VACCINIA VIRUS PROTEIN B14, AN INHIBITOR OF NF-κB ACTIVATION, AND ITS COUNTERPART IN MVA

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DEPARTMENT OF VIROLOGY

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DECLARATION

The work detailed herein is the work of the candidate except where clearly indicated. In particular, the study of WRΔB14+183 in vivo was carried out by Dr Aodhnait S Fahy. In addition, the co-immunoprecipitation of HA-tagged B14 and the IKKβ mutants was carried out in conjunction with Dr Ron A-J Chen. Construction of vΔA49 and vΔA49Rev was carried out by Dr Daniel S Mansur. Lastly, the B14 single-residue mutants were generated by Dr Stephen Graham and Asa Oldring at the Division of Structural Biology (STRUBI) at The University of Oxford.
ABSTRACT

Vaccinia virus (VACV) encodes multiple proteins which modulate NF-κB activation to evade the host immune response. One example is B14, a virulence factor that reduces NF-κB activation via interaction with IKKβ. B14 has orthologues in many VACV strains, including modified virus Ankara (MVA), which lacks many of the immunomodulators present in other VACV strains. Here, the MVA counterpart of B14, protein 183, was characterised. 183R was both removed from MVA and inserted into the B14R locus of a VACV strain Western Reserve (WR) lacking B14R, but in each case there was no change of phenotype. Protein 183 shares 95% amino acid identity with B14 but, unlike B14, was unstable in eukaryotic cells, unless protein degradation was inhibited. Protein 183 did not inhibit NF-κB activation in response to cytokine stimulation, as does B14, nor did it restore the virulence phenotype of WR lacking B14R back to wild type. Therefore, the mutations incurred by 183 during the derivation of MVA have rendered the protein non-functional. However, other MVA immunomodulators remain to be characterised and this thesis describes a bacterial artificial chromosome (BAC) system that may facilitate the improvement of MVA in its role as a vaccine vector.

To further characterise VACV modulation of NF-κB, a VACV WR strain was constructed lacking B14 and another VACV modulator of NF-κB, WR A49. Initial characterisation showed no change with this mutant virus in replication or spread. The effect of the B14 on NF-κB was further characterised by studying the B14-IKKβ interaction using IKKβ and B14 mutants. Residues 300 to 480 of IKKβ were shown to be required for the interaction and a mutant encompassing this region co-purified to a degree with B14 when expressed in E. coli. In addition, mutagenesis showed that B14 residue F130 is required for the interaction of the two proteins.
ACKNOWLEDGMENTS

Firstly, I would like to thank my supervisor, Professor Geoffrey L Smith for his guidance, support and encouragement throughout my time as a PhD student in his group. I am also deeply indebted to Dr Ron A-J Chen for all his help and kindness both during his time in the lab and in the years since. The many members of the Smith Lab were a tremendous resource of knowledge and inspiration. Particular thanks to Aodhnait for always being there, to Claire for keeping me going on many occasions, to Rory for his invaluable help when it was most needed, to Susie for listening to all my ups and downs, to Kim for steering me in the right direction along the way and to Basma for her sincerity and solidarity. Thank you to Mikey P and Michele for their friendship and help. Special thanks to the old team, Lucy, Michael, Sandra and Jenny for their long-standing encouragement and all the editorial training and to Allison for being a true inspiration and greatly missed. All my friends deserve huge thanks for their continuing care and affection, especially over the past few months when I was unable to fully reciprocate their kindness. Finally I would like to thank my parents for their unwavering willingness to make this process easier in any way they could and Francesco for everything, but especially for his extraordinary patience and for always having confidence in me.
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<tr>
<td>2β-Me</td>
<td>2-β-Mercaptoethanol</td>
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<td>3βHSD</td>
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<td>Antibody</td>
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<td>Ampicillin</td>
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<td>ATP</td>
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<td>BAC</td>
<td>Bacterial artificial chromosome</td>
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<td>Bcl</td>
<td>B cell lymphoma</td>
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<td>CD</td>
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<td>HBS</td>
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<td>Multiplicity of infectivity</td>
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<td>NEMO</td>
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<td>NK</td>
<td>Natural killer</td>
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<td>NLS</td>
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<td>Polymerase chain reaction</td>
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<td>Platelet-derived growth factor</td>
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<td>PFU</td>
<td>Plaque-forming unit</td>
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<td>Rabbit kidney cells, ATCC CCL-37</td>
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<td>SOC</td>
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<td>Yaba-like disease virus</td>
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CHAPTER 1: INTRODUCTION

1.1 Poxviruses

VACV is the prototype virus of the Orthopoxvirus (OPV) genus. It is a double-stranded (ds)DNA virus that replicates within the cytoplasm of host cells (Moss, 2006). VACV strains, such as Lister, were used widely in the successful campaign to eradicate smallpox, caused by variola virus (VARV) (Fenner, 1999). Despite the lack of any natural re-emergence of smallpox since 1977, the study of VACV remains an important area. To date, the use of VACV as a candidate live recombinant vaccine vector has been investigated for a variety of infectious diseases. In addition, VACV can provide insight into both host-virus interaction and key processes within eukaryotic cells and the host response to virus infection.

The Poxviridae is composed of the Chordopoxvirinae and Entomopoxvirinae, which infect vertebrates and insects, respectively. The subfamily Chordopoxvirinae consists of eight genera: OPV, Parapoxvirus, Avipoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus, and Yatapoxvirus. OPVs such as VARV, Monkeypox virus (MPXV) and VACV have been studied extensively, particularly VACV due to its role in the eradication of smallpox. The first VACV strain to be sequenced was Copenhagen (COP), which has a genome of 191 kilobases (kb) and encodes about 200 genes (Goebel et al., 1990). The genome contains a central region which encodes mostly essential genes, and approximately 90 of these are conserved across chordopoxviruses. Many of these are required for virus replication and spread. Either side of the central region are variable regions which include both early and late genes (Goebel et al., 1990) encoding proteins with roles in host immune evasion and modulation, viral virulence and host range. At the genome termini there are inverted terminal repeats (ITRs), which are more variable and encode a few non-essential genes.

1.2 VACV use as vaccine

Vaccination remains a highly desirable option for the control of many diseases, both in terms of providing a prophylactic solution leading to eradication of the causative agent, and as one of the most cost-effective control measures for infectious diseases. Recombinant VACV vectors have considerable potential in vaccine technology and
many features of VACV make it highly suitable for use as a recombinant vaccine vector (Moss \textit{et al.}, 1983; Miner & Hruby, 1990). Firstly, techniques enabling the routine construction of VACV recombinants have been developed (Mackett \textit{et al.}, 1984). Secondly, large pieces of DNA (>25 kb) can be inserted into VACV (Smith & Moss, 1983) and infection leads to high levels of expression in many different cells (Panicali & Paoletti, 1982; Moss \textit{et al.}, 1983; Mackett & Smith, 1986). Furthermore, the fact that VACV is stable when freeze-dried, can be produced easily at a relatively low cost and is able to stimulate antibody (Ab) and cell-mediated immunity makes it an attractive option for development as a vaccine vector for many infectious diseases. One approach is to use VACV in a prime-boost strategy whereby a DNA vaccine is administered prior to vaccination with recombinant VACV (expressing the same antigen(s) as the DNA). This strategy induces better levels of CD8+ T cells than using either vector alone against specific epitopes from many infectious agents including human immunodeficiency virus (HIV) (Cooney \textit{et al.}, 1993; Hanke \textit{et al.}, 1998a; Hanke \textit{et al.}, 1998b; Hanke \textit{et al.}, 1999), tuberculosis (Feng \textit{et al.}, 2001) and malaria (Etlinger & Altenburger, 1991; Plebanski \textit{et al.}, 1998; Schneider \textit{et al.}, 1998; Schneider \textit{et al.}, 2001; McConkey \textit{et al.}, 2003).

1.3 \textbf{Modified VACV Ankara (MVA)}

In the later stages of the smallpox eradication campaign, MVA was developed and proved to have an excellent safety record (Fenner, 1999). To address the poor safety record of conventional smallpox vaccines it was used as a smallpox vaccine in over 120,000 individuals without complications (Stickl \textit{et al.}, 1974; Mayr \textit{et al.}, 1978; Mahnel & Mayr, 1994). This highly attenuated strain was generated by more than 570 passages of VACV strain Ankara in chick embryo fibroblasts (CEFs) from the parental virus, chorioallantois vaccinia Ankara (CVA) (Mayr & Munz, 1964; Mayr \textit{et al.}, 1978; Meisinger-Henschel \textit{et al.}, 2007). During this serial passage MVA incurred six major deletions and due to a severe restriction in host range is unable to replicate in most mammalian cells (Meyer \textit{et al.}, 1991). Despite this, MVA is still able to trigger a substantial mammalian immune response (Stickl \textit{et al.}, 1974). In fact, MVA is more immunogenic than a replication competent VACV strains (such as its parent virus CVA and the majority of other strains of VACV) (Belyakov \textit{et al.}, 2003). The host range restriction of MVA and its avirulent but immunogenic profile in animal
models, makes it an attractive candidate for a recombinant human vaccine (Sutter & Moss, 1992). In addition, owing to its avirulence and inability to replicate after in vivo inoculation MVA can be used under biosafety level 1 conditions. It is also suited to some of the newer developments in vaccine technology, such as needle-free routes of administration. Recently a stable, lyophilised, MVA vaccine was developed that can be directly applied to the nostrils of mice without previous reconstitution (Kastenmuller et al., 2009). This vaccine resulted in both systemic Ab and T cell responses of an equivalent stature to those seen with the intramuscular route, and critically the vaccine produced long-lasting protective immunity against lethal bacterial and viral challenges (Kastenmuller et al., 2009).

1.4 The virus life cycle

The VACV life cycle and morphogenesis pathway (Fig. 1.1) are complicated by the existence of two structurally and antigenically distinct infectious forms of virion. Thus, it is simpler to consider the cycle starting with the formation of these two forms. The first and most abundant form is the intracellular mature virus (IMV), which is enclosed in a single membrane and remains within the infected-cell until lysis (Smith et al., 2002). The second infectious form is the extracellular enveloped virus (EEV), which comprises an IMV surrounded by a second lipid membrane populated with several viral proteins (Smith et al., 2002). EEV is exported out of the infected cell before cell death into the extracellular compartment. However, structurally indistinguishable virions are retained on the cell surface and referred to as cell-associated enveloped virus (CEV) (Smith & Law, 2004). The IMV and CEV/EEV forms of VACV exhibit structural and functional differences due to changes in the components of the outer membrane and have different antigenic properties (Boulter & Appleyard, 1973; Belyakov et al., 2003).
VACV IMV enters the cell either by fusion with the plasma membrane or by endocytosis. Transcription of early genes ensues, followed by genome replication and intermediate and late transcription. Virion assembly occurs within the viral factories located in the cytoplasm. The IMV particle is enclosed within a single membrane and most IMV remain within the infected cell until lysis. However, a proportion is wrapped by a double lipid membrane and exported out of the infected cell before cell death. These are called CEV when attached to the cell surface or EEV if released.
1.4.1 Morphogenesis

Both infectious forms originate from progeny virions formed in viral factories in the cytoplasm, which lack most cellular organelles (Joklik & Becker, 1964; Hollinshead et al., 1999). A crescent membrane composed of lipid and protein is the first VACV structure visible by electron microscopy (Dales, 1963; Morgan, 1976). This develops into a full spherical or oval-shaped structure, which encloses the viral genome and is known as an immature virus (IV) (Condit et al., 2006). The proteolytic cleavage of core proteins converts IV into IMV (Moss & Rosenblum, 1973), which has a brick-like shape and a single membrane (Hollinshead et al., 1999). IMV virions are infectious and most of the VACV progeny is released in this form upon cell lysis (Smith et al., 2002). A proportion of virions, however, are transported from the viral factories to the endosomal (Tooze et al., 1993) or trans-Golgi membranes (Hiller & Weber, 1985; Schmelz et al., 1994) via a microtubule-dependent mechanism (Sanderson et al., 2000; Ward, 2005). Here the virion is wrapped by a double membrane to form intracellular enveloped virion (IEV). The formation of IEV requires the viral proteins A27 (Rodriguez & Smith, 1990; Ward, 2005), B5 (Engelstad & Smith, 1993; Wolfe et al., 1993) and F13 (Blasco & Moss, 1991). Both B5 and F13 are component proteins of the wrapping membranes alongside a further six viral proteins A33 (Roper et al., 1996), A34 (Duncan & Smith, 1992), A36 (Parkinson & Smith, 1994), A56 (Shida, 1986), F12 (Zhang et al., 2000), K2 (Brum et al., 2003; Wagenaar & Moss, 2007); and E2 (Domí et al., 2008). Deletion viruses lacking any of these proteins, bar A56 or K2, exhibit a small plaque phenotype indicative of reduced cell-to-cell spread.

1.4.2 Virus egress

The IEV travels via microtubule-associated transport to fuse with the plasma membrane (Geada et al., 2001; Hollinshead et al., 2001; Rietdorf et al., 2001; Ward & Moss, 2001). The resulting double-enveloped virions are positioned on the cell surface and can either be retained as CEV or released to form EEV (Blasco & Moss, 1991). CEV on the cell surface induces signalling pathways resulting in the polymerisation of actin into projections known as actin tails which propel the virion away from the infected cell (Stokes, 1976; Hiller et al., 1979; Blasco & Moss, 1991; Cudmore et al., 1996; Way, 1998; Ward & Moss, 2001). The EEV that are released have a role in
longer range spread (Payne & Kristensson, 1985). In cell culture, EEV spread can occur either by direct infection of adjacent cells (forming standard plaques) or in a unidirectional convection-mediated manner to distal cells (forming comet-shaped plaques) (Boulter & Appleyard, 1973; Law et al., 2002). Interestingly, VACV is able to prevent superinfection. It does so via a K2/A56 protein complex on the cell surface, which binds to the fusion machinery of incoming IMVs to prevent entry into infected cells (Brum et al., 2003; Wagenaar & Moss, 2007).

The additional host membrane of EEV and CEV helps these virions escape host immunity better than IMV. For example, EEV is relatively resistant to complement (Vanderplasschen et al., 1998) due to the presence of host complement control proteins in this membrane whereas IMV is sensitive. In addition, IMV is readily neutralised by Abs that target at least six different surface antigens (A27, L1, H3, D8 and A17 (Putz et al., 2006)), while the additional “cloak” around EEV/CEV evades the potent IMV-specific Ab response. The B5 protein is a target for EEV-specific neutralising Abs, along with A33 in the presence of complement. Therefore, EEV is not easily neutralised (Law & Smith, 2001), enabling further spread throughout the host. Indeed, VACV spread in vivo is believed to be mainly via EEV because VACV strains which form only IMV are avirulent (Smith et al., 2002). Furthermore, Abs to EEV provide more effective protection to OPV challenge than do Abs to IMV (Boulter & Appleyard, 1973).

### 1.4.3 Virus entry

Following adsorption, the membrane(s) encompassing the virion are shed to release the virus core into the cytoplasm. This occurs for IMV via fusion of its single membrane with the plasma membrane or by endocytosis. For CEV/EEV the outer membrane is disrupted first outside the cell to enable fusion of the IMV membrane with the plasma membrane beneath (Law et al., 2006). No cellular receptor has been identified for virions binding to the cell. Although the H3 (Lin et al., 2000), A27 (Chung et al., 1998) and D8 (Hsiao et al., 1999) IMV membrane proteins can bind glycosaminoglycans (GAGs), however, these cellular GAGs are not essential for infection (Carter et al., 2005). Fusion involves nine, highly conserved, non-redundant IMV proteins: A16, A21, A28, G3, G9, H2, J5, L5 (Senkevich et al., 2005) and F9 (Brown et al., 2006). The current model describes two pathways for IMV entry. In the
first the IMV membrane fuses with the plasma membrane in a pH-independent manner to enable the core to enter the cytoplasm (as has been illustrated by electron microscopy) (Carter et al., 2005). In the second, IMV is endocytosed and, thereafter the virus and vesicular membrane fuse in a pH-dependent manner (Townsley et al., 2006). The pathway used depends on both virus strain and cell type (Townsley & Moss, 2007).

Following removal of the IMV membrane, virus cores are transported deeper into the cell on microtubules (Carter et al., 2005). The viral proteins which enable this and the microtubule motor involved in transporting the virions into the cytoplasm have not been identified. The VACV cores transported contain tightly compacted DNA, structural core proteins, and transcriptional enzymes (Broyles, 2003). While the surface of the core comprises closely packed, regularly arranged spikes, which form the palisade (Dubochet et al., 1994). In addition, the core structure is wrapped by a lipid membrane and further virus proteins, to form the IMV. The incoming cores are found aligned along microtubules by indirect immunofluorescence (Mallardo et al., 2001) and accumulate in the perinuclear region of the cytoplasm and partially uncoat to enable initiation of transcription of the immediate and delayed early VACV genes (Broyles, 2003). The transcripts are actively transported into the cytoplasm in an adenosine triphosphate (ATP)-dependent manner (Kates & Beeson, 1970).

1.4.4 Gene transcription

All three classes of VACV genes (early, intermediate and late) are transcribed by the virus-encoded DNA-dependent RNA polymerase, which is packaged in the viral core contained within the infecting virion (Broyles, 2003). The RNA polymerase comprises nine subunits with a combined mass of more than 500 kilo Daltons (kDa) (Broyles, 2003).

1.4.4.1 Early transcription

Approximately half of VACV genes belong to the early class (Oda & Joklik, 1967). Early mRNAs are capped, polyadenylated and of a distinct length. The proteins encoded are involved in DNA replication (Jones & Moss, 1984; Smith et al., 1989a), nucleotide biosynthesis (Smith et al., 1989b), intermediate gene transcription (Sanz & Moss, 1999) and host immune evasion (Moss & Shisler, 2001). Evaluation of many
early promoters has revealed a highly conserved guanosine (G) residue at -21 or -22, flanked by a sequence that is variable but highly adenosine (A)-thymidine (T) rich (Davison & Moss, 1989a). The VACV-encoded early transcription factor (VETF) is essential for early transcription (Broyles & Moss, 1988). VETF is a heterodimer of the viral D6R (Broyles & Fesler, 1990) and A8L gene products (Gershon & Moss, 1990). These proteins are expressed late during infection and are packaged into the viral core and, therefore, are present during early transcription (Gershon & Moss, 1990). The heterodimer interacts with two sites downstream of the transcriptional start site found at nucleotides -12 to -29 and +8 to +10 (Cassetti & Moss, 1996). VETF interacts with the promoter on both sides of the transcription start site but does not block the template at the site of initiation. Once VETF has bound to the promoter the DNA-dependent RNA polymerase is recruited (Broyles & Li, 1993). Concomitant with recruiting the polymerase, VETF sterically hinders the polymerase due to its DNA contacts on the downstream side (Broyles, 2003). VETF has an ATPase activity (Broyles & Moss, 1988) which enables the hydrolysis of ATP to detach VETF from the DNA and thus allow the polymerase to proceed. Many early genes have the termination sequence TTTTTNT, which is recognised as UUUUUNU in RNA and results in the termination of transcription between 20 and 50 nucleotides downstream (Yuen & Moss, 1987).

1.4.4.2 Intermediate gene transcription

Intermediate gene transcription occurs after viral DNA replication has commenced and produces mainly proteins involved in transcription of late genes (Broyles, 2003). Intermediate promoters require the onset of DNA synthesis but lack the TAAATG motif (see section 1.4.4.3) at the transcriptional start site that is characteristic of late promoters (Vos & Stunnenberg, 1988). Intermediate promoters have an initiator element, TAAAT/A at nucleotide -1 to +4 relative to the first A at the transcriptional start site and an A-T-rich upstream element (Baldick et al., 1992). Viral proteins are also required for transcription of intermediate genes such as intermediate transcription factor (ITF)-B, which is the viral capping enzyme (Vos et al., 1991), and ITF-A, a fraction of which was shown to have promoter DNA-melting activity (Vos & Stunnenberg, 1988; Vos et al., 1991). Other identified intermediate co-factors include VACV ITF (VITF)-1, VITF-2 (Rosales et al., 1994) and VITF-3 (Sanz & Moss,
VITF-1 is the 30-kDa subunit of the viral RNA polymerase (Rosales et al., 1994). VITF-3 is a heterodimer of the viral A8L and A23R gene products (Sanz & Moss, 1999).

### 1.4.4.3 Late transcription

The late mRNAs produced are polyadenylated both at 3’ and 5’ ends and are capped and heterogenous in length. Late genes encode structural proteins needed for new virions and enzymes required to initiate the next round of replication (Broyles, 2003). Late promoters have an initiator-like TAAATG element at the start site for transcription and an A-T-rich -upstream element at positions -16 to -11 (Davison & Moss, 1989b). The RNA polymerase initiates on the A triplet (actually on the T triplet on the template strand) and slips repeatedly while attempting to initiate transcription to form a 5’ poly A sequence (Bertholet et al., 1987; Schwer et al., 1987). A newly-synthesized RNA polymerase is required for late transcription (Hooda-Dhingra et al., 1989), which bypasses the dependence of translation on initiation factors (Shirokikh & Spirin, 2008). The minimal set of viral proteins needed for late transcription comprises three intermediate proteins G8, A1 and A2 (Kovacs et al., 1994), and, in addition an early protein H5 has transcription stimulatory activity (Kovacs et al., 1994; Kovacs & Moss, 1996). Furthermore, a cellular factor has been identified, VACV late transcription factor (VLTF)-X, which has some specificity for poly-T tracts and may target late VACV promoters as sites of initiation (Davison & Moss, 1989b).

### 1.4.4.4 Termination

Termination occurs for early transcripts approximately 30-50 nucleotides downstream of the TTTTTNT termination signal (where N is any nucleotide) (Yuen & Moss, 1987). The capping enzyme senses the signal in the RNA form (Uracil (U)UUUUNU) and causes the RNA polymerase to terminate transcription and release the template. In contrast, the 3’ ends of intermediate and most late VACV genes do not contain termination signals (Condit & Niles, 2002).
1.4.5 DNA replication

Poxviruses replicate their DNA in the cytoplasm after early gene transcription (Domi & Beaud, 2000) to form single linear dsDNA genomes of between 130 and 300 kb (Fig. 1.2). The linear DNA is closed with a hairpin loop at each end (Fig. 1.2). DNA replication occurs in distinct cytoplasmic sites (Cairns, 1960) using VACV-encoded proteins. These include a DNA polymerase (E9) (Jones & Moss, 1984; McDonald & Traktman, 1994), an ATP-dependent ligase (A50) (Kerr & Smith, 1989; Colinas et al., 1990) and a type 1 topoisomerase (H7) (Shuman & Moss, 1987).

Replication begins with a nick in one strand adjacent to one or both termini (Esteban et al., 1977; Moyer & Graves, 1981), 1981) (Fig. 1.3). This produces a 3’ hydroxy group which functions as a primer for elongation of the unfolded terminal hairpin (Esteban et al., 1977; Moyer & Graves, 1981). The newly-copied hairpin anneals to itself and the elongation continues along the genome to the opposite terminus resulting in concatemeric replicative intermediates (DeLange, 1989; Merchlinsky & Moss, 1989). The concatemers contain a palindromic structure containing the conserved 20-bp concatemer resolution sequences. The concatemer-resolving enzyme is a Holliday junction (HJ) viral resolvase (Garcia et al., 2000), which creates a four-way HJ recombination intermediate (Fig. 1.3) (Dickie et al., 1987; Merchlinsky et al., 1988). These resolve to unit length genome monomers which are then incorporated into new virions (Merchlinsky & Moss, 1989). A22 is the VACV-encoded *E. coli* RuvC HJ resolvase orthologue (Garcia et al., 2000) and is found as a dimer when bound to the HJ structure or in solution (Garcia et al., 2006). An inducible A22 null mutant VACV was unable to resolve concatemers under non-permissive conditions (Garcia & Moss, 2001).
Figure 1.2 VACV COP genome structure.
The conserved central region is highlighted in grey, either side are the variable regions and the ITRs. From the terminus, the ITR comprises an incompletely base-paired hairpin terminal loop, the concatemer resolution sequence, tandem repeats and also ORFs.
Figure 1.3 VACV genome replication.
A nick is introduced on one strand next to one or both termini. This produces a 3’ hydroxyl that acts as a self-primer (in grey) for elongation into the unfolded hairpin. The newly-copied hairpin (in grey) self anneals and elongation continues along the genome to the opposite terminus. The concatemeric replicative intermediates formed have a HJ-like cruciform structure in supercoiled plasmids. The intermediates resolve into unit length monomers.
1.4.6 Homologous recombination

Homologous recombination occurs in poxvirus-infected cells (Fenner & Comben, 1958), between either two segments of the viral genome or between transfected DNA fragments and the viral genome (Mackett et al., 1982). Recombination can occur by single or by double homologous recombination. The latter must occur when linear DNA molecules are transfected into infected cells, or else non-viable DNA genomes are formed. When a single homologous recombination event occurs between a transfected circular plasmid and the viral genome the circular plasmid is integrated into the viral genome. As the plasmid contains a region of homology with the virus genome integration of the plasmid generates a repeat and thus the genome is unstable. Therefore, the genome subsequently undergoes a further recombination to remove one repeat and reassert genome stability (Ball, 1987). Therefore, plasmids designed to be homologous to specific regions of the genome have been used to manipulate VACV genomes producing VACV variants with viral and non-viral genes inserted or deleted as required (Mackett et al., 1984).

1.5 Poxvirus infection

The effects of poxvirus infection in vitro vary between virus strain and host cell type but include cell rounding and detachment (Bablanian, 1970; Bablanian et al., 1978), changes in membrane permeability (Carrasco & Esteban, 1982), cell motility (Sanderson et al., 2000) and microtubule reorganization and centrosome dysfunction (Ploubidou et al., 2000). Thus, VACV infection alters cell function, metabolism and morphology, collectively termed cytopathic effect (CPE). Many VACV proteins mimic cellular proteins and are involved in manipulating the host to further propagate the virus. Hence, it is unfairly simplistic to consider the CPE observed as a general malfunction of the cell and undirected destruction, but rather the result of a combination of targeted viral strategies which can result in a major effect on many cell-wide processes. In addition, VACV early gene expression coincides with host mRNA translation being selectively inhibited (Bablanian et al., 1991; Lu & Bablanian, 1996). By approximately 6 hours (h) post-infection (p.i.), cellular DNA, mRNA and protein synthesis have gradually decreased to an undetectable level (Buller & Palumbo, 1991).
Poxvirus infections in vivo cause acute infections that are generally effectively cleared from the host within a few weeks (Buller & Palumbo, 1991). The natural host of VACV is not known, but it has a broad host range (Baxby, 1981; McFadden, 2005). In humans, VACV infection via intradermal (i.d.) inoculation (that is, smallpox vaccination) is characterised by localised inflammation and pustule formation, and in some cases vaccine-related complications, such as eczema vaccinatum (in patients with a prior history of eczema) and progressive VACV in some immunocompromised individuals (which could be life-threatening) (Lane et al., 1969). Much important insight has been gained via the use of in vivo murine models. These include the i.d. route of infection which acts as a model for vaccination and the intranasal (i.n.) route of infection which represents a respiratory-acquired systemic infection. Virulence is assessed in the i.n. model via weight loss and signs of illness (Alcami & Smith, 1992). The murine i.d. model results in a localised skin lesion at the site of inoculation (Tscharke & Smith, 1999; Tscharke et al., 2002; Reading & Smith, 2003a). This enables virulence to be assessed by monitoring the size and duration of the lesion. Analysis of lesion tissue has shown infiltration of macrophages, neutrophils, natural killer (NK) cells, and T lymphocytes in both the i.n. (Reading & Smith, 2003a) and i.d. models of infection (Jacobs et al., 2006).

### 1.5.1 Innate immune response

The host immune response to all pathogens, including VACV, is divided into the innate and adaptive response, which in turn is divided into cellular and humoral components. The innate response is crucial in many viral infections as it occurs rapidly and can eliminate infected cells in a non-specific way. It encompasses physical barriers (skin), the complement system, interferons (IFN) as well as NK and γδT cells. In the i.n. model, in addition to the inflammatory cells identified, expression of chemokines, IFNs, nitric oxide (NO) and tumour necrosis factor (TNF)-α was detected (Reading & Smith, 2003a). NO synthase inhibits DNA replication and late protein expression in VACV infection (Karupiah et al., 1993; Harris et al., 1995; Karupiah et al., 1998).

The complement system is part of the humoral arm of the innate immune system and can eliminate pathogens either by lysis or by targeting them for phagocytosis by macrophages and neutrophils through opsonisation (Janeway & Travers, 2001). These
proteins can lyse IMV via the Ab-dependent or independent pathway (Vanderplasschen et al., 1998) and lyse EEV via the classical Ab-dependent pathway (Lustig et al., 2004).

A key element of the host innate immune responses takes the form of intracellular and cell surface receptors which recognise conserved features of microbes. These host sensors are called pattern recognition receptors (PRRs) and the structures they recognise are collectively termed pathogen-associated molecular patterns (PAMPs) (Netea et al., 2004). These molecular patterns can be protein, nucleic acid or lipid and are recognised as being non-self. The recognition of PAMPs by host PRRs results in activation of signalling cascades which upregulate the secretion of cytokines, chemokines and IFNs. One important group of PRRs is the toll-like receptors (TLR). Bioinformatic analyses showed that the intracellular domain of the interleukin (IL)-1 receptor has amino acid similarity with the intracellular domain of TLRs, and this region became known as the Toll/IL-1 (TIR) intracellular receptor domain. TLRs are mainly found within endosomes or at the cell surface of macrophages and dendritic cells (DCs) (Takeda & Akira, 2007). There are thirteen mammalian TLRs which bind and are activated by distinct PAMPs. Upon activation the intracellular TIR domain recruits adaptor proteins: TLRs 1, 2 and 4-9 recruit myeloid differentiation factor 88 (MyD88) and TLRs 3-4 recruit TIR-domain-containing adaptor-inducing IFN-β (TRIF). Signalling cascades are activated by these adaptor molecules (Fig. 1.4) and culminate in the activation of transcription factors including nuclear factor-κB (NF-κB) (see section 1.7 for detailed discussion) and IFN-regulatory factors (IRFs). These transcription factors in turn upregulate the expression of genes encoding pro-inflammatory cytokines, chemokines and IFNs.

Many TLRs have been demonstrated to play a role in countering VACV infection. The lipopeptide-stimulated TLR2/MyD88 pathway is essential to control VACV infection in TLR2−/− mice (Zhu et al., 2007), because TLR2−/− DCs do not induce cytokines following stimulation with VACV as occurs with wild type DCs. In addition, TLR3 is involved in responding to VACV infection, because TLR3−/− mice infected with VACV intranasally lost less weight and had a reduced morbidity (Hutchens et al., 2008). This was explained by the finding that interactions between TLR3 and VACV increase viral replication and enhance the detrimental aspects of the
host immune response to VACV (Hutchens et al., 2008). TLRs 7 and 8 detect nucleic acids in response to VACV infection, although the mechanism remains unknown (Miller et al., 2008). Furthermore, VACV strain CVA has a TLR9-antagonising mechanism in response to TLR9 recognition of DNA localised to the endosome whereas MVA has lost this function (Samuelsson et al., 2008).

IFNs are a group of secreted cytokines that elicit distinct antiviral effects to establish an anti-viral state at the site of infection (Samuel, 1991). This occurs via release of the type I IFN which binds receptors on surrounding cells to activate signal-transduction pathways that trigger the transcription of a diverse set of genes that, in total, establish an antiviral response in target cells (Marie et al., 1998). Consequently, the cell is primed to have an anti-viral state such that upon subsequent infection by a virus, viral RNA is degraded, protein synthesis is inhibited and viral growth restricted (Muller et al., 1994). IFN plays an important role in the control of VACV infection as shown by the increased mortality seen in mice deficient in the IFN-α/β receptor (Panchanathan et al., 2005) and the existence of a VACV-encoded soluble type I IFN inhibitor (Symons et al., 1995) (see section 1.6.3). Type II are also antiviral, namely IFNγ (Gray & Goeddel, 1982). Type III IFNs have an antiviral effect by interacting with a distinct receptor and the expression of murine IFNλ by recombinant VACV attenuated virulence in the i.n. model.

NK cells both secrete IFNγ and exercise a cytotoxic activity against VACV-infected cells (Kiessling et al., 1976; Brutkiewicz et al., 1992) via granzyme and perforin-mediated lysis. They can be activated either by IFNs or via cytokines, such as IL-12 and TNFα secreted by macrophages. NK cells are implicated in control of VACV infection because mice depleted for NK cells experience enhanced disease upon VACV infection (Bukowski et al., 1983). NK cells play a rapid and persistent role in controlling VACV infection in mice and are detected as early as 6 h p.i. (Natuk & Welsh, 1987; Prlic et al., 2005) and a peak infiltration at day 7 (Jacobs et al., 2006) in the i.n. model.

1.5.2 Adaptive immune response

The adaptive immune response is specific to the each pathogen or antigen and cannot, unlike the innate response, provide immediate recourse against infection. Adaptive
immunity may take at least a week to develop and longer than this to reach maximum level, but will result in immunological memory of the specific antigen and thus can provide long-term protection should re-exposure occur. Following VACV infection (vaccination) such memory responses can protect the host from future infection with a related poxvirus (Moss & Shisler, 2001). The adaptive response relies on antigen-specific T and B cells. T cells are subdivided into CD8+ T cells, which have cytotoxic activity, and CD4+ T cells, which direct CD8+ T cells and can prime Ab-producing B cells. However, the adaptive and innate immune responses overlap. Many inflammatory cells recruited to the site of infection, in addition to their role in the innate immune response, form part of the adaptive phase of the immune response. For example, macrophages and DCs are key antigen presenting cells (APC) needed to activate both T and B cells and to initiate specific immune responses. Mice depleted of macrophages are unable to control VACV infections as a consequence of impaired virus clearance and antigen presentation (Werenne et al., 1985; Rodriguez et al., 1991; Karupiah et al., 1996).

VACV infection in mice produces an adaptive response, involving T cell responses that are important for recovery from VACV infection in animal models (Buller & Palumbo, 1991). This was highlighted by the recovery from i.n. infection seen in the absence of Ab via T cell-mediated immunity (Belyakov et al., 2003), although T cell-dependent cytotoxicity via perforin and Fas was not essential to overcoming infection (Kagi et al., 1995). However, the Ab response also plays a role in control of VACV infection, particularly with regards to protection from a secondary challenge. VACV i.n. challenge of previously immunised mice showed that VACV-specific Abs protected from weight loss whereas neither type of T cell was required for protection (Belyakov et al., 2003). The humoral response is also critical in humans and VACV immune globulin is the recommended treatment in the case of severe complications following smallpox vaccination (Hopkins & Lane, 2004).
1.6 VACV immunomodulatory genes

VACV is a useful model for studying host-virus interactions and produces many immunomodulators that facilitate evasion of the host immune system (Seet et al., 2003). Many immunomodulatory proteins mimic host cell receptors or ligands involved in critical cell processes and, as the virus and host have co-evolved, many viral orthologues of cellular proteins have been acquired and adapted to benefit the virus (Bugert & Darai, 2000). Herein the VACV strain WR immunomodulators are used as illustrative examples unless otherwise specified.

1.6.1 MVA immunomodulators

Many of the immune evasion genes are located in the terminal regions of the VACV genome (Buller & Palumbo, 1991). The right terminal region of the MVA genome is more structurally conserved than the left compared to parental virus, CVA, and begins downstream of the RNA polymerase rpo132 subunit gene (A24R) (Meyer et al., 1991). Despite the relatively higher level of conservation in this part of the genome in other VACV strains and OPVs, many of these ORFs are deleted or disrupted in MVA. However, MVA has retained several genes in this region that encode proteins that target components of the host immune system. These include inhibitors of NF-κB activation, apoptosis regulation and the synthesis and function of chemokines and cytokines (Antoine et al., 1998). There is even an enzyme involved in steroid biosynthesis (Antoine et al., 1998). Thus, although infection with MVA results in activation of multiple immune signalling pathways, at least some VACV immunomodulators are retained to undermine the host response (Delaloye et al., 2009). Further understanding of the immunomodulatory mechanisms preserved in MVA could inform and advance the use of this attenuated strain as a recombinant vaccine vector. Therefore, in conjunction with the illustrative examples of WR immunomodulators, the level of conservation of the MVA counterparts will be discussed (Table 1.1). Further characterisation of these predicted immunomodulatory proteins is not just interesting in terms of assessing the means by which this attenuated VACV interacts with the host, but could also highlight genes for targeted removal in order to enhance the attributes of MVA as a recombinant vaccine vector.
1.6.2 Complement

VACV strain WR encodes a secreted protein called VACV complement protein (VCP) that exerts anti-complement activity. The C21L gene product interacts with C3 and C4b and thus can inhibit both the classical and alternative pathways of complement activation (Kotwal & Moss, 1988; Isaacs et al., 1992). In addition, VACV incorporates host complement control proteins such as the cluster of differentiation (CD)55 and CD59 proteins into the EEV outer envelope. These host proteins protect both host tissues and virions from complement lysis (Vanderplasschen et al., 1998). MVA expresses no complement control binding protein equivalent to C21.

1.6.3 Apoptosis

Apoptosis can be triggered by intrinsic and extrinsic signals. The latter is receptor-mediated and is initiated by the binding of ligands to their cognate death receptors, for example, TNF binding to TNF receptor 1. The cell-surface receptors trimerise and initiate an intracellular signalling cascade which results in the cleavage and activation of members of a family of cysteine proteases called caspases which result in apoptosis. In addition, one of these proteins, caspase 8 activates the B cell lymphoma (Bcl)-2 protein Bid which translocates to the mitochondria where it interacts with other pro-apoptotic Bcl-2 proteins to permeabilise the membrane and release additional pro-apoptotic factors. The intrinsic pathway is triggered within the cell and requires mitochondrial involvement to induce apoptosis. Via this pathway stress signals such as DNA damage, dsDNA or virus infection result in mitochondrial release of cytochrome c and subsequent caspase activation. By modulating apoptosis poxviruses can enhance their replication and spread (Taylor & Barry, 2006).

VACV anti-apoptotic mechanisms include two viral serine protease inhibitors (serpins). One, called B13 inhibits caspase 1 and inhibits both TNFα or Fas-induced apoptosis (Kettle et al., 1997). Deletion of B13R did not affect virulence in the i.n. murine model of infection (Kettle et al., 1995) but did cause production of larger lesions than control in the i.d. model (Tscharke et al., 2002).
Table 1.1 Comparison of a range of immunomodulators in VACV strains COP, WR and MVA.

Immunomodulators are grouped approximately by function and those believed to be non-functional due to sequence changes are highlighted in grey. B14, 183 and the COP orthologue are highlighted in dark grey. Adapted from (Antoine et al., 1998) with supplementary information from www.poxvirus.org.

<table>
<thead>
<tr>
<th>Function</th>
<th>COP</th>
<th>WR</th>
<th>MVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocks caspase 1</td>
<td>B13/B14 (disrupted)</td>
<td>B13</td>
<td>Deleted</td>
</tr>
<tr>
<td>Inhibits apoptosis</td>
<td>B23</td>
<td>B22</td>
<td>189</td>
</tr>
<tr>
<td>eIF2α mimic</td>
<td>K3</td>
<td>K3</td>
<td>024</td>
</tr>
<tr>
<td>dsDNA binding protein</td>
<td>E3</td>
<td>E3</td>
<td>050</td>
</tr>
<tr>
<td>Inhibits apoptosis and NF-κB</td>
<td>N1</td>
<td>N1</td>
<td>020 (disrupted)</td>
</tr>
<tr>
<td>Bak/Bax interaction</td>
<td>F1</td>
<td>F1</td>
<td>029</td>
</tr>
<tr>
<td>Unknown</td>
<td>B7</td>
<td>B7</td>
<td>153</td>
</tr>
<tr>
<td>IL-1β receptor</td>
<td>B16 (disrupted)</td>
<td>B15</td>
<td>184</td>
</tr>
<tr>
<td>IFNα/β receptor</td>
<td>B19</td>
<td>B18</td>
<td>187 (disrupted)</td>
</tr>
<tr>
<td>IFNγ receptor</td>
<td>B8</td>
<td>B8</td>
<td>176 (disrupted)</td>
</tr>
<tr>
<td>IL-18 binding protein</td>
<td>C12</td>
<td>C12</td>
<td>008</td>
</tr>
<tr>
<td>Represses NF-κB activation from TLRs</td>
<td>A52</td>
<td>A52</td>
<td>Deleted</td>
</tr>
<tr>
<td>IFNβ (and NF-κB) downregulation</td>
<td>A46</td>
<td>A46</td>
<td>159</td>
</tr>
<tr>
<td>IFNβ (and NF-κB) downregulation</td>
<td>K7</td>
<td>K7</td>
<td>028</td>
</tr>
<tr>
<td>NF-κB downregulation</td>
<td>B15</td>
<td>B14</td>
<td>183</td>
</tr>
<tr>
<td>NF-κB downregulation</td>
<td>A49</td>
<td>A49</td>
<td>165</td>
</tr>
<tr>
<td>NF-κB downregulation</td>
<td>M2</td>
<td>M2</td>
<td>Deleted</td>
</tr>
<tr>
<td>Host range restriction</td>
<td>K1</td>
<td>K1</td>
<td>Truncated</td>
</tr>
<tr>
<td>TNF receptor</td>
<td>A53 (disrupted)</td>
<td>A53 (disrupted)</td>
<td>Deleted</td>
</tr>
<tr>
<td>Chemokine binding</td>
<td>A41</td>
<td>A41</td>
<td>153</td>
</tr>
<tr>
<td>Cell surface immunomodulator</td>
<td>A40</td>
<td>A40</td>
<td>148</td>
</tr>
<tr>
<td>C3 and C4b interaction</td>
<td>C21</td>
<td>006</td>
<td>Deleted</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>A44</td>
<td>A44</td>
<td>157</td>
</tr>
</tbody>
</table>
B13 inhibits caspase 1 to prevent the production of mature IL-1β (Kettle et al., 1997), but is not able to affect the febrile response via inhibition of IL-1β (Kettle et al., 1997). A second serpin, B22, is related to B13, and like the deletion of B13, the deletion of this viral serpin also did not affect virulence in the i.n. model (Kettle et al., 1997). However, while there is no B13 counterpart in MVA there is a B22 orthologue, 189, which shares approximately 90% amino acid sequence identity with WR B22. The first difference between the predicted 189 protein sequence and that of B22 is the presence of an N-terminal extension of 18 residues on 189, whether mRNA encoding this extension is produced has not been confirmed. There are a number of amino acid substitutions but notably most are also present in the parental strain CVA orthologue (encoded by 222R). However, there are four changes and a 12-amino acid deletion in 189 that are not present in the CVA orthologue.

The NIL gene product was initially described as a secreted protein (Kotwal et al., 1989) but later was shown to be an intracellular homodimer expressed early during infection in both i.d. and i.n. models (Bartlett et al., 2002). Overexpression of N1 by transfection inhibited NF-κB activation and this was initially suggested to occur in response to stimulation with IL-1 or TNF via an interaction with the inhibitor of NF-κB (IκB) Kinase (IKK) complex (DiPerna et al., 2004). However, N1 does not interact with the IKK complex (Chen et al., 2008) and inhibits NF-κB activation only in response to TNF-induced signalling (Graham et al., 2008). The role of N1 as a virulence factor is probably partly due to its ability to inhibit apoptosis, shown by the resistance to staurosporine-induced apoptosis of cells transfected or infected with N1 (Coo-ray et al., 2007). In addition, N1 co-precipitates with pro-apoptotic Bcl-2 proteins Bid, Bad and Bax (Cooray et al., 2007). This correlates with the structural similarity seen between the crystal structure of N1 (Cooray et al., 2007) and Bcl-2 members despite a low amino acid sequence similarity between N1 and this family of proteins (Graham et al., 2008). In fact, the N1 structure contains a BH3-like groove like that seen in anti-apoptotic cellular Bcl-2 proteins. MVA encodes an NIL gene, however, due to fragmentation, the encoded protein is only 113 amino acids and unlikely to encode a functional protein (Antoine et al., 1998).

In addition to the WR proteins mentioned, the COP-encoded F1 protein is also a Bcl-2-like protein (Kvansakul et al., 2008). F1 localises via a single transmembrane
domain (Stewart et al., 2005) to the mitochondrial membrane to inhibit the release of cytochrome c (Wasilenko et al., 2005). Deletion of $F1L$ resulted in loss of inhibition of Bak or Bax-induced-apoptosis and biosensor data (Fischer et al., 2006) indicates that F1 interacts with the BH3 domains of Bak and Bax. The F1 interaction directly inhibits activation of the Bak (Wasilenko et al., 2005; Postigo et al., 2006) and interferes with Bim-mediated activation of the pro-apoptotic Bax (Taylor et al., 2006). However, to date, the role of $F1L$ has not been assessed in vivo. F1 is also conserved in MVA, but, there is also a four-amino acid deletion towards the N terminus of the MVA protein not seen in the other VACV strains.

Viral protein E3L exerts an immunomodulatory effect by binding dsRNA, preventing protein kinase R (PKR) activation and thus apoptosis (Chang et al., 1992). The E3 protein has an orthologue in MVA which differs by five amino acid substitutions. MVA lacking E3 is unable to produce late gene transcripts (Ludwig et al., 2006). A further VACV protein encoded by $K3L$ is known to mimic eukaryotic initiation factor (eIF)2α and thus acts as a pseudo substrate for PKR (Davies et al., 1992). This protein also has an MVA counterpart, encoded by $024L$ and differing from WR K3 by one amino acid.

### 1.6.4 Cytokines

The ability of VACV to interfere with the activity of cytokines has been characterised extensively. Among the well-studied examples of VACV proteins known to affect cytokine activity there are those which mimic cellular proteins. These include a secreted viral IL-1β inhibitor (detailed below) B15 (Alcami & Smith, 1992; Spriggs et al., 1992) and B8. The B8 protein is secreted from infected cells and inhibits a component of the host IFN system. B8 expressed by recombinant baculovirus binds to and inhibits the activity of IFN-γ by blocking its interaction with its cellular receptors (Alcami & Smith, 1995). However, MVA does not encode a B8 counterpart, due to gene fragmentation (Blanchard et al., 1998). VACV also encodes B18, a secreted viral IFN I receptor that is related to the IL-1 receptors and is not a class II cytokine receptor family member like the other type I IFN receptors (Symons et al., 1995). Following secretion from infected cells, VACV B18 protein binds and inhibits IFN-α/β binding to cellular receptors on both infected and uninfected cells. Consequently B18 protects cells at the site of infection from the antiviral state induced by IFN-α/β.
(Colamonici et al., 1995; Symons et al., 1995; Alcami et al., 2000). There is an MVA counterpart of B18R gene but the gene is fragmented and non-functional (Antoine et al., 1998; Blanchard et al., 1998).

In addition, another VACV immunomodulator affects IFNγ indirectly by binding IL-18 (Symons et al., 2002; Reading & Smith, 2003b). C12 was identified in our laboratory as a secreted protein that diminishes the production of IL-12-induced IFNγ (Symons et al., 1995) and was identified directly as an IL-18 binding protein by other groups (Smith et al., 2000; Calderara et al., 2001) due to its sequence similarity to secreted cellular IL-18 binding proteins. C12 binds IL-18 and down-regulates IFNγ production. In fact, IFNγ levels were 10- to 20-fold higher in bronchial alveolar lavage from mice infected intranasally with VACV lacking C12L (Reading & Smith, 2003b). IFNγ is a pro-inflammatory cytokine that is secreted by activated macrophages or monocytes. In VACV infections IFNγ is involved in protective immunity and down-regulates NK and T cell activity (Reading & Smith, 2003b). This indicates a role of IL-18 in modulating both innate and the specific adaptive immune responses. IL-18 is also modulated by B13 which prevents caspase I-mediated cleavage of pro-IL-18. MVA encodes a C12 counterpart, it contains some changes in amino acid sequence relative to C12 including a small in-frame deletion, however, the majority of these changes are also seen in the MVA parental virus CVA orthologue.

VACV WR also encodes a soluble IL-1β receptor, B15 (Alcami & Smith, 1992), which competitively inhibits the binding of IL-1β to its receptor. B15 is specific for IL-1β and does not bind IL-1α, (Alcami & Smith, 1992). VACV lacking B15R accelerated signs of illness and mortality in the i.n. model (Alcami & Smith, 1992). Furthermore, insertion of B15 into the COP strain, which does not otherwise encode an IL-1β receptor, prevented the febrile response during systemic infection in the i.n. model (Alcami & Smith, 1996). However, WR containing an inactivated B15R exhibited decreased virulence in an intracranial murine model (Spriggs et al., 1992). This could be explained by the properties of IL-1β, which when present in excess can be harmful to the host in itself (Dinarello, 1994). MVA has retained a copy of B15R, and its deletion from MVA increased the virus-specific CD8+ memory T cell response (Staib et al., 2005). The WR and MVA forms differ only by three conserved substitutions in the second half of the protein. The COP orthologue is non-functional.
due to a frameshift mutation early in the gene. This prevents ribosomes from translating the full length mRNA into protein.

Some poxviruses also encode soluble TNF receptors, four of which, cytokine response modifier (Crm)B (Hu et al., 1994), C (Smith et al., 1996), D (Loparev et al., 1998) and E (Reading et al., 2002) were identified in cowpox (CPXV). Although, most VACV strains do not express TNF receptors three strains Lister, USSR and Evans express both soluble and membrane-bound receptors (Alcami et al., 1999). In other VACV strain such as COP and WR only gene fragments of TNF receptors remain. In addition, MVA does not encode a TNF receptor (Blanchard et al., 1998).

1.6.5 TLR signalling

VACV encodes immunomodulatory proteins to counteract signalling from the TLRs. On binding their activating ligand, TLRs recruit adaptor molecules to their intracellular TIR domains, such as MyD88, TRIF and TIRAP (Fig. 1.4). The signals generated can activate NF-κB, IRF-3 or other transcription factors. VACV proteins A46 and A52 interfere with TLR and IL-1 signalling to inhibit NF-κB activation (Bowie et al., 2000). A52 represses NF-κB activation by interfering with signals emanating from multiple TLRs including TLR-3 (Harte et al., 2003), by interacting with IL-1 receptor-associated kinase (IRAK)2 and TNF receptor-associated factor (TRAF) 6 (Harte et al., 2003). A mutant VACV lacking A52R was attenuated (Harte et al., 2003). A46 also represses NF-κB activation in response to TLR and IL-1 activation and functions by binding MyD88 and two additional TIR adaptor proteins (MyD88-like (Mal)) and TRIF-related adaptor molecule (TRAM) (Stack et al., 2005). Although A46 and A52 have overlapping activity they are not redundant because deletion of either causes reduction in virulence in the i.n. model (Harte et al., 2003; Stack et al., 2005). A52 and A46 were initially predicted to contain TIR domains (Bowie et al., 2000), however, subsequently the Bcl-2 structure of A52 was solved (Graham et al., 2008) and by inference it is also unlikely that A46 contains a TIR domain. There is no counterpart to A52 in MVA, however, there is an orthologue of A46. MVA 159 shares 99 % identity with WR A46. There is only one amino acid substitution in MVA 159 relative to WR A46.
Another VACV protein, K7 has a Bcl-2 fold (Kalverda et al., 2009) and antagonises the host IFN response by interacting with the asparagine-glutamate-alanine-asparagine (DEAD) box RNA helicase (DDX3), thereby suppressing DDX3-mediated IFNβ promoter induction (Schroder et al., 2008). K7 can also bind to TRAF6 and IRAK2 to inhibit NF-κB activation induced by TLR4 (Schroder et al., 2008). K7 has an identical counterpart in MVA, 028 and all strains of VACV.

1.6.6 Chemokines

Chemokines are cytokines that function as chemoattractants for cells involved in the immune response including leukocytes, monocytes, neutrophils. They are secreted by a range of cell types and bind to chemokine receptors (Rossi & Zlotnik, 2000). Chemokines are grouped by the pattern of cysteines near the N terminus, and the major groups are the CC and CXC chemokines where the cysteines are either adjacent or separated by another amino acid, respectively. VACV has two proteins known to bind and inhibit chemokines. The viral chemokine binding protein (CKBP or vCCI) is a secreted protein identified in the supernatant of VACV-infected cells that binds several chemokines (Graham et al., 1997; Smith et al., 1997; Alcami et al., 1998). CKBP has a β-sandwich topology with a negatively-charged CC-chemokine binding site (Carfi et al., 1999). CKBP is expressed by several strains of VACV, such as COP, but it is absent from WR. This strain does, however, encode a structurally-related 30-kDa secreted glycoprotein, A41 (Ng et al., 2001), that binds to CCL21, 25, 26 and 28. A41 also has a β-sandwich structure similar to CKBP, but there are differences in surface loops and electrostatic charge distribution (Bahar et al., 2008). A41 interferes with chemokine-GAG interactions at the cell surface so that a chemokine concentration gradient on the cell endothelial surface is not established and so leukocytes are not recruited (Bahar et al., 2008). The deletion of A41L from WR increases virulence slightly in the i.d. (Ng et al., 2001) and i.n. model (Clark et al., 2006). MVA has retained an A41 counterpart. WR A41 differs from the corresponding COP, MVA and CVA ORFs by six single conserved amino acid changes. An MVA A41 deletion virus provided better protection than wild type MVA against subsequent challenge with WR in immunogenicity studies (Clark et al., 2006). Furthermore, VACV- or foreign epitope-specific splenic CD8+ T cell responses were
enhanced upon subcutaneous immunisation with the MVA A41 deletion virus (Clark et al., 2006).

A further VACV immunomodulatory protein involved in chemokine binding is B7, an 18-kDa glycosylated protein that is expressed late in infection and localised to the ER (Price et al., 2000). A mutant VACV lacking B7R had significantly attenuated virulence in the i.d. murine model. B7 is related to a VARV protein, CrmB, which functions not only as a soluble TNFR but also to bind chemokines via a smallpox virus-encoded chemokine receptor (SECRET) domain. B7 has shares homology with this domain (Alejo et al., 2006). There is a B7 MVA orthologue, 153, which shares a high level of sequence identity with WR B7. The counterpart MVA protein is predicted to have a five residue internal deletion relative to B7.

1.6.7 Further immunomodulators

In addition to expressing proteins that target specific host proteins that function in innate immunity, VACV gene A44L encodes an enzyme, 3-β-hydroxysteroid dehydrogenase (3β-HSD) that synthesizes steroid hormones (Moore & Smith, 1992; Reading et al., 2003). Protein A44 shares 31 % amino acid identity with human 3β-HSD and contributes to VACV virulence by producing glucocorticoids that suppress the local inflammatory response to infection. Removal of the A44L gene reduced virulence in the i.n. model (Moore & Smith, 1992) and increased immunogenicity (Reading et al., 2003). WR A44 has a counterpart in MVA, 157, which has only one amino acid change predicted relative to WR A44.

VACV WR protein A40 has amino acid similarity to C-type animal lectins and has an immunomodulatory function (Wilcock et al., 1999; Jacobs et al., 2006). The precise function of this early protein is not yet clear, but it localises to the membranes of infected cells (Wilcock et al., 1999). A mutant WR with a disrupted A40R gene produced no alteration in either in vitro plaque size or in vivo virulence phenotype in the i.n. model (Wilcock et al., 1999). However, WR lacking A40 produced smaller lesions in the i.d. model (Tscharke et al., 2002). Furthermore, the loss of A40 in the i.d. model resulted in an increase in the recruitment of macrophages and T cells early in infection (Jacobs et al., 2006). In addition, mice immunised with the WR A40 deletion virus were better protected from subsequent challenge with wild type WR
(Susan Jarmin, personal communication). A40 has a counterpart in MVA which differs by only one amino acid substitution.

1.7 NF-κB modulation

As described briefly in section 1.6.5, pro-inflammatory stimuli result in multiple signalling cascades that activate the NF-κB dimeric transcription factor family. NF-κB activation can be triggered by wide-ranging stimuli, including TNFα, and IL-1 (Viatour et al., 2005). The genes under the control of the NF-κB response element are important in a variety of biological processes such as infection, inflammation, transformation, cell proliferation and apoptosis (Viatour et al., 2005).

NF-κB activation results in regulation of numerous genes encoding proteins which are critical for the innate immune response to poxvirus infection (Oie & Pickup, 2001). The genes upregulated encode diverse proteins, including chemokines, cytokines, adhesion molecule, anti-apoptotic proteins and enzymes that facilitate the actions of secondary inflammatory proteins (Ghosh et al., 1998).

Prior to stimulation, NF-κB is retained in the cytoplasm due to an interaction with IkBα which hides the p65 components nuclear localisation signal (NLS) (Hayden & Ghosh, 2004). IkBα is phosphorylated by the IKK complex and thereafter ubiquitinated and degraded by the proteosome (Karin & Ben-Neriah, 2000). NF-κB dimers then translocate to the nucleus where they bind to response elements in the promoters of the many NF-κB target genes and induce transcription (Ghosh & Karin, 2002). All the NF-κB-inducing stimuli result in the activation of various signalling cascades that converge at the IKK complex leading to the activation of NF-κB (Bonizzi & Karin, 2004). In the conventional pathway, this large complex (approximately 700-900 kDa in molecular mass) includes three subunits: IKKα, IKKβ and IKKγ (also known as NF-κB essential modulator (NEMO)). The first two subunits have catalytic functions while IKKγ plays a regulatory role (Bonizzi & Karin, 2004). The active form of the IKK complex phosphorylates two serine residues close to the amino terminal of IkBα, targeting it for degradation and releasing the associated NF-κB dimer (Chen et al., 1996). An additional level of regulation has recently been proposed that would enable certain stimuli to activate only a given subset of NF-κB target genes if required (Shuai, 2006).
Figure 1.4 NF-κB signalling pathways.
Recognition of PAMPs by appropriate receptors stimulates the upregulation of transcription factor activity including the NF-κB family. This occurs via signalling through a range of TLRs which converges on IRAK4 to triggers further signalling cascades to regulate apoptosis and transcription. The latter involves activation of the transforming growth factor β activated kinase (TAK1) complex, which stimulates the p38 mitogen-activated protein kinase (MAPK), JNK and NF-κB pathways. In the NF-κB pathway, TAK1 activates the IKK complex, which then phosphorylates IκBα and thus induces its ubiquination and degradation. This frees the NF-κB dimer to translocates into the nucleus and activate transcription of genes involved in the anti-inflammatory response. In addition, intracellular factors, such as dsRNA, activate RIG-I. This molecule acts via mitochondrial pathways to activate the IKK complex, via IKKε to activate both IRF3/7 and NF-κB transcription factors.
1.7.1 NF-κB and VACV

VACV replication does not require NF-κB and so VACV has evolved many strategies to block NF-κB activation and thereby suppress activation of innate immunity (Oie & Pickup, 2001; Shisler & Jin, 2004). Modulation of NF-κB activation is achieved by a variety of mechanisms, including those involving VACV proteins A46 (Stack et al., 2005), A52 (Harte et al., 2003), K7 (Schroder et al., 2008) (sections 1.6.5) and N1 (DiPerna et al., 2004; Graham et al., 2008) (section 1.6.3). As discussed previously, these proteins modulate the signals leading to activation of the IKK complex to down-regulate NF-κB activation.

Another VACV-encoded modulator of NF-κB is B14 (Chen et al., 2008). The further characterisation of B14 forms a key part of this thesis and its role in NF-κB activation will be discussed at length in 1.7.2. In addition, a further NF-κB-modulatory VACV protein investigated in this work is encoded by A49R. This previously uncharacterised open reading frame (ORF) is predicted to encode a protein, A49, which inhibits the activation of NF-κB (Daniel Mansur, personal communication). This 18.8-kDa protein is conserved across the OPV and located in the cytoplasm. The activation of NF-κB seen with overexpression of cellular components of the NF-κB activation pathway (including TRAF2, and 6, IKKα, IKKβ or p65) was abolished upon co-expression of A49 suggesting a downstream role for A49 in abrogating NF-κB activation. In the presence of A49 IkBα was phosphorylated but NF-κB remained in the cytoplasm (Daniel Mansur, personal communication). A49 is also present in MVA but with three amino acid changes relative to WR A49.

The VACV M2 protein is also a NF-κB modulator. Its function was revealed following study of NF-κB activation seen on infection with an MVA mutant with M2 inserted (MVA does not otherwise encode M2) (Gedey et al., 2006). Infection with MVA activates host NF-κB activity approximately 10-fold relative to that seen with WR in human embryonic kidney cells with SV40 T antigen (HEK 293T) cells but extracellular signal-regulated kinase (ERK)2 phosphorylation was incomplete in MVA-infected compared to WR infected cells (Gedey et al., 2006). The reintroduction of WR M2L into MVA was used to demonstrate that the encoded protein prevents phosphorylation of ERK2 and in consequence NF-κB activation (Gedey et al., 2006). ERK2 belongs to a larger family of MAP kinases, which include
the c-Jun N-terminal kinase/stress-activated protein kinase, p38 kinase, and the highly similar ERK1 protein (Roux & Blenis, 2004). In addition, the NF-κB activation seen after MVA infection was diminished when either MEK/ERK pathway antagonists or a dominant-negative ERK2 mutant protein was present (Gedey et al., 2006).

The VACV K1 protein also prevents NF-κB activation. K1 binds to IκBα and competitively inhibits IκBα host-induced degradation (Shisler & Jin, 2004). K1 is also a host range protein (Perkus et al., 1990) and its orthologue in MVA is truncated and contributes to the restriction in growth of MVA in many mammalian cell lines. This host range defect can be repaired by the stable expression of full-length WR K1 by rabbit kidney cells, ATCC CCL-37 (RK-13) cells which are subsequently permissive for MVA (Sutter et al., 1994).

The WR B14 protein (described in detail in section 1.7.2) is an NF-κB modulator central to this study as is its counterpart in MVA, protein 183. This protein contains two conserved substitutions relative to WR B14. The first change is at residue 27, which is an arginine in WR, but a lysine in MVA and COP. The second substitution is at residue 84, which is an alanine in WR, a valine in MVA but a threonine in COP. Both of the changes seen in MVA were already present in the ancestral strain of MVA, CVA. Overall MVA predicted protein 183 shares 94 % amino acid identity to B14. However, in addition to the single-residue substitutions listed there is also a six-residue deletion in MVA relative to B14 and the COP orthologue. The high-resolution structure available for B14 indicates that helices four, five and six form the backbone of the protein, and the predicted MVA 183 protein lacks six amino acid residues in helix six. It is plausible that this deletion could affect the protein folding or stability and confer loss of function or an altered function. This possibility is made more likely by considering that the six residues are intact in the parental CVA strain, and therefore this deletion was incurred during the 570 passages of MVA in CEFs where there are marked differences in signalling pathways as compared to mammalian cells.
1.7.2 Characterisation of B14 and its role in NF-κB modulation

B14 contributes to virus virulence in vivo and affects the host response to infection (Chen et al., 2006). B14R is a 450-bp gene that encodes a protein of 149 amino acids in length and 17 kDa in size (Chen et al., 2006). It is highly conserved across VACV, OPV and orthologues are also found in Yatapoxviruses, Capripoxviruses, Leporipoxviruses and Suipoxviruses (Fig. 1.5). In fact, all Chordopoxvirinae except Avipoxvirus and Molluscipoxvirus encode a B14-like protein (Chen et al., 2006).

Recently the crystal structure of B14 was solved (Graham et al., 2008) (Fig 1.6) and showed B14 is a Bcl-2 protein like N1 (Aoyagi et al., 2007; Cooray et al., 2007), A52 (Graham et al., 2008), K7 (Kalverda et al., 2009) and F1 (Kvansakul et al., 2008). Proteins F1 (Fischer et al., 2006; Postigo et al., 2006; Taylor et al., 2006) and N1 (Cooray et al., 2007) have a surface groove for binding BH3 peptides of pro-apoptotic Bcl-2 proteins and thereby inhibit apoptosis. In contrast, B14, A52 and K7 lack this groove and are not anti-apoptotic (Graham et al., 2008).

The structure consists of eight α-helical regions joined by short linker sequences with an N-terminal loop. B14 is expressed early at approximately 2 h p.i. and is localised predominantly in the cytoplasm (Chen et al., 2006). A recombinant VACV WR lacking the B14R gene (vΔB14) was constructed showing that B14 is not essential for viral replication and exhibits normal growth kinetics in cell culture (Chen et al., 2006). In a murine i.n. model of infection, vΔB14 induced similar weight loss to control viruses expressing B14 (Chen et al., 2006). However, in the murine i.d. model of infection, vΔB14 induced a smaller lesion size, increased infiltration of cells and decreased virus titres (Chen et al., 2006). The number of both macrophages and T cells rose significantly at eight days p.i. Thus, there is an altered host inflammatory response to VACV WR when B14 is absent.
Figure 1.5 Amino acid alignment of OPV sequences for proteins with ≥95% amino acid identity to VACV strain WR protein B14.

WR is boxed. Incidences of divergences are bold and on a grey background. NB only two VARV strains are presented here due to 100% identity to either the Garcia or Ind3 strain in all other VARV orthologues.
The mechanism by which B14 functions is by inhibiting NF-κB activation in response to stimulation with TNFα, IL-1β, poly(I:C) or phorbol 12-myristate acetate (PMA) (Chen et al., 2008). The B14R deletion virus induced a higher level of phosphorylated IκBα compared to cells infected with wild type WR which implied B14 acts at or downstream of the IKK complex. B14 was found to inhibit NF-κB activation by binding to the IKK complex (Chen et al., 2008). This was demonstrated by B14 co-purifying and co-precipitating with the endogenous IKK complex from both human and mouse cells (Chen et al., 2008). Furthermore, it was shown that the interaction required IKKβ but not IKKα. B14 can inhibit the activation of NF-κB which is induced by overexpression of a constitutively active S276/180E IKKα mutant, but not a constitutively active S177/181E IKKβ mutant, confirming that B14 exerts its effect by association with IKKβ (Chen et al., 2008). Consequently, B14 inhibits the phosphorylation of IκBα so that NF-κB remains complexed with this inhibitor and stays in the cytoplasm (Fig. 1.7). Thus it was proposed that the IKKβ activation loop serine is targeted by B14 and this concept was validated by analysis using Abs specific for phospho-IKKβ (Chen et al., 2008).
Figure 1.6 B14 structure.
Ribbon representation of the crystal structure of B14, α-helices are marked by sequence from the N to C terminus and are coloured from blue (N terminus) to magenta (C terminus) from (Graham et al., 2008).
Figure 1.7 B14 interaction with NF-κB signalling pathway.
Both TNF and PMA bind to cell-surface receptors to activate intracellular signalling pathways. The IKK complex is activated and then phosphorylates IκBα. This targets IκBα for degradation and allows the NF-κB dimer to translocate into the nucleus and activate transcription from the NF-κB response element. When B14 is present it blocks the phosphorylation of IκBα and thus the entry of NF-κB into the nucleus.
1.7.3 Project aims

This project concerned VACV WR protein B14 and its orthologue in MVA.

The project aims were:

(i) To characterise the MVA counterpart protein 183 and establish if it acts in an analogous way to B14 and thus could potentially affect the immunogenicity of an MVA-based vaccine. This involved the construction of an MVA 183 deletion recombinant virus and a WR recombinant virus encoding 183 in the place of B14. In addition, the expression of protein 183 in eukaryotic and prokaryotic cells was investigated.

(ii) To gain further insight into how WR proteins inhibit NF-κB activation, a recombinant WR virus was constructed to analyse the effect of the loss of B14 in concert with another WR protein A49.

(iii) To gain greater understanding of the mechanism of action by which B14 inhibits NF-κB activation. This was studied using truncated IKKβ mutants and single amino acid B14 mutants. The co-purification of the B14-IKKβ complex was also investigated.

(iv) To facilitate the production of additional recombinant MVA viruses a BAC system was developed.
Chapter 2: Materials and methods

2.1 DNA manipulation and analysis methods (I)

2.1.1 Polymerase chain reaction (PCR)

The template for PCR was either 100 ng of plasmid DNA or 2 µl of DNA extracted from a 96-well plate of virus-infected cells by treatment with proteinase K into a total volume of 50 µl (FLUKA Biochemika) (section 2.11.1). The total reaction volume was 50 µl containing high fidelity PCR buffer (Invitrogen), 2 mM MgSO₄, 0.2 mM dNTP mix (Invitrogen), 0.2 µM primer mix and 1 U Platinum HiFi Taq DNA polymerase (Invitrogen). Reactions were performed in a programmable thermocycler using an initial denaturing temperature of 94 °C for 2 min followed by 26 cycles of 94 °C for 30 seconds (s), 45 s annealing (50-60 °C depending on primers used) and finally extension at 68 °C for 10 min. The annealing temperature of the primers was calculated by the formula: Tm = (n(C/G) × 4 °C + n(A/T) × 2 °C) - 5 °C, where G, C, A and T correspond to the 4 different nucleotides in each primer. The primers used for each reaction are shown in Table 2.1. For PCR screening of cloning products and intermediate recombinant viruses, GoTaq polymerase (Promega) was used instead of Platinum HiFi Taq (which has a proofreading capacity) as the high level of fidelity it provides was not required.

2.1.2 Agarose gel electrophoresis

DNA fragments from a PCR or restriction enzyme digest (section 2.1.5) were mixed with 1/6 volume of 6 x loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol CF, 30 % (v/v) glycerol, 50 mM ethylenediaminetetraacetic acid (EDTA) pH 8) before loading onto an 0.8-1 % agarose gel for resolution by electrophoresis. Agarose (BIOLINE) was used at 0.8-1 % in Tris acetate EDTA (TAE, 40 mM Tris-base, 40 mM Na acetate, 1 M EDTA pH8) buffer. EtBr₃ (10 µg/ml, GeneChoice) was added to the agarose solution prior to setting. Electrophoresis was carried out in TAE buffer in a mini gel electrophoresis unit (Bio-Rad) at 100 Volts for 30 min. The resolved DNA was then visualised using an ultraviolet (UV) transilluminator and recorded using the Chemi Doc gel documentation systems (Bio-Rad) with Quantity One software (Bio-Rad).
2.1.3 Pulsed-field agarose gel electrophoresis

Larger DNA fragments from genomic restriction digests were mixed with 6 x loading buffer before loading onto an 0.8-1 % agarose gel for resolution by pulsed-field electrophoresis. Agarose (BIOLINE) was used at 0.8-1 % in 0.5 x Tris Borate EDTA (TBE, 40 mM Tris-base, 40 mM Na acetate, 1 M EDTA pH8) buffer. The gels were run on a Bio-Rad CHEF DR II system in 2 L of 0.5 x TBE at 14 °C for 10 h, the voltage was set at 6 V/cm and pulse time varied between 1 and 6 s. The gel was stained for 1 h with 0.15 µg/ml EtBr\(_3\) (GeneChoice) in 0.5 x TBE and then destained in fresh 0.5 x TBE for 0.5 h. The gel image was captured using a CCD camera over a UV transilluminator.

2.1.4 Isolation of DNA from agarose gels

The DNA band was visualised under low-wavelength UV transillumination and isolated with the QIAquick gel extraction kit (QIAGEN) according to manufacturer’s instructions. The band corresponding to the DNA fragment of interest was excised from the agarose gel with a scalpel and then heated at 50 °C until it dissolved in 3 x (w/v) of kit GC buffer. The DNA was then bound to a spin column filter coated with a silica gel membrane, washed and eluted in 30-50 µl of kit Tris-EDTA (TE) buffer.

2.1.5 Restriction enzyme digestion

Restriction enzymes where indicated (Roche, New England Biolabs) were used at 1 U per 1 µg of DNA with 10 % of the recommended glycerol-based buffer according to the manufacturer’s guidance. The digestions were carried out in a total volume of 20-50 µl for 1 to 12 h at 37 °C.

2.1.6 Quantification of DNA

A NanoDrop ND1000 spectrophotometer (Labtech International) was used to evaluate the concentration of DNA preparations. UV absorbance at 260 nm (1 OD\(_{260}\) = 50 µg/ml dsDNA). The OD\(_{260/280}\) ratio was employed to determine the purity of the nucleic acids and DNA with a ratio value between 1.8 and 2.0 was considered pure.
<table>
<thead>
<tr>
<th>PRIMER NAME</th>
<th>SEQUENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA(F1)</td>
<td>5'-GGAAATCCCTTCGGTTCAACTGGAGATTA</td>
</tr>
<tr>
<td>FB(S2)</td>
<td>5'-GCTCTAGGACTTATTGATACCGTAATTCTACGG</td>
</tr>
<tr>
<td>pB14/WRFAd(R6)</td>
<td>5'-GTACTAAATTTGGCTGGTATCATTACAAATTCAGAACAGGAT</td>
</tr>
<tr>
<td>pB14/WRFBe(F7)</td>
<td>5'-ATATTTCCGGGTATGAGTGATGTTGTATCAGTTTGACATTTTCTC</td>
</tr>
<tr>
<td>B14/MVA(F5)</td>
<td>5'-ATGACGGCCACCTTAGACAC</td>
</tr>
<tr>
<td>B14/MVA(R5)</td>
<td>5'-TCATCAATTCATACGCAGGATAGATGTT</td>
</tr>
<tr>
<td>L-M19/A</td>
<td>5'-ATTGAAATTCGCTCAATAGAAGATACAGA</td>
</tr>
<tr>
<td>R-M19/A</td>
<td>5'-ATTCTCTAGACCCGGGGCTTACTCAATTCAGGAGAGAGG</td>
</tr>
<tr>
<td>L-M19/B</td>
<td>5'-ATTCGCCGGCGCGGCGCGCGCTTACATCTTGGAC</td>
</tr>
<tr>
<td>R-M19/E (e)</td>
<td>5'-ATGGATCTAGCGATGTATACGTATTTATTTG</td>
</tr>
<tr>
<td>M19/LA1622-R-F</td>
<td>5'-CAGGGATCCCAACAAAAAGTTTCAAGAAT</td>
</tr>
<tr>
<td>M19/RA1622-R-R</td>
<td>5'-TGCGCAGCGCCGGCCGGGATTTCTGTGTCTTCTTGAAG</td>
</tr>
<tr>
<td>EarlyF1f</td>
<td>5'-AATTCGGGCTGAGATTCTTTAACATTAGAAT</td>
</tr>
<tr>
<td>F1R</td>
<td>5'-TGGCCGGGGCCCGTCCACAGACGGTAC</td>
</tr>
<tr>
<td>P1F2f</td>
<td>5'-TAGAAAAAGATAGAAAACATTAT</td>
</tr>
<tr>
<td>F2L</td>
<td>5'-TATAGTACTACCTATCTCTTTAT</td>
</tr>
<tr>
<td>GFPI(P21)</td>
<td>5'-AAGCTGACCTCTGAAAGTAC</td>
</tr>
<tr>
<td>AmpF(g93)</td>
<td>5'-AAGATGCTGAAAGATAGTGG</td>
</tr>
<tr>
<td>AmpR(g94)</td>
<td>5'-AGCTGACCTCTGAAAGTAC</td>
</tr>
<tr>
<td>L-FLAG(183)</td>
<td>5'-ATTCTCGAGCCACCATGCGATTACAGGATGACGATTAG</td>
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<tr>
<td>B765-R</td>
<td>5'-GTGATGCTGATGTTTTTTGAGCCCTGGCTCAGGCAGC</td>
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<tr>
<td>New-pcplIKkb300L</td>
<td>5'-AGGAGATATACATTCAAGGCGCCTGGGA</td>
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<tr>
<td>L-Flag-IKkbata</td>
<td>5'-ATAACGCTGTGATACCATTGTACACAGGATGACTAGCAAGCATTGCCCTTCCT</td>
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<tr>
<td>R-IKKβ (NotI)</td>
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<tr>
<td>L-Flag-IKkb(ata230)</td>
<td>5'-ATAACGCTGTGATACCATTGTACACAGGATGACTAGCAAGCATTGCCCTTCCT</td>
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<tr>
<td>L-IKKβ (480N del)</td>
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<tr>
<td>pIKkb732R</td>
<td>5'-TAACATGATCAGGCTGAAACTCTGGTC</td>
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<tr>
<td>B-330L</td>
<td>5'-AGGAGATATACATTCAAGGCGCCTGGGAATCGATGACATCTTAAA</td>
</tr>
</tbody>
</table>

**Table 2.1 Oligonucleotide primers.**
2.1.7 DNA ligation

Ligation was carried out in a total volume of 20 µl containing 1 x buffer (300 mM Tris-HCl pH 7.8, 100 mM MgCl₂, 100 mM dithiothreitol (DTT) and 10 mM ATP), 1 U of T4 DNA ligase (Roche, supplied in 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 1 mM DTT, 0.1 mM EDTA and 50 % glycerol) and the insert and vector DNA in a 3:1 molar ratio. A minimum of 90 ng of vector DNA was used. The reaction mix was incubated at 16 °C for 1 h and then 23 °C for 10 h.

2.1.8 pOPIN cloning

In-Fusion™ 2.0 Dry-Down PCR Cloning Kit (Clontech) (Hamilton et al., 2007) was used to insert PCR products with 15 bp of homology to pOPIN vectors (Berrow et al., 2007) into pOPIN E (Table 2.2) without restriction enzyme cleavage of the insert and without the use of T4 DNA ligase, as outlined in 2.15 and 2.1.7. The PCR product and the linearised cloning vector were added to an In-Fusion™ Dry-Down Mix tube (Clontech) (buffer provided) and incubated for 15 min at 37 °C and then 50 °C before being diluted into 40 µl of TE Buffer pH 8.0 (Qiagen) and transformed into Fusion-Blue™ Competent E. coli (Clontech). The In-Fusion Enzyme, a poxvirus DNA polymerase with 3'-5' exonuclease activity (Hamilton et al., 2007), has an exonuclease activity which excises nucleotides from the 3' ends of the molecules, exposing the overlapping sequence which then anneal to form non-covalently joined molecules that undergo final repair within the E. coli strain to form the vector with insert. The vector used, pOPIN E, was developed at the Oxford Protein Production Facility and provided by Professor D.I. Stuart. pOPIN E contains a C-terminal polyhistidine (His) tag and was linearised by NcoI and PmeI. It enables expression in mammalian, bacterial and insect cells.

2.2 DNA manipulation and analysis methods (II)

2.2.1 Transformation of chemically competent bacteria with plasmid DNA

Chemically competent cells (DH5α E. coli, laboratory-prepared) were thawed on ice. Briefly, the preparation of these cells involved overnight growth in 1 ml of Luria Bertani (LB) broth culture and then inoculating 50 ml of LB with this starter culture.
The 50 ml culture was grown at 37 °C, until the bacteria reached early log phase (optical density (OD)\textsubscript{600} =0.3), and bacteria were then harvested by centrifugation at 3000 rpm (Allegra 6R benchtop centrifuge, GH-3.8A rotor) for 10 min. The pellet was gently resuspended with 1 ml of cold CaCl\textsubscript{2} 100 mM and incubated on ice for 30 min before a second centrifugation step at 3000 rpm (Allegra 6R benchtop centrifuge, GH-3.8A rotor) for 10 min. The pellet was resuspended in 15 % glycerol CaCl\textsubscript{2} 100 mM and incubated overnight at 4 °C and then divided into 100 µl aliquots on dry ice and stored at -70 °C.

To transform the bacteria, a 10 µl aliquot of the DNA ligation reaction was added to 100 µl of these competent cells and incubated on ice for 45 min before undergoing heat-shock for 2 min in a 42 °C water bath. After a 5 min incubation on ice, 100 µl of super optimal catabolite (SOC) medium (Invitrogen) was added and the cells were placed in a shaking incubator at 37 °C for 30-60 min. The cells were then spread onto agar plates containing the appropriate antibiotic for the plasmid transformed and incubated at 37 °C overnight.

### 2.2.2 Transformation of electrocompetent bacteria with plasmid DNA

Electrocompetent cells (BL21/B834 \textit{E. coli}, laboratory prepared) were thawed on ice. Briefly, the preparation of these cells involved overnight growth of a 5 ml of LB culture and then using this to inoculate 500 ml of LB. This culture was grown at 37 °C, until the bacteria reached early log phase (OD\textsubscript{600} =0.3), and was then transferred to an ice water bath for 15-30 min. The bacteria were then harvested by centrifugation at 3000 rpm (Allegra 6R bench-top centrifuge, GH-3.8A rotor) for 20 min at 4 °C. The pellet was gently resuspended with 500 ml of cold H\textsubscript{2}O\textsubscript{2} and the centrifugation step repeated. This process was repeated five times with decreasing volumes of H\textsubscript{2}O to a final volume of 250 ml H\textsubscript{2}O supplemented with 10 % glycerol. A further centrifugation was carried out at 3000 rpm (Allegra 6R bench-top centrifuge, GH-3.8A rotor) for 20 min at 4 °C and the pellet was resuspended in 2 ml of 10 % glycerol H\textsubscript{2}O, aliquoted and stored at -70 °C.

Approximately 1 µg of plasmid DNA was added to 100 µl of laboratory-prepared competent cells and mixed by tapping the tube gently. The mixture was then transferred to a pre-chilled BioRad disposable cuvette. The electroporator was set to
2.5 kV, 25µF and 400 Ohms. The cuvettes were pulsed and 1 ml SOC was added immediately and the mixture transferred to a sterile tube. The cells were incubated for 1 h at 37 °C and a 200 µl spread onto agar containing the appropriate antibiotic and incubated at 37 °C overnight.

2.2.3 Transformation of electrocompetent bacteria with BAC DNA

DH10b electrocompetent cells (Invitrogen) were thawed on ice. Phenol-chloroform-isoamyl extracted BAC DNA (section 2.14.7) from one well of a 6-well plate of infected CEFs (section 2.14.5) was dissolved in 20 µl and then 3 µl was added to 50 µl of competent cells and transferred immediately to a pre-chilled BioRad disposable cuvette. The electroporator was set to 2.5 kV, 25 µF and 400 Ohms. The cuvettes were pulsed and 500 µl of SOC was added immediately and the mixture was transferred to a sterile tube. The cells were incubated for 1 h at 37 °C and then plated out onto agar plates containing chloramphenicol (Cm) and incubated at 37 °C overnight.

2.2.4 Small-scale preparation of plasmid DNA

A 3 ml bacterial culture was grown overnight at 37 °C by inoculating 3 ml of LB with a single bacterial colony. The LB was supplemented with kanamycin (Kan, Sigma) or ampicillin (Amp, Sigma) at 30 µg/ml as appropriate. A 1.5 ml aliquot of the culture was then centrifuged at 13,000 rpm in a microcentrifuge for 1 min, and the DNA was purified using the QIAGEN mini prep method according to the manufacturer’s instructions. Briefly, this involved the lysis of the bacteria by an alkaline lysis method (Birnboim & Doly, 1979). After neutralisation, with the appropriate mini prep kit buffers, the lysate was passed through a spin column containing a silica-gel membrane to which the DNA binds. The column was then washed and the DNA eluted in TE (Qiagen).

2.2.5 Small-scale preparation of BAC DNA

E. coli were transformed with BAC DNA and grown on an agar plate containing the appropriate antibiotic and the resulting colonies were picked. These were grown in 1.5 ml of LB or superbroth (bactotryptone 1.2 %, yeast extract 2.4 %, glycerol 0.4 %, KH₂PO₄ 0.2 %, K₂HPO₄ 1.25 %) containing the appropriate antibiotic overnight at 37 °C in a shaking incubator. A 1.5 ml aliquot of the bacterial culture was collected by
centrifugation at 3000 rpm (Allegra 6R bench-top centrifuge, GH-3.8A rotor) for 10 min and the supernatant was discarded. The bacterial pellet was resuspended in 70 µl of STET (8 % sucrose, 5 % Triton X-100, 50 mM EDTA, 50 mM Tris-Cl, pH 8) and vortexed. Then 200 µl of 10 % sodium dodecyl sulphate (SDS) dissolved in 0.1 M NaOH (alkaline SDS) was added and then mixed by inversion. Following this, 150 µl of 7.5 M ammonium acetate was added and the solutions was mixed by inversion and incubated on ice for 5 min. The sample was spun in a pre-cooled centrifuge (4 °C) for 20 min at 13,000 rpm (Hereaus Sepatech Biofuge 15R, HFA 22.2 rotor). The supernatant was transferred to a clean eppendorf tube and the DNA was precipitated by the addition of 240 µl of isopropanol and mixed by inversion. A further centrifugation step of 10,000 rpm for 5 min in a microcentrifuge at room temperature was undertaken to collect the DNA which was then washed in 200 µl of 70 % ethanol and centrifuged at 10,000 rpm for 3 min in a microcentrifuge. The resulting pellet was allowed to air dry for 15 min and dissolved in 50 µl of TE (Qiagen mini prep kit).

2.2.6 Large-scale preparation of plasmid DNA

A large-scale bacterial culture was grown overnight at 37 °C in a shaking incubator by inoculating 250 ml of LB (containing Kan or Amp at 30 µg/ml) with 100 µl of a 3 ml overnight bacterial culture. The culture was centrifuged at 3000 rpm (Allegra 6R bench-top centrifuge, GH-3.8A rotor) for 15 min and the bacterial pellet was retained for DNA extraction and purification using the QIAGEN Hi-speed maxi prep method. The pellet was resuspended in 10 ml of P1 kit buffer, lysed and neutralised as per the manufacturer’s instructions. The lysate was filtered through a QIA filter cartridge and loaded onto a pre-equilibrated DNA purification column. The DNA was then washed, eluted and precipitated with isopropanol before collection with a QIAprecipitator. Finally, the DNA was washed with ethanol and then eluted with TE buffer (Qiagen).

2.2.7 Large-scale preparation of BAC DNA

A large-scale bacterial culture was grown overnight at 37 °C in a shaking incubator by inoculating 500 ml of LB broth (Cm 30 mg/ml, Sigma) with 500 µl of a 3 ml overnight bacterial culture. The bacterial culture was centrifuged at 3000 rpm (Allegra 6R bench-top centrifuge, GH-3.8A rotor) for 15 min and the pellet was retained for DNA extraction and purification using the QIAGEN low-copy plasmid maxi prep
method. The pellet was resuspended in 20 ml of kit buffer P1, lysed via a modified alkaline lysis method using 20 ml of kit buffer P2 and neutralised with 20 ml of kit buffer P3 and incubated on ice for 30 min. The mixture was centrifuged at 11,000 rpm (Sorvall RC5C plus centrifuge, SLA-3000 rotor) for 30 min at 4 °C. The supernatant was removed rapidly and filtered and applied to a QIAGEN-tip 500 which had been pre-equilibrated with kit buffer QBT (Qiagen). The DNA was washed and eluted with kit buffers as per the manufacturer’s instructions. The DNA was precipitated by adding 0.7 volumes of isopropanol at room temperature and centrifuged for 30 min at 11,000 rpm (Sorvall RC5C plus centrifuge, SS-34 rotor). The DNA pellet was then washed with 2 ml of 70 % ethanol and centrifuged again at 11,000 rpm (Sorvall RC5C plus centrifuge, SS-34 rotor) for 10 min.

An additional method for large-scale purification of BAC DNA was the CsCl gradient method. A large-scale bacterial culture was grown overnight at 37 °C in a shaking incubator by inoculating 500 ml of superbroth (Cm 30 µg/ml, Sigma) with 500 µl of a 5 ml overnight bacterial culture. The bacterial culture was harvested by centrifugation for 20 min at 3000 rpm (Allegra 6R bench-top centrifuge, GH-3.8A rotor) and then resuspended in 40 ml of 50 mM Tris-Cl pH 8, 50 mM glucose, 50 mM EDTA. Lysis was then achieved by adding 80 ml of alkaline SDS to the resuspended pellet while swirling the mixture to ensure even lysis. Immediately following this, 60 ml of 7.5 M ammonium acetate was added and the mixture was cooled on ice for 10 min prior to centrifugation in a pre-chilled centrifuge for 3000 rpm (Allegra 6R bench-top centrifuge, GH-3.8A rotor) at 30 min. The supernatant was retained and then filtered through a clean double-folded muslin cloth into a centrifuge container. The DNA was precipitated by the addition of 100 ml of isopropanol. The mixture was centrifuged at 6000 rpm (Sorvall Legend RT, swinging bucket rotor 7500 6441) for 30 min at room temperature. The resulting pellet was washed with 70 % ethanol and air-dried before being dissolved in 2.2 ml of TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA). This solution (made up to a total weight of 2.62 g with extra TE) was added to 2.8 g of CsCl and 210 µl of EtBr₃ (10 mg/ml, GeneChoice) and the CsCl was dissolved by vortexing and inversion. The mixture was centrifuged in an Optiseal 13 x 33 mm tube (Beckman) at 80,000 rpm for 16 h in a TLN-100 rotor (Beckman TL-100 bench-top ultracentrifuge). Two bands were visible; the upper band contained circular plasmid and the lower band corresponded to the supercoiled plasmid and was removed from the tube via a
blunt-end 3 gauge needle. To remove the EtBr$_3$ from the DNA solution repeated extractions were undertaken with TE-saturated butanol, and subsequently the DNA was precipitated with ethanol and dissolved in TE.

2.2.8 Automated sequencing of DNA

Sequencing was carried out by the Advanced Biotechnology Centre (part of Imperial College London, Hammersmith Campus).

2.2.9 Preparation of glycerol stocks

A small-scale culture (3 ml of LB containing the appropriate antibiotic) was inoculated with a single bacterial colony from an agar plate and grown in a shaking incubator at 37 °C overnight. Equal volumes of the culture and 30 % (v/v) sterile glycerol solution were combined and stored at -70 °C.

2.3 Tissue culture methods

2.3.1 Cell culture

The experimental cell lines used were RK-13, African green monkey kidney cells, ATCC CCL-26 (BS-C-1), HEK 293T, baby hamster kidney (BHK)-21 cells and CEFs. The RK-13, BS-C-1, CEF and HEK 293T cells were grown in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) with 10 % foetal bovine serum heat-treated for 1 h at 56 °C (FBS, Gibco), 50 IU/ml penicillin and 50 µg/ml streptomycin. The BHK-21 cells were grown in modified Eagle’s medium (MEM, Gibco) with 10 % FBS, 10 % essential amino acids (Gibco), 10 % MEM vitamins (Gibco), 50 IU/ml penicillin and 50 µg/ml streptomycin. The cells were incubated in a humidified incubator (Heraeus) with 5 % CO$_2$.

2.3.2 Transfection

FuGENE HD (Roche) was used to transfect DNA into cells to induce transient expression of target genes. The protocol used was as indicated by the manufacturer and the ratio of transfection agent to DNA was 1:3 volumes. The cells were seeded to give about 30-50 % confluency and grown overnight. The medium was replaced with fresh 10 % FBS DMEM 1 h prior to transfection. The FuGENE HD was mixed with
Opti-MEM and incubated at room temperature for 3 min. This solution was then aliquoted into tubes containing the appropriate amounts of plasmid DNA for the approximate number of cells to be transfected and incubated for a further 20 min. The pre-mixed DNA, FuGENE HD and opti-MEM was then added to the cells.

Lipofectamine2000 (Invitrogen) was used to transfect CEFs previously infected at an multiplicity of infection (MOI) of 0.1 for 2 h with MVA, or MVA with HIV clade C antigens inserted into the thymidine kinase (TK) locus (MVAc). This was part of the process of constructing MVA-based recombinant viruses. The Transfection protocol used was as indicated by the manufacturer. After a 10 min pre-incubation of 10 µl of Lipofectamine2000 and 500 µl Opti-MEM, 5 µg of plasmid DNA was added and incubated for 20 min. The mixture was then added to a T25 flask of infected CEFs with 5 ml of fresh 2.5 % FBS DMEM (after removing the inoculum, section 2.14). The transfection medium was removed after 4 h and 8 ml of 2.5 % FBS DMEM was added. The cells were grown for a further 48 h, at which point they were examined by fluorescence microscopy and harvested.

CaPO₄ was used to transfect HEK 293T cells in luminescence-based mammalian protein-protein interactome mapping (LUMIER) and co-immunoprecipitation assays. Cells were seeded to give approximately 50 % confluency and then the medium was replaced with 2.5 % FBS DMEM no more than 1 h prior to transfection. DNA was diluted a hundred fold in water and then CaPO₄ was added drop wise to a total of 10 % volume. The mixture was then incubated for 20 min. After which, an equal volume of HEPES-buffered saline (HBS, 280 mM NaCl, 10 mM KCl, 1.5 mM Na₂HPO₄, 12 mM glucose, 50 mM HEPES, pH 7) was added and a further incubation of 15 min undertaken prior to adding the mixture was added to the cells. After 24 h the cells were washed thoroughly with warm phosphate-buffered saline (PBS) and harvested (sections 2.9 and 2.10).
<table>
<thead>
<tr>
<th>PLASMID</th>
<th>DESCRIPTION</th>
</tr>
</thead>
<tbody>
<tr>
<td>p183</td>
<td>pCB-based, 250-bp flanks of 183 (A,B) EcomGpGFP selection cassette under VACV IE promoter, Amp resistant</td>
</tr>
<tr>
<td>p163</td>
<td>pSH7-based, 250-bp flanks of Sf14R either side of 183R, Amp resistant</td>
</tr>
<tr>
<td>p183</td>
<td>pSH7-based, 250-bp flanks of Sf14R, Amp resistant</td>
</tr>
<tr>
<td>p183His</td>
<td>pET28a-based, 183R with C-terminal His tag, IPTG-inducible, Kan resistant</td>
</tr>
<tr>
<td>p184His</td>
<td>pET28a-based, Sf14R with C-terminal His tag, IPTG-inducible, Kan resistant</td>
</tr>
<tr>
<td>pBI253</td>
<td>pCB-based, ILKØ with C-terminal His tag, IPTG-inducible, Amp resistant</td>
</tr>
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<td>pBΔ100f</td>
<td>pCB-based, ILKØ truncation Δ1-100 with N-terminal FLAG tag, Amp resistant</td>
</tr>
<tr>
<td>pBΔ200f</td>
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<td>pBΔ400f</td>
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<tr>
<td>pBΔ700f</td>
<td>pCB-based, ILKØ truncation Δ700-756 with N-terminal FLAG tag, Amp resistant</td>
</tr>
<tr>
<td>pBΔ300His</td>
<td>pGPN-E based, ILKØ truncation Δ1-300 with C-terminal His tag, Amp resistant</td>
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<tr>
<td>pΔ149</td>
<td>pCB-based, 350-bp flanks of A40R (A,B), distal EcomGpGFP selection cassette under VACV IE promoter, Amp resistant</td>
</tr>
<tr>
<td>pΔ149</td>
<td>pCB-based, 350-bp flanks of A40R (A,B) around A40R, distal EcomGpGFP selection cassette under VACV IE promoter, Amp resistant</td>
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<td>pHA</td>
<td>pCB-based, ILKØ with N-terminal HA tag, Amp resistant</td>
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<tr>
<td>pY35Ef</td>
<td>pCB-based, Sf14R with Y35E substitution and an N-terminal FLAG tag, Amp resistant</td>
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<tr>
<td>pY35Kf</td>
<td>pCB-based, Sf14R with Y35K substitution and an N-terminal FLAG tag, Amp resistant</td>
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<td>pCB-based, VACV WIL with N-terminal FLAG tag, Amp resistant</td>
</tr>
<tr>
<td>pBLuc</td>
<td>pCB-based, ILKØ fused to Renilla luciferase, Amp resistant</td>
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<tr>
<td>pBAC-V1</td>
<td>pBEL0-BAC 11-based, 500-bp flanks of MVA genomic ΔVI, LoxP site, Cm resistant, EcomGpGFP selection cassette under VACV IE promoter</td>
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</table>

Table 2.2 Plasmids.
Description of plasmids used in this study, including source plasmid, inserted features and antibiotic resistance markers
2.4 Protein expression

BL21 (DE3) cells were transformed with pET28a-based (or pET28a- and pOPIN E-based) recombinant plasmid(s) and plated out onto agar containing the appropriate antibiotic(s). A single colony was picked and used to inoculate 5 ml of LB containing the appropriate antibiotic. This was grown at 37 °C overnight and then expanded into a 500 ml culture and grown until the OD_{600} reached 0.4. At this point isopropyl-thio-β-D-galactopyranoside (IPTG) was added (final concentration 1 or 5 mM as indicated in chapter 5) and the culture was grown for a further 24 h. The culture was centrifuged at 3000 rpm (Allegra 6R benchtop centrifuge, GH-3.8A rotor) for 20 min at 4 °C and the pellet was retained and freeze-thawed once. The thawed pellet was then resuspended in 10 ml of lysis buffer (25 mM Tris-CL pH 8.0, 500 mM NaCl, 20 mM imidazole, 1 % Triton-X-100, 2 M non-detergent sulphobetaines, 2 mM β-ME, supplemented with protease inhibitors, Roche) and cooled on ice. The cells were sonicated on ice until they were lysed completely. Benzonanse (Novagen) was added (10 µl) and the mixture was incubated on a roller for 30 min at room temperature before being centrifuged at 13,000 rpm (Sorvall RC5C plus centrifuge, SS-34 rotor) for 30 min. The pellet was retained and stored at -20 °C.

Magic broth: MagicMedia™ E. coli Expression Medium was used according to manufacturer’s instructions.

2.4.1 Incubation with Ni²⁺ beads

A 50 µl aliquot of Ni²⁺ beads was washed with 1 ml of immobilised affinity chromatography (IMAC) binding buffer (30 mM imidazole, 500 mM NaCl, 20 mM Tris (pH 7.9), 2 mM 2-β-mercaptoethanol (2β-Me)) for 5 min on a rotating wheel and then centrifuged at 10,000 rpm (microcentrifuge) for 3 min. This was repeated three times to prepare the beads. E. coli cultures expressing recombinant protein were harvested and the bacterial pellet was lysed (section 2.4). A 1 ml aliquot of the soluble fraction was incubated with the Ni²⁺ bead for 30 min at room temperature. The beads and soluble fraction were spun at 10,000 rpm (microcentrifuge) for 3 min and the beads were resuspended in sample loading buffer (SLB, 50 mM Tris-HCl pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 0.1 % (w/v) bromophenol blue, 100 mM reducing agent (DTT or β-ME)) and denatured by boiling prior to analysis by SDS-
polyacrylamide gel electrophoresis (PAGE) (section 2.5) along with a fresh aliquot of the soluble fraction and the insoluble fraction.

### 2.4.2 Protein purification

Recombinant protein was purified by IMAC and gel filtration. Large (1 L) *E. coli* cultures expressing recombinant proteins were harvested and the resulting bacterial pellet was lysed (section 2.4). The soluble recombinant His-tagged proteins were purified by IMAC on a pre-charged HiTrap His column (Amersham Biosciences). The soluble protein was loaded on to the column in low-salt IMAC binding buffer (30 mM imidazole, 100 mM NaCl, 20 mM Tris pH 7.9, 2 mM 2β-Me). The protein was then eluted via an increasing imidazole gradient (upper limit 500 mM). The protein was further purified by size exclusion chromatography (gel filtration) on a HiLoad 16/60 Superdex 200 column (Amersham Biosciences).

### 2.5 SDS-PAGE

Protein samples were run on a SDS-PAGE according to the Tris/glycine discontinuous buffer system, and using a vertical electrophoresis unit (Mini-PROTEAN II apparatus, Bio-RAD). Samples were denatured by heating at 100 °C for 5 min with 1 x SLB. Gels consisted of a stacking gel layer (125 mM Tris pH 6.8, 5 % acrylamide, 0.1 % SDS, 0.1 % ammonium persulphate and 0.1 % TEMED) and a resolving gel layer (375 mM Tris pH 8.8, 12 % acrylamide, 0.1 % SDS, 0.1 % ammonium persulphate and 0.04 % TEMED). The samples were electrophoresed into the stacking gel at 80 V and resolved by the separating gel at 150 V in protein running buffer (25 mM Tris, pH 8.3, 250 mM glycine, 0.1 % SDS). The samples were electrophoresed alongside pre-stained molecular mass markers (Bio-Rad) in a Tris/glycine buffer (25 mM Tris, 250 mM glycine, 0.1 % SDS). The gels were then processed for coomassie staining or immunoblotting as required.

### 2.6 Coomassie staining

Gels were immersed in at least 5 gel volumes of coomassie stain (0.25 % (w/v) coomassie brilliant blue R-250 (BDH), 10 % (v/v) acetic acid, 45 % (v/v) methanol) for 1 h. After rinsing with water, the gels were immersed in destain (20 % (v/v) methanol/10 % (v/v) acetic acid). The destain buffer was removed when the protein
bands became clearly visible. The gels were dried between 2 sheets of water-soaked cellophane (Bio-Rad) using a Bio-Rad gel drier (model 583) for 2 h at 80 °C.

2.7 Immunoblot analysis

Protein samples were run on SDS-PAGE gels and were subsequently electro-transferred onto a nitrocellulose membrane (Hybond ECL, Amersham) at 20 V for 25 min, using a Mini-Electrophoresis Trans-Blot (Bio-Rad) machine in transfer buffer (25 mM Tris, 190 mM glycine, 0.037 % (w/v) SDS and 20 % (v/v) methanol). The nitrocellulose membrane was then blocked with 5 % (w/v) dried milk powder in PBS. After incubation at room temperature for at least 1 h the membrane was immersed in a fresh aliquot of the blocking buffer, supplemented with the primary Ab, diluted as per Table 2.3 and rocked gently for 1 h. The membrane was then washed three times for intervals of 10 min with 0.1 % (w/v) Tween-20 (Sigma) in PBS. This was followed by a further 1 h incubation with the appropriate secondary Ab (Table 2.3), and then three more washes. Detection of any bound secondary Ab was achieved using enhanced chemiluminescence (ECL) reagents (Amersham/Pierce) as directed by the manufacturers or chemiluminescent HRP substrate (1.25 mM luminol, 0.4 mM p-coumaric acid, 0.1 M Tris pH 8.8, 0.0075 % (v/v) H₂O₂) and exposure to film for 30 s - 10 min.

2.8 Luciferase assays

HEK 293T cells (50 % confluent) were transfected with 100 ng of a plasmid mix containing NF-κB firefly luciferase/TK Renilla-luciferase and 400 ng of expression vector. FuGENE HD was used as described in section 2.3.2. After 18 h transfected cells were stimulated with TNFα (50 ng/ml) for 8 h and then lysed with passive lysis buffer (Promega). Luciferase readings were generated using the Promega Dual-Luciferase® Reporter Assay System as instructed by the manufacturers.
Table 2.3 Antibodies.
Description of Abs used in this study, including their specificity, species of provenance and dilution factor for immunoblotting.

<table>
<thead>
<tr>
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<th>DILUTION FACTOR</th>
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<tr>
<td>αB14</td>
<td>Polyclonal anti-B14</td>
<td>Rabbit</td>
<td>1000</td>
</tr>
<tr>
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<td>Polyclonal anti-A49</td>
<td>Rabbit</td>
<td>1000</td>
</tr>
<tr>
<td>αD8</td>
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<td>Mouse</td>
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<tr>
<td>αVACV</td>
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<td>αTubulin</td>
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<tr>
<td>αRabbit 2'</td>
<td>Anti-rabbit secondary</td>
<td>Goat</td>
<td>2000</td>
</tr>
<tr>
<td>αMouse 2'</td>
<td>Anti-mouse secondary</td>
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<td>2000</td>
</tr>
<tr>
<td>αRabbit 2' Alexa 288</td>
<td>FITC-anti-rabbit secondary</td>
<td>Goat</td>
<td>1000</td>
</tr>
</tbody>
</table>

2.9 LUMIER assays

Cells (HEK 293T) were seeded into 6-well plates and each well was transfected with 1.5 µg of a plasmid encoding IKKβ fused to Renilla-luciferase and 1.5 µg of the indicated plasmid expressing wild type or mutant B14. The CaPO₄ transfection method was used with cells at 50 % confluency and 24 h later the cells were washed carefully with warm PBS and harvested in 200 µl of co-immunoprecipitation buffer (50 mM HEPES pH 7.5, 100 mM NaCl, 10 % (v/v) glycerol supplemented with protease inhibitors, Roche) and incubated on ice for 20 min. The samples were centrifuged at 13500 rpm (Hereaus Sepatech Biofuge 15R, HFA 22.2 rotor) for 20 min at 4 °C and the supernatant was split into three aliquots; 10 µl was immediately assessed for Renilla-luciferase activity using Renilla substrate (1 mg/ml Coelenterazine Free Base in ethanol, Lux Innovate, diluted 1/500 in PBS before use) and 40 µl was denatured and analysed by SDS-PAGE and immunoblotting to assess protein expression levels. The remaining 150 µl was incubated with 20 µl of FLAG-M2 agarose on a rotating wheel at 4 °C for 2 h. The resin was washed thoroughly and
the proteins attached to the FLAG agarose were eluted using FLAG-peptide (50 µg/ml) in a volume of 50 µl per sample. Of this final elution, 20 µl was analysed for Renilla-luciferase activity (as described above.)

2.10 Co-immunoprecipitation

HEK 293T cells (50 % confluent) in 10-cm dishes were transfected with plasmids coding for FLAG-tagged proteins via the CaPO₄ method (section 2.3.2). Approximately 24 h later cells were washed with warm PBS and lysed with 1 ml of co-immunoprecipitation buffer (150 mM NaCl, 20 mM Tris-HCl pH 7.4, 10 mM CaCl₂, 0.1 % Triton-X, 10 % glycerol supplemented with protease inhibitors, Roche). The cells were lysed on ice for 20 min and then were centrifuged at 15,000 rpm (Hereaus Sepatech Biofuge 15R, HFA 22.2 rotor) for 10 min at 4 °C and the supernatant was retained. The supernatant was pre-cleared by rotation with 30 µl of pre-equilibrated protein G beads (GE Healthcare) for 2 h at 4 °C. The samples were centrifuged for 20 min at 15,000 rpm (Hereaus Sepatech Biofuge 15R, HFA 22.2 rotor) at 4 °C and the supernatant was retained. A monoclonal Ab (mAb) to the HA tag (Table 2.3, αHA-ms) was then added to the supernatant and the mixture was rotated overnight at 4 °C. The following morning fresh protein G beads (washed and equilibrated) were added to the Ab-lysate mix for 2 h. The beads were then washed five times before being mixed with 50 µl of SLB, denatured by boiling and analysed, along with the lysate samples, by SDS-PAGE followed by immunoblotting using both FLAG- and HA-tag (Table 2.3, αFLAG and αHA-rb) Ab.

2.10.1 Co-immunoprecipitation of proteins from infected cells

HEK 293T cells (in 10-cm dishes) were transfected with plasmids coding for FLAG-tagged proteins via the FuGene HD method (section 2.3.2). Approximately 16 h later the cells were infected at an MOI of 10 for 1.5 h with vB14-HA that had been purified by sedimentation through a sucrose cushion. After a 6 h incubation, the cells were washed with ice-cold PBS and harvested. The collected cells were lysed with 1 ml of viral co-immunoprecipitation buffer (50 mM HEPES, pH 7.5,100 mM NaCl,1 mM sodium orthovanadate, Nonidet P-40 containing 1 mM phenylmethylsulphonyl fluoride, 0.01 % (v/v) aprotinin, 10 % (v/v) glycerol, supplemented with protease...
inhibitors, Roche) on ice for 20 min. The co-immunoprecipitation protocol outlined in 2.10 was then followed.

2.11 Plasmids for the generation of recombinant VACV WR

2.11.1 Construction of p183

Primers F5 and R5 (Table 2.1) were used to amplify gene 183R by PCR (section 2.1.1) using MVA genomic DNA as a template. The downstream (A) and upstream (B) 250 bp that flank B14R in the WR genome were amplified by PCR using primers F1, R6 and F7, R2 respectively (Fig. 2.1, Table 2.1). The template was a pCI vector (Promega) into which B14R and its flanking sequences had been cloned (Chen et al., 2006). The R6 and F7 primers were engineered so that both fragments A and B would include a small amount of overlapping sequence with B14R. Given the high degree of nucleotide identity between the B14R and 183R genes this enabled the annealing of 183R to B by overlapping PCR using primers F5 and R2 and equal quantities of gel purified 183R and B as template. The resulting MVA-WR chimeric DNA fragment 183R-B was in turn annealed to A using primers F1 and R2 and using equal quantities of gel purified 183R-B and A as template. The resulting fragment, A-183R-B was then purified by agarose gel electrophoresis, digested with EcoRI and NdeI and ligated to the pSJH7 plasmid (Hughes et al., 1991) which had been digested with the same enzymes and gel purified (Table 2.2).
Figure 2.1 Schematic of p183 plasmid construction. The 250 bp immediately up- and downstream of B14R (A and B) in the WR genome were cloned either side of 183R and inserted into pSJH7.
2.11.2 Construction of pΔA49 and pA49

The pΔA49 and pA49 plasmids were constructed Dr D.S. Mansur in this laboratory to generate the vΔA49 and vΔA49Rev viruses referred to in chapter 4. Briefly, pΔA49 was constructed by PCR amplification of the 350-bp flanking regions primers immediately up- and downstream of A49R in the WR genome with appropriate primers (Daniel Mansur, personal communication). The flanks were then joined by overlapping PCR and inserted into a modified pCI plasmid (Promega). This plasmid contained the E. coli guanine xanthine phosphoribotransferase (Ecogpt) gene fused to the enhanced green fluorescent protein (GFP) gene under the control of a constitutively active VACV promoter at a distal site to the A49 flanks. This EcogptGFP fragment was sub-cloned from a fragment in pGEMt (Promega), originally cloned in this laboratory by Miss E Glyde (Fahy et al., 2008). The pA49 plasmid also contained EcogptGFP but instead of only the AB flanks it included the A49R gene surrounded by the flanks A and B. This was generated directly by PCR using the WR genome as template, and cloning into pCI again at a site distal to EcogptGFP.

2.12 Plasmids for the generation of recombinant VACV MVA

The plasmid pΔ183 was designed to construct MVAAΔ183 (Table 2.2). This involved cloning EcogptGFP (described in 2.11.2) surrounded by the 250 bp of genomic DNA either side of the gene(s) targeted for deletion (Staib et al., 2005). Primers MVA-Af and MVA-Ar (Table 2.1) were used to amplify the 250 bp (A) downstream of 183R by PCR (section 2.1.1) using MVA genomic DNA as a template. Subsequently, primers MVA-Bf and MVA-Br were used to amplify the 250 bp (B) upstream of 183R. These flanks were positioned either side of EcogptGFP in pCI.

2.13 Plasmid for the generation of BAC recombinant VACV MVAc

The pBAC-VI plasmid was designed to construct MVAc-BAC (Table 2.2). This involved the synthesis (GENEART) of an EcogptGFP fragment as described above preceding two 500-bp regions of DNA homologous to either side of the MVA genomic deletion VI. These were separable by the inclusion of a PacI site, and thus allowed the linearisation of the plasmid created needed in order to integrate the whole length of the plasmid into the MVAc genome. The synthesised segment was
surrounded by a *Sphi* and a *HindIII* sites in order that it could be directionally cloned into pBELO-BAC11 (supplied by NEB) following digestion with these enzymes (Fig. 2.2).

![Figure 2.2 Schematic of pBAC VI plasmid construction.](image)

pBELO BAC 11 was obtained from NEB and digested with *Sphi* and *HindIII*. GENEART synthesised a pE/L-controlled EcogptGFP adjacent to the F1 and F2 (500 bp flanks of MVA deletion VI). This was excised from the GENEART cloning vector by digestion with *Sphi* and *HindIII* and ligated to the digested pBELO BAC 11 to form pBAC-VI.
2.14 Virus stocks

VACV stocks were stored at -70 °C as master stocks. Cells (RK-13, BS-C-1 and CEFs) were infected out in DMEM supplemented with 2.5 % FBS using aliquoted viral stocks and working stocks, which were sonicated for 30 s prior to use. The parental virus used to constructed the WR recombinant viruses was v∆B14 (Chen et al., 2006). The infectivity of all strains of VACV WR were titrated by plaque assay on BS-C-1 cells. Recombinant MVA-based viruses were generated from MVA and MVAc viruses (HIV clade C GagPolNef and Gp120 antigens, plus inactivated protease and integrase constructs (Gomez et al., 2007)). These parental viruses were titrated in CEFs.

2.14.1 Production of VACV WR recombinants

The modified WR BI4R allele containing 183R was generated using the pSJH7-derived plasmid p183 described above. The mammalian expression vector pSJH7 (Hughes et al., 1991) contained the selection marker Ecogpt (Boyle & Coupar, 1988) to enable selection of recombinant VACV by dominant transient selection (Falkner & Moss, 1990). The p183 plasmid was transfected into a T25 flask of RK-13 cells infected with v∆B14. The MOI was equal to 0.1 and the inoculum volume was 0.5 ml. The infection was carried out for 1.5 h before replacing the inoculum with fresh 2.5 % FBS DMEM and incubating the cells for 48 h. The cells were detached from the flask, collected by centrifugation and re-suspended in 1 ml of 2.5 % FBS DMEM. Ten fold dilutions of the harvested virus preparation were used to infect 6-cm dishes of RK-13 cells, pre-treated the previous day with 25 µg/ml mycophenolic acid (MPA), 15 µg/ml hypoxanthine, 250 µg/ml xanthine (Boyle & Coupar, 1988). After 1.5 h the inoculum was aspirated and the cells overlaid with 1 % low-melting point agarose in 2.5 % FBS DMEM supplemented with the same final concentrations of MPA, xanthine and hypoxanthine as above. After 48 h, a number of intermediate virus plaques were picked into 500 µl of 2.5 % FBS DMEM. The harvested plaques then underwent three cycles of freeze-thaw followed by two cycles of sonication for 30 s. Fifty µl of each sample was used to infect a well in a 96-well plate of RK-13 cells. After 48 h the cells were lysed by treatment with proteinase K according to the manufacturer’s instructions. The proteinase K product (2 µl) was then used as a template for a PCR using primers F1 and R2 to enable screening of the genotypes of the intermediate
viruses. Clones exhibiting bands corresponding to both the parental vΔB14 deletion and the plasmid A-183R-B fragment were then used to repeat the plaque purification method twice. These intermediate viruses containing the transfected plasmid integrated into the virus genome were then used to infect another set of RK-13 cells (6-cm dishes) without the presence of MPA, hypoxanthine or xanthine. This was necessary to resolve the intermediate virus into both the 183 insertion virus (WRΔB14+MVA183) and the 183 deletion virus (WRΔB14ΔMVA183). The drug-free plaque purification was repeated to ensure complete resolution. Finally, virus clones of WRΔB14+MVA183 and WRΔB14ΔMVA183 were used to infect T25 flasks of RK-13 cells until complete CPE was observed. The cell monolayers were then harvested and the associated virus resuspended in 500 µl of 2.5 % FBS DMEM and stored at -70 °C as the virus master stocks.

2.14.2 Production of recombinant VACV MVA

MVAΔ183 was generated using the pΔ183 plasmid (described in section 2.12). This expression vector was engineered to express the selection marker Ecogpt (Boyle & Coupar, 1988) fused in frame to GFP. The inclusion of Ecogpt enabled the intermediate viruses (containing the wild type 183R allele and the full pΔ183 plasmid) to be selected in the presence of MPA, xanthine and hypoxanthine. Due to differences in plaque formation between the WR and MVA strains it was not easy to select recombinant MVA plaques (or foci). For this reason, GFP was incorporated in the selection cassette as it facilitated the visualisation of the recombinant MVA foci (Staib et al., 2005). The pΔ183 plasmid was transfected into T25 flasks of MVA-infected CEFs MOI of 0.1, 1.5 h, 0.5 ml inoculum) and incubated for 48 h. Ten fold dilutions of the harvested virus preparation were used to infect 6-cm dishes of CEFs, pre-treated for 3 h with 25 µg/ml MPA, 15 µg/ml hypoxanthine, 250 µg/ml xanthine (Boyle & Coupar, 1988). After 1.5 h the inoculum was aspirated and the cells were overlaid with 1 % low-melting point agarose in 2.5 % FBS DMEM supplemented with 25 µg/ml MPA, 15 µg/ml hypoxanthine, and 250 µg/ml xanthine. After 48 h, the plates are viewed by fluorescence microscopy and foci of reasonable size were picked into 500 µl of 2.5 % FBS DMEM. This process was repeated three times prior to amplifying the virus clones in CEFs in T25 flasks. Small aliquots (50 µl) of amplified virus clones were used to infect a 96-well plate of CEFs, pre-treated for 3 h with
MPA, hypoxanthine, and xanthine as above. Following 48 h of growth these cells were lysed and the virus DNA was extracted by proteinase K treatment. The DNA obtained was used as template in a PCR to confirm the genotype of the intermediate viruses. Suitable foci were then resolved by three round of plaque purification without MPA, xanthine or hypoxanthine to obtain the deletion viruses required. As the EcoGptGFP cassette was removed from the viral genome at this stage putative deletion viruses were identified by immunostaining the infected cell monolayers with a fluorescein isothiocyanate (FITC)-conjugated secondary Ab to a primary anti-VACV Ab.

The MVAc virus containing the full pBELO-BAC11 plasmid was generated in a similar way but with some alterations. The virus was generated using the pBAC-VI plasmid described above. As with pΔ183, this expression vector contained the selection marker EcoGpt (Boyle & Coupar, 1988) fused in frame to GFP and this enabled selection of recombinant foci. The pBAC-VI plasmid was linearised via PacI digestion between the two deletion VI flanks and then transfected into T25 flasks of MVAc-infected CEFs (MOI of 1, 1.5 h, 0.5 ml inoculum). After 48 h incubation the progeny obtained were amplified by two successive rounds of growth in T25 flasks with liquid overlay in the presence of MPA, hypoxanthine, and xanthine (at the concentrations previously stated) to enrich the proportion of recombinant virus. Following this, three round of foci purification were undertaken as described for the generation of MVAΔ183. Finally the recombinant virus was amplified in a T25 flask and then two T175 flasks to create master and sub master stocks, respectively.

2.14.3 IMV purification by centrifugation through a sucrose cushion

For each virus, ten flasks of RK-13 cells were infected at 0.1 plaque-forming units (PFU)/cell and left until complete CPE was observed. Cells were harvested by tapping the flasks to detach the cells, followed by centrifugation for 5 min at 1500 rpm (Allegra 6R benchtop centrifuge, GH-3.8A rotor). The pellet was resuspended in 10 ml of cold 10 mM Tris pH 9 and left on ice to swell. The cells were dounce-homogenised (25 strokes) on ice to release the IMV. The homogenate was centrifuged at 1500 rpm (Allegra 6R benchtop centrifuge, GH-3.8A rotor) for 5 min to remove
cell nuclei and other debris. The supernatant was retained and the pellet was resubjected to the above process. The two supernatants were then pooled, sonicated and loaded onto 18 ml of 36 % (w/v) sucrose, 10 mM Tris pH 9 in a Beckman Ultra-Clear Tube and centrifuged at 13,500 rpm (Beckman L8M Ultracentrifuge and SW28 rotor) for 80 min at 4 °C. The pellet was washed in 36 ml of 10 mM Tris pH 9 by centrifugation at 13,500 rpm (Beckman L8M Ultracentrifuge and SW28 rotor) for 1 h 20 min. The wash supernatant was discarded and the pellet retained and resuspended in 1 ml 10 mM Tris pH 9. The final product was aliquoted and stored at -70 °C.

2.14.4 IMV purification by centrifugation through a sucrose gradient

Following sucrose cushion purification a further purification step was undertaken via a 29 ml sucrose gradient ranging from 40-24 % (w/v) sucrose, 10 mM Tris pH 9. The product from the sucrose cushion purification was homogenised via three 20 s intervals of sonication and incubation on ice and then gently applied to the sucrose gradient. The gradient was centrifuged at 12,000 rpm (Beckman L8M Ultracentrifuge and SW28 rotor) for 50 min at 4 °C. The concentrated IMV was visible in a white band and the sucrose above this was aspirated. Then the IMV band was removed with a pipette to a fresh centrifuge tube. The solution was then diluted into 2 volumes of 10 mM Tris pH 9 to dilute the preparation and the virus was recovered by centrifugation at 13,500 rpm (Beckman L8M Ultracentrifuge and SW28 rotor) for 80 min at 4 °C. The resulting pellet was resuspended in 500 µl Tris 10 mM Tris pH 9 and stored at -70 °C.

2.14.5 Crude viral genomic DNA purification

Cells (6-well plates) were infected at an MOI of 5, incubated for 24 h and harvested by centrifugation at 1000 rpm (Allegra 6R benchtop centrifuge, GH-3.8A rotor) for 5 min. The cells were washed with ice-cold PBS and resuspended in 50 µl of extraction solution I (0.15 M NaCl, 0.02 M Tris HCl pH 8, 0.01 M EDTA) and then supplemented with 250 µl of extraction solution II (0.02 M Tris HCl pH 8, 0.01 M EDTA, 0.75 % SDS, containing 0.65 mg of proteinase K, Sigma) and incubated for 37 °C for 12 h prior to phenol:chloroform:isoamyl extraction.
2.14.6 Inhibition VACV hairpin resolution prior to DNA extraction

Isatin-β-thio-mericarbazon (IβT) (a kind gift from Professor R Condit, University of Florida, USA) is a drug that blocks late protein synthesis (Cohrs et al., 1989). Subsequently it inhibits the resolution of viral hairpins and thus enables VACV genomic concatemers to be isolated. CEF monolayers were treated with IβT diluted to 45 µM in 2.5 % FBS DMEM following a 1.5 h infection at an MOI of 5 with MVAc-BAC. The IβT was dissolved to 5 mg/ml in acetone for storage at -20 °C and diluted to 1 mg/ml in 0.25 M NaOH just before dilution into 2.5 % FBS DMEM. Infected-cells were harvested 24 h later and viral DNA extracted as per section 2.14.6.

2.14.7 Phenol:chloroform:isoamyl extraction of DNA

DNA was extracted with two volumes of phenol:chloroform:isoamyl (Sigma) and then one volume of chloroform:isoamyl (Sigma). The DNA was then precipitated with 1 ml of ice-cold ethanol supplemented with 100 mM sodium acetate and incubated at -20 °C for 1 h. The DNA pellet was collected by centrifugation at 10,000 rpm (Hereaus Sepatech Biofuge 15R, HFA 22.2 rotor) for 30 min at 4 °C, washed with 1 ml of 70 % ethanol, centrifuged for 10 min at 10,000 rpm (Hereaus Sepatech Biofuge 15R, HFA 22.2 rotor) at 4 °C and air-dried for 15 min. The pellet was then dissolved by the addition of 20, 50 or 100 µl of TE (Qiagen) depending on the subsequent use.

2.14.8 Titration of VACV WR infectivity by plaque assay

The WR viruses were titrated by plaque assay to assess their infectivity. The virus stocks were thawed and sonicated prior to being diluted 10-fold in series in 2.5 % FBS DMEM. From each of these dilutions, 0.5 ml was used to infect confluent BS-C-1 cells grown in 6-well plates in duplicate. The infection was continued for 1.5 h with regular (every 10-15 min) rocking of the plates. Following this, the inocula were removed and replaced with 1.5 ml of 2.5 % FBS DMEM containing 1.5 % carboxymethylcellulose (CMC). After 48 h this overlay was removed and the plates were washed with PBS before staining with 0.01 % (w/v) crystal violet in 15 % (v/v) ethanol.
2.14.9 Titration of VACV MVA infectivity

The MVA viruses were titrated by plaque assay to assess their infectivity. The virus stocks were thawed and sonicated prior to being diluted 10-fold in series in 2.5 % FBS DMEM. From each of these dilutions, 0.5 ml was used to infect confluent CEFs grown in 6-well plates in duplicate. The infection was continued for 1.5 h with regular (every 10-15 min) rocking of the plates. Following this, the inocula were removed and replaced with 1.5 ml of 2.5 % FBS DMEM containing 1.5 % CMC. After 48 h the overlay was removed and an immunostaining protocol was used to visualise the foci. Briefly, plates were washed with PBS before the cells were fixed with 1 ml of a 50 % acetone / 50 % methanol solution for 2 min. The cells were then washed again with PBS and rocked for 1.5 h at room temperature with 1 ml of anti-VACV Ab at a 1 in 1000 dilution in PBS supplemented with 5 % FBS. Cells were washed twice in PBS before the addition of 1 ml of anti-rabbit secondary Ab at 1 in 1000 dilution in PBS supplemented with 5 % FBS. The plates were then rocked for 1 h at room temperature. The cells were then washed with PBS again and stained with 1 ml of 3-3’-diaminobenzidine tetrahydrochloride (DAB) (Sigma) dissolved in a 1 % NiSO$_4$ and 0.01 % H$_2$O$_2$ solution.

2.14.10 Protein degradation assays in VACV infection

Viruses were used to infect cells in the presence or absence of a proteosome inhibitor MG132 (Sigma). CEFs were grown in a 6-well plate and treated, where indicated, 1.5 h prior to infection with 10 mM MG132. The cells were then infected at 5 PFU/cell in 2.5 % FBS DMEM supplemented with 10 mM MG132 where indicated. After a 1.5 h infection the inoculum was removed and replaced with 2 ml of 2.5 % FBS DMEM, containing 10 mM MG132 where indicated. After 6-8 h the medium was aspirated and the cells were washed with PBS and lysed with 0.5 ml SLB per well.

2.14.11 Viral growth kinetics analysis

Viruses were used to infect T25 flasks of confluent cells, BS-C-1s or CEFs for WR and MVA viruses respectively. For the single-step analysis, cells were infected at a MOI of 10 for 1.5 h in 2.5 % FBS DMEM, the inoculums removed and then replaced with 2.5 % FBS DMEM. Cell-associated virus was harvested at time 0 (1.5 h p.i.),
12 h and 24 h by washing with PBS and then scraping the cells into of 5 ml 2.5 % FBS DMEM. Centrifugation at 1000 rpm (Allegra 6R benchtop centrifuge, GH-3.8A rotor) for 5 min produced a pellet which was resuspended in 250 µl 2.5 % FBS DMEM and stored at -20 °C prior to titration. For the multi-step analysis, cells were infected at a MOI of 0.01 for 1.5 h in 2.5 % FBS DMEM, the inocula were removed and replaced with 2.5 % FBS DMEM. Cell-associated virus was harvested at time 0 (1.5 h p.i.), 24, 48 and 72 h by washing and then scraping the cells into of 5 ml 2.5 % FBS DMEM. Centrifugation at 1000 rpm (Allegra 6R benchtop centrifuge, GH-3.8A rotor) for 5 min produced a pellet which was then resuspended in 250 µl 2.5 % FBS DMEM and stored at -20 °C prior to titration.

2.14.12 Focus size analysis

CEFs (in 6-well plates) were infected for 1.5 h at an appropriate MOI to produce 30-50 foci per well. Following removal of the inocula the cells were overlaid with 1.5 % CMC 2.5 FBS % DMEM and incubated at 37 °C for 48-72 h. Following this, the cells were washed and immunostained as per section 2.14.9. Focus length was measured using AxioVision Rel. 4.6 software (Zeiss) and a colour CCD microscope (Axiovert 2000).

2.14.13 Murine i.d. infection

Groups of female C57B/6 mice were anaesthetised and 10 µl of PBS containing $10^4$ PFU of VACV was injected into each ear pinna (Tscharke & Smith, 1999; Tscharke et al., 2002). Mice were between 6 and 8 weeks of age and were examined daily for signs of illness and the diameter of lesions at the site of inoculated was recorded using a micrometer. This work was carried out by Dr A.S. Fahy.
Chapter 3: Characterisation of the 183 protein structure and function

3.1 Bioinformatic analysis of the 183R ORF

VACV strain MVA gene 183R is 432 bp long and is predicted to encode a 143 amino acid polypeptide with a molecular mass of 16.7 kDa and a pI of 4.78. Sequence analyses of the protein revealed no predicted transmembrane or coiled-coil domains, nor any potential secretory signal peptides (www.poxvirus.org). The conservation of 183 is particularly strong across the different strains of VACV (Fig. 3.1), and 183 orthologues are spread across all OPV apart from the Taterapox (TATV). However, there are no known orthologues of the 183 protein outside of OPV. The VACV 183 orthologues have high levels of identity (>95 %) the only notable difference being the six residue deletion in MVA 183, which is not present in any other strain. In fact, the deleted motif (REISAI) is present in all the OPV 183 orthologues with a sequence identity of greater than 95 % (Fig. 3.2). Furthermore, among the OPV orthologues, which have lower levels of identity (60-95 %), although REISAI is not always conserved precisely there are a number of alternatives to the motif and no deletions are seen in 183. These alternative motifs conserve many of the key components of the REISAI motif: for instance, either the arginine and glutamate at the start or the alanine and isoleucine at the end. For example, 009 from CPXV strain Brighton Red (60 % similarity) has a KELRAI motif in place of REISAI, while Yaba-like disease virus (YLDV) strain Davis contains an orthologue with a REAVSM motif at this position. Crucially, none of the orthologues contain a deletion as found in 183, rendering it unique. Furthermore, the genome of the MVA parental virus, CVA, from which MVA was derived during >500 passages in avian cells, revealed that the counterpart of 183 in the parent virus, CVA207 has an intact REISAI sequence (Meisinger-Henschel et al., 2007). The fact that the WR and CVA orthologues of 183 both carry these six residues indicates that the deletion found in MVA 183 very likely was derived during the passages.

The structure of B14 has been solved by X-ray crystallography (Graham et al., 2008) and reveals that the protein is formed by eight α-helices. The fold is similar to that of the cellular Bcl-2 family of proteins, which was not predicted given B14 has a low primary sequence identity with members of this family (Graham et al., 2008). However, this low-level of similarity is not without precedent. The resolved structure
of VACV N1 revealed a homodimeric, predominantly alpha-helical, Bcl-2-like protein despite only low-level amino acid identity (Cooray et al., 2007), and furthermore, the N1 protein has been shown to interact with pro-apoptotic Bcl-2 family proteins (Cooray et al., 2007). N1 and B14, along with other VACV proteins, K7 and A52 are form part of a related family of VACV proteins (Smith et al., 1991). Of these A52 (Graham et al., 2008) and K7 (Kalverda et al., 2009) have also been shown to have Bcl-2-like folds.

It is important to note that despite structural similarity to N1 and cellular Bcl-2 proteins, B14 crucially does not contain a BH-3 binding groove on its surface, and under the conditions tested was found not to negatively regulate apoptosis. There are only three points of difference in the amino acid sequence of 183 and B14 and so it was possible, using a combination of the secondary and tertiary B14 structures, to map the changes in 183 onto the structure of B14. Firstly, helix 1 of B14 contains an arginine, versus a lysine in 183. B14 is actually the exception in this case in comparison to orthologues found in other strains of VACV (except strains Acambis 3 and 3737, Fig. 3.1) though this is a relatively conservative amino acid substitution. There is one further single residue change in helix 5. B14 encodes an alanine, as do the majority of VACV strains, whereas 183 contains a threonine, in common with the Lister and COP strains (Fig. 3.1). Importantly, there is a six-residue deletion in helix six of 183 which is unique to the MVA strains of VACV (MVA, Acambis MVA and MVA I721-075) as discussed previously. The position of this deletion, a short way into the central helix of this small helical protein, suggests that it may result in major rearrangement of the helices around the core. Consequently, the three-dimensional structure of protein 183 may be quite distinct from that of B14 or indeed be unstable due to misfolding.
Figure 3.1 Amino acid alignment of all VACV sequences for 183 orthologues. MVA strains are boxed. Incidences of divergences are indicated by a black background.
### Figure 3.2 Amino acid alignment of OPV proteins with ≥95 % amino acid identity to MVA 183.

The sequence of MVA 183 is boxed. Incidences of divergences are bold and on a black background. *N.B.* only two VARV strains are presented here due to 100 % identity to either the Garcia or Ind3 strain in all other VARV orthologues.
3.2 Construction of MVAΔ183

With knowledge of the immunomodulatory function of B14 (Chen et al., 2006; Chen et al., 2008), I83R was selected for removal from MVA in an attempt to create a more immunogenic vaccine vector. Wild type MVA (received from M Esteban) was used as the parental virus. The genomic structures of the I83R locus of this virus and that of the desired knockout virus are illustrated in (Fig. 3.3). MVAΔ183 was generated using the pCI-derived plasmid pΔ183 described above (Section 2.12). This expression vector was engineered to express Ecogpt (Boyle & Coupar, 1988) fused to GFP (Fahy et al., 2008). The inclusion of Ecogpt enables the intermediate viruses (containing wild type I83R allele and the EcogptGFP selection cassette inserted into pCI) to grow in the presence of MPA, xanthine and hypoxanthine. Due to the block in purine synthesis induced by MPA when Ecogpt is not expressed, virus grown in the presence of MPA must have the plasmid integrated into the virus genome. This can occur via single-site specific recombination event between either of the homologous regions found in both plasmid and genome. Transient dominant selection (Falkner & Moss, 1990), as implemented here, relies on the fact that the recombinant viruses containing the integrated pΔ183 within their genomes (intermediate viruses Fig. 3.1) were unstable due to proximity of direct repeats of the flanking regions. Consequently, after removal of the MPA selection pressure a second single site-specific recombination event occurs. This results in resolution to a virus genome containing the modified sequence contained with the plasmid, or the virus genome returning to wild type.

The pΔ183 plasmid (section 2.12) was transfected into MVA-infected CEFs and the presence of green cells was detected at 2 days p.i. Following a single site-specific recombination event, between either one of the 183 flanks contained in pΔ183 and the corresponding flank within the MVA genome, the whole plasmid sequence integrated into the genome (Fig. 3.3). The cells were harvested and virus therein was amplified in a T25 flask of CEFs under selection pressure from MPA, xanthine and hypoxanthine to enrich for MPA-resistant green recombinant virus. The MPA selection pressure meant that viruses containing pΔ183, and thus expressing Ecogpt, produced foci whereas non-recombinant parental viruses could not. This virus stock was then plated onto fresh monolayers of CEF and green foci were detected by fluorescence microscopy (Fig. 3.4 (i)). Green foci were purified three times. Due to
differences in CPE between the WR and MVA strains it is not easy to select recombinant MVA plaques (known as foci). For this reason the GFP coding region was fused to Ecogpt to enable visualisation of the recombinant MVA foci by fluorescence microscopy (Staib et al., 2005).

The intermediate viruses obtained were unstable due to proximity of direct repeats of the flanking regions. Consequently, on removal of the MPA selection pressure a second recombination event occurred by which the viruses resolved either to MVAΔ183 or the parental MVA. The intermediate viruses that resolved to MVAΔ183 were then plated out at a range of dilutions to obtain discrete foci. This process was repeated three times to obtain an aliquot of the recombinant virus without remnant of the intermediate virus and/or parent virus. Due to loss of EcogptGFP concomitant with resolution to MVAΔ183 the foci were, therefore, no longer identifiable by fluorescence microscopy. Consequently, immunostaining of live cells was used to visualise the resolved MVAΔ183 foci by use of anti-VACV polyclonal primary Ab followed by an anti-rabbit secondary Ab conjugated to the red alexa 488 fluorophore (αRb488). Foci that were positive for alexa 488 but negative for GFP were selected and purified three times (Fig. 3.4 (ii)). The foci harvested were then used to infect a 96-well plate of CEFs. After 48 h each well was treated with proteinase K to extract the viral DNA.
Figure 3.3 Diagrammatic scheme of the generation of the MVAΔ183 virus. CEFs were infected with MVA at an MOI of 0.1 and 1.5 h later the cells were transfected with pΔ183. Following a single site-specific homologous recombination event GFP-positive intermediates were generated which then resolved to the deletion allele present in MVAΔ183 via an additional single recombination event.

Figure 3.4 Fluorescent recombinant MVA foci. CEFs were infected for 1.5 h and overlaid with semi-solid/solid overlay and viewed by fluorescence microscopy. (i) Intermediate MVA focus expressing GFP due to the integration of pΔ183 into genome, following two rounds of MPA selection. (ii) Alexa-488 immunostained recombinant MVA focus not expressing GFP due to resolution of the unstable intermediate following removal of MPA selection pressure.
The extracted DNA was then used as a template for PCR analysis of the 183R locus of MVA (Fig. 3.5). Primers MVA-AL and MVA-BR (Table 2.1) were annealed to the 5' and 3' ends of the flanking regions of 183R. After PCR amplification this produced a 0.9-kb fragment in the wild type virus containing 183R and a 0.5 kb fragment when the gene was removed in the deletion virus MVAΔ183. The PCR product of 0.5 kb represented the two 250-bp flanking regions without 183R as was expected for the deletion virus. In contrast, the 0.9-kb PCR product corresponded to 183R surrounded by the flanking regions was as expected when the parental MVA virus was used as the PCR template. An additional PCR was carried out using primers 93 and 94 (Table 2.1) that bind to the Amp-resistance gene in the pΔ183, but no product was obtained when using MVAΔ183 DNA, confirming plasmid DNA was not carried through the virus modification process. A virus clone was selected and amplified on CEFs in a T25 flask and then in a T175 flask to produce the master and sub master stocks of the MVAΔ183 virus.

**Figure 3.5 PCR analysis of 183R locus.**

(i) A PCR product of approximately 0.5 kb was produced from the MVAΔ183 virus with primers annealing to the flanking regions (A and B). A PCR product of 0.9 kb was produced with the same primers when the wild type MVA virus was used as template. These products correspond to AB alone and 183R surrounded by A and B respectively. (ii) A PCR product of approximately 0.8 kb was produced with pΔ183 with primers annealing to the Amp-resistance gene but no product is seen when MVA Δ183 was used as template. dsDNA size markers were run in parallel.
3.3 Characterisation of MVAΔ183

3.3.1 Focus size differences in MVAΔ183 and parental MVA

Virus stocks were then titrated on CEFs. The foci produced by MVA replication in cell culture differ from those produced by WR and thus the MVA titre cannot be counted accurately by staining the infected monolayers with crystal violet (section 2.14.8). Instead, an immunostaining protocol was used to determine the virus titre (section 2.14.9). Infected CEFs were incubated with an anti-VACV primary Ab and then an anti-rabbit secondary Ab (Table 2.3) prior to DAB staining. The foci were thus counted and virus titre was calculated. It was noted on concurrent titration of MVAA183, MVA and MVAc that the focus size of the mutant virus constructed was larger than those with either the parental virus MVA or the vaccine-adapted MVAc as illustrated in Fig. 3.6. The foci measured for MVA and MVAc were not significantly different (student’s t-test, p= not significant (ns)) (Fig. 3.7). In contrast the mean focus size of MVAA183 was increased by 45 % compared to that of the parent virus MVA, which is a significant difference (Student’s t-test, p<0.001). This difference could have arisen due a change in virus-induced apoptosis, CPE, or actin tail formation. However, this difference was not anticipated following the targeted deletions of I83R from MVA given the immunomodulatory function encoded by its orthologue B14.
Figure 3.6 Focus size of MVAc, MVA and MVAΔ183.
CEFs were seeded into 6-well plates and infected for 1.5 h at an appropriate MOI to produce either 300-500 (left-hand side) or 30-50 (right-hand side) foci per well. Following removal of the inocula the cells were overlaid with 1.5 % CMC 2.5 % FBS DMEM and incubated at 37 °C for 48 h. Following this, the cells were immunostained as per section 2.14.9.
Figure 3.7 Focus length analysis of MVA, MVAc and MVAΔ183.
DAB-stained MVA foci illustrated in Fig. 3.6 were measured using AxioVision Rel 4.6 software (Zeiss) and a colour CCD microscope (Axiovert 2000). For each virus indicated 12 foci were measured and the mean indicated by the horizontal line. Student’s t-test was performed to assess the significance of differences between MVA and MVAc (p=ns) and also MVA and MVAΔ183 (p<0.001).
3.3.2 Genomic digestion of MVA recombinant viruses

The change seen in the size of MVA\(\Delta183\) foci was not predicted from the immunomodulatory function of its WR orthologue B14. The focus size change could either have resulted from a previously uncharacterised function of protein 183 in the context of MVA infection or unintended changes to the MVA genome incurred during the derivation of MVA\(\Delta183\). Given the immunomodulatory function of the WR orthologue B14 the possibility of additional genome changes seemed more plausible and was investigated. Purified virus DNA was digested by \(\text{HindIII}\) and analysed by agarose gel electrophoresis (Fig. 3.8). In addition, DNA from both its parent virus and the modified MVAc were analysed in parallel. The \(\text{HindIII}\) J fragment of MVA (5010 bp from DNA sequence (Antoine et al., 1998)) is altered in MVAc due to the insertion of the HIV genes into the VACV TK locus that lies within this fragment. This extra DNA contains 3 additional \(\text{HindIII}\) sites causing the \(\text{HindIII}\) J fragment to be cut into 4 fragments by \(\text{HindIII}\). In comparison, MVA\(\Delta183\) has a standard-sized J fragment, indicating the parental virus was MVA not MVAc. In addition, the \(\text{HindIII}\) A, B, and C fragments differ between the two viruses and in MVA\(\Delta183\) fragments A and B are larger, and \(\text{HindIII}\) C fragment is smaller. The A and B fragments are located near the right end of the genome while C is found at the opposite terminus (Fig. 3.8 (ii)). These changes suggest that during isolation of this virus there had been a terminal transposition event in which DNA from one end had been copied to the other end. This type of re-arrangement had been reported during the evolution of OPV (Moyer et al., 1980; Moss et al., 1981). Given this finding in combination with the interesting large focus phenotype observed, the genome of this virus will be sequenced to determine what changes have occurred and how the phenotype may be accounted for.
Figure 3.8 HindIII digest of MVAc, MVA and MVAΔ183 genomic DNA.

(i) Approximately 1 µg of the genomic DNA from the indicated viruses was digested with HindIII and then run on a 1 % pulse-field agarose gel for 10 h. (ii) Layout of the HindIII digestion fragments across the MVA genome from left to right. dsDNA size markers were run in parallel.
3.3.3 Growth kinetics analysis of MVA recombinant viruses

Given the major changes seen in the MVAΔ183 genome and the accompanying large focus phenotype, the growth kinetics of the virus were analysed. Cells were infected at an MOI of 10 and the cell-associated virus present at 0, 12 and 24 h p.i. was titrated by immunostaining. No significant difference in titre for MVAΔ183, MVA or MVAc was seen (Fig. 3.9). Therefore, there is no difference in the replication of all three viruses. Thus it can be concluded that the change in focus size seen with MVAΔ183 does not arise from alterations in the replication of the virus.

![Figure 3.9 Single-step growth kinetics of MVA viruses.](image)

CEF in T25 flasks were infected with MVA, MVAc and MVAΔ183 at an MOI of 10 for 1.5 h. The inoculums were removed and cells were washed twice with PBS and then overlaid with 2.5% FBS DMEM. Cell-associated viruses were then harvested immediately (0 h), 12 h and 24 h later. The virus was then titrated in duplicate on confluent 6-well plates of CEFs. The experiment was carried in duplicate and the error bars represent standard deviation from the mean.

3.4 Characterisation of protein 183

3.4.1 Expression in virus-infected cells

To examine the expression of 183, lysates from MVA-infected CEFs were analysed by immunoblotting using the anti-B14 polyclonal Ab (Chen et al., 2006) (Fig. 3.10). Surprisingly, protein 183 was not detected at comparable levels to the B14 produced by WR infection. No changes were detected in the nucleotide sequence of the region upstream of 183R compared to WR B14R suggesting this difference was not due to
changes in promoter activity. So the more likely explanation was that the changes in 183 compared to B14 made the protein less stable (Fig. 3.10). A major pathway for the removal of labile proteins in eukaryotic cells is via ubiquitination and degradation by the proteosome system. To assess whether protein 183 was being turned over by this mechanism a proteosome inhibitor (MG132) (Satheskumar et al., 2009) was added to cells prior to and during infection. MG132 is a short oligopeptidic sequence with an aldehyde electrophilic structure at the C terminus. The electrophilic structure of proteosome inhibitors can be an aldehyde or boronate (reversible inhibitors), or an epoxyketone or vinylsulfone (irreversible inhibitors) (Lee & Goldberg, 1998). MG132 reversibly blocks multiple active sites in the proteosome by forming transition state analogues by interacting with a catalytic hydroxyl/thiol group to forms a reversible hemi(thio)acetal. Protein 183 expression from MVA was detected in the presence of MG132 (Fig. 3.10) but not when the cells were infected but untreated with proteosome inhibitor. These findings indicate that 183 is a labile protein, and imply that the mutations incurred by 183 during the derivation of MVA result in different level of protein stability compared to that of B14.

In addition, CEFs infected with MVAAΔ183 with, and without, MG132 were harvested and the lysates were analysed by SDS-PAGE and immunoblotting (Fig. 3.11). WR- and MVA-infected cell lysates in the presence and absence of MG132 were also analysed as controls. As predicted, WR produced detectable B14 under both conditions whereas MVA yielded detectable protein 183 only with MG132 treatment. However, it is clear that the expression of B14 is reduced in the presence of MG132. This is likely to be due to the ability of MG132 to inhibit VACV late protein expression. Although B14 is expressed early during infection, no reduction in expression is seen by 8 h (Chen et al., 2006). The diminished level of B14 protein with MG132 implies there may also be late expression of B14. In the presence of MG132 only the early transcripts from this promoter would result in protein, therefore, the overall B14 level is reduced. MVAAΔ183 produced no detectable protein 183 in the presence and absence of MG132, confirming the removal of the gene from the viral genome.
Figure 3.10 Expression of protein 183 in virus-infected cells.

CEF's were infected at an MOI of 10 with WR, vΔB14 or MVA. Where indicated cells were pre-treated for 1.5 h with MG132 at 10 µM and this concentration was maintained throughout the infection. Infected cells were incubated for 8 h and lysed (section 2.14.10). Lysates were analysed by SDS-PAGE and immunoblotting. Molecular mass markers were run in parallel.

Figure 3.11 MVA protein 183 is not detected in MVAΔ183-infected cells.

CEF's were infected at an MOI of 10 with WR, MVA, or MVAΔ183. Where indicated cells were pre-treated for 1.5 h with MG132 at 10 µM and this concentration was maintained throughout the infection. Infected cells were incubated for 8 h and lysed (section 2.14.10). Lysates were analysed by SDS-PAGE and immunoblotting. Molecular mass markers were run in parallel with samples.
3.4.2 Expression of recombinant protein 183 in *E. coli*

The purification protocol used to obtain soluble B14 (Chen *et al.*, 2006; Graham *et al.*, 2008) was attempted on a small scale to examine whether 183 could be purified easily to obtain adequate protein for crystallisation trials. BL21 (DE3) cells were transformed with the p183His plasmid. This pET28a-based plasmid encodes the 183 with a C-terminal His tag under an IPTG inducible promoter control and a Kan resistance marker (Table 2.2). The plasmid was grown up sequentially and protein production induced with IPTG when the bacteria reached an OD of 0.4 (section 2.4). After a 5 h growth period the cells were lysed and sonicated. The lysates were centrifuged for 30 min at 3000 rpm (Allegra 6R benchtop centrifuge, GH-3.8A rotor) at 4 °C (Section 2.4) and both the supernatant and pellet were retained and analysed by SDS-PAGE. However, in contrast to B14, 183 was insoluble using this purification strategy (data not shown). Consequently, an auto-induction system was trialled to test if variation in the purification conditions could render protein 183 soluble (Fig. 3.12). MagicMedia™ (Section 2.4) was used at 30 °C and 37 °C, however, just as seen as following IPTG induction, protein 183 remained insoluble. Given the insoluble nature of 183, in contrast to that of B14, further functional characterisation of the protein in a eukaryotic system was deemed necessary, to establish how far-reaching the disparity between the two proteins was, prior to pursuing an alternative purification protocol suitable for insoluble proteins.
Electrocompetent BL21 *E. coli* were transformed with approximately 1 µg of p183His plasmid and plated onto Kan-containing agar. A colony was picked and grown via the MagicMedia™ auto-induction system at both 30 ºC and 37 ºC according to the manufacturer’s instructions. After 24 h growth the cells were harvested and lysed (section 2.4) and the soluble and insoluble fractions analysed by SDS-PAGE and coomassie staining. Cultures grown in the MagicMedia™ and not induced were analysed too. Molecular mass markers were run in parallel.

### 3.5 Assessment of 183 protein function in NF-κB signalling

B14 inhibits NF-κB activation induced by IL-1 or TNFα following co-transfection of a plasmid containing FLAG-tagged B14 (pB14f) and a NF-κB firefly luciferase reporter plasmid (Chen *et al.*, 2008). In view of this, further reporter gene assays were undertaken to examine if protein 183 also inhibited the NF-κB signalling pathway. 183 was cloned with an N-terminal FLAG tag into the mammalian expression vector pCI (Promega) (p183f). The cloning process positioned the gene downstream of a strong human cytomegalovirus immediate early promoter and facilitated the study of the properties and functions of the 183 protein in uninfected cells. HEK 293T cells were transfected with a plasmid encoding firefly luciferase under the control of an NF-κB response element and another plasmid encoding Renilla-luciferase under the control of a herpes simplex virus TK promoter (constitutively active at a low level) as
an internal control. Simultaneously cells were transfected with either pCI or the indicated expression vector (Fig. 3.13) and 18 h later stimulated with TNFα. The reporter gene activity was assessed by firefly luciferase activity. Data generated in the presence of B14 were expressed as the mean fold induction relative to pCI, and all samples were collected in triplicate. Stimulation with TNFα resulted in an approximately 15-fold increase in firefly luciferase activity when the cells had been transfected previously with just pCI. In contrast, in cells transfected with pB14f, stimulation with TNFα resulted in a less than 5-fold increase in firefly luciferase activity. This was not significantly different from the unstimulated cells transfected with either empty vector or pB14f. However, in cells transfected with p183f, stimulation with TNFα resulted in an 18-fold increase in firefly luciferase activity. Thus, the 183 protein does not appear to inhibit NF-κB in response to TNFα stimulation (Fig. 3.13). In fact, there is a marginal enhancement (Student’s t-test; p=0.056) in reporter gene activity compared to empty expression vector although the biological significance of this is unclear. Therefore, 183 does not inhibit TNFα-induced NF-κB activation and is not functionally equivalent to B14 in this regard.

The NF-κB firefly luciferase reporter assays described above were carried out to assess the impact of the protein 183 on NF-κB activity after treatment with TNFα, which is known to activate signalling pathways upstream of NF-κB. However, this experiment also provided indirect information about variation in protein 183 stability in eukaryotic cells depending on the activation state of these NF-κB activating pathways. This was achieved via the use of an immunoblot control, intended to check there was comparable transfection efficiency and correspondent gene expression under the conditions used. Triplicate samples for each transfection condition were pooled and analysed by SDS-PAGE and the resulting immunoblot (Fig. 3.13) revealed that protein 183 expression was readily detected following stimulation with TNFα. However, protein 183 was not seen in cells without stimulation. In addition, the upper half of the membrane was probed with an anti-tubulin mAb (Table 2.3) as a loading control to further indicate the expression level of the indicated expression vector. In conclusion, protein 183 expression is detected following TNFα stimulation but even when more stably expressed it does not down regulate NF-κB activation.
Figure 3.13 Protein 183 does not inhibit NF-κB activation by TNFα.
HEK 293T cells were transfected with either pCI, pB14f or p183f and a firefly luciferase NF-κB reporter plasmid and an internal control plasmid encoding Renilla-luciferase for 16 h. Cells were then stimulated with 50 ng/ml TNFα for 8 h. Cells were lysed and firefly luciferase and Renilla-luciferase expression were assessed. A small aliquot of the cell lysates was retained and analysed by SDS-PAGE and immunoblotting to establish transfection efficiency and protein expression. The reporter gene assay (graph) was carried out in triplicate and the error bars represent standard deviation from the mean.
3.6  Construction of recombinant VACV WR containing 183R at the B14R locus

Another way of investigating if protein 183 had the same or different properties to B14 was to test if loss of B14 from VACV strain WR could be rescued, or compensated for, by the expression of 183, in terms of virus virulence. MVA cannot replicate in many mammalian cell lines, therefore, as a preliminary step in investigating the role of the 183 protein in virus-infected cells, the 183R gene was inserted into the B14R deletion virus vΔB14 (Chen et al., 2006).

A pSJH7-derived plasmid, p183, was used to insert 183R into vΔB14. This plasmid contained 183R flanked by the 250 bp immediately up- and downstream of B14R in the WR genome. These 250-bp flanking regions are denoted A and B. The plasmid was transfected into RK-13 cells and via a single site-specific recombination event, between either one of the flanks in p183 and the corresponding flank within the genome, the whole plasmid sequence integrated into the genome (Fig. 3.14). The recombinant virus progeny were plaque purified three times under selection pressure from MPA, xanthine and hypoxanthine and then grown without MPA so that the intermediate virus resolved by recombination to either WRΔB14+183 or WRΔB14Δ183, in which the B14R locus is identical to the parent virus vΔB14. The intermediate viruses that resolved to WRΔB14Δ183 were purified by three further rounds of plaque purification without MPA, to obtain WRΔB14Δ183. The wild type, parental virus used (vΔB14), intermediate and resolved insertion (WRΔB14+183) and deletion (WRΔB14Δ183) alleles are illustrated in Fig. 3.14
Figure 3.14 Generation of WRΔB14-MVA183.
RK-13 cells were infected with vΔB14 at a MOI of 0.1 and 1.5 h later the cells were transfected with p183. This plasmid contained 183R surrounded by two 250-bp regions A and B corresponding to the 250 bp immediately up and downstream of B14R in the WR genome. Following a single homologous recombination event intermediate viruses were formed and these were selected in the presence of MPA. Subsequently these were resolved in the absence of MPA to either the insertion or deletion allele indicated.
The recombinant viruses generated were analysed by PCR (Fig. 3.15) to ensure that the correct genome structure is present at the B14R/183R locus. The PCR products from WRΔB14-MVA183 were approximately 0.9 kb whereas that of the clone which resolved back to parental genotype, WRΔB14ΔMVA183 was 0.5 kb. These products correspond to 183R surrounded by A and B and AB alone respectively. These are the sizes expected given the modifications carried out as confirmed by PCR products seen with the plasmid (pAB and p183) positive control templates on the right-hand side. These PCR results confirmed that the required changes had been made to the vΔB14 genome. The two recombinant viruses constructed (WRΔB14-MVA183 and WRΔB14ΔMVA183) were amplified in parallel with wild type (WR), the parental virus (vΔB14) and the revertant of the parental virus (vΔB14Rev). Following partial purification of the viruses by sedimentation through a sucrose cushion (section 2.14.3) the virus infectivities were titrated on BS-C-1 cells.

3.7 Characterisation of WRΔB14-MVA183

3.7.1 Expression of 183 in the WR background

The expression of the protein 183 by WRΔB14-MVA183 was examined to assess if it was as highly labile as the protein encoded by 183R in the MVA background or if some viral factor lacking from MVA but present in WR enabled protein 183 to be expressed stably. As described above, cells were infected with WR, vΔB14, WRΔB14-MVA183 or MVA. Infected-cells were then lysed and the lysates analysed.
by SDS-PAGE and immunoblotting with the anti-B14 polyclonal Ab to assess B14/183 expression. The upper part of the membrane was probed with anti-tubulin mAb to serve as a loading control and monoclonal anti-VACV D8 mAb to verify an approximately equivalent level of virus infection with the viruses indicated.

Protein 183 expression was not detected for either the wild type MVA or the WR virus encoding 183R (WRΔB14-183) unless the proteosome was inhibited by MG132 (Fig. 3.16). It is worth noting that the immunoblot using the anti-D8 mAb to assess equivalent level of infection shows reduced signal in the proteosome treated samples as D8 is a late protein and as such its production is predicted to be diminished with proteosome inhibition. This characteristic of D8 also served to act as a control to show whether the proteosome inhibition was successful. MG132 is known to block a post-entry step in VACV replication, the effect is focussed between 2 and 4 h p.i. (Satheshkumar et al., 2009). Viral DNA synthesis is prevented after uncoating and hence virus replication, intermediate and late gene expression are inhibited (Satheshkumar et al., 2009).

Figure 3.16 Expression of the protein 183 by the WRΔB14-183 recombinant virus.
CEFs were infected at an MOI of 10 with WR, vΔB14, WRΔB14-183, MVA. Where indicated cells were pre-treated for 1.5 h with MG132 at 10 µM and this concentration was maintained throughout the infection. Infected cells were incubated for 8 h and lysed (see section 2.4.3). Lysates were analysed by SDS-PAGE and immunoblotting with αB14, αD8 and αTubulin Abs. Molecular mass markers were run in parallel.
3.7.2 Assessment of WRAB14-MVA183 virulence in the murine i.d. model

Due to the stabilisation of the protein 183 seen in the NF-κB assays on stimulation of the cells with TNFα, it was possible that this labile protein was rendered functional in the context of an immune response, although infection of cultured cells did not appear to produce stable 183 protein. As the B14 protein has been previously shown to be a virulence factor in the murine i.d. model (Chen et al., 2006) this method was used to evaluate the contribution of 183R to virus virulence. Three groups of eight female C57B1/6 mice were infected with 10^4 PFU of WR, vΔB14, or WRΔB14-MVA183. Daily examinations recorded the diameters of the lesions at the sites of inoculation up until day 13 and a peak lesion size for all three viruses was observed at day 8 (Fig. 3.17). This work was carried out by Dr A.S. Fahy. The WR wild type virus produced significantly larger lesions from day 4 onwards compared to both vΔB14 and WRΔB14-MVA183, re-affirming the role of B14 as a virulence factor. A comparison of vΔB14 and WRΔB14-MVA183 showed no significant difference at any time point, indicating the presence of the 183R gene at the B14R locus in the WR genome did not restore virulence and was equivalent vΔB14 in the i.d. murine model.

In conclusion, the alterations found in 183R relative to B14R, render the MVA protein unstable when expressed via transient transfection or infection in cell culture, and insoluble when expressed as a recombinant form in E. coli. Furthermore, both the in vitro NF-κB signalling modulation and in vivo virulence factor roles of the B14 protein are not exhibited by 183. In light of this, the re-generation of the MVAΔ183 virus, which had incurred spontaneous genomic alterations described previously in this chapter, was not attempted because 183R was shown to be a non-functioning counterpart of B14, and thus MVA might be considered to already be a B14 knockout virus.
Figure 3.17 Assessment of WRΔB14-MVA183 virulence in the murine i.d. model.
Groups of 8 mice were inoculated with WR, vΔB14, or WRΔB14-MVA183. Lesion diameter was recorded daily for 13 days, following the subsidence in peak lesion size at day 8. Error bars represent standard error from the mean.

3.8 Predicted model of 183 structure
Following the evidence detailed in section 3.7 that 183R encodes a highly labile non-functional version of the B14 protein, further bioinformatic analysis was carried out to model what effect the six residue REISAI deletion and two conservative amino acid substitutions may have on protein fold. The amino acid sequence of the former was modelled on the crystal structure of the later (Graham et al., 2008) using the Modeller program available http://salilab.org/modeller/. The resulting structure is illustrated in Fig. 3.18 and was likely to be an unstable conformation given the alteration seen in one of the central helices.
Figure 3.18 Predicted model of protein 183 structure.
(i) The B14 crystal structure is shown in cyan with the REISAI motif coloured red. (ii) The second structure shows the predicted fold of 183 generated by Modeller, the resultant loss of one of the central helices is highlighted in red and indicated by the black arrow. (iii) The B14/183 primary sequence alignment with B14 secondary structure. The three points of divergence between the 183 and B14 amino acid sequences occur, during the first, fifth and sixth helices of B14, these are highlighted in black.
Chapter 4: B14, in combination with other VACV proteins, acts on the NF-κB pathway

4.1 VACV modulation of NF-κB signalling

The NF-κB transcription factor is a key element in the regulation of many genes involved in the immune response to pathogens (Karin, 2004; Vitour & Meurs, 2007). In unstimulated cells, NF-κB is retained in the cytoplasm via an interaction with IκBα. NF-κB activation is stimulated by a range of inflammatory mediators, including TNFα, IL-1, platelet-derived growth factor (PDGF), lipopolysaccharide (LPS) and hypoxia (Hong et al., 2003). Upon stimulation, these multiple signalling cascades converge on the IKK complex which phosphorylates IκBα which is then degraded by the proteosome (Pasparakis et al., 2002), releasing NF-κB to translocate to the nucleus where it can induce transcription.

VACV encodes multiple proteins to down regulate activation of NF-κB to moderate the host response to infection. These include B14 (Chen et al., 2006), A46 (Stack et al., 2005), A52 (Harte et al., 2003), N1 (Cooray et al., 2007), K7 (Schroder et al., 2008) and A49 (Daniel Mansur, personal communication) as discussed in sections 1.6.3, 1.6.4 and 1.7.1. These VACV proteins interfere with the signalling pathways leading to NF-κB activation following infection as indicated in Fig. 4.1.

Given these multiple mechanisms through which VACV down regulates NF-κB, removing these proteins in concert rather than individually may produce a greater effect on virus function or exert a more substantial change in immunogenicity in vivo. This may be important in the design of improved VACV-based vaccine vectors. Consequently, a series of viruses lacking genes encoding NF-κB modulators has been constructed in sequence. To date, starting with a vΔB14 parent virus A49R and K7L have been removed. Herein, the construction of a virus bearing a double deletion of B14 and A49 (WRΔB14ΔA49) and its revertant (WRΔB14+A49) are described.
Figure 4.1 VACV-encoded proteins and NF-κB signalling
Recognition of PAMPs by appropriate receptors stimulates the upregulation of the NF-κB transcription factor family. Engagement of TLRs by their ligands sends signals which converge on IRAK4. VACV A52, A46, and K7 act upstream of IRAK4 where indicated (section 1.6.5). IRAK4 triggers further signalling cascades, which activate the TAK1 complex to stimulate the p38 MAPK, JNK and NF-κB pathways. TAK1 also activates the IKK complex. VACV N1 acts downstream of TRAF6 but upstream of IKK, the precise molecular target is not known (section 1.6.3). VACV B14 targets IKKβ. The IKK complex phosphorylates IκBα to induce its ubiquitination and degradation. The precise target of VACV A49 is unknown, but it has an effect on the phosphorylation / ubiquitination pathway for IκBα/p65. Thus, the VACV-encoded proteins reduce the amount of NF-κB dimers that translocate into the nucleus and thereby diminish activation of transcription of genes involved in the anti-inflammatory response.
4.2 Generation of WRΔB14ΔA49

A pCI-based plasmid, pΔA49 (see Table 2.2) was designed to remove A49 from the VACV WR genome. The A49R gene was removed from wild type VACV WR by Dr D. S. Mansur and from vΔB14, a virus lacking the B14R gene (Chen et al., 2006), as described here. The A49R gene is conserved among all strains of VACV. A49R also has orthologues in other OPVs including strains of VARV, CPXV, MPXV, TATV, ectromelia virus and camelpox virus. However, no A49R orthologues exist outside the OPV. VACV WR A49R encodes a 161 amino acid protein of 18.8 kDa. It is mainly localised in the cytoplasm and blocks NF-κB activation by affecting the stability of phosphorylated-IκBα and preventing p65 translocation to the nucleus (Daniel Mansur, personal communication). pΔA49 contained the A49R flanking regions A and B to enable site-specific recombination into the VACV genome. The plasmid was engineered to express Ecogpt (Boyle & Coupar, 1988) fused to GFP from a constitutive VACV promoter (section 2.11.2) to allow efficient selection of recombinant virus. RK-13 cells were infected with vΔB14 and subsequently transfected with pΔA49. The plasmid integrated into the vΔB14 genome via a single recombination event between either one of the flanks in pΔA49 and the corresponding sequence within the vΔB14 genome (Fig. 4.2). Although the plaques are easily visible either via neutral red staining or by eye, GFP expression aids detection of recombinant plaques considerably. (Fig. 4.3). The GFP-positive recombinant virus progeny were plaque purified three times under selection pressure from MPA, xanthine and hypoxanthine and then grown without MPA so that the intermediate virus resolved by recombination to obtain WRΔB14ΔA49.

The recombinant viruses generated were analysed by PCR (Fig. 4.4) to ensure that the correct genome structure was present at the A49R locus. The PCR product obtained from WRΔB14ΔA49 using primers specific to external edges of the A49R flanks was approximately 0.7 kb whereas that of the wild type (vΔB14) produced a band of approximately 1.2 kb. The PCR product of 0.7 kb represented the two 350-bp flanking regions without A49R, as expected for the WRΔB14ΔA49 virus. In contrast, the 1.2-kb PCR product represented A49R surrounded by the flanking regions and was formed when vΔB14 was used as the template for PCR. The WRΔB14ΔA49 virus was amplified and purified by sucrose cushion and gradient purification.
RK-13 cells were infected with vΔB14 at an MOI of 0.1 and 1.5 h later were transfected with pΔA49. Following a single site-specific homologous recombination event, GFP-positive intermediate viruses were generated. Subsequently, these resolved via further single homologous recombination to form WRΔB14ΔA49, lacking A49R.

**Figure 4.2 Construction of WRΔB14ΔA49.**

RK-13 cells were infected with vΔB14 at an MOI of 0.1 and 1.5 h later were transfected with pΔA49. Following a single site-specific homologous recombination event, GFP-positive intermediate viruses were generated. Subsequently, these resolved via further single homologous recombination to form WRΔB14ΔA49, lacking A49R.
Figure 4.3 GFP-positive recombinant WR plaque.
RK-13 cells were infected for 1.5 h and the inoculum was replaced with solid overlay and viewed by fluorescence microscopy 48 h later. The image shows an intermediate plaque expressing GFP due to the integration of pΔA49 into the vΔB14 genome.

Figure 4.4 Genotypic analysis of A49R locus of WRΔB14ΔA49 by PCR.
A PCR product of approximately 1.2 kb was produced from WR, vΔB14, or pA49 template DNA with primers annealing to the flanking regions (A and B). A PCR product of 0.7 kb was produced with the same primers when the WRΔB14ΔA49 virus or pΔA49 DNA was used as template. These products correspond to AB and A49R, surrounded by A and B, respectively. dsDNA size markers were run in parallel.
4.3 Generation of WRΔB14+A49

Following the construction of the WRΔB14ΔA49 virus, a revertant WRΔB14+A49 was generated to serve as a control for non-intentional changes to the genome during virus construction. A pCI-based pA49 plasmid was engineered to reinsert the \(A49R\) gene. The plasmid contained \(A49R\) and flanking sequences A and B to enable reintegration of \(A49R\) at the original site, and also expressed Ecgpt (Boyle & Coupar, 1988) fused to GFP from a constitutive VACV promoter to enable selection (section 2.11.2). RK-13 cells were infected with WRΔB14ΔA49 and then transfected with pA49. A single site-specific recombination event between either A or B within pA49 and its counterpart within the WRΔB14ΔA49 genome resulted in the formation of unstable intermediate viruses which had incorporated pA49 (Fig. 4.5). These intermediate viruses were selected for in the presence of MPA and then grown without MPA so that the intermediate virus resolved by recombination to obtain WRΔB14+A49 respectively (Fig. 4.5).

The recombinant virus generated was analysed by PCR (Fig. 4.6) to ensure that the reinsertion of \(A49R\) was achieved. The PCR product arising from WRΔB14+A49, with primers annealing to the edges of the \(A49R\) flanks, was approximately 1.2 kb as expected. The PCR product obtained with the same primers using the WRΔB14ΔA49 virus as the template was approximately 0.7 kb. This was as predicted for a virus where only the 350-bp A and B flanking regions of \(A49R\) remain. The genotype of the revertant was therefore equivalent to that of vΔB14. WRΔB14+A49 was amplified and purified by sucrose cushion and gradient purification.
RK-13 cells were infected with WR∆B14∆A49 at an MOI of 0.1 and 1.5 h later were transfected with pA49. Following a single site-specific homologous recombination event, GFP-positive intermediate viruses were generated which then resolved via further single homologous recombination to the deletion allele present in WR∆B14+A49.

Figure 4.5 Construction of WR∆B14+A49.
4.4 Characterisation of WR viruses with, and without, B14 and A49

4.4.1 Expression of B14 and A49 proteins

The expression of B14 and A49 was examined as an initial step in the characterisation of the recombinant viruses. RK-13 cells were infected overnight with WRΔB14ΔA49, WRΔB14+A49, vΔB14 or wild type WR. Cell lysates were prepared and analysed by SDS-PAGE and immunoblotting with the anti-B14 polyclonal Ab (Fig 4.7). Membranes were also probed with an anti-D8 mAb to determine that infection had been equivalent between viruses, and an anti-tubulin mAb to serve as a loading control. Immunoblotting for B14 indicated that B14 was not present in WRΔB14ΔA49, WRΔB14+A49 or vΔB14 but was expressed, as expected, in wild type WR. The viral lysates were also analysed for A49 expression via SDS-PAGE and immunoblotting with an anti-A49 polyclonal Ab. This confirmed that A49 was expressed in WRΔB14+A49, vΔB14 and wild type WR but not WRΔB14ΔA49. In
summary, both the removal of \(A49R\) from the \(\text{WR}\Delta B14\Delta A49\) virus and its reinsertion into the \(\text{WR}\Delta B14+A49\) virus were successful.

**Figure 4.7 Expression of B14 and A49 proteins.**

CEF, were infected at an MOI of 10 with WR, v\(\Delta B14\), WR\(\Delta B14\Lambda A49\) and WR\(\Delta B14\Lambda A49\) overnight. Cells were lysed and the lysates were analysed by SDS-PAGE and immunoblotting with \(\alpha\)B14, \(\alpha\)A49, \(\alpha\)D8 and \(\alpha\)tubulin Abs as indicated. Molecular size markers were run in parallel.

### 4.4.2 Genomic digest analysis of WR, v\(\Delta B14\), and WR\(\Delta B14\Lambda A49\)

The virus WR\(\Delta B14\Lambda A49\) genome was analysed and compared to parental virus v\(\Delta B14\) to ensure no substantial unintended changes occurred during virus construction. Purified virus DNA was digested by \(\text{HindIII}\) and analysed by agarose gel electrophoresis (Fig. 4.8). \(\text{HindIII}\) cuts at 15 sites within the WR genome, the distinctive 16 band pattern appeared unaltered in the wild type WR, parental v\(\Delta B14\) and double deletion WR\(\Delta B14\Lambda A49\) viruses showing the overall genomic structure is intact. The 450-bp \(B14R\) gene is located in the \(\text{HindIII} B\) fragment and the 489-bp \(A49R\) in the \(\text{HindIII} A\) fragment which are 31178 and 53632 bp long respectively. Consequently, the deletion of either gene or both would not produce a visible difference in the \(\text{HindIII}\) digest pattern. However, given the PCR analysis of the \(A49R\) locus confirming the absence of this gene in WR\(\Delta B14\Lambda A49\), and the immunoblot showing A49 protein expression only from WR\(\Delta B14\Lambda A49\) not WR\(\Delta B14\Lambda A49\), the integrity of the \(A49R\) locus was not assessed further. This assessment of the WR\(\Delta B14\Lambda A49\) genome rendered this virus suitable for use as a parent virus to created viruses lacking additional NF-\(\kappa\)B immunomodulators.
Figure 4.8 *HindIII* digest of WR, vΔB14 and WRΔB14ΔA49 genomic DNA. Approximately 1 µg of genomic DNA from the indicated viruses was digested with *HindIII* for 6 h and run on a 1 % pulse-field agarose gel for 10 h. The positions of the WR genome *HindIII* digestion fragments A-Q are indicated on the right. dsDNA size markers were run in parallel.
4.4.3 Single-step growth kinetics

The growth of the set of recombinant viruses was measured following infection of BS-C-1 cells at 10 PFU/cell. This included the A49 deletion virus (vΔA49) and its revertant (vΔA49Rev) (constructed in our laboratory by Dr D.S. Mansur). No difference was seen in growth kinetics in previous studies with vΔB14 or its revertant (Chen et al., 2006), however, the A49 deletion virus was as yet uncharacterised and it was possible that some greater effect could be produced in the WRΔB14ΔA49 virus. Cell-associated virus present at 0, 12 and 24 h p.i. was titrated by plaque assay. No significant difference was seen in titre at any time point for the WR, vΔB14, WRΔB14ΔA49, WRΔB14+A49, vΔA49, or vΔA49Rev recombinant viruses (Fig. 4.9). Therefore, the deletion of B14R and A49R, individually or in concert, does not affect the replication of the virus under the conditions tested. This is consistent with previous work with vΔB14 and the newly-characterised *in vitro* role for A49 as an immunomodulatory protein.

![Figure 4.9](image.png)

**Figure 4.9 Single-step growth kinetics of recombinant WR viruses.**
BS-C-1 cells in T25 flasks were infected at an MOI of 10 for 1.5 h. The inoculum was removed, cells were washed twice with PBS and 2.5 % FBS DMEM was added. Cell-associated viruses were then harvested immediately (0 h), or 12 h and 24 h later. The viruses were then titrated in duplicate on confluent 6-well plates of BS-C-1 cells. The experiment was carried out in duplicate; error bars represent standard deviation from the mean.
4.4.4 Multi-step growth kinetics

Differences in growth kinetics, due to changes in viral spread or subtle differences in viral replication, can be detected more easily during an infection effected over a longer time course, wherein the cells are infected at a low MOI. Therefore, multi-step kinetics analysis was undertaken to confirm whether the expression of B14 or A49 had an effect on virus spread or replication. No difference was seen in the multi-step growth kinetics in previous studies with vΔB14 or its revertant (Chen et al., 2006). Cells were infected at an MOI of 0.01 and the cell-associated virus present at 0, 24, 48 and 72 h was titrated by plaque assay. No major difference was seen in titre at any time point for the WR, vΔB14, WRΔB14ΔA49, WRΔB14+A49, vΔA49, or vΔA49Rev recombinant viruses (Fig. 4.10).

Figure 4.10 Multi-step growth kinetics of recombinant WR viruses.
BS-C-1 cells in T25 flasks were infected at an MOI of 0.01 for 1.5 h. The inoculum was removed, cells were washed twice with PBS and 2.5 % FBS DMEM was added. Cell-associated viruses were harvested immediately (0 h), or 24 h, 48 h and 72 h later. The viruses were then titrated in duplicate on confluent 6-well plates of BS-C-1s. The experiment was carried out in duplicate; error bars represent standard deviation from the mean.
4.5 Summary

WR\(\Delta B14\Delta A49\) was constructed from v\(\Delta B14\), and subsequently, the revertant WR\(\Delta B14+A49\) was generated. PCR analysis showed the \(A49R\) gene was deleted from WR\(\Delta B14\Delta A49\) and reinserted into WR\(\Delta B14+A49\), while \(B14R\) remained absent from both. SDS-PAGE and immunoblotting showed WR\(\Delta B14\Delta A49\) does not express A49 or B14 whereas WR\(\Delta B14+A49\) expressed A49. \(HindIII\) digestion of genomic DNA of WR\(\Delta B14\Delta A49\) verified that the virus had not undergone any unintended large-scale alterations. Therefore, the WR\(\Delta B14\Delta A49\) virus is a suitable parent virus from which to remove further immunomodulatory genes. Initial characterisation showed no role for A49 alone, or in combination with B14, in either viral replication or spread. The effect of the deletion of both \(A49R\) and \(B14R\) on immune evasion and, in particular, NF-\(\kappa B\) modulation remains to be characterised.
Chapter 5: Further analysis of B14 function and interaction with IKKβ

5.1  B14 interacts with IKKβ

In the unstimulated cell NF-κB family hetero- and homodimers are sequestered with IkBα in the cytoplasm. On stimulation, IkBα is phosphorylated and thereafter ubiquitinated and degraded by the proteosome (Pasparakis et al., 2002). The NF-κB dimers then translocate to the nucleus to bind response elements in the promoters of the many NF-κB target genes and induce transcription. NF-κB activation is mediated by signalling cascades which converge on the IKK complex, which includes IKKα, IKKβ and IKKγ (also known as NEMO). The first two subunits have catalytic functions while IKKγ plays a regulatory role. The active form of the IKK complex phosphorylates two serine residues (S176/180) close to the amino terminus of IkBα, targeting it for degradation (Shuai, 2006).

VACV protein B14 inhibits NF-κB activation via its interaction with the IKK complex. Transient expression of B14 in transfected cells resulted in diminished NF-κB activation following treatment with TNFα, IL-1β, poly (I.C) and PMA (Chen et al., 2008). Assessing the level of NF-κB activation in virus-infected cells is complicated by the multitude of viral factors that are involved. However, it was shown that increased levels of phosphorylated IkBα were present in cells infected with VACV lacking B14R (vΔB14) than with wild type virus, implying B14 inhibits IkBα phosphorylation by the IKK complex. Consistent with this, B14 co-purified and co-precipitated with mouse and human endogenous IKK complex. Knockout mouse cell lines were used to show that the interaction requires IKKβ but not IKKα. Furthermore, B14 inhibited a constitutively active IKKα mutant (S176/180E) but not the comparable IKKβ mutant. Thus it was postulated that B14 interacts with IKKβ to prevent phosphorylation of these serines located in the IKKβ activation loop and this was confirmed using mAbs to phospho-IKKβ.
5.2 Identification of regions of IKKβ responsible for B14 interaction

A better understanding of the B14-IKKβ interaction might indicate how the activity of the IKK complex could be regulated and thus inhibit NF-κB activation during inflammation. In particular, which part of the B14 structure interacts with IKKβ and vice versa required further study. Within the IKK complex, IKKβ is found either as a homodimer or as a heterodimer with IKKα, either of these combinations forming part of the complex’s larger oligomeric structure. The IKKβ molecule itself is divided into five reasonably well-defined predicted structural domains (Delhase et al., 1999; May et al., 2004) (Fig. 5.1), although to date no structure of the molecule has been resolved. The N-terminal 300 amino acids form the kinase domain. Following this, there is a small ubiquitin-like domain from residues 307 to 384 and a leucine zipper motif at residues 458 to 480. Then there is a helix-loop-helix region from residues 603 to 642. Recently, it was reported that disruption of this region can result in a loss of kinase specificity rendering it more broadly active (Shaul et al., 2008). Finally, at the extreme C-terminus there is a small domain involved in the interaction with NEMO, called the NEMO-binding domain (NBD). To date, out of all the IKK complex components, only a small section of NEMO has yielded a structure (Bagneris et al., 2008), and this is complexed to a viral protein. In order to delineate the B14-IKKβ interaction, a series of IKKβ truncations were designed, bearing the previously described domains in mind, to test which parts of IKKβ are needed for the interaction with B14. Ultimately, it is hoped that the identification of a smaller section of IKKβ essential to B14 interaction would enable expression of this complex in E. coli and subsequently determination of the crystal.
Figure 5.1 Structural arrangement of IKKβ truncations.
IKKβ is composed of a kinase domain, ubiquitin-like domain (Ub), leucine zipper (LZ), helix-loop-helix (HLH), and a NBD. The full-length molecule (1-756), and the truncations 1-737, 230-756, 300-756, 480-756 are illustrated.
5.2.1 IKKβ mutants

The IKKβ mutants generated are illustrated in Fig. 5.1. All the mutants were cloned first into the pCI vector fused to an N-terminal FLAG tag. IKKβΔ300 was also cloned into the pOPIN expression system vector E. This vector allows transient expression in prokaryotic systems with a C-terminal His tag (Berrow et al., 2007). The starting point for designing the truncations was the mutant lacking the kinase domain (pβΔ300) because this mutant was still able to form the IKK complex (Delhase et al., 1999). Also, kinase activity can be toxic in E. coli and so any co-purification strategy for the two proteins might need to either lack the kinase domain or have its activity inhibited. In addition, a truncation was generated starting halfway through the kinase domain (pβΔ230) to establish if the kinase region was essential for interaction, and if so which part was important. In addition, a truncation was cloned that lacked the C-terminal and NBD (pβ736Δ), and another that lacked the LZ motif and everything C-terminal of this point (pβΔ480).

5.2.2 Co-immunoprecipitation of B14 and IKKβ truncations

The initial characterisation of the IKKβ mutants was carried out via transient expression of the FLAG-tagged proteins in cells infected subsequently with a WR virus expressing an HA-tagged B14 (vB14-HA) (Chen et al., 2006). Under these conditions it was investigated if the truncated IKKβ molecules would co-immunoprecipitate with B14 as does the full length IKKβ. HEK 293T cells were transfected with pβf, pβΔ300f, pβΔ230f, pβΔ480f, or pβ736Δf using FuGene as described in section 2.3.2. Following an overnight incubation, cells were infected with vB14-HA for 6 h and then lysed, pre-cleared and immunoprecipitated with the anti-FLAG mAb to bind FLAG-tagged IKKβ mutants and any proteins in complex with them (section 2.10.1). Samples were then analysed by SDS-PAGE and immunoblotting and compared to cell lysates prior to immunoprecipitation (Fig. 5.2). The left-hand upper input panel shows that all IKKβ mutants were expressed, albeit at different levels, and the mutants were of the expected sizes 90.7, 63.1, 54.7, 33.1 and 88.4 kDa respectively for pβf, pβΔ300f, pβΔ230f, pβΔ480f, and pβ736Δf. The lower half of the panel illustrates, by use of the anti-HA mAb, that all the virus infections produced HA-tagged B14. The right-hand panel shows the co-immunoprecipitation samples. Here all the IKKβ FLAG-tagged truncations produced bands of the
appropriate size (90.7, 63.1, 54.7, 33.1 and 88.4 kDa as indicated in Fig. 5.2) as the co-immunoprecipitation Ab was anti-FLAG. However, not all the IKKβ truncations pulled down B14 as indicated by the anti-HA immunoblot in the lower half of the left-hand panel. The pβΔ480f truncation mutant did not pull down B14 and thus suggests that the region between residues 1 and 480 is necessary for the IKKβ-B14 interaction. Conversely, the pβΔ300f, pβΔ230, and pβ737Δf truncation mutants all interacted to pull down B14 and as such neither the kinase domain (up to residue 300) nor the NBD (residues from 737 onwards) are required for the interaction. In conclusion, this co-immunoprecipitation data led to a putative B14-interacting region of 300-480 within the IKKβ molecule. This work was carried out in conjunction with Dr R. A-J. Chen.

As stated above, an overriding aim of this work was to obtain soluble B14 in complex with some part of IKKβ to attempt co-purification and crystallisation. Therefore, following these results, the pβΔ300f allele was recloned into the pOPIN E vector described above to form IKKβΔ300His, so that recombinant protein could be produced for structural analysis.
Figure 5.2 Co-immunoprecipitation of IKKβ truncations and vB14-HA. (i) HEK 293T cells were transfected with 5 µg of pβf, pβ∆230, pβ300, pβ∆480 or pβ737∆f and 16 h later were infected with vB14-HA at an MOI of 10. Cells were lysed 6 h later with co-immunoprecipitation buffer (section 2.10), and incubated with anti-FLAG mAb (1/200 dilution) overnight. Protein G sepharose beads were added to separate the mAb-FLAG tag protein complexes from the unbound proteins. Proteins bound to the beads were eluted by the addition of SLB and were analysed by SDS-PAGE and immunoblotting, alongside aliquots of the cell lysate prior to immunoprecipitation. Molecular mass markers were run as indicated. (ii) IKKβ is composed of a kinase domain, Ub, LZ, HLH, and a NBD. The black box indicates the putative B14 target region of IKKβ.
5.2.3 Co-expression of recombinant B14 and IKKβΔ300

To obtain the His-tagged IKKβΔ300 (IKKβΔ300His) truncation in complex with His-tagged recombinant B14 (B14His) the two proteins were co-expressed in the same *E. coli* culture using the pOPIN E-based pβΔ300His and pET28a-based pB14His plasmids (Table 2.2). The pOPIN expression system was advantageous because the backbone vector pOPIN E contains a gene conferring resistance to Amp as a selection marker whereas the pET28a-based vector, used previously to produce soluble B14 for purification and crystallisation, relies on Kan resistance. Therefore, the two plasmids were transformed into BL21 chemically competent *E. coli* and using both antibiotics only bacterial cells that had taken up both plasmids could grow. One of the resulting colonies was picked and amplified in LB, first in a 3 ml culture and then in a set of 50 ml cultures which were used to assess a variety of induction conditions. The cultures were induced when their OD was between 0.4 and 0.8. The growth temperature was either 18, 30 or 37 °C and the concentration of IPTG used to induce expression was either 1 or 5 mM. After 24 h of growth the cultures were harvested by centrifugation and the pellets weighed, and lysed on ice with 2 ml/g of lysis buffer (section 2.4), homogenised and separated into soluble and insoluble fractions. The pellet was resuspended in lysis buffer to a volume equivalent to that of the soluble supernatant fraction. Both sets of samples were analysed by SDS-PAGE and coomassie blue staining (Fig. 5.3). At all conditions surveyed the overall expression and solubility of both proteins was good and did not vary substantially.

This finding was encouraging and consequently a larger scale culture (containing both antibiotics) sufficient to produce enough protein for purification via IMAC was induced. However, no peak was seen following the IMAC purification and when a small sample of the starter culture was assessed it did not, in fact, contain truncated soluble IKKβΔ300His, explaining the lack of a peak following the affinity chromatography. Despite re-transforming the bacteria and reattempting protein expression induction, there was some block to the scaling up of the original small-scale induction.

Following discussion of this problem it was hypothesised that the difference in scale of culture could also have resulted in some difference in the level of aeration of the cultures. The bacterial pellets of the initial expression trials with optimal solubility
had a pinkish appearance and this may have been due to lower oxygen levels in the sealed 250 ml orange-capped centrifuge tubes used as opposed to the 2 L conical flask sealed with a sponge used in the large-scale culture.

Figure 5.3 IKKβΔ300His and B14His co-expression trials in E. coli.
Chemically competent BL21 E. coli were transformed with approximately 1 µg of both pβΔ300His and pB14His and plated onto Kan- and Amp-containing agar. A colony was harvested and amplified in LB with both antibiotics. IPTG at either 5 or 1 mM as indicated was added to induce protein expression during log phase growth at 37, 30, or 18 °C. The cultures were harvested and lysed after 24 h and soluble and insoluble fractions were analysed by SDS-PAGE and coomassie staining. Bands at approximately 20 kDa and 55 kDa correspond to B14His and IKKβΔ300His respectively. These are boxed in white for the soluble fraction of the culture induced with 5 mM IPTG and grown at 37 °C. Molecular mass markers were run as indicated.

Consequently, low oxygen conditions were established in a larger culture by doubling the volume of culture in the 2 L flask to 1 L and reducing shaking to a minimum. Expression was induced over 2 days at room temperature and this was trialled in both BL21 (chemically competent) and B834 (electrocompetent) E. coli. Fifty ml samples were taken prior to harvesting by centrifugation to analyse for solubility before attempting the large scale lysis and IMAC affinity purification step. The 50 ml samples were lysed and analysed as described above, and for both types of bacteria large amounts of soluble B14 were visible on the coomassie-stained gel and smaller amounts of the truncated IKKβ (Fig. 5.4).
Figure 5.4 Expression analysis of low oxygen large-scale culture induction of IKKβΔ300His and B14His.
Chemically competent BL21 and electrocompetent B834 E. coli were transformed with approximately 1 µg of both pβΔ300His and pB14His and plated onto Kan- and Amp-containing agar. Colonies were harvested and amplified in 1 L of LB with both antibiotics. Protein expression was induced by IPTG (1 mM) during log phase growth at 37 °C. After 24 h a 50 ml aliquot was harvested and lysed, while the remaining culture was harvested and the pellet was stored at -20 °C. The soluble and insoluble fractions of the 50 ml aliquot were analysed by SDS-PAGE and coomassie staining. The bands boxed in white at approximately 20 kDa and 55 kDa correspond to B14His and IKKβΔ300His respectively. Molecular mass markers were run as indicated.

Prior to re-attempting protein purification by IMAC it was necessary to determine whether or not the His tags on both proteins were capable of interacting with the affinity resin within the column. It is feasible that the interaction between the two proteins was shielding one or both His tags. To check this was not the case and that both His-tagged proteins were able to bind to the column resin, an aliquot of the soluble fraction was incubated with Ni²⁺ beads (section 2.4.1). Following this, proteins bound to the beads were eluted by the addition of SLB and analysed by SDS-PAGE, along with soluble aliquots not incubated with Ni²⁺ beads and insoluble fractions. This was undertaken for proteins expressed in both BL21 and B834 E. coli. The gel was again stained by coomassie blue (Fig. 5.5) and in both the soluble and insoluble fractions, for both bacteria, there was some soluble truncated IKKβ and B14, although they were accompanied by other contaminating proteins so they did not stand out. However, following the Ni²⁺ bead incubation the two target proteins were selected by the affinity step and consequently are more prominent on the gel. In
summary, both His-tagged proteins are soluble to a degree under these expression conditions and are capable of binding to Ni$^{2+}$ beads. Therefore, they should interact with the IMAC column resin either independently or possibly when bound together to form a complex.

**Figure 5.5 Small-scale purification of IKKβ300His and B14His via Ni$^{2+}$ bead incubation.**

Chemically competent BL21 and electrocompetent B834 *E. coli* were transformed with approximately 1 µg of both pβΔ300His and pB14His and plated onto Kan- and Amp-containing agar. Colonies were harvested and amplified in 1 L of LB with both antibiotics. Protein expression was induced by IPTG (1 mM) during log phase growth at 37 °C. After 24 h a 50 ml aliquot was harvested and lysed, while the remaining culture was harvested and the pellet was stored at -20 °C. The soluble fraction was incubated with pre-equilibrated Ni$^{2+}$ beads for 30 min, the unbound proteins were retained and the bound proteins were eluted by addition of SLB. These fractions, along with soluble aliquots not incubated with Ni$^{2+}$ beads and insoluble fractions, were analysed by SDS-PAGE and coomassie staining. The bands boxed in white at approximately 20 kDa and 55 kDa correspond to B14His and IKKβ300His eluted from the Ni$^{2+}$ beads respectively. Molecular mass markers were run as indicated.
5.2.4 Affinity chromatography and gel filtration

The truncated IKKβ complexed to B14 was purified by an IMAC column. The pellet, resulting from the large-scale induction in B834 cells expressing IKKβΔ300His and B14His, was lysed and the soluble fraction was loaded onto the IMAC column following equilibration, as recommended by the manufacturer. Unfortunately, no protein peaks were seen in the elution profile. As the Ni$^{2+}$ bead incubation had already shown both truncated IKKβ and B14 were capable of binding via their His tags it was deemed worthwhile to modulate some of the conditions of the affinity step to try and procure a peak. Initially, all the conditions were as used for the B14 purification procedure, therefore, it was not that surprising that some modifications would be required when co-purifying B14 in complex with another protein. The first modification was to lower the NaCl concentration in the buffer from 500 to 100 mM. These conditions were used with the soluble fraction from the large-scale induction of BL21 cells, which had also been checked by the Ni$^{2+}$ bead incubation method. This time, the elution profile from the IMAC column revealed two prominent peaks (Fig. 5.6). Aliquots of fractions corresponding to these peaks were taken and denatured with SLB by boiling and then analysed by SDS-PAGE. The coomassie staining revealed the presence of both purified B14His and truncated IKKβΔ300His, although the latter was at a much lower level (Fig. 5.6). The fractions corresponding to the proteins of interest were pooled and run through a gel filtration HiLoad 16/60 Superdex 200 column as a further purification step. The elution profile (Fig. 5.7) again showed two defined peaks, and when a proportion of the relevant fractions were analysed by SDS-PAGE and coomassie staining (Fig. 5.7) a small amount of truncated IKKβ and more B14 were shown to have been purified by this method.
Figure 5.6 IMAC elution profile for soluble IKKβΔ300His and B14His.
(i) The bacterial pellet produced from the large-scale low oxygen BL21 culture was lysed as described in section 2.4 and the soluble fraction was run through a 5 ml IMAC column and eluted with an imidazole gradient. Fractions 164 to 180 on the elution profile were collected. Small aliquots of some of these fractions were added to SLB and analysed by SDS-PAGE and (ii) coomassie staining and (iii) anti-His immunoblotting. A large amount of protein was visible at 20 kDa corresponding to B14His and at 55 kDa corresponding to IKKβΔ300His. Molecular mass markers were run as indicated.
Figure 5.7 Gel filtration elution profile for IMAC purified soluble IKKβΔ300His and B14His.

The peak fractions collected and pooled from the IMAC column elution were run through a HiLoad 16/60 Superdex 200 column. The fractions mapping to the second peak were collected and small aliquots were added to SLB and analysed by SDS-PAGE. Coomassie staining revealed a large amount of protein at 20 kDa corresponding to B14His in almost all fractions but a much smaller amount at 55 kDa corresponding to IKKβΔ300His only in fractions 2B4 and 2B1 (boxed in blue). Molecular mass markers were run as indicated.
5.3 Identification of B14 residues essential to the IKKβ interaction

5.3.1 B14 dimer interface mutants

A feature of the VACV Bcl-2-like proteins N1, A52, and B14 is that they formed dimers during structural studies. For N1 this was also demonstrated biochemically in infected cells (Bartlett et al., 2002). However, the B14 dimers are not thought to form when the protein is expressed in eukaryotic cells. Multi-angle light scattering experiments (Stephen Graham, personal communication) indicated that B14, while capable of forming a homodimer, exists in monomer-dimer equilibrium and, as such, unlike A52 and N1 is not an obligate dimer. Instead it has been postulated that the dimer interaction surface is a protein binding interface, which may be involved in the interaction with cellular targets via a more stable interaction with B14 than that seen in the B14 dimer. To investigate if the dimer interface was involved in the interaction of IKKβ with B14 (in addition to studying which part of IKKβ is involved) the role of specific B14 residues that make up this surface was evaluated. The residues predicted to form part of this interface include F130, T31, L126 and Y35 (Fig. 5.8). A series of five mutants were generated from pB14f by Stephen Graham and Asa Oldring (University of Oxford).

These mutants were F130K, T31K, Y35K, Y35E and L26E, thus the first three have been changed from either a hydrophilic threonine or a large aromatic phenylalanine or tryptophan to a positively-charged lysine. The remaining two have been altered from either an aromatic tryptophan or hydrophobic leucine to the negatively-charged glutamic acid. Initial transfection experiments were carried out with the plasmids encoding the B14 mutants along with an NF-κB-luciferase reporter plasmid by Dr D. S. Mansur in our laboratory. Unlike the wild type B14, the F130K and T31K mutants were not able to abrogate NF-κB activation in response to stimulation with TNFα. The other mutants, however, showed an inhibitory capacity equivalent to that of the wild type (data not shown). To assess whether these mutants, in particular F130K and T31K, are capable of interacting with IKKβ to the same degree as the wild type B14, this interaction with IKKβ was investigated via LUMIER and co-immunoprecipitation assays. A loss of IKKβ interaction ability by these B14 mutants could explain why they failed to down regulate NF-κB activation.
Figure 5.8 B14 dimer interface mutants.
(i) The (non-physiological) B14 homodimer seen in X-ray crystallography studies (Graham et al. 2008). Both B14 chains are rainbow coloured from blue (N-terminus; residue 8) to red (C-terminus; residue 149). (ii) Dimer interface view: Residues T31 and F130 are coloured red and residues Y35 and L126 are coloured green.
5.3.2 Co-immunoprecipitation of B14 dimer mutants and IKKβ

To ascertain whether the B14 mutants, including those which do not inhibit NF-κB activation, are able to bind IKKβ, co-immunoprecipitation of the two molecules was attempted. HEK 293T cells were transfected with plasmids expressing either a mutant or wild type FLAG-tagged B14 and concurrently with HA-tagged IKKβ. After 24 h cells were harvested and lysed (section 2.10) before pre-clearing with protein G beads to remove non-specific binding. A small aliquot was retained at this stage as input. The lysates were then mixed with the anti-HA mAb and subsequently protein G beads were added. Proteins complexed to the beads via a HA tag (either direct or by proxy) were then eluted from the beads by the addition of SLB. These co-immunoprecipitation samples were then analysed by SDS-PAGE and immunoblotting with the anti-FLAG and anti-HA mAbs (Fig. 5.9). The anti-tubulin blot shows a band for all the lysates indicating that a comparable amount of cell lysate was loaded on the gel. The anti-HA blot reveals a band at just under 100 kDa corresponding to IKKβHA for all the lysates showing that expression from pβHA following transfection was successful and equivalent in all cases. Similarly, the anti-FLAG blot shows bands corresponding to FLAG-tagged B14 at approximately 20 kDa for all the lysates, confirming effective expression of all alleles. The co-immunoprecipitation samples all produced a band corresponding to IKKβHA demonstrating that HA-tagged proteins bound effectively to the protein G sepharose-αHA mAb complex. All the co-immunoprecipitation samples apart from that containing the FLAG-tagged F130K B14 mutant produced a band equivalent to FLAG-tagged B14 at approximately 20 kDa due to the interaction of the B14 proteins with HA-tagged IKKβ, which in turn bound to the anti-HA mAb-protein G sepharose beads. Therefore, under the conditions tested, all the B14 mutants are capable of interacting with HA-tagged IKKβ apart from the F130K B14 mutant.
Figure 5.9 Co-immunoprecipitation of IKKβ with B14 mutants.
HEK 293T cells were transfected with 10 µg of pIKKβHA and either pB14f, pF130Kf, pT31Kf, pY35E, Y35K, L126E as indicated. Cells were lysed 24 h later with (section 2.10), pre-cleared with protein G sepharose beads and incubated with αHA mAb (1/200 dilution) overnight. Fresh protein G sepharose beads were added to separate the αHA tag protein complexes from the unbound proteins. Proteins bound to the beads were eluted by the addition of SLB and analysed by SDS-PAGE alongside aliquots of the lysates. The co-immunoprecipitation sample membrane was immunoblotted with αHA and αFLAG mAbs, while the lysate sample membrane was immunoblotted with αtubulin, αHA and αFLAG mAbs. Molecular mass markers were run as indicated.
5.3.3 LUMIER assay of B14 dimer mutants interacting with IKKβ

In addition to the qualitative data obtained via the co-immunoprecipitation method the interaction of the mutant B14 proteins with IKKβ was assessed via a LUMIER assay (Barrios-Rodiles et al., 2005). An IKKβ-Luc allele was used in which the IKKβ protein is fused to Renilla-luciferase (Chen et al., 2008). HEK 293T cells were transfected with this IKKβ-Luc plasmid (pβLuc), and in parallel with one of the B14 mutant or wild type plasmids. In addition, another sample was transfected in parallel with IKKβ-Luc and a plasmid encoding another FLAG-tagged VACV Bcl-2-like protein, N1, to serve as a negative control as there is no interaction between these two proteins (Cooray et al., 2007; Chen et al., 2008). After 24 h the cells were harvested (section 2.9) and the lysate divided into three aliquots. One aliquot was used to assess protein expression by SDS-PAGE and immunoblotting. A second aliquot was analysed for Renilla-luciferase expression to provide a baseline level value, prior to the immunoprecipitation step, whereby the ability of the B14 mutants and IKKβ-Luc to interact was proportional to the increase in Renilla-luciferase expression relative to baseline. The third aliquot was incubated with pre-equilibrated anti-FLAG M2-agarose beads and then any proteins attached to the FLAG beads via a tag were eluted using FLAG peptide. This therefore, would include IKKβ-Luc if it were complexed with either B14 or any of the B14 mutants. Semi-quantitative assessment of how much IKKβ-Luc was bound to any of the FLAG-tagged proteins was assessed by analysing the eluate for Renilla-luciferase expression. The fold binding was thus represented by the ratio of eluate Renilla-luciferase reading to the baseline reading (Fig. 5.10). As expected the FLAG-tagged N1 negative control sample did not elute any IKKβ-Luc as shown by the lack of any increase in the fold binding consistent with prior observation (Chen et al., 2008). In contrast, the B14 wild type protein shows approximately a 40-fold binding increase following elution. The F130K and T31K mutants do seem to have abrogated the interaction between B14 and IKKβ-Luc and thus have lower eluate/input Renilla-luciferase ratio. In addition, the other mutants, Y35K, Y35E and L126K also show reduced fold-binding (6-, 4- and 7.5-fold respectively) to IKKβ-Luc relative to wild type B14. However, the fold-binding for all of the mutants to IKKβ-Luc is higher than that of the negative control N1. In summary, the F130K mutant is not able to inhibit TNFα-induced NF-κB activation nor does it co-purify with IKKβ nor bind comparably to IKKβ-Luc. However, another mutant, which is unable to
inhibit TNFα-induced NF-κB activation, T31K, does co-purify with IKKβ. Although, the binding of this mutant to IKKβ-Luc is reduced compared to the wild type indicating the interaction is not equivalent and this could explain the lack of effect on NF-κB activation. The other three mutants, Y35E, Y35K and L126K, all inhibit NF-κB activation and co-purify with IKKβ. However, as for T31K, the interaction is altered relative to that with wild type B14 as indicated by a reduction in binding to IKKβ-Luc. This indicates that these three residues may be desirable but not essential for B14-IKKβ binding and functional downregulation.

5.4 Conclusions

The interaction between IKKβ and B14 has been further characterised. The region of IKKβ from amino acid 300 to 480 is essential for the interaction. Furthermore, a truncation of IKKβ, including this region, has been shown to co-purify in a soluble form with B14 and thus could, with further study and optimisation, yield adequate quality preparations for structural studies. In addition, it has been shown that mutating either residue F130 or T31 of B14 can diminish the binding affinity of the two proteins to prevent B14 from eliciting its down-regulatory effect on NF-κB activation in vitro. However, how this translates to B14 function as viral virulence factor in vivo has yet to be investigated.
Figure 5.10 LUMIER assay: Semi-quantitative comparison of IKKβ-Luc binding to B14 and single residue B14 mutants.

(i) HEK 293T cells were transfected with 1.5 µg of pβLuc and 1.5 µg of pB14f, pF130Kf, pT31K, pY35E, Y35K, L126E or the negative control pN1f. After 24 h the cells were lysed (see section 2.9) and Renilla-luciferase expression was assessed by luminometer. The lysates were incubated with pre-equilibrated FLAG M2 agarose for 2 h at 4 °C and then FLAG-bound proteins were eluted with FLAG peptide (150 ng/ml) for 0.5 h at 4 °C. The Renilla-luciferase expression of the eluted proteins was assessed by luminometer and the eluate-lysate ratio calculated as an indicator of fold binding.

(ii) Small aliquots of each lysate were analysed by SDS-PAGE and immunoblotting with αIKKβ, αtubulin or αFLAG mAbs. Molecular mass markers were run as indicated.
Chapter 6: BAC VACV system: Construction of further MVA

6.1 BAC VACV system

In chapter 2, it was noted that constructing recombinant MVA can be more challenging than constructing recombinant WR or other less attenuated strains of VACV. Predominantly this is due to the lower number of cell lines that MVA can replicate in and the difference in plaque morphology in monolayers of these cells following MVA infection, as compared to the prominent plaques observed following VACV WR infection (chapter 4). The plaque phenotype of MVA on CEF or BHK-21 cells is such that foci of infected cells are formed instead of the distinctive round plaque seen with WR on other cell types. This makes infected cells more difficult to distinguish from uninfected cells, and a method described in this chapter to overcome this is to express GFP from the virus to facilitate focus picking. However, the foci observed using this method are still difficult to identify compared to VACV WR plaques (see chapter 4). As such, any abnormally large foci have a selective advantage in the focus picking process which may explain why the MVAΔ183 virus generated had undergone a terminal genomic translocation which was associated with a larger focus size phenotype.

In addition to the practical challenges of making a recombinant MVA it is important to consider that the desired end point is to create an MVA that is a more immunogenic. Several MVA deletion mutants lacking individual genes have been created, but few have shown an increase in immunogenicity, but it is possible that deletion of multiple immunomodulators may have a more marked effect, particularly if the deleted genes express proteins that target the same component of innate immunity. Given the problems outlined above regarding generating MVA mutants a new method has been adapted from use with viruses with a circular genome stage during their replication to manipulate the VACV genome (Domi & Moss, 2005; Cottingham et al., 2008). This technology relies on BAC plasmids, which can integrate stably into the virus genome and also be transformed into E. coli following subsequent virus genomic DNA purification. The nature of the BAC VACV system means that multiple changes can be made to the virus genome simultaneously and once the original BAC VACV is generated it eliminates the need for clonal purification by plaque picking.
pBAC-VI is transfected into CEFs that had been infected with MVA. The plasmid recombines with the MVA genome via regions flanking deletion VI, F1 and F2, to integrate into the virus. Virus DNA is then purified from cells infected with this virus and transformed into *E. coli* strain DHB10. The resulting MVA-pBAC-VI plasmid is transfected into BHK-21 cells and infectious progeny produced by infecting these cells with fowlpox virus strain 9 (Fwp9). Fwp9 is non-replicative in BHK-21 cells.

The BAC VACV system works, briefly, by first integrating the BAC plasmid into the VACV genome. Replicative intermediates of viral genomic DNA are then extracted from infected cells and used to transform *E. coli*. This genome can then be checked for fidelity by sequencing and, if needed, modified using recombineering systems (Warming *et al.*, 2005). Finally, the genome is transfected into cells infected with a non-permissive poxvirus (often fowlpox virus) to rescue the virus and produce infectious progeny (Fig. 6.1). This step is necessary due to the reliance of the virus on
its own transcription machinery, meaning that transfection of the genome alone does not produce infectious virus.

6.2 Engineering of pBELO-BAC 11 plasmid

To integrate the BAC into MVA it was necessary to insert this foreign DNA into regions that are non-essential for virus replication. MVA has six well-characterised genomic deletion sites which were produced during its generation from CVA by more than five hundred serial passages in CEFs. All of these sites have deletions of virus DNA without preventing replication in avian cells (Mayr et al., 1978) and, as such, should all be suitable for BAC integration. In addition, for selection of recombinant virus the MVA BAC needs to contain a selection cassette, in this case EcogptGFP under the VACV early/late promoter. The MVA BAC constructed previously by another group lacked the heterologously-expressed antigens HIV clade C antigens inserted at the TK locus and used genomic deletion III for insertion of the BAC plasmid. This site is very close to the MVA counterparts of a number of VACV WR immunomodulatory genes that would be interesting to modify and as such another site was chosen. Genomic deletion VI was selected as a suitable insertion point as it is distal from the immunomodulatory genes identified previously as targets for deletion and has been shown to be a functional site for large inserts (Di Lullo et al., 2009). Deletion VI removes CVA genes 152-155, which are equivalent to the four fragments of the CPXV A-type inclusion gene, and CVA 156L (MVA 137L), which encodes the OPV p4c protein, which is truncated to half its original size in MVA (Antoine et al., 1998) (Fig. 6.2). P4c is a non-essential protein which is involved in the trafficking of viral particles into ATI bodies (McKelvey et al., 2002) and is also truncated in VACV COP. Downstream of the deletion is the intact MVA 135R, which encodes the viral 132-kDa RNA polymerase subunit β chain (Fig. 6.2). Upstream of deletion VI is 138L an intact counterpart of COP of A27L which is involved in the formation of IEV ((Rodriguez & Smith, 1990; Ward, 2005).

To build a plasmid for insertion into deletion VI, 500-bp left and right flanks were designed to be homologous to bp 129186-129686 and 130261-130761 of MVA (Mayr et al., 1978; Antoine et al., 1998) respectively, mapping to the regions either side of deletion VI (Fig. 6.2). In addition, the pBELO-BAC 11 plasmid used contains a Cm
resistance gene to serve as a selection marker in *E. coli* and a loxP site which could allow manipulation of the BAC system via cre recombinase (Domi & Moss, 2005).

**Figure 6.2 Structure of pBELO-11 plasmid and selection cassette.**

pBELO-BAC 11 (NEB) was modified to incorporate EcogptGFP under the control of the VACV early/late (E/L) promoter adjacent to two regions (F1 and F2) of homology to the flanking regions of MVA genomic deletion VI. This plasmid can be linearised using *Pac*I, which digests between F1 and F2 enabling integration by recombination into deletion VI. Upstream of deletion VI is the intact 135*R* which encodes the viral DNA-dependent RNA polymerase 132 kDa β chain. Downstream is the truncated non-functional gene 137*R* encoding a counterpart of the OPV p4c protein.

### 6.2.1 Cloning into pBELO-BAC 11 to form pBAC-VI

A 2383-bp fragment of synthetic DNA was designed and then synthesised by GENEART. This comprised of EcogptGFP under the control of the VACV E/L promoter and two regions designated F1 and F2. These were homologous to 500-bp sections found upstream and downstream of deletion VI in the MVA genome. The 500-bp sections chosen were not contiguous but either side of a 393-spacer region (Fig. 6.2). The sequence was designed so that this segment could be excised using *Sph*I and *Hind*III. Compared to the previous EcogptGFP selection cassette used to construct the viruses (chapters 3 and 4) there were two differences: the *Eco*RI site within GFP was removed by introducing a silent mutation and a *Kpn*I site was removed from the promoter region. This was done to maximise further applications of this selection cassette by widening the cloning options. The plasmid was digested.
using SphI and HindIII to create compatible ends with the EcogptGFP_F2-F1 fragment described above. A clear band of 7507 bp (Fig. 6.3) equivalent to the linearised pBELO-BAC 11 was purified. Simultaneously, the EcogptGFP_F2-F1 fragment was digested with SphI and HindIII and the 2382-bp product (Fig. 6.3) was ligated into pBELO-BAC 11 to form pBAC-VI and transformed into DH5α E. coli. The resulting colonies were screened by mini-prep DNA and digestion with SphI and HindIII (Fig. 6.3) which excised the 2382-bp band inserted.

### 6.2.2 Growth of pBAC-VI and purification

In order for pBAC-VI to recombine and integrate into MVAc it was necessary to first linearise the plasmid by PacI restriction site engineered between the two flanks (Fig. 6.2). Some DNA was lost during the restriction digestion and gel purification steps and so to effectively transfect the linearised plasmid a greater initial maxi prep yield was required. However, BAC plasmids produce lower yields than the high-copy plasmids as they are single-copy. In addition to the overall yield of plasmid DNA being lower, the efficiency of restriction digestion can be reduced. This is due to the contaminating E. coli DNA having many copies of the restriction site for the enzyme in use. A Qiagen maxi prep and a CsCl gradient purification were undertaken. Fig. 6.4 shows the DNA extracted using these methods; the higher band above 10 kb is E. coli DNA, the lower band at 9.5 kb is the pBAC-VI. The CsCl gradient method produced more DNA overall, however, the larger much brighter band corresponds to bacterial DNA and the lower band to pBAC-VI and as such the ratio between the two types of DNA is not greatly altered between the CsCl method and the Qiagen kit method. In addition to these two methods, an altered alkaline lysis mini prep method was also examined (see Chapter 2) and undertaken with multiple samples and the yields were pooled. This pooled preparation was digested with PacI along with the Qiagen kit and CsCl gradient preparations.
Figure 6.3 Construction of pBAC-VI.

(i) The source plasmid supplied by GENEART was digested with SphI and HindIII. This excised a 2382-bp band equivalent to EcogptGFP_F2-F1 selection cassette (boxed in white) (ii) Digestion with SphI and HindIII of pBELO-BAC 11 to a linear fragment of 7507 bp. (iii) Digestion of pBAC-VI with SphI and HindIII produced a 2382-bp band corresponding to the EcogptGFP_F2-F1. dsDNA size mass markers were run as indicated. (iv) Pictorial representation of the directional cloning of EcogptGFP_F2-F1 into pBELO-BAC 11.
Chemically-competent DH5α *E. coli* were transformed with the ligation product pBAC-VI and plated onto Cm-containing agar. A single colony was harvested and amplified in Cm-enriched LB to a final volume of 1000 ml in two 2 L flasks. Following overnight growth, two pellets were obtained by centrifugation. Plasmid was purified from the pellet by either Qiagen maxi prep kit or CsCl gradient maxi prep. Fifty µl of each preparation was run undigested on a 1 % agarose gel (i) Qiagen maxi prep kit purified DNA (ii) CsCl gradient maxi prep purified DNA. Molecular size markers were run as indicated.

**Figure 6.4 Purification of pBAC-VI DNA.**

6.3 Construction of parental BAC VAC virus

6.3.1 Linearisation of pBAC-VI

To generate a recombinant virus containing the linearised pBAC-VI inserted into the deletion VI site, three DNA preparations were used to transfect CEFs using Lipofectamine 2000, which had been infected with MVAc. To establish that the transfection was efficient, and that the pBAC-VI plasmid was capable of producing EcogptGFP, two positive controls were employed. These were the GENEART EcogptGFP_F1F2 fragment (cloned into pUC13) and undigested pooled mini prep DNA of pBAC-VI. The former was used to check that the EcogptGFP was capable of expression, and the latter to assess whether the transfection protocol was efficient for the single-copy pBAC-VI. The pUC13 transfection produced many GFP-positive cells after 48 h as illustrated in Fig. 6.5. Transfection of the mini prep sample of pBAC-VI also resulted in expression of GFP but to a much lower degree (Fig. 6.5). The *PacI*-digested and gel-purified (Qiagen kit) linear pBAC-VI only produced one small foci of GFP-positive cells.
Figure 6.5 CEF cultures after infection with MVAc and transfection with pBAC-VI.
CEF cultures were infected with MVAc at an MOI of 5 were then transfected with approximately 1 µg of the indicated DNA using Lipofectamine 2000. After 48 h cells were inspected for GFP-positive cells by fluorescence microscopy.

Figure 6.6 GFP-positive MVAc-BAC foci.
The virus progeny derived from infection of CEFs with MVAc and transfection with plasmid DNA were amplified in a T25 flask of CEFs in the presence of MPA (25 µg/ml). The progeny were harvested and re-amplified in a further T25 flask of CEFs, again with MPA. After 48 h GFP-positive foci were observed with fluorescence microscopy.
6.3.2 Clonal selection of recombinant MVAc

The cells in the flask containing GFP-positive foci were harvested and the progeny were amplified twice in the presence of MPA to select for virus which had incorporated the pBAC-VI plasmid. Following the second round of amplification many more discernable foci were obtained (Fig. 6.6). Three rounds of focus-picking were carried out in the presence of MPA to purify the MVAc-pBAC-VI (MVAc-BAC). A master stock and working stock were then generated and titrated.

6.3.3 PCR analysis of recombinant MVAc-BAC

Purified DNA was required for PCR analysis of the deletion VI region that was modified during virus isolation. PCRs were performed with primers specific for regions of the EcogptGFP cassette, the F2 region and immediately upstream, and the F1 region and immediately downstream. Bands of the expected sizes were produced for the F1 and F2 regions bordering deletion VI and part of the GFP ORF (Fig. 6.7 (ii)-(iv)), corroborating the GFP-positive nature of the virus and showing that the pBAC-VI plasmid had been integrated into deletion VI. No product was observed when using the downstream F1 primer and the upstream reverse F2 primer with the MVAc-BAC DNA as template (Fig 6.7 (i)), probably because the product of approximately 10 kb may have been too large. However, the wild type MVAc template yielded a band of just over 1.5 kb indicating an alteration had occurred in this site in MVAc-BAC as this product was no longer made.
Figure 6.7 PCR analysis of MVAc-BAC genome at the deletion VI locus.

(i) PCR with primers annealing upstream of F1 and downstream of F2 produced a band of approximately 1.6 kb when MVAc DNA was used as template, but no band was obtained when MVAc-BAC was used as template. (ii) PCR with primers annealing upstream of F1 and at the 3’ terminus of F1 produced a band of 0.5 kb when MVAc-BAC extracted DNA or pBAC-VI were used as template. (iii) PCR with primers annealing to a section of the EcoqptGFP ORF produces a band of 1.25 kb when MVAc-BAC DNA or pBAC-VI were used as template. (iv) PCR with primers annealing upstream of F2 and at the 3’ terminus of F2 produced a band of 0.3 kb when MVAc-BAC DNA or pBAC-VI were used as template.
6.4 MVAc-BAC DNA extraction

For transformation of *E. coli* with the MVAc-BAC genomic DNA, the DNA preparation needs to be in the concatemeric form that is formed during VACV DNA replication (section 1.4.5). BAC technology for modifying virus genomes was pioneered in viruses which have a circular intermediate in their life cycle so that the BAC can function as a plasmid and replicate in *E. coli*. The concatemeric form of the VACV is the only point at which the linear hairpin duplex genome can be considered circular and enables the BAC within it to function as a plasmid.

6.4.1 Use of IβT to inhibit MVAc-BAC hairpin resolution

The compound IβT results in VACV producing longer than wild type transcripts via affecting elongation and termination of transcription. This causes excess production of dsRNA and subsequently blocks late protein synthesis and replication (Cohrs *et al.*, 1989). As a consequence of this viral hairpin resolution is blocked and VACV genomes are retained in the concatemeric form. IβT has been used to enable the purification of VACV-BAC, and notably MVA-BAC, genome concatemers and subsequent transformation into DH10b *E. coli*. CEFs were infected with MVAc-BAC, treated with IβT for 24 h and the viral DNA was extracted. Briefly, this involved lysing infected cells and treating the lysate with proteinase K treatment followed by a phenol-chloroform extraction (described in detail in Chapter 2). The DNA obtained was transformed into DH10b *E. coli* and approximately 50 colonies were obtained on a Cm-containing agar plate. However, initial screening of the alkaline-lysis mini prep samples generated subsequently have not been conclusive. No clear positive or negative information regarding the presence of MVA genes was obtained by PCR using a variety of primers. Similarly, *Hind*III digest of the mini prep samples showed a smeared and indistinct pattern on a pulse-field gel. Following thorough checking of all controls, it is probable the problem with both these screening methods is the quality of DNA obtain in the mini prep samples from this now very large (approximately 185 kb) single-copy plasmid. Consequently, all the colonies were grown in Cm-containing LB to lag phase and stored as glycerol stocks at -80 °C for further screening at such time as a protocol for obtaining higher quality DNA has been optimised.
CHAPTER 7: DISCUSSION

Bioinformatic analyses of B14 counterparts in different VACV strains and other OPVs, showed a high level of conservation. B14 is advantageous for viral propagation in vivo but is not essential for virus replication, as shown by the characterisation of vΔB14 (Chen et al., 2006). MVA is under investigation as a recombinant vaccine vector for a number of infectious diseases, and while much progress has been made (McShane et al., 2004) improvements are required to increase the strength and durability of the immune response. One strategy proposed to achieve this was to modify the vector backbone to alter its intrinsic immunogenicity by removing genes that encode immunomodulatory proteins. As a result of the six genomic deletions incurred throughout its derivation (Meyer et al., 1991), MVA lacks several of the characterised VACV immunomodulators. However, MVA does contain a gene (183R) with high conservation with the VACV strain WR B14R gene and which encodes a virulence factor that modulates NF-κB activation (Chen et al., 2006; Chen et al., 2008). In this study protein 183 was characterised. In addition to investigating the consequences of deleting gene 183R on MVA replication, the interaction of this B14-like protein with IKKβ in the NF-κB signalling pathway was investigated.

7.1. MVA 183R encodes a highly labile protein

This study has provided insight into the expression of the previously uncharacterised 183R gene in MVA. Bioinformatic comparisons revealed that, although there are many VACV WR B14 orthologues across the OPV genus, MVA protein 183 is unique in lacking six amino acid residues which map to a central helix (helix 6) of the crystal structure of B14 (Graham et al., 2008). The location of the six-residue deletion in 183 was considered likely to disturb the stability of the protein fold according to the structure generated by a bioinformatic modelling program. Indeed, analysis of lysates of both MVA-infected cells and cells transfected with FLAG-tagged 183 revealed the expression level of 183 was lower than that of B14. The 183 protein was not detected in the lysates of MVA-infected CEFs except under conditions wherein the proteosome was inhibited, or anti-inflammatory pathways activated. This indicated that the six amino acid deletion in 183 in comparison to B14 had rendered the protein unstable.
Furthermore, an initial objective in characterising protein 183 was to obtain structural information to compare what differences in protein folding may have resulted from the changes in primary sequence compared to B14. However, in contrast to B14, expression of 183 in *E. coli* resulted in only insoluble protein in different induction systems.

It is evident that although the *183R* gene has been retained in MVA, the mutations it incurred have rendered the encoded protein non-functional. Thus in contrast to other mutations that were acquired by MVA during its isolation, such as the six large genomic deletions which led to loss of function of many genes, in the case of *183R*, the encoded protein has been inactivated by a relatively minor mutation. This has implications for other MVA proteins that have small in frame deletions and which may or may not have retained their function (e.g. the B7 and F3 MVA counterparts). It is notable that the gene immediately upstream of *183R*, is fragmented in MVA compared to the B13 serpin of VACV strain WR (Kettle *et al.*, 1995), whereas the downstream gene which encodes the viral IL-1β receptor (Alcami & Smith, 1992) is intact (Antoine *et al.*, 1998; Staib *et al.*, 2005).

### 7.1.1 The NF-κB inhibitory activity of B14 is not retained by protein 183

TNFα binds to its cell surface receptor and initiates a signalling cascade that includes TRAF2/5 and TAK1, to which TAB3 is associated. These TNFα-induced cascades result in the activation of many transcription factors, including NF-κB family members. Despite the high level of sequence conservation between 183 and B14 discussed above, in addition to differences in protein expression, the *in vitro* function of 183 is different to that of B14. After transfection of an NF-κB-luciferase reporter plasmid into cells and stimulation of these cells with TNFα, an increase in luciferase activity was seen, due to upregulation of transcription from the NF-κB response element. Co-transfection of the plasmid encoding B14 resulted in a reduction in this upregulation, because the activation of NF-κB is blocked through the inhibition of IKKβ (Chen *et al.*, 2008). In contrast, upon transfection of the p183f plasmid into mammalian cells, protein 183 expression was detected following TNFα stimulation but it does not down-regulate NF-κB activation. Therefore, the lack of any NF-κB-modulatory function associated to protein 183 is not simply because the protein is not
being stably expressed. TNFα-induced signalling prevents the rapid turnover of protein 183 seen in unstimulated cells. The mechanism by which TNF treatment stabilises protein 183 has not been investigated thoroughly, but TNFα can interfere with normal cellular processes as indicated by the association of its upregulation in a variety of disease states including rheumatoid arthritis, psoriasis, Crohn’s disease, and refractory asthma (Bradley, 2008). Therefore, although 183 has no NF-κB-modulatory function, it is possible that in the context of a systemic immune response, where cytokines such as TNFα are expressed, protein 183 could have a functional role. To assess this, an in vivo characterisation was carried out with VACV WR encoding protein 183 in place of B14.

### 7.1.2 Protein 183 does not restore the wild type phenotype to the vΔB14 virus in vivo

VACV WR can be grown in human cells, whereas MVA has a much more restricted host range and can replicate in very few non-avian cells lines. An advantage in studying WR, rather than MVA, is that due to its ability to replicate in a wider variety of cell types it can cause disease in laboratory animals, for example, in the i.d. model used to characterise B14 as a virulence factor in mice (Chen et al., 2006). For this reason, 183R was inserted into the B14R locus of vΔB14 to form WRΔB14+183. This hybrid virus was then used to investigate if the expression of the 183 protein altered the virulence of vΔB14 in a murine i.d. model (Chen et al., 2008). WRΔB14+183 exhibited equivalent virulence to vΔB14 and thus protein 183 does not restore the wild type WR phenotype to vΔB14 in the i.d. model. Thus in the context of host infection, 183 does not display functional identity to B14.

### 7.1.3 MVAΔ183 incurred a terminal transposition event

In parallel to examining the phenotype conferred by the replacement of B14R with 183R in WR, much useful information could have been gained from an MVA-based virus lacking 183R. Differences between cytokine-induced signalling pathways in avian compared to mammalian cells mean that the modifications incurred by 183 during the generation of MVA in avian cells could have been advantageous for the virus in that host. Consequently, it was proposed that both in vitro and in vivo characterisation of an MVAΔ183 virus could provide valuable information about the
role of 183 in avian and mammalian cells, and most importantly about MVA immunogenicity. An initial aim of this work was to delete 183R from MVA and assess any subsequent changes in replication or immunogenicity. MVAAΔ183 was generated via transient dominant selection using GFP expression and resistance to MPA as the transient selectable markers, and immunostaining to overcome the problems of non-standard small non-round VACV foci of MVA. However, the virus generated had a large-focus phenotype, which was not anticipated following the targeted removal of a proposed immunomodulator. On further examination of the genome of MVAAΔ183 by HindIII digestion it was clear that unintended changes had occurred during virus isolation. Specifically, in addition to loss of the 183R gene there were changes to the terminal regions of the genome that seemed likely to have been generated by a terminal transposition event. Although this mutation was not intended, it did cause a markedly-altered *in vitro* phenotype. It will be interesting to undertake further investigation of this phenotype and to determine its genetic basis by sequencing the virus genome.

Knowing that the 183 protein was unstable in both avian and mammalian cells, was insoluble in *E. coli* unlike B14, and that it did not inhibit NF-κB activation or affect VACV virulence, it seemed that the 183 protein had no phenotype. Therefore, there was little point in making a MVA revertant virus in which the 183R gene was restored to the deletion mutant. Similarly, having found that the MVA183 deletion mutant had additional genetic alterations, although one would normally have repeated the virus isolation to make the correct deletion mutant, in this case it was decided there was little to be gained from this and that the available time would be better spent on other aspects of this project. Indeed, the initial hypothesis, that the removal of 183R from MVA affects immunogenicity, seemed unlikely to be valid.

### 7.1.4 Further bioinformatic analysis of the 183R and the MVA genome

It is noteworthy that the six amino acid residue REISAI is one of only three differences between the B14 and 183 amino acid sequences, and that this deletion is unique to MVA among all OPVs. The genome sequence of VACV strain CVA (the parent of MVA) retains this REISAI motif and, consequently, this deletion must have occurred during the extensive passage of CVA in avian cells during the derivation of MVA. It is more than likely that many of the changes incurred by the genome
throughout this serial passage adapted the virus to growth in avian cells. It was therefore thought plausible that the deletion of the REISAI motif from 183 imbued MVA with some novel function involved in a cell process, such as cytokine signalling, which can differ in avian compared to mammalian cells. However, the lack of expression of protein 183 renders this unlikely, and the possibility is further negated by the bioinformatic analysis, which predicts the REISAI deletion would render the protein unstable, and experimental demonstration that this was true.

As examined in detail in the introduction, MVA lacks many immunomodulatory genes that are preserved in other VACV strains. This is due to both the six genomic deletions it incurred during its derivation and due to multiple deletions and/or frameshift mutations. An example of an immunomodulatory gene that has been largely disrupted is the orthologue of NIL. The ORF encoded by MVA 020L is partially deleted and not predicted to be functional (Antoine et al., 1998). In contrast, the MVA counterpart of WR F1 has a four amino acid deletion and one conservative substitution and is functional. As shown by changes in apoptosis induced upon infection with an F1 deletion MVA virus compared to that induced following wild type infection (Fischer et al., 2006). Therefore, the six amino acid deletion and two conservative substitutions in 183 compared to B14 were not immediately identified as capable of completely abrogating the expression and function of the protein 183 as has been seen in this study.

Further bioinformatic analysis of the MVA genome revealed a number of genes encoding immunomodulatory proteins that contain small deletions relative to all other strains of VACV, including F3L (Froggatt et al., 2007) and B7R (Price et al., 2000), but many of the encoded proteins are as yet uncharacterised. In common with 183R, these deletions are absent in CVA. In addition, no other strain of VACV encodes an orthologue bearing these deletions. Consequently, prior to any study of such MVA genes in the future, validation of expression would be advisable were reagents available, as it is possible that the small internal deletions may have disabled these proteins by destabilising their protein folds.
7.2 WR lacking both B14R and A49R is viable

The next aim of this project was to investigate B14 function in combination with a newly-characterised VACV immunomodulator, A49 (Daniel Mansur, personal communication). The construction of the double deletion virus vΔB14ΔA49 demonstrated that neither gene product is essential to WR alone or in the absence of the other protein. Both the proteins are known to counter the host response to virus infection. They have an inhibitory effect on NF-κB activation induced within cells during infection with WR. Interestingly, other viruses, such as HIV, use NF-κB transcription factors to further their own replication (Roulston et al., 1999; Hiscott et al., 2001). However, the NF-κB transcription factor family are not compatible with OPV promoters because VACV transcription occurs in the cytoplasm not the nucleus. VACV instead affects the NF-κB pathway by reducing its activation and thus modulates the host response to infection.

As described above, B14 is one immunomodulatory protein encoded by WR to modulate the NF-κB pathway (Chen et al., 2008) and it is plausible that B14 acts as a virulence factor in vivo (Chen et al., 2006) via this activity. A49, on the other hand is a newly-characterised immunomodulatory protein encoded by WR that prevents NF-κB family transcription factors from translocating into the nucleus to upregulate transcription from the NF-κB response element. B14 and A49 are just two of a number of VACV proteins known to modulate NF-κB activation including A46 (Stack et al., 2005), A52 (Harte et al., 2003), K7 (Schroder et al., 2008), K1 (Shisler & Jin, 2004) and M2 (Hinthong et al., 2008). Given VACV has evolved multiple mechanisms for modulating NF-κB the targeted removal of just one such immunomodulatory gene is unlikely to produce a marked change in the expression of NF-κB-controlled gene expression and the subsequent immune response. It is possible that the loss of one of the many VACV NF-κB-modulating proteins will be compensated by the others. Therefore, in an attempt to produce a virus with a more markedly altered phenotype, and potentially greater change in immunogenicity, multiple NF-κB immunomodulators were deleted from VACV WR in sequence. The initial ORFs selected for deletion were those predicted to be conserved in MVA so that any change in immunogenicity could potentially be reproduced in MVA. As such, A52, M2, and K1, which are not found in MVA, were not prioritised for deletion. The instability of the 183 protein had not, at this stage, been established and therefore
vΔB14 was the starting point for the multiple deletion virus. Subsequently, A49R was deleted as described in this study and initial characterisation is discussed below. Following the construction of WRΔB14ΔA49 another immunomodulator K7 was removed, again by transient dominant selection. However, the characterisation of this triple deletion virus does not form part of this work. Further work may involve deleting A46 and also the NF-κB-modulators that are not conserved in MVA.

7.2.1 A49 and B14 are not individually or in concert essential to viral replication

Single-step growth kinetics analysis (infection at high MOI) showed no difference between any of the WR viruses, regardless of the presence or absence of B14 or A49. WR lacking A49 (vΔA49), and its revertant (vΔA49Rev) were included in this analysis in addition to the wild type WR, vΔB14, vΔB14ΔA49 and the revertant, vΔB14+A49. These controls were necessary so any changes seen could be attributed to the loss of A49 or B14. However, viral replication in all six viruses was equivalent and as such neither A49, nor B14, play a role in virus replication. Similarly, multi-step growth kinetics analysis (infection at low MOI) showed no differences between any of the six viruses, regardless of the presence or absence of B14 or A49. As for the single-step analysis, WvΔA49 and its revertant were included as controls. The maintenance of normal viral replication and spread, regardless of B14 and A49 expression is not surprising given the previous characterisation of B14 as a virulence factor \textit{in vivo} and its immunomodulatory function and the emerging role of A49 in NF-κB modulation. Furthermore, the kinetics of vΔB14 replication had been shown previously not to deviate from the wild type in a single-step analysis. However, to control for unpredicted roles for A49, or for A49 in combination with B14, this aspect of the virus was investigated. This work was also a necessary prerequisite for the planned \textit{in vivo} analysis of virulence and immunogenicity of vΔB14ΔA49. In addition, the genome of vΔB14ΔA49 was analysed by digestion with HindIII and revealed an unaltered pattern of HindIII fragments compared to WR and vΔB14. Of course, there are differences between the double deletion mutant and controls, but these were not apparent following digestion with HindIII because of the very large size of the HindIII A and B fragments in which genes A49R and B14R reside. This result meant that further NF-κB immunomodulators can in the future be removed from WRΔB14ΔA49 without concern that undesired genomic changes had been incurred.
7.2.2 Future characterisation

A key method to assess the effect of removing both A49 and B14 from WR is to look at virulence *in vivo*. Given the previously characterised reduction in lesion size seen with vAB14 in the i.d. model it would be informative to see if the virulence is further reduced with the additional loss of A49. However, characterisation of the virus in the i.n. model would also be worthwhile as this model allows characterisation of a systemic, rather than local, infection. In addition, it produces a more potent effect and as such could better illustrate the possible greater effect of the loss of two NF-κB immunomodulators from WR.

7.3 B14 interacts with IKKβ

In the unstimulated cell, the NF-κB transcription factor is sequestered in the cytoplasm in complex with IκBα, which occludes the NLS of NF-κB. When IκBα is phosphorylated, it is ubiquitinated and then degraded (Pasparakis *et al.*, 2002) so that NF-κB is released and is transported into the nucleus where it binds to the NF-κB response element, initiating transcription. NF-κB-inducing stimuli result in the activation of various cytoplasmic kinases, which form signalling cascades converging on the IKK complex. The IKK complex (predominantly IKKβ) then phosphorylates IκBα. In the conventional pathway, this large complex (approximately 700-900 kDa) is formed by three subunits: IKKα, IKKβ and IKKγ (also known as NEMO) and other components. The first two subunits have kinase activity whereas IKKγ has a regulatory function.

As discussed, B14 inhibits NF-κB activation upon stimulation with TNFα, but how this was achieved was not clear originally. Subsequently it was shown that the inhibition of NF-κB by B14 occurs by B14 binding to the IKK complex via the IKKβ subunit (Chen *et al.*, 2008). This was clear from the finding that B14 co-purifies and co-precipitates with the endogenous IKK complex from both human and mouse cells. In addition, IKKβ, but not IKKα, is needed for the interaction of B14 with the IKK complex. Further investigation showed that mechanistically, B14 binds IKKβ and inhibits the phosphorylation of IκBα, preventing its degradation and thus the NF-κB dimer remains complexed to IκBα in the cytoplasm. However, which parts of both IKKβ and B14 were needed for this interaction were uncharacterised.
7.3.1 Residues 300-480 are necessary for IKKβ interaction with B14

Within the IKK complex, IKKβ forms homodimers or heterodimers with IKKα as part of the larger oligomeric complex. No structure has yet been solved for IKKβ. In fact, the only structure for a component part of the IKK complex to date is a small fragment of NEMO in complex with another viral protein, vFLIP (Bagneris et al., 2008). IKKβ is composed of five predicted domains (Delhase et al., 1999). Starting from the N terminus there is a 300-amino acid kinase domain, a Ub domain, a LZ, a HLH and the NBD. Given that B14 and IKKβ were shown to interact by co-immunoprecipitation, a series of IKKβ truncations were constructed and tested for interaction with B14 in mammalian cells by immunoprecipitation. A truncated IKKβ, which started at amino acid residue 300 interacted with B14 whereas a fragment comprising residues 480 through to the C terminus did not. Therefore, residues 300 to 480 are essential to the interaction. This excludes the kinase and NBD from involvement in B14 binding. Whether this region alone is sufficient for interaction remains to be assessed, as does which of the Ub, LZ, or HLH domains it encompasses are involved. To this end, smaller truncations of each of these domains and the 300-480 region have been cloned and should enable deduction of the minimum sequence required for the IKKβ-B14 interaction.

7.3.2 Co-purification of IKKβΔ300 and B14 expressed from E. coli

Eventually, identification of the region of IKKβ that is essential and sufficient for binding B14, may enable the 2 proteins to be co-purified and the structure of the complex solved by crystallography. No structure has yet been solved for IKKβ but it may be that the interaction with a highly-soluble viral protein, such as B14, may allow its purification and subsequent structural studies. A problem associated with expressing recombinant IKKβ in E. coli is the toxicity associated with its kinase activity. Therefore, for structural studies truncation of IKKβ to remove the N-terminal kinase domain was desirable, particularly since the remaining protein retained the ability to bind B14. To express this domain for structural studies, a his-tagged allele was cloned into a prokaryotic expression vector and co-transformed into E. coli with His-tagged B14. Initial trials of IPTG-induced expression revealed both soluble truncated IKKβ and B14. However, it was noted that this was only reproducible on a larger scale when the aeration of the cultures was reduced. This is possibly due to the
lower oxidation level altering the cytochrome c pathway in the bacteria as indicated by the pinkish colour of the pellet obtained. However, exactly how this affected the solubility the IKKβ truncation is unclear. Subsequent protein purification by IMAC and size exclusion chromatography produced soluble, His-tagged-truncated IKKβ and B14. However, B14 was present in great excess and the amount of purified IKKβ was too small to proceed towards structural studies. It is plausible that more His-tagged IKKβ could be purified during the IMAC step if there were no competition for the column resin from the abundant His-tagged B14. To overcome this, B14 was recloned into a prokaryotic expression vector without a His tag. Therefore, the purification will rely firstly on the interaction of His-tagged-truncated IKKβ with the IMAC column and secondly between the IKKβ and B14. In addition, if smaller fragments of IKKβ are shown to be sufficient for binding to B14, their purification could be attempted as it is possible smaller fragments will be more easily purified.

### 7.3.3 Dimer mutants affect interaction with IKKβ and NF-κB modulation

In addition to delineating the B14-IKKβ interaction by investigating which domain(s) of IKKβ are required for interaction, further understanding of the B14 protein interaction face has been gained. The crystal structure of B14 showed it was a dimer (Graham et al., 2008). However, B14 is not believed to act as functional homodimer in cells, multi-angle light scattering experiments carried out by Dr Stephen Graham at the University of Oxford indicate that B14, while capable of forming a homodimer, is not an obligate dimer and that the association is weak as indicated by a high dissociation constant. Instead it is thought the protein interface that allows dimerisation enables B14 to interact with cellular proteins, such as IKKβ. Plasmids encoding a series of five B14 variants containing single amino acid changes to residues that form part of this interface were constructed. Initial luciferase assays showed that all the mutants were expressing B14 but that two of the mutants, F130K and T31K, were unable to abrogate the activation of NF-κB in an analogous manner to wild type B14.

LUMIER experiments investigated, in a semi-quantitative manner, the degree of binding of the B14 mutants with IKKβ fused to Renilla-luciferase. All the mutant B14 proteins did not bind to IKKβ in this assay to a comparable degree to wild type B14,
given two of the mutants had lost the ability to inhibit NF-κB activation this assay was not very informative. There may have been underlying reasons why the stability of the IKKβ-B14 interaction was affected by all mutations. Therefore, an additional assay was used to investigate the interaction. The co-immunoprecipitation assays showed that F130K did not bind to IKKβ. However, the ability to co-purify with IKKβ appears unaffected in mutants T31K, Y35K, Y35E and L26E in co-immunoprecipitation experiments. However, of these T31K appears not to be functional in the luciferase assay. This could be due to weakened binding by this mutant or binding in a slightly altered way compromising the B14 function. The other four mutants co-precipitated with IKKβ although preliminary experiments (data not shown) using anti-FLAG mAb, rather than anti-HA mAb, to immunoprecipitate the proteins indicated that the binding may be diminished. Further experiments are needed to confirm that all the B14 mutants bind IKKβ equivalently. However, these initial conclusions indicate that the F130 is involved in B14 binding to IKKβ and subsequent inhibition of NF-κB activation.

7.4 Development of the MVAc-BAC system

Constructing recombinant MVA is associated with challenges not encountered when working with other less attenuated strains of VACV such as WR. MVA is replication-competent in fewer cell lines and the foci produced are harder to distinguish, even with a virus expressing GFP. Consequently, any spontaneous mutations in the virus genome which give rise to larger foci may be isolated during foci picking. Such a mutation(s) occurred during the generation of MVA∆183. Thus, a different strategy to produce recombinant MVA, with potentially less risk of second site mutations was started. The MVAc-BAC virus constructed will allow the modification of the virus genome in the form of a plasmid maintained in E. coli.

MVAc-BAC contains a full-length BAC (pBELO-BAC-11) at genomic deletion VI as well as HIV clade C antigens at the TK locus. DNA was extracted from cells infected with this virus in the presence of IBT to increase the proportion of genome concatemers, which are required for cloning into E. coli. The MVAc-BAC virus DNA was transformed into DH10b E. coli and about 50 clones were obtained. However, the main challenge throughout the construction of this virus was the difficulty in obtaining high-quality pure DNA preparations of the single-copy pBAC-VI plasmid. Currently,
an improved method is required to analyse the MVAc-BAC clones obtained in *E. coli*. Once a full length clone has been obtained, it will be transfected into mammalian cells that are infected with a helper virus (e.g. Fwp9). Since Fwp9 cannot replicate in BHK-21 cells, while MVA can, the only progeny from these cells will be MVA.

There will be many advantages of using MVAc-BAC. Firstly, as it contains the HIV clade C antigens (Gomez *et al.*, 2007) the effect of any targeted deletions can be assessed both in terms of VACV and the foreign antigens. Secondly, multiple deletions can be made simultaneously or in sequence via recombineering protocols (Warming *et al.*, 2005). Greater insight could be gained by removing multiple immunomodulatory genes from MVA as their combined loss could be additive or synergistic. There are examples of single-gene MVA deletion mutants that have had a distinct effect on immunogenicity (Staib *et al.*, 2005) but it is plausible that multiple-deletion virus could produce a more striking change in immunogenicity. This could be more likely if the genes removed interfere with the same part of the immune response, as in the case of the NF-κB-modulator multiple deletion WR virus (Chapter 4).

A further advantage of MVAc-BAC is that it eliminates the need for clonal purification by focus picking when modifying the genome to create targeted deletions or insertions. Modifications are made to the virus genome whilst in the plasmid form by recombineering (Warming *et al.*, 2005). Therefore, the risk of a spontaneous mutation being advantageous to virus growth is minimised. Therefore, the MVA genome can be modified with a lower risk of second site mutations. Lowering this risk is clearly an advantage when constructing mutant VACV, however, it may be even more advantageous when generating recombinant MVA.

Recent work (Suter *et al.*, 2009) has raised questions over the nature of MVA. MVA was proposed to be genetically stable and uniform following the 570 passages by which it was derived from CVA (Mayr *et al.*, 1978). Following this, MVA has been shown to be safe in humans and unable to replicate in human cells lines. However, three MVA isolates, including one used in a combination smallpox vaccine (Stickl *et al.*, 1973) have been shown to exhibit phenotypic differences despite sequencing data indicating they are identical (Suter *et al.*, 2009). Two of the isolates were able to both replicate in human cells and also cause death in immune-compromised mice (Suter *et al.*, 2009). Sequencing of viruses isolated from the mice revealed the isolates to be a
polyclonal mixture of viruses. Importantly, some did not have the characteristic MVA genomic deletions at sites I, I and V, this implies these virus variants were enriched from the isolate used to infect the mice and not a product of spontaneous mutation during passage in the murine host. The authors conclude that the MVA isolates evaluated comprise complex polyclonal mixtures of viruses even though the initial sequencing data showed they shared 100% nucleotide coding sequence. Given the possibility that all MVA isolates derived from the virus isolated after 570 passages in CEFs are polyclonal mixtures the MVAc-BAC has a further advantage. Following the extraction of virus DNA from infected cells, and subsequent transformation into \emph{E. coli}, the MVAc-BAC is in plasmid form and as such is a clone. Therefore, this system not only limits the possibility of second-site mutations to the virus rescue stage but also precludes the enrichment of any minor components if the original isolate of MVA was indeed polyclonal. The BAC strategy obviously does not abrogate the risk of MVA mutation within the vaccinated host system as raised (Suter \textit{et al.}, 2009), but in the immunocompetent human, it might be expected that MVA could not replicate enough due to host restriction factors, to evolve substantial phenotypic changes. However, this possibility should be investigated more thoroughly before further vaccine development in the MVA background.
7.5 Concluding remarks

This thesis provides a characterisation VACV strain MVA protein 183 which was previously uncharacterised. 183 is the counterpart of WR B14, which is a virulence factor and downregulates NF-κB activation. B14 and 183 are highly conserved across OPVs, however, 183 has incurred three changes not found in any other OPV orthologue. Protein 183 is highly labile in both infected and transfected cells and does not act as a virulence factor or downregulate NF-κB. Thus, in conclusion, \textit{I83R} encodes an unstable protein without functionality. Further work detailed in this thesis involved the construction WR\textDelta B14\textDelta A49 and shows that newly-characterised NF-κB modulatory WR protein A49, in combination with B14, is not essential with B14 to viral growth or spread. Additional findings show that B14 downregulates NF-κB by interaction with residues 300-480 of IKKβ. Furthermore, this interaction can be abrogated by the mutating the protein interface of B14, namely by the F130K substitution. Finally, the development of a BAC-based system for manipulating the genome of MVA was described, which could enable faster, less-mutation prone construction of recombinant MVA to remove multiple-deletion viruses which may have an altered immunogenic profile useful to the use of MVA as a vaccine.
REFERENCES


