Mechanism of Action of A238L: An Immune Evasion Protein of African Swine Fever Virus

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Abstract

African swine fever virus is a large double-stranded DNA virus which causes an acute haemorrhagic fever in domestic swine, but is apathogenic in its natural hosts, the warthog and the bushpig, in which the virus persists for long periods. The virus can also persist in, and be transmitted by, soft ticks of the genus Ornithodoros which can incubate the virus for several years and transmit the virus transovarially. Previous studies on the virus have identified a number of viral genes encoding proteins involved in host-cell interactions and immune evasion. One of these proteins, A238L, has been shown to inhibit the NF-κB and calcineurin pathways, two key pathways involved in regulating the immune response. These studies have shown that A238L protein inhibits NF-κB-dependent reporter gene expression, is able to prevent the p65 subunit of NF-κB binding to DNA and that A238L protein co-immunoprecipitates with the p65 protein. The aims of this study were to further investigate the mechanism by which A238L inhibits the NF-κB pathway.

This study shows that p65 is present in the nucleus following ASFV infection, and that the 32 kDa form of A238L accumulates in the nucleus at late times post infection. A238L is unable to move to the nucleus in p65 -/- cell lines, suggesting that A238L binds to NF-κB in the cytoplasm and shuttles to the nucleus in complex with NF-κB. A proportion of A238L remains in the cytoplasm and this is mainly the 28 kDa form of the protein; the cytoplasmic pool of A238L remains when nuclear export is inhibited by leptomycin B suggesting that this cytoplasmic pool is not exported from the nucleus. A negative effect of A238L protein on the rate of cell proliferation was also observed. This effect was similar in cells transfected with a mutant form of A238L which is unable to bind to calcineurin. This suggests that the cause for this effect is via NF-κB inhibition.

In summary, this study provides evidence for a model in which the A238L protein acts to inhibit NF-κB in the nucleus, rather than by sequestering the complex in the cytoplasm.
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1 Introduction

1.1 African swine fever virus introduction

1.1.1 African swine fever disease and pathology

African swine fever (ASF) was first described in East Africa by Montgomery in 1921 (Montgomery, 1921). The symptoms of ASF vary depending on the strain of the virus. Virulent isolates can cause up to 100% mortality. However, ASF disease can be categorised into the following categories: peracute, acute, subacute and chronic (Hess, 1981, Wardley et al., 1983). In the peracute form of the disease, animals die before any symptoms are seen, although haemorrhaging may be visible upon post mortem. The acute form is characterised by a high fever, followed by loss of appetite and the appearance of reddened or cyanotic areas and bloody diarrhoea. Death usually occurs in seven to ten days (Hess, 1981, Wardley et al., 1983).

Subacute infections can vary in appearance and mortality depending on a number of factors such as the age and breed of the infected animal (Hess, 1981, Wardley et al., 1983). Temperature and other symptoms may vary over a period of up to 20 days; some animals display few symptoms, and abortion is sometimes the only consequence of infection (McDaniel, 1978).

Chronic infections can also vary in their presentation. The virus persists for several months and this may cause a range of conditions including pneumonia, arthritis and skin ulcers (Wardley et al., 1983) with hypergammaglobulinaemia a common consequence (Pan et al., 1970). Glomerulonephritis and immune-
complex deposition are also observed (Martin-Fernandez et al., 1991). Post mortem may reveal haemorrhagic lesions (Wardley et al., 1983).

Infection with the ASFV causes a range of pathological changes at a number of sites within infected animals. A pathological study of the livers of infected animals showed no change one day post infection, but a marked deterioration of liver structure by seven days post-infection with a significant amount of cell debris, infected cells, activation of endothelial cells and extensive intravascular coagulation (Gomez-Villamandos et al., 1995b) observed. Disseminated intravascular coagulation was also observed (Villeda et al., 1993).

The lymph nodes of infected animals show haemorrhagic lesions within three days post infection (Carrasco et al., 1997) and high levels of apoptosis are observed, particularly in the T-cell areas of the lymph node. This correlates with a lymphopaenia in the blood due to a reduction in the T lymphocyte population (Carrasco et al., 1996). Haemorrhaging, microthrombi and phagocyte activation are also observed in the renal capillaries (Gomez-Villamandos et al., 1995a) and glomerulonephritis is seen in both acute and subacute forms of the disease (Hervas et al., 1996). Enlargement of the spleen, oedema in the lung with fluid in the thoracic cavity are also observed (Mebus, 1988, Wilkinson, 1989).

Interestingly, the virus also causes changes in platelets, with moderate virulence isolates producing less pronounced changes than infection with highly virulent strains which cause platelet activation and degranulation (Gomez-Villamandos et
al., 1996). This phenomenon is also observed in Ebola virus infection (Fisher-Hoch et al., 1985).

Some of the pathology observed in ASFV infection, may be due to the effect of the immune response on the host. ASFV infects monocytes and macrophages, although lymphocytes are not infected; the infection of these cell types has implications for pathogenesis (Childerstone et al., 1998; Ramiro-Ibanez et al., 1996). Infection has been shown to induce tumour necrosis factor alpha (TNF-α) production (Gomez del Moral et al., 1999). TNF-α is detected in areas where there is viral replication as well as in serum at the onset of symptoms (Gomez del Moral et al., 1999). TNF-α may be responsible for a range of immunopathologies including the disseminated intravascular coagulation, haemorrhage and shock seen in ASF disease (Vassalli, 1992).

The role of TNF-α in haemorrhagic disease is likely to be significant as increased TNF-α levels are seen in the haemorrhagic fevers caused by Junin and Pichinde, with levels of TNF-α correlating with disease severity (Aronson et al., 1995, Heller et al., 1992). High levels of TNF-α and IL-1α are produced by pulmonary intravascular macrophages which show a significant activation following infection with ASFV (Carrasco et al., 2002).

1.1.2 Natural history of the virus

The natural hosts of ASFV are the warthog, *Phacochoerus aethiopicus*, and the bushpig, *Potamochoerus porcus*, in which it causes asymptomatic, persistent infections. The virus can also persist in soft ticks of the genus *Ornithodoros*,
which often live in warthog burrows. The species *O. moubata* is the species found in East, Central and Southern Africa, whereas *O. erraticus* are present on the Iberian Peninsula and in West Africa.

As the warthog and bushpig do not exhibit clinical symptoms, the disease was first noticed following the introduction of domestic pigs to Africa (Montgomery, 1921). Warthog young are born free of the virus and can be infected by bites of infected ticks. No virus could be found in uteri or foetuses of infected sows (Plowright et al., 1969), nor in mammary tissues of lactating animals (Plowright, 1981).

Although clinical signs are not observed in infected bushpigs, there is a low level of virus replication. This results in lymphocyte apoptosis in the lymph nodes and the spleen (Oura et al., 1998).

Virus infection of the tick host, *O. moubata*, was shown not to affect egg-hatching and nymphal feeding rates, but a significant increase in mortality was observed when adult ticks were fed a bloodmeal containing virus (Rennie et al., 2000).

It is interesting to note however, that in some areas of Kenya and Uganda a high proportion of warthogs were found to be infected with ASFV, although *O. moubata* could not be found in their burrows (Pierce, 1974) and in Western Uganda, few ticks were found to be infected (Plowright, 1977, Thomson, 1985).
This suggests that other mechanisms of transmission between warthogs may be important in some areas.

Although new-born warthogs may carry some maternal antibody to ASFV, this does not protect against infection as, in one study, nearly all warthogs were infected by the age of three months (Plowright et al., 1969).

African swine fever was initially a disease of sub-Saharan Africa, but the virus spread to other areas of Africa and to the Iberian Peninsula where disease was endemic for more than 30 years. Infection is thought to have spread from Iberia to other European countries as well as to South America and the Caribbean but the disease has been eradicated from all of these countries apart from Sardinia (Laddomada et al., 1994) (Bech-Nielsen et al., 1995), (Biront et al., 1987) and Malta (Wilkinson et al., 1981), (Tury et al., 1973), (Mebus & Dardiri, 1979, Mebus et al., 1978). ASF affected areas are shown in figure 1.1.

The increase in endemic disease may be due to the attenuation of some virus strains leading to an increased number of carrier animals, rather than a high mortality disease, which was limited in its spread (Thomson, 1985, Wardley et al., 1983).

1.1.3 Virus classification

African swine fever virus was originally classified in its own genus in the family *Iridoviridae* on the basis of its icosahedral morphology and cytoplasmic location (Salas, 1999), but was removed from this family in 1984 (Brown, 1986). The
virus was then placed on its own in a separate genus, African swine fever-like viruses (Dixon et al., 2000). Despite similarities in genome structure and replication cycle to the Poxviridae, the difference in morphology to these viruses led to ASFV being placed as the sole member of a new virus family, the Asfarviridae (Dixon et al., 2000).
Figure 1.1 Geographic distribution of African swine fever virus.
1.1.4 Virus transmission

It is unlikely that the virus is transmitted vertically in warthog populations; a more complicated transmission cycle involving *Ornithodoros* ticks is suggested to maintain the virus in this population (shown in Figure 1.2).

Despite a subclinical infection, ASFV can replicate to high titres in infected warthogs, particularly in the lymph nodes and spleen, and produce viraemia for around a month following infection (Thomson et al., 1980). This viraemic phase may be sufficient to infect ticks that feed on infected animals.

The virus can be transmitted between ticks transovarially (Rennie et al., 2001), transstadially (Endris & Hess, 1992) and sexually (Plowright et al., 1974). A number of experiments have attempted to elucidate the role of other tick species in maintaining and transmitting ASFV. *Ornithodoros savignyi*, another African tick species, is able to transmit the virus transstadially, maintain the virus and infect domestic pigs (Mellor & Wilkinson, 1985). A number of American tick species have been tested for the ability to maintain and transmit ASFV. The blood-feeding *Triatoma gerstaekeri* was found to be able to carry the virus for 41 days, but not transmit it to susceptible animals, while the *Ornithodoros* species *O. coriaceus* and *O. turicata* were found to be able to maintain the virus and transmit it to pigs (Groocock et al., 1980, Hess et al., 1987). Interestingly, the
Figure 1.2 A diagram showing the African swine fever virus transmission cycle. Courtesy Miss Rebecca Nix, Institute for Animal Health, Pirbright.
Ornithodoros puertoricensis Fox species has been found in sites in Haiti and the Dominican Republic (Butler et al., 1985) where ASFV had previously been endemic. This species has been shown to be infected experimentally and to be capable of transmitting the virus transstadially and transovarially, however none of the tick colonies collected from the field were infected with ASFV (Endris et al., 1992, Hess et al., 1987).

1.1.5 The virus particle
The ASFV virion consists of a nucleoprotein core structure of around 70 nm – 100 nm diameter contained within an internal membrane and icosahedral capsid structure of 170 nm -190 nm (Breese & DeBoer, 1966, Carrascosa et al., 1984, Dixon et al., 2000, Salas, 1999). This structure is enveloped by a further lipid membrane to form the extracellular enveloped virus with a diameter of 175 nm-215 nm (Dixon et al., 2000, Salas, 1999). A diagram of the virus particle is shown in figure 1.3.

1.1.7 The viral genome
The genome of African swine fever virus is comprised of a single molecule of linear double-stranded DNA, with a genome size of 170 kbp to 190 kbp depending on the isolate (Dixon et al., 2000, Salas, 1999). The genome is covalently closed at each end by a hairpin loop (Ortin et al., 1979) and contains a terminal inverted repeat (Sogo et al., 1984), both features similar to poxvirus genome structure (Dixon et al., 2000, Geshelin & Berns, 1974, Salas, 1999). The genome structure is shown in figure 1.4.
Figure 1.3 A diagram of the African swine fever virus particle
Figure 1.4 The genome structure of the BA71V strain of African swine fever virus
Taken from Salas, 1999.
The tissue culture adapted strain of the virus, BA71V, has been completely sequenced (Yanez et al., 1995). Analysis of the sequence revealed 151 open reading frames, with a number having identifiable homology to known proteins. About two thirds of the genome of the virulent Malawi field strain has also been sequenced (Dixon et al., 1994, Yozawa et al., 1994). A map of the ASFV genome is shown in figure 1.5.

1.1.7 Viral proteins

Analysis of purified virions has identified 54 proteins which are present in the virus particle (Escribano & Tabares, 1987, Salas, 1999, Tabares et al., 1983). Since the virus replicates in the cytoplasm of infected cells, the virus encodes its own enzymes for RNA and DNA synthesis, and enzymes for RNA synthesis and modification are packaged into the virus particle for use immediately following virus entry into cells (Dixon et al., 2000).

Bioinformatics analysis identified ASFV proteins with similarity to cellular proteins or protein domains from which a function can be suggested (Dixon et al., 2000, Salas, 1999, Yanez et al., 1995). The virus encodes proteins involved in DNA and RNA synthesis and modification: a DNA polymerase α-like protein (Rodriguez et al., 1993b), a DNA polymerase pol X family protein (Garcia-Escudero et al., 2003, Oliveros et al., 1997, Showalter et al., 2001), a DNA ligase (Hammond et al., 1992, Showalter et al., 2001), several RNA polymerase subunits (Yanez et al., 1993a), as well as helicase and topoisomerase family members (Baylis et al., 1992, Baylis et al., 1993, Sussman et al., 1993, Yanez et al., 1993b); proteins involved in post-translational modifications: a ubiquitin-
Figure 1.5 A map of the African swine fever genome. Multigene families are shown in mid-blue and dark blue, structural proteins in red, modifying enzymes in pink, proteins involved in host-interactions and immune evasion are shown in black, proteins involved in replication and nucleotide metabolism are in light blue and genes encoding other proteins are in orange. From Yanez et al., 1995.
conjugating enzyme (Hingamp et al., 1995, Webb et al., 1999) and a serine protein kinase; and a number of proteins involved in immune evasion: a Bcl-2 homologue (Brun et al., 1996, Gangappa et al., 2002, Revilla et al., 1997), an inhibitor of apoptosis (IAP) homologue (Nogal et al., 2001, Rodriguez et al., 2002) and a homologue of IκB (Neilan et al., 1997, Powell et al., 1996). In addition, the virus encodes many proteins without significant homology to other proteins. Unusually for a virus, five different multigene families (MGF) are encoded and these represent about 20% of the total coding information. The function of these is unclear although members of MGFs have been suggested to have a role in macrophage or tick tropism (Yozawa et al., 1994).

Four classes of ASFV proteins have been identified: early proteins which cease to be produced once DNA synthesis has begun, early proteins which are produced for the entire replication cycle, intermediate proteins, and late proteins which are expressed only after DNA synthesis has begun (Rodriguez et al., 1996, Salas, 1999). Synthesis of late proteins can be prevented by treating infected cells with the DNA synthesis inhibitor cytosine arabinoside.

A number of ASFV structural proteins are processed by proteolytic cleavage of precursor polyproteins. The polyprotein pp220 is cleaved at sites with a gly-gly-X motif to form the p150, p37, p34 and p14 proteins (Simon-Mateo et al., 1993). The polyprotein pp62 is cleaved, again at the gly-gly-X consensus sequence to produce a 35 kDa (p35) and a 15 kDa (p15) protein (Simon-Mateo et al., 1997). Some virus proteins are post-translationally modified. These include
the aforementioned pp220 and the virion structural proteins p28 and p13 which are myristoylated (Aguado et al., 1991). A number of viral proteins are phosphorylated, including the proteins p17 and p35, and the major immunogen p32 (Alonso et al., 1997, Prados et al., 1993, Salas, 1999, Salas et al., 1988b).

Three virion proteins, p5, p18 and p58, cross-reacted with anti-ubiquitin sera (Hingamp et al., 1995). The p5 and p18 proteins were later suggested to be monodimeric forms of an unusual lipid-modified form of ubiquitin. Similar proteins are present in membranes of other virus particles including baculovirus (Guarino et al., 1995, Webb et al., 1999).

At least three minor structural glycoproteins were originally detected in ASFV particles (Tabares et al., 1983); however, subsequent work has identified only the CD2v protein as a virion glycoprotein (Ruiz-Gonzalez et al., 1996). ASFV infection of Vero cells was found to induce 19 glycosylated proteins, at least five of which were thought to be virus-encoded (del Val & Vinuela, 1987).

### 1.1.8 Viral replication

A diagram showing the general overview of the ASFV replication cycle is shown in figure 1.6. Specifics of replication are discussed in the following sections.

#### 1.1.8.1 Receptor binding

The host range for ASFV is highly restricted, with only swine and soft ticks becoming infected. Host cell tropism is also restricted and in mammalian hosts
Figure 1.6 The replication cycle of African swine fever virus. Virus particles may lose their outer membrane as they bind to the host cell. Following attachment to the cellular receptor, virus particles are internalised by endocytosis. Fusion of the virus membrane with the endosome releases the virus core into the cytoplasm. Virus genes are transcribed and translated; early genes, intermediate genes, then late genes. Virus particles then assemble at the endoplasmic reticulum and traffic to the plasma membrane where they are released by budding. Virus particles can also be released following cell lysis.
replication of virus occurs only in cells of the monocyte/macrophage lineage (Maurer et al., 1958), and in a small proportion of polymorphonuclear phagocytes (Casal et al., 1984).

Entry of ASFV into Vero cells is mediated by saturable binding sites, of which there are about $10^4$ per cell (Alcami et al., 1989b). The cellular receptor is probably an unglycosylated, lipid-free protein as treatment with enzymes to remove these groups, and prevent the formation of new proteins with these modifications, did not abolish binding of the ASFV attachment protein p12 to cells (Galindo et al., 1997).

Some viruses are able to infect certain cell types, despite being coated by antibody, by using the Fc receptor to mediate entry. In some cases, such as dengue virus infection, secondary infections can cause greater illness due to this antibody-dependent enhancement of infectivity (reviewed in (Porterfield, 1986). As African swine fever virus is still infectious in the presence of high titres of virus-specific antibody, it was possible that virus entry was via the Fc dependent mechanism described. However, ASFV entry into macrophages was shown not to occur via the Fc receptor as virus infection was not inhibited by saturating Fc receptors with antibody, but was inhibited by saturating specific virus receptors with UV inactivated virus (Alcami & Vinuela, 1991).

The p12 protein was identified as a virus attachment protein and shown to bind to sensitive Vero cells, but not to ASFV resistant cell types. This binding was competed out by ASFV particles. Purified p12 protein can inhibit ASFV binding
and infectivity (Angulo et al., 1993). Electron microscopy localized the p12 protein to the outer membrane of the virus particle, external to the capsid structure (Carrascosa et al., 1993).

The attachment of ASFV to cells is most likely a multi-stage process with a number of proteins involved. The ASFV proteins p54 and p30 have been shown to be involved in attachment as purified proteins inhibit ASFV binding in a dose dependent manner and antibodies to these proteins prevent virus attachment (Gomez-Puertas et al., 1998).

The cellular receptor(s) to which these proteins bind is/are unknown, although the CD163 protein was suggested as a possible receptor, since its expression on macrophages correlates with susceptibility to infection and recombinant CD163 binds to virus particles (Sanchez-Torres et al., 2003).

1.1.8.2 Entry

Entry of enveloped viruses into cells requires a fusion event between the virus envelope and a cellular membrane. This fusion event can either take place at the plasma membrane of the cell or with an internal membrane, such as an endosome.

ASFV entry was shown to occur by receptor-mediated endocytosis, which was temperature and energy dependent (Alcamí et al., 1989a, Geraldes & Valdeira, 1985, Valdeira & Geraldes, 1985). Following endocytosis of the particle, the virus envelope fuses with the endosome delivering the virus core particle to the
cytoplasm of the host cell (Valdeira et al., 1998). Studies using a number of chemical inhibitors showed that binding and entry of ASFV was not dependent on the microtubule or actin network, or lysosomal function, but there was a requirement for oxidative phosphorylation (Valdeira et al., 1998). Protease inhibitors prevented viral replication early in infection suggesting that proteolytic cleavage is a necessary step in initiating infection (Valdeira et al., 1998).

Following entry of the virus particle into the cytoplasm, the virus moves to a site next to the nucleus; most likely along microtubules as accumulation next to the nucleus is inhibited by colchicines (Carvalho et al., 1988). This may be mediated by the interaction of the p54 protein of ASFV with the LC8 light chain of the (-) end directed microtubule motor protein dynein (Alonso et al., 2001). An intact microtubule network is required for formation of the perinuclear virus factory (Alonso et al., 2001). However, the p54 protein is a virion membrane protein and may therefore be lost from the particle following fusion of virion membranes with the endosome membrane. Possibly the p54 LC8 interaction is required to recruit membranes to sites of virus assembly and retain immature virus particles in the virus factories (Rodriguez et al. 2004, Alonso et al., 2001). The p54 protein has also been shown to induce apoptosis: possibly by displacing the pro-apoptotic Bim protein from microtubules (Hernaez et al., 2004). This may contribute to the early induction of apoptosis in infected cells which has been shown not to require virus replication (Carrascosa et al., 2002).
1.1.8.3 Transcription and translation

Due to the fact that the virus replicates in the cytoplasm, the DNA dependent RNA polymerase is present in the virus particle along with proteins capable of capping and polyadenylating mRNA (Salas et al., 1981).

Little is known about the molecular regulation of transcription and the promoter/transcription factor interactions involved to initiate transcription, but it is known that gene transcription is tightly regulated and transcription begins only a short distance upstream of the translational start site (Almazan et al., 1992, Rodriguez et al., 1996).

Early genes are expressed immediately following entry of the genome into the host cell, and all the required factors are contained in the virion. The majority of early genes are expressed throughout infection, although expression of some early genes ceases when DNA synthesis begins (Carvalho & Rodrigues-Pousada, 1986, Escribano & Tabares, 1987, Santaren & Vinuela, 1986).

At least two genes have temporal expression characteristics similar to the intermediate genes seen in poxviruses. The I226R gene produces intermediate and late mRNAs, and the I243L gene early, intermediate and late mRNAs, with different transcriptional start sites (Rodriguez et al., 1996).

Late gene expression requires the onset of DNA synthesis and expression of late proteins does not occur when infected cells are treated with the DNA synthesis inhibitor cytosine arabinoside. The promoter region of the late gene p72, the
major capsid protein, has been studied and it was found that the promoter
function was contained within a 41 bp region from -36 nt to +5 nt of the
transcriptional start site (Garcia-Escudero & Vinuela, 2000). The region
contained a TATA box, which was also found in a number of other late
promoters studied (Garcia-Escudero & Vinuela, 2000).

1.1.8.4 DNA replication

Although the replication of African swine fever virus is predominantly
cytoplasmic, there is a role for the nucleus in viral replication; suggested by the
fact that the virus does replicate in enucleated Vero cells, with viral DNA
synthesis inhibited by more than 95% (Ortin & Vinuela, 1977). As virus
replication is not inhibited by the host cell RNA polymerase II inhibitor, α-
amanitin (Salas et al., 1988a), the viral DNA does not require transcription by
host RNA polymerase II in the nucleus.

Garcia-Beato et al. (Garcia-Beato et al., 1992) showed that ASFV DNA synthesis
occurs as a two step process with the primary phase in the nucleus, and the
secondary phase in the cytoplasm. This contrasts with poxviruses which replicate
entirely in the cytoplasm of the infected cell (Moyer, 1987).

The DNA species present in the nucleus are short fragments, with an average
length of approximately 2 kbp, which are localized close to the nuclear
membrane (Rojo et al., 1999). The DNA species found in the cytoplasm are
larger, approximately 25-33% of the genome size, and have been demonstrated
to be precursors to mature DNA by pulse-chase experiments (Rojo et al., 1999).

Rojo et al. (1999) suggest that the evidence does not point to a self-priming mechanism for ASFV DNA replication, as the short DNA species identified do not fit with this hypothesis and radiolabelled full length genomes are not detected from the onset of DNA synthesis. They instead favour a model of DNA synthesis similar to that put forward by Baroudy et al. for Vaccinia virus genome replication (Baroudy et al., 1982). In this model DNA synthesis would begin at both ends with continuous replication without the formation of Okazaki fragments (Costa, 1990). The failure to identify an RNA primase coded by ASFV suggests a possible reason for the requirement of a nuclear stage in replication in order to utilise cellular primase enzymes. The short DNA fragments produced in the nucleus could be used to prime DNA synthesis in the cytoplasm. Replication by this method would produce a dimeric concatemer, with head to head and tail to tail forms, as have been found in ASFV infected cells (Gonzalez et al., 1986). These could be resolved by nicking and ligating within the terminal inverted repeat region.
### 1.1.8.5 Morphogenesis and egress

Assembly of ASFV takes place in a specialized site, adjacent to the nucleus, known as the virus factory. The virus factory is seen from approximately 8 hours post infection (hpi), but increases significantly in size between 12 and 18 hpi (Brookes et al., 1996). Morphologically, the virus factories resemble aggresomes, sites in the cell responsible for the sequestration of misfolded proteins (Heath et al., 2001). A number of characteristics are shared, such as the recruitment of mitochondria, the perinuclear location around the microtubule organising centre, and the envelopment of the structures by a cage of vimentin, an intermediate filament protein (Carvalho et al., 1988, Heath et al., 2001).

Transmission electron microscopy initially revealed that the virus acquired its two inner membranes concurrently with capsid assembly (Arzuza et al., 1992). The virus assembles from membranous structures in the factory which are shaped to form the inner membranes of the virus by the assembly of the vp72 capsid protein on their 'outer' surface (Andres et al., 1997, Cobbold & Wileman, 1998). These membranous structures were shown to derive from the endoplasmic reticulum (ER) (Andres et al., 1998, Cobbold et al., 1996, Rouiller et al., 1998).

The proteins j5R and j13L (the homologue from the Malawi LIL20/1 isolate of p54), which contain putative transmembrane domains, are detected on these structures and may play a role in assembly (Brookes et al., 1998b). The p54 protein has recently been shown to be involved in recruitment of the ER membrane structures to the virus factory (Rodriguez et al., 2004). Depression of p54 expression causes virus assembly to be halted before the recruitment of precursor membranes to sites of assembly (Rodriguez et al., 2004).
Energy for virus morphogenesis is provided by mitochondria which traffic to the virus factory via microtubules and which appear, morphologically and biochemically, to be actively respiring (Rojo et al., 1998). Capsid assembly and membrane wrapping are inhibited when cells are depleted of ATP or calcium (Cobbold et al., 2000).

The core structure of the particle is assembled from the major structural proteins p150, p37, p34, and p14, products of the pp220 polyprotein, and the products of the pp62 polyprotein: p35 and p15. Processing of pp62 requires pp220 and the processing of both polyproteins requires the expression of p72 (Andres et al., 2002a). Inhibition of pp220 expression leads to the formation of icosahedral particles lacking any core structures (Andres et al., 2002b). Further to this, non-myristoylated pp220 mutants do not associate with membranes, as the myristoylated form does, when transfected into cells (Andres et al., 2002b). This finding suggests that the myristoyl moiety may function to attach developing core structures to the inner viral membrane during assembly.

Viral DNA condenses and is inserted into empty particles at a single vertex. This is followed by capsid closure which leads to intermediate particles. Further changes in the nucleoprotein core produce the mature virion (Brookes et al., 1998a).

An interesting feature of ASFV infection is the loss of the trans Golgi network from infected cells (McCrossan et al., 2001). This observation correlates with
ASFV infection causing a significant slow-down transport of cathepsin D to lysosomes, showing that transport through the secretory pathway is inhibited by ASFV infection (McCrossan et al., 2001).

Transport of virus from the factory to the periphery of the cell is along microtubules via an interaction with the (+) end directed motor protein kinesin (Jouvenet et al., 2004). The virus protein E120R is required for this process (Andres et al., 2001).

1.1.9 Immune response to ASFV

A key factor in the pathogenesis of ASFV is the fact that the primary replicative targets of the virus are mononuclear phagocytes which are lysed during infection (Martins et al., 1987-1988, Wardley et al., 1979, Wardley & Wilkinson, 1978). Later studies have shown that infected macrophages become apoptotic at a late stage of infection and this is a likely mechanism for infected cell death. The main target of viral replication is the macrophage, a cell type with an important role in immune response (Casal et al., 1984, Colgrove, 1968, Heuschele et al., 1966).

Pigs which recover from infection with strains of ASFV of lower pathogenicity have a long-lived immunity to challenge with a homologous virus strain.

Early contradictory reports concerning antibody neutralisation of virus have now been resolved and the targets for neutralisation identified. Although early reports suggested that effective neutralizing antibodies were not produced during ASFV infection (Wardley et al., 1987, Wardley et al., 1985), more recent studies have
clearly demonstrated that neutralizing antibodies can play an important role in protection. Tissue culture adaptation of ASFV isolates may be associated with loss of virus neutralization determinants (Zsak et al., 1993), perhaps dependent on the phospholipid composition of the virus particles (Gomez-Puertas et al., 1997). The latter observation, in combination with the employment of high-passage viruses may explain past difficulties in reproducibly demonstrating ASFV neutralizing antibodies. Targets for virus neutralization have been identified as p72, p54 and p30 (Zsak et al., 1993, Gomez-Puertas et al., 1996). Anti-p72 and anti-p54 antibodies inhibit attachment to cells, while anti-p30 antibodies inhibit internalization (Gomez-Puertas et al., 1996). Antibody could also play a role via antibody dependent cellular cytolysis (ADCC) as it has been shown that neutrophil-mediated ADCC reduces virus yield in vitro (Norley & Wardley, 1983).

Some infected pigs develop non-specific hypergammaglobulinaemia. This immune response is insufficient to clear virus in the majority of infected pigs (Pan et al., 1970). It has been shown that ASFV is a potent B cell mitogen, by causing the secretion of factors from infected macrophages, which act to stimulate B cell proliferation (Takamatsu et al., 1999). This B cell mitogenic activity, coupled with loss of appropriate T cell signals to B cells, may be responsible for the observed hypergamma-globulinaemia.

A key observation stressing a role for antibodies in protection against ASFV was the demonstration of protection of pigs against lethal infection through passive transfer of anti-ASFV serum (Onisk et al., 1994). Antibodies may act to limit
infection by neutralisation. Binding of antibodies to infected cells may also
induce complement-mediated lysis of these cells, thus limiting replication. Anti-
ASFV antibody will bind to infected cells from 8 hpi, with antibody
concentrations being sufficient for complement-mediated lysis from 11-12 hpi
(Norley & Wardley, 1982). Further experiments suggested that complement
activation was likely to be via the classical pathway (Norley & Wardley, 1982).

Infection of PBMCs with ASFV in culture has shown a significant inhibition of
mitogen-dependent proliferation. Deletion of the viral homologue of the cellular
CD2 protein prevented this inhibition (Borca et al., 1998).

1.2 Viral immune evasion

Infection of a cell by a virus can cause the activation of a number of host-cell
pathways at a number of different stages. Receptor binding, membrane fusion
events, transcription and genome replication can all trigger signal transduction
cascades, potentially leading to the induction of an antiviral state, both in the
infected cells and in neighbouring cells. It is important therefore, that viruses
disrupt these pathways for a sufficient length of time initially to complete
replication of progeny virions in infected cells and subsequently to suppress an
effective immune response which would lead to virus clearance.

1.2.1 Viral mechanisms to evade host defence

Viruses, from the smallest RNA viruses to the largest DNA viruses, have evolved
a number of mechanisms to modulate the immune response to infection. For
example, Poliovirus, a small single-stranded RNA virus, inhibits TNF-mediated
apoptosis by preventing TNF receptor expression at the cell surface (Neznanov et al., 2001). Larger RNA viruses can also subvert immune responses. Ebola Virus has mechanisms for suppressing expression of MHC class I molecules, 2', 5'-oligoadenylate synthetase, interleukin-6, protein kinase R, interferon regulatory factor-1 and ICAM-1 (Harcourt et al., 1998, Harcourt et al., 1999).

The larger DNA viruses possess a large complement of mechanisms to evade the immune response; this is unsurprising given their large coding capacity. The herpesviruses have evolved ways to evade the immune system at a number of key points. Preventing presentation of antigen via MHC class I molecules is an effective way to hide from immune surveillance and so a large number of viruses attack the MHC class I presentation pathway. HHV-7, for example, prevents expression of MHC class I molecules on the cell surface by diverting the molecules to lysosomes (Hudson et al., 2001). Varicella-Zoster virus downregulates surface MHC class I expression by retaining the molecules in the Golgi apparatus (Abendroth et al., 2001).

Many viruses encode homologues of cellular genes which following their ‘hijacking’ from the host may have been mutated to alter their function. There are examples of poxviruses and herpesviruses which express immunosuppressive cytokines to calm the immune response or which have ‘stolen’ a cytokine receptor gene and modified it so as to make it soluble and able to bind free cytokines before they can act (Bridgeman et al., 2001, Saederup & Mocarski, 2002, Smith et al., 1999, Symons et al., 2002).
Viral proteins also act at other levels of signal transduction pathways. HHV-8 encodes four open reading frames which have homology to cellular interferon regulatory factor proteins: viral (v) IRF-1 (Burysek et al., 1999a), vIRF-2 (Burysek et al., 1999b) and vIRF-3 (Lubyova & Pitha, 2000). These vIRFs have been found to down-regulate genes under the control of interferon responsive promoters. It has been shown that vIRF-2 is transcribed during latency and therefore may play a role in the establishment of latency (Burysek & Pitha, 2001). The M3 protein of murine Herpesvirus-68 is a secreted chemokine receptor which is abundantly produced and capable of binding a number of chemokines before they can bind cellular receptors (Parry et al., 2000, van Berkel et al., 1999). The poxvirus Molluscum contagiosum virus inhibits another key pathway in the antiviral response, the NF-κB pathway (Gil et al., 2001).

1.2.2 African swine fever virus host defence evasion mechanisms

ASFV encodes several genes involved in modulating the immune response (Dixon et al., 2004). The A224L gene of ASFV encodes an IAP (inhibitor of apoptosis) homologue (Chacon et al., 1995). The IAPs are cellular proteins involved in the inhibition of apoptosis and are highly conserved through evolution. The human c-IAPs 1 and 2 have been shown to be associated with TNFR-II in conjunction with TRAF-1 and TRAF-2 (Rothe et al., 1995). It has been shown that c-IAP-1 and c-IAP-2 bind to the effector caspases, caspase-3 and caspase-7, but not to the intermediary caspases -8, -1 or -6 (Roy et al., 1997). Deveraux and co-workers further showed that c-IAP-1 and c-IAP-2 could inhibit the proteolysis of procaspase -3, -6 and -7 by inhibiting caspase-9 activation by
cytochrome c (Deveraux et al., 1998). A brief summary of ASFV immune evasion mechanisms can be seen in figure 1.7.

The A224L protein acts by interacting with the catalytic fragment of caspase-3 (Nogal et al., 2001). It has also been shown that A224L protein can activate the NF-κB pathway by an IKK-dependent mechanism (Rodriguez et al., 2002). Activation of NF-κB causes expression of cellular anti-apoptotic genes which can further suppress apoptotic signals.

The c-type lectin encoded by the EP153R gene has recently been shown to inhibit apoptosis (Hurtado et al., 2004). Infections with virus in which this gene has been deleted show an increased induction of caspase-3 and increased transactivation of genes by p53 compared to infections with parental virus (Hurtado et al., 2004). The p53 protein had previously been reported to be transactivationally active and present in the nucleus of infected cells (Granja et al., 2004).

ASFV also encodes a homologue of the anti-apoptotic gene Bcl-2, A179L. This gene, expressed early and late in infection, acts as a functional homologue of Bcl-2 inhibiting apoptosis by the mitochondria-dependent pathway. The Bcl-2 homologue is 96.6% identical between the Malawi isolate and the BA71V tissue culture adapted strain and contains two highly conserved domains BH1 and BH2 (Revilla et al., 1997). The BH1 and BH2 domains are common to Bcl-2 family
Figure 1.7 Immune evasion mechanisms of African swine fever virus. The figure shows a number of ways in which ASFV acts to modulate the immune response. The dual function of the A238L protein is shown (1): the inhibition of NF-κB activation and NFAT activation, which occurs via the inhibition of calcineurin phosphatase. Inhibition of lymphocyte proliferation (3) and T and B cell apoptosis (6) are observed following ASFV infection. This inhibition of proliferation is not seen following deletion of the viral CD2 homologue. The CD2v protein is also responsible for the haemadsorption (2) seen around infected cells and may be involved in signalling (5). The virus also encodes proteins involved in the inhibition of apoptosis. The adhesion of virus particles to red blood cells (4), aids dissemination of virus in the host. Courtesy Dr. Linda Dixon, IAH Pirbright.
proteins and are responsible for forming homodimers with the pro-apoptotic protein Bax (Oltvai & Korsmeyer, 1994, Williams & Smith, 1993). The ability of Bcl-2 to sequester Bax may provide the anti-apoptotic effect, although the mechanism is still being characterised (Mao et al, 2002).

ASFV also encodes a protein with similarity to the T-cell adhesion receptor CD2 which mediates the haemagglutination around infected cells (Borca et al., 1998, Rodriguez et al., 1993a, Ruiz-Gonzalvo & Coll, 1993). Deletion of this gene has been shown to affect viraemia and onset of symptoms (Borca et al., 1998). Borca and co-workers showed that a deletion of the CD2 gene reduces virus titres 100-1000 fold in lymphoid tissue and delayed viraemia by two to five days. In viraemia following ASFV infection, the majority of virus is associated with the red blood cell fraction. In the case of the CD2 deletion virus, virus was found to be equally distributed between the red blood cell, leukocyte and plasma fractions (Borca et al., 1998). The CD2 gene has also been shown to be important in inhibiting lymphocyte proliferation. Mitogen dependent lymphocyte proliferation was reduced by 90-95% in infected peripheral blood mononuclear cell cultures, whereas infection with CD2v knockout virus did not cause inhibition of proliferation (Borca et al., 1998).

Non-purified recombinant CD2 protein has been used to experimentally vaccinate pigs and has produced some resistance to challenge (Onisk et al., 1994). Viral CD2 has also been produced using a recombinant baculovirus and purified for use as a vaccine (Ruiz-Gonzalvo et al., 1996). When used to immunise pigs, specific antibodies could be detected against the viral CD2. The
immunised pigs were shown to be protected against challenge by a homologous strain of ASFV with levels of viraemia negatively correlating with dose of recombinant CD2v (Ruiz-Gonzalvo et al., 1996).

An additional mechanism of immune evasion was described by Vallée and co-workers. Infection of primary endothelial cells with ASFV caused a downregulation of expression of MHC class I genes in response to interferon-α (Vallee et al., 2001).

Evidence is emerging that a major strategy used by ASFV to evade host defence systems is to interfere with signalling pathways in infected macrophages and thus modulate the transcription of host immunomodulatory genes. The A238L protein, the subject of this thesis, is described below and was the first such protein to be identified. This protein inhibits both activation of NF-κB transcription factors and calcineurin phosphatase. Other candidate proteins with similar functions have been identified. Members of multigene families 360 and 530 have been shown to inhibit interferon-β transcription and possibly also interferon-activated signalling pathways (Afonso et al., 2004). The j4R protein binds to a transcriptional co-activator α-NAC (Goatley et al., 2000), which acts to potentiate c-Jun mediated transcription. The virus-encoded ubiquitin conjugating enzyme binds to the SMCY ARID DNA binding domain containing protein, which has a role in host gene transcription (Bulimo et al., 2000). These virus proteins are thus also predicted to modulate host gene transcription. Since the virus replicates in macrophages, which are important for activating both the immediate innate response to infection and later adaptive immune response,
modulating host gene expression could dramatically affect the host response to infection.

1.3 The role of the macrophage in immune response

Functionally, macrophages have a number of important roles in the immune system. They are important phagocytes (Cohn, 1968). Macrophages migrate towards micro-organisms following a gradient of chemotactic molecules. Pseudopodia extend around the particle, which may be opsonised by antibody or complement proteins, and particle binding, internalization and killing follow (Auger & Ross, 1992).

The cell surface receptors on the macrophage include receptors for IL-1, IL-2, IL-3, IL-4, IFN-α, IFN-β, IFN-γ and TNF-α (Auger & Ross, 1992) which allow the macrophage to respond to both T\(_h\)1 and T\(_h\)2 type responses as well as the acute inflammatory response.

Along with B lymphocytes and dendritic cells, macrophages are able to present antigen to T cells. Degradation of endocytosed particles leads to association of peptides with MHC-II molecules and presentation to T cells (Cresswell, 1985). Activation of T cells also requires the production of IL-1 from the macrophage leading to IL-2 being produced by the T cells which acts in an autocrine fashion to cause T cell proliferation.

Other T cell/macrophage surface protein interactions are involved in T cell activation. Of key importance to their role as professional antigen presenting
cells is the expression of the MHC proteins and CD86 (B7.2) on the macrophage surface (Janeway et al., 1999). Along with other adhesion molecules, these proteins mediate the interaction with T cells, causing activation and proliferation of T cells. The CD86 molecule binds to CD28 on the T cell providing an important costimulatory signal. This signal is especially important in the activation of naïve T cells. If naïve T cells encounter antigen via MHC without the CD86 (CD80 on B cells)/CD28 reaction, anergy is induced in the T cell. This is an important mechanism for the induction of peripheral tolerance (Janeway et al., 1999).

MHC class I is also expressed on the macrophage surface, but peptides presented by this protein are the result of endogenous proteasome-mediated degradation and association with MHC-I molecules in the endoplasmic reticulum (Nuchtern et al., 1989, Yewdell & Bennink, 1989). This allows the macrophage to be subject to CD8⁺ cytotoxic T cell surveillance and killing. The central role of the macrophage in the immune response is shown in figure 1.8.

1.4 The NF-κB pathway

NF-κB was first described as a nuclear factor of B cells that bound to a κ enhancer region of the immunoglobulin gene promoter (Sen & Baltimore, 1986). The NF-κB family of transcription factors comprises homodimers or heterodimers of the polypeptides p105/p50, p65, p100/p52, cRel and RelB. These complexes act as transcriptional transactivators—in some cases suppressors—capable of acting on a range of genes: including those coding for pro-inflammatory cytokines and chemokines, anti-apoptotic genes such as Bel-2 and
Figure 1.8 The central role of the macrophage in the immune response. Shown are the production of cytokines and their effect on the macrophage, and the induction of macrophage cytokine production. The target cells, on which cytokines act, and immune response type are also shown for some of the cytokines produced. The antigen presentation to T cells is shown; peptide is presented via MHC-II to the T cell receptor, with CD4 providing the MHC restriction and the CD28/CD86 interaction the co-stimulatory signal. Adapted from Auger and Ross (1992) and Gordon (1998), with additional information regarding cytokine production and target cells from Janeway et al. (1999).
Bcl-xL (Chen et al., 2000, Tamatani et al., 1999), acute phase proteins and adhesion molecules (Ghosh et al., 1998). Proteins of the NF-κB family are related by the Rel homology domain (RHD), which is involved in regulating activity of the complex via nuclear/cytoplasmic shuttling (Baeuerle & Henkel, 1994, Baldwin, 1996, Ghosh & Karin, 2002). The most abundant, and prototypical, form is p50/p65 (Huxford et al., 1998). Figure 1.9 shows the relationship of NF-κB family members.

There are three classes of proteins which contain an RHD: the NF-κB family proteins, the NFATc proteins and the Ton-EBP tonicity-response proteins (Graef et al., 2001). The formation of the different classes of RHD containing protein is likely to be the result of recombination events. For example, the domains possessing calcineurin binding activity, nuclear localisation and export and substrate binding activity are encoded by just one exon (Graef et al., 2001).

NF-κB complexes are held in the cytoplasm by members of the IκB family: IκB-α, IκB-β, IκB-ε, p105 and p100 (Israel, 2000). These proteins render NF-κB inactive by holding it in the cytoplasm, blocking both the nuclear localisation signal of the p65 subunit of NF-κB and masking the DNA binding domain of the complex (Henkel et al., 1992). A novel member of the IκB family, IκB-ζ, acts in the nucleus to inhibit NF-κB activity (Yamazaki et al., 2001). IκB-ζ localizes in the nucleus via an NLS in its N terminal region and preferentially binds to the p50 subunit of NF-κB thereby preventing DNA binding of p50/p50 homodimers and p50/p65 heterodimers (Yamazaki et al., 2001).
Figure 1.9 Relationship of NF-κB family members. NF-κB refers to a family of proteins containing the Rel homology domain (RHD). This domain contains elements required for DNA binding, dimerisation and association with inhibitor (IkB) family members. The p50 and p52 proteins are produced by proteolytic processing of the p105 and p100 proteins respectively. The IkBs are characterised by the ankyrin repeat regions which mask the NF-κB nuclear localisation signals and prevent binding of NF-κB to target sequences in DNA. From Baldwin, 1996.
Upon cell activation, IκB is phosphorylated at serine residues 32 and 36 by the IκB kinase complex (Karin, 1999). It is likely there are a number of different mechanisms for activating the IKK complex, one of these involves the protein mixed-lineage kinase 3 (MLK3) (Hehner et al., 2000). The phosphorylated IκB is then ubiquitinated and degraded by the proteasome. This exposes the nuclear localization signal and DNA binding domain of the NF-κB complex allowing nuclear translocation and transactivation of NF-κB responsive promoters.

The activation of NF-κB is controlled by a negative feedback loop. The IκB promoter is transactivated by NF-κB. In this way the response to the stimulus is controlled. Upon synthesis IκB accumulates in the nucleus due to its nuclear localisation signal, which is masked when bound to NF-κB. Here, it binds to and exports NF-κB from the nucleus, due to a cytoplasmic localisation sequence in its N terminal region, via a Crm1-dependent mechanism (Huang et al., 2000). The IκB-α/ NF-κB complexes are held in the cytoplasm by interacting with the cytoplasmic protein RasGAP SH3-binding protein 2 (Prigent et al., 2000). A simplified overview of this pathway is shown in figure 1.10.

To avoid premature termination of the activity of NF-κB due to the large amounts of free IκB produced, IκB can be degraded by proteasomes in the nucleus while the stimulus is maintained (Renard et al., 2000).

The activity of NF-κB can be regulated by other mechanisms. It has been shown that the p65 subunit of NF-κB can be reversibly acetylated (Chen et al., 2001,
Chen et al., 2002). The acetylated p65 binds IκB-α very weakly, if at all. The acetylated RelA can be deacetylated by histone deacetylase 3 allowing sequestration by free IκB in the nucleus. The transcriptional activity of p65 is also regulated by phosphorylation (Naumann & Scheidereit, 1994). Calmodulin-dependent protein kinase IV interacts with and phosphorylates p65, thus stimulating gene transcription (Jang et al., 2001).

Further regulation of NF-κB activity is via association of NF-κB with the TATA-binding protein (TBP) of the transcription factor IID (TFIID) complex. TBP is required for efficient gene transcription (Kerr et al., 1993). An interaction of NF-κB with transcription factor IIB (TFIIB) has also been shown (Xu et al., 1993).

Calcium concentration may play a further role in activation of NF-κB. An increase in calcium ion concentration can activate calcineurin phosphatase. Calcineurin has been shown to act in synergy with protein kinase C to activate NF-κB by activating the IκB kinase complex (Trushin et al., 1999).

The crystal structure of NF-κB/IκB-α complexes has been solved by X-ray crystallography to resolutions of 2.7Å (Jacobs & Harrison, 1998) and 2.3Å (Huxford et al., 1998). These data have shown that a stack of six ankyrin repeats within IκB-α face the Rel homology domains of NF-κB. The first two of these repeats obstruct the nuclear localisation signal of NF-κB while ‘the position of the sixth ankyrin repeat shows that full-length IκB-α will occlude the NF-κB DNA-binding cleft’ (Jacobs & Harrison, 1998). The structure of the p50/p65/IκB complex is shown in figure 1.11.
Figure 1.10 A simplified overview showing the activation of NF-κB. Following stimulation of an appropriate receptor, a signal is transduced to the IκB kinase complex. This then phosphorylates IκB targeting it for ubiquitination and degradation by the proteasome. This exposes the nuclear localisation signal and DNA binding domain on the p65 subunit of NF-κB. The dimer, shown here as the prototypical p50/p65 form, then traffics to the nucleus and transactivates target promoters. Adapted from Ghosh & Karin (2002) and Ghosh et al. (1998).
The NF-κB pathway can be activated from within a virus infected cell by activation of protein kinase R (PKR) in response to double-stranded RNAs produced during virus replication. PKR is able to directly activate the IKK complex leading to NF-κB activation (Zamanian-Daryoush et al., 2000).

Proteins of the NF-κB family are also found in invertebrates (Baldwin, 1996). (St. Johnston & Nussleinvolhard, 1992) (Hedengren-Olcott et al., 2004, Hoffmann et al., 1993, Kappler et al., 1993, Reichhart et al., 1992). (Sagisaka et al., 2004). The primary NF-κB homologue in Drosophila is the Dorsal protein. Dorsal is controlled by nuclear/cytoplasmic shuttling in a similar way to NF-κB in mammalian cells, with the IκB-like protein Cactus being responsible for Dorsal regulation (Kidd, 1992, Nicolas et al., 1998, Reichhart et al., 1993).

1.5 The NFAT pathway

Nuclear factor of activated T cells (NFAT) is a family of signalling molecules regulated by calcium ion flux involved in the expression of a number of genes with a number of roles. NFAT signalling is involved in development and morphogenesis of bone, muscle and vasculature as well as signalling in immune cells and lymphocyte activation (Crabtree & Olson, 2002).

A number of viruses interact with or exploit NFAT, or pathways with which it is involved. Herpes simplex virus has been shown to block the activation and nuclear translocation of NFAT (Scott et al., 2001). The virus associated RNAⅡ of human adenovirus has been shown to bind to the NF90 subunit of NFAT and may play a role in regulation of its activity (Liao et al., 1998).
Figure 1.11 Crystal structure of the NF-κB p65/p50 dimer complexed with IkB. The p65 subunit is shown in yellow, p50 in green and IkB in blue. The nuclear localisation sequence (NES) is marked, as are the C termini of p65 and IkB. From Huxford et al., 1998.
The core protein of hepatitis C virus has been shown to activate interleukin-2 transcription via the NFAT pathway (Bergqvist & Rice, 2001) while the nef protein of HIV-1 can activate NFAT to transactivate the provirus LTR which contains two NFAT binding sites (Cron et al., 2000, Manninen et al., 2001, Manninen et al., 2000). The R1 protein of Rhesus monkey rhadinovirus has also been shown to activate NFAT in B cells (Damania et al., 2000).

NFAT is regulated by the cellular phosphatase calcineurin. After stimulation of an appropriate receptor, \([\text{Ca}^{2+}]\) increases and calcineurin is activated. This calcium signalling is essential for T cell activation and it is likely that sustained intracellular \(\text{Ca}^{2+}\) concentrations are required for activation (Negulescu et al., 1994).

The activated calcineurin phosphatase is responsible for the dephosphorylation of NFAT proteins most likely in the cytoplasm. Calcineurin has been shown to dephosphorylate NFAT1 in vitro (Jain et al., 1993, Park et al., 1995) and in cell extracts (Luo et al., 1996); this significantly increases the affinity of NFAT for DNA.

A model for NFAT activation and regulation is shown in figure 1.12. The basic model for NFAT activation is that after an appropriate stimulus (such as activation of the T cell receptor) an increased concentration of cytoplasmic \(\text{Ca}^{2+}\) activates calmodulin which binds to and activates calcineurin phosphatase. Calcineurin then dephosphorylates NFAT exposing a nuclear localization signal and increasing its affinity for DNA. NFAT then translocates to the nucleus where
**Figure 1.12 NFAT activation by calcineurin.** Following an appropriate stimulus, intracellular calcium levels increase. This leads to activation of the cellular phosphatase calcineurin. Calcineurin dephosphorylates NFAT exposing a nuclear localisation signal and DNA binding domain. NFAT then trafficks to the nucleus and transactivates target promoters. Adapted from Rao et al., 1997.
it associates with the API transcription factor (c-Fos and c-Jun) to activate gene expression.

It has been proposed that before activation, NFAT is associated with calcineurin by binding at one, non-catalytic, site. Upon activation, the association of calmodulin with calcineurin changes its conformation allowing binding of NFAT to the active site of calcineurin and causing dephosphorylation of NFAT (García-Cozar et al., 1998).

1.6 The ERK pathway

The extracellular-signal regulated kinases (ERKs) are apart of the family of mitogen activated protein kinases (MAPKs), which also includes the c-Jun amino-terminal kinases (JNKs) and the p38 MAPKs, which are involved in signal transduction from the cytoplasm to the nucleus. The pathways are well conserved from yeasts through to multicellular organisms. Substrates for the downstream effectors include transcription factors, phospholipases and cytoskeletal proteins (Johnson & Lapadat, 2002).

The ERK1 and ERK2 proteins are expressed in the majority of tissues and are involved in the control of meiosis and mitosis (Johnson & Lapadat, 2002). The ERK1 and 2 proteins can be activated through the Ras/Raf pathway by a number of stimuli including growth factors, virus infection and cytokines (Johnson & Lapadat, 2002). Following activation of the pathway, ERK1 and 2 translocate to the nucleus to regulate activation of transcription factors such as c-Fos, c-Jun and c-Myc. Alternatively, they can can activate the RSK protein in the cytoplasm.
leading to its nuclear translocation and RSK-mediated transcription factor activation (Blenis, 1993). The ERK pathway can also be regulated by intracellular calcium levels, with calmodulin (CaM) and CaM dependent protein kinase IV (CaMKIV) able to positively regulate ERK activity (Agell et al., 2002).

The MAPK pathways are also associated with the NF-κB pathway. The p38 MAPK is required for NF-κB-mediated gene transcription (Carter et al., 1999b), with p38 phosphorylating the TATA box-binding protein (TBP) of the TFIID complex and preventing its association with the p65 subunit of NF-κB, inhibiting gene transcription. The ERK and p38 pathways are activated by lipopolysaccharide (Carter et al., 1999b), as is the NF-κB pathway, suggesting that both pathways are activated concurrently and are able to interact to stimulate gene transcription.

The ERK pathway is also able to negatively regulate NF-κB-dependent gene transcription by inhibiting the p38-mediated phosphorylation of TFIID (Carter & Hunninghake, 2000a). These results show that the MAPK pathways play an important role in regulating the transactivational capability of NF-κB.

Interestingly however, the ERK pathway has been shown to act along with NF-κB in inhibiting Fas-mediated apoptosis following treatment with phorbol ester (Engedal & Blomhoff, 2003).
1.7 **The A238L protein**

Analysis of the complete sequence of African swine fever virus revealed a gene, A238L, with similarity to IκB (Yanez et al., 1995). The gene (called 5EL in the Malawi isolate (Neilan et al., 1997)) runs from position 34049-33336 in the BA71V strain of ASFV. The gene encodes a protein containing four ankyrin repeats. The predicted structure of A238L is shown in figure 1.13. It was predicted that this gene may be able to act as a virally encoded modulator of immune mechanisms by acting as an IκB homologue. A sequence comparison of A238L with a number of the IκBs is shown in figure 1.14.

Powell et al (1996) found that A238L did indeed modulate the immune response by interfering with NF-κB mediated gene expression (Powell et al., 1996). Semi-quantitative RT-PCR was used to show that the pro-inflammatory cytokine genes interferon-α, tumour necrosis factor-α and interleukin-8 were down-regulated after ASFV infection. Transfection of a plasmid containing the A238L gene produced a down-regulation of activity of an IL-8 promoter luciferase construct. Mobility shift assays showed that expression of A238L prevented NF-κB binding to DNA. It was later shown that purified recombinant A238L protein (A238Lp) added to nuclear extracts from activated cells was also able to produce these results (Revilla et al., 1998). Interestingly, transforming growth factor (TGF) β production was upregulated following ASFV infection. It has been shown that TGF-β can regulate the function of IRF-7 and cause activation of the IFN-β promoter (Qing et al., 2004).
Figure 1.13 The predicted structure of the A238L protein. The left panel shows the predicted structure of A238L, modelled using the crystal structure of IκB as a template. The ankyrin repeats can be seen in the central region of the protein. The yellow region shows the calcineurin docking motif and red indicates regions that could not be modelled due to insufficient homology. The right panel shows A238L (green) superimposed over the model of IκB-α (blue). Courtesy Dr. Saloua Najjam.
Figure 1.14A The homology of the A238L gene to other IκB sequences. Mad3 is a human IκB found in the nucleus, pig, rat and chick show the porcine, rat and chicken IκB-α sequences. The lines above the alignment show the positions of the ankyrin repeats in IκB. The lines below the alignment show the positions of the ankyrin repeats in A238L. From Revilla et al, 1997.
**Figure 14.4B An alignment of the A238L and IκB-α protein sequences** The nuclear export sequence of IκB is indicated by the black-boxed region. This signal is located at the N-terminus of the protein. Homology between A238L and IκB is confined to the central region of the proteins. No similar sequence to the NES of IκB is present in A238L. The second ankyrin repeat of IκB is indicated by the line below the sequence. This region is responsible for the nuclear localisation of IκB. The sites of IκB phosphorylation are marked by the blue-boxed regions. The calcineurin-docking motif of A238L is indicated by the plum-boxed region. Acidic amino acids are shown in red, hydrophobic amino acids in grey, amido-containing in turquoise, aromatic in olive, hydroxyl containing in pink, proline in green and sulphur containing in yellow.
The A238L gene was shown to be nonessential for virulence in domestic swine by the construction of a gene deletion virus (Neilan et al., 1997). Infection of domestic pigs with A238L knockout virus caused onset of symptoms and disease progression which did not differ from the clinical signs produced by infection with wild-type virus. The A238L knockout and wild-type viruses were also found to replicate to equivalent titres in macrophage cultures (Neilan et al., 1997). Neilan et al also showed that the A238L gene was highly conserved amongst ASFV isolates. The gene was typically 97-100% identical between strains with the Malawi isolate the only strain tested to have a significant difference (86%).

Using the yeast two-hybrid system and by co-precipitation, an interaction between the A238L protein and the cellular protein calcineurin phosphatase was shown. ASFV infection inhibited calcineurin activity and this inhibition was dependent on the presence of the A238L gene (Miskin et al., 1998). A238L interacts with calcineurin by a PxIxIT motif close to the C-terminus of A238L (Miskin et al., 2000) and an 82 amino acid domain from A238L inhibits calcineurin phosphatase activity in vitro (Abrams et al., unpublished data). Thus, in this respect, A238L function resembles that of the immunosuppressive drug cyclosporin A which, in complex with cyclophilin, binds to and inhibits calcineurin phosphatase activity. One of the proteins regulated by calcineurin is NFAT (nuclear factor of activated T cells). The dual function of A238L, inhibition of the NF-κB pathway and calcineurin, is shown in figure 1.15.
Calcineurin (protein phosphatase 2B) is a ubiquitous phosphatase and specifically dephosphorylates proteins involved in a range of pathways. Aside from its role in NFAT activation, calcineurin is also involved in activation of the transcription factor CTF-1 following TGF-β signalling (Alevizopoulos et al., 1997), 1,4,5-trisphosphate receptor signalling (Jayaraman & Marks, 2000) and negative regulation of Elk1 in the MAP (mitogen activated protein) kinase pathway (Tian & Karin, 1999).

In addition to calcineurin, four clones encoding cyclophilin A were identified as binding partners of A238Lp in the yeast two-hybrid system (Miskin et al., 1998), suggesting that A238Lp may also bind to cyclophilin. However this interaction may have required calcineurin which is present in yeast. Cyclophilin A was originally identified as an abundant intracellular protein with a role in protein folding, but further studies showed it could be secreted in response to pro-inflammatory stimuli (Sherry et al., 1992). Cyclophilin was then shown to be a chemoattractant of neutrophils (Sherry et al., 1992), eosinophils (Xu et al., 1992) and T cells (Allain et al., 2002). CD147 has recently been identified as the cellular receptor for cyclophilin A and that signal transduction triggered by binding this receptor results in the activation of ERK (Yurchenko et al., 2002).

The A238L protein has been shown to be synthesized as two molecular weight forms of 28 kDa and 32 kDa in infection (Tait et al., 2000). The 32 kDa form, but not the 28 kDa form, co-precipitated with the p65 subunit of NF-κB.
Figure 1.15 Blocking action of A238L on NF-κB and calcineurin. The dual function of A238L is shown with the binding to NF-κB shown following the degradation of IκB on the left, and calcineurin inhibition on the right.
This contradicts work by Revilla et al (1998) which shows that the 28 kDa form of the protein can co-precipitate with p65. This latter work was, however, performed by transfecting A238L into cells under the control of the CMV IE promoter rather than by infecting with ASFV.

Tait et al (2000) also showed that IκB degradation was required for the A238L protein to bind to NF-κB p65. A238Lp was not able to displace IκB from the NF-κB complex and activation of the NF-κB pathway is required before A238Lp can bind to p65.

Recent work has shown that there is more A238L mRNA transcribed in cells infected with the low virulence NHV isolate compared to infection with the more virulent L60 isolate (Gil et al., 2003). A lower production of the cytokines TNF-α, IL-6, IL-12 and IL-15 was observed following infection with the high virulence isolate compared to the lower virulence isolate (Gil et al., 2003). The lack of a number of genes, including the multigene families 360 and 530, in the low virulence isolate may provide an explanation for this apparent paradox. It has also been shown recently that A238L is also able to inhibit cyclooxygenase-2 (COX-2) via an NFAT-dependent pathway (Granja et al, in press).

1.8 Project aims

Although a large amount of work has been done on the A238L protein, leading to a greater understanding of the mode of action of the protein, the precise mechanism for its function has not yet been explained at the molecular level and
it is not yet clear if the protein has any other functions which have not yet been identified.

One aim of the project was to investigate further the mechanism by which A238L inhibits NF-κB activation. Previous results are contradictory since one report suggested that A238L inhibits NF-κB translocation to the nucleus (Tait et al., 2000) whereas another report (Revilla et al., 1998) suggested that A238L may act within the nucleus to inhibit binding of NF-κB to DNA. The primary aim of this part of the project was to determine at what points in the NF-κB pathway A238L acts to inhibit its activity.

A second aim was to investigate the functional effects of A238L on infected cells. Although A238L is predicted to have a wide effect on host gene transcription and hence on host cell function as yet there is little evidence about the actual functional effects of the A238L protein on host cells.
2 Materials and methods

2.1 Cell culture

African green monkey (*Cercopithecus aethiops*) kidney cells (Vero) were maintained in Dulbecco's modified Eagles medium containing 25 mM HEPES and supplemented with 2 mM glutamine, 50 units/ml penicillin, 50 µg/ml streptomycin and 5% bovine foetal calf serum (FCS) (PAA Laboratories GmbH).

SV40 Transformed African green monkey kidney cells (Cos) were maintained in Dulbecco's modified Eagles medium with supplements as for Vero cells, but with 10% FCS.

Cells were cultured in 175 cm² and 75 cm² Falcon® brand flasks, Falcon® MULTIWELL™ 6 and 24 well plates and incubated at 37 °C in the presence of 5% CO₂. All cell culture flasks and plates were from Becton Dickinson. For cells grown for immunofluorescence, a 13 mm diameter, number 1 thickness glass coverslip (BDH) was placed in the bottom of the required number of wells of a 24 well plate before the addition of medium. Cells were maintained in 175 cm² flasks.

Cells were split from ratios of 1:4 to 1:10 depending on the purpose for which they were intended. Cells for protein gels and Western blotting were split so that they would be 80-90% confluent on the day of the experiment. Cells for immunofluorescence were split to be 60-70% confluent on the day of the experiment.
Cells were split by removing the medium and washing once in trypsin/versene. 5 ml of trypsin/versene was then added to the flask and the cells incubated at 37 °C for 5 minutes. The flask was then agitated until the cells were detached. 15 ml of medium was then added to the flask and the cell suspension pipetted several times to break up cell clumps. An appropriate amount of the cell suspension was then removed and added to fresh medium to seed fresh flasks.

Primary porcine alveolar macrophages (PAMs) were obtained by lung lavage of freshly killed pigs by Dr. Fuquan Zhang (ASFV group, IAH Pirbright). PAMs were cultured in RPMI medium supplemented with 10 % FBS. An immortalized macrophage cell line (IPAM) was also used. These cells were prepared as described in Weingartl (Weingartl et al., 2002). IPAM cells were cultured in RPMI supplemented with 10% FBS and 50 µg/ml gentamycin (Gibco Invitrogen) and 1x non-essential amino acids (Gibco Invitrogen).

2.2 Viruses

2.2.1 Production of virus stocks

Initial stocks of virus were obtained from Dr. Charles Abrams. These were recombinant forms of the Vero cell adapted (BA71V) strain of ASFV. Recombinant viruses had been constructed with an epitope tag (SV5 pk) on the A238L gene (SV5GAL) and an A238L knockout virus (vIKGAL). These viruses had the β-galactosidase genescloned in to enable easy screening of recombinants (Miskin et al., 1998).
Experiments involving the use of primary macrophages as the cellular system required the use of field strains of ASFV for infection. ASFV Malawi (Haresnape, 1984), a high virulence isolate, was the principle isolate used for these experiments. The intermediate virulence Malta isolate (Wilkinson et al., 1981), the low virulence NH68 isolate (Leitao et al., 2001) and the recombinant Rec34 virus (Duarte 2000, PhD thesis University of Lisbon) were also used.

The Rec 34 virus is a recombinant virus based on the backbone of the NHV68 field isolate. This virus has an interrupted lectin-like gene. The CD2v gene of NHV68 has been replaced with the CD2v gene of the Lisbon 60 isolate to produce the Rec34 virus. The CD2v gene in the recombinant virus has a disrupted cytoplasmic tail, but the extracellular domain is intact (Duarte 2000).

These viruses were obtained from the IAH ASFV virus collection apart from the Rec34 and NH68 viruses which were from Dr Margarida Duarte LNIV Lisbon.

Virus stocks were produced by infecting an 80-90% confluent 175 cm² flask with virus. Serum free media were used for all infections. After 4 hours the flasks were washed with serum free medium and 15 ml fresh medium was added with 0.5% FCS. Cells were then incubated at 37 °C in the presence of 5% CO₂. Once cytopathic effect could be observed by light microscopy (on a Nikon model TMS), 5 ml of the medium was used to infect a fresh flask. This was left until almost complete cell detachment was observed. The medium was then transferred to a 50 ml Falcon® tube and centrifuged at 4000 rpm in a Denley BR401 centrifuge at 4 °C for 10 minutes to pellet cell debris. The supernatant
was then decanted and stored at 4 °C for short-term storage or -70 °C for long-term storage.

2.2.2 Titration of virus stocks

Virus stocks were titrated by making use of the β-galactosidase gene encoded by the recombinant viruses and expressed in infected cells. This meant that infected cultures could be detected by removing some of the culture medium and incubating with X-gal. A colour change to blue would indicate that cells in that well were infected.

Virus titrations were performed by culturing Vero cells to 80-90% confluency in 24 well plates. A 10 fold dilution series of the virus stock was prepared and used to infect cells cultured in serum free medium. Infection series were performed in quadruplicate. The wells were then covered in film to prevent aerosol spread between wells. After 48 hours, a portion of medium was removed from each well and added to the corresponding well in another 24 well plate. X-gal (2 μl of 50 mg/ml) was then added to each well and the plate incubated at 37 °C. The colour change to blue could be seen only in the wells in which the virus had not been diluted out and this was used to calculate the virus concentration.

2.2.3 Infections

For experimental virus infections cells were grown to an appropriate confluency, dependant upon the experiment, in medium supplemented with foetal bovine serum. Cells were then washed and cultured in serum free medium and virus added at a multiplicity of infection (MOI) of 5-10. Cells were incubated for 1.5
hours at 37 °C before the medium was removed. The cells were then washed twice with medium and cultured in medium with FCS.

2.3 Plasmid constructs

Constructs containing the A238L gene under a constitutive eukaryotic promoter or its own ASFV promoter were constructed in the ASFV laboratory, Institute for Animal Health, Pirbright. A construct of the A238L gene in pcDNA3 (Invitrogen) was made by Dr. James Miskin. This construct was under the control of the CMV IE promoter. A construct of the A238L gene in pcDNA3 with the WPRE element (Donello et al., 1998, Xu et al., 2003, Zufferey et al., 1999) was cloned by Ms. Rhiannon Silk. The WPRE element increases the efficiency with which the mRNA is exported from the nucleus and thus increases amounts of protein produced. This construct also contains an intron. As ASFV genes are synthesised in the cytoplasm by virus encoded transcription machinery, there is no splicing of these genes. The inclusion of an intron can increase efficiency of protein production by trafficking mRNAs efficiently from the site of transcription in the nucleus via the spliceosome. The A238L gene was also cloned into the pTriEx (Novagen) vector, which also constitutively expresses genes under the control of the CMV IE promoter.

2.4 Transfection of mammalian cells

2.4.1 Transfection using lipofectin

Approximately 1 x 10^5 cells were seeded into 6-well plates and incubated at 37 °C in 5 % CO_2 until 40-60% confluent. For each transfection, 5 μg of DNA was diluted in 100 μl OptiMEM (Life Technologies) and 10 μl Lipofectin
(Invitrogen, 18292-011) was diluted in 100 μl OptiMEM in sterile bijous. The two solutions were then incubated at room temperature (RT) for 45 minutes. The solutions were combined and incubated at RT for 15 minutes. Cells were then washed once with OptiMEM and 0.8 ml per transfection of OptiMEM added to the Lipofectin/DNA complexes. 1 ml of this solution was added to each well of a 6-well plate. Cells were then incubated for 8-24 hours at 37 °C with 5% CO₂ before the medium was replaced with DMEM supplemented with 10% FBS.

2.4.2 Transfection using lipofectamine

Approximately 1 x 10⁵ cells were seeded into 6-well plates and incubated at 37 °C in 5 % CO₂ until 90-95% confluent. For each transfection, 4 μg of DNA was diluted in 250 μl OptiMEM (Life Technologies) and 10 μl Lipofectamine (Invitrogen, 11668-027) was diluted in 250 μl OptiMEM in sterile bijous. The two solutions were then incubated at room temperature (RT) for 5 minutes. The solutions were combined and incubated at RT for 20 minutes. Cells were then washed once with OptiMEM and 0.8 ml per transfection of OptiMEM added to the Lipofectin/DNA complexes. 1 ml of this solution was added to each well of a 6-well plate. Cells were then incubated for 8-24 hours at 37 °C with 5% CO₂ before the medium was replaced with DMEM supplemented with 10% FBS.

2.5 Nuclear/cytoplasmic fractionation

A number of protocols were tested before this one was selected. Other protocols used will be discussed in the appropriate chapters. Cells in 6 well plates were washed twice with ice cold phosphate buffered saline (PBS) and, after the addition of 1 ml PBS, harvested by scraping. Cell suspensions were put into pre-
chilled microcentrifuge tubes on ice. The cell suspension was then spun for 3 minutes at 7000 rpm in an IEC Micromax benchtop centrifuge to pellet the cells. The pellet was then resuspended in 200 μl pre-chilled fractionation buffer (Tris-HCl 150 mM pH 8.8, KCl 10 mM, EDTA 1 mM, NP-40 0.2% v/v, glycerol 10% v/v, phenylmethylsulphonyl fluoride 1 mM, small protease inhibitors [leupeptin, pepstatin, antipain, chymostatin] 1 μg/ml), briefly vortexed then incubated on ice for 10 minutes. The suspension was then centrifuged for 5 minutes at 13,200 rpm to pellet the nuclei. The supernatant was removed and added to 200 μl SDS-PAGE sample buffer (Tris-Hcl pH 6.8 100 mM, sodium dodecyl sulphate 4% w/v, bromophenol blue 0.2% w/v, glycerol 20% v/v, dithiothreitol 100 mM). The nuclear pellet was then harvested in 50 μl of protein gel sample buffer and the DNA sheared by repeated passage through a 21 gauge needle.

2.6 Immunoprecipitation

Following appropriate treatment of cells cultured in 6-well plates, cells were harvested by adding 200 μl NET-N (50 mM Tris-Hcl pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.05% deoxycholate, protease inhibitors [leupeptin, pepstatin, antipain, chymostatin] 1 μg/ml) to each well. Cells were then scraped using tissue culture scrapers and the lysate transferred to a microcentrifuge tube and spun at maximum speed in a benchtop microcentrifuge for 10 minutes. The supernatant was then removed and transferred to a fresh microcentrifuge tube. 2 mg/ml protein A-sepharose resuspended in NET-N was then added and tubes were incubated for 1 hour at 4 °C with rotation. Tubes were then spun at 6000 rpm in a microcentrifuge for 5 seconds and lysates transferred to fresh microcentrifuge tubes.
Specific antibodies were added to precleared lysates to a concentration of 3 mg/ml and tubes incubated for 18 hours at 4 °C with rotation. 2 mg of protein A-sepharose was added to each tube and tubes incubated for 1 hour at 4 °C with rotation. Tubes were then centrifuged at 6000 rpm for 5 minutes in a microcentrifuge and the supernatants discarded. The pellets were then resuspended in 1 ml NET-N and incubated for 2 minutes on ice before being spun at 6000 rpm for 5 seconds. This step was repeated a further two times.

Pellets were then resuspended in 100 μl SDS-PAGE loading buffer and denatured by boiling for 10 minutes. Samples were then spun at 6000 rpm in a microcentrifuge and stored at –20 °C until required.

2.7 SDS polyacrylamide gel electrophoresis

Denaturing gel electrophoresis was performed using a 10% resolving gel (except where specified in text) (40% stock acrylamide solution [acrylamide:N,N'-methylene bisacrylamide, 37.5:1] 25% (v/v), Tris-HCl pH 8.8 0.375M, SDS 0.1% (w/v), ammonium persulphate 0.2% (v/v), TEMED (N,N,N',N'-Tetramethylethylenediamine) 0.0625% (v/v)) and a 4% stacking gel (40% stock acrylamide 10% (v/v), Tris-HCl pH 6.8 100mM, SDS 0.1% (w/v), ammonium persulphate 0.2% (w/v), TEMED 0.1% (v/v). Gels were run at 150 V for approximately 1 ½ hours in the case of mini gels and 120 V for up to 6 hours in the case of large gels. Gels were run in a vertical apparatus containing reservoirs of running buffer (Tris 0.25M, glycine 250mM, SDS 0.1% (w/v)).
2.8 Western blotting

2.8.1 Wet electroblotting

Polyacrylamide gels were removed from the glass plates onto filter paper which had been pre-soaked in Western transfer buffer (methanol 20% v/v, sodium dodecyl sulphate, 0.37% w/v, tris-HCl, 0.58% w/v, glycine 0.29% w/v). Nitrocellulose membrane (Hybond-C extra, Amersham Pharmacia Biotech Ltd.) was placed next to the gel and a further piece of filter paper placed on top of the membrane. The wet-blotting procedure was performed using the Mini Protean II system (Biorad). Blotting was performed for 1 hour at 100 volts. Western blots shown in the following chapters are representative of three independent experiments except where indicated in the text.

2.8.2 Immunodetection

After electroblotting, membranes were blocked in blocking solution (Marvel milk powder, 3% w/v in PBS with 0.1% v/v Tween-20 [polyoxyethylene sorbitan monolaurate]) either for 4 °C overnight or at room temperature for 3 hours. Antibodies were diluted to an appropriate concentration in blocking solution and membranes incubated in antibody solution for 1 hour with shaking at room temperature. Membranes were then washed 3 times in PBS-Tween (Tween-20, 0.05% v/v) before incubation with HRP-conjugated protein A (AbCam, ab-7456-1, 1/2000). Membranes were then washed 4 times in PBS-Tween. Immunoblots were detected by enhanced chemiluminescence reagent (Tris-Hcl 0.1M pH 8.5, para-coumaric acid 200 µM, luminol 1.25 mM). Membranes were incubated with detection reagent for 1 minute before drying and exposure to X-Ray film (Kodak MXB). Films were developed using an X-ograph Imaging Systems Compact X-
4. Digital imaging of gels was performed using a Biorad GS-710 densitometer and Biorad Quantity One software (version 4.1.0).

2.9 Indirect immunofluorescence

2.9.1 Cell culture

Cells for immunofluorescence were cultured as described in section 2.1. Cells were split to a confluency of 20-30% to allow individual cells to be seen clearly.

2.9.2 Immunostaining

Cells were washed twice in Phosphate buffered saline then fixed using either paraformaldehyde or methanol for 20 minutes. Cells were then washed three times in Ca$^{2+}$ and Mg$^{2+}$ PBS and permeabilised for internal staining with PBS containing Triton X-100 (Sigma) 0.2% v/v for 5 minutes. Cells were then washed in PBS plus gelatine (Sigma) 2% w/v. The primary antibody was diluted in PBS-gelatine at a concentration of 1/500 and 200 μl of antibody used per well. Cells were incubated for 20 minutes. The cells were then washed twice in PBS plus Tween-20 (Sigma) 0.2% v/v and three times in PBS-gelatine. The secondary antibodies were fluorochrome conjugated anti-IgG antibodies (Molecular Probes) diluted at 1/500 in PBS-gelatine and incubated for 20 minutes. Cells were then washed twice in PBS-Tween and 3 times in PBS. The coverslips were then floated in distilled water and upturned onto slides (BDH, Superfrost) with mounting medium (Vectashield with DAPI (4'-6 diamidino-2-phenylindole, Vector Laboratories). Nail varnish was used to seal around the edges of the coverslips. Slides were stored at 4 °C.
2.9.3 Microscopy

Immunofluorescence microscopy was performed using a Nikon VFM microscope linked to an Apple Macintosh G3 running Openlab version 2.6 (Improvision, Coventry, Warwickshire UK) software via a Hamamatsu C4742-95 CCD digital camera.

Confocal microscopy was performed using a Leica SP2 confocal laser scanning microscope. Images were analysed using LCS (Leica Confocal Software).

2.10 Bioinformatics

2.10.1 DNA sequence analysis

DNA sequence analysis was performed using the Blast search method. The Blast search at http://www.ncbi.nlm.nih.gov/BLAST/ was used and the discontinuous megablast option was chosen for nucleotide homology searches.

2.10.2 Protein sequence analysis

Protein sequence analysis and alignment was performed using GCG Seqlab software and OMIGA software as well as the web based program Predict NLS (http://maple.bioc.columbia.edu/predictnls/).

2.10.3 Protein structure analysis

A number of web based protein structure prediction programs were used to analyse protein sequences: SMART (http://smart.embl-heidelberg.de/), PFAM (Protein families database of alignments and HMMs,
http://www.sanger.ac.uk/Software/Pfam/), Psipred (http://bioinf.cs.ucl.ac.uk/psipred/).

2.11 Cell proliferation assays

Six-well plates were prepared by drawing and numbering eight 2mm x 2 mm squares on the underside of the plastic. Vero cells were seeded onto the plate and allowed to attach before transfection with lipofectin according to experimental procedure. Transfection treatments were run in duplicate wells.

Four squares from the 16 per each treatment were selected by random number table and the number of cells in each counted 24 hours post-transfection. At appropriate times after the initial (time zero) count, cells in the squares were counted and results were tested for statistical significance using the students t-test.

2.12 Nitric oxide assays

Primary alveolar macrophages were obtained by lung lavage from freshly sacrificed baby pigs (5-10 kg). Cells were then cultured in 24-well plates in RPMI medium supplemented with 10% FBS. Following a 24 hour period of culture at 37 °C in the presence of 5% CO₂, the medium was changed to OptiMEM (Life Technologies) RPMI medium is unsuitable for this assay since it contains high levels of nitrates. Cells were then stimulated with 100nM PMA or infected with ASFV isolates of approximately equivalent titre at an MOI of approximately 5. Equal volumes of each virus isolate were used as virus is
produced in RPMI medium and adding different amounts of RPMI to different samples would make results difficult to interpret.

At appropriate times post stimulation/infection, tissue culture supernatants were harvested and stored at -20 °C until required. Nitric oxide release by macrophages was measured indirectly using the breakdown products nitrate (NO$_3^-$) and nitrite (NO$_2^-$) as an indicator of nitric oxide production.

The assay for nitrate and nitrite following harvest of tissue culture media, was performed using the Stressgen StressXpress nitric oxide detection kit (EKS-300) following the manufacturers instructions.

Briefly, the assay is based around an enzymatic conversion of nitrate to nitrite followed by a colorimetric measurement of nitrite levels by production of an azo dye product following the Griess reaction (Phizackerley & Al-Dabbagh, 1983).
3 Effects of ASFV infection on NF-κB protein levels

3.1 Introduction

ASFV infection has been shown to inhibit PMA induced expression of an NF-κB-dependent luciferase reporter gene and to reduce mRNA levels, late in infection, of NF-κB-dependent transcripts including those encoding TNF-α, IFN-β and IL-6 (see chapter 1 section 1.7 and Powell et al. (1996). The A238L protein provides one mechanism for inhibiting NF-κB activation by binding to the p65/p50 complex. However, in addition to inhibiting activation of pre-existing NF-κB complexes, the virus may have other mechanisms to regulate NF-κB activity, for example, by regulating the level of the NF-κB protein present. This could be achieved by a variety of mechanisms including decreasing the half-life of the protein, or interference with transcription or translation. This could be specific to target genes, or a generalised reduction in host transcription and translation.

Previous studies, using 2D gel electrophoresis to compare protein steady-state levels and synthesis at various times post-infection, have indicated that ASFV infection does not cause a general shut-off of host protein synthesis. However, alterations in relative amounts of both certain host proteins and mRNAs have been observed (Alfonso et al., 2004).

In this chapter, the amount of NF-κB present in cells at various times post-infection was examined. Two ASFV recombinants based on the BA71V isolate were used in these experiments. One had the wild-type A238L deleted and replaced with the A238L gene tagged at the N-terminus with the pk tag from
simian virus 5 (SV5GAL). The other virus had the A238L gene deleted (vIKGAL). Both viruses had the β-galactosidase gene inserted to select recombinant viruses (Miskin et al., 1998).

This study would enable any specific effects of the A238L protein on p50 and p65 protein levels to be estimated. Previous publications have established that these recombinants show similar growth curves and replicate to the same titre as wild-type viruses (Miskin et al., 1998) and have also established that the SV5-tagged A238L protein is functionally active (Miskin et al., 1998).

Initially, protocols were optimised for sample analysis by SDS-PAGE followed by Western blotting. A number of antibodies were tested and the conditions for their use optimised.

### 3.2 Optimisation of cell lines and antibodies

Vero cells were predominantly used for these studies; these are derived from African green monkey kidney. Initial characterisation of NF-κB p65 in these cells, by Western blot, produced bands that were not of the correct size. A number of Vero cell lines were subsequently tested, along with another African green monkey cell line, Cos cells. This revealed one line of Vero cells which produced bands of the correct size corresponding to p65; the Cos cell line also produced a band of the correct size at 65 kDa.

The Vero cell line was chosen for experiments, as the Ba71V strain of ASFV has been adapted to grow in Vero cells, whereas its growth in Cos cells was less
efficient. Further analysis of Cos cells by immunofluorescence showed that the NF-κB p65 protein was constitutively nuclear under its recommended culture conditions of FBS concentration of 5-10%, as assayed by immunofluorescence. Reducing the serum concentration further, in an attempt to maintain p65 in the cytoplasm, had an adverse effect on cell morphology and growth. This made these cells unsuitable as a model system for these studies.

Concurrently with the testing of the cell lines, a number of anti-NF-κB antibodies were tested for their ability to detect p65 and p50 by Western blot. Three anti-p65 antibodies and two anti-p50 antibodies were tested: Santa Cruz anti-p65 sc-109, sc-7151 and sc-372, all rabbit polyclonal; Santa Cruz anti-p50 sc-7178 rabbit polyclonal and sc-1191 goat polyclonal. Two of the anti-p65 antibodies produced bands of the wrong size or produced bands inconsistently. The third (sc-372) reproducibly produced bands of the correct size. One of the anti-p50 antibodies did not produce any bands by Western blot, the other (sc-1191) produced reproducible bands at 50 kDa. The sc-372 anti-p65 antibody and the sc-1191 anti-p50 antibody were also found to work by immunofluorescence.

3.3 Effects of ASFV infection on NF-κB p50 and p65 protein levels

Vero cells were cultured in six-well plates and infected with either SV5GAL or vIKGAL ASFV at an MOI of 10. At 3, 6, 9, 12 and 18 hours post-infection (hpi), cells were harvested in 200 μl SDS-PAGE loading buffer. A mock-infected sample was also harvested at 18 hours post mock-infection. Samples harvested at 18 hpi were taken by harvesting in culture medium and pelleting cells by centrifugation. This would ensure that cells which had detached from the culture
plate were also harvested. Equal volumes of samples were resolved by SDS-PAGE and transferred to nitrocellulose for immunodetection. Figure 3.1 shows expression of viral proteins over time. The membrane was probed with an ASFV hyper-immune serum to show virus structural proteins. Virus proteins were detected from 3 hpi, with no bands visible in mock-infected samples. These bands were of the expected size; the p30 protein was visible early in infection, from 3 hpi, and the p72 structural protein appears later in infection, increasing in amount from 6 hpi. Equivalent levels of viral proteins were seen in SV5GAL and vIKGAL infected cells. Immunofluorescence studies using an antibody to the viral p30 protein showed that approximately 50% cells were infected.

As the cells showed a successful and productive infection, the effects of this infection on levels of NF-κB p50 and p65 proteins were investigated to ascertain the effect of this infection on amounts of these proteins. Figure 3.2 shows levels of NF-κB p50 throughout infection. Bands corresponding to p50 are present at equivalent levels in all lanes; the p105 precursor protein can also be seen in all lanes.

Although no effect of virus infection on p50 protein levels was observed, the effect of infection on p65 was investigated. Figure 3.3A shows levels of NF-κB p65 throughout infection. Bands corresponding to p65 are present in all lanes. Levels of p65 vary through infection compared to levels in mock-infected cells. At 3 hpi following infection with SV5GAL, amounts of p65 protein were greatly increased compared to mock-infected cells. By 6 hpi, amounts of p65 had
Figure 3.1 Virus protein production following a time course of infection of Vero cells with SV5GAL and vlKGAL virus. Vero cells were infected or mock infected with virus and total proteins harvested in 200 μl SDS-PAGE sample loading buffer at 3, 6, 9, 12 and 18 hours post infection. 25 μl was run on an SDS-PAGE gel, transferred to nitro-cellulose and probed with ASFV hyper-immune serum (1/250). An HRP-conjugated protein A was used to visualise proteins by ECL. Figure representative of two experiments.
Figure 3.2 Levels of NF-κB p50 subunit during a time course of infection of Vero cells with SV5GAL and vlKGAL virus. Vero cells were infected or mock infected with virus and harvested in 200 μl SDS-PAGE sample loading buffer at 3, 6, 9, 12 and 18 hours post infection. 25 μl was run on an SDS-PAGE gel, transferred to nitrocellulose and probed with anti-NF-κB p50 (Santa Cruz, sc-1191, 1/500). An HRP-conjugated protein A was used to visualise proteins by ECL. Figure representative of two experiments.
Figure 3.3A Levels of NF-κB p65 subunit during a time course of infection of Vero cells with SV5GAL and vIKGAL virus. Vero cells were infected or mock infected with virus and harvested in 200 μl SDS-PAGE sample loading buffer at 3, 6, 9, 12 and 18 hours post infection. 25 μl was run on an SDS-PAGE gel, transferred to nitrocellulose and probed with anti-NF-κB p65 (Santa Cruz, sc-372, 1/500). An HRP-conjugated protein A was used to visualise proteins by ECL.

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Figure 3.3B Densitometry of bands corresponding to NF-κB p65. Bands corresponding to NF-κB p65 from figure 3.3 were analysed by densitometry. Density values were normalised to a mock-infected value and are expressed as values relative to this.
returned to the level seen in the mock-infected cells, and remained at that level at 
9 hpi. By 12 hpi, amounts of p65 increased to a similar level to that seen at 3 hpi. 
Amounts of p65 at 18 hpi were similar to those seen at 12 hpi. A similar pattern 
of expression was seen in vIKGAL infected cells, although amounts of p65 at 6 
hpi appeared less than the mock-infected cells, and the increase in amounts to 
those seen at 3 hpi occurs at 9 hpi. Amounts of p65 were semi-quantified by 
densitometry and levels of p65 protein relative to the levels in mock-infected 
cells were calculated. The results are shown in figure 3.3B. Relative to the 
amount present in mock-infected cells, the amount of p65 varied by up to 
approximately a two-fold increase or decrease.

Figure 3.3A also shows smaller bands of around 55 kDa present. These bands are 
visible in all lanes in which there is a large amount of p65: the 3, 12 and 18 hpi 
lanes in SV5GAL infection, and the 3, 9, 12 and 18 hpi lanes in vIKGAL 
infection. The bands appear to be slightly more intense in vIKGAL infected cells 
than in SV5GAL infected cells.

To investigate whether an effect of virus infection on levels of protein was 
confined to the NF-κB pathway, the levels of protein in the ERK MAP kinase 
pathway were studied over the course of infection. Figure 3.4 shows the levels of 
ERK throughout infection. Bands corresponding to ERK-1, ERK-2 and 
phosphorylated ERK-1 were visible in all lanes. Amounts of ERK remain 
constant throughout infection with the exception of the band corresponding to 
ERK2, which reduces in amount at 18 hpi in cells infected with SV5GAL and 
vIKGAL and appeared lower at 3 hpi in vIKGAL infected cells. Levels of ERK-
Figure 3.4 Levels of ERK during a time course of infection of Vero cells with SV5GAL and vIKGAL virus. Vero cells were infected or mock infected with virus and harvested in 200 μl SDS-PAGE sample loading buffer at 3, 6, 9, 12 and 18 hours post infection. 25 μl was run on an SDS-PAGE gel, transferred to nitro-cellulose and probed with anti-ERK-1 (Santa Cruz, sc-94, 1/500). An HRP-conjugated protein A was used to visualise proteins by ECL.
1 appeared slightly increased at 3, 12 and 18 hpi in vIKGAL infected cells compared to mock-infected cells.

To confirm that equivalent levels of protein were collected at each time-point, the amount of α-tubulin was assayed at each time-point. As this protein is a cytoskeletal protein and is expressed constitutively, levels of α-tubulin were expected to be constant at all times post-infection. Figure 3.5 shows the levels of α-tubulin throughout infection. Bands corresponding to the 54 kDa α-tubulin protein are visible in all lanes. Protein levels remain approximately constant at all times analysed post-infection, although there appears to be a slight reduction in amount of protein at 18 hpi in both SV5GAL and vIKGAL infected cells. Also, the mobility of the protein is slightly slower in the samples collected at 18 hpi.

To investigate whether the change in NF-κB p65 levels also occurred in macrophages infected with a field strain of ASFV, primary alveolar macrophages were obtained by lung lavage from freshly sacrificed baby pigs and cultured in 6-well plates. Cells were then infected with the Malawi isolate at an MOI of 5 or mock-infected. At 4, 8, 18 and 24 hpi, cells were harvested in 200 μl SDS-PAGE loading buffer. Samples were then resolved by SDS-PAGE, transferred to nitrocellulose and probed with anti-NF-κB p65 antibody. Figure 3.6 shows that p65 is detected at the correct size at all times post-infection. Amounts of protein are equivalent at all times post infection and in mock-infected cells. A smaller band of 55 kDa is visible in infected cell lanes, but not in the mock-infected lane. This smaller band shows an equivalent amount of protein at all times through infection.
Figure 3.5 Levels of α-tubulin during a time course of infection of Vero cells with SV5GAL and vlKGAL virus. Vero cells were infected or mock infected with virus and harvested in 200 µl SDS-PAGE sample loading buffer at 3, 6, 9, 12 and 18 hours post infection. 25 µl was run on an SDS-PAGE gel, transferred to nitro-cellulose and probed with anti-α tubulin (Santa Cruz, sc-5286, 1/500). An HRP-conjugated protein A was used to visualise proteins by ECL. Figure representative of two experiments.
Figure 3.6 Whole cell extracts of macrophages show cleavage of p65 following infection with ASFV. Primary alveolar macrophages were infected with the Malawi isolate of ASFV. At 4, 8, 18 and 24 h.p.i. cells were harvested in 200 µl SDS-PAGE sample buffer. 30 µl sample was resolved by SDS-PAGE, transferred to nitro-cellulose and probed with an anti-NF-κB p65 antibody (Santa Cruz, sc-372, 1/1000). HRP-conjugated protein A was used to detect proteins by ECL. Figure representative of two experiments.
3.6 Discussion

Immunoblotting of infected whole cell extracts over a time course of infection with a hyper-immune ASFV serum demonstrated that Vero cells were infected and produced the expected complement of viral proteins. Comparison of the onset of protein synthesis and intensity of bands produced showed that infection with SV5GAL and vIKGAL of Vero cells is comparable in these experiments. Previous studies (Miskin et al, 1998; Miskin et al., 2000) have shown equivalent growth curves for these viruses.

Relative amounts of NF-κB p50, ERK-1 and ERK-2 and α-tubulin remain approximately constant over a time course of infection. This is consistent with previous data (Alfonso et al., 2004), suggesting that there is no generalised ‘shut off’ of transcription or translation. These data could also be explained by a stabilising effect on these proteins, lengthening their half-lives, or a generalised inhibition of protein degradation, although these mechanisms are unlikely. The slight reduction in amounts of ERK and α-tubulin at 18 hpi may be due to a reduction in membrane integrity, as the cells become apoptotic, allowing the release of proteins into the supernatant.

One interesting observation was the change in amounts of the p65 subunit of NF-κB during infection in Vero cells. This is the subunit responsible for the majority of NF-κB-induced transcriptional transactivation. The increase in p65 levels observed at 3 hours post-infection may result from an activation of host-cell signalling pathways, following virus entry, leading to increased levels of transcription followed by translation of the p65 gene. Alternatively, this may
reflect decreased turnover of the p65 protein. In another study (Zhang et al., unpublished results), a ‘burst’ of pro-inflammatory cytokine gene transcription was observed early in infection of cells followed by a return to basal levels of transcription observed in mock-infected cells. The amount of p65 protein first increased at 3 hpi and then reduced to the level in mock-infected cells at 6 and 9 hours post-infection. These changes were similar to changes in the pattern of pro-inflammatory gene transcription at early stages of infection. However the increase in levels of p65 protein at 12 and 18 hours post-infection compared to mock-infected cells differed from the observed pattern of pro-inflammatory gene transcription which remained at the level in mock-infected cells at late times post-infection. Thus, although a decrease in the amount of p65 protein at 6 and 9 hours post-infection, compared to other times, might have some effect on NF-κB activity in infected cells, it is clear that the relative amounts of NF-κB proteins do not explain the inhibition of NF-κB-dependent gene transcription in ASFV infected cells.

As amounts of other proteins tested remain approximately constant throughout infection, it is likely that the change in p65 levels is a targeted effect, rather than a general modulation of host-cellular transcription and protein synthesis as described previously. Again, this could be explained at a number of levels: increased transcription, change in protein stability and half-life, change in degradation or a change in transcription. Further experiments to analyse the production of p65 mRNA throughout infection would help in the understanding of the stage at which this effect is occurring.
Interestingly, infection of primary macrophages with the Malawi isolate of ASFV produced a different pattern of p65 protein amounts through infection compared to that seen in Vero cells infected with the tissue culture adapted isolate BA71V. In macrophages, relative levels of p65 appear to remain constant throughout infection. Repetition of these experiments using more time-points and with a number of different field strains of the virus would be interesting. The macrophage data presented serves as a caveat for the extrapolation of data from Vero cell infections into cells more relevant in the disease.

The smaller anti-p65 cross-reactive protein bands of approximately 55 kDa that is visible through infection are also of interest. These bands may represent the cleavage of p65 by caspase-3. Caspase-3 cleaves p65 following the onset of apoptosis. Cleavage of p65 produces a dominant negative mutant of p65 of about 55 kDa, which is able to translocate to the nucleus and bind to κB sites in promoters without trans-activating transcription (Levkau et al., 1999). Repetition of these experiments in the presence of chemical inhibitors of caspase-3 would provide further data to support this interpretation of the data.

The p65 sub-unit of NF-κB is cleaved at the number 97 aspartic acid residue to produce a cleaved fragment which migrates at 55 kDa (Levkau et al., 1999). This cleavage is a downstream effect and follows the induction of apoptosis. Following the induction of apoptosis, caspase-3 is also able to cleave IκB-α to produce a dominant-inhibitory form of the protein which is able to inhibit NF-κB (Reuther & Baldwin, 1999). Although only a small amount of p65 is cleaved in infected macrophages, the combination of these two effects may be sufficient to
account for, at least in part, the inhibition of NF-κB-dependent gene transcription in infected cells. The visible cleavage of p65 by 4 hpi in infected macrophages is consistent with published observations in which caspase-3 activation was observed within 2 hpi (Carrascosa et al., 2002). The authors postulated that apoptosis may be induced as a result of virus uncoating. This may explain the low levels of this cleavage product throughout infection. Following an early induction of apoptosis, expression of viral anti-apoptotic genes prevents further stages of apoptosis allowing viral replication.

Although the total amount of p65 changes through infection of Vero cells, these data do not show whether the population of NF-κB that is able to transactivate transcription changes. The data do not show whether the NF-κB is located in the cytoplasm or nucleus, or whether it has the necessary post-translational modifications to the p65 subunit to transactivate transcription. This will be investigated in the following chapters.
4 Effect of ASFV infection on NF-κB and ERK nuclear translocation

4.1 Introduction

The data presented in the previous chapter show that ASFV infection of Vero cells causes some alteration in the relative levels of the p65 subunit of NF-κB, but does not alter levels of the p50 subunit. Thus, the inhibition of NF-κB-dependent gene transcription observed in ASFV infected cells is unlikely to be entirely due to decreased amounts of NF-κB. Instead, it is likely that activation of NF-κB is inhibited. This could be achieved by various mechanisms including:

- inhibition of nuclear translocation of NF-κB,
- inhibition of NF-κB binding to DNA or alteration of NF-κB activation by post-translational modification by phosphorylation or acetylation.

In this chapter the effect of ASFV infection on nuclear/cytoplasmic shuttling of NF-κB was examined. The shuttling of the MAP kinase ERK was also investigated, as this transcription factor is also partially regulated by its sub-cellular location.

Previous work (Powell et al., 1996, Revilla et al., 1998) had shown that A238L inhibited transcriptional trans-activation of an NF-κB dependent reporter gene. However, little was known about the mechanism by which this occurred. Super-shift EMSA assays had indicated that the main form of NF-κB inhibited by A238L was p50/p65 dimers (Revilla et al., 1998). One study (Tait et al., 2000) had suggested that A238L retained NF-κB in the cytoplasm, but this study analysed only one intermediate time-point, 8 hpi. Another study had shown that recombinant A238L protein could, in vitro, inhibit binding of NF-κB to DNA.
and displace NF-κB from preformed complexes with DNA. However, it remained unclear if this mechanism of A238L action occurred in infected cells. The aims for this section of my project were to develop a clean and efficient protocol to produce cytoplasmic and nuclear fractions from adherent cell cultures. Secondly, these methods were used to study the distribution of NF-κB and ERK-1 in ASFV infected cells.

To interpret the effects of ASFV infection in Vero cells, growth conditions for these cells in adherent culture were optimised to ensure that cellular pathways, specifically the NF-κB pathway, were not constitutively activated by serum present in the culture medium.

4.2 Optimisation of nuclear/cytoplasmic fractionation protocol

In most cell types NF-κB in its inactive state is localised in the cytoplasm in association with IκB. Activation by various stimuli results in degradation of IκB and nuclear accumulation of active NF-κB. To determine if A238L may inhibit nuclear translocation of NF-κB, or act within the nucleus to inhibit NF-κB, the amount of NF-κB in the nucleus was assayed following infection with a virus expressing A238L, SV5GAL, and an A238L gene deletion virus, vIKGAL.

A number of methods are available for fractionating cells into nuclear and cytoplasmic fractions, allowing determination of protein location. Three protocols were tested for their ability to produce clean fractions, with cytoplasmic fractions free from nuclear contaminants and vice versa.
Speed of isolation was important to minimise the amount of protein degradation. A commercially available fractionation kit was tested (Pierce, #78833) and a method for producing nuclear extracts for electrophoretic mobility shift assays (EMSA). These methods used two steps of detergent treatments: the first to lyse the plasma membrane and the second to permeabilise the nuclei. Samples were centrifuged at high speed before the second detergent step to pellet the nuclei, allowing removal of the cytoplasmic fraction.

A method using a Dounce homogeniser to lyse cells was also tested. This method used a close-fitting homogeniser in which the space between the homogeniser and the barrel was smaller than that of an average cell, but larger than that of a nucleus. Homogenisation would then open cells mechanically, leaving nuclei intact. Nuclei were pelleted by microcentrifugation leaving the cytoplasmic fraction in the supernatant.

Analysis of the fractions produced by SDS-PAGE and Western blotting (figure 4.1) shows that using cyclophilin A (CypA) as a marker for the cytoplasmic fraction is successful. Bands of the correct size are seen in the lane of purified recombinant CypA and the cytoplasmic fractions produced by the kit and the EMSA method. Bands corresponding to CypA are also seen in the cytoplasmic and nuclear fractions produced by the Dounce homogenisation method. This shows a contamination of the nuclear fraction by cytoplasmic proteins in fractions produced using the Dounce homogeniser. This could be due to insufficient homogenisation of cells or the homogeniser not being a tight enough fit to the barrel.
Although the antibody produced bands of the correct size, the use of PCNA as a marker for the nuclear fraction was unsuccessful. A band corresponding to PCNA was seen in the cytoplasmic fractions prepared by both the kit and the EMSA method, and in the cytoplasmic and nuclear fractions prepared by the Dounce homogeniser. The band visible in the nuclear fraction using the Dounce homogeniser method could be due to cytoplasmic contamination, as seen with the CypA blot as shown in figure 4.1.

As the EMSA method of fractionation was designed to produce extracts for gel shift assays, the harvesting of the nuclear fraction was designed to keep proteins in their active conformations. This step required more handling time and reduced the concentration of nuclear proteins obtained since this extract had to be denatured with SDS-PAGE loading buffer before electrophoresis. The EMSA fractionation protocol was therefore modified to harvest the nuclear fraction directly in SDS-PAGE loading buffer after removal of the cytoplasmic fraction. This method was used for future experiments in preference to the kit method, as the EMSA method was cheaper and the time from harvesting cells to denaturing and storage of samples was shorter.

As the use of anti-PCNA as a nuclear marker was unsuccessful, anti-histone H1 was used as a nuclear marker. Figure 4.2 shows a Western blot using anti-\( \alpha \)-tubulin and anti-histone simultaneously to detect proteins in fractionated cell samples. Bands of 54 kDa, the expected size for \( \alpha \)-tubulin, are seen in the cytoplasmic fractions where 30 \( \mu \)l, 20 \( \mu \)l and 10 \( \mu \)l of sample were run.
Figure 4.1 Testing of three methods for nuclear/cytoplasmic fractionation. Lane x shows purified CypA run as a control. Lanes A are the fractions prepared from a commercially available kit (Pierce #78833, performed following manufacturers instructions), B from a protocol used to produce nuclear fractions for EMSA (described in section 2.5), and C are fractions prepared using a Dounce homogeniser (Cell extracts were harvested in phosphate buffered saline including small protease inhibitors as described in section 2.5. Following homogenisation for 1 minute, samples were transferred to microcentrifuge tubes and spun for 5 minutes at maximum speed in a micro-centrifuge to pellet nuclei. Supernatants were denatured in SDS-PAGE loading buffer and pelleted nuclei harvested in SDS-PAGE loading buffer). Cytoplasmic fractions, labelled C were produced to a final volume of 200 µl and nuclear fractions, labelled N, to a final volume of 20 µl from a single well of a 6-well plate. 20 µl of sample was run per lane.
Figure 4.2 Testing of suitable control antibodies for clean nuclear/cytoplasmic fractionation. Nuclear/cytoplasmic fractions were produced using the modified EMSA nuclear fraction protocol as described in section 2.5. Vero cells were cultured in a T75 flask and fractionated. Cytoplasmic extracts were harvested in 1500 µl and nuclear fractions in 150 µl. Samples were resolved by SDS-PAGE. 30, 20, 10 and 5 µl of extracts were loaded per pair of lanes and transferred to nitrocellulose. Membrane was probed simultaneously with anti-α tubulin (Santa Cruz, sc-5286, 1/500) and anti-histone H1 (Santa Cruz, sc-8030, 1/1000). HRP-conjugated protein A was used to detect proteins by ECL.
No band was visible when 5 μl of sample was run. Bands corresponding to α-tubulin were not detected in nuclear fractions.

Bands migrating at 32 kDa corresponding to histone H1 were observed in the nuclear fractions when 30 μl, 20 μl and 10 μl of sample were analysed, but were not visible in the lane in which 5 μl of sample was analysed. A number of other bands were visible in the lanes corresponding to the nuclear fractions. These could be due to the antibody cross-reacting with other proteins, detection of histone H1 acetylated at a number of sites, or a combination of both. As both antibodies were used together to probe the same membrane, the other bands could also be the result of the anti-α tubulin antibody cross-reacting with a nuclear protein. In later experiments the antibodies were used separately and this demonstrated that the anti-tubulin antibody did not cross-react with nuclear proteins.

Although bands in addition to the 32 kDa histone H1 protein were detected in nuclear fractions, it was decided that the use of these two proteins was acceptable as a fractionation control. However, to simplify interpretation, detection with anti-α tubulin and anti-histone H1 was performed on separate blots. This also resolved the cross-reaction of the anti-α-tubulin antibody with nuclear extracts.

4.3 NF-κB nuclear translocation following ASFV infection

The nuclear localization of NF-κB partially determines whether or not it is capable of transcriptional transactivation and hence could give an indication of
how A238L acts to inhibit NF-κB activation, although there are other regulatory mechanisms involved (see section 1.4).

First, conditions were optimised to maintain NF-κB in the cytoplasm in unstimulated cells. NF-κB can be activated by growth factors, as well as by serum starvation, so the concentration of serum in cell culture medium which did not cause activation of NF-κB was established.

Culturing Vero cells in DMEM supplemented with 2% FBS maintained NF-κB as predominantly cytoplasmic in unstimulated cells as analyzed by immunofluorescence. Figure 4.3 shows that p65 and p50 are maintained in the cytoplasm of Vero cells when cultured under these conditions. Vero cells, at a confluency of 40-60%, were assayed by immunofluorescence using anti-p65 and anti-p50 antibodies. Higher confluency led to activation and nuclear translocation of NF-κB. To maintain consistency, Vero cells of passage number 25-40 were used for NF-κB activation experiments.

The phorbol ester phorbol 12-myristate 13-acetate (PMA) was used to experimentally activate the NF-κB pathway. Initially, a concentration of 10 nM was used, but this gave inconsistent results. A concentration of 100 nM consistently caused nuclear localisation of NF-κB p65 in greater than 95% of cells. When Vero cells were cultured with the conditions described above, NF-κB p65 translocated to the nucleus within 1 hour following PMA stimulation (Figure 4.4).
Figure 4.3 p65 and p50 are predominantly cytoplasmic in unstimulated cells. Vero cells were grown on coverslips in 24-well plates in medium supplemented with 2% FBS. Cells were then fixed in paraformaldehyde and assayed by indirect immunofluorescence. Primary antibodies were rabbit anti-p65 (Santa Cruz, sc-372, 1/200) and goat anti-p50 (Santa Cruz, sc-1191, 1/200). Secondary antibodies were anti-rabbit immunoglobulin G (Molecular Probes, a-21207, 1/200) and anti-goat immunoglobulin G (Molecular Probes, a-11005, 1/200).
Figure 4.4 The p65 subunit of NF-κB translocates to the nucleus following stimulation with a phorbol ester (PMA). Vero cells were grown on coverslips in 24-well plates in medium supplemented with 2% FBS. Cells were then stimulated for 1 hour with 100 nM PMA, fixed in paraformaldehyde and assayed by indirect immunofluorescence. Primary antibody was rabbit anti-p65 (Santa Cruz, sc-372, 1/200). Secondary antibody was anti-rabbit immunoglobulin G (Molecular Probes, a-21207, 1/200)
Vero cells grown on coverslips were infected with the Ba71V strain of ASFV at an MOI of 1. At 4 hpi, cells were fixed in paraformaldehyde and NF-κB p65 location investigated by immunofluorescence. A time of 4 hpi was chosen since preliminary experiments, using non-confocal immunfluorescence microscopy, showed this as the earliest time p65 was detected in the nucleus. Figure 4.5 shows, interestingly, that p65 translocates to the nucleus following ASFV infection. Cells were also stained with an antibody against a virus protein, p30, as a control to show cells were infected.

IPAM cells, an immortalised macrophage cell line (Weingartl et al., 2002), were grown in six well plates and infected with the Malawi isolate of ASFV at an MOI of 5. Virus stocks had been concentrated in filter columns which should reduce the amount of secreted factors present in the tissue culture supernatant. At 3, 6, 9, 12, 18 and 24 hpi, cells were harvested and fractionated into cytoplasmic and nuclear fractions. Samples were resolved by SDS-PAGE, Western blotted, and probed with antibodies against histone H1, NF-κB p65 and ERK-1.

Histone H1 was detected as a 32 kDa protein as expected. Figure 4.6 shows that there is no detectable contamination of the cytoplasmic fraction with nuclear proteins until 18 hpi when small amounts of protein cross-reacting with the anti-histone H1 antibody can be detected in cytoplasmic fractions. At 18 hpi nuclear membrane integrity may be compromised since infected cells can enter apoptosis at late times post-infection. However, this band is not of the expected size and may result from the antibody cross-reacting with another protein.
Figure 4.5 p65 translocates to the nucleus during ASFV infection. Vero cells were grown on coverslips in 24-well plates and infected with the Ba71V strain of ASFV. At four hours post-infection, cells were fixed in paraformaldehyde and assayed by indirect immunofluorescence using confocal microscopy. Image shows a single optical section. Primary antibodies were mouse anti-vp30 (gift from Mr. Mick Denyer, Institute for Animal Health, 1/5) and rabbit anti-p65 (Santa Cruz, Santa Cruz, sc-372, 1/200). Secondary antibodies were anti-rabbit immunoglobulin G (Molecular Probes, a-21207, 1/200) and anti-mouse immunoglobulin G (Molecular Probes, a-11001, 1/200).
Figure 4.6 The translocation of transcription factors in IPAM cells infected with ASFV. IPAM cells were infected with the Malawi isolate of ASFV. At 3, 6, 9, 12, 18 and 24 hours post infection cells were harvested and fractionated into nuclear and cytoplasmic fractions as described in materials and methods. 25 μl of extract was resolved by SDS-PAGE, transferred to nitro-cellulose and probed with either: A, anti-histone H1 (Santa Cruz, 1/500); B, anti-NF-κB p65 (Santa Cruz, 1/500); C, anti Erk-1 (Santa Cruz, 1/500). HRP-conjugated protein A was used to detect bound antibodies by ECL.
Of interest is the finding that p65 is present in the nucleus at all times post-infection analysed in IPAM cells. However, it appears that nuclear levels of p65 protein are reduced at early and mid times (3, 6, 9 and 12 hours) post-infection, compared to mock-infected cells. A similar pattern of nuclear cytoplasmic distribution is seen for ERK-1 and ERK-2. These proteins were detected in the nucleus throughout infection, although reduced levels of these proteins were also detected in the nucleus at early and mid points (3, 6, 9 and 12 hours) post-infection compared to mock-infected cells.

An unusual observation in IPAM cells is the change in the pattern of bands detected by the anti-histone H1 antibody. Two bands, of 32 kDa and 45 kDa were detected in mock-infected cells, with the lower band, of 32 kDa, being more prominent. The lower band could not be detected in cells 3 or 6 hours post-infection, and the amount of the upper band was markedly reduced. Both bands could be seen from 9 hpi onwards, and increased in amount until 18 hpi, but reduced again at 24 hpi.

Figure 4.7 shows the same experiment performed in Vero cells infected with the SV5 pk-tagged A238L (SV5GAL) ASFV virus. NF-κB p65 was detected in the nucleus at all times post infection. At 3, 6, 9 and 12 hpi, nuclear levels of p65 are reduced compared to mock, 18 and 24 hpi, although cytoplasmic levels remain consistent through infection. These results, as expected, agree with those obtained using the wild type Ba71V isolate in Vero cells (data not shown).
Figure 4.7 The translocation of transcription factors in Vero cells infected with SV5GALASFV. Vero cells were infected with the SV5GAL ASFV. At 3, 6, 9, 12, 18 and 24 hours post infection cells were harvested and fractionated into nuclear and cytoplasmic fractions as described in materials and methods. 25 μl of extract was resolved by SDS-PAGE, transferred to nitro-cellulose and probed with either anti-histone H1 (Santa Cruz, 1/500), anti-NF-κB p65 (Santa Cruz, 1/500), anti-Erk-1 (Santa Cruz, 1/500), anti-α tubulin (Santa Cruz, 1/500), anti-SV5 pk tag (Serotec, 1/500) or anti-NF-κB p50 (Santa Cruz, 1/500). HRP-conjugated protein A was used to detect bound antibodies by ECL.
NF-κB p50 was detected in the cytoplasm at all timepoints. Amounts of p50 protein remained quite consistent early in infection, although a significant reduction was observed at 12 hpi. The amount of p50 detected had increased again by 18 hpi. Levels of p50 in nuclear fractions were very low and are barely detectable until 12, 18 and 24 hpi.

Levels of ERK remained consistent in cytoplasmic fractions at all times post-infection. ERK is detectable in the nucleus at all times post-infection, with reduced levels seen in nuclear fractions, 3, 6, 9 and 12 hours post infection compared to mock, 18 and 24 hpi. These results were also the same as obtained with the wild-type Ba71V isolate (data not shown).

Using the anti-pk antibody to detect the A238L protein showed that both forms of A238L were detected in cytoplasmic fractions from 6 hpi. A238L was detected in the nucleus from 12 hpi onwards and the predominant form of A238L detected in the nucleus was the 32 kDa form.

The anti-histone H1 blot showed no contamination of the cytoplasmic fraction with nuclear proteins. The anti-histone blot only shows a faint band of 32 kDa in nuclear fractions throughout infection at all time points. This pattern of histone H1 expression did not alter at different times post-infection of Vero cells. The blot for anti-α-tubulin showed bands of the expected size present in cytoplasmic fractions at all time points, only faint bands were seen in the nuclear fraction throughout infection, indicating a slight contamination of nuclear fractions with cytoplasmic proteins. The increased levels of α-tubulin seen in the nuclear
fraction at 18 and 24 hpi, may be due to nuclear membrane breakdown as a consequence of apoptosis.

In Vero cell infections with the A238L gene deletion virus (vIKGAL) a similar pattern of protein distribution was observed as in Vero cells infected with wild type Ba71V and the Ba71V expressing epitope tagged A238L protein. These data are shown in figure 4.8. The p65 blot following vIKGAL infection shows, in addition to the p65 band of expected molecular weight, a band of 55 kDa; visible at 18 and 24 hours post infection. This may be the cleaved form of p65 produced by caspase-3 cleavage (see section 4.5). This 55 kDa band is visible predominantly in nuclear fractions.

4.4 Effects of A238L on NF-κB p65 post-translational modifications

As discussed previously, in section 1.4, NF-κB activity is further regulated by post-translational modification of the p65 subunit; phosphorylation is required for transcriptional transactivation and acetylation prevents association with IκB hence potentiating transcriptional activation dependent on NF-κB.

To investigate the post-translational modification status of p65 in cells expressing A238L, Vero cells were transfected with a plasmid constitutively expressing the A238L gene under control of the CMV IE promoter. Vero cells were also transfected with an empty vector as a control. Cells were then
Figure 4.8 The translocation of transcription factors in Vero cells infected with vIKGAL ASFV. Vero cells were infected with the vIKGAL ASFV. At 3, 6, 9, 12, 18 and 24 hours post infection cells were harvested and fractionated into nuclear and cytoplasmic fractions as described in materials and methods. 25 µl of extract was resolved by SDS-PAGE, transferred to nitro-cellulose and probed with either anti-histone H1 (Santa Cruz, 1/500), anti-NF-κB p65 (Santa Cruz, 1/500), anti Erk-1 (Santa Cruz, 1/500), anti-α tubulin (Santa Cruz, 1/500) or anti-NF-κB p50 (Santa Cruz, 1/500). HRP-conjugated protein A was used to detect proteins by ECL.
stimulated with 100 nM PMA for 30 or 60 minutes, cell extracts prepared and immunoprecipitations performed using an antibody against p65. Samples were resolved by SDS-PAGE, transferred to nitrocellulose and probed with antibodies to phospho-serine, acetyl groups and p65.

Figure 4.9 (lower panel) shows bands of the expected size which correspond to the p65 subunit of NF-κB. Levels of p65 remained approximately equivalent in the samples harvested at 30 and 60 minutes after mock-stimulation. The top panel shows phosphorylated p65. The intensity of the 65 kDa band increased in empty vector transfected cells 30 minutes post-stimulation before a slight decrease in intensity by 60 minutes post-stimulation. This contrasts with A238L transfected cells in which the increase in intensity does not occur until 60 minutes-post stimulation. The centre panel shows a 65 kDa band in all lanes corresponding to acetylated p65. A slight decrease in intensity was observed in empty vector transfected cells at 30 minutes post-stimulation. In A238L transfected cells, a greater decrease in intensity of the 65 kDa band was observed at 30 minutes post-infection. The intensity did not increase by 60 minutes post-infection as seen in empty vector transfected cells.
Figure 4.9 Analysis of p65 post-translational modifications. Vero cells were transfected with empty pcDNA3 vector or a vector containing the A238L gene. Cells were then stimulated with PMA for 30 or 60 minutes or left unstimulated (M). Following stimulation, cell extracts were prepared and p65 immunoprecipitated using anti-p65 (Santa Cruz, sc-372). Immunoprecipitates were then resolved by SDS-PAGE, transferred to nitro-cellulose and immunodetected with either anti-phosphoserine (Qiagen, Q5, 1/250), anti-pan acetyl (Santa Cruz, sc-8649, 1/250) or anti p65 (Santa Cruz, sc-372, 1/500). HRP-conjugated protein A was used to detect bound antibodies by ECL.
4.5 Discussion

The key finding of this chapter was the observation that the p65 subunit of NF-κB is present in the nucleus at all times post-infection with either SV5GAL or vIKGAL ASFV. Previous work had shown that A238L inhibits NF-κB-dependent reporter gene expression. The demonstration that the 32 kDa form of A238L was co-precipitated with the p65 subunit of NF-κB after IκB degradation suggested that A238L might function as an IκB homologue and bind to NF-κB directly. This previous work had suggested that A238L may sequester NF-κB in the cytoplasm. However, only one time point (8 hpi) post-infection was analysed in these previous experiments and at that time point NF-κB was not detected in the nucleus (Tait et al., 2000). In the present study the amount of p65 present in the nucleus increased at later times post-infection but it was clearly detected in the nucleus even at early times post-infection. The control antibodies used to monitor cross-contamination of nuclear and cytoplasmic fractions confirmed that this was a minor problem observed to a small extent only at later times post-infection. The discrepancy between the results described here and the previous results are most likely due to differences in the sensitivity of detecting p65 in the two sets of experiments in addition to the wider range of time points analysed in the present study.

The finding that p65 translocates to the nucleus in infected cells, suggests that A238L may act within the nucleus to inhibit NF-κB activity rather than by retaining NF-κB in the cytoplasm.
The observation of a 55 kDa product using the anti-p65 antibody at late points post-infection may result from a caspase-3-mediated cleavage of p65 to form the dominant-negative mutant form of p65. The cleaved band is of the correct size for the caspase-3 cleaved p65 protein and its appearance at late times post-infection is consistent with the observed activation of caspase-3 and induction of apoptosis in ASFV infected cells (Carrascosa et al., 2002). The apparent increased amounts of cleaved fragment in vIKGAL infected cells compared to SV5GAL infected cells, suggests that A238L may delay or partially inhibit the cleavage of p65. The caspase-3 cleavage site is in the DNA-binding region of p65 (Kang et al., 2001). If this is the region where A238L is bound, cleavage may be inhibited as access of the caspase-3 protein may be obstructed.

The amounts of p65, p50 and ERK detected in the nucleus were reduced from 3 to 9 hpi compared to mock-infected cells and cells at later times post-infection. These effects were observed in Vero cells infected with SV5GAL or vIKGAL virus and in IPAM cells and were therefore not correlated with expression of A238L. The amount of nuclear ERK was also decreased at these times post-infection compared with later times and with mock-infected cells. This could suggest a more generalised effect on nuclear transport at these times post-infection or that these two key signalling pathways are specifically targeted for inhibition.

Interference with nuclear transport could be a general consequence of ASFV infection. As the virus replicates in factories adjacent to the nucleus and close to the microtubule organising centre, transport of proteins to the nucleus on
microtubules could be impaired. The effects on nuclear accumulation of p65 and ERK are visible from 3 hours post-infection, before formation of the virus factory. However microtubule-mediated transport of proteins may be disrupted even at early times post infection as there is extensive cytoskeletal rearrangement in ASFV infected cells (Carvalho et al., 1988); the beginnings of this reorganisation could lead to impaired trafficking of proteins. Trafficking may also be reduced due to competition between cellular and viral proteins for motor proteins. However nuclear accumulation of p65 and ERK does increase at late times post-infection when virus factories are fully formed, arguing against a general reduction of nuclear transport in virus infected cells.

A possible alternative explanation for the observed reduction in nuclear levels of ERK and NF-κB could be a reduction in levels of these proteins following infection. However, the previous chapter showed that ERK and p50 levels remain constant through infection and p65 levels increase at three hours post-infection.

The consistent levels of histone H1 in the nucleus observed at all time-points post-infection in Vero cells, show that the reduction in amounts of nuclear p65 and ERK protein between 3 and 9 hpi is not an artefact due to poor recovery of nuclear fractions at these times.

The effect could be due to another virus encoded protein. As ASFV has a large coding capacity, it may encode genes with functional redundancy. It is possible that other genes act to inhibit NF-κB at different stages of the pathway. One
possible mechanism could be inhibition of the pathway prior to the degradation of IκB such that complexes are retained in the cytoplasm. For example, the inhibition of calcineurin activity may also be partially responsible for the decrease in NF-κB activity by reducing NF-κB activation by protein kinase C. A further possible mechanism for the NF-κB inhibition could be due to the increase of TGF-β production following ASFV infection (Powell et al., 1996). TGF-β has been shown to lead to increased IκB expression (Arsura et al., 1996) which could reduce NF-κB activity.

The data presented also show that A238L translocates to the nucleus in infected cells. This is particularly evident at late times post-infection (from 12 hpi) and it is mainly the higher molecular weight form of A238L that accumulates in the nucleus. The previous data (Tait et al., 2000) showed that A238L was present in the cytoplasm at 8 hpi. The discrepancy the results presented here and previous results could be explained by the wider range of time points examined in this study and higher sensitivity of detecting A238L.

Analysis of the effect of A238L, expressed from a transfected plasmid, on the post-translational modification of p65 at 30 and 60 minutes post-stimulation, suggests that phosphorylation of p65 may be delayed in cells transfected with A238L, although the data are not yet conclusive. Experiments (data not shown) were performed using post-stimulation time-points of 60 and 120 minutes. At these times, no difference was observed in relative levels of p65 phosphorylation and acetylation between cells transfected with a vector expressing A238L and transfected with the empty vector. A reduction in the rate of p65 phosphorylation
in the presence of A238L would not be unexpected. A238L binding to p65 may impair the ability of the kinase responsible to access the phosphorylation site of p65.

The preliminary results suggesting that levels of p65 acetylation are reduced in A238L transfected cells are also of interest, although, again, these data need to be confirmed. A reduction in acetylated p65 in the nucleus would allow more p65 to be bound by free nuclear IκB-α. This would inhibit NF-κB-mediated transactivation.

The resolution of the data from these experiments is limited by the transfection efficiency. In similar experiments using other transfected plasmids, the transfection efficiency was estimated as 30%-40% by detecting the expressed protein by immunofluorescence. This transfection efficiency may not be sufficient to resolve small differences in modification. A recombinant baculovirus developed in the laboratory by Ms Rhiannon Silk has been used to transfect mammalian cells with over 90% efficiency. Future experiments performed using this system may reveal differences between treatments not observed here.
5 Nuclear translocation of A238Lp

5.1 Introduction

In the previous chapter, data was presented showing that nuclear translocation of NF-κB was not inhibited in ASFV infected cells and that the 32 kDa form of the A238L protein also translocates and accumulates in the nucleus at late times post-infection. Previous preliminary data from Dr. Charles Abrams, shown in figure 5.1, also suggested that A238L accumulates in the nucleus. These results suggest that A238L may function within the nucleus to inhibit NF-κB activity. However, it is not clear how A238L translocates into the nucleus. Since the primary sequence of A238Lp did not reveal a canonical nuclear localization sequence as predicted by the PredictNLS program (http://cubic.bioc.columbia.edu/predictNLS/). An alignment of A238L and IκB is shown in chapter 1 (figure 1.14B). This alignment shows that no sequence of A238L resembles the NLS of IκB. An understanding of the mechanism for this transport might allow a greater understanding of the mechanism by which A238L inhibits NF-κB activation.

5.2 Investigation of A238Lp nuclear transport

Vero cells were grown in six-well plates and infected with the SV5GAL recombinant of ASFV which expresses an epitope tagged (SV5 pk) A238L protein. At 3h, 6h, 8h, 12h and 16h post-infection, the cells were harvested and fractionated to produce cytoplasmic and nuclear fractions. The DNA synthesis inhibitor cytosine arabinoside (AraC) was added to one well. AraC inhibits both virus DNA replication and late virus gene expression, which depends on DNA replication. This was used to confirm that A238Lp expression and nuclear
Figure 5.1 Cytoplasmic/nuclear distribution of A238L through a timecourse of infection I. Vero cells were grown in six-well plates and infected with the SV5-Gal recombinant of ASFV. At various times post infection, cells were harvested and fractionated into nuclear and cytoplasmic components. These were then resolved by SDS-PAGE, transferred to nitrocellulose and probed for the SVS pk tag (Serotec, MCA1360, 1/1000). Secondary antibody was rabbit anti-mouse immunoglobulins (DAKO, P0260, 1/1000).

Figure 5.2 Cytoplasmic/nuclear distribution of A238L through a timecourse of infection II. Vero cells were grown in six-well plates and infected with the SV5-Gal recombinant of ASFV. At various times post infection, cells were harvested and fractionated into nuclear and cytoplasmic components. These were then resolved by SDS-PAGE, transferred to nitrocellulose, and probed for the SVS pk tag (Serotec, MCA1360, 1/500). An HRP-conjugated protein A was used to visualise bound antibodies by ECL.
transport did not require virus DNA and late protein synthesis. Samples were resolved by SDS-PAGE, Western blotted and probed for A238Lp using an antibody to the SV5 pk tag. Protein A conjugated to horseradish peroxidase (HRP) was used to visualise bands by ECL.

Figure 5.2 shows that A238Lp was present from 3 hpi onwards throughout infection. Expression of A238Lp was not prevented by inhibiting DNA synthesis by cytosine arabinoside (AraC), although the amount of A238Lp present in this sample appears slightly less than at the equivalent time-point without AraC. This confirmed that A238L is expressed at early time-points post-infection. The reduced A238Lp levels observed in the presence of AraC suggests that synthesis continues at late time points post-infection.

Due to the higher amounts of protein loaded on gels in this experiment, compared to that in figure 5.1 (and figures 4.7 and 4.8), the two forms of A238Lp have not been clearly resolved. The use of more protein in this experiment does however show that A238Lp is present in the nucleus from 3 hpi. Figure 5.1 also shows a small amount of A238Lp present in the nucleus at 5 hpi.

An interesting observation is that the amount of A238Lp in the nucleus appears less from 3 to 6 hpi, with levels increasing by 8 hpi. The amount of A238Lp seen in the nucleus at 16 hpi may not all be due to active transport to the nucleus. At this late stage in infection, as the cells go into apoptosis, disruption of the nuclear membrane may lead to cytoplasmic populations of proteins moving into the nucleus by diffusion. A small amount of α-tubulin was detected
in nuclear fractions at 18 hpi suggesting that some nuclear diffusion of cytoplasmic proteins may occur at late times post-infection (see chapter 4).

As NF-κB can be activated within minutes of a stimulus, if A238Lp could be found in the viral particle this would allow NF-κB to be inhibited by A238L immediately following viral uncoating. ASFV particles were purified on Percoll gradients and lysed in SDS-PAGE sample buffer. The samples were resolved on an SDS-PAGE gel, Western-blotted and probed with an anti-pk tag antibody. Infected and uninfected Vero cell extracts were run as controls. As a further control, to check for the presence of virus proteins in the sample, blots were probed with pig hyper-immune serum. Figure 5.3 shows that A238L could not be detected in the virus particle. A band corresponding to the lower molecular weight form of A238L was detected in extracts from infected cells, but not in the uninfected cell extracts. The blot with ASFV hyper-immune serum shows that virus structural proteins are present in the purified virus sample.

5.2.1 A238L localisation in p65 knockout cells

One hypothesis for A238Lp transport to the nucleus is that it moves into the nucleus in complex with NF-κB. As the A238L protein is shorter than IkB and has fewer ankyrin repeats, A238Lp binding to NF-κB in the cytoplasm may not mask the NLS of p65. Although IkB does not mask the NLS of the p50 subunit of NF-κB, its NES causes the complex to shuttle out of the nucleus. No typical leucine-rich motif, which may function as an NES, can be found in the A238Lp amino acid sequence (shown in figure 1.14 and appendix I).
Figure 5.3 A238L cannot be detected in the ASFV virus particle. Virus was purified on Percoll gradients and lysed in SDS-PAGE sample buffer. Samples were then resolved by SDS-PAGE, transferred to nitro-cellulose and probed with anti SV5 pk tag (Serotec, MCA11360, 1/250) to detect A238L and pig hyper-immune serum (Gift from Mr. Mick Denyer, Institute for Animal Health, 1/500) as a positive control. Proteins from purified virus is shown (Virus) as well as cell extracts from non-infected (-ve) and infected (+ve) Vero cells were run as controls. An HRP-conjugated protein A was used to visualise bound antibodies by ECL.
Further analysis of A238L and p65 protein movement was performed in a mouse embryonic fibroblast (MEF) cell line. This cell line was a gift from Professor Ron Hay at the University of St. Andrews and was homozygous negative for the p65 subunit of NF-κB. A p65 +/+ was provided as a control.

To confirm that p65 distribution was cytoplasmic in unstimulated MEFs, and to optimize conditions for future experiments, p65 distribution was investigated by indirect immunofluorescence in MEF cells cultured in DMEM supplemented with 10% FBS. Figure 5.4 shows that NF-κB p65 remains cytoplasmic in cells cultured in these conditions. A plasmid encoding A238L under the control of the cytomegalovirus (CMV) immediate early (IE) promoter was transfected into p65 +/+ MEFs. Following transfection and culture, cells were fixed in paraformaldehyde and the localisation of p65 and A238Lp determined by immunofluorescence. Figure 5.5 shows that both p65 and A238Lp remain predominantly cytoplasmic in unstimulated MEFs.

The effect of PMA stimulation on the distribution of these proteins was then investigated. MEF cells were cultured and transfected as above and, following an equivalent incubation period following the addition of transfection reagent, cells were stimulated with 100 nM PMA for one hour. Figure 5.6 shows the redistribution of proteins following PMA stimulation. Compared to figure 5.5, there is a marked increase of p65 and A238Lp in the nucleus.

The same experiment was performed in p65 -/- cells. Cells were grown under identical conditions as in the previous experiment, with exactly the same
Figure 5.4 The p65 subunit of NF-κB is cytoplasmic in unstimulated mouse embryonic fibroblasts. MEFs were grown on coverslips in 24-well plates and maintained in medium supplemented with 10% FBS. Cells were then fixed in paraformaldehyde and assayed by indirect immunofluorescence using confocal microscopy. Image shows a single optical section. Primary antibody was rabbit anti-p65 (Santa Cruz, sc-372, 1/200). Secondary antibody was anti-rabbit immunoglobulin G (Molecular Probes, a-21207, 1/200).
Figure 5.5 A238L is cytoplasmic in unstimulated mouse embryonic fibroblasts. MEFs were grown on coverslips in 24-well plates and maintained in medium supplemented with 10% FBS. Cells were then transfected with an A238L construct expressing A238L under the control of the CMV IE promoter, cultured for 24 hours and fixed in paraformaldehyde and assayed by indirect immunofluorescence using confocal microscopy. Image shows a single optical section. Primary antibodies were rabbit anti-p65 (Santa Cruz, sc-372, 1/200) and mouse anti-SV5 pk-tag (Serotec, MCA1360, 1/200). Secondary antibodies were anti-rabbit immunoglobulin G (Molecular Probes, a-21207, 1/200) and anti-mouse immunoglobulin G (Molecular Probes, a-11001, 1/200).
Figure 5.6 A238L translocates to the nucleus concurrently with p65 in mouse embryonic fibroblasts following stimulation with phorbol ester. MEFs were grown on coverslips in 24-well plates in medium supplemented with 10% FBS. Cells were transfected with an A238L construct and cultured for 24 hours. Following stimulation with 100 nM PMA for 1 hour, cells were fixed in paraformaldehyde and assayed by indirect immunofluorescence using confocal microscopy. Image shows a single optical section. Primary antibodies were rabbit anti-p65 (Santa Cruz, sc-372, 1/200) and mouse anti-SV5 pk-tag (Serotec, MCA1360, 1/200). Secondary antibodies were anti-rabbit immunoglobulin G (Molecular Probes, a-21207, 1/200) and anti-mouse immunoglobulin G (Molecular Probes, a-11001, 1/200).
Figure 5.7 A238L remains cytoplasmic following phorbol ester stimulation in p65 knockout mouse embryonic fibroblasts. MEFs were grown on coverslips in 24-well plates in medium supplemented with 10% FBS. Cells were transfected with an A238L construct and cultured for 24 hours. Following stimulation with 100 nM PMA for 1 hour, cells were fixed in paraformaldehyde and assayed by indirect immunofluorescence using confocal microscopy. Image shows a single optical section. Primary antibody was mouse anti-SV5 pk-tag (Serotec, MCA1360, 1/200). Secondary antibody was anti-mouse immunoglobulin G (Molecular Probes, a-11001, 1/200).
transfection protocol and stimulation methods used. Figure 5.7 shows that A238Lp remains cytoplasmic following PMA stimulation when p65 is not present.

5.2.2 Effect of inhibiting crm 1 mediated nuclear export on A238L localisation

As described previously, A238L does not have a predicted nuclear export signal and is therefore not expected to cause export of NF-κB complexes from the nucleus to the cytoplasm. Further work aimed to characterise the differences between IκB and A238Lp at the level of cellular distribution and shuttling. The effects of the inhibitor of crm-1 mediated nuclear export, leptomycin B (LMB) (Nishi et al., 1994; Wolff et al., 1997), on nuclear accumulation of IκB and A238Lp was examined. Plasmids expressing SV5-PK tagged IκB or A238L were transfected into cells and after overnight incubation cells were stimulated with 100 nm PMA in the presence or absence of LMB for 3 hours. Figure 5.8 shows that following LMB treatment of Vero cells and stimulation with PMA, after three hours all IκB in the cell is present in the nucleus, as is all the p65. This confirmed that LMB treatment had worked as previously reported (Tam et al., 2000) to prevent nuclear export of IκB/NF-κB complexes and cause their nuclear retention. In contrast, figure 5.9 shows that, when a similar experiment was performed following transfection of A238L into cells, a significant fraction of A238Lp remains in the cytoplasm after three hours of LMB and PMA treatment. This shows A238L protein found in the cytoplasm of cells is present because it has not translocated into the nucleus rather and been exported, although the majority of A238Lp seems to translocate to the nucleus.
Figure 5.8 IκB accumulates in the nucleus following stimulation with phorbol ester in the presence of leptomycin B. Vero cells were grown on coverslips in six-well plates and transfected with SV5 pk-tagged IκB-α. Cells were then treated for 3 hours with leptomycin B, fixed in paraformaldehyde and assayed by immunofluorescence using confocal microscopy. Image shows a single optical section. Primary antibodies were rabbit anti-p65 (Santa Cruz, sc-372, 1/200) and mouse anti-SV5 pk tag (Serotec, MCA1360, 1/200). Secondary antibodies were anti-rabbit immunoglobulin G (Molecular Probes, a-21207, 1/200) and anti-mouse immunoglobulin G (Molecular Probes, a-11001, 1/200).
Figure 5.9 Not all A238L accumulates in the nucleus following stimulation with phorbol ester in the presence of leptomycin B. Vero cells were grown on coverslips in six-well plates and transfected with SV5 pk-tagged A238L. Cells were then treated for 3 hours with leptomycin B, fixed in paraformaldehyde and assayed by immunofluorescence using confocal microscopy. Image shows a single optical section. Primary antibodies were rabbit anti-p65 (Santa Cruz, sc-372, 1/200) and mouse anti-SV5 pk tag (Serotec, MCA1360, 1/200). Secondary antibodies were anti-rabbit immunoglobulin G (Molecular Probes, a-21207, 1/200) and anti-mouse immunoglobulin G (Molecular Probes, a-11001, 1/200).
5.3 Mechanism of NF-κB transport

The actual mechanism by which NF-κB shuttles to the nucleus remains poorly defined. A common mechanism of intracellular transport is along microtubules to the microtubule organising centre next to the nucleus. Preliminary experiments, examining p65 localisation following stimulation and ASFV infection, suggested that p65 accumulated in a specific area next to the nucleus prior to its translocation into the nucleus (data not shown).

To investigate whether p65 may move to the nucleus on microtubules, Vero cells were cultured and stimulated with PMA before being fixed and analyzed by immunofluorescence. Cells were stained with anti p65 and anti α-tubulin. Figure 5.10 shows the distribution of p65 and α-tubulin at one and two hours post-stimulation with PMA. The distribution of each protein appears very similar, with a dense accumulation next to the nucleus and a similar distribution through the cytoplasm and around the cell periphery.

Microtubules can be reversibly disrupted by the drug nocodazole which acts to destabilize microtubules and disrupt their polymerisation. Cells were mock-treated or treated with nocodazole prior to stimulation with PMA. Cells were then fixed, stained with anti-p65 and analyzed by immunofluorescence. As can be seen from figure 5.11, disruption of the microtubule network inhibits movement of p65 to the nucleus.
Figure 5.10 Distribution of p65 and α-tubulin following stimulation of Vero cells with phorbol ester. Vero cells were grown on coverslips in 24-well plates in medium supplemented with 2% FBS. Cells were then stimulated for 1 or 2 hours with 100 nM PMA, fixed in paraformaldehyde and assayed by indirect immunofluorescence. Primary antibodies were rabbit anti-p65 (Santa Cruz, sc-372, 1/200) and mouse anti-α tubulin (Santa Cruz, sc-5286, 1/200). Secondary antibodies were anti-mouse immunoglobulin G (Molecular Probes, a-11001, 1/200) and anti-rabbit immunoglobulin G (Molecular Probes, a-21207, 1/200).
Figure 5.11 Nocodazole inhibits p65 translocation to the nucleus. Vero cells were grown on coverslips in 24-well plates in medium supplemented with 2% FBS. Cells were then treated with nocodazole for 30 minutes then stimulated for 1 hour with 100 nM PMA, fixed in paraformaldehyde and assayed by indirect immunofluorescence. Primary antibody was rabbit anti-p65 (Santa Cruz, sc-372, 1/200) and the secondary antibody was anti-rabbit immunoglobulin G (Molecular Probes, a-21207, 1/200).
5.4 Discussion

A238L was observed to translocate to the nucleus in both ASFV infected cells and in cells transfected with a plasmid expressing A238L and stimulated with PMA. This was observed using both cell fractionation and immunofluorescence procedures. Western blotting suggested that the 32 kDa form of A238L is the form of the protein that accumulates in the nucleus late in infection. As explained below, this is of particular interest when compared with the timing of the nuclear localisation of the p65 subunit of NF-κB in ASFV infection described in chapter 4.

The absence of an obvious NLS in the A238L sequence suggested that A238L translocates to the nucleus in complex with a protein(s) which has a functional and exposed NLS. Strong evidence for the nuclear localisation of A238L in complex with NF-κB was provided by the experiments described here, which showed that A238L does not translocate to the nucleus in p65 -/- cells when stimulated with PMA. In the control p65 +/- cells both A238L and p65 translocated to the nucleus following PMA stimulation. This is consistent with a model in which A238L binds to p65, but does not mask the NLS thereby allowing the complex to translocate to the nucleus, but still inhibiting trans-activation of target genes by blocking binding of NF-κB to DNA or inhibiting its post-translational modification or both.

The kinetics of A238Lp movement to the nucleus are also of interest. Using high concentrations of nuclear extracts, A238L pcan be seen in the nucleus from three hours post-infection. However, large amounts of A238Lp are not observed in the
nucleus until 12 hpi. The kinetics of p65 movement to the nucleus are similar, since smaller amounts are detected at early times post-infection and larger amounts at late times post-infection. This is consistent with A238L movement in complex with NF-κB. However, following stimulation of uninfected cells with PMA, NF-κB is observed to rapidly accumulate within the nucleus within 30 minutes. This suggests that in ASFV infected cells there is some mechanism to retain NF-κB in the cytoplasm at earlier times post-infection.

A possible explanation for this observation is that A238L inhibits nuclear transport of NF-κB: perhaps by inhibiting association of p65 with proteins required for transport or anchoring NF-κB to cytoplasmic proteins. However, the finding that p65 and A238L rapidly translocate to the nucleus following PMA stimulation in cells where the A238L gene has been transfected suggests that this is unlikely. Given that levels of A238L are likely to be higher following transfection than infection, it is likely that an inhibition of p65 transport would have been observed in transfected cells. Also, in chapter 3 it was shown that the kinetics of p65 movement to the nucleus are similar in cells infected with ASFV expressing A238L to that in cells infected with the ASFV gene deletion mutant. This suggests that delay in nuclear accumulation of p65 does not depend on A238L.

Previous data (Tait et al., 2000) suggested that A238L is not in a complex with NF-κB until after IκB has been degraded, suggesting that A238L does not displace IκB from the NF-κB complex. In these experiments, partial degradation of IκB was observed by 8 hpi in ASFV infected cells. A possible explanation for
the delayed accumulation of A238L and NF-κB in the nucleus in infected cells, is that ASFV encodes other proteins which inhibit NF-κB activation and act at early times post-infection to retain the NF-κB complex in the cytoplasm.

A notable point is the apparent low level of co-localisation of A238L and NF-κB p65 observed by confocal microscopy. As A238L can be co-immunoprecipitated with p65, it seemed likely that the two proteins would co-localise by immunofluorescence. The low level of co-localisation between these two proteins suggests either a very dynamic interaction, with A238L disassociating and reassociating with p65, or could be explained by A238L localising with other proteins such as calcineurin. Attempts to repeat the co-immunoprecipitation were unsuccessful, again suggesting that the A238L/p65 interaction is of low affinity.

The requirement for an intact microtubule network for p65 transport to the nucleus is not unexpected. A number of proteins, such as p53 (Giannakakou et al., 2000) and the RNA-binding protein Staufen (Kohrmann et al., 1999), have been shown to move within the cytoplasm and to the nucleus using microtubules and their motor proteins dynein and kinesin.

An intriguing finding to add to this hypothesis is the observation that, in HeLa cells, following treatment with nocodazole, NF-κB is activated (Rosette & Karin, 1995). This may be a mechanism for cytoskeletal changes and changes in cellular morphology to cause a change in gene expression. It may also be due to the fact that IκB may interact with the cytoskeleton and disruption of microtubules may cause a release of NF-κB.
It has been observed that, following activation of NF-κB by microtubule disruption, there was a lag phase before the formation of the maximum level of NF-κB/DNA complexes in the cytoplasm (Rosette & Karin, 1995). This may be due to an inhibition in NF-κB transport. This is consistent with a hypothesis in which NF-κB transports to the nucleus along microtubules.
6 Analysis of downstream effects of A238L

6.1 Introduction

The previous chapters have concentrated on better defining the mechanism by which A238L acts to inhibit NF-κB activity. In this chapter, effects of A238L expression on cellular processes dependent on NF-κB and calcineurin pathways are examined. As deletion of A238L from ASFV does not affect virus growth or pathogenesis, it is possible that the virus has complementary genes to compensate for the absence of A238L. It was, therefore, of interest to investigate the effect of A238L expression alone on cells.

As has been discussed earlier, ASFV infection affects the normal movement and/or activation of some host transcription factors. Could ASFV infection or, more specifically, A238Lp have an effect on whole cell growth or function? Further to previous studies, an investigation into the effects of infection of viruses of different virulence may reveal some of the mechanisms important in causing disease of differing severity.

Virus interference with cell cycle progression is common. A number of nuclear replicating DNA viruses, such as adenovirus, force the cell into S phase in order to increase the cellular pool of nucleotides and other precursors to DNA synthesis. For a virus such as ASFV, which replicates in the cytoplasm and encodes its own enzymes for nucleotide metabolism, causing cells to proliferate may not be as necessary.
Observation of previous experiments which involved transfecting A238L into cells under the control of a constitutively active promoter (pcDNA3 containing the CMV IE promoter), seemed to show a reduction in growth rate in those cells which had been transfected with A238L, compared with those which had been mock-transfected, or transfected with an empty vector. Experiments were performed to confirm these observations, and to investigate the mechanism behind them.

This chapter also looks at the production of nitric oxide by primary macrophages following infection with a number of different isolates of ASFV. Nitric oxide, as well as being an important signalling molecule, has an important role in microbicidal activity (Fang, 2004). Nitric oxide is produced rapidly following stimulation, with induction of inducible nitric oxide synthase (iNOS), and acts locally, with a very short half-life.

6.2 Proliferation inhibition experiments

In order to test the initial observation that transfection of A238L inhibited the proliferation of Vero cells, an assay was developed to assess the replication of cells grown in six-well plates. Briefly, eight two-millimetre squares were drawn on the bottom of tissue culture plates. Two wells were used for each experimental treatment and squares were numbered 1-16 for each experimental treatment. Cells were seeded onto dishes and transfected or mock-transfected with the appropriate plasmid. Following a short time of culturing, four squares out of the 16 for each assay were selected using a random number table. Cells in
the chosen squares were counted and this time recorded as time zero. Cells in the selected squares were counted at further times as detailed in the text.

The initial experiment simply compared the effect of A238L transfection on the proliferation of Vero cells compared with non-transfected cells and cells transfected with empty pcDNA3 vector. Figure 6.1 shows a significant (p<0.05) reduction, of approximately 2-fold, in the proliferation of cells transfected with a plasmid (pcDNA3) constitutively expressing A238L, compared to controls.

In order to further confirm this result, the experiment was repeated with an additional two controls. Plasmids expressing the ASFV j4R and l14L genes were transfected into cells and assayed for their ability to inhibit cell proliferation. Figure 6.2 shows that there was no significant difference in cell proliferation between any of the control experiments, with a marked inhibition of proliferation again seen for cells transfected with the plasmid expressing A238L.

To investigate whether this finding was the result of a genuine slow-down of proliferation, or simply a lag effect followed by growth at equivalent rate, assays were repeated with a greater number of time-points, allowing cellular growth curves to be produced. Figure 6.3 shows that all treatments produced similar growth kinetics, with cells transfected with the A238L expression plasmid showing a shallower gradient.
Figure 6.1 Proliferation of Vero cells is inhibited by A238L. Vero cells were transfected with a pcDNA3 construct constitutively expressing A238L, with empty pcDNA vector, or untreated. Numbers of cells in four 2 mm x 2 mm squares, chosen at random from 16 over two wells, were counted following transfection and 30 hours later. Average proliferation for each experimental group was then calculated. Error bars show the standard error of the mean.
Figure 6.2 Proliferation of Vero cells is inhibited by A238L. Vero cells were transfected with a pcDNA3 construct constitutively expressing A238L or the ASFV genes j4R or I14L, with empty pcDNA vector, or untreated. Numbers of cells in four 2 mm x 2 mm squares, chosen at random from 16 over two wells, were counted following transfection and at 30 hours post-transfection. Average proliferation for each experimental group was then calculated. Error bars show the standard error of the mean.
Figure 6.3 Inhibition of proliferation by A238L causes a constant reduction in growth curve. Vero cells were transfected with a pcDNA3 construct constitutively expressing A238L or the ASFV genes j4R or I14L, with empty pcDNA vector, or untreated. Numbers of cells in four 2 mm x 2 mm squares, chosen at random from 16 over two wells, were counted following transfection and at 8, 18, 30 and 48 hours post-transfection. Average proliferation for each experimental group was then calculated. Error bars show the standard error of the mean.
To ascertain whether the amount of A238L had an effect, increasing amounts of A238L expression plasmid were transfected into cells and proliferation assays performed. Figure 6.4 shows the results of these assays. A typical dose response effect was observed with increasing inhibition of cell proliferation as the amount of transfected A238L expression plasmid was increased. Dose dependent response again showed a characteristic ‘flattening’ of the growth curve rather than ‘lag and recovery’ kinetics, as shown in figure 6.5.

6.3 Studies with non-calcineurin-binding mutants

To try and determine the mechanism for the inhibition of proliferation, assays were performed using a plasmid (pcDNA3, Invitrogen) expressing a mutant form of the A238L protein, W13, which is unable to bind calcineurin (Miskin et al., 2000). As can be seen from figure 6.6A, and more clearly in figure 6.6B, there is no significant difference in inhibition between the wild-type A238L protein and the W13 mutant. Since expression of the non-calcineurin binding mutant of A238L inhibited cell proliferation to a similar extent as the wild type protein, this suggests that the inhibition is mediated by the NF-κB inhibitory activity of A238L rather than the calcineurin inhibitory activity.
Figure 6.4 Inhibition of proliferation is dose dependent. Vero cells were transfected with a pcDNA3 construct constitutively expressing A238L. Cells were transfected with 1, 5, 10 or 20 μg of DNA. Numbers of cells in four 2 mm x 2 mm squares, chosen at random from 16 over two wells, were counted following transfection and at 30 hours post-transfection. Average proliferation for each experimental group was then calculated. Error bars show the standard error of the mean.
Figure 6.5 The dose dependent inhibition of proliferation is due to an increased generalised slow-down of cell cycle. The effect of increasing doses of A238L transfected into Vero cells does not cause periods of no growth followed by proliferation at wild-type rate; proliferation inhibition is constant. Error bars show the standard error of the mean.
6.4 Effects of ASFV infection on nitric oxide production

To investigate the functional consequences of ASFV infection on macrophages, nitric oxide release was assayed. As no ASFV field strains with A238L mutations or deletion were available for test, virus isolates of different virulence were assayed for their ability to stimulate nitric oxide production in macrophages.

Primary alveolar macrophages were obtained by lung lavage and cultured in 24-well plates. Cells were cultured in RPMI medium until 1 hour prior to the start of infection. At this time, medium was replaced with OptiMEM (Life Technologies) as the concentration of nitrate is too high in RPMI medium and produces very high background levels in the assays. Cells were then stimulated with 100 nM PMA or infected with either the NH68, Rec34, Malta or Malawi isolate of ASFV for 30 minutes, 1, 2, 4, or 8 hours. The differences between these viruses are detailed in section 2.2.4. Two wells of cells were mock infected as a control. Viruses used for these experiments were purified from macrophage cell culture supernatants in order to remove cytokines and other factors which may be present in differing amounts in culture supernatants. Cells were infected at a multiplicity of infection of 5. Each infection was carried out in duplicate wells and assayed in duplicate without pooling of samples.

Nitric oxide has a short half-life, of seconds to minutes, and degrades to produce nitrite (NO$_2^-$) and nitrate (NO$_3^-$). Figure 6.7 shows the production of nitrite in tissue culture supernatant following PMA stimulation or ASFV infection of
Figure 6.8 Mutants of A238L which are unable to bind calcineurin inhibit proliferation of Vero cells. CaN-binding mutants do not significantly alter the amount of proliferation inhibition caused by A238L. Error bars show SEM (A) and 95% CI (B).
primary alveolar macrophages. There is no significant difference in nitrite production compared to mock infected cells following PMA stimulation or infection with NH68, Malawi or the Malta isolate of ASFV. Cells infected with the Rec34 isolate showed increased levels of nitrite in cell supernatants by 1 hour post-infection. Levels did not increase significantly from 1 hpi to the end of the experiment at 8 hpi. The data show a slight reduction in the amount of nitrite in Rec34 and Malawi infected cells from 1 hpi to 8 hpi, but this is not statistically significant.

Figure 6.8 shows the production of nitrate following stimulation with PMA or ASFV infection. No significant difference in the amount of nitrate produced is observed following PMA stimulation or infection with the Rec34, NH68 and Malta isolates of ASFV. A large increase in nitrate production is observed following infection of macrophages with the Malawi isolate of ASFV. A significant increase was observed within 30 minutes following infection. This level remained approximately constant until the experiment was terminated at 8 hpi. Higher concentrations of NO can lead to an accumulation of other reactive nitrogen oxide intermediates (RNOIs) and higher nitrogen oxide species. Thus, the predominant breakdown products can depend on the initial concentration of NO.
Figure 6.7 Nitrite production by ASFV infected macrophages. Primary alveolar macrophages were cultured and either stimulated with phorbol ester (PMA) or infected with a strain of African swine fever virus. Tissue culture supernatant samples were taken at 30 minutes, 2 hours and 4 hours post PMA stimulation, or 30 minutes 1 hour, 2 hours, 4 hours and 8 hours following infection. Samples were processed using the Stressgen StressXpress nitric oxide detection kit (EKS-300) following the manufacturers instructions. Error bars show 95% CI. The red line indicates the level of nitrate in mock infected samples, with the blue lines the 95% CI.
Figure 6.8 Nitrate production by ASFV infected macrophages. Primary alveolar macrophages were cultured and either stimulated with phorbol ester (PMA) or infected with a strain of African swine fever virus. Tissue culture supernatant samples were taken at 30 minutes, 2 hours and 4 hours post PMA stimulation, or 30 minutes 1 hour, 2 hours, 4 hours and 8 hours following infection. Samples were processed using the Stressgen StressXpress nitric oxide detection kit (EKS-300) following the manufacturers instructions. Error bars show 95% CI. The red line indicates the level of nitrate in mock infected samples, with the blue lines the 95% CI.
6.5 Discussion

The finding that A238L affects cell proliferation was surprising. Differentiated macrophages and monocytes are generally non-replicative, so it is unlikely that ASFV would require a mechanism to prevent proliferation of these cells as a mechanism of inhibiting the immune response.

A possible explanation for the observed inhibition of cell proliferation caused by A238L is the inhibitory effect of A238Lp on NF-κB activation. NF-κB has been shown to regulate expression of the cyclin-D1 gene, which is involved in the transition of G1 to S phase (Hinz et al., 1999, Joyce et al., 1999). Inhibition of NF-κB by could cause a reduction in the transactivation of the cyclin-D1 promoter.

NF-κB has also been linked with control of the cyclin-A gene (Joyce et al., 1999). Trans-activation of the cyclin-A gene by NF-κB is at a much lower level than that of cyclin-D1 and so is less likely to be the cause of this effect. Reduced production of cyclin-D1 in cells would mean that it would take longer for concentrations of this protein to build up to a level where the cell would enter S phase. This would happen in each cell cycle, leading to a general slow-down of proliferation as was observed.

Consistent with this hypothesis is the finding that the mutant form of A238L, which was not able to bind calcineurin, did not abolish or significantly change the level of this inhibition. Had the inhibition of calcineurin been important to this inhibition, deletion of this function of the protein would have produced a
significant effect on the result. This could be further investigated by measuring cyclin D1 transcript levels by semi quantitative RT-PCR or by real-time RT-PCR.

Attempts were made to repeat these experiments in p65 knockout cells. If A238L had an inhibitory effect on these cells it would show that it was via a p65-independent mechanism. Unfortunately, at the confluency required to perform these assays, the p65 knockout MEF cells did not survive transfection. The observation that cells died, most likely by apoptosis, whether they were transfected with a plasmid vector containing A238L or the plasmid vector alone, suggests that the cell death was not due to an effect of A238L on the MEFs.

Previous work transfecting MEFs had been successful; however, these experiments were performed at a higher confluency than required for the proliferation assays. Future experiments could use a tritiated thymidine uptake assay to quantify proliferation. This would allow transfection of cells at a higher confluency, as low cell numbers would not be required in order to accurately count under the microscope.

A further explanation for the perceived inhibition of Vero cells transfected with A238L could be that A238L induces apoptosis in transfected cells. A238L could act as an inducer of apoptosis through NF-κB inhibition, which could inhibit production of the cellular inhibitor of apoptosis (IAP) genes. However, this effect could be balanced by the inhibition of calcineurin (CaN). The inhibition of CaN may inhibit the dephosphorylation, and activation, of the pro-apoptotic protein Bad.
An induction of apoptosis in A238L infected cells would cause an apparent inhibition of cellular proliferation as there would be few cells to proliferate compared with the empty vector transfected cells. However, no differences were observed in the number of cells detached from the culture plate, nor in the morphology of cells, between cells transfected with the empty vector or with A238L.

A useful experimental tool to investigate this further would be FACS analysis of transfected cells. Staining cells with propidium iodide (PI) and for phosphatidyl serine (PS) without first opening cells and counting the numbers of each stained or unstained population would show conclusively whether A238L was affecting, or effecting, apoptosis. Cells which stained double negative would be healthy, cells stained double positive would be dying due to necrosis, or in late stage apoptosis, and apoptotic cell populations would stain PI negative PS positive. Comparison of this final population of cells would allow determination of A238L’s effect on apoptosis.

FACS analysis could also be used to determine if the inhibition in rate of proliferation was the result of a block at any specific stage of the cell cycle. Markers are available to assay populations of cells for their stage of cell cycle progression. Comparison of proportions of cells in different stages of the cycle could reveal a block at one stage of progression as there would be a greater proportion of A238L transfected cells at this stage of the cell cycle.
If an increased number of cells was observed in G₁ following A238L transfection, this would be consistent with a block or inhibition at the G₁ to S phase checkpoint. This would not reveal the mechanism for this block however, as both cyclin-D₁ and cyclin-A control entry into S phase.

The effects of ASFV infection on nitric oxide production are not unsurprising. The fact that Malawi infection produced high nitrate levels, but not nitrite, and Rec34 infection high nitrite infection, but not nitrate, can be explained by the initial concentrations of nitric oxide (NO) as described previously in section 6.4.

A large induction of NO early in infection is unsurprising with Malawi infection. Malawi is a high virulence isolate of ASFV and so a significant cellular activation is not unexpected. The intermediate virulence isolate, Malta, showed no significant induction of NO, nor did the low virulence NH68 isolate. The small NO induction by the Rec34 virus is surprising. The fact that the NO production was limited to the first hour in NH68 infection, and the first 30 minutes in Malawi infection, suggests that, following this initial stage of infection, cells either become tolerant to a particular stimulus, or the pathways involved are inhibited from this time.

The finding that high virulence, highly pathogenic isolates of ASFV induce a strong immune activation suggests that one mechanism for their high pathogenicity is a severe immunopathology.
7 Discussion

7.1 Effects of ASFV infection on NF-κB protein levels

Previous work has shown that transcription of genes dependent on NF-κB is inhibited in ASFV infected cells (Gomez del Moral et al., 1999, Powell et al., 1996). The A238L protein was also shown to inhibit NF-κB-dependent gene transcription when transfected into cells suggesting that it may be responsible for the effect observed in ASFV infected cells (Powell et al., 1996, Revilla et al., 1998). The main aim of this project was to better define how the ASFV A238L protein functions to inhibit activation of the NF-κB transcription factor. A238L also inhibits the cellular phosphatase calcineurin. This activity of A238L has not been examined in this thesis.

The first conclusion drawn, in chapter 3, is that infection of Vero cells with ASFV causes a change in the amount of the p65 subunit of NF-κB during infection; relative amounts of p65 increased by 3 hpi, decreased by 6 hpi and increased again by 12 hpi. Levels of α-tubulin and the p50 subunit of NF-κB were found to remain constant, suggesting that there was no generalised 'shut-off' of host-cell translation. Total amounts of ERK proteins remained approximately constant during infection, although small changes were noticed between the relative amounts of ERK1, ERK2 and the phosphorylated form of ERK1.

The changes in levels of p65 protein are similar in cells infected with A238L pk-tagged wild type ASFV and A238L deletion virus. This suggests that the A238L protein is not responsible for the reduced levels of p65 and that this change is
either a host-cell mediated result of ASFV infection or caused by another virus-encoded protein. Whether alterations in the amount of p65 protein were due to changes in the levels of p65 gene transcription, protein synthesis or turnover was not established. It is clear that the mechanism by which A238L acts to inhibit NF-κB activation is unlikely to involve changes in the amount of NF-κB protein. A similar time-course experiment looking at p65 levels following infection with a non-related virus may show whether this effect is ASFV-mediated or a host-cell response. The p65 promoter region contains three consensus sites for the SP1 transcription factor, but no NF-κB binding sites are present; the promoter is not activated by PMA stimulation (Ueberla et al., 1993). This suggests that ASFV infection modulates other signalling pathways which lead to SP1 activation.

### 7.2 Effects of ASFV infection on NF-κB and ERK nuclear translocation

The data presented in chapter 4 shows that the p65 subunit of NF-κB translocates to the nucleus following ASFV infection. Nuclear translocation of NF-κB occurred with similar kinetics in cells infected with ASFV expressing A238L and with A238L deletion mutant virus suggesting that A238L does not inhibit nuclear translocation of NF-κB. This finding shows that A238Lp does not act by sequestering NF-κB in the nucleus as IκB does.

The observation (in chapter 4) that A238Lp accumulates in the nucleus at late times post-infection with similar kinetics to NF-κB suggested that A238Lp may move to the nucleus with NF-κB. The predominant form of A238L in the nucleus is the 32 kDa form. This is the form of A238Lp that has been shown to co-precipitate with the p65 subunit of NF-κB (Tait et al., 2000).
Although some A238Lp and NF-κB were observed in the nucleus throughout ASFV infection, larger amounts accumulated at late times post-infection from 12 hpi onwards. In contrast, when NF-κB is activated by stimulation of cells with PMA, both A238Lp and NF-κB rapidly accumulate in the nucleus within 1 hour post-stimulation. This suggests that, at early times post-infection, NF-κB may be retained in the cytoplasm in ASFV infected cells. Cytoplasmic retention of NF-κB at early times post-infection does not require the A238L protein since similar results were obtained using ASFV expressing A238Lp and the A238L deletion mutant. Tait et al. (2000) showed that A238Lp does not displace IκB from NF-κB complexes and may replace IκB following stimulation induced degradation. They showed that IκB was partially degraded by 8 hpi. One possibility is that ASFV encodes one or more proteins that inhibit IκB degradation by acting upstream on the signalling pathway that induces its degradation. Other viruses encode proteins which inhibit NF-κB activation by preventing degradation of IκB. For example, poxviruses encode a protein, N1L, which targets the IKK complex and hence prevents phosphorylation and degradation of IκB (DiPerna et al., 2004).

7.3 Nuclear translocation of A238Lp in infection

A proportion of A238Lp was present in the cytoplasm throughout infection. The IκB protein shuttles between the nucleus and cytoplasm providing a mechanism for switching off NF-κB-dependent gene transcription. IκB gene transcription is activated by NF-κB and the newly synthesised IκB translocates into the nucleus
where it binds to NF-κB complexes, displacing them from DNA. A nuclear export signal on IκB leads to transport of IκB/NF-κB complexes to the cytoplasm. One possibility is that the population of A238Lp in the cytoplasm, is at least partly comprised of molecules exported from the nucleus, possibly, as with IκB, in complex with NF-κB.

However, the A238L protein lacks the nuclear export signal -MVKELQEIRL- present from position 45-54 on IκB (Huang et al., 2000). The experiments in chapter 5, using leptomycin B to inhibit CRM1-dependent nuclear export, suggest that A238Lp does not shuttle back to the cytoplasm from the nucleus. Thus the cytoplasmic population of A238L most likely consists of molecules which have not entered the nucleus.

A238L protein is present in two forms throughout infection: a 28 kDa form and a 32 kDa form. It was also shown, in chapter 4, that A238L accumulates in the nucleus at late times post-infection. The predominant form in the nucleus is the 32 kDa form. This is the form of A238Lp that has been shown to co-precipitate with the p65 subunit of NF-κB (Tait et al., 2000).

The finding that p65 is present in the nucleus fits well with the homology between IκB and A238L, and the predicted structure models (see below). A238L is significantly shorter than IκB and contains fewer ankyrin repeats. The ankyrin repeats of A238L most closely resemble those of IκB responsible for masking the DNA binding domain of p65. The finding that p65 translocates to the nucleus in infected cells, along with previous observations, suggests a mechanism...
whereby A238L inhibits NF-κB activity by binding to p65 and masking the DNA binding region of the protein. The smaller size of A238L means the NLS of p65 would remain exposed. This would allow the whole complex to shuttle to the nucleus. Again, this model is consistent with the accumulation of A238L in the nucleus during infection.

7.4 Mechanism of A238Lp nuclear translocation

Chapter 5 further investigated the movement of A238L protein to the nucleus. This observation of A238Lp translocating to the nucleus could be seen by Western blotting of whole populations of cells, as well as by immunofluorescence of individual cells. This would suggest that this is a genuine and reproducible finding. The lack of an obvious nuclear localisation signal on A238Lp raises questions about the mechanism by which A238L traffics to the nucleus. The hypothesis tested was one in which A238Lp translocates into the nucleus in complex with NF-κB.

The finding that A238Lp moves into the nucleus following chemical stimulation of cells when NF-κB is present, but does not in a p65 -/- cell line, provides good evidence that A238Lp does translocate to the nucleus in complex with the p65 subunit of NF-κB.

Experiments using leptomycin B showed that the cytoplasmic population of A238Lp is a population which remains in the cytoplasm and has not been to the nucleus and shuttled out again. If this were the case, the A238L protein would accumulate in the nucleus as IκB does. This observation is consistent with two
populations of A238L in infected cells: one cytoplasmic and one nuclear. One explanation for these two populations could be that the cytoplasmic population is responsible for the inhibition of calcineurin, while the form in the nucleus inhibits NF-κB.

An elegant hypothesis to explain the two populations could be that the cytoplasmic A238Lp, inhibiting calcineurin, is the 28 kDa form, while the nuclear population, inhibiting NF-κB, is the 32 kDa form. This hypothesis would be consistent with the data presented here, that the predominant form of A238L in the nucleus is the 32 kDa form, and data from Tait et al. (Tait et al., 2000), in which the 32 kDa form of A238L was shown to co-immunoprecipitate with NF-κB p65. The findings of Revilla et al. (Revilla et al., 1998), which showed that recombinant A238L protein, produced in *Escherichia coli* and therefore presumably of the 28 kDa unmodified form, prevents p65 DNA binding in vitro, do not apparently fit with this hypothesis. One explanation for the inconsistency is that the modification of A238Lp to produce the 32 kDa form may change the affinity of A238Lp for calcineurin and NF-κB p65. The 28 kDa form of A238Lp may be able to bind to p65, but at a lower affinity than the 32 kDa form. In cells, the 28 kDa form may predominantly bind to calcineurin, but modification to produce the 32 kDa form may change the affinity of A238Lp decreasing its affinity for calcineurin, but increasing its affinity for NF-κB p65. This hypothesis would provide an interesting avenue for further study. As discussed above, the evidence presented here suggests that A238L shuttles to the nucleus in a complex with NF-κB, hence the form of the protein which binds to NF-κB would be predicted to accumulate in the nucleus.
One interesting observation is that in cells transfected with a plasmid expressing A238L, only the lower molecular weight form of the protein could be detected by Western blotting. This is consistent with the findings of other researchers working on this protein. The finding that the two forms can be produced from a single cDNA when expressed from the T7 promoter in cells infected with Vaccinia virus expressing T7 RNA polymerase (Tait et al., 2000), suggests that the 32 kDa form of A238L is the result of a post-translational modification. As this modification is only observed following infection, with either ASFV or Vaccinia, it is likely that the modification is made by a virus activated host-cell modifying enzyme.

Virus infection of cells is known to activate host cell signalling pathways involved in host anti-viral defence responses. These include those induced by initial binding and entry of the virus, those induced by the process of virus replication and at later stages, those induced by cytokines, such as type I interferons and TNF-α, that are produced by the host cell following virus infection. The initial stages of virus infection may activate a variety of pathways depending on the cell receptor to which the virus binds and the mechanism of entry. Virus replication produces double-stranded RNA, which activates PKR kinase in infected cells and binds to Toll-like receptor 4 on the cell surface to activate intracellular signalling pathways. Virus replication may also induce a stress response in infected cells. A recent study has shown that African swine fever virus infection is able to inhibit the induction of the stress-induced transcription factor CHOP/GADD153 (Netherton et al., 2004). This protein is
induced following mitochondrial and endoplasmic reticulum stress: often observed during virus assembly at the ER. As ASFV replication requires ER membrane wrapping, the virus may encode proteins to modulate this response.

Type I interferons induce activation of the JAK/STAT pathway and TNF-α can induce the NF-κB pathway. In future experiments it would be interesting to activate these key signalling pathways in uninfected cells transfected with a plasmid expressing A238L to determine if these caused modification of the A238Lp protein.

The lack of production of the 32 kDa form of A238L in transfected cells is not consequential in the context of the experiments described here. The original experiments (Powell et al., 1996) used a pcDNA3 A238L plasmid transfected into ASFV infected cells to demonstrate that A238L inhibits NF-κB dependent gene transcription. These data were reproduced using the plasmids used in these experiments (R. Silk personal communication). The hypothesis presented above, that both forms of A238Lp can inhibit NF-κB activation but have differing affinities for NF-κB and calcineurin, could explain these observations. Of relevance is the observation that larger amounts of A238Lp are produced in transfected cells compared to ASFV infected cells. The larger amounts of A238Lp in transfected cells could enable NF-κB to be inhibited efficiently even if the 28 kDa form of the protein binds less efficiently.

The fact that the 28 kDa form of A238L can inhibit NF-κB could possibly be due to the Vero cell line these experiments were performed in. The cells which are
naturally infected with ASFV are macrophages and monocytes. The relative levels of host proteins differ between macrophages and Vero cells. A Western blot on whole cell extracts prepared from macrophages and Vero cells showed that there is more calcineurin in macrophages than in Vero cells (data not shown).

If one considers a model whereby the 28 kDa form of A238L has a high affinity for calcineurin and a low affinity for NF-κB, and the opposite is true for the 32 kDa form, then it could be imagined that in macrophages, which contain a higher level of calcineurin, that virtually all of the 28 kDa form of A238L would be bound to calcineurin. Following post-translational modification, A238L could disassociate from calcineurin and bind NF-κB. If there is a smaller amount of calcineurin compared to 28 kDa A238L in Vero cells, then the 28 kDa form of the protein could inhibit NF-κB, albeit with a lower affinity interaction. This hypothesis could explain the low level of co-localisation seen between A238L and p65 in stimulated cells.

If the low level of co-localisation between A238L and p65 observed in A238L transfected cells is due to a dynamic association/disassociation, it may suggest that a small level of p65 transcriptional transactivation is required. One way of regulating the amount of p65-mediated transactivation is to control the affinity of its inhibitor for it. As p65 is important in transactivating the promoter of the IAP genes, perhaps the benefits of allowing a low level of p65-mediated transactivation, with the concomitant production of some pro-inflammatory cytokines, outweigh the disadvantages.
Inhibiting the NF-κB pathway in murine B lymphocytes and 231 murine B lymphoma cells causes apoptosis (Arsura et al., 1996, Lee et al., 1995, Wu et al., 1996). Experiments using a non-degradable IκB have shown that Epstein-Barr virus (EBV)-transformed lymphoblastoid cells, which express high levels of Bcl-2 and Bcl-xL, entered apoptosis (Cahir-McFarland et al., 2000). Caspase inhibitors were unable to prevent cell death.

Activation of the NF-κB pathway is an important step in transformation of lymphocytes by EBV. The finding that blocking the NF-κB pathway in these cells triggers apoptosis, without the requirement for other factors capable of triggering apoptosis (Cahir-McFarland et al., 2000), is interesting. If a basal level of transcription from NF-κB transactivated genes is required at a fundamental level for cell survival, then allowing the transcription of a low level of pro-inflammatory cytokines during infection may be a small concession to make.

The nature of the post-translational modification of A238Lp is not known. In previous studies (Tait et al., 2000), modification by phosphorylation and ubiquitination were investigated but no evidence for these modifications was found. However, modification by phosphorylation cannot be excluded since, in these experiments, even levels of phosphorylation of the major ASFV phosphoprotein, p32, detected by radioactive labelling were very low. The identity and position of the post-translational modification of A238Lp could be resolved in future by purifying large amounts of both forms of A238Lp from two
dimensional protein gels and analysis by mass spectrometry of peptides produced by proteolytic cleavage.

7.5 Suggested model for A238L mechanism of NF-κB inhibition

One of the key findings of this study is that p65 and A238L are present in the nucleus in ASFV infected cells. This observation suggests a mechanism for A238L-mediated inhibition of NF-κB-mediated transactivation.

Previous work has shown that A238Lp can bind to, and co-immunoprecipitate with, p65 (Tait et al., 2000) following the degradation of IκB. This suggests that the NF-κB pathway is activated following ASFV infection. This is very likely as there are a number of steps in the ASFV infectious cycle which could activate the pathway including membrane fusion and cytoskeletal disruption.

The fundamental question surrounding A238L was whether it functioned by sequestering NF-κB in the cytoplasm, or by preventing DNA binding in the nucleus. As both p65 and A238Lp translocate to the nucleus, there is strong evidence for the latter.

As described previously, IκB acts in two ways to inhibit NF-κB: sequestration in the cytoplasm by masking the p65 NLS, and masking of the p65 DNA binding domain. The findings shown above are consistent with A238Lp acting as a partial IκB homologue. As A238Lp contains fewer ankyrin repeats than IκB, it may not be able to mask the NLS and the DNA binding domain simultaneously. A model showing the predicted structure of A238Lp in complex with the
p65/p50 heterodimer is shown in figure 7.1. This model was produced (Dr. Dave Chapman, IAH Pirbright, unpublished results) based on the known structure of IκB complexed to NF-κB. This model predicts the p65 NLS is exposed and not masked by A238Lp. The model predicts that the ankyrin repeats of A238Lp, which share most homology with those in IκB responsible for masking the p65 DNA binding domain, block interaction of p65 with DNA. This seems the most-likely mechanism by which A238L acts to inhibit p65-mediated transactivation.

However, it is possible that A238L may also modify post-translational modification of p65 within the nucleus to inhibit its activity. Preliminary experiments presented in this study suggest that post-translation modifications of p65 are not affected by A238L. In cells transfected with a plasmid expressing A238L the time taken for p65 to be phosphorylated appeared to be increased compared with mock-transfected cells. However the overall level of p65 phosphorylation did not seem to be affected. In these experiments the acetylation of p65 also appeared not to be, or only slightly, affected by A238Lp. These data suggest that A238Lp does not act to inhibit p65-mediated transactivation by preventing the phosphorylation required for transcriptional activation.

The experiments also suggest that A238L does not function by deacetylating p65 which would lead to increased binding of p65 to IκB and removal of NF-κB from the nucleus by IκB. These experiments need to be repeated using a protocol which will enable A238L to be expressed in a higher percentage of cells than achieved using the transfection procedure. One possibility would be to use the baculovirus expressing A238Lp under control of the CMV immediate early
Figure 7.1 The predicted structure of A238Lp in complex with the p65/p50 heterodimer. The p65 NLS is shown in white- unmasked by A238Lp. Courtesy Dr. Dave Chapman, Institute for Animal Health, Pirbright.
promoter which can deliver the gene to greater than 90% of cells (Ms Rhiannon Silk IAH Pirbright, unpublished results).

There are other ways in which NF-κB activity could be inhibited, which have not been investigated in this study. As mentioned previously (section 1.4), NF-κB requires association with the TATA binding protein for efficient transactivation (Carter et al., 1999a). The p38 MAP kinase is capable of phosphorylating TBP, thus preventing its association with p65.

The p38 MAP kinase is also able to positively regulate transactivation by phosphorylating transcription factor IID. The ERK pathway is capable of influencing this pathway by inhibiting the p38-mediated phosphorylation of TFIIID (Carter & Hunninghake, 2000b). An investigation into the activities of these proteins in ASFV infected cells may determine if they are important in NF-κB inhibition.

The predicted structure of A238Lp does not include either the N-terminal or C-terminal domains of the protein, which share insufficient similarity with IκB to enable their structure to be modelled. Mapping of the domain of A238Lp required to inhibit calcineurin has shown an 82 amino acid domain at the C-terminus is sufficient to inhibit greater than 90% of the phosphatase activity (Dr Charles Abrams et al., IAH Pirbright, unpublished results). The function of the N-terminal domain of A238L remains unknown.
7.6 Downstream effects of A238Lp in the host cell

Previous studies on A238L have focused on the mechanism of NF-κB and NFAT inhibition; chapter 6 of this study looked at the potential consequences of inhibiting these pathways. Studies on the effect of A238Lp on cell proliferation showed that A238Lp is capable of causing a significant reduction in the rate of proliferation of Vero cells grown in culture. Repeating these experiments with a mutant form of A238L, which was unable to bind to calcineurin, caused a similar fold inhibition of proliferation. This finding suggests that it is the inhibition of NF-κB which is responsible for the reduction in proliferation; this could be explained by a reduction in NF-κB-mediated transactivation of the cyclin D1 gene promoter.

Assays to quantify the levels of nitric oxide produced in response to infection with isolates of differing variance were also of interest. The finding that the high virulence Malawi isolate produced highest levels of NO breakdown products is not surprising. The low levels of NO produced by the moderate virulence Malta isolate are more surprising. This isolate kills approximately 30% of pigs and a higher level of NO production would be predicted if virulence correlated with the amount of immunomodulatory factors secreted during infection. A more thorough investigation into this assaying production of a large panel of factors may yield a more satisfactory result.

Increased blood levels of nitric oxide can lead to a loss in vascular tone and contribute to shock. NO may also act to cause vasodilation, platelet inhibition and inhibit neutrophil adhesion by interfering with adhesion molecules (Blantz &
Munger, 2002). Ebola virus infection causes an increase in nitrate accumulation (Hensley et al., 2002), suggesting nitric oxide production. In the outbreak of Ebola (Sudan) in Uganda in 2000, blood levels of NO were higher in fatal cases of the disease than in non-fatal cases, levels of NO were also found to correlate with disease severity (Sanchez et al., 2004).

The kinetics of the NO production may perhaps shed light on various virulence determinates of different isolates. As the NO production was predominantly within 30 minutes post-infection, any difference in virulence factors would have to be contained within the virus particle, rather than by virus-encoded proteins, as there would not be sufficient time for viral proteins to be expressed. Possible explanations could be differences in membrane fusion events or a difference in amounts of various proteins present in the virion released after uncoating.

7.7 Future work
Further work is required both to understand the mechanism by which A238L functions and its effects on host cell function. A key outstanding question is to understand the nature and position of the post-translational modification to A238Lp and the mechanism by which this modification is induced. This could enable the modification of A238Lp to be manipulated, either by producing mutant forms of the gene encoding proteins which cannot be modified, or by inhibiting the pathway required to induce modification. This would help the understanding of the role of the two forms of A238Lp.
In theory the hypothesis presented here, that the two forms of A238L have different affinities for calcineurin and the p65 subunit of NF-κB could be investigated by in vitro binding studies using surface plasmon resonance (SPR) to measure affinities each form of the protein for p65 or calcineurin. SPR works as the optical properties of a material are different when it is in a solution, to when it is immobilised. If one protein is immobilised on a carboxymethylated dextran-gold surface, the other protein can be added to this surface and the kinetics of any interaction be quantified. These experiments would require production of each of the two forms of the A238L protein. This may become possible if the nature of the modification is understood. Current attempts to produce recombinant A238L protein have been hampered by the insolubility of the intact protein; expression of smaller fragments of the protein may overcome these problems.

A more thorough investigation of the roles of NF-κB family members during infection would also be of interest. This study focused predominantly on the p65 subunit as this is the major transactivating protein of the prototypical p50/p65 heterodimer. As the p50 protein is capable of acting as a transcriptional repressor, a more detailed investigation into its role in infection would further clarify how NF-κB dependent gene expression is inhibited. An increased presence of p50 in the nucleus could account for the observed inhibition of gene expression. The amounts of p50 in the nucleus, particularly at early times post-infection, were almost undetectable in these studies. Further investigations, perhaps using immunofluorescence methods, may be able to confirm this, or
reveal that p50 is indeed present at these times in greater amounts than revealed by Western blots of fractionated cells.

Other techniques could be used to reveal the status of NF-κB proteins during infections. The present studies concentrated on the nuclear location of p65. These studies could be taken further and nuclear extracts produced and analysed by EMSA. Gel shift assays would reveal whether the p65 present in the nucleus was able to bind DNA. Supershift analysis of complexes could be used to reveal which NF-κB members were involved in the formation of these complexes. These experiments have already been performed in Vero cells (Revilla et al., 1998). In this study, p65/p50 heterodimer binding to DNA was significantly inhibited, whereas only a weak inhibition of p50/p50 binding was observed.

Further experiments are needed to define the effects of A238Lp on host cell function. One possible approach would be to use the recently produced porcine cDNA or oligo microarrays (Dr Fuquan Zhang et al., IAH Pirbright; Qiagen) to investigate effects of A238Lp on host cell gene transcription. This could be carried out by comparing transcription in macrophages infected with either wild-type ASFV or a deletion mutant lacking the A238L gene. Since ASFV may encode other proteins which inhibit NF-κB activation, the effect of A238L may be masked in this analysis. Analysis of the effects of A238L on host cell transcription should also be carried out by expression of A238L on its own in cells.
7.8 Summary

The A238L protein is conserved in all isolates of ASFV investigated. However, when deleted from the virus no effects were observed on either virulence of the virus in domestic pigs or growth kinetics in culture (Neilan et al., 1997; Miskin et al., 1998). Some differences in host cell function have been attributed to A238Lp protein by comparison of cells infected with ASFV expressing A238L and ASFV from which the gene has been deleted. These differences included an inhibition of calcineurin phosphatase activity (Miskin et al., 1998) and inhibition of COX-2 mRNA transcription and hence of prostaglandin production (Granja et al., in press). This latter effect was shown to be mediated by inhibition of NFAT activation. The more obvious ability to detect differences in pathways dependent on calcineurin between cells infected with the wild type ASFV and an A238L gene deletion mutant suggests that the virus may not encode other proteins which can compensate for loss of this A238L function. However, possibly ASFV may encode other proteins which can inhibit NF-κB activation. The failure to detect a difference in pathogenesis in pigs infected with virulent ASFV from which A238L has been deleted either suggests that the pathways inhibited by A238Lp are not involved in induction of pathogenesis or that other compensating proteins are encoded.

Another possibility is that the main role of the A238Lp is in its wildlife hosts in which the virus evolved. ASFV is still an emerging infection in domestic pigs and the virus has had little time to adapt to this host or vice versa.
As ASFV is able to persist in wild pig populations and in ticks, A238L is likely to play a role in maintaining persistence in these species. Ticks contain proteins of the NF-κB family -dorsal and cactus- which are involved in an innate immune system as well as in development. Studies to investigate the effect of A238L deletion on the persistence of virus in individual ticks and tick populations would be very valuable.

Invertebrates also contain calcineurin, but not transcription factors of the NFAT family. Hence inhibition of calcineurin activity in ticks by A238Lp is likely to be important for other pathways rather than activation of NFAT transcription factors.

These studies have revealed the mechanism of NF-κB inhibition by A238L to a greater level than was known previously. Experiments described here provide strong evidence that A238Lp acts to inhibit NF-κB in the nucleus of infected cells, most likely by inhibiting DNA binding of p65, rather than acting to sequester NF-κB in the cytoplasm although other ASFV encoded proteins may act at points upstream in the pathway to delay degradation of IκB.

The movement of A238Lp to the nucleus is likely to be as part of a complex with NF-κB as A238Lp has no obvious nuclear localisation signal and is unable to transport to the nucleus in p65 knockout cells. This movement to the nucleus is likely to be via a microtubule-dependent mechanism.
It is also probable that a number of factors, in addition to role for A238L acting within the nucleus, are involved in inhibiting NF-κB-mediated transactivation: inhibition of calcineurin may impair NF-κB activation via protein kinase C; cytoskeletal rearrangement may inhibit NF-κB transport to the nucleus; other ASFV encoded-proteins may act upstream of IκB degradation to inhibit activation.

A greater understanding of the A238L protein and other proteins involved in immune evasion but could potentially lead to novel control measures through production of engineered attenuated virus vaccines and interference with the maintenance of ASFV in wild pig and tick populations. The A238L protein is the only known protein which can inhibit two key pathways important in activation of host immune responses. Understanding more about A238L’s mode of action and defining functionally important domains in the protein should provide leads for discovery of novel immunomodulatory drugs.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A238Lp</td>
<td>A238L protein</td>
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<tr>
<td>ADCC</td>
<td>Antibody dependent cellular cytolysis</td>
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<td>AraC</td>
<td>Cytosine arabinoside</td>
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<td>ASF</td>
<td>African swine fever</td>
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<td>ASFV</td>
<td>African swine fever virus</td>
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<td>bp</td>
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<td>CaM</td>
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<td>CaMKIV</td>
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<td>CaN</td>
<td>Calcineurin</td>
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<td>CD</td>
<td>Cluster of differentiation</td>
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<td>CFU-GM</td>
<td>Colony forming unit granulocyte macrophage</td>
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<td>CI</td>
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<td>CMV</td>
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<td>ERK</td>
<td>Extracellular-signal regulated kinase</td>
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<td>FBS</td>
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<td>Fibroblast growth factor</td>
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<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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<tr>
<td>HHV</td>
<td>human herpesvirus</td>
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<td>NEMO</td>
<td>NF-κB essential modulator</td>
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<td>NES</td>
<td>nuclear export signal</td>
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<td>nuclear factor of activated T cells</td>
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<td>Acronym</td>
<td>Description</td>
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<tr>
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<tr>
<td>TFIID</td>
<td>transcription factor IID</td>
</tr>
<tr>
<td>TIR</td>
<td>terminal inverted repeats</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>tumour necrosis factor receptor</td>
</tr>
<tr>
<td>TRAF</td>
<td>tumour necrosis factor receptor associated factor</td>
</tr>
<tr>
<td>UV</td>
<td>ultra violet</td>
</tr>
<tr>
<td>v-IRF</td>
<td>viral interferon regulatory factor</td>
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<tr>
<td>WPRE</td>
<td>Woodchuck hepatitis virus post-transcriptional regulatory element</td>
</tr>
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</table>
Appendix I- The amino acid sequence of A238L

1  MEHMFPEREI ENLFVKWIKK HIRNGNLTLF EEFFKTDPWI VNRCDKNGSS
51 VFMWICIYGR IDFLKFLPEQ ESYPGEIINP HRRDKDGNSA LHYLAEEKNH
101 LILEEVLGYF GKNGTKICLP NFNGMTPVMK AAIRGRTSIV LSLIKFGADP
151 TQKDYHRGFT AWDWAVFTGN MELVKSINHD YQKPLYMHFP LYKLDVFHRW
201 FKKKPKIIT GCKNYVEKL PEQPNFLCV KKLKNGYK
Appendix II - Plasmid vectors

pcDNA3
5.4 kb

* There is an ATG upstream of the Xba I site.
References


