

**THE ROLE OF *VACCINIA VIRUS*
GENE *K7R* IN EVASION OF
INNATE IMMUNITY**

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Declaration

The work detailed herein is the work of the candidate except where clearly indicated. In particular, some plasmid construction and luciferase assays were carried out in collaboration with Dr Gabriel Almeida. In addition, the *in vivo* experiment was carried out by Dr Aodhnait Fahy.

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The role of *Vaccinia Virus* gene *K7R* in evasion of innate immunity

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Abstract

This thesis reports an investigation of the role of *Vaccinia virus* (VACV) gene *K7R* in evasion of innate immunity. The K7 protein is an inhibitor of intracellular signalling pathways leading to the activation of the innate immune response and was shown to act by inhibiting TBK1/IKK- ϵ - mediated IRF signalling by targeting DDX3, a cellular RNA helicase (Schroder *et al.*, 2008). To characterise K7 further and study its interaction with DDX3, K7 in complex with fragments of DDX3 was expressed in bacterial cells with the aim of obtaining co-crystals.

The role of *K7R* in the context of VACV infection was also studied. Previous *in vivo* work using a recombinant virus lacking *K7R* had shown that K7 contributes to virulence. However, virulence was retained when a mutation of the initiating methionine codon abrogated K7 protein expression while preserving the nucleic acid sequence. It has been shown here that this is likely to be due to the presence of viral miRNA(s) encoded from the *K7R* sequence. Computational analysis identified putative pre-miRNAs within *K7R* and mutated versions of the *K7R* ORF which contained the predicted pre-miRNA sequences but did not make protein were still able to inhibit the IFN- β , NF- κ B and ISRE-dependent intracellular signalling *in vitro*. Several of these mutated genes were introduced into the *K7R* deletion VACV and their virulence will be examined in a mouse model of infection. The presence of a miRNA also needs to be formally demonstrated using northern blot and/or qPCR analysis.

Finally, there is a strong interest in using attenuated VACV strains such as Modified Vaccinia Ankara (MVA) as vaccine vectors against various pathogens. To investigate whether the removal of immunomodulators from MVA, such as the orthologue of *K7R*, can make the virus more immunogenic, a deletion mutant and its revertant counterpart were made and characterised *in vitro*.

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Abbreviations

6-TG	6-thioguanine
Å	ångström
aa	amino acid
Ab	antibody
Ago	argonaute
APC	antigen presenting cell
AP-1	activator protein 1
AraC	β-D-arabinofuranoside
ATP	adenosine triphosphate
Bcl-2	B-cell leukaemia/lymphoma 2
BH	Bcl-2 homology
BHK-21	baby hamster kidney 21 cells
bp	base pair
BSA	bovine serum albumin
BSC-1	African green monkey kidney cells
C-6 X His	C-terminal 6 histidines tag
CARD	caspase recruitment domain
caspase	<i>cysteine-aspartic acid proteases</i>
CCL	C-C motif chemokine ligand
CD	cluster of differentiation antigen
CEF	chicken embryo fibroblast
CEV	cell-associated enveloped virus
CHO	Chinese hamster ovary cells

CHX	cycloheximide
CMC	carboxymethylcellulose
CMLV	<i>Camelpox virus</i>
COP	Copenhagen
CPE	cytopathic effect
CPXV	<i>Cowpox virus</i>
CTL	cytotoxic T-lymphocyte
CV	column volume
CVA	<i>Chorioallantois virus Ankara</i>
CV-1	African green monkey kidney cells
CXCL	C-X-C motif chemokine ligand
DAB	3,3'-diaminobenzidine tetrahydrochloride
DAI	DNA-dependent activator of IFN-regulatory factors
DAPI	4',6'-diamidino-2-phenylindole
DC	dendritic cell
ddH ₂ O	deionised distilled H ₂ O
DDX	DEAD box protein
DEAD	Asp-Glu-Ala-Asp
DGCR8	DiGeorge syndrome critical region 8
DLS	dynamic light scattering
DMEM	Dulbecco's modified Eagle's medium
dNTP	deoxyribonucleotide triphosphate
ds	double-stranded
DTT	dithiothreitol
EBV	Epstein-Barr virus
<i>E.coli</i>	<i>Escherichia coli</i>

Ecogpt	<i>E. coli</i> guanine xanthine phosphoribosyltransferase
ECL	enhanced chemiluminescence
ECTV	<i>Ectromelia virus</i>
EDTA	ethylene diamine tetraacetic acid
EEV	extracellular enveloped virus
EGFP	enhanced green fluorescent protein
eIF2	eukaryotic initiation factor 2
E/L	early/late (promoter)
EM	electron microscopy
ER	endoplasmic reticulum
ERK	extracellular signal regulated kinase
EV	empty vector
Exp 5	exportin 5
FADD	Fas-associated protein with death domain
FBS	foetal bovine serum
GAF	IFN- γ associated factor
GAG	glycosaminoglycan
GAS	IFN- γ activated sequence
HA	haemagglutinin
HBV	hepatitis B virus
hCMV	human cytomegalovirus
HEK 293T	human embryonic kidney 293 T cells
HeLa	Henrietta Lacks (uterine carcinoma) cell lines
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
His	histidine
HIV	human immunodeficiency virus

HRP	horseradish peroxidase
HPX	hypoxanthine
HSV-1	herpes simplex virus type 1
IEV	intracellular enveloped virus
IFN	interferon
IFNAR	IFN- α receptor
IFNGR	IFN- γ receptor
I κ B	inhibitor of NF- κ B
IKK	inhibitor of nuclear factor- κ B kinase
IL	interleukin
IL-1R	interleukin 1 receptor
IMAC	immobilised metal affinity chromatography
IMV	intracellular immature virus
IPTG	β -D-1-thiogalactopyranoside
IPS	IFN- β promoter stimulator 1
IRAK	IL-1 receptor associated kinase
IRF	IFN regulatory factor
ISG	IFN stimulated gene
ISGF	IFN stimulating gene factor
ISRE	IFN-stimulated regulatory element
ITR	inverted terminal repeat
IV	immature virion
JAK	Janus activated kinase
JNK	cJun N-terminal kinase
kb	kilobases
KDa	kilodalton

KSHV	Kaposi's sarcoma herpes virus
LB	Luria-Bertani broth
LMB	leptomycin B
LPS	lipopolysaccharide
LSDV	<i>Lumpy skin disease virus</i>
luc	luciferase
mAb	monoclonal antibody
MAL	MyD88 adaptor like
MALLS	multiple angle laser light scattering
MAPK	mitogen activated protein kinase
mAU	milli-absorbance unit
Mda5	melanoma-differentiation associated gene 5
MEM	minimal essential medium
MHC	major histocompatibility complex
MHC-I	MHC class 1
MHC-II	MHC class 2
miRNA	microRNA
MTOC	microtubule organising centre
MPA	mycophenolic acid
MPXV	<i>Monkeypox virus</i>
mRNA	messenger RNA
MVA	Modified Vaccinia Ankara
MyD88	myeloid differentiation factor 88
N-6 X His	N-terminal 6 histidine tag
NAP-1	nucleosome assembly protein 1
NEMO	NF- κ B essential modifier

NF-κB	nuclear factor κB
N-HA	N-terminal HA tag
NK	natural killer
NP-40	Nonidet-P 40
OAS	2'-5'-oligoadenylate synthetase
OD	optical density
OPV	<i>Orthopoxvirus</i>
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PBR	Poxviruses Bioinformatics Resources
pfu	plaque forming unit
p.i.	post-infection
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEI	polyethylinimine
PFA	paraformaldehyde
PKR	protein kinase R
PRR	pathogen recognition receptor
RIG-I	retinoic-acid-inducible protein I
RIP-1	receptor interacting protein 1
RISC	microRNA-induced silencing complex
RK-13	rabbit kidney-13 cells
RLH	RIG-like helicase
rLMV	rhesus lymphocryptovirus
RLU	relative light units

RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
RPXV	<i>Rabbitpox virus</i>
rRNA	ribosomal RNA
RT	room temperature
SAP	shrimp alkaline phosphatase
SCR	short consensus repeat
SD	standard deviation
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Serpin	serine protease inhibitor
SV40	simian virus 40
SH	src-homology domain
SHP	SH2-containing phosphatase
SOC	super optimal catabolite
ss	single-stranded
STAT	signal transducers and activators of transcription
SWPV	<i>Swinepox virus</i>
TAB	TAK-binding protein
TAE	Tris Acetate EDTA
TAK	TNF-associated kinase
TATV	<i>Taterapox virus</i>
TANK	TRAF-associated NF- κ B activator
TB	terrific broth
TBK	TRAF-associated NF- κ B activator binding kinase

TEMED	N,N,N,N-tetramethylethylenediamine
TGN	trans-Golgi network
Th	T helper
TIR	Toll/IL-1 receptor
TK	thymidine kinase
TLR	Toll-like receptor
TNF	tumour necrosis factor
TNFR	TNF receptor
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRBP	Tar RNA binding protein
T reg	T regulatory cells
TRIF	TIR-domain containing adaptor inducing IFN- β
tRNA	transfer RNA
Tyk	Tyrosine kinase
U	unit
UTR	untranslated region
UV	ultraviolet
VACV	Vaccinia virus
VARV	Variola virus
VCP	virus complement control protein
vCKBP	viral CC chemokine binding protein
v Δ 028-d	deletion <i>028R</i> virus
v Δ 028	clean deletion <i>028R</i> virus
v028-rev	revertant <i>028R</i> virus
vK7	wild type virus derived at same time of making v Δ K7

vΔK7	deletion <i>K7R</i> virus
vK7-rev	revertant K7 virus
vfsK7-1	frameshifted K7 virus 1
vfsK7-2	frameshifted K7 virus 2
vtrK7	<i>K7R</i> “truncated” virus which has codon 108 mutated to a stop codon
vinvK7	inverted <i>K7R</i> virus
vscrK7	scrambled <i>K7R</i> virus
vK7 ₁₋₁₇₁	K7 ₁₋₁₇₁ virus
vK7 ₁₇₁₋₄₅₀	K7 ₁₇₁₋₄₅₀ virus
vK7 ₁₇₈₋₂₆₉	K7 ₁₇₈₋₂₆₉ virus
vK7 ₂₇₀₋₄₅₀	K7 ₂₇₀₋₄₅₀ virus
Vo	void volume
v/v	volume/volume
WHO	World Health Organisation
WR	Western Reserve
w/v	weight/volume
X	xanthine

Chapter 1: Introduction

Vaccinia virus (VACV), the prototypal member of the *Orthopoxvirus* (OPV) genus of the *Poxviridae*, was the live vaccine used to eradicate smallpox, a highly contagious disease that plagued humanity for thousands of years, killed hundreds of millions of people in the 20th century alone, (Fenner, 1993) and was caused by *Variola virus* (VARV), another OPV. Poxviruses are large, enveloped, double stranded (ds)DNA viruses with genomes between 135 and 300 kb that replicate in the cytoplasm of infected cells (Moss, 2007). They include two sub-families, the *Chordopoxvirinae* and the *Entomopoxvirinae* which infect chordates and insects, respectively. OPVs include VACV, VARV, *Cowpox virus* (CPXV), *Monkeypox virus* (MPXV), *Ectromelia virus* (ECTV), *Camelpox virus* (CMLV), Raccoonpox virus and *Taterapox virus* (TATV).

Vaccination was introduced by Jenner in 1796 after he inoculated a child with CPXV, and a few weeks later challenged the child with smallpox and showed the child was protected (reviewed in (Baxby, 1999)). We now understand that this protection was afforded by the antigenic similarities between CPXV and VARV. Interestingly, in 1939 it was found that all the smallpox vaccines in use in the 20th century were not CPXV but another OPV of unknown origin thereafter named VACV (Downie, 1939). After a vaccination programme driven by the World Health Organisation (WHO), smallpox was eradicated in 1977 (Fenner *et al.*, 1988). Therefore, VACV was the first vaccine to eradicate a human disease. It was also the first animal virus observed by microscopy (Buist, 1886). VACV is the most intensively studied poxvirus and analysis of the many immunodulatory proteins it encodes has provided new insights into virus-host interactions. Additionally, VACV has been developed as a live recombinant vaccine vector (Mackett *et al.*, 1982; Panicali & Paoletti, 1982) against a variety of infectious agents and cancer (Cebere *et al.*, 2006; Harrop *et al.*, 2007). However, because the different VACV strains used in the vaccination campaign were not perfectly safe (Lane *et al.*, 1969), there is a need to improve vaccine safety. One approach to do this is to identify and remove virus genes encoding proteins contributing to virulence such as those interfering with the host immune response to infection (Clark *et al.*, 2006).

1.1 Genome organisation

VACV strain Copenhagen (COP) was the first VACV strain to be sequenced and contains approximately 200 tightly packed open reading frames (ORFs) (Goebel *et al.*, 1990). The central region of the VACV genome generally includes essential genes encoding proteins involved in viral transcription, DNA replication and structural components of virions (Gubser *et al.*, 2004). Approximately 90 genes from this central region are conserved between all chordopoxviruses (Upton *et al.*, 2003). In contrast, the genes located towards the termini are much less conserved, are non-essential for virus replication, and encode proteins that affect virus host range, immunomodulation or virulence (Witteck, 1982). Some of these genes encode viral mimics of host proteins (Bugert & Darai, 2000). VACV genes do not have introns, and there are no reports of splicing, consistent with the cytoplasmic location of virus mRNAs. The left and right termini of the genome contain inverted terminal repeats (ITRs) and genes in these regions are diploid (Witteck *et al.*, 1980). At the genomic termini, the two DNA strands are connected covalently by AT-rich incompletely base-paired hairpin loops that are important for DNA replication (Baroudy *et al.*, 1982). Adjacent to the hairpin loops, there is a highly conserved sequence (ATTTAGTGTCTAGAAAAAAA) that is essential for the resolution of concatemeric DNA intermediates formed during DNA replication (Merchlinsky & Moss, 1989). There are also sets of short, tandemly repeated sequences of variable length (Witteck & Moss, 1980). The ITRs are variable in length due to deletions, repetitions and transpositions (Witteck, 1982).

1.2 Virion structure

There are different forms of VACV particles and these were first described by electron microscopy (EM) (Morgan *et al.*, 1954; Dales & Siminovitch, 1961). When observed by EM VACV virions appear brick-shaped and are approximately 360 x 270 x 250 nm (Cyrklaff *et al.*, 2005). The most abundant infectious form of virus is the intracellular mature virus (IMV) which consists of an internal biconcave core surrounded by heterogeneous material called lateral bodies and a single lipid membrane bilayer (Dales & Siminovitch, 1961; Hollinshead *et al.*, 1999). The core contains the viral genome, structural proteins and enzymes required for the transcription of genes expressed early in the VACV replication cycle (reviewed in (Condit *et al.*, 2006)). The extracellular enveloped virus (EEV) is an IMV which has been wrapped with an extra membrane derived from either the trans-Golgi network (TGN) or early endosomes. This additional envelope

contains at least 6 virus proteins (A33, A34, A56, K2, B5 and F13) (Roberts & Smith, 2008). The E2 protein has also recently been shown to be associated with EEV, although it is not a transmembrane protein and is also associated with IMV, so unlike other proteins mentioned above is not EEV specific (Domi *et al.*, 2008).

1.3 Life cycle of VACV

1.3.1 Overview

The life cycle of VACV consists of virus binding, entry, uncoating, gene expression, DNA replication, virion assembly and virion release. An overview of the VACV life cycle is depicted in Fig. 1.1.

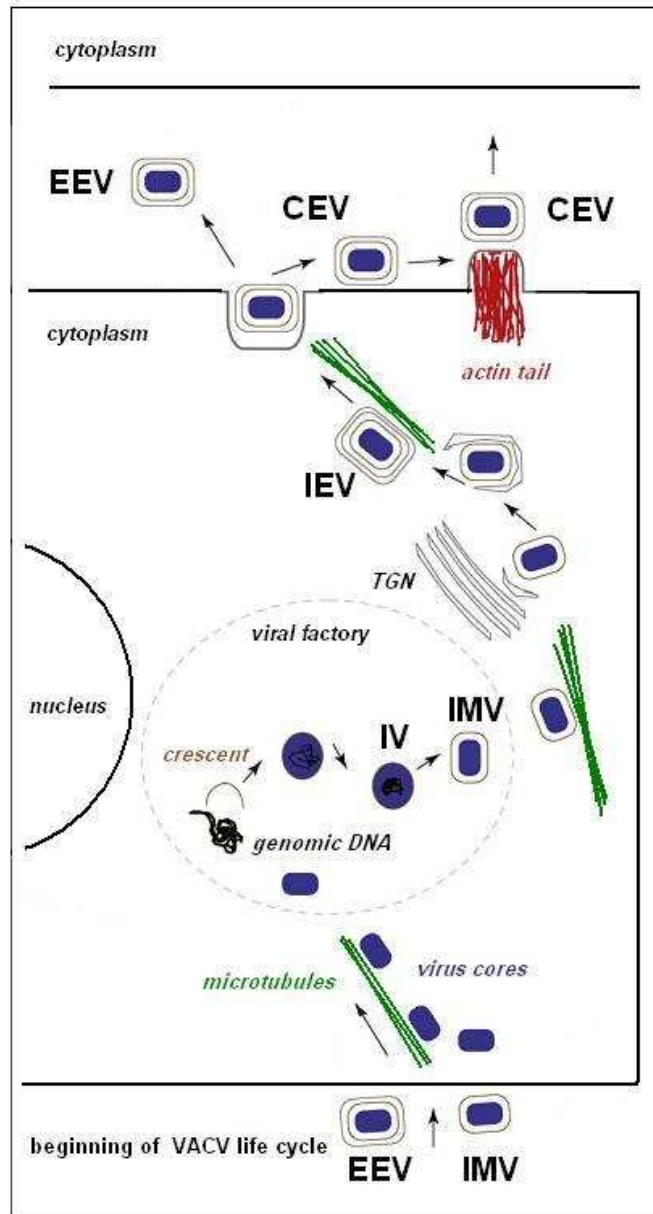


Fig. 1.1. VACV life cycle. EEVs or IMVs bind to the cell surface and lose their membrane(s). Virus cores are then released into the cytoplasm and are transported on microtubules to perinuclear sites. Early mRNAs are transcribed, the cores are uncoated and DNA is replicated. IVs are assembled and processed to produce IMVs in viral factories. Some IMVs are transported on microtubules and are wrapped by membranes from the early endosomes or the TGN to form IEVs. IEVs are then transported to the cell surface on microtubules. The outer membrane of IEVs fuses with the plasma membrane forming CEVs at the cell surface. Polymerisation of an actin tail below the CEV can occur to push the CEV to a neighbouring cell or EEV is released. (Adapted from (Roberts & Smith, 2008)).

1.3.2 VACV binding and entry

The first step of infection is the binding of VACV to permissive cells. When studying VACV binding and entry it is important to define which form of virus, IMV or EEV, is being studied because these have different surface proteins and are surrounded by different numbers of membranes. It is established that IMV and EEV bind to different receptors (Vanderplasschen *et al.*, 1998a) although the nature of these remain unknown. A claim that VACV binds to the epidermal growth factor receptor (Brown *et al.*, 1985) has been refuted (Eppstein *et al.*, 1985). IMV attachment to cells is partially mediated through viral membrane proteins such as D8, H3 and A27 which bind glycoaminoglycans (GAGs), though none of them is essential individually (Chung *et al.*, 1998; Hsiao *et al.*, 1998; 1999; Lin *et al.*, 2000). The binding of IMVs to cells via GAGs was also shown to be cell-type specific and non-essential for IMV entry (Carter *et al.*, 2005). In addition, on some cell types, A26 was found to bind laminins and this could also facilitate entry (Chiu *et al.*, 2007).

After binding, the IMV and EEV particles must penetrate into the cytosol and lose their membrane(s) in the process. The IMV enters by fusion with either the plasma membrane in a pH-independent manner (Carter *et al.*, 2005) or by endocytosis followed by fusion with the endosomal membrane in a low pH-dependent manner (Townesley *et al.*, 2006). The use of each pathway is determined by the virus strain and cell-type (Moss, 2007). In addition, binding of IMV to cells can induce cell blebbing on infected cells, which was claimed to mimic apoptotic bodies allowing entry by macropinocytosis (Mercer & Helenius, 2008). However, some of these data were refuted by Laliberte and Moss (2009) who confirmed the importance of phospholipids present on IMVs for VACV entry, but showed that this was not phosphatidylserine specific (Laliberte & Moss, 2009).

After binding to cells, a membrane fusion complex consisting of eight IMV transmembrane proteins (A16, A21, A28, G3, G9, H2, J5, and L5) is responsible for the fusion of the virus and cell membrane (Senkevich *et al.*, 2005). More recently, it was demonstrated that F9 and I2 are also required for entry (Brown *et al.*, 2006; Nichols *et al.*, 2008). In contrast, EEVs are surrounded by an additional membrane and both membranes must be shed before replication can commence. An explanation of this conundrum was provided by Law *et al.*, (2006), who demonstrated that when an EEV binds to the cell surface, at the point of contact its outer member is disrupted in a non-fusogenic manner. This enables the IMV particle within to make contact with the plasma membrane, fuse and enter (Law *et al.*, 2006). In addition, it has been proposed that EEV particles can be endocytosed and the outer membrane disrupted by low pH in

endosomes (Ichihashi, 1996; Vanderplasschen *et al.*, 1998a; Townsley & Moss, 2007). Consequently, the IMV within the EEV becomes exposed and enters the cell. Viral cores are then transported to perinuclear sites on microtubules (Carter *et al.*, 2003) and enzymes present in the core lead to the transcription of early genes.

1.3.3 VACV gene expression

VACV mRNAs are capped and polyadenylated like eukaryotic mRNAs (Wei & Moss, 1975). The VACV transcriptional complex includes the virally-encoded DNA-dependent RNA polymerase, transcription factors, a capping enzyme and a poly (A) polymerase (Broyles & Moss, 1986). VACV genes are expressed in a strictly controlled manner and there are early, intermediate and late classes (reviewed in (Broyles, 2003)) with the expression of genes in each class determined by specific transcription factors (Sanz & Moss, 1999). Notably, VACV genes can also be expressed constitutively by combining early and late promoters (Vos & Stunnenberg, 1988; Garces *et al.*, 1993). Recently, a new sub-class of 35 immediate-early genes was identified and defined as being expressed by 30 min post-infection (p.i.), though a genetic basis for this classification is lacking (Assarsson *et al.*, 2008). Genes in this category are implicated in immune evasion and DNA replication. VACV early genes include genes encoding proteins involved in DNA replication, virulence factors, and factors required for intermediate gene transcription (Broyles, 2003). Early genes are expressed by 1 h p.i. and their expression peaks at 2 h p.i. Only a few genes are expressed at the intermediate stage. They include factors required for the expression of late genes and these are expressed at 1.5 h p.i., reach a peak at 2 h but decline rapidly later (Baldick *et al.*, 1992). Late genes mainly encode virus structural proteins and factors required for early gene transcription that are packaged in virus cores. These genes start to be expressed between 2 and 4 h p.i. and their expression continues to 24 h p.i. (Assarsson *et al.*, 2008). Promoters of early, intermediate and late genes contain specific consensus sequences. These are schematised and described in Fig 1.2. Additionally, for early genes, transcription termination occurs approximately 20-50 nucleotides downstream of a T₅NT sequence with N being any base (Rohrmann *et al.*, 1986; Yuen & Moss, 1987; Piacente *et al.*, 2008). Interestingly, late gene mRNAs are long and heterogeneous in length with undefined 3'ends because the late transcriptional machinery does not recognise the early transcription termination signal (Yuen & Moss, 1987).

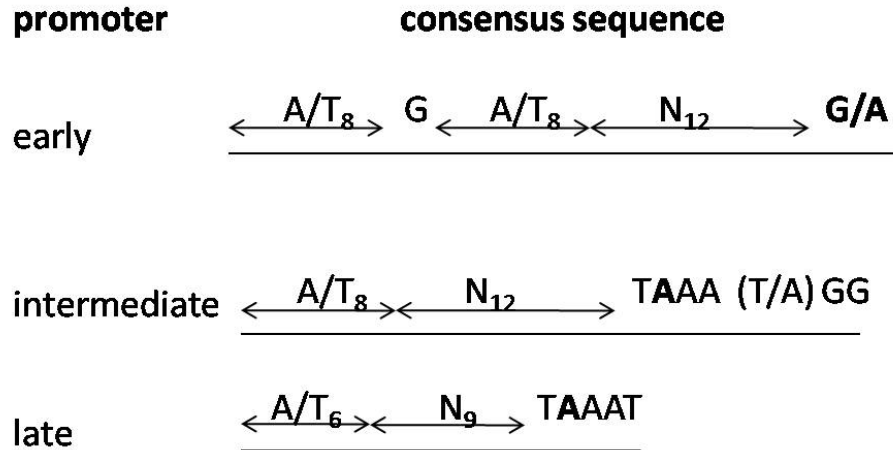


Fig. 1.2. Consensus sequence elements in VACV early, intermediate and late promoters. ‘N’ indicates positions where any of the four nucleotides may be present and the letter in bold indicates the transcriptional start site. Numbers represent the number of continuously repeating residues (adapted from (Broyles, 2003)).

1.3.4 VACV DNA replication

The arrest of early mRNA transcription is associated with uncoating of the core and DNA release (Woodson, 1967). The replication of viral genome then starts and takes place in viral factories formed in the cytoplasm (Cairns, 1960; Harford *et al.*, 1966). This event also signals the start of intermediate and late mRNAs production. DNA replication starts 1 to 2 h p.i. and yields approximately 10,000 genomic copies per cell, about half of which are incorporated into new virions (Salzman, 1960; Joklik & Becker, 1964). The viral DNA polymerase (E9), nucleoside triphosphatase (D11), serine threonine protein kinase (B1) and DNA glycosylase (D4) are required for genomic DNA replication and were identified by studies of temperature sensitive VACV mutants that were impaired in DNA synthesis (Lackner *et al.*, 2003).

The currently proposed model for VACV replication starts with the introduction of a nick in a single DNA strand near either of the terminal genomic hairpins loops to expose a free 3’ hydroxyl group (DeLange & McFadden, 1990). This serves as a self-primer for the VACV DNA polymerase that copies the template to the end of the incompletely base-paired terminal hairpin. The old and nascent strands are then denatured and refolded to form two hairpin termini. The 3’OH of the newly formed hairpin loop is then itself used as a primer allowing replication to

continue along the rest of the genome. During VACV DNA replication, concatemers are formed in head-to-head or tail-to-tail fashion. These are then resolved into unit length genomes by enzymes that recognise a palindromic structure including the ATTTAGTGTCTAGAAAAAAA concatemer resolution sequence (Merchinsky & Moss, 1989).

1.3.5 VACV assembly, transport and release

The initial step of virus assembly is the appearance of crescent-shaped structures within viral factories which later extend to form the non-infectious immature virus (IV). The IV particle contains the virus core, DNA genome and enzymes required for transcription of early genes. IMVs are formed from IVs when the DNA-nucleoprotein complex condenses and several capsid proteins are cleaved proteolytically (Moss & Rosenblum, 1973), reviewed in (Condit *et al.*, 2006).

Some IMVs are transported on microtubules (Sanderson *et al.*, 2000; Ward, 2005) to sites close to the cellular microtubule organising centre (MTOC) where they are wrapped in an endosome or TGN-derived double layer of membrane to form intracellular enveloped virus (IEV) (Tooze *et al.*, 1993; Schmelz *et al.*, 1994). IEVs are also transported on microtubules to the cell periphery (Geada *et al.*, 2001; Hollinshead *et al.*, 2001; Rietdorf *et al.*, 2001; Ward & Moss, 2001) where the outer membrane fuses with the plasma membrane to form cell-associated enveloped virus (CEV) by exocytosis (Smith & Law, 2004). Some CEVs induce actin tail formation beneath the plasma membrane and this pushes these virions away from the cell and into neighbouring cells (Frischknecht *et al.*, 1999), while others are released into the medium as EEVs that mediate long-range spread of virus (Law & Smith, 2004).

1.4 Effect of VACV infection on cells

Cells infected with VACV undergo morphological, metabolic and functional changes and the combination of these events results in cell death and is called the cytopathic effect (CPE) (Bablanian, 1970). This includes rounding and cell-cell dissociation which are dependent on early viral gene expression (Bablanian *et al.*, 1978) as well as cytoskeleton alterations affecting microtubules, actin and intermediate filaments (Meyer *et al.*, 1981; Ferreira *et al.*, 1994; Ploubidou *et al.*, 2000; Schepis *et al.*, 2006). Additionally, VACV infection induces cell motility

after early gene expression and the development of projections by extension and condensation of lamellipodia from the cell body (Sanderson *et al.*, 1998).

VACV infection also causes downregulation of host DNA replication, RNA transcription and protein synthesis. Cellular DNA replication is inhibited by 1-2 h p.i. (des Gouttes Olgiati *et al.*, 1976). Transcription of cellular genes by RNA polymerase II is inhibited and cellular mRNAs are also rapidly degraded (Puckett & Moss, 1983; Rice & Roberts, 1983). Host mRNA translation is also negatively regulated by mRNA decapping activity of the VACV D10 protein which exposes the RNA to exonucleases (Parrish *et al.*, 2007). DNA microarray studies have also demonstrated that expression of host genes involved in essential cellular processes including replication, transcription, translation, apoptosis and proteosomal degradation are downregulated after VACV infection (Guerra *et al.*, 2003).

1.5 Cell restriction and host range genes

VACV has a relatively broad host range in cell culture and can replicate in most mammalian cells except Chinese hamster ovary cells (CHO) (Moss, 1974; McFadden, 2005). In CHO cells, the arrest in replication occurs at the stage of intermediate gene expression (Ramsey-Ewing & Moss, 1998). VACV genes *K1L* and *C7L* are host range genes that are needed for replication in human cells (Gillard *et al.*, 1986; Rosel *et al.*, 1986; Perkus *et al.*, 1990). Interestingly, *K1L* mutants are also unable to replicate in rabbit kidney cells 13 (RK13) due to a blockage in the expression of early proteins but can replicate in chicken embryo fibroblasts (CEFs) and baby hamster kidney 21 (BHK-21) cells (Ramsey-Ewing & Moss, 1995; Carroll & Moss, 1997).

VACV genes *E3L* and *K3L* encode proteins that inhibit the action of the interferon (IFN)-induced protein kinase R (PKR) (Davies *et al.*, 1993; Rivas *et al.*, 1998) are also host range genes. The replication of an *E3L* deletion mutant was restricted in Vero and Henrietta Lacks (HeLa) cells (Beattie *et al.*, 1996) and a lack of *K3L* led to incomplete replication in BHK-21 cells (Langland & Jacobs, 2002). The absence of PKR expression in HeLa cells led to the restoration of replication of *E3L* mutants in those cells (Zhang *et al.*, 2008).

1.6 Overview of host immune responses to viral infection

The host immune response to infection can be divided into the innate and acquired components. The innate response is rapid and deployed in a non-antigen specific manner and does not confer long lasting protective immunity but acts as the first line of defence against pathogens (Tosi, 2005). Innate immune induction relies also on pattern-recognition receptors (PRRs) that recognise conserved pathogens-associated molecular patterns (PAMPs) as non-self. PRRs include membrane receptors such as the Toll-like receptors (TLRs) and various intracellular sensors such as retinoic-acid-inducible (RIG)-like helicases (RLH) (reviewed in (Medzhitov, 2009)) or the DNA-dependent activator of IFN (DAI) (Takaoka *et al.*, 2007). PRRs are particularly expressed in epithelial or immune cells (e.g. macrophages and dendritic cells (DCs)) but some are present in many cell types. PAMPs can be nucleic acid, proteins or lipids and their detection leads to induction of anti-viral (or other pathogen) innate immune signalling pathways. These pathways which are summarised in Fig. 1.3 consist of a cascade of biochemical events that result in the activation of transcription factors that include the activator protein 1 (AP-1), nuclear factor kappa B (NF- κ B) and IFN regulatory factors (IRF) families. These regulate expression of IFNs, chemokines and inflammatory cytokines, which orchestrate the inflammatory and immune response against viruses. IFNs are produced by infected cells and act in an autocrine and paracrine way to establish an anti-viral state through induction of genes such as PKR, oligoadenylate synthetase (OAS) and MX. These restrict virus replication and spread by targeting processes such as translation. Cytokines and chemokines recruit and activate the effectors of the innate immune response which include antigen presenting cells (APCs like DCs and macrophages) and natural killer cells (NK) cells. NK cells play a key role in anti-viral immunity by lysing infected cells when they detect alteration or downregulation of major histocompatibility complex (MHC) class I (MHC-I) molecules (Vivier *et al.*, 2008). Another key effector of innate immunity is the alternative pathway of the complement system which recognises PAMPs directly and consists of a biochemical cascade of cleavages leading to lysis of virion or infected cells, or resulting in targetting them for phagocytosis by opsonisation (Atkinson & Frank, 2006).

The adaptive immune response is directed at specific antigens from pathogens and encompasses the cell-mediated and humoral responses, which are mediated by T and B cells, respectively. APCs present antigens to T cells in local lymph nodes, resulting in the activation of adaptive immunity. Helper T-cells (cluster of differentiation antigen (CD)4+) can recognise antigens presented on MHC class II (MHC-II) molecules and, in the presence of specific cytokines,

differentiate into two subtypes. These are T helper (Th) 1 and 2 and these lead to the induction of cell-mediated and humoral responses, respectively (reviewed in (Chtanova & Mackay, 2001)). Cell-mediated immunity includes cytotoxic T-cells (CD8+) which recognise peptide epitopes presented on an MHC-I molecules and induce the death of virus-infected cells. Humoral immunity is mediated by B cells producing antibodies (Ab)s specific to foreign antigens. Abs can directly neutralise pathogens and also mediate their destruction by opsonisation leading to uptake by phagosomes or by recruitment and activation of complement.

Fig. 1.3. Antiviral innate immune induction signalling pathways. This diagram depicts a simplified representation of signalling that leads to the induction of the anti-viral innate immune response. The pathways mediated by TLR-2/IL-1, TLR-3, RIG-I and DAI are illustrated. Engagement of these PRRs by PAMPs leads to the recruitment of PRR adaptor proteins such as myeloid differentiation factor 88 (MyD88), TIR domain-containing adapter-inducing IFN- β (TRIF) and IFN- β promoter stimulator 1 (IPS-1) and biochemical cascades involving several intermediate proteins lead to the activation of the AP-1, NF- κ B and IRF-3 families of transcription factors. This activation is achieved after these transcription factors are phosphorylated and translocated to the nucleus where they bind the promoters of IFN and pro-inflammatory genes (e.g. IL-6, tumour necrosis factor (TNF)- α) and activate their expression. These cytokines are released from cells and can bind neighbouring ones. IFN- β can bind the IFNAR (R1 and R2) to induce the activation of many IFN-stimulated regulatory element (ISRE)-dependent anti-viral genes (e.g. PKR, MX-1) as indicated. Other cytokines such as TNF- α and IL-1 can also bind their receptors on cells and induce signalling to activate the expression of pro-inflammatory genes. 2' 5' OAS = 2'-5'-oligoadenylate synthetase; FADD = Fas-associated protein with death domain; I κ B = inhibitor of NF- κ B; IKK = inhibitor of nuclear factor- κ B kinase; IL-1R = interleukin 1 receptor; IRAK2 = IL-1 receptor associated kinase; ISGF3 = IFN stimulating gene factor 3; Jak1 = Janus activated kinase 1; MAPK = mitogen activated protein kinase; NAP-1 = nucleosome assembly protein 1; R1 and R2 = IFN receptors 1 and 2; RIP-1 = receptor interacting protein 1; STAT = signal transducers and activators of transcription; TAB = TAK-binding protein; TAK = TNF-associated kinase; TBK1 = TRAF-associated NF- κ B activator binding kinase 1; TNFR = tumour necrosis factor receptor; TRAF2/6 = TNFR associated factor 2 or 6; Tyk2 = Tyrosine kinase 2. Others abbreviations are as defined in the preceding text. Adapted from (Meurs & Breiman, 2007).

1.7 PRRs

1.7.1 TLRs and VACV infection

TLRs are a family of at least twelve proteins in mammals containing a Toll/interleukin (IL)-1 receptor (TIR) homology domain and each one of them recognises specific PAMPs (see (Kumar *et al.*, 2009) for recent review). Once a TLR engages with a PAMP, its TIR domain recruits an

adaptor protein itself containing a TIR domain such as MyD88 (all TLRs except TLR3) and TRIF (for TLRs 3 and 4). The activation of these adaptors induces signalling cascades involving several proteins to lead to the activation of NF κ B, IRF or AP-1 transcription factors as exemplified in Fig 1.3 for TLR2 and TLR3. Work with TLR2 knock-out mice has shown that the secretion of pro-inflammatory cytokines by DCs in the context of VACV infection is TLR2/MyD88-dependent and this is required for innate immune induction to control VACV infection (Zhu *et al.*, 2007). TLR3 recognises dsRNA, which means it could recognise hybridised VACV complementary transcripts. TLR3 is located in the luminal side of the endosome and VACV RNA is cytoplasmic but it can be envisaged that after phagocytosis of infected cells by DCs and macrophages, phagosomes fuse with endosomes which could allow detection of viral RNA originating from infected cells. Interestingly, when TLR3 knock-out mice were infected intranasally with VACV they suffered a less severe infection than their wild-type counterparts and this was attributed to the role of TLR3 in the induction of IL-6 and TNF- α which may promote adverse effects of VACV *in vivo* (Hutchens *et al.*, 2008a). In contrast, TLR4 was implicated in protection against VACV in an intranasal *in vivo* mouse model using wild-type mice and mice with a non-functional TLR4 mutant (Hutchens *et al.*, 2008b). Finally, TLR9 which is located in the endosomal membrane like TLR3 has been shown to be important for recognition of VACV by DCs (Samuelsson *et al.*, 2008).

1.7.2 Intracellular PRRs

Intracellular PRRs capable of detecting viral infections include DNA-sensing molecules such as DAI (Takaoka *et al.*, 2007), or RLHs such as RIG-I and melanoma differentiation-associated gene (Mda)5 (Asahina *et al.*, 2008). DAI binds Z-DNA in the cytoplasm and undergoes homodimerisation and phosphorylation leading to the recruitment of TRAF-associated NF- κ B activator binding kinase (TBK)1 and IRF3 (Wang *et al.*, 2008). RIG-I and Mda5 contain two N-terminal cysteine-aspartic acid protease (caspase) activation and recruitment domains (CARD) and a C-terminal RNA helicase domain. Binding of RNA triggers a conformational change which allows association with the adaptor molecule IPS-1 through its own CARD domain (Kawai *et al.*, 2005). Subsequently, IPS-1 associates with TRAF3 which recruits and activates TBK1 and IKK- ϵ which in turn activate IRF3 and IRF7 (Kawai *et al.*, 2005). The DAI and RIG-I pathways described here are both illustrated in Fig. 1.3.

Additionally the dsRNA-dependent PKR plays a key role in the anti-viral response. It contains two dsRNA binding domains and a serine/threonine kinase domain that phosphorylates eukaryotic initiation factor (eIF)2 α leading to translational arrest of cellular and viral mRNAs (Garcia *et al.*, 2007). Additionally, PKR seems to play an adaptor role (independent of RNA binding) in various pathways leading to NF- κ B activation (Deb *et al.*, 2001; Takada *et al.*, 2007). In particular PKR acts downstream of TLR3 signalling when recruited by TAK1 (Williams, 2001).

The 2'-5'-OAS is another dsRNA dependent enzyme with a key anti-viral role. It converts adenosine triphosphate (ATP) to 2'-5'-linked oligoadenylates with a 2'-5' phosphodiester bond instead of a 3'-5' one (Hovanessian, 1991). These small molecules activate ribonuclease (RNase) L which cleaves mRNAs, tRNA, rRNAs and leads to inhibition of protein synthesis (Silverman, 2007).

It is worth noting that neither PKR nor 2'-5' OAS has been conclusively implicated in the primary response to cytoplasmic dsRNA.

1.8 IFNs

IFNs are important in host defence against virus infection. They were discovered over 50 years ago as an agent that “interfered” with viral replication (Isaacs & Lindenmann, 1957). IFNs are a family of structurally related cytokines sub-divided into three classes: IFN types I, II and III and are involved in innate and adaptive immune anti-viral responses. Type I IFNs include several subtypes of IFN- α , IFN- β and several others, and IFN- γ is the only type II IFN (reviewed in (Pestka *et al.*, 2004)). Type III IFNs were discovered more recently and induce similar effects to type I IFNs but they are less widely expressed and bind a different receptor (Kotenko *et al.*, 2003). Type I IFNs are induced through PRR signalling and once secreted from cells bind a receptor composed of two subunits IFNAR1 and IFNAR2. The binding of IFN type I to the IFNARs leads to the induction of over 300 IFN-stimulated genes (ISGs) which include PRRs (for instance, RIG-I, Mda5 and TLR3), antiviral proteins (for instance, PKR and 2'-5' OAS) as well chemokines (for instance C-X-C motif ligand (CXCL)10 and C-C motif ligand (CCL)5). In the first phase of the IFN response, IFN- β is produced and released from cells to bind the type I IFN receptors. This then triggers a second phase in which various subtypes of IFN- α are secreted in a

positive feedback loop. IFN- γ is secreted by T cells to enhance the Th1 response, and by macrophages and NK cells to activate other immune cells.

1.8.1 IFN-induced signalling pathway

The intracellular domains of the IFNARs are associated with Tyk2 and Jak1 which are activated when IFNs bind their receptors. Once activated, these kinases phosphorylate STAT1 and STAT2 (Darnell *et al.*, 1994). Tyrosine phosphorylation of STATs leads to the formation of ISGF3 or IFN- γ -activated factor (GAF) complexes. ISGF3 is a heterotrimeric complex of tyrosine-phosphorylated STAT1, STAT2 and another transcription factor, IRF9 (or p48), whereas GAF is a homodimer of phosphorylated STAT1. GAF and ISGF3 are translocated to the nucleus where they bind IFN- γ -activated sequence (GAS) (Decker *et al.*, 1991; Lew *et al.*, 1991) and ISRE, respectively (Kessler *et al.*, 1990). This results in transcription of ISGs. The ISRE induction pathway is illustrated in Fig.1.3. There are also alternative pathways of ISRE induction through other signal transducers in a STAT-dependent or -independent manner and such components include extracellular signal regulated kinase (ERK)2 (David *et al.*, 1995) or Src homology-2 domain containing phosphatase (SHP)-2 (You *et al.*, 1999). The p38 MAPK pathway also induces IFN type I mediated signals (Katsoulidis *et al.*, 2005). Although they bind different receptors, type III IFNs trigger similar signalling pathways to the type I IFNs. IFN- γ binds its own dimeric receptor IFN- γ receptor (IFNGRI/II) which associates with Jak1 and Jak2 and mainly signals through GAF (Pestka *et al.*, 2004).

1.9 NF- κ B pathway

The NF- κ B family is a group of evolutionary conserved transcription factors that regulate expression of genes involved in various physiological processes including the inflammatory response, cell proliferation and apoptosis (reviewed in (Ghosh & Hayden, 2008)). In mammalian cells, these include p50 (NF- κ B1), p52 (NF- κ B2), p65 (REL-A), REL-B and REL and these components can homo or heterodimerise through the Rel homology domain they all share. In the absence of stimulation, dimers are bound to members of the inhibitor of NF- κ B (I κ B) family (of which I κ B α is the prototypical member) which retains them in an inactive form in the cytoplasm. P50 and p52 are derived from precursors (P100 and p105, NF- κ B2 and NF- κ B1, respectively)

which also act as I κ B proteins. NF- κ B activation occurs after stimulation with pro-inflammatory cytokines (e.g. TNF- α), microbial products (e.g. LPS), viruses or radiation to trigger signalling cascades. The pathway of NF- κ B activation after TNF- α and IL-1 stimulation is illustrated in Fig. 1.3. All signals then converge on the IKK complex which comprises two effector subunits (IKK- α and IKK- β) and one regulatory subunit (IKK- γ , NF- κ B essential modulator, NEMO). In the classical pathway, IKK- β is activated and phosphorylates I κ B which is then ubiquitinated and targeted for degradation by the proteasome. This causes release of the NF- κ B dimer which is then translocated to the nucleus where it binds to κ B sites in the promoters of target genes and activates their expression. In the alternative pathway, which is triggered by certain TNF family members, IKK- α activates and phosphorylates p100 which leads to its ubiquitination and the degradation of its C-terminal I κ B half by the proteasome. The NF- κ B dimer is then released and transported to the nucleus. The NF- κ B combination of different homo and heterodimers are selectively regulated by I κ B proteins, and this a primary source of transcriptional specificity (reviewed in (Hoffmann *et al.*, 2006)).

1.10 VACV immunomodulation

In response to the host immune response, VACV has acquired a wide range of proteins that interfere with its induction or activity. These VACV immunomodulators can be divided into two categories. Firstly, there are secreted proteins targeting IFNs, cytokines and chemokines outside the cell, for instance, VACV protein A41 binds chemokines (Ng *et al.*, 2001; Clark *et al.*, 2006). Secondly, there are intracellular proteins targeting signalling pathways and apoptosis, or synthesizing steroid hormones e.g. VACV protein A44 is a steroid biosynthetic enzyme (Moore & Smith, 1992; Reading *et al.*, 2003a). These will be described in more detail in the following sections.

1.10.1 Complement regulation

The VACV *C21L* gene from WR encodes a secreted protein called a VACV complement control protein (VCP), which is mainly composed of four short consensus repeats (SCRs) that are typical of complement regulators (Kotwal & Moss, 1988b; Kotwal *et al.*, 1990; McKenzie *et al.*, 1992; Mullick *et al.*, 2005). This protein inhibits the classical and alternative pathways of complement

activation by binding C3b and C4b components of complement, and contributes to VACV virulence (Isaacs *et al.*, 1992; Sahu *et al.*, 1998). B5, a component of the EEV outer membrane also has SCRs but there is currently no evidence that it has complement inhibitory activity (Takahashi-Nishimaki *et al.*, 1991). However, EEV forms of VACV are resistant to complement-induced neutralisation due to the incorporation of cellular regulators of complement to the outer membrane (Vanderplasschen *et al.*, 1998b).

1.10.2 Inhibition of IFNs and cytokines by VACV-encoded proteins

VACV can also interfere with a wide range of cytokines including type I and II IFNs, IL-1 related cytokines (IL-1 β , IL-18,) as well as TNF- α and CC chemokines.

B18 is a type I IFN-binding protein that is present in the cell supernatant and on the surface of infected cells (Colamonici *et al.*, 1995; Symons *et al.*, 1995; Alcamí *et al.*, 2000). This protein binds to IFN- α/β with high affinity, thus preventing IFN signalling. It also binds both infected and uninfected cells and protects them from the anti-viral effects of type I IFN thus allowing viral replication in those cells (Symons *et al.*, 1995; Alcamí *et al.*, 2000). *B8R* encodes a secreted protein with sequence similarity to the binding domain of cellular IFN- γ receptors and which binds to and inhibits the activity of IFN- γ by preventing its interaction with cellular receptors (Alcamí & Smith, 1995). B8 can inhibit the biological activities of human, bovine, equine, ovine and rat IFN- γ but not murine IFN- γ (Alcamí & Smith, 1995; Mossman *et al.*, 1995; Symons *et al.*, 2002b). Consistently, loss of B8 did not affect VACV virulence in mice (Symons *et al.*, 2002b). IL-18 works synergistically with IL-12 to induce IFN- γ which promotes a Th1 response with cytotoxic T cells and NK cell activation (Arend *et al.*, 2008). VACV encodes an IL-18-binding protein from the *C12L* gene (Smith *et al.*, 2000) which inhibits IL-18 activity thus inhibiting IFN- γ secretion by activated macrophages and it also promotes virulence (Symons *et al.*, 2002a; Reading & Smith, 2003). Most VACV strains do not express a TNF receptor, but three strains (USSR, Lister and Evans) encode a soluble and cell surface TNFR called CrmE (Saraiva & Alcamí, 2001; Reading *et al.*, 2002). CrmE binds and inhibits TNF- α and contributes to virulence in an *in vivo* murine model using VACV USSR (Reading *et al.*, 2002). Finally, VACV encodes an IL-1 receptor from gene *B15R* in VACV strain WR that binds IL-1 β but not IL-1 α (Alcamí & Smith, 1992). This receptor binds human or mouse IL-1 β with high affinity and prevents activation of the febrile response in mice infected intranasally (Alcamí & Smith, 1992).

All inhibitors described here are secreted proteins but VACV also encodes for H1 an intracellular protein which inhibits IFN signalling by dephosphorylating STAT1 (Mann *et al.*, 2008).

1.10.3 Chemokine inhibitors

Chemokines are small heparin-binding proteins that play an important role in the host defence against viruses by mobilising leucocytes to sites of infection. They are classified into C, CC, CXC or CX₃C subfamilies based upon the positions of conserved cysteines (Ransohoff, 2009). Several VACV strains encode a CC chemokine binding protein (vCKBP) (Graham *et al.*, 1997; Alcami *et al.*, 1998). vCKBP binds a wide range of CC chemokines (including CCL2, CCL3, CCL5 and CCL11) with high affinity to block recruitment of inflammatory cells in response to chemokines (Alcami *et al.*, 1998). The presence of vCKBP affected the outcome of infection with VACV in mice by reducing recruitment of inflammatory cells to lungs of VACV-infected mice (Reading *et al.*, 2003b). VACV *A4IL* which shares sequence similarity with vCKBP, binds of some CC chemokines (CCL21, CCL25, CCL26, and CCL28) with moderate affinity to inhibit their interaction with GAGs at the cell surface (Clark *et al.*, 2006; Bahar *et al.*, 2008).

1.10.4 PKR and OAS regulation

The VACV E3 protein prevents the activation of PKR (Chang *et al.*, 1992) and OAS (Rivas *et al.*, 1998) by binding dsRNA thus inhibiting the IFN-mediated antiviral response (Beattie *et al.*, 1995). Additionally, VACV encodes K3 which is a mimic of the N-terminal domain of eIF2 α (Beattie *et al.*, 1991). K3 acts as a pseudo substrate for PKR so that the phosphorylation of eIF2 α is prevented and protein synthesis can continue (Davies *et al.*, 1992; Kawagishi-Kobayashi *et al.*, 1997). Deletion of either K3 or E3 increases the sensitivity of VACV to IFN (Beattie *et al.*, 1995).

1.10.5 Apoptosis inhibitors

Apoptosis, or programmed cell death, is a process that can lead to elimination of virus-infected cells to restrict viral replication and spread and therefore is part of the innate immune response (reviewed in (Elmore, 2007)). Apoptosis can be induced by extracellular (for instance TNF or Fas binding) and intracellular stimuli (sensed by the mitochondrion, ER or Golgi complex) that cause

DNA damage or mitochondrial dysfunction. Apoptosis is mediated by cellular caspases and B-cell lymphoma 2 (Bcl-2) proteins. Caspases are present as inactive pro-enzymes that are activated in a proteolytic cascade by external stimuli leading to cell death (Thornberry, 1999). Alternatively, caspase activation can begin at the mitochondrion where internal apoptosis stimuli converge and result in the loss of mitochondrial membrane potential to lead to the release of proteins that initiate caspase activation (Budihardjo *et al.*, 1999). Bcl-2 proteins act as homo or heterodimers to regulate the release of both pro- and anti-apoptotic molecules from the mitochondrion. They are characterised by up to four sequences termed Bcl-2 homology (BH) (1-4) domains. The balance between apoptosis and cell survival is reliant on the relative levels of pro- and anti-apoptotic proteins.

VACV modulates apoptosis in several ways (Taylor & Barry, 2006). VACV strain WR B13 is a serpin that protects virus-infected cells from TNF- and Fas-mediated apoptosis and inhibits cleavage of pro-IL-1 β to its mature form (Kettle *et al.*, 1997). B22 is another VACV-encoded serpin which regulates apoptotic cascades by acting as pseudo substrate for target proteases (Kotwal & Moss, 1988a; Smith *et al.*, 1989; Kettle *et al.*, 1995). VACV proteins N1 and F1 inhibit the activity of pro-apoptotic Bcl-2 proteins. N1 is a virulence factor (Bartlett *et al.*, 2002) and is a Bcl-2-like protein that interacts with pro-apoptotic Bcl-2s and protects infected cells from staurosporine induced-apoptosis (Cooray *et al.*, 2007). F1 localises to the mitochondria where it inhibits the release of cytochrome c, a key intermediate of apoptosis (Wasilenko *et al.*, 2003). F1 is also a Bcl-2 protein and interacts with the BH3 domain of Bcl-2 proteins to inhibit apoptosis (Fischer *et al.*, 2006; Kvensakul *et al.*, 2008).

1.10.6 Steroid hormone synthesis

VACV gene *A44L* encodes a 3 β -hydroysteroid dehydrogenase which contributes to virulence as it synthesises steroid hormones that inhibit the inflammatory response (Moore & Smith, 1992; Reading *et al.*, 2003a). Mice infected intranasally with a deletion *A44L* virus displayed enhanced IFN- γ production and increased cytotoxic T cell activation (Reading *et al.*, 2003a).

1.10.7 Intracellular inhibitors of TLR signalling

VACV encodes many intracellular inhibitors of innate immune signalling pathways, including several that function downstream of TLRs such as A46 and A52 (Bowie *et al.*, 2000). A52

inhibits NF- κ B activation by interacting with IRAK2 and TRAF6 (Harte *et al.*, 2003; Keating *et al.*, 2007). A46 was predicted to contain a TIR domain (Bowie *et al.*, 2000) and targets the host TIR adaptors MyD88 and TRIF to lead to inhibition of NF- κ B activation (Stack *et al.*, 2005). In addition, A46 can also inhibit MyD88 adaptor like (MAL) and TRIF-related adaptor molecule (TRAM) which leads to TRIF-dependent IRF3 inhibition. However, it now seems unlikely that A46 has a TIR domain because A46 and A52 are related and A52 has a Bcl-2 fold (Graham *et al.*, 2008) and A46 is also predicted to be a Bcl-2 protein. B14 is a virulence factor (Chen *et al.*, 2006) and blocks intracellular signalling from IL-1R, TLR and TNFR by binding to the IKK- β subunit of the IKK complex leading to inhibition of NF- κ B (Chen *et al.*, 2008). N1 is both an apoptosis inhibitor (Cooray *et al.*, 2007) and an inhibitor of the NF- κ B pathway, at least downstream of the IL-1 receptor (DiPerna *et al.*, 2004; Graham *et al.*, 2008). The exact target for N1 remains unknown but it is believed to act somewhere between TRAF6 and the IKK complex (Graham *et al.*, 2008). M2 and K1 are two additional VACV proteins modulating the NF- κ B pathway. M2 inhibits NF- κ B activation by inhibiting the phosphorylation of ERK2 (Gedey *et al.*, 2006) however it must be noted that this protein is located exclusively in the ER unlike all the others discussed here (Hinthong *et al.*, 2008). K1 blocks NF- κ B by preventing its nuclear translocation through inhibiting I κ B α degradation (Shisler & Jin, 2004). K7 inhibits the TLR-dependent and -independent induction of IFN- β by interacting with the DEAD-box protein 3 (DDX3) RNA helicase to prevent TBK1 and IKK- ϵ from activating IRF3 and is also an inhibitor of NF- κ B (Schroder *et al.*, 2008). K7 will be discussed in more details at the end of this introduction. These signalling inhibitors are indicated in Fig 1.4 at the position in the pathways where they cause inhibition.

Some of these intracellular signalling inhibitors namely A46, A52, N1, B14, K7 are members of a family of VACV proteins first described in 1991 (Smith *et al.*, 1991). The structures of several of these proteins (N1, B14, A52 and K7) have been determined and are all Bcl-2 family members (Cooray *et al.*, 2007; Graham *et al.*, 2008; Kalverda *et al.*, 2009) but have important differences. N1 has an open groove on the surface that allows the binding of BH3 domains from pro-apoptotic Bcl-2 proteins (Cooray *et al.*, 2007). In contrast, A52, B14 and K7 have lost the surface groove suggesting they are not anti-apoptotic and this has been confirmed experimentally for B14 and A52 (Graham *et al.*, 2008). Instead, these proteins function as inhibitors of innate immune signalling pathways and the stable Bcl-2 fold is exploited by VACV to provide a surface for binding host signalling molecules.

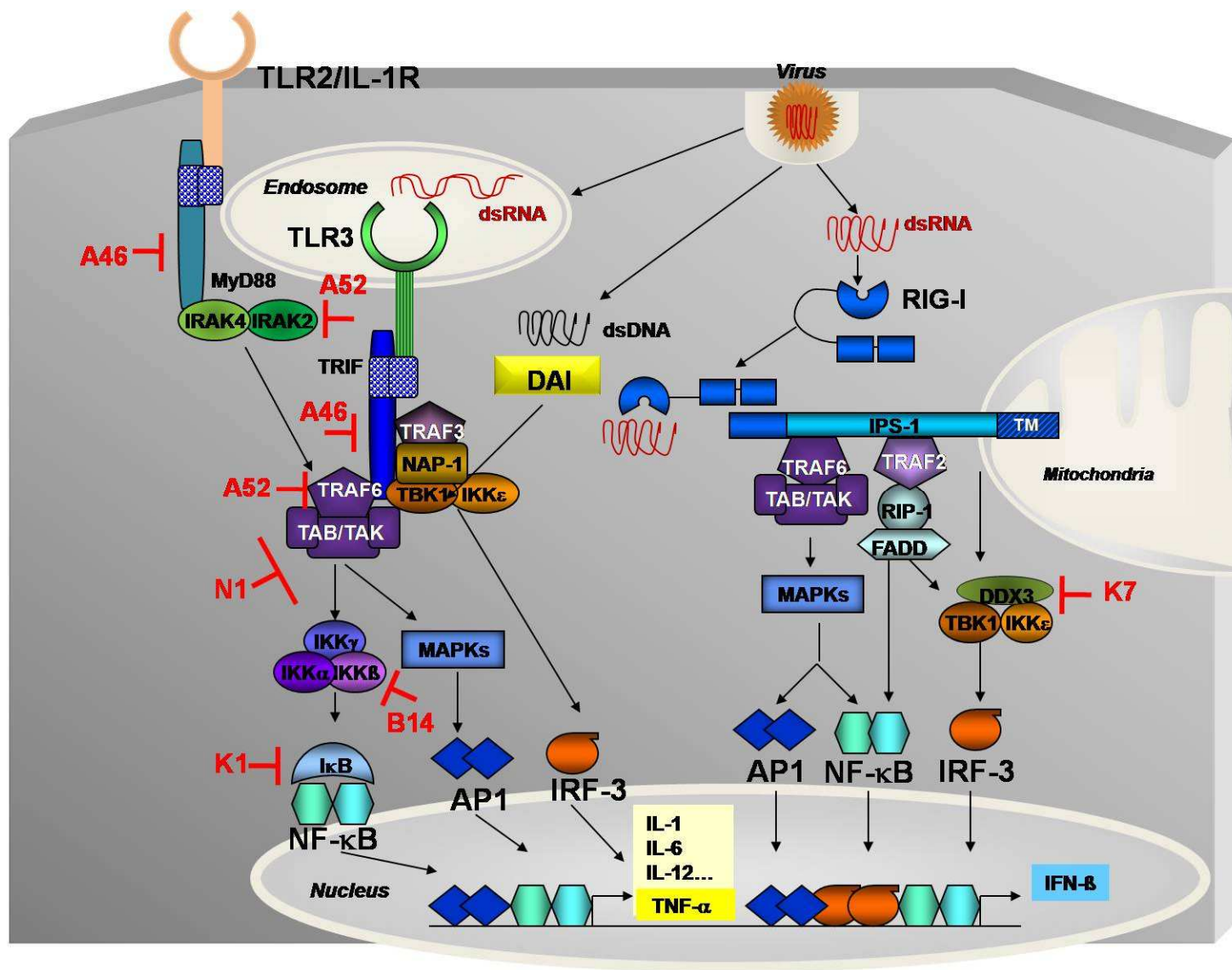


Fig. 1.4. VACV intracellular modulators of innate immune signalling pathways. VACV immunomodulatory proteins are shown at the sites where they have been demonstrated to inhibit the pathways. M2 is an ER membrane protein that has also been shown to block NF- κ B activation but is not represented on the figure (see text).

1.10.8 VACV and inhibition of peptide antigen presentation

Protein A35 from VACV WR contributes to virulence when the virus is administered by the intranasal or intraperitoneal route to mice (Roper, 2006; Rehm *et al.*, 2010). It was also found that A35 localized to endosomes and reduced the amount of antigenic peptide displayed by MHC-II molecules (Rehm *et al.*, 2009). In addition, A35 decreased VACV-specific T cell responses *in vivo* (Rehm *et al.*, 2009).

1.10.9 Summary of VACV immunomodulators

protein	function
E3	binds dsRNA preventing PKR and OAS activation and inhibition of protein synthesis and IFN antiviral response (Beattie <i>et al.</i> , 1995; Rivas <i>et al.</i> , 1998)
K3	eIF2 mimic which acts as pseudo substrate for PKR, enabling protein synthesis to continue (Beattie <i>et al.</i> , 1995; Kawagishi-Kobayashi <i>et al.</i> , 1997)
B13	serpin, inhibits caspase 1 and thereby apoptosis, and cleavage of pro-IL-1 β to mature cytokine (Kettle <i>et al.</i> , 1997)
B22	serpin inhibiting apoptosis (Kettle <i>et al.</i> , 1995)
F1	interferes with cytochrome c release from mitochondria and interacts with BH3 domain of Bcl-2 proteins to inhibit apoptosis (Kvansakul <i>et al.</i> , 2008)
N1	inhibits apoptosis and NF- κ B activation at the level of TRAF6 or downstream components but upstream of IKK complex (DiPerna <i>et al.</i> , 2004; Cooray <i>et al.</i> , 2007; Graham <i>et al.</i> , 2008)
A46	inhibits TLR-induced NF- κ B and IRF3 activation by targeting TIR-containing PRR adaptors e.g. MyD88 (Stack <i>et al.</i> , 2005)

A52	inhibits TLR-induced NF- κ B activation by targeting IRAK2 and TRAF6 (Harte <i>et al.</i> , 2003; Keating <i>et al.</i> , 2007)
K7	inhibits TLR-dependent and independent IRF-3/7 signalling through TBK1/IKK ϵ by binding DDX3 and inhibits TLR-induced NF- κ B activation by targeting IRAK2 and TRAF6 (Schroder <i>et al.</i> , 2008)
K1	inhibits NF- κ B activation by suppressing I κ B α degradation (Shisler & Jin, 2004)
M2	inhibits NF- κ B activation and is localised in ER (Gedey <i>et al.</i> , 2006)
B14	inhibits NF- κ B by targeting IKK- β (Chen <i>et al.</i> , 2008)
A44	3- β -hydroxysteroid dehydrogenase which synthesises steroid hormones inhibiting the inflammatory response (Moore & Smith, 1992; Reading <i>et al.</i> , 2003a)
A35	localises to endosomes and inhibits MHC-II antigen presentation (Rehm <i>et al.</i> , 2009)
H1	inhibits IFN signalling by dephosphorylating STAT1 (Mann <i>et al.</i> , 2008)
B18	binds and inhibits IFN- α/β (Alcami <i>et al.</i> , 2000)
B8	binds and inhibits IFN- γ (Alcami & Smith, 1995)
B15	binds and inhibits IL-1 β (Alcami & Smith, 1992)
C21	inhibits the classic and alternative pathways of complement activation by binding C3 and C4b (Mullick <i>et al.</i> , 2005)
C12	binds IL-18 and inhibits IL-18 -induced IFN- γ production (Reading & Smith, 2003)
A41	binds some CC cytokines (CCL21, CCL25, CCL26, and CCL28) with moderate affinity to inhibit their interaction with GAGs at the cell surface (Bahar <i>et al.</i> , 2008)
vCKBP	binds CCL2, CCL3, CCL5 and CCL11 and blocks recruitment of inflammatory cells to lungs of VACV-infected mice (Reading <i>et al.</i> , 2003b)

Table 1.1. Summary of VACV WR immunomodulatory proteins and their functions.

Shaded area indicates extracellular molecules

1.11 Biogenesis of MicroRNAs

MicroRNAs (miRNAs) are short (approximately 20-22 nt long) single stranded (ss)RNA sequences that regulate post-transcriptional gene expression. In human cells, these are initially transcribed by RNA polymerase II to produce capped and polyadenylated RNA precursors of variable length called primary miRNAs (pri-miRNAs) (Cai *et al.*, 2004; Lee *et al.*, 2004). These are then cleaved in the nucleus by RNase III enzyme Drosha and its dsRNA binding co-factor DiGeorge syndrome critical region 8 (DGCR8) into pre-miRNAs consisting of approximately 70 nt hairpin loops with characteristic 2 -nt 3' overhangs (Lee *et al.*, 2003; Denli *et al.*, 2004; Gregory *et al.*, 2004). Pre-miRNAs are exported from the nucleus and released into the cytoplasm by exportin 5 and its co-factor Ran which recognise the overhangs (Yi *et al.*, 2003). Pre-miRNAs are then cleaved by the RNase III enzyme Dicer and its co-factor Tar RNA binding protein (TRBP) to remove the terminal loop and produce duplex intermediate miRNAs (Hutvagner *et al.*, 2001; Chendrimada *et al.*, 2005). These duplexes are then loaded into the miRNA induced silencing complex (RISC), which is incompletely defined but whose core components are the Argonaute family proteins (Ago 1-4). Here, the miRNA strand of the duplex which exhibits less stable base pairing at the 5' end is cleaved into a ssRNA mature form and the other strand is released and degraded (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003; Gregory *et al.*, 2005).

The effects of miRNAs on mRNA translation are mediated by the binding of their seed region (nt 2-8 from the 5' end) to a homologous sequence in the 3' untranslated region (UTR) of the target mRNA. This primes RISC to act by either cleaving and degrading the target mRNA if there is extensive sequence identity between the miRNA and this target (Zeng *et al.*, 2003), or, more commonly, by inhibiting translation when there is only partial sequence complementarity (Zeng *et al.*, 2002). The miRNA biogenesis pathway is illustrated in Fig. 1.5.

MiRNAs regulate the expression of genes involved in wide range of processes including development, differentiation, cancer and disease and their expression is often tissue specific (Bartel, 2004). Single miRNAs can target multiple genes and single genes can be targeted by several different miRNAs (Krek *et al.*, 2005). MiRNAs can also be encoded from intron, exons or non-coding regions (Bartel, 2004; Rodriguez *et al.*, 2004). Approximately half of mammalian miRNA are thought to be within known transcriptional units and approximately 36 % are part of clusters within either intergenic or intronic regions (Rodriguez *et al.*, 2004; Olena & Patton, 2010). Exonic miRNAs are the rarest (Olena & Patton, 2010).

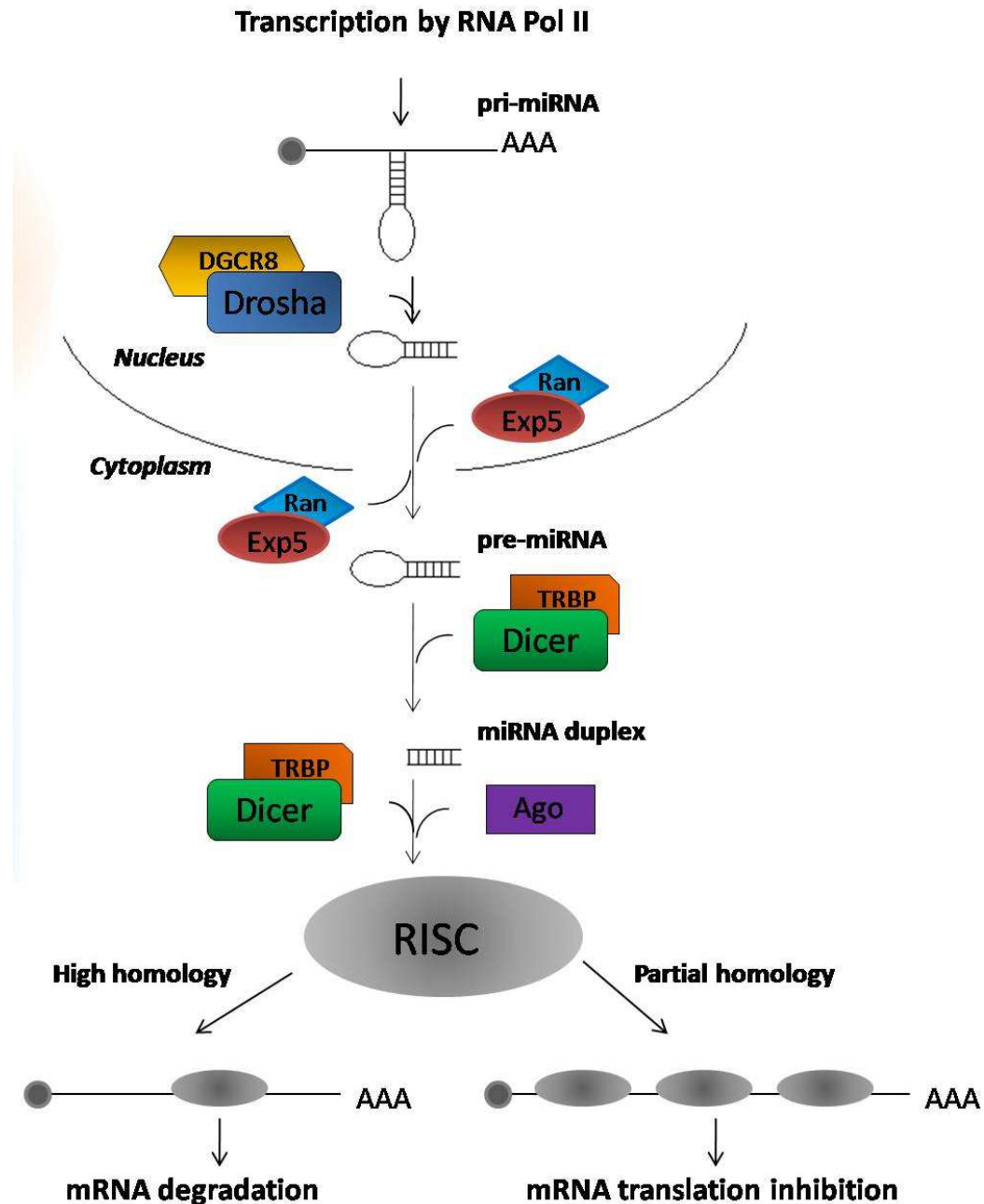


Fig. 1.5. Biogenesis of human miRNAs. This diagram is a simplified overview of the miRNA biogenesis pathway. Pri-miRNAs are transcribed by RNA polymerase II and cleaved by Drosha–DGCR8 in the nucleus. The resulting hairpin, the pre-miRNA, is exported from the nucleus by Exportin-5 (Exp5) and its co-factor Ran. In the cytoplasm, the RNase Dicer and co-factor TRBP cleave the pre-miRNA hairpin to its mature miRNAs. The functional strand of the mature miRNA is loaded together with Ago proteins into RISC. RISC can silence target mRNAs through mRNA cleavage or translational repression (adapted from (Cullen, 2006)).

1.12 MicroRNAs and the immune response

MiRNAs are differentially regulated in immune cells (Chen *et al.*, 2004). Several host miRNAs such as miR-146a, miR-155 and miR-9 were found to be differentially expressed in response to TIR and TNF- α receptor activation. These miRNAs downregulated TIR signalling components, including NF- κ B1 (by miR-9), TRAF6, IL-1 and IRAK1 (all by miR-146a), TAB2 and IKK- ϵ (by miR-155) (Taganov *et al.*, 2006; O'Connell *et al.*, 2007; Tili *et al.*, 2007; Bazzoni *et al.*, 2009; Ceppi *et al.*, 2009). Therefore, miRNAs can inhibit the activation of innate immune signalling pathways by negative regulation of their components. Others, such as miR-233 and the miR-17-19 cluster have been implicated in myeloid proliferation and differentiation (Fontana *et al.*, 2007; Johnnidis *et al.*, 2008). The maturation of DCs and their pathogen binding ability is also affected by miR-155 (Martinez-Nunez *et al.*, 2009). Additionally, work carried out in conditional Dicer knock-out mice showed that miRNAs were required for T-cell proliferation and CD4⁺ T regulatory (T reg) cells maturation (Cobb *et al.*, 2005; Cobb *et al.*, 2006) and affected B-cell function (Koralov *et al.*, 2008). Less is known about the roles of individual miRNAs but it has been demonstrated that miR-155 and miR-181 are important for differentiation and proliferation of CD4⁺ T reg cells and the production of CD4⁺ and CD8⁺ T-cells respectively (Li *et al.*, 2007; Rodriguez *et al.*, 2007; Thai *et al.*, 2007). MiR-106a was also shown to regulate IL-10 expression (Sharma *et al.*, 2009).

1.13 Virus-encoded miRNAs and their functions

Virus-encoded miRNAs were first discovered by Pfeffer *et al.*, (2004) (Pfeffer *et al.*, 2004) using a combination of computational and experimental studies from Epstein-Barr virus (EBV)-infected B cells. The miRNAs prediction programme used by Pfeffer *et al.* (2004) was especially designed for this purpose because established miRNA prediction programmes rely on conservation of miRNAs between species. Some of the hits they obtained were verified experimentally by cloning of small RNA, sequencing, and northern blotting (Pfeffer *et al.*, 2004).

To date, the miRNA registry at miRbase (www.mirbase.org) (version 14) lists 176 viral miRNAs encoded from 19 different viruses. Interestingly, 171 of those miRNAs are encoded by herpes viruses. Indeed, all herpes viruses examined encode miRNAs (Cullen, 2006). miRNAs are expressed by human herpes viruses such as EBV (Cai *et al.*, 2006; Grundhoff *et al.*, 2006) or

Kaposi's sarcoma-associated herpes virus (KSHV) (Cai *et al.*, 2005) but they have also been identified in simian (Schafer *et al.*, 2007), murine (Buck *et al.*, 2007; Dolken *et al.*, 2007) and avian herpes viruses (Yao *et al.*, 2007). In addition to herpes viruses, miRNAs are also expressed by two other virus families namely, polyomaviruses and adenoviruses. For instance, miRNAs have been found in simian virus 40 (SV40) (Sullivan *et al.*, 2005) and adenovirus type 5 (Xu *et al.*, 2007). It has been reported that retroviruses and flaviviruses, which have an RNA genome, do not encode miRNAs (Pfeffer *et al.*, 2004; Lin & Cullen, 2007) but this was later refuted by a report of HIV-1-encoded miRNAs and the matter remains controversial (Ouellet *et al.*, 2008). Additionally, miRNA precursor sequences have been predicted for poxviruses (Pfeffer *et al.*, 2004) though their existence has not been validated nor ruled out. Furthermore, these viruses replicate in the cytoplasm and as the initial step in miRNA biogenesis is the cleavage of the pre-miRNA loop by Drosha in the nucleus, it is unclear how any miRNA from these viruses would be processed.

Targets for all virus miRNAs discovered to date are still largely unknown. Interestingly, three viruses have been found to share seed sequence identity with human miR-155 and are considered miR-155 orthologues (Gottwein *et al.*, 2007; Skalsky *et al.*, 2007; Zhao *et al.*, 2009). Other virus-encoded miRNAs have been found to regulate the expression of virus-encoded genes. In some cases, viral miRNA are transcribed anti-sense to a virus protein encoding region making the RNA of the opposite strand an obvious target (Cullen, 2006). In herpes viruses, virus miRNA targeting the expression of viral lytic transcripts enables the maintenance of a latent state of infection (Umbach & Cullen, 2009). For instance, herpes simplex virus type 1 (HSV-1) miR-H2 is transcribed anti-sense to ICP0, an immediate early gene which encodes a protein important for starting productive replication (Umbach *et al.*, 2008). However, validated virus targets of virus-encoded miRNAs have mainly been viral regulatory protein-encoding genes (Umbach & Cullen, 2009). Examples of viral targets of virus miRNAs include SV40 miR-S1 which downregulates expression of the viral T antigen (Sullivan *et al.*, 2009) and EBV miR-Bart-2 which regulates expression of EBV-encoded DNA polymerase BALF5 (Barth *et al.*, 2008). Cellular targets of virus miRNAs discovered to date have been apoptosis regulators or host immune response modulators (Cullen, 2009). For instance, KSHV miR-K5, K9 and K13 target apoptotic factor BCLAF1 (Ziegelbauer *et al.*, 2009) and EBV miR-BART5 targets PUMA a host pro-apoptotic factor thus promoting host-cell survival (Choy *et al.*, 2008). Host immune response gene targets of virus miRNA include the cytokine CXCL-11, a T-cell chemoattractant, targeted by miR-BHRF1-3 from EBV (Xia *et al.*, 2008) and MIC B a ligand for the NKG2D receptor, found

primarily on NK cells, which is targeted by human cytomegalovirus (hCMV) miR-UL112-1 (Stern-Ginossar *et al.*, 2007). Interestingly, miR-UL112-1 also targets the hCMV *IE1* gene which encodes an immediate early gene required for the activation of early gene transcription (Murphy *et al.*, 2008).

Recently, the conservation of virus-encoded miRNA within different γ -herpes virus species was analysed (Walz *et al.*, 2009). Overall, miRNAs from these viruses are not evolutionary conserved between species, but there are two exceptions. For instance, 7 miRNAs are conserved between EBV and the closely related rhesus lymphocryptovirus (rLMV) out of a total of 25 and 16 miRNA they are known to encode respectively (Cai *et al.*, 2006; Walz *et al.*, 2009). Overall, the sequences of those miRNAs are not conserved even though their genomic locations are (Walz *et al.*, 2009). This appears to also be the case for HSV-1 (an α -herpes virus) and polyomavirus miRNAs (Cullen, 2009).

1.14 VACV as a vaccine vector

VACV was first developed as an expression vector in 1982 (Mackett *et al.*, 1982; Panicali & Paoletti, 1982). Subsequently, recombinant VACVs expressing foreign antigens were shown to induce immune responses to these antigens and protect against the organism from which the gene was derived (Panicali *et al.*, 1983; Smith *et al.*, 1983). VACV also has a large capacity for foreign DNA (Smith & Moss, 1983) which enabled the simultaneous expression of multiple foreign proteins (Perkus *et al.*, 1985). Additionally, infection with those recombinant viruses led to high levels of expression in different cell types (Mackett & Smith, 1986). The main obstacle to developing VACV as a vaccine vector was the poor safety record of the strains used for smallpox vaccination (Lane *et al.*, 1969). Smallpox vaccination with the conventional VACV strains led to complications in vaccinees with immunodeficiencies or certain skin disorders (Lane *et al.*, 1969; Lane & Goldstein, 2003; Moore *et al.*, 2006). Therefore, attenuated strains like Modified Vaccinia virus Ankara (MVA) (Hochstein-Mintzel *et al.*, 1972), LC16m8 (Kidokoro *et al.*, 2005) or NYVAC (Tartaglia *et al.*, 1992) have been proposed as alternative vectors but it is uncertain if these induce protection against smallpox.

1.15 VACV MVA

MVA was derived from its parent *Chorioallantois vaccinia Ankara* (CVA) strain by over 570 passages in CEFs (Mayr *et al.*, 1975). This produced an attenuated virus (Hochstein-Mintzel *et al.*, 1972) that had lost approximately 30 kb in six large genomic deletions referred to as deletions I to VI (Meyer *et al.*, 1991). In addition, many virus genes were disrupted or mutated (Antoine *et al.*, 1998). The majority of ORFs at the left terminus of the genome were deleted, fragmented or truncated. This region contains four large deletion corresponding to VACV strain Copenhagen genes *C22L-C19L*, *C16L-C12L*, *C5L-N1L* and *M1L-K1L* (Antoine *et al.*, 1998). Towards the right end of the genome, the regions corresponding to VACV strain Copenhagen genes *A51R-A55R*, *B25R-B28R* and the *B20R* ORF were deleted. In contrast, most of the genes in the central region of the MVA genome are intact except three genes (corresponding to *F5L*, *F11L* and *O1L*) which are non-essential (Antoine *et al.*, 1998). The recent availability of the CVA genome sequence has also enabled the exact mapping of the six major deletions between MVA and CVA (Meisinger-Henschel *et al.*, 2007).

These large genomic changes in MVA have led to a restriction in host range. The two “classical” host range genes in VACV needed for replication in human cells are *K1L* which is partially deleted in MVA and *C7L* which is intact and has 100 % amino acid sequence identity with its VACV WR orthologue (Perkus *et al.*, 1990; Antoine *et al.*, 1998). Despite the presence of an intact *C7L* orthologue, MVA replication is abortive or greatly restricted in human cells even though it can infect them normally *in vitro* (Sutter & Moss, 1992; Blanchard *et al.*, 1998). Consequently, the infection does not spread in human cells and MVA is highly attenuated in man. That is also the case with most mammalian cell lines but the Syrian hamster BHK-21 cell line (Carroll & Moss, 1997; Drexler *et al.*, 1998) and the rat IEC-6 cell line (Okeke *et al.*, 2006) are fully permissive for MVA. Additionally, it was recently found that MVA could also replicate in immortalised cell lines derived from foetal Egyptian fruit bats (Jordan *et al.*, 2009). In contrast, MVA replicates well in some avian cell types such as CEFs (Mayr *et al.*, 1978; Altenburger *et al.*, 1989; Carroll & Moss, 1997). Interestingly, MVA lost the ability to replicate in the highly permissive CEFs when modified to lack the MVA orthologue of another known host range gene *E3L* (Langland & Jacobs, 2002; Hornemann *et al.*, 2003).

1.16 VACV MVA as a vaccine vector

Currently, there is strong interest in genetically engineering MVA to make it a recombinant vaccine vector for HIV, malaria and other diseases (Gherardi & Esteban, 2005; Cebere *et al.*, 2006; Dunachie *et al.*, 2006; Ondondo *et al.*, 2006). MVA vectors expressing tumour antigens have also been used for cancer immunotherapy (Harrop *et al.*, 2007; Song *et al.*, 2007) or suicide gene therapy (Erbs *et al.*, 2007). Other applications include the use of MVA vectors for high level protein expression in mammalian cells (Hebben *et al.*, 2007).

MVA is an ideal vaccine strain because of its safety and good immunogenicity. It was used in more than 120 000 primary vaccinees in Germany without any significant adverse effects (Mayr *et al.*, 1978). This was also supported by more recent studies using a third generation smallpox vaccine clone derived from MVA which confirmed its safely profile in immunocompromised individuals (Vollmar *et al.*, 2006). MVA can also express foreign proteins at a high level (Sutter & Moss, 1992) and is immunogenic despite being replication defective in human cells (Sutter *et al.*, 1994). MVA is as immunogenic as the conventional replication-competent VACV vaccine strain, Dryvax (Davies *et al.*, 2007) if not more so (Gomez *et al.*, 2001; Earl *et al.*, 2004; Coulibaly *et al.*, 2005). However, the mechanisms by which MVA elicits the immune response are poorly understood. VACV infection is abortive and induces apoptosis in DCs (Jenne *et al.*, 2000) but this process was found to be accelerated after MVA infection compared to infection with a replication competent strain (Chahroudi *et al.*, 2006). This was the first indication that enhanced immunogenicity of MVA compared to other strains may be a result of cross presentation whereas direct priming is known to be more usually the case with replication competent strains (Chahroudi *et al.*, 2006). Differences in antigen presentation between MVA and other VACV strains were later studied by Gasteiger *et al.* (2007) and cytotoxic T-lymphocyte (CTL) responses against MVA were found to be dominated by cross-priming (Gasteiger *et al.*, 2007). Additionally, a recent study in mice showed that delivery route of MVA (intradermal vs. intramuscular) impacts on the quality of the immune response, as the former yielded better antigen distribution and APC recruitment (Abadie *et al.*, 2009). These studies highlight the need for a better understanding of how MVA elicits the immune response to design more immunogenic vaccines and better antigen delivery routes.

1.17 VACV MVA and immunomodulation

MVA lacks several immunomodulators present in other VACV strains such as the soluble receptors for TNF, IFN- γ , or IFN- α/β but others remain (Antoine *et al.*, 1998; Blanchard *et al.*, 1998). Additionally, some MVA orthologues of other VACV immunomodulators are partially deleted, disrupted or mutated, for instance N1 and B14 orthologues (Antoine *et al.*, 1998). The orthologue of N1 is fragmented and the protein it encodes (008) has three deletions towards its C terminus (Antoine *et al.*, 1998) but based on the known structure of N1 it is unlikely to be expressed stably. The MVA orthologue of B14, called 183 has a couple of conserved amino acid substitutions but also has a 6 amino acid internal deletion that renders the protein unstable and non-functional (Laura McCoy, personal communication). In contrast, the sequences of VACV WR K7 and its MVA orthologue (028) are identical. Similarly, the sequence of VACV WR A46 and its orthologue MVA 159 differ by a single amino acid substitution (from poxvirus.org). Additionally, the sequences of A35 in COP and MVA are the same, though the VACV WR orthologue, which was recently characterised (Rehm *et al.*, 2009), has a single amino acid substitution at position 150. The deletion of specific genes encoding immunomodulatory proteins from VACV strain MVA can increase immunogenicity (Staib *et al.*, 2005; Clark *et al.*, 2006) and therefore more immunogenic MVA vaccines can be created by targeted removal of additional immunomodulatory genes. For instance, the K7 or A35 orthologues would be ideal candidates for removal as they are highly conserved in MVA. Therefore, part of this project was aimed at deleting the *K7R* orthologue from MVA. The MVA used in this project is called MVA-C and has human immunodeficiency virus (HIV) clade C Env/Gag-Pol-Nef sequence inserted in the thymidine kinase (TK) locus of wild type MVA (Gomez *et al.*, 2007).

In summary, table 1.2 lists the orthologues of known immunomodulatory genes and the state of their conservation in MVA.

VACV WR protein	VACV MVA protein	Amino acid sequence condition in MVA	% sequence identity
E3	50	7 conserved aa substitutions	97
K3	11	2 aa substitutions, one is conserved	95
B13	-	deleted	NA
B22	189	18 aa extension, 15 aa substitutions, 12 aa deletion and some discordance compared to VACV COP orthologue	84 (with VACV COP)
F1	29	4 aa deletion at N-terminus and 1 conserved aa substitution	97
N1	20	internal 4 aa deletion and 20 aa substitutions all at the C terminus; unlikely to be stable	81
A46	159	single conserved aa substitution	99
A52	-	deleted	NA
K7	28	identical	100
K1	-	deleted	NA
M2	-	deleted	NA
B14	183	internal 6 aa deletion and 1 conserved aa substitution	94
A44	157	single aa substitution	97.99
A35	146	single aa substitution	99
B18	-	fragmented, no protein produced	NA
B8	-	gene fragmented, no functional protein is produced	NA
H1	91	single conserved aa substitution	99
B15	184	no change	100
C21	-	deleted	NA
C12	8	19 aa substitutions and a 6 aa internal deletion	84
A41	157	6 aa substitutions, 5 of which are conserved	97

Table1.2. Conservation of VACV immunomodulatory proteins between WR and MVA strains. aa = amino acid.

1.18 VACV K7 overview

This project was focused on *K7R* and the K7 protein it encodes. K7 is highly conserved amongst VACV strains and OPVs suggesting an important function. Within the VACV proteome K7 is most closely related to A52 with which it shares 26 % amino acid sequence identity and 45 % similarity (see 3.1 for details).

K7 in VACV WR was previously studied in the Smith lab by Dr Stuart Lucas (unpublished results). A deletion mutant lacking the *K7R* gene (Δ K7) and a revertant virus where the *K7R* gene was re-inserted into Δ K7 (Δ K7-rev) were made. Growth of Δ K7 in cell culture was measured after infection at 10 plaque forming units (pfu)/cell and its replication compared to that of the wild-type virus (Δ K7) and Δ K7-rev and all three viruses produced equivalent titres. However, when cells were infected at 0.01 pfu/cell, slightly lower titres were obtained from Δ K7-infected cells at 48 and 72 h p.i. As there were no significant differences in the size of plaques formed on infected cells by the three viruses, this difference was believed to be due to enhanced cellular defence mechanisms in the absence of K7 rather than reduced cell to cell spread efficiency.

To assess virus virulence, Δ K7 was tested *in vivo* in both the intradermal (Tschärke & Smith, 1999) and the intranasal (Williamson *et al.*, 1990) mouse models of infection and was found to be attenuated compared to wild type and revertant controls. In mice infected intradermally, the size of the resulting lesions was monitored and mice infected with Δ K7 displayed a reduced lesion size compared to those infected with Δ K7 and Δ K7-rev. This difference was statistically significant from days 7-11, 13 and 14 p.i. In the intranasal model, Δ K7 induced substantially less weight loss from days 4-13 p.i. Additionally, virus titres in infected lungs were significantly lower at 6 days p.i and there was an increase in lymphocytes (CD45 +) infiltrating into the lung. This increase in CD45+ correlated with an increase in CD8+ T-cells. Collectively, all these data indicated that K7 contributes to virulence.

However, it seems that loss of the K7 protein was not the primary cause of the attenuation observed in those experiments. This was because a virus that contained the K7 gene but was engineered not to make the K7 protein was as virulent as wild type. Specifically, an extra nucleotide was introduced into the *K7R* translational initiation codon, thus mutating the ATG codon to ATAG so that the protein was not produced. Consequently, confirming and

investigating how *K7R* nucleic acid contributes to virulence formed a significant part of this project.

The function of K7 has also been studied by Dr Andrew Bowie and co-workers in Trinity College Dublin (Schroder *et al.*, 2008). They showed that expression of K7 in transfected cells inhibited a broader range of innate signalling pathways than the related A52. Like A52, K7 co-immunoprecipitated with TRAF6 and IRAK2 and inhibited the induction of NF- κ B via TLRs, but unlike A52, K7 also inhibited non-TLR induced NF- κ B activation. Additionally, K7 inhibited TLR-dependent and TLR-independent activation of IRF3. The latter effect was mediated by an interaction with the host DEAD-box protein DDX3, an RNA helicase (Yedavalli *et al.*, 2004) which led to the discovery of a novel role for DDX3 in innate immune signalling pathways. DDX3 had previously been shown to play a role in cellular transcription and translational control, RNA splicing and export and cell cycle control (Rosner & Rinkevich, 2007). In this case, DDX3 was found to be phosphorylated by IKK- ϵ and/or TBK1 that are the two components upstream of IRF3 and IRF7 and essential for their activation, but K7 binding to DDX3 inhibited their activation (Schroder *et al.*, 2008; Soulat *et al.*, 2008). Interestingly, it is also known that hepatitis B virus (HBV) and HIV encode proteins that target DDX3 (Kwong *et al.*, 2005; Ariumi *et al.*, 2007).

Collectively, these data indicate that the K7 protein is an immunomodulator and suggest that the nucleic acid transcribed from *K7R* may contribute to virulence even when it does not encode protein.

1.19 Project aims

This study aimed to further characterise K7 from VACV WR strain, and investigated its role in VACV infection. Characterisation of K7 included delineating its conservation through the OPV genus, its expression profile and localisation within the context of a viral infection. In addition, the possible role of *K7R* nucleic acid in virus virulence *in vivo* and immunomodulatory effects *in vitro* were investigated. Co-expression of K7 with its binding partner DDX3 to produce soluble complexes for crystallisation trials was also attempted. Finally, MVA lacking 028R, the *K7R* orthologue was constructed and characterised.

The experimental results are arranged in the following order:

Chapter 3: Analysis of the K7 protein

Chapter 4: Expression and purification of K7 protein and its binding partner DDX3 in a bacterial expression system for crystallisation trials

Chapter 5: Investigating the possible contribution of *K7R* nucleic acid to virulence in VACV WR

Chapter 6: Construction and *in vitro* characterisation of MVA-C viruses lacking *K7R* orthologue *028R*

Chapter 2: Materials and methods

2.1. Molecular cloning

2.1.1 Restriction digestion of plasmid DNA

DNA was digested with restriction enzymes (New England Biolabs or Roche) at a minimum of 1 unit (U) of restriction enzyme per μg of DNA and no more than 10 % (v/v) of restriction enzyme per reaction to minimise glycerol content. DNA was digested for 1-2 h at 37 °C.

2.1.2 Agarose gel electrophoresis

DNA was resolved by electrophoresis on 1 or 2 % (w/v) agarose gels made up in Tris-acetate-ethylenediaminetetraacetic acid (EDTA) (TAE) (40 mM Tris pH 8, 20 mM acetic acid, 1 mM EDTA) with 10,000 X SYBRsafe dye (Invitrogen). Samples were mixed at a ratio of 5:1 with 6 X loading buffer (10 mM Tris pH 7.6, 0.03 % (w/v) bromophenol blue, 0.03 % (w/v) xylene cyanol FF, 0.15 % (w/v) orange G (inorganic synthetic azo chemical compound), 60 % glycerol, 60 mM EDTA) before loading. The DNA fragments were visualized and photographed using the SYBRsafe imager (Invitrogen), a 300 nm blue light transilluminator, to avoid UV damage to DNA that occurs when using a short wave UV transilluminator. The DNA bands of interest were excised with a clean scalpel and the DNA was purified using the QIAquick gel extraction kit (QIAGEN) according to manufacturer's instructions. Briefly, the gel slice was dissolved in 3 X (w/v) buffer QG by heating at 50 °C until the gel dissolved. The DNA was then adsorbed onto a silica gel membrane in a spin column at high salt concentration and pH < 7.5 which were provided by the QG buffer. Impurities were then washed away by centrifugation at 13,000 rotations per minute (rpm) for 1 min in a benchtop microcentrifuge (Heraeus) and pure DNA was eluted in deionised distilled H₂O (ddH₂O).

2.1.3 Dephosphorylation of 5' ends of DNA fragments

Dephosphorylation of the 5' ends of linearised plasmid vectors was performed using 1 U of shrimp alkaline phosphatase (SAP, Amersham Biosciences) per μg DNA in 20 mM Tris-HCl pH 8.0, 10 mM MgCl_2 for 1 h at 37 °C. The reaction was terminated by heating at 65 °C for 15 min to inactivate the phosphatase.

2.1.4 Klenow reaction

Linear DNA fragments containing overhanging sticky ends that were generated by restriction enzyme digestion were made blunt ended where necessary by using the Klenow enzyme (large fragment of DNA polymerase I from *E. coli*). The DNA was mixed with nick translation buffer (50 mM Tris-HCl pH 7.5, 10 mM MgSO_4 , 0.1 mM dithiothreitol (DTT), 500 $\mu\text{g}/\text{ml}$ in H_2O), 80 μM deoxyribonucleotide triphosphate (dNTP) and 1 U of Klenow enzyme (Roche). After 30 min incubation at room temperature (RT), the reaction was stopped by adding EDTA to a concentration of 20 mM.

2.1.5 DNA ligations

DNA ligations were carried out at 1:3 vector to insert molar ratio with 100-200 ng of vector DNA and 1 U of T4 ligase (Rapid DNA ligation kit, Roche) per reaction in T4 DNA ligase buffer. The reaction was incubated at RT for 5 to 10 min.

2.1.6 Transformation of plasmids into chemically competent cells

Transformation competent *E. coli* cells (Nova blue DH5 α , Novagen) were stored at -80 °C and then thawed on ice before use. No more than 10 % total volume of a DNA ligation reaction (section 2.1.5) was added to a 20 μl aliquot of competent cells and incubated on ice for 30 min. The cells were then heat-shocked at 42 °C for 30 s before recovery on ice for 2 min. Usually 80 μl of super optimal catabolite (SOC) medium was added, the mix was spread on LB (Luria-Bertani) agar dishes containing the relevant antibiotic (typically ampicillin (50 $\mu\text{g}/\text{ml}$) and/or kanamycin (30 $\mu\text{g}/\text{ml}$)) and incubated at 37 °C overnight.

2.1.7 Preparation of plasmid DNA

A 5 ml LB broth culture supplemented with the relevant antibiotic(s) was inoculated with a single bacterial colony and was incubated overnight at 37 °C with shaking at 200 rpm. Plasmid DNA was extracted from the pellet of 1.5 ml of the overnight culture by alkaline lysis. Briefly, the pellet was lysed by resuspending in 150 µl P1 buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, RNase A 100 µg/ml) in an Eppendorf tube. Then 150 µl of buffer P2 (0.2 M NaOH, 1 % sodium dodecyl sulphate (SDS)) was added and the tube was shaken gently and incubated for 5 min at RT. Afterwards, 150 µl of P3 buffer (2.55 M KOAc pH 4.8) was added and the tube was shaken thoroughly and centrifuged at 13,000 rpm for 5 min in a benchtop microcentrifuge (Heraeus). The supernatant (approximately 450 µl) was then transferred to another tube and 1 ml of ethanol was added to it to precipitate the DNA. The DNA was collected by centrifugation, washed with 150 µl of 70 % ethanol and dried in a speed vacuum (Savant). The DNA pellet was dissolved in 40 µl of ddH₂O. To obtain a larger amount of highly purified and concentrated DNA, a Maxi-prep kit (Qiagen) was used starting from a 250 ml culture of the DH5α *E. coli* strain harbouring the plasmid clone of interest according to the manufacturer's instructions. The principle of this procedure was also based on DNA extraction from bacteria by alkaline lysis. DNA was dissolved in 1 ml of TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and its concentration determined by spectrophotometry by measuring UV absorbance at 260 nm using the Nanodrop ND-1000 (Labtech International) (1 OD₂₆₀ = 50 µg/ml dsDNA). The purity of the nucleic acids was assessed by the OD_{260/280} ratio. A ratio between 1.8 and 1.9 is considered as highly pure DNA.

2.1.8 Screening of plasmid clones

The presence of the correct DNA fragment after cloning was verified by restriction enzyme digestion and PCR. The plasmid DNA was digested with the appropriate restriction enzyme(s) followed by agarose gel electrophoresis. For PCR analysis see section 2.1.10.

2.1.9 Preparation of glycerol stocks

A 5 ml LB culture supplemented with the appropriate antibiotic(s) was inoculated with a single bacterial colony and was incubated overnight at 37 °C with shaking at 200 rpm. A 0.5 ml sample of this culture and an equal volume of 30 % (v/v) sterile glycerol in LB solution were mixed and frozen at -80 °C. When required, this frozen glycerol stock of bacteria was scraped using a sterile

loop and streaked out on an LB agar plate containing the appropriate antibiotic(s) and incubated overnight at 37 °C to obtain isolated colonies.

2.1.10 Standard PCR

Platinum *Pfx* high-fidelity DNA polymerase (Invitrogen) was used to amplify DNA used for cloning. Gotaq (Roche) or Taq (Invitrogen) polymerases were used for diagnostic screening of DNA. A standard PCR in a 50 µl reaction volume contained 10 to 100 ng of template DNA, oligonucleotide primers (0.1-1 µM each), 200 µM dNTPs, 1.5 mM MgCl₂, PCR buffer (20 mM Tris-HCl pH 8.4, 50 mM KCl) and 2 U of thermal polymerase. Typically, the template DNA was denatured at 95 °C for 3 min, followed by 25 cycles of denaturing at 95 °C (30 s), annealing at 50 °C (30 s) and extension at 72 °C (for a length of time equal to 1 min per kb of desired extension product). A final extension at 72 °C for 5-10 min followed. DNA from PCRs was analysed by agarose gel electrophoresis, visualised and excised from the gel as described in section 2.1.2 and purified using the Qiagen Gel Extraction kit. When not electrophoresed on an agarose gel, the DNA was purified using the Qiagen PCR purification kit. The DNA purification principle in this kit was the same as for the gel extraction kit.

2.1.11 DNA ligation with PCR fragments

Blunt-ended ligations were carried out using the Zero Blunt TOPO Cloning Kit (Invitrogen) where the plasmid vector (pCR4 Blunt-TOPO) is supplied linearised with VACV topoisomerase I bound to the 3' ends, ready for cloning blunt-ended products. The vector and the fresh PCR product insert were mixed gently in 1.2 mM NaCl, 10 mM MgCl₂ for 5 min at RT. The reaction mix was then placed on ice and transformed into competent bacterial cells.

2.1.12 Oligonucleotides

PCR primers were produced by Sigma-Aldrich, UK or IDT. Lyophilised primers were dissolved in to yield a master stock with a concentration of 100 µM. Working stocks were diluted to 10 µM in ddH₂O and stored at -20 °C. Below is a list of all primers used.

oligonucleotide name	5' to 3' sequence	sequence bound
Fl-1	ACTTCTTCCATGTCCAATATG	028R left flank
Fl-3	ACCCCTATATCATCTAGAGACTATCTCACAAAAGACAG	028R left flank
Fl-2 m	ATTAGCGGCCGCACGAAGTTAATACTATACTAATG	028R right flank
Fl-4 m	ATTATCTAGATACAGATCTGATGACCGACG	028R right flank
039U	AACTTCTAGATTACCACTTCTTCCATGTCC	K7R left flank
039D	TGATGAATTCGGGGTTGGGTGTAAGATTGG	K7R right flank
1-171F4	GGAAGACTGTACAAGGAATTGTAATGATATAGGGGTCTTCATAACG	K7R left flank with K7R ₁₋₁₇₁ overlap
1-171R3	CGTTATGAAGACCCCTATATCA TTACAATTCCTTGTACAGTCTTCC	K7R ₁₋₁₇₁ with K7R right flank overlap
ScrR1	CGTAGTCCAACTTTGTGGCCATGACTATCTCACAAAAGACAGTAG	K7R left flank with K7R scrambled overlap
ScrF2	CTACTGTCTTTTGTGAGATAGTCATGGCCACAAAGTTGGACTACG	K7R scrambled with K7R left flank overlap
ScrR3	CGTTATGAAGACCCCTATATCATCAGTTTAACTTCTTCTCAAGG	K7R scrambled with K7R right flank overlap
ScrF4	CCTTGAGAAGAAGTTAAACTGATGATATAGGGGTCTTCATAACG	K7R right flank with K7R scrambled overlap
InvR1	AGAAAAAAAAATTGAATTGAGACTATCTCACAAAAGACAGTAG	K7R left flank with K7R inverted overlap
InvF2	CTACTGTCTTTTGTGAGATAGTCTCAATTCAATTTTTTTTCT	K7R inverted with K7R left flank overlap
InvR3	CGTTATGAAGACCCCTATATCAATGGCGACTAAATTAGATTA	K7R inverted with K7R right flank overlap
InvF4	TAATCTAATTTAGTCGCCATTGATATAGGGGTCTTCATAACG	K7R right flank with K7R inverted overlap
270-450R1	CTTTCCTGATCATGGACTTTTCATGACTATCTCACAAAAGACAGTAG	K7R left flank with K7R ₂₇₀₋₄₅₀ overlap

270-450F2	CTACTGTCTTTTGTGAGATAGTCATGAAAAGTCCATGATCAGGAAAG	<i>K7R</i> ₂₇₀₋₄₅₀ with <i>K7R</i> left flank overlap
VMR1	GTACCGTATAGCGACATCATCGAGACTATCTCACAAAAGACAGTAG	<i>K7R</i> left flank with <i>K7R</i> ₁₇₈₋₂₆₉ overlap
VMF2	CTACTGTCTTTTGTGAGATAGTCTCGATGATGTCGCTATACGGTAC	<i>K7R</i> ₁₇₈₋₂₆₉ with <i>K7R</i> left flank overlap
VMR3	CGTTATGAAGACCCCTATATCAGTAAAATTGATGTAGTGGTC	<i>K7R</i> ₁₇₈₋₂₆₉ with <i>K7R</i> right flank overlap
VMF4	GACCACTACATCAATTTTACTGATATAGGGGTCTTCATAACG	<i>K7R</i> right flank with <i>K7R</i> ₁₇₈₋₂₆₉ overlap
172-450R1	GCGACATCATCGAACTTCATGACTATCTCACAAAAGACAGTAG	<i>K7R</i> left flank with <i>K7R</i> ₁₇₁₋₄₅₀ overlap
172-450F2	CTACTGTCTTTTGTGAGATAGTCATGAAGTTCGATGATGTCGC	<i>K7R</i> ₁₇₁₋₄₅₀ with <i>K7R</i> left flank overlap
p91	CAAGGTGAAGCAGTGGCC	HIV-C antigen
p92	CGGCCTTGCCGATCTTGG	HIV-C antigen
K7 N-His fwd	AAGTTCTGTTTCAGGGCCCGGCGACTAAATTAGATTATGAGGATGC	<i>K7R</i> with N-6 X His
K7 N-His rev	ATGGTCTAGAAAGCTTTAATTCAATTTTTTTTCTAGAAAAAGTATCAAGATG	<i>K7R</i> with N-6 X His
K7 C-His fwd	AGGAGATATACCATGGCGACTAAATTAGATTATGAGGATGC	<i>K7R</i> with C-6 X His
K7 C-His rev	GTGATGGTGATGTTTATTCAATTTTTTTTCTAGAAAAAGTATCAAGATG	<i>K7R</i> with C-6 X His

Table. 2.1. Oligonucleotides used for PCR. The exact context for the use of these oligonucleotides will be described in Results. N-6 X His and C-6 X His = N-terminal and C-terminal 6 histidine tag.

2.1.13 Automated DNA sequencing

DNA samples were prepared by adding 3 pmol of sequencing primer to 3 µg of extracted DNA. Sequencing was carried at the MRC Clinical Sciences Centre, Hammersmith Hospital. Results were analysed using Chromas and Clustal X programmes.

2.2. Protein expression and purification

2.2.1 Bacterial protein expression systems

Plasmid pET28a (Novagen) containing the *K7R* ORF with a N-6 X His tag was introduced into *E. coli* B834 DE3 cells by transformation. *K7R* was under the control of bacteriophage T7 promoter and its expression only started after inducing T7 RNA polymerase in host B834 cells with β-D-1-thiogalactopyranoside (IPTG) (pET system manual, 11th Edition, Novagen). Later, pOPIN E and F vectors developed by Prof David Stuart, OPPF, Wellcome Trust Centre for Human Genetics, University of Oxford, were used instead (Berrow *et al.*, 2007). The pOPIN vectors made it possible to cleave the histidine tags more easily by using 3-C protease (PreScission protease, GE healthcare) rather than the less efficient thrombin that is required when using pET-28a. The K7 protein expressed had either a C- or N-6 X His tag. A single well-isolated bacterial colony from an LB agar plate was used to inoculate 5 ml of LB broth supplemented with the appropriate antibiotic(s) and this culture was incubated at 37 °C with shaking at 200 rpm overnight. The antibiotics used to select for bacteria were ampicillin (100 µg/ml) when using pOPIN vectors and kanamycin (30 µg/ml) when using pET-28a. The overnight culture was used to inoculate 250 ml of Terrific Broth (TB) autoinduction medium (Overnight Express Instant TB medium, Novagen) that was incubated at 23 °C for two days with shaking at 200 rpm or 37 °C for 6 h followed by overnight incubation at 25 °C. When using TB, induction with IPTG during the exponential phase of bacterial growth is not required. This is due to the presence of several metabolisable components in the medium that automatically induce protein expression from *lac* promoters (pET system manual, 11th Edition, Novagen). The cells were harvested by centrifugation at 3,000 rpm in an Allegra 6R centrifuge (Beckmann Coulter) for 20 min at 4 °C. When not processed immediately, pellets were stored at -80 °C.

In later experiments, the co-expression of DDX3 5-167 or DDX3 22-409 from pET-28a with K7 pOPINE was required. In this case, B834 cells were co-transformed with the two plasmids and bacterial colonies were selected in the presence of both kanamycin and carbenecillin. LB was also used instead of TB medium and cultures were induced with IPTG during the exponential growth phase for 3 h at 37 °C or overnight at 30 °C. Cultures were then processed as described previously.

2.2.2 Bacterial pellet lysis for protein extraction

A pellet containing the bacteria from a 250 ml culture (section 2.2.1) was lysed in 10-14 ml of lysis buffer (25 mM Tris pH 8, 500 mM NaCl, 20 mM imidazole, 1 % Nonidet-P 40 (NP-40), 2 mM β -mercaptoethanol and 1 protease inhibitor tablet per 10 ml, Roche) and was sonicated five times for 30 s with incubations on ice for 30 s between each sonication. The tube containing the lysate was rotated in the presence of benzonase nuclease (1000 X, 25 U/ μ l, Novagen) for 30 min at RT to digest contaminating DNA. The lysate was then centrifuged at 11,000 rpm in an Optima L-100 ultracentrifuge using an SW41-TI rotor (Beckmann Coulter) for 30 min at 4 °C. After centrifugation, soluble and insoluble fractions were obtained and separated into different 50 ml Falcon tubes. Samples of these two fractions were analysed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Coomassie blue staining or immunoblotting (see sections 2.2.4; 2.2.5 and 2.2.6).

2.2.3 Purification of His-tagged protein

The soluble protein fraction obtained after lysing the bacterial pellet (section 2.2.2) was filtered and loaded on to a 5 ml HisTrap column (GE Healthcare). The column was washed with 50 ml (10 column volumes, (CV)) binding buffer (20 mM Tris-HCl, 500 mM NaCl, 40 mM imidazole, pH 7.5) to remove non-specific protein and then bound protein was eluted with 15 CV using a gradient of increasing imidazole concentration (from 40 to 500 mM imidazole) with elution buffer (20 mM Tris-HCl, 500 mM NaCl, 500 mM imidazole, pH 7.5). Fractions of interest were analysed by SDS-PAGE followed by immunoblotting or staining with Coomassie blue to check for protein content. Fractions containing recombinant protein were combined and concentrated using a Centricon YM-10 to no more than 1-2 ml. This was applied to a Superdex 75 16/60 column (GE Healthcare) for gel filtration and eluted with 1.2 CV gel filtration buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 50 mM NaCl, pH 8). Again, fractions were analysed for protein by SDS-PAGE followed by staining with Coomassie blue or immunoblotting.

2.2.4 SDS-PAGE

Proteins were resolved by electrophoresis on a 5 % polyacrylamide stacking gel (125 mM Tris pH 6.8, 5 % (v/v) acrylamide mix (Protogel, National Diagnostics) 0.1 % (w/v) SDS, 0.1 % (w/v) ammonium persulphate and 0.04 % v/v N,N,N',N'-tetramethylethylenediamine (TEMED)) and a resolving gel (12 or 15 % (v/v) acrylamide mix, 375 mM Tris pH 8.8, 0.1 % (w/v) SDS, 0.1 % (w/v) ammonium persulphate, 0.04 % (v/v) TEMED) by electrophoresis, in a Tris-glycine running buffer (24 mM Tris-Base, 192 mM glycine, 1 % (w/v) SDS) using a Mini-PROTEAN II apparatus (BioRad). The prestained molecular mass marker used was Precision-Plus (BioRad). Protein samples were mixed at a ratio of 5:1 with 6 X Laemmli loading buffer (300 mM Tris pH 6.8, 12 % (w/v) SDS, 60 % (v/v) glycerol, 0.6 % bromophenol blue) supplemented with 100 mM DTT before use. Samples were heated at 85 °C for 3 min and run at 80 V through the stacking gel and then at 160 V through the separating gel.

2.2.5 Coomassie blue staining

Polyacrylamide gels were stained for 30 min in Coomassie blue solution (10 % (v/v) acetic acid, 45 % (v/v) methanol, 0.25 % (w/v) Coomassie brilliant blue (BDH)) and then destained in 10 % (v/v) acetic acid, 25 % methanol solution. Gels were equilibrated in water and dried between 2 sheets of pre-wet cellophane membrane (Bio-Rad) in a hot air dryer (GelAir drying system, Bio-Rad).

2.2.6 Immunoblotting

After electrophoresis, polyacrylamide gels were equilibrated for 15 min in transfer buffer (190 mM glycine, 25 mM Tris pH 9, 0.1 % SDS, and 20 % (v/v) methanol) and transferred to Hyperbond enhanced chemiluminescence (ECL) 0.2 µM pore size nitrocellulose membranes (GE Healthcare) at 18 V for 25 min. The membrane was incubated in blocking buffer (5 % milk powder (w/v) (Fluka), phosphate-buffered saline (PBS), 0.1 % Tween) for 1 h at RT or overnight at 4 °C. Then the primary Ab was added to fresh blocking buffer and incubated with the membrane for 1 h at RT or overnight at 4 °C. The membrane was washed 3 times in 0.1 % Tween PBS and incubated with horseradish peroxidase (HRP)-labelled secondary Ab in blocking buffer for 45 min at RT. After washing again 3 times with 0.1 % Tween PBS, ECL Plus Western blotting detection reagent (GE Healthcare) was added to the membrane for 4 min at RT. The membrane was then drained of ECL and exposed on X-ray film (GE healthcare).

2.3 Cell culture

2.3.1 Cell lines

Chicken embryo fibroblasts (CEFs) were used for the construction, growth, and titration of recombinant MVA-C viruses. CEFs were purchased from the Institute for Animal Health, Compton. CEFs used were either primary or had been passaged no more than three times. Baby hamster kidney (BHK)-21 cells were used for titration of virus stocks and for characterisation of recombinant MVA-C viruses. Henrietta Lacks (HeLa) uterine carcinoma cells were used for some transfections and for some infections with VACV WR. Human embryonic kidney cells 293T (HEK 293T) were used to carry out luciferase reporter assays. BSC-1, CV-1 (both African green monkey kidney cells), D98OR (subclone of HeLa cells) and rabbit kidney 13 cells (RK-13) were used for infection and plaque assays with VACV WR.

2.3.2 Cell line maintenance and passage

All cells were maintained in medium supplemented with 50 U/ml penicillin, 50 µg/ml streptomycin (Gibco BRL) and 10 % heat-treated foetal bovine serum (FBS, heat-treated at 56 °C for 30 min, Harlan Sera-Lab). CEFs (prepared from 10-day old embryos at Institute for Animal Health, Compton), HEK 293T (CRL-1573), BSC-1 (ATCC CCL-26), RK-13, (ATCC CCL-37), CV-1 (ATCC CCL-70) and D98OR cells (ATCC CCL18.3) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL). BHK-21 (ATCC CCL-10) were cultured in modified Eagle's medium (MEM, Gibco BRL) supplemented with MEM vitamins (Gibco) and MEM non-essential amino acids (Sigma). HeLa cells (ECACC CCL-2) were cultured in MEM with MEM non-essential amino acids. The cells were incubated at 37 °C (or a different temperature where appropriate) in a humidified incubator with 5 % CO₂. HEK 293T-Rex-based (Invitrogen) stable inducible cell lines were maintained in DMEM supplemented with blasticidin (50 µg/ml) and zeocin (100 µg/ml). Doxycycline (2 µg/ml) was used to induce expression of the gene of interest.

2.3.3 Transfections

Lipofectamine 2000 transfection reagent (Invitrogen) was used for transfections of CEFs infected with MVA-C. Typically, 10 µl of lipofectamine 2000 was added to 250 µl of Opti-MEM medium (Gibco BRL) in one tube and 5 µg of DNA to 250 µl of Opti-MEM in another

tube. These tubes were incubated for 10 min at RT. The two solutions were then mixed and incubated for 20 min at RT. This mix and 5 ml of DMEM (with 2.5 % FBS) were added to a T25 flask of CEFs previously infected with MVA-C (see section 2.4.1 for infection protocol). The cells were incubated for 4-5 h, the transfection reagent was removed and replaced by DMEM supplemented with 2.5 % FBS. The cells were incubated for 2 days.

Polyethylenimine (PEI, 10 mg/ml) transfection reagent (Sigma-Aldrich) was used for all other transfections. Briefly, cells were seeded at 70 % confluency. After 24 or 48 h, the culture medium was replaced by pre-warmed DMEM containing 2 % FBS. Plasmid DNA was added to serum-free Opti-Mem and then PEI was added directly into the DNA mixture at a ratio of 1:2 of μg DNA to μL PEI. The reagents were vortexed briefly and incubated for 30 min at RT before adding to the cells.

2.3.4 Preparation of lysates of mammalian cells

Lysis of mammalian cells for analysis of proteins by SDS-PAGE was carried out using a Triton X buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, 10 % glycerol, 1 % Triton X-100, 0.05 % NP-40, 500 X protease inhibitor tablet (Roche), filtered). The cells were washed with PBS and the lysis buffer was added and the cells scraped with a cell scraper. The harvested lysates were stored at -20°C .

2.3.5 Luciferase assays

HEK 293T cells were seeded at 2×10^5 per well of a 96-well plate two days prior to transfection. The NF- κB -firefly-luciferase reporter plasmid (NF- κB -luc), ISRE-firefly-luciferase reporter plasmid (ISRE-luc) and a TK-Renilla-luciferase (TK-Renilla luc) plasmid were all obtained from the Promega PathDetect system. The IFN- β -firefly-luciferase reporter (IFN- β -luc) plasmid was a gift from Prof Bowie's group, Trinity College Dublin. Cells were transfected with 60 ng of NF- κB -luc/ISRE-luc/IFN- β -luc, 10 ng of TK-Renilla-luc, and 60 ng of the different *K7R* plasmids with PEI (Sigma-Aldrich). After 24 h, cell were treated with 250 ng/ml TNF- α , 1000 U/ml IFN- α and 100 $\mu\text{g}/\text{ml}$ poly I:C with 250 ng/ml TNF- α for 6 h for the NF- κB , ISRE and IFN- β luciferase reporter assays, respectively. Cells were then harvested in passive lysis buffer (Promega) and freeze-thawed to lyse the cells. Firefly luciferase activity was measured by adding firefly luciferase reagent (8 mg/ml luciferin (Lux Biotechnology), 270 μM acetyl co-enzyme A (Sigma-Aldrich), 530 μM ATP, 33 mM DTT, 0.1 mM EDTA, 2.67 mM MgSO_4 , 20 mM Tricine) to the cell lysates. Renilla luciferase

activity was measured by adding renilla luciferase reagent (2 µg/ml Coelenterazine (Lux Biotechnology) in PBS) to the cell lysates. The relative stimulation of reporter luciferase activity was measured as relative light units (RLUs) in a Luminoskan Luminometer (Labsystems). Reporter activity was calculated by normalizing firefly luciferase activity with Renilla luciferase activity. In all cases, data shown are from one of at least three independent experiments with similar qualitative results. Experiments were performed in triplicate and data are expressed as means ± SD.

2.3.6 Immunofluorescent staining

HeLa cells used for scanning confocal microscopy were grown on 22-mm sterilised glass coverslips (borosilicate glass, BDH) in 6-well plates. After 24 h cells were infected at 1 pfu/cell overnight with virus, or were transfected with plasmids of interest for 6, 12, 16, 20 or 24 h. At various times p.i or post-transfection, cells were washed twice in ice-cold PBS and fixed with 4 % paraformaldehyde (PFA) in 250 mM HEPES in PBS for 20 min at RT. Coverslips were then washed twice in PBS and quenched for 5 min with 50 mM NH₄Cl. After an additional two washes in PBS, cells were permeabilised in 0.1 % (w/v) Triton X-100 (Sigma-Aldrich) in PBS for 7 min. The cells were then blocked for 1 h at RT with PBS, 10 % FBS and incubated for 1 h with primary Ab diluted in the same blocking buffer. This was carried out by depositing each coverslip on 100 µL of the appropriate diluted Ab solution on parafilm and covering it with a darked-out moisture chamber. Slides were then washed three times with blocking buffer and stained with Alexa 488 or 546-conjugated secondary Abs (Invitrogen) at RT for 30 min in the same set-up as before. Coverslips were then washed three times in blocking buffer, three times in PBS, and once in ddH₂O before being mounted on glass slides in Mowiol-4',6-diamidino-2-phenylindole (DAPI) mounting medium. Slides were examined with a Zeiss LSM5 Pascal confocal microscope using Zeiss LSM software.

2.3.7 Cell fractionation

HeLa cells in 14.5-cm dishes were infected at 5 pfu/cell overnight with different recombinant viruses. Cells were gently washed twice with ice-cold lysis buffer (20 mM HEPES pH 7.8, 0.5 mM DTT, 0.5 mM MgCl₂) and then 20 ml of the same buffer were added per dish and incubated on ice for 10 min to allow the cells to swell. The buffer was then removed and dishes were tilted to help remove any residual buffer. Cells were gently scraped, collected and dounced on ice (25 strokes) in a 1 ml dounce homogeniser with 50 µL protease inhibitor cocktail (1 tablet in 10 ml ddH₂O, Roche). Lysates were then transferred to Eppendorf tubes

and centrifuged at 3,200 rpm at 4 °C for 2 min. Supernatants were transferred to new tubes and represented the cytoplasmic fractions. Nuclei were washed in PBS by gently resuspending the pellet, centrifuging as before and carefully removing the supernatant. This was repeated at least four times to avoid cytoplasmic contamination in the nuclear fractions. Nuclear resuspension buffer (100 µL, 50 mM Tris-HCl pH 8, 0.5 mM MgCl₂, and 20 mM iodoacetamide, supplemented with Roche protease inhibitor tablets) was then added to each sample and nuclei were sonicated until well disrupted. Samples were supplemented with 6 X protein gel loading buffer and boiled for 5 min before use.

2.4 Virus

2.4.1 VACV infection of CEFs or mammalian cells

Cells were washed once in PBS prior to infection. Typically, virus was added in 0.5 ml of DMEM with 2.5 % FBS in a well of a 6-well plate and 2 ml in a T25 flask. Infection was carried out for 90 min and the plates or flasks were rocked every 20 min to prevent cells drying out. The medium was then removed and replaced with either a solid overlay containing 1 % low melting temperature agarose (Agarose type VII, Sigma) in DMEM with 2.5 % FBS, or a semi-solid overlay containing 1.5 % carboxymethylcellulose (CMC) in DMEM with 2.5 % FBS, or liquid DMEM with 2.5 % FBS overlay. When using a solid overlay, the cells are placed in the 37 °C, 5 % CO₂ incubator after the agar had set.

2.4.2 Making recombinant WR viruses

Recombinant VACVs strain WR were made by transient dominant selection (Falkner & Moss, 1990) as detailed in chapter 5. The targeting plasmid contained a selection cassette of *E. coli* guanine phosphoribosyltransferase fused to enhanced green fluorescent protein (Ecogpt-EGFP) under the control of a VACV synthetic early/late (E/L) promoter (Boyle & Coupar, 1988; Davison & Moss, 1990). This plasmid was transfected into CV-1 cells in a T25 flask that had been infected at 0.1 pfu/cell for 90 min with the relevant parental virus. Recombinant viruses that had integrated the plasmid into the virus genome were distinguished from the parental virus by growth on cells incubated with 50 µg/ml mycophenolic acid (MPA), 250 µg/ml xanthine (X) and 15 µg/ml hypoxanthine (HPX) and by visual observation of expression of EGFP using fluorescent microscopy (Zeiss Axiovert 200M inverted light microscope, EGFP filter set, 10 X magnification). This provided positive selection for viruses

that have integrated the plasmid at the *K7R* locus and therefore express Ecogpt-EGFP. In some cases, the targeting plasmid used contained only Ecogpt. In this case, recombinant viruses were also obtained by growth on cells with MPA selection and plaques were visualised after overlaying cells with solid agar overlay supplemented with neutral red stain (50 µg/ml). In both cases, recombinant viruses were plaque purified a further 2-3 times and screened by PCR to ensure intermediate viruses that had integrated the plasmid at the *K7R* locus were obtained. Typically, plaques were picked into 0.5 ml and stored frozen at -80 °C until use. Usually 0.01 – 0.2 ml of this was used to infect pre-treated monolayers of BSC-1 cells. Again, green plaques were picked after 2-3 days and the process repeated. Intermediate viruses were then passaged in BSC-1 cells without drug until viruses were fully resolved as monitored by PCR for the loss of the targeting cassette. Alternatively, intermediate viral clones were first grown in D98OR cells in the presence of 6-thioguanine (6-TG), to select against expression of Ecogpt before plaque purifying on BSC-1 without drug selection. Virus genotypes were analysed by PCR using primers homologous to the flanking regions of *K7R*. Viral genomic DNA was obtained after Proteinase K digestion of infected cells (see section 2.4.6) and the PCRs were carried using Gotaq polymerase as described in section 2.1.10.

2.4.3 Production of recombinant MVA-C viruses

MVA-C (Gomez *et al.*, 2007) recombinant viruses were made using a modified version of an established method (Staib *et al.*, 2005) or by transient dominant selection as detailed in chapter 6. In both cases, a T25 flask of CEFs was infected with MVA-C or its derivatives at 0.1 pfu/cell for 90 min and was then transfected with a targeting plasmid containing an Ecogpt-EGFP fusion protein. The recombinant viruses that had integrated the plasmid containing Ecogpt-EGFP were distinguished from the parental virus by growth on CEFs in the presence of MPA, (50 µg/ml), X (250 µg/ml) and HPX (15 µg/ml) and by visual observation of expression of EGFP using fluorescent microscopy (Zeiss Axiovert 200M inverted light microscope, EGFP filter set, 10 X magnification). To ensure intermediate viruses (recombinants expressing Ecogpt-EGFP) were obtained, a further 2 or 3 rounds of foci purification were carried out. This step was carried out as described for WR plaques in 2.4.2. Intermediate viruses were then amplified by infecting a T25 in drug free medium. To obtain resolved recombinant viruses, the virus stock harvested from T25 flasks was diluted and used to infect CEFs for 2 days under semi-solid overlay without drug. Viruses that have resolved and lost Ecogpt-EGFP were identified by staining cells with anti-VACV rabbit polyclonal serum, followed by rabbit secondary immunofluorescent (red) Ab (see section 2.4.4) and lack of EGFP expression when observed by microscopy. Virus genotypes were

analysed by PCR using primers homologous to the flanking regions of *028R*. Viral genomic DNA was obtained after Proteinase K digestion of infected cells (see section 2.4.6) and the PCRs were carried using Gotaq polymerase as described in section 2.1.10.

2.4.4 Immunostaining of MVA-C recombinant foci

After incubating infected CEFs or BHK-21s for 2 or 3 days, the semi-solid overlay was aspirated and the cells were washed twice gently with PBS and incubated for 90 min at RT with anti-VACV rabbit polyclonal serum ((Law & Smith, 2001), 1:1000 dilution in 2.5 % FBS, PBS). Cells were then washed twice in PBS and incubated for 60 min with anti-rabbit Alexa 546 (red) fluor-labelled secondary Ab (1:1000 dilution in 2.5 % FBS, PBS, prepared by Mr Mike Hollinshead using the Alexa protein labelling kit, Molecular Probes). Cells were washed once with PBS and observed by microscopy. EGFP negative foci were picked after the cells were covered with solid agar overlay (see section 2.4.1).

2.4.5 Immunotitration of MVA strains

After incubating infected cells with virus for 2 or 3 days, the semi-solid overlay was aspirated and the cells were washed twice gently with PBS. The cells were fixed for 2 min with 1:1 methanol/acetone solution, washed twice with PBS and incubated for 90 min with the anti-VACV rabbit polyclonal serum. Cells were then washed twice in PBS and incubated for 60 min with anti-rabbit HRP-labelled secondary Ab (1:1000 dilution in 2.5 % FBS, PBS, Sigma). Cells were then washed twice in PBS and stained for 15 min with 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate (1 tablet per 10 ml of deionised water with and 0.03 % NiSO_4 and 0.03 % H_2O_2). The plates were washed with PBS and dried before counting the dark brown foci obtained.

2.4.6 Proteinase K treatment of infected cells to obtain virus DNA for PCR analysis

Confluent cells in 96-well plates were infected with 50 μl (10 % v/v) of a plaque or focus isolate in DMEM supplemented with 2.5 % FBS for 48 h. Cells were then washed once in PBS and typically mixed with 50 μl of proteinase K reaction solution (0.05 mg/ml proteinase K enzyme (Fluka) in PCR buffer (Invitrogen)) and freeze-thawed once. Proteinase K reactions were transferred to 0.2 ml PCR tubes. A thermocycler (Corbett Research) was used to heat the tubes at 56 °C for 20 min for digestion by proteinase K and then at 85 °C for 10 min to inactivate the enzyme.

2.4.7 Growth kinetic analysis of recombinant MVA-C viruses

Monolayers of CEFs in T25 flasks were infected with 10 or 0.01 pfu/cell for measurement of single or multiple growth kinetics, respectively. After infection for 90 min, the virus inoculum was removed, cells were washed twice in PBS to remove unbound virus and were incubated with 4 ml DMEM containing 2.5 % FBS. At the indicated times p.i., the culture medium was removed and centrifuged at 1,500 rpm at 4 °C for 10 min to collect detached cells. Attached cells were scraped into the growth medium, added to the cells collected from the culture supernatant, freeze-thawed 3 times, sonicated for 30 sec and then the cell-associated virus (including IMV, IEV and CEV) titres were determined by foci assay using BHK-21s. The titre of virus released into the medium (mostly EEV but with some IMV released from broken cells) was also determined.

2.4.8 Analysis of foci formed by recombinant MVA-Cs

Confluent monolayers of CEFs or 70 % confluent monolayers of BHK-21 were infected with approximately 50 pfu per well of a 6-well plate. Infected cells were overlaid with 1.5 % CMC in DMEM with 2.5 % FBS and incubated for three days and then immunostained with anti-VACV Ab. Foci ($n=20$ per virus) were chosen randomly and photographed using Zeiss AxioCam and Axiovert software. Using this software a line was drawn across the length of each focus and its length in μM was noted.

2.4.9 Virus purification by centrifugation through 36 % w/v sucrose cushions

For VACV WR purification, 10 T175 flasks of confluent RK-13 cells were infected at 0.1 pfu/cell for three days. For VACV MVA-C purification, 12-15 T175 flasks of confluent CEFs or BHK-21 cells were infected instead. Infected cells were harvested by either tapping the flasks a few times (RK-13s only) or scrapping and collected by centrifugation at 1,500 rpm for 15 min. The cell pellets were resuspended in chilled 10 mM Tris pH 9.0 buffer (1 ml per flask of cells initially infected) and left on ice for 15 min for the cells to swell. Virus was released by lysing the cells on ice using a dounce homogeniser (25 strokes) and the cell nuclei and debris were removed by centrifugation at 1,500 rpm for 5 min at 4 °C. The pellet was resuspended in 10 ml of 10 mM Tris pH 9.0, re-dounced and re-centrifuged as before. Supernatants were pooled, sonicated briefly and loaded carefully, taking care not to mix the supernatant and the sucrose, onto 18 ml of 36 % (w/v) sucrose solution in 10 mM Tris pH 9.0

buffer in 25 x 89 mm centrifuge tubes (Beckman Ultra-Clear Tubes). The tubes were centrifuged at 32,900 g for 80 min at 4 °C using a Beckman L8M Ultracentrifuge and SW28 rotor. The supernatant was removed by aspiration and the pellet was resuspended in 1 ml 10 mM Tris pH 9.0 buffer.

2.4.10 Virus purification by sucrose density gradient centrifugation

Only virus that has been purified as described in 2.4.9 can be further purified by centrifugation through a 24-40 % (w/v) sucrose gradient. Gradients were prepared a day in advance by layering 5.8 ml of each of 40, 36, 32, 28 and 24 % (w/v) sucrose in 10 mM Tris pH 9 solutions in 25 x 89 mm tubes taking care not to mix the different layers and were stored at 4 °C overnight. The purified virus was then layered on the sucrose gradient and centrifuged at 26,000 g for 50 min at 4 °C. The white band containing the virus was collected carefully. The solution corresponding to the collected virus band (approximately 10 ml) was then topped up to 36 ml with 10 mM Tris pH 9 and transferred to new centrifuge tubes and collected by centrifugation at 32,900 g for 80 min. Both centrifugations were carried out using the Beckman L8M Ultracentrifuge and the SW28 rotor. The final pellet was resuspended in a small volume (0.5-1 ml) of 10 mM Tris pH 9, sonicated, aliquoted and stored at -80 °C.

2.4.11 Phenol/chloroform extraction of DNA

The purified virus was resuspended in a volume of 0.6 ml in 10 mM Tris pH 9 and 50 µL 1 M Tris (pH 7.8), 50 µL 10 % SDS, 100 µL 60 % sucrose and 200 µL Proteinase K were added to it and incubated for 4 h at 37 °C. The genomic DNA was extracted by adding 1 volume of 25:24:1 phenol:chloroform:isoamyl alcohol mixture, mixing the phases thoroughly but gently by inversion, spinning in a microfuge at 3,000 rpm for 2 min, and pipetting the top (aqueous) phase into a new tube using blunt-end pipette tips to avoid shearing DNA and taking care not to disturb the lower organic phase or the interface between the two phases. The organic phase was then discarded and the last step repeated with the aqueous phase. The aqueous phase was then removed and 1 volume of a 24:1 part mixture (chloroform:isoamyl alcohol) was added, the tube was inverted to mix the solutions and was centrifuged as before. The aqueous phase was removed and DNA was recovered by ethanol precipitation.

2.4.12 Ethanol precipitation of DNA

DNA was extracted from aqueous solutions by the addition of sodium acetate (pH 5.2) to a final concentration of 0.1 M, followed by the addition of 2.5 volumes of absolute ethanol. Tubes were inverted gently several times to mix and were incubated at -20 °C overnight. DNA was recovered by centrifugation (13,000 rpm, 30 min and 4 °C) and the pellet obtained was washed once with 70 % ethanol and re-centrifuged. The DNA pellets were air dried until no moisture was visible inside the tube and were dissolved overnight at 4 °C in 100 µL sterile ddH₂O.

Chapter 3: Analysis of K7 protein

3.1 Bioinformatics analysis of K7

WR gene *K7R* encodes K7, a 149 amino acid protein with a predicted molecular mass of 17.5 kDa (Poxviruses Bioinformatics Resources (PBR) Centre www.poxvirus.org). To investigate how conserved K7 is in poxviruses, the sequences of K7 orthologues available at [poxvirus.org](http://www.poxvirus.org) were aligned using Clustal W (<http://www.ebi.ac.uk/clustalw/>). All strains of VACV for which sequence data were available showed a *K7R* open reading frame encoding a highly conserved K7 protein. VACV strains included WR and MVA, which were used in this project, as well as, COP, Lister, Duke, LC16m8, and rabbitpox Utrecht (RPXV-UTR). The K7 protein was identical in all strains except RPXV Utrecht that contained three amino acid changes, two of which were conserved. This computational analysis showing conservation of K7 sequences was consistent with experimental investigation of K7 protein expression by different VACV and CPXV strains using an anti-K7 Ab. This demonstrated that in addition to the strains for which sequence data were available (WR, MVA, COP, Lister, rabbitpox Utrecht, 3737, Acambis 3, Acambis 2000, Acambis 3000 MVA, CVA, Duke, LC16m8, LC16mO, Lister VACV107, MVA I721, and Tian Tan) an additional 10 strains (USSR, Evans, Wyeth, buffalopox, Kings's Institute, Patwad, Dairen, IHD-J, IHD-W and Tashkent) all expressed a K7 protein of similar size (Dr. Stuart Lucas unpublished data). Therefore, a total of 27 VACV strains are known or predicted to encode K7.

Next, the VACV WR K7 sequence was compared to K7 orthologues within other OPVs. K7 orthologues were predicted in VARV, MXPV, CPXV, CMLV, TATV and horsepox (HSPV) but not in ECTV. ECTV strains Moscow and Naval contain a nucleic acid sequence with 95 % identity to *K7R* but several mutations at the beginning of the sequence (first 60 bp) followed by a stop codon mean that no K7 protein is encoded. The peptide sequence identity between all K7 orthologues found in OPVs was ≥ 93.96 % and sequence similarity was ≥ 96.58 %. A K7 amino acid sequence alignment with representative orthologues from each OPV species is shown in Fig. 3.1. This strong K7 sequence conservation within OPVs despite the location of *K7R* in the less conserved terminal regions of the genome suggests an important function for K7.

		*	20	*	40	*	60	*	
CMLV-CMS-35R	:	MATKSDYEDAVFYFVDD	-EI	CSRDSIIDLIDEYITWRNHV	I	VFNKDITSCGRLYKELMKFDD	AAIRYYG	INKINE	: 74
VARV-BGD75maj-C4R	:	MATKSDYEDAVFYFVDD	-EI	CSRDSIIDLIDEYITWRNHV	I	VFNKDITSCGRLYKELMKFDD	AAIRYYG	IDKINE	: 75
HSPV-MNR76-040	:	MATKLDYEDAVFYFVDD	DKI	CSRDSIIDLIDEYITWRNHV	I	VFNKDITSCGRLYKELMKFDD	VAIRYYG	IDKINE	: 75
VACV-MVA-028R	:	MATKLDYEDAVFYFVDD	DKI	CSRDSIIDLIDEYITWRNHV	I	VFNKDITSCGRLYKELMKFDD	VAIRYYG	IDKINE	: 75
VACV-WR039R	:	MATKLDYEDAVFYFVDD	DKI	CSRDSIIDLIDEYITWRNHV	I	VFNKDITSCGRLYKELMKFDD	VAIRYYG	IDKINE	: 75
VACV-Cop-K7R	:	MATKLDYEDAVFYFVDD	DKI	CSRDSIIDLIDEYITWRNHV	I	VFNKDITSCGRLYKELMKFDD	VAIRYYG	IDKINE	: 75
RPXVutr028	:	MATKSDYEDAVFYFVDD	DKI	CSRDSIIDLIDEYITWRNHV	I	VFNKDITSCGRLYKELMKFDD	AAIQYYG	IDKINE	: 75
CPXV-BR-046	:	MATKSDYEDAVFYFVDD	DEL	CSRDSIIDLIDEYITWRNHV	I	VFNKDITSCGRLYKELMKFDD	VAIRYYG	IDKINE	: 75
TATV-DAH68-040	:	MATKSDYEDAVFYFVDD	-EI	CSRDSIIDLIDEYITWRNHV	I	VFNKDITSCGRLYKELMTEDD	VAIRYYC	IDKINE	: 74
MPXV-ZAR-C6R	:	MTKSDYEDAVFYFVDD	DEL	CSRDSIIDLIDEYITWRNHV	I	VFNKDITSCGRLYKELIKFDD	AAIRYYG	IDKINE	: 75
		MaTK	DYEDAVFYFVDDd	i	CSRDSIIDLIDEYITWRNHv	I	VFNKDITSCGRLYKELmkFDD	AirYYgIdKINE	

		80	*	100	*	120	*	140	
CMLV-CMS-35R	:	IVEAMSEGDHYINLT	EV	HDQESLFATIGICAKIT	EH	WGYKKISESRFQSLGNITDL	ITDDNINILILFLEKKLN	:	148
VARV-BGD75maj-C4R	:	IVEAMSEGDHYINLT	EV	HDQESLFATIGICAKIT	EH	WGYKKISESRFQSLGNITDL	MTDDNINILILFLEKKLN	:	149
HSPV-MNR76-040	:	IVEAMSEGDHYINFT	KV	HDQESLFATIGICAKIT	EH	WGYKKISESRFQSLGNITDL	MTDDNINILILFLEKKLN	:	149
VACV-MVA-028R	:	IVEAMSEGDHYINFT	KV	HDQESLFATIGICAKIT	EH	WGYKKISESRFQSLGNITDL	MTDDNINILILFLEKKLN	:	149
VACV-WR039R	:	IVEAMSEGDHYINFT	KV	HDQESLFATIGICAKIT	EH	WGYKKISESRFQSLGNITDL	MTDDNINILILFLEKKLN	:	149
VACV-Cop-K7R	:	IVEAMSEGDHYINFT	KV	HDQESLFATIGICAKIT	EH	WGYKKISESRFQSLGNITDL	MTDDNINILILFLEKKLN	:	149
RPXVutr028	:	IVEAMSEGDHYINFT	KV	HDQESLFATIGICAKIT	EH	WGYKKISESRFQSLGNITDL	MTDDNINILILFLEKKLN	:	149
CPXV-BR-046	:	IVETMSEGDHYINLT	EV	HDQESLFATIGICAKIT	EH	WGYKKISESRFQSLGNITDL	MTDDNINILILFLEKKLN	:	149
TATV-DAH68-040	:	IVEAMSEGDHYINLT	EV	HDQESLFATIGICAKIT	EH	WGYKKISESRFQSLGNITDL	MTDDNINILILFLEKN--	:	146
MPXV-ZAR-C6R	:	IVEAMSEGDHYINLT	EV	HDQESLFATIGICAKIT	AE	HWGYKKISESRFQSLGNITDL	MTDDNINILILFLEKKLN	:	149
		IVEaMSEGDHYIN	T	VHDQESLFATIGICAKIt	EH	WGYKKISESRfQSLGNITDLm	TDDNINILILFLEKkln		

Fig. 3.1. Amino acid alignment of K7 orthologues in OPVs. CPXV-BR = cowpox virus strain Brighton; TATV-DAH68 = taterapox virus strain Dahomey 1968; CMLV-CMS = camelpox virus strain CMS; HSPV-MNR76 = horsepox virus strain MNR76; VAR-BGD75maj = variola major virus strain Bangladesh 1975 and MPXV-ZAR = monkeypox virus strain Zaire. The sequences were retrieved from the PBR Centre, aligned using Clustal W and visualised using GENEDOC. Black background = 100 % amino acid identity between sequences; dark grey = 80-99 % amino acid identity between sequences; light grey = 60-79% amino acid identity between sequences. The last row of letters indicates the consensus sequence.

Within other chordopoxvirus genera, K7 had significant sequence similarity with proteins predicted to be encoded by swinepox (SWPV) ORF 133 (genus *Suipoxvirus*) and lumpy skin disease virus (LSDV) ORF 136 (genus *Capripoxvirus*). The former shared 25 % amino acid identity and 50 % similarity with K7 and the latter only 24.6 % identity and 47 % similarity. A Blast similarity search (www.ncbi.nlm.nih.gov/blast/) gave no hits for K7 outside poxviruses meaning it does not have any cellular orthologues.

In addition to the K7 orthologues identified in VACV strains, other OPVs and chordopoxviruses, K7 shows similarity to other VACV proteins including A52. VACV WR A52 and K7 sequences share 26 % amino acid identity and 45 % similarity. Notably, K7 also shares some of the intracellular targets of A52, namely TRAF6 and IRAK2, but it is a broader inhibitor of innate immune signalling than A52 because it inhibits both TLR-dependent and independent pathways (Harte *et al.*, 2003; Schroder *et al.*, 2008). Additionally, K7 and A52 are members of a family of poxvirus proteins first described in 1991 (Smith *et al.*, 1991) and which also includes VACV proteins, A46, A52, C6, N1, F1 and B14. The structures of several of these proteins (N1, B14, A52, K7 and F1) have been determined and are all Bcl-2 family members (Cooray *et al.*, 2007; Graham *et al.*, 2008; Kvansakul *et al.*, 2008; Kalverda *et al.*, 2009). Although these proteins have a similar structures, the sequence identity between them is low (< 20 %) but the amino acid physiochemical properties are conserved (Fig. 3.2). For instance, 40 % of K7's peptide sequence is physiochemically conserved in other proteins of this family. Fig. 3.2 also indicates that the N terminus of these proteins is variable in length. In addition, these proteins share other biochemical properties: they are small, acidic (low pI), non-hydrophobic and are also known or predicted to be mainly alpha-helical. The structures of N1, B14, A52, K7 and F1 also indicate important differences. N1 and F1 have an open groove on the surface that allows the binding of BH3 domains from pro-apoptotic Bcl-2

proteins and both are known to inhibit apoptosis. In contrast, A52, B14 and K7 have lost the surface groove suggesting they are not anti-apoptotic and this has been confirmed experimentally for B14 and A52 (Graham *et al.*, 2008). Instead these proteins function as inhibitors of innate immune signalling pathways and the stable Bcl-2 fold is exploited by VACV to provide a surface for binding host signalling molecules. The properties of these VACV Bcl-2 like proteins including, pI, molecular mass, and conservation in MVA as well as sequence identity to K7 are summarised in Table 3.1.

Within MVA there is no A52 orthologue because the corresponding region was deleted from the MVA genome during extensive passage in CEFs (Antoine *et al.*, 1998). In contrast, MVA encodes an orthologue of VACV WR A46 (called 159) with which it shares 99 % amino acid identity and has a single conserved amino acid substitution. F1 and its MVA orthologue 029 shared 97 % amino acid identity and 029 has a 4 amino acid N-terminal deletion and a conserved amino acid substitution. MVA does not encode a full length orthologue of a N1 due to gene fragmentation so that the corresponding protein (008) has three deletions towards its C terminus (Antoine *et al.*, 1998). Based on the published crystal structure of N1, it is highly likely the remaining peptide (008) will be unstable. The MVA orthologue of B14, called 183 has a couple of conserved amino acid substitutions but also has a 6 amino acid internal deletion that renders the protein unstable and non-functional (Laura McCoy, personal communication). Finally, VACV WR C6 is uncharacterised but has 97 % identity with its MVA counterpart, 019, which has 3 conserved amino acid substitutions but also a 6 amino acid extension at the C terminus (Antoine *et al.*, 1998).

```

      *      20      *      40      *      60      *      80
K7 : -----MATKLDYEDAVFYF-----VDDDKICSR-----DSIIDL : 29
A52 : -----MDIKIDISISGDKFTVTTRRENEERKKYLPLQKKTDTVIKFDYLEYDDLDR-----DEMFTI : 59
A46 : MAFDISVNASKTINALVYFSTQQNKLVIRNEVNDTHYTVFEFDRKWVDTEFISYNRHNDTIEIRGVLPPEETNIGCAVNTPVSMITYL : 85
B14 : -----MTANFSTHVFSF-----QHCGRRLTSI-----DDVRQC : 29
C6 : -----MNAYNKADSFSLSE-----DSIKDV : 20
N1 : -----MRTL : 4
F1 : MISMFCNNIVDYVDDIDNGIVQDIEDEASNNVDHDYVYPLPEIMVYREFDKSTMILDYLLSTER-----DHVMMA : 69

      *      100      *      120      *      140      *      160      *
K7 : IDEYITWRNHVIVFNKDKITSCGRLYKELMKFDDVAIRYVG--IDKIN-EIVEAMSE--GDHYINFTKVHDQ-----ESLFATI : 102
A52 : LEEYFMYRGLLGLR----IKYGRLFNEIKKFDNDAAEQFGT--IEELK-QKLRLNSEEGADNFIDYIKVQKQDIVKLTIVYDCISMI : 138
A46 : YNKYSFKLILAEYIRHRNTISGNIYSALMTLDDLAIKQVGD-IDLLFNEKLKVDSDSGLFDFVNFVKDMIC-----CDSRIVV : 162
B14 : LTEYIYWSSYAYRNR--QCAGQLYSTLLSFRDDAELVFID-IRELVKNMPWDDVADCAEIRCYIPDEQK----TIREISAIL : 105
C6 : IHDYICWLSMTDEMR--PSIGNVFEKAMETFEKIDAVRYVDGNIYELAKDINAMSEFGFIRSLQTIASKKDK-----LTVYGTM : 95
N1 : LIRYILWRNDNDQTY----YNDDFKKLMLLDELVD---DGPVCTLIKNMRMTLSDGELLDRLNQEVNNIE-----DAKRMI : 73
F1 : VRYYSKQRLDDLYRLPTKTRSYIDIINIYCDKVSNDINRMNIMYMASTKSEFVYDINNEVNTILMDN-----KGLGVRL : 147
      Y

      180      *      200      *      220      *      240      *
K7 : GICAKITEHWG-YKKISESRFQSLGNITDLMT-DDNTNIIILFLEKILN----- : 149
A52 : GLCACVVDVWR-NEKLF SRWKYCLRAIKLFIN-DHMLDKIKSILQNLVYVEMS----- : 190
A46 : ALSSLVSKHWE-LTNKKYRCMALAEHISDSIP-ISELSRLRYNLCKYLRGHTESTEDKEDYEEDDESSTCSAVTDRETDV : 240
B14 : GLCAYAAATYWGGEDHPTSNLSNALFVMLEMLN-YVDYNIIFRRMN----- : 149
C6 : GILSIVVDINKGCDISNIKFAAGIILMEYIFDDTDMSHLKVALYRRIQRRD VDR----- : 151
N1 : AISAKVARDIG--ERSEIRWEESFTILFRMIE--TYFDDIMIDLYGEK----- : 117
F1 : ATISFITELGRLRCMNPVETIKMFTLLSHTICDDYFVDYITDISPPNTIPNTSTREYLLKLLGTAIMFATYKTLKYMIG : 226

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Fig. 3.2. Amino acid alignment of VACV Bcl-2 like proteins from strain WR. The sequences were retrieved from the PBR Centre, aligned using Clustal W and visualised using GENEDOC. Black background = hydrophobic amino acid (aa); grey background and blue letter = aromatic aa; grey background and red letter = aliphatic aa; green background = polar aa; blue background = charged aa; yellow background and green letter = small aa and blue letter = aa A, G or S in particular.

VACV Bcl-2 like proteins (WR)	orthologue in MVA	pI	predicted molecular mass kDa	function	structure	peptide sequence identity with K7
K7	028, identical sequence	4.7501	17.5	inhibits NF- κ B and IRF3/7 activation	Bcl-2-like with no BH3 binding groove	100 %
B14	183, two conserved substitutions and a 6 aa deletion	4.82	17.38	inhibits NF- κ B by targeting IKK- β	Bcl-2-like with no BH3 binding groove	22 %
A52	deleted	5.4474	22.71	inhibits NF- κ B by targeting IRAK-2	Bcl-2-like with no BH3 binding groove	22 %
A46	159, single conserved aa substitution	4.99	27.62	inhibits NF- κ B by targeting MyD88 and TRIF	predicted to be mainly alpha-helical	20 %
N1	020, several substitutions and three small aa deletions at C terminus	4.48	13.9	inhibits apoptosis and NF- κ B activation	Bcl-2-like with open BH3 binding groove	18 %
F1	029, 4 aa deletion at N terminus and 1 conserved aa substitution	4.58	26.4	inhibits apoptosis	Bcl-2-like with open BH3 binding groove	12 %
C6	019, 3 conserved aa substitutions and a 5 aa extension at C terminus	4.96	17.33	unknown	predicted to be mainly alpha-helical	15 %

Table 3.1. Summary of VACV WR Bcl-2 like proteins biochemical properties, function and conservation in MVA. The table indicates the pI, predicted molecular mass, function (if known) and structure (if known), conservation in MVA and % sequence identity with K7 of the proteins listed. aa = amino acid

3.2 Localisation of K7 protein in mammalian cells

3.2.1 Localisation of K7 protein in mammalian cells by immunofluorescence

The location of the K7 protein was examined by immunofluorescence in HeLa cells transfected with a plasmid expressing an N-terminally Haemagglutinin (HA)-tagged (N-HA) K7 (pCI-HA-K7) or empty vector control (pCI) (Fig. 3.3). This showed that K7 is cytoplasmic and has no obvious co-localisation to specific organelles. These experiments were repeated using an anti-K7 polyclonal Ab raised against recombinant K7 expressed in *E. coli* (Harlan labs) but the Ab was found not to detect K7 protein by immunofluorescence.

The finding that K7 is cytoplasmic when expressed transiently contradicts an earlier report (Schroder *et al.*, 2008)) that K7 is located in both the cytoplasm and the nucleus. Some of those experiments were also carried out using a plasmid expressing N-HA K7 but it must be noted that cells were stained and observed 48 h post-transfection whereas data shown here were generated with cells transfected for 6 h. Further investigation showed that K7 was still mainly cytoplasmic at 20 or 24 h post-transfection, when the level of K7 increased, but some K7 was also seen in the nucleus (results not shown). Taken together, these data suggest that some K7 can be detected in the nucleus between 20 and 48 h post-transfection when high levels of the protein are present. However, the biological relevance of this is uncertain given that K7 is an early protein whose expression is detected 1 h p.i. and steadily increases up to 24 h p.i. (e.g. shown in Fig. 3.6 and Fig. 5.20).

Consequently, the localisation of K7 in the context of VACV infection was also investigated. This was carried out in HeLa cells after infection for 24 h with a virus producing N-HA K7 protein (vK7-HA) or with vΔK7 as a control. Fig. 3.4 shows that K7 is predominantly cytoplasmic and excluded from the nucleus during infection. There was no significant

background in cells infected with $\nu\Delta K7$ that were identified by anti-B5 staining. In conclusion, K7 is located in the cytoplasm during infection.

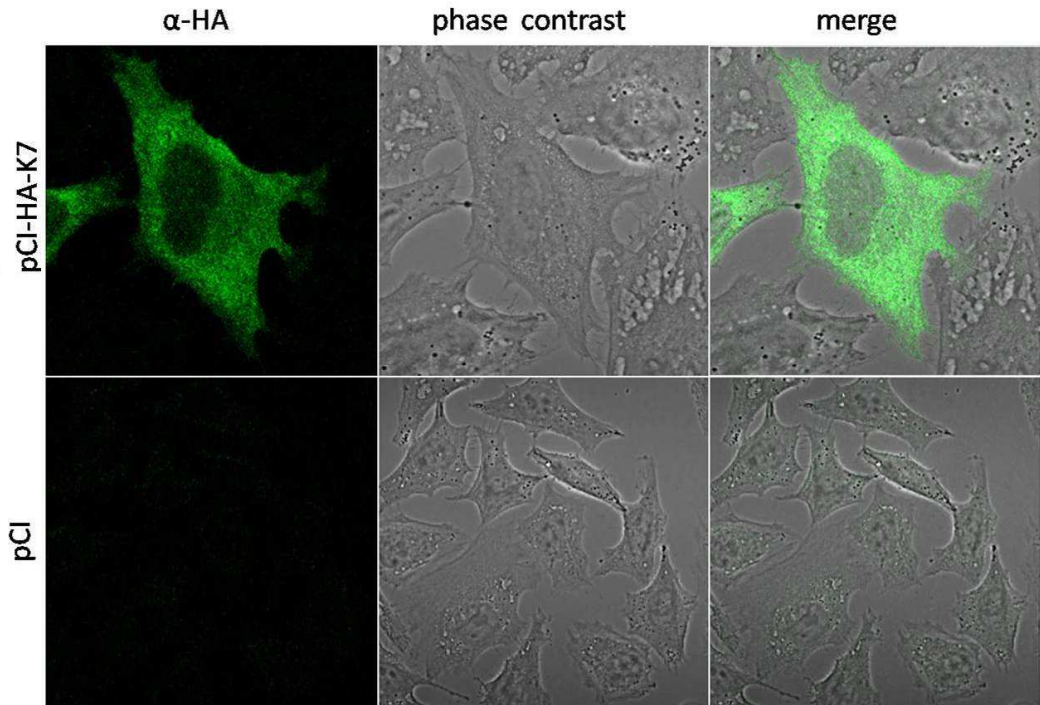


Fig. 3.3. Sub-cellular localisation of K7. HeLa cells were transfected with pCI or pCI-HA-K7 as indicated for 6 h. Cells were then fixed and permeabilised as described in Materials and Methods 2.3.6 and stained with anti-HA mouse monoclonal Ab (mAb) followed by staining with anti-mouse Alexa 488-conjugated (green) secondary Ab. Images were recorded by laser scanning confocal microscopy. Phase contrast and merge images are also shown as indicated. The scaling is $0.14\ \mu\text{m} \times 0.14\ \mu\text{m}$ for each of the three top panels and $0.29\ \mu\text{m} \times 0.29\ \mu\text{m}$ for the three lower panels.

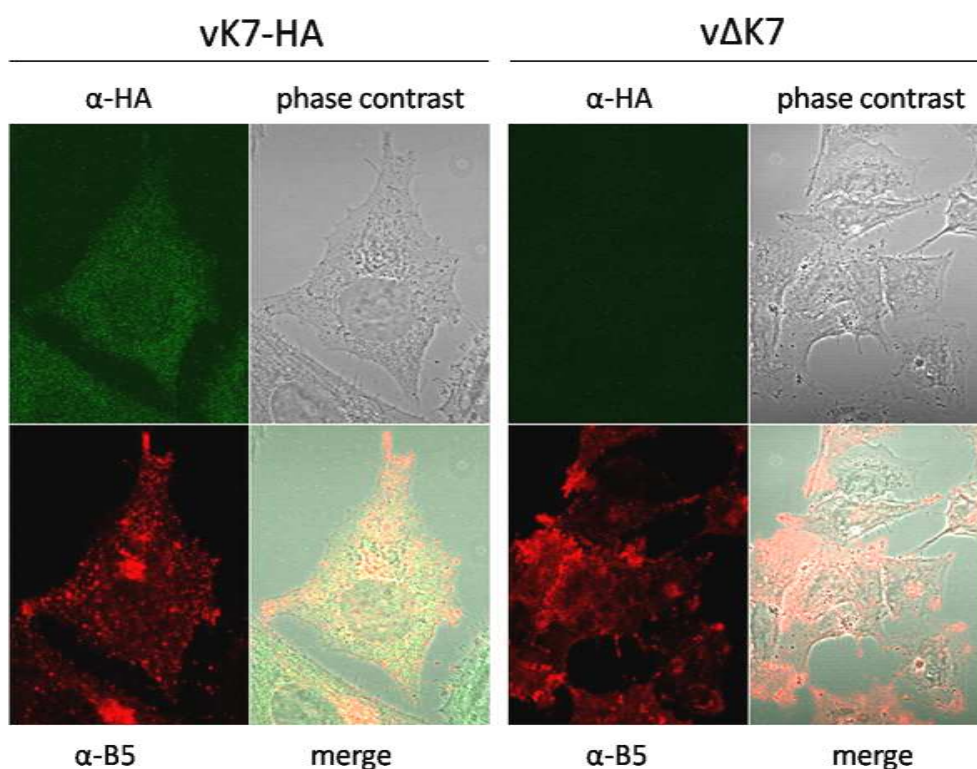


Fig. 3.4. Localisation of K7 in VACV-infected cells. HeLa cells were infected with vK7HA or vΔK7 as indicated at 1 pfu/cell for 16 h. Cells were then fixed and permeabilised as in Fig. 3.3 and stained with anti-B5 and anti-HA Abs. Cells were then stained with anti-rat Alexa 546 (red) and anti-mouse Alexa 488 (green)-conjugated secondary Abs and images were recorded by laser scanning confocal microscopy. Phase contrast and merge images are also shown as indicated. The scaling is 0.10 μm x 0.10 μm for each of the four left panels and 0.16 μm x 0.16 μm for the four right panels.

3.2.2 Localisation of K7 in VACV-infected mammalian cells by immunoblotting

The sub-cellular localisation of K7 was also investigated by biochemical fractionation of cells followed by immunoblotting. HeLa cells were mock-infected or infected with vK7, vΔK7, vfsK7-1, and vK7-HA. A full description of vfsK7-1 will be provided in chapter 5. Briefly, it is a virus in which an extra nucleotide was introduced into the *K7R* translational initiation codon thus mutating the ATG codon to ATAG and consequently it was shown not to produce K7 protein. Cell lysates were then fractionated into nuclear and cytoplasmic (Materials and Methods 2.3.7) before they were analysed by SDS-PAGE followed by immunoblotting (Fig. 3.5). A 4-fold greater proportion of the nuclear fraction was loaded onto the gel than for cytoplasmic fraction. Cellular proteins, tubulin and lamins A and C, known to localise to

either the cytoplasm or nucleus, respectively, were used as controls, together with the VACV IMV protein, D8. Fig. 3.5 shows that tubulin and lamins A and C are expressed in the expected cellular compartments. The control D8 protein was seen in both cytoplasmic and nuclear samples and indicated that infections were carried at similar levels for all the viruses. K7 was detected in cytoplasmic fractions from the viruses that express it (vK7 and vK7HA) but was absent in the fractions from the vΔK7, vfsK7 and mock-infected samples. A very faint band corresponding to the size of K7 was detected in the nuclear fraction after overexposure but this was similar to an equally faint band of the size of tubulin in the nuclear fraction samples after overexposure (result not shown) indicating a small amount of cytoplasmic contamination in nuclear samples. Therefore this experiment confirms that K7 is cytoplasmic during infection.

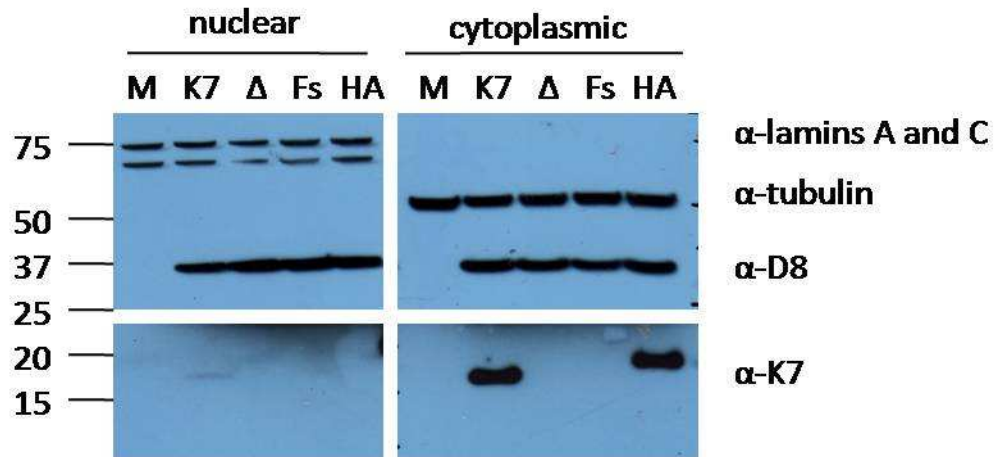


Fig. 3.5. Determination of K7 localisation by immunoblotting. Cells were mock-infected or infected at 10 pfu/cell with the vK7 (K7), vΔK7 (Δ), vfsK7-2 (Fs) and vK7HA (HA) for 16 h and then lysed. Cell lysates were harvested and fractionated into cytoplasmic and nuclear fractions and analysed by SDS-PAGE (12 % gel) and immunoblotting with the indicated Abs. The positions of molecular mass markers are shown in kDa.

3.3 Temporal expression of K7 during VACV infection

The temporal expression of K7 during VACV infection was investigated by infecting cells at 5 pfu/cell in the presence or absence of β-D-arabinofuranoside (AraC), an inhibitor of viral

DNA replication and thereby of intermediate and late gene expression. In addition to AraC, treatment with cycloheximide (CHX, a translation inhibitor) was included to demonstrate that the K7 protein seen early during infection was the product of new protein synthesis rather than being introduced into the cell by the infecting virions. Infected samples were lysed at different times p.i. and lysates were analysed by immunoblotting (Fig. 3.6). The VACV D8 protein was only expressed in the absence of AraC, as expected, for it is known to be expressed only late during infection (Niles & Seto, 1988). By contrast, K7 was detected from 1 h p.i., and its level increased up until 8 h p.i. In the presence of AraC, K7 expression was reduced but not absent, indicating that K7 is expressed both early and late during infection.

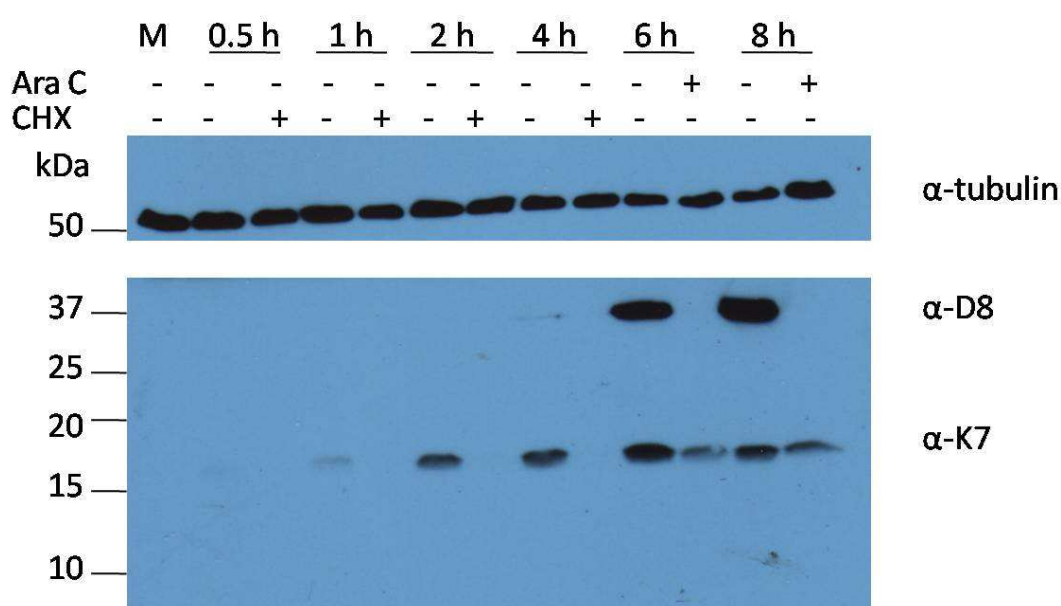


Fig. 3.6. Temporal expression of K7 during VACV WR infection. HeLa cells were mock-infected (M) or infected with VACV WR at 10 pfu/cell in the presence (+) or absence (-) of 40 μ g/ml AraC or in the presence or absence of 100 μ g/mL CHX. At the indicated times p.i., the cells were washed once with PBS and lysed by addition of Triton X based cell lysis buffer (see Materials and Methods 2.3.4). Samples were analysed by SDS-PAGE (12 % gel) and immunoblotting with anti- α -tubulin, anti-D8 and anti-K7 Abs. The positions of molecular mass markers are shown in kDa.

Chapter 4: Expression and purification of K7 protein in a bacterial expression system for crystallisation trials

To gain further insight into the mechanism of action of the K7 protein we sought to determine its structure in collaboration with David Stuart's group at the University of Oxford. All protein expression and purification steps were undertaken at Imperial College London and protein crystallisation trials and collection of diffraction data were carried out by Dr Stephen Graham from Prof Stuart's group at the University of Oxford.

Preliminary work carried out by Dr Stuart Lucas in this lab showed that the K7 protein from VACV strain WR was insoluble when expressed from pET-28a in Rosetta *E. coli* cells that were grown in LB and induced by addition of IPTG. This was done using DNA encoding an N-6 X His K7 that had been cloned into pET-28a by Dr Lucas.

4.1 Expression and purification of K7 from a bacterial expression system

4.1.1 Expression of soluble K7 protein from pET-28a in TB broth

To obtain soluble protein, B834 *E. coli* harbouring pET-28a-K7 were cultured for two days at RT. Other *E. coli* strains such as BL-21 were tested but this strain was found to give the highest expression. Bacterial cells were collected by centrifugation, lysed and the soluble and insoluble fractions obtained were analysed by SDS-PAGE followed by staining with Coomassie blue and immunoblotting using an anti-4 X histidine (His) Ab. Both soluble and insoluble K7 were obtained (Fig. 4.1a). Optimisation of conditions for K7 soluble expression showed that incubation at 23 °C for 48 h gave the highest yield of protein (results not shown). Soluble protein was affinity purified via its N-terminal His tag by immobilised metal affinity chromatography (IMAC) (Fig. 4.1b). Non-specific protein was washed away using wash buffer and bound protein was eluted with an imidazole gradient. A peak in UV absorbance at 280 nm was seen and samples from fractions corresponding to this peak were analysed by SDS-PAGE followed Coomassie blue staining or immunoblotting (Fig. 4.1c). Soluble K7 protein was observed on the stained gel in all peak lanes and this was also seen on the immunoblot. These fractions were pooled, concentrated and applied to a Superdex 75 10/30 column (analytical grade) for gel filtration.

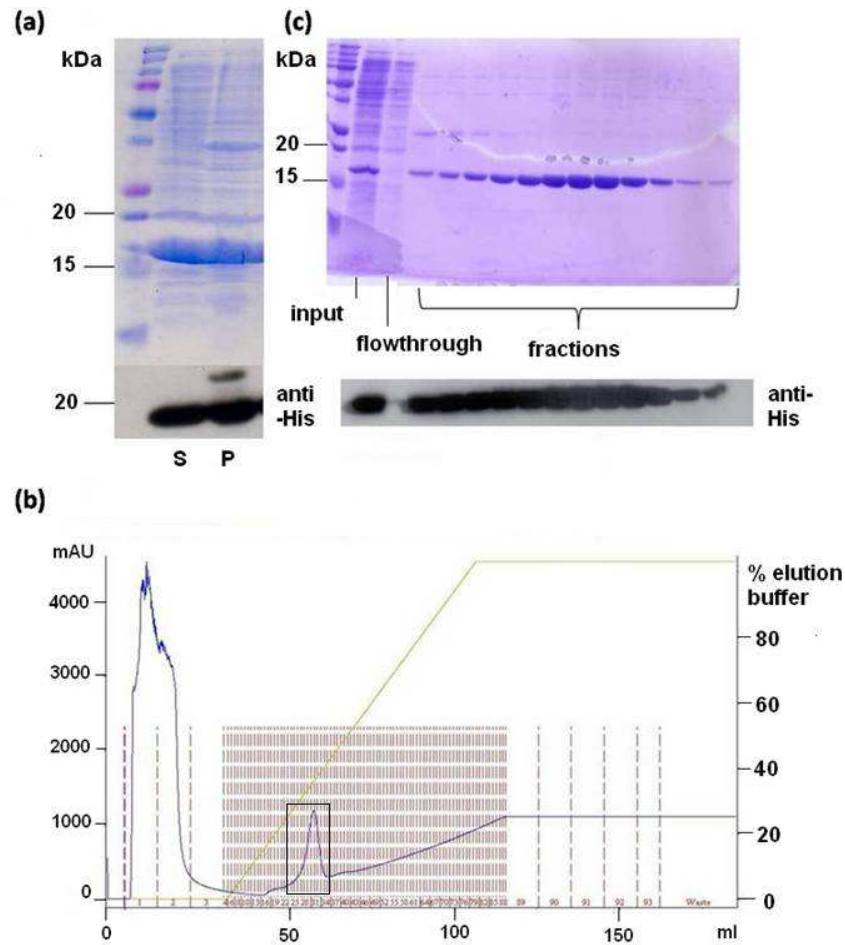


Fig. 4.1. Purification of His-tagged K7 protein expressed from pET-28a by IMAC. (a) N-6 X His K7 was expressed from pET-28a in B834 *E. coli* and the soluble and insoluble protein fractions were harvested as described in Materials and Methods 2.2.2. Samples of soluble (S) and insoluble (P) fractions were analysed by SDS-PAGE (15 % gel) followed by staining with Coomassie blue and immunoblotting using anti-4 X His Ab. (b) Soluble N-6 X His K7 was purified by IMAC using a HisTrap column as described in Materials and Methods 2.2.3. The blue line indicates the UV absorbance at 280 nm and the green line indicates the percentage v/v of elution buffer flowing through the HisTrap column. The different fractions collected during this experiment are indicated in red. The x-axis indicates the volume of buffer that has entered the column since the beginning the run. The left y-axis indicates the UV absorbance in milli-absorbance units (mAU) and the right y-axis indicates the percentage of elution buffer (v/v) entering the column. (c) Fractions corresponding to the peak in UV absorbance at 280 nm (after the washing step, indicated by a black box in (b)) were analysed for the presence of N-6 X His K7 by SDS-PAGE (15 % gel) followed by staining with Coomassie blue or immunoblotting using anti-4 X His Ab. A sample of the input and a sample of the flowthrough from the initial washing step were also tested as indicated. The positions of molecular mass markers are shown in kDa.

Fig. 4.2a shows that several peaks of UV absorbance at 280 nm were obtained and samples of corresponding fractions were analysed by SDS-PAGE and Coomassie blue staining (Fig. 4.2b). Fractions from the middle peak contained soluble purified His-tagged K7 protein. Samples from other peaks did not contain any K7 protein and no other bands were visible. The first small peak appears to correspond to the void volume (V_0) of the gel filtration column (elution volume of any molecules too big to enter the largest pores in the gel matrix) and the last large peak may correspond to the elution of imidazole that was present in the elution buffer in the protein solution from the Histrap purification step. In subsequent repeat experiments, the gel filtration buffer used initially which had 200 mM NaCl was replaced with one with 50 mM NaCl, because K7 was found to precipitate in presence of higher salt concentrations and the larger Superdex 75 16/60 gel filtration column (preparation grade) was used. Protease inhibitors were also left out as these can interfere with crystallisation and proteases should no longer be present after the affinity purification step.

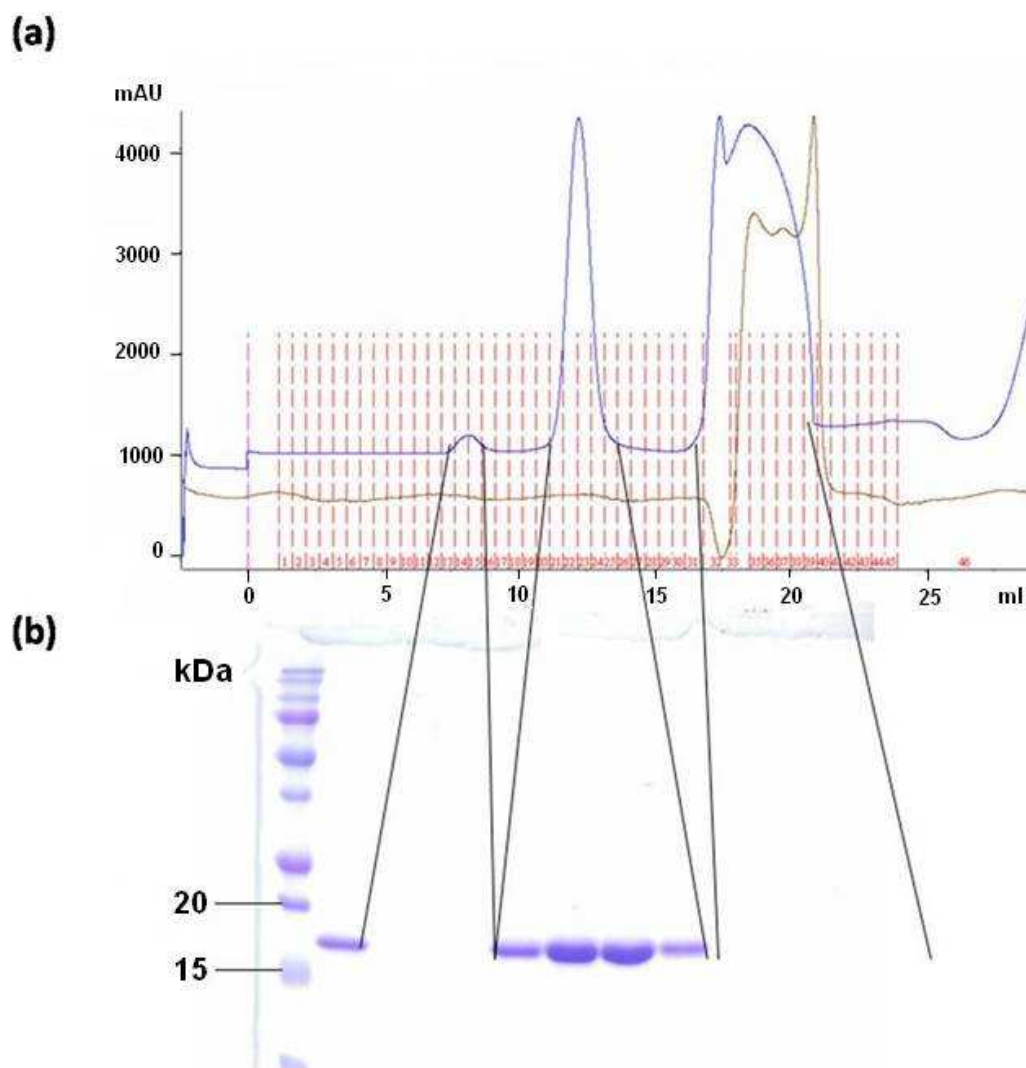


Fig. 4.2. Purification of His-tagged K7 protein expressed from pET-28a by gel filtration.

(a) N-6 X His K7 was purified by IMAC as described in Fig. 4.1, was applied to a Superdex 75 10/300 column in 0.5 ml and eluted in 20 mM Tris-HCl pH 8, 200 mM NaCl as described in Materials and Methods 2.2.3. The blue line indicates the UV absorbance at 280 nm and the brown line indicates the conductance. The different fractions collected during this run are indicated in red. The x-axis indicates the volume of buffer that has entered the column since the beginning of the run and the y-axis indicates the absorbance in mAU. (b) Fractions corresponding to different peaks in UV absorbance at 280 nm in (a) were analysed for the presence of N-6 X His K7 by SDS-PAGE (15 % gel) followed by staining with Coomassie blue as indicated. The positions of molecular mass markers are shown in kDa.

Over the course of these experiments, yields of up to 28 mg of protein were obtained from a single 500 ml culture. Soluble K7 protein obtained was sent to collaborators where it was found to be monodisperse in solution by Dynamic Light Scattering (DLS) analysis. This technique is used to determine the size distribution of protein in solution. K7 protein solution was concentrated to 5 mg/mL and crystallisation trials were set up and some small crystals were being obtained reproducibly (Fig. 4.3) but these were not of good enough quality for X-ray crystallography. These crystals may have been inherently disordered and it was thought that removing the N-6 X His tag from protein may bring some improvement. This cleavage was done using thrombin but was incomplete despite several attempts and tagged and untagged K7 protein could not be separated by IMAC (results not shown).

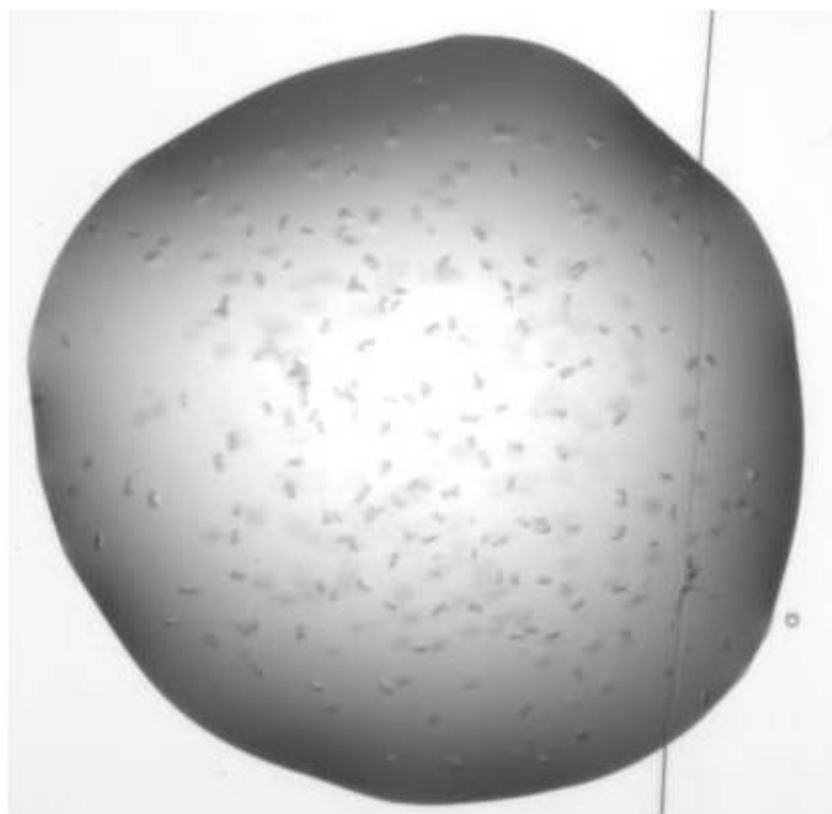


Fig. 4.3. Crystallisation of N-6 X His K7 expressed from pET-28a vector. N-6 X His K7 was purified by IMAC and size exclusion chromatography as described in Fig. 4.1 and 4.2 (except that the gel filtration buffer used was 10 mM HEPES, 50 mM NaCl, pH 8) and was sent to Prof. D Stuart's group for crystallisation trials. The purified protein was concentrated from 1 mg/ml to 5 mg/ml and used to set up 96-well microfluidics crystallisation experiments and small crystals of K7 protein were obtained and are shown in this photograph.

4.1.2 Expression of soluble K7 protein from pOPIN E and F vectors in TB broth

As thrombin cleavage of the His-tag of protein expressed from pET-28a vector was incomplete, recombinant protein was expressed from pOPIN E and pOPIN F vectors. These were developed at the OPPF, Wellcome Trust Centre for Human Genetics, University of Oxford and express proteins that have either a C or N-6 X His tag which can be cleaved completely by using 3-C protease (N-terminal tag) or carboxypeptidase A (C-terminal tag). The *K7R* sequence was cloned into pOPIN F linearised with *KpnI* and *HindIII* to generate the N-6 X His allele and into pOPIN E linearised with *NcoI* and *PmeI* to generate the C-6 X His version. The sequences were generated by PCR using VACV WR genomic DNA as template and primers K7 N-His fwd and rev or K7 C-His fwd and rev (See Materials and Methods 2.1.12). Cloning of the PCR product into plasmids was carried out using In-Fusion™ Dry-Down PCR Cloning Kit where a proprietary enzyme is used to carry out ligation-independent cloning (Clontech, www.clontech.com/images/pt/PT3754-1.pdf). This is based on a recombination between the pOPIN vectors that were designed to contain universal 15 bp sequences and their homologous sequences also present in PCR primers.

The expression of these versions of K7 was carried out in TB and the incubation conditions were the same as described when using pET-28a. This His-tagged protein was also affinity purified as before (Fig. 4.4) and purified by size exclusion chromatography on a Superdex 75 16/60 column (Fig. 4.5). As with K7 protein expressed from the pET-28a vector, soluble and insoluble K7 protein was obtained from B834 cells as seen by SDS-PAGE followed by staining with Coomassie blue (Fig. 4.4a) and when the soluble fraction was loaded on a Histrap column, a peak in UV absorbance at 280 nm was seen (Fig 4.4b). Samples from that peak were found to contain K7 protein (results not shown) and were pooled and concentrated before gel filtration. The latter yielded a large peak of UV absorbance at 280 nm (Fig. 4.5a) and its corresponding fractions contained soluble K7 protein as seen in Fig. 4.5b. Final protein yields of up to 6 mg were obtained from a 250 ml culture of C-6 X His-K7. This clone was the one used most frequently as only 1 mg of protein was obtained with N-terminal his-tagged protein. However, it is unknown why these yields are much lower (nearly 30 fold less) than those obtained with N-6 X His K7 expressed from pET-28a.

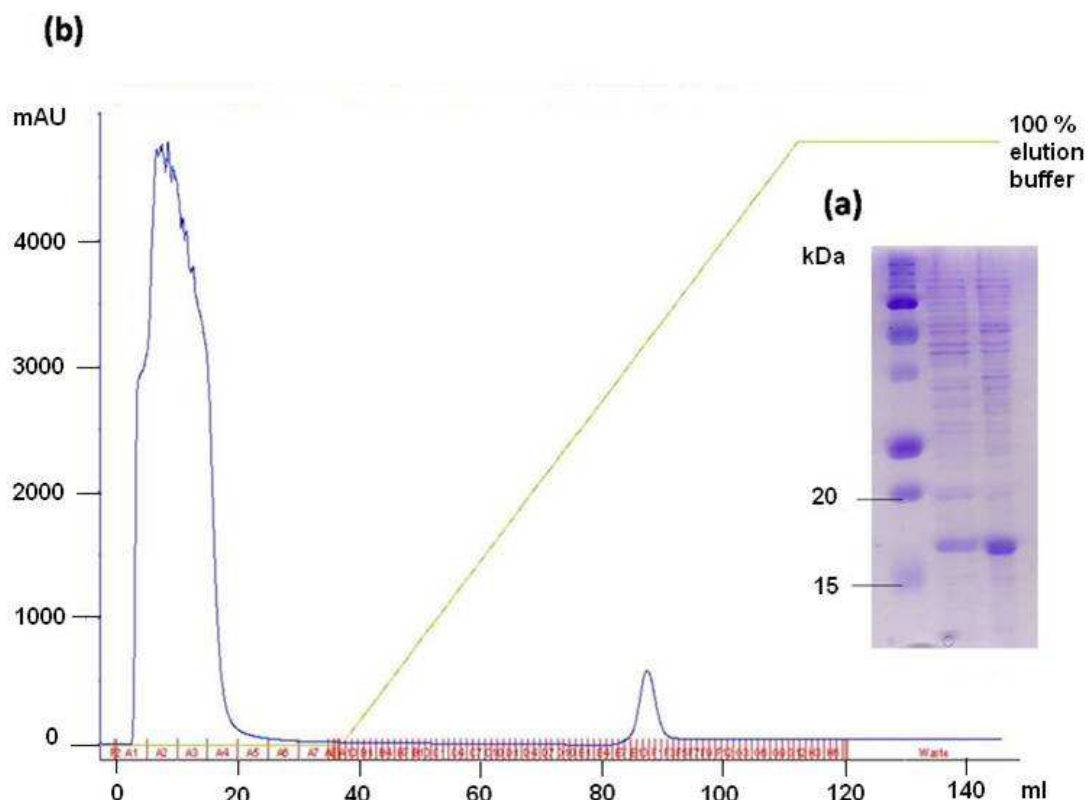
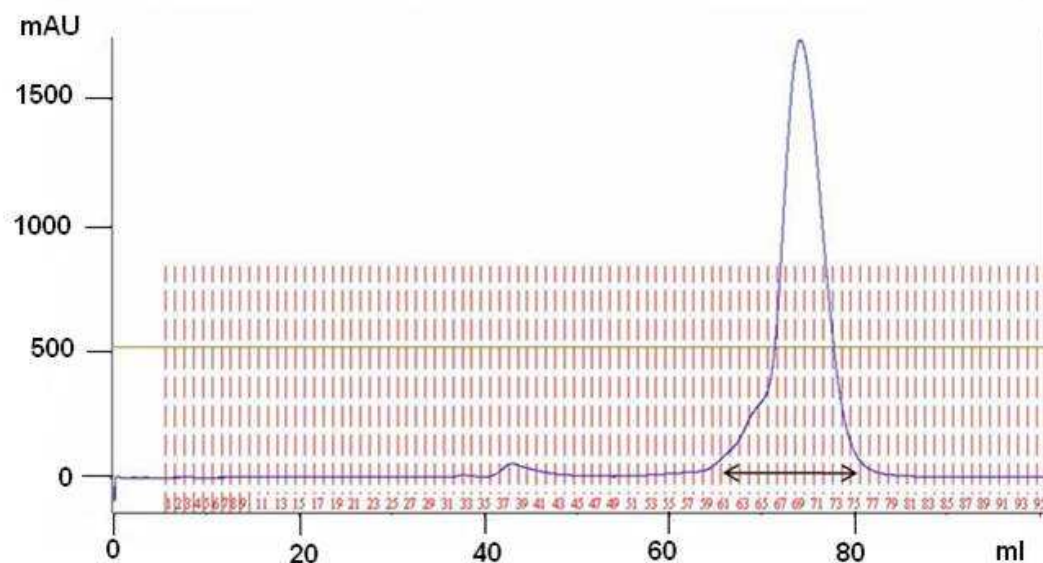


Fig. 4.4. Purification of His-tagged K7 protein expressed from pOPIN E by IMAC. (a) C-6 X His K7 was produced from pOPIN E in B834 *E. coli* and the soluble and insoluble protein fractions were harvested as described in Materials and Methods 2.2.2. Samples of soluble (S) and insoluble (P) fraction were analysed by SDS-PAGE (15 % gel) which was later stained with Coomassie blue. The positions of molecular mass markers are shown in kDa. (b) Soluble C-6 X His K7 protein was purified by IMAC as described in Fig. 4.1b. The blue line indicates the UV absorbance at 280 nm and the green line indicates the percentage v/v of elution buffer flowing through the Histrap column. The different fractions collected during this run are also indicated in red. The x-axis indicates the volume of buffer that has entered the column since the beginning the run. The left y-axis indicates the absorbance in mAU and the right y-axis indicates the percentage of elution buffer (v/v) entering the column.

(a)



(b)

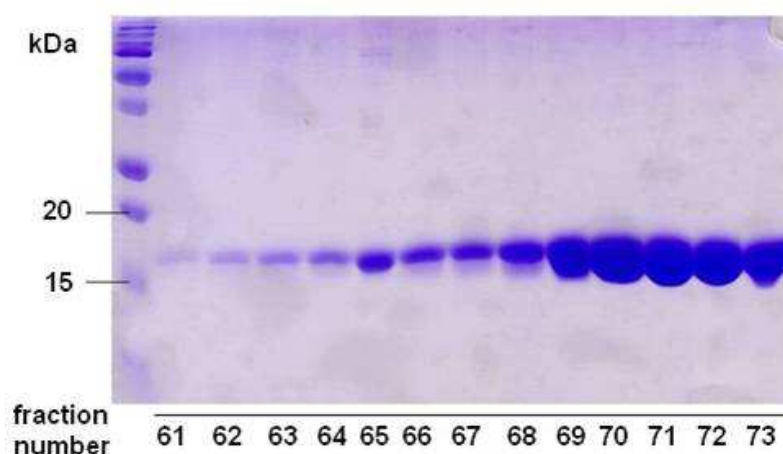


Fig. 4.5. Purification of His-tagged K7 protein expressed from pOPIN E by gel filtration.

(a) C-6 X His K7 was purified by IMAC as described in Fig. 4.4b, was concentrated to 1-2 ml and was applied to a Superdex 75 16/60 column and eluted in 10 mM HEPES 50 mM NaCl as described in Materials and Methods 2.3.3. The blue line indicates the UV absorbance at 280 nm and the different fractions collected during this experiment are indicated in red. The x-axis indicates the volume of buffer that has entered the column since the beginning the run and the y-axis indicates the absorbance in mAU. (b) Fractions corresponding to a peak in UV absorbance at 280 nm (indicated with an arrow in (a)) were analysed for the presence of C-6 X His K7 by SDS-PAGE (15 % gel) followed by staining with Coomassie blue. The positions of molecular mass markers are shown in kDa.

The pure soluble protein was taken to Oxford for crystallisation trials. The His tag was removed from K7 produced after expression from pOPIN E by Dr Graham in Oxford before setting up crystallisation trays. Although small protein crystals, the largest of which was 134 microns, were obtained (Fig. 4.6), the diffraction of these crystals only yielded a resolution of 10 ångström (Å), which is not enough to resolve the crystal structure of K7. Normally a resolution in the range of 2-3 Å would be required.

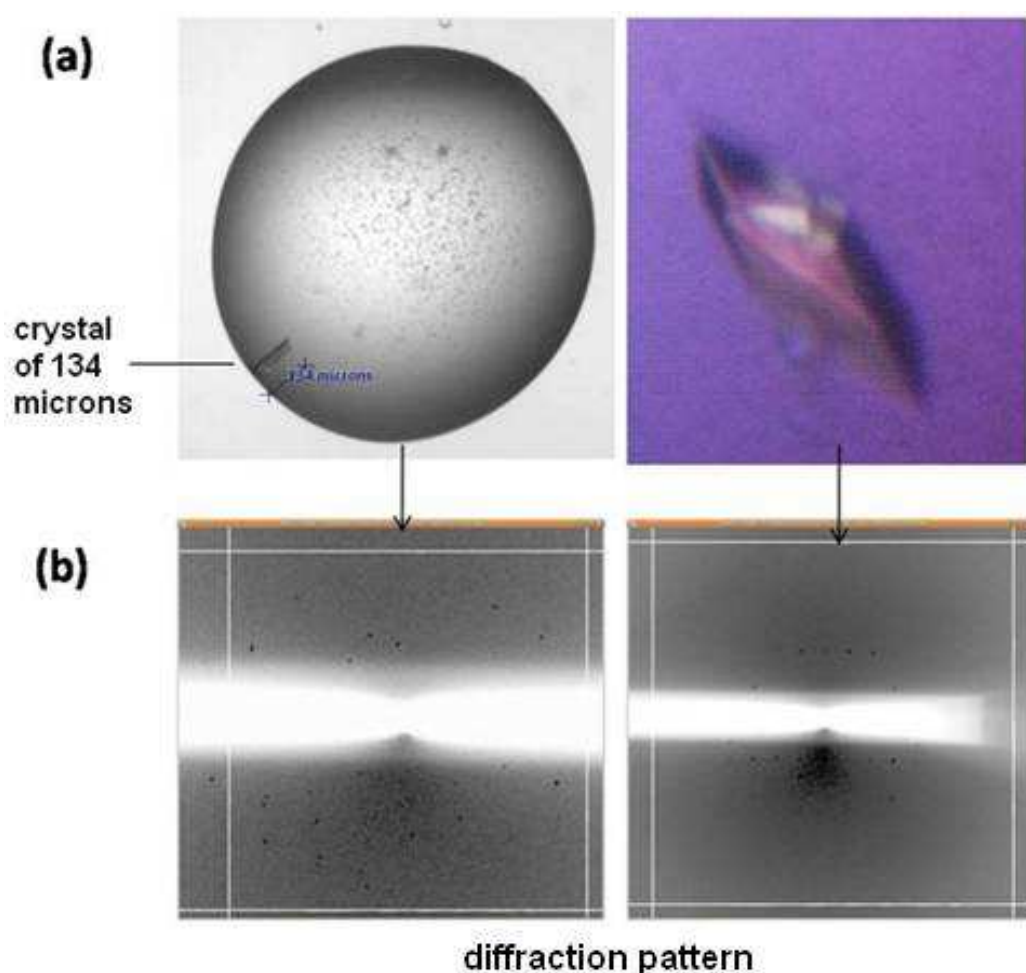


Fig. 4.6. Crystallisation of C-6 X His K7 expressed from pOPIN E vector. C-6 X His K7 expressed from pOPIN E was purified by IMAC and size exclusion chromatography as described in Fig. 4.4 and Fig. 4.5 and was sent to Prof. D Stuart's group for crystallisation trials. The purified protein was concentrated and used to set up 96-well microfluidics crystallisation experiments. Several crystals were obtained a few days later, one of which was 134 microns (b) X-ray diffraction was carried out on these crystals at Diamond light source, Didcot, UK and the diffraction pattern obtained from them is shown in (b). The resolution obtained was 10 Å.

Thousands of crystallisation experiments using different conditions were carried out but only crystals that yielded low resolution diffraction were obtained. This suggested an inherent disorder within the crystals formed. Therefore, it was decided to attempt to co-purify K7 with its known binding partner DDX3. Work published by Prof A. Bowie's lab in Trinity College Dublin showed that DDX3, a cellular DEAD box helicase interacts with K7 and that K7's inhibition of IRF-3 signalling is mediated by its interaction with DDX3 (Kalverda *et al.*, 2009). Co-expression of the two proteins might enable formation of crystals that are more ordered and yield higher resolution diffraction data. Data generated from a co-crystal structure would also be more informative and help explain how K7 modifies DDX3 to lead to signalling inhibition.

4.2. Expression and purification of K7 protein in complex with N-terminal fragments of its binding partner DDX3

Binding studies carried out in Prof Bowie's laboratory show that N- or C-terminally truncated K7 (K7₁₋₁₀₉ and K7₄₁₋₁₄₉) no longer binds DDX3 and that the N-terminus of DDX3 is required for K7 binding. Therefore, work was carried out using full length C-6 X His K7 expressed from pOPIN E as before. Initial work towards a co-structure was carried out by Dr A. Khan, also in Dublin, who found that N-terminal fragments of DDX3 (5-167 and 22-409) bind K7 and in contrast to the full length protein, yield soluble protein when expressed in bacteria. Two pET-28a plasmids expressing DDX3₅₋₁₆₇ and DDX3₂₂₋₄₀₉ with an N-6 X His tag were obtained from Dr Khan and the protein expression and purification work was continued at Imperial College London. The co-expression of either DDX3 fragments with K7 by co-transformation into B834 cells was attempted because pET-28a plasmids contain a kanamycin resistance gene whereas the pOPIN plasmid from which K7 is expressed contains an ampicillin resistance gene. Competent B834 cells were co-transformed and plated on LB agar plates supplemented with both antibiotics. Bacterial cultures were induced with 0.1 mM IPTG for 3 h at 37 °C (Fig. 4.7a) or overnight at 30 °C (Fig. 4.7b) and were collected by centrifugation and lysed as described in section 4.1.1. The resulting soluble and insoluble fractions were analysed by SDS-PAGE followed by staining with Coomassie blue (Fig. 4.7).

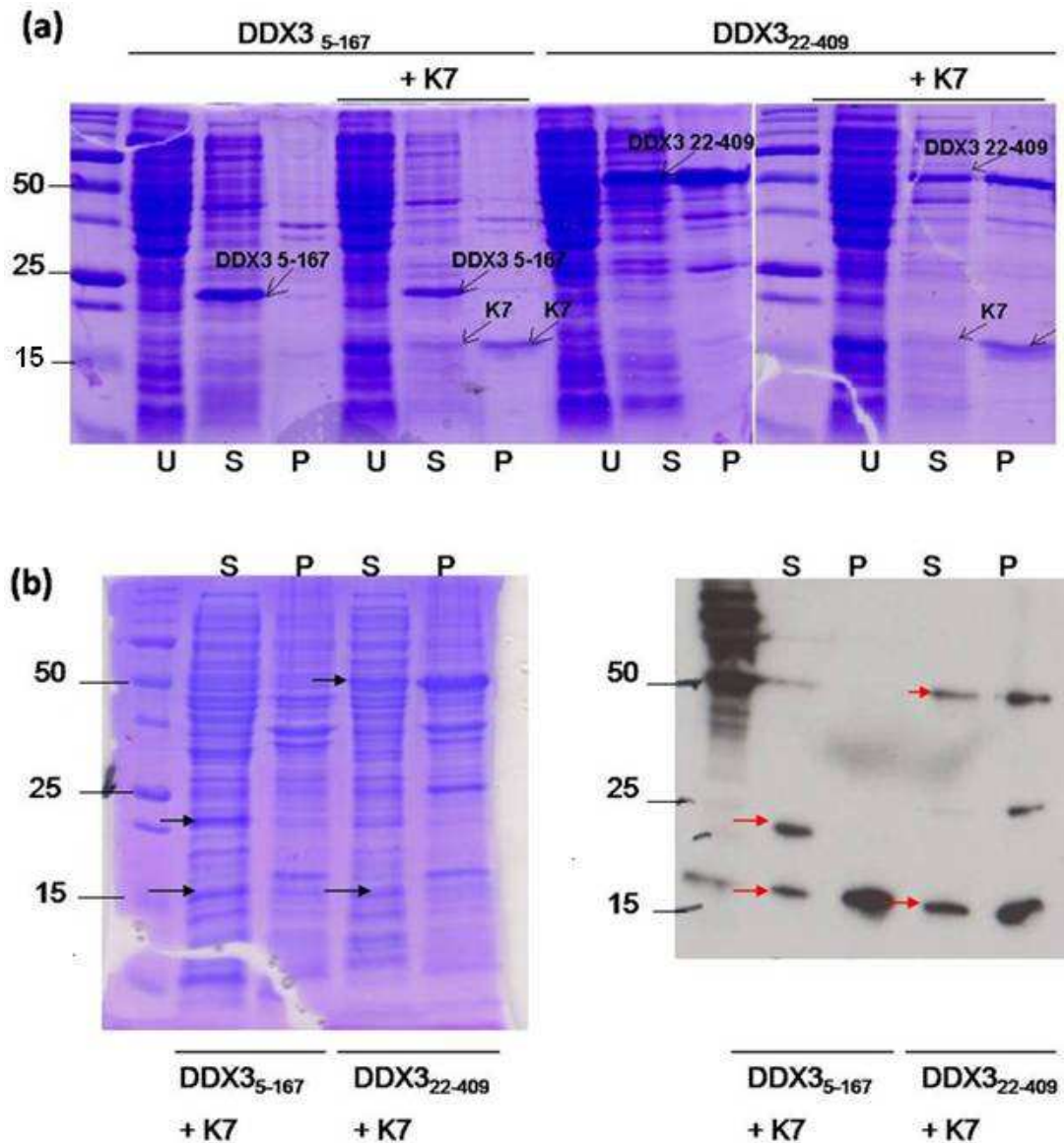


Fig. 4.7. Co-expression of DDX3₅₋₁₆₇ and K7 or DDX3₂₂₋₄₀₉ and K7 from B834 *E. coli*. (a) N-6 X His DDX3 fragments were expressed from pET-28a in B834 *E. coli* with or without co-production of C-6 X His- K7. Bacterial cultures were incubated at 37 °C until the OD₆₀₀ reached 0.4 and were then induced with 0.1 mM IPTG for 3 h at 37 °C. Samples of soluble (S) and insoluble (P) fractions as well as uninduced (U) whole lysate controls were harvested as described in Materials and Methods 2.2.2 and were analysed by SDS-PAGE (15 % gel) followed by staining with Coomassie blue. (b) DDX3 fragments and K7 protein were co-produced as before but induced with IPTG overnight at 30 °C. Samples were run on SDS-PAGE (15 % gel) and analysed by staining with Coomassie blue (left) and immunoblotting using anti-4 X His Ab (right). The positions of molecular mass markers are shown in kDa.

DDX3₅₋₁₆₇ and DDX3₂₂₋₄₀₉ have predicted molecular masses of approximately 20 and 45 kDa and the strong bands observed in the soluble fraction samples on the Coomassie blue stained gel in Fig. 4.7a were slightly larger than this. However these bands were also seen by immunoblotting with anti-4 X His Ab (Fig. 4.7b) confirming that they are DDX3 protein fragments. Under these induction conditions, K7 (approximately 18 kDa) was observed in both soluble and insoluble fractions. When cultures were induced at 30 °C overnight the ratio of K7 to DDX3₅₋₁₆₇ protein appeared to have increased suggesting that the stoichiometry of the two proteins was closer to 1:1 so this incubation condition was used in subsequent experiments. However, with DDX3₂₂₋₄₀₉ these conditions reduced solubility (Fig. 4.7b).

Both DDX3₅₋₁₆₇ with K7 and DDX3₂₂₋₄₀₉ with K7 were purified by IMAC (Fig. 4.8a and 4.10a) and subjected to gel filtration as above. IMAC fractions were analysed by SDS-PAGE followed by staining with Coomassie blue. Some lanes were found to contain both or either of the two proteins, but mostly both proteins were in the same fractions (Fig. 4.8b and 4.10b). However, because they both have a His tag this does not imply that both proteins are necessarily in complex.

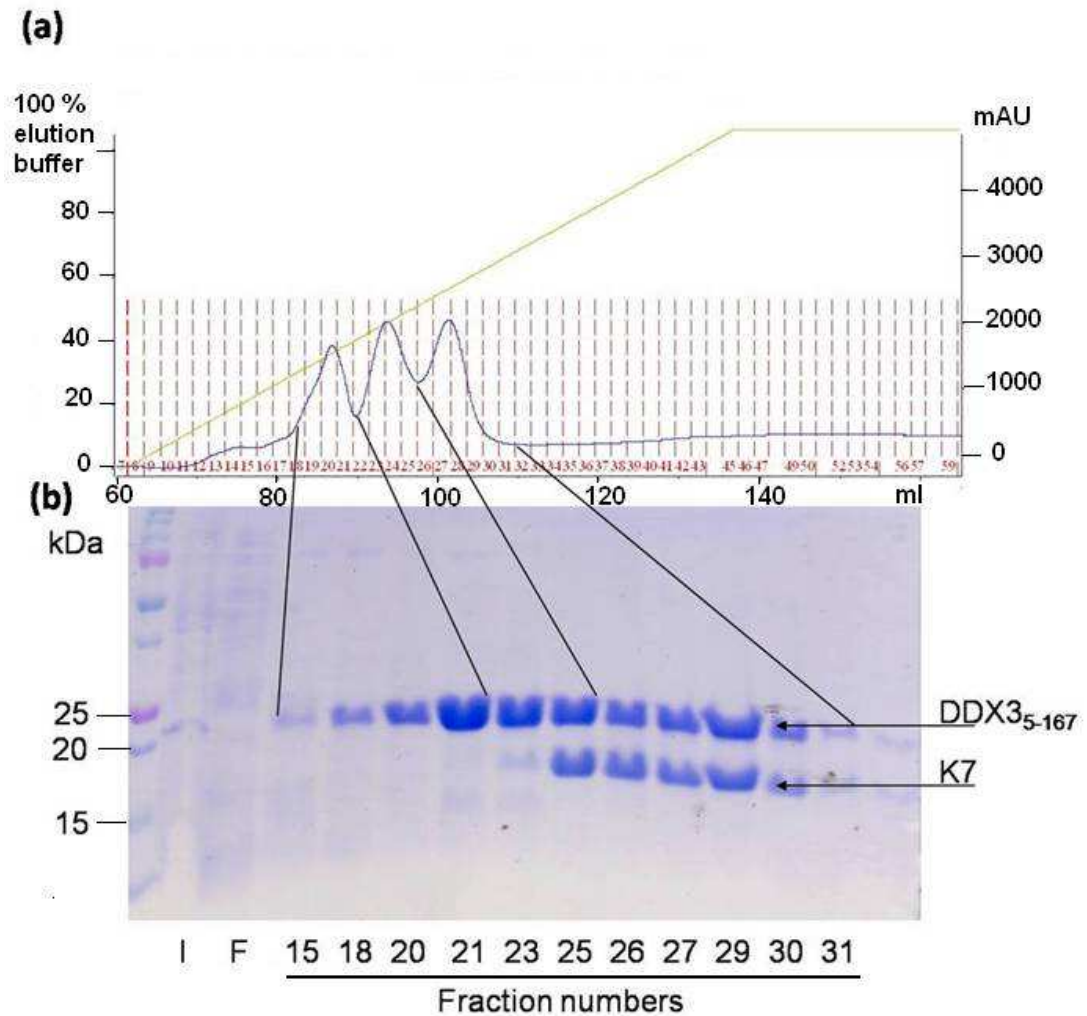


Fig. 4.8. Purification of N-6 X His DDX3₅₋₁₆₇ and C-6 X His K7 by IMAC. (a) Soluble N-6 X His DDX3₅₋₁₆₇ and C-6 X His proteins described in Fig. 4.7 were purified by IMAC as described in Materials and Methods 2.2.3. The blue line indicates the UV absorbance at 280 nm and the green line indicates the percentage v/v of elution buffer flowing through the Histrap column. The different fractions collected during this run are also indicated in red. The x-axis indicates the volume of buffer that has entered the column since the beginning the run. The right y-axis indicates the absorbance in mAU and the left y-axis indicates the percentage of elution buffer (v/v) entering the column. (b) Some of the fractions corresponding to a peak in UV absorbance at 280 nm (after the washing step, fractions 15-31) were tested for the presence N-6 X His DDX3₅₋₁₆₇ and N-6 X His K7 by SDS-PAGE (15 % gel) and staining with Coomassie blue as indicated. The positions of molecular mass markers are shown in kDa.

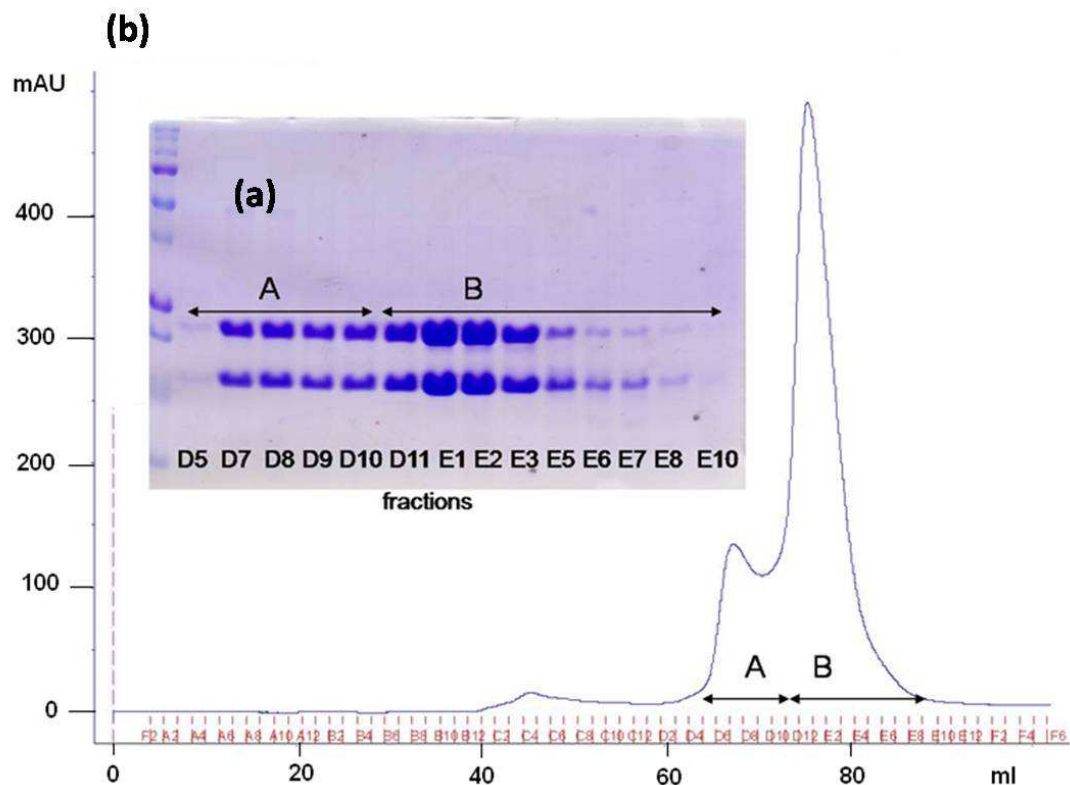


Fig. 4.9. Purification of recombinant N-6 X His DDX3₅₋₁₆₇ and C-6 X His K7 protein by gel filtration. (a) N-6 X His DDX3₅₋₁₆₇ and C-6 X His K7 were purified by IMAC as described in Fig. 4.8 and were applied to a Superdex 200 16/60 column and eluted in 10 mM HEPES 50 mM NaCl as described in Materials and Methods 2.2.3. The blue line indicates the UV absorbance at 280 nm and the different fractions collected during this experiment are indicated in red. The x-axis indicates the volume of buffer that has entered the column since the beginning of the run and the y-axis indicates the absorbance in mAU. Two different peaks of UV absorbance at 280 nm were obtained and were designated A and B as indicated (b) Fractions corresponding to peaks A and B were tested for the presence of N-6 X His DDX3₅₋₁₆₇ and C-6 X His K7 by SDS-PAGE (15 % gel) followed by staining with Coomassie blue. The letter and number designating each gel filtration fraction tested in each lane are indicated. The positions of molecular mass markers are shown in kDa.

Samples obtained were pooled, concentrated and applied in 1-2 ml to a Superdex 200 16/60 column for gel filtration. Fractions corresponding to a peak at 280 nm were analysed by SDS-PAGE followed by Coomassie blue staining. In Fig. 4.9b two distinct but overlapping 280 nm peaks were formed (designated A and B). Both contain the two proteins (Fig. 4.9a) so this suggested that different types of complexes may have formed and that it is not necessarily a 1:1 complex between DDX3₅₋₁₆₇ with K7. Samples from peaks A and B were pooled

separately and sent to Oxford where multiple angle laser light scattering (MALLS) was carried out on the samples. This technique enables the determination of the molar mass of proteins in solution. Samples A and B ran as a single species of 40 kDa suggesting that the stoichiometry of the complex is 1:1 however sample B appeared to be the most monodisperse. It was possible that these would also include DDX3 or K7 dimers, but this was not followed up, because the NMR structure of K7 became available (Kalverda *et al.*, 2009) at this time and it was shown conclusively that K7 is a monomer in solution. Proteins from samples B were concentrated by Dr Graham to 7 mg/mL and used to set-up some crystallisation trays; unfortunately, no crystals were obtained.

By contrast, in Fig. 4.11b the gel filtration of the DDX3₂₂₋₄₀₉ and K7 sample yielded a multitude of peaks at 280 nm (A-E) all which contained both proteins as shown by Coomassie staining in Fig. 4.11a. This suggested the sample was very heterogeneous as it was eluting at different stages of the run. Samples from each of those peaks were sent to Dr Graham who carried out MALLS and the samples were all found to be polydisperse even though peaks A and B contained some DDX3/K7 complex, meaning that they were totally unsuitable for crystallisation trials.

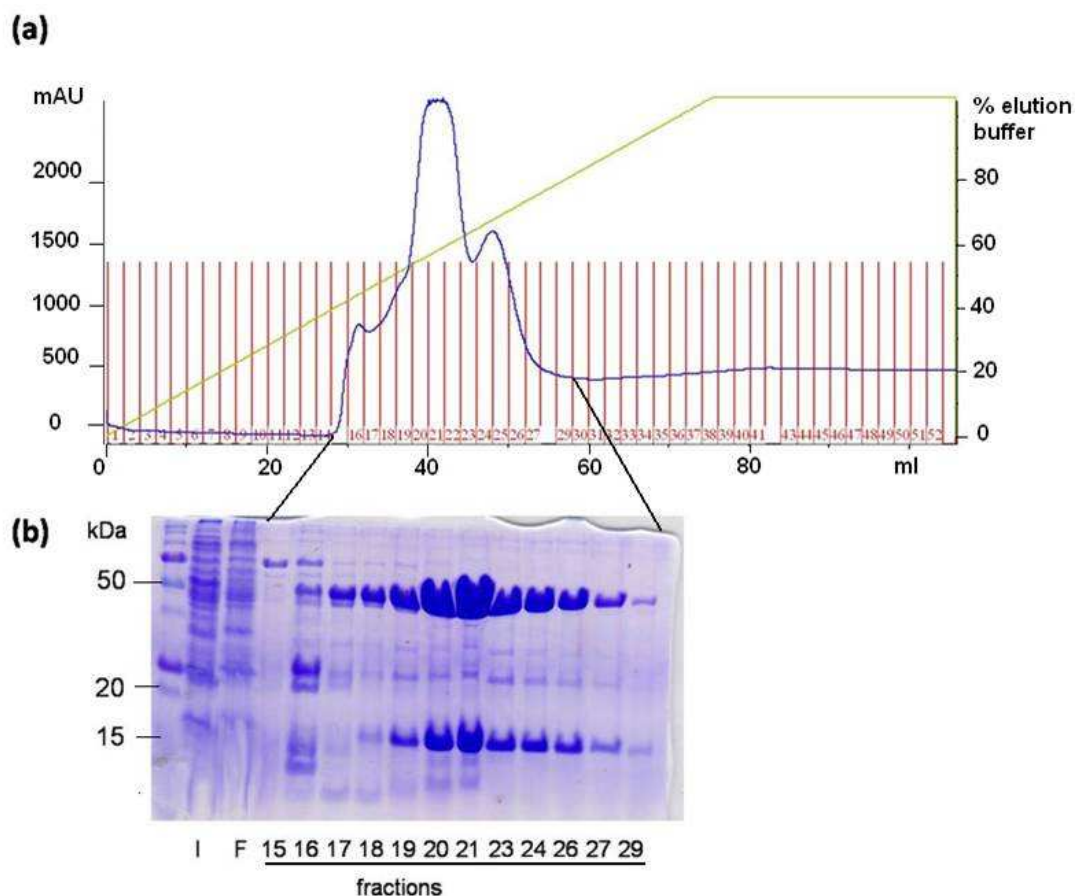


Fig. 4.10. Purification of N-6 X His DDX3₂₂₋₄₀₉ and C-6 X His K7 by IMAC. (a) Soluble N-6 X His DDX3₂₂₋₄₀₉ and C-6 X His proteins described in Fig. 4.7 were purified by IMAC, as described in Materials and Methods 2.2.3. The blue line indicates the UV absorbance at 280 nm and the green line indicates the percentage v/v of elution buffer flowing through the Histrap column. The different fractions collected during this run are also indicated in red. The x-axis indicates the volume of buffer that has entered the column since the beginning the run. The left y-axis indicates the absorbance in mAU and the right y-axis indicates the percentage of elution buffer (v/v) entering the column. (b) Some of the fractions corresponding to a peak in absorbance at 280 nm (after the washing step, fractions 15-29) were tested for the presence of N-6 X His DDX3₂₂₋₄₀₉ and N-6 X His K7 by SDS-PAGE (15 % gel) followed by staining with Coomassie blue as indicated. (I) is a sample of the input and (F) is a sample of the flow through from the initial washing step. The positions of molecular mass markers are shown in kDa.

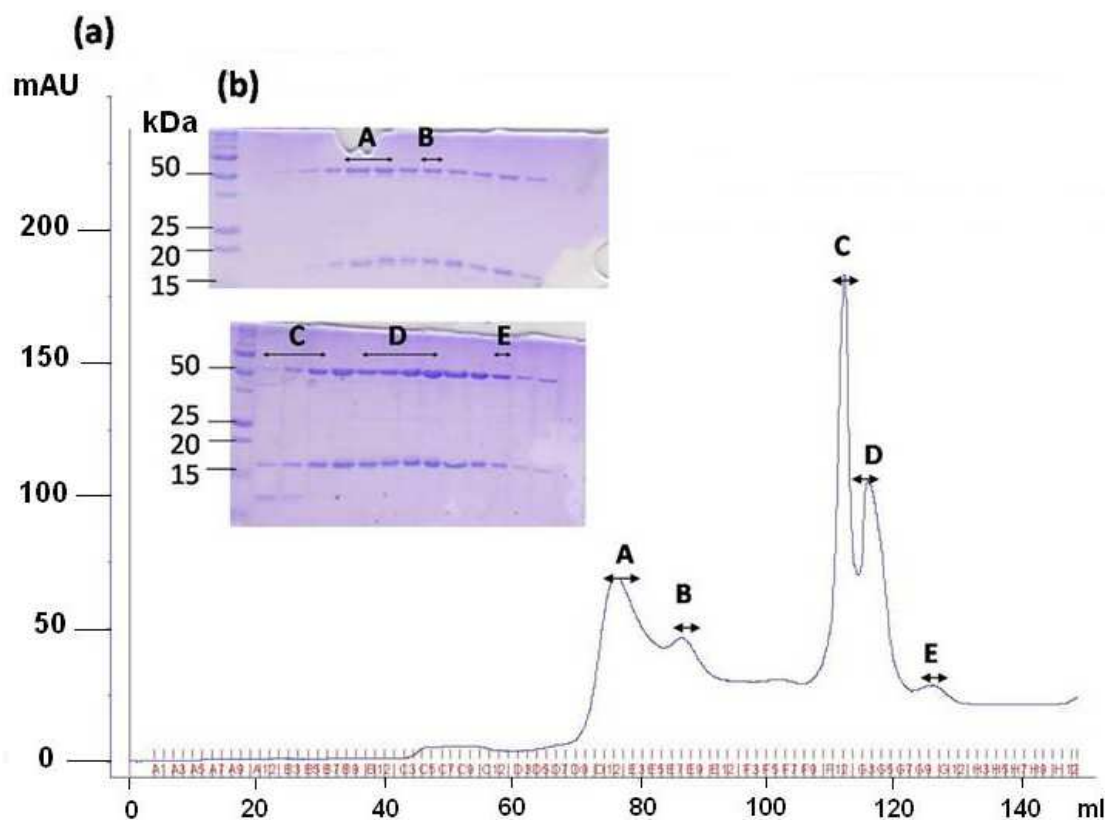


Fig. 4.11. Purification of recombinant N-6 X His DDX3₂₂₋₄₀₉ and C-6 X His K7 protein by gel filtration. (a) N-6 X His DDX3₂₂₋₄₀₉ and C-6 X His K7 were purified by IMAC as described in Fig 4.10 and applied to a Superdex 200 16/60 column and eluted in 10 mM HEPES 50 mM NaCl as described in Materials and Methods 2.2.3. Several peaks of UV absorbance at 280 nm were obtained and were designated A-E as indicated. The blue line indicates the UV absorbance at 280 nm and the different fractions collected during this experiment are indicated in red. The x-axis indicates the volume of buffer that has entered the column since the beginning of the run and the y-axis indicates the absorbance in mAU. (b) Fractions surrounding and including those corresponding to peaks A-E of UV absorbance at 280 nm were tested for the presence of N-6 X His DDX3₂₂₋₄₀₉ and C-6 X His K7 by SDS-PAGE (15 % gel) followed by staining with Coomassie blue. The positions of molecular mass markers are shown in kDa.

4.3 Conclusions

In conclusion, it was possible to purify complexes of DDX3₅₋₁₆₇ with K7 or DDX3₂₂₋₄₀₉ with K7. The former yielded samples that were monodisperse in solution but unfortunately no crystals were obtained despite thousands of crystallisation experiments being set up. For the latter experiment, purified samples were too polydisperse for use in crystallisation. One possible way to improve this situation could be the use of untagged DDX3 protein meaning that only complexed DDX3 and K7 would be affinity purified before the size exclusion chromatography step, therefore excluding monomer contaminants. Alternatively, based on the findings of (Kalverda *et al.*, 2009) that the first 8 amino acids of K7 are unstructured and are not required for binding DDX3 the use of an N-terminally truncated K7 could help yield ordered crystals. However, this publication also showed that the N-terminus of DDX3 is itself highly disordered but that residues 61-90 are sufficient for K7 binding. Therefore a better approach could be to work on crystallising K7 with this short peptide.

Chapter 5: Investigating the possible contribution of *K7R* sequence to virulence in VACV WR

Previous work in this laboratory by Dr. Stuart Lucas demonstrated that deletion of the *K7R* gene from VACV strain WR caused attenuation in a mouse intranasal infection model (Williamson *et al.*, 1990) compared to parental and revertant controls. As shown in Fig. 5.1a, mice infected with vΔK7 lost less weight than those infected with controls from three days p.i. As a further control, an additional recombinant virus was constructed by Dr. Lucas in which an extra nucleotide was introduced into the *K7R* translational initiation codon thus mutating the ATG codon to ATAG. As expected, PCR and sequence analysis demonstrated that this new virus (vfsK7-1) contained the mutant ATAG codon and immunoblotting with a polyclonal anti-K7 Ab raised against the full length protein did not detect any K7 protein (results not shown). The virulence of this virus was then tested by intranasal infection and compared to vK7, vΔK7 and vK7-rev, a revertant virus in which the *K7R* gene was re-introduced at its natural locus in vΔK7. Surprisingly, it was found that the vfsK7-1-infected mice lost weight to a similar extent to vK7 or vK7-rev-infected ones and significantly more than those infected with vΔK7, demonstrating that despite not making the K7 protein this virus retained virulence (Fig. 5.1b). Dr Lucas also made a recombinant VACV virus in which a stop codon was inserted in place of codon 108 of the *K7R* sequence (called vtrK7) and it was found that this virus too did not express K7 protein and its virulence profile was the same as that of vfsK7 (results not shown). These data provided an interesting scenario: namely, that the nucleic acid of the *K7R* gene, but not the protein, was contributing to virulence, despite the report that the K7 protein inhibits multiple innate immune signalling pathways and binds to host protein DDX3 (Schroder *et al.*, 2008).

Disclaimer: Work shown in this chapter was carried out in a collaborative manner with Dr Gabriel Almeida. I was responsible for the making of all new recombinant viruses and also cloned some of the plasmids used in luciferase assays. Dr Almeida cloned the other *K7R* alleles. The luciferase assays were a joint effort between us. The *in vivo* work shown was carried out by Aodhnait Fahy.

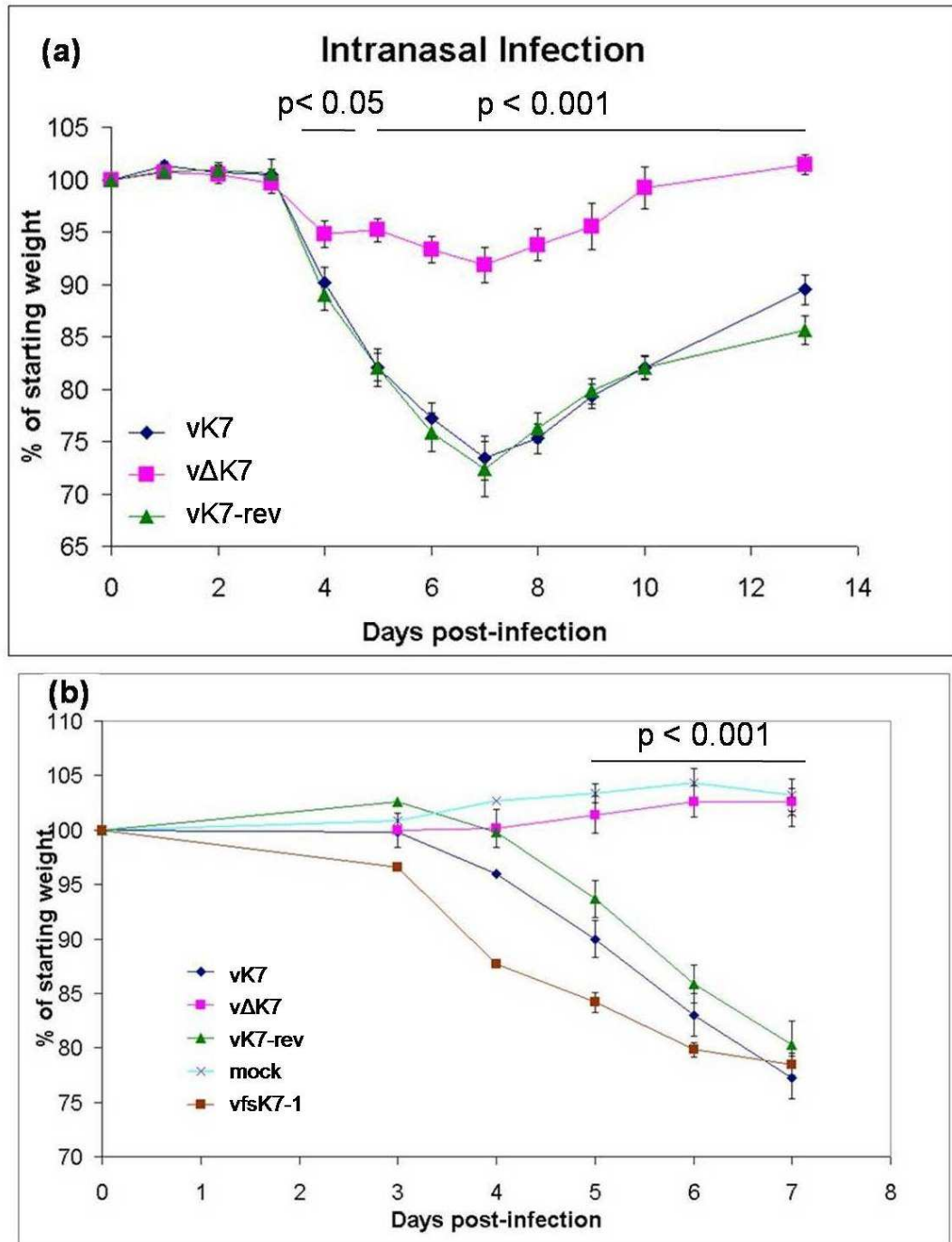


Fig. 5.1. Evaluation of the virulence of *K7R* recombinant viruses. Mice were infected intranasally ($n=5$) with 10^4 pfu of vK7, vΔK7 and vK7-rev (a) or with vK7, vΔK7, vK7-rev and vfsK7-1 (b). Mice were weighed daily and the mean weight of each group of animals on each day is expressed as a percentage of the mean weight of the same group of animals on day zero. These data are presented as mean $\pm$ SD. A two-tailed student T-test was carried out between all different mice groups for each day and significant p values between vΔK7-infected mice and each of vK7, vK7-rev and vfsK7-1-infected mice are indicated. Work shown here was performed by Dr Stuart Lucas.

5.1 Construction and characterisation of a new recombinant virus with frameshifted *K7R* sequence

5.1.1 Construction of a new recombinant virus with frameshifted *K7R* sequence

The finding that vfsK7-1 retained virulence despite not making the K7 protein was surprising. Although it seemed unlikely that this phenotype was caused by a mutation elsewhere in the virus genome that increased virulence, it was important to eliminate this possibility before investigating how the *K7R* nucleic acid might contribute to virulence. To rule out secondary site mutations as the cause of retention of virulence with vfsK7-1, another vfsK7, hereafter called vfsK7-2 was made independently. The same frameshifted sequence was re-inserted into the *K7R* locus of $\nu\Delta K7$ by transient dominant selection (Falkner & Moss, 1990) as shown in Fig. 5.2. Cells were infected with $\nu\Delta K7$ and transfected with pSTU6fs, a plasmid constructed by Dr. Lucas by cloning the *K7R* sequence and flanks into the multiple cloning site of pSJH7 plasmid (Hughes *et al.*, 1991). pSJH7 contains the *Ecogpt* gene downstream of a VACV E/L promoter (Boyle & Coupar, 1988) and is suitable for selection of recombinant VACVs by transient dominant selection. The extra adenine within the *K7R* ATG was added by site-directed mutagenesis. After infection-transfection, a single homologous recombination event between one of *K7R* flanks in the plasmid and its counterpart in the virus genome leads to integration of the whole plasmid into the virus genome. This virus can be distinguished from parental virus because it expresses *Ecogpt* and so is able to replicate in the presence of MPA, whereas parental virus cannot (Boyle & Coupar, 1988). The plasmid within the virus genome is maintained in the presence of MPA and such viruses are termed intermediate because they contain repeated DNA sequences that are unstable if the drug selection is removed. Recombination between the repeated sequences can generate either vfsK7-2 or parental $\nu\Delta K7$. Details of the protocol for making recombinant viruses are given in 2.4.2 of Materials and Methods. MPA-resistant intermediate viruses were plaque purified 3 times in the presence of drug and then plaque purified three times without MPA to allow resolution. The final virus obtained was called vfsK7-2.

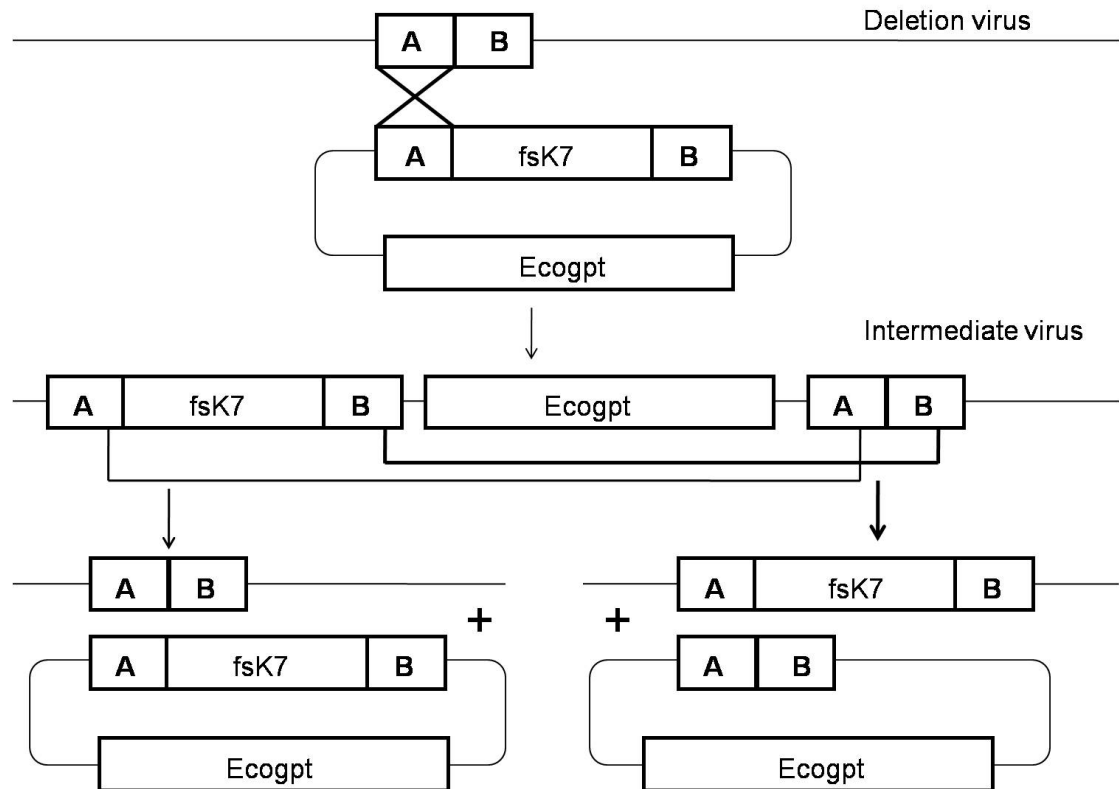


Fig. 5.2. Construction of vfsK7-2 by transient dominant selection. vΔK7-infected cells were transfected with pSTU6fs containing fsK7 and *K7R* flanking regions (A and B) and the dominant selectable marker Ecogpt. Recombination between a *K7R* flank sequence in the plasmid and corresponding sequences in the virus genome generated an intermediate virus with direct repeated sequences, and these Ecogpt-expressing recombinants were selected in the presence of MPA. After removing the selective pressure for Ecogpt, the intermediate virus resolved by the recombination between the repeat sequences as illustrated to generate either vΔK7 (parental virus) and pSTU6fs or vfsK7-2 and a pSTU6fs that had lost the fsK7 sequence but retained the *K7R* flanks.

5.1.2 Viral genome analysis by PCR

The genotype of individual plaque isolates at the *K7R* locus was then analysed by PCR. This was carried out using primers 039U and 039D (Materials and Methods 2.1.12) which bind to the *K7R* flanks as shown in Fig. 5.3a. When *K7R* is present, the expected PCR product size of *K7R* and flanks is 1.1 kilobase (kb) pairs. In vΔK7, the expected size decreases to 660 bp. Fig. 5.3b shows that clone 4 yielded a 1.1 kb product (indicated by an arrow) meaning it has

resolved to the vfsK7-2 genotype, whereas the other clones have resolved to vΔK7 genotype. In most cases a fainter band of the size expected from the alternative allele is also observed and this indicates that the isolate is not yet pure but a mix of resolved and intermediate virus. Consequently, two additional rounds of plaque purification were carried out with a vfsK7-2 and a vΔK7, called vΔK7-2. These two viruses would be genetically most closely related as they were derived from the same intermediate parent virus. The genomes of plaque purified viruses were analysed by PCR as before and Fig. 5.3c shows that the genomes of vfsK7-2 (clones 1-5) and vΔK7-2 (clones 6 and 7) yielded PCR products of the expected sizes.

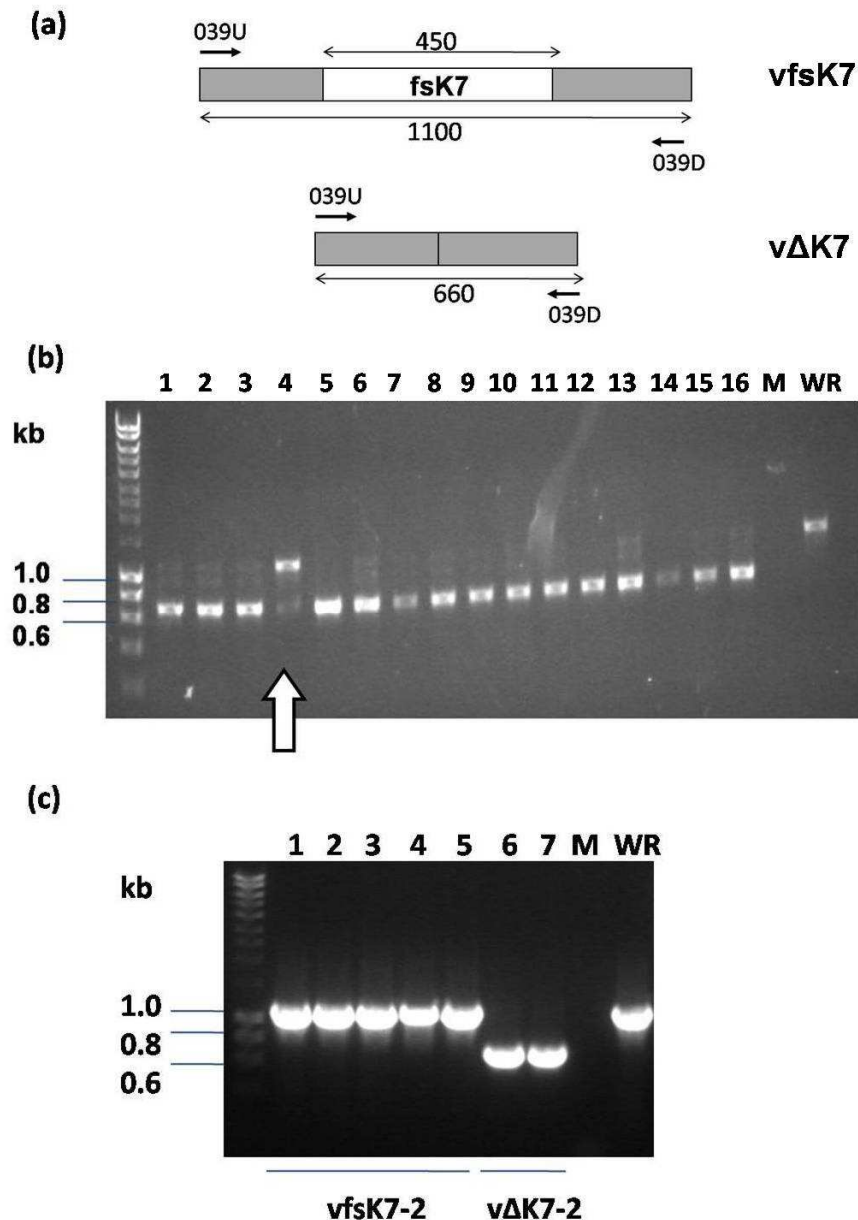


Fig. 5.3. Analysis of recombinant VACV genomes by PCR. (a) The diagram depicts the vfsK7-2 and vΔK7 alleles and indicates the expected product sizes after a PCR using those templates with primers 039U and 039D. (b) Virus DNA was extracted from cells that had been mock-infected (M) or infected with the numbered plaque isolates. This DNA was used as template for PCR with 039U and 039D primers. (c) Virus DNA was extracted from cells that had been mock-infected (M) or infected with the indicated plaque purified viruses and these DNAs were used as templates for PCRs using primers 039U and 039D. PCR products were analysed by agarose gel electrophoresis. The positions of DNA size markers in kb pairs are indicated.

5.1.3 K7 protein expression from *K7R* recombinant viruses

K7 protein expression was analysed by immunoblotting using extracts of virus-infected cells. K7 was only expressed by vK7 and vK7-rev, and not by vΔK7, vfsK7-1, vtrK7 and vfsK7-2 as expected (Fig 5.4). Cell extracts were also immunoblotted with a mAb against the IMV surface protein D8 and this confirmed that all viruses had infected the cells. In conclusion, this immunoblot confirms that K7 is not expressed from viruses with a frameshifted *K7R* sequence. Since the anti-K7 Ab was raised against the entire K7 protein expressed in bacteria, this result also made it unlikely that any smaller K7 protein was expressed by translational initiation from a downstream AUG codon in-frame with the *K7R* ORF.

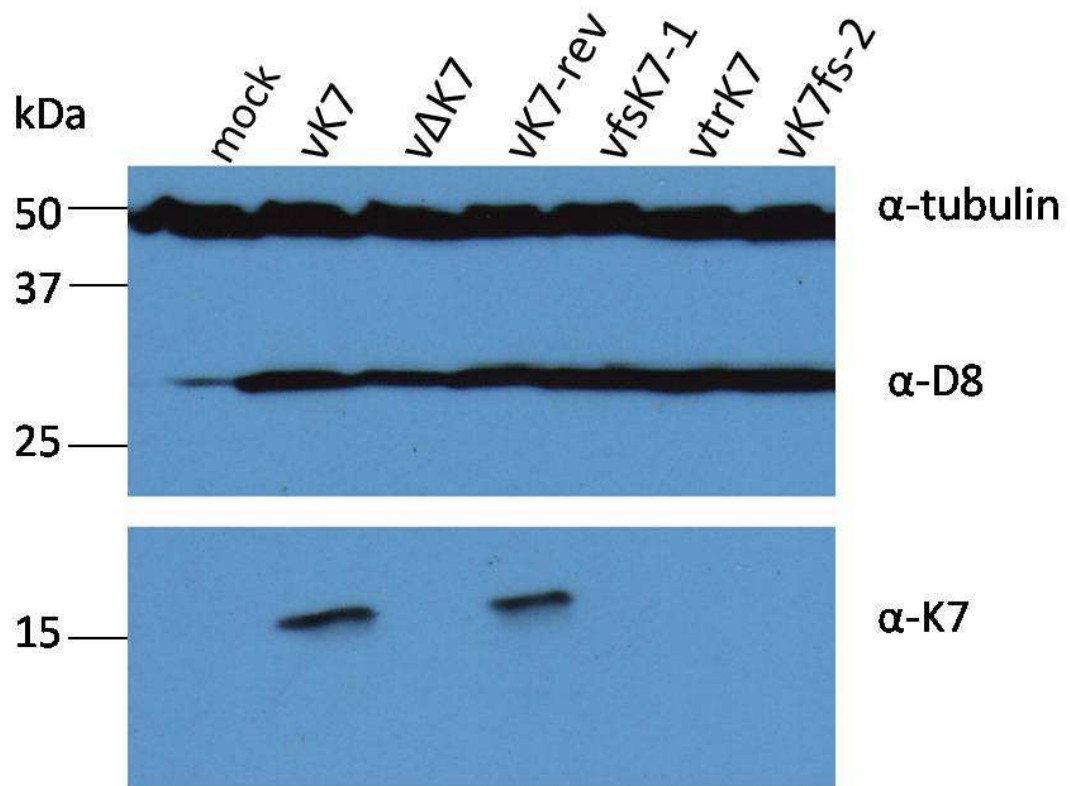


Fig. 5.4. Analysis of K7 protein expression by recombinant viruses by immunoblotting. BSC-1 cells were infected with the indicated viruses at 1 pfu/cell overnight or mock-infected and then cell lysates prepared as described in Materials and Methods 2.3.4. Lysates were analysed by SDS-PAGE (15 % gel) followed by immunoblotting with the indicated Abs. The sizes of molecular mass markers are indicated in kDa.

5.1.4 *In vivo* intranasal challenge experiment repeat (Aodhnait Fahy)

vfsK7-2 described in section 5.1 was amplified and purified by centrifugation through a 36 % sucrose cushion as described in Materials and Methods 2.4.9 and titrated in duplicate. The virulence of the recombinant virus was then compared to controls by intranasal infection of mice. The titre of infectious virus administered to mice was measured by plaque assay and found to be similar for all viruses. Fig. 5.5 shows mice infected with vΔK7 lost significantly less weight than those infected with any of the other viruses from 5 days p.i. There were no significant differences between mice infected with vΔK7 or vΔK7-2 and the results for vΔK7-2 are not shown for clarity. Mice infected with vfsK7-1 or vfsK7-2 lost weight to a similar extent to each other and to vK7. However, vΔK7-infected mice lost significantly less weight than those infected with vK7, VfsK7-1 and vfsK7-2 from 6-10 days after infection. Those two vfsK7-1 viruses made independently had the same phenotype and this confirms that virulence is maintained in the presence of K7 nucleic acid despite absence of K7 protein and that this phenotype is very unlikely to be due to a secondary site mutation.

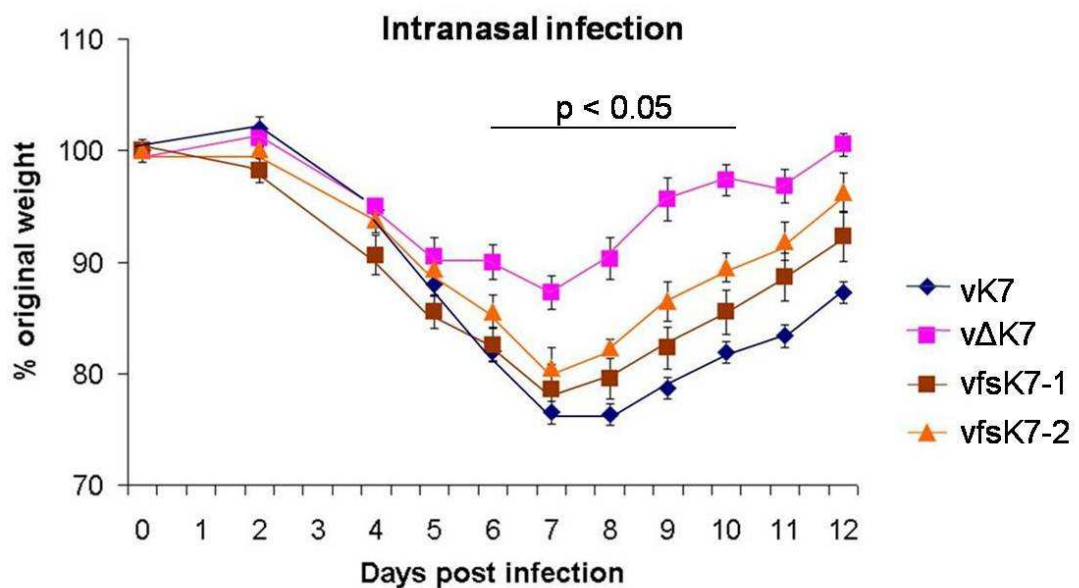


Fig. 5.5. Evaluation of the virulence of vK7fs-2. Mice were infected intranasally ($n=5$) with 10^4 pfu of vK7, vΔK7, vfsK7-1 and vfsK7-2. Mouse weights were monitored daily and data were generated and are presented as described in Fig. 5.1. A two-tailed student T-test was carried out between all different mice groups for each day and significant p values between vΔK7-infected mice and each of vK7, vfsK7-1 and vfsK7-2-infected mice are indicated. These data are presented as the indicated mean $\pm$ SD.

5.2 Possible explanations of the vfsK7 virulent phenotype

5.2.1 F1 expression levels are similar between vK7 and vΔK7

Several hypotheses that could explain the virulent phenotype of vfsK7-1 were investigated. One possibility was that the expression of neighbouring genes was disrupted by the deletion of the whole *K7R* gene sequence in vΔK7 virus and this was responsible for attenuation. Consequently, the levels of expression of *FIL*, the gene downstream of *K7R*, were analysed in vK7, vΔK7, vK7-rev, vfsK7-1, vtrK7 and the new vfsK7-2 virus by immunoblotting using lysates of infected BSC-1 cells. The results in Fig. 5.6 show that only vK7 and vK7-rev express the K7 protein, as expected, and the levels of F1 are similar between all viruses. Similarly the level of the D8 IMV protein was equivalent between viruses indicating the infections had been similar for all viruses. Therefore the attenuated phenotype of the vΔK7 virus was not due to disruption of F1 expression.

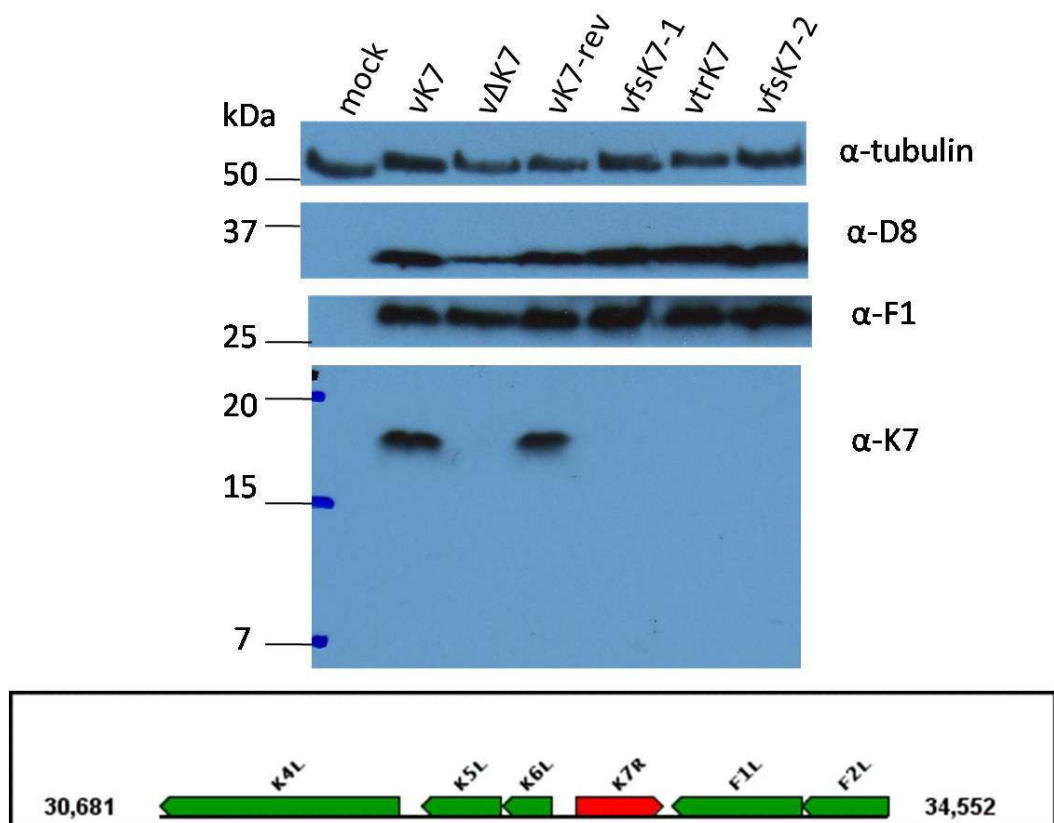


Fig. 5.6. Analysis of F1 protein expression by immunoblotting. BSC-1 cells were infected with the indicated viruses at 1 pfu/cell overnight or mock-infected and cell lysates were prepared as described in Materials and Methods 2.3.4. Lysates were analysed by SDS-PAGE (15 % gel) followed by immunoblotting with the indicated Abs. The sizes of molecular mass markers are indicated in kDa. The diagram below the immunoblot is a schematic representation of the VACV WR genome around the *K7R* locus and was reproduced from www.poxvirus.org.

K6L is the gene upstream of *K7R*. *K6L* is expressed immediate-early (Assarsson *et al.*, 2008) and is predicted to encode an 81 amino acid peptide. Its expression level in different viruses was not analysed, because there was no Ab available, however the intergenic region between *K7R* and *K6L* is predicted to contain the *K6L* promoter based on its orientation in the VACV genome. This region is unaltered in vΔK7 and contains a putative promoter sequence based on the consensus sequence for early genes (Broyles, 2003). Therefore it is unlikely that the expression of *K6L* would be affected in vΔK7.

In conclusion, it is improbable that the expression of *K6L* or *FIL* genes is affected in vΔK7.

5.2.2 Is a smaller K7 protein expressed by vK7fs?

In the presence of the mutated translation initiation codon (ATAG), it was possible that ribosomes might initiate translation from an alternative downstream AUG start codon, either in the same or different reading frame. In the other 2 reading frames no peptide greater than 15 amino acids could be produced because of the presence of multiple translational termination codons in the A:T rich VACV DNA. In the K7 reading frame the next potential initiation codon is present at bases 171-173 and if this was used a polypeptide of 10 kDa would be produced. However, this seemed unlikely as there was no strong Kozak consensus sequence around this ATG. In addition, there are 7 AUGs present in other reading frames that a ribosome scanning down the mRNA would encounter before reaching the AUG codon at 171-3. Immunoblotting also showed that during infection no such protein was detected (Fig. 5.7). It is therefore possible that this 10 kDa protein is not produced by the virus. The Ab used for expression detection by immunoblotting was polyclonal and raised against the whole 17.5 kDa K7 protein. However, it cannot be completely ruled out that this 10 kDa protein is simply not detected by the Ab or is unstable once made.

To address the former possibility K7₁₇₁₋₄₅₀ with an N-terminal HA tag (N-HA) called K7₁₇₁₋₄₅₀ was amplified and cloned into the *XhoI* and *NotI* sites of pCI, a mammalian expression vector with a constitutively active hCMV promoter. Another plasmid containing the frameshifted *K7R* sequence with an N-HA tag (fsK7) was constructed similarly. These plasmids were then transfected into mammalian cells alongside an empty vector plasmid and an N-HA K7 plasmid (K7), previously cloned by Dr Stuart Lucas, and samples were analysed by immunoblotting with anti-K7 and anti-HA Ab (Fig. 5.7a). Full length K7 protein was detected by both Abs in cells transfected with K7 plasmid but no other bands were detected by either Abs in any of the other samples. Expression of the loading control α -tubulin was detected to similar levels in all samples. In conclusion, no smaller K7 protein expression was detected from the N-HA fsK7 or K7₁₇₁₋₄₅₀ transfected cells in those conditions.

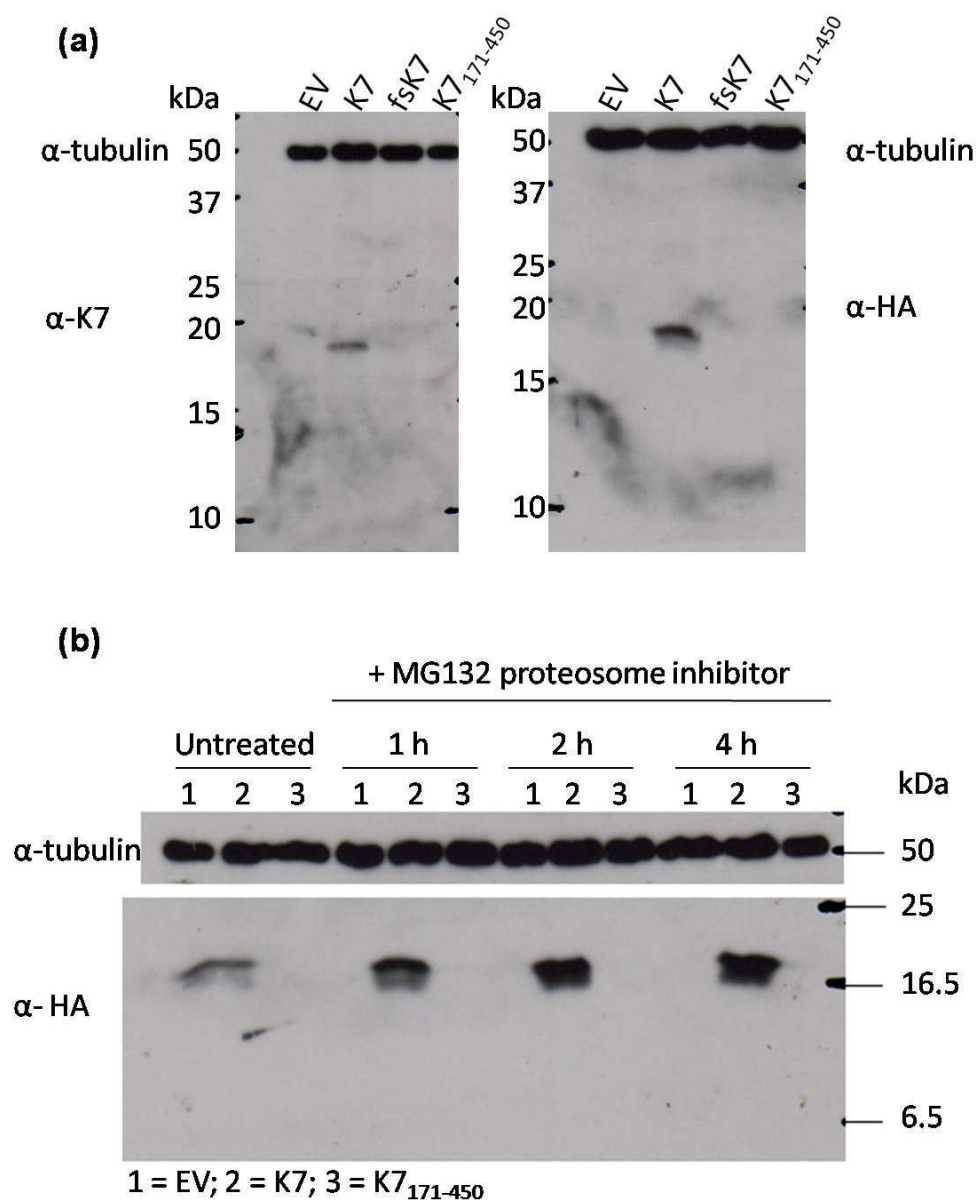
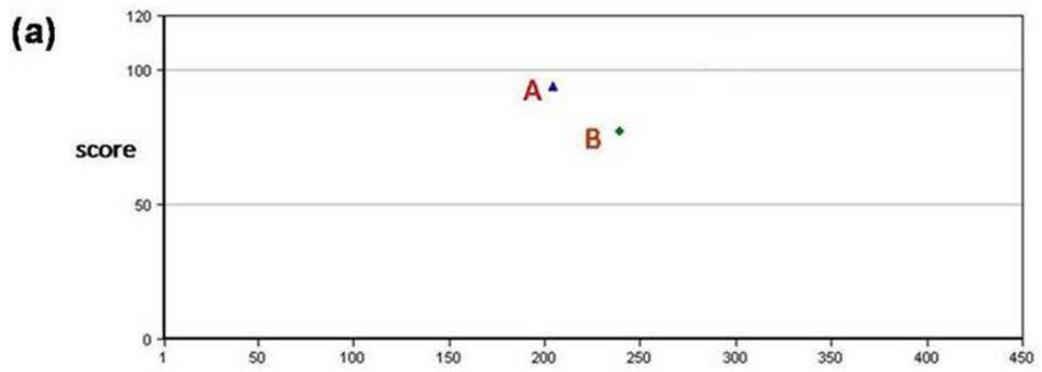


Fig. 5.7. Analysis of transient expression of K7₁₇₁₋₄₅₀ by immunoblotting. (a) HEK 293T cells were transfected with an empty vector (EV) or K7 or fsK7 or K7₁₇₁₋₄₅₀ plasmids for 24 h and cell lysates were prepared as described in Materials and Methods 2.3.4. Lysates were analysed by immunoblotting with anti-K7, anti-HA or anti-alpha tubulin Abs. (b) The experiment described in (a) was repeated but where indicated the transfected cells were treated with the proteasome inhibitor MG132 (5 µg/mL) at 24 h post-transfection for the indicated times before they were lysed. Lysates were analysed by immunoblotting with anti-HA and anti-alpha tubulin Abs. The sizes of molecular mass markers are indicated in kDa.

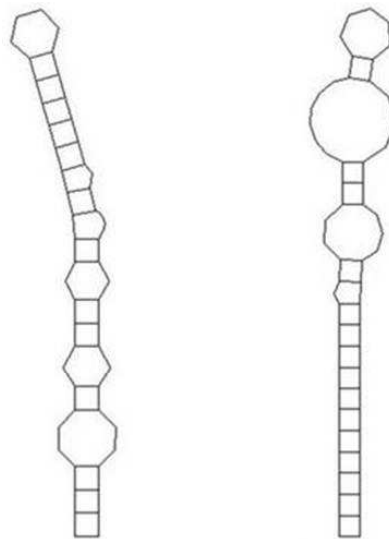
However, it was possible that the putative 10-kDa K7 protein was made but was unstable and targeted for proteosomal degradation. In this regard it was known that the K7 protein was likely to be a Bcl-2 like protein because of similarity to other Bcl-2 proteins as described in section 3.1. These proteins are small, globular α -helical proteins. If the region of K7 represented by the putative 10 kDa protein was aligned with the other VACV Bcl-2 family members for whom structural data was available, it was evident that the truncated protein would lack α -helix 1 and most of α -helix 2 which are predicted to contribute to overall protein stability. Consequently, it was quite likely that the putative 10-kDa protein would be unstable. Therefore, the last experiment was repeated in the presence of the proteasome inhibitor MG132 (5 μ g/mL) inhibitor for 0, 1, 2, or 4 h the day following transfection and lysates were analysed by immunoblotting with anti-HA Ab (Fig. 5.7b). The figure shows that only N-HA K7 was detected by the anti-HA Ab and no protein was detected in the other samples as before. This indicated that no K7 protein was expressed from nucleotides 171-450 of the *K7R* gene.

5.3 Bioinformatics analysis of *K7R* sequence with regard to possible miRNA encoding regions

The retention of virulence by vfsK7-2 raised the possibility that the viral RNA was contributing to virulence, possibly by the production of miRNAs. To address this possibility, computational analysis to identify putative pre-miRNAs hairpin loops within the *K7R* sequence was carried out. The VMir programme (Grundhoff *et al.*, 2006) was developed to predict viral pre-miRNAs and this was used with the recommended default settings. This identified two possible pre-microRNAs within the *K7R* gene: one in the sense-strand (mRNA) and one for the negative-strand RNA sequence (Fig. 5.8a and 5.8b). The scores obtained were 94 and 77 respectively. To put this into context, Grundhoff and colleagues (2006) have indicated that with a cut-off score of 110, 98 % of a set of human reference miRNA precursors passed the filter meaning that scores above this cut-off are more likely to be biologically relevant. An alternative programme, MiR-abela developed by Zavolan's lab (who was the first to discover miRNAs in viruses) (Pfeffer *et al.*, 2004) was available at http://www.mirz.unibas.ch/cgi/pred_miRNA_genes.cgi and was also used to analyse *K7R* and this also predicted miRNAs within the K7 sequence (Fig. 5.8a). Indeed, the predictions made by these two programmes were partially overlapping, which was encouraging as schematised in Fig 5.8c. Both programmes predicted the possible presence of a hairpin loop between positions 179-233 on the positive stand and there is some overlap for the second pre- miRNA prediction (nucleotides 212-269) on the anti-sense strand.



(b) **VACV WR K7R nucleotides**



(c) **A : 179-233 (+)** **B : 212-269 (-)**

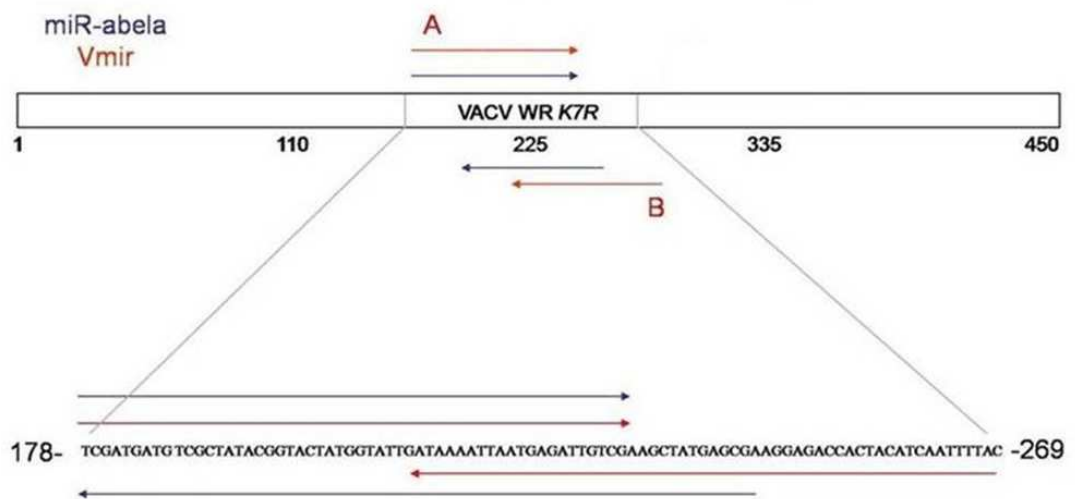


Fig. 5.8. Pre-miRNA hairpin loop predictions for VACV WR K7R. (a) Predictions were carried out using Vmir (Grundhoff *et al.*, 2006). The graph indicates the position in the nucleotide sequence of K7R of the predicted pre-miRNAs and their score. Numbers shown represent the positions of the hairpin loop with the 450-bp K7R. (+) = sense and (-) = anti-sense RNA strands. (b) Schematic representation of hairpin loops predicted by programme Vmir. (c) Illustration of the positions of pre-miRNA hairpin loops predictions made by Vmir (in red) and MiR-abela (in blue) within K7R (Pfeffer *et al.*, 2004). The nucleotide sequence for K7R bases 178-269, the region where pre-miRNAs were predicted, is also shown.

To determine if the predicted miRNAs within the K7 sequence were conserved in other VACV strains and other OPVs, a DNA sequence alignment was carried out using Clustal W using K7R orthologue sequences retrieved from www.poxvirus.org. This alignment focused on the sequence where pre-miRNA hairpin loops were predicted by the two computational programmes used (hairpins A and B). This sequence (positions 178-269) was conserved within OPVs as illustrated in Fig. 5.9. Notably, this sequence was also conserved in ECTV which does not express a K7R orthologue because of an early stop codon in the corresponding sequence. This indicated that these putative miRNAs could also be encoded in other OPVs, and in the case of ECTV they were retained despite the K7R open reading frame being disrupted.

```

          *          20          *          40          *          60          *          80          *
VACV_WR   : TCGATGATGTCGCTATACGCTACTATGGTATTGATAAAATTAATGAGATTGTCGAAGCTATGAGCGAAGGAGACCACTACATCAATTTTAC : 91
VACV_MVA  : TCGATGATGTCGCTATACGCTACTATGGTATTGATAAAATTAATGAGATTGTCGAAGCTATGAGCGAAGGAGACCACTACATCAATTTTAC : 91
MPXV_ZAR  : TCGATGATGCCGCTATACGCTACTATGGTATTGATAAAATTAATGAGATTGTCGAGGCTATGAGTGAAGGAGACCACTACATCAATCTTAC : 91
VARV_AFG  : TCGATGATGCTGCTATACGCTACTATGGTATTGATAAAATTAATGAGATTGTCGAGGCTATGAGTGAAGGAGACCACTACATCAATCTTAC : 91
CMLV_M96  : TCGATGATGCTGCTATACGCTACTATGGTATTGATAAAATTAATGAGATTGTCGAGGCTATGAGCGAAGGAGACCACTACATCAATCTTAC : 91
TATV_DAH68 : TCGATGATGTTGCTATACGCTACTATGGAATTGATAAAATTAATGAGATTGTCGAGGCTATGAGCGAAGGAGACCACTACATCAATCTTAC : 91
CPXV_BR   : TCGATGATGTCGCTATACGCTACTATGGAATTGATAAAATTAATGAGATTGTCGAGACTATGAGCGAAGGAGACCACTACATCAATCTTAC : 91
ECTV      : TCGATGATGTTGCTATACGCTACTATGGAATTGATAAAATTAATGAGATTATCGAGGCTATGAGCGAAGGAGACCACTACATCAATCTTAC : 91
          TCGATGATG  GCTATACGgTACTATgGtATTgATAAAATTAATGAGATTgTCGAaggCTATGAGcGAAGGAGACCACTACATCAATcTtAC

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Fig. 5.9. DNA sequence alignment for position 178 to 269 of VACV WR *K7R* with *K7R* orthologues. The alignment was carried out using Clustal W. VARV_AFG = VARV strain Afghanistan and CMLV_M96 = CMLV strain M96. Dark background = identical nucleotides between sequences. Grey background = 80-99%% identical nucleotides between sequences.

So far the only way of assaying whether the different *K7R* alleles had a biological effect was by analysis *in vivo*. Consequently, setting up an *in vitro* assay that would enable the testing of the contribution of K7 RNA rather than K7 protein was attempted and this will be addressed in the next section.

5.4 Inhibition of IFN- β promoter activation by K7 nucleic acid or protein

K7 was reported to inhibit innate immune signalling pathways, including the activation of the IFN- β promoter (Schroder *et al.*, 2008), yet does not contribute to VACV virulence in the *in vivo* model tested. Therefore, it was possible that the RNA was also contributing to inhibition of innate immune signalling pathways. To investigate this, the contribution of K7 RNA and protein to inhibition of activation of the IFN- β promoter was studied using K7 alleles that would make either RNA and protein, or only RNA. The N-HA K7, fsK7 and K7₁₇₁₋₄₅₀ and empty vector were co-transfected with the IFN- β -luc plasmid and the TK-renilla-luc as described in Materials and Methods section 2.3.5. After 22 h, the cells were stimulated with both poly I:C and TNF- α for 6 h, as this was found to give good activation of the IFN- β promoter, whereas under the conditions tested either stimulus alone did not give a sufficient level of induction. Firefly luciferase data were normalised by dividing them by TK-renilla luciferase readings and data were expressed as the mean fold induction relative to mean empty vector control (Fig 5.10). These data show that the presence of all *K7R* alleles tested inhibited IFN- β activation induced by poly:IC and TNF- α . This showed that i) it was possible to use this *in vitro* assay to assess the biological effect of different K7 alleles, and ii) that alleles that do not express protein have biological activity.

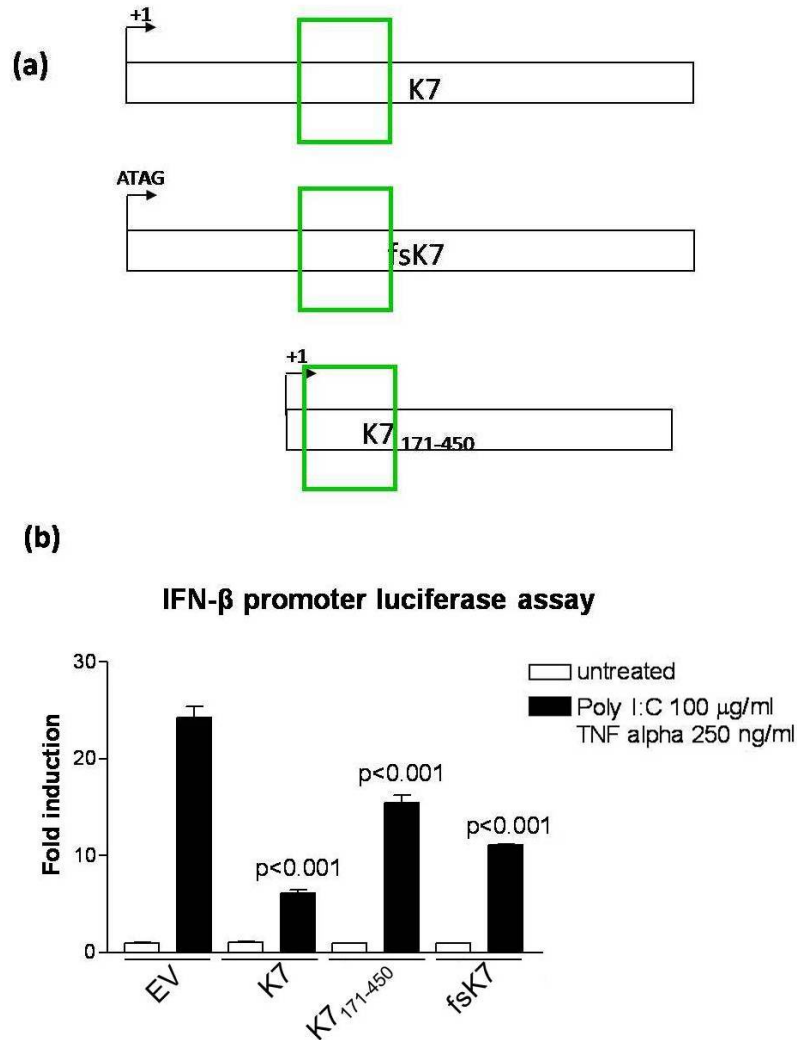


Fig. 5.10. Analysis of the effect of K7, K7fs and K7₁₇₁₋₄₅₀ on IFN- β activation by luciferase reporter assay. (a) Schematic representation of the different *K7R* alleles tested in the IFN- β promoter luciferase reporter assay. The green boxes represent the region predicted to encode miRNAs. (b) HEK 293T cells were co-transfected with plasmids K7, fsK7, K7₁₇₁₋₄₅₀ and empty vector (EV) together with IFN- β -luc and TK-renilla-luc. At 22 h post-transfection, cells were stimulated with TNF- α and poly I:C for 6 h at indicated concentrations. Firefly luciferase activity was measured and data were normalised against TK-renilla luciferase data. Data are expressed as a mean fold induction compared to the normalised mean data in cells expressing empty vector control. Data shown are from one of at least 3 independent experiments with similar qualitative results and are presented as mean fold induction $\pm$ SD. A two-tailed student T-test was carried out and significant p values between treated samples and EV treated samples are shown.

Interestingly, all three alleles contained nucleotides 178-269 of the *K7R* sequence, which is the stretch predicted to encode pre-miRNA hairpin loops by two different bioinformatics programmes. To investigate whether or not this nucleic acid is sufficient to inhibit IFN- β gene expression several new *K7R* alleles were designed and cloned into pCI. All alleles used in this project are illustrated and described in Table 5.1. These included K7 inverted (invK7), K7 scrambled (scrK7), K7₁₋₁₇₁, K7₃₀₀₋₄₅₀ K7₁₇₈₋₂₆₉, K7₂₃₈₋₄₅₀ and K7₂₇₀₋₄₅₀.

K7 allele	presence of predicted miRNAs	K7 protein expression
	yes	yes
	yes	no
	yes	no
	yes	no
	no	yes
	no	no
	no	no
	yes	no
	partially	no
	no	no

Table 5.1. Overview of different *K7R* alleles cloned into pCI. The table lists the different *K7R* alleles constructed and indicates whether K7 protein can be produced and if the sequence is predicted to encode for miRNAs for each allele. Numbers indicate which section of the 450-bp *K7R* sequence is present in the allele and green boxes represent the region predicted to encode miRNAs. K7= full length gene; fsK7 = full length gene starting at ATAG instead of ATG; invK7 = inverted full length gene; scrK7 = mutated *K7R* described in Fig. 5.11; K7₁₋₁₇₁ = bases 1 to 171 of *K7R*; K7₃₀₀₋₄₅₀ = bases 300 to 540 of *K7R*; K7₁₇₈₋₂₆₉ = bases 178 to 269 of *K7R*; K7₂₃₈₋₄₅₀ = bases 238 to 450 of *K7R* and K7₂₇₀₋₄₅₀ = bases 270 to 450 of *K7R*.

The invK7 contained the *K7R* sequence in the opposite orientation and was made to exclude the possibility of any internal initiation of translation while a full length RNA sequence is still transcribed.

scrK7 has a *K7R* nucleotide sequence mutated so the mRNA would still encode an identical protein but this was no longer predicted to encode miRNAs. This was achieved by designing mutations at every third base when possible. The sequence was also designed so that the overall A-T content was maintained. The scrK7 sequence is shown in Fig. 5.11 and is compared to the K7 sequence in an alignment. This allele was constructed to investigate the contribution of the K7 protein to IFN- β promoter activation, or other activity, in absence of putative miRNAs.

```

      *      20      *      40      *      60      *
K7      : ATGGCGACTAAATTAGATTATGAGGATGCTGTTTTTACTTTGTGGATGATGATAAAATATGTAGTCGCGACTCC : 75
scrK7   : ATGGCCACAAAGTTGACTACGAAGACGCAGTATTCTATTTCGTCGACGACGACAAGATTGTCAGCCGGGATTTCG : 75

      80      *      100      *      120      *      140      *
K7      : ATCATCGATCTAATAGATGAATATATTACGTGGAGAAATCATGTTATAGTGTTTAAACAAAGATATTACCAGTTGT : 150
scrK7   : ATTATAGACCTTATCGACGAGTACATAACCTGGAGGAACCACGTAATTGTCTTCAATAAGGACATAACGAGCTGC : 150

      160      *      180      *      200      *      220
K7      : GGAAGACTGTACAAGGAATTGATGAAGTTTCGATGATGTGCTATACGGTACTATGGTATTGATAAAATTAATGAG : 225
scrK7   : GGTAGGCTCTATAAAGAGTTAATGAAATTTGACGACGTGGCAATTGCTATTACGGAATAGACAAGATAAACGAA : 225

      *      240      *      260      *      280      *      300
K7      : ATTGTCGAAGCTATGAGCGAAGGAGACCACCTACATCAATTTTACAAAAGTCCATGATCAGGAAAGTTTATTCGCT : 300
scrK7   : ATAGTGAGGCAATGAGTGAGGGTGATCATTTATATAAACTTCACTAAGGTGCACGACCAAGAGAGCTTGTTTGCA : 300

      *      320      *      340      *      360      *
K7      : ACCATAGGAATATGTGCTAAAATCACTGAACATGGGGGATACAAAAAGATTTCAGAATCTAGATTCCAATCATTG : 375
scrK7   : ACGATTGGTATTTGCGCAAAGATAACAGAGCACTGGGGTTATAAGAAAATATCTGAGTCAAGCTTTCAGTCTTTA : 375

      380      *      400      *      420      *      440      *
K7      : GCAAACATTACAGATCTGATGACCGACGATAATATAAACATCTTGATACTTTTTCTAGAAAAAAATTGAATTGA : 450
scrK7   : GCTAATATAACTGACCTCATGACGGATGACAACATTAATATATTAATCTATTCTTGAAGAAGTTAAACTGA : 450

```

Fig. 5.11. DNA sequence alignment of K7 and scrK7 gene sequences. Alignment generated using Clustal W. The sequences shown encode the same polypeptide. Dark background = identical nucleotides for both sequences.

Another allele $K7_{178-269}$ containing only the region predicted to encode two miRNAs (putative hairpins A and B in Fig. 5.8) was also constructed.

In addition, a set of truncated alleles of $K7R$ were also designed to exclude the 178-269 region of $K7R$ and these included $K7_{1-171}$, $K7_{300-450}$ and $K7_{270-450}$. These would indicate whether the inhibition of IFN- β observed is linked to the presence of this RNA sequence.

Finally, $K7_{238-450}$ was constructed to encode only one predicted pre-miRNA sequence (hairpin B in Fig 5.8) and to see whether this is sufficient to inhibit IFN- β promoter activation.

IFN- β promoter luciferase reporter assays were carried out in HEK 293T as described previously and representative examples of data obtained are shown in Fig. 5.12. In Fig. 5.12a and b, scrK7 and $K7_{238-450}$ alleles are shown to inhibit IFN- β promoter activation to a level similar to that observed with the K7 wild-type control. This inhibition is not complete but statistically significant ($p < 0.001$). In Fig. 5.12c-f the IFN- β promoter fold activation measured for K7, scrK7 and $K7_{178-269}$ transfected samples was significantly lower than that of empty vector control. In contrast, that of $K7_{270-450}$, $K7_{1-171}$ or $K7_{300-450}$ was not reduced, meaning RNA transcribed from these sequences is not sufficient to inhibit IFN- β promoter activation. These three alleles do not contain the sequences predicted to encode miRNAs. All these inhibitors except scrK7 contained the 178-269 sequence predicted to encode pre-miRNA hairpin loops. ScrK7 which produces K7 protein from a mutated transcribed RNA also inhibited IFN- β activation. This suggests that both the protein and the RNA transcribed have activity and that transcription of $K7_{178-269}$ is sufficient to yield an inhibitory effect on IFN- β gene activation.

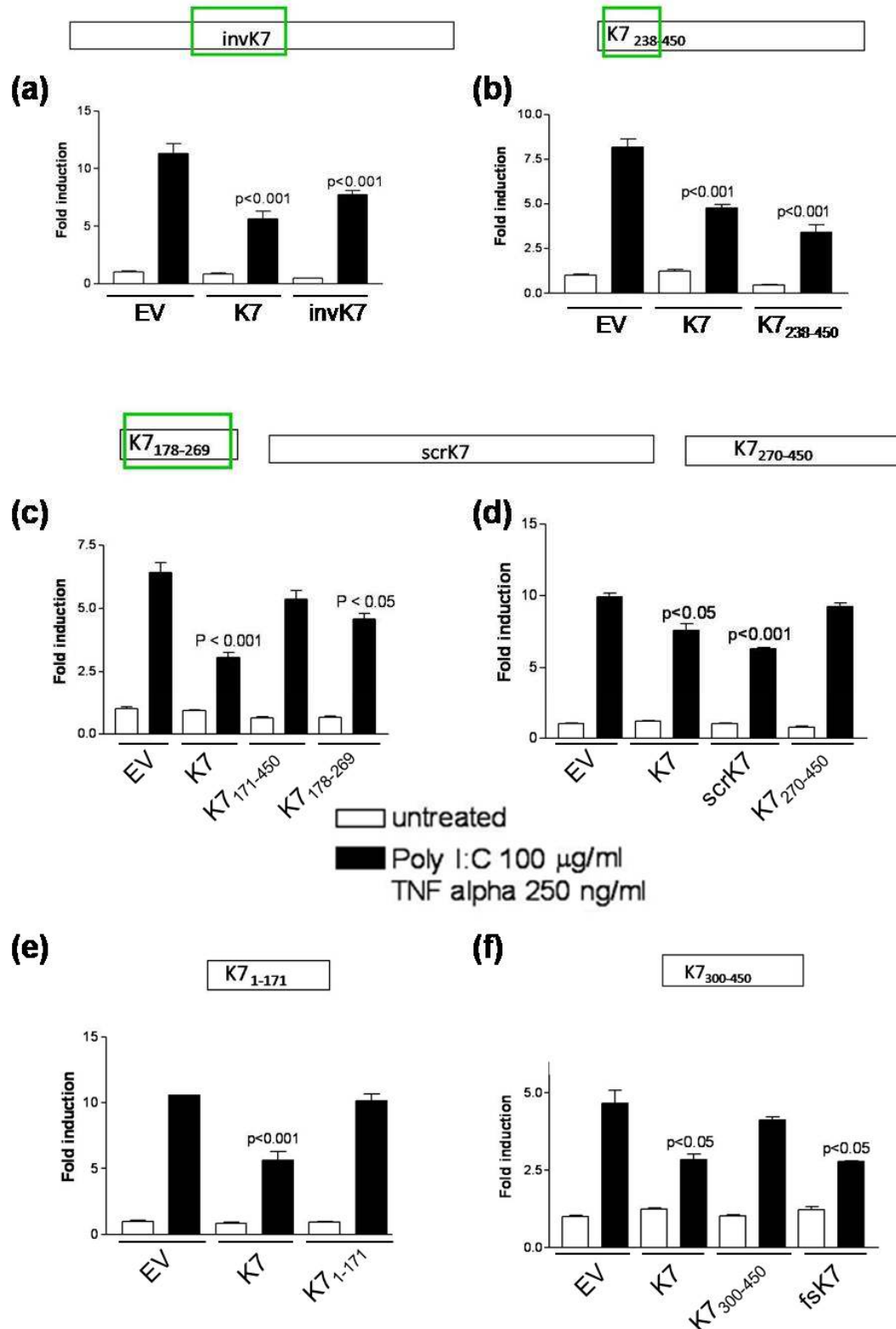


Fig. 5.12. Analysis of the effect of different *K7R* alleles on IFN- β activation. A representation of each different *K7R* allele is shown in above data from the experiment in which it was tested. The green boxes represent the region predicted to encode miRNAs. HEK 293T cells were co-transfected with the indicated plasmids and empty vector (EV) together with IFN- β -luc and TK-renilla-luc. At 22 h post-transfection, cells were stimulated with TNF- α and poly I:C at the indicated concentrations. Samples were treated and data are presented as described in Fig. 5.10. Data shown are from one of at least 3 independent experiments with similar qualitative results and are presented as mean fold induction $\pm$ SD. A two-tailed student T-test was carried out and significant p values between treated samples and EV treated samples are shown.

In conclusion, RNA containing K7₁₇₈₋₂₆₉ transcribed from a transfected plasmid is sufficient to partially inhibit IFN- β promoter activation. In contrast, *K7R* sequences not containing the region predicted to encode a miRNA were not able to do so with the exception of scrK7 which expressed the K7 protein from a mutated K7 sequence. It is also noteworthy, that immunoblotting experiments indicated the levels of K7 expressed by the scrK7 allele were significantly higher than those encoded by *K7R* (data not shown). Therefore this suggests that both K7 protein and part of the *K7R* mRNA are sufficient to modulate this pathway.

5.5 Analysis of effect of *K7R* nucleic acid on other gene reporter assays

5.5.1 Effect of fsK7 on NF- κ B luciferase reporter assay

Schroder *et al.*, (2008) reported that K7 inhibits activation of NF- κ B as well as IFN- β . However, it is possible that this effect could be mediated by the K7 RNA rather than the protein. Consequently, the effects of the K7 protein and *K7R* nucleic acid sequence on NF- κ B dependent gene expression were analysed by a luciferase reporter assay as described in 2.3.5. Cells were transfected with empty vector, N-HA K7, fsK7, scrK7 and a plasmid expressing VACV WR protein B14 together with NF- κ B-luc and TK-renilla-luc plasmids. B14 was used as a positive control because it is a known inhibitor of this pathway (Chen *et al.*, 2008). Cells were stimulated with TNF- α and data were generated and analysed as described in section 5.4. Results in Fig. 5.13 show that K7, fsK7, scrK7 and B14 all significantly inhibit the activation of NF- κ B promoter in response to TNF- α . Thus, it appears that both K7 protein and *K7R* RNA contribute to inhibition

of NF- κ B-dependent gene expression. However, it would be preferable to repeat the experiment by transfecting with an allele that would not inhibit this pathway to act as a negative control.

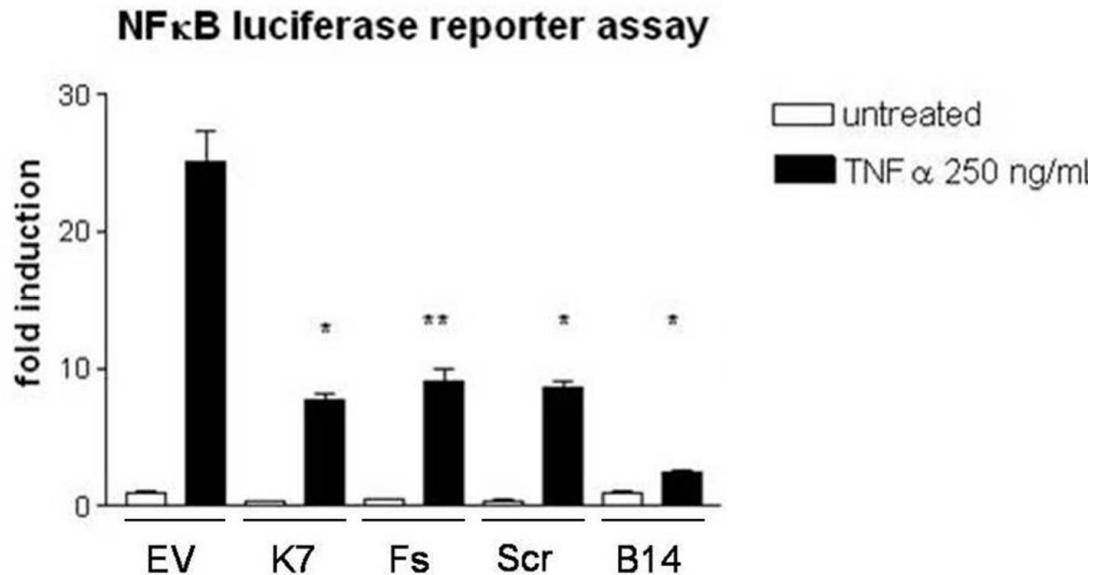


Fig. 5.13. Analysis of the effect of different *K7R* alleles on NF- κ B activation by luciferase reporter assay. HEK 293T cells were co-transfected with either empty vector (EV), K7, fsK7 (Fs), scrK7 (Scr) or HA-B14 (B14) together with NF- κ B-luc and TK-renilla-luc plasmids. At 22 h post transfection the cells were stimulated with 250 ng/ml TNF- α for 6 h. Data were generated and are presented as described in Fig. 5.10. Data shown are from one of at least 3 independent experiments with similar qualitative results and are presented as mean fold induction $\pm$ SD. A two-tailed student T-test was carried out and significant p values between each treated sample group and EV treated samples are shown and * = $p < 0.05$ and ** = $p < 0.01$. The variance of K7, Scr and B14-transfected and IFN-treated samples was significantly different from the variance of EV-transfected and IFN- treated samples therefore a Welch's correction was applied before obtaining the final p value.

5.5.2 Effect of fsK7 on ISRE promoter luciferase reporter assay

Aodhnait Fahy showed previously that both VACV WR proteins K7 and C16 inhibit ISRE promoter activation using luciferase assays with IFN- α stimulation for 6 h (Aodhnait Fahy PhD thesis). Consequently, the effect of K7 protein and *K7R* nucleic acid sequence on this pathway

were analysed by reporter luciferase assay as described in 2.3.5. Cells were transfected with empty vector, N-HA K7, fsK7, scrK7 and untagged C16 expressed from the same plasmid vector, pCI together with ISRE-luc and TK-renilla-luc plasmids. Cells were stimulated with IFN- α and data were generated and analysed as described in section 5.4. Fig. 5.14 shows that N-HA K7, fsK7, scrK7 and C16 all significantly inhibit the mean fold activation of ISRE promoter in response to IFN- α . Thus, it appears that both K7 protein and *K7R* RNA contribute to inhibition of ISRE-dependent gene expression. However, it would be preferable to repeat the experiment by transfecting with an allele that would not inhibit this pathway to act as a negative control.

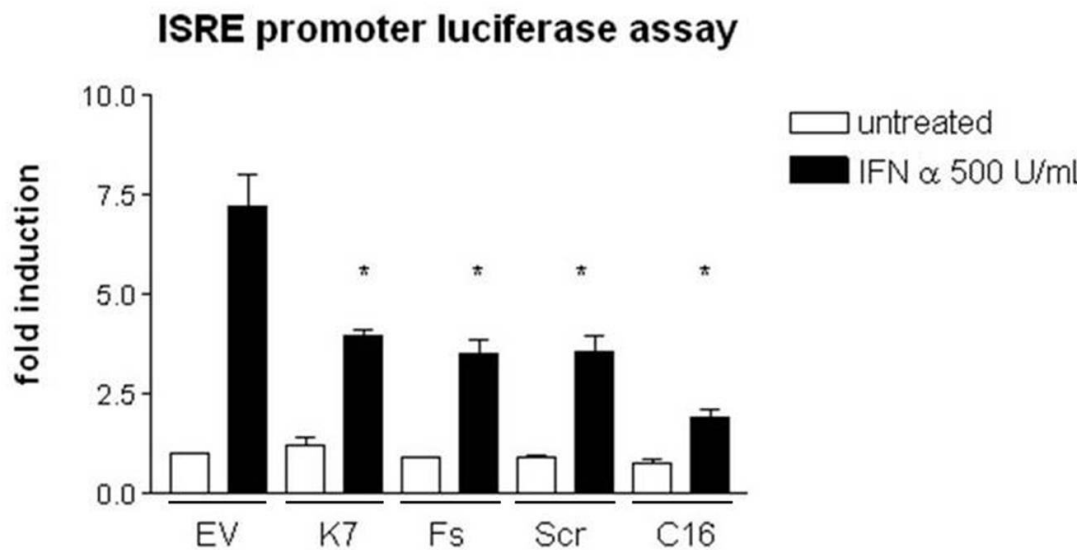


Fig. 5.14. Analysis of the effect of different *K7R* alleles on ISRE activation by luciferase reporter assay. HEK 293T cells were co-transfected as in Fig. 5.13 except that i) the ISRE-luc reporter was used instead of an IFN- β -luc reporter, ii) a C16-expressing plasmid was included as a positive control, and iii) cells were stimulated with 500 U/ml IFN- α for 6 h, 22 h post-transfection, instead of TNF- α . Data were generated and are presented as detailed in Fig. 5.10. Data shown are from one of at least 3 independent experiments with similar qualitative results and are presented as mean fold induction $\pm$ SD. A two-tailed student T-test was carried out and significant p values between treated samples and EV treated samples are shown and * = $p < 0.05$. The variance of C16-transfected and IFN-treated samples was significantly different from the variance of EV-transfected and IFN- treated samples therefore a Welch's correction was applied before obtaining the final p value.

5.5.3 *K7R* sequence and protein both inhibit the ISRE pathway through a STAT1 - independent mechanism

In light of the inhibition of ISRE promoter activation following stimulation with type I IFN, the position within the signalling pathway at which K7 and K7 RNA acted was investigated. STAT1 is phosphorylated after type I IFNs bind to their receptor (see Fig. 1.3). This is shown in Fig. 5.15. These experiments were done using HEK 293T cells lines that express K7 or fsK7 or scrK7 inducibly upon addition of doxycycline. These cells lines were produced in T-Rex HEK 293T cells (Invitrogen) by Amanda Cunningham (K7 cell line) and Gabriel Almeida (fsK7 and scrK7 cell lines) and all the alleles had a C-terminal streptavidin plus FLAG tag. A similar cell line expressing another VACV protein, C6 was also used as a negative control because this protein has previously been shown not to inhibit ISRE pathway activation by luciferase reporter assays (unpublished data from our lab). Cells were induced with doxycycline overnight and treated the next day for 15 min with 1000 U/ml IFN- α . Cells were then lysed and lysates were analysed by immunoblotting (Fig. 5.15). No K7 protein was detected in the fsK7 cell line but it was seen in samples induced with doxycycline in the K7 and scrK7 cell lines when probing with anti-FLAG Ab. The levels of α -tubulin control protein were similar in all samples. Expression of C6 was also detected from C6 cell line samples in the presence of doxycycline. Samples were also immunoblotted for phosphorylated STAT1 (at tyrosine 701) and this was found only in samples that had been treated with IFN. However, the level of phospho-STAT1 was similar regardless of whether doxycycline was present. The levels of total STAT1 protein were also similar for all samples of a given cell line. This indicated that the presence of K7 protein and or *K7R* mRNA was not affecting STAT1 phosphorylation.

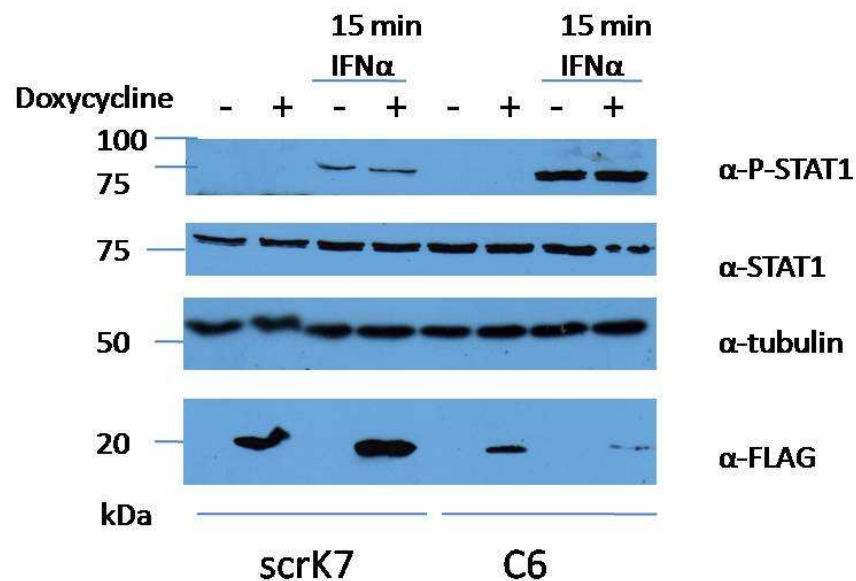
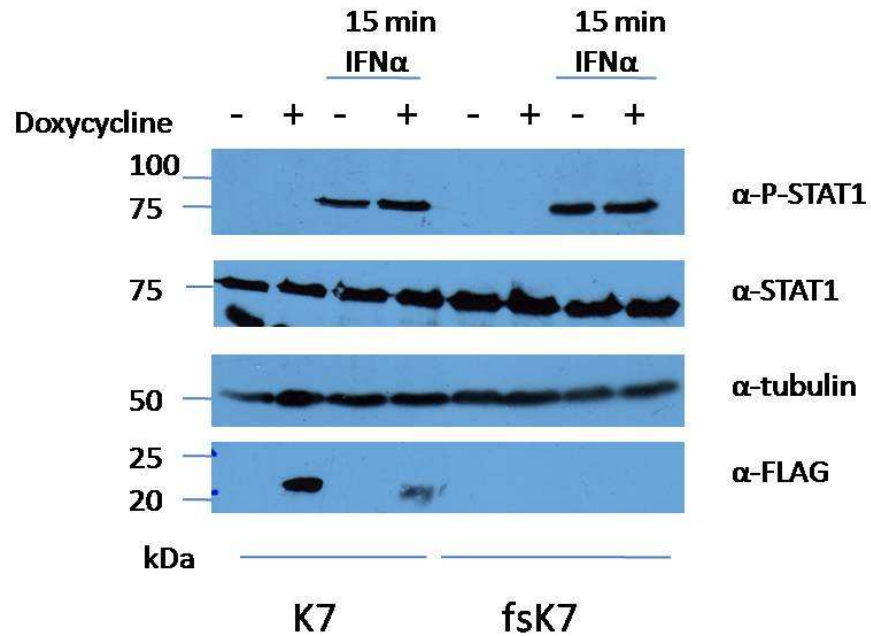


Fig. 5.15. Expression of phosphorylated STAT1 from IFN- α -treated cells expressing K7, fsK7, scrK7 or C6 in a doxycycline-inducible manner. Cells from each different cell line were induced with doxycycline at 2 μ g/ml (+) or left uninduced (-) overnight and were then treated with 1000 U/mL of IFN- α for 15 min or left untreated as indicated. Samples were lysed in the lysis buffer described in Materials and Methods 2.3.4 supplemented with phosphatase inhibitor tablets (Roche) and were analysed by immunoblotting with the indicated Abs. The sizes of molecular mass markers are indicated in kDa.

In conclusion, the presence of both K7 protein and K7 RNA expressed from the *K7R* sequences does not lead to inhibition of STAT1 phosphorylation and so these alleles may be modulating ISRE promoter activation by blocking components further downstream in the IFN pathway.

5.6 Construction of VACV WR recombinant viruses with different *K7R* alleles

Fig. 5.12 shows that *K7R* mRNA can inhibit IFN- β gene expression *in vitro*, and therefore several new recombinant VACV WR viruses were constructed to test if these alleles affected virus virulence *in vivo*. New *K7R* alleles were re-inserted into the *K7R* locus of v Δ K7 and these included vK7₁₇₁₋₄₅₀, vK7₁₇₈₋₂₆₉, vK7₁₋₁₇₁, vK7₂₇₀₋₄₅₀, vinvK7 and vscrK7. These new viruses will be tested in the *in vivo* mouse intranasal model to determine whether vK7₁₇₈₋₂₆₈ retains virulence, now that it has been shown that RNA transcribed from that allele inhibits innate immune signalling. The other viruses would act as various negative or positive controls. As shown in Fig. 5.16, K7₁₇₁₋₄₅₀ and invK7 alleles still contained the sequences predicted to encode miRNAs, so it is hypothesised that virulence would be comparable to that of vK7 and vfsK7 *in vivo*. Alleles K7₁₋₁₇₁ and K7₂₇₀₋₄₅₀ do not contain the region shown to be sufficient for the *in vitro* phenotype and it is expected these viruses would be attenuated. vscrK7 would contain a mutated *K7R* RNA sequence but produce K7 protein and so would enable the contribution of K7 protein to virulence to be made in the absence of K7 miRNA.

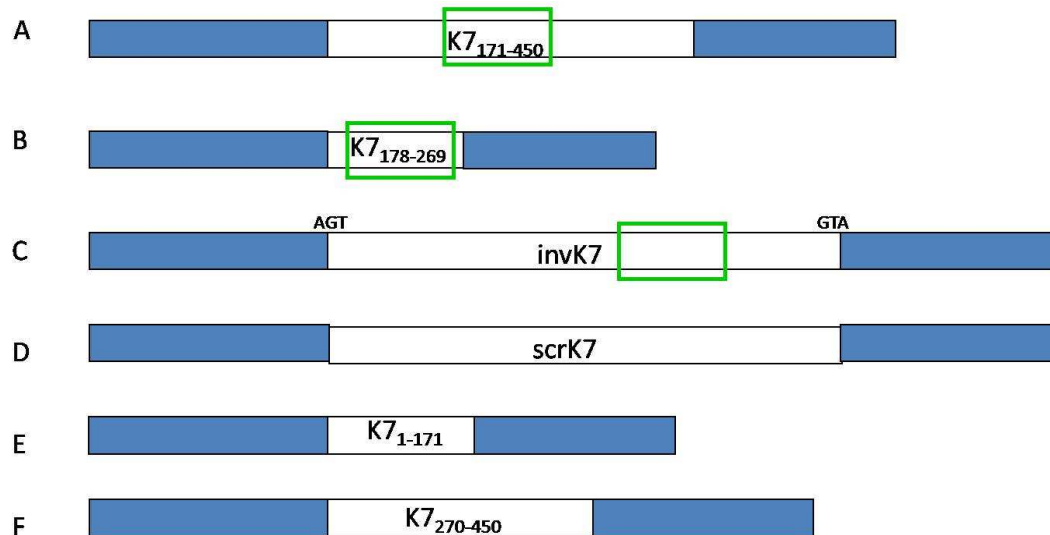


Fig. 5.16. *K7R* alleles re-introduced into $v\Delta K7$ at the *K7R* locus by transient dominant selection. Different *K7R* alleles and *K7R* flanks (in blue) designated A to F are illustrated. The green box indicates the section of the K7 sequence predicted to encode miRNAs. A to F were each subcloned into pUC13 Ecogpt-EGFP as shown in Fig. 5.17 and the resultant plasmids were used to generate recombinant viruses by transient dominant selection.

These six new viruses were made by transient dominant selection. Unlike the earlier viruses the transient dominant selectable marker was an Ecogpt-EGFP fusion protein that enabled selection of viruses by virtue of resistance to MPA and visual selection due to EGFP expression by fluorescent microscopy. The various alleles flanked by the upstream and downstream sequences of the *K7R* locus were cloned into the *EcoRI* site in pUC13 Ecogpt-EGFP. These different alleles were amplified by overlap extension PCR as illustrated in Fig. 5.17.

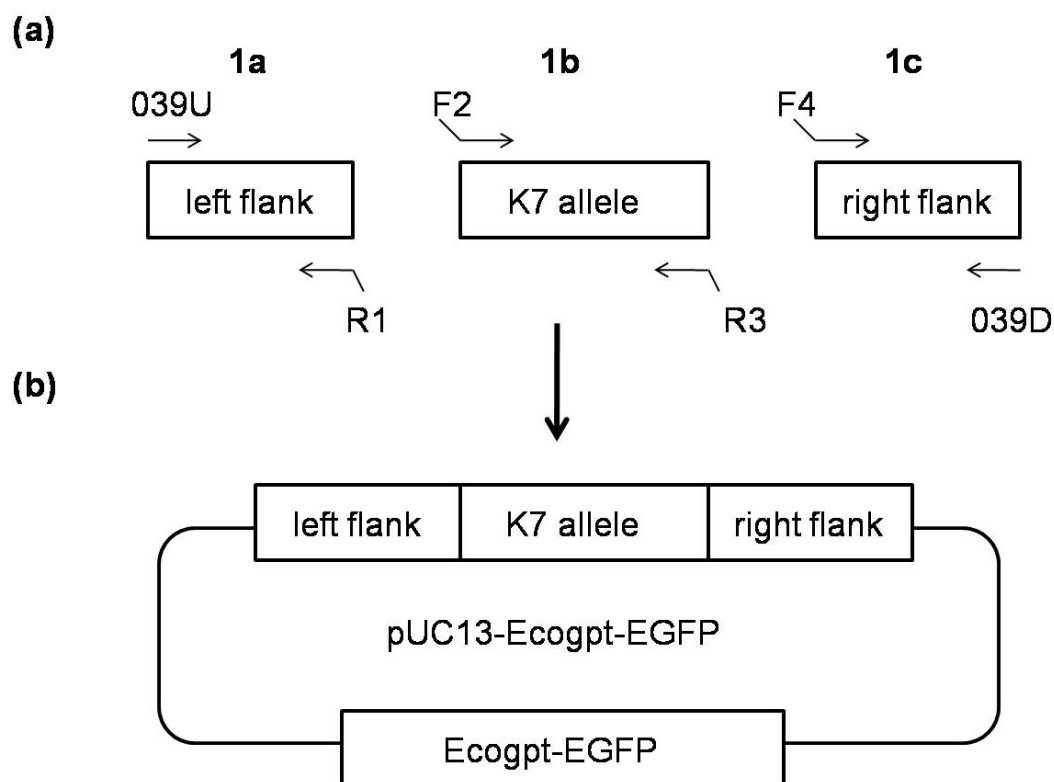


Fig. 5.17. Cloning of plasmids for re-introducing *K7R* alleles into $v\Delta K7$ at the *K7R* locus by transient dominant selection. (a) The diagram is a generic depiction of the various fragments needed and the primers used to amplify alleles A to F that were shown in Fig. 5.16. These fragments were assembled by overlap extension PCR. The left flank of *K7R* was amplified using primers 039U and R1 and that PCR is designated 1a. The K7 allele of interest was amplified using primers F2 and R3 by a PCR called 1b. The right flank of *K7R* was amplified using primers F4 and 039D in a PCR called 1c. Primers R1, F2, R3 and F4 vary from allele to allele but this generic terminology is used for clarity and a full list of primers is given in Materials and Methods section 2.1.12. Products from PCRs 1a and 1b were then used as templates for a PCR with primers 039U and R3 to yield a product consisting of the left flank and the allele of interest. This product was then used with the right flank as templates for a PCR with 039U and 039D. This final PCR yielded one of the alleles marked A-F. However, for A, E and F only one overlap extension PCR was necessary as there is no break between one end of the allele's sequence and one of the *K7R* flanks and the protocol was modified accordingly. (b) Alleles A to F were cloned into the commercial TOPO pCR4 intermediate vector directly and then subcloned into pUC13 EcoGpt-EGFP at the *EcoRI* site to yield the plasmids illustrated.

Recombinant viruses were constructed and isolated as described in section 5.1. After the viruses were plaque purified, their genomic DNA was extracted from infected cells (see Materials and Methods 2.4.6) and their genomes analysed by PCR using primers homologous to the *K7R* flanks as schematised in Fig. 5.18a. For $\Delta K7$, only the flanks can be amplified and the product size expected is 660 bp. For $vK7_{171-450}$, $vK7_{178-269}$, $K7_{1-171}$, $K7_{270-450}$ templates, the expected sizes increase by 279, 90, 171 and 180 bp respectively. InvK7 and scrK7 alleles are the same length as wild type K7 (450 bp) so the PCR product is expected to be 1.1 kb. Fig. 5.18b confirms that the six new recombinant viruses DNA template yielded PCR products of the expected sizes.

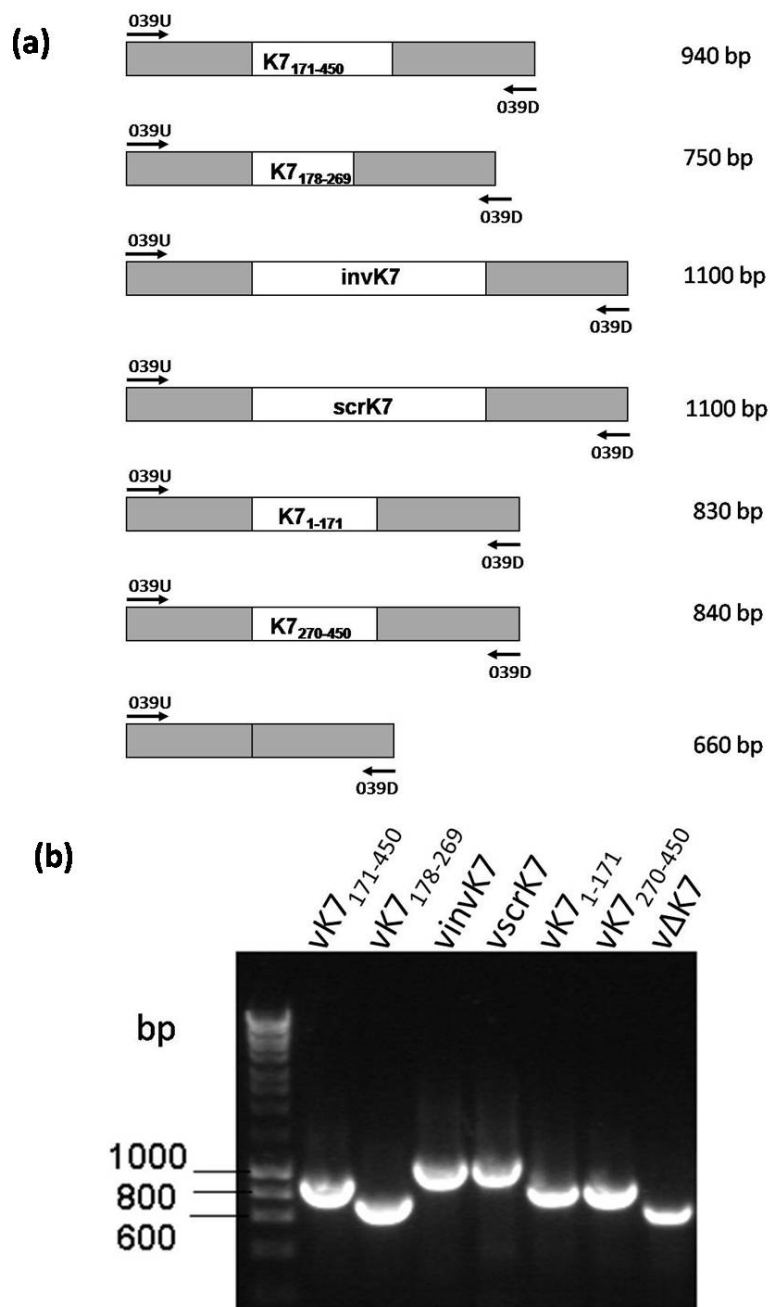


Fig. 5.18. Analysis of recombinant VACV genomes by PCR. (a) The diagram illustrates *K7R* alleles re-introduced into vΔK7 and indicates the expected product size for each allele by PCR with primers 039U and 039D. (b) Virus DNA was extracted from cells that had been infected with the indicated viruses and used as template for PCR with primers 039U and 039D. PCR products were analysed by agarose gel electrophoresis. The positions of DNA size markers in bp are indicated.

The genomes of the new recombinant *K7R* viruses described in this chapter were analysed after *HindIII* restriction digestion to check if any gross genomic modifications have occurred during their isolation. Each virus was purified by sucrose density gradient centrifugation (Materials and Methods section 2.4.10), virus DNA was extracted and then this was digested with *HindIII*. The digested genomic DNA was then analysed by agarose gel electrophoresis (Fig. 5.19). All the viruses had the same restriction digest pattern with the exception of the *HindIII* K fragment which is normally 4.526 kb in VACV WR. This fragment includes the *K7R* locus and so a slight reduction in its size compared to WR was expected for some of the recombinant viruses. In the deletion virus, the loss of the 450-bp *K7R* would make *HindIII* K fragment size practically the same as *HindIII* L fragment meaning it would not be seen on the gel. This is indeed what was found for vΔK7 and vK7₁₇₈₋₂₆₉ in which only 90 nt of *K7R* remain. It was also evident that DNA co-migrating with the *HindIII* L fragment was also more intense than wild type, indicating the presence of additional DNA. No change in *HindIII* K fragment was seen in the genome of vK7fs-2, vinvK7 and vscrK7 as expected as these viruses contain a full length *K7R* sequence, or, in the case of vscrK7, contains a mutated sequence of the same size as wild type. For the remaining viruses the shift in *HindIII* K fragment size is in line with the designed reduction in *K7R* sequence for that virus. In conclusion, none of the recombinant viruses contained gross genomic modifications.

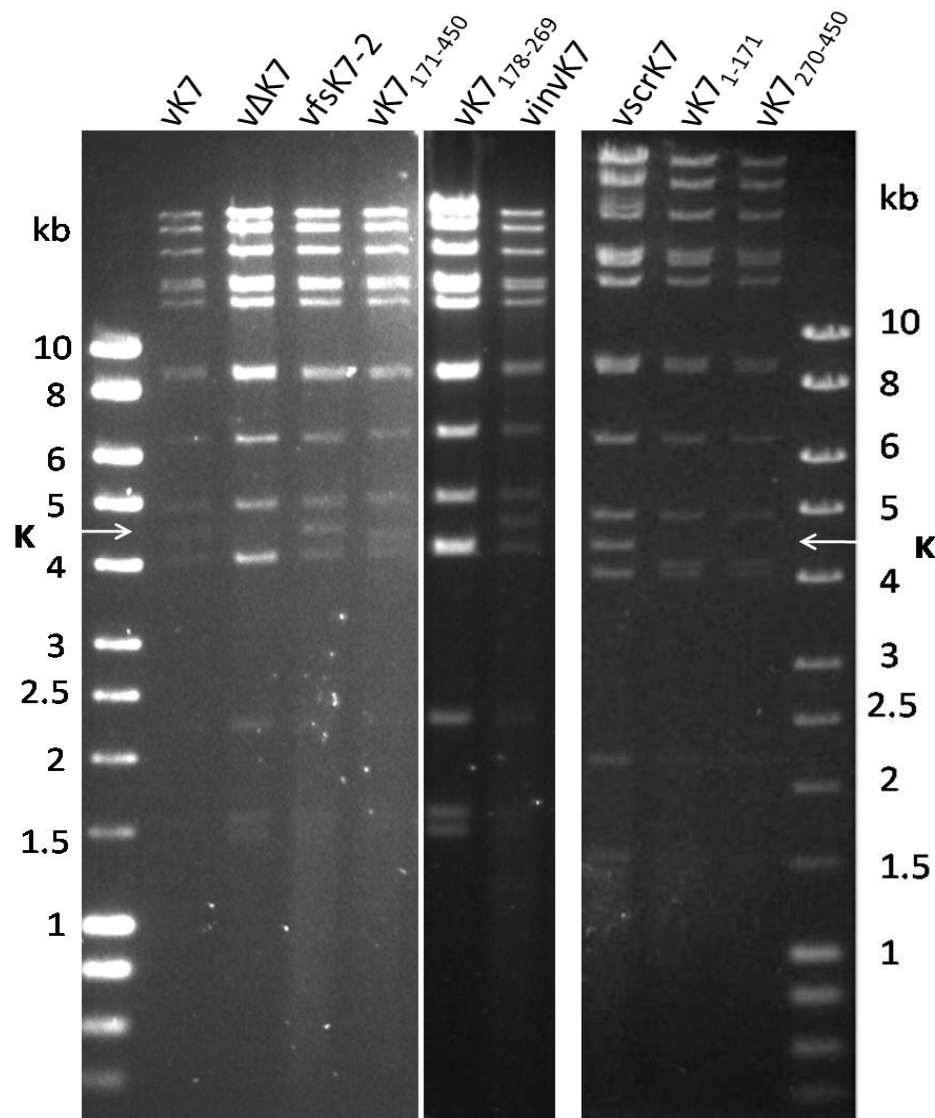


Fig. 5.19. Characterisation of the genomes of recombinant VACVs. Genomic DNA was extracted from purified virus stocks for the indicated viruses and 1 μ g of each genomic DNA was digested with *Hind*III and the resulting DNA fragments were resolved on a 0.7 % agarose gel overnight at 4 °C. The gel was then stained in ethidium bromide for 90 min prior to photography under UV light. The positions of molecular size markers are indicated in kb. K= VACV WR *Hind*III K fragment.

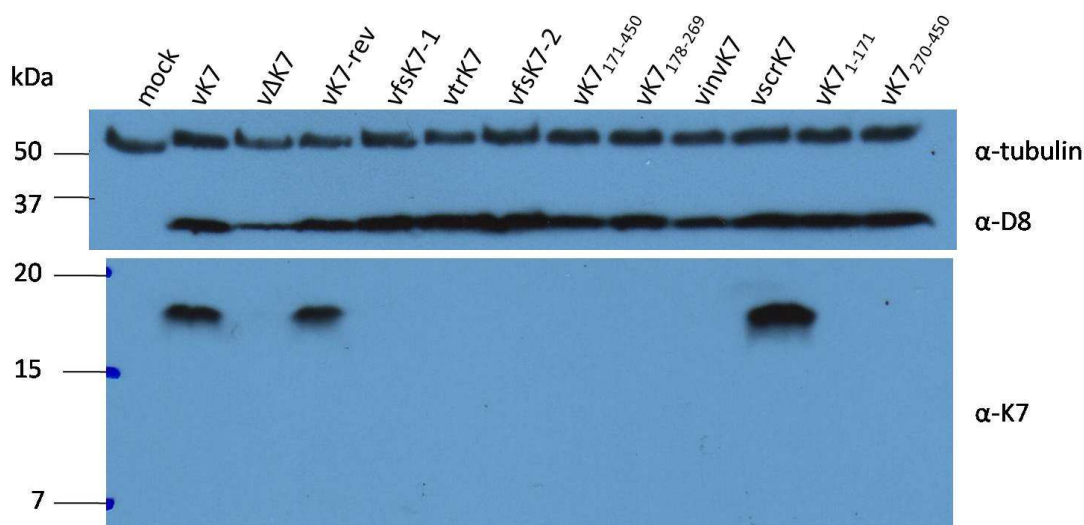


Fig. 5.20. Expression of K7 by recombinant viruses. BSC-1 cells were infected with the indicated K7 viruses at 1 pfu/cell overnight. Cells were then lysed as described in Materials and Methods 2.3.4 and lysates were analysed by SDS-PAGE (15 % gel) followed by immunoblotting with the indicated Abs. The sizes of molecular mass markers are indicated in kDa.

The expression of K7 by these viruses was then examined. BSC-1 cells were infected overnight with the indicated viruses and cell lysates were analysed by immunoblotting for K7 expression (Fig. 5.20). Expression of K7 was seen in vK7, vK7-rev and vscrK7, but none of the other viruses, as expected. Immunoblotting with a mAb against the IMV D8 protein demonstrated that each virus had infected the cells. Interestingly, it appeared that the level of K7 expression by vK7scr might be slightly increased compared to wild type but this was equivocal because the expression of D8 control in that sample was also slightly higher. This was investigated further with samples harvested at different times p.i. with vK7, vΔK7, vfsK7-2 and vscrK7 (Fig. 5.21). The expression of the D8 control protein was detected from 4 h p.i. and its levels were similar at the same timepoint for samples infected by different viruses. K7 was detected earlier (from 2 h p.i.) in samples infected with vK7 or vscrK7 and their levels of expression were similar. In conclusion, K7 is expressed at similar levels from vK7 and vscrK7.

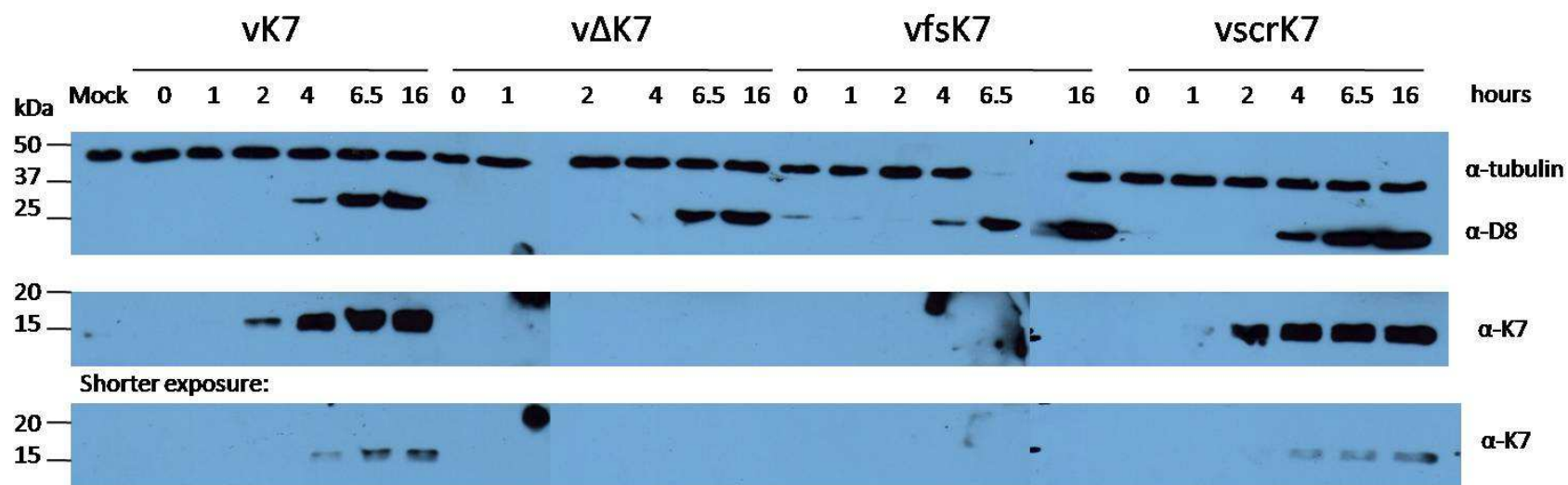


Fig. 5.21. Expression of K7 by vK7 and vscrK7 at different times post infection. BSC-1 cells were infected with the indicated viruses at 5 pfu/cell for the indicated time. Cells were then lysed as described in Materials and Methods 2.3.4 and lysates were analysed by SDS-PAGE (12 % gel) followed by immunoblotting with the indicated antibodies. The sizes of molecular mass markers are indicated in kDa.

Chapter 6: Construction and *in vitro* characterisation of MVA-C viruses lacking K7 orthologue 028R

VACV WR *K7R* encodes an immunomodulatory protein and its orthologue in MVA, *028R* is predicted to encode a protein with the same sequence and activity. It is known that the targeted removal of immunomodulators from MVA can make the virus more immunogenic (Staib *et al.*, 2005; Clark *et al.*, 2006). Therefore, a more immunogenic MVA vaccine could be created by the removal of *028R*. This project has undertaken the first steps to test that hypothesis.

Consequently, *028R* was deleted from MVA. Initially, a deletion *028R* virus (vΔ028-d) was obtained using a technique based on a double recombination between a transfected plasmid and the MVA genome and in which a short sequence is left within the MVA genome at the *028R* locus (section 6.1.1). Subsequently, this repeat was removed by transient dominant selection to form a complete, clean deletion virus (vΔ028). Finally, a revertant virus (v028-rev) was produced in which the *028R* gene was restored to its natural locus, again by transient dominant selection (section 6.1.2). The parental strain of MVA used in this project is called MVA-C and has the HIV clade C Env/Gag-Pol-Nef sequence inserted in the TK locus of wild type MVA (Gomez *et al.*, 2007). This is illustrated in Fig. 6.1.

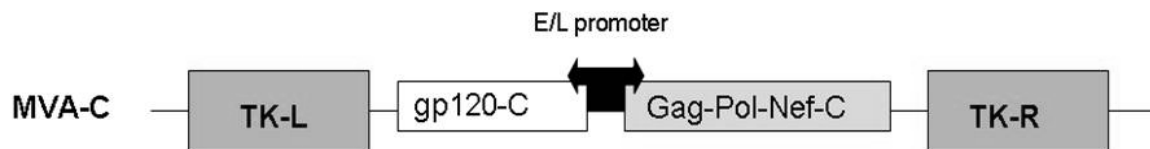


Fig. 6.1. Structure of TK gene locus of MVA-C containing HIV clade C gp120-C (Env) and Gag-Pol-Nef. The expression of these antigens is driven by two VACV synthetic E/L promoters in a back-to-back orientation. TK-L (left) and TK-R (right) are the TK flanking regions.

6.1 Construction of MVA-C vΔ028-d, vΔ028 and v028-rev

6.1.1 Construction of vΔ028-d

6.1.1.1 Cloning the vector for making vΔ028-d

A plasmid required for the removal of *028R* from MVA-C was constructed. This vector, based on pCR4 blunt TOPO (Invitrogen), contains the Ecogpt-EGFP chimeric coding sequence driven by a VACV promoter and flanked by short repeats (R1 and R2). This whole region was flanked by VACV DNA taken from upstream and downstream of the *028R* gene. The regions flanking *028R* were engineered to include the 3' end of the *028R* gene because it contains a potential transcription termination site (T₅NT) for the gene downstream of *028R* (see Fig. 6.2). Fig. 6.3 illustrates the cloning process for creating the pCR4 (A-R1-Ecogpt-EGFP-R2-B) vector used to make vΔ028-d.

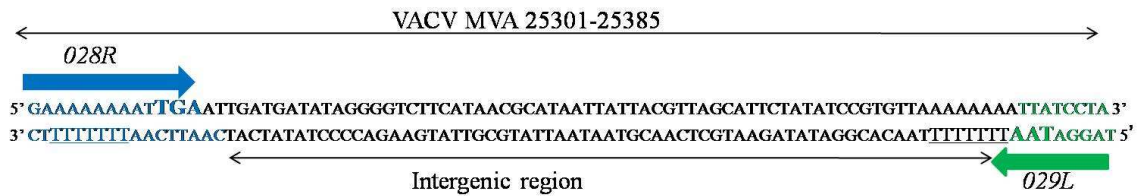


Fig. 6.2. Potential early transcription termination sequences for *029L*. The primers used to generate the flanking regions of *028R* were designed not to delete part of the *028R* open reading frame that contains an additional potential transcription termination sequence (T₅NT) for the neighbouring *029L* gene. One of those sequences is positioned between the two genes and the other is at the end of *028R* and both are underlined. *029L* runs in the direction opposite to *028R* so the T₅NT consensus region of interest corresponds to AAAAAAA in the *028R* coding strand. Therefore, the downstream flanking region of *028R* cloned into plasmids included the last 17 nucleotides of *028R*. The last 17 nucleotides of the *028R* coding region are in blue, the sequence between the two genes is in black and the last few bases of *029L* coding region are in green. The stop codons for *028R* and *029L* are in large bold letters on the coding strand for each gene.

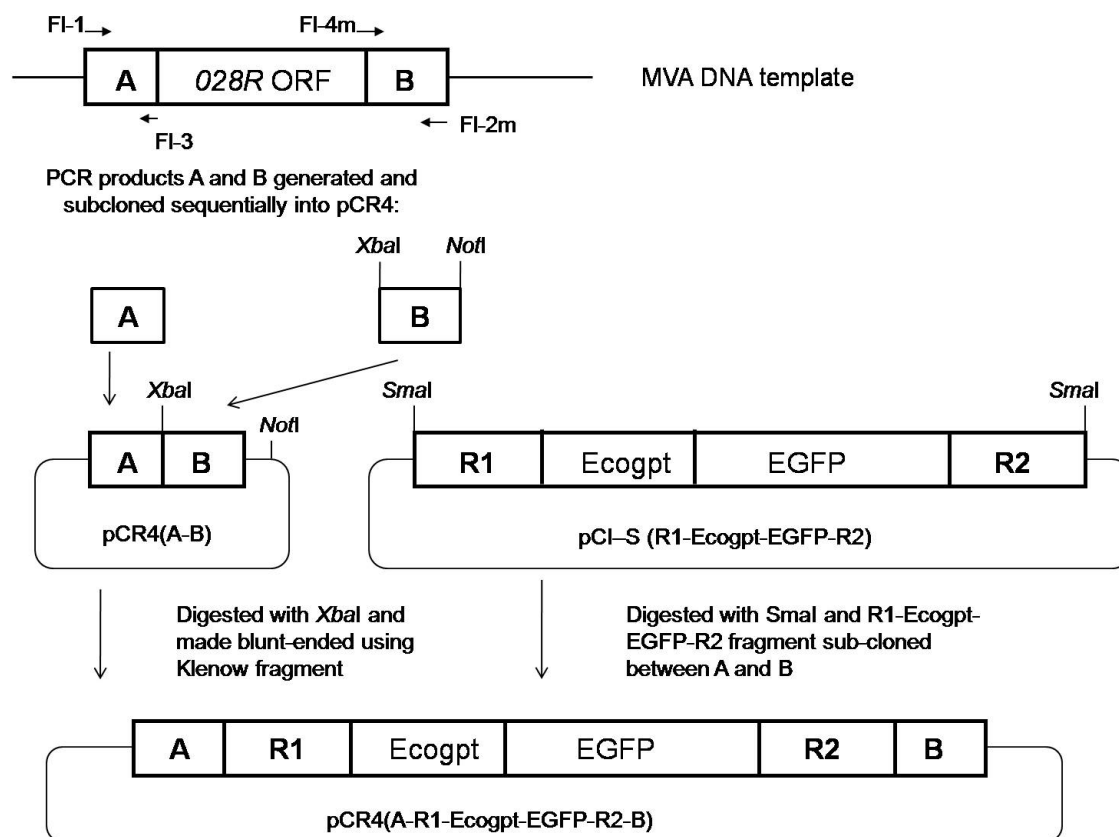


Fig. 6.3. Construction of the plasmid vector for making MVA-C v Δ 028-d. The flanking regions of *028R* in MVA (-320 bp upstream of *028R* to the beginning of the open reading frame and +354 downstream of *028R* to base 433 of *028R*) were each amplified using primers FI-1 and FI-3 for the 5' flank (A) and FI-4m and FI-2m for the 3' flank (B). The template used was MVA genomic DNA extracted from infected cells. An *XbaI* site was also introduced to enable insertion of DNA between the flanks. Flank A was cloned directly into pCR4 Blunt-TOPO (Invitrogen). Subsequently, flank B was cloned between the *XbaI* and *NotI* restriction sites in pCR4. This vector (pCR4 (A-B)) was sequenced to check that no mutations had been introduced into flanks A and B during PCR. It was then linearised with *XbaI* and the sticky ends rendered blunt-ended using the Klenow fragment of DNA polymerase. The R1-Ecogpt-EGFP-R2 fragment was excised from a parent vector made by Dr Mike Pütz (pCI-S R1-Ecogpt-EGFP-R2) with *SmaI* and ligated to the linearised pCR4-(A-B) vector.

6.1.1.2 Gene knock-out strategy based on double homologous recombination

Gene *028R* was initially knocked out from MVA-C using a modified version of an established method (Staib *et al.*, 2005) because it was the strategy agreed by the consortium of laboratories funded through the same grant from the Gates Foundation. This method consisted of infecting cells with the parental virus (MVA-C) and then transfecting these cells with a plasmid containing sequences flanking the *028R* gene separated by a cassette comprising the Ecogpt-EGFP fusion protein under the control of a synthetic VACV E/L promoter. This cassette was flanked by repeat sequences (R1 and R2) derived from the *Lac Z* gene of *E. coli* (Fig. 6.4). The VACV E/L promoter was described previously (Davison & Moss, 1990). The Ecogpt-EGFP sequence was obtained from vector pSEL-Ecogpt-EGFP, created by Emily Glyde (MRes Thesis, 2005). The *E. coli* gene *Ecogpt* confers resistance of VACV to MPA (Boyle & Coupar, 1988). Within the infected cells a double homologous recombinant event between the regions flanking the *028R* gene in the plasmid and the virus genome replaced the *028R* gene with the repeats and Ecogpt-EGFP cassette. Given that only about 0.1 % of progeny virus is recombinant, selective methods were needed to distinguish these from parental virus. The recombinant viruses containing Ecogpt-EGFP were distinguished from the parental virus by growth on CEFs in the presence of MPA, X and HPX (Boyle & Coupar, 1988; Falkner & Moss, 1990) and by visual observation of expression of EGFP using fluorescent microscopy. The MPA-resistant green virus isolated was termed an intermediate virus because it contains the R1 and R2 repeats that make it unstable. Once the selective pressure for retention of Ecogpt was removed, a recombination between these repeats caused excision of one repeat and the intervening DNA. Viruses that have undergone such recombination and have lost Ecogpt-EGFP were identified after plaque assays by staining cells with anti-VACV rabbit polyclonal serum, followed by rabbit secondary fluorescent Ab. Lack of EGFP expression was confirmed by microscopy. The genome of the deletion virus (vΔ028-d) obtained contains a single R repeat in place of the targeted *028R* gene (Fig. 6.4).

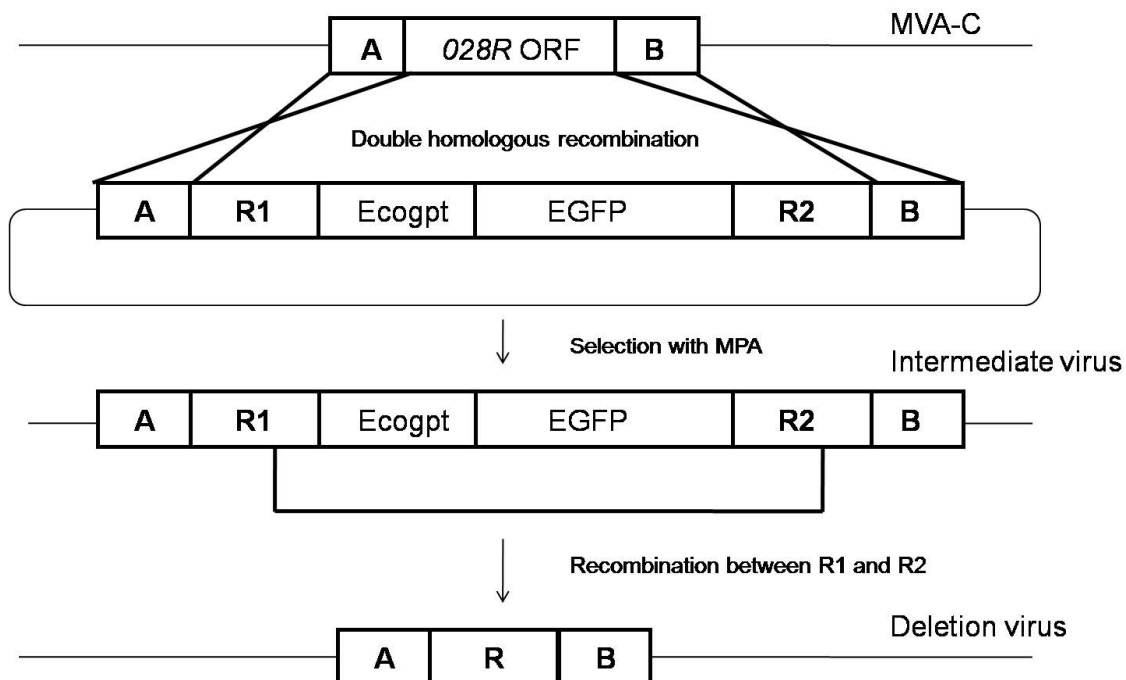


Fig. 6.4. Overview of construction of vΔ028-d. MVA-C-infected cells were transfected with pCR4(A-R1-Ecogpt-EGFP-R2-B) which was described in Fig. 6.3. A double homologous recombination event between the *028R* flank sequences in the plasmid and corresponding sequences in the virus genome replaced the target gene with the R1 and R2 repeats and Ecogpt-EGFP cassette. These Ecogpt-EGFP-expressing recombinants were selected in the presence of MPA and by visual observation of EGFP expression by fluorescent microscopy. When the selective pressure for retention of Ecogpt was removed; a recombination between the repeats caused excision of one repeat and the intervening DNA. This recombination event generated vΔ028-d with a single repeat sequence remaining at the *028R* locus.

6.1.1.3 Making recombinant MVA-C viruses

Primary CEFs were infected with MVA-C and then the targeting plasmid was transfected into the cells. After 48 h, CEFs were observed by fluorescent microscopy. If expression of EGFP in cells was observed, the infection-transfection was harvested. Fig. 6.5 shows fluorescent cells 48 h after a typical infection-transfection.

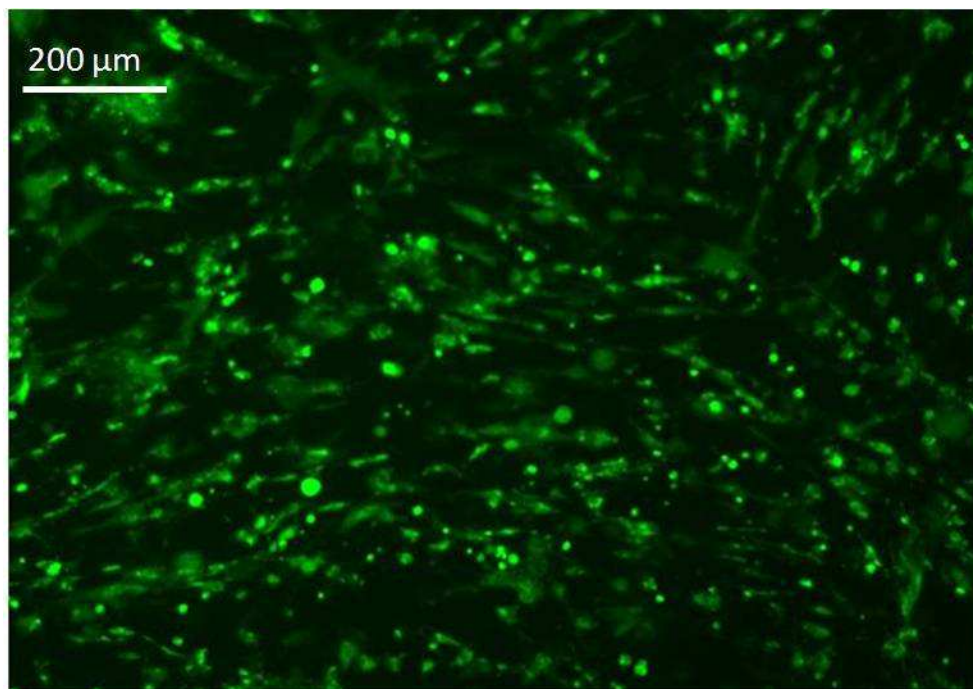


Fig. 6.5. Expression of EGFP in CEFs. CEFs were infected with MVA-C at 0.01 pfu/cell for 90 min. Cells were then transfected with 5 μg of pCR4(A-R1-Ecogpt-EGFP-R2-B) and incubated for 48 h before being visualised by fluorescent microscopy (Zeiss Axiovert 200M inverted light microscope, EGFP filter set, 10 X magnification).

To obtain single, well-isolated foci of recombinant viruses distinct from the parental virus, CEFs were pre-treated with MPA, HPX and X before being infected with different dilutions of virus derived from the infection-transfection. This provided positive selection for viruses that have integrated the plasmid at the *028R* locus and therefore express Ecogpt-EGFP. After infection for 2-3 days small green foci were detected by fluorescence microscopy and marked prior to picking from plates under solid overlay. Examples of such foci can be seen in Fig. 6.6a.

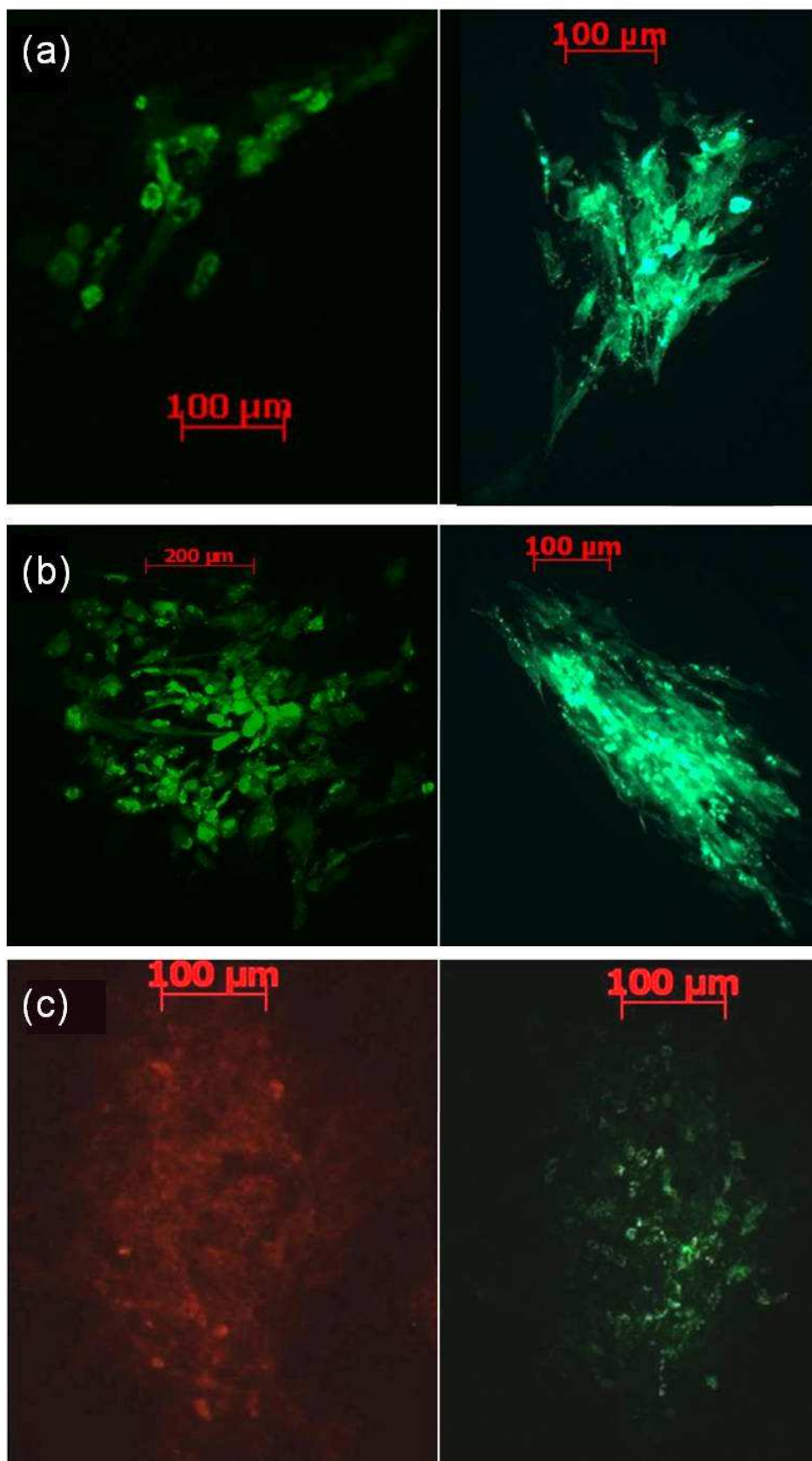


Fig. 6.6. Observation of EGFP positive foci by florescent microscopy. (a) The two foci were formed on CEFs infected with MVA-C expressing Ecogpt-EGFP and were visualised by fluorescent microscopy. Foci shown were obtained after a plaque assay on CEFs in the presence of MPA using virus harvested from the infection-transfection. (b) The two foci shown were obtained after virus isolates shown in (a) were picked and used to infect new CEFs in the presence of MPA. (c) Intermediate viruses were amplified in CEFs without MPA for 2-3 days and harvested. These virus stocks were used to carry out plaques assays on CEFs. Infected monolayers were then stained with anti-VACV Ab followed by Alexa 546-conjugated (red fluorophore) secondary Ab and observed by fluorescent microscopy. The two images shown are of the same focus observed under the Alexa 546 and EGFP filter sets. The fluorescent microscope used was the Zeiss Axiovert 200M inverted light microscope at 10 X magnification.

To ensure that intermediate viruses (recombinant viruses that have integrated the cassette and lost the *028R* gene from its locus) had been obtained, a further two rounds of foci purification with drug selection were carried out. Typically, foci were picked into 0.5 ml and stored frozen at -80° C until use. Usually 0.1 – 0.2 ml of this was used to infect pre-treated CEFs. Again, green foci were picked after 2-3 days and the process repeated. Images of typical foci are in Fig. 6.6b. Subsequently, the foci obtained after three rounds of selection were amplified in T25 flasks of CEFs for 3-4 days in drug-free liquid medium. The quality and confluency of CEFs was important to obtain foci in all experiments.

To obtain resolved recombinant viruses, the virus stock harvested from T25 flasks was diluted and used to infect CEFs for 2 days under semi-solid overlay without drug. Then EGFP-negative foci were selected. To visualise these, the semi-solid overlay was removed, and the plates were stained with rabbit anti-VACV Ab. Bound Ab was detected with anti-rabbit-Alexa red fluor labelled secondary Ab (Fig. 6.6c). The foci were then used to infect more CEFs without drugs. This process was repeated 3 times and the genotype of the final plaque isolate was determined by PCR.

6.1.2 Construction of the clean Δ 028R and rev028R viruses

6.1.2.1 Cloning the vectors for making v Δ 028 and v028-rev

To clone the plasmid required for making v Δ 028, the 028R flanks were excised from pCR4 (A-B) shown in Fig. 6.3 using *EcoRI*, rendered blunt-ended using the Klenow fragment of DNA polymerase and ligated into the *SmaI* site in the MCS of pCI-S Ecogpt-EGFP. The latter plasmid contains the Ecogpt-EGFP cassette downstream of the E/L VACV promoter. To clone the plasmid required for making the v028-rev virus, the 028R sequence and flanking region was excised from pCR4 (A-028R-B) and ligated into pCI-S Ecogpt-EGFP. This process is illustrated in Fig. 6.7.

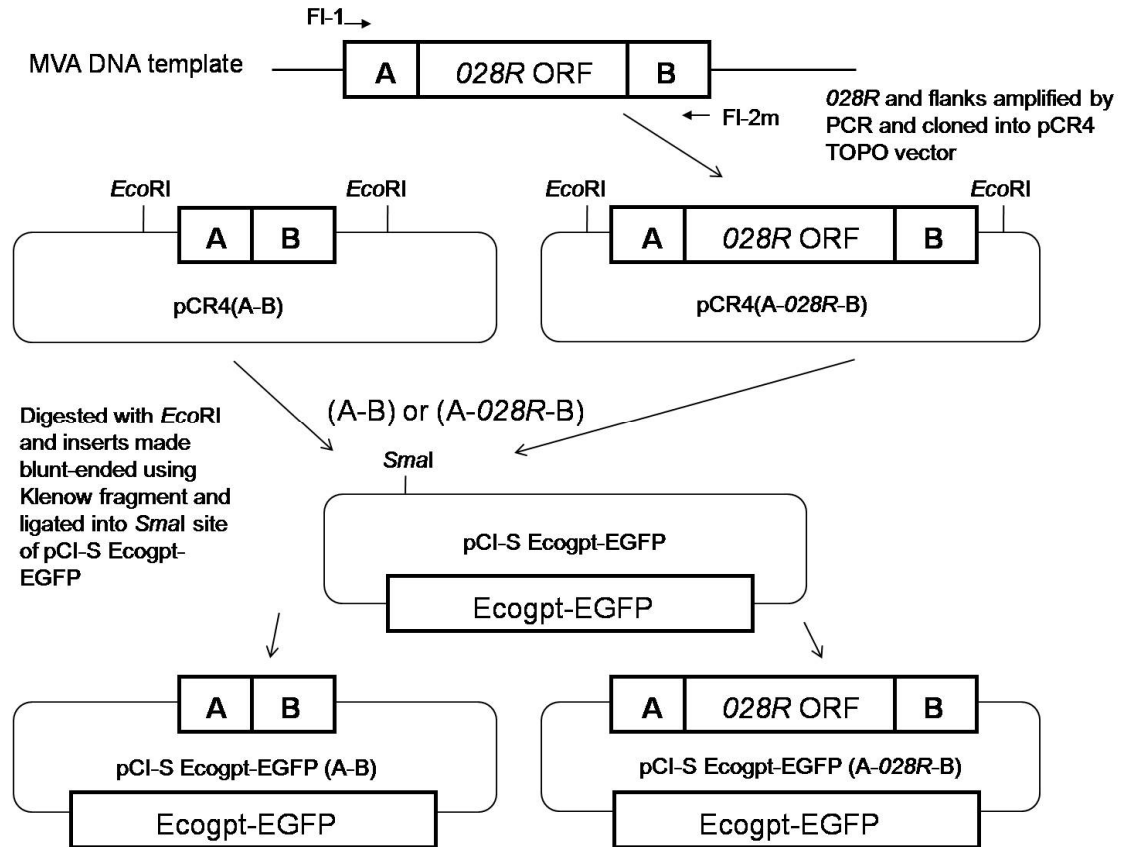


Fig. 6.7. Construction of the plasmid vectors for making vΔ028 and v028-rev. *028R* and flanking regions in MVA (-320 bp upstream of *028R* to +354 downstream of *028R*) were amplified from MVA genomic template using primers FI-1 and FI-2m. A and B are the upstream and downstream flanks of *028R*. The PCR product obtained was cloned into pCR4 by “TOPO cloning” (Invitrogen). Vector pCR4 (A-028R-B) was sequenced and then pCR4(A-B) and pCR4(A-028R-B) were digested with *Eco*RI and the A-B and A-028R-B fragments were rendered blunt-ended using the Klenow fragment DNA polymerase. These were then subcloned into the *Sma*I site of pCI-S Ecogpt-EGFP.

6.1.2.2 Construction of recombinant viruses by transient dominant selection

The strategy used to construct the v Δ 028 was transient dominant selection. This method also consists of transfecting CEFs with a plasmid containing the same Ecogpt-EGFP cassette but in this instance this sequence is situated distal to the sequence of the gene and its flanks. Thus, a single homologous recombination event between one of *028R* flanks in the plasmid with its virus counterpart leads to the whole plasmid being integrated into the virus genome. Its presence within the genome is maintained in the presence of MPA and intermediate viruses are obtained. The presence of flanking region repeats at the site of insertion of the plasmid make the intermediate virus unstable, and growth on CEFs in absence of selective pressure to maintain Ecogpt, causes the spontaneous loss of the plasmid DNA by recombination to form v Δ 028 or parental v Δ 028-d (Fig. 6.8).

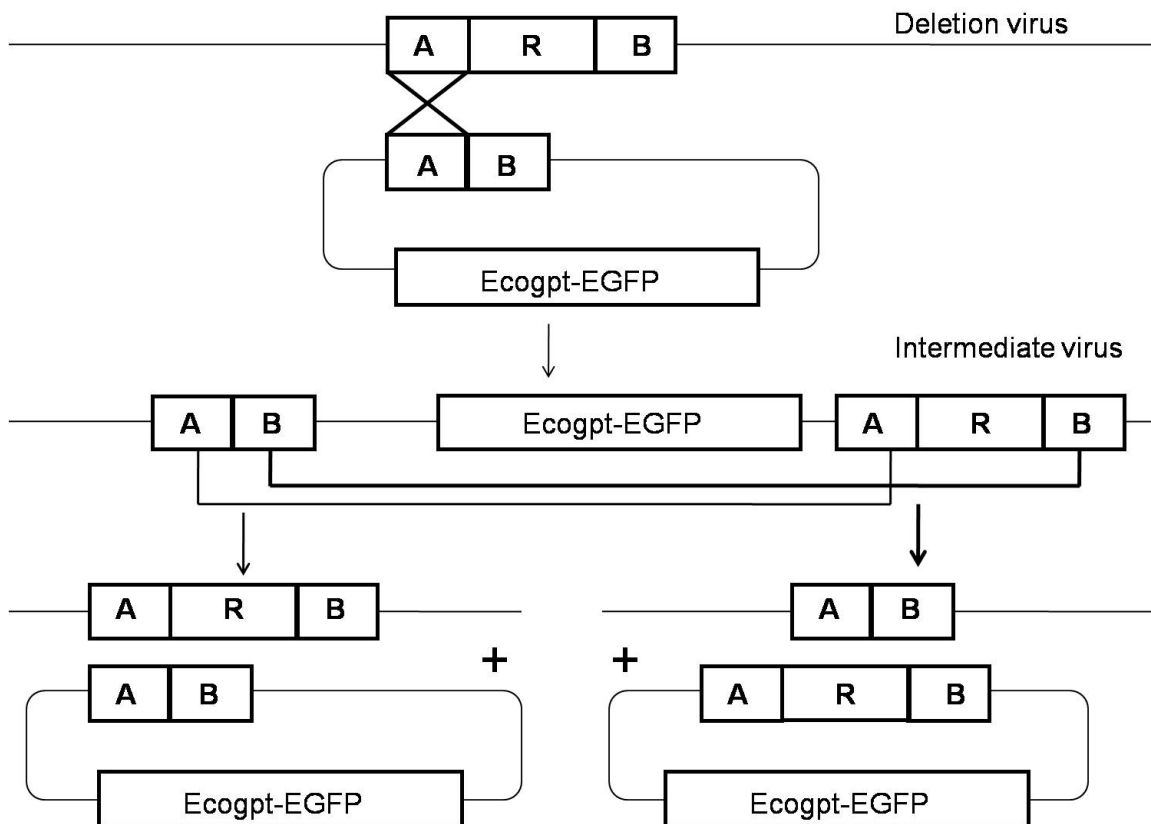


Fig. 6.8. Overview of construction of vΔ028 from vΔ028-d by transient dominant selection. Cells infected with vΔ028-d were transfected with pCI-S Ecogpt-EGFP (A-B) containing *028R* flanking regions and the dominant selectable marker Ecogpt-EGFP. Recombination between a *028R* flank sequence in the plasmid and corresponding sequence in the virus genome generated an intermediate virus with direct repeated sequences and these Ecogpt-EGFP-expressing recombinants were selected in the presence of MPA after visual observation of expression of EGFP under fluorescent microscopy. After removing the selective pressure for Ecogpt, the intermediate virus resolved by recombination between the repeat sequences as illustrated to generate either vΔ028-d (parental virus) and pCI-S Ecogpt-EGFP (A-B) or the clean *028R* deletion mutant vΔ028 and pCI-S Ecogpt-EGFP (A-R-B).

After v Δ 028 was obtained, the *028R* gene was re-inserted into this deletion mutant to form v028-rev. The purpose of the revertant virus was to ensure that any phenotypic differences between the parental and deletion viruses are attributed to genetic modifications in the *028R* gene locus rather than to possible second site mutations. The strategy to construct v028-rev was also transient dominant selection and is illustrated in Fig. 6.9. When flanking region repeats recombine in the intermediate virus, either parental v Δ 028 or v028-rev is formed.

Both v Δ 028 and v028-rev were made and obtained as described in 6.1.1.3.

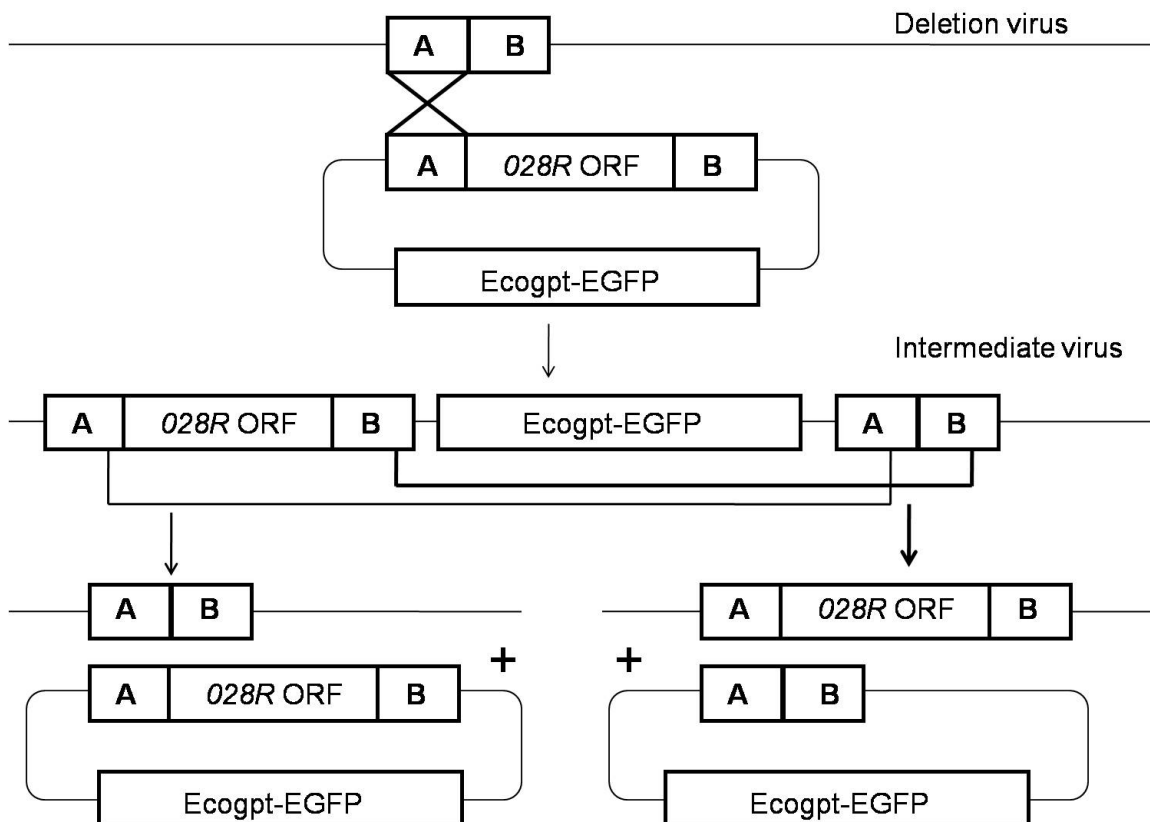


Fig. 6.9. Overview of construction of v028-rev in from vΔ028 by transient dominant selection. Cells infected with vΔ028 were transfected with pCI-S Ecogpt-EGFP (A-028R-B) containing 028R and flanking regions and the dominant selectable marker Ecogpt-EGFP. Recombination between a 028R flank sequence in the plasmid and corresponding sequence in the virus genome generated an intermediate virus with direct repeated sequences and these recombinants were selected as described in Fig. 6.8. After removing the selective pressure for Ecogpt, the intermediate virus resolved by recombination between the repeat sequences as illustrated to generate either vΔ028 (parental virus) and pCI-S Ecogpt-EGFP (A-028R-B) or v028-rev and pCI-S Ecogpt-EGFP (A-B) that had lost the 028R sequence but retained the 028R flanks.

6.1.3 Viral genome analysis by PCR

The genotypes of the different viral clones were analysed by PCR using primers to the flanking regions of the *028R* gene. Genomic DNA was obtained from infected cells. The PCR product expected for wild type and revertant viruses is 1.1 kb, whereas the size for the 2 deletion mutants vΔ028-d and vΔ028 was 900 and 660 bp, respectively, depending whether the *Lac Z* repeat was present (see Fig. 6.10a). The PCR products obtained from each virus DNA are shown in Fig. 6.10b and were of the expected sizes for all viruses. Another PCR was carried out to verify the presence of HIV-C DNA in the TK locus of the MVA-C virus and derivatives. The PCR was carried out using primers p91 and p92 that anneal within the Gap-Pol-Nef sequence and yield a 1.8-kb product (Fig. 6.10c). A 1.8-kb band was obtained in all MVA-C viruses but not from DNA of MVA and mock-infected preparations. The HIV-C gene sequence is therefore present in MVA-C viruses tested (Fig. 6.10d).

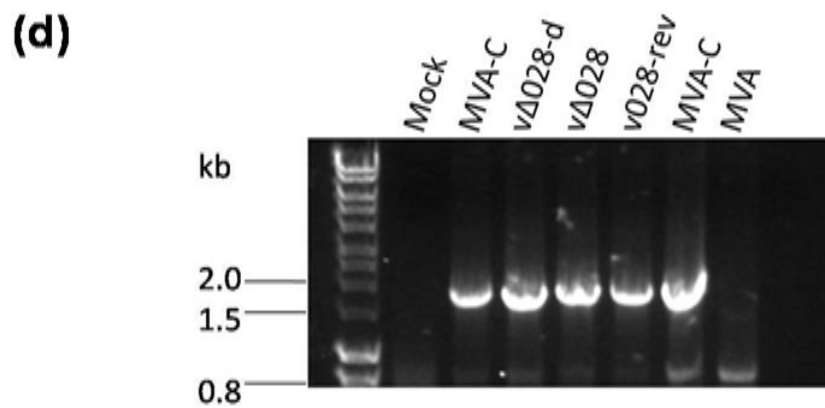
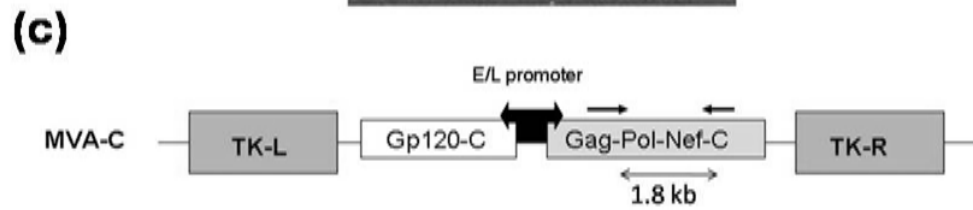
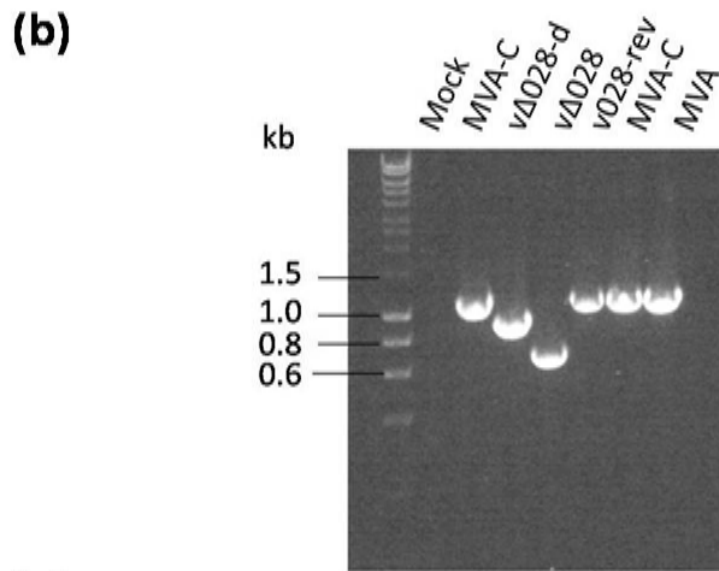
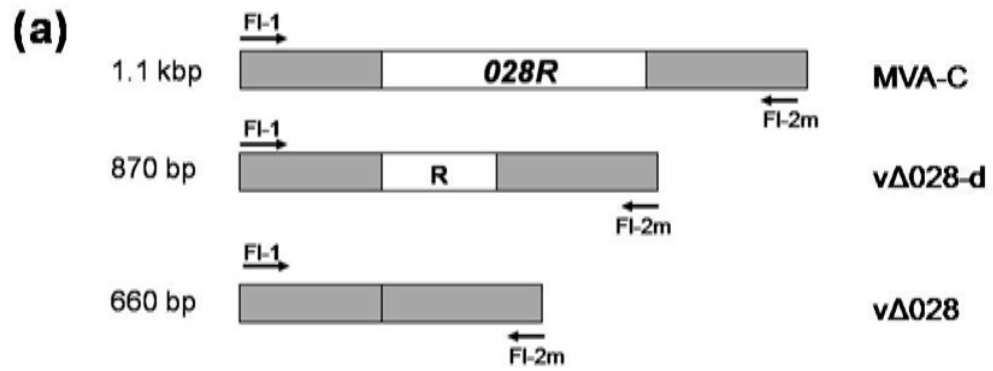
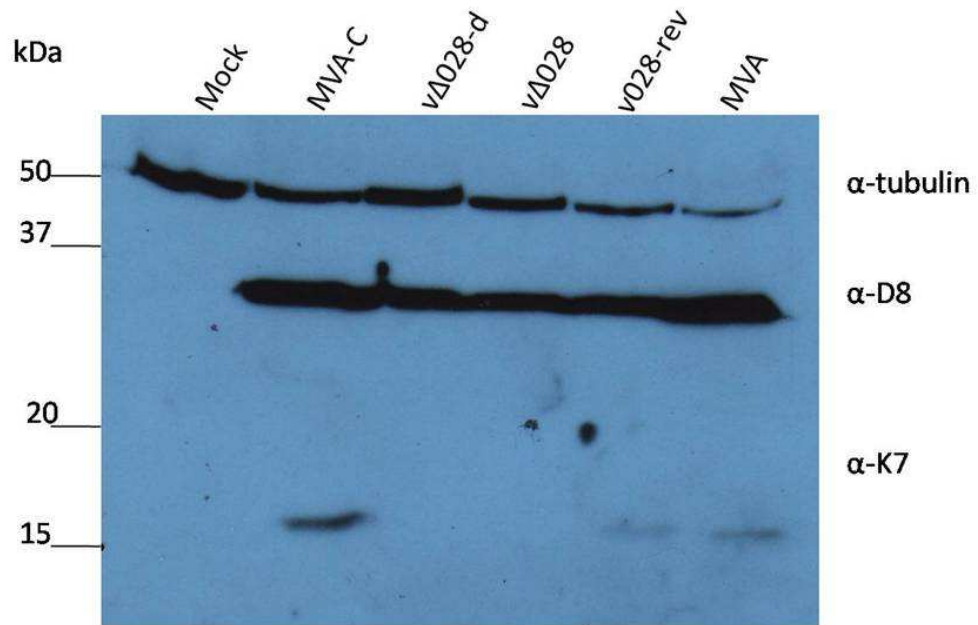


Fig. 6.10. Analysis of recombinant MVA-C genomes by PCR. (a) The diagram illustrates the *028R* locus in MVA-C, vΔ028-d and vΔ028 and indicates the expected product size for each allele by PCR with primers Fl-1 and Fl-2m. (b) Virus DNA was extracted from cells that had been infected with the indicated viruses and used as template for PCR with primers Fl-1 and Fl-2m. PCR products were analysed by agarose gel electrophoresis. (c) The diagram illustrates the structure of TK gene locus of MVA-C containing HIV clade C gp120-C (Env) and Gag-Pol-Nef and the arrows indicate where primers p91 and p92 bind and the expected size of the PCR product amplified using those primers. (d) DNA template from the indicated viruses was used for PCR with primers p91 and p92. PCR products were analysed by agarose gel electrophoresis. In b) and d) the positions of DNA size markers in kb are indicated.

6.1.4 K7 and HIV-C protein expression

K7 protein expression was analysed by immunoblotting using extracts of virus-infected cells. K7 was only expressed by MVA, MVA-C and v028-rev, and not by the deletion mutants, as expected (Fig. 6.11a). Cell extracts were also immunoblotted with a mAb against the IMV surface protein D8 and this confirmed that all viruses had infected the cells. Lastly, expression of the HIV-C gp120 and Gag proteins was investigated by immunoblotting with HIV specific sera and all MVA-Cs expressed the HIV antigen as expected (Fig. 6.11b).

(a)



(b)

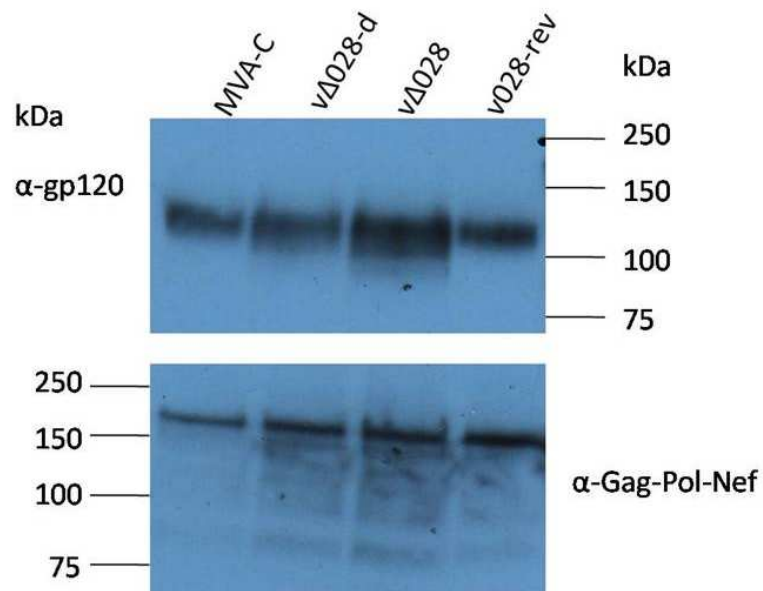


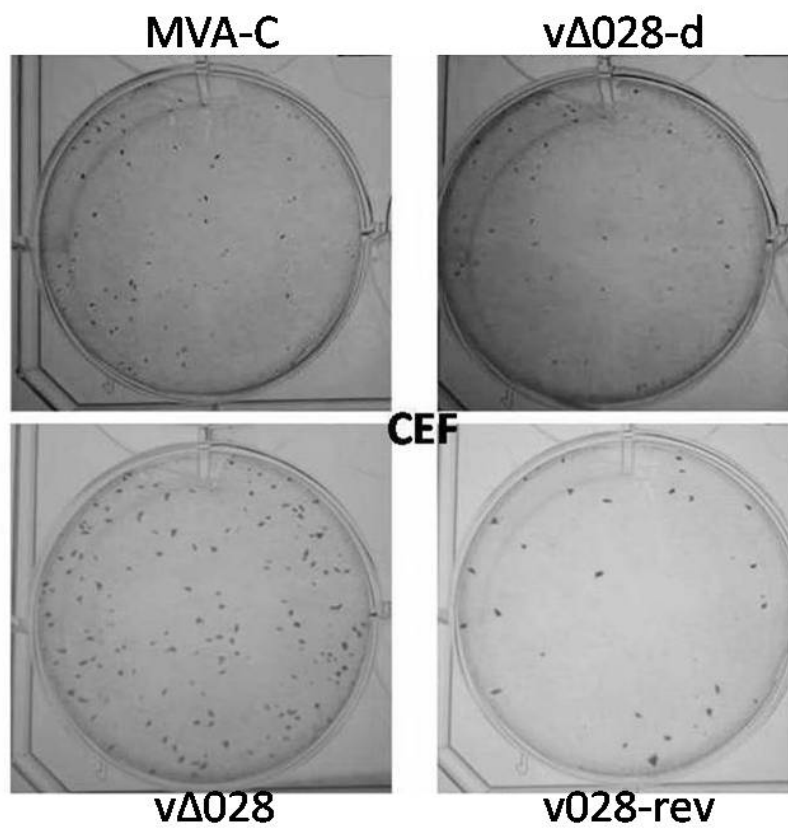
Fig. 6.11. Expression of K7 and HIV-C antigens by MVA-C viruses. (a) CEFs were infected with the indicated viruses at 1 pfu/cell overnight and cells were lysed. Lysates were analysed by SDS-PAGE (15 % gel) followed by immunoblotting with the indicated Abs. (b) The same cell lysates were analysed by SDS-PAGE (10 % gel) followed by immunoblotting with the indicated Abs. The sizes of molecular mass markers are indicated in kDa.

6.2. *In vitro* characterisation of 028R recombinant viruses

6.2.1 Foci size analysis

The size of foci produced by the three recombinant 028R MVA-C viruses was compared to that of parental MVA-C virus on CEFs and BHK-21s. After infection, monolayers were overlaid with semi-solid medium to prevent the formation of secondary foci and 72 h later, foci were visualised by immunostaining. After initial inspection, the foci of MVA-C and vΔ028-d looked visibly smaller than those of vΔ028 and v028-rev on CEFs (Fig. 6.12a), but there were no obvious differences observed on BHK-21s (Fig. 6.12b). The monolayers were then observed by light microscopy and the length of each focus measured ($n=20$ per virus). Length of foci formed by each virus was compared to that of the other viruses by the Student's *t* test. A range of focus sizes for each virus (Fig. 6.13 lower panel) was found and overall the median size increased for each new virus made compared to its parent virus. Despite this range, there were significant differences ($p < 0.0001$) in mean foci size between either the vΔ028 or v028-rev compared to each of the other two viruses on CEFs. However, there were no significant differences in mean foci size between MVA-C and vΔ028-d or between vΔ028-d and v028-rev (Fig. 6.13). In contrast, no repeatable significant differences were found between foci lengths measured on BHK-21.

(a)



(b)

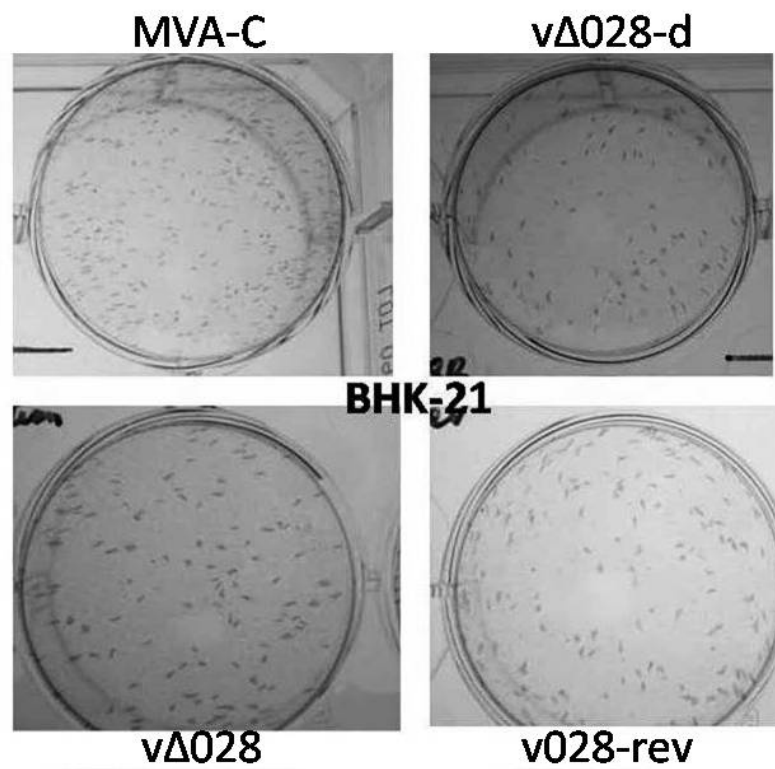


Fig. 6.12. Immunostained foci formed by MVA-C recombinant viruses. Confluent monolayers of CEFs (a) and 70 % confluent monolayers of BHK-21 (b) were infected with the indicated viruses and overlaid with semi-solid medium for three days. Monolayers were immunostained using polyclonal rabbit anti-VACV Ab as described in Materials and Methods section 2.4.4 and photographed.

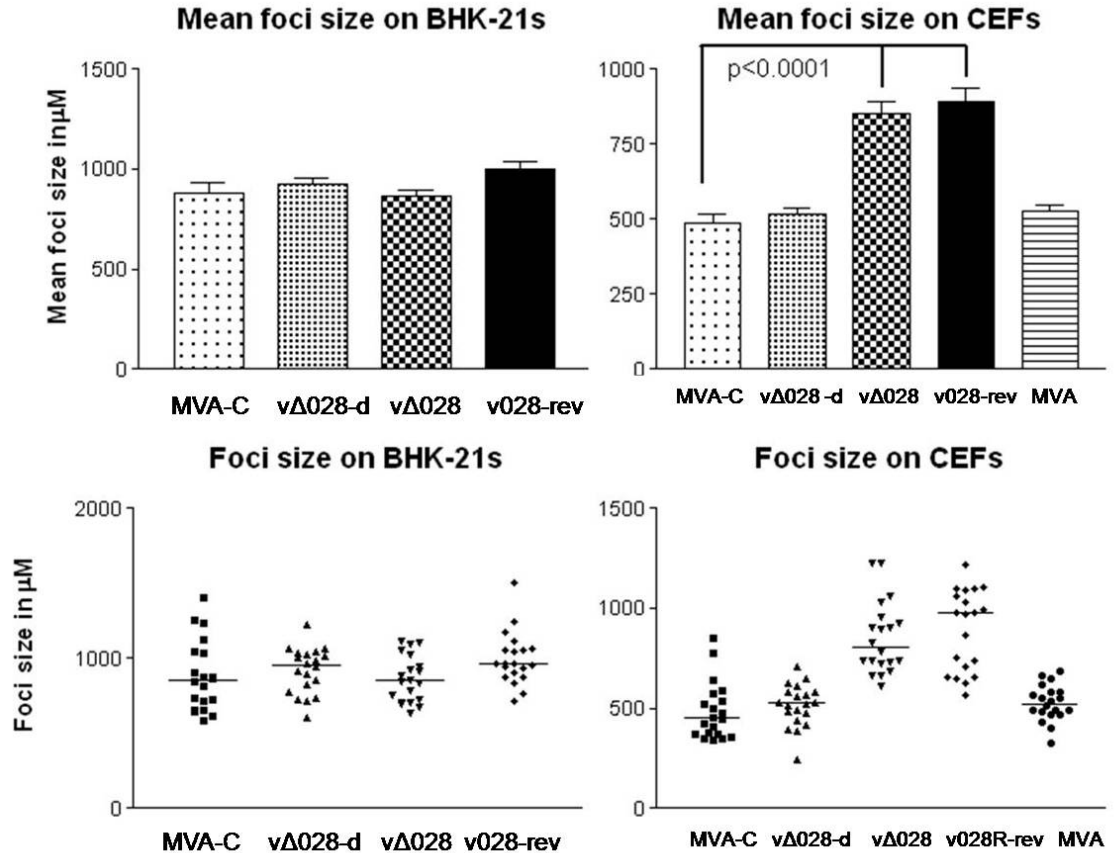


Fig. 6.13. Foci sizes formed by the recombinant MVA-Cs. Foci such as those shown in Fig. 6.12. were viewed under a light microscope (Zeiss Axiovert 200M inverted light microscope, 10 X magnification) and foci ($n=20$ per virus) were chosen randomly and photographed using Zeiss AxioCam and Axiovert software. Using this software a line was drawn across the length of each focus and its length in μM was noted. In the top panels, results are shown as the mean $\pm$ SD and in the lower panel, individual points represent each focus and the bar is the median. Data shown are from one of 2 and 3 independent experiments on BHK-21 and CEFs, respectively, with similar results.

The focal phenotype observed on CEFs could be attributed to differences in spreading efficiency from cell to cell which could be caused by genetic differences between the viruses acquired during virus making. This phenotype was only observed on CEFs; therefore it seems to be a host specific phenotype. The viruses were obtained sequentially from the MVA-C backbone in the following order: vΔ028-d, vΔ028 and v028-rev. As vΔ028 and v028-rev yield bigger foci, any genetic change should have occurred when making vΔ028 from vΔ028-d. During virus construction, the natural tendency is to pick bigger foci which might lead to selection of rare mutant viruses with increased focus size. This could have happened while making vΔ028. The possibility that a genetic difference was responsible for this focus phenotype was investigated in the next section.

6.2.2 Virus genomic DNA restriction digests

To check for genetic alterations in the recombinant viruses, virus genomic DNA was extracted from virus stocks that had been purified by sucrose density gradient centrifugation and was used for restriction enzyme digest by *HindIII* or *XhoI* (Fig. 6.14 and 6.15). The genomic DNA was then studied by agarose gel electrophoresis. When VACV MVA was digested with *HindIII*, 15 DNA fragments were obtained (Mayr et al, 1991) and were named A to Q representing fragments in order of decreasing size as listed in the table on Fig. 6.14.

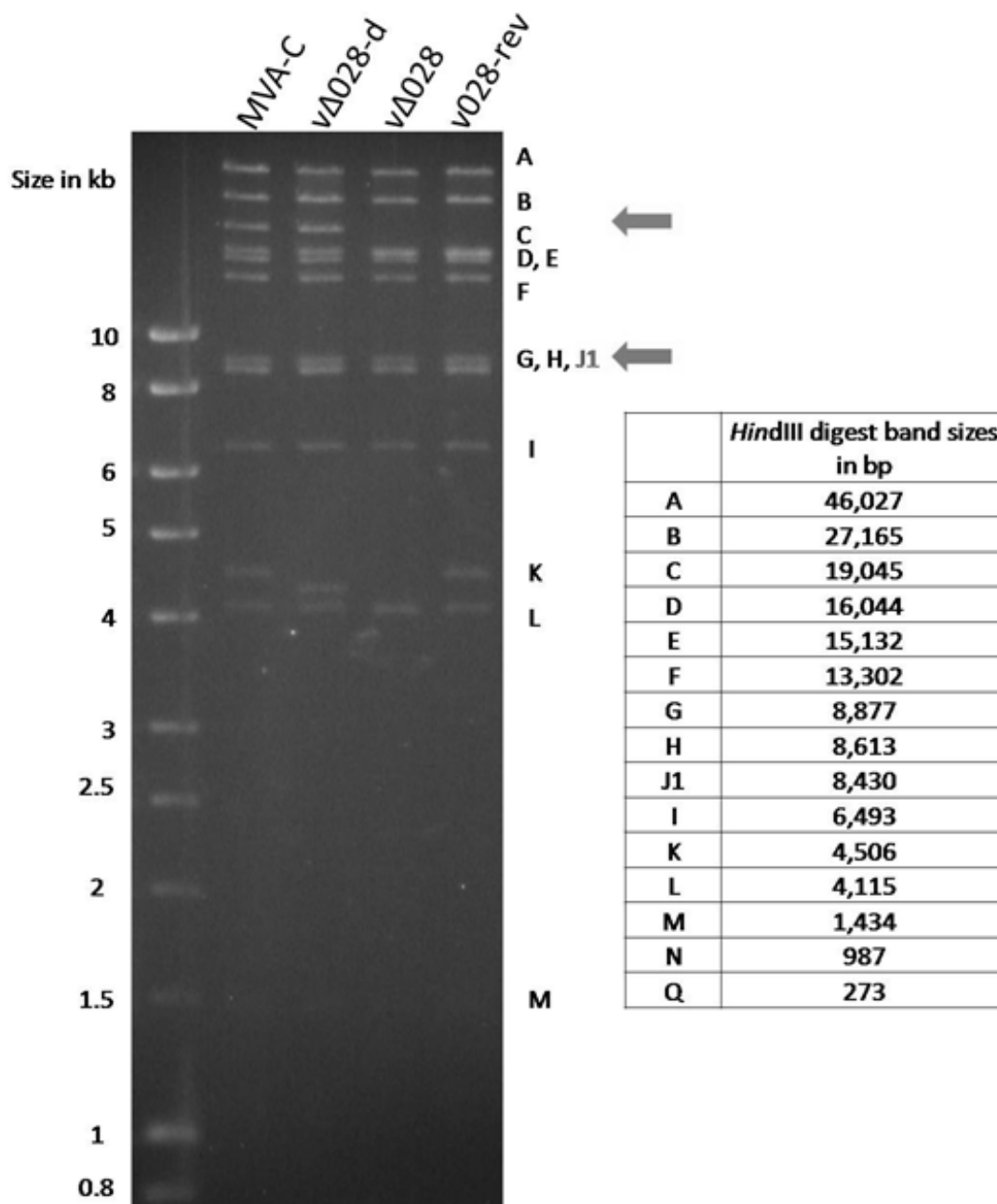


Fig. 6.14. Analysis of the genomic DNA of recombinant MVA-C viruses digested with *Hind*III. Genomic DNA was extracted from purified virus stocks for the indicated viruses and 1 µg of each genomic DNA was digested with *Hind*III and the resulting DNA fragments were resolved on a 0.7 % agarose gel overnight at 4 °C. The gel was then stained in ethidium bromide for 90 min prior to photography under UV light. The positions of molecular size markers are indicated in kb. The table lists the letters designating each of the genomic DNA fragments obtained after digestion with *Hind*III and their respective sizes in bp.

The *Hind*III K fragment which contains the *028R* locus was found to be of a smaller size for the vΔ028-d and vΔ028, as expected because the knock-out leads to the loss of about 250 and 450 bps respectively. The *Hind*III J band is normally 5 kb in MVA and contains the *TK* gene but for MVA-C viruses an approximately 6.1 kb sequence of HIV-C antigens are present in that locus and the *TK* sequence (534 bp) and part of its flanks (159 bp downstream and 182 bp upstream) totalling 875 bp were removed. This new sequence contains several *Hind*III sites and after restriction, a band of 8.6 kb can be seen below the *Hind*III H fragment, but other products are too small to be seen on the gel as expected. Notably a distinct genetic difference was also found between MVA-C/vΔ028-d and vΔ028/v028-rev. The *Hind*III C fragment was absent in the vΔ028 and v028-rev viruses yet it was intact in the parental MVA-C and the vΔ028-d. This fragment corresponds to the 20 kb at the left end of the VACV MVA genome. With the exception of the *Hind*III K fragment described previously, no other changes were seen in the *Hind*III digestion profile between the viruses.

The genomic DNA of these viruses was also digested with *Xho*I. In MVA this restriction yields an *Xho*I H fragment of 5.3 kb. As the *Xho*I H fragment contains the *TK* gene, it is therefore modified in MVA-C where an additional 6.1 kb HIV-C antigen sequences are present and the *TK* sequence with a small part of its flanks removed (approximately 0.8 kb). After digestion with *Xho*I bands of approximately 4.3, 4.1, 1.5 and 0.8 kb are obtained, the first three of which were visible on the gel for all the viruses. In addition, there was no visible *Xho*I F fragment in vΔ028 and v028-rev. As with the *Hind*III C fragment, the *Xho*I F band corresponds to the left end of VACV MVA genome and is approximately 16 kb. All other fragments seen on the gel are of the same size for all viruses, the largest of which is the *Xho*I A band (approximately 43 kb) meaning there is no visible evidence of a transposition of the *Xho*I F fragment.

Thus, the analysis of restriction digest of the VACV MVA-C viruses genomes by using two different enzymes, suggests that a deletion may have occurred at the left end of the genome during the construction of the recombinant virus.

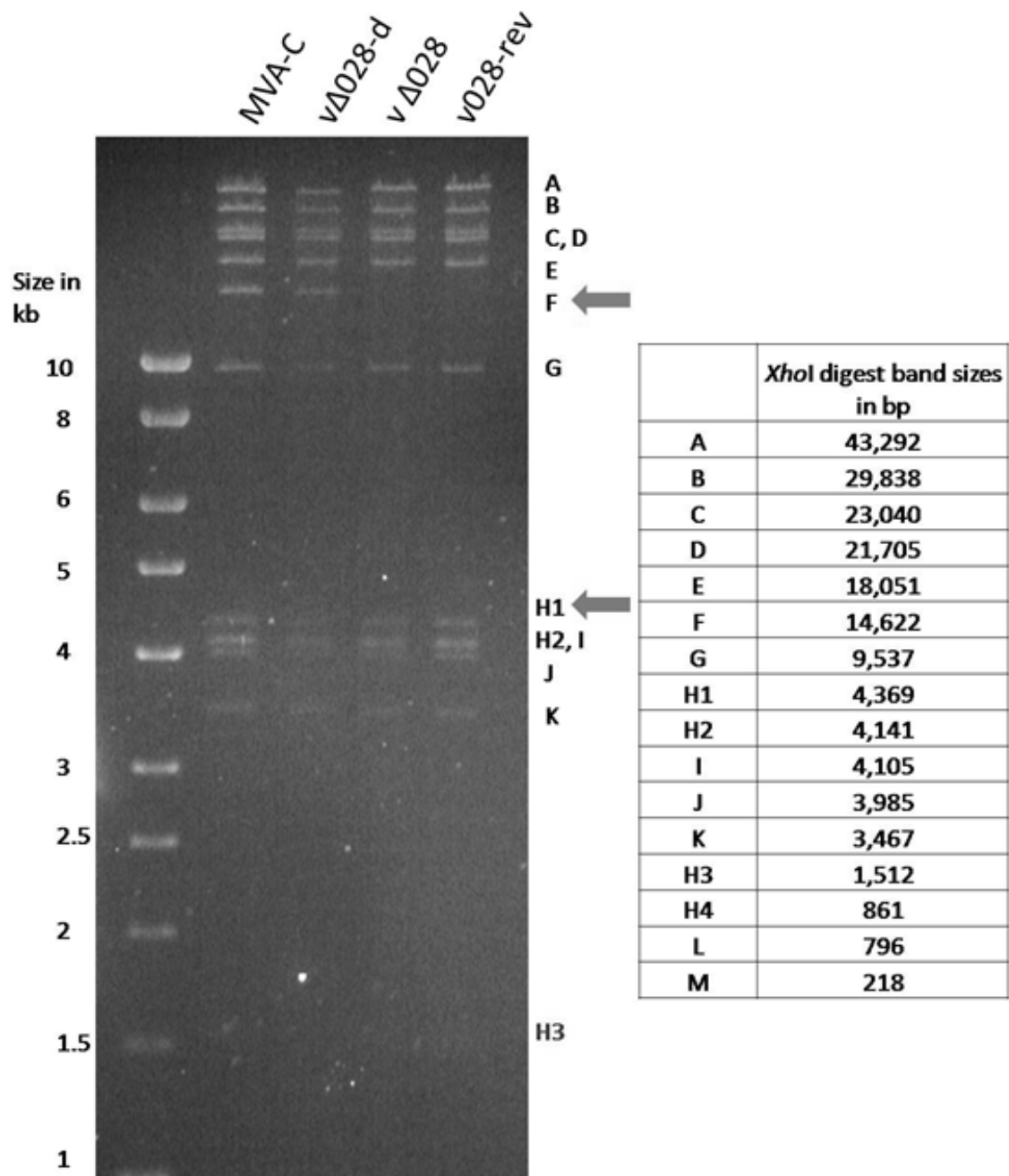


Fig. 6.15. Analysis of the genomic DNA of recombinant MVA-C viruses digested with *XhoI*. Genomic DNA was extracted from purified virus for the indicated viruses and 1 µg of each genomic DNA was digested with *XhoI* and the resulting DNA fragments were resolved on a 0.7 % agarose gel overnight at 4 °C. The gel was then stained in ethidium bromide for 90 min prior to photography under UV light. The positions of molecular size markers are indicated in kb. The table lists the letters designating each of genomic DNA fragments obtained after digestion with *XhoI* and their respective sizes in bp.

6.2.3 Single and multiple growth kinetics

To look for possible differences in replication and growth kinetics between viruses, the growth of each virus was compared on CEFs cells after infection at 10 or 0.01 pfu/cell (Fig. 6.16). At the indicated time after infection, the medium was collected and detached cells were collected by centrifugation. These cells were pooled with the adherent cells that were scraped from the dish. The infectious virions from the clarified culture medium or from the cells were defined as medium or cell-associated viruses, respectively. The amount of virus in the medium provides a good estimate of EEV production, although a small proportion of this may represent IMV released through cell lysis. The cell-associated virus represents mainly IMV but also with IEV and CEV.

In the single growth kinetics experiment (infection at 10 pfu/cell) the titres for MVA-C and v Δ 028-d were significantly lower ($p < 0.001$) than those of v Δ 028 and v028-rev (student's T-test). This was the case for both cell-associated and medium-associated viruses. This suggested that there are differences in replication efficiency between the viruses. In contrast, there were no significant differences between titres of viruses in the multiple growth kinetics experiment (0.01 pfu/cell). This was surprising as it was expected that any differences in replication during single step growth conditions would be more marked during multiple rounds of replication.

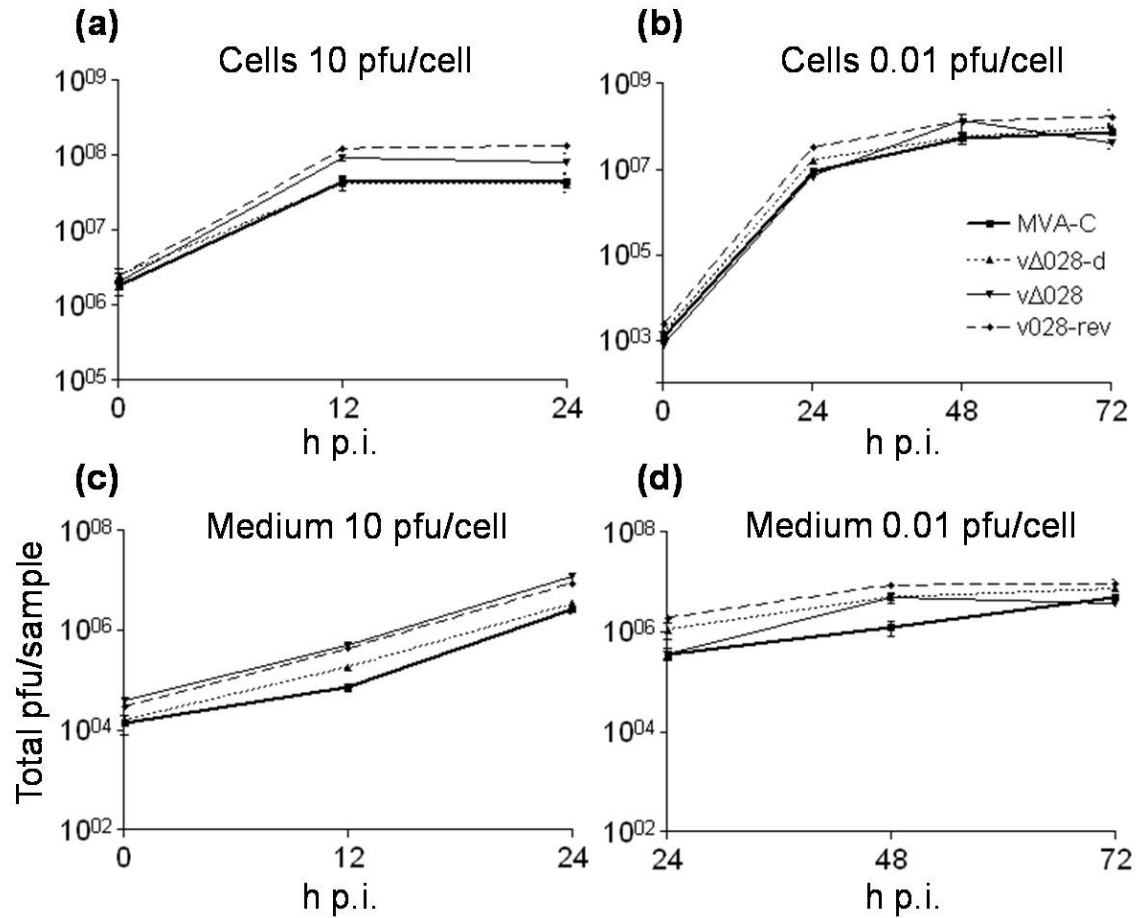


Fig. 6.16. Growth kinetics of the MVA-C recombinant viruses. (a-b) A single growth kinetics experiment was carried out with recombinant MVA-C viruses. CEFs were infected with the indicated viruses at 10 pfu/cell. At 0, 12 and 24 h p.i. virus present in the cell lysates (cells), (a) or in the clarified supernatants (medium (b)) was collected and was titrated on BHK-21 monolayers. The experiment was performed in duplicate and results are shown as the mean $\pm$ SD. (c-d) A multiple growth kinetics experiment was carried out with recombinant MVA-C viruses. CEFs were infected with the indicated viruses at 0.01 pfu/cell. At 0, 12, 24, 48 and 72 h p.i. virus was harvested and titrated as for the single growth kinetics experiment. Results for cell-associated virus are in (cells (c)) and those for medium virus are in (medium (d)). Each experiment was performed in duplicate and results are shown as the mean $\pm$ SD.

The discrepancy between single and multiple step growth conditions needs addressing by additional experiments and without those data it is uncertain if there are replication differences between the viruses.

In conclusion, differences in replication observed in the single growth kinetics experiment do not appear to be linked to the differences in foci size though this remains to be validated. However, evidence of a genetic difference between the viruses was found and this may have caused this phenotype. The focal size phenotype observed could be due to variation in genes involved in tropism between these viruses as the phenotype was observed on CEFs but not on BHK-21s. In this regard it is known that the *HindIII* C fragment, which was shown to be different between the viruses, encodes some host range genes such as *018L*, the MVA orthologue of *C7L* (Perkus *et al.*, 1990). However, the exact genetic differences between the viruses may only be discovered if their whole genomes are sequenced.

Chapter 7: Discussion

The *K7R* gene from VACV WR is located near the left terminus of the VACV genome and is predicted to encode a 149 amino acid protein with a molecular mass of 17.5 kDa (www.poxvirus.org). Bioinformatic analysis of the K7 peptide sequence indicated that it is identical to VACV strains WR and MVA and only VACV strain RPXV shows differences. Additional bioinformatic analysis showed that K7 is also highly conserved amongst OPVs CMLV, CPXV, HSPV, MPXV, VARV and TATV. A notable exception is ECTV that retains the comparable gene although it is not predicted to encode the K7 protein. The high amino acid sequence identity between K7 orthologues despite the location of *K7R* in the less conserved terminal region of the genome suggests an important function for K7. Additionally, the pre-miRNA sequences predicted in the *K7R* ORF are also conserved amongst OPVs including ECTV which does not encode K7 protein.

K7 was expressed early and late during infection and this expression profile was consistent with a genome-wide transcriptional analysis that detected *K7R* mRNA by 30 min p.i. and it was present until 24 h p.i. (Assarsson *et al.*, 2008). As an early protein, K7 expression is independent from viral DNA replication and this would be consistent with the immunomodulatory role described for K7 (Schroder *et al.*, 2008) to protect infected cells as quickly as possible from host immune responses.

Immunofluorescent microscopy indicated that K7 was cytoplasmic when expressed ectopically and this contradicted a report that K7 was located both in the cytoplasm and the nucleus (Schroder *et al.*, 2008). However, Schroder *et al* analysed the localisation of K7 48 h post-transfection whereas data in this thesis were obtained 6 h post-transfection. Analysis of K7 localisation at later times 24 h post transfection indicated that a small amount of K7 was nuclear (data not shown). This suggested that K7 can be detected in the nucleus when present at high levels but the biological relevance of this is uncertain. Firstly, 24 h p.i. with VACV WR, K7 was detected in the cytoplasm with no obvious nuclear localisation both by immunofluorescence and by sub-cellular fractionation. Secondly, K7 is an early protein whose expression steadily increases until 24 p.i. and its reported immunomodulatory functions would be most relevant early after infection. Finally, K7 was shown to inhibit IRF3/IRF7 activation at the level of TBK1/IKK- ϵ through its interaction with its binding partner DDX3 and K7 would only be in a position to modulate that signalling pathway by being in the cytoplasm (Schroder *et al.*, 2008). K7 and

DDX3 co-localise at discrete sites in the cytoplasm though they also co-localise in the nucleus in the presence of leptomycin B (LMB) which blocks CRM-1 dependent nuclear export (Meissner *et al.*, 2004; Schroder *et al.*, 2008). Nonetheless, to rule out the presence of K7 in the nucleus unequivocally, immunofluorescence experiments should be repeated in the presence of LMB to allow putative nuclear K7 to accumulate there and indicate if K7 shuttles between the cytoplasm and the nucleus. Nuclear association of K7 could be linked to another yet unknown function and/or be the result of an interaction with an unknown binding partner. VACV replicates in the cytoplasm and encodes few nuclear proteins, and these can be found in both nucleus and cytoplasm such as C16 (Fahy *et al.*, 2008) and E3 (Yuwen *et al.*, 1993). However, other VACV proteins including A46, A52, N1, B14 and K1, which like K7 inhibit NF- κ B signalling, function within the cytoplasm (Bowie *et al.*, 2000; Bartlett *et al.*, 2002; Shisler & Jin, 2004; Chen *et al.*, 2008).

Some of these VACV intracellular signalling inhibitors including A52, N1, B14 and K7 are known to be Bcl-2 like proteins. A46 is also predicted to be one. These proteins share only low amino acid sequence identity but the structures of N1, B14, A52 and K7 have been determined and shown to be Bcl-2 family members (Cooray *et al.*, 2007; Graham *et al.*, 2008; Kalverda *et al.*, 2009). However, at the start of this project, the structure of N1 was the only one known. Subsequently, determining the crystal structure of K7 in collaboration with Prof David Stuart's group at the University of Oxford was attempted to gain further insight into its mechanism of intracellular signalling inhibition. N-His K7 was purified by IMAC followed by size exclusion chromatography and sent to collaborators to set up crystallisation screens. K7 was found to be monodisperse in solution and small crystals were formed reproducibly but were not of good enough quality for X-ray diffraction. As the presence of an N-His tag could be causing the formation of disordered crystals, K7 protein was expressed from alternative vectors to enable the efficient cleavage of the His tag from its N or C terminus. Some crystals were formed from these purified proteins and their quality was an improvement on what had been obtained previously, but unfortunately these did not diffract beyond 10 Å resolution (structures are usually solved from 2-3 Å resolution diffraction data). Additionally, at this time, the NMR structure of K7 was solved by another group (Kalverda *et al.*, 2009). K7 was confirmed to have an α -helical fold belonging to the Bcl-2 family of proteins. The structure solved was K7 8-149, an N-terminally truncated version which yielded better NMR spectra. Proteins of the Bcl-2 family are constituted of a bundle of alpha helices termed α 1- α 7 (Graham *et al.*, 2008). Unlike other solved structures of

Bcl-2 like proteins in VACV, K7 was shown to have a loop region between $\alpha 3$ and $\alpha 5$ instead of an $\alpha 4$ helix (amino acids 84-93) (Kalverda *et al.*, 2009), though at that stage the functional implications of that were unclear. Anti-apoptotic Bcl-2 proteins regulate apoptotic pathways by binding the BH3 motifs of pro-apoptotic Bcl-2 proteins via a surface groove that is opened in the ligand bound state to enable hydrophobic contact with BH3 motifs (Cory *et al.*, 2003). Consistent with this, N1 which has been shown to be an anti-apoptotic protein, has an open BH3 groove that binds cellular Bcl-2 proteins Bad, Bax and Bid (Cooray *et al.*, 2007). However, the K7 structure did not reveal a BH3 binding groove as with B14 and A52 and this is in agreement with the fact that these proteins have no effect on apoptosis (Graham *et al.*, 2008; Schroder *et al.*, 2008). Additionally, K7 was a monomer in solution whereas A52, B14 and N1 are dimeric (Cooray *et al.*, 2007; Graham *et al.*, 2008; Kalverda *et al.*, 2009). Notably, the N terminus of K7 (amino acids 8-23) was unstructured and this may explain the difficulty in obtaining ordered crystals that diffracted at an appropriate resolution.

Consequently, it was decided to attempt to co-purify K7 with its known binding partner DDX3 because a complex of the two proteins could lead to the formation of a stable complex and therefore crystals that diffract better. Additionally, this structure would give more insight into K7's mechanism of intracellular signalling inhibition. K7 was co-expressed with either DDX3₅₋₁₆₇ or DDX3₂₂₋₄₀₉ which included the N-terminal fragment of DDX3 (DDX3₁₋₁₃₉) shown to bind K7 and mediate the induction of IRF3/IRF7 (Schroder *et al.*, 2008) and which were soluble when expressed in bacteria (Dr Amir Khan, personal communication). Notably, this N terminus was not included in the published structure of DDX3 which was determined using DDX3₁₆₈₋₅₈₂ which only comprises the two conserved tandem, RecA helicase domains typical of DEAD box helicases like DDX3 (Hogbom *et al.*, 2007). Complexes of DDX3₅₋₁₆₇ with K7 or DDX3₂₂₋₄₀₉ with K7 were purified. The former yielded samples that were monodisperse in solution but unfortunately no crystals were obtained. For the latter experiment, purified samples were too polydisperse for use in crystallisation. This may have been improved by the use of untagged DDX3 protein meaning only complexed DDX3 and K7 would be affinity purified, therefore excluding monomeric contaminants. Additionally, since the first 8 amino acids of K7 are unstructured and are not required for binding DDX3 (Kalverda *et al.*, 2009) the use of an N-terminally truncated K7 may have helped in the formation of ordered crystals. However, it was also demonstrated that the N terminus of DDX3 is itself highly disordered but that residues 61-90 are sufficient for K7 binding (Kalverda *et al.*, 2009). Therefore a better approach could be to work on crystallising K7 with this short peptide.

Recently, this idea was followed up by the same group and the crystal structure of K7 in complex with such a DDX3 peptide was obtained (Oda *et al.*, 2009). That work indicated DDX3₈₁₋₉₀ had a similar affinity for K7 compared to near full length DDX3₅₋₅₈₀ and the crystal structure of K7₆₋₁₄₉ in complex with DDX3₈₂₋₈₈ was obtained. Interestingly, the structure revealed that K7 lacked both $\alpha 4$ and $\alpha 6$ of the 7 canonical helices found in Bcl-2 like proteins and it appears that the corresponding region forms a deep hydrophobic pocket where the DDX3 peptide is bound (Oda *et al.*, 2009). Interestingly, this region corresponds to the surface of A52 that enables dimer formation and which is conserved within the VACV Bcl-2 family. In contrast, K7 does not seem to form homodimers and this surface can therefore interact with DDX3. Overall the DDX3 peptide crystallised is believed to form a projection into a hydrophobic pocket within K7 thus occupying the $\alpha 6$ position of the Bcl-2 canonical fold (Oda *et al.*, 2009). Additionally, mutagenesis experiments indicated that DDX3₈₂₋₈₈ contains two phenylalanine side chains (84 and 85) which are essential for DDX3/K7 binding and DDX3 activation of IRF3 and type I IFN induction (Oda *et al.*, 2009). The study has therefore demonstrated that K7 had evolved as a monomer that binds DDX3 in contrast to the other dimeric members of VACV Bcl-2 like family. This also explains why A52 which has sequence similarity with K7 shares some of its targets such as TRAF6 and IRAK2 but does not bind DDX3 (Schroder *et al.*, 2008). Consequently, K7 interaction with these proteins must be mediated through a different region which contains the same epitopes as A52. So far the only information available is that a peptide from the $\alpha 4$ - $\alpha 5$ loop of A52 inhibits NF- κ B activation following TLR-dependent stimulation (McCoy *et al.*, 2005) but more work is needed to determine IRAK2 and TRAF6 binding sites. A peptide of the corresponding region of K7 could be used to investigate whether the effect is the same and mutagenesis experiments could be carried out on the K7 structure.

Unpublished data from this lab has shown that Δ K7 was attenuated in intranasal and intradermal mouse infection models (Williamson *et al.*, 1990; Tschärke & Smith, 1999). However, when an additional control virus (vfsK7-1) containing a *K7R* sequence with an additional nucleotide mutating the translational initiation codon from ATG to ATAG was tested *in vivo*, it was found to retain virulence and was phenotypically similar to wild type virus (vK7). Second site mutations as a cause of this phenotype were unlikely because these would be increasing rather than decreasing virulence, but to address this possibility a new virus (vfsK7-2) was made independently by transient dominant selection and shown to retain virulence *in vivo* as well. The genotype of this

virus at the *K7R* locus was confirmed by PCR using primers to the flanks of *K7R*, and immunoblotting confirmed that the virus did not produce K7 protein infected cells.

Since the anti-K7 Ab used was raised against the full length protein it was deemed unlikely that any smaller K7 protein was expressed by translational initiation from an alternative downstream AUG codon in-frame with the *K7R* ORF. Moreover, the next potential initiation codon within *K7R* was not surrounded by a strong Kozak consensus sequence. Nonetheless, it could not be completely ruled out that this smaller protein was either not detected by the K7 Ab or that it was unstable. However, no such protein was detected from cells transfected with a plasmid containing a *K7R* sequence starting at that putative downstream initiation codon with an HA epitope sequence when immunoblotting with anti-HA Ab. Based on structural data, the putative smaller protein would lack $\alpha 1$ and most of $\alpha 2$ of the canonical α -helices forming the Bcl-2 like fold and is therefore likely to be unstable. However, no smaller K7 protein was detected even when transfections were repeated in the presence of proteasome inhibitor.

Another hypothesis to explain the retention of virulence of vK7fs was that the attenuation of v Δ K7 was due to disruption of expression of neighbouring gene(s) *K6L* or *FIL* rather than to the absence of K7. The predicted promoter for *K6L* is outside the *K7R* ORF meaning its expression is unlikely to be affected. In addition, immunoblotting with anti-F1 Ab indicated that F1 expression in v Δ K7 was similar to that in vK7 or vK7fs.

Taken together, these data suggested that the viral K7 RNA was contributing to virulence, possibly by the production of miRNAs. Using two different virus-encoded miRNA prediction programmes (Pfeffer *et al.*, 2004; Grundhoff *et al.*, 2006), two putative pre-miRNA hairpin loops were identified by both programmes within the 178-269 region of *K7R* and the sequences predicted by the two programmes were mostly overlapping. Once these hits were obtained, attempts were made to predict potential targets of *K7R* encoded miRNAs. However, these were not successful as the mature miRNA sequence that would be processed from these hairpins is not yet known and miRNA target prediction programmes require knowledge of the seed sequence of the mature miRNA to give results.

An *in vitro* experiment that would enable testing the biological effect of K7 RNA rather than K7 protein was set up because hitherto the only way to investigate this phenotype was *in vivo*. K7 has been reported to be a broad inhibitor of intracellular signalling pathways leading to NF- κ B and IRF3/7 activation which then induce IFN- β (Schroder *et al.*, 2008) and some of these effects may be mediated by the K7 RNA rather than protein. The IFN- β promoter is composed of AP-1, NF-

κ B and IRF3 binding sites and therefore its activation could be diminished if any of these transcription factors was inhibited. The contribution of K7 RNA and protein to inhibition of the IFN- β promoter was studied by reporter assay using K7 alleles transcribed from transfected plasmids that would make either K7 RNA and protein, or only RNA. It was found that some alleles still had the ability to inhibit that pathway, although they did not express the protein. Several more alleles were then designed in a bid to identify the region of the K7 RNA which was sufficient to yield a biological effect. RNA containing K7₁₇₈₋₂₆₉, predicted to encode miRNAs, was sufficient to inhibit IFN- β promoter activation. In contrast, *K7R* truncated sequences not containing this region were not able to do so. Interestingly, scrK7 which expressed the K7 protein from a mutated K7 nucleotide sequence, but was not predicted to make a miRNA was able to inhibit IFN- β promoter activation too. Therefore both the K7 protein and part of the *K7R* mRNA are sufficient to modulate this pathway.

It is also noteworthy, that immunoblotting experiments indicated the levels of K7 expressed by the scrK7 allele were significantly higher than those encoded by *K7R* (data not shown). A possible explanation is that given scrK7 is not predicted to encode miRNAs all of its mRNA would be used for translation to K7 protein whereas a significant part wild-type K7 mRNA would be processed into pre-miRNA and not available for translation into protein.

K7's reported broad inhibition of intracellular immune signalling pathways (Schroder *et al.*, 2008) could be an indication of the combined effects of both K7 RNA and protein. They may have different targets but which lead to the same end-results. IFN- β , NF- κ B and ISRE pathways were tested using *K7R* which produced both K7 RNA and K7 protein and therefore should be repeated using alleles that only produce RNA or the protein but not miRNA (Schroder *et al.*, 2008). K7 RNA and protein may target different components upstream of IFN- β promoter and this was investigated by studying different pathways independently. For example, K7 was reported to inhibit IL-1, TLR3 and TLR4 induced activation of NF- κ B (Schroder *et al.*, 2008). Interestingly, K7 also inhibits NF- κ B gene activation after TNF- α stimulus. The effects of the K7 protein and *K7R* nucleic acid sequence on NF- κ B-dependent gene expression were analysed by a luciferase reporter assay where NF- κ B gene expression was induced by TNF- α stimulus. Interestingly, both K7 protein and *K7R* RNA contributed to inhibition of NF- κ B-dependent gene expression in this case. The same results were obtained after stimulation with IL-1 β (Gabriel Almeida, personal communication). However, these experiments need repeating by transfecting with an allele that would not inhibit this pathway to act as a negative control.

Previously, it was shown in this lab that K7 can inhibit the ISRE promoter in a reporter assay after stimulation with IFN- α for 6 h (Aodhnait Fahy PhD thesis). This relatively early timepoint was used to ensure the Jak/STAT pathway of ISRE-dependent gene expression is activated directly with no positive feedback effect of IFN- β induction. Curiously, both K7 protein and *K7R* RNA were found to downregulate IFN- α -induced ISRE gene expression just as they did with the NF- κ B promoter reporter assay described earlier. In both experiments, the positive controls, which were other VACV proteins, behaved as expected but these results also highlighted the need for repeating these reporter assays with an appropriate negative control. Nonetheless, the possible mechanism of K7 RNA and protein downregulation of ISRE gene expression was investigated further by analysing the phosphorylation of STAT1, a modulator which transduces the IFN- α signal. The effect of K7 RNA or protein on STAT1 phosphorylation was tested using doxycycline-inducible cell lines expressing K7, fsK7 or scrK7 alleles or a negative control cell line which were IFN- α -treated. Neither K7 protein nor K7 RNA led to inhibition of STAT1 phosphorylation, at least not at the time point and IFN- α concentration tested, so these alleles may be modulating ISRE promoter activation by blocking components further downstream in the IFN pathway. IFN- α binds IFNAR, induces the catalytic activation of JAK that interacts with and phosphorylates the STAT1 protein, allowing interaction with STAT2 and IRF9, and the translocation of this complex (referred to as ISGF3) into the nucleus, where it activates the transcription of genes containing an ISRE-promoter sequence (Lew *et al.*, 1991). Consequently, phosphorylation or translocation patterns of STAT2 and IRF9 should also be analysed. Additionally, it would be of interest to test whether translocation of STAT1 into the nucleus is inhibited in any of the K7 cell lines. Moreover, it cannot be ruled out that K7 RNA and/or protein inhibit an alternative pathway of ISRE induction such as the p38 MAPK pathway (Katsoulidis *et al.*, 2005) although, preliminary data have shown that K7 can still inhibit ISRE-dependent expression in the presence of a MAPK inhibitor, suggesting it does not affect that pathway (Aodhnait Fahy, PhD thesis).

Additional recombinant VACVs were constructed by insertion of various *K7R* alleles into the *K7R* locus of v Δ K7 and these included vK7₁₇₁₋₄₅₀, vK7₁₇₈₋₂₆₉, vK7₁₋₁₇₁, vK7₂₇₀₋₄₅₀, vinvK7 and vscrK7. PCR analysis with primers to the *K7R* flanks and genomic DNA restriction digestion analysis demonstrated that these viruses were as expected at the *K7R* locus and did not have major genetic alterations elsewhere in the genome. These new viruses will be tested in the *in vivo* mouse intranasal model to determine in particular whether vK7₁₇₈₋₂₆₈ retains virulence, now that it has been shown that RNA transcribed from that allele is sufficient to inhibit innate immune

signalling. Interestingly, in contrast to the transfection model, the levels of K7 produced from vK7 and vscrK7 were similar.

Both *in vivo* and *in vitro* data gathered to date suggest a functional role for K7 RNA. It is hypothesised that this could be explained by the presence of miRNA encoded by this virus because the sequence predicted to encode miRNAs was sufficient to yield a phenotype *in vitro*. Therefore, the next essential experimental step would be to validate the presence of these miRNAs. This can be carried by northern blotting using probes corresponding to the predicted sequence or by qRT-PCR using primers annealing to those regions (Pfeffer *et al.*, 2004; Grundhoff *et al.*, 2006; Reichenstein *et al.*, 2009). Encouragingly, recent qRT-PCR data from this lab have confirmed the presence of a miRNA which would be processed from one of the two hairpin loops (K7₂₁₂₋₂₆₉, negative sense RNA) predicted from K7R during infection but nothing corresponding to the other predicted hairpin loop (K7₁₇₉₋₂₃₃ positive sense RNA) was detected (Gabriel Almeida, personal communication). The presence of this miRNA in infected cells should now be confirmed by northern blotting.

MiRNAs have only relatively recently been found to be encoded by members of the herpesvirus, polyomaviruses and adenovirus families ((Pfeffer *et al.*, 2004; Sullivan *et al.*, 2005; Xu *et al.*, 2007)). From an evolutionary point of view, virus-encoded miRNAs offer many advantages. They provide a highly specific way of down-regulating expression of host genes, are not antigenic and due to the very short length and requirement for only limited homology with their target they can rapidly acquire mutations to downregulate new target genes (reviewed in (Cullen, 2006)). It is interesting that viruses have evolved to encode their own miRNAs rather than target protein components of the host miRNA biogenesis pathway. A plausible explanation for this is that mature miRNAs have a long half life (days) and would already be present in host cell at the time infection, so that even if a virus produced a protein that inhibited further synthesis of host miRNAs, the levels of existing miRNAs would remain for the virus replication cycle (Cullen, 2009). Therefore, the production of miRNAs by the virus would be more effective than encoding proteins targeting the miRNA biogenesis pathway. However, poxviruses replicate in the cytoplasm and as the initial step in miRNA biogenesis is the cleavage of the pre-miRNA loop by Drosha in the nucleus it is unclear how any miRNA from these viruses would be processed. So far, miRNA precursor sequences have been predicted in poxvirus genomes (Pfeffer *et al.*, 2004) but their existence not validated. An interesting hypothesis would be that some cellular proteins

could be recruited to the cytoplasm or to virus factories. Alternatively, VACV mRNAs could be transported to the nucleus for processing or could enter the cellular miRNA biogenesis pathway at a later stage. *In situ* hybridisation experiments using probes against K7 mRNA may help to clarify whether or not any of it is transported to the nucleus.

MVA has been developed as a vaccine vector against various infections and diseases (Gherardi & Esteban, 2005; Cebere *et al.*, 2006; Dunachie *et al.*, 2006; Ondondo *et al.*, 2006) and it has been demonstrated that the removal of some of the immunomodulatory proteins it encodes can make it more immunogenic (Staib *et al.*, 2005; Clark *et al.*, 2006). MVA 028R, the orthologue of K7R was deemed a good candidate for removal because it is predicted to encode a protein with the same sequence and activity as the protein made by VACV WR. Consequently, analysis of the role of 028, the MVA orthologue of K7 was carried out using a set of recombinant viruses namely, vΔ028-d, vΔ028 and v028-rev. All of these viruses were derived from MVA-C which has an HIV-C Env/Gag-Pol-Nef sequence inserted in the TK locus of wild type MVA (Gomez *et al.*, 2007). VΔ028-d was constructed using a targeting method based on a double homologous recombination between a plasmid containing the 028R flanking regions and the 028R locus. The protocol was a modified version of an established method (Staib *et al.*, 2005) as required by the consortium of laboratories funded through the same grant from the Gates Foundation and resulted in the insertion of a short heterologous sequence in between the 028R flanks. This sequence was removed, to give vΔ028, using a transient dominant selection method which involves selection of single recombination events (Falkner & Moss, 1990). A revertant virus (v028-rev) was also obtained with this method. Genotypes of the different recombinant viruses were confirmed by PCR analysis with primers binding in the 028R flanks. Additionally, no 028 protein was detected from vΔ028-d or vΔ028 as expected and the levels of expression of HIV-C antigens were similar for all recombinants.

Interestingly, differences were observed when the sizes of foci formed by the three recombinant viruses were compared with those formed by parental MVA-C. Even after initial inspection, the foci formed on CEFs by vΔ028 and v028-rev were visibly bigger than their MVA-C and vΔ028-d counterparts. In contrast, there were no visible differences in foci sizes formed on BHK-21s. Sizes of foci formed by vΔ028 and v028-rev were then confirmed to be significantly different from those formed by MVA-C and vΔ028-d counterparts after they were measured precisely. However, there were no significant differences in the size of foci formed between the four viruses

on BHK-21 cells. Notably, these differences were found despite the high dispersion of foci size within each virus group and the median focus size for each group steadily increased from MVA-C to vΔ028-d to vΔ028 to v028-rev respectively. Notably this was the sequence in which they were derived from the MVA-C backbone. During virus construction, the natural tendency is to pick bigger foci which might lead to selection of rare mutant viruses with increased focus size. Given that vΔ028 and v028-rev yield bigger foci, any genetic change should have occurred when making vΔ028 from vΔ028-d.

Subsequently, restriction enzyme mapping showed there were indeed unexpected genetic differences between these viruses. While MVA-C and vΔ028-d had the predicted genome structures after digestion with *HindIII* or *XhoI*, the restriction profiles of vΔ028-d and v-028-rev were altered for the *HindIII* C fragment or *XhoI* F fragment. No additional bands were visible on the restriction profile of vΔ028 or v028-rev and none of the visible bands appeared larger than expected. Consequently, in order to test whether the immunogenicity of MVA is improved in the absence of *028R*, the vΔ028 and v028-rev viruses would have to be remade.

The focal phenotype observed could be attributed to acquisition of genetic differences during virus construction. This may have led to differences in replication or spreading efficiency from cell to cell therefore the single and multiple growth kinetics of these viruses were analysed. The single growth kinetics experiment suggested there were some significant differences in replication between the virus forming bigger foci and the others. However, this could not be confirmed by the multiple growth kinetics experiment which indicated that there were no significant differences in cell to cell spread between viruses. Therefore, the results were conflicting and the experiments need repeating. Nonetheless, the focal phenotype was only observed on CEFs, therefore it seems likely to be host-specific. Interestingly, the *HindIII* C fragment, which was altered in the genomes of the viruses which formed bigger foci on CEFs, is known to encode the orthologue of *C7L* a gene affecting host range (Perkus *et al.*, 1990) and vaccinia growth factor (VGF) which helps virus replication and the survival of infected cells (Andrade *et al.*, 2004; Postigo *et al.*, 2009). Interestingly, a recent publication has shown that *C7L* is not required for replication in BHK-21 (Backes *et al.*, 2009) and so could also not be required for replication on CEFs. However, the exact genetic differences between those viruses can only be conclusively determined by sequencing their whole genomes and only then can genetic differences be linked with the phenotype observed. Differences found could then be studied further and may lead to a better understanding of MVA biology and determinants of host range.

Concluding remarks

This thesis reports an investigation of the role of VACV gene *K7R* in evasion of innate immunity. For this, various approaches were used including bacterial expression of K7 in complex with its binding partner DDX3 for use in crystallisation trials, construction of recombinant viruses with several mutated alleles of *K7R*, *in vivo* characterisation and functional *in vitro* assays. Although deletion of the whole *K7R* ORF decreases virulence, abrogation of protein expression by a mutation of the initiating methionine was found not to cause viral attenuation by itself. Bioinformatic and functional analysis shown here suggested that this is likely due to the presence of a miRNA in K7. A virus in which the *K7R* ORF is reduced to the sequence predicted to encode the miRNA was made and its virulence *in vivo* will soon be assessed. Formal identification of the miRNA by northern Blot and/or qPCR analysis will also be carried out. A deletion of the *K7R* orthologue in MVA strain was also made as part of a larger project aiming at making safer and more immunogenic vaccine vectors by removing MVA immunomodulatory genes.

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