
| p72-1 | p72, 5' end | GAGCTCGTCGCGCTGACTGCCAAAGGG |
| p72-2 | p72, 3' end | GTCGACAAAAAGCTCCTCCTGATGCCAT |
| Luc2 | Luciferase, 3' end | GTTACATTACAATTGGACTTT |

Table 3.3 Sequence of the primers used to amplify the UBCv gene and verify the construction of plasmid PH3.

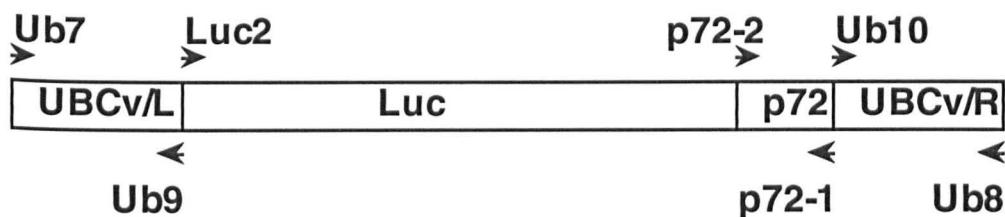
The oligonucleotides were complementary to the UBCv gene from the BA71V ASFV isolate (UB7-UB10) (Rodriguez *et al.*, 1992b), the 250 bp region upstream of the Malawi LIL20/1 structural protein VP72 (p72) (p72-1 & p72-2), or the luciferase gene in plasmid pGEM-luc (Promega) (Luc2). I/S: insertion site of the luciferase reporter in UBCv (nucleotide position 300 in the BA71V UBCv sequence).

| No | Primers | | Fragment amplified | Expected size (bp) | Observed size (bp) |
|----|-------------|--|---------------------|--------------------|--------------------|
| P1 | UB8 UB9 | | UBCvL | 260 | 250 |
| P2 | UB8 p72-1 | | UBCvL+Luc+p72 | 2185 | 2150 |
| P3 | UB8 UB7 | | UBCvL+Luc+p72+UBCvR | 2530 | 2500 |
| P4 | Luc2 p72-1 | | Luc+p72 | 1925 | 1950 |
| P5 | Luc2 UB7 | | Luc+p72+UBCvR | 2270 | 2250 |
| P6 | p72-2 p72-1 | | p72 | 250 | 250 |
| P7 | p72-2 UB7 | | p72+UBCvR | 595 | 600 |
| P8 | UB10 UB7 | | UBCvR | 345 | 350 |

Table 3.4 Sizes of the PCR products amplified from the PH3 plasmid.

The components of plasmid PH3 were amplified by PCR (P1 to P8) using the primers described in table 3.3. The expected and observed size of the PCR products are indicated. UBCvL/R: left and right UBCv arms, p72: ASFV VP72 promoter, Luc: luciferase gene.

A



B

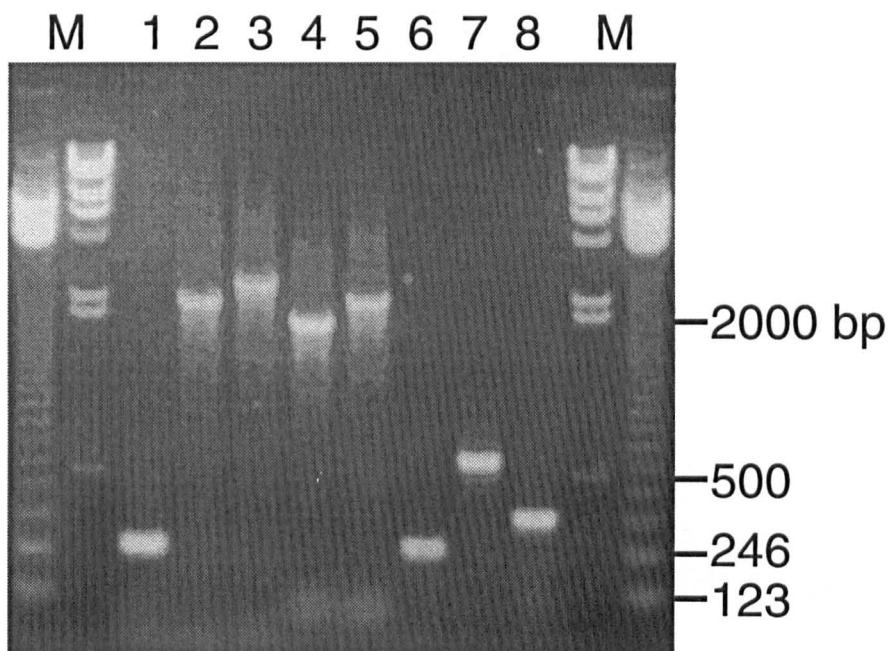
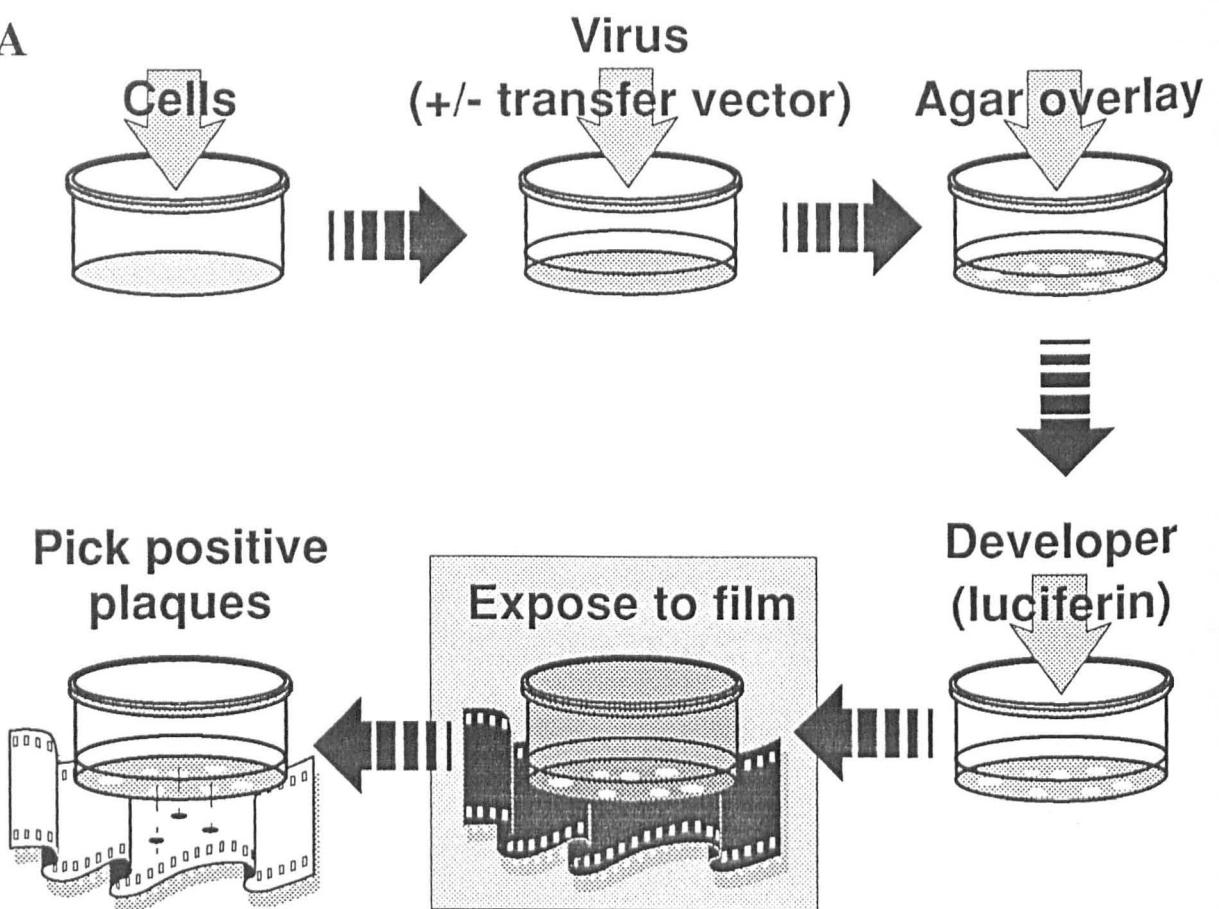


Figure 3.19 Verification of the PH3 construct by PCR amplifications.

Eight PCR amplifications, P1 to P8, were carried out with plasmid PH3 as template DNA (see table 3.4). The PCR primers were complementary to locations on plasmid PH3 indicated in panel A. Panel B: analysis by 1% agarose electrophoresis of the P1-P8 PCR products (lanes 1 to 8 respectively). The sizes of the DNA markers (M) are indicated to the right.

A



B

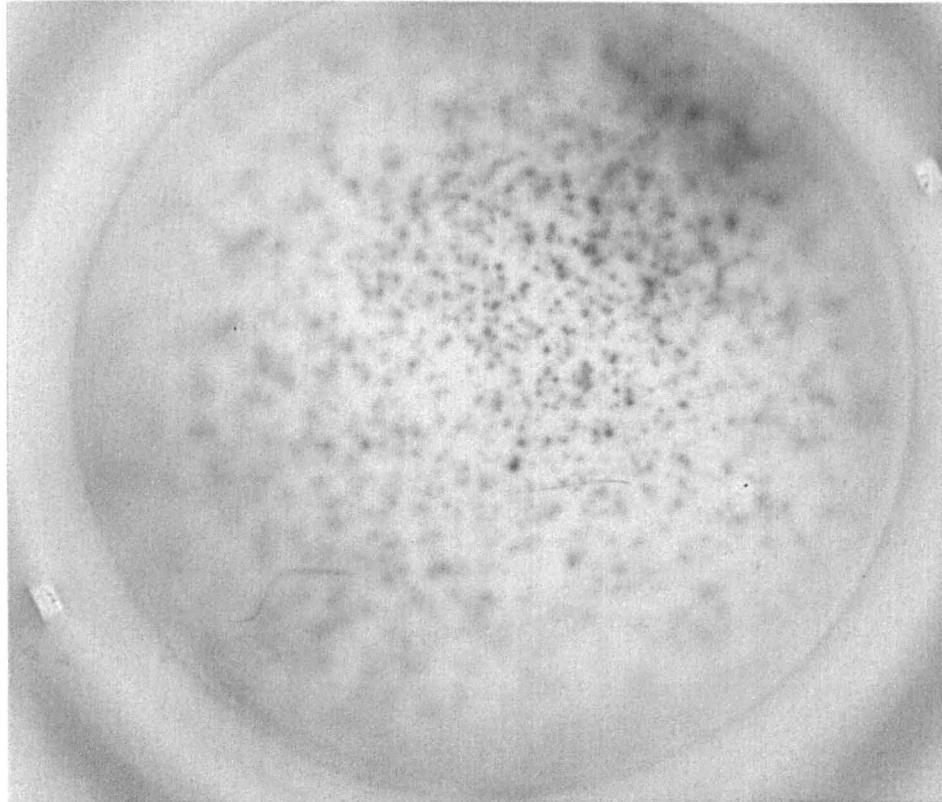


Figure 3.20 Detection of ASFV luminescent plaques transiently expressing the luciferase reporter

Figure 3.20 Detection of ASFV luminescent plaques transiently expressing the luciferase reporter (facing page).

A diagram of the method used for the detection of luminescent ASFV plaques is shown in panel A. IBRS2 cells were grown to confluence in 25 mm diameter tissue culture inserts with 0.45 μm pores (Falcon). The cells were infected with ASFV (Uganda-A, 0.1 multiplicity of infection) for one hour and then transfected for 5 hours with 2 μg of PH3 plasmid DNA (Lipofectin, Gibco BRL). The cells were overlayed with 1% agar and incubated at 37°C for a further 16 hours. The cells were then incubated with beetle luciferin and a plasma membrane permeabilizing agent (nigericin, Sigma) before exposing the inserts to high sensitivity film (Tmax 400ASA, Kodak) for 1 hour at 37°C. The film was developed (HC110, Kodak) showing the presence of luminescent ASFV plaques (panel B).

A technique was also developed for the detection of ASFV plaques expressing the luciferase reporter (figure 3.20, A). Using this method, luminescent plaques could be detected in ASFV infected cells transfected with plasmid PH3 (figure 3.20, B).

Progeny viruses of ASFV infected cells transfected with plasmid PH3 were harvested and passaged in fresh cells. The presence of ASFV mutants in the progeny was tested by luminescence plaque assays and scintillation counting. Despite repeated attempts, no luciferase activity was detected in passages after the initial transfection with the PH3 plasmid (data not shown). The failure to isolate a mutant ASFV with a luciferase disrupted UBCv gene suggested that the UBCv gene was essential for ASFV replication.

3.4 Discussion

The UBCv enzyme was detected by Western blotting both in primary pig macrophages infected with the virulent ASFV Malawi LIL20/1 field isolate and in tissue culture cells infected with attenuated Uganda-A and BA71V ASFV isolates. These findings supported the hypothesis that the UBCv gene apparently present in all ASFV isolates was functional (§ 2.1.2). The UBCv enzyme was expressed both in the early and late phases of ASFV replication, in agreement with the reported presence of UBCv mRNA in early and late BA71V infected cells (Rodriguez *et al.*, 1992b). Furthermore the intracellular concentration of UBCv increased throughout the replication of ASFV which indicated that the UBCv protein had a long half-life. This suggested that UBCv does not target itself for proteolysis as the UBCv auto-ubiquitination activity observed *in vitro* might have suggested. Moreover, no ladder of products above the UBCv enzyme band was detected in infected cells, indicating that UBCv is probably not auto-ubiquitinated *in vivo*.

The UBCv enzyme was faintly detected by immunofluorescence in the cytoplasm of ASFV infected cells only late in infection. This staining was typical of soluble cytosolic antigens which are difficult to resolve by immunofluorescence. A higher resolution was achieved by electron microscopy which confirmed that UBCv was not associated with any subcellular structure. The finding that UBCv was present in ASFV particles by both immunogold labelling and immunoblotting was surprising

since UBCv was not detected by immunofluorescence in high concentration in viral factories where virion morphogenesis takes place. The presence of UBCv in ASFV particles might be spurious and not essential for its function since many other cytosolic proteins such as actin and β -tubulins are also detected in purified virus particles (see §1.2.1). Alternatively, UBCv may be packaged inside ASFV virions and may not be accessible to antibodies.

It was not possible to inhibit UBCv expression in ASFV infected cells using antisense oligonucleotides. The concentration of viral mRNA might have been too high for the antisense oligonucleotides to have any notable effect. Alternatively the oligodeoxynucleotides-mRNA heteroduplexes might have been dissociated by the putative helicases of unknown function or activity encoded by the ASFV genome (Baylis *et al.*, 1993b; Nellen and Lichtenstein, 1993; Roberts *et al.*, 1993).

The late phase of ASFV replication appeared to be impaired at the non-permissive temperature in a cell line (TS20) with a thermosensitive E1 enzyme which is defective in the ubiquitin conjugation pathway. The partial inhibition of ASFV replication might be explained by the incomplete shut-off of E1 enzyme activity at the non-permissive temperature (Kulka *et al.*, 1988). The defect of ASFV replication in TS20 cells was consistent with the UBCv enzyme playing an important role in ASFV replication, although the possibility that this defect might be the result of the inhibition of one or more of the host's cell UBC enzymes could not be ruled out. Since early ASFV replication was apparently unaffected in TS20 cells at the non-permissive temperature, it was unlikely that UBCv played a role in early replication events such as virus uncoating.

An ASFV mutant with the UBCv gene disrupted by the luciferase reporter gene could not be isolated. This experiment was therefore inconclusive since only a positive result would have allowed a definite conclusion to be drawn. In addition, since there was no unequivocal evidence for the successful insertion of the luciferase reporter in a known non-essential region of the ASFV genome (Vydelingum, personal communication), it cannot be definitely concluded that the UBCv gene is essential for virus replication in tissue culture.

On the basis of the UBCv *in vivo* analysis, it is not possible to assign a specific role to the UBCv enzyme in ASFV replication. It seems more likely however that UBCv participates in late ASFV replication since the enzyme accumulates at this

late stage and early replication was not apparently inhibited in cells defective in ubiquitin conjugation. Possible roles for UBCv late in replication are in viral DNA replication, late gene expression, virion morphogenesis or virus release. The results obtained also do not eliminate a possible host cell modulating function for UBCv.

Chapter 4

Characterization of ubiquitin conjugates in ASFV particles

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An important part in both the biochemical and functional characterization of the UBCv enzyme is the identification of its physiological substrates. Apart from exhaustive *in vitro* testing of potential substrates of both viral and cellular origin (using the system described in §2.3), this aim could be achieved by searching for novel ubiquitin conjugates in ASFV infected cells. Ubiquitin conjugates can be detected in cell extracts by immunological techniques using commercial anti-ubiquitin antisera that recognize both free and conjugated ubiquitin (Haas and Bright, 1985; Haas, 1988; Magnani *et al.*, 1991). Any novel ubiquitinated proteins thus identified in infected cells would be potential substrates of the UBCv enzyme which could then be characterized further.

4.1 Analysis of ubiquitin conjugates during ASFV replication

4.1.1 Presence of ubiquitin conjugates in ASFV infected cells

The ubiquitin conjugates present in ASFV infected tissue culture cells were examined at various times post-infection following separation by SDS/PAGE by immunoblotting using a commercial anti-ubiquitin antiserum (Dakopatts). This antiserum is raised in rabbits against bovine ubiquitin conjugated to the keyhole limpet haemocyanin carrier protein and recognizes ubiquitin either free or conjugated to cellular proteins.

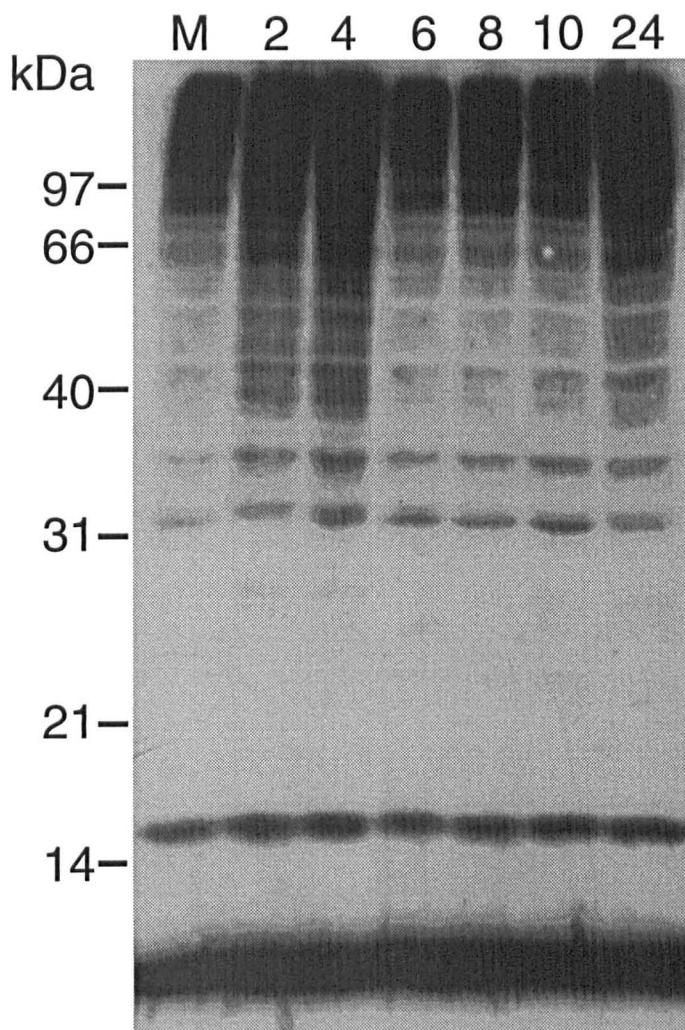


Figure 4.1 Analysis of ubiquitin conjugates in ASFV infected cells.
Tissue culture IBRS2 cells were infected with the Uganda-A ASFV isolate. At 2,4,6,8,10 and 24 hours post-infection, infected cell extracts were analysed by 12.5% SDS/PAGE followed by Western blotting. The blot was probed with affinity purified anti-ubiquitin antiserum (Dakopatts) and bound antibodies were detected by ECL. M: mock infected cells. Molecular weight markers (in kDa) are indicated to the left.

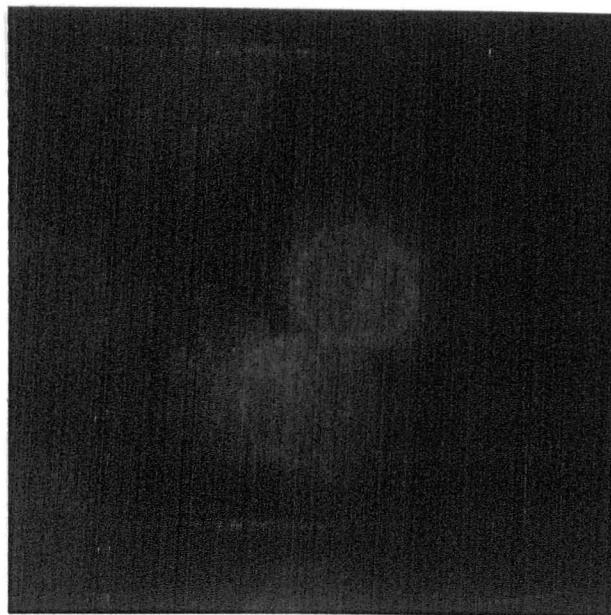
The anti-ubiquitin immunoblot of ASFV infected cell extracts is shown in figure 4.1. The same broad range of anti-ubiquitin immuno-reactive proteins was detected at all time points post-infection. These ubiquitin conjugates were similar to those present in mock infected cells, indicating that they were normal cellular ubiquitin conjugates and were not specifically induced by virus infection. Nonetheless, a significant increase in the overall intensity of anti-ubiquitin immunoreactivity above 30 kDa was observed early and very late in ASFV infection (2 to 4 and 24 hours post-infection).

A duplicate blot of the infected cell extracts was also reacted with anti-ASFV antiserum (see §3.1.2 figure 3.6, A) which confirmed that normal ASFV induced protein expression had taken place up to 10 (early) and 24 (late) hours post-infection. Since the total amount of protein loaded was the same in each lane of these gels, variations in levels of ubiquitin conjugates did not result from variations in quantities of protein loaded. These results showed that although the pattern of ubiquitin conjugates was unaltered in ASFV infected cells, a non-specific increase in the concentration of high molecular weight ubiquitin conjugates was observed during early and very late ASFV replication.

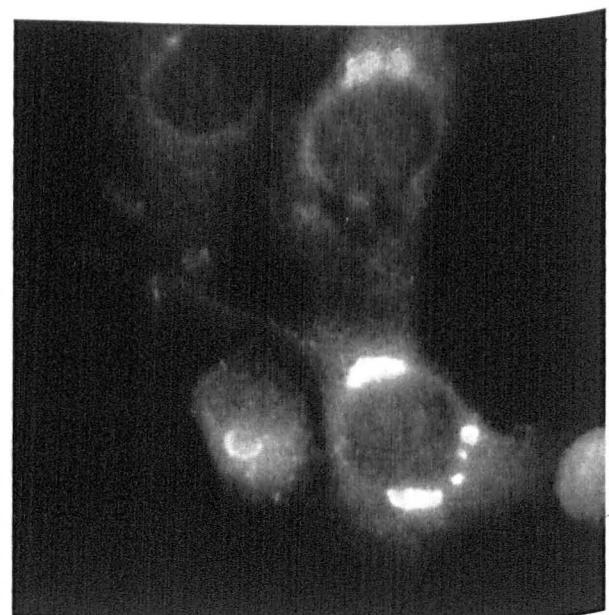
4.1.2 Subcellular localization of ubiquitin conjugates in ASFV infected cells

Tissue culture cells were infected with ASFV and observed by immunofluorescence using anti-ubiquitin antiserum. The cytoplasm of mock infected cells was weakly stained (figure 4.2, A), whereas prominent perinuclear structures reminiscent of ASFV viral factories were intensely stained by anti-ubiquitin antibodies late in ASFV infection (figure 4.2, B).

Fixed and permeabilized ASFV infected cells were also probed with rabbit anti-ubiquitin antiserum together with a mouse monoclonal antibody that recognizes the ASFV structural protein VP72 which is found in viral factories (Carrascosa *et al.*, 1986). The bound primary antibodies were then detected using FITC-conjugated anti-rabbit IgG and rhodamine-conjugated anti-mouse IgG antisera. The double staining



A



B



C



D

Figure 4.2 Indirect anti-ubiquitin immunofluorescence of ASFV infected cells.

Figure 4.2 Indirect anti-ubiquitin immunofluorescence of ASFV infected cells (facing page).

Tissue culture IBRS2 cells were either mock infected (panel A) or infected with the Uganda-A ASFV isolate (panels B, C and D). At 16 hours post-infection, the cells were fixed and permeabilized in ice cold acetone/methanol. In panels A, B and C, the fixed cells were probed with anti-ubiquitin rabbit antiserum followed by FITC conjugated secondary antibody. Panel D shows the same field as panel C but probed with anti-VP72 mouse monoclonal antibodies followed by rhodamine conjugated secondary antibodies. The photographs were taken with a Vickers incidence fluorescence microscope (x400 magnification).

showed that the anti-ubiquitin staining observed late in ASFV replication (figure 4.2, C) co-localized with the viral factories identified by the anti-VP72 antibodies (figure 4.2, D). The speckled anti-VP72 staining of the cytoplasm of ASFV infected cells was not seen with the anti-ubiquitin antiserum and probably represented mature virus particles migrating to the plasma membrane.

Thin sections of ASFV infected cells were probed with gold labelled anti-ubiquitin antibodies and observed by transmission electron microscopy (TEM). The gold labelled anti-ubiquitin antibodies were associated with ASFV virions in viral factories (figure 4.3, A). In contrast to the immunofluorescence analysis, TEM also showed that mature ASFV virions migrating to the plasma membrane (figure 4.3, B) and enveloped extracellular virions (figure 4.3, C) were decorated by the anti-ubiquitin antibodies.

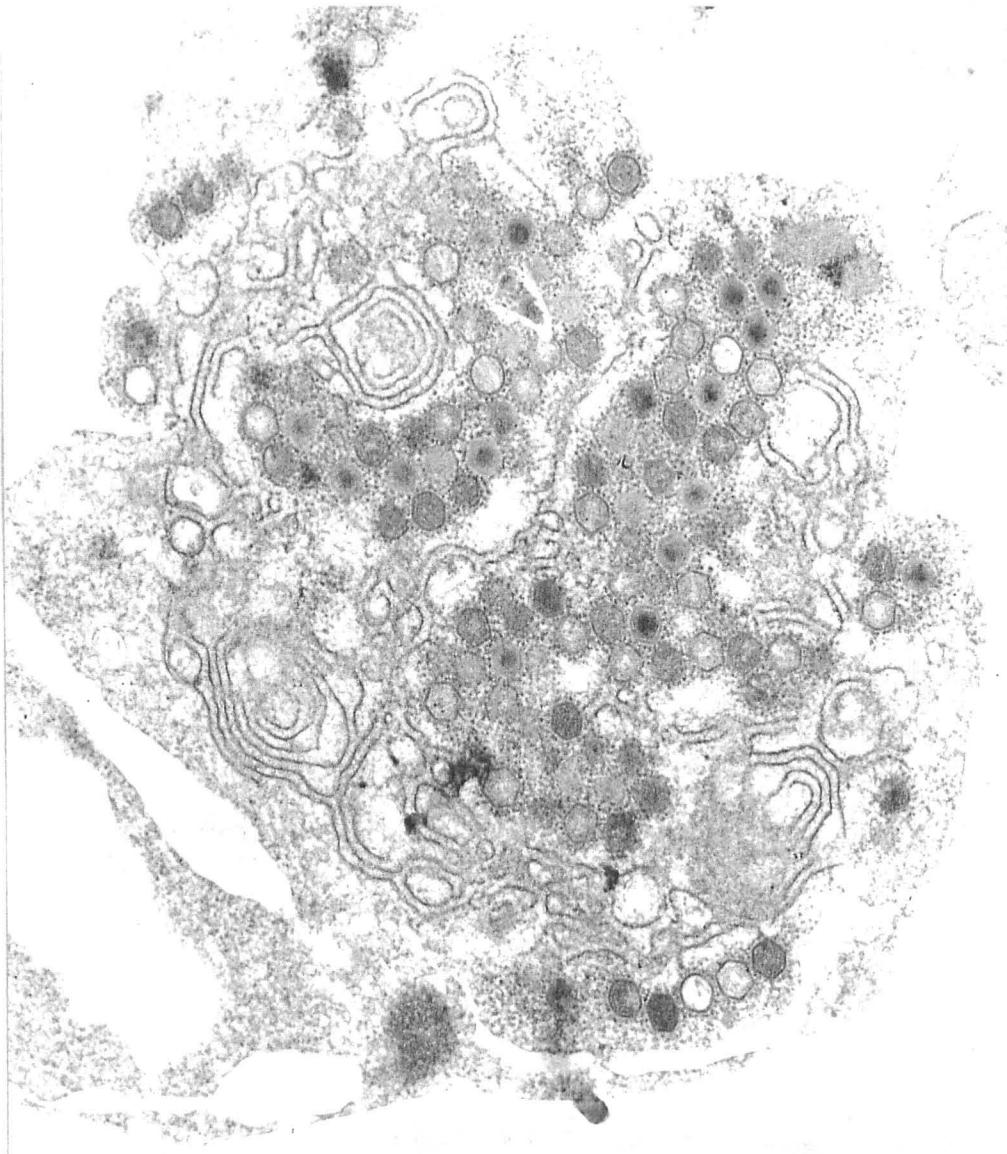
The position relative to the virus capsid of 115 gold particles was measured for 52 virions with a clear hexagonal outline (figure 4.4). This showed that 70% of the gold labelled anti-ubiquitin antibodies were situated inside the virus capsid. The most frequent location for anti-ubiquitin antibodies was either between the virion nucleo-protein core and the internal membrane or in the virus capsid. The ubiquitin conjugate locations identified by the gold labelled antibodies are similar to those determined for the VP37 core/internal membrane protein and the VP72 capsid protein of the BA71V ASFV isolate (Carrascosa *et al.*, 1986).

The majority of the ubiquitin antigens were therefore encapsidated in the virions which might explain why intact mature ASFV particles migrating to the cell membrane could not be detected with the anti-ubiquitin antibodies by immunofluorescence. However, strong staining of viral factories with anti-ubiquitin antiserum could be observed by immunofluorescence, probably because of the presence in these factories of immature virions with incomplete capsids that were permeable to the anti-ubiquitin antibodies.

4.1.3 Presence of ubiquitin conjugates in purified ASFV particles

The presence of ubiquitin conjugates in viral factories and mature virions suggested that ASFV structural proteins might be ubiquitinated. To investigate this possibility, proteins in purified extracellular ASFV particles (Percoll purified, see §3.2.2) were

A



C

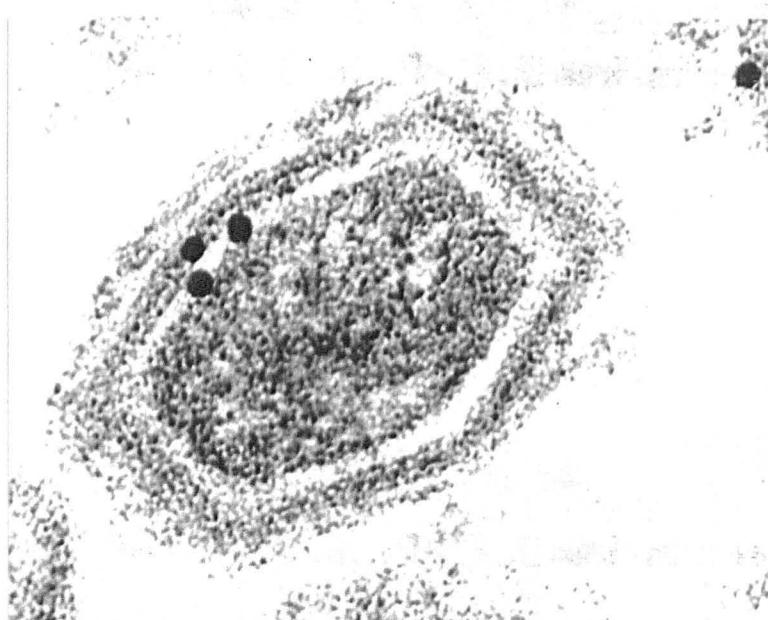


Figure 4.3 Anti-ubiquitin immunogold labelling of ASFV infected cells.

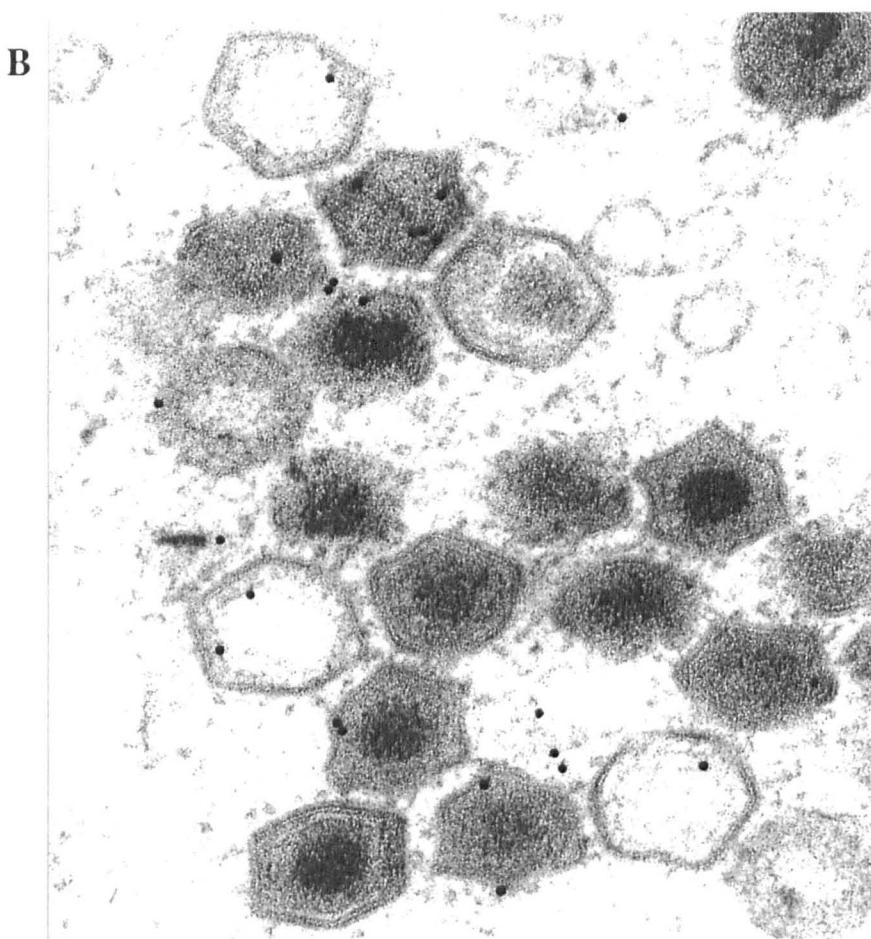


Figure 4.3 Anti-ubiquitin immunogold labelling of ASFV infected cells.

Tissue culture IBRS2 cells were infected with the Uganda-A ASFV isolate. At 16 hours post-infection the cells were fixed and embedded in araldite. Thin sections were probed with goat anti-ubiquitin antiserum followed by gold labelled secondary antibodies. The details of a virus factory (x32000, facing page panel A), mature intracellular virions (x13000, panel B) and an extracellular ASFV particle (x320000, panel C, facing page) are shown. The photographs were taken using a Philips EM410 electron microscope.

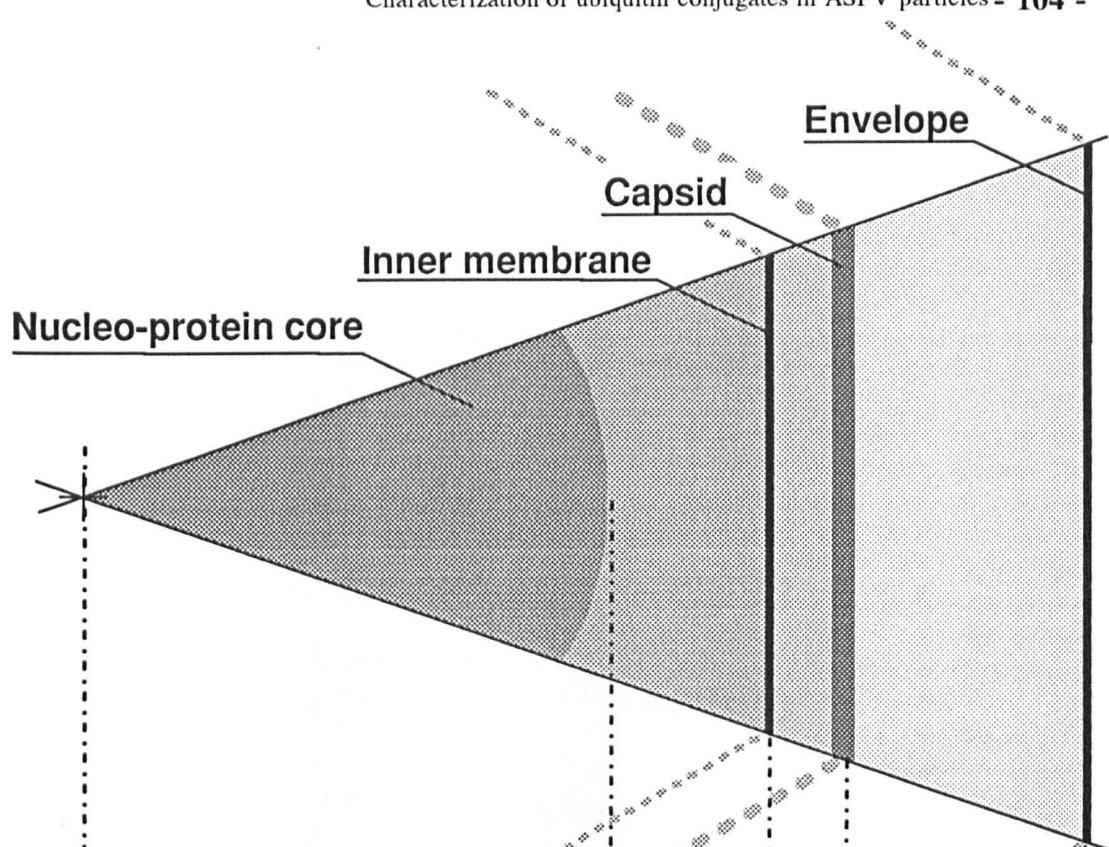
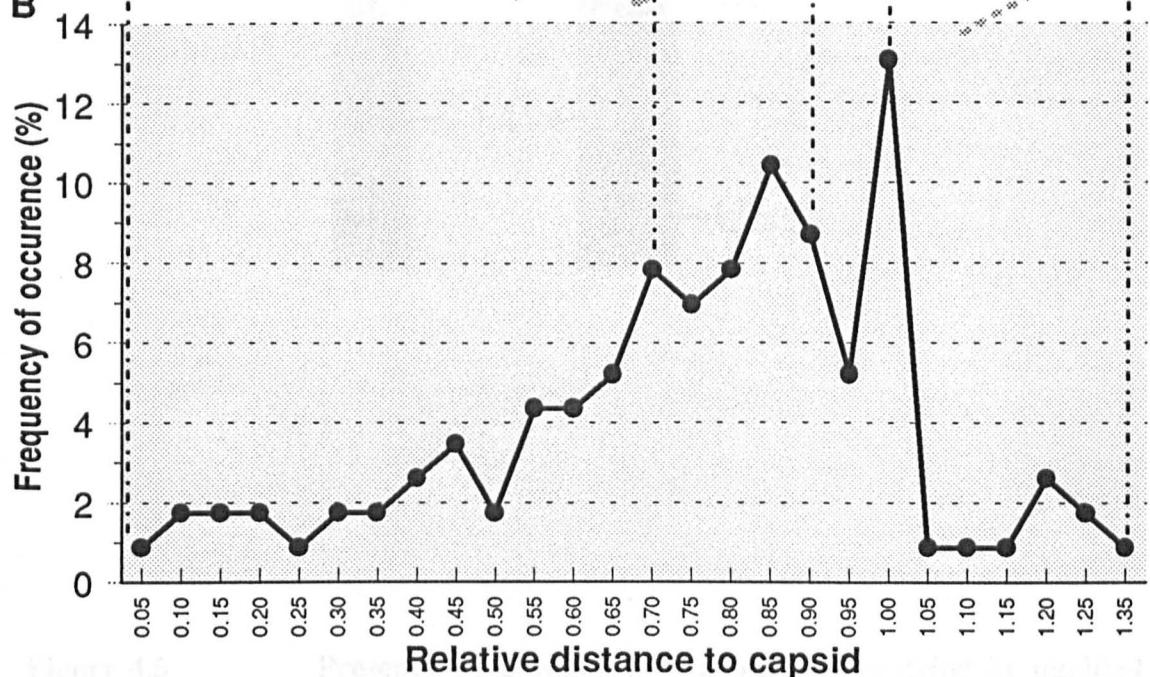
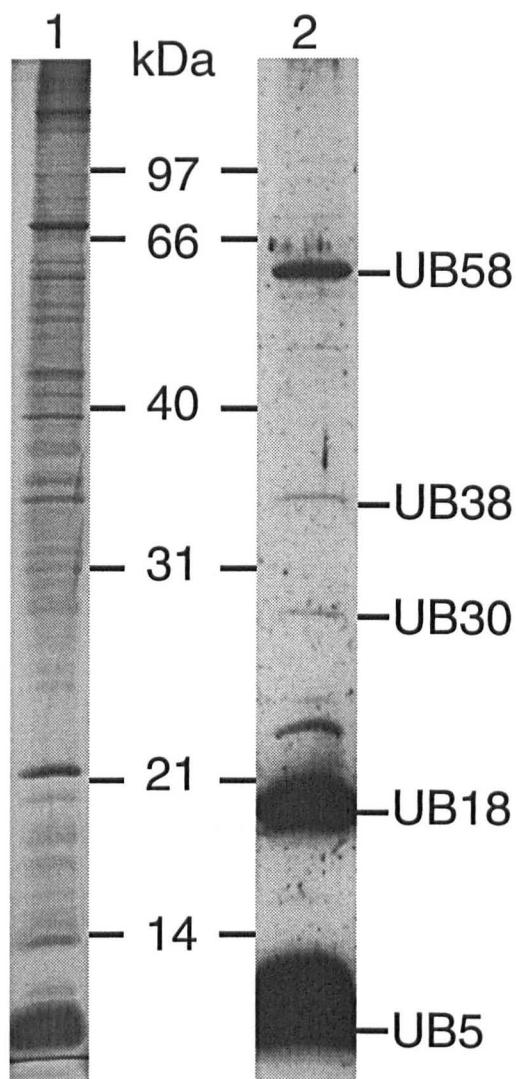
A**B**

Figure 4.4 Position relative to the ASFV capsid of gold labelled anti-ubiquitin antibodies.

The relative position of 115 gold particles was measured in 52 ASFV virions. A relative position of zero refers to the centre of the virus particle and a relative position of 1 corresponds to the virus capsid (panel A). In panel B the observed frequency (in percentage) of the relative positions of the gold particles is represented as a line graph.

**Figure 4.5**

Presence of ubiquitinated structural proteins in purified extracellular ASFV virus particles.

Percoll purified ASFV extracellular particles (see §3.2.2) were separated by 12.5% SDS/PAGE. The separated proteins were either Western blotted and probed with anti-ubiquitin antiserum (lane 2) or silver stained (lane 1). Bound antibodies on the protein blot were detected by ECL. The positions of the ubiquitin conjugates described in §4.1.3 are shown on the right. The position of molecular weight markers (in kDa) are indicated to the left.

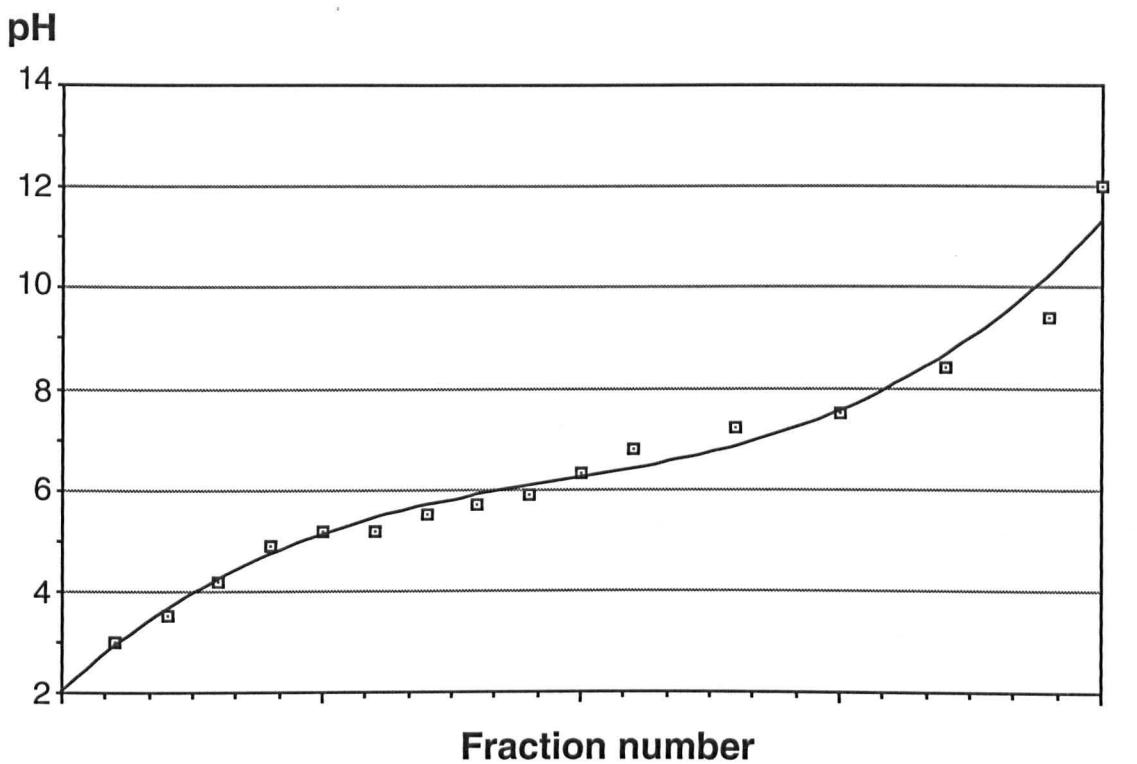
separated by SDS/PAGE and analyzed by immunoblotting using anti-ubiquitin antiserum (figure 4.5, lane 2). Three strongly immuno-reactive proteins were detected with apparent molecular weights of 58, 18 and 5 kDa. Four additional proteins (51, 38, 30 and 23 kDa) cross-reacted weakly with the anti-ubiquitin antiserum. The 58, 38, 18 and 5 kDa anti-ubiquitin immuno-reactive proteins (hereafter referred to as UB58, UB38, UB18 and UB5 respectively) were the most likely candidates for genuine ubiquitin conjugates since they were consistently detected with five anti-ubiquitin antisera tested, irrespective of the batch or manufacturer (data not shown).

The UB5 protein was likely to correspond to free ubiquitin since ubiquitin was shown to migrate with an apparent molecular weight of 5.5 kDa in SDS/PAGE (Ciechanover *et al.*, 1978; Wilkinson, KD, 1988). The silver staining of purified ASFV particle proteins (figure 4.5, lane 1) suggested that UB5 was an abundant component of purified ASFV particles. The high molecular weight ubiquitin conjugates (UB18 to UB58), although only minor ASFV structural proteins, were potential substrates for the UBCv enzyme and were therefore further characterized.

4.2 Characterization of the ubiquitin conjugates in ASFV virions

4.2.1 Preliminary attempts to purify ubiquitinated ASFV structural proteins

Possible approaches to further characterize the ASFV ubiquitinated structural proteins include protein purification followed either by N-terminal protein sequencing or by the production of specific antisera. The antisera could then be used to screen expression libraries containing either virus or cellular DNA and sequences of inserts in immunoreacting clones could be determined to obtain the amino acid sequence of the encoded protein. Protein sequences obtained using either method could be used to search databases to identify these substrates. Both these methods require substantial amounts of the proteins of interest purified to near homogeneity. To achieve this, three semi-preparative purification techniques were tested to obtain large quantities of purified ASFV ubiquitinated structural proteins.



1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

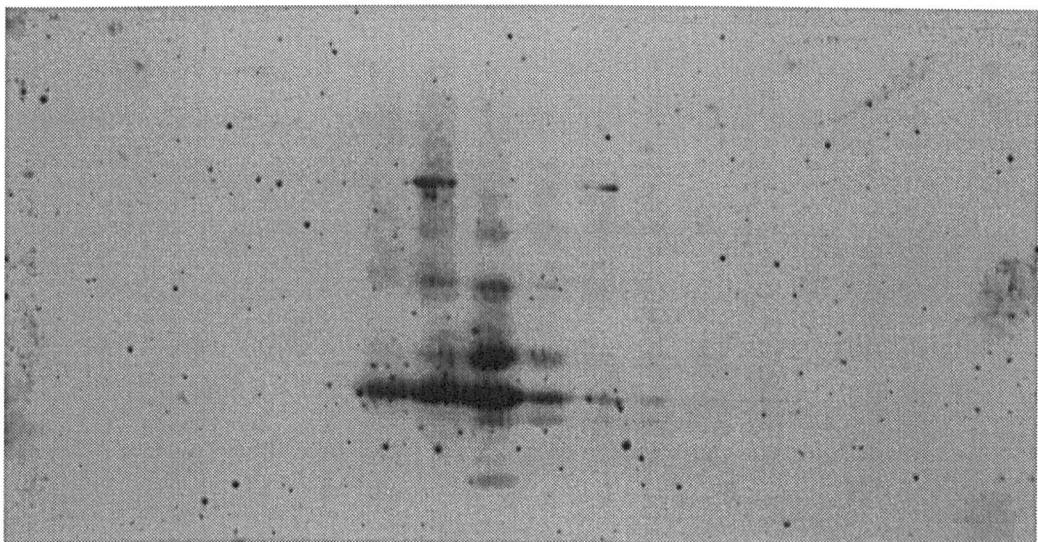


Figure 4.6 Preparative liquid phase isoelectric focusing of ASFV structural proteins.

Percoll purified extracellular ASFV particles (see §3.2.2) were solubilized and subjected to liquid phase IEF (Rotofor, Biolog). The pH of the 20 focused fractions collected is indicated in panel A. The proteins in each of the 20 fractions were analysed by 12% SDS/PAGE followed by Western blotting. The blotted proteins were probed with anti-ubiquitin antiserum and bound antibodies were detected by ECL (panel B).

The first method used preparative liquid phase isoelectric focusing (IEF) (Rotofor, Biorad). Percoll purified ASFV particles were solubilized in 8 M urea / 2% NP40 and the resulting structural proteins were separated according to their isoelectric point (pI) into 20 fractions ranging from pH3 to pH11 (figure 4.6, A). The 20 fractions were separated by SDS/PAGE and analyzed by immunoblotting using anti-ubiquitin antiserum (figure 4.6, B). This showed that the ASFV ubiquitin conjugates were focused in fractions 8 to 10 (pI 6.3 to 7.5). Since the majority of ASFV structural proteins also focused in these neutral fractions (data not shown), purification of ubiquitin conjugates from other virion proteins was not sufficient and this method was not pursued further.

The use of anti-ubiquitin affinity chromatography offered an alternative semi-preparative approach for the purification of ASFV ubiquitin conjugates. An affinity column was prepared by conjugating anti-ubiquitin antibodies to a crosslinked agarose matrix (Sulfolink, Pierce). Approximately 70 µg of anti-ubiquitin antibodies were linked to 1 ml of the gel matrix. The ASFV structural proteins were solubilized by sonicating Percoll purified ASFV particles in a disruption buffer containing 10% NP40 and 1 M NaCl. The solubilized ASFV structural proteins were then concentrated and re-diluted in PBS buffer to remove the NP40 and NaCl before loading on the anti-ubiquitin column. The column was washed with a low salt buffer (0.2 M NaCl) and bound proteins were eluted with a high ionic strength buffer (2 M NaCl). The elution of proteins was monitored by measuring the UV absorbance (OD_{280}) of the column effluent (figure 4.7).

The protein content of the wash and eluate fractions was analysed by SDS/PAGE followed by silver staining. Surprisingly, although a strong absorbance peak (0.5 OD_{280}) was recorded following the low salt wash, no proteins could be detected in this fraction by silver staining (figure 4.8, lane 2). The UV absorbance spectrum of the wash fraction revealed an absorbance maximum for $\lambda = 260$ nm (data not shown) which indicated that the wash material probably consisted entirely of DNA.

In contrast to the wash fraction, several proteins were detected in the fraction eluted with the high salt buffer (figure 4.8, lane 3). The two most abundant proteins in the eluate migrated with apparent molecular weights of 12 and 140 kDa. A further

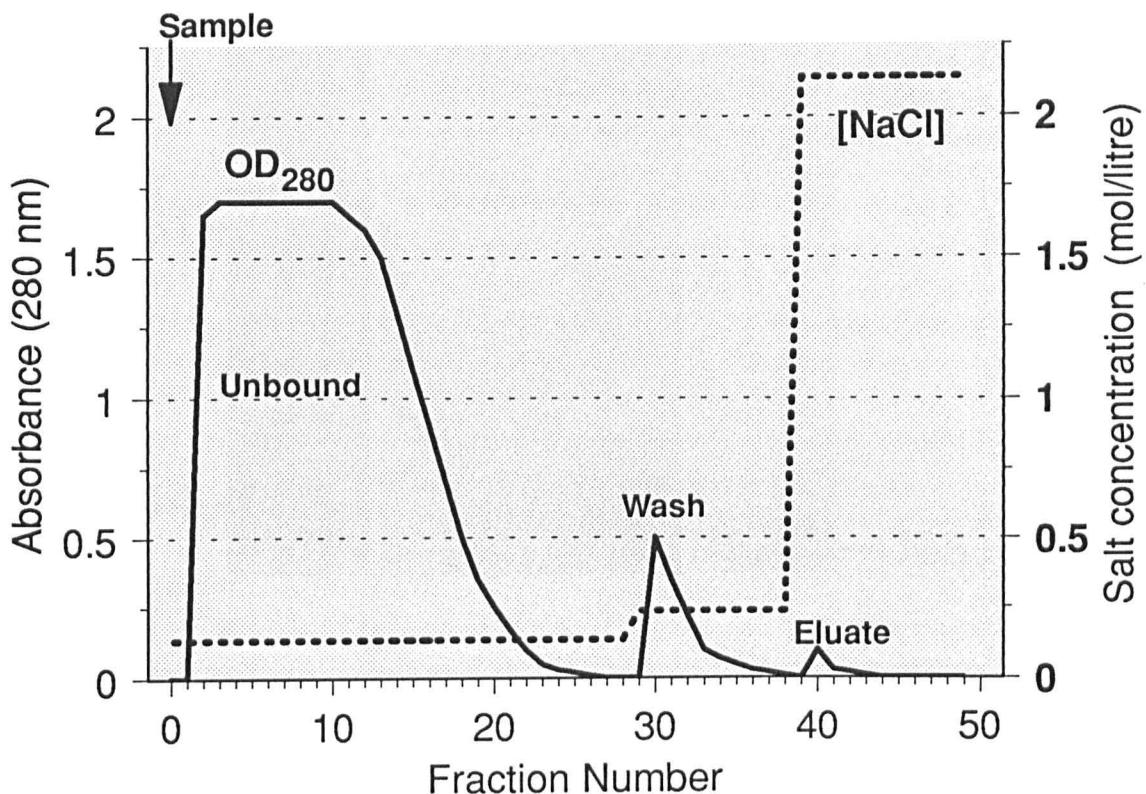


Figure 4.7 Purification of ASFV structural proteins by anti-ubiquitin affinity chromatography.

Percoll purified extracellular ASFV particles (see §3.2.2) were solubilized and applied on a chromatography column consisting of anti-ubiquitin antibodies immobilized on an agarose gel matrix. The loaded column was washed with increasing concentrations of NaCl in PBS ([NaCl], right axis) and the protein content of the column effluent was monitored by UV absorbance (OD_{280} , left axis). The absorbance peaks corresponding to unbound ASFV structural proteins (unbound), non-specific bound proteins (wash) and anti-ubiquitin specific ASFV proteins (eluate) are indicated.

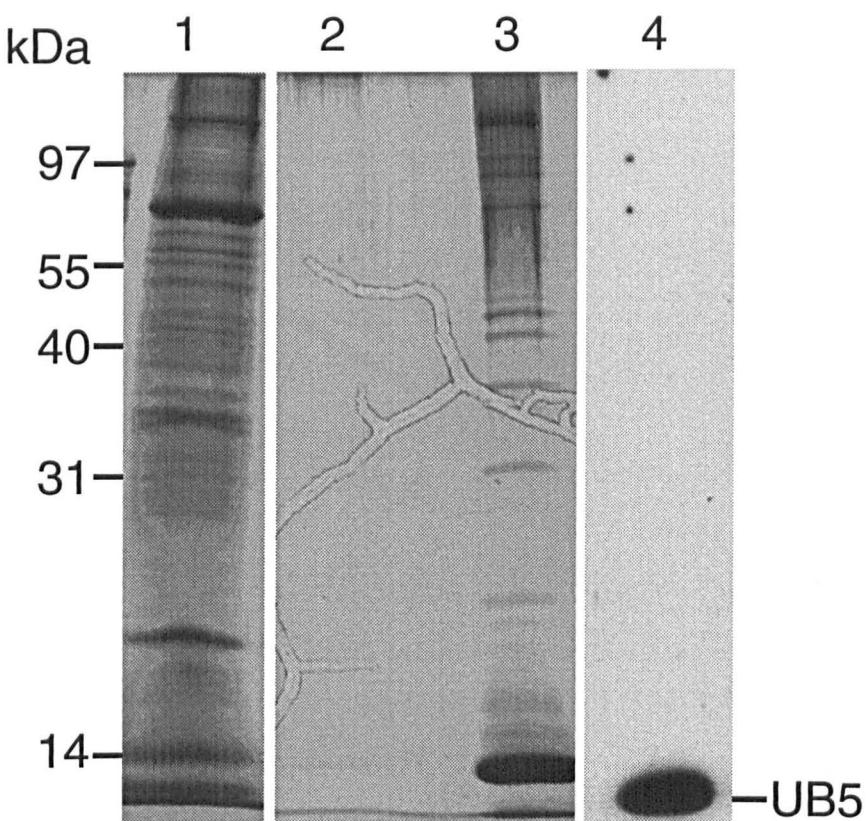


Figure 4.8 Analysis of the ASFV structural proteins eluted from the anti-ubiquitin affinity column.

The anti-ubiquitin affinity purified proteins contained in the wash (lane 2) and eluate (lane 3) fractions were analysed by 12.5% SDS/PAGE followed by silver staining. The silver stain of total ASFV proteins is shown in lane 1. The proteins contained in the eluate fraction were also analysed by immunoblotting using anti-ubiquitin antiserum (lane 4). The positions of molecular weight markers (in kDa) are indicated to the left.

12 minor proteins were also eluted with the high salt buffer (5, 16, 18, 23, 30, 38, 43, 46, 58, 72, 90, and 100 kDa). The diffuse silver staining in the upper portion of the eluate lane was typical of DNA contamination. This was confirmed by the UV absorption spectrum of the eluate fraction which showed that DNA ($\lambda_{\text{max}}=260$ nm) accounted for 75% of the absorption measured at 280 nm (data not shown).

The ASFV structural proteins purified by anti-ubiquitin affinity chromatography differed from those previously detected in Western blots and only the 5 kDa affinity purified protein (likely to represent free ubiquitin) was recognized by anti-ubiquitin immunoblotting of the eluate fraction (figure 4.8, lane 4). This variation was probably the result of the differing antigenic properties of native compared to SDS denatured ubiquitin conjugates. Nonetheless, some of the minor affinity purified proteins migrated with molecular weights similar to those previously observed in anti-ubiquitin immunoblots of whole virus (namely UB18, UB30, UB38 and UB58). These might not have been present in sufficient quantity in the affinity chromatography eluate to be efficiently detected by immunoblotting.

The eluate fractions from four successive affinity purifications (each using 500 μg of solubilized ASFV virions) were pooled which produced approximately 10 μg of anti-ubiquitin affinity purified proteins. The purified proteins were separated by SDS/PAGE, transferred to a PVDF membrane and stained with Coomassie blue. The protein bands, although faint, were individually excised and subjected to automated N-terminal protein sequencing (Model 473A, Applied Biosystems). As was feared from the weak signal obtained by Coomassie staining, the amounts of blotted proteins proved insufficient to recover protein sequence data by the Edman degradation method. This approach to the purification of ASFV ubiquitinated structural proteins was abandoned since the poor yield, which probably resulted from the relatively low affinity interaction between the proteins and anti-ubiquitin antibodies, meant that prohibitively large amounts of antibodies would have to be used.

4.2.2 Detergent extraction of ubiquitinated ASFV structural proteins

The ASFV attachment protein VP12 which was shown to be situated outside the virus capsid (Carrascosa *et al.*, 1993) was partially purified from extracellular ASFV virions by gentle extraction with the non-ionic detergent *n*-octyl- β -D-glucopyranoside

(OG) (Carrascosa *et al.*, 1991; Alcami *et al.*, 1992). Since electron microscopy suggested that a subset of the ubiquitinated ASFV structural proteins were also located outside the virus capsid, it was likely that some ubiquitin conjugates would be released from ASFV virions by treatment with OG detergent.

A suspension of Percoll purified ASFV extracellular particles in PBS (1 mg.ml⁻¹) was incubated with various concentrations of OG for 16 hours. The suspension was then centrifuged on a 25% sucrose cushion to pellet subviral ASFV particles and the soluble proteins contained in the supernatant were concentrated and analyzed by SDS/PAGE.

The separated proteins were silver stained (figure 4.9, A) and, as expected, the number of structural proteins present in the supernatant increased as the concentration of OG detergent used was increased. The supernatant proteins were also analyzed by immunoblotting using anti-ASFV antiserum (figure 4.9, B) indicating that most immuno-reactive proteins detected in whole virus were gradually released after OG treatment.

The VP72 ASFV structural protein was the only protein extracted at OG concentrations below 0.25%, whereas most ASFV structural proteins were extracted when the concentration of OG exceeded 0.5%. The supernatant obtained after treatment with 0.25% OG contained 9 major proteins of molecular weights 10.5, 12, 14, 18, 21, 34, 35, 72 and 150 kDa. The 12 kDa protein was probably the previously characterized VP12 ASFV attachment protein described above. Thus the mild 0.25% OG treatment was likely to extract only ASFV structural proteins located in or outside the ASFV capsid.

The OG extraction of ubiquitin conjugates was monitored by immunoblotting using anti-ubiquitin antiserum (figure 4.9, C). The four ASFV ubiquitin conjugates (UB5 to UB58) were all extracted with detergent concentrations of 1% and above, but only UB18 was significantly extracted by the mild 0.25% OG detergent treatment although small amounts of UB58 were also extracted at this concentration. It was therefore likely that UB18 was located either in or outside the capsid, whereas the other ASFV ubiquitin conjugates (including free ubiquitin) were probably encapsidated in the virion.

The single 18 kDa protein detected in the supernatant of ASFV particles treated with 0.25% OG and analyzed by silver staining (figure 4.10, lane 2) was

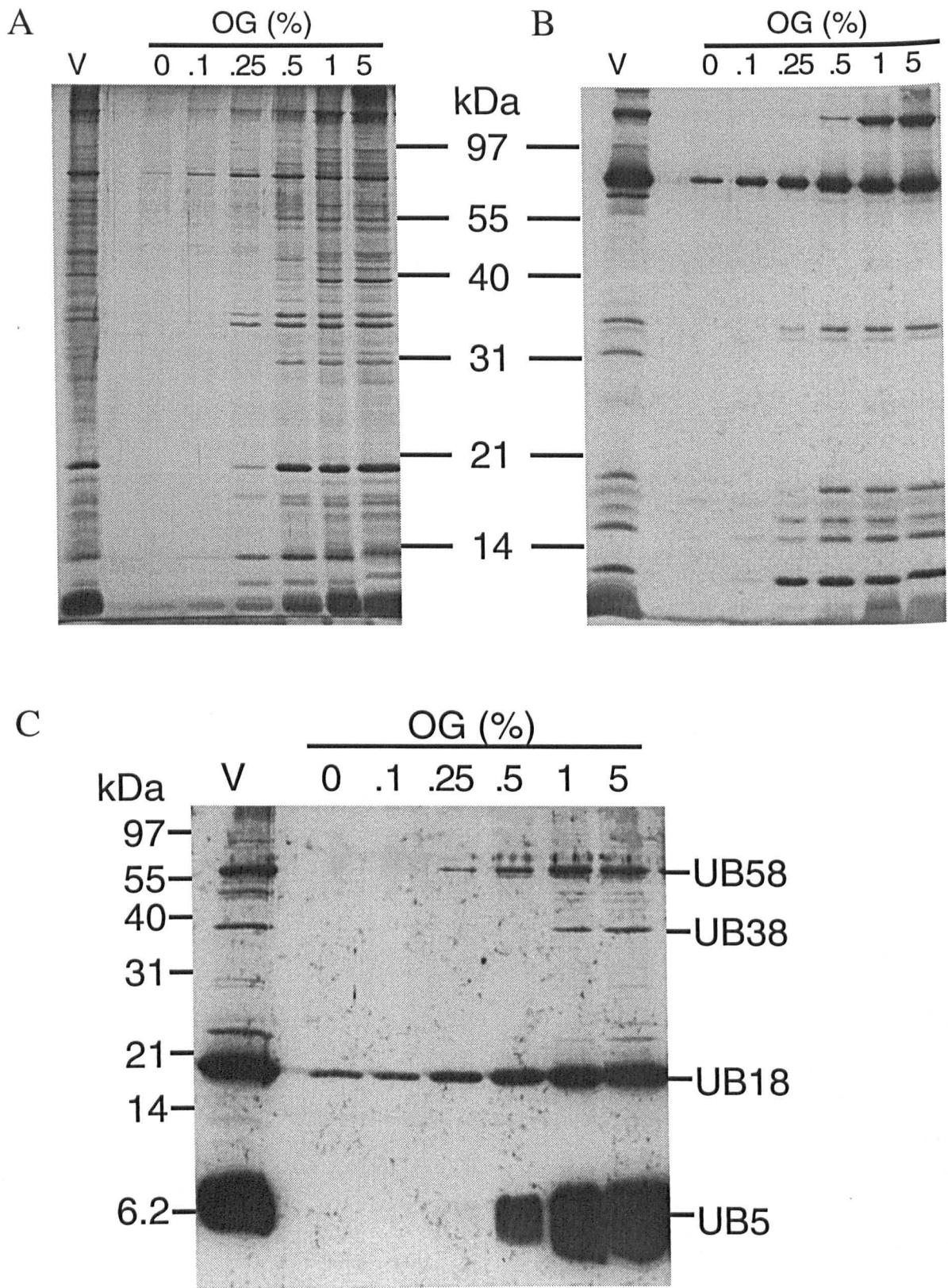


Figure 4.9 Extraction of ASFV structural proteins with the non-ionic detergent octyl-glucopyranoside

Figure 4.9 Extraction of ASFV structural proteins with the non-ionic detergent *n*-octyl- β -D-glucopyranoside (OG) (facing page).

Percoll purified extracellular ASFV particles (see §3.2.2) were either untreated (V) or incubated with various concentrations of OG in PBS (0, 0.1, 0.25, 0.5, 1, and 5% OG). Particulate material was removed by centrifugation and the solubilized proteins were analysed by 12.5% (panels A and B) or 17.5% (panel C) SDS/PAGE. The separated proteins were silver stained (panel A) or Western blotted and probed with either anti-ASFV hyperimmune serum (panel B) or anti-ubiquitin antiserum (panel C). The bound antibodies were detected by ECL. The positions of the ubiquitin conjugates are indicated to the right and the position of molecular weight markers (in kDa) are indicated to the left.

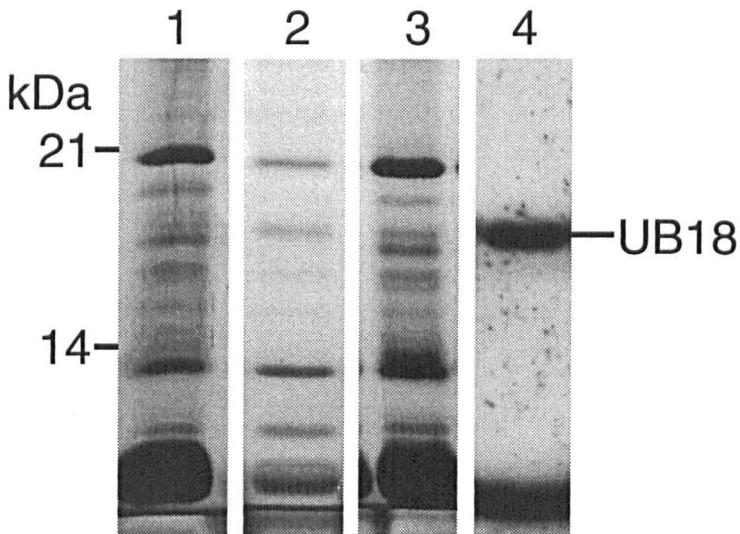


Figure 4.10 Purification of the UB18 ubiquitin conjugate by gentle extraction with 0.25% n-octyl- β -D-glucopyranoside.

Detail of the 10 to 20 kDa region of the 12.5% SDS/PAGE analysis of the detergent extraction of ASFV structural proteins presented in figure 4.9. Lane 1: silver stain of total ASFV structural proteins; lane 2: silver stain of the 0.25% OG supernatant; lane 3: silver stain of the 1% OG supernatant; lane 4: anti-ubiquitin immunoblot of the 0.25% OG supernatant. The positions of UB18 and molecular weight markers (in kDa) are indicated.

probably the UB18 ubiquitin conjugate detected by immunoblotting (figure 4.10, lane 4). This 18 kDa protein (UB18) was well separated by SDS/PAGE from other proteins in the 0.25% OG fraction although some closely migrating virus proteins were present in the 1% OG fraction (figure 4.10, lane 3). At least 25% of total UB18 was present in this fraction as estimated by the intensity of silver staining and immunoreactivity. Thus OG extraction of ASFV structural proteins followed by SDS/PAGE could be used to purify sufficient quantities of UB18 from ASFV virions for further characterization. This method was however not suited for the purification of other virus ubiquitin conjugates (UB5, UB38 and UB58) since these were only released, together with most of the virus structural proteins, when high OG concentrations were used.

4.2.3 Identification of the putative ubiquitinated ASFV structural protein UB18

A large scale extraction with 0.25% OG was carried out starting with 2 mg of Percoll purified Uganda-A ASFV particles which produced approximately 250 µg of OG extracted structural proteins. The detergent extracted proteins were separated by SDS/PAGE and blotted onto a PVDF membrane. The blotted proteins were either stained with Coomassie blue or probed with anti-ubiquitin antiserum which showed that a protein clearly stained with Coomassie blue migrated at the same position as the UB18 anti-ubiquitin immunoreactive protein (data not shown). The Coomassie stained band corresponding to UB18 was excised from the blot and subjected to automated N-terminal protein sequencing (Model 473A, Applied Biosystems). If UB18 consisted of a ubiquitin polypeptide covalently linked via its C-terminus to an internal lysine of an unknown protein (referred to as USB1), then the N-termini of both ubiquitin and its conjugated USB1 protein should be available for sequencing.

The first seven cycles of Edman degradation applied to UB18 (table 4.1) each generated one clearly predominant amino acid presumably corresponding to USB1. For the first five cycles a secondary residue was also detected that corresponded to the known N-terminal sequence of mammalian ubiquitin (Wilkinson, 1988).

| | Primary | | Secondary | |
|-------|---------|-----------|-----------|-----------|
| Cycle | Residue | Raw yield | Residue | Raw yield |
| 1 | Pro | 32.5 | Met | 26 |
| 2 | Ser | 22.6 | Gln | 6.3 |
| 3 | Asp | 15.2 | Ile | 2.4 |
| 4 | Met | 8.8 | Phe | 3.1 |
| 5 | Lys | 7.6 | Val | 0.84 |
| 6 | Gln | 10.6 | - | - |
| 7 | Phe | 8.3 | - | - |

Table 4.1 Identity of the first 10 amino acid residues detected during the N-terminal sequencing of UB18.

The UB18 protein was blotted on a PVDF membrane and subjected to 10 cycles of Edman degradation (Model 473A, Applied Biosystems). The amino acids generated were identified by their retention factor during reverse phase HPLC. The column 'Primary' shows the most abundant amino acid for each cycle and the column 'Secondary' shows amino acids detected in smaller quantity that correspond to the sequence of ubiquitin. The amount in pmol of each residue (adjusted by subtracting the background read for the previous residue) estimated by UV absorbance is given in the column 'Raw yield'.

The observed molar ratio between the amino acid raw yields of ubiquitin and USB1 was as low as 1:10 which was inconsistent with the 1:1 molar ratio expected of a mono-ubiquitinated USB1 protein (or the 2:1 ratio expected of a bi-ubiquitinated USB1 protein). This phenomenon was similar to that reported during the N-terminal protein sequencing of the ubiquitinated lymphocyte homing receptor (Siegelman *et al.*, 1986) and could be attributed to an incomplete blocking of the ubiquitin N-terminus either prior to its integration into the UB18 conjugate or during subsequent purifications of UB18. Alternatively this discrepancy in the molar ratios might have indicated that the weaker ubiquitin signal was the result of unspecific contamination with free ubiquitin. The presence of a non-specific free ubiquitin contaminant in the blot was unlikely since no ubiquitin sequence was detected during the N-terminal sequencing of the 12 kDa ASFV attachment protein excised from the same blot (Kytes and Bailey, personal communication).

The plot of the amino acid raw yields against the residue number (figure 4.11) showed that the repetitive yield for the ubiquitin sequence was significantly lower than for the USB1 sequence (46% versus 80%) and the starting amounts of ubiquitin and USB1 was extrapolated to approximately 41 and 32 pmoles respectively. The figure of 41 pmoles for ubiquitin was probably somewhat overestimated as a result of the artificially high quantity of Met¹ attributable to contaminant proteins which share an N-terminal methionine. Thus although the sequencing of ubiquitin was less efficient than that of USB1, both were probably roughly equimolar in UB18 consistent with it being a mono-ubiquitinated USB1 conjugate.

The characterization by N-terminal sequencing of the iso-2-cytochrome-c ubiquitin conjugate (Sokolik and Cohen, 1991) showed that the raw amino acid yields of the lysine residues to which ubiquitin was conjugated was significantly lower than expected. This was due to the retention in the blot of the lysines at the fork of the branched heterologous protein which were linked via their ϵ amino group to the C-terminus of ubiquitin. The raw yield of the Lys⁵ of USB1 was not significantly lower than expected, indicating that ubiquitin was probably not conjugated to this lysine site in USB1.

The sequence of the N-terminal 7 amino acids of the USB1 polypeptide was used to screen protein sequence databases. A single 100% amino acid match was

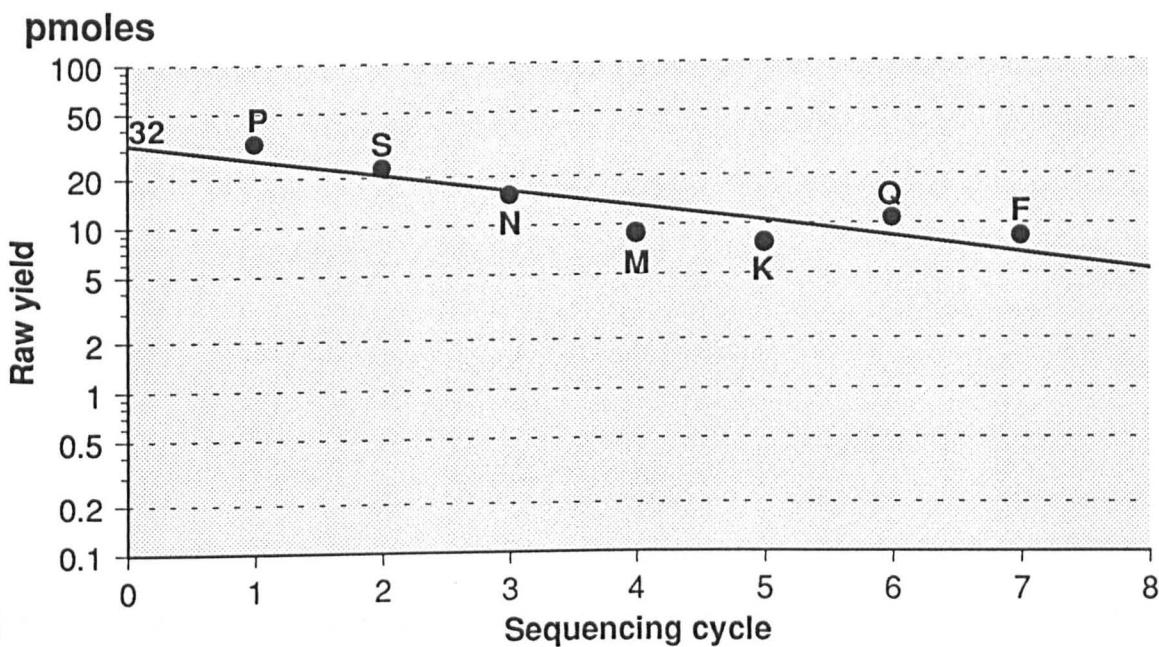
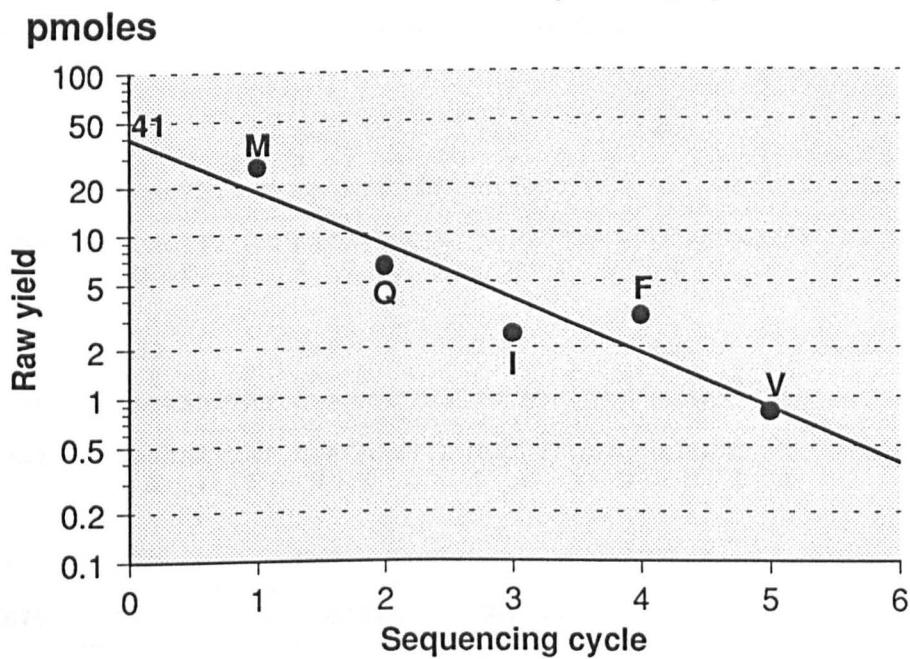
A**B**

Figure 4.11 Plots of the raw yields of the USB1 and ubiquitin sequences against the sequencing cycle number.

The raw amino acid yields (table 4.1) of the USB1 (panel A) and ubiquitin (panel B) sequences are plotted against the sequencing cycle number. The repetitive yield corresponds to the slope of the line which correlates most closely to the experimental raw yields and the starting amounts of protein are extrapolated from cycle 0. The identity of the amino acids are indicated.

| | | | | | | |
|--------------|--|-----|-----|-----|--------|-------------|
| | 0 | 10 | 20 | 30 | 40 | |
| | | | | | USB1 → | M K |
| E75 | CATTCAATT TAAAATTATA AAATAATAAG AAGAT.CCCT CTAATATGAA | | | | | |
| BA71V | CATTCAATT TAAAATTATA AAATAATAAG AAGATGCCCT CTAATATGAA | | | | | |
| | | | | | USB1 → | M P S N M K |
| | M <- VP32 | | | | | |
| | 50 | 60 | 70 | 80 | 90 | |
| E75 | Q F C K I S V W L Q H D P D L L | | | | | |
| | ACAGTTTGCG AAGATTCTG TATGGCTGCA G...CACGAT CCAGATTAT | | | | | |
| BA71V | ACAGTTTGCG AAGATTCTG TATGGCTGCA GCAGCACGAT CCAGATTAT | | | | | |
| | Q F C K I S V W L Q Q H D P D L L | | | | | |
| | 100 | 110 | 120 | 130 | 140 | |
| E75 | E I I N N L C M L G N L S A A K | | | | | |
| | TAGAAATTAT CAACAACTTA TGTATGCTTG GCAATTATC CGCGGCAAAG | | | | | |
| BA71V | TAGAAATTAT CAACAACTTA TGTATGCTTG GCAATTATC C ... | | | | | |
| | E I I N N L C M L G N L S ... | | | | | |
| | 150 | 160 | | | | |
| E75 | Y K H G V T G ... | | | | | |
| | TACAAACACCG GAGTTACCGG CAT ... | | | | | |

Figure 4.12 Sequence comparison between the N-terminus of UB18 and the predicted translation product of the USB1 ORF encoded by ASFV. The nucleotide sequences of the USB1 ORF from the BA71V and E75 ASFV isolates are shown together with their predicted translation products. The N-terminal protein sequence of USB1 determined experimentally is shown in bold. The sequence of the putative promoter is shown in bold.

found with the N-terminus of the predicted translation product of an ASFV open reading frame situated in the conserved central region of the genome of the BA71V isolate (figure 4.12). This ORF had not been previously described and was only partially contained in the upstream region of the sequence determined for the otherwise unrelated ASFV C'204L gene (Prados *et al.*, 1993). An essentially similar partial USB1 ORF was also present in the genome of the E75 ASFV isolate (Alfonso *et al.*, 1992). However, a single G/C deletion in the E75 sequence of USB1 (corresponding to the initiation ATG in the BA71V sequence) indicated that the first E75 initiation methionine would correspond to Met⁴ in the Ba71V sequence. Consequently the predicted N-terminus of the polypeptide translated from the E75 USB1 ORF would not provide a complete match with the N-terminal sequence of USB1 determined experimentally.

The sequence of C'204L from the BA71V ASFV isolate published by Prados and co-workers included the first 108 bp of the 5' end of the USB1 ORF, the stop codon of which was presumably further downstream. The USB1 ORF was encoded on the complementary strand of the ASFV genome and was preceded by an A/T rich upstream sequence (including a TAAAAT motif 10 bp upstream of the initiation codon) which agreed with the typical structure of ASFV promoters. These observations suggested that the UB18 ubiquitin conjugate identified in ASFV virions was a ubiquitinated virus protein encoded by the USB1 ORF found in the genome of the BA71V ASFV isolate.

4.3 Discussion

The first step in the search for a substrate for UBCv was to compare the ubiquitin conjugates present in non-infected and ASFV infected cells. The anti-ubiquitin immunoblotting suggested that ASFV replication did not lead to the production of any visible novel ubiquitin conjugates. Some novel ubiquitin conjugates present in low amounts however might not have been detected because of the low sensitivity of the anti-ubiquitin antisérum. Furthermore the often short half-life inherent to ubiquitin conjugates might have contributed to the elusiveness of potential UBCv substrates targeted for degradation.

Although no novel ubiquitin conjugates were detected in ASFV infected cells, a significant increase in the overall concentration of cellular ubiquitin conjugates was observed early in ASFV replication. Such a phenomenon is unlikely to result from an inhibition of ubiquitin dependent proteolysis since it would then be expected that many previously degraded cellular ubiquitinated proteins would be detected following their stabilization. The observed increase in ubiquitin conjugate concentration is more likely to be attributed to a general stimulation of ubiquitin conjugation. This hypothesis is supported by the observation that the levels of cellular ubiquitin mRNA increase during early ASFV infection probably as a result of cell stress (Ferreira *et al.*, 1993) which is known to induce the expression of the genes for ubiquitin and several UBC enzymes (Jentsch *et al.*, 1991; Jentsch, 1992).

The anti-ubiquitin immunofluorescence analysis of ASFV infected cells showed that the subcellular localization of ubiquitin conjugates was dramatically altered during infection. The anti-ubiquitin immunoreactivity appeared highly concentrated in viral factories late during ASFV replication which indicated a possible association of ubiquitin conjugates with virions. This was confirmed by anti-ubiquitin immunoblotting of purified ASFV extracellular particles which suggested that free ubiquitin and several ubiquitin conjugates (UB18, UB38 and UB58) were present in virions. Furthermore, electron microscopy of ASFV particles probed with immunogold labelled anti-ubiquitin antiserum suggested that most ubiquitin conjugates were located inside the virus capsid, although some ubiquitin conjugates were also observed in the capsid. The position in the virion of these ubiquitin conjugates suggest that they may participate in the capsid structure or in the close interaction between the capsid and the virus lipid membranes.

The treatment of ASFV particles with detergent indicated that UB18 was readily extracted from virions, making it a prime candidate for the ubiquitin conjugates localized by electron microscopy in the capsid. The characteristics exhibited by UB18 are similar to those of the ASFV capsid protein VP72 and attachment protein VP12 which suggests that UB18 might also be involved in early infection events (e.g. virus entry or uncoating). The N-terminus of the UB18 ASFV structural protein was sequenced indicating that it probably consisted of a mono-ubiquitinated USB1 protein. The N-terminal 7 amino acid sequence of USB1 matched exactly the translation product of a previously partially sequenced ASFV ORF. The

complete sequence of this putative USB1 gene will have to be determined in order to compare the predicted molecular weight of its product to that observed for UB18. If one ubiquitin polypeptide of 8 kDa is conjugated to USB1 then the translation product of the USB1 gene is expected to have a Mw of approximately 10 kDa. Should the predicted molecular weight of the product of the USB1 ORF be greater than 10 kDa then it is likely that the observed UB18 structural protein is the result of proteolytic processing. The N-terminal proline amino acid of USB1 determined experimentally is in an identical position to that of the Mos protein kinase which was shown to be degraded in *Xenopus* eggs via the ubiquitin dependent pathway (Nishizawa *et al.*, 1992, 1993). It is therefore possible that USB1 is ubiquitinated by the N-end rule pathway although it is not clear how UB18 would then avoid being degraded to its amino acid constituents by the ubiquitin dependent protease.

The ASFV ubiquitinated structural proteins such as UB18 are novel ubiquitin conjugates detected in ASFV virions and are therefore potential substrates for the UBCv enzyme. However, to demonstrate that these ubiquitinated virus structural proteins are not substrates of host cell UBC enzymes, it will be necessary to show that these ubiquitin conjugates are not formed during infection with an ASFV mutant with an inactive UBCv enzyme. It will also be interesting to determine if USB1 can be ubiquitinated by UBCv *in vitro*.

Chapter 5

General discussion and future prospects

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Analysis of the nucleotide sequence of the ORF encoding the UBCv enzyme showed it had the characteristics of a functional ASFV gene and that it was probably not a recently acquired gene (§2.1). This view is supported by the detection of UBCv transcripts in ASFV infected cells (Rodriguez *et al.*, 1992b) and by the apparent presence of the UBCv gene in all the ASFV isolates tested. Furthermore, although the predicted protein sequence of UBCv has diverged considerably from all other known eukaryotic UBC enzymes (§2.2), the residues necessary for the enzyme's catalytic activity are present since , as demonstrated in §2.3.3, *E. coli* expressed UBCv enzyme is active *in vitro*. The presence in the predicted UBCv protein sequence of a C-terminal extension to the catalytic core, together with the ability of UBCv to ubiquitinate histones *in vitro*, suggests that UBCv recognizes its substrates without a trans-acting E3 enzyme. UBCv poly-ubiquitinated substrates *in vitro*, including UBCv itself and histones. This might indicate that its physiological substrates are targeted for ubiquitin-dependent degradation. However, the UBCv auto-ubiquitination property observed *in vitro* may be artefactual since auto-ubiquitination of UBCv was not detected in ASFV infected cells by Western blotting using anti-UBCv antiserum (§3.1.2). Results obtained *in vitro* may not reflect the *in vivo* enzyme activity and must be treated with caution. The potential proteins targeted by UBCv for degradation might include virus capsid proteins during uncoating, short lived virus regulatory proteins or cellular proteins which might include those involved in the host cell cytoprotective response. However, analysis of the total cellular ubiquitin conjugates present in ASFV infected cells did not detect novel ubiquitin conjugates generated during infection, although an increase in pre-existing ubiquitin

conjugates was observed (§4.1.1). This general increase in ubiquitin conjugation may result from stress induced activation of the cellular ubiquitin pathway. The failure to detect novel ubiquitin conjugates could be misleading since poly-ubiquitinated proteins, which are intrinsically unstable, often go undetected (Ciechanover *et al.*, 1991a). This obstacle might be overcome by adding, to ATP depleted ASFV infected cells, some non-hydrolysable ATP analogues which support ubiquitin conjugation but inhibit degradation of ubiquitin conjugates (Johnston and Cohen, 1991). The putative virus induced ubiquitinated proteins would then be stabilized and could then be detected by immunoblotting using anti-ubiquitin antiserum. This approach could be extended to *in vitro* assays, containing radiolabelled ubiquitin and recombinant UBCv, and using ASFV infected cell lysates as a source of potential substrates.

The UBCv enzyme is a persistent early cytosolic protein which is expressed throughout the replication of ASFV (§3.1 and §3.2). The presence of UBCv during early infection suggests that it may take part in early replication events, such as in the preparation of the virus genome for replication. By analogy to the proposed role of eukaryotic UBC enzymes in controlling the structure and replication of chromatin (possibly through ubiquitination of histones, Mori, M. *et al.*, 1993), it is conceivable that the modification by UBCv of viral DNA-associated proteins is a necessary step for the replication of the ASFV genome. Alternatively, UBCv might play a role in the formation of virus factories, possibly by participating in the alteration of the host cell cytoskeleton (Murti *et al.*, 1988b). However, the involvement of UBCv in these early events is unlikely since early ASFV replication seems to proceed normally in TS20 cells which are grown at the non-permissive temperature to inactivate the E1 enzyme (§3.3.2). An exact evaluation of the impairment of ASFV replication in these mutant cells could provide useful hints on the role of UBCv. Important data which could be obtained from ASFV infected TS20 cells would include the analysis of virus DNA synthesis, late ASFV gene expression (possibly using transfected reporter genes driven by a late promoter to detect the onset of late gene expression) and the release as well as the infectivity of progeny virus particles. Nonetheless, it must be noted that the impaired ASFV replication phenotype observed in the TS20 cells might result from inhibition of the cellular ubiquitin conjugation pathway rather than of UBCv specifically.

Since some persistent early ASFV proteins are known to participate in virus assembly (Santaren and Vinuela, 1986; Escribano and Tabares, 1987; Camacho and Vinuela, 1991; Prados *et al.*, 1993), it is possible that, rather than an early event, the UBCv enzyme is implicated in a late replication event such as virus morphogenesis. The ubiquitination of virus structural proteins by UBCv might be necessary for their integration into virions and may involve a chaperone-like role for ubiquitin. Alternatively, ubiquitination of virus DNA-associated proteins might allow packaging of the virus genome in virions. However, the UBCv enzyme would then be expected to be located in the virus factories where virus assembly takes place, rather than throughout the cytosol as shown experimentally (§3.2.1). A role for ubiquitination in virus assembly is also suggested by the findings that high concentrations of ubiquitin conjugates are located in virus factories (§4.1.2) and that several ASFV structural proteins in extracellular virus particles are ubiquitinated (§4.1.3). These proteins may be substrates of UBCv, although it cannot be ruled out that these ubiquitinations are carried out by cellular UBC enzymes. Finally, the presence of the UBCv enzyme in purified virus particles (§3.2.2) might indicate that UBCv has a role immediately upon virus entry (i.e. before translation of early virus proteins), such as in virus uncoating. This would imply that, as well as free ubiquitin which is found in virions (§4.1.3), an E1 enzyme would have to be packaged in virions. Furthermore, unless the ubiquitination by UBCv of the substrate protein is in itself sufficient to promote uncoating, an immediate early role for UBCv in virus uncoating would imply that virus particles also contain the ubiquitin-dependent 26S protease. This hypothesis could be tested by searching for ubiquitin conjugation activity in purified virus particles *in vitro*. If ubiquitin conjugation activity was detected in purified virions supplemented with ATP and labelled ubiquitin, this would demonstrate that an E1 enzyme is also packaged in virus particles. If structural protein degradation is observed upon addition of purified ubiquitin-dependent 26S protease to this virion *in vitro* system, this might strongly suggest that UBCv directed ubiquitination participates in virus uncoating.

The pattern of ubiquitinated ASFV structural proteins detected by immunoblotting in virus particles does not appear to correspond to poly-ubiquitinated forms of the same protein substrate, since ladders of conjugates differing in size by that of ubiquitin monomers (8 kDa) were not observed (§4.1.3). It is more likely that

several distinct structural proteins are ubiquitinated. Some of these ubiquitin conjugates are located close to the virus nucleo-protein core whereas others are located more externally in the virus capsid (§4.1.3). The N-terminal sequence of one such capsid ubiquitin conjugate (UB18) confirmed that it is a mono-ubiquitin conjugate and showed that it is a virus encoded protein. Little is known about the function of mono-ubiquitin conjugates which include histones, although they do not seem to be good substrates for ubiquitin-dependent degradation. Since ubiquitin is a substrate for certain cellular UBC enzymes, it is possible that the single ubiquitin tag of UB18 could act, upon virus entry, as a primer for the formation by cellular UBC enzymes of a poly-ubiquitin chain. This could lead to the degradation of UB18 and participate in virus uncoating, although, if this is the case, it is unclear how UB18 could escape degradation in the cytoplasm during virus assembly in the previous replication cycle. The ASFV gene encoding the ubiquitinated UB18 structural protein can now be sequenced and subcloned in a bacterial expression vector. The production of recombinant UB18 will allow its expression, location and post-translational modification to be followed in ASFV infected cells and virus particles using antibodies raised against recombinant UB18. In addition, recombinant UB18 could be used to determine whether it is a substrate for UBCv *in vitro*. A similar approach to that used to characterize UB18 might also be applicable to the other ubiquitin conjugates detected in ASFV virus particles.

The study of the phenotype of an ASFV mutant with an inactivated UBCv gene could provide valuable information about the function of UBCv. Although the generation of mutants is routinely carried out with vaccinia virus, the mutagenesis of ASFV is still in its infancy. The attempts to replace the UBCv gene in ASFV with a gene disrupted with the luciferase reporter were unsuccessful (§3.3.3). Since a positive control consisting of the luciferase reporter inserted in a known non-essential region of the ASFV genome was not available, this experiment did not conclusively demonstrate that the UBCv gene is essential for ASFV infection. It is possible that using a transfer vector with longer homologous flanking regions disrupted by a selectable reporter (such as neomycin resistance or guanine phosphoribosyltransferase) would increase the efficiency of both recombination and mutant isolation and enable the UBCv gene to be disrupted. However if the UBCv gene is essential for virus replication, it would not be possible to generate a null mutant and a conditional lethal

mutant would have to be generated. This could be achieved by placing the UBCv gene under control of an inducible promoter using the *lac* operon/*lac* repressor system used in vaccinia virus (Zhang *et al.*, 1992). Conditional expression is achieved by inserting the *lac* operator adjacent to the transcription start site of the targeted gene in a recombinant virus that constitutively expresses the *E. coli lac* repressor. The expression of the targeted gene is then dependent on the addition of IPTG. Since this strategy would require the generation of a double recombinant, a simpler and perhaps more flexible approach would consist of constructing an ASFV mutant with a gene encoding a thermosensitive UBCv enzyme. It has been shown that the substitution of a single conserved proline residue of UBC enzymes (corresponding to Pro⁶¹ in the sequence of UBCv) to a serine residue results in thermolabile UBC^{ts} enzymes (Ellison *et al.*, 1991). A UBCv^{ts} gene could be obtained by site directed mutagenesis of the cloned wild type UBCv gene and the mutated gene product activity could then be tested *in vitro* at various temperatures. If the engineered UBCv^{ts} enzyme was indeed thermolabile, the genomic UBCv gene could be replaced by the UBCv^{ts} gene by homologous recombination, thus allowing UBCv activity to be shut off at any stage of the ASFV replication cycle by a shift to the non-permissive temperature.

Alternatively, an ASFV UBCv null mutant could be generated by disrupting the UBCv gene by insertion with a reporter gene if the host cell constitutively expresses UBCv. A cell line expressing the UBCv gene under control of a eukaryotic promoter such as the SV40 promoter could be generated using standard techniques (Ausubel *et al.*, 1991b). The cell line expressing UBCv would complement the deficiency of the ASFV UBCv null mutant, thus enabling such a lethal mutation to be constructed. The phenotype of the null mutant could then be studied in a wild type cell line not expressing UBCv. In addition, the cell line expressing UBCv could be useful to determine whether UBCv interferes with any of the host cell's metabolic functions. This approach could also be applied to investigate the possible involvement of UBCv in disturbing the host immune response. Indeed the natural host cells for ASFV infection are principally monocytes and macrophages and the latter play an important part in antigen presentation to T and B cells. Since the ubiquitin system is involved in antigen processing, it is tempting to speculate that the ASFV encoded UBCv enzyme might affect this process and might therefore be a factor responsible for the peculiar host immune response to ASFV. Although it is not clear how UBCv

could disrupt the ubiquitin-dependent class I antigen processing, this hypothesis could be tested by expressing UBCv in macrophages or in tissue culture cells manipulated (as described by Michalek *et al.*, 1993) to be artificially class I antigen-presenting (APC). These UBCv expressing APC cells could then be evaluated for their ability to present antigens by measuring T cell stimulation.

The ultimate aim of this study was to determine the function of the UBCv enzyme in the life cycle of ASFV. Although it is clear that the precise role of the UBCv enzyme is still elusive, the results presented here have narrowed the range of potential UBCv functions as well as identified several potential substrates for the enzyme and provide a basis on which to design further experiments.

Chapter 6

Materials and methods

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6.1 Materials

6.1.1 Suppliers

Unless stated otherwise, all the chemicals and reagents were purchased from BDH Chemicals or from Sigma Chemical Company and were of analytical grade or of the highest grade available. The restriction endonucleases and other molecular biology enzymes were obtained from Boehringer Mannheim or from Promega and the reactions were carried out using the recommended buffers supplied by the manufacturer. The radioisotopes were from New England Nuclear (NEN, Dupont). The conjugated antisera were purchased from Dako (Dakopatts, Denmark) and from Amersham International. The phosphorothioate oligonucleotides were purchased from the Oswel DNA Service (Department of Chemistry, University of Edinburgh). The media for cell culture were purchased from Gibco BRL Life Technologies.

The following items were generously provided as gifts:

| | |
|---|---|
| <u>E. Vinuela</u> | Anti-VP72 monoclonal antibodies, clone 17L.D3, |
| Universidad Autonoma, Madrid, Spain. | ASFV BA71V isolate, plasmid clones containing BA71V DNA. |
| <u>Prof. R.J. Mayer</u> | TS20 and E36 cell lines, |
| Dept. Biochem., Nottingham, UK. | Iodinated ubiquitin. |
| <u>Dr. J.M. Hammond</u> | p72-Luc DNA construct. |
| IAH, Pirbright, UK. | |

6.1.2 Standard buffers

The compositions of standard buffers were as follow:

| | |
|---|--|
| Blocking solution | M9 minimal medium |
| 5% dried low-fat skimmed milk (Sainsbury's) in PBS-T | 22 mM glucose 90 mM Na ₂ HPO ₄ 8.5 mM NaCl |
| Buffer C | 22 mM KH ₂ PO ₄ |
| 120 mM NaCl | 18.7 mM NH ₄ Cl |
| 5 mM Tris-HCl pH 7.5 | |
| 2% NP40 | NETN 50 mM Tris-HCl pH7.5 |
| Gel destain | 150 mM NaCl |
| 7.5% acetic acid | 5 mM EDTA |
| 25% methanol | 0.05% NP40 |
| Gel stain | Orange G (10x) |
| 25% isopropanol | 0.25% orange G |
| 10% acetic acid | 30% glycerol |
| 0.05% Coomassie blue | |
| IPTN buffer | PBS (Phosphate buffered Saline) |
| 0.5 M NaCl | 136 mM NaCl |
| 20 mM Tris-HCl pH7.5 | 2.7 mM KCl |
| 2 mM EDTA pH8.0 | 10 mM Na ₂ HPO ₄ |
| 0.2% NP40 | 17 mM KH ₂ PO ₄ |
| | pH7.4 (adjusted with HCl) |
| LB (Luria-Bertani) medium | PBS-T |
| 1% tryptone | 0.1% Tween 20 in PBS |
| 0.5% yeast extract | |
| 172 mM NaCl | |

| | |
|--------------------------------|--------------------------------|
| PCR Buffer | TAE (50x) |
| 10 mM Tris-HCl pH8.3 | 2 M Tris-base |
| 50 mM potassium chloride | 4 M sodium acetate |
| 1.5 mM MgCl ₂ | 2 mM EDTA |
| 0.01% w/v gelatin | pH8.0 |
| | |
| SDS/PAGE running buffer | TBS |
| 25 mM Tris-base | 140 mM NaCl |
| 200 mM glycine | 20 mM Tris-HCl pH7.5 |
| 0.1% SDS | |
| | |
| Sample Buffer | TE |
| 2% SDS | 10 mM Tris-HCl pH7.5 |
| 20% glycerol | 1 mM EDTA |
| 0.01% bromophenol blue | pH8.0 |
| 62.5 mM Tris-HCl pH6.8 | |
| 100 mM DTT or 5% | TFB |
| βmercaptoethanol | 10 mM Mes |
| | 100 mM RbCl |
| | 45 mM MnCl ₂ |
| Solution I | 10 mM CaCl ₂ |
| 50 mM glucose | 3 mM hexaminecobaltic chloride |
| 25 mM Tris-HCl pH8 | 100 mM Tris-HCl, pH 8.5 |
| 10 mM EDTA | |
| | Transfer buffer |
| Solution II | 40 mM glycine |
| 200 mM NaOH | 48 mM Tris-base |
| 1% SDS | 0.1% SDS |
| | 20% methanol |
| | |
| Solution III | |
| 3 M potassium acetate | |
| 11.5% glacial acetic acid | |

Versene-Trypsin

136 mM NaCl

5.3 mM KCl

5.5 mM glucose

6.5 mM NaHCO₃

0.02% trypsin

0.01% versene (EDTA)

0.1% phenol red

6.1.3 Viruses

The Malawi LIL20/1 ASFV isolate was used in all pig monocyte and macrophage infection experiments. The Malawi LIL20/1 isolate was isolated from ticks collected in Malawi (Lilongwe, Central Region) following outbreaks in November 1983 (Haresnape *et al.*, 1988). The Malawi LIL20/1 ASFV isolate has been passaged twice in pigs, maintaining its high virulence characteristics, and was grown in primary pig monocytes cultures.

The Uganda-A ASFV isolate was used in all IBRS2 tissue culture infection experiments. The Uganda-A isolate was derived from the UGA59 ASFV field isolate (isolated in Uganda in 1959 from the spleen of an infected pig, Detray, DE, 1960) that was adapted to grow in IBRS2 tissue culture cells. The passage number of the Uganda-A isolate in IBRS2 cells is not known.

The BA71V ASFV isolate was used in all VERO tissue culture infection experiments. The BA71V isolate was derived from the BA71-5 field ASFV isolate (isolated in 1971 from the spleen of an infected pig in Badajoz, Spain) that was adapted to grow in VERO cells (Enjuanes *et al.*, 1976a; Ley *et al.*, 1984).

6.2 Methods

6.2.1 Nucleotide and amino acid sequence analysis

The analysis of the DNA and predicted protein sequences was carried out using the University of Wisconsin GCG software package (Genetics Computer Group, Inc.) (Devereux *et al.*, 1984). The PHYLLIP PHYlogeny Inference Package (version 3.5c, produced by Joe Felsenstein, University of Washington) was used to make the phylogenetic analysis of UBC enzymes.

6.2.2 DNA manipulations

All the DNA manipulations including polymerase chain reaction, restriction enzyme digestion, DNA ligation, plasmid transformation in *E. coli*, *E. coli* plasmid preparation and analysis on agarose gels were performed according to methods adapted from Sambrook *et al.*, 1989.

Polymerase chain reaction

The polymerase chain reactions (PCR) were performed on a thermal cycler (MJ Research Inc.) programmed for a 5 minutes denaturing step at 95°C, followed by 25 cycles consisting of a 2 minutes denaturing step at 92°C, a 2 minutes hybridization step at 50°C and a 3 minutes primer extension step at 72°C. The PCR reactions contained 50 ng of template DNA, 5 units of Taq polymerase (Boehringer), 200 µM of dNTP's and 100 pmoles of the oligonucleotide primers in a final volume of 100 µl PCR buffer. A 50 µl mineral oil overlay was added to prevent evaporation during the amplification process. A 5 µl aliquot of amplified DNA was analyzed by 0.6% agarose gel electrophoresis and visualised under short wavelength UV light. The remainder of the amplified DNA was purified with Magic PCR Purification kits (Promega) and resuspended in 50 µl TE.

Agarose gel electrophoresis

The DNA samples in 1x orange G were analyzed by electrophoresis in 0.6%-1% agarose gels containing 1xTAE buffer and $1 \mu\text{g.ml}^{-1}$ ethidium bromide. The DNA was visualised under UV light.

Extraction of DNA from low melting point (LMP) agarose gels

A 50 ml 0.8% LMP agarose gel containing $1 \mu\text{g.ml}^{-1}$ ethidium bromide was prepared in 1xTAE buffer and run for 2-3 hours at 80 Volts. The DNA band was visualised under long wavelength UV light and excised from the gel with a fresh scalpel blade. The DNA was extracted from the gel slice using the Geneclean kit (Bio101) and resuspended in 50 μl of TE.

Small scale plasmid purification (mini-prep)

A single bacterial colony was inoculated into 5 ml LB containing $50 \mu\text{g.ml}^{-1}$ ampicillin and cultured overnight at 37°C with vigorous shaking. 1.5 ml of culture was pelleted in a microfuge for 2 minutes and the supernatant discarded. The pellet was resuspended by vortexing in 100 μl solution I and held at room temperature for 5 minutes. 200 μl of solution II was added and mixed by inversion before incubating on ice for 5 minutes. 150 μl of solution III was added and the tube was vortexed gently to mix the contents. After a further 5 minutes on ice the mixture was pelleted in a microfuge for 5 minutes at 4°C. The supernatant was transferred to a fresh tube and extracted once with an equal volume of phenol, once with an equal volume of chloroform and once with an equal volume of ether. The DNA was precipitated by the addition of 2 volumes of ethanol, holding for 20 minutes at room temperature and pelleted for 5 minutes in a microfuge. The resulting pellet was resuspended in 50 μl of TE containing $10 \mu\text{g.ml}^{-1}$ RNase A and stored at -20°C until required.

Large scale plasmid purification (maxi-prep)

500 ml of LB medium containing $50 \mu\text{g.ml}^{-1}$ ampicillin was inoculated with a single colony of *E. coli* and cultured at 37°C overnight with vigorous shaking. The cells were pelleted at 6000 rpm for 20 minutes in a Sorvall RC-5B centrifuge (SS34 rotor), resuspended in 10 ml of solution I and held at room temperature for 5 minutes. 20 ml of solution II was added, the solution mixed and held on ice for 10 minutes, 15

ml solution III was then added and the mixture held on ice for a further 10 minutes. The mixture was pelleted at 13,000 rpm for 20 minutes at 4°C in a Sorvall RC-5B centrifuge (SS34 rotor), and the supernatant decanted. The DNA in the supernatant was precipitated by the addition of 0.6 volumes of isopropanol then incubated at room temperature for 20 minutes and pelleted at 10,000 rpm for 30 minutes at room temperature in a Sorvall RC-5B centrifuge (SS34 rotor). The DNA pellet was resuspended in 8 ml of TE containing 8 g CsCl and 8 mg ethidium bromide and was transferred to a quickseal ultracentrifuge tube (Beckman). The tube was filled with liquid paraffin, heat sealed and centrifuged overnight at 55,000 rpm at 20°C in a Beckman L8-90 ultracentrifuge (70.1 Ti rotor). The supercoiled plasmid DNA (the lower of 2 bands visualised under long wavelength UV light) was collected using a hypodermic needle and syringe. The ethidium bromide was removed from the solution by extraction with an equal volume of CsCl saturated isopropanol, and the DNA was precipitated by adding 3 volumes of ethanol and incubation at room temperature for 30 minutes. The DNA was pelleted in a microfuge for 5 minutes, resuspended in 500 µl of TE and the OD₂₆₀ nm was determined. The concentration was adjusted to 500 µg.ml⁻¹ with TE and the DNA was stored at -20°C until required. Alternatively the Magic Maxiprep columns (Promega) were used according to the manufacturer's instructions.

Preparation of competent *E. coli*

A single bacterial colony was seeded into 25 ml of LB medium and incubated at 37°C with agitation until the OD₆₀₀ nm of the culture was between 0.4 and 0.6. The bacteria were pelleted and resuspended in 2.5 ml of TFB and held on ice for 5 minutes. 100 µl of dimethylformamide (DMF) was added and the bacteria were incubated for a further 5 minutes on ice. 100 µl of 2.25 M DTT in 40 mM potassium acetate was then added and the cells were held on ice for a further 5 minutes. Finally, 100 µl DMF was added and the cells were held on ice until required (up to 8 hours).

Transformation of competent cells

200 µl of competent cells were mixed with the plasmid DNA and held on ice for 45 minutes. The mixture was then heat shocked at 42°C for 2 minutes and incubated at 37°C in 500 µl of LB for 1 hour. The transformation mix was then plated out onto

LB agar containing the necessary antibiotics and reagents required for plasmid selection.

Preparation of synthetic oligonucleotides

The oligonucleotides were synthesized by Dr. M. Ryan and Dr. P. Thomas (IAH, Pirbright) using an automated DNA synthesizer (Model 381A, Applied Biosystems). The oligonucleotides were removed from the support column by 3 washes of 300 µl each of undiluted ammonia solution. The washes were pooled and incubated at 56°C for 18 hours followed by rapid cooling on ice and rotary evaporation of the ammonia. The dried pellet was resuspended in 500 µl distilled water and the DNA concentration was adjusted to 10 pmol.µl⁻¹. The oligonucleotides were stored at -20°C until required.

6.2.3 Radioactive labelling of *E. coli* proteins

The TG1 *E. coli* strain (lac I^a) was used for all experiments. An *E. coli* colony harbouring plasmid PH1 or plasmid pkk233-2 was grown overnight in 5 ml of M9 minimal medium. The cells were then spun down and resuspended in 1 ml of M9 medium. After 1 hr incubation at 37°C, IPTG was added to a final concentration of 1 mM. The labelling of proteins was achieved by adding 100 µCi of [³⁵S]methionine to the cells, incubating for 5 minutes before pelleting and lysing the cells in 100 µl of sample buffer containing 100 mM DTT. The samples were boiled for 5 minutes prior to SDS/PAGE analysis.

6.2.4 Protein analysis by SDS/PAGE

Proteins in sample buffer were analyzed on 12.5%, 15%, 17.5%, 20% or gradient 7-20% resolving SDS-polyacrylamide gels, usually 0.75 mm or 1.5 mm thick 15 x 17 cm slab gels (Gibco BRL Life Technologies), with a 4% stacking gel, according to a method adapted from Laemmli, 1970. The gels were run with 1x running buffer at 20-70 mA at room temperature except for the analysis of ubiquitin thiolester bonds where gels were run at +4°C. The proteins separated by SDS/PAGE were stained with coomassie blue by soaking the gel in stain solution for 1 hour then in destain

solution overnight. The gels were silver stained with the Silver Stain Plus kit (Bio-Rad) or the Silver Stain kit (Stratagene) following the manufacturer's instructions. The gels were dried on Whatman 3MM paper on a heated (80°C) slab drier, or were dried at room temperature between two sheets of clear cellulose. The gels containing [³⁵S]methionine labelled proteins were fluorographed in 1 M sodium salicylate for 30 minutes before drying and autoradiography was carried out with Kodak intensifying screens on Fuji X-Ray film at -70°C.

6.2.5 Two-dimensional analysis of proteins

The isoelectric focusing (IEF) first dimension separation was carried out in 12 cm long tube gels (1.5 mm inside diameter). The ampholytes used were a mix (4:1 v/v ratio) of 40% ampholytes pH 5/7 (Sigma Chemicals) and 40% ampholytes pH 3/10 (Bio-Rad). The tube gels contained 3.325% acrylamide / 0.175% bisacrylamide / 9 M urea / 2% NP40 / 5% ampholytes / 0.1% ammonium persulfate (APS) / 0.075% TEMED and were polymerized overnight. The anode solution consisted of 15 mM orthophosphoric acid and the cathode solution was 50 mM sodium hydroxide. The protein samples (purified ASFV particles, 5 µg of protein per tube gel) were solubilized in 9 M urea / 100 mM DTT / 4% NP40 / 2% ampholites / 0.1% SDS and were loaded on tube gels pre-run at 175 V for 15 minutes, 200 V for 30 minutes and 400 V for 1 hour. The loaded tube gels were run at 700 V for 19 hours with external cooling of the buffers. The tube gels were then extracted, incubated in sample buffer (for a maximum of 5 minutes) and overlayed on a standard 12.5% SDS/PAGE slab gel with a single well. The second dimension was run as described in §6.2.4 (protein analysis by SDS/PAGE).

6.2.6 Purification of the ubiquitin conjugating (E1) enzyme from pig brain tissue

This procedure is derived from the methods described by Ciechanover *et al.*, 1982 and Hershko *et al.*, 1983. Half a piglet brain (30 g) was homogenized on ice for 1 minute at maximum speed in 150 ml (20% suspension) of 20 mM Tris-HCl / 1 mM DTT / 1% glycerol / 3 mM potassium phosphate buffer pH 7.1. The homogenate was

centrifuged at 30,000 g for 30 minutes at 4°C. A batch ion-exchange chromatography was achieved by adding to the supernatant 150 g of pre swollen DEAE cellulose (Whatman) previously equilibrated with 3 mM potassium phosphate / 1 mM DTT pH 7.0. After agitation for 30 minutes on ice, the slurry was centrifuged 5 min at 1800 rpm and the supernatant was decanted (fraction I). The pellet was washed three times with 300 ml of 3 mM potassium phosphate buffer / 20 mM KCl / 1 mM DTT pH 7.0. The bound proteins were eluted by adding 100 ml of 20 mM Tris-HCl / 1 mM DTT / 0.5 M KCL pH 7.2. The slurry was agitated 10 minutes on ice and centrifuged 10 minutes at 2200 rpm. The supernatant was dialysed overnight at +4°C against 8 litres of 20 mM Tris-HCl / 0.2 mM DTT / 100 mM MgCl₂ pH 7.4 to produce pig brain fraction II.

The fraction II was concentrated with spin concentrators (Centriprep-10, Amicon) to 6 ml and adjusted to 20 mM ATP. A ubiquitin affinity column was constructed by conjugating ubiquitin (Sigma Chemical Company) to a cross-linked agarose matrix (Sepharose-4B, Pharmacia LKB). Ubiquitin was conjugated (5 mg.ml⁻¹ gel) to the cyanogen bromide pre-activated sepharose gel according to the manufacturer's instructions. The concentrated fraction II was loaded on the ubiquitin affinity column equilibrated with 5 mM ATP / 10 mM MgCl₂ / 50 mM Tris-HCl / 0.2 mM DTT pH7.5. The loaded column was incubated for 5 minutes at room temperature and then washed with 6 ml of 50 mM Tris-HCl pH 7.5. The ubiquitin activating enzyme (E1) was specifically eluted with 6 ml of 50 mM Tris-HCl / 2 mM AMP / 0.04 mM NaPPi pH 7.5. The AMP/PPi E1 eluate was then concentrated 10 times with spin concentrators (Centriprep-30, Amicon) and stored in 50 µl aliquots at -70°C.

6.2.7 Preparation of recombinant UBCv for *in vitro* assays

An *E. coli* colony harbouring plasmid PH1 was grown in 1 litre of LB medium at 37°C with agitation to an optical density (OD₆₀₀) of 0.3. The UBCv expression was then induced by adding IPTG to a final concentration of 1 mM. The culture was incubated a further 90 minutes at 37°C with agitation. The cells were then pelleted and resuspended in 10 ml of 50 mM Tris-HCl pH 7.5 / 0.1 mM DTT / 1 mM EDTA. The cells were lysed by freeze/thawing three times and centrifuged at 20,000

rpm (Beckman SWTi) for 30 minutes at +4°C. A fraction II was prepared from the supernatant (as described for E1 in §6.2.6) by chromatography on a 20 ml DEAE cellulose column (100 ml wash followed by a 50 ml elution). The dialysed fraction II was concentrated 25 times with spin concentrators (Centriprep-10, Amicon) and used for UBCv activity assays.

6.2.8 UBCv thiolester assay

A 60 µl mixture containing 10 µl of *E. coli* fraction II (UBCv), 15 µl of affinity purified E1, 0.1 units of inorganic pyrophosphatase (Boehringer) and [¹²⁵I]ubiquitin (3.10⁶ cpm) in 5 mM DTT / 5 mM MgCl₂ / 10 mM ATP / 50 mM Tris-HCl pH 7.5 was incubated at 37°C for 15 minutes. The reaction was stopped by adding sample buffer. The products of the reaction were analyzed by SDS/PAGE with or without boiling with 2% β-mercaptoethanol for 3 minutes prior to loading. The E1 thiolester assay was carried out using the same conditions without adding UBCv.

6.2.9 Ubiquitin conjugation assays

The conjugation assays were carried out in the same way as the thiolester assays except that 50 µg of a substrate protein was added to some reactions and the incubation time was extended to 60 minutes. The reaction mixtures were boiled in 2% β-mercaptoethanol for 3 minutes prior to analysis by SDS/PAGE.

6.2.10 Synthesis of oligopeptides

The oligopeptides used for rabbit immunizations were synthesized by Mr R. Staple, Mrs J. Oxtoby and Dr T.R. Doel (IAH, Pirbright) using an automated Beckman System 990 Synthesizer. The dehydrated peptides were resolubilized in sterile double distilled water and stored at -20°C.

6.2.11 Purification of recombinant UBCv for rabbit immunizations

Recombinant UBCv was purified from a 1 litre culture of an *E. coli* colony containing plasmid PH2. The fusion protein purification protocol was performed essentially as described by Ausubel *et al.*, 1991a. Briefly, a 1 litre culture (LB medium / 50 µg.ml⁻¹ ampicillin) of bacteria containing plasmid PH2 in mid-exponential phase (0.3 OD₆₀₀) was induced with 0.1 mM IPTG and incubated for a further 3 hours at 37°C with vigorous agitation. The cells were then pelleted, the medium was discarded and the cells were resuspended in 10 ml of ice cold PBS. The cells were lysed by sonication and Triton X-100 was added to a final concentration of 1%. The lysate was thoroughly mixed and the cellular debris was pelleted by centrifugation at 10,000 g. The supernatant was decanted and mixed for 5 minutes with 1 ml of a 50% glutathione-agarose slurry (Promega). The agarose beads were then pelleted by centrifugation in a microfuge, the supernatant was discarded and the beads were resuspended in 50 ml of ice-cold PBS. The beads were washed again twice with 50 ml of ice-cold PBS. The beads were then washed twice in 10 ml of ice-cold 1% Triton X-100 in PBS and were washed twice in cleavage buffer (2.5 mM CaCl₂ / 50 mM Tris-HCl pH7.5 / 150 mM NaCl). Finally the beads were resuspended in 500 µl of cleavage buffer containing 50 µg of thrombin protease (Boehringer Mannheim). The slurry was incubated for 1 hour at room temperature before pelleting the beads in a microfuge. The supernatant was decanted (fraction 1) and the beads were washed four times with 500 µl of cleavage buffer (fractions 2-5). All the fractions (containing purified recombinant UBCv) were stored at -20°C until required.

6.2.12 Rabbit immunizations

The inoculations of rabbits were carried out by Mr L. Pullen (IAH, Pirbright). For the raising of anti-peptide serum, the rabbits first received (day 1) 150 µg of peptide in Freund's complete adjuvant, by both intramuscular (IM) and subcutaneous (SC) injections. The rabbits then received two 150 µg IM and IC boost injections in Freund's incomplete adjuvant at 21 and 42 days. The rabbits were bled on day 63 and the clotted blood was centrifuged at 5000 x g before aliquoting the serum supernatant

at -20°C. The raising of antisera against recombinant UBCv in rabbits was carried in the same way, using doses of either 15 µg (LD8) or 100 µg (LD9) of purified UBCv protein.

6.2.13 Affinity purification of anti-peptide antisera

The anti-peptide rabbit antisera were purified by affinity chromatography. The preparation of the affinity peptide-sepharose columns and the purification of antibodies were carried out using the ProtOn Kit 1 (Multiple Peptide Systems) following the manufacturer's instructions.

6.2.14 Purification of pig monocytes and macrophages

The monocytes and macrophages were isolated from pig peripheral white blood cells by virtue of their adherence to tissue culture plates. Normal pig blood was collected in heparin (10 i.u./ml), dispensed into 25 ml aliquots and stored on ice for 5 minutes before being centrifuged at 1800 rpm for 10 minutes at 4°C in an IEC Centra-7R centrifuge (IEC 210 rotor). The buffy coat was removed with a 5 ml pipette and mixed with 5 ml of ice cold Eagles medium. Two buffy coat suspensions were then carefully layered onto 15 ml of Lympho-Paque (Nycomed Pharmaceuticals) in a 50 ml Falcon tube and centrifuged at 1800 rpm for 30 minutes at 4°C (as above). The interface white blood cell fraction was collected with a 10 ml pipette and washed twice in 20 ml of ice cold Eagles by resuspending the cells and centrifuging at 1800 rpm for 5 minutes at 4°C (as above). The remaining erythrocytes were then flash lysed by briefly resuspended cells in 10 ml of sterile distilled water and rapidly adding 10 ml of 2 x Eagles. Finally, the leucocytes were resuspended to a concentration of 10^7 cells.ml⁻¹ in ice cold Eagles supplemented with 10% porcine serum. The suspension was dispensed in 5 ml aliquots into 6 cm diameter petri dishes and incubated at 37°C overnight in a 5% CO₂ incubator. The medium was then decanted and non-adherent cells washed off with warm Eagles plus 10% porcine serum. The media was replenished and the remaining cell population were incubated as above until required. All media contained antibiotics (30 µg.ml⁻¹ benzyl penicillin and 1 µg.ml⁻¹ streptomycin) as well as a fungicide (2.5 µg.ml⁻¹ amphotericin B).

6.2.15 Cultivation of tissue culture cells

The IBRS2 and VERO tissue culture cells were grown as monolayers in Dulbeccos modified Eagles medium (DMEM) supplemented with 10% foetal calf serum in a humidified atmosphere containing 5% CO₂ and maintained at 37°C. The TS20 and E36 tissue culture cells were grown as monolayers in alpha-MEM medium supplemented with 10% foetal calf serum in a humidified atmosphere containing 10% CO₂ and maintained at 32°C. All the media contained antibiotics (30 µg.ml⁻¹ benzyl penicillin and 1 µg.ml⁻¹ streptomycin) as well as a fungicide (2.5 µg.ml⁻¹ amphotericin B).

Confluent cell sheets were sub-cultivated by decanting the medium, washing the cells with Ca²⁺/Mg²⁺ free PBS and adding 0.05 ml.cm⁻² of versene/trypsin. After 5-10 minutes incubation the cells became detached and were resuspended in the appropriate medium. The IBRS2 and VERO cells were routinely split 1:5, whereas TS20 and E36 cells were split between 1:10 and 1:15. All cells were cultivated in plastic dishes or flasks (Falcon).

6.2.16 Time courses of ASFV infection

The 6 cm diameter petri dishes containing the target cells were washed with warm medium and incubated for one hour at 37°C (32°C or 40.5°C with TS20 and E36 cells) in 1 ml of serum free medium containing ASFV at a multiplicity of infection (MOI) of 10 to insure synchronized infection. With pig monocytes and macrophages, the infection was carried out with the ASFV isolate Malawi LIL20/1, whereas the Uganda-A isolate was used with IBRS2, TS20 and E36 cells and the BA71V isolate was used with VERO tissue culture cells. The dishes were then washed with warm PBS and incubated with 5 ml of medium supplemented with 10% serum until the desired time point was reached. The dishes were then washed with warm medium before lysing the cells with 300 µl of buffer C. The cell lysates in buffer C were stored at -20°C until required. The frozen cells lysates were then rapidly thawed and spun down before aliquots were mixed in sample buffer for SDS/PAGE analysis.

6.2.17 Immunoprecipitation analysis

Cells were infected as described (§6.2.16) and were pulse labelled with [³⁵S]methionine at various times post-infection. One hour before collection, the pig monocyte and macrophage dishes were washed with warm methionine free Eagles and incubated for one hour at 37°C in the presence of 100 µCi of [³⁵S]methionine in 1 ml of methionine free Eagles. The cells were then washed with warm methionine free Eagles before being lysed in 300 µl of buffer C. The cell lysates were pelleted in a microfuge for 5 minutes and the supernatant stored at -70°C until required.

The immunoprecipitation was carried out by adding 10 µl of antiserum and 500 µl of IPTN buffer to 100 µl of the labelled cell extract. The contents were mixed by inversion and incubated at room temperature for one hour before gently mixing with 20 µl of NETN washed *Staphylococcus aureus* pansorbin. The mixture was incubated for a further 30 minutes at room temperature and then centrifuged 5 minutes. The supernatant was discarded and the pellet was washed successively in 500 µl of NETN and 500 µl of TBS. Finally, the pellet was resuspended in sample buffer and analyzed by SDS/PAGE and fluorography.

6.2.18 Immunoblotting analysis

The proteins to be analyzed by immunoblotting (blotting of proteins for N-terminal sequencing is described in §6.2.28) were first separated by SDS/PAGE (§6.2.4) and were then transferred by Western blotting to a nitrocellulose membrane (Hybond C-Super, 0.45 µm pores, Amersham). The transfer was carried out on a Bio-Rad semi-dry transblotter using Transfer buffer under a 0.8 mA.cm⁻² current for 1 to 1.5 hours. The blot was then washed in PBS, air dried and stored at 4°C until required. When the blot was to be probed with anti-ubiquitin antiserum, the pre-wetted membrane was briefly autoclaved before use to enhance detection sensitivity (Swerdlow *et al.*, 1986).

The protein blots were stained with gold using the Proto-Gold kit (BioCell Research Laboratories) according to the manufacturer's instructions. Immunodetection was carried out by first incubating the blots in Blocking solution (§6.1.2) for 1 hour at 37°C with gentle agitation, followed by incubation in the same conditions with primary antiserum diluted in fresh blocking solution. The dilutions of primary antisera

were 1/200 for anti-UBCv serum (LD9) and 1:25 to 1:50 for anti-ubiquitin serum (Sigma or Dako). The blots were washed 3 times in PBS-T and incubated as above with secondary antiserum (HRP-conjugated anti-rabbit IgG, Dako) diluted 1:2000 in blocking solution. The blot was then thoroughly washed 5 times in PBS-T. The detection of bound HRP-conjugated antibodies was carried out using enhanced chemiluminescence (ECL kit, Amersham) according to the manufacturer's instructions.

6.2.19 Immunofluorescence of ASFV infected cells

The tissue culture cells were grown to near confluence on glass (IBRS2 and VERO) or plastic (TS20 and E36) chamber slides (Gibco-BRL Life Technologies). Mock or ASFV infected cells were washed in PBS and fixed in ice cold methanol/acetone (1/1 v/v) for 5 minutes. The fixed slides were air dried and preferably used immediately for immunofluorescence, or were stored shortly at +4°C until required.

The slides were rehydrated in PBS for 15 minutes and incubated with foetal calf serum (FCS) for 15 minutes at 37°C. The slides were then incubated for one hour at 37°C with primary antiserum diluted in PBS-T plus 10% FCS. Primary antiserum dilutions were 1:50 for anti-UBCv (LD9), 1:20 for anti-ubiquitin (Sigma), 1:10 for anti-DNA (Boehringer). The slides were then washed 3 times in PBS-T and incubated for 30 minutes at 37°C with secondary antiserum at a 1:40 dilution in PBS-T plus 10% FCS. The secondary antisera were either FITC-conjugated (Dako or Boehringer) or TR-conjugated (Texas Red, Amersham). For double staining, the primary antiserum was composed of a mixture of rabbit anti-ubiquitin and mouse anti-VP72 (1:50 dilution of 17L.D3 monoclonal supernatant) and the secondary antiserum was a mixture of FITC-antirabbit and TR-antimouse antisera. Finally, the slides were washed 5 times for 5 minutes in PBS-T and a coverslip was mounted with 10% PBS, 2% DABCO (anti FITC fading agent) in glycerol. The slides were observed with a Vickers incident fluorescence microscope at a 40x10 magnification. The photography was carried out with Fujichrome 400 ASA film with 15 seconds to 2 minutes exposures.

6.2.20 Immunogold labelling and electron microscopy

IBRS2 cells infected with ASFV were fixed for 1 hour at 37°C in 2% formaldehyde / 0.5% glutaraldehyde (Bio-Rad) / 1% sucrose / 2 mM CaCl₂ in 0.1 M sodium cacodylate buffer pH7.3. The cells were scraped off the dishes, pelleted in a microfuge and washed 3 times in 0.1 M cacodylate buffer pH7.3 / 3% sucrose / 2 mM CaCl₂. The cells were stored in the wash buffer at 4°C. All the subsequent manipulations were carried out by Dr T. Self (Nottingham University). The fixed cells were embedded in araldite, thin sliced, probed with gold labelled anti-ubiquitin antibodies and observed by electron microscopy (Philips EM410).

6.2.21 Purification of extracellular ASFV particles

The culture adapted ASFV Uganda-A isolate was seeded (at a multiplicity of infection of approximately 0.01) on confluent IBRS2 cells in 850 cm² roller bottles (Falcon). The cells were incubated at 37°C until 100% cytopathic effect (CPE) was observed (usually three to four days). All subsequent operations were performed at 4°C. The cell debris were pelleted by centrifugation at 2000 rpm for 30 minutes in a Mistral 6L centrifuge (rotor No.59563). The pellet was discarded and the supernatant centrifuged for a further 60 minutes at 18,000 rpm in a Beckman L8-M ultracentrifuge (Type 19R rotor). The virus pellet was resuspended in 50 ml of PBS and Percoll (Pharmacia) was added to a final concentration of 45% (w/v). The Percoll gradient was centrifuged for 30 minutes at 20,000 rpm in a Beckman L8-M ultracentrifuge (Type 42.1 rotor). The lower band containing the virus was collected and its Percoll concentration re-adjusted to 45%. The second gradient was respun as above and the virus band collected. The refraction index of this band was 1.343, corresponding to 53% Percoll indicating a density of approximately 1.09 g.ml⁻¹. This preparation was diluted in PBS and spun at 30,000 rpm for 30 minutes (as above). Percoll forms a solid pellet, whereas the virus forms a loose pellet which can be collected separately. The virus thus collected was resuspended in PBS and the last operation was repeated. Finally, the virus was resuspended in PBS and stored at -70°C. The protein concentration in the purified virus preparation was approximately 1 mg.ml⁻¹ (Protein Assay kit, Bio-Rad).

6.2.22 Liquid phase preparative isoelectric focusing (IEF)

Preparative IEF of purified virus was carried out using the Rotofor system (Bio-Rad) essentially as recommended by the manufacturer. Briefly, 1.4 ml of purified virus was dissolved by 15 seconds sonication (Soniprobe model 7530A, Dawe Instruments) in 10 ml of IEF running solution (8 M urea, 10 mM DTT, 2% NP40, 2% ampholites (Biolyte pH 3-10, Bio-Rad)). The Rotofor cell was pre-equilibrated twice with 55 ml of distilled water for 5 minutes, followed by 1.5 hours pre-focusing with 40 ml of IEF running solution at 12 W (550 V, 22 mA). The 10 ml of solubilized virus was loaded in the Rotofor cell central port and focusing was achieved in 1 hour 40 minutes at 12 W. The final voltage stabilized at 1045 V. The 20 fractions of 3 ml each were harvested and their pH measured with pH papers (Whatman). 400 μ l of 5 M NaCl was added to each fraction before dialysis in Visking tubing (24 Å pores) against 5 litres of PBS at 4°C. The buffer was changed at 1, 20, 23 and 26 hours. The PBS buffer was then exchanged for 20% polyethylene glycol (PEG 6000, Kochlight) in PBS and the Rotofor fractions were dialysed for a further 16 hours. Finally, 300 μ l of sample buffer was added to the concentrated 600 μ l fractions before storing at -20°C.

6.2.23 ASFV infections in the presence of antisense oligonucleotides

VERO cells were grown to near confluence in 6 well plates (Falcon) and incubated overnight in DMEM supplemented with 1% foetal calf serum (FCS) and containing 25 μ M of the antisense phosphorothioate oligonucleotides. The media was then decanted and the cells were incubated with the BA71V ASFV isolate (MOI of 1) in DMEM for 1 hour. The infectious media was then decanted and replaced with DMEM / 1% FCS / 25 μ M antisense oligonucleotides. The cells were incubated for a further 4, 8 or 24 hours before removing the media and lysing the cells with sample buffer. The cell lysates were harvested and stored at -20°C until required.

6.2.24 Generation of ASFV mutants by homologous double recombination

IBRS2 or VERO cells were grown to near confluence in tissue culture inserts (0.45 µm pores, Falcon). The medium was decanted and the cells were incubated for 1 hour with the Uganda-A (for IBRS2 cells) or BA71V (for VERO cells) ASFV isolates (at an MOI of 10). The infectious medium was then decanted and the cells were washed twice with warm DMEM. The cells were then transfected by lipofection (Lipofectin, Gibco BRL Life Technologies) with the transfer vector DNA (plasmid PH3 DNA purified with Magic Maxipreps, Promega). For each tissue culture insert, 2.5 µg of PH3 plasmid DNA in 50 µl of sterile water was mixed with 50 µl of diluted Lipofectin (10 µl Lipofectin added to 40 µl of sterile water). The DNA/Lipofectin mix was incubated at room temperature for 15 minutes before being added to the infected cells covered with 200 µl of DMEM. The tissue culture inserts were then incubated at 37°C for 4-5 hours before removing the media and replacing it with DMEM supplemented with 1% foetal calf serum. The infected/transfected cells were then incubated at 37°C until over 75% CPE was observed (usually 2 to 3 days). The medium was then collected, the cellular debris was pelleted and the virus in the supernatant containing potential ASFV recombinants was used (at a 1:10 dilution in DMEM) to infect fresh tissue culture cells. The cells infected in the second round were then tested for luciferase activity as described in §6.2.25 (below).

6.2.25 Detection of luciferase activity in ASFV plaques

Cells in tissue culture inserts were incubated for 1 hour with progeny ASFV collected from cells transfected with the PH3 transfer vector. The infectious medium was then decanted and replaced with an agar overlay (DMEM / 1% low melting point agarose / 1% foetal calf serum). The overlayed infected cells were then incubated for 24-48 hours at 37°C before 1 ml of developer medium was added to each insert. The developer medium was composed of DMEM supplemented with 3.5 mM KCl / 60 mM NaCl / 2.6 mM MgSO₄ / 1 µM nigericin / 0.6 mM ATP / 40 mM DTT / 5 µM beetle luciferin (Promega) and adjusted to pH7.5 with sodium bicarbonate. The tissue culture inserts were then immediately laid in a dark room on 400 ASA black and

white film (Tmax, Kodak). The film was exposed to the tissue culture inserts for 30 minutes and was then developed with HC110 (Kodak) and fixed with Hypam (Ilford).

Essentially the same procedure was used to detect luciferase activity in ASFV infected cells transiently expressing the reporter gene (i.e. in ASFV infected cells transfected with the PH3 transfer vector).

The Luciferase Assay System (Promega) was used according to the manufacturer's instructions to measure the luciferase activity in ASFV infected cells. Briefly, infected cells were washed twice in warm PBS and lysed with a minimal volume of ice cold lysis buffer (1% Triton X-100 / 10% glycerol / 25 mM Tris-phosphate pH7.8 / 2 mM DTT / 2 mM 1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid). The cellular debris was pelleted in a microfuge and for each assay, 20 μ l of the supernatant was mixed with 100 μ l of 530 μ M ATP / 470 μ M luciferin / 270 μ M coenzyme A / 33.3 mM DTT / 0.1 mM EDTA / 2.67 mM MgSO₄ / 1 mM (MgCO₃)Mg(OH)₂ · 5H₂O / 20 mM tricine. The photoemission of the reaction mixture was immediately measured in counts per minute (cpm) in the [³H] window of a scintillation counter (Model 1209 Rackbeta, LKB Wallac).

6.2.26 Affinity chromatography of ASFV ubiquitinated structural proteins

The anti-ubiquitin affinity column was constructed by conjugating 2.8 mg of anti-ubiquitin antibodies (purified immunoglobulin fraction, Dako) through disulfide bonds to a 1 ml cross-linked agarose gel (Sulfolink, Pierce). The anti-ubiquitin antibodies were partially reduced in 3 ml of 0.1 M phosphate buffer pH6 containing 50 mM β -mercaptoethylamine and 5 mM EDTA. The reducing reaction was allowed to proceed for 2 hours at 37°C before the reaction mixture was applied on a desalting column (P6 matrix, Biorad) equilibrated with 50 mM tris pH8.8 / 5 mM EDTA. Using this procedure, only the two hinge disulfide bonds of the antibody molecules are reduced (Pierce). Approximately 250 μ g of reduced immunoglobulins were collected from the column effluent and were mixed with 2 ml of Sulfolink slurry (1 ml of gel). The conjugation of the antibodies to the Sulfolink gel was performed according to the manufacturer's instructions. Approximately 70 μ g of anti-ubiquitin antibodies were linked to the gel (binding efficiency of 30%). The gel was then packed in a plastic

column and washed with 20 ml of PBS.

The affinity purification of ubiquitinated ASFV structural proteins was carried out with 500 µg of Percoll purified ASFV particles. The structural proteins were solubilized by sonicating ASFV particles in a PBS buffer containing 1 M NaCl / 2% NP40. The solubilized proteins were then repeatedly concentrated in spin concentrators with 3 kDa molecular weight cut-off membranes (Centricon-3, Amicon) and diluted in PBS until the concentration of NaCl was lowered to 140 mM. The ASFV structural proteins (in 0.5 ml of PBS) were then loaded on the anti-ubiquitin affinity column equilibrated with PBS. The optical absorbance at $\lambda=280$ nm (OD_{280}) of the effluent was monitored on-line and 0.5 ml fractions were collected. The flow rate was maintained at $0.05\text{ ml} \cdot \text{min}^{-1}$ with a peristaltic pump until all the protein sample was loaded. The flow rate was then set at $0.5\text{ ml} \cdot \text{min}^{-1}$ and the column was washed with PBS until the OD_{280} returned to baseline. The column was then washed with 0.1 M NaCl in PBS until the OD_{280} returned to baseline, followed by 2 M NaCl in PBS. The effluent fractions corresponding to the various OD_{280} peaks were then concentrated in spin concentrators (Centricon-3, Amicon) and rediluted in sample buffer ready for analysis by SDS/PAGE.

6.2.27 Detergent extraction of ASFV structural proteins

Percoll purified ASFV particles ($250\text{ }\mu\text{g.ml}^{-1}$ of protein) were incubated with 0.1% to 5% *n*-octyl- β -D-glucopyranoside (OG) in PBS for 2 hours at 4°C. The detergent treated samples were then carefully layered onto a 25% sucrose (in PBS) cushion and centrifuged at 20,000 rpm in a Beckman SW50.1 rotor for 15 minutes. The supernatants, free of ASFV particles, were then concentrated in spin concentrators (Centricon-3, Amicon) and rediluted in sample buffer ready for SDS/PAGE analysis.

6.2.28 Purification of proteins for N-terminal sequencing

Proteins in sample buffer were loaded on 17.5% SDS/PAGE 1.5 mm thick slab gels that had been allowed to polymerize overnight. The gels were run overnight at a constant voltage of 75 V and the cathode buffer contained 0.1 mM thioglycolate. The gels were then transferred using a tank electroblotter (Transphor TE series, Hoefer

Scientific Instruments) to a PVDF membrane (Immobilon P, Millipore). The transfer buffer was composed of 10 mM CAPS pH11 in 10% methanol, and the transfer was carried out overnight at a constant voltage of 25 V with external cooling of the buffer. Once the transfer was completed, the membrane was washed in double distilled water and the blotted proteins were stained by incubating the PVDF membrane in 0.025% coomassie blue in 40% methanol / 0.1% acetic acid for 1-5 minutes. The membrane was then destained in 50% methanol until the protein bands were clearly visible. The stained bands corresponding to the proteins of interest were then carefully cut out and submitted to N-terminal sequencing.

6.2.29 N-terminal sequencing of ASFV structural proteins

The N-terminal sequencing (Edman degradation) of ASFV structural proteins blotted to PVDF membranes was carried out by Drs J. Kytes and K. Bailey (Nottingham University) using an automated sequencer (Model 473A, Applied Biosystems).

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