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Characterisation of the Single-Stranded DNA binding protein
encoded by Kaposi's Sarcoma HerpesVirus

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A thesis presented for the degree of Doctor of Philosophy

in

The Faculty of Biomedical and Life Sciences

at the University of Glasgow

Institute of Virology

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Summary

The work presented in this thesis is on the characterisation of the single stranded DNA (ssDNA) binding protein of the human herpesvirus, Kaposi's sarcoma herpesvirus (KSHV), encoded by the ORF6 gene. There is a core set of six proteins conserved throughout the herpesvirus family that are required for viral DNA replication and are active at the replication fork. These proteins function as a DNA polymerase and its processivity factor, a trimeric helicase-primase complex and a single-stranded DNA binding protein. In KSHV these functions are carried out by the proteins expressed by the following genes: ORF9 (DNA polymerase), ORF59 (processivity factor), ORF56 (helicase), ORF44 (primase), ORF40/41 (helicase-primase associated factor) and ORF6 (single-stranded DNA binding protein). Current models of herpesvirus DNA synthesis are based mostly upon knowledge of the herpes simplex virus type 1 (HSV-1) replication fork proteins, which have been more extensively studied than any of their homologues in other herpesviruses. The DNA replication proteins in KSHV have not been well studied and therefore their roles in KSHV DNA synthesis have been largely predicted by analogy with their HSV-1 counterparts.

To date there has been no published characterisation of the KSHV ssDNA binding protein (pORF6). The work completed for this thesis describes the over-expression and purification of pORF6 and characterisation of its binding to ssDNA.

A recombinant baculovirus expressing pORF6 was constructed and used to infect *Spodoptera frugiperda* cells. The protein was purified from these cells using heparin sulphate and mono-Q columns. Following successful purification of pORF6 it was

established that it could bind to ssDNA, using an electrophoretic mobility shift assay (EMSA) and surface plasmon resonance measurements on a BIAcore instrument. The number of nucleotides required for pORF6 to bind to ssDNA and the effect of NaCl on binding were also investigated, revealing that optimal binding occurred at 150 mM NaCl. Fourteen nucleotides were required for pORF6 to bind, however, binding became more efficient as the length was increased to twenty.

ICP8, the HSV-1 homologue of pORF6 has been shown to bind to ssDNA in a cooperative manner. The binding mechanism of pORF6 to a ssDNA of thirty-two nucleotides was investigated, again using an EMSA and surface plasmon resonance. This revealed that pORF6 may also bind to single-stranded DNA cooperatively. A comparison of ICP8 and pORF6 binding to ssDNA using the BIAcore indicated that they bind to single-stranded DNA with a similar affinity.

A panel of monoclonal antibodies was generated against pORF6 and was tested by western blotting, immune-fluorescence and immune-precipitation, using *Spodoptera frugiperda* cells infected with a recombinant baculovirus expressing pORF6. Human B-cells infected with KSHV that had been induced into their lytic cycle were also used in immune-fluorescence assays, which revealed pORF6 to be present in globular areas within the cell, reminiscent of herpesvirus DNA replication compartments.

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Declaration

Unless otherwise stated all work in this thesis was carried out by the author.

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List of abbreviations

aa	Amino acid
AcNPV	<i>Autographa californica</i> nuclear polyhedrosis virus
AIDS	Acquired immune deficiency syndrome
ATP	Adenosine 5' triphosphate
ATPase	Adenosine 5' triphosphatase
bp	Base pairs
BSA	Bovine serum albumin
Ci	Curies
CIP	Calf intestinal phosphatase
CPE	Cytopathic effect
CREB	Cyclic AMP response element binding factor
C-terminal	Carboxy-terminal
dH₂O	Deionized water
DBP	DNA binding protein
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
°C	Degrees Celcius
DMSO	Dimethyl sulphoxide
dsDNA	Double-stranded DNA
dT	Deoxythymidine
DTT	Dithiothreitol
EBV	Epstein-Barr Virus
<i>E. coli</i>	<i>Escherichia coli</i>
ECL	Enhanced-chemoluminescence
EDTA	ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EMSA	Electrophoretic mobility shift assay
EtBr	Ethidium bromide
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
GFP	Green fluorescent protein
HCMV	Human cytomegalovirus

HEPES	(N-[2-hydroxyethyl] piperazine-N'-[2-ethane sulphonic acid])
HIV	Human immunodeficiency virus
hpi	hours post-infection
HHV6/7	Human herpesvirus 6/7
HRP	Horse-radish peroxidase
HSV-1/2	Herpes simplex virus-1/2
HVS	Herpesvirus saimiri
ICP8	Infected cell protein 8
IPTG	Isopropyl- β -D-thio-galactoside
IR	Internal repeat
IRS/L	Inverted repeat short/long
kbp	Kilobase pairs
kDa	Kilodaltons
KSHV	Kaposi's sarcoma-associated herpesvirus
LANA-1	Latency associated nuclear antigen 1
LB	Luria-Bertani medium
LUR	Long unique region
M	Molar
MAb	Monoclonal antibody
mA	Milliampere
ml	Milliliter
mg	Milligram
mm	Millimetre
mM	Milli-molar
mRNA	Messenger RNA
MW	Molecular weight
ND10	Nuclear domain 10
NP40	Nonidet P40
ng	Nanograms
nm	Nanometer
nM	Nanomolar
N-terminal	Amino terminal
nt	Nucleotide

OB-fold	Oligonucleotide/oligosaccharide binding fold
OBP	Origin binding protein
OD	Optical density
ORF	Open reading frame
%	Percent
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu	Plaque forming units
pH	Potential of hydrogen
pM	Pico molar
PML	Promyelocytic leukemia protein
PMSF	Phenylmethanesulphonylfluoride
PODs	PML oncogenic domains
pORF6	protein expressed from KSHV ORF6 gene
RNA	Ribonucleic acid
RPA	Replication protein A
rpm	Revolutions per minute
RT	Room temperature
RU	Response units
SCP	Smallest capsid protein
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
<i>Sf</i>	<i>Spodoptera frugiperda</i>
ssDNA	Single-stranded DNA
SSB	Single stranded DNA binding protein
TAE	Tris, acetate, EDTA
TBE	Tris, boric acid, EDTA
TBS	Tris-buffered saline
TRS/L	Terminal repeat short/long
U_L	Unique long region
U_S	Unique short region
UV	Ultraviolet
V	Volts

VZV	Varicella-Zoster virus
v/v	volume per volume (ratio)
w/v	weight per volume (ratio)
µg	microgram
µl	microlitre

Amino acids

alanine	A	leucine	L
arginine	R	lysine	K
asparagine	N	methionine	M
aspartate	D	phenylalanine	F
cysteine	C	proline	P
glutamate	E	serine	S
glutamine	Q	threonine	T
glycine	G	tryptophan	W
histidine	H	tyrosine	Y
isoleucine	I	valine	V

Nucleotide Bases

adenine	A
cytosine	C
guanine	G
thymine	T
uracil	U

Chapter 1

Introduction

1.1 Introduction

The work presented in this thesis is on the characterisation of the ssDNA binding protein of the human herpesvirus, Kaposi's sarcoma-associated herpesvirus (KSHV), encoded by the ORF6 gene. The introduction will begin with a discussion of some of the general properties of the herpesvirus family. More detailed descriptions of herpesvirus DNA replication and other ssDNA binding proteins will follow.

1.2 The Herpesviruses

1.2.1 Herpesvirus characteristics

The herpesviruses are a large and diverse family of over 100 viruses that have been identified in mammals, birds, fish, amphibians and reptiles. They share a variety of common characteristics, including their genome type, virion morphology, basic mode of replication and the ability to establish latent infection in their natural hosts. The herpesvirus-common characteristics have been summarised by Roizman *et al.* (1992) and Davison & Clements (1998).

Herpesviruses possess large, linear double-stranded DNA genomes and hence have the capacity to encode many viral proteins and enzymes. The length of DNA ranges from approximately 120 to 250 kbp. Herpesvirus virions are large and complex with a common morphology consisting of four elements: core, capsid, tegument and envelope (Dargan, 1986) (figure 1.1). The core consists of the dsDNA genome, which is packaged into an icosahedral capsid (Widly *et al.*, 1960; Furlong *et al.*, 1972). The nucleocapsid is surrounded by an amorphous layer known as the tegument (Roizman and Furlong, 1974). The tegument is enclosed within an envelope consisting of a lipid bilayer, derived from golgi membranes, which contains viral glycoproteins (Spear and Roizman, 1972). The number of different viral glycoproteins on the virus particle varies between different

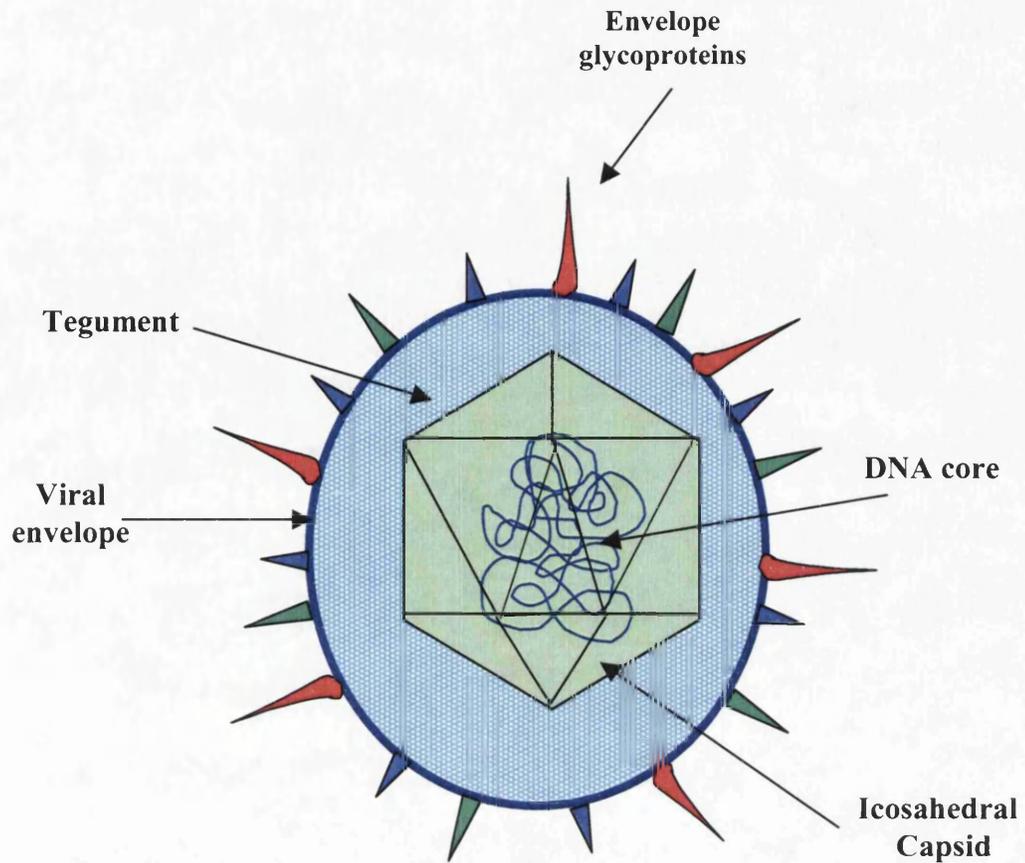


Figure 1.1 Generalised structure of a Herpesvirus virion

A schematic representation of a herpesvirus particle virion is shown, with the DNA core, icosahedral capsid, tegument layer and lipid envelope indicated. The viral envelope also contains various glycoproteins which protrude from the surface. The number of envelope glycoproteins varies amongst the herpesviruses.

herpesviruses.

Herpesviruses encode a variety of enzymes involved in nucleotide metabolism (thymidine kinase, thymidylate synthase, dUTPase, ribonucleotide reductase), which enable them to replicate in resting cells (Kit and Dubs, 1965; Pyles *et al.*, 1992; Bacetti *et al.*, 1986; Brown *et al.*, 1995). They also produce viral enzymes involved in DNA synthesis (e.g. DNA polymerase, DNA helicase, primase).

Herpesviruses are able to alter the cellular environment to suit their needs. These alterations include shutting off or stimulating host cell molecular synthesis, inhibiting or inducing host cell replication, or immortalizing the host cell. Although many herpesviruses encode genes that can transform cells in experimental systems, in only a few cases is it a part of the *in vivo* life cycle, e.g. the immortalization of B cells by Epstein Barr Virus (EBV) in the process of establishing latency (Miller *et al.*, 1982).

The lytic life cycles of the herpesviruses are similar. Virus entry to the host cell is through glycoprotein-mediated binding and fusion with the cell membrane (Sears *et al.*, 1991; Spear, 1993). The nucleocapsid complex is then transported to the nuclear pore, where the viral genome is released into the nucleus (Sodiek *et al.*, 1997). Replication of the viral genome and assembly of progeny nucleocapsids takes place in the nucleus (Furlong *et al.*, 1972). After encapsidation of the genomic DNA, the nucleocapsids are released from the nucleus by budding through the inner nuclear membrane (Vlazny *et al.*, 1982; Vernon *et al.*, 1981). It is thought that virions acquire their final envelope from post-endoplasmic reticulum cytoplasmic compartments (Skepper *et al.*, 2001). Mature virions are thought to exit the cell by a process of exocytosis. Ultimately, production of infectious progeny virus is usually accompanied by destruction of the infected cell. DNA

replication is mediated largely by viral enzymes but is dependent on host cell enzymes for several functions.

Herpesvirus genome arrangements consist of various combinations of unique sequences and repeated elements. These repeated elements may be present either internally or at the genome termini. This gives rise to a variety of distinct genome organisations and sizes depending on the sequence arrangements and the copy number of the repeated regions, respectively. Hence, herpesvirus genomes have been classified into seven groups according to genome arrangement, as described by Roizman *et al.*(1992) and Davison & McGeoch (1995). The structures of the seven genome classes are represented in Figure 1.2.

Group 0 genomes have a single unique coding region with no repeat units (e.g. tree shrew herpesvirus). Group 1 genomes have single direct repeats at each terminus (e.g. human herpes virus 6). The genomes of group 2 have multiple repeats at each terminus (e.g. KSHV). Group 3 genomes also have multiple repeats at each terminus but additionally have internal copies of these repeats in the opposite orientation, resulting in a genome with two unique regions (e.g. cottontail rabbit herpesvirus). Group 4 genomes consist of two unique regions separated by a group of internal repeats. Like groups 2 and 3, each end of the genome is also flanked by groups of direct terminal repeats but these are unrelated to the internal repeats (e.g. EBV). The genomes of group 5 have two unique regions, each region being flanked by a pair of unrelated inverted repeats, but those flanking the U_L are very short (Varicella-zoster virus). Group 6 genomes are related to the group 5 genomes but have much longer repeats flanking the U_L region. They also contain α sequence repeats that are found in the same orientation at each terminus and in inverted orientation between the internal repeats flanking U_L and U_S (e.g.

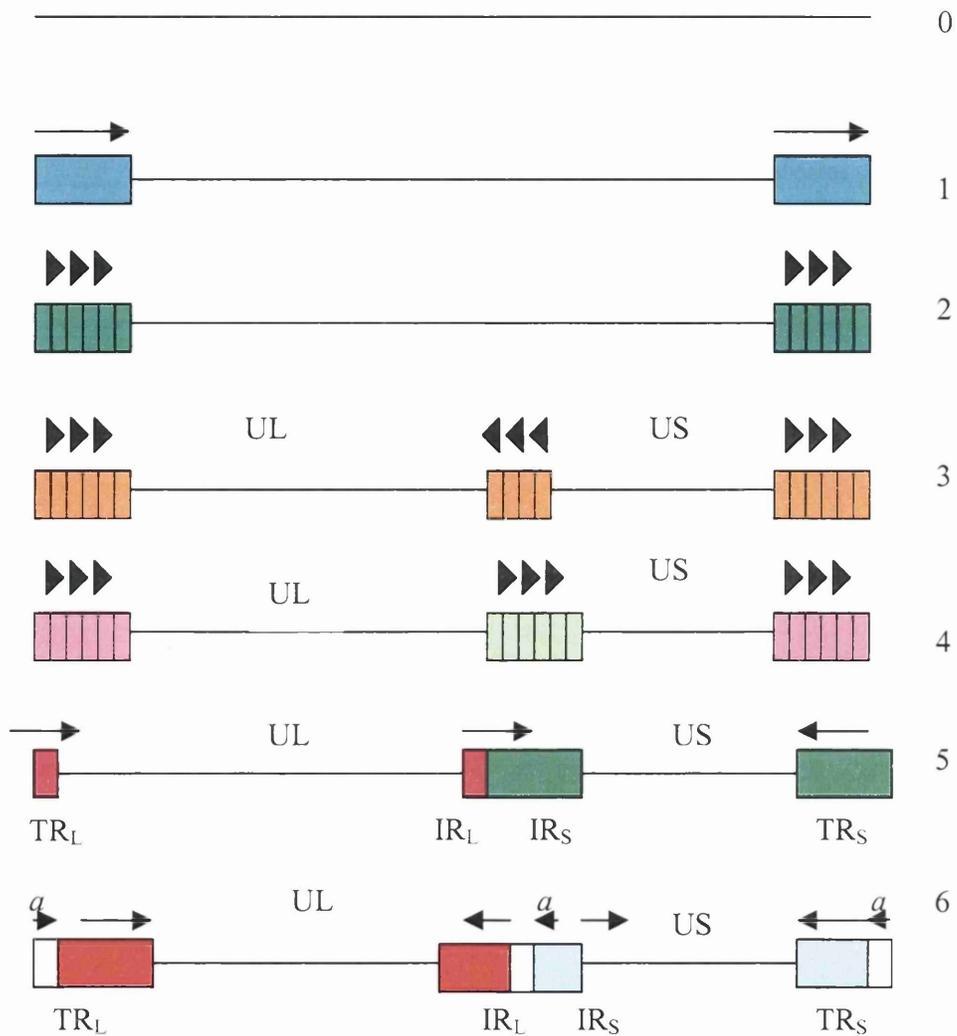


Figure 1.2 Herpesvirus genome structures

A schematic representation of the various genome structures of the herpesviruses. Unique regions are represented as single lines. Repeat regions are shown as boxes with their relative orientation indicated by an arrow (Roizman *et al.*, 1992; Davison and McGeoch, 1985). The a sequence of the group 6 viruses contains the minimal packaging and cleavage sequences.

herpes simplex virus-1).

The presence of long inverted repeats flanking U_S in the group 5 genomes results in the U_S region being able to invert through recombination. This results in the group 5 genomes existing as a mixed population of two isomers. The increased size of the U_L repeats in group 6 genomes means that both the U_L and the U_S regions can invert at high frequency resulting in four genomic isomers (Hayward *et al.*, 1975).

A distinctive feature of the herpesviruses is their ability to establish latent infections in their natural hosts, an effective mechanism of evading the host immune system. Following primary infection, viral DNA takes the form of a closed circular molecule, which is present at low copy number. Few, if any viral proteins are expressed. In dividing cells, the viral DNA is replicated via specific viral origins and cellular replication proteins, and is then segregated into the daughter cells (Yates *et al.*, 1984; Yates *et al.*, 1985; Hu *et al.*, 2002). Such latent infections typically last for the entire life of the host. The cell type in which latency is established varies between the different herpesviruses e.g. latent HSV-1 is found in neurons of dorsal root ganglia whereas latent EBV is primarily found in B lymphocytes. Reactivation to productive infections may occur sporadically throughout the life span of the host.

1.2.2 Herpesvirus classification

The mammalian and avian herpesviruses comprise three subfamilies (Roizman *et al.*, 1981). Classification has traditionally been made on the basis of differing biological properties, such as host range, length of reproductive cycle, cytopathology and site of latent infection. However, increasingly genome sequence data are being used for the purpose of herpesvirus phylogeny (e.g. McGeoch *et al.*, 1995). In most cases, the original classifications have been substantiated by the groupings, which have now been made on the basis of comparison of sequence data. The updated herpesvirus classifications were published in the current International Committee on Taxonomy of Viruses report (Minson *et al.*, 2000). The herpesvirus sub-families are as follows:

Alphaherpesvirinae

These are typically neurotropic viruses with a short reproductive cycle. They spread rapidly in culture and have a wide host range *in vitro*. They are highly cytolytic and some members have been shown to establish latent infections in sensory ganglia. This subfamily contains the genera *Simplexvirus*, *Varicellovirus*, “Marek’s disease like virus” and “Infectious laryngotracheitis-like virus”

Betaherpesvirinae

The reproductive cycle of these viruses is long and the infection progresses slowly in culture. Infected cells often become enlarged and fuse to form multinucleate cells called cytomegalia. Another characteristic is a restricted host range. Latent infections have been associated with cells of the monocyte series. This subfamily contains the genera *Cytomegalovirus*, *Muromegalovirus* and *Roseolovirus*.

Gammaherpesvirinae

Gammaherpesviruses are generally lymphotropic and often establish latency in T or B lymphocytes. Host range in cell culture and length of reproductive cycle is variable, as is the resulting cytopathology. Productive infections are associated with the development of lymphoproliferative disorders. Some members also cause lytic infection in epithelioid and fibroblastic cells. The subfamily contains two genera: *Lymphocryptovirus* and *Rhadinovirus*.

1.2.3 Human herpesviruses

Human herpesviruses are generally ubiquitous, with large proportions of the world-wide population having been exposed to and latently infected by one or more of these viruses. They are usually spread by close contact via bodily secretions. Eight herpesviruses that infect humans have been identified to date. Generally, primary herpesvirus infection in immunocompetent hosts does not result in severe or fatal disease. However, herpesvirus infections in certain susceptible populations, such as immunocompromised individuals, may cause serious illness.

Three alphaherpesviruses are known to infect humans. Herpes simplex viruses 1 and 2 (genus *Simplexvirus*) are closely related. Both are associated with mucosal infections and establish latency in sensory ganglia. HSV-1 is primarily associated with mucosal infections of the mouth and throat and may cause symptoms including fever and more commonly, oral lesions (cold sores), but is also associated to a lesser extent with genital mucosal infections. Conversely, HSV-2 is primarily associated with mucosal infections of the genitalia and to a lesser extent the mouth and throat. Both viruses can reactivate,

which is manifested by lesions in the skin. Varicella-zoster virus (genus *Varicellovirus*) is the third human alphaherpesvirus and the causative agent of chicken pox during primary infection. VZV establishes latency in sensory neurones and reactivation results in shingles.

Human cytomegalovirus (genus *Cytomegalovirus*) is a betaherpesvirus that causes widespread infection in humans. As with other human herpesviruses, primary infection is usually asymptomatic but severe disease can occur in susceptible individuals infected with HCMV. Human herpesvirus-6 and human herpesvirus-7 (genus *Roseolovirus*) are more recently identified human betaherpesviruses. Both are associated with febrile illnesses in children and post-transplant disease in immunosuppressed transplant recipients. Human herpesvirus-6 has been found in a latent state in macrophages (Levy, 1997) whereas the site of latency for human herpesvirus-7 is unclear.

The final two human herpesviruses are members of the *Gammaherpesvirinae*. EBV (genus *Lymphocryptovirus*) was the first human gammaherpesvirus to be identified and is the causative agent of infectious mononucleosis. EBV establishes latency in B-lymphocytes and has been associated with malignancies including Burkitt's lymphoma, Hodgkin's disease and nasopharyngeal carcinoma. Human herpesvirus-8 (genus *Rhadinovirus*), also known as Kaposi's sarcoma-associated herpesvirus (KSHV), is the most recently identified human herpesvirus. It is the causative agent of Kaposi's sarcoma (KS) and is also associated with two other human cancers, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD).

1.3 KSHV

1.3.1 Discovery and epidemiology of KSHV

KSHV was first identified from an AIDS-KS skin lesion in 1994 using representational difference analysis (Chang *et al.*, 1994). Within two years of its discovery the KSHV genome was sequenced from a PEL-derived cell line and from a KS lesion (Russo *et al.*, 1996; Neipel *et al.*, 1997).

Unlike most human herpesviruses, KSHV is not ubiquitous but is distributed according to a combination of geographic and behavioural risk factors. In northern Europe and northern America less than 10% of the population have been infected with KSHV (Iscovich *et al.*, 2000). The rates of infection in Mediterranean countries are higher and, within Europe, KS occurs most frequently in elderly males of Mediterranean ethnicity (Simpson *et al.*, 1996). In some areas of Africa, adult populations have infection rates of over 50% (Gao *et al.*, 1996).

KSHV seroprevalence in high-risk populations is much greater than in the general population. Homosexual men can have asymptomatic infection rates approaching 40% and KS is more frequent among HIV negative homosexual men than the general male population (Gao *et al.*, 1996; Martin *et al.*, 1998, Blackbourn *et al.*, 1999). In many developed countries women are much less likely to be infected than men (Keddes *et al.*, 1997). Epidemiologic studies show that only a small percentage of healthy adults who are infected with KSHV will develop symptomatic disease. Symptomatic disease primarily occurs among individuals with immune suppression e.g HIV positive individuals, chemotherapy and transplant patients, and the elderly.

1.3.2 KSHV disease manifestations

Three diseases have been associated with KSHV infection so far. Kaposi's Sarcoma is a vascular tumour composed of proliferating spindle cells, thought to be derived from an endothelial lineage. PEL is a B-cell lymphoma, which occurs as malignant effusions in visceral cavities, although malignant cells can be found in adjacent visceral organs and in the peripheral circulation. MCD is a reactive lymphadenopathy, involving enlargement of lymph nodes.

1.3.3 Types of KS

Four clinical categories of KS have been described. These are classical KS (found predominantly in the Mediterranean), endemic KS, epidemic KS (AIDS-KS) and immuno-suppression associated KS. Endemic KS in sub-Saharan Africa is responsible for up to 12% of all malignancies and has the worst disease prognosis of all the KS types (Armes, 1989). KS in childhood is virtually nonexistent in North America or Europe but it occurs in endemic areas of Africa. Childhood KS is aggressive and rapidly fatal. Immuno-suppression KS is often associated with transplant patients with 1 in 500 patients developing the disease.

1.3.4 KSHV transmission

KSHV is mainly spread through sexual contact, with gay and bisexual men being most susceptible to infection in the developed world (Martin *et al.*, 1998, Blackbourn *et al.*, 1999). In Africa KSHV is spread via casual contact between children and young adults.

The exact means of transmission is not known but it may be associated with oral contact (Pauk *et al.*, 2000).

1.3.5 Treatment of KSHV

As herpesvirus DNA replication is largely autonomous from the host cell replicative machinery, this stage of the life cycle is a suitable target for antivirals. Herpesvirus DNA replication enzymes are sufficiently distinct from the cellular replication enzymes to allow specific targeting of viral functions. The majority of anti-herpetic drugs are nucleoside analogues.

Some of the drugs that were developed to treat other herpesvirus infections (HSV-1 and HCMV) successfully prevent KS, but it is not clear whether they treat existing tumours (Kedes and Ganem, 1997). KSHV is sensitive to both gancyclovir and cidofovir, but is insensitive to acyclovir. Treatment of the underlying immune deficiency appears to be the best current approach for controlling KSHV associated diseases. In cases of AIDS-KS, treatment with an effective antiretroviral therapy can cause KS tumours to regress. When individuals receiving chemotherapy have their medication withdrawn, tumour regression is also observed.

1.4 KSHV molecular biology

1.4.1 Genome structure

KSHV is the only human member of the *Rhadinovirus* genus discovered to date (Moore *et al.*, 1996). It was first sequenced using cosmid and phage genomic libraries from the BC-1 cell line and 81 ORFs were identified (Russo *et al.*, 1996). Other genes were

subsequently discovered by Neipel *et al.* (1997) and Sarid *et al.* (1998). The structure of KSHV is similar to herpesvirus saimiri (HVS) as it has a long unique region (LUR) flanked by terminal repeats (TR). The long unique region of the KSHV genome is about 145 kb in length and contains at least 87 genes. Its sequence is composed of about 54.5% G+C and includes all identified KSHV ORFs. The TR regions consist of multiple tandem 801-bp direct repeat units. These have 84.5% G+C content with potential packaging and cleavage sites (Russo *et al.*, 1996). The genomes of KSHV and HVS are essentially co-linear and KSHV genes are named after their HVS counterparts. KSHV genes that are not homologous to genes of HVS are given a K prefix (e.g. ORF K1 to K15). Like HVS, KSHV encodes thymidine kinase, thymidylate synthase and dihydrofolate reductase. It is the only human herpesvirus, which contains all three of these genes. In addition to this, KSHV is also unusual in possessing a large number of genes encoding homologues of host genes. Many of the host genes that are expressed by KSHV are induced by other herpesviruses, most notably EBV. For example, KSHV encodes a homologue of cyclin D2 whereas EBV LMP-1 induces cyclin D2 in B-cells, which do not normally express this protein (Davis *et al.*, 1997; Arvanitakis *et al.*, 1995). The acquisition of host genes may enable KSHV to control host cell processes and avoid anti-viral responses.

1.4.2 Virus structure

Like other herpesviruses, KSHV has a nucleocapsid core surrounded by a tegument and a lipid envelope. Three-dimensional reconstruction of the KSHV capsid imaged by electron microscopy revealed a size and structural organisation similar to previously studied herpesviruses (Trus *et al.*, 2001). Because of the difficulties in transmitting the

virus *in vitro*, little is known about the mode of cell entry or the cellular receptor for KSHV.

1.4.3 KSHV Gene Expression

As with other herpesviruses, KSHV gene expression can be classified into distinct kinetic stages. The temporal sequence of gene expression has been monitored by Northern blot and microarray analyses (Sun *et al.*, 1999, Jenner *et al.*, 2001).

Immediate early genes are expressed in the absence of de novo protein synthesis. Early gene expression is dependent on the products of immediate early genes binding to their promoters and driving early gene expression. Late gene expression begins at the onset of viral DNA replication. Latent gene expression occurs when the virus is in its latent cycle.

Latent gene expression

During latency few viral genes are expressed. Because KSHV is latent in the majority of cells that it infects, the genes that are expressed in latency may have a major role in the pathogenesis of KSHV associated cancer. Genes shown to be expressed during latency include v-cyclin, v-FLIP and Kaposin (Davis *et al.*, 1997; Sturzl *et al.*, 1999; Muralidhar *et al.*, 1998). LANA-1 is also expressed which acts to tether the viral episomes to the host chromosomes (Rainbow *et al.*, 1997; Kedes *et al.*, 1997, Fejer *et al.*, 2003).

Immediate early gene expression

A number of immediate early transcripts have been identified including those from the ORF50 gene (Zhu *et al.*, 1999). Sequence comparison indicated that the ORF50 gene is

a homologue of the EBV BRLF1 gene, which encodes the EBV transactivator protein Rta (Sun *et al.*, 1998). The ORF50 product has been demonstrated to activate the KSHV viral lytic cycle (Lukac *et al.*, 1998; Gradoville *et al.*, 2000, Xu *et al.*, 2005).

Early gene expression

The ORF50 protein activates the expression of the ORFK8 gene (Chen *et al.*, 2000; Lukac *et al.*, 1998). The product of K8 is a bZip protein that shows homology to Zta, an EBV transactivator and origin binding protein (Fixman *et al.* 1995). Other early genes expressed are regulators of gene expression e.g. ORF57 and ORF45 (Zhu *et al.*, 1999).

Delayed early gene expression

The delayed early gene products include the conserved replication fork proteins that are required for viral DNA replication. These are the products of ORF6, the ssDNA binding protein; ORF9, the DNA polymerase; ORF59, the polymerase processivity factor; ORF56, the primase; ORF44, the helicase and ORF40/41, the primase associated factor. Although little is known about the lytic DNA replication of KSHV these proteins were shown to be able to substitute for their EBV counterparts, driving replication from the EBV origin of lytic replication (oriLyt) (Wu *et al.*, 2001).

Late gene expression

The late genes encode primarily structural proteins that are present at the late stage of the viral lytic cycle. Examples are the products from the K8.1A and K8.1B genes. Differential splicing of the K8.1 transcript gives rise to these two proteins. They are glycoproteins that are associated with the virion envelopes and the surfaces of transfected BCBL-1 and COS-1 cells (Zhu *et al.*, 1999b).

1.5 Herpesvirus DNA replication

1.5.1 Pathway of DNA replication in HSV-1

HSV-1 input genomes locate to structures in the cell nucleus called ND10 domains. As the lytic cycle of the virus progresses these structures become disrupted through the action of the immediate early viral protein ICP0 (Everett *et al.*, 1998; Everett and Maul, 1994). Pre-replicative sites form at the disrupted ND10 domains. These mature into replication compartments, which represent the sites of active viral DNA replication. Expression of the DNA replication proteins is followed by their localising into the nucleus, assembling onto the parental viral DNA in the pre-replicative sites and replicating the viral DNA (Lukonis and Weller, 1996; Lukonis and Weller, 1997; Liptak *et al.*, 1996; Zhong and Hayward, 1997; Taylor *et al.*, 2003; Taylor and Knipe, 2004).

The current model for HSV-1 DNA replication proposes that initial rounds of replication proceed via a theta-like mechanism, followed by a rolling-circle mode of replication at later times in infection (reviewed by Boehmer & Lehman, 1997). It had been reported that linear HSV-1 genomes were rapidly circularised following infection, probably via direct ligation of the complementary single overhanging nucleotides at their termini (Mocarski & Roizman, 1982; Poffenberger and Roizman, 1985; Garber *et al.*, 1993). Circularisation overcomes the problems of replicating the genomic termini and a theta-like mechanism is consistent with exponential accumulation of viral DNA during early stages of DNA synthesis (Zhang *et al.*, 1994). A rolling-circle mechanism of DNA replication later on in infection has been suggested by the prevalence of “endless” DNA, which restriction enzyme analysis indicated to consist of tandem head-to-tail concatemers (Jacob *et al.*, 1979; Severini *et al.*, 1994; Zhang *et al.*, 1994).

However, Jackson and Deluca (2003) have challenged this model. They failed to observe circularisation of HSV-1 genomes during productive infection and also observed that ICP0, which is known to be involved in the reactivation from latency, inhibited the formation of circular genomes. They suggest that HSV-1 may actually replicate its genome via a linear method. The genomic termini would be replicated following homologous recombination and concatemer formation similar to T4 and T7 bacteriophage (Leider and Mosig, 1982; Richardson, 1983). Further evidence is required to support this model, and the circularisation of the genome during lytic infection has not yet been definitely excluded.

Once the viral DNA has been replicated, encapsidation occurs by a process in which cleavage of HSV progeny DNA concatemers into unit-length genomes and packaging of the DNA into the capsid are tightly linked (Varmuza and Smiley, 1985; Ladin *et al.*, 1982; Deiss *et al.*, 1986).

1.5.2 DNA synthesis

DNA synthesis begins at defined sites within herpesvirus genomes, known as origins of replication. There is considerable variation in the size, sequence and structure of origins amongst the human herpesviruses suggesting that different mechanisms may be employed in the initiation of DNA synthesis. In contrast, the ORFs encoding essential replication fork proteins are amongst the conserved genes found in all herpesviruses, and once a replication fork has been established, DNA synthesis is presumed to proceed via a common mechanism. HSV-1 was the first herpesvirus in which the origins of replication and the proteins essential for viral DNA replication were identified. Having been extensively studied, it has become the model system for the study of herpesvirus DNA

replication and much of our knowledge regarding DNA replication in other herpesviruses has been inferred by analogy to HSV-1. Of the gammaherpesviruses, DNA lytic replication has been most extensively studied in EBV. As it is a comparatively close relative of KSHV its DNA lytic replication is also discussed.

The seven HSV-1 proteins found to be essential for origin-dependent viral DNA replication comprise an origin binding protein and six proteins that function at the replication fork. The replication fork proteins function as a ssDNA-binding protein, a heterodimeric DNA polymerase holoenzyme and a heterotrimeric complex possessing helicase and primase activity. Homologues of all of these six proteins, have been identified in all mammalian and avian herpesviruses sequenced to date, including the human herpesviruses EBV (Baer *et al.*, 1984), HCMV (Chee *et al.*, 1990), VZV (Davison & Scott, 1986) and KSHV (Wu *et al.*, 2001). The names and functions of the six conserved DNA replication proteins in HSV-1, and their proposed homologues in the human herpesviruses, are listed in Table 1.1.

1.5.3 Origins of replication

The lytic origins (oriLyt) of herpesviruses belonging to different subfamilies differ significantly and the OBP, unlike the other replication proteins, is not conserved. The alphaherpesviruses and the members of the roseolovirus genus of the betaherpesviruses have a distinct OBP that initiates DNA synthesis (Inoue *et al.*, 1994; Stow, 1982; Weller *et al.*, 1985; Elias & Lehman, 1988; Olivo *et al.*, 1988). The oriLyt of the gammaherpesviruses contains regions that are involved in transcriptional activation, and the OBP has roles both in transcriptional activation and the initiation of DNA synthesis (Schepers *et al.*, 1993; Schepers *et al.*, 1996; Lin *et al.*, 1999).

Function	HSV-1 (α)	VZV (α)	HCMV (β)	HHV-6 & 7 (β)	EBV (γ)	KSHV (γ)
ssDNA binding protein	UL29 (ICP8)	UL29	UL57	U41	BALF2	ORF6
DNA polymerase catalytic s.u	UL30	UL28	UL54	U38	BALF5	ORF30
DNA polymerase processivity s.u	UL42	UL51	UL44	U27	BMFR1	ORF42
Helicase	UL5	UL55	UL105	U77	BBLF4	ORF44
Primase	UL52	UL6	UL70	U43	BSLF1	ORF56
Helicase-primase accessory protein	UL8	UL52	UL102	U74	BBLF2/3	ORF40/41

Table 1.1 Conserved herpesvirus DNA replication proteins
Six core replication fork proteins are conserved throughout the herpesviridae. The table gives the names and functions of the conserved replication fork proteins of the human herpesviruses. s.u = subunit

1.5.4 HSV-1 origins of replication

HSV-1 contains two different origins of replication, known as ori_S and ori_L (Stow, 1982; Weller *et al.*, 1985). ori_S is located in the inverted repeat sequence (TRs/IRs) which flanks the Us segment and hence two copies are present within the genome. One copy of ori_L is present in the centre of the U_L region. Although they vary in size, the sequences of ori_S and ori_L are very similar. Each contains palindromic sequences centred around a region consisting of A and T residues only. The core region of ori_S is approximately 80 bp and contains an imperfect 45 bp palindrome with a central 18 bp A+T region. Within and adjacent to the palindromic sequence flanking the A+T region of ori_S are three related sequences, box I, box II and box III, which are binding sites for the HSV-1 origin-binding protein, UL9. Box I (CGTTCGCACT) has the highest affinity for UL9, with a five-fold higher affinity than box II (TGCTCGCACT) and 1000-fold higher affinity than box III (CGTTCTCACT) (Olivo *et al.*, 1988; Koff & Tegtmeyer, 1988; Elias *et al.*, 1990; Hazuda *et al.*, 1991). ori_L is larger and more symmetrical than ori_S and contains a perfect 144 bp palindrome with a 20 bp A+T central region. ori_L contains one box I and one box III sequence on each arm of the palindromic sequence. Hence, ori_L contains two very high affinity UL9-binding sites.

The functional significance of the presence of three origins of replication within the HSV-1 genome and the structural differences between ori_S and ori_L is not known. There appears to be a degree of redundancy between ori_S and ori_L as mutant viruses lacking either ori_L or both copies of ori_S grow as well as wild type virus in cultured cells (Polvino-Bodnar *et al.*, 1987; Igarashi *et al.*, 1993). It is possible that they are differently activated depending on cell type. For example, ori_L contains a functional glucocorticoid receptor binding site, which is involved in stimulation of its activity by dexamethosone

in nerve growth factor-differentiated PC-12 cells (Hardwicke and Schaffer, 1997).

1.5.5 HCMV origin of lytic replication

The HCMV lytic origin comprises a sequence spanning approximately 2.4 kbp near to the centre of U_L. This region contains the highest content of inverted and direct repeats in the HCMV genome (Anders *et al.*, 1992; Masse *et al.*, 1992). It includes various repeated sequence motifs, transcription factor binding sites including ATF, CREB and Sp-1, and also an AT rich segment. An oligopyrimidine sequence is also necessary for oriLyt function.

The possible role of transcription units in HCMV oriLyt function has been investigated (Huang *et al.*, 1996). Of particular interest was the identification of a series of short, non-polyadenylated transcripts called the smallest replicator transcript (SRT). Spanning approximately 0.22 kbp, with a common 5' terminus and heterogeneous 3' ends, SRTs are early transcripts driven by an upstream promoter. The structure of SRT suggests that it is not an mRNA, leading to the suggestion that it may have a role in the initiation of DNA synthesis. Recently a strong promoter with bidirectional activity was located within the core region of the oriLyt, which is required for efficient amplification of oriLyt (Xu *et al.*, 2004).

1.5.6 EBV Origin of lytic replication

The EBV genome has two copies of oriLyt containing two essential core elements and an auxiliary domain. One core element contains Zta responsive elements (ZREs) for the

transactivator protein Zta, which were shown to be absolutely required in a transient-transfection assay (Hammerschmidt and Sugden, 1988; Schepers *et al.*, 1993; Schepers *et al.*, 1996). The other core element contains two AT rich palindromes and an adjacent polypurine-polypyridimine tract. The auxiliary domain is an enhancer region containing DNA binding sites for Rta (viral transactivator) and Zta. Rta is not essential for oriLyt dependent DNA synthesis but it has a significant stimulatory effect on replication efficiency (Fixman *et al.*, 1995). Whilst the core region ZREs are essential for replication, the enhancer region ZREs are dispensable (Schepers *et al.*, 1996). The regions of Zta that are critical for the Zta-mediated DNA replication are nearly identical to regions that have previously been identified to be crucial for transcriptional activation of Zta dependent promoters.

1.5.7 Essential DNA replication proteins in HSV-1

The identification of the HSV-1 origins of replication enabled investigation of the identity of the viral-encoded proteins involved in DNA synthesis. A series of plasmids containing cloned fragments of the HSV-1 genome were tested for their ability to support replication of a plasmid containing an HSV-1 origin of replication when all were co-transfected into cells. This led to the identification of six fragments of HSV-1 DNA, which supplied the necessary trans-acting functions required to replicate the transfected origin (Challberg, 1986). Systematic sub-cloning of these fragments resulted in the identification of seven viral genes, which were necessary and sufficient for origin-dependent replication (Wu *et al.*, 1988). The results of the transient assay were in agreement with detailed mapping of DNA negative mutant HSV-1 viruses (reviewed by Boehmer & Lehman, 1997) and were confirmed by the demonstration that replication of an origin-containing plasmid is supported by infection of *Sf* cells with recombinant

baculoviruses expressing the seven replication proteins (Stow, 1992).

The HSV-1 DNA polymerase isolated from HSV-1 infected cells is a heterodimer consisting of the UL30 and UL42 proteins (Vaughan *et al.*, 1985). UL30 (pol) is the catalytic sub-unit, which has been extensively studied. It shares sequence similarity to other DNA polymerases, and hence has also been studied as a model eukaryotic DNA polymerase. In addition to its polymerase function, UL30 possesses a 3'→5' exonuclease activity and RNase H activity (Knopf, 1979; O'Donnell *et al.*, 1987; Crute & Lehman, 1989). These properties are presumed to confer a proof-reading activity and the ability to remove RNA primers during the processing of Okazaki fragments.

UL42 is a phosphoprotein with sequence-independent ds DNA-binding activity that associates with UL30 and serves to increase its processivity (Gallo *et al.*, 1988; Hernandez & Lehman, 1990; Gallo *et al.*, 1989; Gottlieb *et al.*, 1990). It is monomeric in solution and binds to ssDNA as a monomer (Randell and Coen, 2004). It was thought that by interacting with UL30 and ds DNA simultaneously, UL42 tethers UL30 to the template, enabling the synthesis of long DNA chains (Gottlieb & Challberg, 1994). However, Chaudri and Parris (2002) suggest that the interaction between UL30 and UL42 may change the conformation of UL30 so that UL30 has a more closed conformation around the DNA. This would result in UL30 having a stronger attachment to the DNA and would therefore increase processivity. The interaction between UL30 and UL42 is mediated by a short sequence (35aa) at the C-terminus of UL30 and appears to be critical for DNA replication. Deletion of this sequence has no effect on the DNA polymerase activity of UL30 but abolishes its ability to support long chain synthesis (Digard *et al.*, 1993; Tenney *et al.*, 1993) and origin-dependent DNA replication (Stow, 1993).

Essential helicase and primase functions in HSV-1 infected cells are provided by a heterotrimeric complex comprised of the UL5, UL52 and UL8 proteins (Crute *et al.*, 1989). The 5'→3' helicase has associated ATPase and GTPase activities (Crute *et al.* Lehman, 1991). The primase exhibits strong sequence preference for the synthesis of short oligoribonucleotide primers of between 8-10 bases (Tenney *et al.*, 1995; Crute & Lehman, 1991). The UL5 protein contains six motifs characteristic of helicases and UL52 contains a sequence motif similar to that found in other DNA primases (McGeoch *et al.*, 1988), hence the helicase and primase functions have been assigned to the UL5 and UL52 subunits, respectively. A sub-assembly of the UL5 and UL52 proteins displays both helicase and primase functions (Calder & Stow, 1990; Dodson & Lehman, 1991). In addition, site-directed mutagenesis studies have demonstrated that the helicase and primase active sites reside within the UL5 and UL52 components, respectively (Zhu & Weller, 1992; Klinedinst & Challberg, 1994), although neither protein alone exhibits significant enzymatic activity. More recently it was shown that the two proteins demonstrate an interdependence for DNA binding and that the UL52 protein may have a more active role in helicase activity than previously thought (Biswas and Weller, 2001).

The role of the UL8 protein was initially unclear, as it does not appear to perform any obvious enzymatic functions and does not bind to DNA (Parry *et al.*, 1993). Several studies have now indicated it is likely that UL8 has several auxiliary roles at the replication fork. It is known to be necessary for efficient primer utilisation by the polymerase in a model of lagging strand synthesis (Sherman *et al.*, 1992), to stimulate primer synthesis (Tenney *et al.*, 1994) and is required for efficient nuclear translocation of the helicase-primase complex (Calder *et al.*, 1992; Marsden *et al.*, 1996). UL8 is known to interact with several other replication proteins, indicating a multifunctional role

at the replication fork.

UL9 functions as an origin-binding protein, involved in the initiation of DNA replication. It exists as a homodimer in solution, binds to specific sequences present in HSV-1 origins (Elias & Lehman, 1988; Olivo *et al.*, 1988) and possesses DNA-dependent ATPase and helicase activities (Fierer & Chalberg, 1992; Boehmer *et al.*, 1993). The amino-terminal portion of the protein mediates the helicase, ATPase and dimerisation activities whilst the carboxy-terminal domain is involved in sequence-specific DNA binding. The non-sequence specific helicase activity of UL9 appears to be required for DNA synthesis as the introduction of mutations into the helicase motifs renders the protein unable to support DNA replication (Martinez *et al.*, 1992). Binding of UL9 to sites I and II in ori_S is co-operative, indicating that an interaction occurs between UL9 protein(s) bound at each site (Elias *et al.*, 1990). The helicase activity of UL9 is stimulated by the ssDNA-binding protein, ICP8, by its interaction with UL9 (Fierer & Chalberg, 1992; Lee and Lehman 1997). An interaction has also been demonstrated between UL9 and UL42, which also stimulates the helicase activity of UL9 (Monahan *et al.*, 1998; Trego and Parris, 2003).

ICP8 (UL29) is the ssDNA binding protein in HSV-1. As it is the homologue of KSHV pORF6 and it has been extensively studied, a detailed review of it is given in section 1.7.2.

1.5.8 Essential DNA replication proteins in EBV

Homologues of six of the core replication proteins found in HSV-1 are also encoded by EBV (Fixman *et al.*, 1992). These are the DNA polymerase (BALF5) and its accessory

factor (BMRF1), the ssDNA binding protein (BALF2), the helicase (BBLF4), the primase (BSLF1) and the primase-associated protein (BBLF2/3). Their functions are sufficiently conserved that the HSV-1 homologues in the presence of Zta and Rta can drive replication from EBV oriLyt (Fixman *et al.*, 1995).

The polymerase and polymerase accessory protein have been shown to interact by co-immunoprecipitation, and together mediate DNA replication with strand displacement in model replication systems (Kiehl and Dorsky, 1991; Lin *et al.*, 1991; Tsurumi *et al.*, 1993; Tsurumi *et al.*, 1997). The holoenzyme exhibits both 5' to 3' DNA polymerase and 3' to 5' exonuclease activities (Tsurumi, 1991).

BALF2 is the homologue of pORF6. It binds with limited cooperativity to ssDNA and functions to melt out secondary structure from ssDNA, which facilitates the activity of the DNA polymerase (BALF5). The presence of BALF2 eliminates pausing by BALF5 during DNA replication (Tsurumi *et al.*, 1996). BALF2 has been found to contact the DNA polymerase (Zeng *et al.*, 1997) and also possibly the helicase-primase complex (Gao *et al.*, 1998). The latter interaction has been shown to be necessary in HSV-1 for localising the ssDNA binding protein (ICP8) to pre-replicative sites (described in section 1.5.11) (Liptak *et al.*, 1996; Lukonis *et al.*, 1997).

In addition to being a transcriptional activator, Zta also acts as the OBP. A cotransfection assay in EBV negative cells showed that Zta (in addition to the six conserved replication fork proteins) was absolutely required for replication from EBV oriLyt (Fixman *et al.*, 1995). Mutation of the four Zta binding sites in the promoter region abolished oriLyt-dependent replication (Schepers *et al.*, 1996). An interaction between Zta and the helicase-primase complex has been demonstrated (Gao *et al.*, 1998). This interaction

between the helicase protein of the helicase-primase complex and Zta has been mapped to aa 22 to 86 of the Zta protein, (Liao *et al.*, 2001).

Interactions between the helicase-primase subunits were demonstrated by immunofluorescence assays. Expressed individually, the proteins showed either mixed nuclear and cytoplasmic, or cytoplasmic staining. When all three proteins were transfected, nuclear localisation of all three was observed (Gao *et al.*, 1998). Co-immunoprecipitation experiments revealed that each component of the BBLF4-BSLF1-BBLF2/3 complex directly interacts with the other two (Yokoyama *et al.*, 1999).

In addition to interacting with Zta, the helicase-primase has also been shown to interact with the polymerase through both the BSLF1 and BBLF2/3 subunits. (Fujii *et al.*, 2000). The polymerase interacts with its accessory factor, which in turn binds to Zta (Zhang *et al.*, 1996). Co-immunoprecipitation assays have also demonstrated an interaction between the helicase-primase associated factor and the cellular zinc finger protein ZBKR1, which binds to EBV oriLyt. It has been suggested that ZBKR1 may be acting as a tethering point on oriLyt for the replication complex, through its interaction with the helicase-primase complex. (Liao *et al.*, 2005).

1.5.9 Host cell-encoded functions involved in HSV-1 DNA replication

HSV-1 DNA replication is largely autonomous from the host cell, however, several essential functions are not encoded by HSV-1 and are therefore assumed to be provided by host cell enzymes. For example, a topoisomerase is expected to be required to remove supercoils from replicating DNA, and a DNA ligase for joining the Okazaki fragments produced during lagging strand synthesis.

1.5.10 Model for origin unwinding and initiation of DNA replication in HSV-1

Using the information that has been gathered regarding the HSV-1 origins, replication proteins and how they interact with each other, a model for the unwinding of the replication origin and establishment of a replication fork has been proposed (Boehmer & Lehman, 1997 and Stow, 2000), however, it should be noted that not all the events proposed have been substantiated experimentally. Although the initial events in unwinding the replication origins may vary amongst the different herpesviruses, the subsequent recruitment of DNA replication proteins and establishment of a replication fork may follow a similar pathway to that which is proposed for HSV-1. Steps 1-3 are illustrated in figure 1.3.

- 1) The initial step involves the binding of UL9 to recognition sites (box I and/or II) on either side of the AT region at the centre of ori_S and ori_L .
- 2) Bound UL9 proteins interact with each other, possibly mediated by a leucine zipper motif within the N-terminal region of UL9. This interaction results in a distortion of the DNA helix in the AT region between the binding sites. Specifically, it has been suggested that the intervening DNA is held in a loop configuration as a result of the interaction (Koff *et al.*, 1991).
- 3) The interaction between UL9 and ICP8 serves to recruit ICP8 to the origin, where it binds to the distorted DNA in the AT region. ICP8 stimulates the sequence independent helicase activity of UL9 and increases its processivity allowing unwinding of the duplex DNA adjacent to the origin. The interaction between UL9 bound to the opposite sides of the origin is maintained during initial unwinding and ssDNA extruded from the UL9-ICP8 complex is stabilised by coating with ICP8. Sequence specific unwinding of an HSV-1 origin by UL9 has been demonstrated only in the presence of ICP8, indicating the importance of the UL9-ICP8 interaction

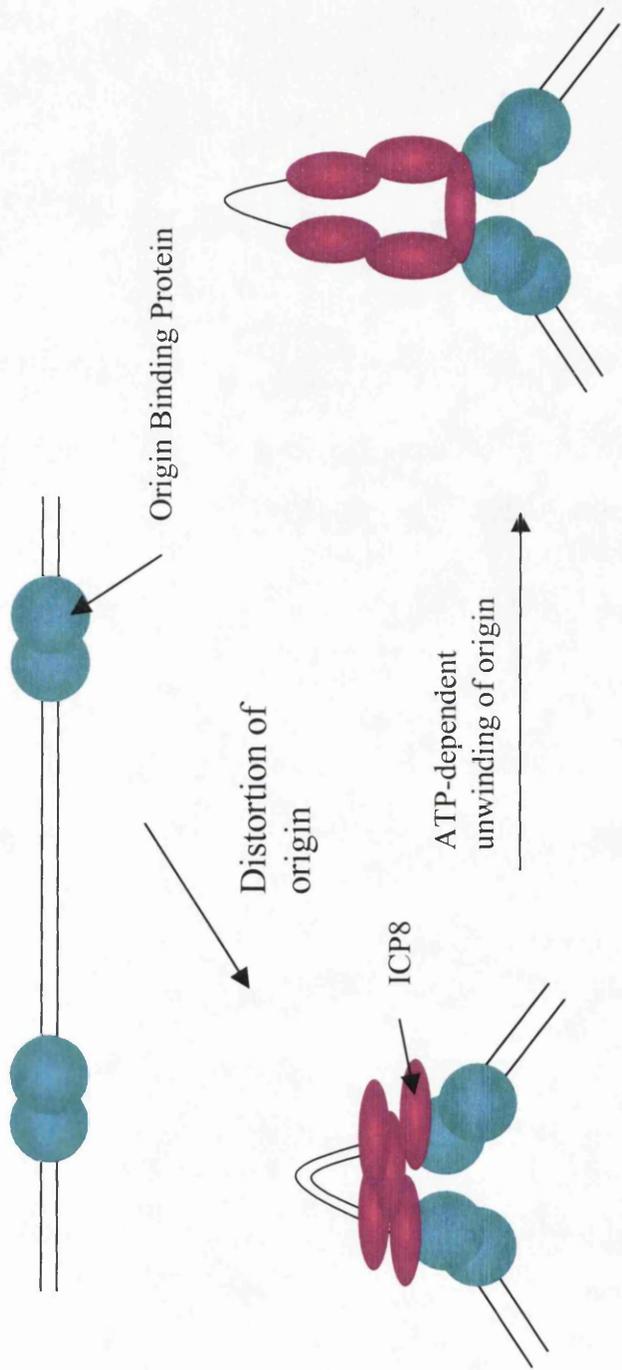


Figure 1.3 Model for the role of ICP8 during initiation of DNA replication

ICP8 binds to the already bound OBP UL9 and assists in the ATP-dependent unwinding of the origin.

to the unwinding process (Lee & Lehman, 1997).

- 4) Once unwinding of the duplex DNA has begun, recruitment of the helicase-primase and polymerase functions is necessary for DNA synthesis to commence. It is postulated that the arrival of the helicase-primase and polymerase enzyme complexes result in either the displacement of UL9, or disruption of the interaction between UL9 proteins, allowing the origin to take the form of a replication bubble. It is probable that the helicase-primase heterotrimer is recruited via protein-protein interactions involving the UL8 component, as UL8 physically interacts with UL9 (McLean *et al.*, 1994) and an interaction with ICP8 is also strongly suggested (Falkenberg *et al.*, 1997; Tanguy Le Gac *et al.*, 1996).
- 5) The helicase-primase complex then directs the synthesis of short RNA primers on both unwound DNA strands for elongation by the DNA polymerase holoenzyme.
- 6) Recruitment of the polymerase holoenzyme to the sites of unwound and RNA-primed DNA is possibly mediated through interactions of the polymerase holoenzyme with UL9 and or the UL8 subunit of the helicase-primase complex. The polymerase accessory protein, UL42, specifically interacts with the N-terminal region of UL9 (Monahan *et al.*, 1998) and this interaction may also contribute to the proposed dissociation of UL9 to allow formation of a replication bubble. The other interaction, which may be involved in recruitment of polymerase occurs between UL30 (catalytic subunit) and the UL8 subunit of the helicase-primase complex. Characterisation studies indicate that the region spanning the C-terminal 32 amino acids of UL8 is involved in the interaction (Marsden *et al.*, 1997). It is interesting to note, therefore, that a UL8 mutant lacking the C-terminal 33 residues does not support origin-dependent replication (Barnard *et al.*, 1997), supporting the hypothesis that the UL8-UL30 interaction may be crucial for DNA synthesis.
- 7) Once polymerase has been recruited to the replication fork, the RNA primers are

extended and bi-directional DNA synthesis is established. The helicase-primase complex proceeds to further unwind the duplex DNA and synthesise primers for lagging strand synthesis as the replication fork moves away from the origin.

1.5.11 HSV-1 Replication fork

As replication progresses the remaining six DNA replication proteins (UL5/UL52/UL8, UL30/UL42 and ICP8) may function together as a multiprotein complex co-ordinating DNA synthesis on the two strands. A complex of these six proteins was shown to be able to carry out rolling-circle type DNA synthesis on circular plasmid templates (Skaliter and Lehman, 1994).

A model for the DNA replication fork has been proposed in which the lagging strand is looped in a manner such that the leading and lagging strand DNA polymerases can both move along the DNA in the same direction as the replication fork. In the case of HSV-1 this would allow a UL5-UL8-UL52 trimer to unwind the duplex DNA and prime lagging strand DNA synthesis (Figure 1.4) (Stow, 2000). The UL8-ICP8 interaction may serve to direct the helicase-primase complex to regions of ssDNA and to modulate the enzymatic activities of the complex. ICP8 stimulates the DNA-dependent ATPase and helicase functions of the complex (Hamatake *et al.*, 1997), and efficient helicase-primase activities on ICP8-coated templates are dependent on the presence of UL8 (Falkenberg *et al.*, 1997; Tanguy Le Gac *et al.*, 1996). The UL8-UL30 interaction probably has a role in the co-ordination of DNA unwinding and leading strand synthesis and also co-ordinates the synthesis of RNA primers with lagging strand synthesis.

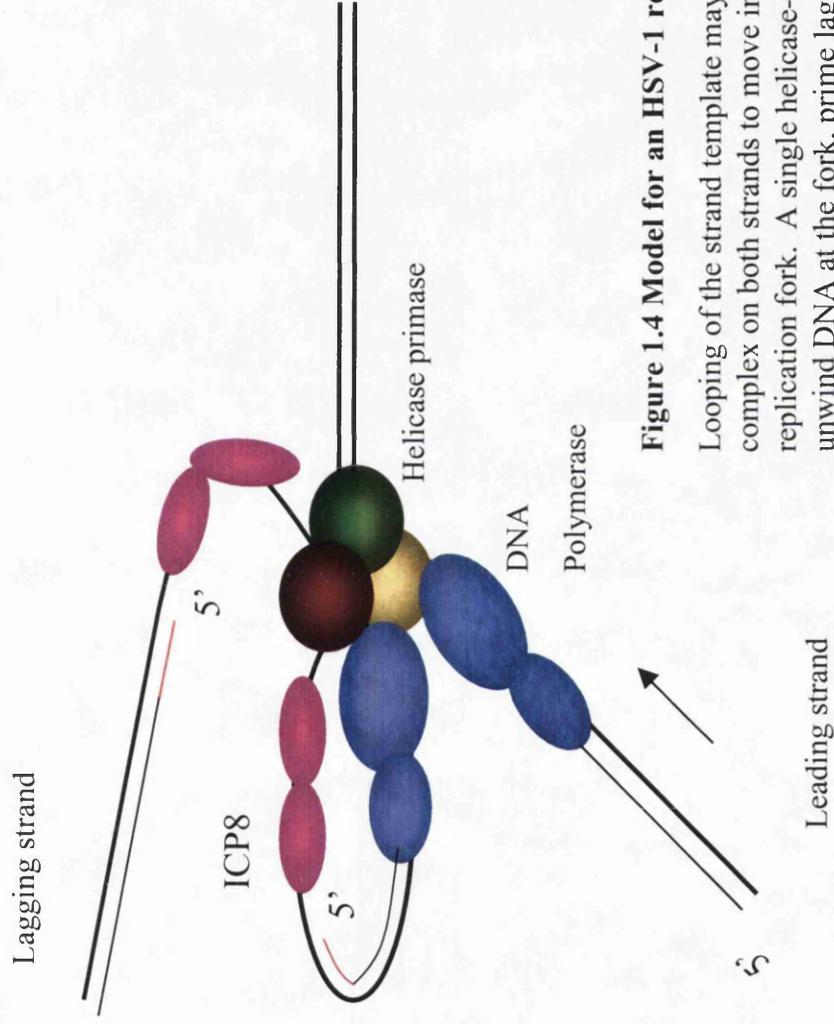


Figure 1.4 Model for an HSV-1 replication fork

Looping of the strand template may allow the DNA polymerase complex on both strands to move in the direction of the replication fork. A single helicase-primase complex could unwind DNA at the fork, prime lagging strand DNA and establish interactions with the leading and lagging strand DNA polymerases. RNA primers are shown in red. Template DNA is shown as thick lines, newly synthesised DNA as thin lines (adapted from Stow, 2000)

1.5.12 Recombination events in HSV-1

During replication of the HSV-1 genome events such as DNA damage may lead to double-stranded breaks and the collapse of the replication fork. To repair these and continue replication a recombination strategy may be employed. Recombination is also responsible for the genomic inversions during replication in which the U_L and the U_S components invert their relative orientation. Homologous recombination between co-infecting viruses has also been well documented (Schaffer *et al.*, 1974; Honess *et al.*, 1980; Umene, 1985; Brown *et al.*, 1992). Two viral proteins that have been implicated in these processes are the alkaline nuclease (UL12) and the ssDNA binding protein (ICP8). Possible mechanisms for recombination are discussed in section 1.7.2

1.6 KSHV lytic DNA replication

1.6.1 KSHV origin of lytic replication

Two duplicated copies of the DNA replication origin (oriLyt) have been identified (AuCoin *et al.*, 2002). They are located between K4.2 and K5 and between K12 and ORF71. The two ori-Lyt share an almost identical 1.1 kb sequence and 600 bp GC rich repeats. The whole 1.7 kb DNA sequences are necessary and sufficient for lytic replication (Lin *et al.*, 2003). Within these sequences the most important *cis*-acting elements have been shown to be eight C/EBP (CCAAT/enhancer binding protein) binding motifs, arranged as four spaced palindromes; an 18 bp AT palindromic sequence where local unwinding of ds-DNA during initiation of replication is thought to occur, and an ORF50/Rta-dependent promoter that is composed of Rta response element and a TATA box. These lytic origins closely resemble the lytic origin of the rhesus macaque rhadinovirus (Pari *et al.*, 2001) with both having GC-rich regions adjacent to an AT-rich domain.

The lytic origin and the *trans*-acting factors involved in KSHV DNA replication were investigated further by AuCoin *et al.* (2004). The use of site directed mutant plasmids in a transfection replication assay revealed that the AT-rich sequences, three AP1 transcription factor binding sites and a downstream component containing an ORF50 response element and a TATA box were necessary for efficient replication of oriLyt (figure 1.5). The organisation of the lytic origin is similar to that of EBV and the AP1 transcription binding sites in EBV function as Zta response elements. The ORF50 response elements were further investigated in this study. The use of luciferase reporter constructs containing the ORF50 response element revealed that they were responsive to K-Rta (ORF50 product) and that it is a region of active transcription within the viral genome. The EBV oriLyt also contains two Rta binding sites.

1.6.2 KSHV DNA replication proteins

Although a homologue of the OBP in HSV-1 (UL9) has not been identified, the HHV8 encoded protein- K8, has been shown to bind to the oriLyt and may be serving as an initiator protein. K8 is thought to be a homologue of Zta (OBP for EBV). The two proteins have limited sequence homology, corresponding genomic locations, similar splicing patterns and are both members of the bZIP family (Lin *et al.*, 1999). Although no direct interaction has yet been proven between K8 and the other KSHV replication proteins, K8 has been shown to co-localise with a KSHV viral DNA replication complex comprising the KSHV homologues of the six conserved herpesvirus replication fork proteins (Wu *et al.*, 2001). These are the protein products of ORF6, the ssDNA binding protein (SSB); ORF9, the DNA polymerase (Pol); ORF59, the polymerase processivity factor (PPF); ORF56, the helicase (HEL); ORF44, the primase (PRI) and ORF40/41 the helicase-primase associated factor (PAF).

Wu *et al.* (2001) also investigated the nuclear location of these proteins and the formation of KSHV replication compartments. When expressed individually in Vero cells, SSB and PPF were transported to the nucleus and diffuse nuclear staining was observed. POL, HEL and PRI were localised in the cytoplasm, whereas PAF gave a mixed but predominantly cytoplasmic staining. Cotransfection of POL with PPF was sufficient to translocate POL to the nucleus. Complete nuclear staining of PAF required all six proteins to be present.

In the presence of all six core replication proteins pseudo-replication compartments that excluded cellular DNA were formed. On co-transfection with a plasmid containing the EBV oriLyt and Zta, newly synthesised EBV oriLyt plasmid DNA was observed, confirming that these pseudo replication compartments could accommodate DNA replication and that the EBV and KSHV replication fork proteins were interchangeable. Inspection of KSHV infected cells undergoing lytic infection demonstrated similar replication compartment structures that contained newly synthesised DNA and co-localised with K8 (Wu *et al.*, 2001).

A cotransfection replication assay demonstrated that in addition to the core conserved herpesvirus DNA replication proteins, K-Rta and K8 were also required for replication of KSHV oriLyt to be detected. The regions of K8 that were essential for DNA replication were explored. Mutation of the leucines within the leucine zipper motif demonstrated that this region of the protein is essential for its activity and for efficient oriLyt dependent DNA replication. This study demonstrated that although K8 may be the primary origin binding protein, K-Rta may also have a role in DNA replication from oriLyt (Aucoin *et al.*, 2004).

A recent study using a KSHV mutant that had a large deletion in the ORF50 gene (K-Rta) further demonstrated that K-Rta is required for lytic viral reactivation and also the transactivation of viral genes contributing to DNA replication (Xu *et al.*, 2005). This is in contrast to EBV where the K8 homologue Zta drives the lytic cycle. Zta also performs essential transactivation and DNA replication functions within EBV oriLyt whereas K8 does not appear to have a corresponding transactivation function.

To date, the only interaction between KSHV replication proteins that has been studied in detail, is between the polymerase (Pol) and its processivity factor (PPF). To determine whether the interaction between the two proteins was functionally significant, the DNA synthesis activity of pol in the presence and absence of PF was investigated using primed M13 ssDNA as the template in an *in vitro* DNA synthesis assay (Lin *et al.*, 1998). In the absence of PF, Pol could only incorporate a few nucleotides. PF was required for pol to synthesize extended DNA products. The effect of the PF from HSV-1 (UL42) on KSHV pol was also tested. UL42 was not able to confer processivity on pol demonstrating that the functional interaction between KSHV pol and PF is specific.

To confirm that pol and PF physically interacted the two were successfully co-immunoprecipitated (Chan and Chandran, 2000). The pol binding domain of PF was then investigated using PF deletion mutants demonstrating an essential role for amino acids 10-27 in the interaction with pol.

1.7 Single stranded DNA binding proteins

1.7.1 General

KSHV pORF6 belongs to the family of single-stranded DNA binding proteins (SSB).

The most extensively studied of this class are SSB from *E.coli*, DBP (DNA binding protein) from adenovirus, gp 32 (gene 32 protein product) from bacteriophage T4, gp 2.5 (gene 2.5 protein product) from bacteriophage T7 and RPA (Replication protein A), the eukaryotic SSB. These proteins are involved in recombination, repair and replication. They bind to ssDNA in preference to dsDNA in a sequence independent manner.

Recognition of DNA is mediated by their OB-fold (apart from the adenovirus DBP, the only SSB identified to date without an OB-fold). The OB-fold is a small structural motif originally named for its oligonucleotide/oligosaccharide binding properties (Murzin, 1993). They range in length from 70 to 150 amino acids and there is only a low degree of sequence similarity among them (Wutte *et al.*, 2003). However, the fold has a distinct topology. It consists of two three-stranded anti-parallel β -sheets, where strand 1 is shared by both sheets. The proteins interact with nucleic acids mostly through stacking interactions with aromatic amino acid side chains, electrostatic charge interactions and packing interactions with hydrophobic side chains or the aliphatic portions of polar groups such as lysine and arginine (Kelly *et al.*, 1976; Prigodich *et al.*, 1984; Curth *et al.*, 1993; Kim and Richardson, 1993; Kallenpoulos *et al.*, 1995).

The adenovirus DBP and T4 gp 32 are monomeric up to a certain concentration where they self-associate to form dimers and higher molecular weight aggregates (Schechter *et al.*, 1980; Alberts and Frey, 1970; Carroll *et al.* 1972). Gp 2.5 (T7) exists as a dimer (Kim and Richardson, 1994) and the *E.coli* SSB assembles as a homotetramer (Molineux

et al., 1974; Weiner *et al.*, 1975). The eukaryotic RPA is a heterotrimeric protein (Wold and Kelly, 1988).

Binding to ssDNA is in a cooperative fashion and facilitates their activity. (Alberts and Frey, 1970; Kuil *et al.*, 1989; Lohman and Overman, 1985; Kim and Wold, 1994). Helix destabilisation is promoted since the binding of one molecule to the exposed ssDNA region, increases the affinity of others, helping to prevent renaturation of the two strands. During elongation of the DNA, covering of the ssDNA template not only aids the polymerase by removing secondary structure from the DNA but it also protects the DNA from nuclease digestion (figure 1.6). Gp 2.5 may stimulate the T7 DNA polymerase, not just by removing secondary structure from DNA, but also by a direct physical interaction (Kim *et al.*, 1992; He *et al.*, 2003). An interaction has also been demonstrated between eukaryotic RPA and DNA polymerase alpha (Dornreiter *et al.* 1992).

The C-terminal region of the adenovirus DBP appears to be important for protein-protein interactions that aid cooperative binding. DBP has a C-terminal arm that hooks onto a second DBP monomer, which results in the formation of long protein chains along the ssDNA (Tucker, 1994). Conversely, the region required for cooperative binding in gp 32 (T4) lies at the very N-terminus of the protein (Spicer *et al.*, 1979; Lonberg *et al.*, 1981). The cooperative binding domain of the *E. coli* SSB also lies in the N-terminal region (Williams *et al.*, 1984).

Another feature of gp32 (T7) is its ability to bind to RNA and possibly control gene expression at the level of translation. The specificity of gp32 for gp32 mRNA and not other T4 mRNA lies in a uniquely structured region that spans 50nt (Krisch *et al.*, 1982).

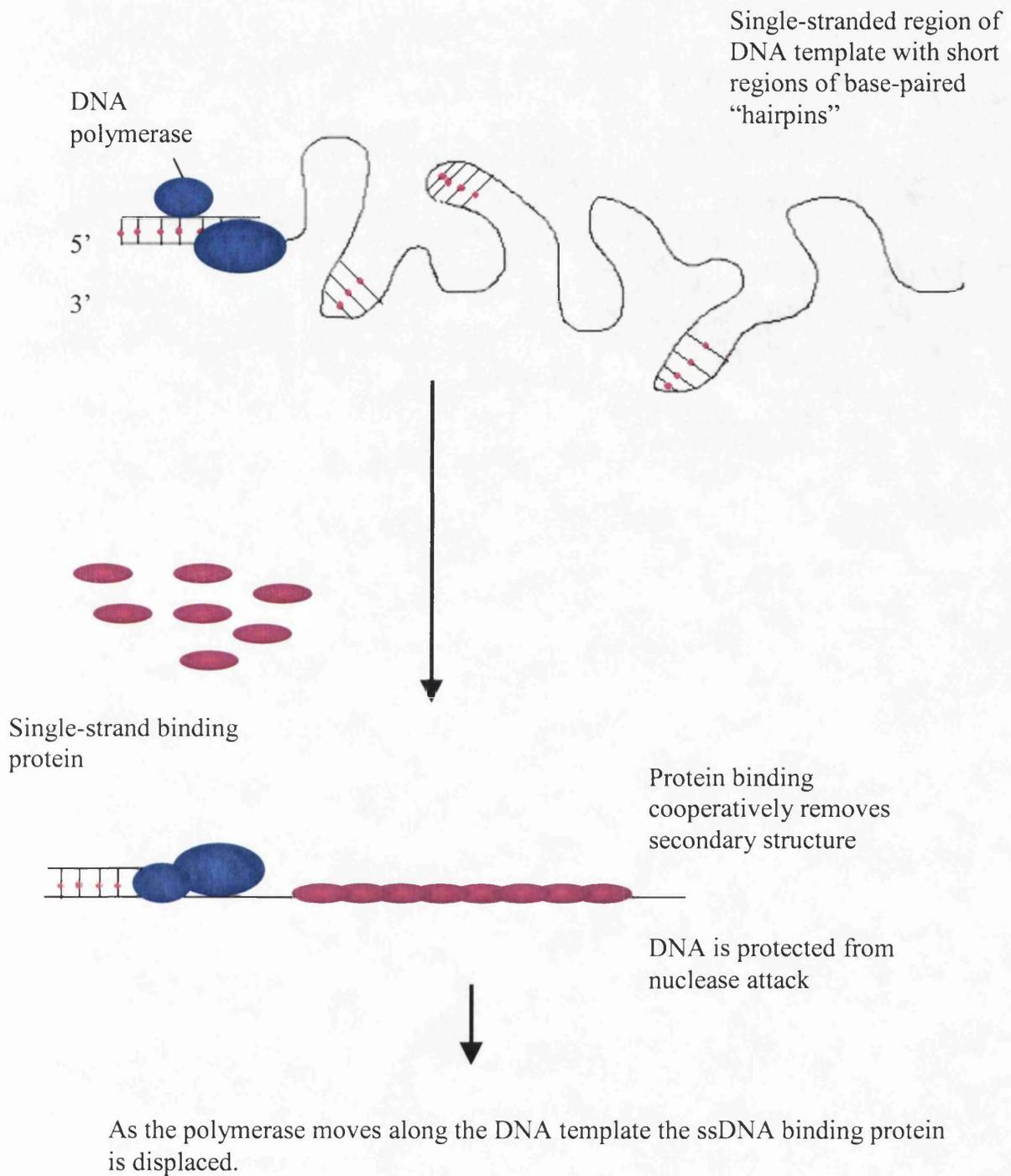


Figure 1.6 The effect of single-strand DNA-binding proteins on the structure of single-stranded DNA

As each protein molecule prefers to bind next to a previously bound molecule, long rows of the protein form on a DNA single strand. This cooperative binding removes secondary structure from the DNA template and facilitates the DNA polymerisation process.

Gp 32 has a higher affinity for ssDNA than its mRNA ensuring that all ssDNA is saturated before it will bind to its mRNA to control its own expression level (Krisch *et al.*, 1974; Russel *et al.*, 1976; Newport *et al.*, 1981). SSB (*E.coli*) and DBP (adenovirus) also bind to RNA with a lower affinity than they bind to ssDNA and may also have a role in controlling their own expression levels (Molinuex *et al.*, 1975; Cleghon and Klessig, 1986). Eukaryotic RPA can also bind to RNA but again with a lower affinity than it binds to ssDNA (Kim *et al.*, 1992).

SSBs also have a role in recombination and repair. Recombination and replication are coupled events, and homologous recombination is a strategy used to repair double-stranded breaks in the DNA. The SSB of *E.coli* aids the RecA protein in strand exchange by removing secondary structure from the DNA (Kowalczyowski *et al.*, 1987; Lavery and Kowalczyowski, 1992). T4 gp32 has been demonstrated to aid strand exchange (George *et al.*, 2001) and T7 gp2.5 has been shown to be involved in DNA annealing (Kong and Richardson, 1996; Kong and Richardson, 1998). The adenovirus DBP enhances DNA renaturation (Zijderveld *et al.*, 1993). RPA has been shown to be required for strand exchange. (Moore *et al.* 1991).

1.7.2 ICP8

ICP8 is the HSV-1 homologue of KSHV pORF6, the protein studied in this thesis, and is the most extensively studied of the SSBs from the herpesviruses. It is a zinc metalloprotein with a MW of approximately 128 kDa (Ruyechan, 1983). The zinc enhances structural integrity and is not involved in binding to DNA (Gupte *et al.*, 1991). Like other proteins in this family (SSBs) ICP8 has a role in recombination, repair and

replication. It has helix destabilising properties and has a higher affinity for ssDNA than ds-DNA (Boehmer and Lehman, 1993; Purifoy and Powell, 1976).

During initiation of replication ICP8 is believed to bind to the C-terminal domain of the OBP at the origin of replication and assists in the ATP-dependent unwinding of the origin (Boehmer and Lehman, 1993; Boehmer *et al.*, 1994; Makhov *et al.*, 1996; Lee and Lehman, 1997) (figure 1.3). As elongation of the DNA proceeds, ICP8 is proposed to act at the replication forks to hold the DNA in an extended conformation, facilitating the activity of the DNA polymerase and also protecting exposed ssDNA from nuclease attack. ICP8 also interacts with the UL8 subunit of the helicase-primase and stimulates the helicase activity of this complex (Tanguy le Gac *et al.*, 1996; Hamatake *et al.*, 1997).

Whilst a direct physical interaction between ICP8 and the HSV-1 polymerase has not been demonstrated, a functional interaction was shown by ICP8 stimulating the enzymatic activity of the DNA polymerase (O'Donnell *et al.*, 1987). ICP8 is also required for the polymerase to localise to pre-replicative sites within the HSV-1 infected cell, demonstrating another functional interaction (Bush *et al.*, 1991).

Like other SSBs, aromatic and basic residues mediate protein-nucleic acid contacts via stacking and electrostatic interactions (Ruyechan and Olson, 1992). The binding site size has been reported to be between 12-40 nt per monomer (Ruyechan, 1983; O'Donnell *et al.*, 1987; Gustafasson *et al.*, 1995; Bortner *et al.*, 1993; Makhov *et al.*, 1996; Dudas and Ruyechan, 1998). This lack of agreement may be due to differences in experimental technique, concentration of protein used or differences between protein preparations. The use of deletion mutants and photoaffinity labelling has placed the minimum region for binding between amino acids 332 to 902 (Leinbach and Heath, 1989; White and

Boehmer; 1999). Within this stretch lies the predicted zinc finger motif between amino acids 499 and 512 (Gupte *et al.*, 1991).

ICP8 binds to ssDNA in a cooperative manner, which induces a conformational change in the protein (Ruyechan, 1983; Lee and Knipe, 1985; Dudas *et al.*, 2001). The region of the protein that is responsible for mediating the protein-protein interactions required for cooperative binding includes the C-terminal 60-residues (Mapelli *et al.*, 2000). The C-terminus additionally contains a nuclear localisation signal (Gao and Knipe, 1992). Binding to ssDNA is optimal at 150mM NaCl and neutral pH (Ruyechan and Weir, 1984).

The crystal structure of ICP8 has recently been resolved (Mapelli *et al.*, 2005). The protein preparation used was not the whole protein (1196aa) as this had previously failed to yield well diffracting crystals, but a deletion mutant lacking the C-terminal 60 residues. The structure is composed of a large N-terminal domain (9-1038) and a smaller C-terminal α -helical domain (figure 1.7). The N-terminal domain is described as consisting of head, neck and shoulder regions. The neck region contains a structure similar to the OB fold, which mediates ssDNA binding and has been found in all SSBs described to date (except for adenovirus SSB). Although the C-terminus was missing 60 residues, the C-terminal domain fits loosely into a concave surface on the back of the N-terminal domain, revealing the structure that confers co-operativity of ICP8 binding to ssDNA. It is thought that the C-terminal 60 residues would reach round and bind to a hydrophobic region on the N-terminal domain, stabilising this interaction.

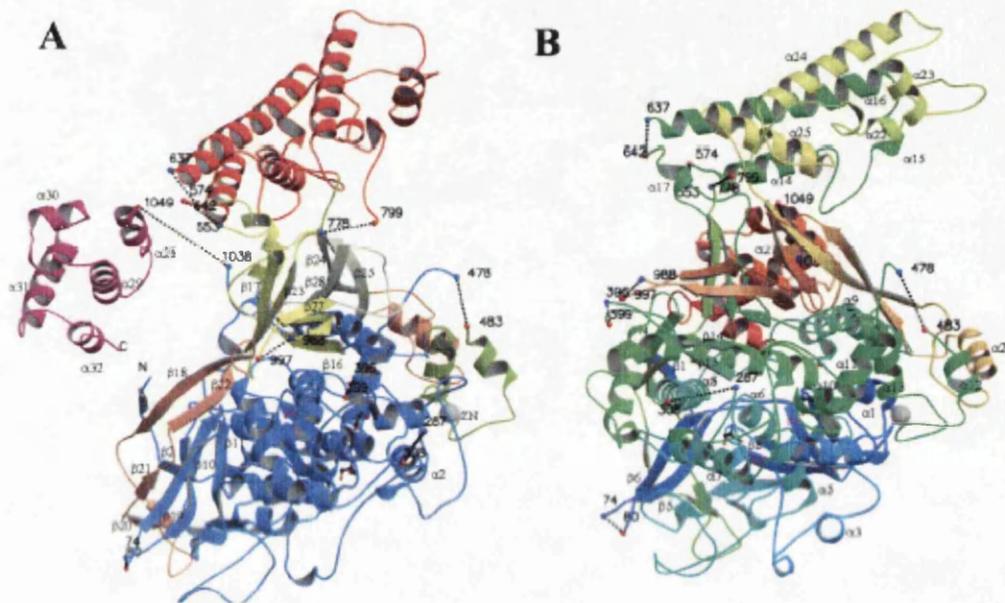


Figure 1.7 Structure of ICP8

A Overall view of the ICP8 structure. Dotted lines represent disordered regions with blue and red balls signifying the N and C-terminal ends of the disordered regions. The shoulder region is blue, the zinc binding region is green, the part of the polypeptide chain linking the neck and shoulders as a single folding unit is orange. The neck is coloured yellow (front) and grey (back). The head is red and the C-terminal helical domain is purple.

B The structure rotated 60° along x-axis relative to A. In this orientation the C-terminus is behind the neck. (from Mapelli *et al.*, 2005).

As previously mentioned, ICP8 and UL12 together are capable of catalysing an *in vitro* strand exchange reaction. Further studies demonstrated that ICP8 has a strong recombinase activity and it has been likened to other recombinases such as RecA, UvsX and Rad 51 (Reuven *et al.*, 2004). Like these recombinases, ICP8-ssDNA filaments are helical, and in solution, in the absence of DNA it forms helical self-filaments.

ICP8, in conjunction with the viral helicase-primase, has been shown to catalyse strand exchange (Nimonkar and Boehmer, 2002). In contrast to the work from Reuven *et al.* (2004) this was UL12 independent. Also demonstrated was its ability to promote the assimilation of ssDNA into homologous supercoiled DNA, resulting in the formation of a displacement loop (Nimonkar and Boehmer, 2003). Renaturation of complementary DNA strands by ICP8 has also been shown (Dutch and Lehman, 1993).

A co-immune-precipitation study of HSV-1 infected cells, using ICP8 monoclonal antibodies revealed a number of cellular proteins that co-precipitated with ICP8 (Taylor and Knipe, 2004). The majority of the proteins were components of cellular complexes that coordinate DNA recombination and repair. HSV-1 may recruit some of these cellular proteins to participate in HSV-1 replication and also the repair of damaged viral DNA that may arise during replication.

ICP8 also has a role in viral transcription, affecting it in at least two ways. It can repress transcription from input parental viral genomes and can also stimulate late gene transcription (Godowski and Knipe, 1985; Gao and Knipe 1991). An interaction between ICP8 and ICP27 has been established (Zhou and Knipe, 2002; Olesky *et al.*, 2005).

ICP27 stimulates expression of some early viral genes and transcription of some late viral genes. The two proteins were also shown to associate with cellular RNA

polymerase II, which is responsible for transcription of all of the viral genes. It is hypothesised that the ICP27-ICP8 interaction plays a role in the stimulation of late gene transcription.

A direct interaction between ICP8 and RNA has recently been demonstrated which provides a possible molecular basis for the role of ICP8 in the regulation of viral gene expression. (Boehmer, 2004). Its ability to form R-loops may also be related to its role in recombination. R-loops are triple stranded structures formed between RNA and duplex DNA in which the RNA strand displaces the DNA strand of identical sequence (Kabak *et al.*, 1979). The RecA protein from *E.coli* uses R-loops to initiate recombinatorial repair of DNA breaks (Kasahara *et al.*, 2000; Zeitsev and Kowalczykowski, 2000)

1.8 Aims

There has been little work carried out so far on the core set of six replication proteins that are required for the DNA replication of KSHV. To date the protein product of the KSHV ORF6 gene (pORF6) had not been purified and characterised. The aims of this project were to firstly over-express and purify pORF6 and then to characterise its binding to ssDNA.

Initial work involved the generation of a baculovirus expression system in which the protein could be expressed to a high level. Successful expression led to the purification of the protein by column chromatography. Preliminary experiments demonstrated that the purified pORF6 could bind to ssDNA. Characteristics of binding were then investigated by electrophoretic mobility shift assay (EMSA) and surface plasmon resonance. A panel of monoclonal antibodies (MAbs) against pORF6 were also generated and characterised, and studies were initiated to examine the effects of the MAbs on the interaction of pORF6 with ssDNA.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and reagents

All chemicals were obtained from Sigma Chemicals Company or BDH Laboratory Supplies with the following exceptions;

Amersham Pharmacia Biotech (APB)	ECL western blotting detection reagents, Rainbow protein MW markers
BIAcore	Surface plasmon resonance chips and buffer
Roche	Tris base, Nonidet-P40, complete EDTA-free protease inhibitor tablets
Bio-Rad Laboratories	Ammonium persulphate, 30 % acrylamide/bis-acrylamide solution, protein assay dye reagent concentrate
Calbiochem Corporation	Clelland's reagent (DTT)
Prolabo	Methanol
New England Biolabs	Lambda DNA- <i>BstE</i> II digest markers
Smithkline Beecham Research	Ampicillin

2.1.2 Enzymes

Enzymes were obtained from:

New England Biolabs, USA : T4 polynucleotide kinase, T4 DNA ligase, calf intestinal phosphatase

Roche: *Bam*HI, *Eco*RV

2.1.3 Antibodies

Antibodies were supplied by the following:

Sigma Chemical Company	Goat anti-mouse IgG-HRP conjugate, Goat anti-mouse IgG-FITC conjugate, Goat anti-mouse IgG-Cy5 conjugate, Goat anti-rabbit IgG-HRP conjugate
Dr Susan Graham, MRC Virology Unit	Ab726 (Polyclonal anti-peptide antibody made against C-terminus of pORF6)
Dr Terry McDonald	Made the peptide corresponding to the C-terminus of pORF6 used to make Ab726.

2.1.4 Radiochemicals

γ -³²P-ATP and ³⁵S-Methionine and were supplied by NEN.

2.1.5 Plasmids

Plasmid pACLL29.1 was described previously (Livingston and Jones, 1989). Cos 86 was obtained from Dr Andrew Davison, MRC Virology Unit, Glasgow.

2.1.6 Oligonucleotides

Oligonucleotides used for PCR amplification and sequencing of ORF6 were obtained from MWG-Biotech AG.

Oligonucleotides used for EMSAs were obtained from Sigma-Genosys.

2.2 Miscellaneous materials

Other materials used in experimental work were obtained from the following;

Amersham Pharmacia Biotech	Nitrocellulose membrane
Medicell International Ltd	Dialysis membrane
Dynex Technologies Inc, USA	Immulon 1 microtitre plates
Whatman International Ltd	3MM filter paper, anion exchange paper
Kodak Ltd	X-omat film
UKC Chemical Laboratories	Citifluor

AE buffer	100 mM Tris-HCl (pH 8), 100 mM NaCl, 2 mM EDTA, 0.5% (v/v) deoxycholate, 1% (v/v) NP40, 10% (v/v) glycerol, 1 complete protease inhibitor tablet per 50 ml
Cell Fix solution	-20°C acetone/methanol (1:2) solution
Cell freezing Mix	Appropriate cell medium + 10% DMSO
Cell Permeabilisation Solution	19 ml PBS, 1 ml 10% NP40, 2 g sucrose
Coomassie blue gel stain	5% (v/v) methanol, 7% (v/v) acetic acid, 0.2 % Coomassie brilliant blue
DNA loading buffer (5x)	4% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol
ELISA washing buffer (10x)	1.45 M NaCl, 75 mM Na ₂ HPO ₄ , 28 mM NaH ₂ PO ₄ , 0.5% (v/v) Tween-20
Gel destain	5% (v/v) methanol, 7% (v/v) acetic acid, 88% (v/v) water
IP wash	100 mM Tris-HCl (pH 8), 100 mM NaCl, 2 mM EDTA
PBS	170 mM NaCl, 3.4 mM KCl, 10 mM Na ₂ HPO ₄ , 1.8 mM KH ₂ PO ₄ , 6.8 mM CaCl ₂ , 4.9 mM MgCl ₂ (pH 7.2)
PBS-Tween	PBS with 0.001% Tween-20
Resolving gel buffer	0.74 M Tris-HCl (pH 8.0), 1% SDS
SDS-PAGE sample buffer	6% SDS, 30% stacking gel buffer, 30% glycerol, 210 mM β-mercaptoethanol, 0.3% bromophenol blue
SDS-PAGE tank buffer	52 mM Tris, 53 mM glycine, 0.1% SDS
Stacking gel buffer	0.122 M Tris-HCl (pH 6.7), 0.1% SDS
TAE	40 mM Tris-acetate, 1 mM EDTA
TBS	20 mM Tris-HCl (pH 7.5), 500 mM NaCl
Towbin transfer buffer	25 mM Tris base, 192 mM glycine, 20% methanol (v/v), 0.01% SDS (w/v)

2.4 Cells and cell culture

2.4.1 Bacterial cells and culture

E. coli strain DH5 α was used for the maintenance and propagation of plasmid DNA.

Bacterial cells were grown in LB. Agar plates were made using 1.5 % (w/v) agar in LB.

When necessary, media and agar plates were supplemented with the appropriate antibiotics.

2.4.2 Eukaryotic cells

All cell media and supplements were obtained from Gibco, BRL. The following cell lines were used in experimental work. The *Sf* cells were obtained from the cytology department in the Institute of Virology. The B cell lines were obtained from Dr David Blackbourn from the Institute of Virology.

<i>Spodoptera frugiperda</i> -21 (<i>Sf</i>)	Insect cell line derived from worm ovarian tissue (Vaughn <i>et al.</i> , 1977).
BCBL1	B cell line latently infected with KSHV established from a human primary effusion lymphoma (Drexler <i>et al.</i> , 1999).
BJAB	EBV negative B-lymphoma cell line. (Source: American type culture collection)

2.5 Cell Culture

2.5.1 Insect cell culture

Sf cells were grown in 175cm² plastic flasks containing 50 ml TC-100 medium supplemented with 5% FCS, 1% penicillin-streptomycin and incubated at 28°C without CO₂. Cells were passaged by removing the existing medium then shaking the cells into a small amount of fresh *Sf* medium added to the vessel. Harvested cells were typically split 1:4 into fresh flasks.

2.5.2 B-cell culture

B cells were grown in 80 cm² plastic flasks containing 20ml RPMI medium supplemented with 10% FCS, 1% L-glutamine, 1% non-essential amino acids, 1% neomycin. Cells were passaged every 5 days by diluting with fresh medium, typically 1:4, and transferred into fresh flasks.

2.5.3 Hybridoma cell culture

Hybridoma cells were cultured in 96 well plates in Dulbecco's medium supplemented with HAT (0.1 mM sodium hypoxanthine, 0.4 μM aminopterin, 0.01 mM thymidine), 10% FCS, 1% L-glutamine, 1% penicillin-streptomycin. On the appearance of a single colony the cells were transferred into 24 well plates. Cells were subsequently cultured into 25cm² flasks and then 80cm² flasks.

2.6 DNA Manipulation

2.6.1 PCR amplification of ORF6

PCR amplification was performed on an Eppendorf Mastercycler PCR machine.

Reaction mixtures were subjected to an initial denaturation step of 95°C for 5 minutes, then allowed to cycle 30 times through the following sequence of temperatures; 1) template denaturation at 95°C for 1 minute, 2) primer annealing at 55°C for 1 minute, 3) DNA polymerisation for 4 minutes at 72°C. Specimens were then held at 4°C.

Reaction mixtures contained primers (100ng), 10 × vent buffer, 10mM dNTP mix, vent DNA polymerase (10 units), cos 86 (100ng) (cosmid containing pORF6 gene) in 50 µl.

The primers used to amplify the ORF6 gene from Cos 86 were:

5'-GGCCCCGATCCATGGCGCTAAAGGGCACACAAA-3' (forward)

5'-GGCCCCGATCCCTACAAATCCAGGTCAGAGAGC-3' (reverse)

The stop and start codons are shown in red and the *Bam*HI sites are underlined.

2.6.2 Restriction enzyme digestion of DNA

Restriction enzyme digestions were carried out using commercial enzymes and corresponding buffers. Typically, 0.5-2 µg of DNA was digested in a final volume of 10-20 µl using an excess of enzyme (5-10 units/digest) and the corresponding buffer at the recommended temperature for 1-2 hours.

2.6.3 Agarose gel electrophoresis of DNA

DNA samples were mixed with a 1/5 volume of DNA loading buffer and loaded into wells in horizontal 1% agarose gels made in 1x TAE containing 0.5 µg/ml EtBr.

Electrophoresis was carried out using Bio-Rad sub-cell DNA gel electrophoresis systems with the gel submerged in 1x TAE also containing EtBr for approximately 40 minutes at 70 V. DNA was visualised using either a short wave or long wave UV transilluminator, as appropriate.

2.6.4 Purification of DNA from non-denaturing agarose gels

DNA fragments resolved by agarose gel electrophoresis were visualised under long-wave UV illumination and the required bands were cut from the gel. The DNA was purified using the GeneClean kit by Bio 101 Inc., according to the manufacturer's instructions and using supplied materials. The volume of the gel slices was determined and 3x volumes of NaI were added. The gel was melted by incubating at 45°C for 5-10 minutes. A 10 µl volume of DNA-binding glassmilk was added and the solution was incubated at room temperature, with mixing, for approximately 15 minutes. The glassmilk was pelleted at 13 000 rpm for 5 secs and washed using 400 µl of wash solution. This wash was repeated twice. After the final wash, tubes were left open at room temperature for 10 minutes to ensure ethanol from the wash solution had evaporated. The DNA was re-suspended in dH₂O, approximately 20 µl for ~ 5 µg of DNA.

2.6.5 DNA ligation reactions

Ligation of DNA fragments and linearised plasmid DNA was carried out as follows. Fragment and plasmid DNA were mixed such that the fragment or insert DNA was in molar excess over the plasmid DNA by approximately 3-fold. One unit of bacteriophage T4 DNA ligase enzyme was added along with an appropriate amount of 10X ligase enzyme buffer in a final volume of 20 µl. Reactions were incubated overnight at room temperature. The plasmid was subjected to treatment using calf intestinal phosphatase (CIP) enzyme prior to the ligation, in order to prevent recircularisation during the ligation reaction. Typically, 10 units of CIP along with phosphatase buffer was added to 1 µg of

restriction enzyme-digested plasmid DNA in a final volume of 20 μ l and incubated at 37°C for one hour.

2.6.6 Preparation of competent DH5 α *E. coli* cells

A single colony of DH5 α *E. coli* cells was used to inoculate 5ml of LB and grown overnight at 37°C in an orbital shaker. The next day, 1 ml of the overnight culture was diluted in 50 ml LB and allowed to grow for 2 hours at 37°C, with shaking. Cells were chilled on ice for 20 minutes and then pelleted at 3000 rpm (microfuge) for 5 minutes at 4°C. The pellet was resuspended in 25 ml 0.1 M CaCl₂ and incubated on ice for 30 minutes. The cells were pelleted again at 3000 rpm (microfuge) for 5 minutes at 4°C and finally resuspended in 4 ml 0.1 M CaCl₂. Cells were stored at 4°C for at least one hour before use in transformation reactions.

2.6.7 Transformation of competent DH5 α *E. coli* cells

Approximately 100 ng of DNA (either unmodified plasmid DNA or that from ligation reactions) was mixed with 200 μ l of competent cells and incubated on ice for 30 minutes. Competent cell/DNA mixtures were then subjected to “heat-shock” by incubating at 42°C for 90 seconds. 800 μ l of LB was added immediately and samples were incubated at 37°C for 1 hour, with shaking. Cells were then pelleted at 13000 rpm in a microfuge for 30 seconds and 800 μ l of the supernatant discarded. The cell pellet was resuspended in the remaining 200 μ l of medium and then spread onto LB agar plates, containing

ampicillin (50 µg/ ml) using a sterile plastic spreader. Plates were incubated overnight at 37°C.

In the case of cells transformed using DNA from ligation reactions, individual bacterial colonies that had grown were picked from agar plates into 5 ml of sterile LB containing the appropriate antibiotic (to which the transformed plasmid confers resistance) and shaken overnight at 37°C. The following day, small-scale preparations of plasmid DNA were made.

2.6.8 Small scale preparation of plasmid DNA (mini-prep)

Bacteria contained in 10 ml of overnight culture were pelleted by centrifugation at 13000 rpm (microfuge) for 30 seconds and the supernatant discarded. Plasmid DNA was then isolated using a 'Perfect Prep' kit (5'→3', Inc) according to the manufacturers instructions and using reagents as supplied. Plasmid DNA was eluted from the column using 60 µl of dH₂O at 65°C, and stored at -20°C.

2.6.9 Large-scale preparation of plasmid DNA (maxi-prep)

A single colony of bacteria was used to inoculate 100 ml of LB containing the appropriate antibiotic, using a sterile loop. Cultures were grown in 500 ml flasks. The cells were pelleted by centrifugation at 3000 rpm for 15 minutes and the supernatant was discarded. The DNA was extracted using a Qiagen maxi-prep kit following the manufacturers instructions.

2.6.10 DNA sequencing

An ABI automated sequencer was used for sequencing of double-stranded recombinant plasmid DNA, using the dideoxy method of Sanger, (1977). Sequencing was carried out by Aidan Dolan. Alignment of the DNA sequence was carried out using Gap4 (Staden *et al.*, 1998).

2.7 Analysis of Proteins

2.7.1 SDS-PAGE

Complex protein mixtures were resolved using SDS-PAGE. The Bio-Rad Mini Protean II gel apparatus was used to prepare and run mini gels. Usually 10 % polyacrylamide resolving gels (acrylamide:bisacrylamide 37.5:1) were prepared in 1x running gel buffer and poured between vertical mini-gel plates. A 7.5% polyacrylamide stacking gel (acrylamide:bisacrylamide 19:1) prepared in 1x stacking gel buffer was polymerised on top of the resolving gel. Prior to loading into gel wells, protein samples were mixed with 1/3 volume of SDS-PAGE sample buffer and boiled for 5 minutes. Gels were electrophoresed at 180 mA for 40-60 minutes, until the dye front reached the bottom of the gel. Proteins were detected by staining gels in 0.2 % Coomassie blue stain for 10 minutes followed by de-staining or transferred onto nitrocellulose membrane by western blotting for detection using antibodies.

2.7.2 Western Blotting

Proteins were transferred from polyacrylamide gels onto nitrocellulose membranes according to the method of Towbin *et al.* (1979). Gels were placed on top of Whatman 3 MM paper presoaked in Towbin transfer buffer. A sheet of nitrocellulose membrane followed by another sheet of 3 mm paper (both pre-soaked) were placed on top of the gel. This assembly was transferred into a Bio-Rad mini trans-blot cartridge and tank as instructed. Electro-blotting was carried out in Towbin transfer buffer for 1 hour at 200 mA.

2.7.3 Detection of proteins on nitrocellulose membrane using antibodies

Nitrocellulose membranes were immersed in blocking buffer, consisting of 5% dried milk in PBS, and agitated for 1 hour at room temperature or overnight at 4°C. Blocking buffer was rinsed off using PBS containing 0.001% Tween-20 (PBS-Tween) and then the membranes were incubated with primary antibody, either in sealed plastic bags or suitable plastic containers, at room temperature for 1-2 hours. After three 5 minute washes in PBS-Tween, the membranes were incubated with either anti-mouse or anti-rabbit IgG horseradish peroxidase-conjugate antibody (depending on the source of primary antibody), diluted 1:1000 in PBS-Tween, at room temperature for 1 hour, with agitation. Unbound secondary antibody was removed by three 5 minute washes using PBS-Tween. Membranes were then transferred onto glass plates and treated using Amersham ECL western blotting reagents according to the manufacturer's instructions. After incubation, the nitrocellulose was covered with transparent film and then exposed to Kodak X-Omat film, which was processed using a Konica 101 SRA film developer.

2.7.4 Quantification of proteins

Protein concentrations were determined using the Bio-Rad protein assay kit (micro-assay), which is based on the Bradford dye-binding protein assay (Bradford, 1976), according to the manufacturers instructions. A standard curve of protein concentration against absorbance at 595 nm was produced, using BSA as the standard protein at the following concentrations ($\mu\text{g/ml}$); 50, 100, 200, 400, 800 and 1000. The absorbance of the sample proteins was measured and protein concentration calculated from the standard curve. OD was read at 280 nM.

2.8 Generation of recombinant Baculovirus

2.8.1 Transfection

The recombinant baculovirus was made using a BacPAK kit (Clontech). *Sf* cells were set up in 35ml dishes at of 1×10^6 cells/plate (2 mls) and were incubated at 28°C overnight.

The following day two tubes were prepared containing: tube 1) 0.5 μg of viral DNA (linearised baculovirus DNA) and 2 μg of plasmid DNA (pID1), made up to a volume of 500 μl with TC100 without supplements; tube 2) 15 μl of lipofectin and 485 μl of TC100 (without supplements). The contents of the two tubes were mixed together and incubated at room temperature for 15 minutes. The growth media from the 35 ml dishes was removed and the cells were washed twice with TC100 without supplements. 1 ml of the DNA/liposome mix was then added to the plates, which were incubated for 4 hours at 28°C. The cells were washed with TC100 (complete). 2mls of TC100 (complete) was then added to the dishes, which were subsequently incubated for 3 days at 28°C.

2.8.2 Screening progeny from transfection (Plaque assay)

Sf cell monolayers set up in 35ml dishes at 1×10^6 cells/plate (2 mls) were incubated at 28°C overnight. The transfected cells (see above) were harvested with their media and were placed into bijoux bottles and sonicated. Serial 10-fold dilutions were prepared in TC100 without supplements. These were neat, 10^{-1} , 10^{-2} , 10^{-3} and 10^{-4} . The medium from the *Sf* cells set up the day before was removed and 200µl of the diluted virus was added. This was adsorbed for 1 hour at room temperature. The inoculum was then removed and 2mls of 3% LGT agar diluted 1:1 with complete TC100 was added. Once set, 1ml of complete TC100 was added. The plates were then incubated for 3-5 days until plaques had formed.

2.8.3 Picking plaques

The medium left on the agarose was removed and 1ml of neutral red diluted 1/25 with complete TC100 was added. The plates were incubated at 28°C for 3 hours. Next the neutral red was removed and the clear plaques were picked using sterile yellow tips and placed into 500µl of complete TC100 in bijoux bottles. The agar plug was pipetted out of the tip and was then vortexed thoroughly. Half of this solution was frozen at -70°C and the other half was used to inoculate a cell suspension containing 5×10^5 *Sf* cells in 1 ml of medium per well in a 24 well plate. As controls two of the wells were left uninoculated. The cells were incubated for 4 days at 28°C and were then checked for CPE compared to the control cells.

2.8.4 Screening for recombinants expressing ORF6 protein

Western blot

The cells and media were harvested from each well into eppendorf tubes and were spun at 13000 rpm (microfuge) for 5 minutes. The supernatant was removed and stored in an eppendorf tube at -70°C. The cell pellet was washed twice with PBS with intervening 2 minute spins at 13000 rpm. It was then resuspended in 30 µl of SDS-PAGE sample buffer. This was run on a SDS-PAGE minigel and a western blot performed to determine which samples were positive for the ORF6 protein. The blot was probed with polyclonal antibody Ab726 which had been made against the very C-terminus of the ORF6 protein.

³⁵S-methionine labelling

24-well dishes were seeded with 1×10^6 *Sf* cells/well in 1 ml medium. At approximately 70% confluency, they were infected with 100 µl of virus from the picked plaques, or parental PAK6 baculovirus at a MOI of 10. Virus was adsorbed for 1 hour at 28°C, then fresh medium was added and the cells were incubated at 28°C overnight. The next day, the medium was replaced with 500 µl of methionine-free *Sf* cell medium containing 30 µCi per well of ³⁵S-methionine. Cells were incubated at 28°C overnight. The medium was removed and the cells were harvested in 500 µl of cold TBS and then pelleted by centrifugation at 6000 rpm (microfuge). The supernatant was discarded and the cells were washed twice more in 500µl TBS. The cells were centrifuged again at low speed, the supernatant was removed and the pellet was resuspended in SDS-PAGE sample buffer, boiled for 5 minutes and analysed by SDS-PAGE. The gel was exposed to Kodak X-Omat film, which was processed using a Konica 101 SRA film developer.

2.8.5 Preparation of stocks of AcNPV-ORF6

Large scale stocks of baculovirus AcNPV-ORF6 expressing pORF6 were generated. 60mm dishes were seeded with 2.5×10^6 *Sf* cells in 5mls of complete TC100. The following day the media was removed and the cells were infected with 300 μ l of the supernatant that had previously been stored at -70°C (2.8.4). This was adsorbed for 1 hour then 2 mls of complete TC100 was added and the plates were incubated at 28°C for 4 days. The cells and media were then harvested and sonicated. Large flasks were set up with *Sf* cells. When the cells were $\sim 70\%$ confluent they were infected with 1ml of the virus from the 60 mm dishes. This was adsorbed for 1 hour then 20 mls of TC100 was added and the cells were incubated at 28°C for 3 days. The cells and media from the flasks were then harvested and stored in bijoux bottles at -70°C . This was the original stock. An elite stock was made from this using the same method but using 5 flasks for and then a working stock was made from the elite stock also using 5 flasks. The working stock was concentrated by pelleting the virus from the supernatant at 12 000 rpm at 4°C in a Sorvall SLA1500 rotor for 2 hours. The pellet was resuspended in 3mls of complete media and stored at -70°C . Virus was titrated by a plaque assay, as described in section 2.8.2.

2.8.6 Sterility of viral stocks

The sterility of viral stocks was checked by streaking samples onto blood agar plates. The plates were incubated at 31°C for up to 5 days and any viral stocks containing bacterial contamination were discarded.

2.9 Purification of ORF6 protein

2.9.1 Infection of *Sf* cells with baculovirus AcNPV-ORF6

Typically, 10 large flasks of *Sf* cells at approximately 70% confluency (3.5×10^7 cells/flask) were infected with recombinant baculovirus AcNPV-ORF6 at a MOI of 10. Virus was adsorbed for 1 hour, and then 20 ml medium was added to the flasks, which were then incubated for 48hrs at 28°C.

2.9.2 Harvesting of infected *Sf* cells and extraction of protein

Cells from the flasks were harvested into the existing cell medium by shaking and transferred to Falcon 225 ml conical centrifuge bottles. The cells were pelleted by centrifugation at 3000 rpm for 7 minutes at 4°C (Sorvall RT-7 Benchtop centrifuge) and the supernatant discarded. The pellet was washed 3 times by resuspension in 80 ml ice-cold TBS followed by centrifugation at 3000 rpm for 7 minutes at 4°C, each time the supernatant being discarded. The cells were kept on ice throughout this procedure. Following the final wash, cell pellets were resuspended in 10 mls of extraction buffer (20 mM Hepes (pH7.6), 0.5 mM DTT, 0.5 mM MgCl₂, 10 mM NaHSO₃, 1 protease inhibitor tablet per 50 ml) then transferred to a dounce homogeniser. The cells were lysed by 10 strokes in the homogeniser and pelleted at 4000 rpm and the supernatant discarded. The pellet was resuspended in 10 mls of high NaCl extraction buffer (20 mM Hepes (pH7.6), 3.4 M NaCl, 0.5 mM DTT, 0.5 mM MgCl₂, 10 mM NaHSO₃, 1 protease inhibitor tablet per 50mls) and incubated on ice for 10 minutes. The lysed cell suspension was transferred to Sorvall 35 ml centrifuge tubes and centrifuged at 40000 rpm for 1 hour at 4°C in a Sorvall T865 ultracentrifuge rotor. The final supernatant was carefully decanted and kept on ice.

2.9.3 Ion exchange chromatography

All chromatography was carried out using the AKTA 900 purification system.

pORF6 was purified from the insect cell lysate by a two-step chromatography process.

The first column was a heparin sulphate column (5 ml). Heparin sulphate is a high capacity cation exchanger. The column was firstly equilibrated with hepes buffer (20 mM hepes (pH 7.6), 0.5 mM DTT, 0.5 mM EDTA, 100 mM NaCl, 10% glycerol).

Clarified cell extract that had been dialysed overnight against hepes buffer, was loaded onto the column at a flow rate of 2 ml/minute. The column was washed with 5 column volumes of hepes buffer. Finally a 0-1M NaCl gradient in hepes buffer was applied over 20 column volumes at a flow of 5 ml/minute. Fractions of 1ml were collected, 50 µl was taken from each fraction for use in subsequent analysis and the remainder was kept at 4°C.

Fractions eluted from the column were analysed by SDS-PAGE and staining with Coomassie blue. Fractions containing pORF6 were pooled and dialysed overnight against Tris buffer (see below).

The second column was a monoQ column (1 ml), which is a strong anion exchanger.

The column was firstly equilibrated with Tris buffer (20 mM Tris-HCl (pH8), 0.5 mM DTT, 0.5 mM EDTA, 100 mM NaCl, 10% glycerol). The pooled and dialysed fractions from the previous column were loaded onto the column. The column was washed with 5 column volumes of Tris buffer. A 0-1M NaCl gradient in Tris buffer was applied over 50 column volumes at a flow rate of 1ml/minute. Fractions of 1ml were collected and analysed on a Coomassie blue stained SDS-PAGE gel. Peak fractions were aliquoted into 50 µl volumes and frozen at -70°C.

2.9.4 Gel filtration chromatography of pORF6

A 24 ml superose column 12 HR 10/30 (Amersham) was used to determine the size of pORF6 in solution. Tris running buffer (20 mM Tris-HCl (pH8), 0.5 mM DTT, 0.5 mM EDTA, 100 mM NaCl, 10% glycerol) was used at a flow rate of 0.2 ml/min. 200µl of Blue dextran, Aldolase and BSA each at a concentration of 1mg/ml were firstly loaded onto the column, as molecular markers. These were eluted with 1.5 column volumes and a second identical run was started using 200 µl of pORF6 (2 mg/ml). 0.5 ml fractions were collected and analysed by western blot for the presence of pORF6.

2.10 Purification of MAb52

A protein G column (5 ml) was used to purify MAb52. The MAb52 supernatant was dialysed overnight in a 20 mM sodium phosphate buffer (pH 7). The column was equilibrated with the same buffer. The dialysed supernatant was applied to the column. Any unbound protein was washed out with 5 column volumes of running buffer. Bound protein was eluted with a 0.1M glycine buffer (pH 2.7) over 5 column volumes. The 0.5 ml fractions were eluted into eppendorfs containing 40 µl 1M Tris (pH 9) to neutralise the solution.

2.11.Generation of ORF6-specific MAbs

2.11.1 Preparation of immunogen

Protein pORF6 was purified from insect cell lysate by two-column chromatography as described in section 2.9.3. Protein of at least 95% purity was used for immunisation.

2.11.2 Immunisation schedule

Female Balb/c mice were immunised subcutaneously initially using 20µg of soluble pORF6 emulsified in Freund's complete adjuvant. This was followed by three booster injections of 20 µg pORF6 emulsified in Freund's incomplete adjuvant, at two week intervals. Sera from test bleeds were titrated against pORF6 in ELISA to ascertain which animals exhibited the best antibody response. In preparation for the fusion, the 2 best-responding mice were given final intra-peritoneal boosts of 60 µg pORF6 emulsified in Freund's incomplete adjuvant.

2.11.3 Preparation of spleen cells for fusion

Mice were killed by cervical dislocation and the spleens removed immediately and placed in sterile DMEM medium on ice. Spleen cells were isolated by puncturing the spleen surface several times with a 26-G needle and injecting sterile medium into the spleen using another 26-G needle and syringe, forcing cells out through the perforations. The extracted cells were pelleted at 1400 rpm (Sorvall RTH-250 rotor) for 10 minutes at 4°C , the supernatant was removed and 10ml of sterile DMEM was used to resuspend the cells, which were then counted.

2.11.4 Preparation of Myeloma Cells

Confluent Sp2/0-Ag14 cells were harvested by shaking into their existing medium and then pelleted at 1400 rpm (Sorvall RTH-250 rotor) for 10 minutes at 4°C. The cell pellet was stored on ice. Some of the supernatant was retained for use in the conditioned medium to be added subsequently to the cells following fusion.

2.11.5 Fusion protocol

1 x 10⁷ myeloma cells and 1 x 10⁸ spleen cells, were mixed in a 50 ml tube and pelleted at 1400 rpm (Sorvall RTH-250 rotor) for 5 minutes. The supernatant was removed and the cell pellet tapped loose. Fusion of the splenocytes and myeloma cells was achieved by adding 1 ml of 50% PEG (1 ml of PEG mixed with 1 ml of DMEM at 37°C) to the cells and mixing gently. After 1 minute, the PEG was diluted 1:2 using 1 ml of DMEM. Dilution of the PEG was repeated by adding a further 4, 8 and 16 ml DMEM at 2, 3 and 4 minutes respectively. The cells were then centrifuged at 1400 rpm (Sorvall RTH-250 rotor) for 15 minutes and resuspended in conditioned HAT medium, comprising 75% fresh HAT medium and 25% pre-conditioned Sp2/0-Ag14 cell medium (medium in which Sp-2 cells had previously been grown) at a final concentration of 10⁷ Sp2/0-Ag14 cells per 100 ml. The cell suspension was distributed into 96 well microtitre plates by adding 150 µl per well, and incubated at 37°C in a humidified incubator with 5% CO₂.

2.11.6 HAT selection and maintenance of fused myeloma/spleen cells

The cells were checked after 3 days to ensure the HAT medium was killing the Sp2/0-Ag14 cells. At 7-10 days following the fusion, the wells were monitored for the appearance of large colonies of hybrid myeloma/spleen cells. The supernatant from wells containing single colonies of diameter approximately one third of the well was screened for reactivity against ORF6 by ELISA, as described below.

2.11.7 ELISA screening of hybridoma cell supernatant

Immulon 1 microtitre plates were coated with 200 ng/well of purified pORF6 diluted in PBS at 37°C overnight, then blocked using 2% BSA in PBS (100 µl/well) for 1 hour at 37°C. Hybridoma cell supernatant (50 µl) was added to the wells and incubated at 37°C

for one hour. The plates were washed 6x in PBS + 0.005% Tween 20 and were tapped dry before adding 50 µl/well of anti-mouse-HRP conjugate secondary antibody and incubating at room temperature for 1 hour. Unbound conjugate was removed by washing 6x using PBS/Tween-20 and the plates tapped dry. 100 µl per well of ABTS-peroxidase substrate was added and the colour change in each well after twenty minutes was measured by reading the optical density at 405 nm. pORF6-reactive supernatant was designated as that producing a reading more than twice that produced by control supernatant from Sp-2 cells.

2.11.8 Propagation of positive-secreting hybridoma cell lines

Cells secreting positive antibody were transferred to small flasks and topped up with fresh HAT medium. Once the cells had reached confluency in the small flasks, the supernatant was tested again for reactivity against ORF6 to ensure they were still secreting ORF6-reactive antibodies. If positive for anti-ORF6 antibody, cells were harvested and transferred to medium flasks and, ultimately, large flasks. Once cells were confluent in large flasks, the supernatant medium was collected and frozen in aliquots at -20°C. The hybridoma cells were aliquoted in HAT medium containing 10% DMSO and frozen down for long-term storage in liquid nitrogen.

2.12 Testing of MAbs

2.12.1 Testing reactivity of MAbs against ORF6 in western blotting

Cell extracts from *Sf* cells infected with the recombinant baculovirus AcNPV-ORF6 or PAK6 baculovirus were used. The cell extracts were subjected to SDS-PAGE and transferred to nitrocellulose as described in section 2.7.2. The nitrocellulose membranes

were cut into 0.5 mm strips and incubated in blocking buffer overnight at 4°C.

Individual strips were incubated with 1 ml of a single hybridoma cell supernatant at room temperature for 1 hour. The remainder of the western blot was carried out as described in 2.7.3.

Cell extracts from B cells infected with KSHV were also used. Cells were induced by adding sodium butyrate (1 mM). At various times after induction the cells were pelleted in a microfuge at 6000 rpm for 5 minutes and lysed in a cell lysis buffer (100 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 1% NP40, 0.5% sodium deoxycholate, 0.5 mM PMSF). The cell extracts were used in a western blot as described in sections 2.7.1-2.7.3.

2.12.2 Testing reactivity of MAbs to ORF6 in immunofluorescence

Sf cells were set up in 35 ml dishes at 1×10^6 cells/ml (2 ml) and were incubated at 28°C overnight. The next day, the medium was removed and the cells were infected with recombinant baculovirus AcNPV-ORF6 or PAK6 baculovirus at a MOI of 10 and incubation continued at 28°C. After 48 hours, the cells were washed 3 times using PBS-Tween. The cells were then resuspended in PBS and placed onto a slide using a cytospin (800 rpm for 3 min) and fixed by soaking in -20°C acetone/methanol (1:2) solution at -20°C for 20 minutes. After a further 3 washes with PBS-Tween, blocking solution (1% FCS in PBS-Tween) was added and left at 37°C for 1 hour. This solution was removed and 400 µl of undiluted supernatant medium from monoclonal hybridoma cells was added. After 1 hour at 37°C, the cells were washed 3 times using PBS-Tween (1%). A 1:200 dilution of α-mouse-FITC conjugate was added to the cells (150 µl) and incubated at RT for 1 hour. The cells were again washed 3 times using PBS-Tween. Coverslips were mounted onto a small drop of Citifluor solution (UKC), covering the cells.

Examination of immunolabelled cells was performed using a Zeiss LSM 510 confocal microscope attached to a computer operating the appropriate LSM 510 software.

Immunofluorescence of B cells infected with KSHV was carried out using a similar method except the cells were placed onto the microscope slide by pelleting the cells (2ml at 7×10^5 /ml), resuspending in 20 μ l of PBS and spotting onto the slide which was then air dried.

2.12.3 Screening MABs for reactivity against pORF6 by immunoprecipitation: expression of radiolabelled pORF6 protein

24-well dishes were seeded with 1×10^6 *Sf* cells/well in 1 ml medium. At approximately 70% confluency, they were infected with AcNPV-ORF6 or PAK6 baculovirus at a MOI of 10. Virus was adsorbed for 1 hour at 28°C, then fresh medium was added and the cells were incubated at 28°C overnight. The next day, the medium was replaced with 500 μ l of methionine-free *Sf* cell medium containing 30 μ Ci per well of 35 S-methionine. Cells were incubated at 28°C overnight. The medium was removed and the cells were harvested in 500 μ l of cold TBS and pelleted by centrifugation at 6000 rpm (microfuge). The supernatant was discarded and the cells were washed twice more in 500 μ l TBS. The cells were centrifuged again at low speed, the supernatant was removed and the pellet was kept on ice. Proteins were extracted by resuspending the cell pellet in 150 μ l of cold AE buffer and incubating on ice for 20 minutes. Extracts were then centrifuged at 35000 rpm for 30 minutes at 4°C in a Beckman TLA-100.2 rotor (Beckman TLA-100 benchtop ultracentrifuge). Small samples of the supernatant containing extracted proteins from AcNPV-ORF6 and PAK6-infected cells were analysed by SDS-PAGE to

check that pORF6 had been expressed. The gel were dried and exposed to a phosphorimage screen.

2.12.4 Immunoprecipitation of pORF6 from insect cell extracts

Proteins were extracted in cold AE buffer as described above. Cell extracts were mixed with 100 μ l of MAb (undiluted hybridoma cell supernatant) for 2.5 hours at 4°C. 50 μ l of a 50% suspension of Protein A-sepharose beads in buffer AE was then added and mixed for a further 1.5 hours at 4°C. Samples were then centrifuged at 6000 rpm in a microfuge for 2 minutes to pellet the protein A sepharose beads and the supernatant was discarded. The beads were then washed to remove any proteins not specifically bound. The beads were re-suspended in 500 μ l of cold AE buffer and centrifuged at 6000 rpm in a microfuge for 2 minutes. The supernatant was discarded and the beads were washed twice. After the final wash, the pelleted beads were mixed with 50 μ l of SDS-PAGE sample buffer and boiled for 5 minutes to dissociate the bound proteins. The beads were briefly centrifuged again at 6000 rpm and the supernatant was analysed by SDS-PAGE, together with a whole insect cell lysate sample to allow identification of the pORF6 protein band. Following electrophoresis, gels were vacuum dried at 80°C for 1 hour and exposed to a phosphorimage screen.

2.13 DNA-Protein binding assay

2.13.1 Preparation of radio labelled DNA

Single stranded DNA (poly dT) was 5' labelled using T4 bacteriophage polynucleotide kinase and γ -³²P ATP (30 μ Ci per reaction) in 1 \times buffer (70 mM Tri-HCl (pH 7.6), 10 mM MgCl₂, 5 mM DTT) in a volume of 30 μ l for 30 mins at 37°C. Labelled DNA was stored at 4°C.

2.13.2 Electrophoretic Mobility Shift Assay (EMSA)

A 5% non-denaturing polyacrylamide gel (30:1 acrylamide : bisacrylamide) was prepared and pre-run for 1 hr at 100-150 V using 0.5 \times TBE as tank buffer. Binding reactions were set up in 1.5 ml eppendorf tubes containing the specified amounts of pORF6 and ³²P labelled poly dT in 1 \times buffer (20 mM tris, 0.5 mM DTT, 0.5 mM EDTA, 100 mM NaCl, 10% glycerol) in a volume of 30 μ l. These were incubated for 30 min at 37°C. The samples were loaded onto the gel without sample loading buffer.

Bromophenol blue containing sample buffer was loaded onto a separate lane to observe how far the gel had run. The gel was run for approximately 4 hours at 100-150 V in a 4°C room until the dye front was two thirds of the way to the bottom of the gel. The gel was then placed onto DE81 anion exchanger chromatography paper (Whatman), which in turn was placed onto a sheet of 3MM paper and a sheet of transparent film was placed on top of the gel. The gel was vacuum dried at 80°C for 1 hour and then exposed to a phosphorimage screen.

The phosphorimages were quantified using the Bio-rad quantity one software. Areas of the same size were highlighted around the bands to be quantified and volume analysis was carried out to determine the relative counts of radioactivity in the selected regions.

2.14 Surface plasmon resonance

Surface plasmon resonance was performed using the BIAcore 2000 in conjunction with chips and buffers supplied by BIAcore. A streptavidin chip, which consisted of four flow cells coated with streptavidin was employed. The chip was conditioned by three 1 min injections of 1M NaCl in 50 mM NaOH at a flow rate of 20 µl/min. Biotin 5' end-labelled DNA dissolved in buffer (10 mM Hepes pH 7.4, 150 mM NaCl, 3.4 mM EDTA, 0.005% v/v surfactant P20) was loaded onto the chip to about 100 response units. Two injections of 50 µl of free biotin (1 mg/ml) were then flowed over the cell to block any free streptavidin sites. One of the flow cells had no DNA loaded onto it, and was used as a reference. The 5' biotin end labelled oligonucleotides used were poly (dT)₁₅ and an oligodeoxyribonucleotide of 32 nt with the sequence GAACGCGAAGCGAAGCGTTCGCACTTCGTCCC which was kindly donated by Dr Graeme Thompson (MRC Virology Unit, Glasgow).

For all experiments the BIAcore 2000 was run at 37°C. A series of dilutions of pORF6 were made using the running buffer and were individually flowed across the chip, at a flow rate of 70 µl/min. Association and dissociation times were 180 seconds. Both the flow cells, with and without DNA were used. In between injections of pORF6, the chip was regenerated with injections of 0.5 M NaCl to remove all bound protein.

The data was analysed using the BIAevaluation software.

Chapter 3

Expression and purification of pORF6

3.1 Introduction

The aim of the work described in this section was to express and purify the product of KSHV ORF6 (pORF6) for use in subsequent experiments including the generation of monoclonal antibodies. The recombinant baculovirus expression system was selected for this purpose as it had previously been successfully employed to express the SSBs of other human herpesviruses (Boehmer and Lehman, 1993a; Tsurumi *et al.*, 1998), and it has advantages being a eukaryotic expression system. Column chromatography was carried out to purify pORF6 to near homogeneity, and was also employed to investigate the multimerisation state of the purified pORF6 in solution.

3.2 Construction of a recombinant baculovirus expressing ORF6

To construct the recombinant baculovirus, the ORF6 gene was firstly PCR amplified and cloned into the baculovirus transfer vector, pAcCL29.1 (figure 3.1), under the control of the strong polyhedrin promoter. PCR amplification of the ORF6 gene was from Cos86 DNA, a cosmid containing nucleotides 1-7437 of the KSHV genome, which was kindly supplied by Dr Andrew Davison (MRC Virology Unit, Glasgow). The primers were designed to amplify a 3.4 kbp fragment spanning nucleotides 3210-6611 containing the complete ORF6 gene (accession number U93872). *Bam*HI sites were incorporated into the primers to facilitate cloning into the transfer vector.

The PCR reaction was performed as described in methods (section 2.6.1) and the products digested with *Bam*HI. Plasmid pAcCL29.1 was digested with *Bam*HI in the presence of CIP to prevent recircularization during ligation. The digested PCR product and linearized pAcCL29.1 were run on an agarose gel (figure 3.2). The bands were excised and the DNA purified as described (section 2.6.4). The gel purified DNAs were

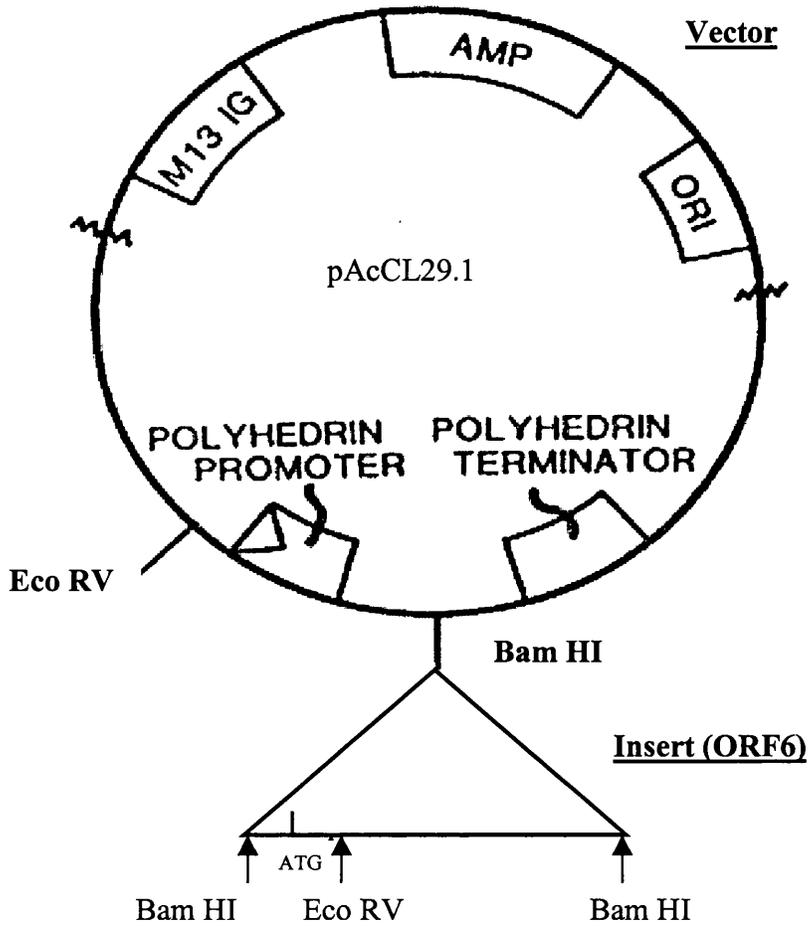


Figure 3.1 pAcCL29.1 transfer vector and insert

The ORF6 gene was inserted into the Bam HI site. The Bam HI and Eco RV sites on the insert are indicated by an arrow. The start codon is indicated with a red line. The Eco RV site in the plasmid and the unique site in the ORF6 gene allows orientation of the cloned inserts to be determined. AMP represents an ampicillin resistance gene and ORI represents the plasmid origin of replication.

ligated and the products were used to transform competent *E. coli* strain DH5α cells. The transformed cells were plated out and ampicillin resistant colonies selected. Twelve colonies were picked, grown in 10 ml liquid cultures and small scale plasmid preparations were made.

To identify plasmids containing the 3.4 kbp insert, DNA samples were cleaved with *Bam*HI and the fragments resolved on an agarose gel alongside *Bst*EII cleaved bacteriophage lambda DNA markers (figure 3.3a). The gel shows DNA from 2 colonies which produced two fragments of the sizes expected for the vector and PCR-amplified fragment.

Plasmids containing the ORF6 gene in the correct orientation were identified by digestion with *Eco*RV, which cleaves approximately 80 bp upstream of the polyhedron promoter and asymmetrically within the ORF6 gene (figure 3.1). Plasmids containing the ORF6 gene in the correct orientation should generate fragments of approximately 10.3 kbp and 755 bp, whilst insertion in the opposite orientation would yield fragments of approximately 8.2 kbp and 2.5 kbp. Figure 3.3b shows the digest patterns for two representative colonies. The fragments generated are consistent with the insert being in the wrong orientation in colony 1, but in the correct orientation in colony 2.

A colony containing the insert in the correct orientation (colony 2) was selected, named pID1, and a large scale preparation of DNA made. To confirm that no errors had occurred during PCR, the sequence of the entire insert was determined. This sequence was identical to the DNA sequence (accession number U93872) except for two nucleotide substitutions: a C to T change at position 207, and an A to G change at position 1149. These substitutions change codon 68 of the ORF6 gene from AAC to

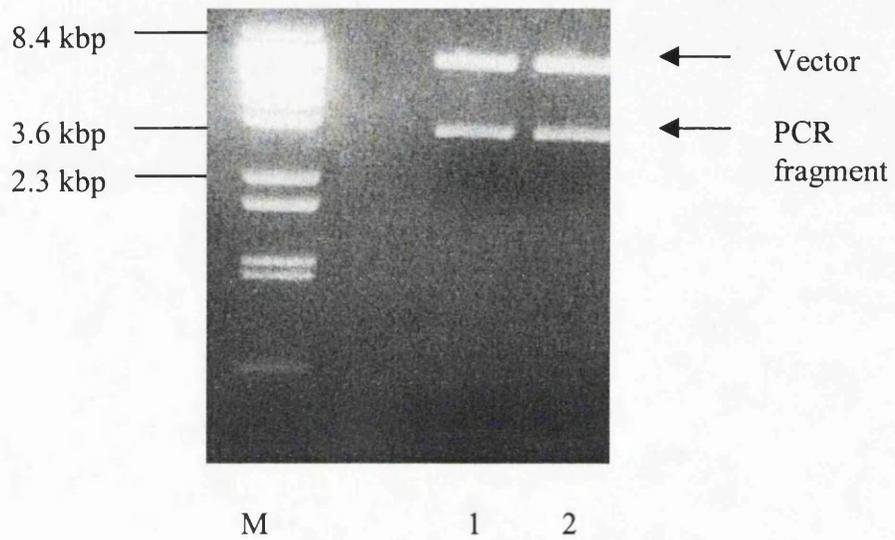


Figure 3.3a Analysis of plasmid DNA from transformants

*Bam*HI digests of DNA from two colonies run alongside lambda DNA *Bst*EII molecular weight markers (lane M) on a 1% agarose gel. The appropriate marker band sizes are indicated on the left.

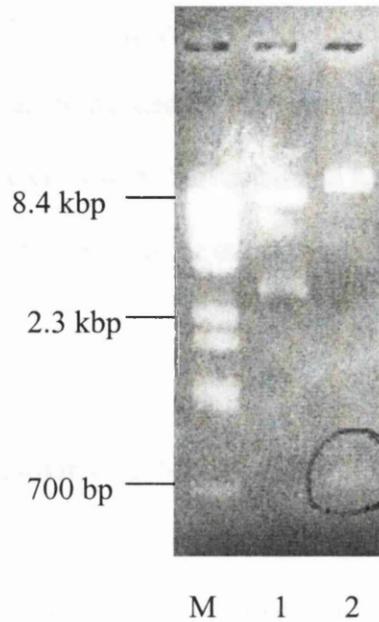


Figure 3.3b Digestion of plasmids containing ORF6 insert with *EcoRV*

DNA from colonies 1 and 2 (same as figure 3.3a) cleaved with *EcoRV*. The fragments were resolved on a 1% agarose gel. Lane M contains lambda DNA *BstEII* molecular weight markers and the sizes of the appropriate bands are indicated on the left. The 755 bp fragment diagnostic of insertion in the correct orientation is circled in lane 2.

AAT, and codon 383 from ACA to ACT, but in each case the encoded amino acid remains the same (asparagine and threonine, respectively).

To generate the recombinant baculovirus, plasmid pID1 was co-transfected into *Sf* cells with linearized DNA of the baculovirus PAK6 (clontech BacPAK Baculovirus expression system) as recommended by the manufacturers. The cells were incubated for 3 days at 28°C, progeny virus were harvested and titrated on monolayers of *Sf* cells. Plaques were picked and examined for expression of pORF6.

3.3 Recombinant baculovirus AcMNPV-ORF6 expresses the ORF6 protein (pORF6)

To select which recombinant baculovirus was to be used for production of ORF6 protein (pORF6), individual plaque isolates were first tested for expression pORF6. *Sf* cells that had been infected with the recombinant baculoviruses were labelled with ³⁵S-methionine, for the identification of expressed proteins. The labelled cell extracts were run on an SDS-PAGE gel, which was then exposed to Kodak X-Omat film, which was subsequently developed (figure 3.4). As a control *Sf* cells infected with the parental baculovirus (PAK6) were also used. This experiment demonstrated that the three recombinant baculoviruses were all expressing a protein, which correlated with that of the predicted size of pORF6 (126 kDa). Isolate number nine expressed it more strongly than the others.

The parental baculovirus also expressed a protein of a similar size, β-galactosidase (114 kDa). To confirm that it was indeed pORF6 that had been expressed by the recombinant baculoviruses and not another protein of a similar size, a western blot was

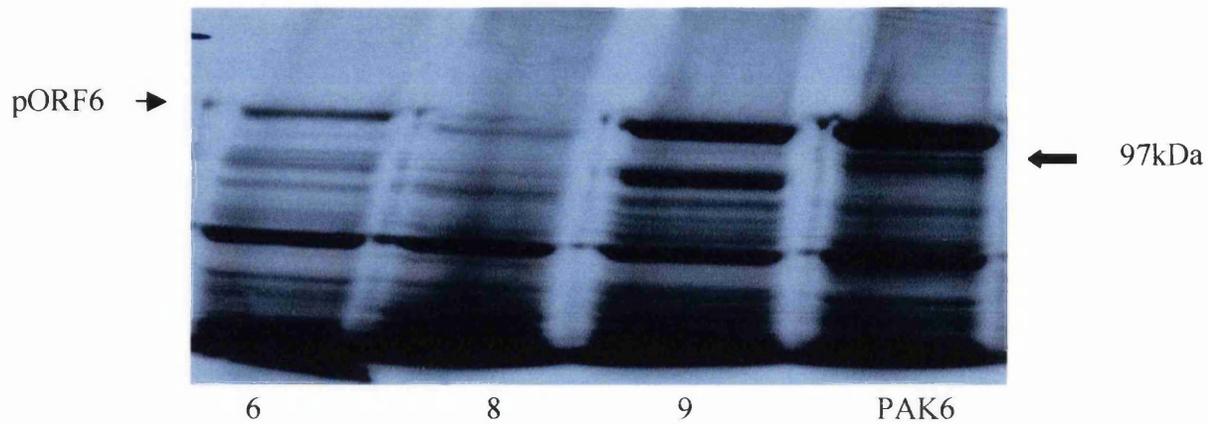


Figure 3.4 ^{35}S labelling of *Sf* cells infected with recombinant (ORF6) and parental baculovirus.

Sf cells infected with a recombinant or parental baculovirus were labelled with ^{35}S -methionone. The cells were then boiled for 5 minutes in SDS-PAGE sample buffer and resolved by SDS-PAGE. The gel was then exposed to Kodak X-Omat film, which was processed using a Konica 101 SRA film developer. Numbers 6, 8 and 9 represent different isolates. PAK6 is the parental baculovirus. pORF6 is ~126 kDa. The location of the 97 kDa marker is indicated.

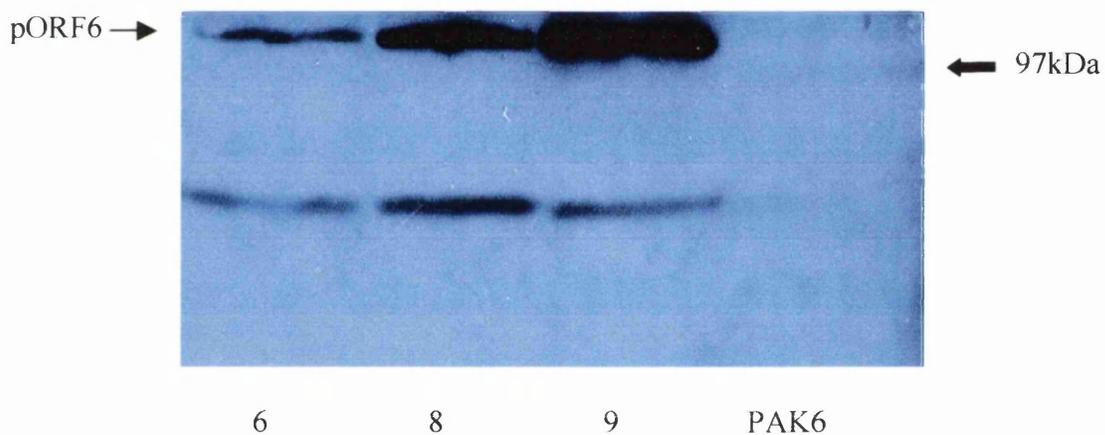


Figure 3.5 Western blot of cell extract from *Sf* cells infected with recombinant (ORF6) and parental baculovirus

Sf cells that had been infected with a recombinant baculovirus were boiled for 5 minutes in SDS-PAGE sample buffer and resolved by SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and a western blot was carried out using an anti-peptide polyclonal antibody (Ab726) made against the very C-terminus of pORF6. Numbers 6, 8 and 9 represent different isolates. PAK6 is the parental baculovirus. pORF6 is 126 kDa. The location of the 97 kDa marker is indicated.

carried out using an anti-peptide polyclonal antibody that had been made against the C-terminal 15 aa of pORF6 (Ab726) (figure 3.5). Again, *Sf* cells infected with the parental baculovirus were used as a control. This confirmed that all three isolates were expressing pORF6 as the antibody reacted against a protein of the correct size and no reactivity was seen in the control. A breakdown product of pORF6 was also detected. The antibody reacted most strongly against isolate number nine. As this was the baculovirus that was expressing pORF6 at the highest level this was chosen for the generation of ORF6 protein. Large scale stocks of this virus (named AcNPV-ORF6) were produced and titrated for use in subsequent experiments.

3.4 Purification of pORF6

For characterisation of pORF6, purified protein was required. Ten large flasks of *Sf* cells at approximately 70% confluency were infected with the recombinant baculovirus (AcNPV-ORF6). After a three day incubation period, the cells were harvested and lysed. Column chromatography was employed for the purification. The cell extract was dialysed overnight against the column running buffer and applied to the first column, a heparin sulphate column. A 0-1M NaCl gradient was run and 1 ml fractions were collected. Samples of each fraction were analysed by SDS-PAGE. pORF6 eluted from the column at approximately 0.3 M NaCl in seventeen 1 ml fractions corresponding to a major peak in the UV absorbance trace (figure 3.6). A stained gel of the peak fractions is present in figure 3.7 which shows that pORF6 is the major protein in these fractions.

The peak fractions were pooled and dialysed overnight against the running buffer of the second column, a mono-Q column, to which it was applied. A protein peak was observed at 0.3M NaCl (figure 3.8) and confirmed as pORF6 by SDS-PAGE. In addition

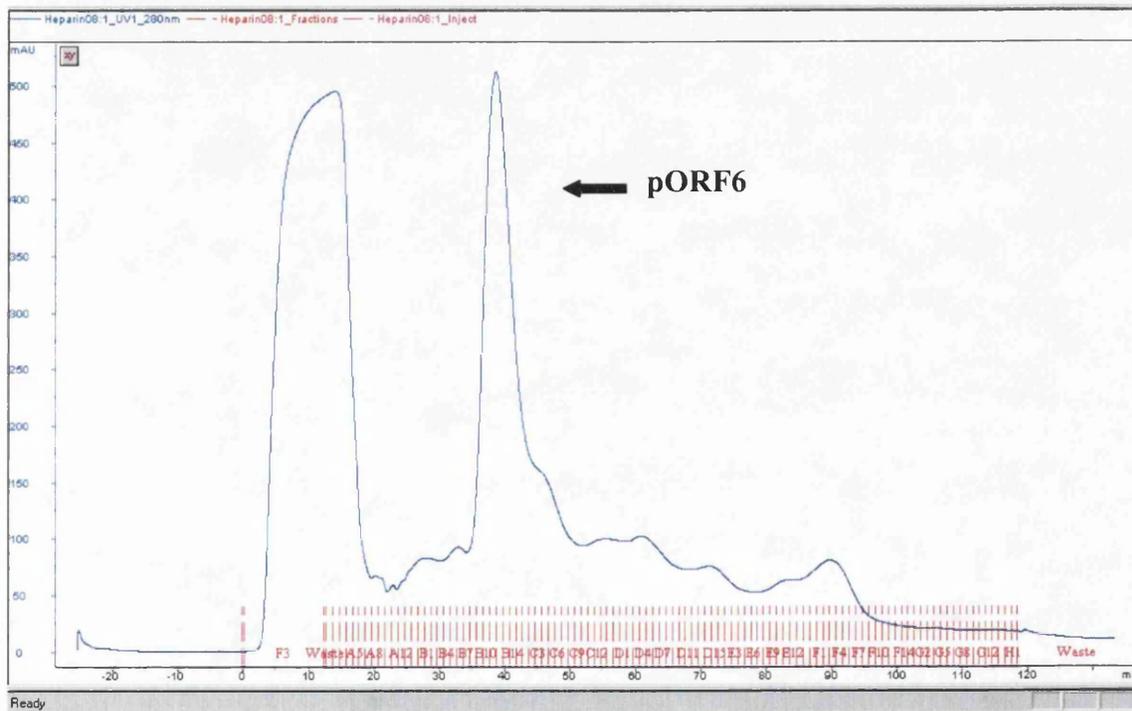


Figure 3.6 Heparin sulphate column

After being dialysed against the column running buffer the *sf* cell extract containing pORF6 was loaded onto the column. Protein that bound to the column was eluted with a NaCl gradient (150mM-1M). pORF6 was eluted with 0.3M NaCl. The trace shows the UV absorbance of fractions and the position of the peak of pORF6 is labelled.

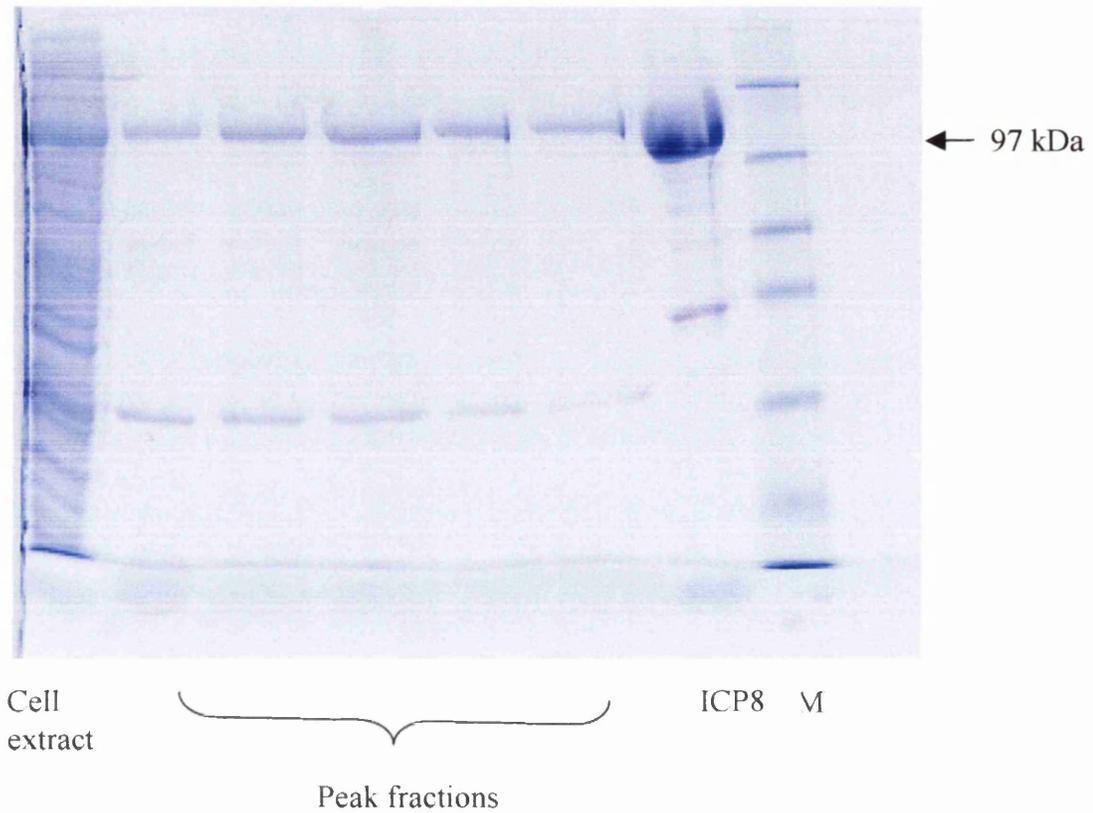


Figure 3.7 Analysis of pORF6 fractions collected from the heparin sulphate column by SDS-PAGE

Sf cells were infected with a recombinant baculovirus expressing pORF6. The cell extract was applied to a heparin sulphate column and peak fractions were collected. These fractions were analysed by SDS-PAGE along with cell extract, purified ICP8 and markers. The gel was then stained with Coomassie blue.

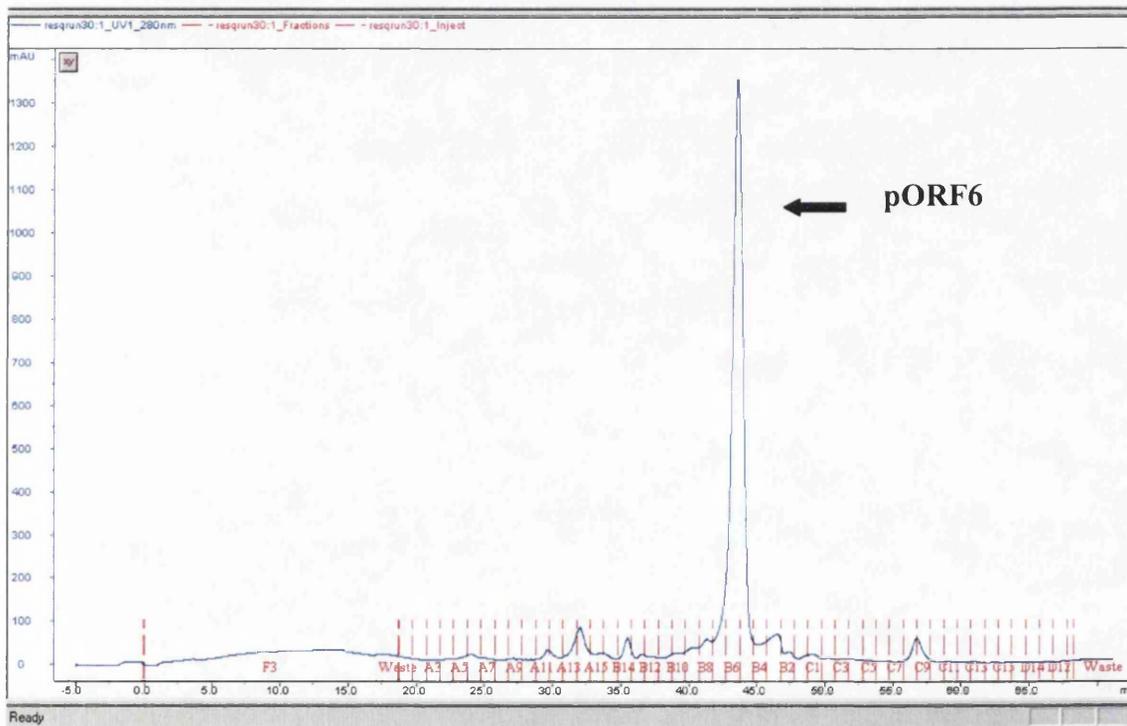


Figure 3.8 Mono-Q column

The peak fractions from the heparin sulphate column were pooled and dialysed against the column running buffer for this column. They were then loaded onto the column. Protein that bound to the column was eluted with a NaCl gradient (150mM-1M). pORF6 was eluted with 0.3M NaCl. The trace shows the UV absorbance of fractions and the position of the peak of pORF6 is labelled.

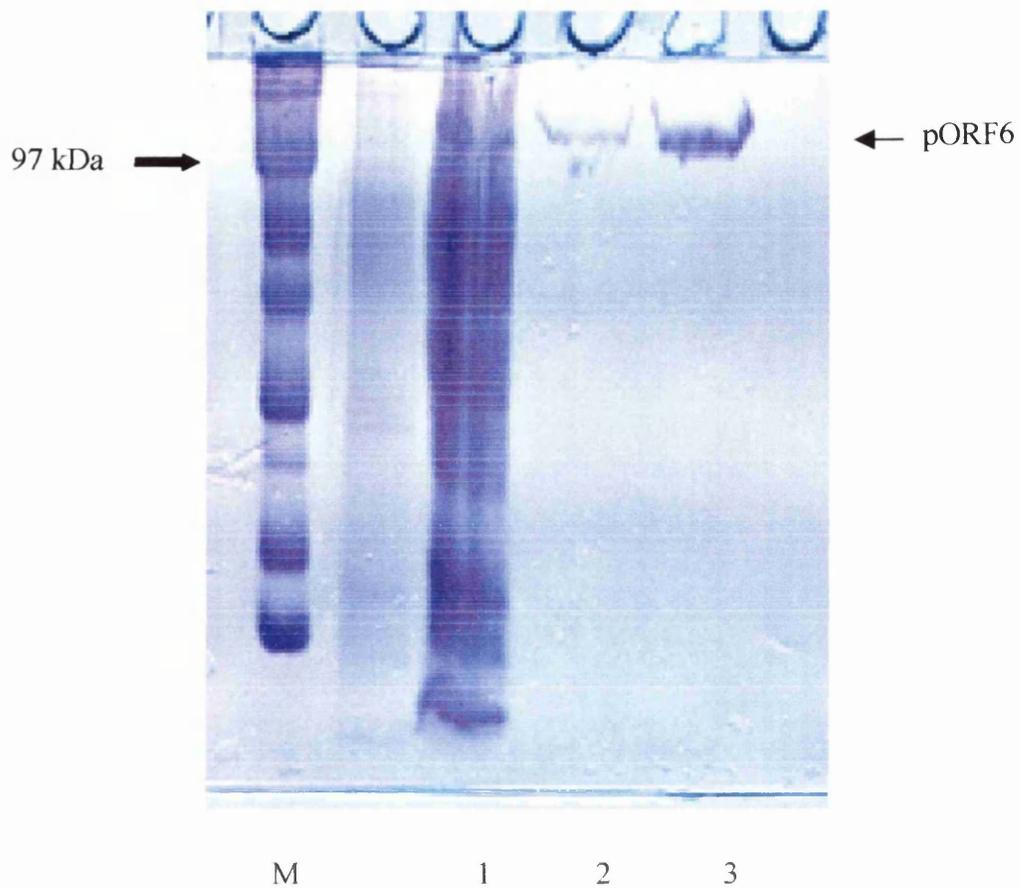


Figure 3.9 Purification of pORF6

pORF6 was purified using a two-step column chromatography method. Samples from each stage were resolved on a 10% acrylamide gel and stained with Coomassie blue. M= markers (high molecular weight rainbow markers (Amersham)). 1= nuclear extract. 2= Pooled peak fractions from heparin sulphate column. 3= Pooled peak fractions from the monoQ column. pORF6 is ~126 kDa. The 97 kDa marker is indicated.

to further purifying the protein (figure 3.9) this procedure also concentrated it, as the protein was eluted in a smaller amount of buffer (3ml).

Figure 3.9 shows an example of the peak fractions containing pORF6 collected from the two columns. The fractions from the heparin sulphate column had additional faint bands that could be seen on the gel but not on the scanned version. There were no additional bands seen after purification with the monoQ column. This demonstrates that the protein has been purified to near homogeneity. Several preparations of pORF6 were made throughout the project. The concentration of pORF6 was ~2mg/ml, as determined by a Bradford assay.

3.5 Confirmation that pORF6 has been purified

To confirm that it was indeed pORF6 that had been purified and not another protein, samples of purified pORF6 and ICP8 (HSV-1) (20 µg of each) were western blotted and the membrane was reacted with Ab726 (figure 3.10). A strong band of the correct size was seen in the lane with purified pORF6, whereas there was no reaction in the lane with ICP8. This result indicates that pORF6 was purified successfully and that the purification process has removed the breakdown fragments (figure 3.5). It also showed that the antibody did not cross-react with ICP8.

Ab726 was made against a branched peptide consisting of the 15 C-terminal amino acids of pORF6 (figure 3.11). Figure 3.12 shows this sequence aligned with the 15 C-terminal amino acids of ICP8. Although there are a few conserved amino acids and some of the amino acids are conserved in their properties, this is not sufficient for a cross reaction of Ab726 with HSV-1 ICP8.

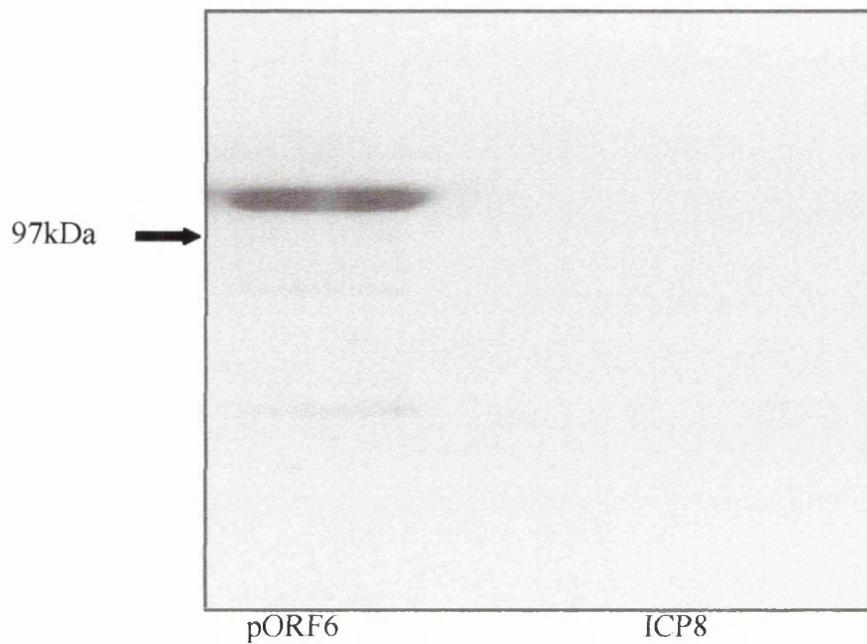


Figure 3.10 Western blot of purified pORF6

pORF6 that had been purified by column chromatography was resolved by SDS-PAGE (20 μg). It was then transferred onto nitrocellulose paper and a western blot was carried out using an anti-peptide polyclonal antibody made against the very C-terminus of pORF6. As a control purified ICP8 was also used (20 μg).

3.6 Mass spectrometry of pORF6

For additional confirmation that pORF6 had been purified mass spectrometry was employed. Mass spectrometry of a tryptic digest of the purified protein was carried out by Dr Robin Antrobus (University of St. Andrews). The peptide masses were submitted to an on-line search engine (Mascot, Matrix science). It identified the protein as the KSHV-ORF6 product, as 37 of the peptides matched to this sequence (figure 3.13). Search scores above 74 are considered significant and the search score for KSHV was 359. This was the only match that scored above 74 confirming that pORF6 had been purified.

3.7 Purified pORF6 is monomeric in solution

ICP8 (SSB from HSV-1) has been shown to be monomeric in solution up to concentrations of 5mg/ml, by the use of size exclusion chromatography and ultracentrifugation (Mapelli *et al.*, 2000). Both T4 gene 32 protein and the adenovirus DBP exist as monomers in solution, but only up to a concentration of ~ 0.1mg/ml (Alberts and Frey, 1970; Schechter *et al.*, 1980). Above this concentration, self-association of the proteins occurs, creating dimers and sometimes higher molecular weight species, depending on the conditions.

To establish whether ORF6 is monomeric in solution, a superose 12 sizing column (24 ml) was used. The column was first calibrated with proteins of known molecular weight. These were aldolase (150kDa) and BSA (67kDa). Blue dextran (2000kDa) was also used to mark the exclusion volume of the column, which is 300kDa. Molecules of a greater molecular weight than this are eluted at the same point. The aldolase and the BSA were

10	20	30	40	50	60
MAL	ENIG	PCGY	HNFP	LGNG	FSLPLLHGLT
KGPQ	SAAPT	LYAY	IGEAS	YPEAK	
TL	GT	LT	SL	KV	
70	80	90	100	110	120
VE	AVH	ASV	EAI	FQ	EKLCRESREL
SDF	HKKID	KLTSY	IVFH	IFQ	
PLNV	DATT	HR	NTHL	GKL	
K					
130	140	150	160	170	180
FG	HKG	CPQL	FMA	KER	PVPSQ
STF	TLWS	PCANE	VIVTE	LYGG	TPPVH
VEEQ	SPEA	IEI	GF	KLV	
190	200	210	220	230	240
IG	LYD	RAQ	PD	IFT	VKD
EHQ	DEDL	ELCR	ISRY	GIAQ	VST
AFK	FGPS	FYN	LHDS	ALR	VIQA
IP					
250	260	270	280	290	300
SER	KIP	FPQC	GST	VAEL	SFIEGPQDSC
QFV	KL	CASR	LMVID	GMSY	
HQD	VQAK	RGTD	SL	YGL	
QY	AKD				
310	320	330	340	350	360
EV	FENC	LRA	QAL	AANS	VAKLPQKNQR
LN	ETPD	LEVW	HIGA	VLYL	
YD	AR	HAE	QLF	TR	
WPI					
370	380	390	400	410	420
GD	LQH	ATV	FKG	GSS	YASSFSPHLL
AN	GLG	KENG	VPSA	YTLQ	
MY	YLSE	ASA	LD	HLA	
SFY					
430	440	450	460	470	480
AR	PHH	YNV	APS	GQC	LFYRMKDRFP
M	KNT	VVDY	QMC	PAVC	
Y	NSQS	VGTA	DLCQ	INT	
QFL					
490	500	510	520	530	540
PV	YVIT	NDLE	TFR	PVE	WQLCQNITEK
LS	GTAG	ILGN	EEEG	DAPK	
N	TY	FNA	GN	YTY	
KRDP					
550	560	570	580	590	600
LA	DAL	PSF	DST	FIN	NFREN
SM	RTL	VKVF	VEA	CMIK	IKSVH
ISEGG	LIVDI	FKGI	ELLK	N	
610	620	630	640	650	660
HIL	WQAP	LYY	QD	YEQ	VPSEWLKMHF
Q	PCPV	KSL	ICLT	DNP	
FAC	FLT	LLTVI	SCMM	AVGI	
NVY					
670	680	690	700	710	720
QT	CFD	ELK	CDL	GGM	VRIAR
M	KGAI	I	FD	FAP	MLMV
NFKGA	TGG	QSMF	DAAI	ARMQ	
730	740	750	760	770	780
PK	IFSN	IQAG	QRDS	YMK	TLFPSTK
T	STG	FMKP	YVGGP	FLNAL	HTSA
IKNRI	AES	PAS	GP	HK	
790	800	810	820	830	840
LY	TKN	GEHL	VKAS	INVL	LTSYAKIKLN
L	PIL	TELC	SQAF	DLVP	
WHK	PGVS	CNY	FEE	DPDT	
I					
Q					

850	860	870	880	890	900
SSILRACGQT	QFYATTL SCL	SPVTQLVPAE	EYPHVLGPVG	LSSPDEYRAK	VAGRSVTIVQ
910	920	930	940	950	960
STLKQAVSTN	GRLRPIITVP	LVVNKYTGSN	GNTNVFHCAN	LGYFSGRGVD	RNLRPESVPF
970	980	990	1000	1010	1020
KKNNVSSMLR	KRHVIMTPLV	DRLVKRIVGI	NSGEFEAEAV	KRSVQNVLED	RDNP NLPKTV
1030	1040	1050	1060	1070	1080
VLELVKPPRW	SSCASL TEED	VIYYLG PYAV	LGDEVLSLLS	TVGQAGVPWT	AEGVASVIQD
1090	1100	1110	1120	1130	
IIDDCELOFV	GPEEPCLIQG	QSVVEELFPS	PGVPSLTVGK	KRKIASLLSD	LDL

Figure 3.13 Peptide matches from the mass spectrometry of purified pORF6

Mass spectrometry of purified pORF6 was carried out by Dr Robin Antrobus (University of St. Andrews). The peptide masses were submitted to an on-line search engine (Mascot, Matrix science). Thirty-seven of the peptides matched to the ORF6 sequence. These are shown in red.

eluted from the column at 12.32ml and 18.98ml, respectively (fig. 3.14). The pORF6 that was loaded onto the column was at a concentration of ~ 2mg/ml. The peak of pORF6 was eluted at 15.01ml, which lies in between the two markers. The peak is asymmetrical with a smaller co-joining peak eluting just before the main peak, which is discussed below.

To confirm that pORF6 was eluted at this point, fractions were collected and a western blot was carried out using Ab726 (figure 3.15). pORF6 has a predicted monomeric molecular weight of 126kDa. As it is eluted after the 150Kda marker this suggests that it is monomeric in solution. However, the asymmetric shape of the peak suggests there may be an equilibrium between monomers and dimers of pORF6, with the monomeric form being favoured. There was no evidence of a peak corresponding to a stable dimer of pORF6.

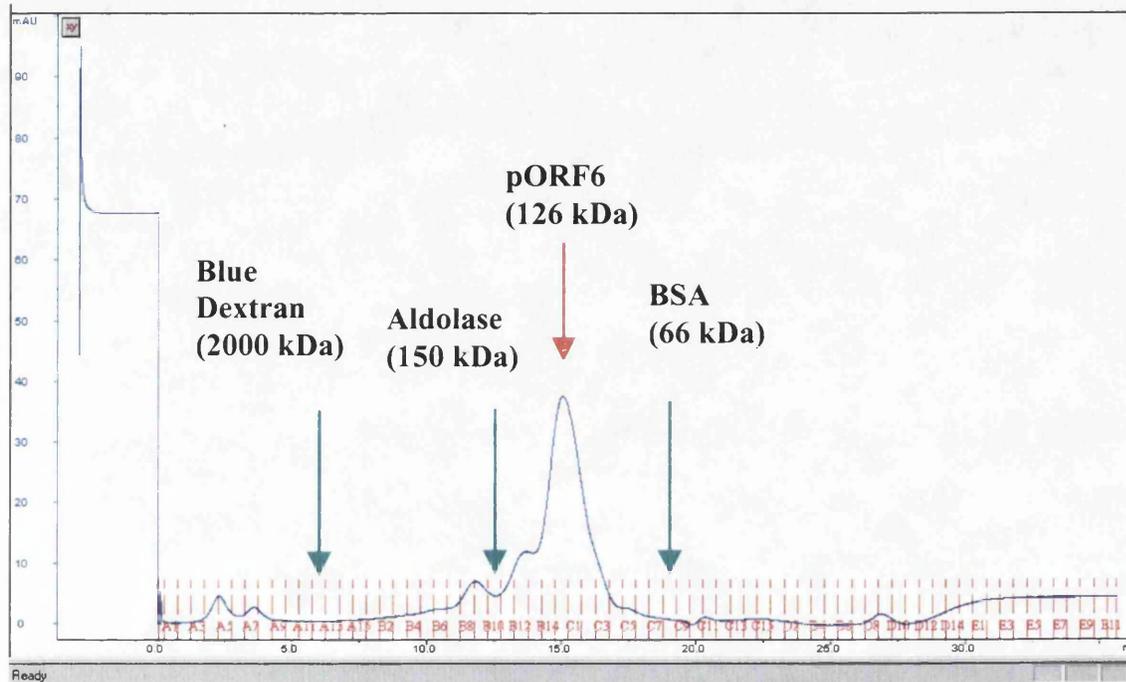


Figure 3.14 Superose 12 column

200 μ l of pORF6 at a concentration of \sim 2mg/ml was loaded onto the column. The fractions (0.5 ml) were collected and saved. The UV absorbance trace for the pORF6 run is shown. The green arrows indicate where the markers were eluted on an identical run. The main peak of pORF6 is indicated.

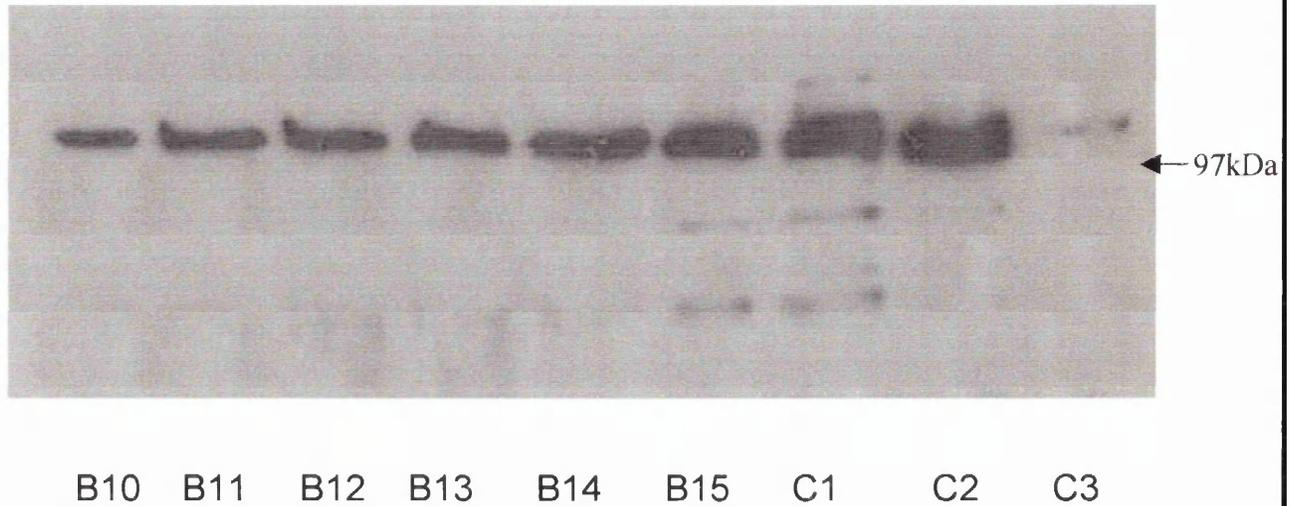


Figure 3.15 Western blot against eluted fractions from superpose 12 column. Peak fractions were collected from the superose 12 column and were resolved by SDS-PAGE. The proteins were transferred onto a nitrocellulose membrane and a western blot was carried out using an anti-peptide polyclonal antibody that had been made against the very C-terminus of pORF6 (Ab726). The label underneath indicates which fraction is loaded onto the lane. pORF6 is 126 kDa. The location of the 97 kDa marker is indicated.

3.8 Discussion

A recombinant baculovirus was successfully constructed that expresses pORF6 (AcNPV-ORF6). After infection of *Sf* cells with this baculovirus, pORF6 was successfully over-expressed and purified to near homogeneity, by a two-step chromatography process.

This resulted in pORF6 being produced at a concentration of ~2mg/ml.

The baculovirus system was used to over-express pORF6 as it is a eukaryotic system, and therefore post-translational modifications take place. The HSV-1 homologue of pORF6, ICP8, has been shown to be phosphorylated and this may also be true for pORF6. The purification method that was used was chosen as ICP8 (HSV-1) had been purified successfully by similar methods (Bohmer and Lehman, 1993; Dudas and Ruyechan, 1998). An advantage of this purification method is that attachment of a tag to the protein (e.g. a His tag) is not required. It is not known how the attachment of such tags would affect the folding or the function of the purified protein.

An anti-peptide antibody (Ab726) against pORF6 was used to confirm that pORF6 had been purified and not another protein of a similar size. This antibody had also been used to verify that the recombinant baculovirus constructed (AcNPV-ORF6) expressed pORF6. Mass spectrometry of the purified protein further confirmed the identity of the protein as pORF6, the ssDNA binding protein from KSHV.

To investigate the state of pORF6 in solution the protein was loaded onto a sizing column at a concentration of ~2mg/ml. However, running the sample through the column led to an inevitable dilution of the protein since the volume loaded onto the column was 200µl and pORF6 was eluted in 2mls of buffer it was diluted approximately

tenfold. It can therefore be concluded that pORF6 is monomeric up to 0.2mg/ml but may form higher order aggregates at higher concentrations.

As pORF6 had been purified and its state in solution had been investigated, it could now be used in experiments that characterise its binding to ssDNA. The following chapters describe such experiments.

Chapter 4
EMSA analysis of pORF6 binding to
ssDNA

4.1 Introduction

Once the ORF6 protein had been successfully over-expressed and purified to homogeneity, it was necessary to ascertain whether the purified protein was functional. As other ssDNA binding proteins have been shown to bind to ssDNA in a cooperative manner, experiments were also carried out to characterise the binding of pORF6 to ssDNA. The experiments described in this chapter used an electrophoretic mobility shift assay (EMSA). The basis of the EMSA is that protein-DNA complexes remain intact when fractionated by gel electrophoresis and migrate as distinct bands, more slowly than the free DNA fragment.

This method was employed to investigate pORF6 binding to ssDNA since the binding of ICP8 (HSV-1) to ssDNA had been successfully studied using EMSA (Mapelli *et al.*, 2000). An advantage of this method is that a qualitative result is gained by visual inspection with free and bound DNA being clearly distinguishable. For quantitative analysis, it is possible to determine the relative amount of radioactivity present in different DNA bands in the gel (volume analysis).

Volume analysis was carried out using the phosphorimager and associated software. Areas of the same size were highlighted around the DNA that needed to be determined and the amount of radioactivity within these selected areas quantified.

4.2 Purified pORF6 binds to ssDNA

To establish whether purified pORF6 could bind to ssDNA, an EMSA was employed using ^{32}P labelled $(\text{dT})_{35}$ single-stranded oligonucleotides. After the pORF6 had been incubated with the labelled DNA the protein-DNA complexes were resolved on a gel, which was dried and exposed to a phosphorimage screen (figure 4.1a). Lane 1 has protein only loaded onto it, lane 2 contains the binding assay with both DNA and pORF6 and lane 3 the probe in absence of the protein.

In the absence of protein a fast migrating band corresponding to free DNA was seen (lane 3). Two additional slower migrating bands were observed when pORF6 was present (lane 2). These correspond to protein-DNA complexes and demonstrate that purified pORF6 can bind to ssDNA.

To gain further information on the process, the gel was stained with Coomassie blue (figure 4.1b). The lane with protein only (lane 1) shows most of the protein to be present as a slow migrating smear. There is also a band of protein that migrates more quickly, which may be a breakdown product. Incubation of pORF6 with the labelled probe (lane 2) resulted in the appearance of two slower migrating bands (A and B), which correspond to the protein-DNA complex in figure 4.1. Bands A and B may represent two or one molecules, respectively, of pORF6 bound to the probe. Alternatively, they may represent binding of intact pORF6 and a smaller breakdown product.

The results in these figures show that the majority of the protein is capable of binding to DNA and that the preparation of protein is therefore highly active.

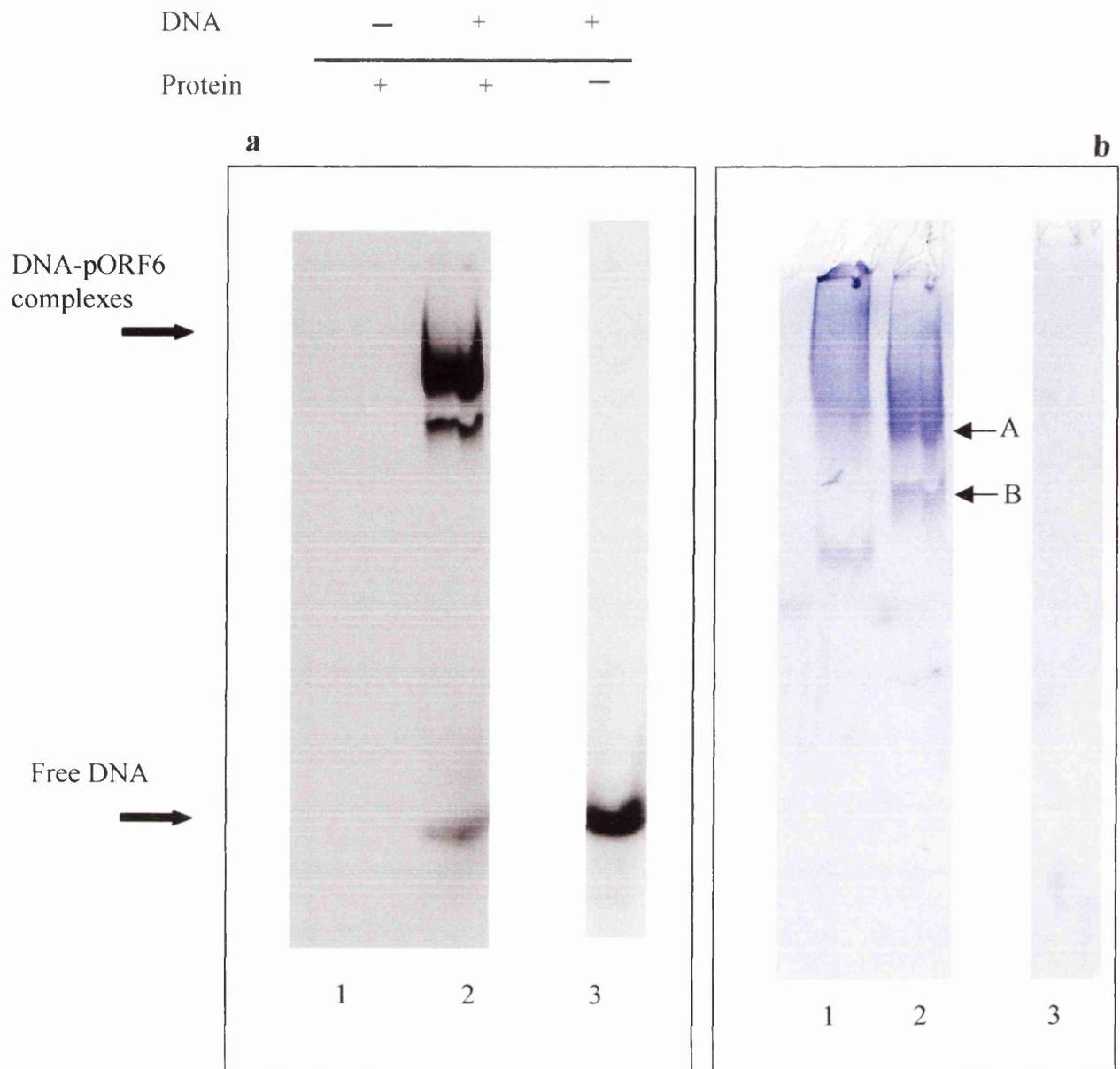


Figure 4.1a pORF6 binds to ssDNA

pORF6 was incubated with ³²P labelled ssDNA (dT)₃₅ for 30mins at 37°C. 60 pmole of labelled DNA was used in each reaction (30µl), except for lane 1 which was a control with pORF6 only (140 pmole) and no DNA. Lane 2 has DNA and pORF6 (140 pmole). Lane 3 has DNA only. The DNA-protein complexes were resolved by EMSA. The gel was then stained with Coomassie blue, dried and exposed to a phosphoimage screen. DNA bound by pORF6 and free DNA are labelled.

Figure 4.1b pORF6 binds to ss-DNA

Coomassie blue staining of the gel shown in figure 4.1a

4.3 pORF6 requires between 14 and 20 nucleotides to bind to ssDNA

The estimated DNA binding site size for ICP8 (HSV-1 homologue of pORF6) is between 12 and 40 nt, based on experiments investigating strand displacement and annealing activities (Boehmer and Lehman, 1993), nuclease protection (O'Donnell *et al.*, 1987) and electron microscopy (Ruyechan and Weir, 1984). To investigate the length of DNA required for pORF6 to bind, an EMSA using ³²P labelled DNA was again employed. Oligo-dT probes of 10, 12, 14, 20 and 28 nt (equimolar amounts) were incubated with pORF6 and the complexes were then resolved on a gel. Figure 4.2 shows the result of a representative experiment.

Binding of pORF6 to ssDNA occurred on lengths from 14 nt upwards. No binding was observed on oligonucleotide lengths shorter than this. Although binding to DNA occurred with the oligonucleotide length of 14 nt, the ability of the protein to bind to the DNA increased substantially when the DNA length was increased to 20 nt. As the length of DNA was further increased, binding of pORF6 to the DNA did not appear to increase, demonstrating that the 20 nt oligonucleotide was adequate for pORF6 to bind efficiently. Although the minimum number of nucleotides required for pORF6 to bind to ssDNA in this assay is fourteen, this clearly represents only a weak interaction with between 14 and 20 nt being required for full activity.

4.4 The effect of NaCl on pORF6 binding to ssDNA

It is thought that protein-DNA interactions are particularly sensitive to changes in salt concentration due to the cations interacting with the nucleic acid. An investigation into the effect of the NaCl concentration on ICP8 binding to ssDNA revealed that optimal

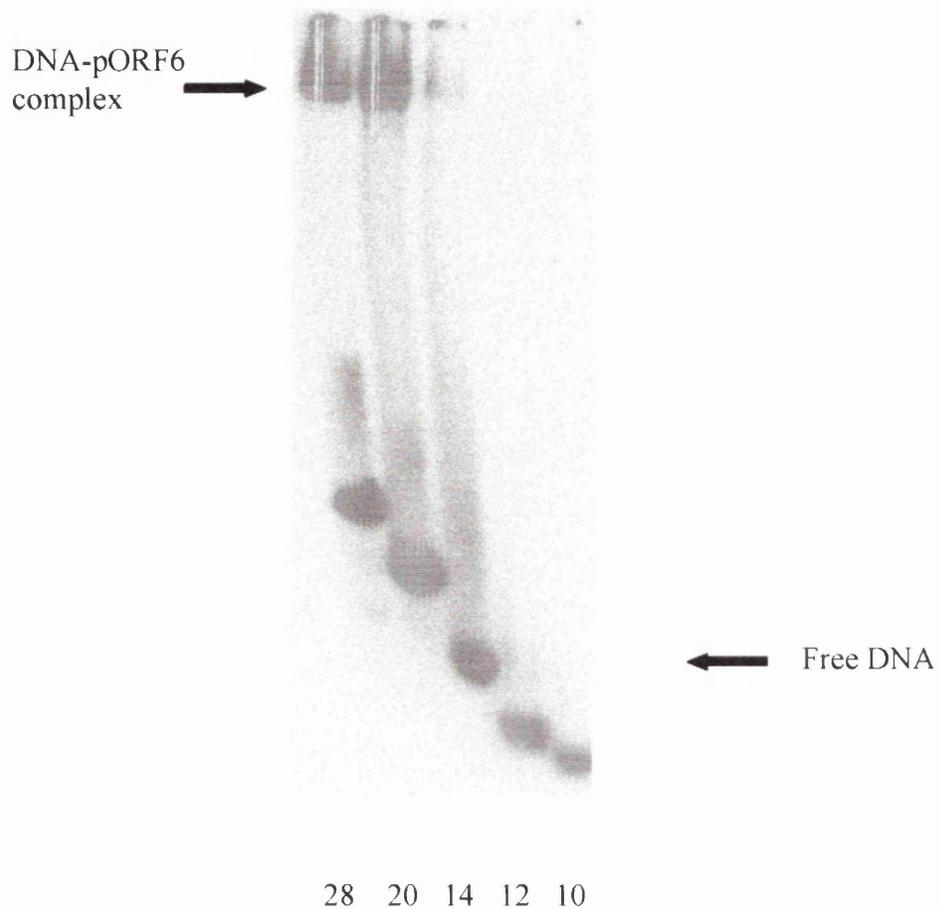


Figure 4.2 pORF6 requires greater than 12 nt to bind to ssDNA

pORF6 was incubated with ^{32}P labelled ssDNA (poly dT) of the indicated lengths (nucleotides) for 30mins at 37°C. Each 30 μl reaction contained 100pmole of DNA and 60pmole of pORF6. The DNA-protein complexes were resolved by EMSA. The gel was then dried and exposed to a phosphorimage screen. DNA bound by pORF6 and free DNA are labelled

binding occurred at 150mM NaCl with a sharp decrease in binding at 300mM (Lee and Knipe, 1985). Binding further decreased as the NaCl concentration was increased to 1M.

The effect of the NaCl concentration on the binding of pORF6 to ss-DNA was analysed by EMSA. A number of incubations were set up which were identical, except for the concentrations of NaCl which were 50mM, 100mM, 150mM, 300mM, 500mM and 1000mM. The complexes were resolved by EMSA. A representative example of this experiment is shown in figure 4.3a.

At high NaCl concentrations the mobility of the free DNA probe was affected. Binding of pORF6 to ssDNA was quantified by the amount of labelled DNA in the shifted band, which was determined by using the volume analysis program. Figure 4.3b shows a graph of these results. Maximal binding of pORF6 to ssDNA occurs at 150mM NaCl, the same as was observed for ICP8, by Lee and Knipe (1985), using a filter binding assay.

However, the capacity of pORF6 to bind to ssDNA did not decrease as sharply at 300mM NaCl as was found for ICP8. At this NaCl concentration the binding capacity of ICP8 had decreased by approximately two thirds. The concentration of NaCl required to elute pORF6 from a ssDNA column is 300mM, so it was expected that the amount of shifted complex would have been reduced dramatically. In addition, binding did not decrease as much as expected at the highest concentration of NaCl (1M).

These observations may be due to some dilution of the sample occurring when loading onto the gel, allowing increased binding of the protein to the DNA.

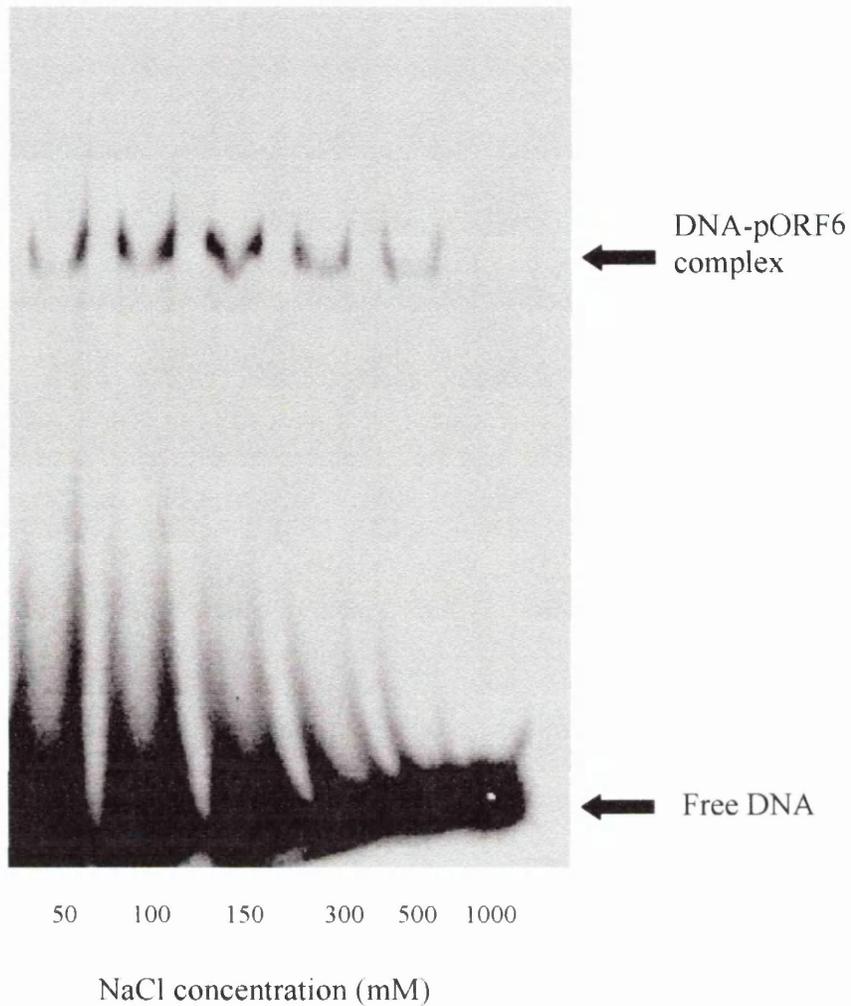


Figure 4.3a Effect of NaCl on pORF6 binding to ssDNA

pORF6 was incubated with ^{32}P labelled ssDNA (dT)₂₀ for 30mins at 37°C, in buffer containing the indicated final concentrations of NaCl. The DNA-protein complexes were resolved by EMSA. The gel was then dried and exposed to a phosphorimager screen. In each 30µl reaction 100pmole of DNA and 60pmole of pORF6 was used. DNA bound by pORF6 and free DNA are labelled.

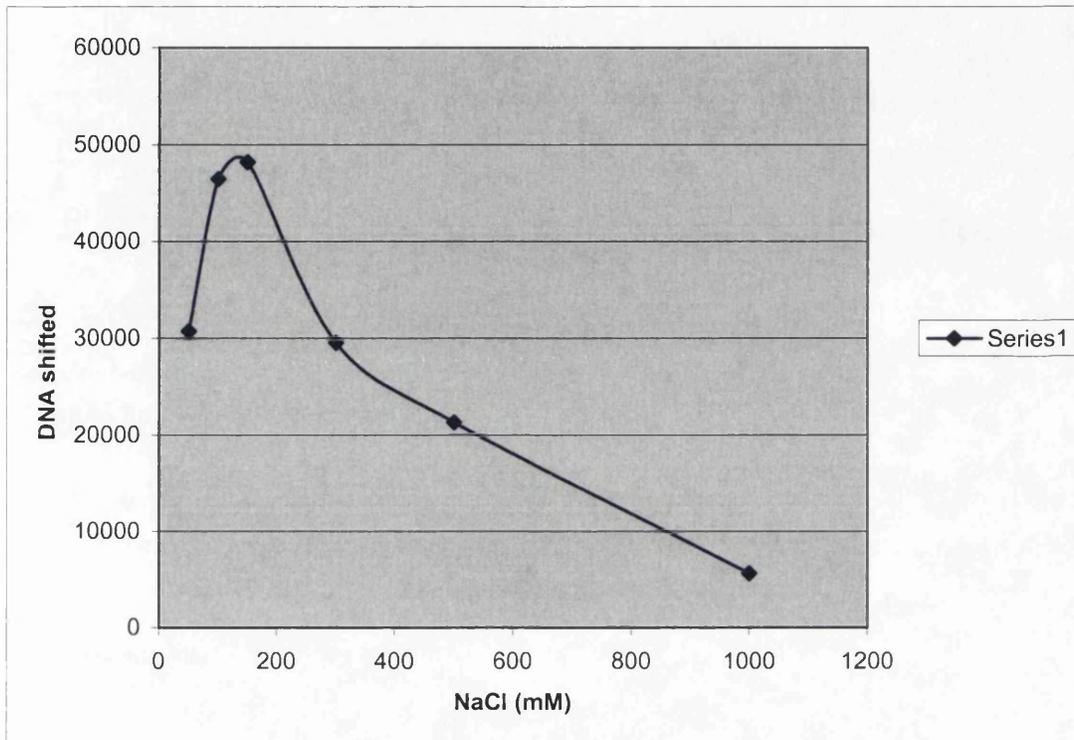


Figure 4.3b Effect of NaCl on pORF6 binding to ssDNA

The amount of DNA shifted in the EMSA in figure 4.3a was determined by volume analysis, as relative counts. This was plotted as a function of NaCl concentration.

4.5 Titration of pORF6

Many ssDNA binding proteins have been found to bind to long ssDNA molecules cooperatively. This arises from direct protein-protein interactions between nearest neighbours (Alberts and Frey, 1970; Ruyechan, 1983).

The aim of the next experiment was to determine whether pORF6 binds to ssDNA in a cooperative manner. If pORF6 binds cooperatively to ssDNA it is expected that the relationship between the amount of DNA bound and the concentration of pORF6 would not be linear. Above a certain concentration of pORF6, the amount of DNA shifted would be more sensitive to an increase in concentration due to the second molecule of protein binding having a higher affinity for the DNA, if a cooperative binding model is occurring.

To investigate the binding of pORF6 to ssDNA a titration was carried out using fixed amounts of ssDNA ((dT)₆₀) labelled with ³²P and varying the concentration of pORF6. This length of DNA was used as it should accommodate more than one molecule of pORF6, since it was shown previously that pORF6 can bind to a minimum of 14nt in this type of assay. In each reaction there was 3 pmole of DNA. The pORF6 quantity ranged from 0 to 30 pmole. After incubation at 37°C, protein-DNA complexes were resolved by EMSA (figure 4.4a). Three shifted bands of DNA were observed, which probably represent three, two and one molecules of pORF6 binding to a molecule of ssDNA. This is indicated by a schematic diagram next to the figure.

The total amount of DNA shifted at the different concentrations of pORF6 used was calculated by densitometry and was plotted on a graph (figure 4.4b). The graph has a sigmoidal shape which is indicative of cooperative binding.

Inspection of the figure 4.4a also shows that the ssDNA probe used contains breakdown products. On comparing these breakdown products with a range of oligonucleotides that had been run on another gel under the same conditions, the smallest of these breakdown products was estimated to be approximately 15 nt in length. These shorter fragments will accommodate fewer ORF6 molecules than the 60-mer. With increasing pORF6 concentration it can be seen that the longer DNA molecules are completely shifted into protein-DNA complexes before the smaller DNA molecules. This is again consistent with cooperative binding i.e. free pORF6 molecules bind with higher affinity to a protein-DNA complex (provided there is space) than to free DNA.

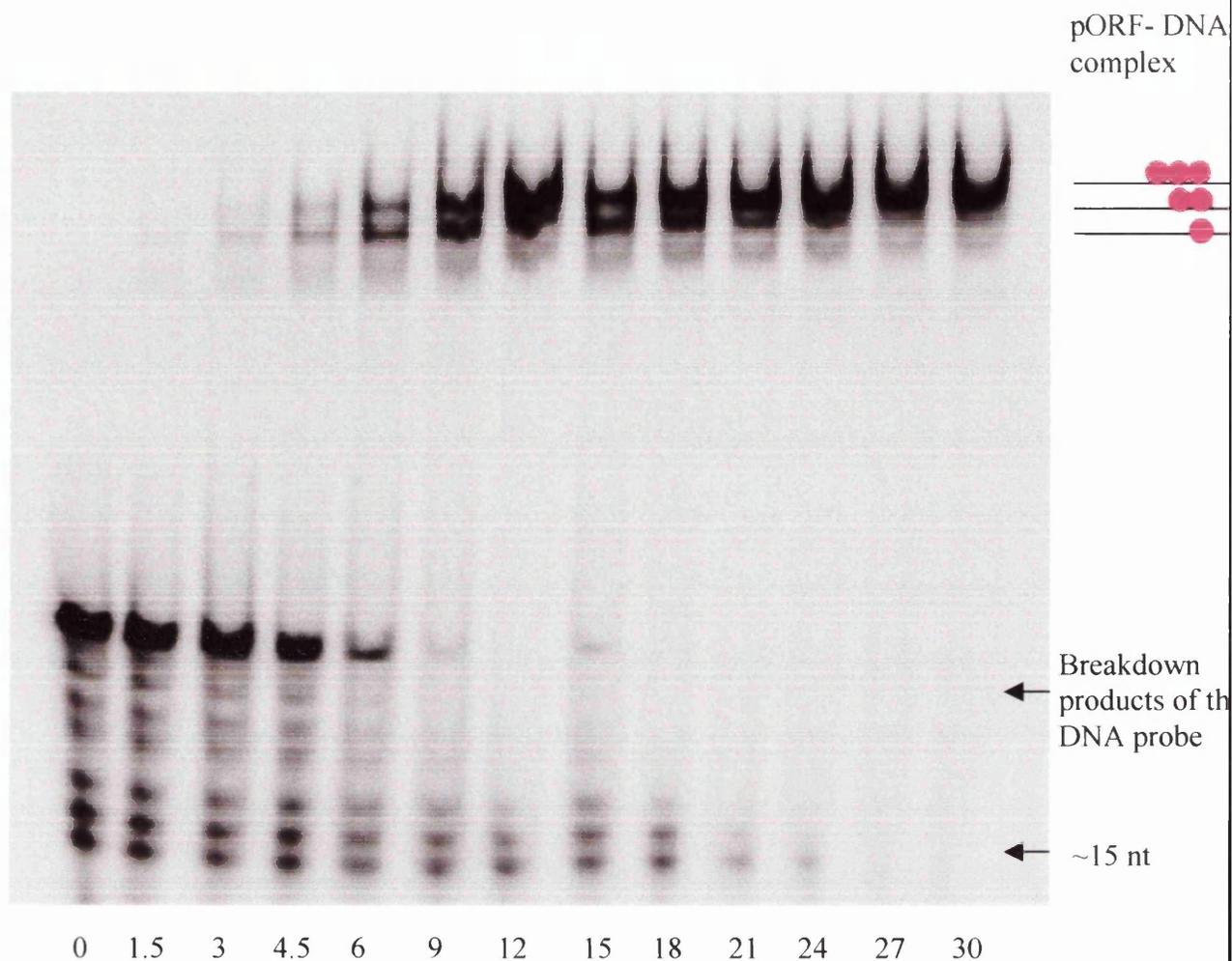


Figure 4.4a Titration of pORF6 binding to ssDNA

Different amounts of pORF6 were incubated with 3 pmole ^{32}P labelled ssDNA $(\text{dT})_{60}$. The amount of protein in each reaction is shown underneath in pmol. The DNA-protein complexes were resolved by EMSA. The gel was then dried and exposed to a phosphorimage screen. The breakdown products of the DNA probe are indicated with the approximate size of the smallest breakdown product shown. A schematic is shown of the number of molecules of pORF6 possibly binding to the ssDNA in the shifted complexes.

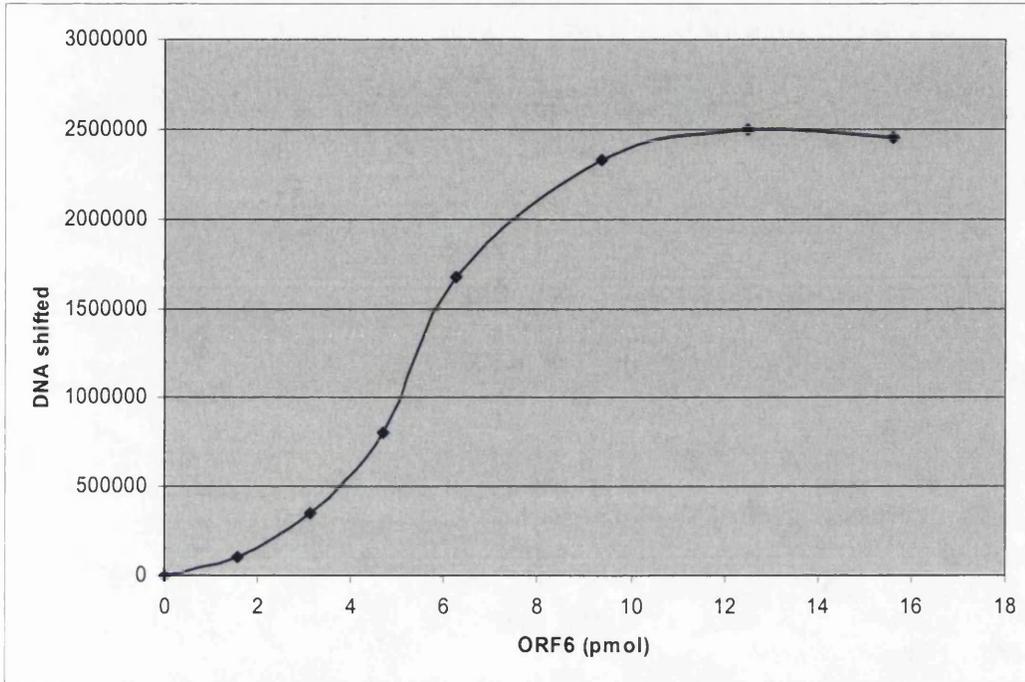


Figure 4.4b Titration of pORF6 binding to ssDNA

The amount of DNA shifted in figure 4.4a was determined by volume analysis, as relative counts. This was plotted as a function of amount of protein. Amounts of pORF6 > 15 pmole are not shown.

4.6 Discussion

The work described in this chapter produced results that gave both qualitative and quantitative information on the binding of pORF6 to ssDNA. Firstly it demonstrated that the pORF6 that had been over expressed and purified could bind to ssDNA and that the majority of molecules could participate in the formation of a protein-DNA complex and was therefore functional (figure 4.1).

The DNA molecules used in these experiments were dT homopolymers. These were used to ensure that there would be no secondary structure formed in the DNA, which may have interfered with binding. Future experiments could use heteropolymers to investigate whether this makes a difference to pORF6 binding to ssDNA, since this is what pORF6 would bind to *in vivo*. Another difference that could be made when conducting future experiments would be to use ficoll in the binding buffer instead of glycerol. Both of these agents are used in protein buffers to stabilise the protein. However, the use of ficoll apparently reduces the smearing of the edges of the DNA bands that have been bound by the protein (Garner and Revzin 1991). This would make quantification of the phosphorimage easier and more accurate.

One of the quantitative results for the binding of pORF6 to ssDNA was the observation of the minimum number of nucleotides required for pORF6 to bind to ssDNA being between 14 and 20 nt. As mentioned previously, the number of nt required for ICP8 to bind to ssDNA varied depending on the type of assay used, being between 12 and 40 nt. It would be interesting to carry out these assays using pORF6 to see if the same variations occurred.

NaCl concentration has an effect on the binding of SSBs to DNA. Two binding modes have been identified in the *E.coli* SSB when bound to ss-DNA, that are dependent on NaCl concentration (Lohman and Overman, 1985). As NaCl concentration increases the DNA binding site size also increases from 33nt to 65nt. Cooperativity of binding to ss-DNA of the *E.coli* SSB also changes with NaCl concentration, with the level of cooperative binding decreasing as NaCl is increased (Lohman *et al.*, 1986). At the lower NaCl concentrations (below 10mM) unlimited cooperative binding is observed with long chains of protein binding to the ss-DNA. At the higher NaCl concentration (above 200mM) limited cooperative binding is observed with the protein binding to the DNA as dimers. These observations suggest that increasing NaCl concentration may affect the protein-protein as well as DNA-protein interactions. Kowalczykowski *et al.* (1981) demonstrated that the binding constant of gp32 (T4 bacteriophage) to polynucleotides decreased as the NaCl concentration was raised above 200mM. The cooperativity parameter however did not change. A similar phenomenon was also shown for DBP from the adenovirus (Kuil *et al.*, 1989).

A change in NaCl also had an effect on the binding of pORF6 to ssDNA (figure 4.3). Although binding was observed at low NaCl, 150mM NaCl was required for maximal binding. As the concentration of NaCl was increased above 150mM the ability to bind to DNA decreased. Since figure 4.3 was performed using (dT)₂₀ it is not possible to determine whether NaCl concentration affected cooperativity. It would be interesting to repeat this experiment with the (dT)₆₀ probe used in figure 4.4 and to quantify the total amount of shifted DNA and whether NaCl affects the relative binding to the shorter breakdown products in the same way as to the (dT)₆₀.

ssDNA binding proteins characteristically bind to ssDNA in a cooperative manner. It is thought to aid their role in recombination, repair and replication. During initiation of replication, as an area of DNA becomes unwound to reveal ssDNA the SSB begins to bind to the area. Additional molecules then bind with greater affinity, keeping the DNA strands apart. As the replication fork advances, cooperative binding of the protein to the DNA results in a protein chain being formed along the lagging DNA strand. (Ruyechan, 1983; Alberts and Frey, 1970; Kuil *et al.*, 1989; Boehmer and Nimonkar, 2003). This holds the DNA in an extended conformation, which facilitates the polymerase activity and also protects the ssDNA from nuclease digestion.

The titration of pORF6 with ssDNA revealed that pORF6 may bind to ssDNA in a cooperative manner, consistent with its proposed role during DNA replication. This experiment needs to be repeated to confirm that pORF6 binds cooperatively. If this is confirmed, future work could also employ deletion and point mutations within the protein to identify the residues involved in cooperative binding.

A deletion mutant of ICP8 was constructed that had the 60 C-terminal amino acids deleted and also had two internal cysteine residues changed to serines. The ability of the protein to bind to oligo- dT probes of 14, 20, 28 and 35 was investigated by EMSA (Mapelli *et al.*, 2000). The analysis indicated that neither removal of the 60 C-terminal amino acids or the two internal mutations affected the intrinsic ability of ICP8 to bind to ssDNA. However, the C-terminal deletion mutants exhibited a loss of cooperativity on the longer DNA molecules (20-35 nt). A similar phenomenon was observed in a deletion mutant of the human mitochondrial SSB that had the 60 C-terminal amino acids removed (Curth *et al.* 1994). Again the implication is that the C terminus is involved in the molecular mechanism of cooperativity. Hooking of the 17 residue C-terminal region of

the adenovirus DBP to the nearest neighbour on the protein bound to ssDNA has been observed by crystallography and is presumed to be the mechanism by which the protein binds cooperatively (Tucker *et al.*, 1994).

After confirmation that pORF6 binds to ssDNA cooperatively, it would be interesting to make a similar deletion to pORF6 and to investigate its effect on its binding to ssDNA to see if the C-terminus of this protein, like some of its homologues is involved in cooperative binding. If deleting the 60 C-terminal had the same effect as it had on ICP8, additional deletion mutants could be made to further locate the region critical for cooperative binding e.g. 50, 40, 30 etc amino acids deleted.

Chapter 5
BIAcore analysis of pORF6 binding to
ssDNA

5.1 Introduction

This chapter describes work that was carried out using a BIAcore 2000, which utilizes surface plasmon resonance (SPR) and continuous flow technology to monitor molecular interactions in real time. This is an advantage over other methods that investigate binding, which only give a 'snap shot' of the events occurring during association, equilibrium and dissociation. Using the BIAcore, the entire events of association, equilibrium and dissociation can be monitored, although it should be noted that the equilibrium stage seen is not a true equilibrium but is actually a steady state, as it is an open system that is being continually supplied with ligand dissolved in buffer. This approach was used to investigate further the interaction of pORF6 binding with ssDNA.

Surface plasmon resonance is a phenomenon that occurs when light is reflected off thin metal films. A fraction of the light energy incident at a sharply defined angle can interact with the delocalised electrons in the metal film (plasmon) thus reducing the reflected light intensity. The precise angle of incidence at which this occurs is determined by a number of factors, but in the BIAcore the principal determinant is the refractive index close to the *backside* of the metal film. Target molecules are immobilised on this surface (ligand) and bound by molecules in a mobile phase (analyte) running along a flow cell. If binding occurs to the immobilised target, the local refractive index changes, leading to a change in SPR angle, which can be monitored in real-time by detecting changes in the intensity of the reflected light. The change in SPR angle can be related to a resonance signal indicative of the mass bound to the chip and plotted over time to produce a sensorgram.

The essential components of the BIAcore system are the sensor chip where the interaction takes place, the optical system responsible for the generation and the detection of the SPR signal and the liquid handling system for the transport of samples to the sensor surface (sensor chip).

As mentioned above, one of the molecules of interest is immobilized to the surface of the chip. The other molecule is free in solution, which flows over the chip surface. As molecules from the solution bind to the immobilized ligand, the resonance angle changes and a response is registered. Figure 5.1 shows a diagram outlining the events.

The raw data are presented as a real-time graph of response units (RU) against time and is referred to as a sensorgram. During injection of analyte, changes in signal result from two processes: association to and dissociation from the surface. At the end of injection, running buffer continues to flow over the chip; at this stage the change in signal results from dissociation only. Figure 5.2 depicts the details of a sensorgram.

The experiments detailed in this chapter were carried out by immobilizing ssDNA to the chip and injecting a solution containing pORF6 over it. The chips used were coated with streptavidin. The ssDNA was adhered to the chips by using ssDNA with a 5'biotin end label and taking advantage of the strong interaction between streptavidin and biotin ($K_D = 10^{-14}$ M). It is important to ensure that the chip is regenerated properly between each injection of analyte and that the baseline returns to its initial level. This was achieved by injecting a 0.5M NaCl solution over the chip, which eluted any protein remaining on the chip. For all experiments the BIAcore was set to 37°C.

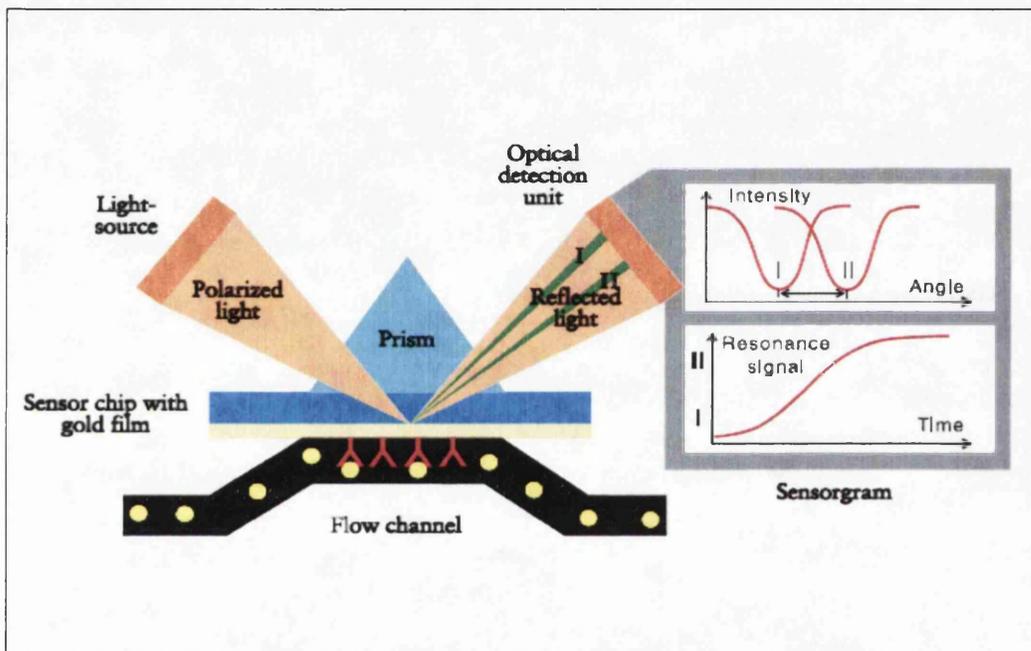


Figure 5.1 Surface plasmon resonance on the BIAcore

A diagram showing the details of events on the BIAcore. The analyte is flowed over the cell in which the ligand has been adhered. Binding of the analyte to the ligand causes a change in the angle of reflection of the polarised light beam that is applied to the gold film on the sensor chip. This is converted into a resonance signal. Adapted from www.biocore.com

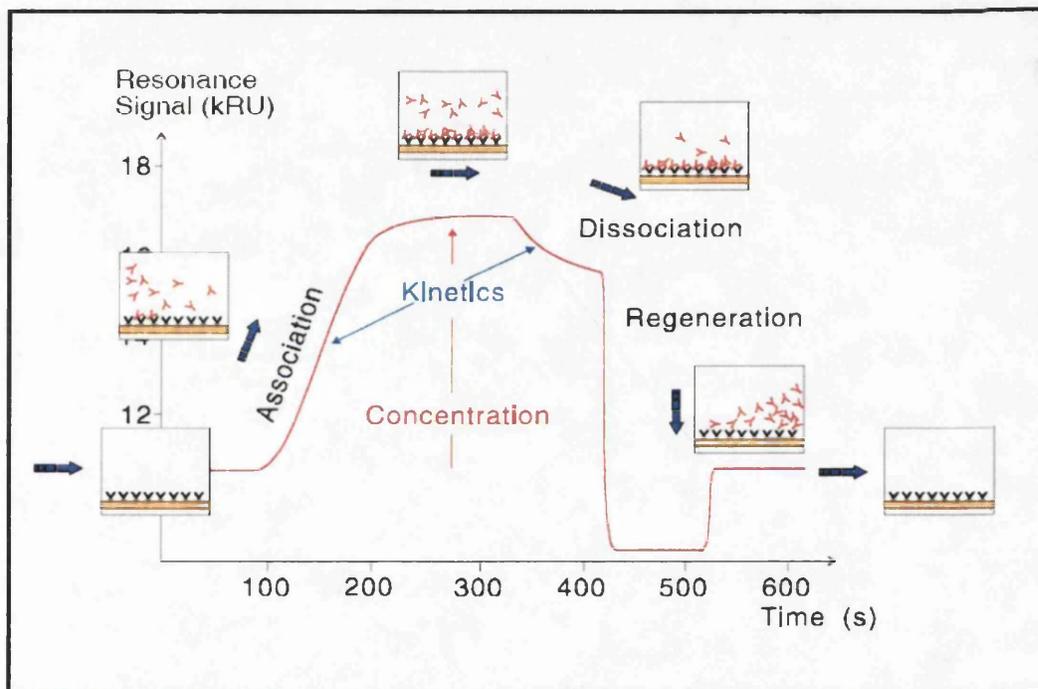


Figure 5.2 Details of a curve produced using the BIAcore

Shown is a schematic of a curve created using the BIAcore with the different areas labelled
 Adapted from www.biocore.com.

As a control, one of the flow cells on the chip was not reacted with the DNA ligand. The same dilutions of pORF6 were also applied to this flow cell and the signal generated was subtracted from the flow cell containing DNA.

The majority of work was carried out on the BIAcore 2000. One of the advantages this has over the BIAcore 1000 is that the same injection of protein is flowed over both the cell with DNA attached, and the control cell, and the signal generated by the control cell is automatically subtracted from the cell with DNA bound. It is important that this is done as the buffer by itself produces a signal. Figure 5.3 shows an example with the signal from both the experimental and control cell and the automatic subtraction to give the signal representing specific binding.

5.2 pORF6 binding to ssDNA

It was firstly determined whether binding of pORF6 to ssDNA could be detected by surface plasmon resonance using the BIAcore. An oligonucleotide of 32nt that was 5' biotin labelled, was attached to one of the flow cells on a streptavidin chip. This was carried out by injecting a DNA solution in running buffer over the chip until approximately 100 response units of DNA had been attached. Free biotin was then flowed across the cell to block any free streptavidin sites, which may have produced background binding. The following concentrations of pORF6 were flowed across the DNA-containing and control flow cells: 160nM, 80nM, 40nM, 20nM, 10nM and 5nM. The serial dilutions of pORF6 were made using the running buffer, which was a hepes buffer. A regeneration injection of 0.5M NaCl was flowed across between each protein application. The curves from the sensorgram were aligned using the BIAevaluation software.

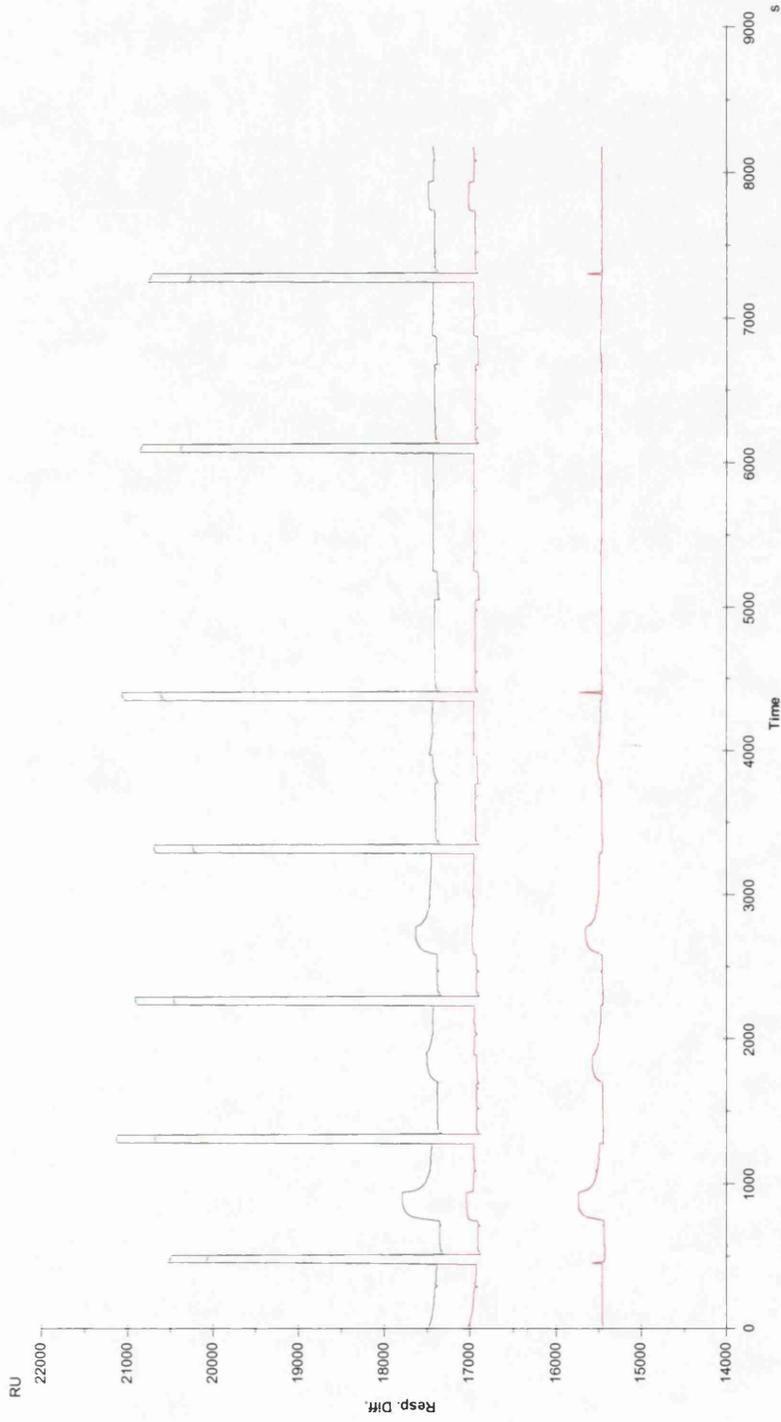


Figure 5.3 Raw data produced from pORF6 binding to ssDNA

Shown are the three traces produced from applying pORF6 to the flow cells with and without DNA adhered. The green trace shows the signal from the cell with DNA, the pink one shows the signal from the control cell (no DNA). The red trace is the signal produced after subtraction of the control cell signal from that produced from the cell with DNA.

Preliminary experiments used an association time of 120 seconds and did not reach steady state (Figure 5.4). However, the data demonstrated that pORF6 does bind to ssDNA in this assay and that the initial rate of binding increases as concentration of pORF6 increases. Label A indicates when the ligand was applied (association and dissociation occurring) and label B shows where only buffer is applied (dissociation is occurring).

The experiment with the ssDNA was repeated with an association time of 180 seconds, which allowed a steady state to be reached. The dissociation time during which running buffer minus analyte was applied was also 180 seconds. Figure 5.5a presents the raw data from the experiment as a sensorgram showing several sequential applications. The points at which the chip was regenerated with NaCl are indicated on the figure. Figure 5.5b shows the transformed data for this experiment.

This figure again demonstrates that the initial rate of binding increases as the concentration of pORF6 is increased. It also shows that the steady state level increases with increasing pORF6 indicating that more pORF6 is bound as its concentration increases.

To investigate the relationship between the concentration of pORF6 and the amount of pORF6 bound to ssDNA at equilibrium (steady state), a graph was plotted of the two variables (figure 5.6). The data used for this graph was derived from the curves shown in figure 5.5b.

The shape of the curve appears sigmoidal suggesting that pORF6 may be binding to ssDNA in a cooperative manner in this assay. This is reminiscent of the data obtained in

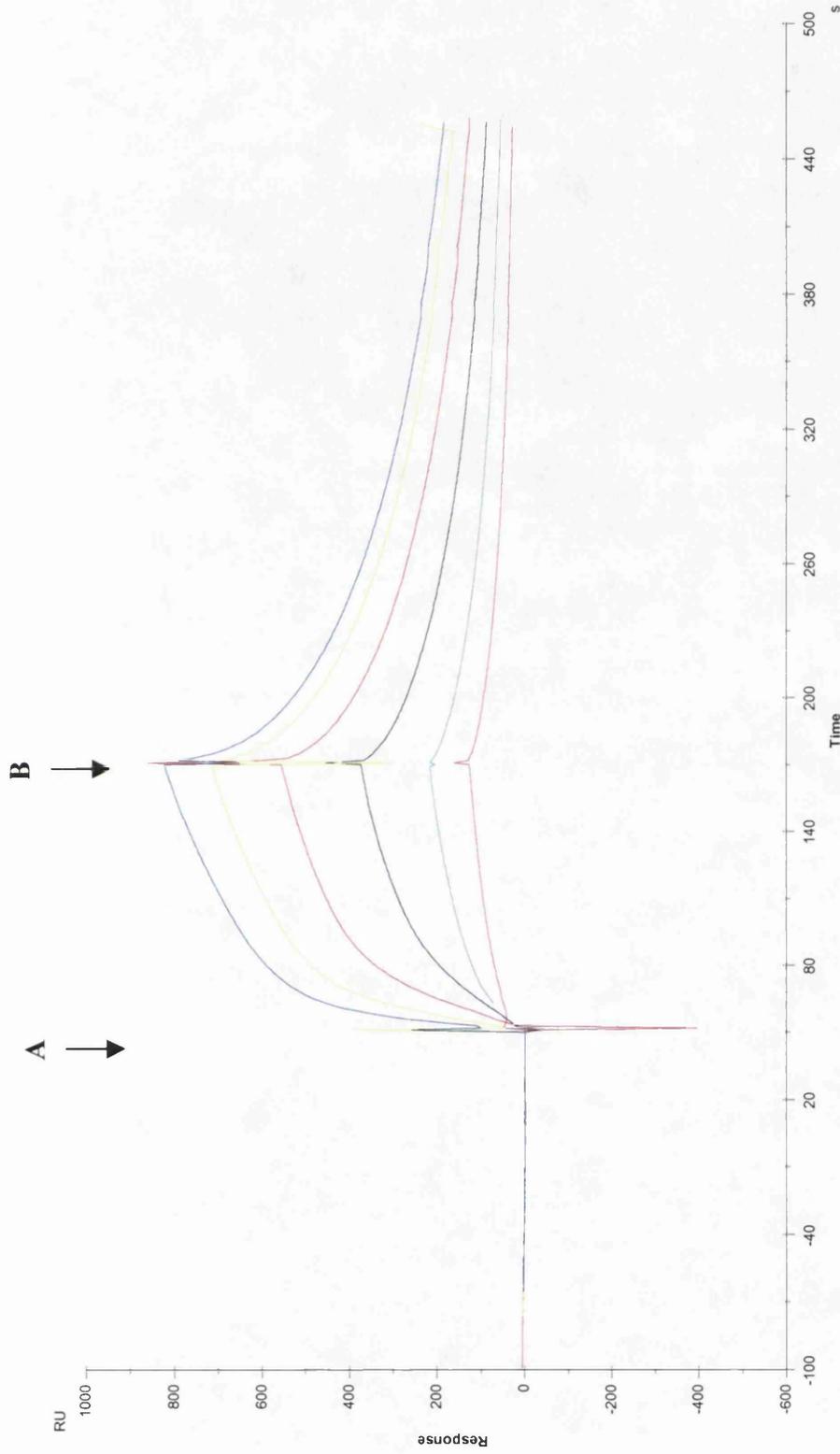


Figure 5.4 pORF6 binding to ssDNA using the BIAcore

The curves shown were generated on the BIAcore from pORF6 binding to ssDNA (32nt). Concentrations of pORF6 used were (nM): 160 (blue), 80 (yellow), 40 (red), 20 (dark blue), 10 (light blue), 5 (pink). A indicates where protein is applied. B indicates where buffer only is applied.

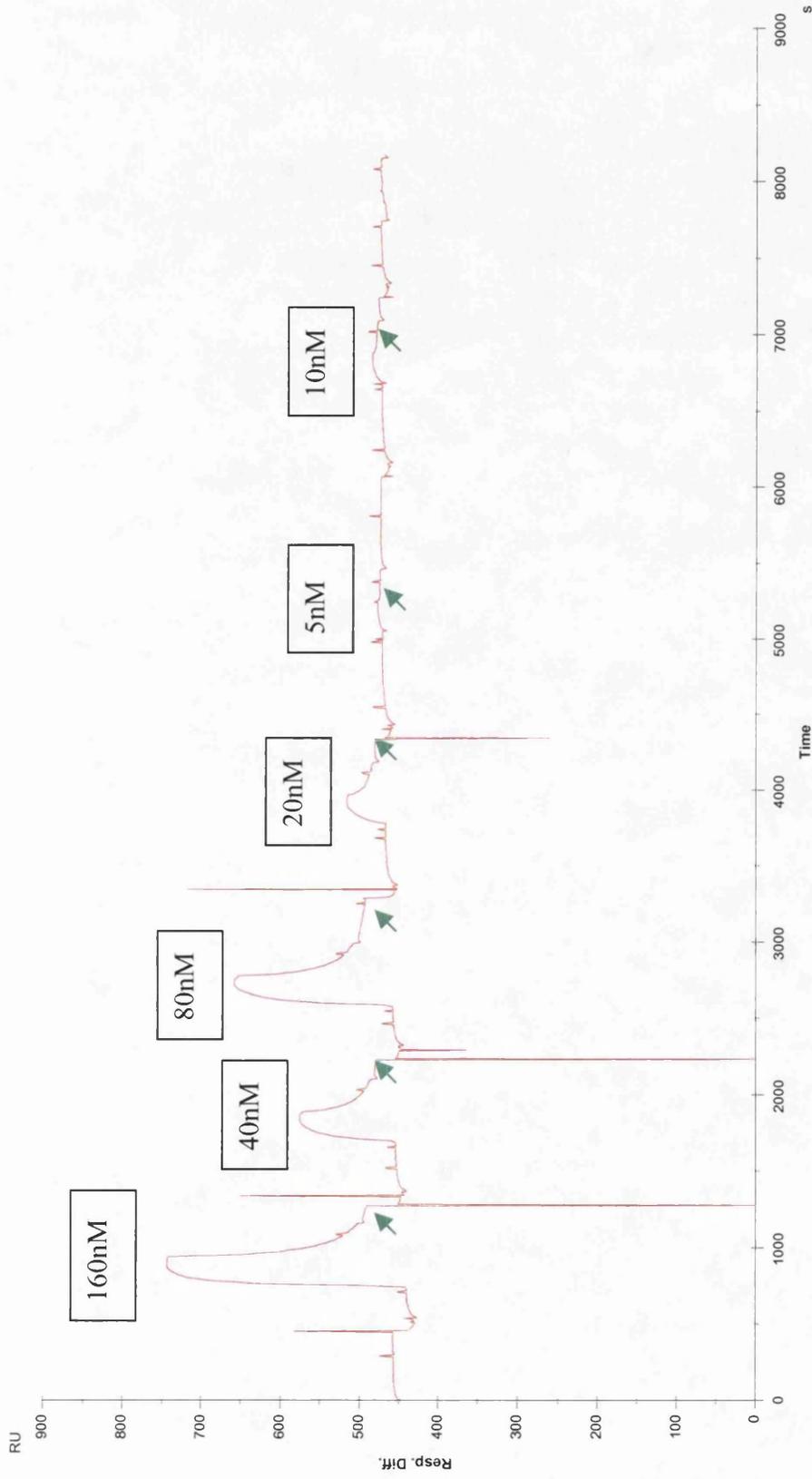


Figure 5.5a A sensorgram produced on the BIAcore from pORF6 binding to ssDNA

A DNA strand of 32nt (seq) was adhered to a flow cell to 100 response units. The following concentrations of pORF6 were then applied (nM): 160, 80, 40, 20, 10, 5 at a rate of 70 μ l/minute from which the above data was generated. The curve created from each concentration of pORF6 is indicated. Green arrows indicate where NaCl was applied to regenerate the chip.

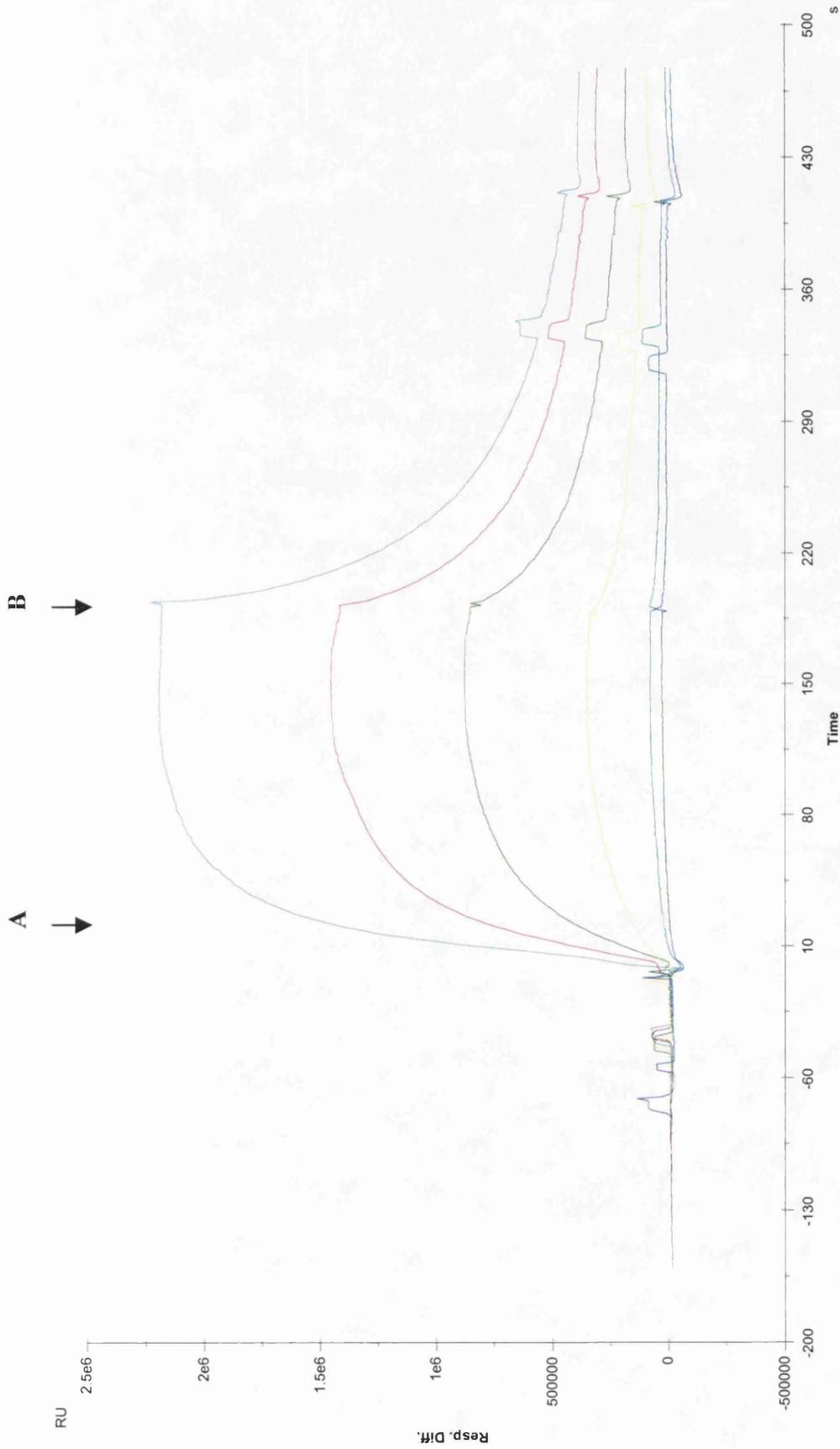


Figure 5.5b pORF6 binding to ssDNA

The data shown is the curves from figure 5.5a (pORF6 binding to ssDNA-32 nt) aligned using the BIAevaluation software. Concentrations of pORF6 used were (nM): 160 (light blue), 80 (pink), 40 (green), 20 (yellow), 10 (dark blue), 5 (blue). B indicates where protein was applied. A indicates where buffer only was applied

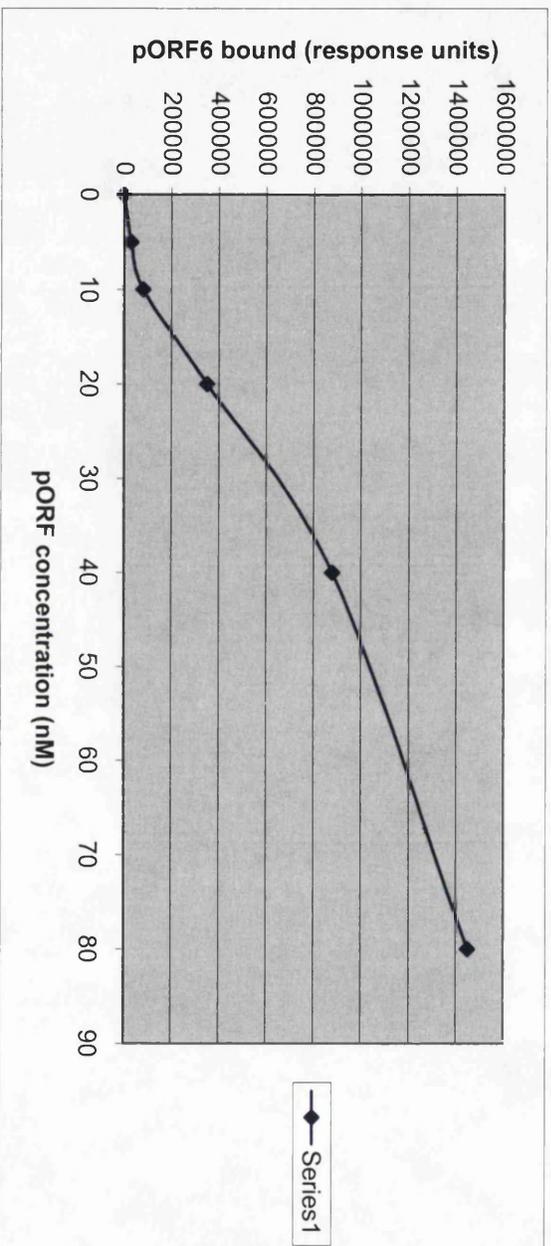


Figure 5.6 pORF6 binding to ssDNA

A series of curves of pORF6 binding to ssDNA (32nt) at different concentrations of pORF6 were generated using the BIAcore (concentrations of pORF6 were (nM): 80, 40, 20, 10, 5). The relative amount of pORF6 bound to the ssDNA (response units) at equilibrium was plotted as a function of pORF6 concentration.

mobility shift assays shown in figure 4.4b, which also demonstrated a sigmoidal response between the amounts of pORF6 binding to ssDNA at equilibrium, and the pORF6 concentration. Again further experiments would need to be done to strengthen the conclusion that pORF6 binds cooperatively. It should be noted that binding had not reached saturation by 80 nM pORF6 and further experiments using higher concentrations of pORF6 would be required to determine whether the signal observed with 160 nM corresponds to saturation. It would also be informative to include concentrations in between the points already used.

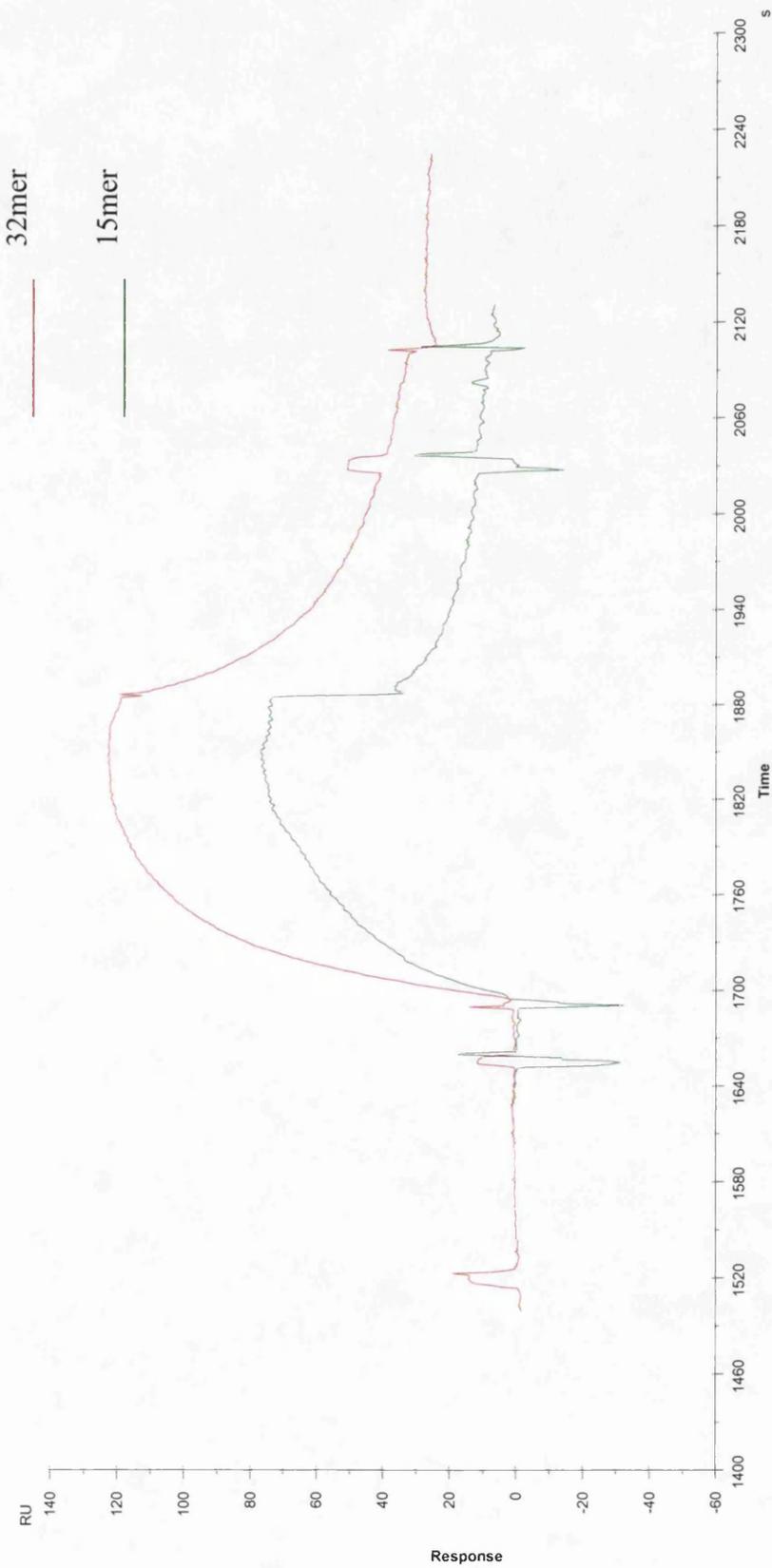
5.3 A comparison of pORF6 binding to oligonucleotides of 15 and 32 nt in length

As two molecules of pORF6 can bind to a ssDNA probe of 32 nt, it was decided to investigate binding of pORF6 to a DNA length that would accommodate only one molecule of pORF6. A 5' biotin end labelled oligonucleotide of 15 nt was attached to a chip to approximately 100 response units and pORF6 was flowed across it. The raw data from the binding of pORF6 to the 15 nt DNA strand was aligned and compared with raw data from pORF6 binding to the 32 nt DNA strand. The curves were aligned using the BIAevaluation software. Figure 5.7 shows the curves generated with 80nM pORF6 applied to the flow cells with both the 32 nt and the 15 nt DNA strands attached.

The initial rate of association of pORF6 with the ssDNA is greater for the 32 nt DNA strand than for the 15 nt strand. Inspection of the dissociation phase shows the slope for the 15 nt DNA to be much steeper than for the 32nt DNA. The majority of pORF6 appears to dissociate rapidly from the 15 nt DNA whereas it dissociates at a much slower rate from the 32 nt DNA.

Figure 5.7 Comparison of pORF6 binding to ssDNA of 15 and 32 nucleotides in length

Shown are the curves generated by the BIAcore for 80 nM pORF6 binding to ssDNA of 32 and 15 nucleotides in length. The curves were aligned using the BIAevaluation software.



pORF6 therefore has a greater affinity for the 32 nt DNA strand than for the 15 nt strand DNA. This is probably because two molecules are binding to the 32mer and binding of the second molecule occurs cooperatively whereas only one binds to the 15mer. However, the mobility shift assay in figure 4.2 demonstrated that although pORF6 binds to ssDNA of 14 nt, it binds with a greater capacity to ssDNA of 20 nt. Thus a ssDNA of 15 nt may not be sufficiently long to enable optimum binding of a single pORF6 molecule. In addition, because the ssDNA is linked to the chip surface its full length may not be available for unhindered binding of pORF6.

5.4 A comparison of ICP8 and pORF6 binding to ssDNA

It was next decided to compare ICP8 and pORF6 binding to ssDNA. As ICP8 is the SSB from HSV-1 it was expected that its binding to ssDNA would be similar. ICP8 was purified using the same methods as were used for purifying pORF6 (section 3.8). Binding was investigated using the 32nt ssDNA probe. A series of dilutions of ICP8 were made and these were individually applied to the flow cell. Curves generated using the same concentration of each protein (pORF6 and ICP8) were aligned and compared with each other. Figures 5.8a and 5.8b show the aligned curves for 40 nM and 80 nM applied protein, respectively.

The initial association rates for both proteins were similar, but ICP8 did not reach equilibrium in this time period, suggesting that the association rate for pORF6 may be greater. However, the dissociation rate of pORF6 from ssDNA in both cases appeared to be greater than for ICP8. The overall affinity of each protein for ssDNA is therefore likely to be similar. To establish this, the affinity constant would need to be calculated. For simple bimolecular interactions the affinity constant $K_D = k_d/k_a$. However, the

interactions of ICP8 and pORF6 with a 32 nt ssDNA strand are more complex since more than one molecule can interact with DNA and binding is cooperative, and K_D values calculated by the above formula are not meaningful. However, consistent with the two proteins exhibiting similar overall affinities it can be seen that the steady state binding levels were probably also very similar, although ICP8 had not quite reached equilibrium.

Figure 5.8a A comparison of pORF6 and ICP8 binding to ssDNA

Shown are the curves generated by the BIAcore from pORF6 and ICP8 binding to ssDNA (32 nt). 40nM of each protein was used. The curves were aligned using the BIAevaluation software.

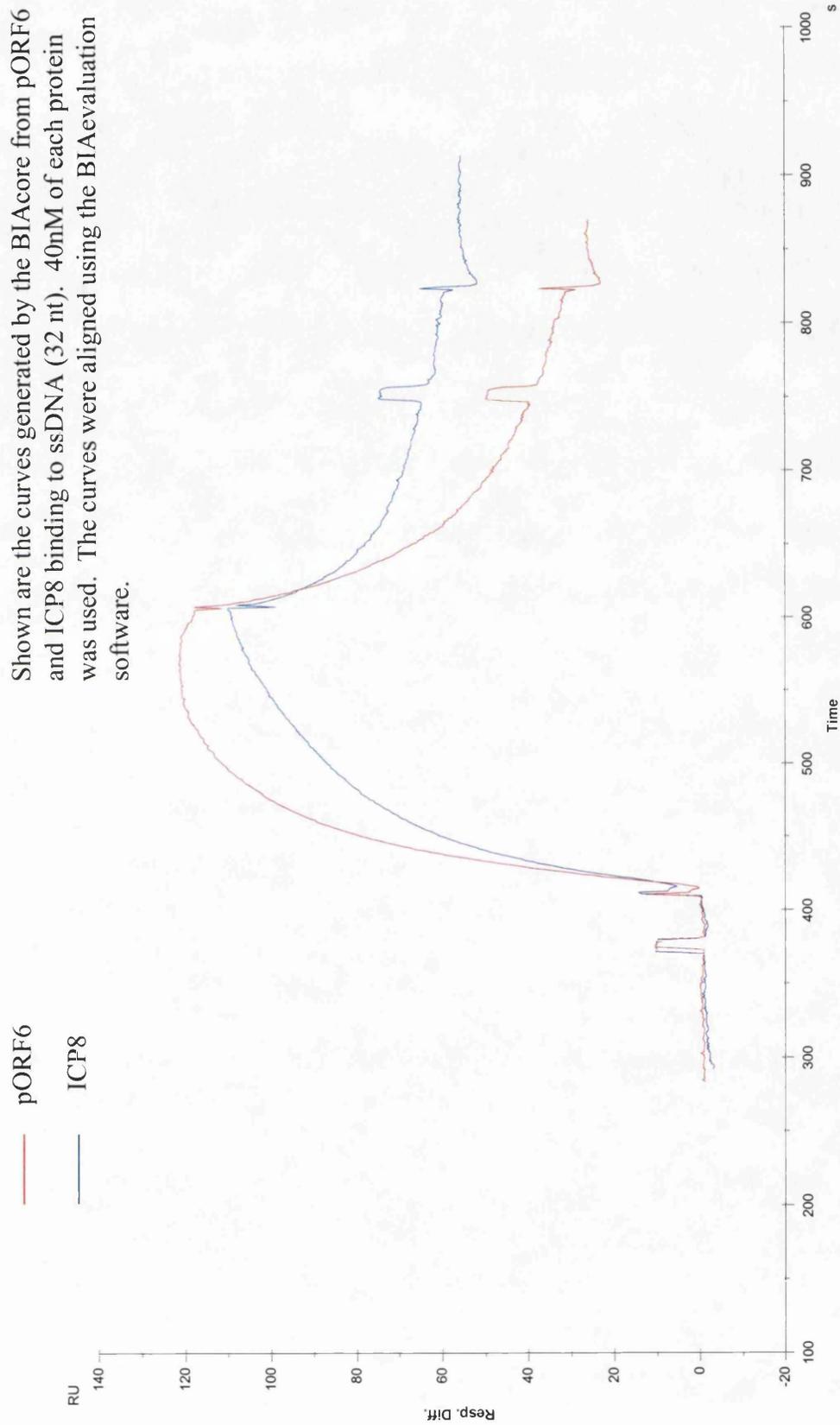
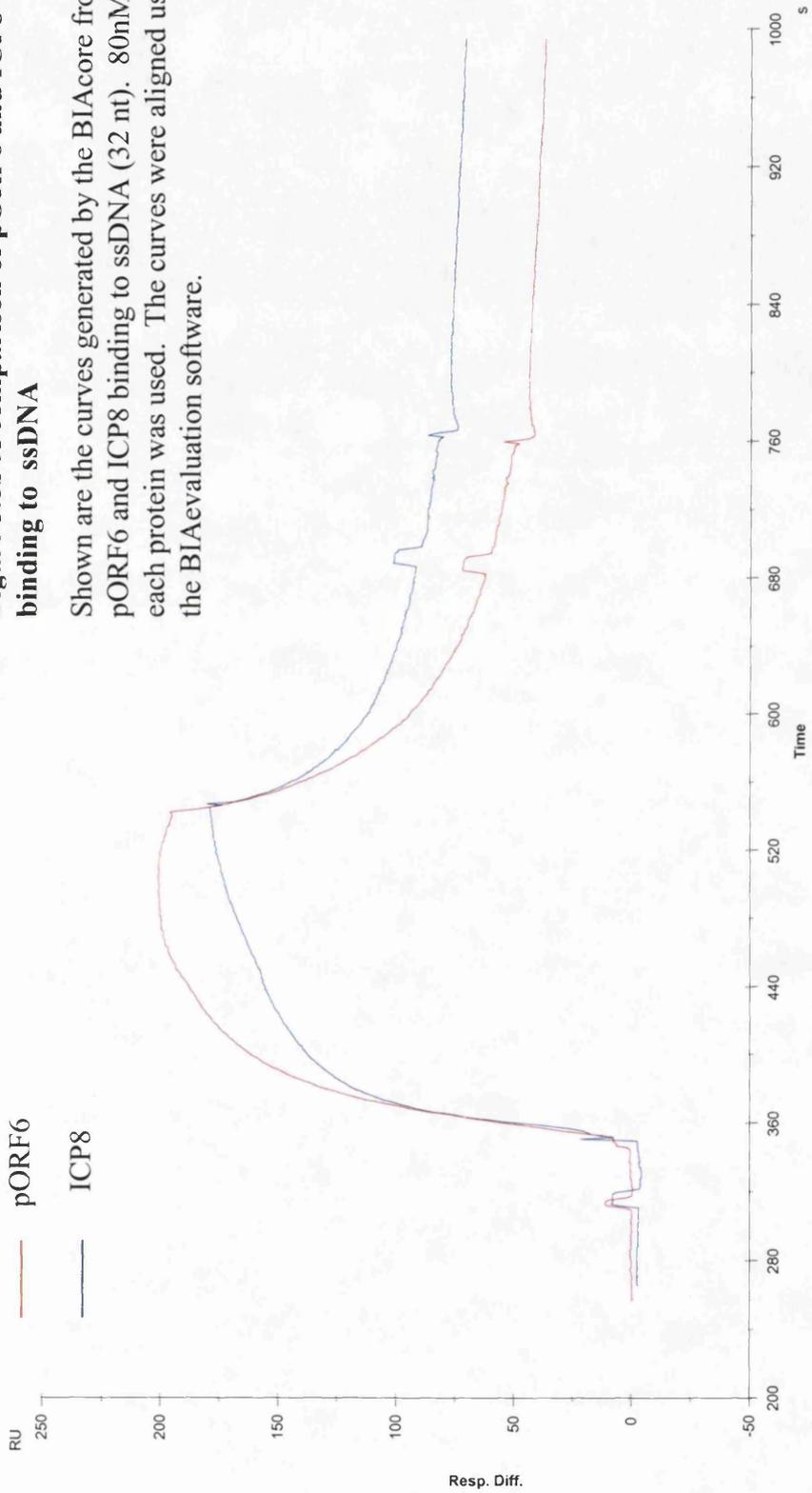


Figure 5.8b A comparison of pORF6 and ICP8 binding to ssDNA

Shown are the curves generated by the BIAcore from pORF6 and ICP8 binding to ssDNA (32 nt). 80nM of each protein was used. The curves were aligned using the BIAevaluation software.



5.5 Discussion

This section of work further investigated pORF6 binding to ssDNA and it provided additional evidence that the protein may bind to ssDNA in a cooperative manner, although this work needs repeating and extending to corroborate this. A comparison of pORF6 binding to ssDNA strands of 32 and 15 nt demonstrated a higher affinity for the 32nt strand. However, unlike the 15 nt strand the 32 nt DNA strand used was not a homopolymer. This work needs be repeated with a 32 nt poly dT strand to confirm that the difference is due solely to the length of the ssDNA strand rather than its composition or sequence.

In the experiment comparing the binding of pORF6 and ICP8 to ssDNA (figure 5.8), good data was not achieved at all of the concentrations of ICP8 used. This work could be repeated and extended to different lengths of ssDNA strands, and the relationship of steady-state binding levels to ICP8 protein concentration investigated as in figures 5.6 and 5.7 to gain a more complete comparison of the two proteins. Nethertheless the data presented in figure 5.8 suggests that there are differences between the kinetics of interaction of the two proteins with ssDNA. Even though their overall affinities for ssDNA appear similar it is not clear whether the differences reflect between the protein-DNA or protein-protein interactions or both.

Initially pORF6 binding to dsDNA was also investigated. On repeating this experiment to achieve equilibrium pORF6 did not bind to the dsDNA. This may be due to the DNA having been degraded on storage. Future work would investigate the binding of pORF6 to dsDNA using the BIAcore and comparing it to ssDNA.

One of the advantages of using the BIAcore system to study binding rates is that the whole event of association, equilibrium and dissociation is monitored. Using the BIAcore to investigate DNA-protein interactions does not impose structural constraints on either of the molecules being investigated if the DNA is bound to the chip. Binding the DNA to the chip via a 5'biotin label should not alter the structure of the DNA or inhibit its dynamic movement. This contrasts with the situation when the BIAcore is used to characterise protein-protein interactions during which one of the proteins is attached to the chip. This is usually achieved by binding free amine groups on the protein to a carboxymethylated dextran chip. A disadvantage of this is that the protein is no longer dynamic which may inhibit conformation changes associated with binding to another molecule. The orientation in which the protein is fixed to the chip may also be inappropriate, resulting in the masking of a binding site.

Chapter 6
Generation and characterisation of pORF6
specific MAbs

6.1 Introduction

The purification of native pORF6 was described in chapter 3. This chapter describes the use of that protein to generate a panel of monoclonal antibodies and their preliminary characterisation.

Antibodies are an important tool in the study of proteins and their function. Monoclonal antibodies (MAbs) are especially useful as their highly specific and unique binding properties can be exploited for many purposes. A further advantage is that they can be produced repeatedly and in limitless quantities. Producing MAbs would be expected to give rise to a range of antibodies, each with unique individual specificities, but collectively, with reactivity to a variety of epitopes on the ORF6 protein. The production of a panel of MAbs is also more likely to result in a selection of antibodies, which are suitable for a wider range of applications. This is an important consideration, as polyclonal antiserum does not always suit every application. Also, once epitope-mapping of a monoclonal antibody has been carried out, its unique specificity to a defined region can often be employed in relating protein structure to function.

Having isolated MAbs, it is necessary to characterise their properties to determine their affinities and the applications in which they may be useful. This is an important objective as the hybridoma cell lines used in this study were selected and isolated on the basis of screening against pORF6 in ELISA only. Immunochemical techniques used to test the reactivities to pORF6 of each monoclonal were western blotting, immune precipitation and immunofluorescence.

Principles of monoclonal antibody production

MAbs are secreted from single clones of hybridoma cells. These cells are created following the fusion of mutant myeloma cells that have lost the ability to produce hypoxanthine phosphoribosyltransferase (HPRT) and therefore cannot synthesise purines via the salvage pathway, and antibody-producing immune lymphocytes. Polyethylene glycol (PEG) is the agent used to promote membrane fusion between the cells in a process first described by Kohler & Milstein (1975). Hybridoma cells inherit both immortality from the myeloma cells and antibody-producing capability from the B lymphocytes. Following fusion, the hybrid cells are selected from the mixture of spleen cells, myeloma cells and hybrids by the addition of HAT (hypoxanthine, aminopterin, thymidine) to the culture medium. Aminopterin is an antibiotic that inhibits *de novo* nucleic acid synthesis by blocking purine and pyrimidine synthesis. However in normal cells, this pathway can be bypassed by using the salvage pathway, which requires the substrates hypoxanthine and thymidine for purine and pyrimidine synthesis, respectively. As the mutant myeloma cells are deficient in the salvage pathway, they do not survive. Neither do the unfused spleen cells due to their limited life span in culture. Hence the only cells that survive are hybrid cells that have inherited the ability to utilise the salvage pathway from the spleen cells and long-term viability from the myeloma cells. Some of these hybrid cells will also have antibody producing capacity of the splenic lymphocytes. The culture supernatant from single colonies of the hybridoma cells is then tested for the presence of the antibodies of the desired reactivity. Cell lines positive for the secretion of antibody are propagated and frozen down at -140°C for long-term storage.

6.2 Generation of pORF6 MAbs

pORF6 was purified as described in Materials and Methods and mice were immunised as described in methods. 33 hybridoma cell lines secreting pORF6-specific antibodies were isolated following the fusion of Sp2/0-Ag14 myeloma cells and spleen cells from the immunised mice. Positive cell lines were identified by using the hybridoma supernatant in an ELISA. An OD reading was taken and the supernatant was deemed positive if it produced a reading more than twice than that produced by control supernatant from Sp-2 cells.

6.3 Reactivity of pORF6-specific MAbs to pORF6 over-expressed in *Sf* cells in western blotting assays

For western blot analysis of the MAbs that had been raised against pORF6, cell extract was made from *Sf* cells that had been infected with a baculovirus (AcNPV-ORF6), expressing pORF6. As a positive control, polyclonal antibody 726 (anti-peptide antibody raised against the C –terminus of pORF6) was used. Negative controls were western blots using cells infected with the parental baculovirus (PAK6) and a control MAb against a different protein, UL8 (HSV-1).

The thirty-three MAbs that had been positive in an ELISA were tested and twenty-two of these reacted positively in a western blot. As some breakdown of the ORF6 protein used on the blot had occurred, different patterns of reactivity were observed with the MAbs suggesting reaction with different regions of the protein. Representative reactions are shown in figure 6.1. By comparing the pattern of the western blots with that of a western blot using Ab726 (figure 6.1a), which reacts against the very C-terminus of pORF6, three patterns of reactivity could

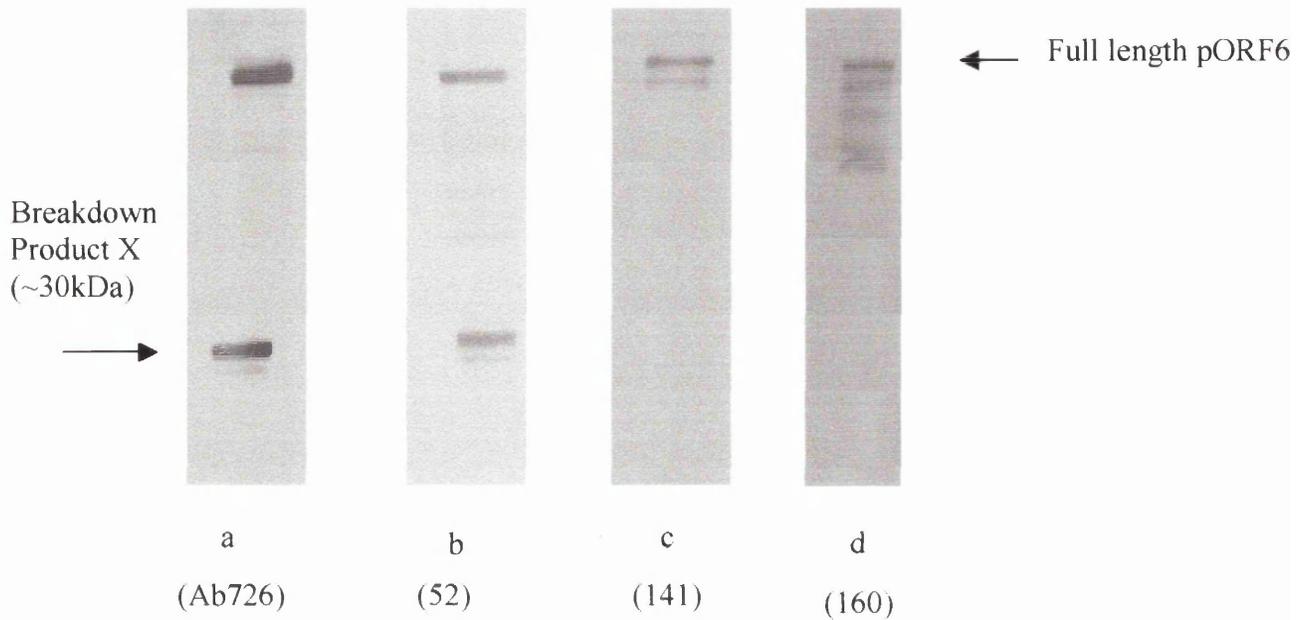


Figure 6.1 Reactivity of pORF6-specific MAb to pORF6 over-expressed in *Sf* cells in western blotting

Cell extract from *Sf* cells that had been infected with AcORF6 was resolved by SDS-PAGE and blotted nitrocellulose which was then cut into strips. The strips were incubated in 1ml of MAbs (undiluted supernatant from individual hybridoma cell lines), Ab726 or control hybridoma cell supernatant. Strips were then washed and incubated with either anti-mouse HRP-conjugated antibody or anti-rabbit HRP-conjugated antibody, as appropriate. After further washing, strips were treated with ECL reagents and exposed to photographic film.

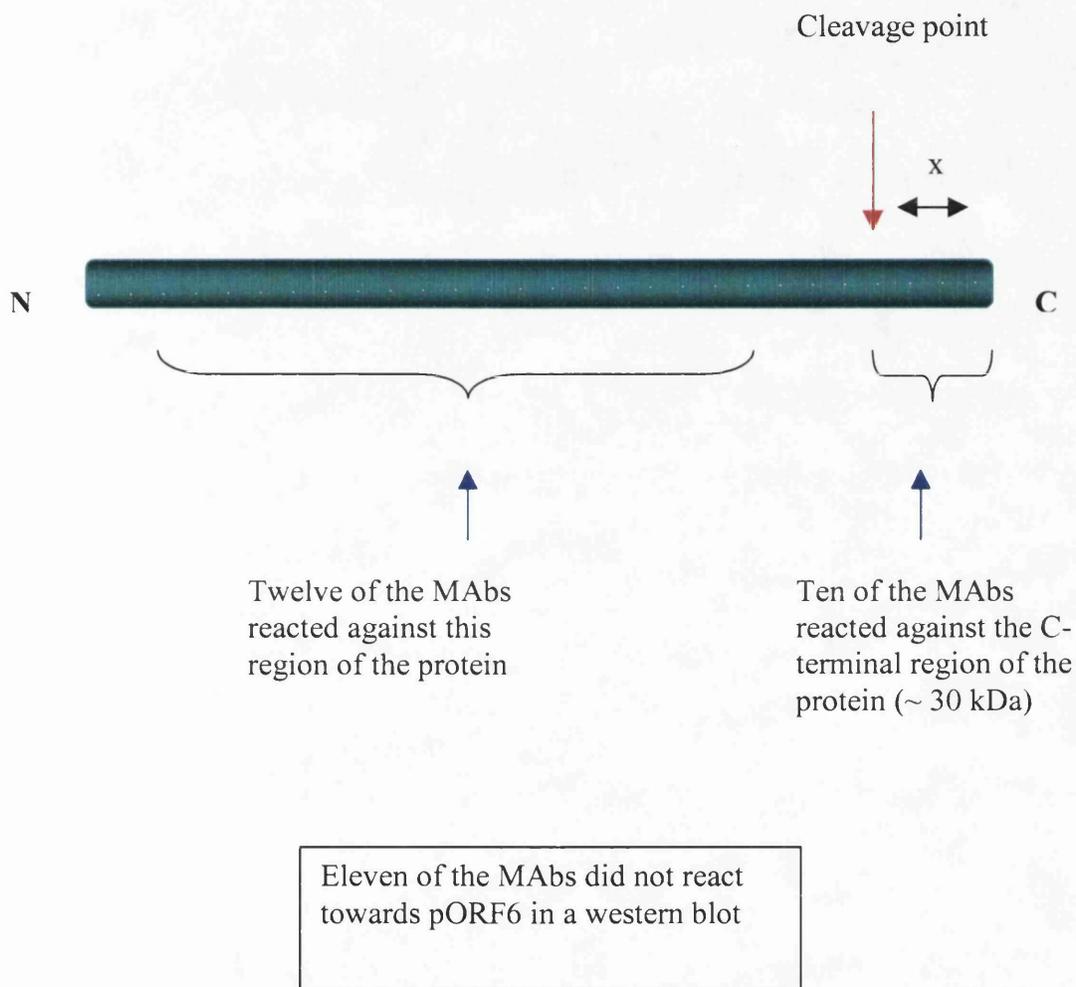


Figure 6.2 Regions of pORF6 that the MAbs react towards in a western blot

A schematic diagram of the pORF6 protein showing the putative regions with which the MAbs react.

be deduced. One group of the MAbs, exemplified by MAb 52, gave a similar reaction pattern to that of Ab726, as a strong reaction to the full length protein and a strong reaction to a breakdown product ($x \sim 30\text{kD}$) towards the bottom of the blot, was observed (figure 6.1-b). This leads to the suggestion that these MAbs are reacting to an epitope near the C-terminus of pORF6.

Another set of the MAbs (exemplified by MAb 141) reacted to the whole protein and also to breakdown products not much smaller than pORF6, while other MAbs (exemplified by MAb 160) reacted to both these and intermediate size fragments (figure 6.1- c&d). It can be deduced that both these sets of MAbs interact with epitopes that lie outside the C-terminal $\sim 30\text{kDa}$. Figure 6.2 summarises the reactivity of the MAbs towards pORF6 in a western blot.

6.4 Reactivity of pORF6-specific MAb 15 to pORF6 in HHV8 infected B-cells in western blotting assays

As many of the MAbs could react with pORF6 that had been over-expressed in *Sf* cells, a western blot assay was carried out using cells that were infected with KSHV to ascertain whether levels of pORF6 in a natural infection were sufficient to be detected. A B-cell line (BCBL1) latently infected with KSHV, which had been established from a primary effusion lymphoma, was used. To induce the virus into lytic infection, sodium butyrate was added. At 24, 48 and 72 hours post induction cell extracts were made and analysed in a western blot (figure 6.3). As controls, cell extracts of uninduced cells were also made at these times and a B-cell line not infected with KSHV (BJAB) was also used.

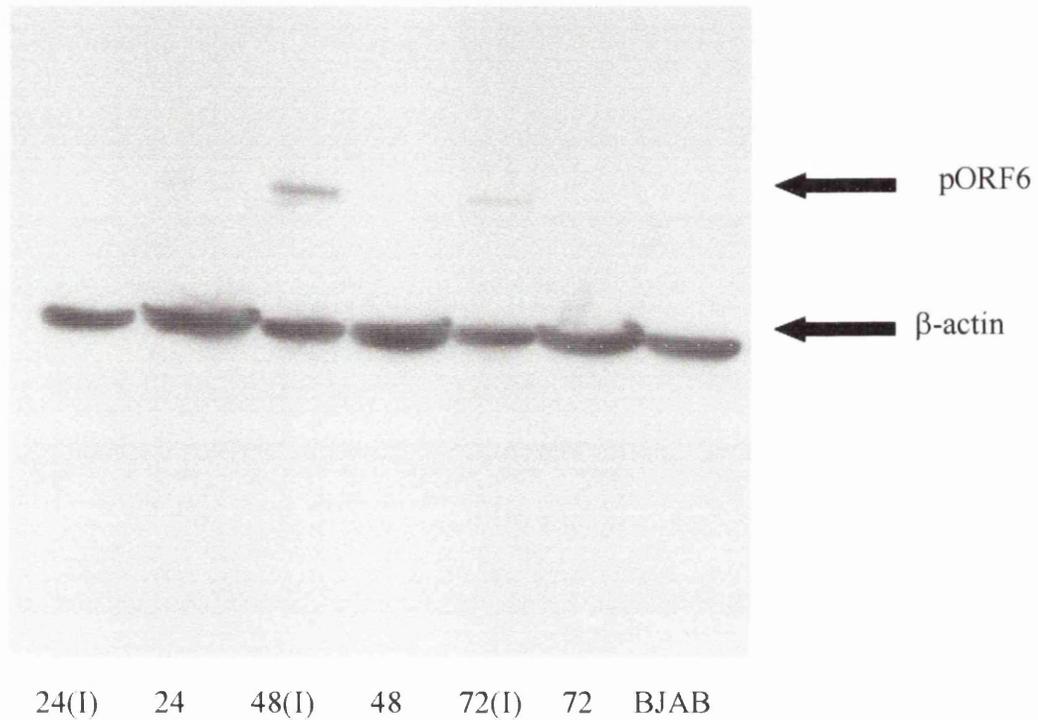


Figure 6.3 Reactivity of pORF6-specific MAb with pORF6 in HHV8 infected B-cells. B cells that were latently infected with HHV8 were induced with sodium butyrate. At the indicated times (h) post induction cell extracts were made. The proteins were resolved by SDS-PAGE and blotted onto nitrocellulose. The nitrocellulose was incubated in 1ml of MAb (undiluted supernatant from individual hybridoma cell lines). The membrane was then washed and incubated with anti-mouse HRP-conjugated antibody. After further washing, the blot was treated with ECL reagents and exposed to photographic film. As controls, uninduced cells and also a B-cell line uninfected with HHV8 were used (BJAB). The numbers indicate hours after induction the cell extracts were made. Cells that were induced are represented with (I) after the number. An anti- β -actin MAb was also used to confirm that a similar amount of cells had been used in each lane.

A western blot of these samples was reacted with MAb 15 (figure 6.3). pORF6 was detected at 48h and 72h in the induced samples but not at any time in the uninduced or BJAB controls. To confirm the same amount of cells had been loaded onto the wells an antibody against β -actin was also used. After chemical induction, KSHV early genes can be detected at about 13 hours (Sun *et al.*, 1999) and so pORF6 had probably not accumulated to a high enough level for the Mab to detect by 24h. The decrease of pORF6 at 72 hours is probably due to cell viability decreasing as sodium butyrate is toxic to the cells, and an associated breakdown of pORF6.

6.5 Reactivity of pORF6-specific MAbs to pORF6 over-expressed in *Sf* cells in immune-fluorescence assays

The MAbs were also tested to see if they could react to pORF6 over-expressed in *Sf* cells, in an immune-fluorescence assay. The cells, which had been infected with AcNPV-ORF6 for 48hrs were mounted onto slides using a cytopsin. The cells were then fixed, permeabilised and incubated with the MAbs and subsequently an anti-mouse-FITC conjugated antibody. Controls used were *Sf* cells infected with the parental baculovirus (PAK6) and a control Mab was also used (anti-HSV-1 UL8). The stained cells were visualised using the LSM 510 (Zeiss) confocal microscope. Figure 6.4 shows the pattern obtained with one of the positive MAbs, MAb 199. In total eighteen out of the thirty-three antibodies gave a positive result. The pORF6 was located in the nuclei of infected *Sf* cells in agreement with its previously demonstrated intrinsically nuclear location in Vero cells (Wu *et al.*, 2001).

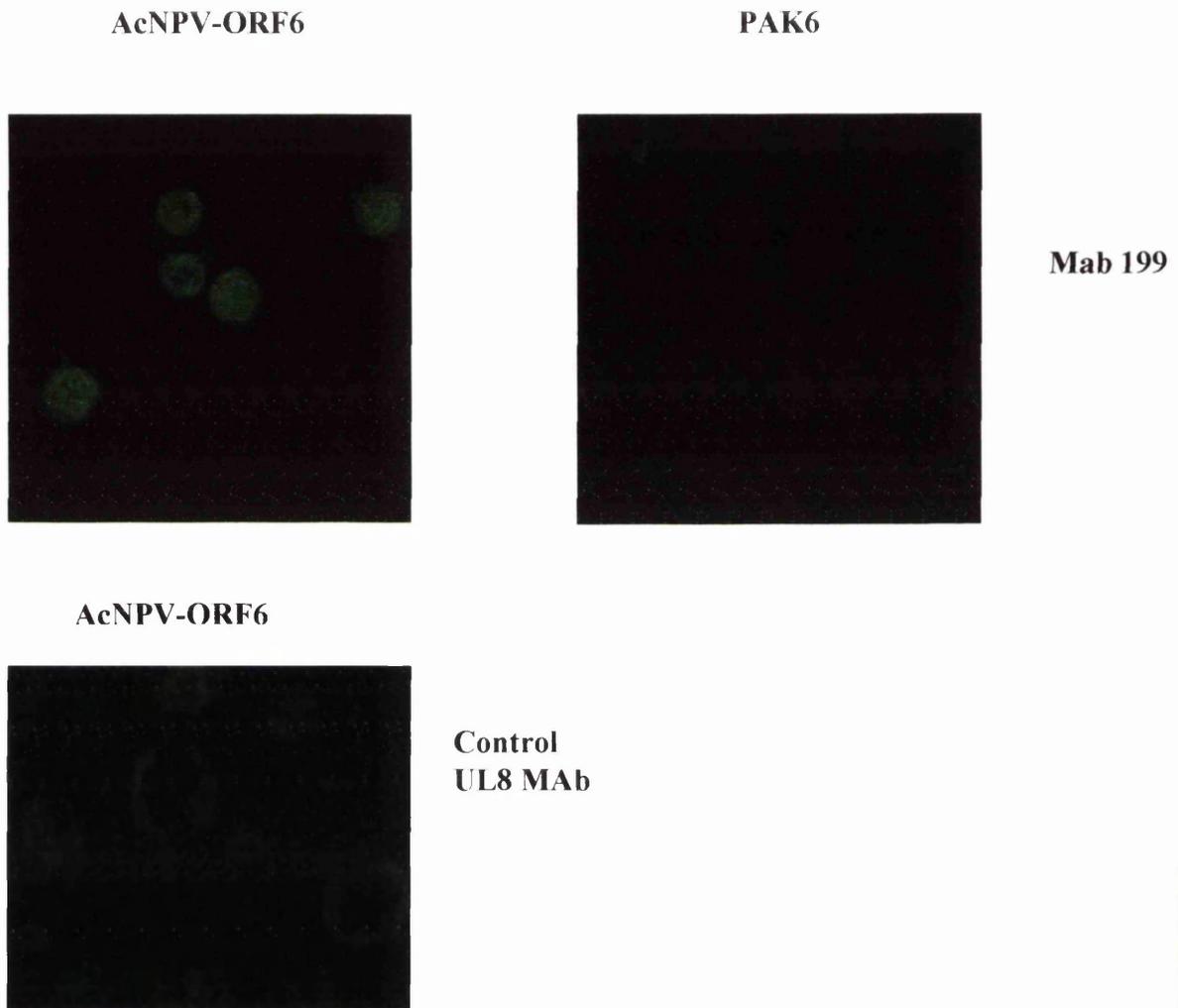


Figure 6.4 Reactivity of pORF6-specific Mabs with pORF6 over-expressed in *Sf* cells in immune-fluorescence

Sf cells were infected with a recombinant baculovirus expressing pORF6 or the parental baculovirus (control). After 48hrs infection they were mounted onto slides using a cytopspin, fixed and permeabilised and incubated with MAbs. After washing the cells were incubated with an anti-mouse-FITC conjugated antibody and visualised using a confocal microscope (LSM 510, Zeiss). AcNPV-ORF6 infected cells were also reacted with a control MAb against the HSV-1 UL8.

6.6 Reactivity of pORF6-specific MAbs with pORF6 in HHV8 infected B-cells in immune-fluorescence assays

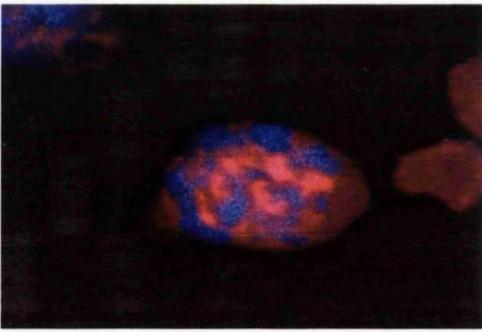
As some of the MAbs were positive by immune-fluorescence for interaction with pORF6 over-expressed in *Sf* cells, it was next determined if similar reactivity could be observed in cells infected with KSHV. Again B-cells latently infected with KSHV were used. Cells were induced using sodium butyrate (20-40% are usually induced) and after 48 hours they were mounted onto a slide, fixed with acetone/methanol and then incubated with MAbs 15 and 199. They were then incubated with anti-mouse-Cy5 conjugated antibody. A Cy5 conjugated antibody was used in this experiment as the virus expresses GFP, which fluoresces at the same wavelength as FITC. MAbs 15 and 199 reacted with pORF6 in this assay (figures 6.5 a and 6.5b) and nuclear staining was again observed (panels B and D, figure 6.5a; panel B figure 6.5b).

To visualise the nucleus more clearly, the cells were also stained with DAPI, which binds to DNA. pORF6 was found in globular areas, typical of herpesvirus replication compartments in agreement with previous data demonstrating that KSHV replicates in such structures (Wu *et al.*, 2001). The DAPI staining seemed to be confined largely to regions outside those containing pORF6 (figure 6.5a, A-C and figure 6.5b, A-C). In the case of HSV-1, cellular chromatin is pushed aside and becomes condensed as the viral replication compartments expand (Monier *et al.*, 2000). This may also be occurring here and could account for the appearance of the cellular DNA. Presumably there will be viral DNA within the replication compartments, but this may not be visibly stained by the DAPI because there is a large excess of stained cellular DNA.

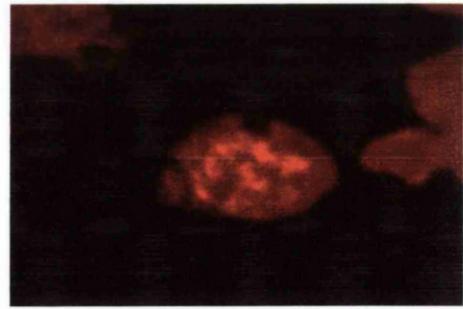
Figure 6.5a Reactivity of pORF6-specific MAbs to pORF6 in KSHV infected B-cells in immune-fluorescence assays

B-cells that were latently infected with KSHV were induced into lytic infection by adding sodium butyrate. At 48hrs they were mounted onto slides, fixed with methanol/acetone and then incubated with pORF6- MAb15. After washing they were incubated with anti-mouse-Cy5 conjugated antibody. Following further washing they were stained with DAPI. As a control cells that were uninduced were used. A control MAb (against HSV-1 UL8) was also used on induced cells. A-C induced cells incubated with MAb15 (A,merged image; B, Cy5; C, DAPI); D, induced cells incubated with MAb15, showing Cy5 staining; E, uninduced cells incubated with MAb 15 showing Cy5 staining; F, induced cells incubated with control antibody (UL8).

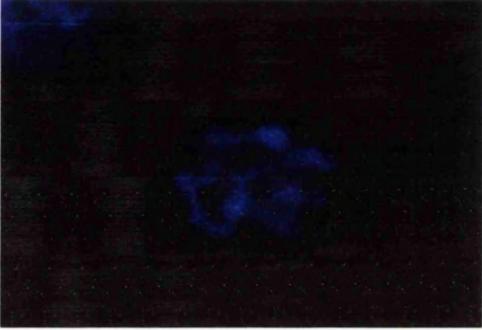
A



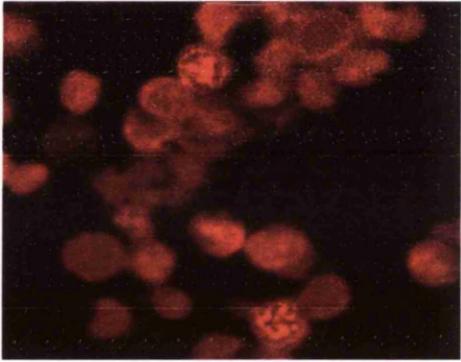
B



C



D



E



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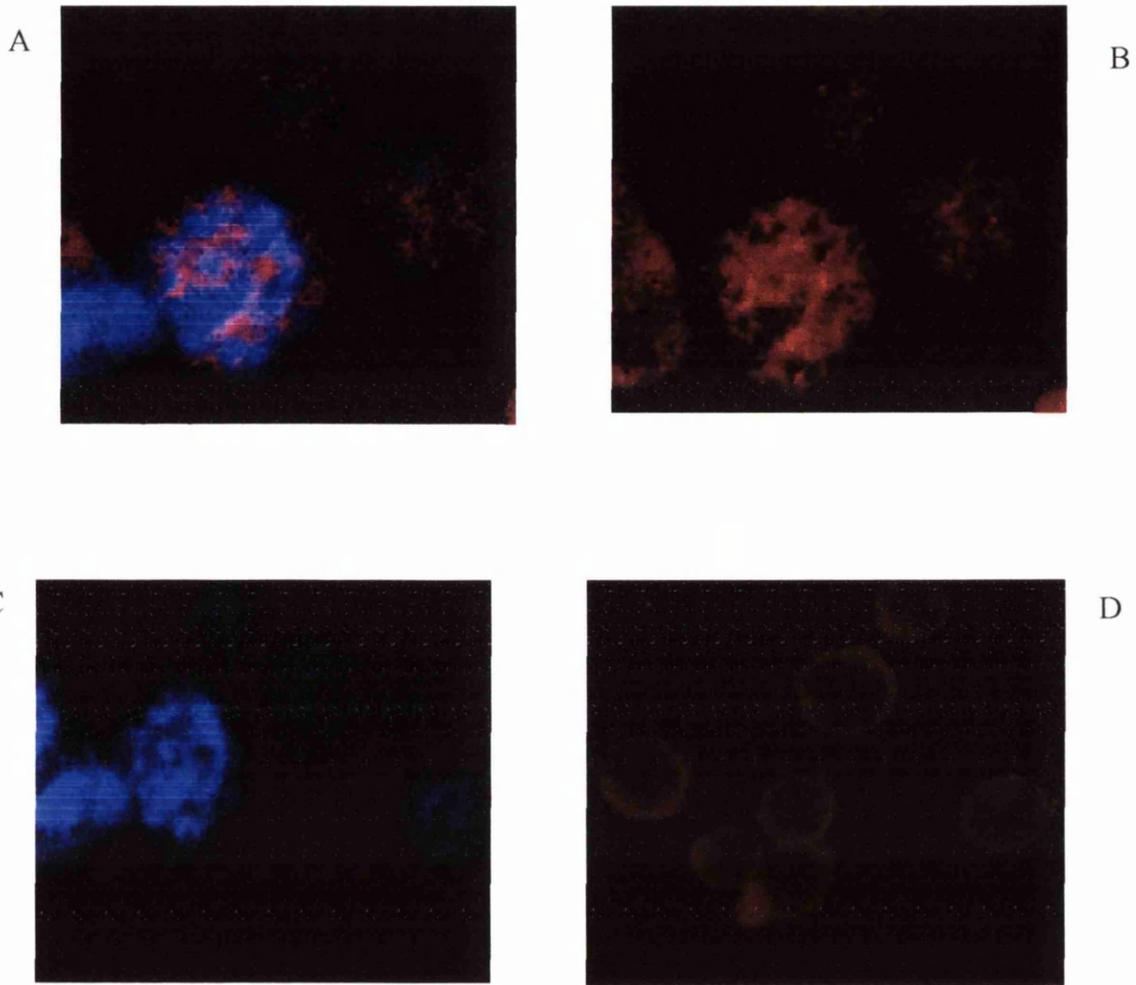


Figure 6.5b Reactivity of pORF6-specific MAbs to pORF6 in KSHV infected B-cells in immune-fluorescence assays

B-cells that were latently infected with KSHV were induced into lytic infection by adding sodium butyrate. At 48hrs they were mounted onto slides, fixed with methanol/acetone and then incubated with pORF6- MAb199. After washing they were incubated with anti-mouse-Cy5 conjugated antibody. Following further washing they were stained with DAPI. As a control, cells that were uninduced were used. A-C induced cells incubated with MAb199 (A, merged image; B, Cy5; C, DAPI staining); D- uninduced cells incubated with MAb 15 showing Cy5 staining

6.7 Reactivity of pORF6-specific MAbs with pORF6 over-expressed in *Sf* cells in immune-precipitation assays

Sf cells were infected with recombinant baculovirus AcNPV-ORF6. After 24 hours the medium was replaced with methionine-free medium containing ^{35}S -methionine and incubated for a further 24 hours. Cells were harvested and washed in TBS, then proteins were extracted in cold AE buffer and incubated with MAbs at 4°C . A suspension of protein A sepharose beads was added. After mixing for 1 hour the beads were pelleted by centrifugation, washed in AE buffer boiled in SDS-PAGE buffer. The beads were pelleted by centrifugation and the supernatant was analysed by SDS-PAGE. The gels were dried and then exposed to a phosphorimage screen. A representative experiment using some of the MAbs is shown in figure 6.6. As a control *Sf* cells infected with the parental baculovirus (PAK6) were also used. Some of the Mabs reacted strongly e.g. 199 while others reacted weakly e.g. 1 and 15 or not at all e.g. 120. In total, ten of the Mabs were able to immune-precipitate pORF6.

6.8 A protein in the hybridoma supernatant binds to ssDNA

To give more information on the binding of pORF6 to ssDNA, it was decided to test the effect of the MAbs in an EMSA with pORF6 and ssDNA. This would be expected to result in a supershift of the DNA-pORF6 complex, or, depending on which part of the protein the antibody bound, to, might result in pORF6 no longer being able to interact with ssDNA. Such an approach would help to elucidate the region of pORF6 involved in ssDNA binding. MAb52 was chosen for an initial experiment as it had reacted well in the immune assays. Binding assays were set up with MAb, pORF6 and ^{32}P -labelled poly (dT)₃₅. After incubation complexes were resolved by on an acrylamide gel, which was dried and exposed to a phosphorimage screen (figure 6.7).

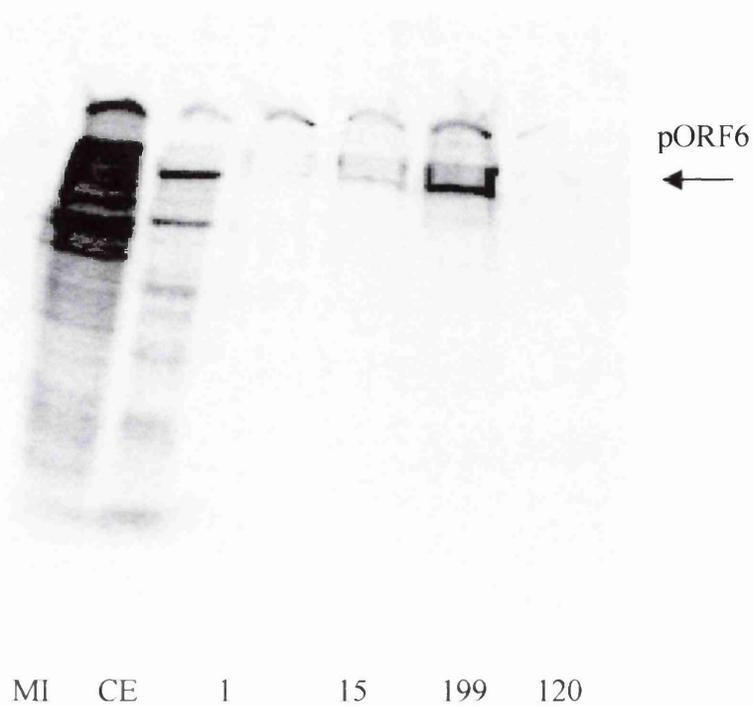


Figure 6.6 Reactivity of pORF6-specific MAbs in immune-precipitation assays

Sf cells were infected with recombinant baculovirus AcNPV-ORF6. After 24 hours the medium was replaced with methionine-free medium containing ^{35}S -methionine and incubated for a further 24 hours. Extracts were prepared and immune-precipitated with the MAbs. Samples of extract from mock-infected (MI) and infected (CE) cells were analysed alongside the immunoprecipitates by SDS-PAGE. A phosphorimage of the dried gel is shown. The numbers correlate to the Mab used.

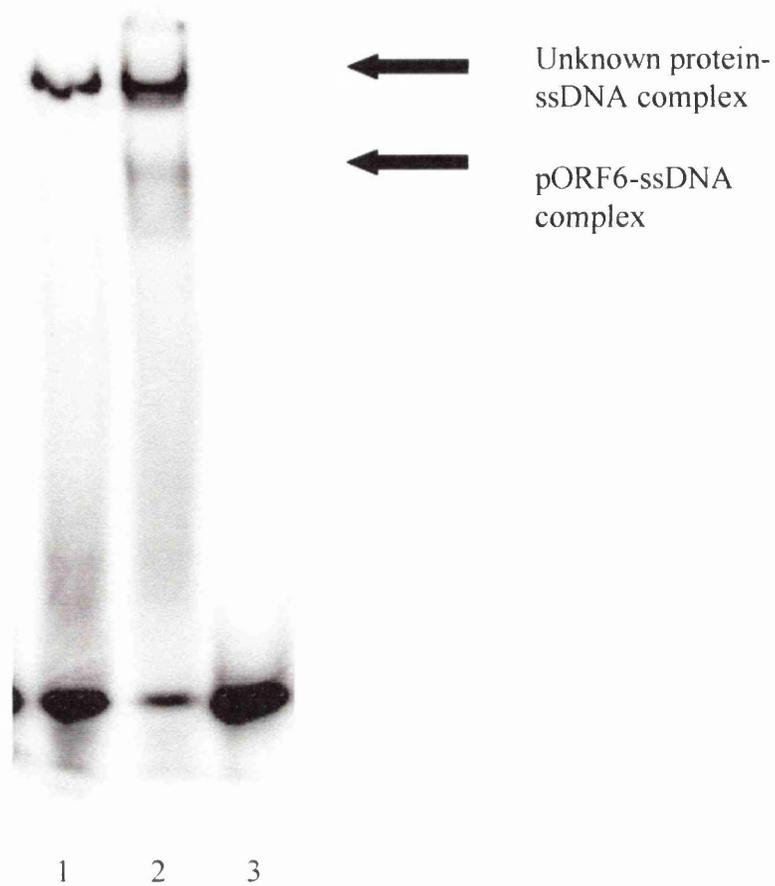


Figure 6.7 A protein in the hybridoma supernatant binds to ssDNA

Binding assays were carried out containing **1-** MAb52 and (dT)₃₅ **2-** MAb52, pORF6 and (dT)₃₅ **3-** (dT)₃₅ for 30 mins at 37°C. Prior to addition of DNA the MAb and pORF6 were incubated for 1 hour at 37°C. The DNA-protein complexes were resolved by EMSA and the gel was then dried and exposed to a phosphorimage screen.

On inspecting the lane 2, which contained all the components, a novel additional band migrating more slowly than the pORF6 complex was apparent suggesting that the antibody might be binding to, and further reducing the mobility of the complex.

However, the control with MAb and ssDNA only, revealed that the slower migrating band represented a protein from the hybridoma supernatant (probably from the foetal calf serum) binding to the DNA. The MAb was therefore purified from the supernatant medium in an attempt to remove this binding activity.

6.9 Purification of MAb 52 raised against pORF6

MAb 52 was purified using a protein G column. Thirty mls of the hybridoma supernatant was dialysed overnight in the protein G column running buffer and applied to the column. The MAb was eluted using a glycine buffer (pH 2.7) into tubes containing 1M TRIS-HCl (pH 9) to neutralise the eluant. Figure 6.8 shows a chromatogram of the purification. The antibody was eluted in three 0.5ml fractions. To verify the purity of the antibody, samples were resolved by SDS-PAGE and the gel was then stained with Coomassie blue (figure 6.9). The presence of heavy and light chains of the antibody confirmed that the antibody had been purified to near homogeneity.

Unfortunately due to time constraints the EMSA experiments with the purified MAb were not completed. This is work that could be done in the future.

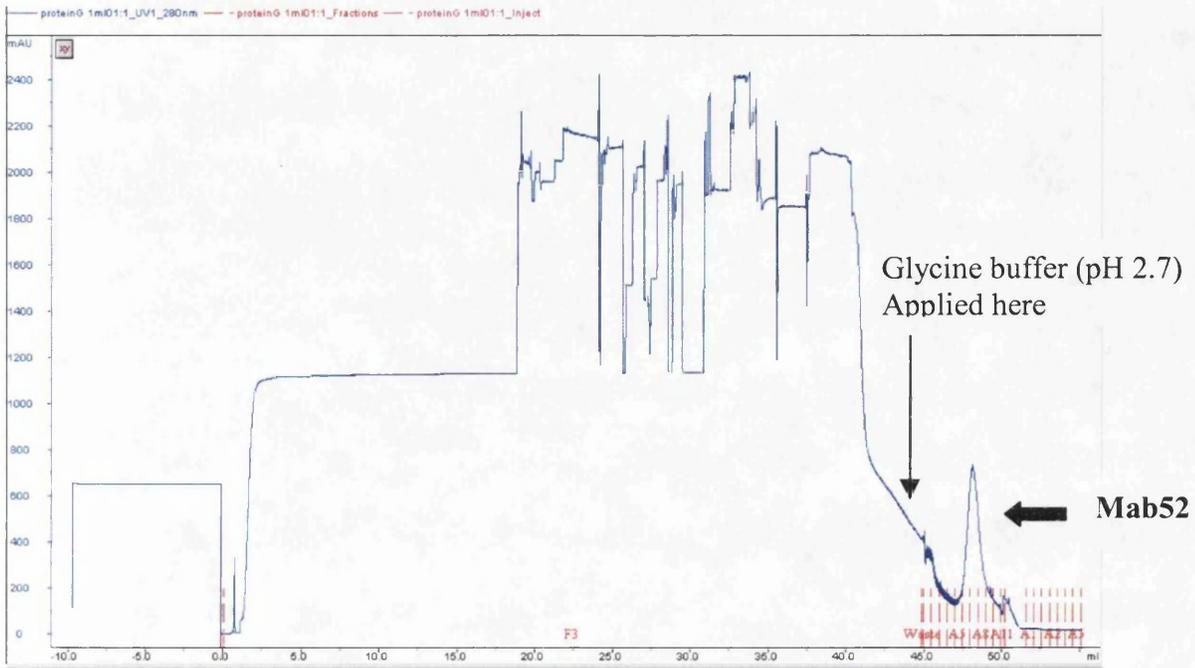


Figure 6.8 Purification of Mab52 raised against pORF6

Thirty mls of dialysed hybridoma supernatant was loaded onto the protein G column using a sodium phosphate buffer. Mab 52 was eluted from the column using a glycine buffer (pH 2.7). The antibody was eluted in three 0.5 ml fractions. The trace shows the UV absorbance of fractions and the position of the peak of eluted antibody.

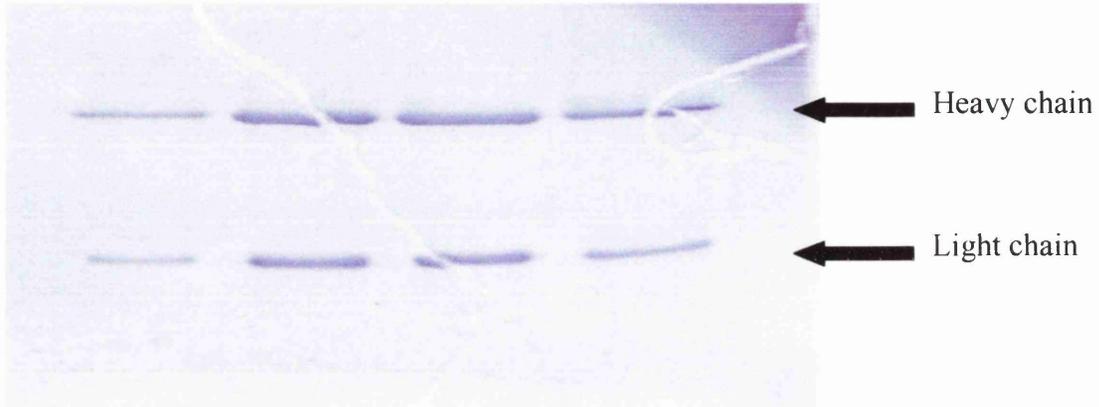


Figure 6.9 Purification of Mab raised against pORF6

The peak fractions from the purification (figure 6.8) were resolved by SDS-PAGE. The gel was then stained with Coomassie blue. As it is a denaturing and reducing gel the heavy and the light chains of the antibody become separated.

6.10 Discussion

This chapter described the work that was carried out with MAbs raised against pORF6. The MAbs were tested in different assays that had pORF6 in different states i.e. in the western blot it is denatured whereas in the immune-precipitation it is in its native state. The immune-fluorescence assay also used pORF6 in its native state but inside an intact cell. The number of positive results was greatest with the western blot. As pORF6 denatured in this assay, the epitopes recognised by the MAbs may have been more accessible.

It would be useful to use the MAbs that may be reacting to the N-terminal region of pORF6 in the DNA binding reactions. As mentioned previously, the C-terminal region of HSV-1 ICP8 does not affect the intrinsic binding ability of ICP8 to ssDNA, but the cooperative nature of binding. The ability of ICP8 to bind to DNA may be due to a region in the N-terminus, which may also be true for pORF6. Any of the MAbs which blocked DNA binding could be epitope mapped and this might provide some information on the nature and location of the DNA binding site.

The antibodies that could immune-precipitate ORF6 could also be used in a co-immune-precipitation assay to identify the proteins that pORF6 interacts with. ICP8 has been shown to bind to the origin binding protein of HSV-1. An interaction with the helicase-primase associated factor has also been demonstrated. The origin binding protein function of KSHV is speculated to be carried out by the K8 protein. If an interaction between pORF6 and K8 could be demonstrated, this would be further support for K8 in this role. An attempt was made to construct a baculovirus expressing K8 that could be

used for co-immune-precipitation assays. Unfortunately repeated attempts to clone K8 were unsuccessful and this was left in order to concentrate on other work. It is also possible that certain MAbs might block a particular protein-protein interaction, and in this instance epitope mapping might provide information on the region of pORF6 involved in the interaction.

The study of the formation of replication compartments in KSHV could also be carried out using the MAbs that have a positive reaction to pORF6 in immuno-fluorescence. KSHV positive cells that have been induced into lytic replication could be fixed and studied at a range of times and the progression of the replication compartment formation and maturation could be followed.

Chapter 7

Discussion

7.1 General Discussion

The set of DNA replication proteins required for origin-dependent KSHV DNA replication includes six core replication fork proteins that are conserved amongst the herpesvirus family (Wu *et al.*, 2001). So far the KSHV core replication proteins have not been well characterised. The aim of this project was to purify and characterise one of these core KSHV proteins, the ssDNA binding protein, pORF6. To date there has been no work published reporting the binding of pORF6 to ssDNA.

In this thesis the ability of pORF6 to bind to ssDNA was firstly established and then further investigated. This included determining the number of nucleotides required for pORF6 to bind to ssDNA, the effect of NaCl concentration on pORF6 binding, and establishing whether, like its homologue in HSV-1, ICP8, it binds to ssDNA in a cooperative manner (Ruyechan, 1983; Lee and Knipe, 1985; Dudas *et al.*, 2001). This involved using two different DNA binding assays, EMSA and surface plasmon resonance (BIAcore).

The results demonstrated that pORF6 binds to ssDNA in both DNA binding assays. Like its homologue, ICP8, and other SSBs, pORF6 may bind cooperatively to ssDNA, as was demonstrated in both the EMSA and in the data generated from the BIAcore.

Cooperative binding would ensure that during viral DNA replication, KSHV ssDNA becomes covered by pORF6. This would not only aid the DNA polymerase by removing secondary structure but also protect the ssDNA from nuclease attack. As stated before this work should be repeated and extended to verify that pORF6 binds cooperatively to ssDNA.

The work carried out using the BIAcore also compared ICP8 and pORF6 binding to ssDNA, and demonstrated that each protein binds to ssDNA with a similar affinity. pORF6 associates with ssDNA at a slightly greater rate but is also dissociates from ssDNA slightly faster. The work using the BIAcore also compared pORF6 binding to ssDNA of 32 nt and 15 nt, and demonstrated a higher affinity for the 32 nt strand. This is probably because two molecules of pORF6 can bind cooperatively to the 32 nt DNA. However, this result could also be explained by 15 nt being too short for optimal binding of a single molecule of pORF6.

Previous publications have reported that the number of nucleotides required for ICP8 to bind to ssDNA lies within the range 12-40 nt (Ruyechan, 1983; O'Donnell *et al.*, 1987 Gustafsson *et al.*, 1995; Bortner *et al.*, 1993; Makov *et al.*, 1996; Dudas and Ruyechan, 1998.) These differences may depend in part on the experimental procedure used to determine this parameter. Also the occluded binding site (the length of DNA covered or rendered inaccessible when the protein is bound) and the interaction site (the length of DNA that directly interacts with the protein) may be different. The work for this thesis found that the minimal number of nucleotides required for pORF6 to bind to ssDNA in an EMSA was 14. However, an increase to 20 nt led to an increase in the level of pORF6 binding. It may be that 14 nt is not enough for the whole protein to bind to with 20 nt allowing the whole protein to bind.

As previously mentioned, protein-DNA interactions are thought to be particularly sensitive to a change in NaCl concentration due to the cations interacting with the nucleic acid. A study on ICP8 investigated the effect of NaCl concentration on ICP8 binding to

ssDNA using a filter-binding assay (Ruyechan and Weir, 1984). Optimal binding was observed at 150mM NaCl and at 300mM NaCl the binding of ICP8 to ssDNA was dramatically reduced.

The effect of NaCl concentration on pORF6 binding to ssDNA was investigated by an EMSA in this study. Similar to HSV-1 ICP8, optimal binding occurred at 150mM NaCl. However, pORF6 was less sensitive to an increase in NaCl concentration than ICP8. This may be because of a dilution effect of loading the samples onto the gel. It would be informative to study the effect of NaCl on the binding of pORF6 and other SSBs, using the same method for each. This would establish whether any differences observed were because of real differences in the binding of these proteins to ssDNA or because different assays have been used. The results from the BIAcore indicated that although the pORF6 and ICP8 bind to ssDNA with a similar affinity the kinetics of binding are not identical. It is possible that this may be indicative of subtle differences between the functioning of the two proteins in infected cells e.g. the phase of viral DNA synthesis in KSHV infected cells is longer than during HSV-1 infection.

The panel of MAbs made against pORF6 were tested in immune assays using *Sf* cells infected with a recombinant baculovirus expressing pORF6, to establish which were positive for each assay. These were western blot, immune fluorescence and immune-precipitation assays. The results indicated that more MAbs may recognise linear than conformational epitopes as the western blot assay gave the most positive results.

Two of the MAbs that were positive in these assays were used in a western blot and immune fluorescence assays against KSHV infected B cells. The pORF6 expressed

during KSHV infection was detected by these antibodies demonstrating that reactivity is not confined to pORF6 over-expressed in *Sf* cells. The immune fluorescence assay showed pORF6 to be in globular areas that may represent the replication compartments in which herpesviruses DNA synthesis is known to occur (Lukonis and Weller, 1996). It would be interesting to fix cells at several time points after induction and to monitor the development of the KSHV replication compartments with these antibodies.

Interactions of pORF6 with ssDNA and the other replication fork proteins could also be characterised using these MAbs. It would be useful to define the region of pORF6 recognised by each MAb, by epitope mapping. The MAbs could then be used to determine the regions of pORF6 critical for its intrinsic binding to ssDNA, and also the region important for co-operative binding. Using these MAbs in co-immune precipitation assays with extracts from cells infected with KSHV could identify interactions between pORF6 and the other KSHV replication fork proteins or other KSHV proteins.

One potential interaction that could be tested for between pORF6 and the OBP of KSHV. The protein that is thought to play the role of OBP in KSHV is K8 (Lin *et al.*, 1999; AuCoin *et al.*, 2004). In HSV-1, ICP8 (SSB) and UL9 (OBP) have a functional interaction that stimulates the helicase activity of UL9 (Boehmer and Lehman, 1993). If K8 is the OBP of KSHV then it may also have a functional interaction with pORF6. If an interaction were to be established between these two proteins it would be more evidence that K8 plays the major role in origin recognition and the initiation of viral DNA synthesis.

The C-terminal region of ICP8 and also of adenovirus DBP is involved in cooperative binding of these proteins to ssDNA. Deletion mutants of ICP8 established that the C-terminal 60 residues are critical for cooperativity (Mapelli *et al.*, 2000). The crystal structure of this protein reveals that the C-terminal domain fits loosely into a concave surface on the back of the N-terminal domain (Mapelli *et al.*, 2004). The crystal structure of adenovirus DBP also reveals that it has a C-terminal 'arm' that hooks onto a second DBP monomer, which results in the formation of long protein chains along the ssDNA (Tucker, 1994). If pORF6 has a similar structure to ICP8 and DBP in that it has a C-terminal 'arm' that links onto the next molecule then a MAb binding to this region may abolish cooperativity. Deletions made in the C-terminus of pORF6 would provide more information on the mechanism of its cooperative binding.

The structure of ICP8 and pORF6

As mentioned previously the crystal structure of the homologous HSV protein, ICP8, has been solved. The protein consists of a large N-terminal region and a smaller C-terminal region (Mapelli *et al.*, 2005). The N-terminal region is described as consisting of head, neck and shoulder regions (see figure 1.7). The head region consists of eight helices, the front of the neck contains a five-stranded β -sheet and two helices (structurally similar to an OB-fold), the back of the neck has a three-stranded β -sheet and the shoulder contains an α -helical and β -sheet region. The C-terminal domain is entirely helical and is connected to the N-terminal domain by a disordered linker.

The neck region (530-1028) is proposed to be involved in DNA binding, since there are a number of aromatic and positively charged residues that are exposed. This is the same region of ICP8 that has been previously suggested for this function based on mutagenesis studies (Leinbach and Heath, 1989; White and Boehmer, 1999). The exposed residues

EBV --MQGAQTS**EDNL**GSQSQPGPCGYIMFYPLATYPLREVAT**EG**TGYAGHRCLTV**PL**LCGIT
 KSHV MALKGPQTL**ENIG**SAAPT**GP**CGYLMAYLTHNFPIGEASL**EG**NGYPEAKVFS**PL**LHGLT
 HHV6 -----MAD**NET**VVSAPVSTA**AW**IMVFPKDKELLDVLSV**SL**MERNNSPIV**IS**PLMNLIT
 HHV7 -----MAD**NET**VVSAP**IC**TAAWLMILPK**EQ**KLIEILTT**SL**MEKRKSVV**IS**PLLNLT
 HCMV -----MS**HE**ELTAL**AP**VG**PA**AF**MF**SRLNA**EQ**EILAT**SL**CDRSSSVV**IA**PLAGLT
 HSV1 -----MET**KP**KATTIKV**PG**PLGYV**AR**AC**SE**GI**EL**LAL**S**ARSGDS**DV**AV**AP**LVVGLT
 HSV2 ----MDTK**P**KTT**TT**TVK**V**PG**MG**YV**GR**AC**PA**EG**LE**LL**SL**SARSGDAD**V**AV**AP**LVVGLT
 VZV ----MEN---TQKT**V**TV**VT**GP**LG**YV**AC**RVEDLD**LE**IE**IS**FAARSTSD**DL**ALL**EL**MRNLIT

56

EBV **V**PGFSIN**V**KALHRR----PDPN**CG**LLRA**SS**--YHRDI**Y**V**NA**HM**V**PP**IF**EGPG**-**LE**AL**
 KSHV **V**SD**PL**N**V**KAVHKK----IDATTAS**V**LT**SS**--YHREAI**V**FN**TH**LFQ**PI**FQ**KG**-**LE**KL
 HHV6 **V**N**DF**ST**TV**K**PI**TN----FGG-TILT**IS**--FMPV**CF**FG**GT**EQ**LV**GMA**ED**HGD**IR**L
 HHV7 **V**N**DF**F**PT**V**K**PIIN----YGG-TVIT**IS**--FMPV**CF**FG**GT**D**V**L**KE**A**ED**HGN**DK**L
 HCMV **V**A**DE**F**V**SV**RT**FPVLC----YDG-GVLT**IS**--FC**PF**FAL**Y**F**HT**Q**GI**V**AF**ED**HG**D**V**HR
 HSV1 **V**SG**FE**AN**V**AV**V**GS**RT**TGLGGTAVSL**LP**SHYSSSV**V**Y**GG**RHLDPST**QA**PN**-**L**TR**L
 HSV2 **V**SG**FE**AN**V**AV**V**GS**TT**GLGGTAVSL**LP**SHYSPSV**V**Y**GG**RHLAPST**QA**PN**-**L**TR**L
 VZV **V**K**TE**TS**SL**AV**VS**GARTTGLAGAGITL**LT**TS**H**F**Y**PS**V**F**VG**GG**K**H**V**L**P**SS**AA**PN**-**L**TR**A

115

EBV **CG**ET**EV**FGY**DA**SAL**PRE**-SS**K**PG--D**FF**PEG-L**DP**SAY**LG**AV**IT**E**AF****CR**LYSG**N**LV
 KSHV **CR**ES**EL**EG**FS**T**F**VE**Q**H**K**GT**L**WSP--E**AG**P**QL**-PC**AN**E**IF**MA**VI**V**TE**GF**CR**LYGG**K**LV
 HHV6 **CR**QT**Q**K**F**HL**Q**S**F**VE**T**ARK**V**DI**K**--AL**S**AV**G**K**D**AD**SV**ICH**V**AC**GN**GF**LL**FA**GL**LI
 HHV7 **CR**QT**E**K**EN**L**Q**E**F**V**V**NG**R**K**P**V**D**IG--KI**CS**V**GR**N**AD**D**V**L**CH**I**V**V**GN**GF**LL**FA**GL**LI
 HCMV **CR**ED**A****Q**K**Y**AL**E**AY**MP**E**AD**R**VP**T**DL**A--AL**CA**AV**G**C**Q**AS**ET**V**H**V**V**V**GN**GL**FL**FA**GL**LI
 HSV1 **CR**EA**R**h**F**EG**F**SD**Y**T**PR**PG**DL**K**H**ET**T**GEAL**CE**RL**GL**DP**DR**ALL**YL**V**V**TEGF**RA**VC**IN**T**NT**F
 HSV2 **CR**EA**R**h**F**EG**F**SD**Y**AP**RP**CD**L**K**H**ET**T**GDAL**CE**RL**GL**DP**DR**ALL**YL**V**V**TEGF**RA**VC**IN**T**NT**F
 VZV **C**NA**A****ER**EG**F**S**R**C**Q**GP**P**V**D**GA**V**ET**T**GA**IE**IC**TR**L**GL**E**P**ENT**IL**YL**V**V**T**AL**F**RA**V**F**MC**N**V**F

175

EBV A**IP**SL**K**Q**E**V**AV**Q**S**AS**V**RV**PL**Y**D**KE**VE**-----PEG**V**P**QL**R**Q**E**Y**NS**D**
 KSHV **P**V**PS**Q**TT**P**V**H**IG**E**H**Q**AF**K**IP**ED**ED**LE**G**-----P**S**RA**Q**EL**CR**E**Y**NP**D**
 HHV6 **PC**VE**EQ**I**Q**V**Q**VE**YS**CV**KI**PL**MS**AT**LE**E-----TE**ET**IS**LS**SC**TE**F**IQ**ER**GS**FL**PA**
 HHV7 **PC**VE**EQ**I**Q**V**Q**VE**CL**AI**KI**PL**MS**AT**LE**E-----SE**ET**LC**ID**T**CT**E**F**IQ**EN**GS**Y**AP**Q**
 HCMV **PC**VE**EA**TT**V**RL**H**G**EA**VR**VP**LP**PT**EN**SL**Q---LD**AE**AD**EV**SL**D**ARS**AF**VE**AR**GL**Y**V**PA**
 HSV1 **L**HL**GG**S**D**K**V**T**IG**GA**EV**HR**IP**V**Y**PL**Q**EM**PD**FS**R**V**IA**E**PF**NAN**HR**S**IG**E**K**F**TY**PL**PF**FN**RP**
 HSV2 **L**HL**GG**MD**K**V**T**IG**DA**EV**HR**IP**V**Y**PL**Q**EM**PD**FS**R**V**IA**AD**PF**NC**N**HR**S**IG**EN**F**NY**PL**PF**FN**RP
 VZV **L**HY**GG**LD**I**V**H**IN**H**GD**V**IR**IE**LF**FP**V**Q**LE**MP**D**V**N**RL**VP**DF**N**TH**RS**IG**EG**F**V**Y**PT**PT**Y**NT**G

235

EBV **L**S**R**CM**HE**AL**Y**T**GL**A**QA**L**V**RR**V**G**K**L**V**EL**LE**K**Q**SL**Q**D**Q**AK**V**AK**V**AP**L**KE**FP**---AST**IS**--
 KSHV **I**S**R**YL**H**DS**IF**T**G**IA**QA**L**V**K**D**V**ST**V**IQ**AS**ER**Q**F**V**H**D**Q**Y**KI**P**KI**V**Q**AK**D**FP---QC**AS**R--
 HHV6 **L**SE**TL**F**Y**V**V**T**SW**GT**LF**SN**T**KE**L**ID**AG**L**K**Q**FT**Q**D**GE**Q**TV**K**L**AP**H**K**Y**L**G**IS**G**Q**K**IS**--
 HHV7 **I**SE**V**L**F**Y**L**I**F**T**SW**GM**TL**FN**NT**LE**L**I**K**AG**L**K**Q**F**I**Q**D**TE**Q**TV**K**L**AP**N**K**Y**H**G**I**P**G**Q**K**LS--
 HCMV **V**SE**TL**F**Y**V**V**Y**T**SW**C**Q**SL**F**SE**PR**V**L**IE**A**AL**R**Q**F**V**H**D**S**Q**Q**SV**K**L**AP**H**K**R**Y**L**G**Y**MS**Q**RLS--
 HSV1 **L**N**R**LL**FE**AV**V**G**PA**AV**AL**CR**N**V**D**AV**AR**AA**HL**AF**DE**N**H**EG**AA**L**PA**D**IT**FT**A**FE**AS**Q**G**--**K**
 HSV2 **L**AR**LL**FE**AV**V**G**PA**AV**AL**AR**N**V**D**AV**AR**AA**HL**AF**DE**N**H**EG**AA**L**PA**D**IT**FT**A**FE**AS**Q**G--**K**
 VZV **L**CH**L**I**H**DC**V**I**AP**MA**VA**L**VR**N**V**T**AV**AR**GA**HL**AF**DE**N**H**EG**AV**L**PP**D**IT**Y**TY**F**Q**SS**SS**G**TT

294

EBV -----HP**DS**GA**LM**I**V**DS**AA**CEL**AV**S**Y**AP**AM**LE**AS**H**ET**P**AS**L**NY**DS**W**PL**F**AD
 KSHV -----GT**D**GS**T**LM**VI**DS**LV**AE**L**MS**Y**GL**S**F**IE**GP**Q**DS**CE**V**L**NY**DT**W**PI**F**EN**
 HHV6 -----A**VE**K**D**FL**ML**LV**DS**V**V**T**EL**S**F**SH**V**AE**Y**L**DS**V**Y**D**PS**Q**IM**N**F**ND**W**PI**IR**N
 HHV7 -----P**IE**K**D**HL**ML**LV**DA**V**IT**EL**TF**S**Y**TA**E**Y**L**DS**I**Y**EN**N**Q**IM**N**F**SE**W**PI**IK**S**
 HCMV -----S**LE**K**D**HL**ML**SD**AV**VC**EL**AF**S**F**AS**V**F**DS**AY**Q**PA**ES**ML**F**SE**W**PL**V**T**N
 HSV1 **T**PR**G**GR--D**GG**G**K**GA**AG**GF**EQ**RL**AS**V**M**AG**DA**AL**AL**ES**IV**S**MA**V**F**DE**PP**TD**IS**AW**PL**F**EG**
 HSV2 **P**QR**G**AR--D**AG**N**K**GP**AG**GF**EQ**RL**AS**V**M**AG**DA**AL**AL**ES**IV**S**MA**V**F**DE**PP**DT**IT**W**PL**L**EG**
 VZV **T**ARG**A**R(5)ST**SK**PS**PG**GF**ER**RL**AS**IM**AA**DT**AL**HA**EV**IF**NT**GI**YE**ET**PT**DI**KE**W**PM**F**IG**

351



EBV CEGPEARVAALHRYNASLAPHVSTQIEATSVLYVSGVSKSTGQ-----GKESLF
 KSHV CETPDARLRALAEVWHAEQALHIGAQLFAASVLYLTRVAKLPQKNQ-----RGDANMY
 HHV6 SETHAERMAQLTNLKLHLSSHLAVLIAPPSIYCSKLAIFPNVKQAFNS--VMTQELLL
 HHV7 AETHEEKIVELKCLRHLSSHVAALVFAASIIYSNKLAYISNTKQAFNS--AITQETLL
 HCMV ATDHRDLIRALTELKLHLSTHVAALVFAASVLYQHRVLYLQSSARHPSAGGTASQETLL
 HSV1 QDTAARANAVGAYLARAAGLVGAMVSTSAHLTEVDDAGPADPK-----DHSK-PSF
 HSV2 QETPAARAGAVGAYLARAAGLVGAMVSTSAHLTEVDDAGPADPK-----DHSK-PSF
 VZV MEGTLRPLNALGSYARVAVGIVGAMVSPSALYLTVEEDSGMTEAK-----DGGPGPSF

406



EBV NSFYMT HGLGLTQEGTWDPCR RPCFSGWGGPDVGTNGPGNYAVE L VYAASFSPNLLAR
 KSHV NSFYLQ HGLGLYSEATV KENGASAFKGVVPSALDGSS---YTLQ L YASSFSPHLLAR
 HHV6 RSLSF CNALSSLSDDVYNDNRK--IIKCDSTSGKDDK---FSAN L YACATSPQLLSF
 HHV7 RSIQFC NSLSSLNEDFYNDARK--LIKCNSSPCKEDK---FSAF L YACATCPQILSH
 HCMV KAIQFT NGLSACEDVYNDARK--VLKFGQAPLKDER---YGPQ L LVCGTCPQIVSG
 HSV1 YRFFLV PGTHTVAANPQVDREGHVVPGFEGRPTAPLVGGTQEFAGE L MLCGFSPALLAK
 HSV2 YRFFLV PGTHTVAANPQLDREGHVVPGFEGRPTAPLVGGTQEFAGE L MLCGFSPALLAK
 VZV NRFYQF AGPHLAANPQTRDRDGHVLS-----SQSTGSSNTEFSVDYL L IICGFAPLLAR

466



EBV YAYYLQFCQGKSSLTVPVETGYSYVAG-AAASPMCSLCEGRAPAVCLNLLFFLLRDLFPP
 KSHV MCYYLQFLPHHKNTNSQSYNVVDYVGT-AAAPSQMODLQGGQCPAVCINLIFYMKDFPP
 HHV6 VVWNLNRMSVYNAG-NAHEIYNHLVN-CSSN-LCEFCGDKCCQSCIGRAMVIVGTLP
 HHV7 IIWNLNRMSIYNTN-CGNSEIYNHIVN-CSSN-LCEFCGDKCCQSCIGRAMVIVGTLP
 HCMV FVWYENRVSIVNTGLSGSSTLNHLVG-CAAG-LCEACGGTCCHTCYQAFVIVRTLPV
 HSV1 MLFYLERCDGAVIVGRQEMDVFRYVADSNQTDVPCNLCTFDRHACVHTLMLLRAHPK
 HSV2 MLFYLERCDGGVIVGRQEMDVFRYVADSGQTDVPCNLCTFETRACVHTLMLLRAHPK
 VZV LLFYLERCDAGAFVTGG-HGDALKYVVTGTFDSEIPCSLCEKHTRPVCAHTVHLLRQMPR

526



EBV VMSTQR RDPYV IISGASGSYNETFLNFLNFIDKEDDQRPDDEP-----YTY
 KSHV VLSNVK RDPYVITGTAGTYNDLEILNFATFRERE EEGNPVEDAP-----KYTY
 HHV6 IPKNVKE PLVMSMFSRYVAE VILSFGGRKPVSELKEIGKDQNN-----TSLDLGKF
 HHV7 ISKTTKKEPIVMTMFSRFLADV VLSFGKKGVNESKDPMKEAQT-----TPSLDFKF
 HCMV VPKQPKKEPCVITVQSRFLNDV VILSFGRRYNVDAKDGGLDGKG(46)MLGASVDTYR
 HSV1 FASAARGAIGVFGTMNSMYSDCV LNYAAFSA LKR-ADGSETAR----TIMQETYAAT
 HSV2 FASAARGAIGVFGTMNSMYSDCV LNYAAFSA LKR-ADGSE NTR----TIMQETYAAT
 VZV FGQATRQPIGVFGTMNSQYSDCPLNYAPYLILRKP GDQTEAAK----ATMQDTYATL

582



EBV WQLNQNLLE-RLSRLGIDAEG-KLEKEPHGPRDFVVMFKDVAADVAEVQFMNSMAKN-
 KSHV WQLCQNITE-KLASMGI SEGGDALRTLIVDIPSFVKVFKGIDSTVEAELLKF INCMIKN-
 HHV6 VSQIFDYCK-KNSLIDPVTGEDTFNVR--SKKDFVSIHGLTQCI ECVSR CIVE MRR--
 HHV7 LGMIHDYCK-KNNLIDAITGEDNLNFK--SQNDFVNMINDLIQCI EAVSKCISEMRK--
 HCMV LNRILDYCR-KMRLIDPVTGEDTFSAH--GKSDFFVAVFSALNK FVDD EALGFVSEVR--
 HSV1 ERVMAELETQYVDQAVPTAMGRLETIITNREALHTVVNNVRQVVDREVEQLMRNLVEGR
 HSV2 ERVMAEALQYVDQAVPTALGRLETIIGNREALHTVVNNIKQLVDREVEQLMRNLIEGR
 VZV ERLFIDLEQERLLDRGAPCSSEGLSSVIVDHPTRFRILDTLRARIEQTTTQFMKVLVETR

642



EBV NITYKDLVKSCYHVMQYSCNIFAQPAQIFITQLFYRSLTILDISIPICMCEYENDNPGL
 KSHV NYNFIENIKSVHHILQFACNVWQAPCPVFLTLTYKSLTIVLIDICHTSCMMEYEQDNPAV
 HHV6 TQTP EQIENCLQSFNVDTFYATAFSPFLTFSYKVIITVNLALAIIVASGHVVDPRPCT
 HHV7 TQTS EQIENCLQSFNIDTTL S LAFSPFFVFTYYKVIITVNLALAIIGTYVVDPRPCT
 HCMV LKSS DEVAGATQAFNLDLNPYAVAFQELLAYAYFRSVFYIINVALITATSYIVDNPLT
 HSV1 NFKF DGLGEANHAMSLTLDPYACGPCPLLQLLGRRSNLAVYDLAISQCHGVFAGQSVE
 HSV2 NFKF DGLAEANHAMSLSLDPYTCGPCPLLQLLARRSNLAVYDLAISQCHGVFAGQSVE
 VZV DYKIEGLSEATHSMALTFDPSYSGAFCPITNFLVKRTHLAVYDLAISQCHCVFYGQQVE

702



EBV QSPPEWLKGHY TLCTNFRSLAIDK VLTAKAEKVHGEPTCDLPDLLDAALQGRVYGRR
 KSHV IVPSEWLKMHF TMWTNFKGACFDK AITGGELKIVHQSMFCDLFDTDAAIGGMFAPAR
 HHV6 NSISKWLQVQY SLYGTFFHSSYLKK FLNTRTVKVASNVDMEQILDCDLYKSGKYVKT
 HHV7 NLLSKWLMQQY SLYGAFYNSHFKK FLNMKTVKIASNVDMEQYIDFNLFKSGKYAKTS
 HCMV TNLVSKWMTQHF SIHGAFSTSSRK FLFTKQIKSSKNSDHDRLDFRLYAQGTAVVP
 HSV1 RN---FRNQF PVLRRRVMDMFNN FLSAKTLTVLSEGAACAPSLTAGQTAPAESS 758
 HSV2 RN---FRNQF PVLRRRVMDLFNN FLSAKTLTVLSEGAACAPSLTAGQTAPAESS
 VZV RN---FRNQF PVLRRRFVDLFNG FISTRSTVTVLSEGP-VSAPNPTLGQDAPAGRT



EBV LPVRMSKVLMLCPRNIKIIN VVFTGEN-----AALQNSFIKSTTRRENYIINPY
 KSHV MQVRIAAAMLVPKTIKIIN IIFSNTG-----AESIQAGFMKPPASQRDSYIVG PY
 HHV6 IQAKLCLSMQCLRDFRIIN PFNKSSK-----TAHNNPYFKKNVKHKKNPLS CI
 HHV7 IQAKLCLSMQCLKDFRVIN PFNKPNK-----NTQNNPFKK-VKQKKNPLS CL
 HCMV MEIKLSLSVPTLIMVRVIN PIYRAGK-----GNAGSVFFRRDHVPRRNPAPK CL
 HSV1 FEGDVA VTLGFPKELRVIS VLFAGASANASEAAKARVASLQSAQKPKDKRVDILL PL
 HSV2 FEGDVA VTLGFPKELRVIS VLFAGASANASEAAKARVASLQSAQKPKDKRVDILL PL 818
 VZV FDGDLAVSVEVIRDIRVIN VVFSGNCTNLSEAAARARLVGLASAYQRQEKRVMDMLHAL



EBV MKFNTYKTLFF-----DTKLSSLYLWHNFSRRRSVVPVSGASAEYSDLALFVDGGS
 KSHV MKFNLAKTLFF-----STKTSALYLWHKIGQTTKNPILPGVSGEHLTELCNYVKASS
 HHV6 SFLFKYDKLFF-----NVKISCLELWQRFLLNNVPKTLDIGNPEVKTFIKFAFSIT
 HHV7 SFLFKYERLFF-----NLKISCLEFWQRIILLNNMPKIDIGNVEDMRSPKFTFRVT
 HCMV GFLLYRHERLFF-----ECGLPCLQFPQKVCNALPKNVPIGDMGEFNAFVKFLVAVT
 HSV1 GFLDKQFAAIFPENGKPPGSNQPQWFWTALQRNQLPARLLS---REDIETIAFIKKFS
 HSV2 GFLDKQFAAIFPENGKPPGSNQPQWFWTALQRNQLPARLLS---REDIETIAFIKRFS 873
 VZV GFLDKQFGLLFFRGMPPNSKSPNQWFWTLLQRNQPADKLT---HEEITIAAVKRFT



EBV RAHEESNVIDVVGNLVTYAKQRLN AITKACGQTQFYI SLIQGLVP-RTQSVPARDYPH
 KSHV QAFEINVLDLVDTLTSYAKIKLNSSI RACGQTQFYATT SCLSP-VTQLVPAEYYPH
 HHV6 NTYDEIDLDIQECLSTFIDCYFH KFLSALG-FHDYLTSEHGLTS-KLVTONPVLFV
 HHV7 NSYDEIDLDIQECLSFIEYFHKLLSVLG-YRDYLTSEHALTS-KLVPQNPMLFPV
 HCMV ADYQEHDLDDVAEDCVLSYVESRFH KFLCYG-FKDYIGSEHGLTT-RLTTQNHAFPH
 HSV1 LDYGAINFINLAPNNVSELAMYMA QILRYCDHSTYFINTLTAIAGSRRPPSVQAAA 935
 HSV2 LDYGAINFINLAPNNVSELAMYMA QILRYCDHSTYFINTLTAIAGSRRPPSVQAAA
 VZV EYAAINFINLPTCIGELAQFYMA LILKYCDHSQYLINTLTSIITGARRPRDPSSVLH



EBV VLG-TRAVESAAAYAEATSSLTATTVCAATDCLS-----QVCKA PVRTLPVTIN YT
 KSHV VLG-PVGLSSPDYRAKVAGRSVTIVQSTLQAVS-----TNGRLPIITVPLVNN YT
 HHV6 VLDKQPKFSSIQYLIVYVKKLVLDGVPNPVIASLSKEPNFGTIFTS SLVTFGLTLE FV
 HHV7 FLKEHPTFSSVQYVMHVKKLVGNLKEPMTASLTKEPNFGSIFTG SIITFGLMIE FV
 HCMV VLGASPRFSSPAFALHVKGLKTAGVPAPMAATVARESILVRSVFEH SLVTVPVSVE YA
 HSV1 WSAQ---GGAGLAGARALMDAVDAHGAWTSMFASCNLLRPVMAA PMVVLGLSIS YY 992
 HSV2 WAPQ---GGAGLAGARALMDSLDAHGAWTSMFASCNLLRPVMAA PMVVLGLSIS YY
 VZV WIRKDVTSAAIDITQAKALLEKTENPELWTTAFTSTHLVRAAMNQ PMVVLGISIS YH



EBV GVNGNNTFEQANLGYFMRGVDRNLQAPGAGLRKQAGGSSMRKKFVF TPTLGLTVKR
 KSHV GSNGTINVEHCANLGYFSRGVDRNLRPES-VPFKKNVSSMLRKRHVIIMTPLVDRLVKR
 HHV6 SLAN-REYQFQLGWIGSGVDRNLNPTS---SALQDFRFRQKTIITKFSSEVIVK-
 HHV7 SVAS-RDYEHFQLGWIASGVDRNLNPPS---SGLQDFRFRQKRVITKLCDIIVK-
 HCMV GINNSKEIYQFQIGYFSNGVERSLNVSS---MSGQDYRFRQRYLLTRLADVLIK-
 HSV1 GMAGNDRVQANWASLMGKNACPILLIFD-----RTRKFVLCPRAGFVCA 1040
 HSV2 GMAGNDRVQANWASLLGKNACPILLIFD-----RTRKFVLCPRAGFVCA-
 VZV GAAGNRRVQANWASLNGKNVCPILLIFD-----RTRRFILCPRGGFICP-



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EBV      RTQAAAT-TYEIENIRAGLEAIIISQKQEEDCVFDVVCNLDAMGEACASLTRDIAEYLLGR
KSHV     IVGINSGEFEAEAVKRSVQNVLEDRDNPPLPKTVVLELVKHLGSSCASLTEEVIYYLGP
HHV6     KVRREAIMFDTEVVKGKVLIVENLTND--IDPELLIIAEVMRDREDKPTMDMLFFVDG
HHV7     KVKREAIYDVEVIRGKVLNIIESLSNS--VNPELLILAEVMKDRDSDKPTMDMLFFVDG
HCMV     RSRRENVLFDADLIKNRVMLALDAENLD--CDPEVMAVYEILSVREEIPASDVLFFVDG
HSV1     -ASSLGGGAHESSLCEQLRGIIE--GGAAVASSV FVATVKSLGPRQTQQLQIE WLALLED 1098
HSV2     -ASSLGGGAHESSLCEQLRGIIE--GGAAVASSV FVATVKSLGPRQTQQLQIE WLALLED
VZV      -VTGPSSGNRETTLSDQVRGIIIVS--GGAMVQLAIYATVVRVAVGARQAQHMADF WLSLTDD

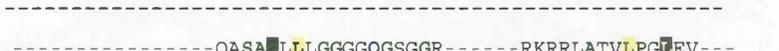
```



```

EBV      FSVLADSVLETLATIASSGIE-WTAAEARDFLEGVWGGPGAQDNFISVAEVPVSTAS---
KSHV     YAVLGDVLSLLSTVGGQAGVP-WTAEGVASVIQDIIDD---CELQFVGPPEPCLIQG---
HHV6     REAFAASIMLKLNLHLDVDMNVKDFSIITNLQSVFETVSS-----NDAPVYDFSEILAE---
HHV7     REPFAKSVMNKIQLHTDLNVHDFSLSTLLSVFEEQVE-----DSAAIYDFSELLVEG---
HCMV     CEAFASASLMDKFAALQEQGVDFSLLENLRRVLDADAQRLTDAAGGEVHDL SALFAPSGV(
HSV1     -EYFSEEMMELTARALERNGEWS TDAALEVAHEAEALVSQLG---NAGEVFNFGDFG-- 1152
HSV2     -EYFSEEMMEFTTRALERGHGEWS TDAALEVAHEAEALVSQLG---AAGEVFNFGDFG--
VZV      -EFAARDLELHQIIQTLETPTWIVEGALEAVKILDEKTTAGDGETPTNLA FNFDSCPE-

```



```

EBV      -----QASALLLGGGGQSGGR-----RKRLATVLPGEV---
KSHV     -----QSVVEELFPSPGVP SLTVG-----KRRKIASLLSDLDL---
HHV6     -----DDQGNVLKCDETETETDEP-----MTKKNR---
HHV7     -----NEQGFILKCEETEHENEPP-----SLKKAR---
HCMV     15) SVAGNSICFVGPGETGGCFVLNAGEDEAGGVG(6) GGSGLLPKRSR-----
HSV1     ---CEDDNATPFG--GPGAPPAFAGRKRAFHGDDP---FGE-GPPDKKGDFTLDML 1196
HSV2     ---DEDDHAASFGGLAAAAAAGVARKRAFHGDDP---FGE-GPPEKK-DFTLDML
VZV      ---SHDTTSNVLNISGSNIS--STVPGLKRPPEDDEL---FDLSGIPIKHGNTMEMI

```

Figure 7.1 An alignment of the SSBs from the human herpesviruses

The SSBs from the eight human herpesviruses were aligned using ClustalW and annotated using CHROMA. The virus from which the protein is from is indicated on the left hand side. Conserved amino acids involved in DNA binding in ICP8 (HSV-1) are highlighted with a red arrow. Conserved residues that form a hydrophobic patch on the N-terminus of ICP8 are highlighted with a blue arrow. Conserved residues that are involved in zinc binding are indicated with a light green arrow. A FNF motif conserved among the alpha herpesviruses is highlighted with a pink line below the alignment. The secondary structure of ICP8 is shown above the alignment. Horizontal cylinders indicate α -helices, horizontal arrows indicate β -sheet. Elements are coloured dark pink for the head, blue and orange for the shoulder, light pink and grey for the neck, green for the zinc binding loop, mauve for the C-terminal helical domain. The dashed line indicates the region that was absent from the crystal structure. The number at the end of each line indicates the residue number of ICP8 at the end of the line. The colour code for the amino acids is shown on the next page.

The colour code is as follows:

Y

letter with a grey background indicates conserved residues

DE

basic residues

ST

Ser/thr

ILV

aliphatic

HKR

positive

FHWY

aromatic

DEHKR

charged

ACDGNPSTV

small

CDEHKNQRST

polar

ACFGHILMTVWY

hydrophobic

that may be involved in DNA binding are Tyr⁵⁴³, Asn⁵⁵¹, Arg⁷⁷², Lys⁷⁷⁴, Arg⁷⁷⁶, Tyr⁹⁸⁸, Phe⁹⁹⁸ and Asn¹⁰⁰². Figure 7.1 shows an alignment of the SSBs from the eight human herpesviruses. The sequences were aligned using ClustalW and annotated using CHROMA (Goodstadt and Ponting, 2001), which colours conserved regions. The above mentioned residues are fairly well conserved within these herpesviruses and have been highlighted with a red arrow below the sequence. Arg⁷⁷² is not conserved between ICP8 and pORF6 but the replacement amino acid, lysine, also contains a basic side chain and would be able to contribute in the same electrostatic interactions with DNA as arginine. Also shown in the figure above the alignment is the secondary structure of ICP8 determined by crystallography (Mapelli *et al.*, 2005). This secondary structure is likely to be largely conserved throughout human herpesvirus SSBs.

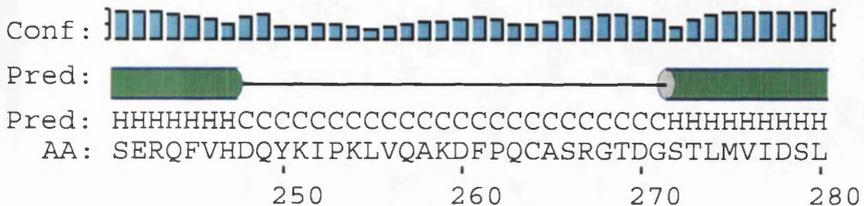
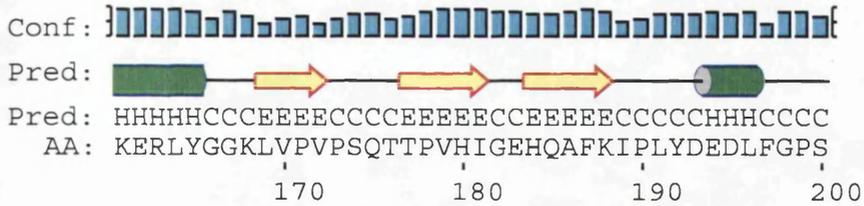
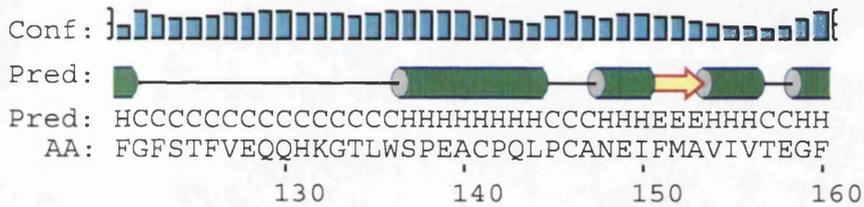
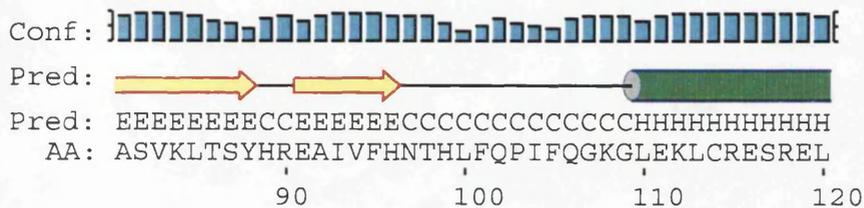
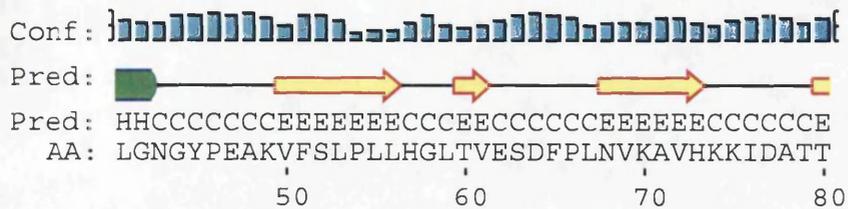
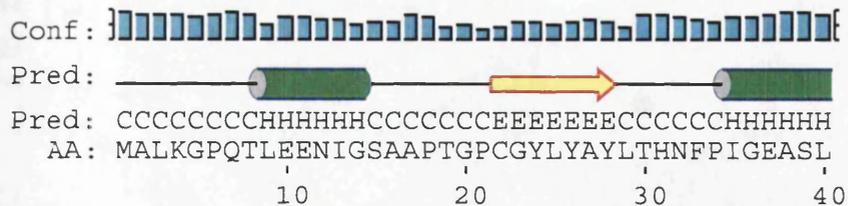
ICP8 is a zinc metalloprotein containing one zinc atom per molecule that as predicted is coordinated by three cysteines (Cys⁴⁹⁹, Cys⁵⁰², Cys⁵¹⁰). These amino acids are conserved in the human herpesviruses and have been highlighted with a light green arrow. Also conserved is Thr⁵¹³, which further stabilizes the zinc loop in ICP8. These residues most likely have the same function in pORF6 as in ICP8.

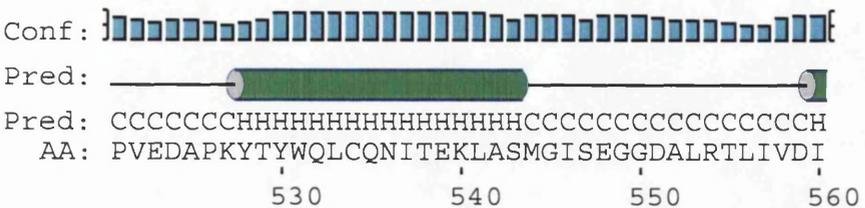
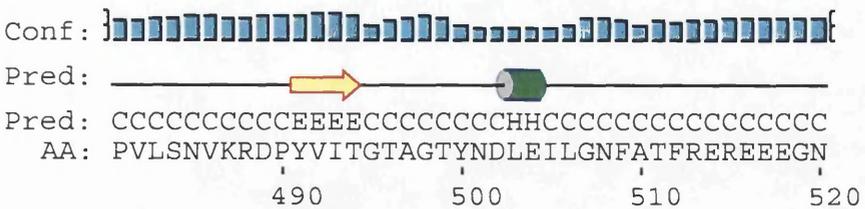
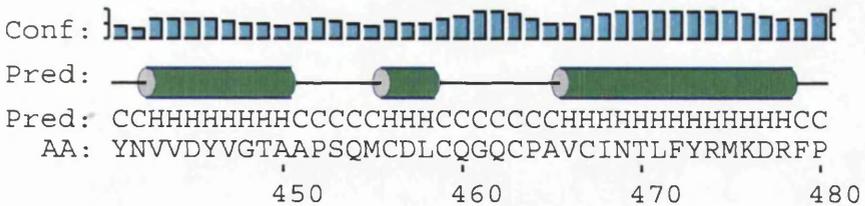
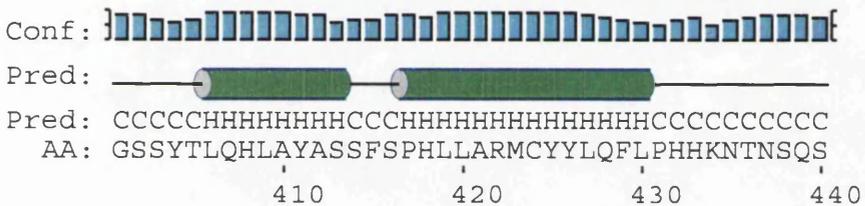
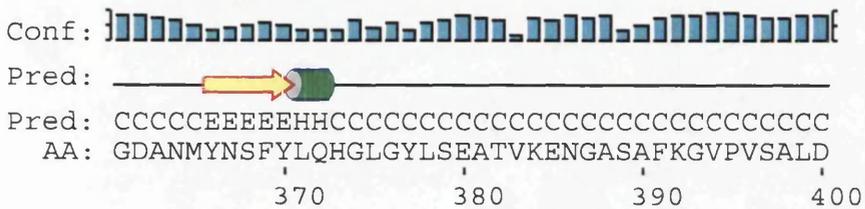
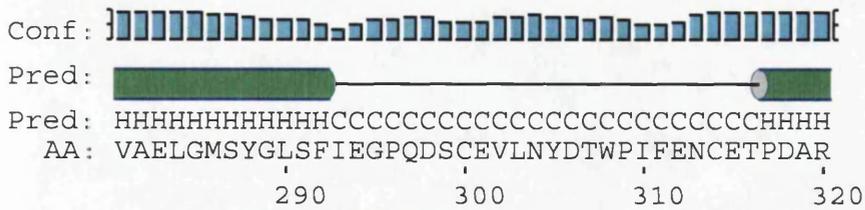
The C-terminus of ICP8 has been shown to be important for cooperative binding. It is thought that it interacts with a hydrophobic patch in the head region of another molecule of ICP8 that is formed by Phe⁸²⁷, Phe⁸⁴³, Trp⁸⁴⁴, Leu⁸⁵⁷ and Ile⁸⁶⁵. These residues have been highlighted with a blue arrow in figure 7.1. The phenylalanine⁸²⁷ and the tryptophan are conserved within the human herpesviruses. Phe⁸⁴³ and Leu⁸⁵⁷ are not conserved but in pORF6 the replacements, Leu⁸⁴³ and Pro⁸⁵⁷ are also hydrophobic. Ile⁸⁶⁵ is also not conserved and pORF6 is the only herpesvirus SSB in which the replacement amino acid does not have a similar side chain chemistry. The other proteins also contain

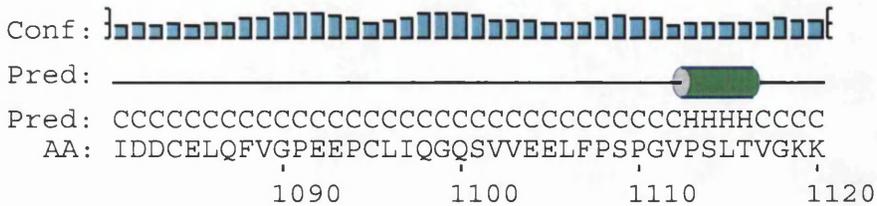
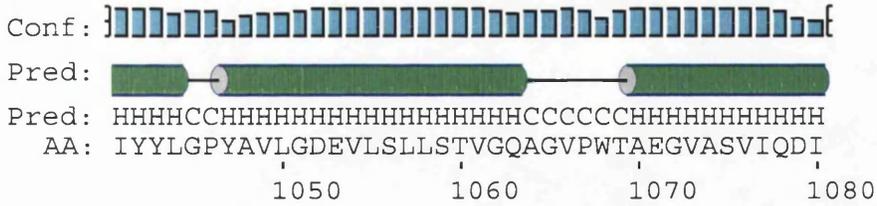
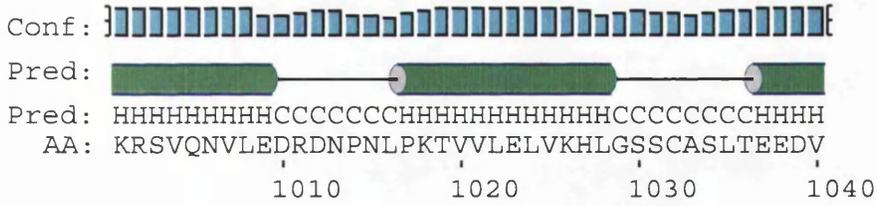
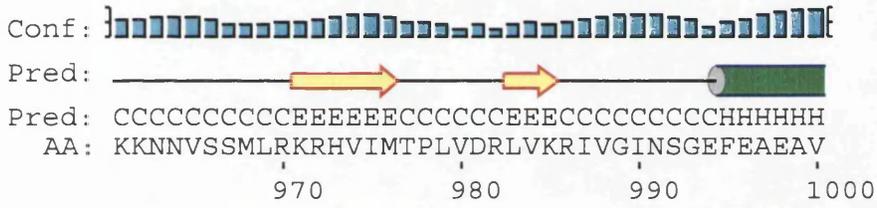
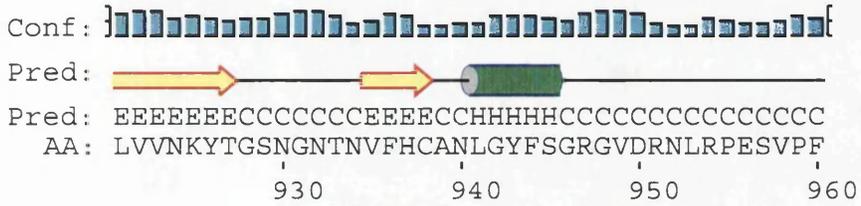
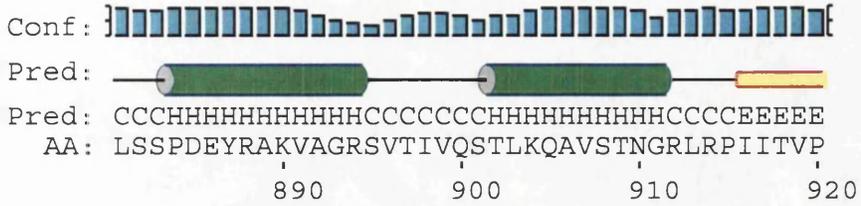
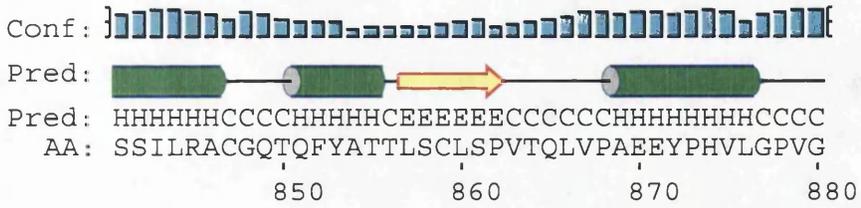
a hydrophobic residue at this point but pORF6 contains a cysteine. However, there is still a hydrophobic patch created by the other conserved amino acids that could interact with the C-terminus of an adjacent pORF6 molecule.

The extreme C-terminus of the human herpesvirus SSBs does not appear to have much sequence conservation. However, there is a FNF motif that is conserved within the alpha human herpesviruses that is thought to interact with the hydrophobic patch on the N-terminus of the next protein molecule. This is highlighted with a pink line underneath the alignment in figure 7.1. The other viral proteins shown in the alignment do not contain this motif but they do have two hydrophobic residues in the same region, which could also interact with the hydrophobic patch on the N-terminus of an adjacent protein molecule.

Figure 7.2 shows the secondary structure prediction of pORF6 generated using PSIPRED (Jones, 1999). Overall this is very similar to the actual structure of ICP8 for the corresponding regions of the alignment (figure 7.1). In ICP8, the region containing the amino acids involved in zinc binding has an α -helical structure (499-513 (figure 7.1-light green arrows)). The corresponding region containing the conserved residues in pORF6 is also predicted to be α -helical (455-470). The sequences containing the amino acids involved in DNA binding in ICP8 form a loop region (543-541) and two β -sheets (772-776 and 998-1002) (figure 7.1-red arrows). The first of these regions mainly consists of a loop structure in ICP8 but in pORF6 it is also predicted to contain a small α -helical structure (499-509). However, the two β -sheet regions in ICP8 have a calculated corresponding β -strand predictions in pORF6 (725-732 and 925-939). The hydrophobic patch in the head region of ICP8 that binds to the C-terminus of an adjacent molecule is α -helical with loop regions (827-865 (figure 7.1-blue arrows). The corresponding region







Conf: 
 Pred: 
 Pred: CCCCCCCCCC
 AA: RKIASLLSDLDL
 |
 1130

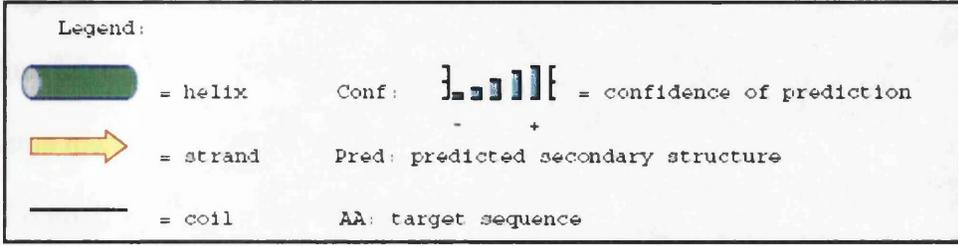


Figure 7.2 Secondary structure prediction of pORF6

The pORF6 amino acid sequence was analysed using the PSIPRED protein secondary structure prediction programme <http://insulin.brunel.ac.uk/cgi-bin/psipred> (Jones 1999). The programme assigns an amino acid residue to a secondary structure motif within the context of the surrounding amino acids. The figure shows which amino acids are likely to form α -helices, β -sheets or non-specific coiled loops.

in pORF6 is also predicted to be α -helical with loop areas (772-792). In addition, the C-terminus of ICP8 is α -helical, which is also the predicted structure of the C-terminus of pORF6.

The regions of pORF6 discussed above that contain the conserved important functional residues have the same secondary structure as ICP8 consistent with them functioning in the same manner in the binding with ssDNA. Similarly, the cooperative nature of pORF6 binding with ssDNA may like ICP8, be due to a C-terminal 'arm' linking onto the next molecule and consistent with this, the predicted structure in this region of pORF6 is very similar to the ICP8 structure. The information gained from the alignment and secondary structure prediction provides a greater understanding of how pORF6 may be binding to ssDNA. In addition, knowledge of the structure of ICP8 and the identification of residues involved in important functions of the protein provides information that could be used to design mutations in pORF6 to further characterise its binding to ssDNA.

Investigating the DNA replication proteins of the herpesviruses not only provides additional information on how these viruses replicate, but also characterises a potential target for anti-viral therapy. The disruption of protein-protein interactions between the DNA replication proteins of the herpesviruses represents a novel anti-viral strategy. The interaction between the HSV-1 DNA polymerase (UL30) and its processivity factor (UL42) can be disrupted by short peptides corresponding to the C-terminus of UL30 (Marsden *et al.*, 1994; Digard *et al.*, 1995; Bridges *et al.*, 2000), which inhibits the ability of UL30 to synthesise long DNA chains. Loregian *et al.* (1999) demonstrated that a peptide corresponding to the C-terminal 27 residues of UL30 fused to the B subunit of

E.coli enterotoxin can enter the nucleus of HSV-1 infected Vero cells and inhibit viral replication. A peptide corresponding to the C-terminus of the HCMV DNA polymerase also blocks the interaction between the HCMV polymerase and its accessory factor and specifically inhibited the stimulation of the polymerase by the accessory factor (Loregian *et al.*, 2003). However, using a peptide as an anti-viral drug imposes the potential problem of an immune response from the host. More recently small molecules that also inhibit the interaction between UL30 and UL42 have been identified (Pilger *et al.*, 2004). One of these molecules had more potent anti-viral than cytotoxic activity. Molecules of this type may have better potential as anti-viral drugs than peptides as they are less likely to cause an immune response.

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