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The Multifunctional ORF57 Protein of Kaposi's Sarcoma Associated Herpesvirus

A thesis presented for the Degree of
Doctor of Philosophy

by

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in

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Abstract

The aim of this work was to identify proteins, both cellular and viral, that interact in infected cells with the Kaposi's sarcoma-associated herpesvirus (KSHV) regulatory protein ORF57. In the light of this information, functional assays were performed to characterise various activities of the ORF57 protein.

KSHV, the most recently identified herpesvirus to infect humans and associated with cancer, encodes ORF57, an immediate early (IE) regulatory protein. ORF57 is the only KSHV IE gene that has a counterpart in all herpesviruses sequenced so far. Studies of this protein, that may have similar functions throughout the *Herpesviridae*, are important to answer key questions about herpesvirus biology and the development of effective therapies.

During lytic virus infection, expression of different classes of KHSV genes is temporally regulated. Two of the IE proteins, ORF50 and ORF57, are central to this regulation and are essential for viral growth. ORF50 causes the switch from the viral latent to lytic cycle and is required for establishment of latency. ORF57 regulates viral gene expression at the transcriptional and post-transcriptional levels. Transcriptionally, it is capable of acting on its own or in combination with ORF50; post-transcriptionally, ORF57 enhances expression of viral genes and represses expression of some intron-containing genes.

This study shows that ORF57 (like its herpes simplex virus ICP27 counterpart) in fusion protein pull down assays and coimmunoprecipitation assays interacts with four cellular proteins using extracts from KSHV-infected cells. These are: heterogeneous nuclear ribonucleoprotein K (hnRNP K), protein kinase casein kinase 2 (CK2), RNA export factor binding protein (REF) and splicing associated protein 145 (SAP145).

CK2 was present in complexes with hnRNPK or SAP145, only in the presence of ORF57. Interestingly, the CK2 activity that came down with anti-ORF57 antibody could phosphorylate ORF57 protein itself and certain other proteins, including hnRNPK present in the coimmunoprecipitated complex. ORF57 phosphorylation by CK2 increased its affinity for hnRNP K. CK2 activity could be important for regulation of the various interactions, and act to control them depending on the phosphorylation status of ORF57

and its partners.

A further ORF57 interaction was identified with the viral transcriptional transactivator ORF50 protein, and immunofluorescence on virus-infected cells showed that ORF57 partially colocalised with ORF50, in accordance with the involvement of these two IE proteins in activities together. In functional assays, ORF57 protein was shown to augment activation of the ORF50 promoter by ORF50 protein, and this activity may reflect a requirement for ORF57 at an early stage of lytic infection to amplify environmental stimuli thereby facilitating viral replication.

The interaction of ORF57 with several proteins known to be involved at several stages of gene expression point to it being a multifunctional protein; and how these partner proteins contribute to ORF57's various functions can be inferred from a knowledge of their activities. An association with hnRNP K may account for ORF57's transcriptional regulation of viral gene expression acting either on its own or with ORF50. Much of the ORF57 activity is seen at the post-transcriptional level which also could be attributed to the action of hnRNP K. Immunofluorescence data demonstrated that ORF57 colocalises in punctate spots in the nucleus with hnRNP K, and reporter assays have shown that ORF57 and hnRNP K are functionally linked.

Promotion of viral mRNA export acts to enhance gene expression and may occur via the association of ORF57 with REF; immunofluorescence data demonstrated that ORF57 colocalised in punctate spots in the cell nucleus with REF. This study, for the first time, shows that ORF57 acts directly as a nuclear RNA export factor in somatic cells, and does not use a CRM1 dependent route. Rather, ORF57-mediated RNA export shares certain components of the nuclear export pathway utilised by cellular mRNAs but may exploit more than one export route at different stages of infection.

The ORF57 interaction with SAP145 may cause partial or selective inhibition of splicing, allowing the splicing out of introns in viral transcripts yet inhibiting cellular pre-mRNA processing. Unlike with HSV ICP27 protein, the splicing p32 protein does not interact with ORF57 and this may support selective splicing inhibition in KHSV. Inhibition of cellular RNA splicing would indirectly block cellular mRNA export, freeing up REF protein to interact with ORF57.

Finally, the multiple interactions of ORF57 are likely to be facilitated by its ability to self

interact, enabling the formation of large complexes. These protein:protein and protein:nucleic interactions could however be dynamic, changing at different intracellular locations or times post-infection to facilitate the various functions of ORF57. The present study has allowed insights as to how ORF57 exerts its various activities and will help in the understanding of how viral transcription, RNA processing and RNA export are interlinked in KHSV-infected cells.

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"To steal ideas from one person is plagiarism,

to steal ideas from many is research"

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This thesis is dedicated to my mother, father and family,

for always being there for me…….

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Abbreviations

^{32}P	phosphorus-32 radioisotope
^{35}S	sulphur-35 radioisotope
A	adenine or absorbance or amps
aa	amino acid
Ab	antibody
AHV-2	alcelaphine herpesvirus-2
AIDS	acquired immune deficiency syndrome
APS	ammonium persulphate
ASF/SF2	alternative splicing factor / splicing factor 2
ATP	adenosine-5'-triphosphate
BCBL	body cavity based lymphoma
β-gal	beta-galactosidase
BHK	baby hamster kidney
BHV-4	bovine herpesvirus 4
bp	base pair
BPS	branch point sequence
BSA	bovine serum albumin
bZIP	basic region-leucine zipper DNA binding domain
C	cytosine or carboxy (-terminal end of protein)
CAT	chloramphenicol acetyltransferase
CBP	CREB-binding protein
Ci	Curie
CIP	calf intestinal phosphatase
CK2	protein kinase casein kinase 2
cryoEM	cryoelectron microscopy
CTD	RNA polymerase II carboxy terminal domain
CTE	constitutive transport element
DEN	Dengue virus
DMEM	Dulbecco's Modified Eagles Medium
DMSO	dimethyl sulphoxide
DMVECs	human dermal microvascular endothelial cells
DNA	deoxyribonucleic acid
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DTT	dithiothreitol
E. coli	Escherichia coli
EBV	Epstein-Barr virus

ECL	*enhanced chemiluminescence*
EDTA	*ethylenediaminetetra-acetic acid*
EHV 1-4	*equid/equine herpesvirus 1-4*
EJC	*splice exon-junction complex*
FCS/FBS	*foetal calf/ new born bovine serum*
FITC	*fluorescein isothiocyanate*
FL	*full-length*
g	*gram or glycoprotein*
G	*guanine*
GMEM	*Glasgow Modified Eagle's Medium*
GST	*glutathione-S-transferase*
GTP	*guanosine-5'-triphosphate*
h	*hour*
HCMV	*human cytomegalovirus*
Hep C	*hepatitis C virus*
HEPES	*N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid*
HHV-1 to 8	*human herpesvirus 1-8*
HIV-1	*human immunodeficency virus type 1*
HLA	*human leukocyte antigen*
hnRNP	*heterogeneous nuclear ribonucleoprotein*
HPV	*human papilloma virus*
HSV-1 / 2	*herpes simplex virus type 1/ type 2*
HuT	*human T-cell growth media*
HVS	*herpesvirus saimiri*
Ind	*induced BCBL-1 cells by addition of TPA*
ICP	*infected cell protein*
IE	*immediate early*
IFA	*immunofluorescence assays*
IFN-α	*interferon-α*
IgG	*immunoglobulin G*
IP	*immunoprecipitation*
IPTG	*isopropyl β-D-thiogalactopyranoside*
ISRE	*interferon stimulatory response element*
kb	*kilo-bases*
kDa	*kilo-daltons*
KH	*hnRNP K homology*
KNS	*hnRNP K nuclear shuttling signal*
KPK	*hnRNP K protein kinase*
KS	*Kaposi's sarcoma*

KSHV	Kaposi's sarcoma-associated herpesvirus
l	litre
LANA	latency-associated nuclear antigen
LMB	leptomycin B
LOX	erythroid 15-lipoxygenase
LRR	leucine rich repeat region
LUR	long unique region
M	molar
μ	micro
m	milli
Mab	monoclonal antibody
MAPK	mitogen-activated protein kinase
MBP	maltose binding protein
MCD	multicentric Castleman's disease
MCMV	Murine cytomegalovirus
MDV	Marek's disease virus
β-ME	β-mercaptoethanol
MHC	major histocompatibility complex
MHV-68	murine gammaherpesvirus-68
MIP I/II	macrophage inflammatory protein I/II
mi	mock infected
min	minutes
mRNA	messenger RNA
mRNPs	messenger ribonucleoproteins
n	nano
N	amino (-terminal end of protein)
NES	nuclear export signal
NLS	nuclear localisation signal
NP40	nonidet P-40
NPC	nuclear pore complex
Oct-1	octamer-binding protein
OD	optical density
ORF	open reading frame
REs	response elements
p	pico
PAA	phosphono acetic acid
PAGE	polyacrylamide gel electrophoresis
PAN	polyadenylated nuclear
PBMCs	peripheral blood mononuclear cells

PBS	*phosphate-buffered saline*
PEL	*primary effusion lymphoma*
PKC	*protein kinase C*
PMA	*phorbol-myristic acid*
PMSF	*phenylmethylsuphonyl fluoride*
Pol-8	*DNA polymerase -8*
PPF	*polymerase processivity factor*
pre-mRNA	*precursor mRNA*
PRV	*pseudorabies virus*
PVDF	*polyvinylidene difluoride*
RBD	*RNA binding domains*
REFs	*RNA and export factor binding proteins*
RFMHV	*retroperitoneal fibromatosis herpesvirus*
RGG	*arginine rich RNA binding motif*
RNA	*ribonucleic acid*
RNA Pol II/III	*RNA polymerase II/III*
RNP	*ribonucleoprotein*
RRE	*HIV-1 Rev-response element*
RRV	*rhesus rhadinovirus*
RRL	*rabbit reticulocyte lysate (used for in vitro protein synthesis)*
RT-PCR	*reverse transcriptase-polymerase chain reaction*
SAP	*splicing-associated protein*
SDS	*sodium dodecyl sulphate*
snRNA	*small nuclear RNA*
snRNP	*small nuclear ribonucleoprotein particle*
SV40	*simian virus 40*
T	*thymine/ Tween-20/large T antigen of SV40*
TBP	*TATA-binding protein*
tDMVECs	*transformed dermal micro-vascular endothelial cells*
TE	*Tris-EDTA*
TEMED	*N, N, N', N'-tetramethylethylenediamine*
TF	*transcription factor*
TPA	*12-O-tetradecanoyl phorbol-13-acetate*
TR	*terminal repeat*
TREX	*transcription/export complex*
TRITC	*tetramethylrhodamine isothiocyanate*
U	*uridine or units*
U2AF	*U2 associated factor*
Un	*uninduced BCBL-1 cells*

UV	*ultra-violet*
V	*volts*
v/cTK	*viral/cellular thymidine kinase*
VEGFR-3	*vascular endothelial growth factor receptor-3*
vFLIP	*viral FLICE inhibitory protein*
vGPCR	*viral G-protein-coupled receptor*
vIL-6	*viral interleukin-6*
vIRFs	*viral interferon regulatory factors*
VP	*virion polypeptide*
VZV	*varicella-zoster virus*
w/v	*weight/volume*
wt	*wild type*
WT1	*Wilm's tumour protein*

Amino acid symbols

One letter symbol	Three letter symbol	Amino acid (aa)
A	Ala	Alanine
C	Cys	Cysteine
D	Asp	Aspartic acid
E	Glu	Glutamic acid
F	Phe	Phenylalanine
G	Gly	Glycine
H	His	Histidine
I	Ile	Isoleucine
K	Lys	Lysine
L	Leu	Leucine
M	Met	Methionine
N	Asn	Asparagine
P	Pro	Proline
Q	Gln	Glutamine
R	Arg	Arginine
S	Ser	Serine
T	Thr	Threonine
V	Val	Valine
W	Trp	Tryptophan
Y	Tyr	Tyrosine
start codon	ATG	
stop codon	TAA TAG TGA	

Chapter 1: Introduction

Chapter 1 Part A1. The Herpesviridae

1A1 Family Herpesviridae: General features

The involvement of herpesviruses in a range of prominent medical or veterinary diseases makes them one of the most important virus families. Their ubiquitous occurrence, genetic complexity, evolutional diversity, and differing biological properties have motivated great research efforts worldwide. Approximately 100 herpesviruses have been identified so far and at least one herpesvirus has been described in most animal species including fish, amphibians, reptiles, birds and especially mammals, including cattle, pigs and man (Roizman, 1993). Herpesviruses differ widely in their pathogenic potential but share the ability, after primary infection, to establish latent infections throughout the lifetime of the host. To date, eight different herpesviruses whose natural host is man have been identified. Herpesviruses of humans, their properties and the diseases they cause are shown in Table 1A1.

A large proportion of people worldwide has been exposed to, and may be infected with, one or more herpesviruses. Generally, the pathogenesis of these viruses is controlled by an intact immune system but they cause life-threatening diseases in immunocompromised individuals, and certain ones are implicated in different types of cancer. Thus, with increased use of immunosuppressive drugs for organ transplant and the advent of AIDS, herpesvirus infections of man pose a growing problem.

1A1.1 Distinctive structural charateristics

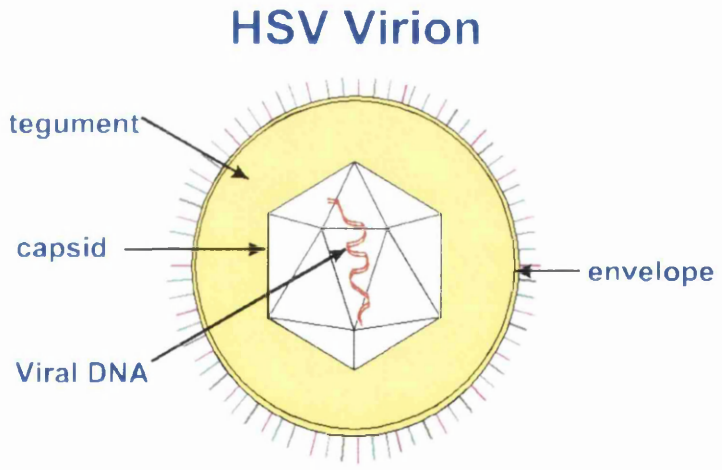
Herpes virions vary considerably in size from 120 nm to 300 nm in diameter, but all have a similar structure. Classification as a member of the *Herpesviridae* is based on the architecture of the virion (Fig 1A1.1). All herpes virions consist of a core containing a double stranded linear DNA genome encased in an icosahedral capsid (Wildy, 1960). An amorphous, sometimes asymmetric, material surrounds the capsid and has been designated the tegument (Roizman & Furlong, 1974). Surrounding the tegument is an envelope containing viral glycoprotein spikes on its surface (Spear & Roizman, 1972). Virion structure is reviewed in Rixon & McLauchlan (1993). Most investigations of virion structure have been carried out with HSV-1, but recent studies have indicated that the KSHV and HSV-1 structures are similar (Wu *et al.*, 2000a; Trus *et al.*, 2001).

Table 1A1. Human herpesviruses

Common name	Subfamily	G+C %	DNA Size (kbp)	Common disease/symptoms
HSV-1	α	68.3	152	cold sores + other occasional symptoms
HSV-2	α	69	152	genital lesions
VZV	α	46	125	chicken pox/ shingles
EBV	γ	60	172	infectious mononucleosis (glandular fever), Burkitt's lymphoma + other cancers.
HCMV	β	57	229	mononucleosis
HHV-6	β	42	162	exanthum subitum or roseola infantum
HHV-7	β	45	145	none known
KSHV	γ	53	140.5	Kaposi's sarcoma, PEL, MCD

Abbreviations: Herpes Simplex virus (HSV), Varicella-Zoster virus (VZV), Epstein-Barr virus (EBV), Human Cytomegalovirus (HCMV), Human Herpesviruses (HHV) Kaposi's Sarcoma-Associated Herpesvirus (KSHV) Primary Effusion Lymphoma (PEL), Multicentric Castleman's Disease (MCD).

Fig 1A1.2. Schematic representation of a herpesvirus virion showing structural components



Double-stranded DNA is encased in an icosohedral capsid, which is surrounded by the tegument proteins and viral envelope, containing glycoprotein spikes.

From Clements & Brown (1997).

1A1.2 Biological properties

Four significant biological properties are shared by all herpesviruses:

(i) A large array of virally encoded enzymes is used for nucleic acid metabolism.

(ii) The synthesis of viral DNA and assembly of capsids occurs in the nucleus.

(iii) Production of infectious virus progeny causes destruction of the host cell.

(iv) Virus can remain latent in its natural host.

Biological variation between members occurs at the level of range of host species, speed of multiplication in the host, host cell specificity and clinical manifestations.

1A1.3 Classification of herpesviruses

Classification is based upon biological properties and divides the *Herpesviridae* family into three sub-families (*Alpha-, Beta-* and *Gammaherpesvirinae*) (Roizman *et al.*, 1981).

1A1.3.1 Alphaherpesvirinae

e.g. HSV-1, VZV.

All members of this subfamily have a variable host range, a relatively short reproductive cycle, show rapid spread in culture, efficient destruction of cells and establish latent infections primarily but not exclusively in sensory ganglia.

1A1.3.2 Betaherpesvirinae

e.g. HCMV.

These viruses generally have a restricted host range, a long reproductive cycle and infect cells slowly in culture. Infected cells frequently become enlarged and latency occurs in secretary glands, lymphoreticular cells, kidneys and other tissues.

1A1.3.3 Gammaherpesvirinae

e.g. Members of the *Gammaherpesvirinae* include KSHV, herpesvirus saimiri (HVS), EBV, bovine herpesvirus 4 (BHV-4), equine herpesvirus 2 (EHV-2), and murine gammaherpesvirus-68 (MHV-68).

Gammaherpesviruses are all lymphotropic: able to infect lymphocytes and also frequently associated with lymphomas, particularly in non-native hosts (Neipel *et al.*, 1998). Viruses are specific for either B or T lymphocytes, infection is frequently found without production of infectious progeny. They can infect some endothelial, epithelial and fibroblastic cells *in vitro*. Latency often occurs in the lymphoid tissue. Gammaherpesviruses are subdivided into two genera: *Lymphocryptovirus*, or gamma-1

herpesviruses and *Rhadinovirus*, or gamma-2 herpesviruses. EBV is classified as gamma-1 whereas KSHV and HVS as gamma-2 herpesviruses. I shall be describing KSHV in detail.

KSHV shares a number of characteristics with all other herpesviruses: it replicates in the cell nucleus, undergoes prolonged latency in cells as a circular episomal form, and possess homologs to cellular enzymes (e.g., DNA polymerase, thymidine kinase, ribonucleotide reductase) involved in DNA replication and nucleic acid metabolism (Roizman, 1993). KSHV belongs to the *Rhadinovirus* genus on the basis of the nearness of its encoded amino acid sequence to corresponding sequences of other gamma-2 viruses and its overall genomic organization (a unique sequence bounded by tandem reiterations and with a characteristic gene complement and order of blocks of structural genes) (Chang *et al.*, 1994; Moore *et al.*, 1996b; Russo *et al.*, 1996; Neipel *et al.*, 1998) (see Section 1A2.9 for other gamma-2 herpesviruses).

1A1.4 Herpesvirus DNAs

Herpesvirus DNAs, when extracted from virions, are double-stranded linear molecules that can circularise upon release into host cell nuclei. Between different herpesviruses, DNA size and base composition can vary. The DNA size ranges between 120 kbp to 230 kbp, base composition varies from 31 % G+C to 75% G+C (see Table 1A1). Differences in the genome size of any one herpesvirus seems to be minimal but significant, terminal and internal repeated sequences, spontaneous deletions and recombination contribute some variation (Roizman, 1996) (see also Section 1A2.6.1 for the KSHV genome).

1A2 The Gamma-2 herpesvirus: KSHV

1A2.1 Background

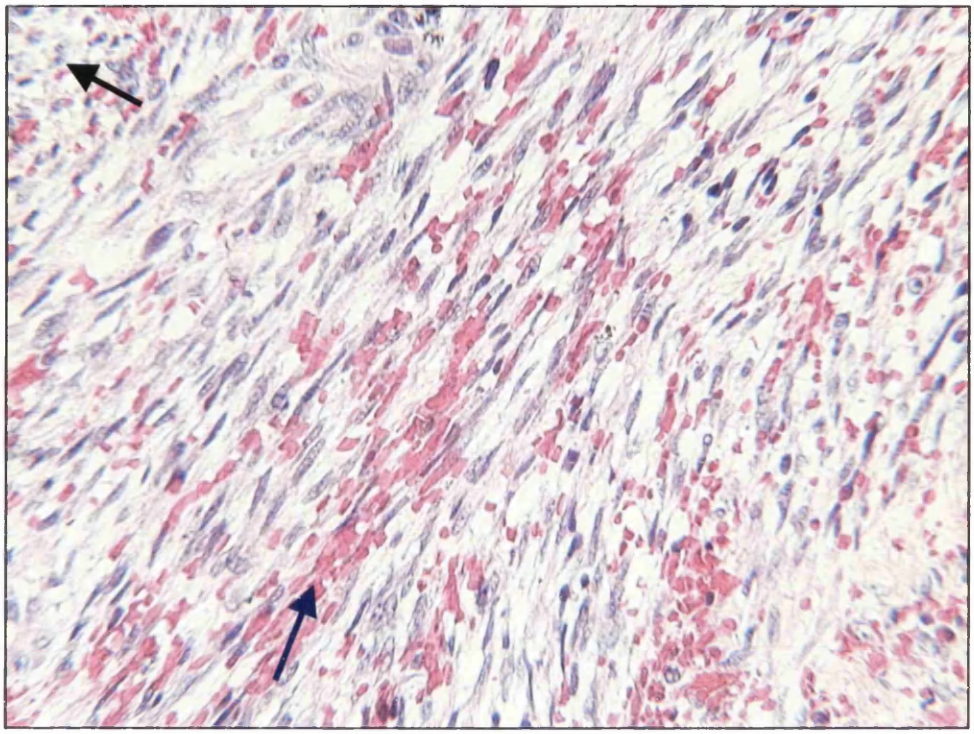
KSHV, the most recently identified herpesvirus infecting humans is the eighth described human herpesvirus and has the formal designation Human Herpesvirus 8 (HHV-8) but here I am using the common name, KSHV. Regardless of its role in human disease, the discovery of this herpesvirus is unique because it was accomplished using molecular biology techniques without direct viral culture (Chang *et al.*, 1994). Since its discovery KSHV has become the focus of intensive research; there has been great interest in its pathogenicity, its origins, and its cellular gene homologues. KSHV represents the opportunity to study a new human tumourigenic virus. The biology of KSHV, its association with KS, characterisation and functions of some of its genes (with special reference to ORF57) are presented here.

1A2.2 Kaposi's sarcoma (KS)

KS was first described in 1872 by Moritz Kaposi, a dermatologist from Vienna as "idiopathic multiple pigmented sarcoma of the skin" (Kaposi, 1872). KS lesions of the skin are a red-brown colour caused by the presence of numerous aberrantly formed blood vessel networks lined by spindle cells. The synchronously appearing lesions over the surface of the skin may arise through expansion of circulating progenitor cells (Judde *et al.*, 2000). These progenitor cells may become transformed tissue and develop into spindle cells (Rabkin *et al.*, 1997). Tumour lesions are usually restricted to the skin with a predilection for distal localization in the extremities and visceral organs but in some individuals, the malignancy appears at multiple sites. The most prominent features of the final nodular stage of KS are characteristic, well-defined interwoven bundles of spindle cells of endothelial origin (Fig 1A2.2).

The origin and clonality of these KS cells still remains very controversial. Some studies showed that nodular KS lesions display both monoclonal and polyclonal pattern based on terminal repeats of the virus genome, suggesting that KS begins as a polyclonal disease with subsequent evolution to a monoclonal process and KSHV infection precedes tumour growth (Judde *et al.*, 2000) whereas other studies suggest that not all KS lesions are clonal. Some studies concluded that both spindle and endothelial components of KS were of vascular rather than lymphatic origin (Armes, 1989). Alternatively, Dupin *et al.* (1999) suggested that spindle cells are a proliferation of either lymphatic endothelium

Fig 1A2.2. KSHV expression in KS lung tissue section



Haematoxylin and eosin stained lung tissue section of KS (original magnification x250)

Normal lung tissue (→), KS lesion in lung (→).

(Kindly provided by D.J. Blackbourn, Institute of Virology, University of Glasgow, UK)

or of immature endothelial cells and looked for increased expression of vascular endothelial growth factor receptor-3 (VEGFR-3) with presence of KSHV infection (Dupin *et al.*, 1999). VEGFR-3 is specific for lymphatic or proliferating endothelial cells and its overexpression appears to be a robust marker for the immunohistological diagnosis of early KS. This receptor is expressed on most endothelial cells during early embryogenesis but is subsequently restricted to the lymphatic endothelium of adult tissues (Dupin *et al.*, 1999).

1A2.3 Epidemiological forms of KS

KS occurs as four clinical types varying in disease prognosis including classical KS, endemic KS, epidemic KS (including acquired immune defficiency syndrome-associated KS [AIDS-KS]), and immunosuppression-/organ transplant- associated KS (Armes, 1989).

Classical KS is a well-known rare disease of elderly Mediteranian men with endemic areas in southeastern European countries and present in older males of Jewish background and has no known contributing environmental factor. A proportion of classical KS may occur among human immunodeficiency virus (HIV)-negative gay men, who appear to be a higher risk for disease than HIV-negative heterosexual men (Hjalgrim *et al.*, 1996). Endemic KS has a particularly high incidence in Central African countries including the Republic of Congo, Uganda, and Zambia which have the highest incidence rates in the world. African KS occurs more commonly in men than in women, occurs in all age groups and has no known precipitating environmental factor (Ziegler & Katongole-Mbidde, 1996). Both these types of KS are not typically associated with immune deficiency whereas the remaining two types are accompanied by immune impairment.

AIDS-associated KS is the most recently described and clinically aggressive subtype of KS, first recognised in USA in 1980's and often involving skin, lymph, and mucosal tissues. Gay men are at highest risk for AIDS-KS, although other at-risk groups, including women, develop disease less frequently (Grulich & Kaldor, 1996). Gay men with AIDS early in the AIDS epidemic had a 50% lifetime rate of developing KS (Katz *et al.*, 1994). Posttransplant or immunosuppression-related KS is distinct in that the occurrence of KS is highly related to iatrogenically induced immunosuppression. KS in iatrogenically immunosuppressed patients tends to be clinically aggressive; however discontinuation of immunosuppressive therapy is associated with remission of disease (Penn, 1988; Besnard *et al.*, 1996).

1A2.4 The discovery of KSHV

The association of KS with HIV-1-positive and immunosuppressed patients, its multicentric appearance and tendency to regress spontaneously, suggested the involvement of a sexually transmissible infectious agent. Beral *et al.,* (1990) investigated the epidemiological data of KS and HIV-1 infection reported to the Centers for Disease Control, until 1989. They concluded that neither immunosuppression nor HIV-1 infection could be the sole cause of KS in HIV-1 infected individuals (Beral *et al.*, 1990; Beral *et al.*, 1991; Beral *et al.*, 1992). Investigations by researchers in several laboratories over long periods for the presence of possible causative agents such as HCMV, hepatitis B virus (HBV), HHV-6, mycoplasma penetrans, human papilloma virus (HPV) in KS, remained negative until 1994 (Jahan *et al.*, 1989; Van den Berg *et al.*, 1989; Holmberg, 1990; Johnston *et al.*, 1990; Rappersberger *et al.*, 1990; Huang *et al.*, 1992).

Chang *et al.,* (1994) used DNA subtractive hybridization techniques (Lisitsyn & Wigler, 1993) and by representational difference analysis searched for DNA sequences present in KS lesions and absent in uninvolved tissues. From these experiments, two small fragments of the KSHV genome, designated KS330Bam and KS631Bam, were initially discovered in some 90% of DNA samples from AIDS-KS lesions and shown to be related to gammaherpesviruses (Chang *et al.*, 1994). These results were confirmed and expanded by PCR analyses (Ambroziak *et al.*, 1995).

1A2.5 Evidence for association of KSHV with disease

KSHV is associated with three neoplastic disorders: KS, primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD).

1A2.5.1 KS

The endemic cases of KS found in sub-Saharan Africa are responsible for up to 12% of all malignancies. Many groups have reported that the KSHV genome can be detected by Southern blot or PCR in all epidemiological forms and clinical stages of KS (Chang *et al.*, 1994; Moore & Chang, 1995; Boshoff *et al.*, 1995; Dupin *et al.*, 1995; Schalling *et al.*, 1995; Su *et al.*, 1995; Besnard *et al.*, 1996; Ziegler & Katongole-Mbidde, 1996; Chang *et al.*, 1996; Buonaguro *et al.*, 1996; Chuck *et al.*, 1996; Luppi *et al.*, 1996; Noel *et al.*, 1996; Gaidano *et al.*, 1996b; Dictor *et al.*, 1996; Lebbe *et al.*, 1997).

KSHV has also been detected in non-involved tissue from KS patients, including brain sensory ganglia, nasal fluid saliva, skin, lymphoid tissue, and peripheral blood mononuclear cells (PBMCs) (Ambroziak *et al.*, 1995; Whitby *et al.*, 1995; Lebbe *et al.*, 1995; Gaidano *et al.*, 1996b; Dictor *et al.*, 1996; Moore *et al.*, 1996c; Lefrere *et al.*, 1996;

Humphrey *et al.*, 1996; Monini *et al.*, 1996; Gupta *et al.*, 1996; Corbellino *et al.*, 1996; Brambilla *et al.*, 1996; Viviano *et al.*, 1997; Howard *et al.*, 1997; Smith *et al.*, 1997; Blackbourn *et al.*, 1998).

Several epidemiological studies support the association of KSHV with KS (Gao *et al.*, 1996; Kedes *et al.*, 1996; Lennette *et al.*, 1996; Simpson *et al.*, 1996; Rainbow *et al.*, 1997; Zhu *et al.*, 1999b). Serologic studies using indirect immunofluorescence assays (IFA) to determine the presence of specific antibodies were carried out with sera from patients with AIDS-KS and control patients with HIV infection or AIDS. Nuclear staining was present in AIDS-KS sera that was absent in control sera, indicating reactivity towards KSHV antibodies and not EBV antibodies. Paired sera taken 8 to 14 months prior to KS onset and post-KS onset from two patients, showed an eight-fold increase in titre. These serologic studies showed a strong correlation between development of KS and infection with KSHV. Despite clinical and epidemiological differences, the four subtypes of KS are histologically indistinguishable and the utility of differentiating KS into subtypes is becoming less apparent.

1A2.5.2 PELs

KSHV genomes are also consistently found in PEL, also termed 'body-cavity-associated lymphomas' (BCBL) (Cesarman *et al.*, 1995a; Pastore *et al.*, 1995; Nador *et al.*, 1996; Gaidano *et al.*, 1996b; Otsuki *et al.*, 1996; Ansari *et al.*, 1996; Carbone *et al.*, 1996; Komanduri *et al.*, 1996; Gessain *et al.*, 1997), a rare form of AIDS-related B-cell lymphoma also present in HIV-negative individuals which is characterized by malignant effusions in the pleural or abdominal cavity, immunological gene rearrangement, lack of most surface markers and, unlike Burkitt's lymphoma, a lack of *c-myc* rearrangement (reviewed in Jaffe (1996)). Most cases of PEL are dually infected with EBV and KSHV (Cesarman *et al.*, 1995a; Nador *et al.*, 1996; Gessain *et al.*, 1997) but occasional cases of EBV-negative/KSHV-positive PEL have been reported (Cesarman *et al.*, 1996a; Renne *et al.*, 1996b; Carbone *et al.*, 1996; Strauchen *et al.*, 1996).

Several latently infected PEL cell lines have been established that are an important resource for laboratory studies of the virus. The first characterised KSHV harbouring PEL cell line, designated HBL-6 and BC-1 was independently established by two laboratories from a single BCBL tumour in an AIDS patient and is coinfected with EBV (Cesarman *et al.*, 1995b; Gaidano *et al.*, 1996a). Most PEL cells retain the KSHV genome as a latent episome (Cesarman *et al.*, 1995a), as shown by *in situ* hybridisation (Cesarman *et al.*, 1995b) and pulsed field gel electrophoresis (Moore *et al.*, 1996b), with multiple (40 to 150) copies present per cell.

1A2.5.3 MCD

MCD is an atypical lymphoproliferative disorder, which occurs in two histological variants, the hyaline-vascular variant and the plasma cell variant. A characteristic of MCD is close association with KS that occurs during the clinical course of most HIV-associated MCD cases and in some HIV-negative patients. Most cases of MCD in HIV-infected patients, in particular the plasma cell variant, contain detectable KSHV (Soulier *et al.*, 1995; Gessain *et al.*, 1996). In contrast, KSHV is much less frequently detected in MCD of HIV-negative patients (Soulier *et al.*, 1995; Gessain *et al.*, 1996).

1A2.6 Characterisation of KSHV

1A2.6.1 Sequence and genome structure

The nearly complete nucleotide sequence of KSHV was determined from the BC-1 PEL cell line (Russo *et al.*, 1996) and from a KS biopsy specimen (Neipel *et al.*, 1997b). KSHV has a genome structure characteristic of other rhadinoviruses. A long unique region (LUR) of 140.5 kb with lower-G+C content (53.3% G+C, L-DNA) is flanked by two terminal repeats (TR) consisting of several 801 bp direct repeats of high G+C content (85% G+C, H-DNA) (Neipel *et al.*, 1997b). The overall G+C contents of some gammaherpesviruses, such as rhesus rhadinovirus (RRV) (59.9% G+C content for a 10.6-kb sequence) (Desrosiers *et al.*, 1997) and EHV-2 (58% G+C content) (Telford *et al.*, 1995) are similar but, they are lower in HVS (34.5%) (Albrecht *et al.*, 1992), BHV-4 (41.4%) (Broll *et al.*, 1999; Zimmermann *et al.*, 2001) and MHV-68 (46%) (Virgin *et al.*, 1997).

Encapsidated linear DNA isolated from BCBL-1 virions has been estimated to be 165 kb in size (Renne *et al.*, 1996a; Renne *et al.*, 1996b; Zhong *et al.*, 1996). The KSHV genome is colinear with the HVS genome and the nomenclature for viral genes corresponds to that previously adopted for HVS. KSHV ORFs sharing sequence homology with HVS (ORFs 2-75) are assigned the HVS ORF number (Albrecht *et al.*, 1992). Genes that are unique to KSHV have been designated with a 'K' prefix and numbered sequentially starting at ORFK1 and extending to ORFK14. Like other herpesvirus genomes, that of KSHV contains genes encoding proteins which are required for replication and assembly of new virus progeny. All the presently known ORFs (more than 81) are encoded within the LUR (see Fig 1A2.6.1) (Russo *et al.*, 1996).

1A2.6.2 KSHV Virions

Cell lines treated (i.e. induced) with chemicals such as phorbol ester 12-*O*-tetradecanoyl

phorbol-13-acetate (TPA) can produce KSHV virions that have formed the basis for ultrastructural studies (Renne *et al.*, 1996a; Said *et al.*, 1996). KSHV virions have morphological features typical of other herpesviruses, with a diameter of approximately 110 nm (Renne *et al.*, 1996a). Nucleocapsids with an electron-dense core are found in the nuclei of TPA-induced lymphoma cells and enveloped virions are found in the cytoplasm (Renne *et al.*, 1996a; Said *et al.*, 1996). Similar particles have also been observed in KS lesions in a few cells with spindle-shaped morphology (Walter *et al.*, 1984; Ioachim, 1995; Orenstein *et al.*, 1997; Said *et al.*, 1997) and were reported earlier, before description of KSHV (Ioachim *et al.*, 1992). Log-phase cultures of BCBL-1 cells induced with TPA were used to obtain KSHV capsids for electron cryomicroscopy (cryoEM) images and image computer reconstruction. The KSHV virion is 120-150 nm (Fig 1A2.6.2) and the 3D structure of KSHV capsids, reconstructed from electron cryoEM images, revealed that the capsids have a polyhedral shape with characteristic capsomer protrusions (Wu *et al.*, 2000a; Trus *et al.*, 2001).

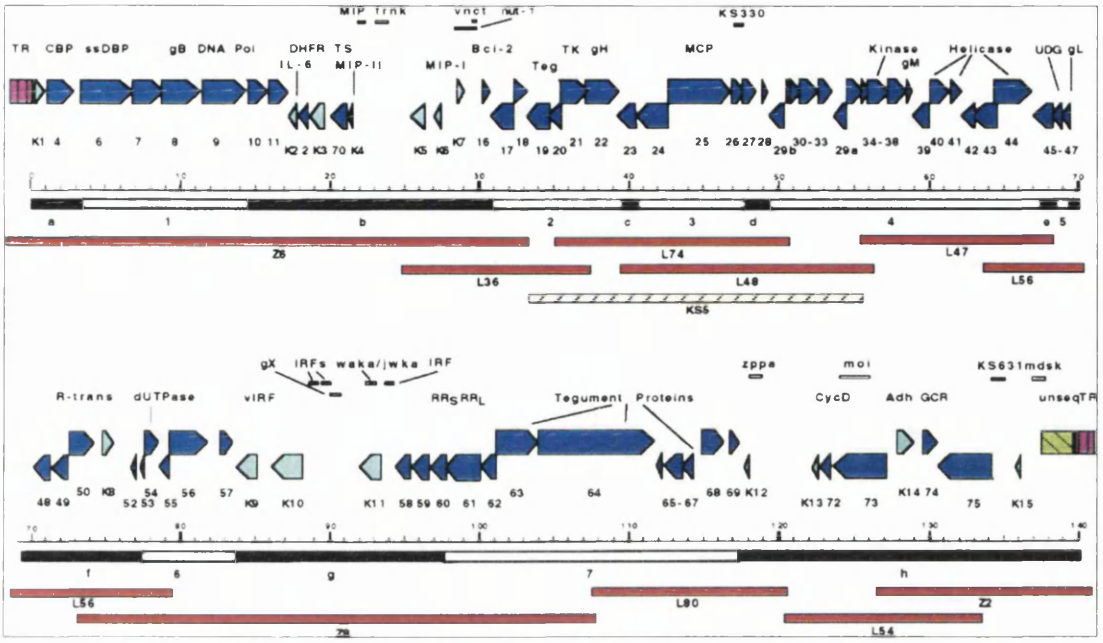
1*A2.6.3 Sequence- and strain- variability*

Most of the KSHV genome is highly conserved in isolates or sequences obtained from KS biopsies or PEL cell lines and from different geographical regions. A comparison of a 20 kb genomic region obtained from a KS lesion (Moore *et al.*, 1996b) and a PEL cell line (Russo *et al.*, 1996) showed only 0.1% nucleotide variation. A nearly complete genomic sequence from a KS biopsy (Neipel *et al.*, 1997b)) is highly homologous to the complete genomic sequence from a PEL cell line (Russo *et al.*, 1996), with the exception of both genome ends.

Comparison of several recently available complete or partial KSHV sequences suggests that ORFK1 of KSHV may exhibit variability of up to 19% difference in the protein sequence. ORFK1 is located at the 'left' end of KSHV genome, and encodes a type 1 membrane glycoprotein bearing a functional immunoreceptor tyrosine-based activation motif-like sequence (Russo *et al.*, 1996; Neipel *et al.*, 1997b; Lagunoff & Ganem, 1997; Lee *et al.*, 1998; Nicholas *et al.*, 1998; Lagunoff *et al.*, 1999; McGeoch & Davison, 1999).

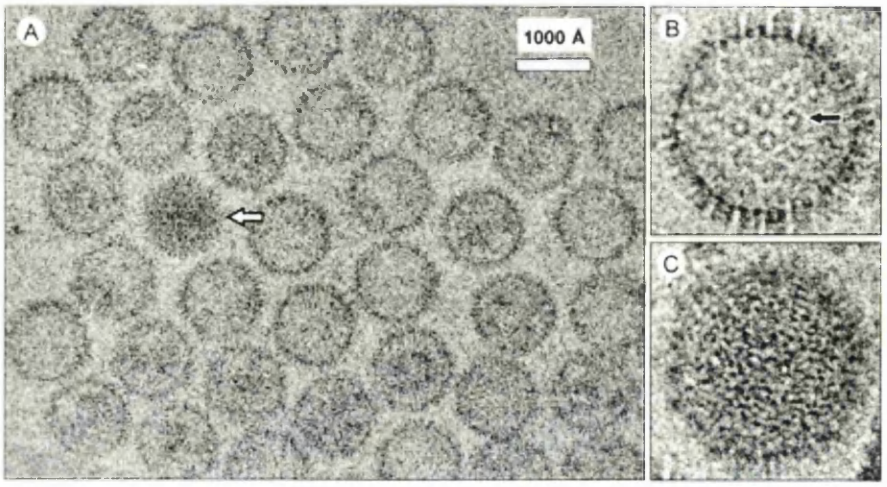
Zong *et al.*, (1999) sequenced the genes for the highly variable 289 amino acid ORFK1 membrane protein from more than 60 different KSHV samples and demonstrated that they display up to 30% amino acid variability and cluster into four very different

Fig 1A2.6.1. Original KSHV genome map



The KSHV genome derived from the BC-1 cell was shown to contain this 140.5-kb coding LUR, which is flanked by multiple G + C-rich 801-bp TR sequences. These TR structures are believed to facilitate circularisation of the genome as seen in the episomal state during viral latency. The light blue shaded ORFs in between the conserved blocks illustrate regions (marked as a-h and shown in black) that are unique to KSHV and encode proteins that can mimic cell cycle regulation and signal transduction proteins. Conserved herpesvirus genes are grouped into the seven numbered blocks (1-7) and are depicted by white bars. From Russo *et al.* (1996).

Fig 1A2.6.2. CryoEM of KSHV capsids



CryoEM of KSHV capsids. (A) A micrograph area of ice-embedded KSHV capsids showing predominantly intermediate capsids and one full capsid (arrow). The enlarged view of an intermediate capsid (B) shows the capsomers (arrow) that form a characteristic hexagonal pattern with adjacent capsomers. The full-capsid (C) image reveals the characteristic "fingerprint" pattern. From Wu *et al.* (2000).

major evolutionary subgroups referred to as A, B, C, and D. These authors further subdivided the ORFK1 protein pattern into a total of 13 distinct variants based on amino acid differences totalling 5% or greater or on the presence of common in-frame deletions within the VR2 domain and assigned additional numerical groups (e.g., A1 to A5, C1 to C5, and D1 or D2). Most United States AIDS-associated KS samples are A1, A4, or C3 variants whereas most classic KS cases from the Middle East, Asia, Europe, and United States are C2 variants. In contrast, samples from Africa are predominantly of the B subtype (plus occasional A5 variants) and the rare D subtype appears to be of Pacific Island origin. These results implied that the major subtypes of KSHV correlate with the modern human population divisions that occurred via migrations from Africa first to southern Asia and Oceania 60,000 years ago and second to Europe and northern Asia some 35,000 years ago (Zong *et al.*, 1999).

Further studies explored the patterns of KSHV strain differences and the possibility that strain differences are associated with different disease states. Poole *et al.* (1999) evaluated variability of overall genotype patterns and the potential for chimeric genomes at six loci across KSHV DNA, including three segments in the central conserved portions of the genome and three at the extreme right-hand end of the genome. These results confirmed that the three or four major subtypes (referred to as A, B, C, and D) can also be recognised within the more conserved genome portions (such as ORF26, T0.7/K12, and ORF75). Two diverged alternative allelic forms (referred to as predominant [P] and minor [M]) of the ORFK15 region at the right-hand end of the genome that appears to be highly divergent in some isolates were also identified (Poole *et al.*, 1999). A recent study by Kakoola *et al.* (2001) reported about the recombination in KSHV strains from Uganda. Phylogenetic analysis of ORFK1 indicated that majority of KS patients were infected by the B subtype, several by the A5 subtype and one by a variant of the C subtype. Analysis of the ORFK15 indicated that the P allele was predominant with only a single strain bearing the M allele (Kakoola *et al.*, 2001). DNA and amino acid sequences of the two K15 versions are so distinct that their relationship is equivalent to that of a pair of homologous genes from two highly diverged viruses; this is presumed to be the result of recombination between a KSHV strain and a distant gamma-2 relative, presently unidentified (McGeoch & Davison, 1999).

1A2.6. 4 In vitro culture and cytopathicity

It has been difficult to culture KSHV efficiently till now. Latently infected PEL cell lines can be induced to enter the lytic cycle by addition of the phorbol ester TPA (Renne *et al.*, 1996b) and produce virions. Attempts to transmit KSHV particles to various lymphoid

and epithelial cells have produced inefficient and low level transmission and long-term serial propagation of KSHV in tissue culture have proved to be difficult (Renne *et al.*, 1998). Blackbourn *et al.* (2000) provided further biological evidence in cell culture for the host range of KSHV in primary CD19+ B cells, macrophages, endothelial cells and some epithelial cells demonstrating that cells of different lineage can be infected with KSHV (Blackbourn *et al.*, 2000). Infection of umbilical cord blood monocytes (UCMC) did not yield transformed established lymphoblastoid cell lines and virus infection only persisted for 4-7 days. However, long-term KSHV infection of UCMC could be achieved mainly by coinfection with EBV (Blackbourn *et al.*, 2000).

Limited lytic replication and serial transmission have been achieved in 293 cells, an adenovirus-transformed human embryonal kidney epithelial cell line (Foreman *et al.*, 1997). 293 cells are semi-permissive for KSHV infection but do not permit transmission of mature virions to infect other cell types. Nevertheless it has been possible to culture KSHV directly from KS lesions (Foreman *et al.*, 1997) and saliva (Vieira *et al.*, 1997) in 293 cells, confirming the presence of KSHV virions in tumour samples. While 293 cells show some promise as a culture system, they are not currently a practical means of cultivating virus for genetic studies.

The first evidence for *in vitro* growth promotion of endothelial cells by KSHV with implications for neoplastic transformation showed that infection of primary endothelial cells gave extended life span and survival (Flore *et al.*, 1998), but the virus was not present in all cells and virus driven growth of uninfected cells would be possible only by a paracrine mechanism. An *in vitro* model utilizing transformed dermal micro-vascular endothelial cells (tDMVECs) immortalized with the HPV16 E6 and E7 genes and latently infected with KSHV was recently developed that supported significant KSHV infection (Moses *et al.*, 1999). The majority of tDMVEC cells expressed the latency-associated nuclear antigen (LANA) encoded by ORF73, and TPA-induced tDMVECs expressed the lytic-cycle associated genes ORF59 and ORFK8.1A/B. Infected endothelial cells developed a spindle shape resembling that of KS lesional cells, demonstrating the ability of KSHV to induce phenotypic changes that resemble characteristics of KS spindle cells *in vivo* (Moses *et al.*, 1999).

A PEL derived cell line, JSC-1, that yields highly infectious KSHV supernatant virions more efficient at infecting human dermal microvascular endothelial cells (DMVEC) was established from the ascitic fluid of an HIV-positive patient (Cannon *et al.*, 2000). JSC-1 virus can induce cultured primary DMVECs to form colonies of proliferating latently infected spindle-shaped cells all of which express LANA protein (Ciufo *et al.*, 2001).

Recently DMVECs immortalised by expression of telomerase (TIME cells), were readily infected by KSHV virions, leading to establishment of latent infection (Lagunoff *et al.*, 2002). Latently infected TIME cells do not undergo major morphological changes or growth transformation, and infection is lost from the culture upon serial passage (Lagunoff *et al.*, 2002).

KSHV also established a productive infection in primary human keratinocytes and viral particles produced from keratinocytes infected other primary cells, such as human endothelial cells (Cerimele *et al.*, 2001).

1A2.7 KSHV epidemiology

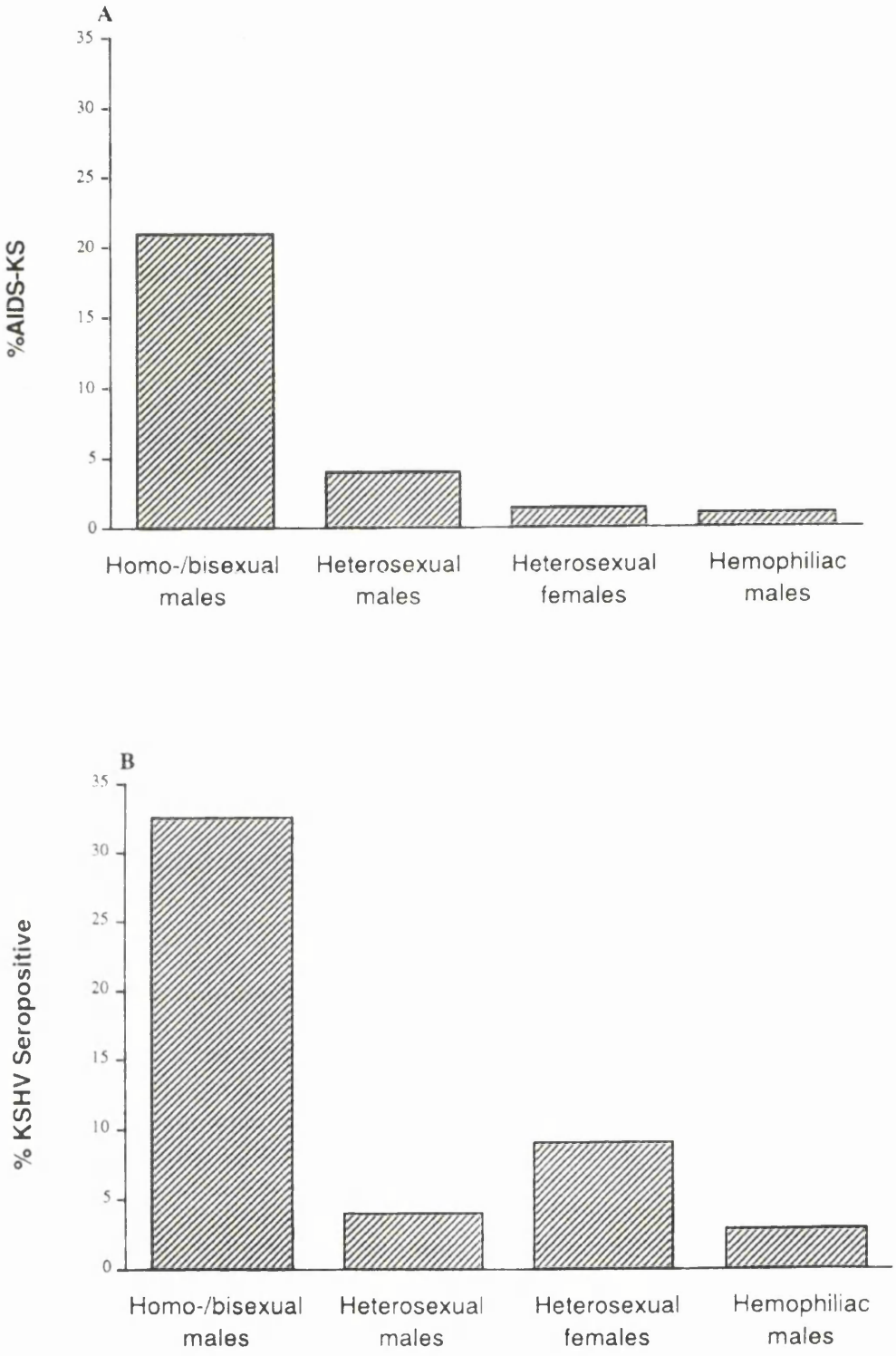
KSHV seroprevalence rates in high-risk populations are generally related to KS rates in these populations (Fig 1A2.7) (Sarid *et al.*, 1999). For example, based on 1989 AIDS surveillance data, 21% of homosexual or bisexual men with AIDS in the United States had KS at AIDS-defining diagnosis compared to only 1% of hemophiliacs with AIDS (Beral *et al.*, 1990). Antibody studies conducted in 1996 suggest 30-35% of homosexual or bisexual men with AIDS in the United States are KSHV-infected (Gao *et al.*, 1996; Kedes *et al.*, 1996) compared to 0-3% of HIV-positive hemophiliacs (Gao *et al.*, 1996; Kedes *et al.*, 1996). A population-based study of gay men in San Francisco from 1984 found 47.7% prevalence among HIV-infected men and 8.7% prevalence among HIV-uninfected men (Martin *et al.*, 1998).

1A2.8 KSHV relatives

KSHV is the first member of the *Rhadinovirus* genus found to infect humans and is closely related to HVS, a lymphomagenic rhadinovirus of squirrel monkeys that causes fulminant T cell lymphoma in New World monkeys (Albrecht *et al.*, 1992) and to MHV-68 (Efstathiou *et al.*, 1990). Closely related rhadinoviruses have also been detected in captive macaques, and rhadinovirus of rhesus monkeys, RRV (Desrosiers *et al.*, 1997), and retroperitoneal fibromatosis herpesvirus (RFMHV) (Rose *et al.*, 1997) are additional members that infect primates.

Three new distinct members of the rhadinoviruses have been found in chimpanzees and in a gorilla. Theses are more closely related to KSHV than to any other virus of rhadinovirus genus described so far. These new viruses tentatively named PanRHV1a and PanRHV1b (Lacoste *et al.*, 2000) and *Pan troglodytes* rhadinovirus-1 (PtRV-1) (Greensill *et al.*, 2000a) for the chimpanzee (*Pan troglodytes*) rhadino-herpesvirus and GorRHV1 for the gorilla virus, cluster together with KSHV on a distinct branch (Lacoste *et al.*, 2000).

Fig 1A2.7. A comparison of the frequency of KS and KSHV



A comparison of the frequency of KS and KSHV in selected high-risk populations. (A) Percentage of AIDS patients by HIV risk category (Beral *et al.*, 1990) who were reported to have KS at primary diagnosis from United States surveillance data. Similar trends in KSHV seropositivity (B) are seen for LANA seroreactivity in homosexual and bisexual men and women attending a sexually transmitted disease clinic, and HIV-positive hemophiliacs (Gao *et al.*, 1996; Kedes *et al.*, 1996). From Sarid *et al.* (1999).

Two distinct new gamma-2 herpesviruses were also reported (Greensill *et al.*, 2000b) in African green monkeys; serological screening and PCR for the viral DNA polymerase gene detected sequences for *Chlorocebus* rhadinovirus 1 (ChRV1) and ChRV2. ChRV1 is more closely related to KSHV and RFHV, whereas ChRV2 is closest to RRV. These findings suggest the existence of two distinct rhadinovirus lineages, represented by the KSHV/RFHV/ChRV1 group and the RRV/ChRV2 group respectively, in at least two Old World monkey species (Greensill *et al.*, 2000b).

1A3 KSHV Molecular Biology

1A3.1 KSHV gene expression profile

In latency, the spectrum of viral gene expression is minimised (Renne *et al.*, 1996b; Zhong *et al.*, 1996; Sarid *et al.*, 1998) limiting potential targets for host immune responses, and the latent virus relies primarily on cellular replication machinery for genomic replication and repair. When the life cycle switches from a quiescent latent phase to the production of whole virions, it is referred to as the lytic phase since it is postulated to lead to cell lysis and death. Latent herpesvirus infections in cultured cells can shift spontaneously into an active lytic replication cycle or be induced to shift into lytic replication by chemical agents such as the phorbol ester TPA or sodium butyrate (Miller *et al.*, 1996; Miller *et al.*, 1997).

KSHV transcript expression has been classified into four distinct kinetic stages: latent, IE, early and late (Sarid *et al.*, 1998; Sun *et al.*, 1999). Latent gene expression occurs constitutively throughout the viral life cycle. After TPA induction, the IE gene mRNA amounts remain constant even after the addition of cycloheximide and transcripts are expressed without *de novo* protein synthesis. Early gene expression is dependent on IE expression and is independent of viral DNA synthesis on the basis of inhibition of expression by cycloheximide but not by the antagonist of the viral DNA polymerase, PAA. Late gene expression begins at the onset of viral DNA replication and is inhibited by PAA.

A map of actively transcribed regions of KSHV in BC-1 cells was constructed by Northern hybridisation using DNA probes extending across the viral genome (Sarid *et al.*, 1998; Sun *et al.*, 1999). As the BC-1 cell line is known to have a tighter control of latency (Russo *et al.*, 1996) than other PEL cell lines, it is easier to distinguish during experiments between chemically induced promotion of the lytic cycle and spontaneous reactivation.

KSHV displays a temporal order of gene expression (Sarid *et al.*, 1998; Sun *et al.*, 1999). Representatives of each kinetic gene class are shown in Table 1A3.1 and some are discussed briefly below.

1A3.1.1 KSHV gene expression products I: Immediate early ORFs

Two of the earliest genes to be transcribed in KSHV-infected B cells are ORF50 and ORF57. The ORF50 product was able to transactivate expression of delayed early viral

Table 1A3.1. Temporal cascade of KSHV gene expression in PELs

Transcript Class	ORFs	Putative function
Latent	71 (vFLIP)	Anti-apoptotic
Latent	73 (LANA)	Latent nuclear antigen
Immediate Early	**50 (Rta)***	**Transcriptional Transactivator**
Immediate Early	**57(Mta)***	**Transcriptional/post-transcriptional Regulator**
Immediate Early	K9 (vIRF)	Interferon Regulatory Factor
Early	K8 (k-bZIP/RAP)	Transactivator
Early	K4 (vMIPII)	Angiogenesis
Early	16 (vBcl2)	Potentially tumourigenic
Early	K2 (vIL-6)	Potentially tumourigenic
Delayed Early	59 (PPF-8)	DNA polymerase processivity factor
Delayed Early	70	Thymidylate synthase
Delayed Early	37	Alkaline exonuclease
Late	29	DNA packaging protein
Late	K8.1A	Glycoprotein
Late	26	Minor capsid protein
Late	65 (sVCA)	Small viral capsid protein

Examples of different classes of KSHV ORFs with encoded proteins in parenthesis. ***Genes studied in detail in the present study**. From Russo *et al.* (1996); Zhong *et al.* (1996); Sarid *et al.* (1998); Sun *et al.* (1999).

genes (Lukac *et al.*, 1998; Sun *et al.*, 1998; Lukac *et al.*, 1999). Following TPA stimulation, ORF57 was expressed together with the ORFK8, K3 and K5 gene products. Expression of IE genes ORF50 and ORF57, the involvement of ORF50 in reactivation of lytic replication and their roles in transactivation of early and delayed early genes are discussed (see Sections 1A4 and 1B1).

The ORFK8 gene product (Kb-ZIP) contains a b-ZIP domain which is common in transactivators and DNA-binding proteins (Lin *et al.*, 1998; Seaman *et al.*, 1999; Zhu *et al.*, 1999a). This gene product was termed K-bZIP and shown to contain a heptad repeat of four leucines and one isoleucine and to homo-dimerise at the C terminus. The N-terminal portion of K-bZIP protein is derived from ORFK8 which, through in-frame splicing, adjoins the ZIP domain. The K-bZIP protein has significant similarity to the EBV BZLF1 protein in terms of its similar genomic location, splicing pattern and 37 % amino acid sequence homology. Expression of the K-bZIP transcripts was blocked by cycloheximide but not by PAA, indicating that it is an early gene (Lin *et al.*, 1998). Evidence shows that the K-bZIP represses the transcriptional activity of p53 which is required for apoptosis of the host cell and there is interaction between the bZIP domain of K-bZIP and the carboxy-terminal region of p53 (Park *et al.*, 2000).

To avoid the T-cell mediated immune response, KSHV encodes K3 and K5 zinc finger membrane proteins (containing a C_4HC_3 motif) which remove major histocompatibility complex class I molecules from the cell surface (Ishido *et al.*, 2000). Both are expressed during the early lytic cycle of viral replication (Nicholas *et al.*, 1997a; Sun *et al.*, 1999). K3 and K5 displayed different specificities in downregulation of HLA allotypes and dramatically downregulated class I molecules by inducing their rapid endocytosis.

1A3.1.2 KSHV expression products II: Early ORFs

Polyadenylated nuclear (PAN) RNA, also known as T1.1 or nut-1 RNA, is believed to be a marker of viral lytic cycle (Sun *et al.*, 1999). The T1.1-kb PAN RNA is the most abundant lytic cycle transcript but does not express any known protein. This RNA species is expressed in only a few cells within KS biopsies as the majority of cells within KS lesions are latently infected. Zhong *et al.*, (1996) have proposed a number of functions for this abundant RNA in KS, it might play a structural role in the nucleus, possess RNA catalytic functions as a ribozyme or could associate with small ribonuclear proteins with roles in RNA transport or processing.

1A3.1.3 KSHV expression products III: Delayed early ORFs

The KSHV ORF 59 gene product belongs to the delayed early class of proteins involved in viral DNA replication as a DNA polymerase-associated processivity factor (Chan *et al.*, 1998). KSHV ORF 59 gene is homologous to HSV-1 UL42, HCMV UL44, HHV-6 U27, and EBV BMRF-1 genes encoding accessory proteins essential for viral DNA replication. ORF59 encodes the polymerase processivity factor (PPF) protein that enables the KSHV-encoded DNA polymerase (Pol-8) to produce DNA products in excess of 7,000 nucleotides (Lin *et al.*, 1998). The processivity function of PPF is correlated with both Pol-8-binding and dsDNA-binding activities (Chan & Chandran, 2000).

1A3.1.4 KSHV expression products IV: Late ORFs

Glycoproteins (gpK8.1A and gpK8.1B) are associated with the virion envelope and the surface of transfected BCBL-1 and COS-1 cells. Detecting expression of these glycoproteins in infected cells is possible with monoclonal antibodies (Chandran *et al.*, 1998) and indicates completion of the lytic viral cycle.

Interaction of KSHV glycoprotein B (gB, ORF8) and gpK8.1A with ubiquitous cell surface heparin sulphate molecules was demonstrated (Akula *et al.*, 2001; Wang *et al.*, 2001a). Recently Akula *et al.,* provided evidence implicating the glycoprotein B RGD (Arg-Gly-Asp) motif in binding the α3β1 (CD 49c/29) integrin molecule as one of the *in vitro* cellular receptors involved in KSHV entry (Akula *et al.*, 2002). KSHV infectivity was inhibited by peptides with RGD amino acids, antibodies against RGD-dependent α3 and β1 integrins, and by soluble α3β1 integrin and virus infectivity was increased by the expression of human α3 integrins in cells. Virus binding studies suggested KSHV interacts with α3β1 integrin after cell attachment: anti-gB Abs immunoprecipitated the -α3 and -β1 complexes indicating gB specifically binds to the α3 and β1 chains of integrin and implicating α3β1 in KSHV entry (Akula *et al.*, 2002).

1A3.2 KSHV gene expression profile in PELs

Analysis of the expression patterns of viral genes during different phases of infection is an important approach to understanding KSHV biology. The first genome-wide analysis of KSHV gene expression in PELs was performed by Renne and colleagues (1996) using cDNA Southern hybridisation against genomic clones (Renne *et al.*, 1996b). Other investigators also studied KSHV gene expression in the BC-1 cell line (Sarid *et al.*, 1998; Sun *et al.*, 1999).

KSHV gene expression is strongly restricted in PEL cells under normal growth

conditions, although up to 5% of the cell population shows spontaneous reactivation, and the genome is present as monomeric circular episomes (Renne *et al.*, 1996b) analogous to the state of EBV DNA in latently infected lymphoblastoid cell lines or lines derived from Burkitt's lymphoma (Kieff, 1995b; Kieff, 1995a).

Only a limited repertoire of KSHV genes is expressed in most uninduced PEL cell lines (Renne *et al.*, 1996b; Sun *et al.*, 1999). This group comprises the 0.7 kb mRNA for ORFK12 (Renne *et al.*, 1996b), the 2.2kb mRNA for vcylin (ORF72) and vFLIP (ORF13/ORF71) (Dittmer *et al.*, 1998; Sarid *et al.*, 1998), the 6kb mRNA encoding ORF73/LANA (Dittmer *et al.*, 1998; Sarid *et al.*, 1998), and another 6kb unknown mRNA located in the region of ORFK14 and ORF75 (Sarid *et al.*, 1998). A putative enhancer/promoter has been identified immediately upstream of ORF73/LANA (Nicholas *et al.*, 1998; Sarid *et al.*, 1998). Apart from ORFK12, the expression of these genes in PEL cell lines is not upregulated by TPA or sodium butyrate treatment (Sarid *et al.*, 1998; Sun *et al.*, 1998). The three genes encoded by ORFs 71, 72, and 73 [viral FLICE inhibitory protein (v-FLIP), vcyclin, and immunogenic LANA] are transcribed as a polycistronic transcript in the PEL cell line BCP-1 either uninduced or induced with sodium butyrate (Talbot *et al.*, 1999).

A second class of genes is expressed in unstimulated PEL cell lines at a low level and can be further upregulated by TPA or n-butyrate. Genes encoding structural proteins are not expressed in the majority of unstimulated PEL cells but are inducible and therefore grouped into a third class of lytic genes. This group includes virus encoded viral interferon regulatory factor (vIRF, ORFK9), macrophage inflammatory protein (MIP) I/MIP 1A (ORFK6, ORFK4), MIP II/MIP 1B (ORFK4.1), viral interleukin-6 (vIL-6) (ORFK2) a non-translated nuclear RNA PAN, vBcl-2 and a G-protein-coupled receptor homologue (vGPCR), (Moore *et al.*, 1996a; Zhong *et al.*, 1996; Nicholas *et al.*, 1997a; Nicholas *et al.*, 1997b; Yen-Moore *et al.*, 2000). Several putative promoters for these genes have been identified and both monocistronic unspliced and spliced mRNAs are encoded. The KSHV-encoded IRF (vIRF; ORF K9) inhibits responses to interferons (Gao *et al.*, 1997; Zimring *et al.*, 1998) also IRF-1-mediated transactivation, and stable expression of vIRF in NIH3T3 or Rat-1 cells results in cellular transformation (Gao *et al.*, 1997; Li *et al.*, 1998).

Several KSHV encoded homologues of cellular genes, including vIL-6, vBcl2 (ORFK16) (Sarid *et al.*, 1997), vMIP I and vGPCR (Kirshner *et al.*, 1999), are expressed as viral lytic cycle products in cultured PEL cells. Expression of v-Ox2 (ORF K14) and vGPCR ORF74, present on a bicistronic message, appears to be predominantly restricted to the lytic replication cycle (Talbot *et al.*, 1999). By immunohistochemistry, PEL cells

and KSHV-infected B-cells in lymphatic tissue, but not KS cells, express vIL-6, an inducible gene (Moore *et al.*, 1996a). Transcripts from ORFs 72, 73, 74 and 75 expressed in latent and lytic cycles were identified in PELs (BC-1 and BC-2) (Cesarman *et al.*, 1996b).

DNA arrays provides a means to measure the expression of hundreds or thousands of genes simultaneously and allows high-throughput characterization of samples (reviewed in Lockhart & Winzeler (2000)). A nylon membrane-based DNA array that enables simultaneous measurement of the expression level of almost every known KSHV ORF was constructed (Fig 1A3.2) (Jenner *et al.*, 2001). The BC-3 cell line was used to study the KSHV expression during latency and after the lytic induction and cluster analysis was used to arrange genes according to their expression profile. This method groups together genes that may be involved in a common process and arranges them in a temporal order consistent with the known stages of herpesvirus replication. Cluster analysis revealed a correlation between expression and assigned gene function and the feasibility of this approach was demonstrated by the discovery of a novel transcribed ORF (K10.7) with homology to known IRFs. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis confirmed the array data, suggesting that KSHV encodes four full-length IRF-related proteins.

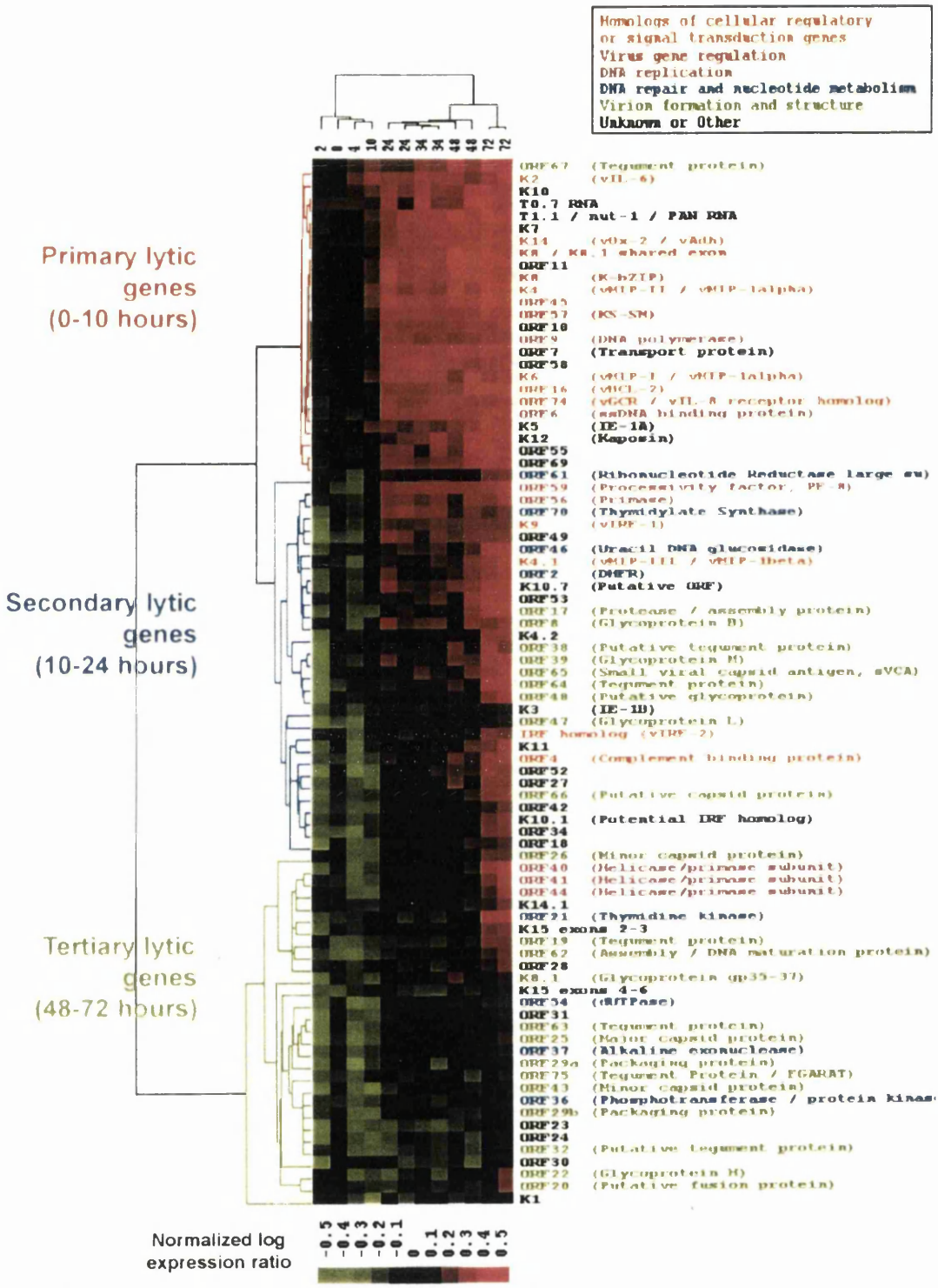
PEL parallels KS in the pattern of latent and lytic cycle viral gene expression but the predominant infected cell type is a B cell. e.g. latency-associated nuclear antigen 2 (LANA 2), is encoded by ORF K10.5, which is expressed in KSHV-infected hematopoietic tissues, including PEL and CD but not in KS lesions (Rivas *et al.*, 2001).Thus there are some differences in gene expression between KS spindle cells and KSHV infected B-cells and further differences are likely to be found.

1A3.3 KSHV gene expression profile in KS lesions/cells

The first analysis of KSHV gene expression in KS tissues was performed by Zhong and colleagues (1996), using cDNA Southern hybridisation against genomic clones (Zhong *et al.*, 1996). These authors identified two small transcripts that represented the bulk of the virus-specific RNA transcribed in KS tumour tissue.

In situ hybridisation was used to detect viral transcripts in a KS lesion and showed that KS spindle cells within the lesion were transcribing a latency-associated transcript termed T0.7 but only 1 to 3 % of the cells were transcribing T1.1 the lytic RNA transcript (Staskus *et al.*, 1997). *In situ* hybridization (Davis *et al.*, 1997; Staskus *et al.*, 1997; Sturzl *et al.*, 1997) and LANA immunohistochemistry have also confirmed KSHV gene expression in spindle cells within KS tumours (Rainbow *et al.*, 1997) and showed

Fig 1A3.2. Hierarchical clustering of KSHV genes after TPA induction



Hierarchical clustering of genes and samples after the induction of lytic replication with TPA. The genes are ordered using a self-organizing map algorithm (Eisen *et al.*, 1998). The normalized log expression ratio is color coded according to the scale at the bottom. ORFs and corresponding gene names are listed on the right and color coded according to putative function shown by the key above. The dendrogram on the left represents relatedness of the patterns of gene expression. The three major branches are color coded according to the class of genes they represent and the times at which expression is first detected: primary lytic genes (0 to 10 h), secondary lytic genes (10 to 24 h), and tertiary lytic genes (48 to 72 h). Each column represents a sample taken at different times in hours after TPA induction (labeled above). The dendrogram at the top relates the samples according to the pattern of gene expression. From Jenner *et al.* (2001).

the distribution of cell types infected latently by KSHV in patch, plaque and nodular KS, MCD and also in a lymph node of a patient with PEL (Dupin *et al.*, 1999).

Only a limited repertoire of KSHV genes is expressed in the majority of spindle cells of KS lesions (Zhong *et al.*, 1996; Davis *et al.*, 1997; Staskus *et al.*, 1997; Sturzl *et al.*, 1997; Nicholas *et al.*, 1997a; Sun *et al.*, 1999; Yen-Moore *et al.*, 2000). This group comprises the 0.7 kb mRNA for ORF K12 ((Zhong *et al.*, 1996; Staskus *et al.*, 1997), the 2.2kb mRNA for vcyclin (ORF72) and vFLIP (ORF13/ORF71) (Davis *et al.*, 1997; Kellam *et al.*, 1997; Rainbow *et al.*, 1997), the 6kb mRNA encoding ORF73/LANA (Kellam *et al.*, 1997; Rainbow *et al.*, 1997). During latent-phase expression in KS lesions, only viral cyclin D and viral FLIP are expressed (Davis *et al.*, 1997; Dittmer *et al.*, 1998; Reed *et al.*, 1998). Both are encoded on single RNA transcripts; one contains coding sequences for both proteins, and the other also contains the coding region for LANA (Kedes *et al.*, 1997; Kellam *et al.*, 1997; Rainbow *et al.*, 1997; Dittmer *et al.*, 1998) suggesting that vFLIP and vcyclin are expressed in majority of KS lesions with LANA expressing in only some KS spindle cells.

In KS tissues, only a small population of cells express inducible genes and are believed to correspond to lytically infected cells (Staskus *et al.*, 1997; Sturzl *et al.*, 1997). vBcl-2 is expressed in some KS cells and has an anti-apoptotic role (Sarid *et al.*, 1997). ORF74, a KSHV homologue of a GPCR, is expressed in some KS tissues and induces expression of the angiogenic cytokine VEGF and cell growth. Expression of ORF74 (vGPCR) is generally restricted to lytic phase replication and a small minority of cells (Guo *et al.*, 1997; Arvanitakis *et al.*, 1997; Bais *et al.*, 1998; Kirshner *et al.*, 1999; Yen-Moore *et al.*, 2000). Transcripts from ORFs 72, 73, 74 and 75 expressed in latent and lytic stage were identified in KS tissues by RT-PCR although KSHV transcripts appeared to be more abundant in the PELs than in KS tissues, consistent with the higher genomic number in the PELs (Cesarman *et al.*, 1996b).

The expression of lytic KSHV genes has been documented in monocytes/macrophages (Blasig *et al.*, 1997). In addition, the presence of linear KSHV genomes in PBMC, suggesting the presence of productively infected cells, has also been reported (Decker *et al.*, 1996). Some of these productively infected cells have the appearance of spindle cells (Staskus *et al.*, 1997).

1A3.4 Potential KSHV tumour inducing gene products

What is the role of KSHV in the induction of KS? The close epidemiological association of KSHV and KS triggered a search for putative viral oncogenes. Due to the lack of a KSHV animal model or an efficient cell culture system researchers have relied on

heterologous expression and rodent fibroblast transformation assays. Homologues of cellular genes encoded by KSHV proposed to play a causative role in the diseases associated with this virus are shown in Table 1A3.4 (Neipel *et al.*, 1997a; Neipel & Fleckenstein, 1999). Recently a KSHV artificial bacterial chromosome has been constructed to generate recombinant viruses (Zhou *et al.*, 2002).

Some of the viral genes, including vGPCR, ORFK9 (vIRF-1), vIL-6, Bcl-2, ORFK1, ORFK12, and MIPs have been shown to transform NIH 3T3 fibroblasts in transfection experiments (Gao *et al.*, 1997; Bais *et al.*, 1998; Lee *et al.*, 1998; Li *et al.*, 1998; Muralidhar *et al.*, 1998). However with the possible exception of a K12 encoded protein, these genes are lytic-phase genes that are not expressed in the majority of cells in KS lesions.

Signalling by KSHV GPCR leads to cell transformation and tumourigenicity, and induces a switch to an angiogenic phenotype mediated by VEGF (Bais *et al.*, 1998). GPCR can activate two protein kinases, JNK and p38MAPK, by triggering signalling cascades similar to inflammatory cytokine induction. Thus KSHV GPCR is a viral oncogene that can exploit cell signalling pathways to induce transformation and angiogenesis in KSHV-mediated oncogenesis (Bais *et al.*, 1998).

The vIRF-1 protein is believed to interfere with the antiproliferative action of IRF. The other IRF homologues may have tumourigenic potential but require to be studied in greater detail. One of these homologues LANA 2, encoded by ORF K10.5, is expressed in KSHV-infected hematopoietic tissues, including PEL and CD but not KS lesions and appears to have arisen from a gene duplication of a captured cellular IRF gene. LANA 2 was shown to be a potent inhibitor of p53-induced transcription in reporter assays and may contribute to KSHV tumourigenesis in hematopoietic tissue (Rivas *et al.*, 2001).

Among the most compelling cellular homologues to induce the diseased state is viral IL-6 that has a hydrophobic 19-amino acid secretory signal having 24.8 % amino acid identity and 62.6 % similarity to its cellular counterpart (Moore *et al.*, 1996a). These investigators showed enhanced expression of this homologue in 43 % of non-induced BCP-1 cells. The vIL-6 protein is predominantly expressed in lymphoproliferative disorders and is a candidate for both promoting cell proliferation and preventing apoptosis. Previous evidence demonstrated that AIDS-KS spindle cells produce and proliferate in response to IL-6 (Miles *et al.*, 1990) thus strengthening the evidence of this cytokine and also its viral counterpart in inducing KS.

Table 1A3.4. KSHV genes putatively relevant for KSHV pathogenesis

ORF	Gene product	Experimental evidence	Putative function in infection or viral persistance	Possible role in KS pathogenesis	Expression pattern
K1	Non-conserved transmembrane glycoprotein	Transformation of Rat1 cells in HVS: lymphocyte immortalisation, lymphoma induction	? Controlled amplification of natural habitat	Positional and (possibly) functional analogue to other rhadinoviral oncoprotein	Productive (?)
K2	Viral IL-6	Proliferation of B-cells IL6R α chain not required	Controlled para/autocrine implification of natural host cells	Paracrine growth stimulation of spindle cells	Productive
VMIPs (K4, K4.1, K5)	Viral macrophage inflammatory proteins	Binding to both CC and CXC receptors, attraction of eosinophils induction of angiogenesis	Amplification of natural habitat	Angiogenesis, attraction of Th2 lymphocytes	Productive
K9	Interferon response factor homologue	Transformation of 3T3 cells tumours in nude mice	Counteracting IFN-mediated virus suppression	Interfering with the antiproliferative action of IRF	Productive
K12	?	Transformation of Rat-3 cells w. low efficiency tumour in nude mice	*Unknown*	Transformation	Latent
ORF16	vbcl-2	Anti-apoptotic activity	Counteracting elimination of persistently infected cells	Stabilising productively infected cells	Productive
ORF71	vFLIP	*None*	Counteracting elimination of persistently infected cells	Stabilising latently infected cells	Latent
ORF72	v-Cyclin		Proliferation of latently infected cells	Dysregulated cell cycle progresion, inhibits cell cycle arrest by p53 accumulation of somaticmutations	Latent
ORF73	LANA	Maintenance of plasmid with TR sequences and origin of replication from KSHV genome in cell lines	Interaction with p53, retinoblastoma protein, Episome maintainanace	*Unknown*	Latent
ORF74	vIL-8R/ vGPCR		Amplification of natural habitat	Angiogenesis, transformation of endothelial cells	Productive
K15	Integral membrane protein	*None*	*Unknown*		?

Abbreviations. ORF: open reading frame; IL-6: interleukin-6; vFLIP: viral FLICE inhibitory protein; LANA: latent nuclear antigen; vIL-8R/vGPCR: viral interleukin-8 receptor homologue/viral G-protein coupled receptor; HVS: herpesvirus saimiri; IL6Rα: interleukin-6 receptor α-chain (gp80); VEGF: vascular endothelial growth factor; TR: terminal repeat; IFN: interferon; IRF: interferon responsive factor. From Neipel & Fleckenstein (1999).

A novel viral Bcl-2 homolog from KSHV was identified through cross hybridisation and sequence analysis comparison with EBV and HVS (Sarid *et al.*, 1997; Cheng *et al.*, 1997). The Bcl-2 protein family is characterised by the ability to suppress cell death and represents a unique type of protooncogene that extends cell survival by inhibiting apoptosis rather than promoting cell proliferation (Korsmeyer, 1992). Overexpression of vBcl-2 blocked apoptosis as efficiently as Bcl-2, Bcl-$_{XL}$, or the EBV homolog BHRF1 (Cheng *et al.*, 1997).

The vMIP I and vMIP II cellular homologues are found late and early respectively in the KSHV life cycle and act as cellular proliferative and angiogenesis factors. Viral MIP II binds to the CC-chemokine receptor CCR3 and to the CXC-chemokine receptors. This is pertinent, as the CCR3 is known to be important in the chemoattraction of both eosinophil granulocytes and Th-2 lymphocytes, both involved in KS lesion morphology. Together with promoting infiltration of inflammatory cells, the vMIP I and vMIP II are inducers of angiogenesis in a chorioallantoic assay (Boshoff *et al.*, 1997).

Although several oncogenes can be identified in cell culture systems, serologic evidence also suggests that KSHV infection is necessary for the development of KS but it has been possible to transform only some cultured cells with virus (Flore *et al.*, 1998; Moses *et al.*, 1999; Cerimele *et al.*, 2001; Ciufo *et al.*, 2001), and only a few KSHV carrying tumour cell lines (PELs only) have been isolated from patients. Transformation of endothelial cells by KSHV as well as paracrine mechanisms that are induced by this virus may be critical in the pathogenesis of KS (Flore *et al.*, 1998).

1A4 KSHV ORF50

1A4.1 Expression of ORF50 protein

The ORF50 gene product is also known as Rta/R protein. Sequence comparison indicated the ORF50 gene was a homologue of the EBV BRLF1 gene (Hardwick *et al.*, 1988; Sun *et al.*, 1998) that acts in concert with the ZEBRA protein in activating early EBV genes in B cells and the lytic cascade in epithelial cells. It is also homologous with other gammaherpesvirus transactivators including HVS transactivator R/ORF50 protein (Nicholas *et al.*, 1991; Whitehouse *et al.*, 1997a). The HVS ORF50 produces two transcripts: ORF50a, which is spliced, and ORF50b that is shorter and unspliced. A C-terminal activation domain of ORF50b interacts with the basal transcription factor TATA-binding protein TBP (Hall *et al.*, 1999). ORF50, an IE gene product, was

competent to initiate reactivation of the KSHV lytic cycle (Sun *et al.*, 1998) and activated vIL-6 and ORFK8 mRNA. ORF50 mRNAs are composed of three spliced variants ranging from 3.6 to 3.8 kb (Zhu *et al.*, 1999a).

An ORF50 expression plasmid when transfected into a PEL cell line stimulated the complete KSHV life cycle, from induction of delayed early and late genes leading to the release of DNAse resistant viral DNA (Gradoville *et al.*, 2000). Rta (ORF50 homologue) of the murine gammaherpesvirus MHV-68, alone was also able to disrupt latency, reactivate the complete lytic cycle replication, and drive the lytic cycle to completion (Wu *et al.*, 2000b).

ORF50 protein is required for viral reactivation by all known chemical inducers (e.g. TPA and sodium butyrate) (Figure 1A4.1) (Lukac *et al.*, 1998; Sun *et al.*, 1998) Transient transfection studies with plasmids containing ORF50 gene fragments examined their ability to transactivate a selection of KSHV delayed early and late promoters. ORF50 protein strongly activated all delayed ealy promoters, but only weakly activated late promoters (Lukac *et al.*, 1998). The ORF50 gene product also upregulated the promoter for ORF57.

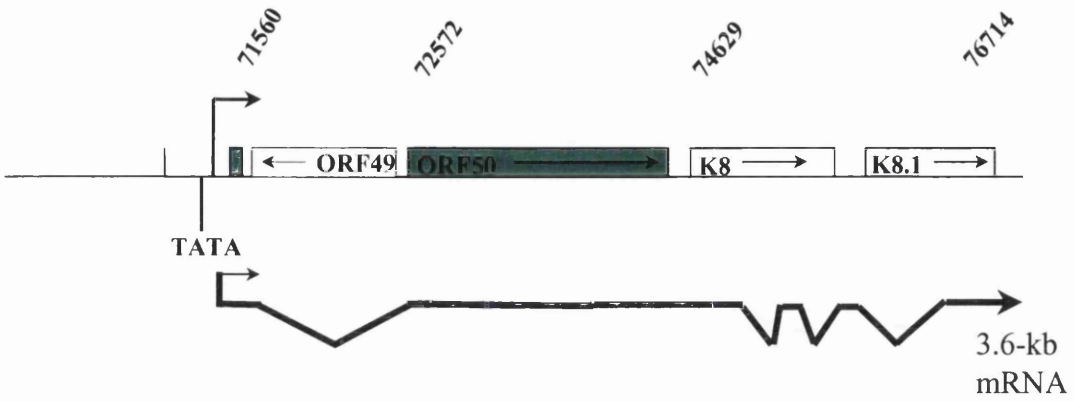
1A4.2 Autoactivation of ORF50

The ORF50 promoter is responsive to the ORF50 protein. Transient transfection experiments in BCBL-1 cells indicated that ORF50 positively autoregulates its own expression by some three-fold (Seaman *et al.*, 1999). Similarly in a PEL cell line, HH-B2 ORF50 protein stimulated expression of its own mRNA (Gradoville *et al.*, 2000) and in CV-1 cells ORF50 induced expression by seven-fold from the ORF50 promoter (Wang *et al.*, 2001b). Transfection of an ORF50 expression plasmid into PEL cells latently infected with KSHV and into 293 cells activated the expression of ORF50 transcripts from endogenous viral genomes and from co-transfected cells.

1A4.3 ORF50 as a transcriptional activator of KSHV gene expression

Transient transfection studies with ORF50 expression vector showed that ORF50 upregulated the expression of several downstream target KSHV genes, such as ORF57, K8 (KbZIP), K9 (vIRF), PAN, K5 (thymidine kinase, TK), K12 (Kaposin), ORF6 (single-stranded DNA binding protein, SSB), ORF59 (PPF), K14 (vOX-2), vGPCR, and vIL6, finally leading to virus production. The ORF50 promoter element required for autoregulation binds an octamer-binding protein (Oct-1), indicating that the regulation

Fig 1A4.1. ORF50 genomic locus



The ORFs located between the indicated nucleotides in the KSHV genome are shown with the arrow indicating the direction of transcription. Exon 1 (shaded) is separated by a 959 nt intron from exon 2. Exon 2 includes coding sequences from the downstream K8 and K8.1 genes. From Lukac *et al.* (1998).

was through a non-ORF50 DNA binding mechanism (Sakakibara *et al.*, 2001). Mutations in this binding motif inhibited upregulation of the ORF50 promoter by ORF50 protein but there was some autoactivation when the motif was deleted, indicating that autoactivation is not entirely dependent on Oct-1 (Sakakibara *et al.*, 2001).

Some promoters such as TK and vIRF are activated indirectly and depend on Sp1 sites (Zhang *et al.*, 1998; Chen *et al.*, 2000b), AP-1 sites were also involved in activation of vIRF (Chen *et al.*, 2000b). In another study, an ORF50 DNA-binding element consisting of a conserved 12-bp palindromic sequence and less conserved sequences immediately 3', shared by the ORF57 and k-bZIP promoters, was identified. Transfer of this element to TATA boxes of heterologous promoters confered high-level responsiveness to ORF50, indicating it was both necessary and sufficient for activation. The strong concordance of DNA binding *in vitro* with transcriptional activation *in vivo* strongly implies that sequence-specific DNA binding is necessary for ORF50-mediated activation via this element (Lukac *et al.*, 2001).

While the PAN, ORF57 and K8 gene promoters are activated by sequence-specific DNA binding of ORF50; the strong ORF50 responsive element in pPAN does not share obvious homology with the responsive regions of pORF57 and pK8 (Lukac *et al.*, 2001; Song *et al.*, 2001). ORF50 binding sequences on PAN were mapped to a 16 bp region and an additional 7 bp on both sides of this were also important (Song *et al.*, 2002). In another study, PAN and K12 genes were shown to be activated by ORF50 via a large, highly conserved response element. These response elements are bound by similar cellular proteins, suggesting their regulation by a complex mechanism involving binding by ORF50 protein and related cellular activators/repressors (Chang *et al.*, 2002).

1A4.4 Interaction of ORF50 with cellular proteins

CREB-binding protein (CBP) and histone deacetylase were reported to interact with ORF50 and regulate its function: ORF50 represses p53-induced apoptosis through a CBP-related mechanism (Gwack *et al.*, 2001). ORF50 also interacts with Stat3 and stimulated transcription of Stat-driven genes. Stat3 was recruited to the nucleus and ORF50 induced the dimerisation of Stat3 monomers in the absence of Stat3 phosphorylation (Gwack *et al.*, 2002).

A cellular protein referred to as human hypothetical protein MGC2663 (Genebank) and displaying sequence similarities with members of the Krueppel-associated box-zinc finger proteins interacted with ORF50 in the yeast-two-hybrid system, coimmunoprecipitations and pull down assays (Wang *et al.*, 2001b). MGC2663

has been named K-RBP (KSHV-RTA/ORF50 binding protein) (Wang *et al.*, 2001b) and its expression was detected in all primate cell lines tested, it was neither stimulated nor inhibited by ORF50. MGC2663 synergizes with ORF50 to activate viral transcription, and its overexpression in the presence of ORF50 further enhances ORF50 transactivation of several responsive viral promoters (Wang *et al.*, 2001b).

Chapter 1 Part B. KSHV ORF57

1B1 ORF57 and its properties

1B1.1 General features

The ORF57 gene product has other names such as KS-SM, or Mta protein, here I shall be using ORF57. The presence of a homologue in every herpesvirus of mammals and birds sequenced so far suggests that aspects of its regulatory role are maintained throughout the family; some homologues have been shown to act post-transcriptionally affecting RNA splicing and transport. The homologues include HSV-1 ICP27 (Sandri-Goldin & Mendoza, 1992; Smith *et al.*, 1992), EBV Mta (also known as BMLF1, M, SM, or EB2) (Buisson *et al.*, 1999), HCMV UL69 (Winkler *et al.*, 1994), VZV ORF4 (Defechereux *et al.*, 1997) and HVS ORF57/IE52 (Nicholas *et al.*, 1988). Overall, the identity at the amino acid level is approximately 30% among the gammaherpesvirus KSHV ORF57, EBV Mta/SM and HVS ORF57/IE52 proteins. The HVS ORF57 product has trans-regulatory functions that appear to be mediated at the post-transcriptional level (Whitehouse *et al.*, 1998b) and as such is functionally homologous to the 63 kDa IE phosphoprotein ICP27 which has been extensively studied in the prototype alphaherpesvirus, HSV-1 ICP27 (reviewed in Phelan & Clements (1998) Sandri-Goldin (1998a)) is one of two HSV-1 IE proteins essential for lytic virus replication whose functions include downregulation of intron-containing transcripts and upregulation of certain late messages (Sandri-Goldin & Mendoza, 1992; Smith *et al.*, 1992). Studies of ICP27 have highlighted the multifunctional nature of this protein, which acts at both transcriptional and post-transcriptional levels. Acting post-transcriptionally, ICP27, which has a punctate nuclear distribution when detected by IFA (Phelan *et al.*, 1993), binds RNA *in vivo,* enhances RNA 3' processing, inhibits pre-mRNA splicing and facilitates the nuclear export of HSV-1 transcripts. KSHV ORF57 is a lytic gene expressed between 2-4 h after TPA induction of BCBL-1 cells, immediately following the appearance of ORF50 but prior to most delayed early gene products (Lukac *et al.*, 1999).

1B1.2 ORF57 is a spliced gene

The previously published sequence for KSHV ORF57 begins at nucleotide 82717 and terminates at nucleotide 83544, encoding a protein of 275 amino acid residues (Russo *et al.*, 1996). The KSHV sequence upstream of the published ORF57 contains 749

nucleotides unassigned to any identified KSHV ORF. Within this region are 5 ATG codons, the first four clustered within a region of 33 nucleotides, are in frame with each other and with the downstream ORF57 but are separated from it by a single stop codon. This ATG cluster begins at nucleotide 82069 at 648 nucleotides upstream of the published ORF57 initiation codon.

Contrary to the previously published KSHV sequence (Russo *et al.*, 1996), the ORF57 gene is spliced and ORF57 protein is expressed very early in infection from a 1.7-kb spliced RNA bearing several in-frame ATG codons (Bello *et al.*, 1999; Gupta *et al.*, 2000; Kirshner *et al.*, 2000). The ORF57 gene contains two coding exons, a single 108 bp intron with an ATG at nucleotide 82069 and is predicted to encode a protein of 455 amino acid residues (Bello *et al.*, 1999; Gupta *et al.*, 2000; Kirshner *et al.*, 2000). For several gammaherpesviruses such as HVS, alcelaphine herpesvirus-2 (AHV-2), BHV-4, and MHV-68 the upstream coding exon is 16-17 amino acids and contains 3-4 methionine residues.

1B1.3 ORF57 acts at the posttranscriptional level to regulate gene expression

The homologous HSV-1 ICP27, EBV Mta, and HVS ORF57 genes all activate expression of other genes via posttranscriptional mechanisms (Buisson *et al.*, 1989; Sandri-Goldin & Mendoza, 1992; Phelan & Clements, 1998; Ruvolo *et al.*, 1998; Whitehouse *et al.*, 1998b).

Cotransfection of KSHV ORF57 cDNA into BJAB cells led to a 4 to 10-fold increase in CAT activity and, similar to EBV Mta, it was capable of activating CAT transcribed from a variety of promoters such as the EBV latent promoter Wp, the CMV IE promoter or the SV40 late promoter (Gupta *et al.*, 2000). As nuclear levels of CAT mRNA increased, ORF57-mediated activation may not simply reflect enhanced cytoplasmic transport of CAT mRNA. In nuclear run-on transcription assays, no difference in CAT transcript initiation rate was observed in BJAB cells cotransfected with CMV-CAT and ORF57 as compared to control transfected cells indicating that the activation is posttranscriptional, although it is not possible to rule out direct activation of other promoters (Gupta *et al.*, 2000).

In transient reporter gene assays, ORF57 expression had little effect on expression of the luciferase reporter gene driven by a wide variety of KSHV delayed early and latent gene promoters and heterologous promoters. Thus ORF57 is not a broad-spectrum activator such as adenovirus E1A or HSV ICP0. Nonetheless, the accumulation of some viral RNAs (e.g., those for ORF59 and PAN) was strongly augmented in the presence of ORF57 in a manner that suggested posttranscriptional regulation (Kirshner *et*

al., 2000).

1B1.3.1 Activation by ORF57 is gene-dependent

The activating effect of HSV-1 ICP27 and EBV Mta is gene dependent. ICP27 does not activate all HSV genes equally (Rice & Knipe, 1988; Soliman *et al.*, 1997). Similarly, EBV Mta has been shown not to activate β-galactosidase, luciferase, or certain lytic EBV genes in cotransfection assays (Kenney *et al.*, 1989b; Markovitz *et al.*, 1989; Semmes *et al.*, 1998). These differences may be due to multiple factors, including the sequence of the gene itself, as well as the sequence of the 3' untranslated region (Key *et al.*, 1998; Ruvolo *et al.*, 1998; Semmes *et al.*, 1998; Buisson *et al.*, 1999). KSHV ORF57 did not activate luciferase expression from an intronless SV40 promoter luciferase reporter construct (Gupta *et al.*, 2000).

Expression of EBV BMRF1, a DNA Pol Accessory factor and a delayed early gene involved in DNA replication, was enhanced by EBV Mta protein (Semmes *et al.*, 1998) and HSV ICP27 (Uprichard & Knipe, 1996). KSHV ORF59 (equivalent to BMRF1) cloned downstream from the HCMV IE promoter gave almost 20-fold higher levels of mRNA and protein in the presence of ORF57 than in its absence. Since the HCMV IE promoter is not significantly upregulated by ORF57, the bulk of this upregulation is posttranscriptional. Similar levels of induction were also observed with PAN cloned downstream of the HCMV promoter in presence of ORF57. However other KSHV-containing transcripts (e.g., vGPCR and K5) driven by HCMV promoter were unaffected, indicating that posttranscriptional upregulation by ORF57 is transcript specific (Kirshner *et al.*, 2000).

ORF57 synergistically augments the effect of ORF50 on the PAN promoter. Addition of even low levels of ORF57 to ORF50 resulted in a striking (40-to 50-fold) further upregulation of expression from an (intronless) PAN-promoter driven luciferase reporter over that generated by ORF50 alone (Kirshner *et al.*, 2000). A dominant negative ORF50 mutant lacking a transcriptional activation domain but retaining dimerisation capacity specifically inhibited the stimulation of gene expression by ORF50+ORF57, indicating that the synergy requires the transactivation activity of ORF50. This further upregulation was not due to increased expression of ORF50 further activating the PAN promoter as ORF50 mRNA and protein levels did not increase upon addition of ORF57, and this effect could reflect a posttranslational enhancement of ORF50 transcriptional activity (Kirshner *et al.*, 2000).

The synergistic ORF50+ORF57 effect is promoter specific, as with an ORF50-responsive promoter TK intronless and intron-containing reporters were not

synergistically upregulated by ORF50+ORF57 whereas expression from an intronless Kaposin promoter reporter was further induced by 25-fold relative to an reporter with the intron-containing construct (versus 80- to 100-fold for the intronless promoter PAN promoter relative to intron-containing PAN promoter) (Kirshner *et al.*, 2000).

The cumulative effect of KSHV ORF57 on expression of a specific gene likely depends on several gene-specific factors including the presence or absence of introns as well as the 3' untranslated region and the coding sequence. Like its counterparts in HSV, EBV, and HVS, KSHV ORF57 may play an important role in the activation of other viral lytic genes, particularly those that are expressed as unspliced mRNAs, to enhance the lytic cascade.

1B1.3.2 ORF57 and splicing factors, effects on host cell splicing

KSHV EGFP-ORF57 fusion protein exhibited a punctate nuclear distribution that co-localised with the cellular splicing factor SC-35 (Bello *et al.*, 1999) consistent with similar observations with ICP27 (Phelan *et al.*, 1993) and HVS ORF57 (Cooper *et al.*, 1999).

ICP27, HVS ORF57, and EBV Mta inhibit the expression of genes containing introns perhaps due to interference with the normal processing of intron-containing pre-mRNAs (Buisson *et al.*, 1999). In cotransfection experiments with intron-containing reporter constructs, ORF57 resulted in a slight decrease in reporter activity (Gupta *et al.*, 2000). Thus introns in the target gene appears to interfere with ORF57-mediated activation, an effect that may be important in selectively enhancing expression of certain KSHV genes, which are intronless. However the inhibition was not marked as seen with EBV Mta (Ruvolo *et al.*, 1998).

The data argue against an ORF57-encoded activity that globally impairs splicing or actively represses expression from intron-containing genes. However it does not exclude the possibility that some viral genes might display intron-dependent responses to ORF57 (Kirshner *et al.*, 2000).

1B1.3.3 ORF57 nucleocytoplasmic shuttling and transport of mRNAs

An EGFP-ORF57 fusion protein exhibited a punctate nuclear distribution in Hep-2 cells but an N-terminal deletion exhibited a predominantly cytoplasmic distribution, indicating that a nuclear localisation signal resides between amino acids 1-180 (Bello *et al.*, 1999). Cos-7 cells transfected with influenza hemagglutinin epitope (HA)-tagged ORF57 also showed a nuclear speckled pattern similar to that seen with ICP27 (Phelan & Clements, 1997) and EBV Mta (Gupta *et al.*, 2000). When CV-1 cells were transfected with an

ORF57 expression vector, intense staining was observed only in the nuclei (Kirshner *et al.*, 2000).

ORF57 can shuttle between the nucleus and cytoplasm (Bello *et al.*, 1999) but unlike ICP27 (Phelan & Clements, 1997; Sandri-Goldin, 1998b), does not shuttle in the presence of actinomycin D (Pinol-Roma & Dreyfuss, 1992). Shuttling in the heterokaryon assay but not after actinomycin D treatment of transfected cells is observed with many shuttling proteins, including all the nucleocytoplasmic transport receptors such as cyclin B1 (Hagting *et al.*, 1998); to demonstrate shuttling following actinomycin D treatment, protein re-import into the nucleus has to be transcription-dependent.

Expression of ORF57 led to a 6-fold increase in cytoplasmic CAT poly (A)+ mRNA versus a 2.6-fold increase in nuclear CAT poly (A)+ mRNA suggesting that ORF57 may facilitate nucleocytoplasmic transport of target mRNAs (Gupta *et al.*, 2000) but no direct evidence of a role for ORF57 in nuclear mRNA export was shown. Unlike with EBV Mta (Boyle *et al.*, 1999), no cytoplasmic translocation of ORF57 was observed upon overexpression of the cellular protein CRM1 with ORF57 (Gupta *et al.*, 2000).

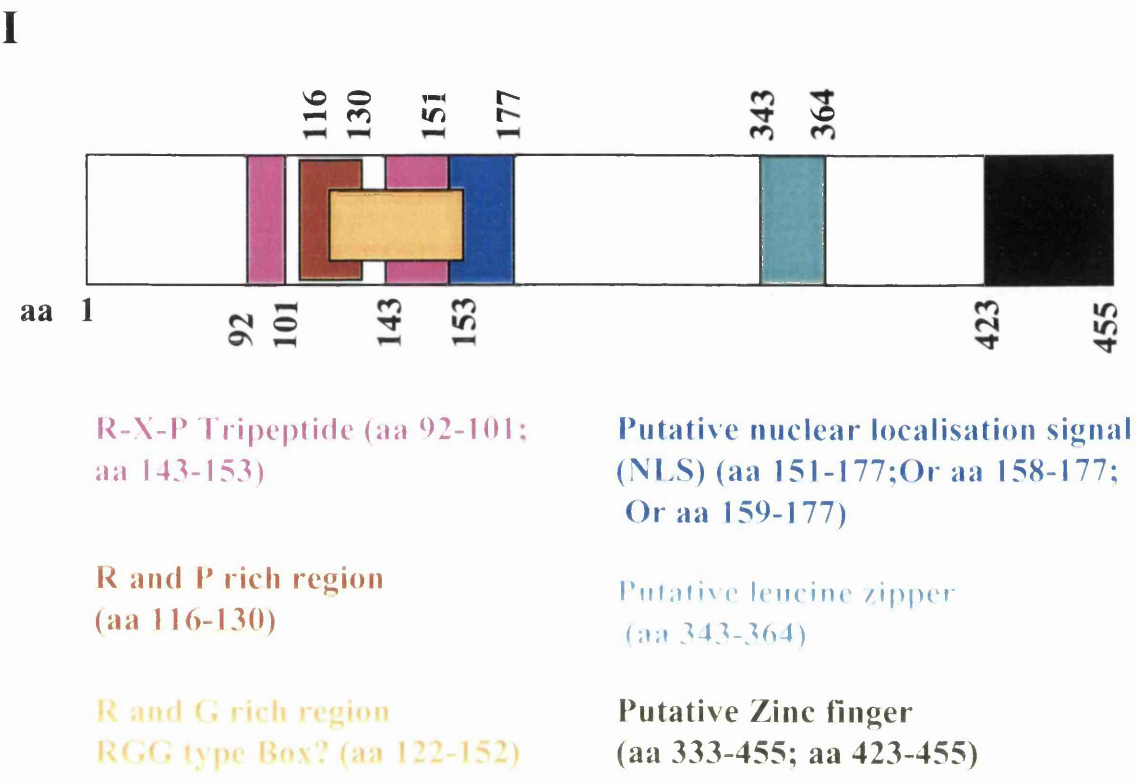
1B1.4 Putative functional domains of ORF57 protein

The domain organisation of ORF57 has not yet been fully characterised. On the basis of amino acid homology with its herpesvirus counterparts several putative domains are present (Fig 1B1.4 Panel I). As in ICP27 (Hibbard & Sandri-Goldin, 1995), in ORF57 Arg-Gly dipeptides or an Arg and Gly rich RGG box type domain (between aa 122-152) characteristics of many RNA-binding proteins (Manley & Tacke, 1996) are present: there are two Arg-X-Pro tripeptide type domains (between aa 92-101 and aa 143-153) and an Arg-Pro rich region (aa 116-130) (Fig 1B1.4 Panel I).

HVS ORF57, contains an arginine-rich nuclear localisation signal (NLS) demonstrated to interact with karyopherin α family members, importin α1 and α5 (Goodwin & Whitehouse, 2001) which forms a heterodimeric complex with importin β a transport adapter molecule binding to the nuclear pore complex via a direct interaction with specific nucleoporins (Gorlich *et al.*, 1996; Weis *et al.*, 1996). This interaction could be necessary for the efficient nuclear export of HVS RNA. Interestingly an ORF57 N-terminal deletion construct localised to the cytoplasm whereas full-length protein localised to the nucleus (Bello *et al.*, 1999) indicating the possibility of a NLS between amino acids 1-180. Analysis of this region reveals several arginine-rich potential NLS (between aa 151-177 or 158-177 or 159-177) (Fig 1B1.4 Panel I).

The C-terminal and middle regions contains a greater degree of similarity amongst the ORF57 homologues (Brown *et al.*, 1995). Metal chelate affinity

Fig 1B1.4 Panel I. Putative functional domains of ORF57



Panel I. On the basis of amino acid homology, ORF57 contains a number of putative functional domains which are colour coded.

chromatography showed that ICP27 (aa 407-512) binds zinc *in vitro* (Vaughan *et al.*, 1992) and mutation analysis (Vaughan *et al.*, 1992) demonstrated that the same region encodes a potential zinc finger motif ($C_{(483)}$-X_4-$C_{(488)}$-X_{13}-$H_{(502)}$-X_5-$C_{(508)}$). This motif resembles a type of zinc finger which is involved in protein:protein interactions e.g. GATA-1 binding to FOG (Fox *et al.*, 1998; Mackay & Crossley, 1998).

Based on amino acid similarity, ORF57 aa 333-455 contains two positionally conserved motifs: a zinc finger-like domain $C_{(333)}$-X_{89}-$H_{(423)}$-X_3-$C_{(427)}$-X_4-$C_{(432)}$ or, $H_{(423)}$-X_3-$C_{(427)}$-X_4-$C_{(432)}$ conserved in all herpesviruses (Vaughan *et al.*, 1992) and a hydrophobic GLFF domain (aa 448-451) highly conserved in gammaherpesviruses.

For HVS ORF57 the zinc finger region is required both for transactivation and repression and the GLFF domain is required for transactivation not repression (Goodwin *et al.*, 2000). Similarly the zinc finger containing C-terminal region is responsible for the gene activation and repression effects exerted by ICP27 (Hardwicke *et al.*, 1989) (McMahan & Schaffer, 1990). Later studies showed that activation is correlated with increased polyadenylation and that repression is correlated with inhibition of splicing (Chapman *et al.*, 1992; McLauchlan *et al.*, 1992; Sandri-Goldin & Mendoza, 1992; Hardwicke & Sandri-Goldin, 1994). Based on amino acid similarity, the ORF57 C-terminal region may also possess activation and repression activity.

The ICP27 C-terminal region is required for redistribution of snRNPs (Hibbard & Sandri-Goldin, 1995) and in anti-Sm coimmunoprecipitations the region required for coimmunoprecipitation with ICP27 was aa 450-504 (Sandri-Goldin & Hibbard, 1996). Self-interaction of ICP27 requires the residues that make up the zinc finger (Zhi *et al.*, 1999). Similarly in HVS ORF57 the zinc finger-like domain is required for the intense SC-35 nuclear staining (Goodwin *et al.*, 2000). Based on amino acid homology the ORF57 C-terminal region may also be involved in self-interaction (Section 3C) and/or inhibition of splicing.

Several differences are also present that may be functionally important such as ORF57 does not seem to contain a leucine-rich nuclear export signal (NES) potentially capable of binding CRM1 (Wen *et al.*, 1995; Ossareh-Nazari *et al.*, 1997; Fornerod *et al.*, 1997a; Fukuda *et al.*, 1997) and involved in export of viral mRNAs as found in EBV Mta, HVS ORF57 and HSV ICP27 (Nicholas *et al.*, 1988; Sandri-Goldin, 1998a; Boyle *et al.*, 1999). Although, in ORF57 there are two regions with homology to other known NESs: $L_{(369)}$NFRGGLLL$_{(377)}$ similar to NES of HDM2 protein (L--NFRGGLLL) and $L_{(375)}$LLAFVVLTI$_{(383)}$ similar to NES of vertebrate PKI protein (L-L-LAFVLTI). Unlike its homologues in other herpesviruses, ORF57 contains a leucine zipper motif (generally found in DNA binding proteins) between aa 343-364 with a possible role in self-

interaction or DNA binding (Kouzarides & Ziff, 1988; Landschulz *et al.*, 1988) (Fig 1B1.4 Panel I). The region encompassing the putative zinc finger motif is predicted to form α-helical secondary structures (Fig 1B1.4 Panel II) that are characteristics of the dimerisation motifs in well characterised helix-loop-helix proteins (Murre *et al.*, 1989) as well as leucine zipper DNA binding protein such as GCN4 (Ellenberger *et al.*, 1992).

The secondary structure of ORF57 was predicted by the program "insulin (PSI PRED)" using the website: *www.Run@insulin.brunel.ac.uk/cgi-bin/psipred/graphics/nph-view.cgi?*. PSIPRED program prediction confidence range was between 0 (low) and 9 (high) and on that graphic scale ORF57 secondary structure is shown in Fig 1B1.4 Panel II. All ORF57 helices were predicted to form internal or externl loops using the same website by MEMSTAT2 program and no transmembrane loops were predicted. ORF57 structure is predicted to form a large number of helices in the middle region and all the conserved amino acids between homologues also form helices.

1B1.5 ORF57 and DNA replication

The KSHV primase-associated factor (PAF, ORF40/41) together with primase (ORF56) and helicase (ORF44) required the presence of all five other viral replication proteins for efficient nuclear translocation. As a replication-associated component, ORF57 also contributed although less efficiently to PAF nuclear translocation (Wu *et al.*, 2001).

ICP27 is essential for DNA replication (Sacks *et al.*, 1985) and also partially co-localises with replication compartments (Zhong & Hayward, 1997). Recently ICP27 was shown to coimmunoprecipitate with ssDNA-binding protein (ICP8) and RNA polymerase II holoenzyme, and association of both was independent of viral DNA synthesis (Zhou & Knipe, 2002). Infection with ICP27 gene mutant viruses revealed that ICP27 is required for the association of ICP8 with RNA polymerase II, while studies with ICP8 gene deletion mutants showed no apparent role for ICP8 in the association of ICP27 with RNA polymerase II. These authers suggested that the ICP27 RNA polymerase II interaction reflects its role in stimulating early and late gene expression and/or its role in inhibiting host transcription, and that the interaction of ICP8 with RNA polymerase II reflects a role in stimulating viral transcription (Zhou & Knipe, 2002). Other Mta family proteins such as ORF57 are not known to directly interact with replication proteins (Wu *et al.*, 2001).

1B2 ORF57 homologues

ORF57 protein is the only KSHV IE gene which has homologues throughout the *Herpesviridae* and this suggests that aspects of its regulatory role are maintained

Fig 1B1.4 Panel II. Predicted secondary structure of ORF57

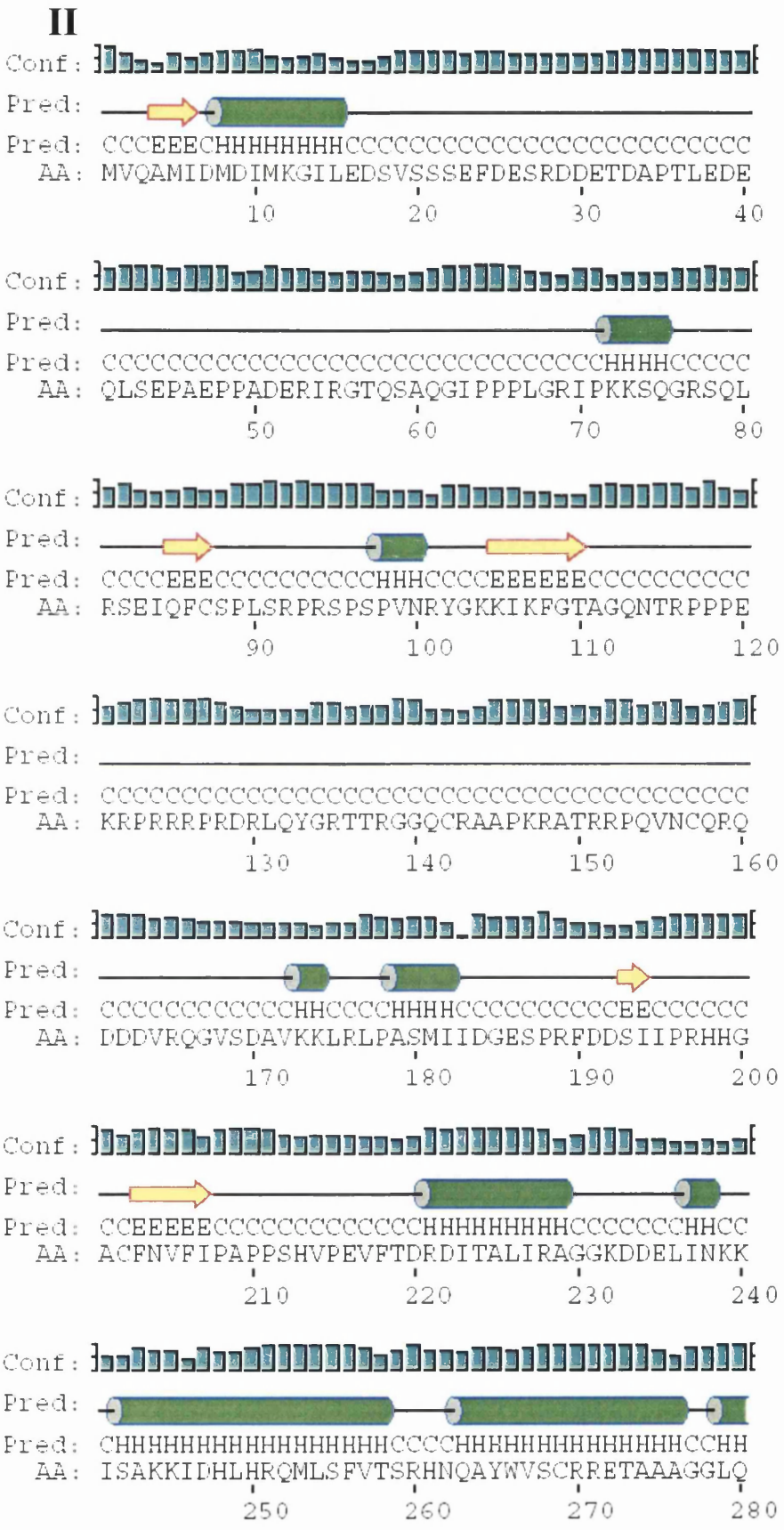
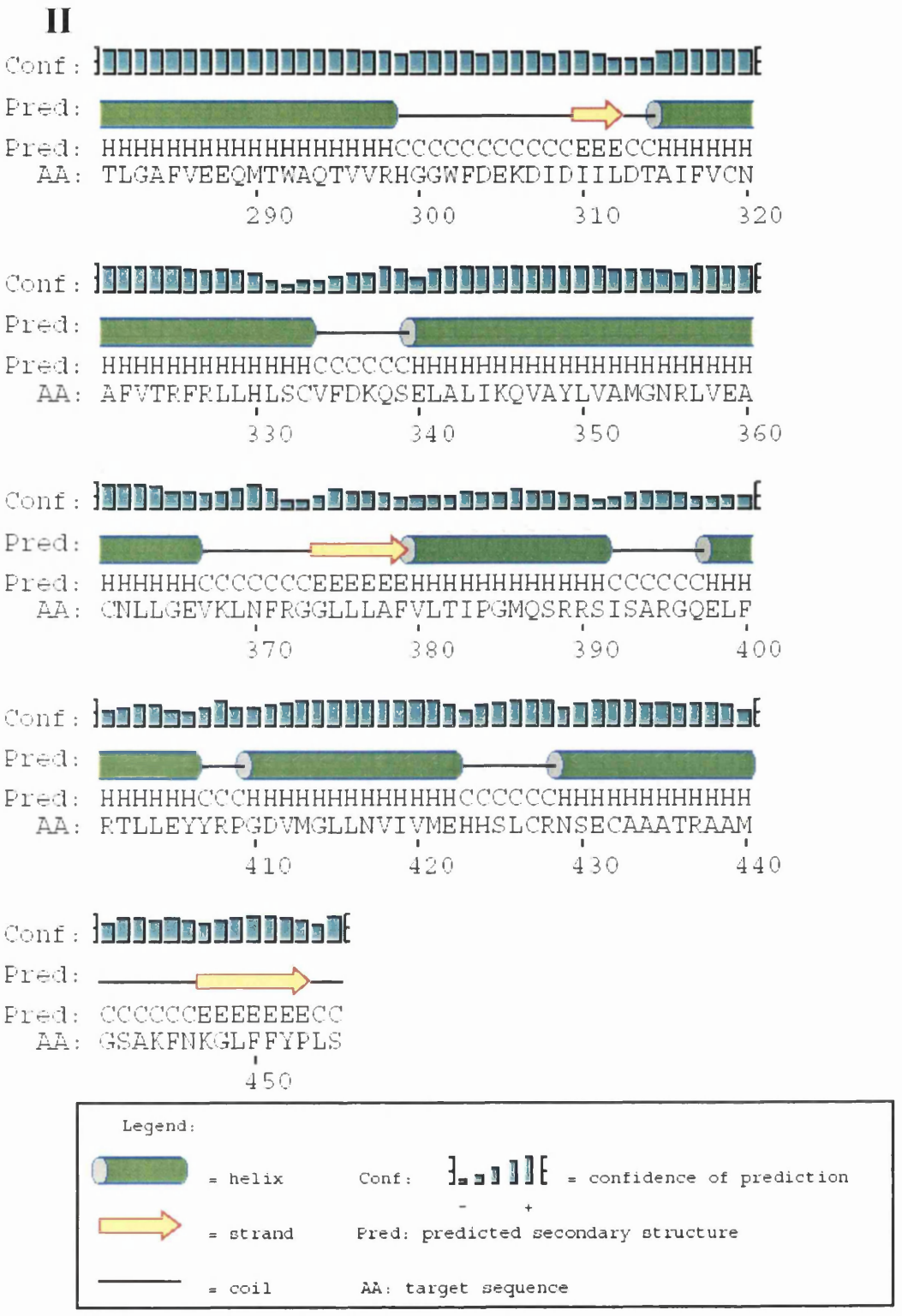


Fig 1B1.4 Panel II. Predicted secondary structure of ORF57 (continued)



Panel II. Secondary structure of ORF57 was predicted by running "Insulin" using the Website: WWW. Run@insulin.brunel.ac.uk/cgi-bin/psipred/graphics/ nph-view.cgi?
All helices were predicted to form inside or outside loops, no transmembrane helices were predicted by MEMSAT2 program using the same Website.

PSIPRED program prediction confidence range (0=low ∃, 9=high E).

throughout the herpesvirus family. Comparisons of functional and structural characteristics of ORF57 with its herpesvirus counterparts aids understanding of each homologue's function, and the evolution of the protein during the emergence of the different herpesviruses.

Sequence alignments of ORF57 homologues in the herpesvirus subfamilies are shown in Fig 1B2 Panel I, II and III.

Proceeding from the N to C termini, the gammaherpesvirus protein comprise of

- An N-terminal exon consisting 16-18 amino acids
- An acidic region
- A basic region
- A linker
- A conserved domain

The alphaherpesvirus proteins do not contain the N-terminal region from an additional exon and the betaherpesvirus proteins also do not have this structure. The betaherpesviruses contain a large non-conserved C-terminal extension of variable length containing an acidic domain. Throughout the herpesviruses, the amino termini are not conserved and contain acidic and basic residues however the central region is conserved with certain residues conserved in alpha-, beta-, and gamma-herpesviruses (Fig 1B2 Panel I, II and III).

1B2.1 HSV-1 ICP27

HSV-1 ICP27 is a pleiotropic regulator that binds RNA (Ingram *et al.*, 1996; Mears & Rice, 1996) and shuttles between the nucleus and the cytoplasm (Phelan & Clements, 1997; Soliman *et al.*, 1997; Mears & Rice, 1998; Sandri-Goldin, 1998a). Temperature sensitive mutations have shown that ICP27 is essential for lytic viral replication and is required for inhibition of host cell pre mRNA splicing, an activity that contributes to host shutoff and to the down regulation of intron-containing genes in transient assays (Hardwicke & Sandri-Goldin, 1994; Hardy & Sandri-Goldin, 1994; Sandri-Goldin & Hibbard, 1996). ICP27 promotes RNA 3' processing at weak viral poly (A) sites (McLauchlan *et al.*, 1989; McGregor *et al.*, 1996). Viruses mutant in ICP27 show reduced levels of viral mRNAs, are defective in viral DNA replication (Rice & Knipe, 1988; Sekulovich *et al.*, 1988; McCarthy *et al.*, 1989; Rice & Knipe, 1990) and fail to efficiently suppress cellular gene expression (Hardwicke & Sandri-Goldin, 1994). ICP27 interacts with cellular proteins heterogeneous nuclear ribonucleoprotein K (hnRNP K), protein kinase casein kinase 2 (CK2), p32 and splicing associated protein 145 (SAP145) (Fig 1B2.1 Panel I) (Wadd *et al.*, 1999; Bryant *et al.*, 2000; Bryant *et al.*, 2001).

Fig 1B2 Panel I. Amino acid conservation of ORF57 homologues in gammaherpesviruses

Proceeding from the N to C terminus, the protein consists of:

- A 16-20 residue region from exon 1
- An acidic region
- A basic region
- A linker
- A conserved domain

Acidic and basic residues are in colour for all but the conserved domains. The charged regions are, as expected, highly hydrophilic. Bold residues are conserved among homologues.

(Amino acid sequences were aligned using program: PILEUP. Figure kindly provided by A. Davison, MRC Virology Unit, Glasgow, UK).

Notes

The HVS sequence has been corrected on the basis of data supplied by Helmut Fickenscher.
The EHV-2 protein appears to have the same structure as other gamma-2s, but there are complexities (from additional splicing or frame shift errors) that make the situation difficult to interpret. Hence this protein has been omitted. There is a sequence available for ILTV (an alpha), but it is somewhat suspect at its C terminus and has been omitted. It is more dissimilar from the other alphas than they are from each other. Regions outside the conserved domain cannot be aligned properly.

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Gammaherpesviruses

HHV-8        MVQAMIDMDIM.KGILE/DSVSSSEFDESRDDETDAPTLEDEQLSEPAEPPADERIRGTQSAQGIPPPLGRIPKKSQ
HVS          MAQAMVTNCQM.EDIIE/GISSDDDFDSSDSSSDEEESDTSPQIMKSDVTMASPPSTPEPSPDVSASTSNLKRERQR
BHV-4        MAQAMLTMDCM.REIIE/DLSSDIDSFSGGESIDMESELEEGEIESDTNSSKPPPPQDLSKPPMMRIPRKRVASPDN
AHV-1        MAQQAIVTMSALRRTME/VSDSGDVSIDISAEDSNDSFHLEESVDDCMDDCKPNNRPNPISMKPAKRRVFMVPKRER
MHV-68       MAQQMLEAGAL.DQMME/GLPSDFDFDTSDEEGELSDSPPVEEPTGPVRDVVYEPDPLFDDPPPTPSPDVKPPSPKA
EBV       MVPSQRLSRTSSISSNEDPA/ESHILELEAVSDTNTDCDLDPMEGSEEHSTDGEISSSEEEDEDPTPAHAIPARPSSVVI

HHV-8     GRSQLRSEIQFCSPLSRPRSPSPVNRYGKKIKFGTAGQNTRPPPEKRPRRRPRDRLQYGRTTRGGQCRAAPKRATRRPQV
HVS       SPITWEHQSPLSRVYRSPSPMRFGKRPRISSNSTSRSCKTSWADRVREAAAQRRPSRPFRKPYSHPRNGPLRNGPPRAPP
BHV-4     ERMEYRSPLNRTYPPPFTERYGKRRRLTAGRPNWSGRVNEDKGRYRRRGLSDNKTIRHTQASIKDEVAVSLRKMKIPTGM
AHV-1     SKTPVQHTSPLNRLYPNVVLGKQHGYKQRPAPSAPSRRPQPYSARKDSAAKPQSTPSNQNPLTELLKNVDPAIASRITEM
MHV-68    RKRALSPEIVHNSPLLRDTTKYEPAPKRSYSYHPRRSPQRENANQKQKRGPDSRRPNRWNQKSQKQYWSPKPLLDYSKIP
EBV       TPTSASFVIPRKKWDLQDKTVTLHRSPLCRDEDEKEETGNSSYTRGHKRRRGEVHGCTDESYGKRRHLPPGARAPRAPRA

HHV-8     NCQRQDDDVRQGVSDAVKKLRLPASMIIDGESPRFDDSIIPRHHGACFNVFIPAPPSHVP.................EVF
HVS       LLKLFDISILPKSGEPKLFLPVPSLPCQEA.....................................................EKT
BHV-4     IRRAGEKPFDETLLSSGGPGRYSVFLPRAPEFKL.................................................ERY
AHV-1     RIPRSMLRTPSGQPFAHWLMPSAEDSSKFINVNPVNMEV.....................................EEH
MHV-68    RAEYKNAKLLVPTTGKLRP.....................................................EFY
EBV       PRVPRAPRSPRAPRSNRATRGPRSESRGAGRSTRKQARQERSQRPLPNKPWFDMSLVKPVSKITFVTLPSPLASLTLEPI

HHV-8     TDRDITALIRAGGKDDELIN.KKISAKKIDHLHRQMLSFVTSRHNQAYWVSCRRETAAAGGLQTLGAFVEEQMTWAQTVV
HVS       NDKYVLAMAQRAMHDVPISS.KQLTANLLPVKFKPLLSIVRYTPNYYYWVSMRKETIASANLCTVAAFLDESLCWGQQYL
BHV-4     TDKLVSSLVEKGGENGAGIS.KKLSHLKLSSNFSVIHSFLNKSINYHYWVCLRKETMGSCGLTSLMLFLEETCCWAQLCT
AHV-1     VNVVVRRCTEWALISSRLQD.KSISTKYLAENFYDLRDFAQRSINKSAWINLRREAIANAGFVNLCAFADEMMMWLQLNL
MHV-68    TDRFVDAIIQNAARNCPVSE.KAVSLKNIEESFKLLNSFFNSGINKDHWLSTRYFAIFNNGLVVLTHMLDEQLAWAYACL
EBV       QDPFLQSMLAVAAHPEIGAWQKVQPRHELRRSYKTLREFFTKSTNKDTWLDARMQAIQNAGLCTLVAMLEETIFWLQEIT

HHV-8     RHGGWFDEKDIDIILDTAIFVCNAFVTRFRLL.HLSCVFDKQSELALIKQVAYLVAMGNRLVEACNLLGEVKLNFRGGLL
HVS       KNDFIFSENGKDIILDTSSALLSQLVHKIKMLPFCHCLMQTTPQDHIVKQVCYLIASNNRILDAVRYLQTSVIKSPIVLL
BHV-4     SNDVSINGFSNDIILNSANFLSVQIMFKLRSL.VMPCFAREAHNISLVKQLGYLVSTTNKIQTAASLIRELKLDTKLCLL
AHV-1     NNQGSWKACREDIILTGAPDMCFHALQKVRAF..IKCFLRERHQRALVNALCHIICFEGGIKQAATLCQELFFDFKVGLM
MHV-68    KHGRELP..TDDILMSTSEKLSQQLVIKLIEV..IKCIEKDGIFSRILKGVADAVCLKAQFLRGMITLKRTPCSLPMYTL
EBV       YHGDLPLAPAEDILLACAMSLSKVILTKLKEL..APCFLPNTRDYNFVKQLFYITCATARQNKVVETLSSSYVKQPLCLL

HHV-8     LAFVLTIPGM.QSRRSISARGQELFRTLLEYYRPGDVMGLLNVIVMEHHSLCRNSECAAATRAAMGSAKFNKGLFFYPLS
HVS       LAYAVCLPAA.IICTKNETQLYSHCMRILKEYRPGDVMNILHESLTQHLNKCPSSTCAYTTRAIVGTKANTTGLFFLPTQ
BHV-4     AAFAIVVPTL.LETDKTEHGTYAFFMQYINRYRPGCIMSLYNDVISSHSRECTSRLCIANTRALAGTKDKTKGLFFCPI
AHV-1     VLYFLTPYAFLYSHTIPQCNFGGYFSKCVAQYTPGAVTGLLNSAIEDHYKDCTSQDCTNLITAIVSPETSNKGLLFFPLPM
MHV-68    FVYVLTIPTL..RTRVIRDPLLTQCKDVVLKYQPGDCITLLKAALNCHQCNKDCDKCKYILDPLLGQTHRTKGVFFVCE
EBV       AAYAAVAPAYINANCRRRHDEVEFLGHYIKNYNPGTLSSLLTEAVETHTRDCRSASCSRLVRAILSPGTGSLGLFFVPGLNQ
```

Fig 1B2 Panel II. Amino acid conservation of ORF57 homologues in alphaherpesviruses

Proceeding from the N to C terminus, the protein consists of:
An acidic region, a basic region, a linker, and a C terminal conserved domain.
Acidic and basic residues are in colour for all but the conserved domains. The charged regions are, as expected, highly hydrophilic. Bold residues are conserved among homologues.

```
HSV-1     MATDIDMLIDLGLDLSDSDLDEDPPE.PAESRRDDLESDSSGECSSSDEDMEDPHGEDGPEPILDAAPPAVRPSRPEDPG
HSV-2     MATDIDMLIDLGLDLSDSELEEDALERDEEGRRDDPESDSSGECSSSDEDMEDPCGDGGAEAI.DAAIPKGPPARPEDAG
EHV-1                                                        MALSSVSSCEPMEDEMSIMGSDTEDNFTGGDTCA
EHV-4                                                        MALSSVSSCEPMDDEMSIMGSDTDDTL.GGSCVE
BHV-1                                                              MADPEIATLSTASESDDLSLFGSDRE
PRV
VZV                                                                                  MASASIPTD
MDV                                                    MSVDAFSRESDDMMSLLDYDFIEGSSSDENAEVTEMETSAK

HSV-1     VPSTQTPRPTERQGPNDPQPAPHSVWSRLGARRPSCSPEQHGGKVARLQPPPTKAQPARGGRRGRRRGRGRGGPGAADGL
HSV-2     TPEASTPRPAARRGADDPPPATTGVWSRLGTRRSASPREPHGGKVARIQPPSTKAPHPRGGRRGRRRGRGRYGPGGADST
EHV-1     EATRGLVNKSAFVPTQTVGTVSALRNVVGDPPKSVVVSFSASPQRAQPSNPKSERPAFGHGRRNRRRPFRRNNWKQQQRG
EHV-4     AAQSAVVNKRAFEMSESTGTMSTIRNVVSEVPKSLVVSFAASPKNPKPQNTTSERSAFPHGRKNRRRPFRRNNWK..QRA
BHV-1     EDDEAPSLAPALRSVVGQVRKRKLEGAEDEPMPAEPPGEGAASGDGGPAEAPPARRARVRPRRPRRRPRRRQPAGEQRSR
PRV         MEDSGNSSGSEASRSGSEERRPVRERLGSRPPERRPVRARLGAIRRRRGGRGGRAARQALRQRRRQQQQQQRQQQHQR
VZV       PDVSTICEDFMNLLPDEPSDDFALEVTDWANDEAIGSTPGEDSTTSRTVYVERTADTAYNPRYSKRRHGRRESYHHNRPK
MDV       TANNKNEVLFAPPCTQELLTERPSPDSKNSQGDDDSNSIYGNVIRDAQHSASRYATRCLDNAIPRKRLRLANLTVDSACI

HSV-1     SDPRRRAPRTNRNPGGPRPGAGWTDGPGAPHGEAWRGSEQPDPPGGQRTRGVRQAPPPLMTLAIAPPPADPRAPAPERKA
HSV-2     PKPRRRVSRNAHNQGGRHPASARTDGPGATHGEARRGGEQLDVSGGPRPRGTRQAPPPLMALSLTPPHADGRAPVPERKA
EHV-1     WEKPEPENVPARQSAGSWPKRSSLPVHMRLGQRGGDSSSADSGHGGAGPSDRWRFKTRTQSVARVHRNRRRGNANHGSNT
EHV-4     WEKQSQEAAPANQGSRNWPKRSSMPVHMRLGHRSGDFQSADAGHCTAGPSGGWRFKTRTHSASRVYHNRQRGNTNKSGNA
BHV-1     GPAAKREAALATSSHGGGGAAARSIGSSLRLARSLAEAAQ RATAERVTAVFAGARLDLMRPVQNGGFRAAGV.......
PRV       RRQEADRPDGGPD APPDRLSESARAAVSATHA RVGATRVNELFASARHDLSRPVFNDGFRAAGS..............
VZV       TLVVVLPDSNHHGGRDVETGYARIERGHRRSSRSYNTQSSRKHRDRSLSNRRRRPTTPPAMTTGERNDQTHDESYRLRFS
MDV       SQTKRPHGTGNRKQYHRRNFPMSPTSQEKIHLRLHNRLGSRSEKQQRSLNYDRRLQEGHHRRRFYSERRIYDQNHSHHRT

HSV-1     PAADTIDATTRLVLRSISERAAVDRISESFGRSAQVMHDPFGGQPFPAAN..........SPWAPVLAGQGG.PFDAETR
HSV-2     PSADTIDPAVRAVLRSISERAAVERISESFGRSALVMQDPFGGMPFPAAN..........SPWAPVLATQAG.GFDAETR
EHV-1     PGRSAGDRLNAAAAASSIADVCRRVTSSRIGEMFHGARETLTTPVKNGGFRAENS......SPWAPVLGFGSD.QFNPEAR
EHV-4     SSSRSGDRLNAAAAANAIADVSKRVTSSRISDMFHGARETLTSPVKNGGFRAEHS......SPWSPVLNFGLE.QFNPEGR
BHV-1     ..........................................................SPWAAVLDFGAE.QFVPEGR
PRV       ..........................................................SPWAAVLEFGAE.QFTPDGR
VZV       KRDARRERIRKEYDIPVDRITGRAIEVVSTAGASVTIDSVRHLDETIEKLVVRYATIQEGDSWASGGCFPGI......KQ
MDV       HDIRVPLEKYRVSRQHDLPVHEELNEILQREKHRLASISNECDFRVSSK..........NRWAAVLTFSSNAESTLCGP

HSV-1     RVSWETLVAHGPSLYRTFAGNPRAASTAKAMRDCVLRQENFIEALASADETLAWCKMCIHHNLPLRPQDPIIGTTAAVLD
HSV-2     RVSWETLVAHGPSLYRTFAANPRAASTAKAMRDCVLRQENLIEALASADETLAWCKMCIHHNLPLRPQDPIIGTAAAVLE
EHV-1     RITWDTLVEHGVNLYKLFEVRSHAAEAARSLRDAVMRGENLLEALASADETLSWCKMIVTKNLPMRTRDPIISSSVALLD
EHV-4     RITWDTLVTHGENLYKLFEVRSHAAEAARSLRDLVMRGENLLEALASADETISWCKMIITKNLPMRTRDPIIHSSIALLE
BHV-1     RVTWETLMFHGRDLYRMFEVRPHAAQAARALRDLVLRSANLVDALASADECLTWCKFIATKNLRLRTKDPIVATAGAVLE
PRV       RVTWETLMFHGADLHRLFEVRPHATEAARVLREMVLLNEGLTESLASADETLTWVKLILTKGLTLRTLDPIVATAGAVLQ
VZV       NTSWPELMLYGHELYRTFESYKMDSRIARALRERVIRGESLIEALESADELLTWIKMLAAKNLPIYTNNPIVATSKSLLE
MDV       QITWEYLLHAGPELRNTFEIRPRISLQASAAREAVLRGESFIAALGSAEETLSWLKLHAVLKLRLVNHDPIFKTAGAVLD

HSV-1     NLATRLRPFLQCYLKAR....GLCGLDELCSRRRLADIKDIASFVFVILARLANRVERGVAEIDYATLGVGVGEKMHFYL
HSV-2     NLATRLRPFLQCYLKAR....GLCGLDDLCSRRRLSDIKDIASFVLVILARLANRVERGVSEIDYTTVGVGAGETMHFYI
EHV-1     NLRLKLEPFMRCYLSSS....GSPTLAELCDHQRLSDVACVPTFMFVMLARIARAVGSGAETVSRDALGPD.GRVLADYV
EHV-4     NLRLKLEPFMRCYLSSS....GSPTLAELCDHQRLSDVACVPTFMFVTLARIARAVGSGAEAVSPDALGPA.GHALANYV
BHV-1     NLRLKLAPFLRCYLRGR....GLPSLEELCAARRLSLATCPASYMFVMLARLSRAVRSGAECVPLLEVTVG.DAPFEEYI
PRV       NLRLKLGPFLRCYLRDT.......PVDELVRRRRLRDVRCIVTYTLVMLARIARVVERGSSCVLPEDLGDS.PVPLEEYV
VZV       NLKLKLGPFVRCLLLNRDNDLGSRTLPELLRQQRFSDITCITTYMFVMIARIANIVVRGSKFVEYDDISCN.VQVLQEYT
MDV       NLRLKLAPIMMCKYGTE.....KRSMGDMLRRSAPEDINDSLTLCLILLSRIRRVMHRTSGSKYSYMIDPR..GCMIDYV

HSV-1     PGACMAGLIEILDTHRQECSSRVCEL.TASHIVAPPYVHGKYFYCNSLF
HSV-2     PGACMAGLIEILDTHRQECSSRVCEL.TASHTIAPLYVHGKYFYCNSLF
EHV-1     PGACLAGTLEAIDAHKRRCKADTCSL.VSAYTLVPVYLHGKYFYCNQIF
EHV-4     PGTCLAGTLEAIDLHKRRCKESTCSL.VSSYTLVPVYLHGKYFYCNQIF
BHV-1     PGTCVAGLIDALDTHKQACDSMTCKL.VANFTLVPVYMHGKYFYCNEIF
PRV       PGACLGGIMDALDSHKTGCDAPTCRL.TCSYTLVPVYMHGKYFYCNHLF
VZV       PGSCLAGVLEALITHQRECGRVECTLSTWAGHLSDARPYGKYFKCSTFNC
MDV       PGECMTNILRYVDAHTRRCSDPACNL.YISCTLMPIYIHGRYFYCNTLFGM
```

Fig 1B2 Panel III. Amino acid conservation of ORF57 homologues in betaherpesviruses

Acidic and basic residues are in colour for all but the conserved domains. The charged regions are, as expected, highly hydrophilic. Bold residues are conserved among homologues.

Abbreviations: HHV-8/KSHV: human herpesvirus-8/Kaposi's sarcoma associated herpesvirus; HVS: herpesvirus saimiri; BHV-4: bovine herpesvirus-4; AHV-1: alcelaphir herpesvirus-1; murine gammaherpesvirus-68; EBV: Epstein-Barr virus; HSV-1: herpes simplex virus-1; HSV-2: herpes simplex virus-2; EHV-1: equine herpesvirus-1; EHV-4: equine herpesvirus-4; BHV-1: bovine herpesvirus-1; PRV: pseudorabiesvirus; VZV: varicella zoster virus; MDV: Marek's disease virus; HHV-6: human herpesvirus-6; HHV- human herpesvirus-7; MCMV: murine cytomegalovirus; HCMV: human cytomegaloviru

Self-interaction of ICP27 *in vivo* has been shown by IFA, coimmunoprecipitations and yeast two-hybrid assay and C-terminal cystine-histidine-rich zinc-finger-like region was required for the self-association (Wadd *et al.*, 1999; Zhi *et al.*, 1999).

However, the effect of ICP27 on splicing does not seem to be critical for virus replication (Soliman *et al.*, 1997). ICP27-dependent cytoplasmic accumulation of unspliced α-globin RNA can be uncoupled from the inhibitory effects of ICP27 on splicing (Ellison *et al.*, 2000). In *Xenopus* oocytes also no inhibition of splicing or export of cellular transcripts was observed in the presence of ICP27 (Koffa *et al.*, 2001). Splicing inhibition may be indirect via a direct effect on RNA export i.e., ICP27 may efficiently compete for REF and host cell splicing is shut down due to less REF availability or there may be a direct inhibitory effect mediated via ICP27+SAP145/p32 interactions. ICP27 appears to promote the export of intronless viral RNAs but not spliced viral transcripts (Soliman *et al.*, 1997; Sandri-Goldin, 1998a).

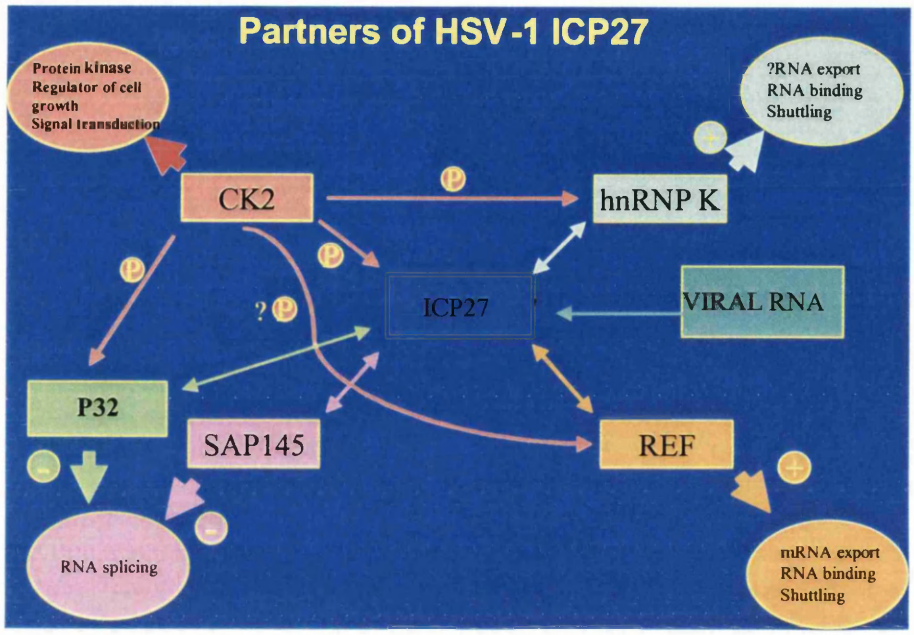
ICP27 has also been proposed to affect nuclear export of viral RNAs via CRM1-dependent and -independent pathways (Soliman & Silverstein, 2000). However recently ICP27 was shown to dramatically stimulate the export of intronless viral mRNAs in *Xenopus laevis* oocytes. Use of inhibitors showed that ICP27 neither shuttles nor exports viral mRNA via the CRM1 pathway. Instead, ICP27-mediated viral RNA export requires REF and TAP, factors involved in cellular mRNA export. It was proposed that ICP27 associates with viral mRNAs and recruits TAP via its interaction with REF proteins, allowing the inefficiently exported viral mRNAs to access the TAP-mediated cellular export pathway (Fig 1B2.1 Panel I & II). ICP27 protein was also shown to be exported independently without the viral RNA in this system (Koffa *et al.*, 2001).

1B2.2 HVS ORF57

HVS is the prototype gamma-2 herpesvirus. The HVS ORF57 protein is a 52 kDa multifunctional regulatory protein. Transactivation of late viral genes by ORF57 occurs independently of target gene promoter sequences and appears to be mediated at a posttranscriptional level. HVS ORF57 can activate and repress expression from a range of both early and late HVS promoters, and levels of mRNA transcribed from intronless genes do not correlate with the increase/decrease in reporter CAT activity, indicative of a posttranscriptional mechanism. Repression of gene expression was dependent on the presence of an intron within the reporter coding region.

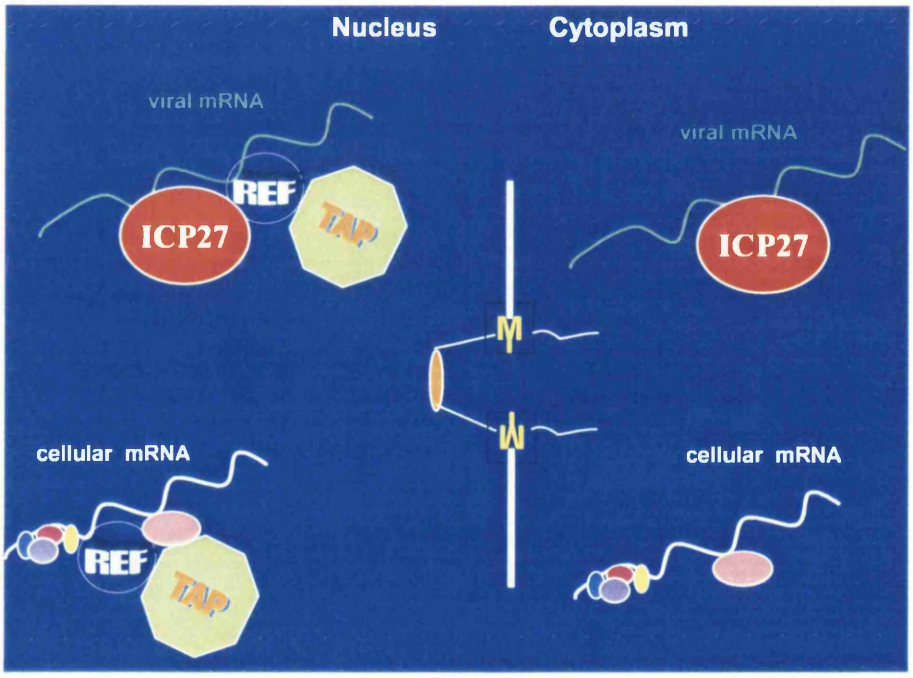
The HVS-ORF57 C terminus contains both transactivation and repression domains. Like HSV-1, HVS infection results in the redistribution of SC-35 and snRNP U2 spliceosome components into distinct nuclear aggregations, ORF57 gene product is

Fig 1B2.1 Panel I. HSV-1 ICP27 partner proteins



HSV-1 ICP27 interacts with cellular proteins such as hnRNP K, CK2, SAP145, p32 and REF which are involved at various stages of gene expression and it may perform its role via interaction with these proteins. ICP27 also binds viral RNA (Figure provided by Barklie Clements, Institute of Virology, University of Glasgow, UK).

Fig 1B2.1 Panel II. Export pathway utilised by HSV-1 and cellular mRNAs



ICP27-mediated viral RNA export requires REF and TAP, factors involved in cellular mRNA export. It was proposed that ICP27 associates with viral mRNAs and recruits TAP via its interaction with REF proteins, allowing the inefficiently exported viral mRNAs to access tha TAP-mediated cellular export pathway (Koffa *et al.*, 2001).

enough to cause this redistribution and co-localises with the redistributed spliceosome components (Cooper *et al.*, 1999).

HVS ORF57 protein has the ability to bind viral RNA, shuttle between the nucleus and the cytoplasm, and is required for efficient cytoplasmic accumulation of virus mRNA. This suggests that HVS ORF57 may play an important role in mediating the nuclear export of viral transcripts (Goodwin *et al.*, 1999). Recently the nucleocytoplasmic shuttling mechanism utilised was investigated, HVS ORF57 interacts with importin α isoforms 1 and 5 and an N-terminal arginine-rich sequence, functioning as a NLS, was required for this interaction. The HVS ORF57 protein was also responsible for the redistribution of importin α into the nucleoli. Finally, the importin α interaction was also suggested to be required for efficient viral RNA export (Goodwin & Whitehouse, 2001).

1B2.3 EBV Mta

Mta expressed at early times in EBV lytic infection was originally described as a promiscuous transcription factor, as it activates transient expression of reporter genes placed under the control of different promoters (Lieberman *et al.*, 1986). However, Mta acts in *trans* by a posttranscriptional mechanism, which is reporter gene dependent (Kenney *et al.*, 1989a). Like ICP27, Mta binds RNA and regulates gene expression via control of splicing and RNA export (Ruvolo *et al.*, 1998). Mta inhibits cytoplasmic accumulation of polyadenylated RNAs when they are generated by the use of cryptic 5' splice sites, and induces cytoplasmic accumulation of both intronless and intron-containing RNAs generated by use of constitutive splice sites (Buisson *et al.*, 1999).

Thus via EBV Mta expression of many early and late intronless viral genes may be enhanced, while expression of certain intron containing IE and latent genes may be repressed. EBV Mta binds RNA, co-localises with SC35 and shuttles between the nucleus and the cytoplasm (Semmes *et al.*, 1998). Thus it has properties of an RNA export protein that specifically increases cytoplasmic accumulation of unspliced EBV viral RNAs (Semmes *et al.*, 1998). Both the *trans*-activation function and shuttling of EBV Mta are reported to be dependent on association with CRM1 *in vivo* (Boyle *et al.*, 1999). Mta associated *in vivo* with other components of the CRM1 export pathway, including the small GTPase Ran and the nucleoporin Nup214. Mutation of a leucine-rich region (LRR) or addition of LMB both inhibited CRM1 mediated cytoplasmic translocation and Mta activity suggesting that Mta is not merely a soluble carrier protein for RNA but rather is bound directly to intranuclear proteins, possibly including the nuclear pore complex (Boyle *et al.*, 1999). However, in another report Mta was shown to efficiently promote

the nuclear export of unspliced RNA via a CRM1 independent pathway (Farjot *et al.*, 2000). Site-directed mutagenesis and domain swapping experiments indicated that the region, which matches the consensus sequence for the leucine-rich NES, is not an NES per se. Mta nucleocytoplasmic shuttling, unlike Rev shuttling, was not affected by LMB. Overexpression of an N-terminal deletion mutant of Nup214 (also known as Δcan) blocked both Rev- and Mta-dependent nuclear export of RNA, indicating that this major cell nucleoporin is involved in the nuclear export by both proteins (Farjot *et al.*, 2000).

1B2.4 VZV ORF4/IE4

HSV-1 ICP27 and the VZV ORF 4 IE gene product (Defechereux *et al.*, 1997) are homologues (Moriuchi *et al.*, 1994). Although ORF4 did not complement an ICP27-defective HSV-1 mutant (Moriuchi *et al.*, 1994), and is predominantly cytoplasmic, as opposed to KSHV ORF57 or ICP27, the N-terminal region of ORF4 can efficiently replace that of ICP27 (Moriuchi *et al.*, 1995). Unlike ICP27, ORF4 does not exhibit any *trans*-repressing activities but is capable of stimulating gene expression either alone or in synergy with the major VZV regulatory protein ORF62/IE7 (Inchauspe *et al.*, 1989; Perera *et al.*, 1992; Defechereux *et al.*, 1993). VZV ORF4 does not bear an RGG box but instead has three arginine-rich regions and three potential bZIP sequences located near an arginine-rich region. No potential NES has been located within the ORF4 N-terminal region.

ORF4 self interaction mainly occurred through the central and the C-terminal regions of the protein. Regions important for dimerisation were also shown to be necessary for transactivation. ORF4 was also shown to possess nucleocytoplasmic shuttling ability, partly via a CRM1-dependent mechanism and both the central and C-terminal regions were involved in its nuclear export (Baudoux *et al.*, 2000).

ORF4 is reported to be a transcriptional activator that requires the presence of an upstream element within the promoter to mediate transcription (Perera *et al.*, 1994). Analysis of ORF4-mediated transactivation in transient transfection assays suggests that it is a multifunctional protein which can stimulate heterologous and autologus gene expression through more than one mechanism, one of which involves post-transcriptional regulation (Defechereux *et al.*, 1997). Unlike ICP27 (Yao & Courtney, 1992), ORF4 is a component of the VZV virion (Moriuchi *et al.*, 1994).

ORF4 can act as a major coactivator of transactivation mediated through ORF62, thus ORF4 and ORF62 activate gene expression synergistically (Inchauspe *et al.*, 1989) and its intracellular localization is affected by ORF62 (Defechereux *et al.*, 1996). As the ORF4 and ORF62 proteins are both present in the VZV virion tegument, this suggests

that their action occurs at the earliest stages of VZV replication (Kinchington *et al.*, 1995). ORF4 and ORF62 form stable complexes in solutions under stringent salt conditions. A direct physical interaction was shown between ORF4 and a less phosphorylated form of ORF62 which appears important for virion assembly as well as regulation of gene expression in VZV-infected cells (Spengler *et al.*, 2000).

1B2.5 BHV-1 BICP27

The BHV-1 homologue of KSHV ORF57, BICP27, is expressed with early kinetics and accumulates in nuclei. Transient expression assays using target genes differing only in their poly (A) sites showed that BICP27, like ICP27, might be involved in increasing the processing efficiency of mRNA containing weak poly (A) sites (Singh *et al.*, 1996).

1B2.6 HCMV UL69

The KSHV ORF57 homolog of the β-herpesvirus HCMV is the multifunctional nuclear phosphoprotein UL69 (Winkler *et al.*, 1994). Like VZV ORF4, UL69 of HCMV is located in the virion (Winkler & Stamminger, 1996). In contrast to HSV-1 ICP27, the UL69 gene product is expressed with early-late kinetics; it can function as a transactivator of several viral and cellular promoters (Winkler *et al.*, 1994).

UL69 interacts with the cellular factor hSPT6, which has been implicated in the regulation of chromatin structure and transcriptional elongation (Winkler *et al.*, 2000). UL69 is also involved in cell cycle control, since it induces infected or transfected cells to accumulate in the G1 phase (Lu & Shenk, 1999; Hayashi *et al.*, 2000). Despite the sequence homology between UL69 and ICP27, there is evidence for major functional differences between the two proteins as UL69 does not exert any measurable negative regulation that is dependent on the presence of introns (Winkler *et al.*, 1994).

Recently, UL69 has been shown to be able to shuttle between the nucleus and the cytoplasm and this shuttling activity was not affected by LMB, indicating that the trafficking does not involve binding to CRM1. A short, transferable NES, comprising 28 amino acids, which is distinct from that of HIV-1 Rev and other shuttling motifs was identified and suggests that the activity of UL69 is linked to a novel, so far uncharacterized nuclear export pathway (Lischka *et al.*, 2001).

1B3 Nuclear export of mRNAs to the cytoplasm

To exit the nucleus, mature mRNA must pass through the nuclear envelope, through protein lined channels called nuclear pore complexes (NPCs). RNA within cells is associated with proteins as ribonucleoprotein particles (RNPs), and it is these RNP

complexes that are transported. Once exported, the transport proteins need to be re-imported into the nucleus to pick up a new load. The signals within proteins that cause nuclear localisation are NLSs and those that allow export of proteins are NESs. The classical NLS, first identified in simian virus 40 (Kalderon *et al.*, 1984), consists of a short stretch of basic amino acids and is found in a large number of viral and cellular nuclear proteins. Two other classes of NLS are known, the M9 and KNS domains and these two sequences also function as NESs; the M9 NLS is unique in being RNA pol II transcription-dependent (Pinol-Roma & Dreyfuss, 1991). Three different classes of NES are known, the Rev-like NES (Bogerd *et al.*, 1996), the M9 domain (Siomi & Dreyfuss, 1995) and the bi-directional KNS domain (Michael *et al.*, 1997). The model for transport of proteins containing these signals requires that signal-containing proteins bind to receptors that then translocate through the NPC, as illustrated in Fig.1B3 Panel I (reviewed in Izaurralde & Adam (1998)). Most transport processes are thought to require the small GTPase Ran. Various classes of RNAs use different types of these signals and, as far as is known, they do not compete with each other, and utilise separate pathways to receptors in the nuclear membrane for exit from the nucleus (Fig 1B3 Panel II).

1B3.1 Nuclear export signals

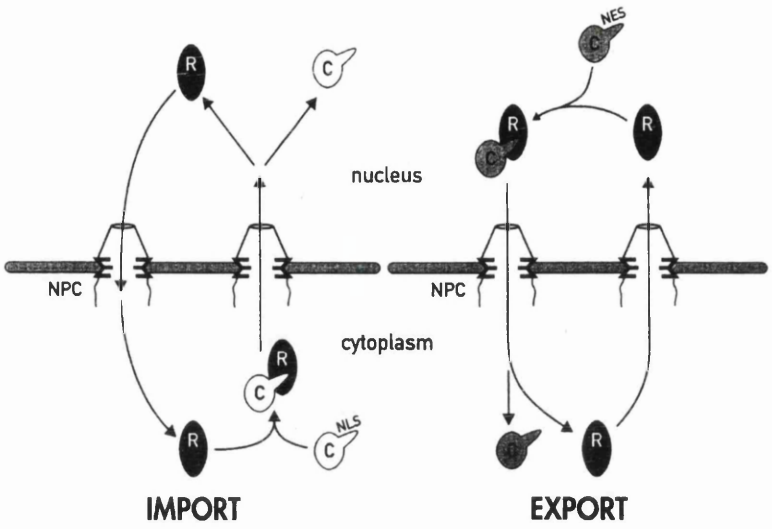
1B3.1.1 Rev-like NES

This leucine-rich sequence is found not only in HIV-1 Rev, a protein which promotes the export of partially and unspliced viral RNAs (see Section 1B4.1), but also in several cellular proteins including TFIII A which is involved in 5S RNA export (Fischer *et al.*, 1995; Fridell *et al.*, 1996). The export receptor for the Rev-like NES has been identified as CRM1 (Fornerod *et al.*, 1997a). Consistent with being a receptor, CRM1 is a Ran-binding protein, and interacts with nucleoporins Nup214 and Nup88 (Ossareh-Nazari *et al.*, 1997; Fornerod *et al.*, 1997a; Fornerod *et al.*, 1997b). The cytotoxin LMB inhibits Rev-NES mediated transport (Wolff *et al.*, 1997). In higher eukaryotes, mRNA transport is not inhibited by LMB (Fornerod *et al.*, 1997a), indicating that cellular mRNAs are not exported via this pathway.

1B3.1.2 M9 domain

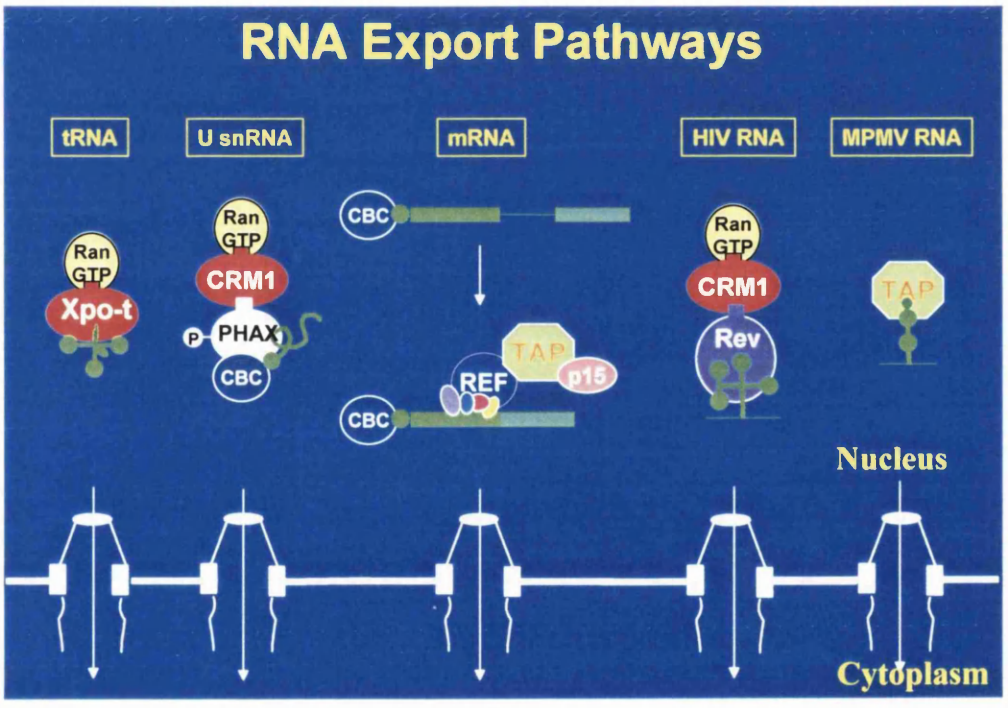
This sequence consists of a glycine-rich sequence of 38 amino acids and is required for the export and import of hnRNP A1 (Michael *et al.*, 1995). As mRNAs are coated with numerous hnRNP molecules and because hnRNP A1 remains bound to the mRNA during translocation, it has been suggested that hnRNP A1 promotes export of mRNA via the

Fig1B3 Panel I. Shuttling receptor model



Nuclear export and import are mediated by shuttling receptors (R), which recognise and bind to nuclear localisation sequences (NLS) or export sequences (NES). An import receptor binds its cargo (C) in the cytoplasm, translocates though the nuclear pore complex (NPC), and releases the cargo into the nucleoplasm. The receptor is then recycled back to the cytoplasm to initiate another round of import. Conversely, an export receptor binds its cargo in the nucleoplasm and releases it in the cytoplasm.
From Izaurralde & Adam (1998).

Fig 1A3 Panel II. Various RNA export pathways



Showing different types of RNAs utilising various receptor-export adaptor molecules such as exportin, PHAX-CRM-1, REF/TAP, Rev-CRM-1 and CTE-TAP, for nuclear export; MPMV: Mason Pfizer monkey virus (Stutz & Rosbash, 1998; Cullen, 2000; Conti & Izaurralde, 2001).

M9 signal (Michael *et al.*, 1995). The export receptor for the M9 domain is not known. hnRNP A1 re-import into the nucleus is transcription-dependent; this is consistent with hnRNP A1 exporting mRNA as, in the absence of transcription, energy would not be wasted re-importing export factors (Michael *et al.*, 1995). Export of some mRNAs is not inhibited by an excess of hnRNP A1 (Saavedra *et al.*, 1997), implying that mRNA export may not occur by a single mechanism. Export of individual mRNA could depend on the pattern of associated hnRNP proteins and the type of export signals they carry.

1B3.1.3 KNS domain

Identified in hnRNP K (Michael *et al.*, 1997). It is not known which, if any, mRNA utilises the KNS for export (see Section 1C1 on hnRNP K)

1B3.1.4 HNS domain

In one of the minor hnRNPs HUR, a nuclear-cytoplasmic shuttling sequence was identified (Fan & Steitz, 1998).

1B3.2 Nuclear retention and export

Binding of export factors is probably the first step in the commitment of a mRNA to a transport pathway, however it is not sufficient for export. For example, unspliced mRNAs associated with hnRNP A1 are not exported (Legrain & Rosbash, 1989). This observation lead to a suggestion that the removal of other factors that cause nuclear retention is also required. Evidence for such factors came from the identification of a dominant nuclear retention signal present in hnRNP C (Nakielny & Dreyfuss, 1996).

1B4 RNA processing/export in other viral systems

Different classes of RNA are transported through separate pathways, and export of each of these classes is saturable, indicating the involvement of specific limiting factors (Jarmolowski *et al.*, 1994; Fischer *et al.*, 1995; Pasquinelli *et al.*, 1997; Saavedra *et al.*, 1997). Full details of the mRNA export pathway and how different viruses subvert the cellular export machinery for their own RNAs, have not yet been elucidated. An intriguing question regarding the functioning of virus-encoded nucleocytoplasmic shuttle proteins is the mechanism(s) they utilize for transport through the nuclear pore complex. Soluble transport receptors mediate macromolecular trafficking into and out of the nucleus (reviewed in Jarmolowski *et al.* (1994); Mattaj & Englmeier (1998); Gorlich & Kutay (1999)). These receptors bind specific proteins or RNA cargoes and interact with nuclear pore proteins, which eventually allow the translocation of the receptor through

NPC. *1B4.1 HIV-1*

In HIV and other complex retroviruses, the export of intron-containing RNAs is mediated through the action of specific elements in these RNAs in conjunction with virus-encoded proteins that bind directly to these sequences (reviewed in Pollard & Malim (1998)). CRM1 interacts with nucleoporins and is the bridging protein for the interactions of NES-containing proteins and the NPC. CRM1 mediates the nuclear export of a variety of protein and RNA substrates, including incompletely spliced HIV-1 mRNAs via the HIV-1 Rev export factor and cellular U-rich small nuclear RNAs (UsnRNAs), but is dispensable for export of tRNA and most cellular mRNAs (Fornerod *et al.*, 1997a).

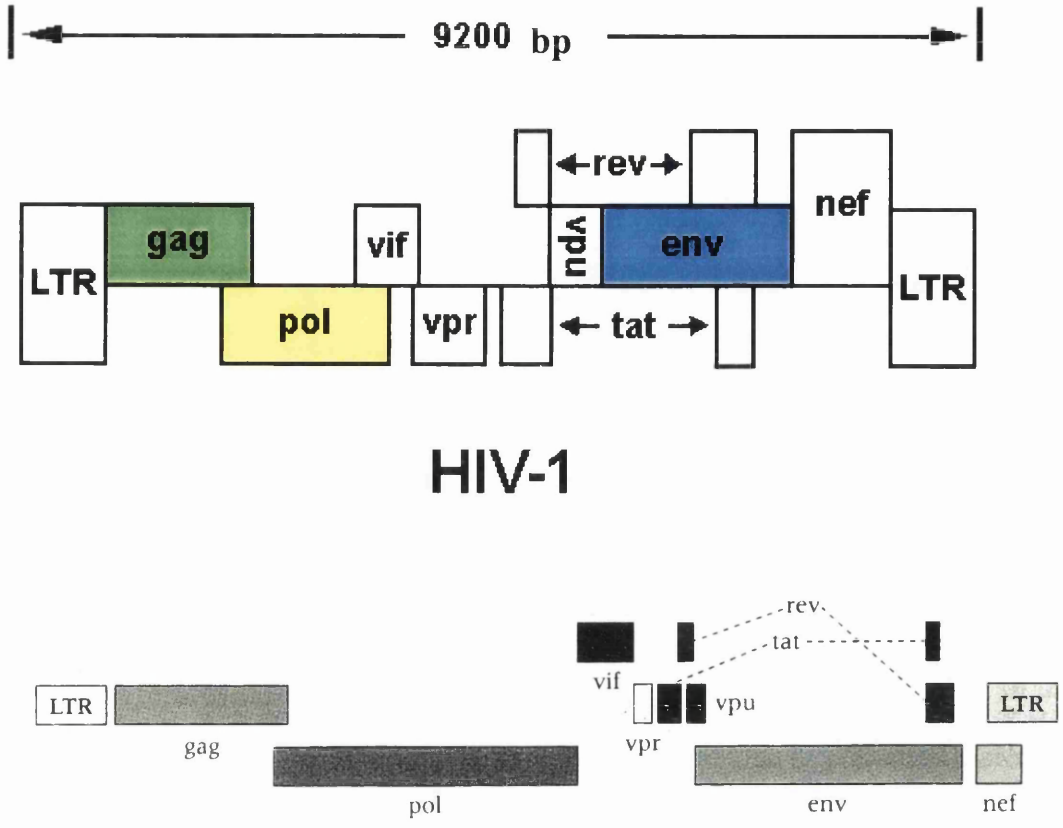
1B4.1.1 HIV-1-Rev and RNA export

HIV-1 encodes a range of mRNA species derived by partial or complete splicing of a single primary transcript (Fig. 1B4.1.1 Panel I). If a cellular mRNA contains an intron, there is interaction with a subset of splicing factors which commits the transcript to being spliced and exported or, if the signal is redundant, to degradation. This presents a problem for HIV-1 gene regulation as for production of some proteins splice sites must be recognised by the splicing machinery and for others the splicing machinery must be by-passed, while RNA export must still occur (reviewed by Cullen, (1998)). This problem is solved by action of the HIV-1 Rev protein. Produced early in infection from a transcript which is fully spliced, it facilitates expression of late viral proteins produced from a primary transcript which is partially- or un-spliced. Domains of HIV-1 Rev are shown in Fig. 1B4.1.1 Panel II.

1B4.1.2 HIV-1 Rev and nuclear export of viral transcripts

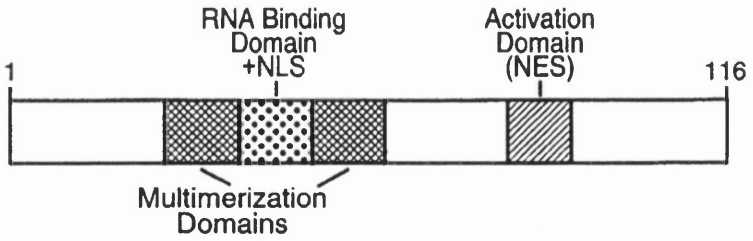
HIV-1 Rev acts to transport incompletely and unspliced HIV-1 RNAs that contain the *cis* acting Rev response element (RRE) (Malim *et al.*, 1989b). This activity requires Rev to shuttle between the nucleus and the cytoplasm, exporting viral transcripts and then being reimported. Rev export is mediated by a leucine-rich NES (Fig. 1B4.1.1 Panel II) (Wen *et al.*, 1995; Fischer *et al.*, 1995) that requires the cellular co-factor Rab (Bogerd *et al.*, 1995). The Rev-Rab-RNA complex then binds to the export receptor CRM1 and exits the nucleus. Once in the cytoplasm, the complex dissociates, presumably exposing the NLS, and Rev is reimported into the nucleus. In this way HIV-1 transcripts bound to Rev are exported from the nucleus. This shuttling process requires the GTP bound form of Ran (Richards *et al.*, 1997). The model is consistent with the shuttling receptor model in Fig. 1B3 Panel I.

Fig 1B4.1.1 Panel I. Genomic organisation of HIV-1



The genome is flanked by terminal repeats (LTR). All mRNAs are derived from one precursor RNA, dotted lines join exonic sequences of genes Rev and Tat, expressed after complete splicing of the precursor RNA. Nef is also expressed after complete splicing, Env, Vif, Vpr and Vpu are expressed from mRNAs derived by partial splicing of the precursor RNA, while Gag and Pol are expressed from unspliced precursor RNA.

Fig 1B4.1.1 Panel II. Functional domains of HIV-1 Rev



RNA binding domain and NLS (aa 34-50); activation domain and leu-rich NES (aa 75-84); multimerisation domain (aa 12-60). From Cullen (1998).

1B4.1.3 HIV-1 Rev and splicing

HIV-1 Rev has a second functional domain (aa 34-50) which binds RNA, and contributes to its ability to oligomerise; this domain inhibits splicing of RRE-containing transcripts *in vitro* (Kjems *et al.*, 1991; Kjems & Sharp, 1993). Exogenously added p32 protein is seen to specifically relieve this inhibition of splicing *in vitro*, and a model (Fig. 1B4.1.3 Panel I) of Rev bound to RRE interacting with p32, which is associated to ASF at the 5' splice site, therefore stabilising the interaction of U1 snRNP with the 5' splice site and inhibiting assembly of functional spliceosomes, has been proposed (Tange *et al.*, 1996). As the Rev-ASF -p32 interaction has also been implicated in mRNA export via the Rev NES (Gilmartin *et al.*, 1992; Yu *et al.*, 1995b; Tange *et al.*, 1996), it is unclear whether inhibition of splicing is a direct or an indirect effect.

Expression of the SR protein SC35 is also upregulated during HIV-1 infection (Maldarelli *et al.*, 1998). *In vitro* and *in vivo* alteration of the SC35 amount can influence the selection of alternative splice sites (Fu & Maniatis, 1992; Wang & Manley, 1995). As HIV-1 transcripts contain multiple 5' and 3' splice sites, some of which are cryptic, utilisation of alternative splicing sites would influence the relative abundance of viral transcripts. It is not known if increase in SC35 alters the pattern of splicing in HIV-1 but it seems a likely scenario. In the spliceosome, normally SC35/ASF and p32 are associated and Rev by recruiting SC35 could influence the selection of viral splice sites.

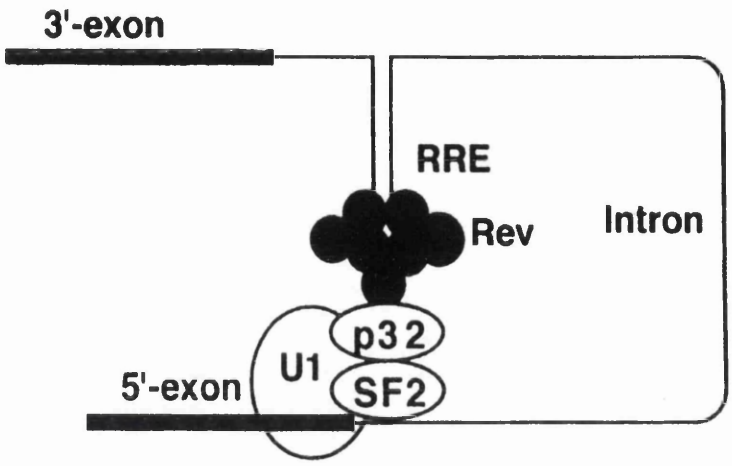
A summary of effects of HIV-Rev on splicing and mRNA export is shown in Fig. 1B4.1.3 Panel II.

1B4.2 Adenoviruses

Although transcriptional control is important in adenovirus infection, the virus uses only nine viral promoters and post-transcriptional control mechanisms are needed to produce the much higher number of viral proteins. Adenovirus infection produces changes in the activity of cellular splicing and polyadenylation factors and as infection proceeds the pattern of processing alters, towards the production of smaller mRNAs (reviewed in Leppard (1997)). Two viral proteins, ORF6 and 55K, are essential for the transport of viral late mRNAs, in a way substantially similar to HIV-1 Rev (Williams & Leppard, 1996). The same proteins are required to prevent commitment of host cell mRNA to the export pathway, but whether the two events are linked is not known.

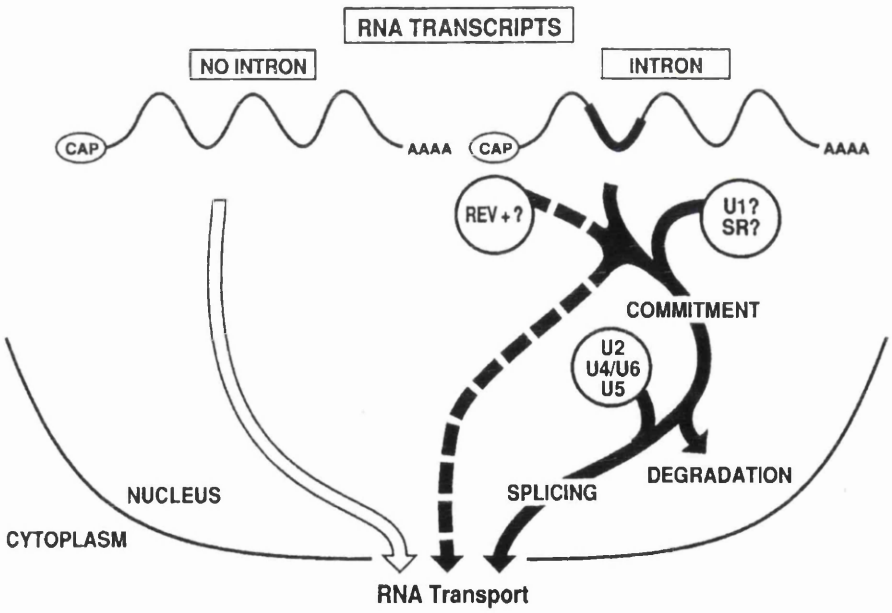
Of particular interest is the redistribution of snRNPs (Bridge *et al.*, 1993). In contrast to HSV-1 transcripts that are not spliced, adenovirus transcripts are extensively spliced. At early times, splicing factors have a general distribution but at later times

Fig 1B4.1.3 Panel I. Putative model for the p32-Rev interaction



Rev protein bound to RRE interacts with p32 protein associated with ASF/SF2 at the 5' splice site. This interaction could stabilise the interaction of the U1 snRNP with the 5' splice site and inhibit assembly of functional spliceosomes. The arrested complexes may subsequently function as a substrate for Rev-mediated nuclear export. From Tange *et al.* (1996).

Fig 1B4.1.3 Panel II. HIV-1 Rev action



While fully spliced RNAs are readily exported from the nucleus, RNAs containing introns are retained in the nucleus by commitment factors, such as the U1 snRNP or SR proteins, until fully spliced or degraded. Rev induces the nuclear export of Rev response element containing viral RNAs and thereby either prevents or reverses nuclear retention. From Cullen (1998).

(when there is increased splicing) they are recruited into virus transcription and processing sites. Following this, splicing factors form large foci, similar to those observed in HSV-1 infected cells, but corresponding to an increase in, rather than a block in, splicing. These foci contain late viral mRNA enriched in exon sequences, i.e., which may already be spliced. Although their role is not clear, these foci could reflect host splicing machinery/transport pathways being saturated with viral transcripts and so "backing up" in the nucleus or may reflect increased recycling of splicing factors or they may be in some way related to the block on nuclear export of host RNA. The redistribution of snRNPs with viral late transcripts may act to disrupt host mRNA export. A yet unknown viral protein may drive nuclear reorganisation or reorganisation may be secondary to disruption of another RNA processing event.

1B4.3 Influenza

The genome of influenza A virus consists of eight single-stranded RNAs of negative sense, present as ribonucleoproteins. Each segment is transcribed as an individual unit in the nucleus of infected cells and a significant amount of viral gene regulation occurs post-transcriptionally.

1B4.3.1 NS1 in splicing

NS1 protein both inhibits cellular mRNA splicing (Qiu *et al.*, 1995) and alters the usage of 5' splice sites in alternatively spliced transcripts (Fortes *et al.*, 1994). Interaction of NS1 with U6 snRNA (Qiu *et al.*, 1995) may inhibit splicing by preventing base pairing between U6, U2 and U4 snRNAs, and 5' site selection may be regulated by an alteration of activity of soluble splicing factors such as ASF, perhaps by phosphorylation. In line with these observations, NS1 protein expression alters the nuclear localisation of splicing factors (Fortes *et al.*, 1995), reorganising them in a similar way to that in HSV infected cells. The reorganised snRNPs in influenza-infected cells are thought to represent dysfunctional aggregates of splicing factors. Given the similarities of reorganisation in HSV-1 and adenovirus, it is not unreasonable to suggest that NS1 interacts with cellular proteins to disrupt/modulate splicing.

1B4.3.2 NS1 in polyadenylation and mRNA export

NS1 is required for the nuclear retention of cellular poly $(A)^+$ mRNAs, where they are degraded (Katze & Krug, 1984) allowing viral polymerase access to cap snatching substrates (Katze & Krug, 1984). NS1 binds to poly (A) (Qiu & Krug, 1994), to the cleavage/polyadenylation specificity factor -30 subunit (Nemeroff *et al.*, 1998) and to

poly A binding protein II (Chen *et al.*, 1999), inhibiting both polyadenylation and cleavage of the 3' end of mRNA; the combined approaches cause nuclear retention of cellular pre mRNA. As NS1 has been shown to be transdominant over the Rev-NES (Chen *et al.*, 1998b), this points to a mechanism involving specific nuclear retention rather than disruption of export. As splicing, polyadenylation and transport are linked, redistribution of splicing factors may also be the cause of nuclear retention or, alternatively, redistribution may be secondary to inhibition of transport.

1B4.3.3 NS2 mediates nuclear export of viral mRNAs

NS2 protein can substitute for HIV-1-Rev and mediate nuclear export of a heterologous protein crosslinked to it, and in addition injection of antibodies against NS2 prevents viral mRNA reaching the cytoplasm (O'Neill *et al.*, 1998). NS2 can also interact with nucleoporins (O'Neill *et al.*, 1998) acting like HIV-1 Rev as a mediator of viral mRNA export. Transport of viral mRNAs can be blocked by methylation or phosphorylation inhibitors (Vogel *et al.*, 1994), suggesting that these processes also are important.

1B4.4 Summary of viral post-transcriptional gene regulation

Examination of post-transcriptional regulation in the viral systems discussed shows that ICP27 and its homologs are not unusual in their effects and comparisons reveal common themes in viral disruption of RNA processing, namely:

(i) Reorganisation of cellular snRNPs, directly due to modulation of splicing or as secondary to alterations in the activity of cellular splicing factors.

(ii) Use of an alternative pathway(s) for mRNA export, often via a viral protein, concurrent with disruption of cellular mRNA export.

(iii) Viral induced changes in phosphorylation play a role in changes of function/location of cellular processing factors

(iv) Effects on different RNA processing events are often linked.

(v) Control is often exerted at several points in processing by a multifunctional viral protein.

1B5 Multifunctional proteins

There are multifunctional proteins that are involved in transcriptional and post-transcriptional processes and provide further evidence for links between them. The list of these multifunctional proteins is growing (reviewed in Ladomery (1997)). Some well characterised examples include Y box proteins, Wilm's tumour protein (WT1), TFIIA, La protein and hnRNP K (see also Section 1C1).

1B5.1 Y box proteins

All Y box proteins contain a cold shock domain thought to switch on genes required for the cold shock response and to act as an RNA chaperone, favouring translation at low temperature. Y box proteins have a number of other domains, and are implicated in the upregulation of cell proliferation genes, repression of MHC class II genes and in binding to and masking of cytoplasmic mRNA in *Xenopus* oocytes; this masking prevents cytoplasmic translation before the correct time in development. A similar phenomenon occurs in the developing haploid spermatid, and Y box protein can be detected binding to mRNA in mouse spermatids. Finally, Y box proteins bind to somatic mRNP and may therefore have a general role in mRNA packaging. YB-1 protein has been shown to modulate transcription through binding single-stranded polypyrimidine-rich DNA sequences and by interacting with transcription factors (Denisenko *et al.*, 1996; Michelotti *et al.*, 1996; Du *et al.*, 1998). YB-1 is known to be involved in repressing and activating transcription (Du *et al.*, 1998; Swamynathan *et al.*, 1998) and is thought to activate transcription of human polyomavirus JC by recruiting another trans-activator (p65) to the viral promoter (Raj *et al.*, 1996). YB-1 can act on a single gene, the matrix metalloproteinase 2 gene, in a positive or negative manner that is dependent upon cellular context (Mertens *et al.*, 1997). Direct interaction between YB-1 and hnRNP K has been reported (Shnyreva *et al.*, 2000).

1B5.2 The Wilm's tumour gene product (WT1)

WT1, first identified as a tumour suppressor, produces a protein, which can exist as sixteen known isoforms. It is a transcriptional activator and binds to DNA. The mouse cell line M15 contains at least two WT1 isoforms, with or without three amino acids lysine, threonine and serine (KTS). The KTS^+ form is found preferentially in nuclear speckles and colocalises with snRNPs, suggesting a role in splicing, while the KTS^- isoform preferentially colocalises with the transcription factors SP1 and TFIIB. Thus, WT1 may exert effects at the transcriptional and post-transcriptional levels via these isoforms (Call *et al.*, 1990; Gessler *et al.*, 1990; Ellisen, 2002; Algar, 2002).

1B6 Cross-talk between mRNA processing events

The co-localisation of various gene expression machineries suggests that there is some level of cross talk between them. The splicing machinery has been shown to be associated with the RNA polymerase II carboxy terminal domain (CTD) (Steinmetz, 1997). This role for CTD may ensure that the nascent transcript is presented directly to the pre-mRNA processing machinery even as its synthesis continues and allows co-ordination of

transcription and RNA processing. The mRNA processing reactions of capping, splicing, and polyadenylation are now believed to occur co-transcriptionally. They not only influence one another's efficiency and specificity, but are also coordinated by transcription. Phosphorylated CTD of RNA polymerase II provides molecular contacts to couple mRNA processing reactions with transcriptional elongation and termination (Proudfoot *et al.*, 2002).

Another example of cross talk is that splicing can be linked to polyadenylation. *In vitro,* a functional polyadenylation signal can enhance splicing of the 5' terminal intron and *vice versa*, implying that excision of most 3' terminal intron of pre-mRNA and polyadenylation are functionally linked (Niwa *et al.*, 1990). A recent report suggests that a transcription/export complex containing components from transcription and export machineries is specifically recruited to activated genes during transcription and travels the entire length of the gene with RNA polymerase II thus coupling transcription to mRNA export (Strasser *et al.*, 2002).

Thus, interactions between transcriptional/posttranscriptional regulators, components of the splicing machinery, splicing factors, phosphorylating kinases and export components likely reflect the close coordination and mechanistic coupling of the events required to produce, export and possibly translate functional mRNAs (Cole, 2001).

Chapter 1 Part C. ORF57 partner proteins

This study identifies cellular proteins that interact with ORF57, all of which have proposed roles in RNA transcription/processing. The KSHV ORF50 protein is also found to interact in a complex with ORF57. Characteristics of the cellular proteins are summarised below.

1C1 HnRNP K

HnRNP K protein has an apparent molecular weight of 65 kDa and was first discovered as a component of the hnRNP particle (Matunis *et al.*, 1992) although a large fraction of hnRNP K is not associated. It can be found as at least five alternatively spliced isoforms (Dejgaard *et al.*, 1994), the relevance of which is unclear. A function for hnRNP K in the hnRNP particle has yet to be defined. It has a wide intracellular distribution and can be easily obtained from cytoplasmic and nuclear extracts (Dejgaard *et al.*, 1994; Ostrowski *et al.*, 1994). HnRNP K is a multifunctional protein that has multiple domains (Fig. 1C1 Panel I).

HnRNP K interacts with a large repertoire of mRNAs (Ostrowski *et al.*, 2000; Ostrowski *et al.*, 2001) with a preference for poly (rC) RNA (Matunis *et al.*, 1992). The binding of RNA and DNA is mediated via one or more of hnRNP K homology (KH) domains (Siomi *et al.*, 1994), which has three copies of the conserved 65-70 aa motif. Phosphorylation of hnRNP K decreases its binding to RNA (Dejgaard *et al.*, 1994). Immunoprecipitated hnRNPK protein-mRNA complexes were disrupted by tyrosine phosphorylation, suggesting that the in vivo binding of K protein to mRNA may be responsive to the extracellular signals that activate tyrosine kinase (Ostrowski *et al.*, 2000).

HnRNP K contains a novel 38 aa long KNS shuttling motif, which mediates bi-directional nucleocytoplasmic transport; hnRNP K shuttling, and unlike that of hnRNP A1 which is mediated by the M9 motif, is transcription-independent. However, hnRNP K also contains a classical NLS which when deleted renders hnRNP K dependent on RNA polymerase II transcription for nuclear localisation. Thus, hnRNP K can access a transcription-dependent pathway, raising the possibility that hnRNP K shuttles with mRNA and the tie to transcription would prevent wasteful reimport of export factors. HnRNP K has not been shown to utilise any of the known nuclear import receptors (Michael *et al.*, 1997).

HnRNP K also binds to DNA which is the preferred ligand over RNA *in vitro*

(Ostrowski *et al.*, 1994; Tomonaga & Levens, 1995; Michelotti *et al.*, 1996). This binding is sequence specific to G+C rich sequences and the protein has been suggested to be a transcription factor. For example, hnRNP K binds to the (CCCC/GGGG) tract present in the CT motif found in *c-myc* promoter P1 and transactivates transcription from this promoter *in vitro* (Takimoto *et al.*, 1993). The CT element consists of four imperfect direct repeats of the sequence CCCTCCCCA, and a fifth repeat downstream of the first four, which acts as a *cis* enhancing element for c-*myc in vivo* (DesJardins & Hay, 1993). HnRNP K can interact with TBP, and when hnRNP K and TBP are over-expressed *in vivo,* transcription from a CT element dependent reporter is synergistically activated (Michelotti *et al.*, 1996). Chemical and enzymatic probes selective for single-stranded DNA indicate that the CT element of the c-*myc* promoter adopts an under-wound and flexible conformation in the presence of hnRNP K (Tomonaga *et al.*, 1998), which may allow bending of DNA to bring together *cis* elements facilitating transcription (Fig. 1C1 Panel II).

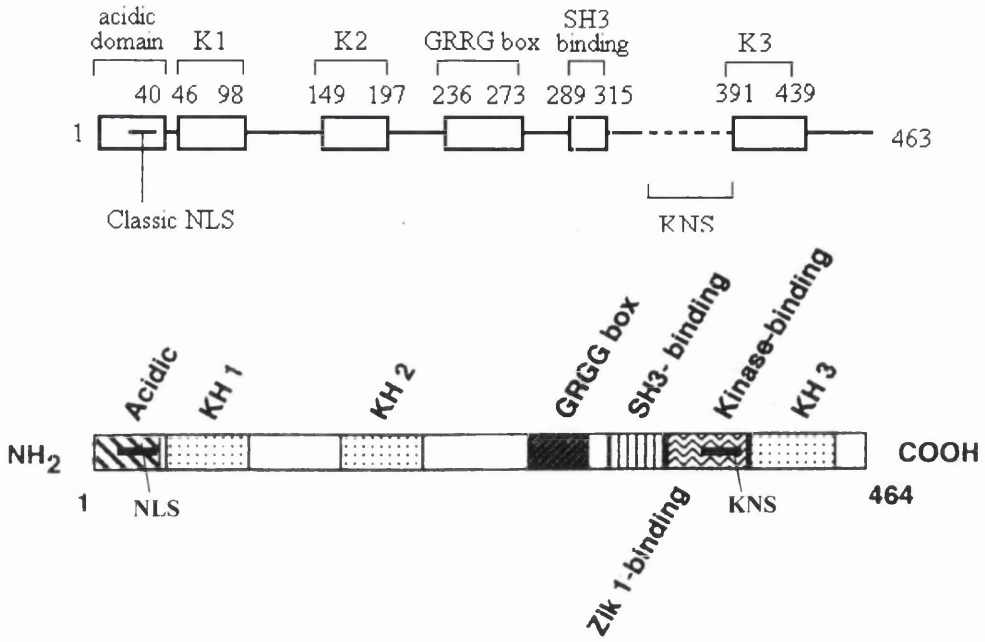
Using chimeric constructs and a variety of different promoters, hnRNP K can regulate a number of genes (Lee *et al.*, 1996). For example, when the adenovirus E2 promoter is used, hnRNP K lowered rather than activated transcription, reminiscent of Y-box proteins. As well as interacting with TBP, hnRNP K interacts with the transcriptional repressor Zik1 and this is blocked by binding of poly (A) RNA (Denisenko *et al.*, 1996), raising the possibility that RNA binding can regulate transcription.

HnRNP K has been shown to interact directly with YB-1. Both proteins separately were able to transactivate transcription from a polypyrimidine rich-promoter; however this effect was reduced when hnRNP K and YB-1 were co-expressed suggesting a functional interaction between them (Shnyreva *et al.*, 2000).

Interaction of hnRNP K with both nucleic acid and protein partners is regulated by extracellular signal-induced phophorylation cascades (Schullery *et al.*, 1999; Ostrowski *et al.*, 2000); hnRNP K is phosphorylated by and interacts with hnRNP K protein kinase (KPK). Phosphorylation of hnRNP K by KPK is DNA- or RNA-dependent and interleukin 1 responsive. KPK activity towards hnRNP K is activated by phosphorylation (Van Seuningen *et al.*, 1995a). HnRNP K can also interact with signalling molecules such as c-*src* (Weng *et al.*, 1994; Van Seuningen *et al.*, 1995a) and *vav* (Bustelo *et al.*, 1995) via SH3 domains. Binding of KPK and c-src may be able to occur concurrently as the regions involved in binding lie adjacent to each other. KPK can be phosphorylated by c-src *in vitro* (Van Seuningen *et al.*, 1995a).

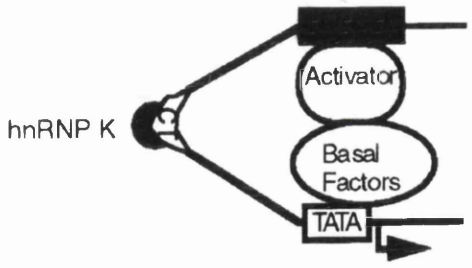
The hnRNP K protein was shown to be phosphorylated *in vitro* by protein kinase Cδ (PKCδ) at Ser^{302} and by other PKCs. Mutations of Ser^{302} decreased the level of

Fig 1C1 Panel I. Domains of hnRNP K



NLS: nuclear localisation signal (aa 21-37), KH1-KH3: KH -RNA binding domains (aa 46-98, 149-197, and 391-439), GRRG box: classic RNA binding domain (aa 236-273), KNS: novel bi-directional nuclear export signal (aa 323-390), SH3: proline-rich interaction site for proteins containing an SH3 domain (aa 289-315). From Bomsztyk *et al.* (1997).

Fig 1C1 Panel II. Possible mechanism of action of hnRNP K



HnRNP K binds to the CT element of c-*myc*, may cause bending of DNA, bringing together activator proteins and basal transcription factors, which are bound to spatially separate promoter and enhancer/repressor regions. From Tomonaga *et al.* (1998).

phosphorylation of exogenously expressed K protein in TPA treated COS cells, suggesting that Ser^{302} is a site for PKC-mediated phosphorylation *in vivo*. The ability of PKCδ to bind and phosphorylate K protein may not only serve to alter the activity of K protein itself, but K protein may also bridge PKCδ to other K protein molecular partners and thus facilitate molecular cross-talk (Schullery *et al.*, 1999).

The hnRNP K protein also is tyrosine-phosphorylated *in vitro* by Src and Lck. Treatment with oxidative stress inducers, H_2O_2/Na_3VO_4, stimulated K protein tyrosine phosphorylation in cultured cells and in intact livers. Tyrosine phosphorylation increased K binding of Lck and the proto-oncoprotein Vav *in vitro* and oxidative stress increased the association of K protein with Lck and Vav, suggesting that tyrosine phosphorylation regulates K protein-protein interactions and the recruitment of these effectors *in vivo* (Ostrowski *et al.*, 2000).

HnRNP K has been implicated in regulation of translation (Bomsztyk *et al.*, 1997). It interacts with the elongation factor-1a and, together with hnRNP E1 (PCBP-1 or αCP1), interacts with and causes translational silencing of erythroid 15-lipoxygenase (LOX) mRNA both *in vitro* and *in vivo* (Ostareck *et al.*, 1997). *In vitro* data suggests this is achieved by specific inhibition of 80S ribosome assembly on LOX mRNA and it inhibits both cap-mediated and internal ribosomal entry site-mediated translation.

HnRNP K protein bound RNA sequences encoded by different loci spanning nearly the entire mitochondrial genome and was associated with several processed mitochondrial transcripts, providing evidence that K protein acts within functional modules responsible for expression of genes in mitochondria (Ostrowski *et al.*, 2002).

The pleiotropic cellular effects of insulin action are mediated by multiple-signalling pathways that respond to the binding of insulin to its cell-surface receptor (Saltiel, 1996). But the mechanisms responsible for the diversity of the insulin-induced effects on gene expression are less well understood. Treatment of hepatocyte cultures with insulin increased K protein tyrosine phosphorylation, altered K protein interaction with RNA and DNA *in vitro* and revealed preferential *in vivo* K protein binding of a subset of transcripts, including the insulin-inducible c-fos mRNA (Ostrowski *et al.*, 2001). Habelhah *et al.* (2001) established that mitogen-activated protein kinase/ extracellular-signal-regulated kinase (MAPK) causes phosphorylation-dependent cellular localization of hnRNP K, required for its ability to silence mRNA translation. A model for hnRNP K protein function is emerging where K protein could serve to link signalling cascades to nucleic acid-directed process within multiple functional modules responsible for expression of many genes (Ostrowski *et al.*, 2001; Habelhah *et al.*, 2001). This

general model for K protein action is likely to be shared by signal transduction-responsive nucleic acid-binding factors such as YB-1 (Shnyreva *et al.*, 2000; Chen *et al.*, 2000a).

It has been suggested that hnRNP K provides a molecular docking platform for proteins to interact. If this were true, formation of hnRNP K multimers would provide the capacity to interact with transcriptional activators and repressors, KPK, signalling molecules and possibly mRNA processing factors simultaneously in a way that is regulated by nucleic acid. In turn, interaction with different kinases would be expected to alter phosphorylation of hnRNP K and its partner proteins. This arrangement would then provide a cross talk mechanism between transcription, signalling, and mRNA processing.

1C1.1 Regulation of gene expression by hnRNP K in viruses

Dengue (DEN) virus, a member of the Flaviviridae, is the primary cause of illness such as dengue fever, dengue hemorrhagic fever, and dengue shock syndrome. In mature DEN virus particles, the core structural protein forms a nucleocapsid complex with genomic RNA. Recently hnRNP K was shown to interact with the C-terminal hydrophilic region of the core protein (aa 73-100). Binding assays indicated that the DEN core protein-hnRNP K interaction was abolished in presence of hnRNP K cognate nucleic acids such as poly (C), LOX-R, and CT3 oligonucleotides. HnRNP K serves as a repressor of C/EBPβ-mediated gene expression (Miau *et al.*, 1998). Coexpression of DEN virus full-length core protein restored the C/EBPβ activity that was repressed by hnRNP K, indicating that the biochemical interaction between hnRNP K and DEN core protein may modulate hnRNP K-mediated transcriptional activity (Chang *et al.*, 2001).

Similarly, hepatitis C core protein can relieve hnRNP K's repression of the human TK gene (Hsieh *et al.*, 1998). Binding of core occurs in a region of hnRNP K that causes disruption of hnRNP K binding to other cellular factors, and this may be how suppression is lifted. In addition, the binding of core protein to a transcription factor (hnRNP K) may account for its ability to modulate the promoter activities of other genes. Hepatitis C core is not only involved in transcriptional regulation, it is a multifunctional protein which besides being a viral nucleocapsid protein has RNA binding ability, can oligomerise, binds to the tails of cytoplasmic lymphotoxin β receptor and tumour necrosis factor receptor and sensitises cells to tumour necrosis factor and *fas* mediated cell death. Core has also been linked to cellular lipid metabolism (McLauchlan, 2000).

The progressive life cycle of HPV is linked to the differentiation of the stratified epithelium. Late viral protein L2 is restricted to terminally differentiated epithelial cells in the superficial layers of the squamous epithelium, while mRNA can be found in the

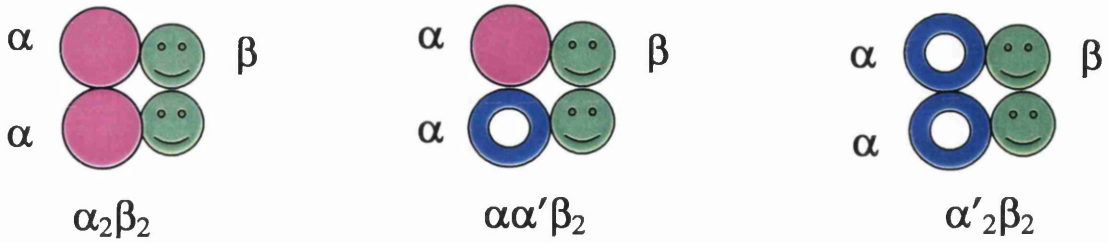
lower levels. Recombinant hnRNP K, overexpressed and purified, binds to HPV-16 L2 mRNA in a sequence specific manner. This binding efficiently inhibits translation of L2 mRNA *in vitro* (Collier *et al.*, 1998). HPV-16 therefore may utilise hnRNP K to control gene expression at the level of translation.

1C2 Protein kinase CK2

Protein kinase CK2 (formerly known as casein kinase 2) is a ubiquitously expressed pleiotropic and probably constitutively active serine/threonine kinase that can use either ATP or GTP as phosphoryl donor (dual-co-substrate) to phosphorylate serine or threonine residues that are N-terminal to acidic amino acids. The capability of CK2 also to phosphorylate tyrosine has been reported in recent years. (Chardot *et al.*, 1995; Wilson *et al.*, 1997; Marin *et al.*, 1999). The holoenzyme form of CK2 is a heterotetramer that can exist in 3 forms: $\alpha_2\beta_2$, $\alpha'_2\beta_2$ or $\alpha\alpha'\beta_2$ but CK2β-free pools of CK2α also exist and there is increasing evidence that the isolated subunits can exist *in vivo* under certain circumstances (Pinna & Meggio, 1997). The α and α' are the catalytic subunits and β is the regulatory subunit (Fig 1C2 Panel I). Human CK2α is normally 391 amino acids long and has a molecular mass of 45.1 kDa. The regulatory CK2β is 218 amino acids long with a molecular mass of 28 kDa (Fig 1C2 Panel II). *In vitro* the CK2 holoenzyme forms spontaneously from the individual subunits by self-assembly mediated by dimerisation of the two CK2β chains (Graham & Litchfield, 2000). The individual subunits have recently been shown to have their own activities which may go some way to explaining CK2's many and varied roles (reviewed in Guerra & Issinger (1999)). CK2β seems to play at least a trifunctional role: it confers stability to the holoenzyme (Meggio *et al.*, 1992), it increases enzyme activity (Grankowski *et al.*, 1991), and it determines substrate specificity (Meggio *et al.*, 1992; Bidwai *et al.*, 1992). The catalytic α-subunit is regulated not only by the β-subunit but also by polyanions though very little is known about this subunit. CK2 is known to phosphorylate and in some cases modulate the activity of over 160 putative protein substrates (Pinna & Meggio, 1997), including transcription factors e.g., Sp1 (Pugh & Tjian, 1990), signalling molecules e.g. A-Raf kinase (Hagemann *et al.*, 1997), ribosomal proteins e.g. L5 (Kim *et al.*, 1996), and a considerable number of viral proteins.

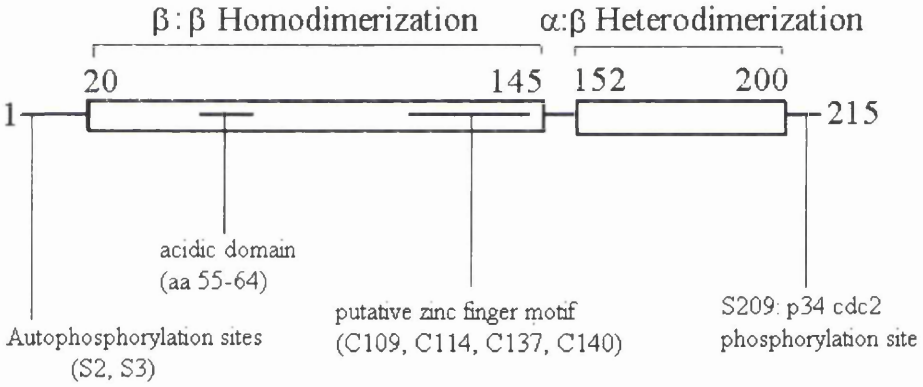
The crystal structure of fully active tetrameric form of human protein kinase CK2 obtained at 3.1 A^0 resolution showed that the regulatory subunits form a stable dimer linking the two catalytic subunits, which make no direct contact with one another (Niefind *et al.*, 2001). Each catalytic subunit interacts with both regulatory chains,

Fig 1C2 Panel I. Representation of CK2 tetrameric holoenzyme



A schematic representation of the CK2 holoenzyme with either two α catalytic and two β regulatory subunits, or one α and one α' and two β subunits or two α' and two β subunits to form a functional holoenzyme. (Figure by M. Koffa, Institute of Virology, University of Glasgow, UK).

Fig 1C2 Panel II. Domains of the CK2 β subunit



CK2 β possess autophosphorylation sites at its N terminus, aa 20-145 are involved in homodimerisation with another β subunit whereas aa 152-200 are involved in heterodimerisation with the α/α' subunits.

predominantly via an extended C-terminal tail of the β subunit (Niefind *et al.*, 2001).

Recently RNA polymerase III transcription was shown to be repressed in response to DNA damage by downregulation of TFIIIB, a core component of the polymerase III transcriptional machinery (Ghavidel & Schultz, 2001). Active CK2 associates with and normally activates the TBP subunit of TFIIIB and is required for its efficient recruitment to the promoter *in vitro*. The β regulatory subunit that binds to TBP is required for high TBP-associated CK2 activity and polymerase III transcription in unstressed cells. Transcriptional repression induced by DNA damage coincides with downregulation of TBP-associated CK2 and dissociation of catalytic subunits from TBP-CK2 complexes. Therefore, CK2 is the terminal effector in a signalling pathway that represses polymerase III transcription when the genome integrity is compromised (Ghavidel & Schultz, 2001).

Cells transfected with constructs expressing CK2α or CK2αβ showed significant resistance to chemical-mediated apoptosis, commensurate with a corresponding CK2 elevation in the nuclear matrix; transfection with CK2β did not demonstrate this effect. These data suggest that besides the commonly appreciated function of CK2 in cell growth, it might also have a role in protecting cells against apoptosis (Guo *et al.*, 2001).

CK2 phosphotransferase activity and protein levels are commonly elevated in solid human tumours and transformed cell lines (Heriche & Chambaz, 1998). Furthermore CK2 overexpression along with c-Myc induces lymphomas in mice (Seldin & Leder, 1995). In summary, from its substrates CK2 is implicated in the regulation of carcinogenesis, viral tumourigenesis, signal transduction, transcriptional control, apoptosis, cell cycle and other key biological processes (reviewed in Guerra & Issinger (1999)).

1C2.1 CK2 and viruses

Many viruses exploit CK2 to phosphorylate their proteins. CK2 phosphorylation is important in HIV-1 infection where phosphorylation of Vpu has been correlated with its ability to interact with the CD4 receptor (Willey *et al.*, 1992; Margottin *et al.*, 1996), a crucial step in viral infection. HIV-1 Rev activates CK2, and activated CK2 phosphorylates several cellular and viral proteins in HIV-1 infected cells (Ohtsuki *et al.*, 1998). Rev is phosphorylated *in vitro* by CK2 at Ser-5 and Ser-8 and these sites are also phosphorylated *in vivo*. Phosphorylation of Rev significantly reduces its binding to the RRE, suggesting that CK2 may affect nuclear export of HIV-1 transcripts. CK2 phosphorylates Rev by a unique mechanism, notably it is fully dependent on the regulatory β-subunit, relying on the integrity of an acidic stretch which downregulates

phosphorylation of other substrates and this is inhibited in a dose-dependent manner by polyamines and other polycationic effectors that normally stimulate CK2 activity. CK2 phosphorylation of Rev relies on conformational features of distinct regions that are also required for the transactivator's biological activity (Marin *et al.*, 2000).

CK2 has been shown to phosphorylate a number of viral proteins (Table 1C2) and to modulate their behaviour. On examination of this list two points are obvious. Firstly, for the majority of viruses investigated the CK2 target protein is involved in transcription. e.g. P proteins of Mononegavirales (non-segmented negative strand viruses), hepatitis delta and influenza. Secondly, in the same virus different proteins are modified by CK2 (at different time points or simultaneously during infection). For example, in HSV-1 the structural proteins VP22 and VP16, the R1 subunit of ribonucleotide reductase and glycoprotein E are all CK2 phosphorylated.

The CK2 β subunit has been shown to bind and potentiate phosphorylation of HSV-1 ICP27 protein suggesting that some of the characteristics attributed to the viral ICP27 protein could be due to it its interaction with this protein (Wadd *et al.*, 1999).

In addition to its ability to phosphorylate viral proteins, CK2 has been shown to nucleotidylate the HSV-1 IE protein ICP22 (Mitchell *et al.*, 1997), ICP27 is also nucleotidylated (Blaho *et al.*, 1993) a modification that has been suggested to be carried out by CK2. Thus CK2 may play a general role in the processing/regulation of viral/cellular proteins involved in HSV-1 infection.

1C3 REF

The REF proteins (RNA and export factor binding proteins, also known as Aly in murines, or Yra-1p in yeast) belong to a conserved superfamily of RNA binding proteins containing ribonucleoprotein-type RNA binding domains (RBD) (Burd & Dreyfuss, 1994) that are required for mRNA export (Strasser & Hurt, 2000; Stutz *et al.*, 2000; Rodrigues *et al.*, 2001). The distinguishing feature of the REF family is the presence of two highly conserved motifs at their N and C termini: REF-N and REF-C boxes. Between the conserved motifs and the RBD, REF proteins have Arg-Gly-rich regions of variable length (N-vr and C-vr), which are related to the RGG boxes present in many hnRNP proteins (Fig 1C3) (Burd & Dreyfuss, 1994; Stutz *et al.*, 2000). REFs bind RNA non-specifically (Stutz *et al.*, 2000). Yra-1p, is an essential nuclear protein first identified from its RNA annealing activity (Portman *et al.*, 1997). Recently Yra-1p was shown to be involved in the export of mRNA from the nucleus (Strasser & Hurt, 2000; Stutz *et al.*, 2000).

Table 1C2. Viral proteins that are phosphorylated by CK2

Virus	*Viral protein*	*Function*
Borna disease	P protein	Transcriptional co-factor
Canine distemper	P protein	Transcriptional co-factor
Measles	P protein	Transcriptional co-factor
Vesicular stomatitis	P protein	Transcriptional co-factor
Respiratory syncytial	P protein	Transcriptional co-factor
Bovine papilloma	E1	ATP dependent helicase
Hepatitis delta	Small HDAg	genome replication
EBV	ZEBRA protein	transcriptional activator
EBV	EBNA-2	immortalisation of B-cells
Influenza	Polymerase	RNA polymerase
Polyoma	VP1	major capsid protein
HSV	VP22	viral replication
HSV	ICP27	Posttranscriptional regulator
HSV	VP16	transcriptional activator
HSV	IE22	regulation of transcription
HSV	R1 subunit	ribonucleotide reductase
HSV	Glycoprotein E	blocking some host responses
VZV	Glycoprotein I	AP-1 recruitment to Golgi
VZV	Gene 63 protein	nuclear protein
HIV	Vpu	membrane protein
HIV	Rev	viral RNA processing
Human papilloma	E7	oncoprotein
SV40	Large T	oncoprotein

Although REF proteins have been implicated in the export of cellular mRNAs, it is still unclear whether they bind directly to mRNA. Human REF is a component of a 335 kDa protein complex that is recruited to mRNAs by the spliceosome 20-24 nts upstream of the 5' splice site during splicing in a sequence-independent manner (Le Hir *et al.*, 2000a; Zhou *et al.*, 2000; Le Hir *et al.*, 2000b; Le Hir *et al.*, 2001). But REFs can also associate with intronless mRNAs, independent of splicing with variable efficiency that is influenced by both the sequence and the length of the transcript, and promote export of unspliced mRNAs (Rodrigues *et al.*, 2001).

In mouse, REFs are encoded by at least three different genes (Burd & Dreyfuss, 1994; Portman *et al.*, 1997; Stutz *et al.*, 2000) and they differ at multiple positions in the variable regions due to deletions and/or amino acid changes (Stutz *et al.*, 2000). Expression of multiple splice variants further increases the complexity of the murine family (Stutz *et al.*, 2000). Murine REF1-II is generated by alternative splicing of REF1-I also named Aly (Bruhn *et al.*, 1997) and lacks the N-terminal variable region (see Fig 1C3), whereas murine REF2-I and REF2-II differ by a single amino acid insertion in REF2-I (Q198) (Stutz *et al.*, 2000). REF1-I was first identified as a protein interacting with LEF-1, a transcription factor that participates in the regulation of the T-cell receptor α enhancer (Bruhn *et al.*, 1997). Subequently, human REF (also called BEF) was shown to increase transcriptional activation by proteins containing a basic region-leucine zipper DNA binding domain (bZIP) (Virbasius *et al.*, 1999). Thus REFs may participate in multiple steps of mRNA biogenesis including transcription and transport.

REFs have been proposed to participate in mRNA export by recruiting export adaptor TAP (known as Mex67p in yeast) to cellular mRNPs (Le Hir *et al.*, 2000a; Strasser & Hurt, 2000; Stutz *et al.*, 2000; Le Hir *et al.*, 2001). Recently REFs were shown to be directly implicated in the export of mRNAs from the nucleus. The variable regions are necessary for binding of REFs to RNA and TAP. Antibodies specific to REF inhibit mRNA nuclear export in *Xenopus* oocytes and this inhibition is independent of pre-mRNA splicing or nuclear export of constitutive transport element (CTE)-containing RNAs from simian type D retroviruses, which is directly mediated by TAP (Gruter *et al.*, 1998). Recombinant REFs stimulated the export of mRNAs that are otherwise exported inefficiently. Thus REF proteins play a critical role in mRNA nuclear export, acting downstream of splicing and upstream of TAP, and spliced and unspliced mRNAs use common export factors to reach the cytoplasm (Rodrigues *et al.*, 2001).

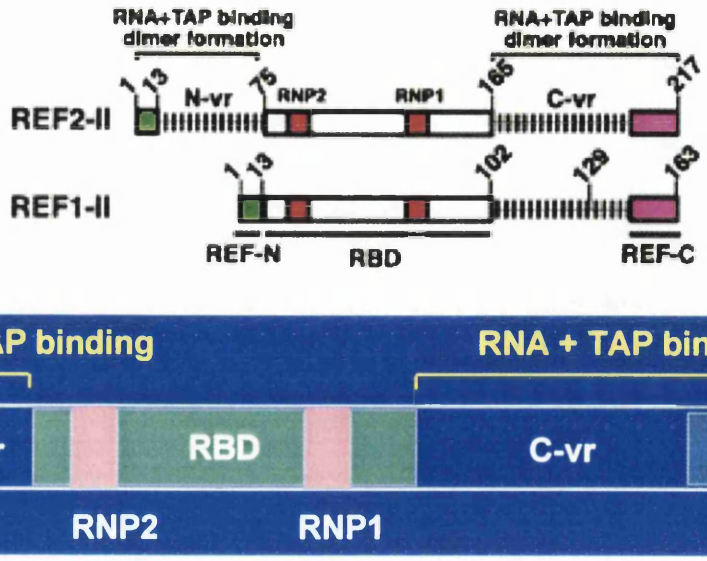
1C4 TAP/Mex67p/NXF1

Metazoan TAP and its yeast orthologue Mex67p are members of an evolutionarily conserved protein family, the NXF family, implicated in mRNA export from the nucleus (Herold *et al.*, 2000). Mex67p and the *Caenorhabditis elegans* protein NXF1 are essential for the export of bulk polyadenylated RNAs to the cytoplasm (Segref *et al.*, 1997; Tan *et al.*, 2000). Human TAP is required for the export of simian type D retroviral unspliced RNAs, binding to the the CTE and interacting with the NPC (Gruter *et al.*, 1998; Braun *et al.*, 1999; Kang & Cullen, 1999; Kang *et al.*, 2000); NPC reviewed in Ryan & Wente (2000). In *Xenopus* oocytes, titration of TAP with an excess of CTE RNA prevents cellular mRNAs from exiting the nucleus indicating a role for TAP in cellular mRNA nuclear export (Pasquinelli *et al.*, 1997; Saavedra *et al.*, 1997; Gruter *et al.*, 1998; Braun *et al.*, 2001). Although TAP binds the retroviral CTE specifically (Braun *et al.*, 1999) the interaction of TAP and its yeast homologue Mex67p with cellular RNAs is likely to be mediated by protein-protein interactions, including via REFs that are required for mRNA export (Strasser & Hurt, 2000; Stutz *et al.*, 2000; Rodrigues *et al.*, 2001).

TAP partners include various nucleoporins (Katahira *et al.*, 1999; Bachi *et al.*, 2000); p15 (also called NXT1) a protein related to nuclear transport factor 2 (NTF2) (Katahira *et al.*, 1999; Black *et al.*, 1999); transportin, which mediates TAP nuclear import (Bachi *et al.*, 2000); and several mRNP-associated proteins, such as E1B-AP5, RAE1 (Bachi *et al.*, 2000), and members of the REF/Yra1p family of proteins (Strasser & Hurt, 2000; Stutz *et al.*, 2000). Binding of TAP to these mRNP-associated proteins is mediated by its N-terminal domain (Bachi *et al.*, 2000; Stutz *et al.*, 2000) which includes an RBD and four LRRs. TAP also exhibits general RNA binding affinity with specific binding to the CTE RNA (Fig 1C4) (Gruter *et al.*, 1998; Braun *et al.*, 1999; Liker *et al.*, 2000).

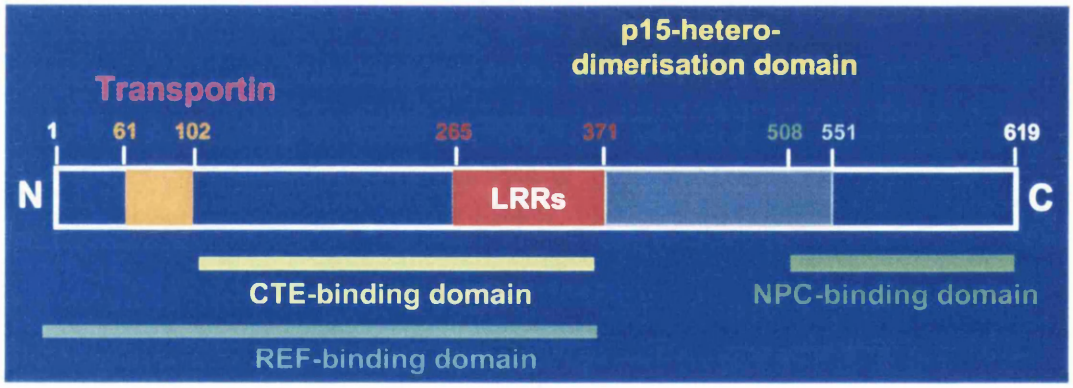
TAP, an export receptor protein that is not an importin β family member, failed to interact with CRM1/Xpo1 in yeast two-hybrid screen but did make direct contact with the FG-repeat domain of Nup214 and possibly other nucleoporins (Katahira *et al.*, 1999). An uncharacterised human protein hCG1, which is related to Nup42p/yeast Rip1p, was also found to interact with TAP via its FG-repeat domain. Nucleoporin binding by TAP *in vitro* and *in vivo* is mediated by a domain located at the extreme C-terminal end and this region is necessary and sufficient for the localization of TAP to the nuclear rim (Bear *et al.*, 1999; Bachi *et al.*, 2000; Herold *et al.*, 2000); the FG repeat-containing fragments of Nup214 have been shown to interact with TAP (Katahira *et al.*, 1999; Bachi *et al.*, 2000).

Fig 1C3. Domain diagram of REF



Domain organization of REF proteins (Stutz *et al.*, 2000). RBD with the conserved RNP1 and RNP2 motifs; REF-N and REF-C, conserved N- and C-terminal motifs; N-vr and C-vr represent the N- and C-terminal variable regions specific to each member of the family REF variable regions are required for TAP and RNA binding. Numbers indicate the position in the amino acid sequence. From Rodrigues *et al.* (2001).

Fig 1C4. Domain diagrams of TAP



Domain organisation of various interacting regions of TAP protein. Amino acids 102-371 are involved in binding with constitutive transport element (CTE) containing RNAs of simian type D retroviruses and aa 508-619 are involved in binding with nuclear pore complex assembly (NPC). The leucine rich repeat region (LRR) is found between aa 265-371.

Thus TAP represents a different class of export receptors, which directly interact with components of the NPC and this does not need a bridging protein, such as CRM1 used by HIV-1 Rev. Based on the similarities of this domain to the ubiquitin-associated domain, it was predicted that residues located in a conserved loop were implicated in TAP-nucleoporin interaction (Suyama *et al.*, 2000). TAP via an NTF2-like domain heterodimerises with p15 protein (Suyama *et al.*, 2000) which forms homodimers, p15 also heterodimerises with the NTF-2 like domain of NXF proteins (see Fig 1C4) (Bachi *et al.*, 2000; Herold *et al.*, 2000; Suyama *et al.*, 2000). Overexpression of full length TAP with p15 bypasses nuclear retention and stimulates export of a variety of mRNAs normally not exported efficiently, and the LRR and NTF2-like domains are essential for this (Braun *et al.*, 2001).

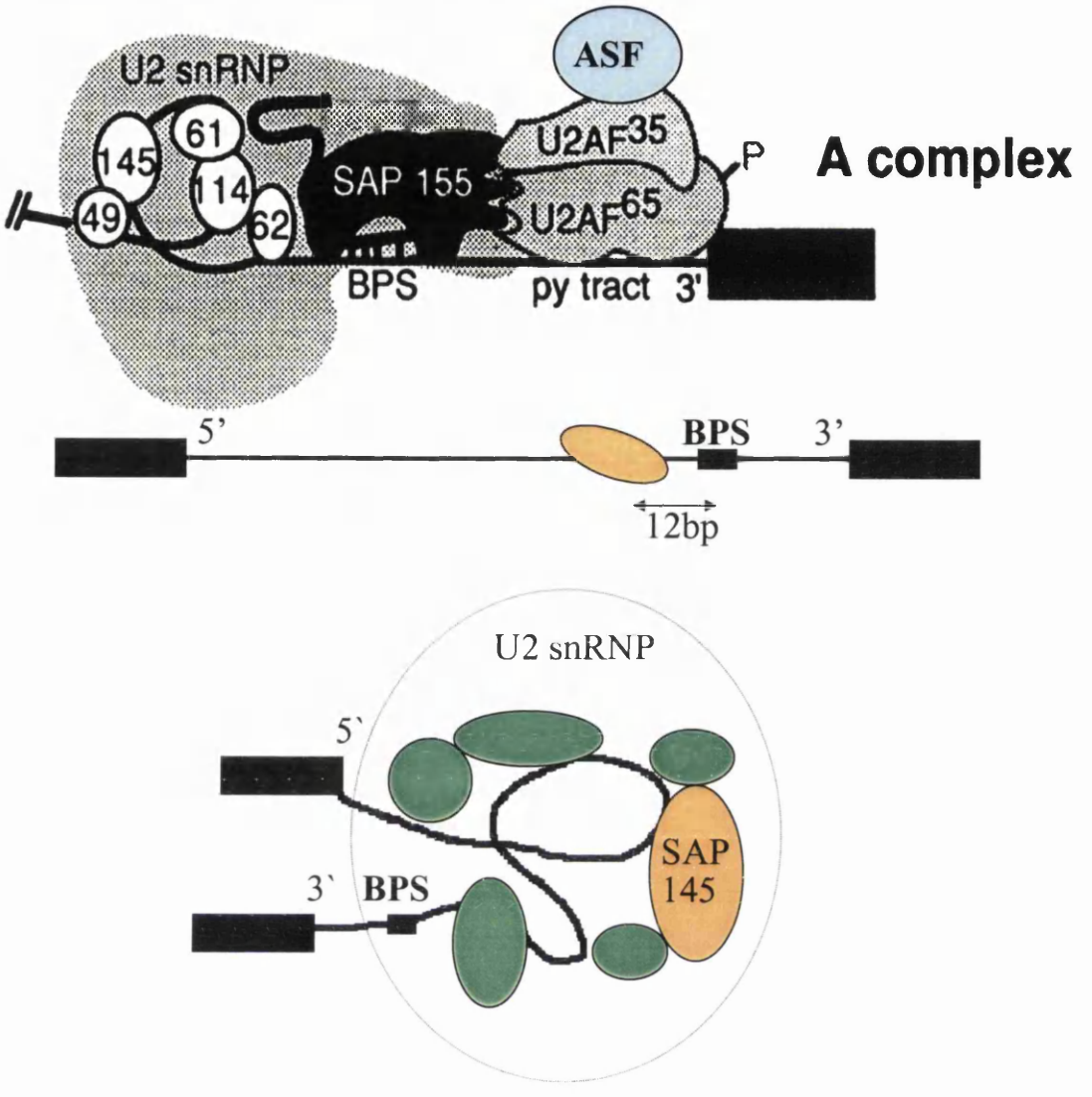
1C5 SAP145

Spliceosomal complexes assemble on pre-mRNA in the order E, A, B, and C, with the catalytic step taking place in the C complex (reviewed in Kramer (1996)). Step 1 is a nucleophilic attack on the 5' splice site (ss) by the branch-site adenosine, which is bulged from a duplex between U2snRNA and the branchpoint sequence (BPS). This duplex is established in the A complex concomitant with binding of six U2 snRNP proteins (SAPs 49, 61, 62, 114, 145, and 155) near the BPS. These proteins are components of two multiprotein complexes, SF3a, and SF3b which are required for A complex assembly (reviewed in Kramer (1996); Reed (1996)). SF3a consists of three subunits, $SF3a^{60}$, $SF3a^{66}$, $SF3a^{120}$ (corresponding to SAPs 61, 62, and 114, respectively), and SF3b consists of at least four subunits $SF3b^{50}$, $SF3b^{130}$, $SF3b^{145}$, and $SF3b^{155}$ (SAPs 49, 130, 145, and 155, respectively). The SF3a subunits are all essential in yeast.

In the mammalian A complex, the SF3a/b subunits cross-link to a 25-nucleotide region in the pre-mRNA located immediately upstream of the BPS (Gozani *et al.*, 1996). Although the binding of these proteins occurs in a sequence-independent manner, the interaction of the SF3a and SF3b subunits with this site is absolutely essential for tethering the U2 snRNP to the branch site. These RNA protein interactions are thought to function in part to anchor U2 snRNP to the BPS (Gozani *et al.*, 1996).

Splicing associated protein 145 is one of the seven splicing associated polypeptides found within the U2 snRNP where it acts to tether the snRNP to the intron of pre-mRNA (Fig. 1C5) (Champion-Arnaud & Reed, 1994). Yeast SAP 145, identified by its similarity to human SAP (Gozani *et al.*, 1996), corresponds to the CUS1 gene,

Fig 1C5. Position of SAP145 in the U2 snRNP



Splicing Diagram

SAP145 interacts with pre-mRNA and other spliceosome proteins to tether the snRNP U2 complex to the intron. Shown also are the positions of U2AF subunits and ASF/SF2 bound to the pyrimidine tracts (py tract) in the intron near the 3' splice site. In the mammalian A complex, the SF3a/b subunits cross-link to a 25-nucleotide region in the pre-mRNA located immediately upstream of the branch point sequence (BPS) (Gozani *et al.*, 1996). Although the binding of these proteins occurs in a sequence-independent manner, the interaction of the SF3a and SF3b subunits with this site is absolutely essential for tethering the U2 snRNP to the branch site. These RNA protein interactions are thought to function in part to anchor U2 snRNP to the BPS (Gozani *et al.*, 1996). 5' and 3' represents the beginning and end of intron boundaries respectively as 5' splice site and 3' splice site. Filled black rectangular boxes represent exon regions separated by thin lines representing the intron.

isolated as a suppressor of a U2 snRNA mutation (Wells *et al.*, 1996). CUS1 is essential in yeast and is required for A complex assembly (Wells *et al.*, 1996). It appears that the primary role of SAP145 is to mediate binding of the U2 snRNP to the pre-mRNA.

Cyclin E-cdk2 is a critical regulator of cell cycle progression from G_1 into S phase in mammalian cells. Cyclin E-specific antibodies coimmunoprecipitated a number of cyclin E-associated proteins from cell lysates, including three SAPs: SAP 145, SAP 155, SAP 114, as well as snRNP core proteins B' and B linking pre-mRNA splicing to the cell cycle machinery (Seghezzi *et al.*, 1998).

SAP 145 binds HSV-1 ICP27 protein suggesting that some of the characteristics attributed to the viral ICP27 protein for splicing inhibition and host cell shut off could be due to this interaction (Bryant *et al.*, 2001).

1C6 P32

P32 was first isolated as a protein tightly associated with ASF purified from HeLa cells (Krainer *et al.*, 1990). ASF is a member of the SR family of splicing factors and it has a role in the enhancement of splicing and the regulation of alternative splicing (reviewed in Fu (1995)). ASF shuttles between the nucleus and the cytoplasm and its phosphorylation state regulates this activity (Caceres *et al.*, 1998). The phosphorylation status of other SR proteins affects their function in pre-mRNA recognition (Cao *et al.*, 1997), spliceosome assembly (Xiao & Manley, 1997), splicing catalysis (Kanopka *et al.*, 1998), subcellular localisation (Caceres *et al.*, 1998) and organelle distribution (Misteli & Spector, 1996; Caceres *et al.*, 1997). P32 may exert its effect on splicing by modulating ASF function (Petersen-Mahrt *et al.*, 1999); it inhibits phosphorylation of and RNA binding by ASF and seems to inactivate ASF as a splicing repressor or activator protein (Petersen-Mahrt *et al.*, 1999).

Despite this, the distribution of p32, like its function, is controversial. P32 is reported to have a mitochondrial distribution (Muta *et al.*, 1997; Matthews & Russell, 1998) but can also be found in the nucleus as granules and tubules (Matthews & Russell, 1998). Further, the distribution of p32 is altered during adenovirus infection where, with viral core protein V, it redistributes to the nucleus (Matthews & Russell, 1998).

P32 interacts with a variety of cellular and viral proteins adding to the confusion regarding its function. Interacting proteins include lamin B receptor (Simos & Georgatos, 1994), TFIIB (Yu *et al.*, 1995a), the globular head domain of the plasma complement component C1q, whose haemolytic activity is inhibited as a result (Ghebrehiwet *et al.*, 1994), high molecular weight kininogen and factor XII (Herwald *et al.*, 1996), vitronectin

(Lim *et al.*, 1996), hyaluronic acid (Deb & Datta, 1996), HSV-1 ORF-P protein (Bruni & Roizman, 1996), EBV EBNA I protein (Wang *et al.*, 1997; Chen *et al.*, 1998a) adenovirus polypeptide V (Matthews & Russell, 1998), and HIV-1 proteins Rev and Tat (Luo *et al.*, 1994; Yu *et al.*, 1995b; Tange *et al.*, 1996) (see Section 1B4.1 on HIV-1). Together, these interactions have suggested a role for p32 not only in splicing (Luo *et al.*, 1994; Yu *et al.*, 1995b; Tange *et al.*, 1996; Petersen-Mahrt *et al.*, 1999) but also in nucleocytoplasmic transport (Matthews & Russell, 1998; Petersen-Mahrt *et al.*, 1999), and transport from the mitochondria (Matthews & Russell, 1998; Jiang *et al.*, 1999) and in maintaining oxidative phosphorylation (Muta *et al.*, 1997).

The interaction of p32 with Rev which is bound to ASF at the 5'-splice site may stabilise the association of U1 snRNP with the 5'-splice site and inhibit assembly of a functional spliceosome (Tange *et al.*, 1996). The dysfunctional spliceosome could then function as a substrate for Rev-mediated nuclear transport. However, p32 also appears to bridge the HIV-1 protein Tat to the general transcription machinery via its interaction with TFIIB and appears to act synergistically with Tat to stimulate transcription (Yu *et al.*, 1995b), suggesting that p32 plays multiple roles in HIV-1 replication. Similarly deletion of the regions of EBNA-1 required for interaction with p32 abrogated the transcriptional activity of EBNA-1, implicating p32 as a possible mediator of EBNA-1-dependent transactivation.

P32 has also been shown to bind the HSV-1 ICP27 protein suggesting that some of the characteristics attributed to ICP27 for splicing inhibition and/or host cell shut off could be due to this. Also p32 exists predominantly in the cytoplasm of uninfected cells but redistributes to the nucleus in the presence of ICP27 alone and during HSV-1 infection (Bryant *et al.*, 2000).

Chapter 1 Part D. Significance of interactions between ORF57 and cellular/viral proteins: aims of the study

KSHV is a newly described human virus associated with tumours, therefore it represents a new opportunity to investigate possible mechanisms of virus pathogenesis and tumorigenesis. Recent findings indicate that the ORF57 protein and its homologue HSV-1 ICP27 possess common properties. ORF57 is a key protein in KHSV infection so understanding its action is important to an understanding of KSHV biology. In addition, ORF57 has homologues in every member of the *Herpesviridae* so knowledge of its activities will have implications for other herpesviruses.

Studies of these homologues are likely to facilitate important insights into protein function in the context of the different virus/host relationships, indicating whether they possess similar, additional or different structures and functions and how they interact with cellular/viral proteins and nucleic acid. Any key viral protein, especially one so conserved, is a candidate for antiviral therapy. Drugs designed to disrupt ORF57 protein:protein and/or protein:nucleic acid interactions are an obvious potential offshoot of this kind of study. Finally, KHSV via ORF57 interferes with and utilises host cellular mechansims for its own replication, therefore its study might be expected to shed considerable light on host cell processes.

The aim of this work was to identify proteins, both cellular and viral, that interact in infected cells with the Kaposi's sarcoma-associated herpesvirus regulatory protein ORF57. In the light of this information, functional assays were performed to characterise various activities of ORF57 protein.

Chapter 2: Materials and Methods

Chapter 2A. Materials

2A1 Construction of recombinant plasmids

The following plasmids were generated:

2A1.1 pGEX-57 FL and pGEX-57 small (N-terminal deletion of aa 1-180)

Full length ORF57 (aa 1-455) as cDNA and ORF57 DNA 2nd exon (N-terminal deletion containing aa 181-455) were cloned as N-terminal fusions into the *Eco*RI sites of pGEX-5X-3 (Pharmacia Biotech) and pGEX-2T (N+1) from available constructs called pKS4 (GFP-cORF57 FL) and pKS1 (GFP-ORF57 small) (Bello *et al.*, 1999) respectively. These will be referred to as GST-57 (FL) and GST-57 (small). The ORF57 fragment DNAs were excised with *Eco*RI, agarose gel purified and ligated with *Eco*RI-digested, CIP-treated, gel purified pGEX-5X-3 and pGEX-2T (N+1) vectors. The ligation mix was transformed into DH5α cells. Inserts of the appropriate clones were confirmed by DNA sequencing and clones were transformed into protease-deficient BL21 cells for expression.

2A1.2 pCITE-gORF57 (FL) and pCITE-cORF57 (FL)

Full length ORF57 genomic and ORF57 cDNA using *Bam*HI (PM1 oligo, Table 2A1) and *Xho*I (PM2 oligo, Table 2A1) were cloned as S-tagged N-terminal fusions into the corresponding sites of pCITE-4b- (+) (Novagen) from available constructs called pKS3 (Bello *et al.*, 1999) and pKS4 respectively to obtain *in vitro* transcribed-translated ^{35}S-labelled-ORF57 protein. Products synthesised *in vitro* from these constructs will be referred to as ^{35}S-labelled-ORF57 (FL). ORF57 fragments (genomic and cDNA) were PCR amplified from pKS3 and pKS4 clone using pfu polymerase at 60°C with 30 cycles. PCR amplified blunt ended products were digested with *Bam*HI and *Xho*I, agarose gel purified and ligated with gel purified *Bam*HI- and *Xho*I- digested pCITE-4b (+). The ligation mix was transformed into DH5α cells. Inserts of the appropriate clones were confirmed by DNA sequencing and pCITE-cORF57 (FL) was used to express *in vitro* transcribed-translated ^{35}S-labelled-ORF57 (FL).

Table 2A1. Oligonucleotides used for plasmid construction

Name	Nucleotide sequence	Source
PM1, 34 mer	GCGGGATCCCATGGTACAAGCAATGATAGACATG	Inst Virology, Glas Univ, UK.
PM2, 32 mer	GCGCGCTCGAGTTAAGAAAGTGGATAAAAGAA	Inst Virology, Glas Univ, UK.
57N1F, 27 mer	TCACCAGGATCCATGGTACAAGCAATG	M Biotech, UK
57N17F, 27 mer	AAGGGCGGATCCATGGACTCTGTGTCC	M Biotech, UK
57N181F, 30 mer	CTCCCTGGATCCATGATAATTGACGGTGAG	M Biotech, UK
57N329F, 30 mer	CCAGATTTGGATCCATGCATCTTTCCTGCG	M Biotech, UK
57N387F, 24 mer	ACTATCGGATCCATGCAGAGTCGC	M Biotech, UK
57N215R, 26 mer	GTCCGTCTCGAGCTACGGGACGTGGG	M Biotech, UK
57N328R, 29 mer	AACGCACTCGAGCTAAAGTAATCTAAATC	M Biotech, UK
57N455R, 26 mer	CGGTTTGGCTCGAGTTAAGAAAGTGG	M Biotech, UK

2A1.3 GAL4 BD-ORF57 (FL and small) constructs

Full length cDNA of ORF57 (aa 1-455) and 2nd exon of ORF57 DNA (aa 181-455), using *Bam*HI (PM1 oligo, Table 2A1) and *Xho*I (PM2 oligo, Table 2A1), were amplified from pKS4 and cloned into the corresponding sites of pGBKT7 (Clontech, yeast 2-hybrid system III). These will be referred to as ^{35}S-labelled-pGBKT7-ORF57 (FL) and ^{35}S-labelled-pGBKT7-ORF57 (small) and express ORF57 as a c-myc-tagged-N-terminal fusion proteins. These were also used to synthesise *in vitro* ^{35}S-labelled-ORF57 proteins.

2A1.4 pCMV-RevM10-cORF57

Plasmids expressing Rev and RevM10 proteins were generated by replacing the GFP coding sequence from the pEGFP-C1 plasmid by the HA-Rev and HA-RevM10 coding sequences respectively using the *Age*I and *Eco*RI sites (Braun *et al.*, 2001). To test the role of ORF57 in an RNA export assay, a construct expressing RevM10-ORF57 was generated by excising full-length ORF57 cDNA from the pKS4 construct as *Xho*I-*Sal*I fragment and subcloning into *Xho*I-*Sal*I-digested pCMV-RevM10 plasmid as a C-terminal fusion protein. This is referred to as RevM10-ORF57 and used in RNA export assays.

2A1.5 pcDNA4/HisMax(B)-gORF57, pcDNA4/HisMax(B)-cORF57 (FL) and deletion constructs

ORF57 full-length genomic and cDNA were PCR amplified from pKS3 (Bello *et al.*, 1999) and pKS4 clones, respectively, using *Bam*HI and *Xho*I oligos (Table 2A1) and cloned into pcDNA4/HisMax(B) vector (Invitrogen). To clone various deletion mutants of ORF57, five oligos spanning the desired length of ORF57 DNA were designed with *Bam*HI and *Xho*I sites (Table 2A1). Using these oligos, ORF57 fragments of specified lengths were amplified by PCR, digested with *Bam*HI-*Xho*I, agarose gel purified and ligated with *Bam*HI-*Xho*I digested pcDNA4/HisMax C vector. The ligation mix was transformed into *E. coli*, DH5α cells. Inserts of the appropriate clones were confirmed by the DNA sequencing and clones were used to synthesise *in vitro* transcribed-translated ^{35}S-labelled-ORF57. These constructs are referred to as ^{35}S-labelled-pcDNA4-ORF57 (FL) and deletion mutants containing aa 1-455, 17-455, 1-215, 181-328, 329-455 and 387-455.

All plasmid DNAs were purified using the Qiagen miniprep kit for sequencing and QIAfilter Plasmid Maxi Kit (Qiagen) for bulk DNA preparation.

pKS1 recombinant: This plasmid contains the 2nd exon of ORF57 DNA (aa 181-455) cloned into the *Eco*RI site of pEGFP-C1 (Clontech) (Bello *et al.*, 1999).

pKS3 recombinant: This plasmid contains ORF57 full-length genomic DNA cloned into the *Eco*RI site of pEGFP-C1 (Clontech) (Bello *et al.*, 1999).

pKS4 recombinant: This plasmid contains ORF57 full-length cDNA cloned into the *Eco*RI site of pEGFP-C1 (constructed by L. Bello, Institute of Virology, Glasgow UK).

pCITE-ICP27 recombinant: This plasmid expresses ^{35}S-labelled ICP27 (FL) (Koffa *et al.*, 2001).

The acknowledged investigators provided the following plasmids

pGEX-27: (Mears & Rice, 1996); provided by S. Rice, University of Minnesota, USA. Expresses the fusion protein GST-ICP27 and referred to as GST-27.

pGEX-5X-1-hnRNP K/GST-K: (Michelotti *et al.*, 1996); provided by D. Levens, National Institutes of Health, USA. Expresses the fusion protein GST-hnRNP K (full length), referred to as GST-K.

pGEX-K and deletion mutants: (Van Seuningen *et al.*, 1995b; Michelotti *et al.*, 1996); provided by K. Bomsztyk, University of Washington, USA. All hnRNP K deletion mutants are in pGEX-KT.

pGEX-145: (Seghezzi *et al.*, 1998); provided by R. Reed, Harvard University, USA. Expresses the fusion protein GST-SAP145 (full-length), referred to as GST-SAP145.

pGEX-HA-CK2α, pGEX4T1-CK2α′, pMALc2-CK2β, pRSET-CK2α and their deletion mutants: (Heriche *et al.*, 1997; Wadd *et al.*, 1999; Leroy *et al.*, 1999); provided by O. Filhol, INSERM, Grenoble, France.

pGST-REF2-I (murine), its deletion mutants and pcDNA3.1mycHisB-REF2-I: (Koffa *et al.*, 2001); provided by S. Wilson, UMIST, UK. Murine REF2-I cloned into the *Bam*HI-*Xba*I sites of pcDNA3.1mycHisB (Invitrogen).

pGEXCS-REF1-II expressing GST-REF1-II, its deletion mutants (aa 14-102 and aa 103-163) and pRSETB-(His) REF1-II (aa 1-163 [FL] and aa 103-163): (Stutz *et al.*, 2000; Rodrigues *et al.*, 2001); provided by E. Izaurralde, EMBL, Germany. Murine REF1-II cDNA fragment cloned into *Nco*I-*Bam*HI sites of pGEXCS.

pcDNA3-gORF50 and pcDNA3-FLc50: (Lukac *et al.*, 1998; Lukac *et al.*, 1999); provided by D. Ganem, HHMI, University of California, USA.

pcDNA3/N-Flag-hnRNP K: (Hsieh *et al.*, 1998); provided by M. Lai, HHMI, University of Southern California School of Medicine, USA. HnRNP K with a "Flag" tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) at the N-terminus was cloned into *Eco*RI sites of pcDNA3 and is referred as Flag-hnRNP K.

pGL3-CT3wt-c-fos and CT3mut-c-fos promoter luciferase reporter plasmids: (Michelotti *et al.*, 1996; Shnyreva *et al.*, 2000); provided by K. Bomsztyk, University of Washington, USA.

pGL3-pORF50 promoter luciferase reporter plasmids, pRLSV-40, pGL3 basic, pGL3 control, pISRE-promoter luciferase plasmids (Promega): Provided by D.J. Blackbourn, Institute of Virology, University of Glasgow, UK. Various lengths of ORF50 promoter region were PCR amplified with oligos containing *Sac*I-*Bgl*II sites and cloned into the respective sites of pGL3.

pCMV128 (CAT-RRE), pCH110 (β-gal, Pharmacia), pCMV-HA-Rev, pCMV-HA-RevM10, pEGFP-C1-TAP (GFP-TAP), pEGFP-N3-zzp15-1 (zz p15-1), pRCMV-REF2-II: (Braun *et al.*, 2001); provided by E. Izaurralde, EMBL, Germany. REF2-II cDNA excised from the pGEXCS-REF2-II construct as *Nar*I-*Bam*HI fragment and inserted into the *Acc*I-*Bam*HI sites of pEGFP-C1.

pCMV-27: (Bryant *et al.*, 2000) The plasmid pCMV63 consists of the full length ICP27 (IE63) cloned downstream of a HCMV immediate early promoter in pCMV10 vector, constructed by N. Rethmeier, Institute of Virology, University of Glasgow, UK.

2A2 Enzymes

Restriction enzymes were obtained from Boehringer Mannheim or New England Biolabs. T4 DNA ligase (10U/μl) and calf intestinal phosphatase, CIP (10000U/ml) were from Boehringer Mannheim. Lysozyme (10mg/ml stock) came from Sigma and RNAse ONE™ ribonuclease (10U/μl) was obtained from Promega. Purified CK2 subunits ($\alpha_2\beta_2$ at 0.2mg/ml, α at 3.6mg/ml, $\alpha'_2\beta_2$ at 40μg/ml) were a gift from O. Filhol and CK2 holoenzyme was from Boehringer Mannheim.

2A3 CK2 Peptide substrate

The protein kinase CK2 peptide substrate (Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu) was synthesised by Calbiochem.

2A4 Bacteria and bacterial culture media

2A4.1 Bacteria

E.coli strain DH5α was used for maintenance and propagation of plasmid DNA. Fusion protein expression from prokaryotic vectors utilised *E. coli* strain BL21 (Studier *et al.*, 1990). For optimisation of GST-fusion protein expression, *E. coli* BL21, BL21 (DE3) pLysS), TOPP, XL-1 blue (Stratagene), TG-1 and Novablue (Novagen) cells were used.

2A4.2 Bacterial culture media

All strains were grown either in L-Broth (10g NaCl, 10g Bactopeptone, 5g yeast extract in 1l water, pH 7.5) or in 2YT broth (5g NaCl, 16g Bactotryptone, 10g yeast extract in 1l water). Agar plates were 1.5 % (w/v) agar in L-broth. Where necessary, media and LB agar plates were supplemented with antibiotic: 50 μg/ml kanamycin (for pRSET, pEGFP transformed cells) or 100 μg/ml ampicillin for all other plasmid-containing bacteria.

2A5 Cells and tissue culture media

2A5.1.1 BCBL-1 cells

This is a primary effusion lymphoma cell line derived from the body cavity-based lymphoma of an AIDS patient containing latent episomal KSHV (Renne *et al.*, 1996b). These cells are positive for KSHV but negative for EBV. KSHV infection is latent and can be induced to lytic replication by adding the phorbol ester, TPA. TPA acts via the protein kinase C pathway to activate the AP-1 family of transcription factors that stimulate gene expression (Angel *et al.*, 1987). BCBL-1 cells were grown in HuT (Human T-cell growth) medium (RPMI 1640 BioWhittaker, 10% FCS, 1% L-glutamine and 1% penicillin-streptomycin solution).

2A5.1.2 BJAB cells

This cell line is a PEL derived body cavity based lymphoma cell line, uninfected both for KSHV and/or EBV. BJAB cells were grown in HuT medium.

2A5.1.3 BHK-21 C13 cells

A fibroblastic line derived from baby hamster kidney cells (Macpherson & Stoker, 1962), BHK C13 cells were grown in Glasgow Modified Eagle's Medium (GMEM) supplemented with 10% new born calf serum, 10% tryptose phosphate broth, 100 units/ml penicillin and 0.01% streptomycin.

2A5.1.4 HeLa cells

An epithelial cell line derived from a human cervical carcinoma, HeLa cells were grown in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 2.5% foetal calf serum, 2.5% new born calf serum and antibiotics as above.

2A5.1.5 Vero cells

Derived from African green monkey kidney, Vero cells were grown in GMEM supplemented with 10% foetal calf serum and antibiotics as above.

2A5.1.6 293 cells

Derived from human embryonic kidney, 293 cells were grown in DMEM supplemented with 10% foetal calf serum, 1% L-glutamine, 1% non-essential amino acids and antibiotics as above.

2A5.1.7 293-T cells

Derived from human embryonic kidney and transfected with SV40 large T antigen, 293-T cells were grown in DMEM supplemented with 10% foetal calf serum, 1% L-glutamine, 1% non-essential amino acids and antibiotics as above.

Unless otherwise stated all cell culture media were obtained from GIBCO.

2A5.2 Radioactive media

The media used were (i) [^{35}S]-L-methionine medium (RPMI 1640 with L-glutamine, containing 20% of normal methionine level (GIBCO, Cat. No. 11876-026), 2% newborn calf serum, antibiotics as above and 150μCi/ml [^{35}S]-L-methionine) (ii) [α-^{32}P]-orthophosphate medium (DMEM without sodium pyruvate (GIBCO, Cat. No. 32430-027) or RPMI 1640 with L-glutamine, containing 20% of normal phosphate level (GIBCO, Cat no. 11877-032), 2% new born calf serum, antibiotics as above and 150μCi/ml [α-^{32}P]-orthophosphate-dATP). For methionine or orthophosphate starvation of cells the media minus the radioactive isotopes were added for 1 h prior to labelling.

2A6 Viruses

Viruses used were:

2A6.1 KSHV

The genome is maintained as episome in BCBL-1 cell line and adding phorbol esters such as 20ng/ml TPA or sodium butyrate can induce replication.

2A6.2 HSV-1 strain 17^{+}

HSV-1 wild type (wt) virus (Brown *et al*, 1973).

2A7 Antisera and monoclonal antibodies

The antisera and monoclonal antibodies used and their sources are listed in Table 2A7.

Table 2A7. Antibodies used

Abbreviations: ND, not done. NP, not published. IP, immunoprecipitation. IF, immunofluoresence. W.blot, Western blot.

Antisera	Type	W. blot	IP	IF	References	Source
PRIMARY ANTIBODIES	species	dilution	µl used	dilution		
ORF57 (GH)	Rabbit	1:2500	5µl	1:250	NP	G. Hayward, Johns Hopkins School of Med, Baltimore, USA
ORF50	Rabbit	1:2500	5µl	1:250	NP	G. Hayward, Johns Hopkins School of Med, Baltimore, USA
ICP27 (#1113)	Mouse	1:2000	5µl	1:100	Ackermann *et al.* (1984)	Goodwin Institute for Cancer Research, Florida, USA
HnRNP K (#54)	Rabbit	1:10000	1µl	1:100	Van Seuningen *et al.* (1995)	K. Bomsztyk, Dept. Med. Washington Univ, Seattle, USA
HnRNP A1	Mouse	1:1000	ND	1:100	Kamma *et al.* (1995)	G. Dreyfuss, HH Medical Institute, Philadelphia, USA
CK2 α subunit	Rabbit	1:2000	ND	ND	NP	O. Filhol, Inserm, Grenoble, France
CK2 α' subunit	Goat	1:1000	5µl	1:50	NP	Santa Cruz Biotechnology
CK2 β subunit	Rabbit	1:2000	5µl	1.100	NP	O. Filhol, Inserm, Grenoble, France
ORF57 N terminus ((#718, 719)	Rabbit	1.250	5µl	ND	This study	Inst. Virology, Glasgow Univ, UK
ORF57 C terminus ((#720, 721)	Rabbit	1.250	5µl	ND	This study	Inst. Virology, Glasgow Univ, UK
REF (KJ 70)	Rabbit	1:2000	5µl	ND	Rodrigues *et al.* (2001)	E. Izaurralde, EMBL, Heidelberg, Germany
TAP (KJ 60)	Rabbit	1:2000	5µl	ND	Braun *et al.* (1999)	E. Izaurralde, EMBL, Heidelberg, Germany
Y14	Rabbit	1:500	ND	ND	NP	E. Izaurralde, EMBL, Heidelberg, Germany
SAP 145	Rabbit	1:1000	5µl	1:150	Seghezzi *et al.* (1998)	R. Reed, Harvard Univ, Boston, USA
p32	Mouse	1:200	ND	ND	Bryant *et al.* (2000)	J. Scott, Inst. Virology, Glasgow Univ, UK
c-myc	Rabbit	ND	ND	1:200		Sigma
GFP	Mouse	1:1000	ND	ND		Clontech
GFP	Rabbit	ND	3µl	ND		Clontech
HA	Mouse	1:1000	ND	ND		Sigma
MBP	Rabbit	1:1000	ND	ND		New England Biolabs
SECONDARY ANTIBODIES						
Anti-Mouse HRP Conjugate	Goat	1:1000	ND	ND		Sigma
Protein-A HRP Conjugate		1:1000	ND	ND		Sigma
Anti-Mouse FITC Conjugate	Goat	ND	ND	1:100		Sigma
Anti-Rabbit TRITC Conjugate	Goat	ND	ND	1:100		Sigma
Anti-Rabbit cy3 Conjugate	Goat	ND	ND	1:2000		Sigma
Anti-Rabbit cy5 Conjugate	Goat	ND	ND	1:200		Sigma

2A8 Radiochemicals

Radiochemicals were from Dupont or NEN at the following specific activities

γ–[^{32}P] ATP	6000Ci/mmol	10mCi/ml
[^{35}S]-L-methionine	1175Ci/mmol	10mCi/ml
[α-^{32}P]-orthophosphate	8500Ci/mmol	5mCi/ml
α–[^{32}P] ATP	800Ci/mmol	10mCi/ml
Acetyl Co-A	2.83Ci/mmol	0.05mCi/ml

2A9 Common solutions

30% acrylamide mix	29% (w/v) acrylamide, 1% (w/v) N, N'-methylene bis-acrylamide.
5x agarose gel loading buffer	1xTAE, 1% SDS (w/v), 50% glycerol (v/v), 1mg/ml bromophenol blue.
Bradford's reagent	0.01% (w/v) Coomassie Brilliant Blue, 0.0003% (w/v) SDS, 4.75%(v/v) ethanol, 8.5% (v/v) phosphoric acid.
Coomassie stain	0.02% (w/v) Coomassie Brilliant Blue 50% (v/v) methanol, 43% (v/v) water, 7% (v/v) acetic acid.
Destain	5%(v/v) methanol, 7% (v/v) acetic acid, 88% (v/v) water.
Giemsa stain	1.5% (w/v) suspension of Giemsa in glycerol, heated at 56°C for 2h and diluted with an equal volume of methanol.
PBS-A	170mM NaCl, 3.4mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4, pH7.2.
PBS complete	PBS A plus $CaCl_2.2H_2O$ and $MgCl_2.6H_2O$ each at 1g/l.
PBS-T	PBS-A plus 0.05% (v/v) Tween 20.
3x Protein gel loading buffer	150mM Tris HCl, 300mM dithiothreitol, 6% (w/v) SDS, 0.3% (w/v) bromophenol blue, 30% (v/v) glycerol, pH 6.8.
Protein gel running buffer	0.05M Tris base, 0.05M glycine, 0.1% (w/v) SDS
Reduced glutathione buffer	75mM HEPES, 150mM NaCl, 20-40mM reduced glutathione, 5mM DTT, 0.1% (v/v) Triton-X100, pH 7.4
50x TAE	0.2M Tris base, 0.05M EDTA (pH 8.0), pH with acetic acid.
TE buffer	10mM Tris HCl, 1mM EDTA, pH 8.0.
Towbin (blotting buffer)	25mM Tris base, 192mM glycine, 20%(v/v) methanol.
Transfer tank buffer	50mM Tris base, 50mM glycine, 20% (v/v) methanol, 0.01% (w/v) SDS.

Trypsin	0.25% (w/v) trypsin in Tris-saline containing phenol red, adjusted to pH 7.5 with $NaHCO_3$
Versene	0.6mM EDTA in PBS A, 0.002% phenol red
Water	Sterile deionised water obtained from a "Milli-Ro 60 plus" deioniser (Millipore, USA) and sterilised by autoclaving.

2A10 Chemicals and reagents

All chemicals and reagents were from BDH Chemicals UK or from Sigma Chemical Co. unless otherwise stated.

Amersham:	ECL Western blotting reagents, nitrocellulose membrane, Rainbow molecular weight markers
Beecham Research:	Ampicillin sodium B.P. (Penbritin®)
Bio-Rad:	TEMED, ammonium persulphate, Coomassie brilliant blue
Dupont:	En^{3}hance
Fluka:	Formaldehyde, formamide
Joseph Mills Ltd.:	Ultra pure ethanol, methanol
Prolabo:	Butanol, glacial acetic acid, glycerol, isopropanol
Pierce:	Phosphocellulose units, spinZyme™ format
Roche:	Protease inhibitor cocktail

Chapter 2B. Methods

2B1 Preparation and transformation of competent *E.coli* cells

Plasmids were grown and maintained in *E.coli* strain DH5α, and plasmids incorporating glutathione S-transferase (GST)- or Maltose binding protein (MBP)- tags were transformed into *E.coli* strains BL21, BL21 (DE3) pLysS, TG-1, Novablue or TOPP cells immediately before protein expression. Standard methods of preparing competent *E.coli* cells and of plasmid transformation were used. A 1ml overnight culture was inoculated into 100 ml L-broth and grown for about 3 h at 37°C in a shaking incubator until the optical density (OD) at 630 nm was approximately 0.4. The culture was then cooled on ice and cells pelleted by centrifugation at 5000 rpm for 10 min at 4°C in Sorvall RT 6000B refrigerated centrifuge. Cells were resuspended in 50 ml cold 50 mM $CaCl_2$ and left on ice for 30 min, then pelleted as before and resuspended in 10 ml cold 50 mM $CaCl_2$, 15% glycerol. 200µl aliquots were snap frozen and stored at -70 C. About 5-10ng plasmid DNA was added to a 200 µl aliquot of competent *E.coli* cells, which was thawed on ice. The mixture was incubated on ice for 30 min prior to heat shock in a 42°C water bath for 2 min. 800µl of L-broth was added to the cells, which were shaken for 60 min at 37°C before plating onto LB agar plates containing appropriate antibiotic (Section 2A4). Plates were incubated overnight at 37°C. For transformation of commercial competent cells, manufacturer's instructions were followed.

2B2 Polyclonal peptide antisera to ORF57

Two peptides, one located close to the N terminus (peptide J 15 mer: EQLSEPAEPPADERI) and another one close to the C terminus (peptide L 15 mer: GSAKFNKGLFFYPLS) were synthesized. They were purified by HPLC and used to immunise two rabbits each. 200 µg/rabbit of each peptide in PBS was mixed with Freund's complete adjuvant by vortexing and passing through a 26G needle; 500µl of each peptide emulsion was inoculated subcutaneouly into rabbits at ~4-5 places in the Central Research Facility, University of Glasgow. After 21 days, fresh peptide emulsion was prepared for each peptide using incomplete Freund's adjuvant and a first booster was given; 16 days later a second booster was given. On day 14, a first test bleed was collected from each rabbit and next day a third booster was given. Between boosters, test bleed were collected and checked for the titres and specificity by ELISA, Western blotting, and immunoprecipitations. In total five boosters were given and the final bleed

collected. ELISA plates were coated with 200 ng peptide antigen/well and kept 16 h at 20°C for binding. Plates were washed in PBS-T and blocked with blocking solution (2 % BSA in PBS) for 1 h at 37°C. Fifty µl each of different dilutions (1:50 to 1:3200 at doubling dilutions) of primary antibodies (test and pre-immune sera) were added for 1 h at 37°C. Plates were washed and 50 µl of 1:1000 dilution of protein-A-HRP conjugated secondary antibody added for 45 min at 37°C. After washings, 50 µl of substrate (10 mg ABTS in 20 ml 0.1M citrate buffer pH 5.0 + 30 µl H_2O_2 fresh) was added and plates kept in the dark for colour development, stop solution was added and plates read at 405 nm in a Dynax Revelation 3.2 reader.

2B2.1 Testing the specificity and cross reactivity of peptide antisera by immunoblotting

Recombinant ORF57 (GST-57 and GST alone) or induced BCBL-1 cell extracts were run on an SDS-PAGE, transferred to a nitrocellulose membrane by Western blotting, and then dilutions of peptide antiserum were tested by immunoblotting for their reactivity. To prepare an antiserum which is pre-adsorbed with the antigen for estimating the specificity and cross reactivity of the antisera, 200 µg/100 µl of each peptide solution (2mg/ml) was incubated with 5 µl of appropriate antiserum and 50 µl of protein-A-Sepharose beads for 1 h at 37°C. Antigen-antibody complexes were precipitated by centrifugation and the supernatant used in immunoblotting.

2B2.2 Purification of peptide antisera

Anti-ORF57 peptide antisera were affinity purified using a Hi-Trap Protein-G column on the AKTA purifier superloop system (Amersham-Pharmacia Biotech). The column was washed with 200 volumes of start buffer and the rabbit antiserum was diluted in 10 volumes of start buffer (20 mM sodium phosphate buffer), filtered through a 0.22 µM filter (Millipore) and applied. The run was started using Method Editor: Protein-G at 280 nm, wash with 10 column volumes (CV) elution rate set to 1 ml/min and elution with 20 CV of elution buffer (100 mM glycine pH 2.7) at <5 M Pa pressure. Following elution of antibody the low pH (2.7) of the elution buffer was neutralised by the addition of 100 µl of neutralisation buffer (1 M Tris pH 9.0), followed by quick mixing to avoid precipitation. All the buffers used were filtered through a 0.22 µM filter (Millipore). After the run was over (total time ~1.3 h) 0.1 % sodium azide was added to relevant antibody fractions and stored at 4°C.

2B3 Cell Culture

2B3.1 Growth and maintenance of uninfected cell lines

Mammalian cells were passaged in sterile disposable 175cm^2 plastic flasks in the appropriate media (Section 2A5) and were grown at 37°C in a humidified incubator under 5% CO_2. Confluent layers were harvested by washing the monolayer twice in 25ml trypsin: versene (1:4; supplied by Institute of Virology Media Services) and resuspending in 10ml medium. For continual passage, BHK cells and HeLa cells were split in a 1:10 ratio every 4-5 days, Vero cells 1:6 every 4-5 days, 293 and 293-T cells 1:10 every 6-7 days (feeding fresh medium on the 3rd day).

BJAB cells grow in culture in HuT media as clumps in suspension and they were split in a 1:10 ratio every four days.

2B3.2 Growth and maintenance of virally infected cell lines

2B3.2 1 Maintenance of BCBL-1 cells

These cells grow individually in suspension. Cells were collected from a flask and pelleted by centrifugation at 2000rpm for 5 min and passaged by splitting at 1:5 ratio ($2x10^5$ cells/ml) in HuT medium. These were then kept at 37°C in 5% CO_2 and reached confluency in approximately 4 days.

2B3.3 Freezing/storage of cells

When cells were frozen in liquid nitrogen, the medium used was 90% FCS and 10% DMSO made fresh and allowed to chill before adding to cells. Cells were harvested by pelleting, at $4x10^6$ cells/ml, resuspended in 1.5 ml freezing media and stored at -70°C for 16 h prior to transfer into liquid nitrogen storage.

2B3.4 Sterility checks

An inoculum from each culture was streaked on a blood agar plate (to test for bacterial contamination) or brain heart infusion agar plate (to test for fungal contamination), each incubated at 37°C or room temp respectively for 1 week and inspected for growth. Mycoplasma contamination, although harder to detect, was regarded as present if blood agar plates discoloured and all cell lines were checked regularly at our mycoplasma detection facility.

2B3.5 Induction of the KSHV lytic cycle in BCBL-1 cells and radiolabelling

Suspension cultures, grown in 175cm^2 flasks, almost reaching confluency (1x10^6/ml), were split at 0.2x10^6 cells/ml (1:5) and induced using TPA at 20ng/ml final concentration on the same day or left uninduced; unless otherwise stated induction was for 72 h. Cells for immunofluorescence were handled similarly. On some occasions, induced and uninduced cells were labelled with [^{35}S]-L-methionine or [α-^{32}P]-orthophosphate for 2-6 h. (Section 2A5.3). Cells were pelleted and resuspended in methionine- or orthophosphate- deficient media at 2x10^{7}cells/10ml for 1 h starvation and either left uninduced or induced with TPA. Then cells were pelleted and resuspended in [^{35}S]-L-methionine medium (150μCi/ml) or [α-^{32}P]-orthophosphate medium (150μCi/ml) at 2x10^{7}cells/10ml. Harvesting was after 6 h for early proteins. Alternatively, cells were induced unlabelled for 48 h then labelled for 2-6 h for late protein extracts. After labelling, cells were washed 3x with PBS and soluble protein cell extracts (Section 2B3.7) prepared by adding 2x10^{7}cells/800 μl lysis buffer.

2B3.6 Transfection of cells

2B3.6.1 Chemical transfection of mammalian cells

Mammalian (293, 293-T, HeLa, BHK and Vero) cells were transfected with various plasmids DNAs. A 175cm^2 confluent flask of cells (~1x10^7 cells) was treated with trypsin:versene (Section 2B3.1), and cells were pelleted and resuspended in 10 ml serum and antibiotic free DMEM. A 100μl aliquot was diluted 10x and counted. The cells were then resuspended at 5x10^6 per 750μl medium. Cells were either plated onto six-well dishes (6x10^5 cells/well) or for immunofluorescence added to a 35mm dish with 3 coverslips (3x10^5 cells/well) and left for 24h, swirling every 10min in the first hour. Next day, approximately 50-60% confluent cells were transfected using Fugene/Polyfect transfection reagent (Qiagen) following the manufacturer's instructions, and harvested as transfected and untransfected cells after 48 h for protein expression and reporter gene expression assays or after 24h for fixing for immunofluorescence (Section 2B8).

2B3.6.2 Electroporation of BJAB or BCBL-1 cells

BCBL-1 or BJAB cells were plated at 0.2x10^6 cells/ml one day prior to transfection to be in growth phase for DNA uptake. Cells harvested by centrifugation were washed once in PBS-A, counted and aliquoted at 4x10^6 cells for BJAB and 2x10^6 cells for BCBL-1 cells

in 0.45 ml RPMI 1640 media (minus antibiotics and FCS), transferred to a 0.4 cm electroporation cuvette containing usually 10 μg plasmid DNA, mixed slowly avoiding air bubbles and kept on ice for 10 min. The cells were electroporated at 0.25kV, 0.95μF x1000 (time constant should be about 30 sec) using a BioRad Gene Pulser. Following incubation on ice again for 10 min, cells below the 'foam' were resuspended gently in 3ml complete HuT media (no antibiotics) in a well swirling every 10 min for 1 h and the medium changed after 5-6 h. Then TPA was added or cells were left uninduced. Cells were harvested after 16 h treatment for reporter assays or immunofluorescence (Section 2B8) and after 42 h for cell protein extracts (Section 2B3.7).

2B3.7 Whole cell extract preparation

Cells from 90mm dishes (~ $2x10^7$ cells) were washed, pelletted by centrifugation at 15000 rpm/5 min/4°C (Sorvall RT 6000B) and resuspended in 800 μl HEPES extract buffer (50 mM HEPES, 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, 1 % Triton-X-100, 0.1 % Nonidet P-40, 0.5 mM PMSF and protease inhibitor cocktail, pH 7.5), lysed by sonication for 30-60 sec in a soni-bath (Ultrasonic Bath, Kerry) or by passing 5x through a syringe with a 26-gauge needle, and left rotating at 4°C/30 min for solubilisation. Debris was pelletted by microcentrifugation at 15000 rpm for 30 min at 4°C; the soluble protein extracts (supernatants) were either used directly or stored at -70°C.

For coimmunoprecipitation assays, cells were harvested in immunoprecipitation (IP) binding buffer (50 mM Tris-HCl, 50 mM NaCl, 1 mM EDTA, 1.0 % Triton-X-100, 0.5 % Nonidet P-40, 0.5 mM PMSF and protease inhibitor cocktail, pH 7.5) and extracts were prepared as above.

For protein kinase CK2 assays, following GST pull downs or coimmunoprecipitations, cells were harvested in IP binding buffer containing 2.5 mM $MgCl_2$, 0.5 mM DTT, 20 mM β-glycerophosphate, 0.1 mM $NaVO_3$, 0.1 mM Na_2MoO_4, 30 mM p-nitrophenyl-phosphate and 10 mM NaF and extracts prepared as above.

On occasion, cell extracts were treated with 10U RNAse (ONE^{TM} Ribonuclease Promega) at 37°C for 15 min.

To standardise the amount of protein added to coimmunoprecipitations or pull down assays, the total protein content of soluble extracts was determined by Bradford's assay

(Bradford, 1976) and protein concentration adjusted to 2mg/ml. 10µl of protein sample was diluted in 90µl dH_2O and mixed with 1ml of Bradford's reagent. After 10min at 20°C the OD of the solution was measured at 595 nm, and converted to mg protein/ml by comparison to a standard curve generated using known quantities of bovine serum albumin (BSA).

2B4 Investigation of protein: protein interactions

There are several ways to investigate protein: protein interactions which include the yeast-two-hybrid assay, mammalian-two-hybrid, Far Western blotting technique, fusion protein based pull down assays and coimmunoprecipitation assay. Each has certain advantages and disadvantages and evidence for any protein: protein interaction should use more than one procedure.

The yeast-two-hybrid system is a sensitive way to screen for potential interacting proteins, giving the investigator possibilities to confirm or discredit these in other assays such as pull downs and coimmunoprecipitations. One aim of this study was to look for the interaction of ORF57 protein with known partner proteins of the alphaherpesvirus ICP27 protein. A combination of pull down assays and coimmunoprecipitations was used to test these interactions.

2B4.1 Pull down assays

Pull down assays using a protein such as GST- or MBP- fused to the protein of interest can be used to identify new protein:protein interactions or to confirm those identified in other systems. The procedure can be scaled up to isolate proteins of unknown identity, and the test protein can be mutated relatively easily. The GST tag has the advantage over MBP of being smaller in size and uncharged (unlike the His-tag), making it easier to express larger proteins as fusions (optimum size for expression of a protein including the 26 kDa GST tag in bacteria being ~60kDa).

The gene for the protein of interest is cloned in frame with the GST or malE (which encodes the MBP gene from the parasite helminth *Schistosoma japonicum*) into a pGEX vector (Pharmacia Biotech) or pMAL vector (New England Biolabs). A protease-deficient strain of *E. coli*, such as BL21, is used to express fusion proteins. The fusion protein is immobilised and purified by binding to glutathione attached to Sepharose 4B beads (Smith & Johnson, 1988) or amylose resin. The matrix is then washed to remove

non-specifically bound bacterial proteins, and subsequently mixed with cellular extracts allowing proteins which interact with the target protein to bind. Interacting proteins can be partially purified by a simple centrifugation step; hence this is a "pull down" assay (Fig. 2B4.1).

Expression from pGEX is under control of the *tac* promoter which is induced using the lactose analogue isopropyl β-D-thiogalactoside (IPTG) and from pMAL is under the same control. Beads are washed several times to remove non-specifically bound cellular proteins and interacting proteins can be eluted off the matrix using reduced glutathione solution for GST fusion proteins or maltose solution for MBP proteins. Proteins are analysed by SDS polyacrylamide gel electrophoresis followed by Western blotting or autoradiography.

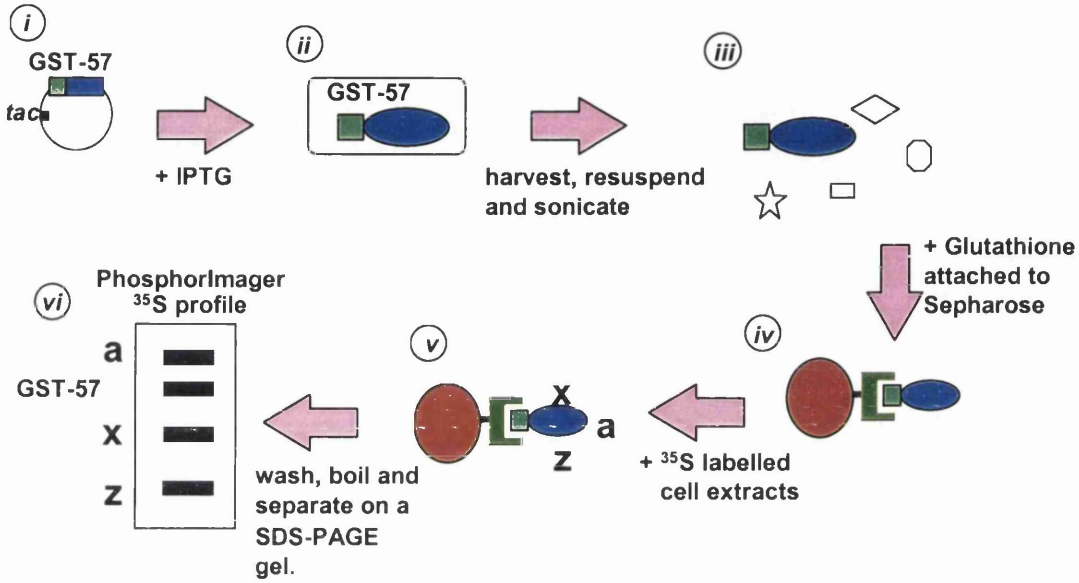
Optimisation of several factors is needed to maximise expression of a particular fusion protein. Decreasing the temperature will decrease the total yield but will also reduce the action of proteolytic enzymes and can increase the solubility. The IPTG concentration may affect the yield of fusion protein product and increasing the time of induction affects yield but also increases the time proteases can act. Salt and detergent concentrations, along with target and interacting protein concentrations also affect which proteins are pulled down.

2B4.2 Expression of fusion proteins and pull down assays

Initial attempts to express GST-57 by a standard method previously used for expression of GST-ICP27 (Bryant, 2000) were not very successful. The GST-57 (FL) fusion protein was expressed in a highly insoluble form with very little full-length ORF57 and it did not bind well to glutathione beads. Smaller bands seen on the Coomassie brilliant blue stained gel were confirmed as ORF57 truncations by Western blotting using anti-ORF57 Ab. Results of one such attempt are shown in Fig 2B4.2 lanes 5 to 7 and 13.

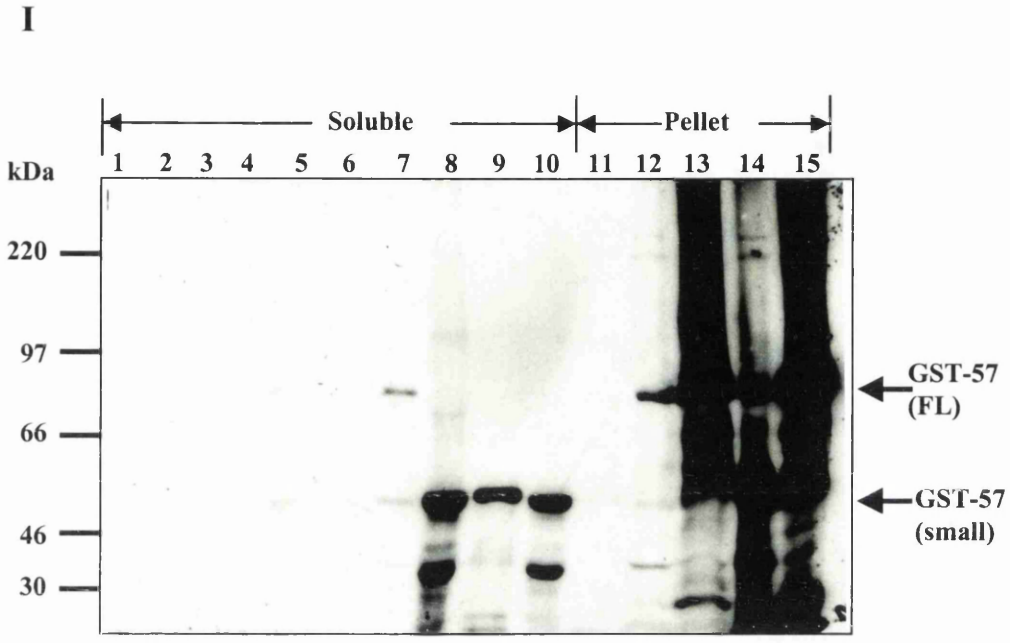
Use of urea made the GST-57 (FL) fusion protein soluble but during dialysis it again came out of solution (lane 15). Expression of another GST-57 construct (referred to as GST-ORF57 small), with a 180 aa deletion at the N terminus was also tried (Fig 2B4.2 lanes 8 to 10 and 14).

Fig 2B4.1. GST pull down assay



E. coli strain BL21 were transformed with GST-ORF57 (or other GST-/MBP-fusion proteins) expressing plasmid and grown to an OD_{600} of 0.4 (i). GST-ORF57 expression was induced by the addition of IPTG (ii), after 3h, bacteria were harvested, resuspended in a small volume and split open by sonication (iii). GST-ORF57 was purified from bacterial proteins by the addition of glutathione attached to Sepharose beads (iv). The pull down assay was performed by the addition of ^{35}S-labelled cellular extracts to purified GST-ORF57 bound onto beads. After washing, the proteins which interacted with GST-ORF57 remained in a complex with the beads (v). The complex was boiled to denature the proteins and break protein:protein interactions, and proteins separated by SDS-PAGE electrophoresis (vi). Labelled proteins were visualised by phosphorimaging and an estimation of interacting protein size made by comparison with molecular weight markers simultaneously run on the gel. Adapted from Bryant (2000).

Fig 2B4.2. GST-57 fusion protein (FL) is insoluble and forms a part of the cell debris pellet



Panel I. Western blot with anti-ORF57 Ab. GST-57 (FL and small) and GST proteins were expressed, run on a 10% SDS-PAGE gel and visualised by Coomassie staining. The smaller bands in lanes 5, 7, 12, 13, and 15 represent degradation products/truncations of GST-57 (FL) and smaller bands in lanes 8, 9, 10 and 14 represent degradation products/truncations of GST-57 (small). Samples used in **lanes: 1.** Rainbow marker for Western blot **2.** GST control (soluble) **3.** GST control (bead bound) **4.** GST control (unbound in wash) **5.** GST-57 (FL) ~76 kDa (soluble) **6.** GST-57 (FL) ~76 kDa (bead bound) **7.** GST-57 (FL) ~76 kDa (unbound in wash) **8.** GST-57 (small) ~56kDa (soluble) **9.** GST-57 (small) ~56kDa (bead bound) **10.** GST-57 (small) ~56kDa (unbound in wash) **11.** GST control (induced pellet) **12.** GST-57 (FL) ~76 kDa (uninduced pellet) **13.** GST-57 (FL) ~76 kDa (induced pellet) **14.** GST-57 (small) ~56kDa (induced pellet) **15.** Precipitate from detergent solubilised GST-57 (FL) after dialysis.

GST-57 (small) fusion protein expressed more efficiently than GST-57 (FL), was mostly soluble and bound to glutathione beads better than GST-57 (FL) (lanes 8 to 10 and 14). Expression of the GST-57 (small) construct also resulted in 2-3 smaller truncations.

As a good expression of full-length GST-57 fusion protein was required, each of the variables in expression process was altered. Effects of temperature (25/28/30/37°C), IPTG concentration (0.1/0.2/0.5/1.0 mM), method of lysis (sonication/lysozyme/freeze-thaw/Bug buster reagent), time of induction (16 h/6 h/3 h), and various strains of bacteria (BL21/BL21 (DE3) plysS/Noablue (DE3)/TOPP cells, Stratagene) were assessed and a different culture medium (2YT broth) was tried. Because of the insoluble nature of the fusion protein, several solubility enhancing reagents/detergents/denaturing agents (10% glycerol, 0.1-1.0% Triton-X-100, urea and SDS) were used to enhance solubility.

At the end of optimisation, standardised conditions (28°C/16 h induction/1.0 mM IPTG /sonication with lysozyme addition/BL21 (DE3) plysS or TOPP cells/500 µl glutathione beads per 5 ml cell lysate) were used to produce GST-57 (FL) fusion protein. After optimisation, a significant amount of full-length product was detectable both by Western blotting and Coomassie brilliant blue staining (see Fig. 3A2 Panel I lane 1), and GST-57 (small) also expressed well (see Fig. 3A2 Panel I lane 2). Specific truncated products were still observed in GST-57 (FL) fusion protein preparation, but there were significant amounts of full-length GST-57 protein present.

2B4.2.1 Optimal expression of GST-57 fusion protein

The final protocol used was as follows:

E.coli BL21 or other protease-deficient cells were transformed with plasmids expressing GST or MBP fusion proteins or GST or MBP alone and grown on LB plates containing ampicillin (Section 2A4.2). Single colonies were picked and inoculated into 10ml LB broth containing ampicillin, the culture was grown overnight and a 1:50 dilution (GST-57 FL) or 1:100 dilution (GST-57 small or other fusion proteins) into 100 ml LB broth containing ampicillin was made. After further growth at 30°C at an OD_{600} of 0.4-0.6,

fusion protein expression was induced by adding 1.0 mM IPTG for 16 h at 28°C. Bacteria were harvested by centrifugation at 3,000 rpm for 10 min, the pellet was washed once in PBS and resuspended in 5ml modified NETN lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 0.5 mM PMSF, 10% (w/v) glycerol, 0.5% Triton X-100, 5 mM β-ME and protease inhibitor cocktail; pH 8.0) containing 100μg/ml of lysozyme and incubated on ice for 15 min. DTT was added to 5 mM, Sarkosyl to 1.5%, the cell pellet was vortexed for 5 sec and sonicated on ice using a soni-probe (Branson sonifier 450). Then 20% Triton X-100 was added to 2%, the solution was kept at 4°C for 30 min for solubilization of proteins and cell debris was removed by centrifugation (15,000 rpm/4°C/30 min/ Sorvall SS34 rotor). Fusion proteins were purified by binding to pre-swollen glutathione-Sepharose 4B beads or amylose resin as appropriate. Beads and supernatant containing soluble protein (5 ml *E.coli* lysate/1000 μl beads) were mixed for 4 h at 4°C. After washing 3x using PBS+1% Triton X-100 and once with PBS at 500 g for 5 min at 4°C, the bound protein was eluted off the beads using reduced glutathione solution 3x (Section 2A9).

For binding assays, fusion protein-bound beads were directly resuspended in an equal volume of NETN buffer to give a 50% slurry and mixed with ~200 μg cell extracts (Section 2B3.7) for 2h at 4°C in 5ml binding buffer (50 mM Tris-HCl, 50 mM NaCl, 0.5% Nonidet P-40, 0.5 mM PMSF with protease inhibitor cocktail; pH 8.0). Approximately equal amounts of GST and GST-57 or other fusion proteins were used in pull down assays as judged by Coomassie stained gels of the fusion proteins bound onto beads. After binding, the beads were washed 4x in pull down wash buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% Nonidet P-40, 1% Triton X-100, pH 8.0) and bound proteins were eluted by heating the beads in SDS gel loading buffer at 100°C for 5 min, separated by SDS PAGE, and visualised using a phosphorimaging system (Section 2B6.4) or Western blotting (Section 2B6.2 and 3).

For examination of ORF57 self interaction and with ICP27, either TPA-induced or uninduced BCBL-1 cell extracts or ^{35}S-labelled-ORF57 or ^{35}S-labelled-ICP27 synthesised *in vitro* were mixed with GST-57 or GST alone at 4°C for 3 h. Following washing with binding buffer, beads were then washed overnight in 50 μl of high stringency buffer (50 mM HEPES, 1M NaCl, 0.1% Nonidet P-40 with the addition of protease inhibitor cocktail, pH 7.5) to elute bound proteins.

To determine *in vitro* phosphorylation of ORF57 by protein kinase CK2 or direct physical interaction between ORF57 and both CK2 subunits, 40µl GST-57 or GST bound beads were mixed with 0.2µg purified CK2 ($\alpha_2\beta_2$ recombinant holoenzyme) or MBP-CK2 β subunit or His-CK2 α subunit. These data were analysed using a phosphorimager or Western blotting with ORF57 and CK2 subunit antibodies.

Histidine-tagged REF1-II was expressed in BL21 (DE3) PlysS *E.coli* cells and purified on Talon resin, then dialysed against buffer (50 mM sodium phosphate, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, pH 7.4) and further purified on a SP-Sepharose column using a 0-1.0 M NaCl gradient in buffer.

2B4.3 Pull downs using rMp32- or rMGO- Sepharose affinity chromatography columns

Affinity columns produced by D. Matthews (Matthews & Russell, 1998) were used to look at possibility of interaction between ORF57 with cellular protein p32. 100µl cell extracts harvested 6 h, 48 h or 72 h post TPA induction were mixed with 40µl of p32- or control glucose oxidase-Sepharose beads (rMp32/rMGO) at 4°C for 2h. Beads were washed 10x with medium stringency washing buffer (PBS A; 200 mM NaCl, 0.1% NP-40), pelleted before elution with 35µl warm protein gel loading buffer, then the supernatants were boiled for 5min. Samples were loaded onto SDS PAGE for analysis by Coomassie staining or Western blotting. In some cases, wash buffers of higher stringency (500 mM NaCl) replaced the medium stringency washing buffer. (PBS-A; 350 mM NaCl 0.1% NP-40).

2B4.4 The GST pull down assay using in vitro synthesised radiolabelled proteins

Various ^{35}S-labelled proteins were produced using the *in vitro* transcription/translation TNT kit (Promega) as per the manufacturer's instructions using 1-5 µg of template DNA. For GST pull downs, binding reactions were carried out in 500 µl of *in vitro* pull down binding buffer (10 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 10% glycerol buffer, pH 8.0), using ~5 µg of GST fusion protein bound to 20 µl of glutathione-Sepharose, together with 5 µl of ^{35}S-labelled protein (spun at 12,000 rpm, 4°C for 10min to remove insoluble proteins). For GST-TAP [purified GST-TAP protein (Braun *et al.*, 1999; Braun *et al.*, 2001) was provided by E. Izaurralde, EMBL, Germany], binding reactions were carried in 500 µl of PBS. In some experiments RNAse was added to the reactions. Proteins were eluted from the resin using elution buffer (50 mM Tris-HCl, 20- 40 mM

reduced glutathione pH 8.0).

2B5 Coimmunoprecipitation assays

Due to their high specificity towards the antigen antibodies can be used to isolate specific antigens and their interacting partner proteins from complex mixtures such as cell lysates (Fig. 2B5). These antigen-antibody complexes can be precipitated and pelleted using a "sandwich" reagent, *Staphylococcus aureus* protein A/G which interacts with IgG, coupled to Sepharose beads. Identification of the immunoprecipitated antigen and other co-purifying proteins can be achieved by separation of the complex on an SDS-PAGE gel, followed by detection of radiolabelled proteins by autoradiography or non-labelled proteins by Western blotting.

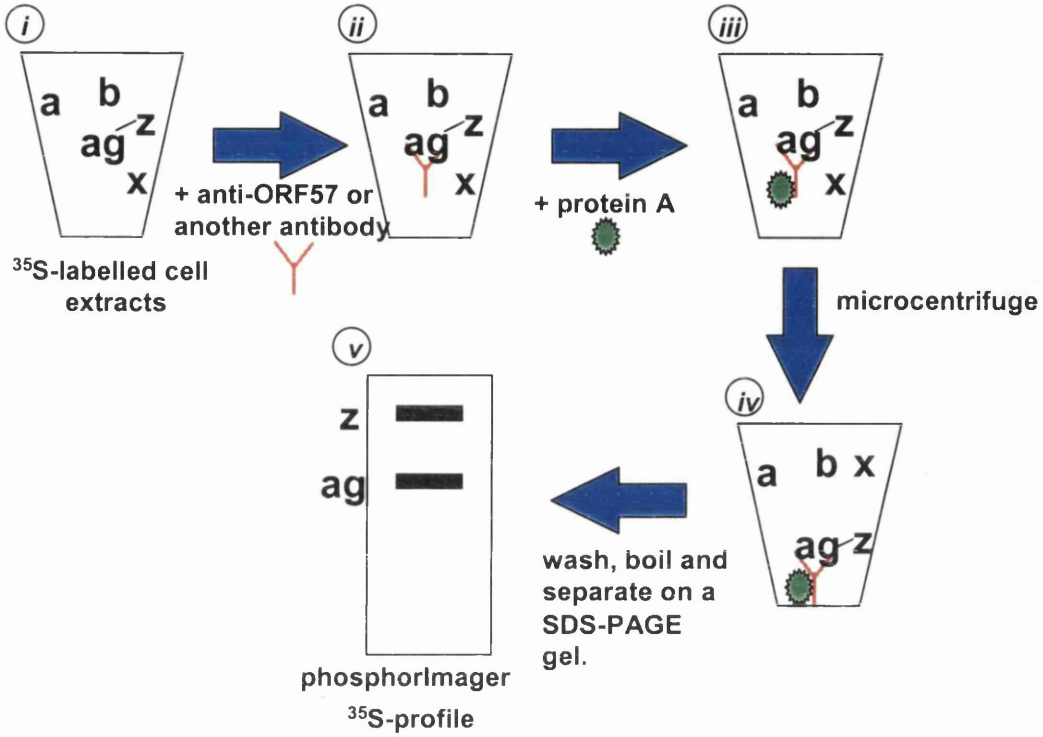
Both polyclonal antisera and Mabs can be used, however like every technique there are certain limitations. These include an antibody masking an interaction site or a protein: protein interaction masking the antibody binding site. In this respect, polyclonal antisera are better due to the availability of a range of epitopes. However if they work in immunoprecipitations, Mabs often give a cleaner result. Pre-immune serum was used as control in all reactions.

Salt and detergent concentrations in immunoprecipitation and wash buffers can affect the ability of proteins to interact, so these conditions were optimised for various interactions. The amounts of immunoprecipitated proteins are too low to detect by silver staining and, unlike GST pull downs, because of complex nature of antigen: antibody interactions it is not easy to scale up immunoprecipitations.

2B5.1 Using ORF57 polyclonal antiserum

Various cell extracts (~200μg) were mixed with 5μl ORF57 antisera in 300μl buffer E (100 mM Tris-HCl, 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 0.1% Nonidet P-40, 0.5% Na deoxycholate, 0.5 mM PMSF and protease inhibitor cocktail; pH 8.0) for 3-16 h at 4°C. 75μl protein A-Sepharose (made up at 50% (w/v) and washed in buffer E) was then added and samples rotated for 1h at 4°C. After pelleting, the beads were washed 5x in cold IPP150 buffer (10 mM Tris-HCl, 150mM NaCl, 1% Triton X-100, pH8.0), 50μl

Fig 2B5. Coimmunoprecipitation Assay



The appropriate antibody (anti-ORF57 or any other Ab shown above in red as Y) was incubated with radiolabelled cell extracts (i). Antibody binds to its antigen (ag), also bound to the antigen are any proteins (a, b, z and x) which interact with it (ii). Protein A-Sepharose was added, it binds to the antibody forming a complex of the antibody, antigen and interacting protein (iii). This was separated from the soluble cell extract by centrifugation (iv). After washing to remove non-specific binding proteins, the precipitate was boiled to denature the proteins and disrupt protein:protein interactions and proteins separated by SDS-PAGE electrophoresis (v). Labelled proteins were visualised by phosphorimaging and an estimation of interacting protein size made by comparison with molecular weight markers simultaneously run on the gel. From Bryant (2000).

warm protein gel loading buffer (without DTT or β-ME) was added to elute bound proteins. After centrifugation, DTT was added to the supernatants to 1mM, samples were placed in a boiling bath for 5 min and coimmunoprecipitated proteins separated by SDS-PAGE (Section 2B6.1). For CK2 activity assays protein gel loading buffer was not added and samples were processed as described in Section 2B7.

2B5.2 Using ORF50 polyclonal antiserum

5µl of polyclonal antiserum against ORF50 was mixed with 200µg nuclear extract and incubated at 4°C for 4h in a final volume of 300µl low salt binding buffer (10 mM Tris-HCl, 50 mM NaCl, 5% glycerol, 1 mM EDTA, 4 mM DTT, 0.5 mM PMSF and protease inhibitor cocktail; pH 8.0). 30µl protein A-Sepharose was added and the incubation was continued for 2 h at 4°C with constant agitation. The beads were washed and eluted as above.

2B5.3 Using hnRNP K antiserum

Beads for immunprecipitation were made up by mixing protein A/G-Sepharose beads with anti-hnRNP K polyclonal antibody (Table 2A7) or preimmune serum in binding buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 0.5 mM PMSF and protease inhibitor cocktail; pH 7.5) at 4°C for 2 h (1µl serum/10µl beads). Immunoprecipitation was carried out by mixing the immune or preimmune serum coated beads with cell extracts (~200µg protein) for 4h in binding buffer at 4°C with washes and elution as above. Again for CK2 activity assays, protein gel loading buffer was not added and samples were processed as described in Section 2B6.

2B5.4 Using SAP145 and REF/TAP antisera

The procedure followed was as for ORF57 antiserum but used 5µl of anti-SAP145 polyclonal serum or anti-REF (KJ70)/anti-TAP (KJ60) polyclonal serum (Table 2A7) in 300µl binding buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5 % Nonidet P-40, 0.5% Na deoxycholate, 0.5 mM PMSF and protease inhibitor cocktail; pH 7.5).

2B6 Detection of proteins by electrophoresis, Western blotting and autoradiography

2B6.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

Proteins were resolved by electrophoresis on SDS polyacrylamide mini or larger gels (Laemmli, 1970) using Bio-Rad mini gel electrophoresis tanks or a large Bio-Rad Protean II cell. 8-15% acrylamide/bis-acrylamide concentration was used in gels to resolve

proteins of different molecular weights. For larger gels, amounts were 50 ml resolving gel and 10 ml stacking gel and for mini gels 7.0 ml resolving gel and 3.0 ml stacking gel.

2B6.2 Electroblotting of proteins to nitrocellulose membrane; immunodetection

Proteins resolved by SDS-PAGE were transferred to nitrocellulose (Towbin *et al.*, 1979) using a Bio-Rad transblot cell. The gel was put directly in contact with an appropriate sized sheet of nitrocellulose (Hybond-ECL Amersham) avoiding any air bubbles and both were sandwiched between Whatman 3mm paper of the correct size on each side. This was in turn sandwiched between sponges provided by Bio-Rad and finally all were placed in the blotting cassette and the transfer carried out at 200-250 mA for 1-3 h. After transfer, the presence of proteins was detected using specific antibodies. For blocking non-specific binding, membranes were incubated in PBS-A + 5% dried milk for 1 h at 20°C with one change of buffer after 30min, or at 4°C for 16 h. Membranes were washed 3x 10 min at 20°C in PBS-T, before incubation for 1-2 h at 20°C on a shaker in 20 ml of appropriately diluted primary antibody (Table 2A7) in PBS-T. Blots were washed 3x 10 min at 20°C in PBS-T, and protein-A horseradish peroxidase conjugate or anti-mouse IgG whole molecule conjugate added at a 1/1000 dilution in PBS-T. After 40 min incubation at 20°C, with agitation, blots were washed as previously and proteins were detected using the Amersham Enhanced Chemiluminescence system. The blots wrapped in cling film were exposed to Kodak X-OMAT S film.

2B6.4 Phosphorimage analysis and autoradiography of ^{35}S- and ^{32}P- labelled proteins

Proteins labelled with radioactive [^{35}S]-L-methionine, [α-^{32}P]-orthophosphate or [α-^{32}P]-ATP were detected by conventional exposure to Kodak X-OMAT film at -70°C or using a Bio-Rad Molecular Imager® FX and associated Bio-Rad Quantity One software by phosphorimaging. Images were prepared for printing using Adobe Photoshop software.

2B7 ORF57 phosphorylation and CK2 activity

2B7.1 In vitro phosphorylation of recombinant ORF57 and pulled down and coimmunoprecipitated proteins by CK2

Approximately 10 µg (15 µl of the 50 % matrix slurry) of fusion protein beads GST-57 (FL and small) or GST alone were mixed with 3 µl of human recombinant CK2 holoenzyme from *E. coli* ($\alpha_2\beta_2$ holoenzyme from Boehringer Mannheim) in 30 µl of phosphorylation kinase buffer (20 mM Tris-HCl, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA and 100µM γ–[^{32}P] ATP (1µl) per reaction, pH 7.5). Reactions were

carried out for 30 min at 25°C either in the presence or absence of 100 μM 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB), a CK2 inhibitor that acts *in vitro* and *in vivo* (Zandomeni & Weinmann, 1984; Blaydes & Hupp, 1998). GST-ICP27 protein was used as a positive control substrate and GST protein coated beads were used as a negative control. After incubation, protein gel loading buffer was added, samples were heated for 5 min at 100°C and proteins were analysed by SDS-PAGE.

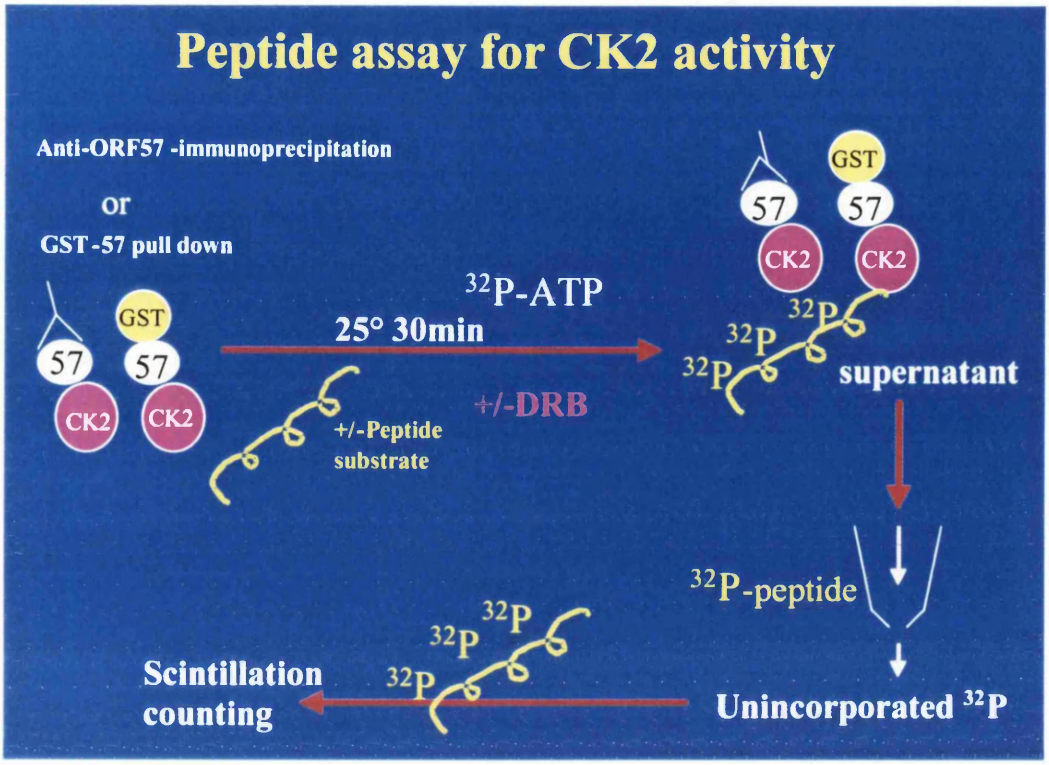
2B7.2 Peptide substrate assay for CK2 activity

CK2 activity in cell extracts was analysed after pull down with GST-57 (FL and small) or GST then determining incorporation of $^{32}PO_4$ into a CK2-specific artificial peptide substrate (Fig. 2B7.2). 75 μl of the 50% matrix slurry of GST-fusion protein (or 75μl relevant antibody coated protein-A/G beads) was added to 300 μl of cell extract and incubated 4-16 h at 4°C, with constant agitation. The matrix was washed 4x in wash buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, pH 8.0) and pulled down beads or immunoprecipitated complexes on the beads or infected whole cell extracts suspended in 30 μl CK2 reaction buffer (50 mM Tris, 20mM $MgCl_2$, 20 mM β-glycerophosphate, 0.1 mM $NaVO_3$, 2mM DTT and 10μCi γ–[^{32}P] ATP per reaction, pH 8.2), either with or without 0.1mM peptide substrate Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu (Kuenzel & Krebs, 1985). In some cases, the CK2 inhibitor DRB was added. Reactions were carried out at 25°C for 30 min, and after centrifugation the supernatant was applied to a SpinZyme™ column (Pierce), which was then washed with 75 mM phosphoric acid. Phosphorylated peptide substrate which bound to the column was detected by liquid scintillation counting (Beckman LS 5000 CE scintillation counter) and results expressed as counts per minute (cpm).

2B7.5 In vivo phosphorylation of ORF57 and its partner proteins

[^{32}P]-orthophosphate labelling was carried out either in the presence or absence of 5 and 10μM DRB. As a positive control for *in vivo* labelling of proteins both uninduced and induced cells were also labelled with [^{35}S]-L-methionine (Section 2A5.3) using 40μCi of [^{35}S]-L-methionine per 30 mm dish under identical conditions without addition of DRB. After labelling, cells were washed 3x with PBS, and soluble protein cell extracts (Section 2B3.7) were made (by adding 2x10^{7}cells/800 μl IP binding buffer) and used in immunoprecipitation assays.

Fig 2B7.2. CK2 Peptide assay



Coimmunoprecipitates (i) were incubated at 25°C for 30min in the presence of CK2 artificial peptide substrate and γ-[^{32}P]-ATP. Presence of CK2 in the coimmunoprecipitate allows phosphorylation of the peptide with γ-[^{32}P]-ATP (ii). The mixture was applied to a SpinZyme column (iii). Radioactive peptide was retained on the column. After washing the column was placed in a scintillation vial with scintillant and counted (iv). Counts per min of ^{32}P reflected phosphorylation of the peptide and therefore CK2 activity in the immunoprecipitate. Controls without peptide and with the addition of CK2 inhibitor DRB were also included. Adapted from Bryant (2000).

2B8 Localization of proteins in cells by confocal microscopy

The localisation of ORF57 and interacting partner proteins was studied by indirect immunofluorescence and confocal microscopy.

2B8.1 Immunofluorescence Assay

2B8.1.1 On mammalian cells growing in monolayers

Transfected (Section 2B3.6.1) or untransfected mammalian cells grown on 13 mm glass coverslips in 24-well Nunc Linbro plates, were washed 3x with PBS-A and fixed for 10 min at 20°C with 2% sucrose, 5% formaldehyde in PBS-A. After fixing, cells were washed 3x in PBS-A, permeabilised for 10 min at 20°C with 0.5% NP40, 10% sucrose in PBS-A and again washed 3x in PBS-A. 20µl aliquots of the relevant primary antibody diluted to the appropriate concentration (Table 2A7) in PBS A + 1% calf serum were then added to each coverslip for 60 min at 20°C. Cells were washed 3x in PBS and incubation with 20µl secondary antibody diluted appropriately in PBS-A carried out for 30 min at 20°C. For immunofluorescence and confocal microscopy, fluorescein isothiocyanate isomer (FITC), tetramethylrhodamine isothiocyanate (TRITC), cy3 or cy5 conjugated secondary antibodies were used. Cells were washed a further 3x in PBS-A before a final wash in H_2O; then they were air dried and mounted on glass slides with mounting media, Glycerol in PBS (Sigma).

2B8.1.2 On BCBL-1 and BJAB cells growing in suspension cultures

2.0 ml $2x10^6$ BCBL-1 or BJAB cells, transfected by electroporation (Section 2B3.6.2) with appropriate plasmid DNAs and induced with TPA (Section 2B3.5) or untransfected and uninduced were taken, diluted with addition of 11 ml PBS-A and pelleted at 1300 rpm (800 g in Heraeus HR400) for 5 min. The cell pellet was washed, resuspended gently and washed 2x in 13 ml PBS-A.The cell pellet was resuspended in 2 ml PBS-A and counted with Trypan blue (1:1 dilution). Cells were suspended in PBS-A + 0.5% FBS at $10x10^6$ cells/ml and spotted 2µl/well for IFA. The slides were air dried and fixed by adding to a pre-chilled bath of acetone: methanol (1:1) at -20°C for 5 min. Slides were air-dried and either used directly for permeabilisation or stored at 4°C for up to a maximum 15 days. To stain the cells with Abs for IFA, either slides stored at 4°C were incubated in PBS-A for 20 min for rehydration or fresh slides were used directly and boundaries were drawn around the wells using a hypodermic pen. Permeabilisation solution (0.2 % Triton X-100 in PBS-A) was added for 30 min at 20°C and, to avoid non-

specific binding of antibodies to antigen, cells were blocked for 15 min with PBS-A+3 % BSA. Then 12 µl primary antibody (diluted in PBS-A + 3% BSA) was added and slides incubated at 37°C in a humidified chamber for 30 min- 60 min. Cells were washed 3x in PBS-A + 0.1 % BSA and incubated with appropriate conjugated secondary antibody (either FITC, or cy5 conjugated) diluted in PBS-A+3% BSA for 30 min at 37°C in a humidified chamber. Washed in PBS-A+0.1 % BSA and finally washed once in sterile distilled water. Slides were air dried, mounted with 4 drops of Citifluor, covered with a 22x50 mm square cover slip and sealed with clear nail varnish.

2B8.2 Confocal Microscopy

Cells were examined with a Zeiss LSM 510 confocal microscope system, with 2 lasers giving excitation at 488 nm (FITC/GFP), and 633 nm (cy5) and a Zeiss Axioplan microscope using a 63X oil immersion objective lens, numerical aperture 1.4. Data were processed with LSM 510 software and then exported for preparation for printing using Adobe Photoshop.

2B9 Functional assays

2B9.1 CT3 c-fos promoter driven luciferase reporter assays for interaction of ORF57 and hnRNP K

A day prior to transfections cells were seeded at $6x10^5$ cells/well (for 293 cells) or at $4x10^5$ cells/well (for HeLa cells) in six-well dishes. At 50% confluency, cells were transfected (Section 2B3.6.1) with a plasmid DNA mixture. The DNA mixture consisted of either 0.25µg of the pGL3-CT3 wt-c-fos or pGL3-CT3mut-c-fos luciferase reporter plasmid and 0.75µg FLAG-hnRNP K expression vector and 0.85µg ORF57 expression vector (pKS3 or pKS4) per well. 10 ng of pRL-SV40 plasmid (Promega) was cotransfected to normalize transfection efficiency in a dual luciferase assay. The total amount of plasmid DNA transfected in each sample was held constant by adding the appropriate amount of the corresponding pcDNA3.1 parental plasmid without the insert, and was brought to a total of 2.0µg (for 293 cells) and 1.5µg (for HeLa cells). Cells were harvested 48 h after transfection and frozen at -70°C for at least 2 h. Luciferase activity was measured by a luminometer (Turner Designs) using the Dual Luciferase^R Reporter Assay Kit (Promega) that measures both *Firefly* and *Renilla* luciferase activities. Protein expression levels were analysed by Western blot using anti-GFP or anti-ORF57 and anti-hnRNP K antibodies. Each plasmid was tested in duplicate in one experiment and experiments were repeated at least three times. Standard deviations of the replicates are

shown as error bars.

2B9.2 CAT assays to test the role of ORF57 in nuclear RNA export

2B9.2.1 ORF57 used directly as an RNA export factor in transient transfection assays

An ORF57 protein expression vector was cotransfected with a reporter plasmid (pCMV128 CAT-RRE) encoding an inefficiently spliced CAT transcript into 293 and 293-T cells at $6x10^5$ cells/well. The transfected DNA mixture consisted of 0.25μg CAT reporter plasmid, 0.5 μg GFP-gORF57 or GFP-cORF57. Transfection efficiency as β-galactosidase (β-gal) expression was determined by including 0.5 μg of pCH110 plasmid (Pharmacia). The total amount of GFP coding DNA transfected in each sample was held constant by adding the appropriate amont of the parental plasmid without the insert (EGFP-C1), and total DNA concentration was brought to 2.0 μg by adding pcDNA3.1 empty vector (Invitrogen) as necessary. Cells were harvested 48 h after transfection and CAT activity was measured. Protein expression levels were analysed by Western blot using anti-GFP or anti-HA antibodies. Each experiment was repeated at least three times in duplicate. Standard deviations of the replicates are shown as error bars.

2B9.2.2 ORF57 used as a RevM10- fusion for tethering directly onto RNA export-cargo in transient transfection assays

ORF57 was fused in frame with pCMV-RevM10 at the C-terminus. The HIV RevM10 protein export deficient mutant is still capable of binding to the HIV RRE present within an inefficiently spliced CAT transcript in pCMV128. The transfected DNA mixture consisted of 0.25 μg of CAT reporter plasmid pCMV128, 0.5 μg of plasmid pCMV-RevM10-cORF57 and/or 0.5 μg of plasmids pEGFP-C1-gORF57, pEGFP-C1-TAP, pEGFP-N3zzp15 and/or pRCMV-REF2-II. Transfection efficiency was determined by including 0.5 μg of pCH110 plasmid. Plasmids pCMV-Rev and pCMV-RevM10 were used as positive and negative controls respectively. Again, the total amount of plasmid DNA transfected in each sample was held constant by adding appropriate amounts of the pEGFP-C1 parental plasmid and final DNA concentration was brought to a total of 2.5 μg by adding pcDNA3.1 empty vector. To examine the route for RNA export, leptomycin B was added at 5 nM 12 h after the transfections and cells were harvested at 48 h.

2B9.2.3 Measurement of CAT activity (diffusion based)

CAT activity was measured according to the published method (Morency *et al.*, 1987; Braun *et al.*, 2001). In brief, monolayers were washed with PBS-A, and 600 μl TEN

buffer (40 mM Tris-HCl pH 7.5, 1 mM EDTA, 150 mM NaCl) was added. The cells were scraped, spun down and the pellet was resuspended in 400 µl of 100 mM Tris (pH 7.8). The cells were stored at -70°C or directly lysed by freezing/thawing. After centrifugation at 12,000 rpm for 10 min at 4°C, the supernatant was used for β-gal and CAT assays. Only CAT assay samples were heated at 70°C for 10 min to inactivate endogenous cell acetylases. Fresh reaction cocktail (100µl/sample- 100 mM Tris pH 7.8, 5 mg/ml-chloramphenicol (Boehringer Mannheim), 3 µl/ml- acetyl-1-^{3}H Coenzyme A (50 µCi, 200 mCi/mmol, Dupont) was mixed with 100 µl heat inactivated cell extract in a scintillation vial, overlayed with 3 ml Econofluor (Dupont), and vials were capped and incubated at 37°C. Counts (cpm) were taken by liquid scintillation counter (Beckman LS 5000 CE). Values of CAT activity were normalised for transfection efficiency by comparing with the β-gal assay values for the same samples.

2B9.2.4 Measurement of β-gal activity

Cotransfection of pCH110 plasmid (Pharmacia) with CAT reporter gene plasmid provides cell extracts that can be assayed simultaneously for both CAT and β-gal activities. Samples obtained as above were treated using β-Galactosidase Enzyme Assay System (Promega) in a 96-well plate assay and activity was measured by absorbance of the samples (comparing with β-Galactosidase standards) at 405nm in a plate reader (spectrophotometer).

2B9.3 ORF50 promoter driven luciferase reporter assays to examine the functional significance of the ORF57+ORF50 interaction

2B9.3.1 Transient transfection assays

To map the ORF50 promoter various lengths of the region upstream of the ORF50 translation initiation codon were cloned in front of *Firefly* luciferase reporter gene into the pGL3-Basic vector (D.J. Blackbourn, unpublished data). 293 cells were transfected with 0.25µg of ORF50 promoter plasmids (containing lengths ranging from -250bp to -3000bp of 5' upstream DNA and designated as pORF50- length of the ORF50 promoter region in bp), 10 ng of pRL-SV40 control plasmid, increasing amounts of ORF57 (pKS3, pcDNA4-gORF57 or pcDNA-cORF57), with or without ORF50 as indicated. The total amount of DNA in each transfection was kept constant by adding empty pEGFP-C1 or CMV expression plasmid (pcDNA vector).

In some experiments, a promoter containing interferon stimulatory response element linked to luciferase (pISRE) was used to test the specificity of ORF57+ORF50 effects on a heterologous promoter. Interferon-alpha (IFN-α) was added to the pISRE transfected cells at 200 units/ml 5 h post-transfection. In the assays, final luciferase activity derived from the reporter plasmids was determined after normalizing firefly luciferase activity to Renilla luciferase activity from a cotransfected pRL-SV40 control. All experiments were performed in duplicate and repeated at least three times. Standard deviations of the replicates are shown as error bars.

Results and Discussion

Chapter 3: Protein: protein interactions of ORF57

This project was aimed at identifying cellular and viral gene products which interact with the ORF57 protein and thus provide more information about the roles played by ORF57 in the KSHV life cycle. Known cellular protein partners of the alphaherpesvirus HSV-1 ICP27 protein, previously identified in our laboratory by yeast-two-hybrid assay and then confirmed by protein binding assays and coimmunoprecipitations, were tested for interaction with KSHV ORF57 protein to assess the conservation of these interactions, thus examining the possibility of a similar role played by these homologous herpesvirus proteins. There are various ways to investigate protein: protein interactions; to confirm the probable interactions and to reveal interacting partners of ORF57, fusion protein pull down assays and coimmunoprecipitations were used in this study.

For ICP27, cellular partner proteins identified in yeast two-hybrid screen performed by (Wadd, 2000) confirmed by pull downs and immunoprecipitations are shown in Table 3.

Protein interacting with ICP27 in the Yeast-2-hybrid (Y-2-H) screen	**Clones showing sequence homology to the proteins in Y-2-H screen**
hnRNP K	1
CK2β subunit	51
Aly I/ REF	4
SAP145	7
p32	6
other proteins	7

Table 3. Using ICP27 (containing aa's 10-512) as a bait, 2.3 x 10^6 individual clones were screened from a HeLa cell library. Out of total 76 clones, different partner proteins were identified on the basis of sequence homology (Wadd, 2000).

My project involved the confirmation and characterisation of the cellular protein interactions of ORF57, and also examining the interaction of ORF57 with another viral protein ORF50 in TPA-induced KSHV-infected cells. The profile of interacting proteins

produced by the GST pull down assays and coimmunoprecipitations is presented here.

3.1 Expression kinetics of ORF57 in TPA-induced BCBL-1 cells

Western blotting of TPA-induced BCBL-1 at various times after TPA induction with anti-ORF57 Ab showed that ORF57 protein is expressed as early as 2-4 h post-TPA induction (Fig 3.1 see lanes 3 & 4) although non-specific minor bands are also present in uninduced and TPA induced cells (lanes 1-12). Up to 48 h, anti-ORF57 Ab interacted with one major band of ~50-52 kDa, corresponding to ORF57 in TPA-induced cells (compare lane 7 with 9 and 11 with 10), between 48 and 72 h another faster migrating band of ~48-50 kDa appeared (compare lanes 11 with 13, 15 & 16). Thus it appears that at late times ORF57 is processed/cleaved to generate a smaller product. Anti-ORF57 Ab did not interact with the major protein band in uninduced BCBL-1 cell extracts (lanes 1, 9, 10 & 12) and the top bands seen in lane 12 is due to spillage of some TPA-induced extract from lane 13 to lane 12 during loading the samples on gel.

3A Pull down assays

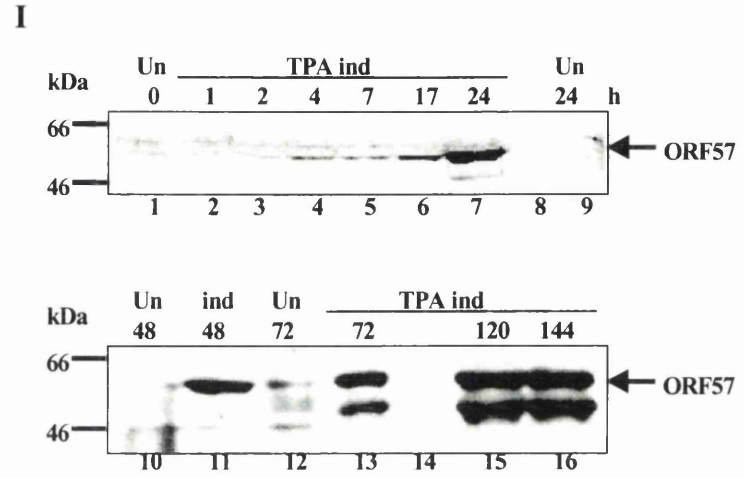
3A1 Expression of ORF57 synthesised *in vitro by* transcription/translation

^{35}S-labelled ORF57 protein synthesized *in vitro* for protein interaction experiments was obtained using pCITE-4b-ORF57 (FL) and pGBKT7-ORF57 (FL and small) recombinants (Section 2B4.4). Using pCITE-ORF57 recombinant a single band of 63 kDa was obtained (approximately 12 kDa larger than full-length ORF57 protein due to the N-terminal tag's fusion) that was recognised by Western blotting (Fig. 3A1 Panel I lane 3) using anti-ORF57 Ab (Table 2A7). The predicted size of ORF57 produced *in vitro* is same as the expected size of ORF57 protein band in TPA-induced BCBL-1 cells. Using pGBKT7-ORF57 recombinants, ^{35}S-labelled-ORF57 (FL and small) protein was synthesized *in vitro* and analysed by phosphorimaging and autoradiography (Sections 2B6.1 and 2B6.4) as shown in Fig. 3A1 Panel II lanes 1-6.

3A2 Expression of fusion protein GST-57 in *E.coli*

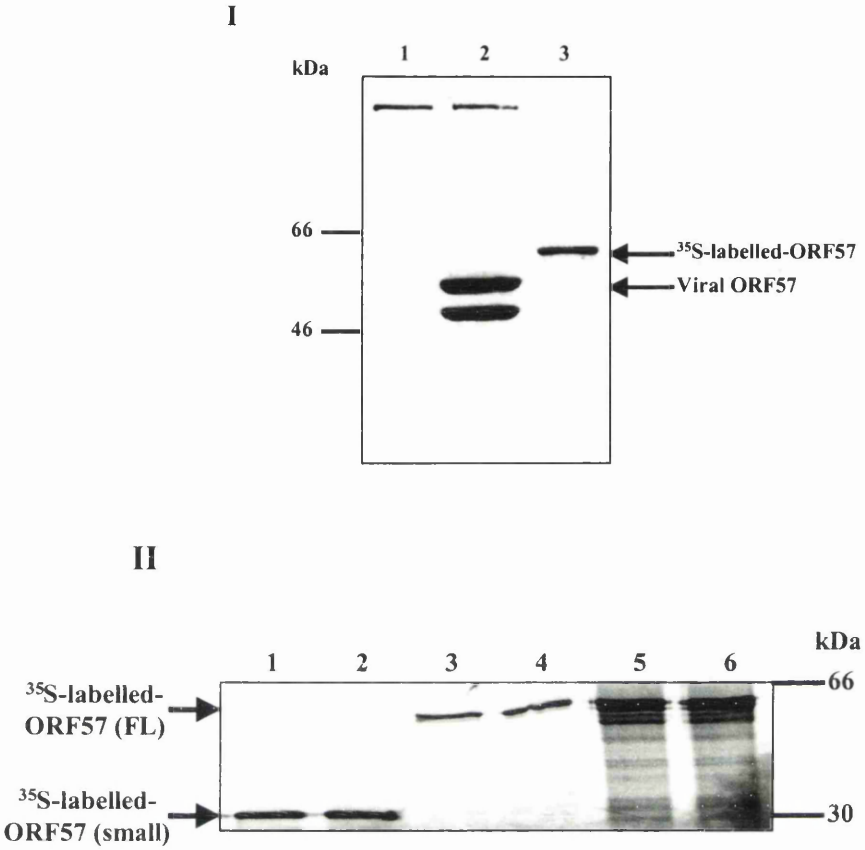
To produce ORF57 as a GST fusion protein, a protease deficient strain of *E.coli*, (BL21) was used to minimise proteolytic degradation. Protein expression from the pGEX vector is under the control of the *tac* promotor. To achieve significant expression of full-length GST-57 fusion protein each of the variables affecting expression was altered in turn to increase the amount of full-length product (see Section 2B4.1 and 2B4.2). After optimisation, a significant amount of full-length GST-ORF57 plus various truncation

Fig 3.1. ORF57 is expressed early between 2-4 h post TPA induction, after 72 h a second faster migrating band appears



Panel I. Western blot for ORF57 with anti-ORF57 Ab of TPA-induced BCBL-1 cells at various times after TPA induction (1 to 144 h) and uninduced BCBL-1 cell extracts showing that ORF57 protein is expressed as early as 2-4 h post-TPA induction. BCBL-1 suspension cultures, grown in 30 mm dishes, almost reaching confluency ($1x10^6$/ml), were split at $0.2x10^6$ cells/ml (1:5) and induced using TPA at 20ng/ml final concentration on the same day or left uninduced. Samples were collected at indicated time points post-induction, cells were pelleted, lysed in SDS loading buffer, separated by SDS-PAGE, and analysed by Western blotting with anti-ORF57 or anti-ORF50 polyclonal antibody. Samples used in **lanes: 1.** 0 h uninduced BCBL-1 extract **2.** 1 h TPA-induced BCBL-1 extract **3.** 2 h TPA-induced BCBL-1 extract **4.** 4 h TPA-induced BCBL-1 extract **5.** 7 h TPA-induced BCBL-1 extract **6.** 17 h TPA-induced BCBL-1 extract **7.** 24 h TPA-induced BCBL-1 extract **8.** empty **9.** 24 h uninduced BCBL-1 extract **10.** 48 h uninduced BCBL-1 extract **11.** 48 h TPA-induced BCBL-1 extract **12.** 72 h uninduced BCBL-1 extract **13.** 72 h TPA-induced BCBL-1 extract **14.** empty **15.** 120 h TPA-induced BCBL-1 extract **16.** 144 h TPA-induced BCBL-1 extract.

Fig 3A1. Expression of in vitro transcribed-translated 35S-labelled-ORF57 (FL and small)

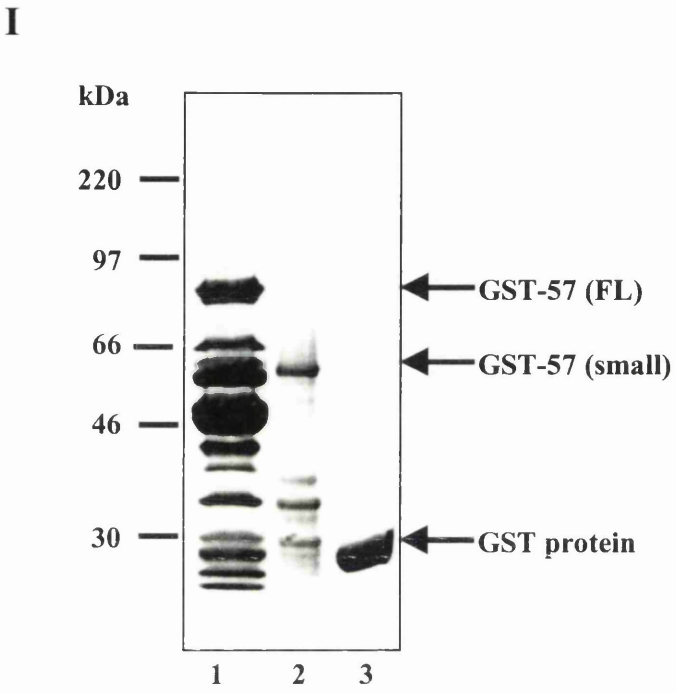


^{35}S-labelled ORF57 proteins (ORF57 FL [aa 1-455] and ORF57 small [N-terminal deletion, aa 181-455]) were produced using the *in vitro* transcription/translation TNT kit (Promega) as per the manufacturer's instructions using 1-5 μg of ORF57 (FL and small) template cDNAs. Aliquots of expressed proteins were mixed with SDS loading buffer, separated by SDS-PAGE. Gel was either dried and exposed to phosphorimager screen for analysis of labelled proteins or transferred to nitrocellulose membrane and Western blotted for ORF57 using anti-ORF57 Ab.

Panel I. Western blot for ORF57 with anti-ORF57 Ab. ^{35}S-labelled-ORF57 protein was synthesised *in vitro* using pCITE-4b-ORF57 (FL) recombinant and analysed by Western blotting. Shown in **lanes: 1.** Uninduced BCBL-1 cell extract **2.** TPA-induced BCBL-1 cell extract **3.** *In vitro* synthesised ^{35}S-labelled-ORF57 protein from pCITE-ORF57 (FL).

Panel II. Phosphorimager analysis showing *in vitro* synthesised ^{35}S-labelled-ORF57 protein (FL and small). ^{35}S-labelled-ORF57 protein was synthesised *in vitro* using pGBKT7-ORF57 (FL and small) recombinants and analysed by phosphorimaging and autoradiography. Shown in **lanes: 1.** *In vitro* synthesised ^{35}S-labelled-ORF57 from pGBKS-ORF57 (small) **2.** ^{35}S-labelled-ORF57 from pGBKS-ORF57 (small) **3.** ^{35}S-labelled-ORF57 from pGBKS-ORF57 (FL) **4.** ^{35}S-labelled-ORF57 from pGBKS-ORF57 (FL) **5.** ^{35}S-labelled-ORF57 from pCITE-ORF57 (FL) **6.** ^{35}S-labelled-ORF57 from pCITE-ORF57 (FL).

Fig 3A2. Expression of GST-57 (FL and small) and GST protein



Panel I. GST-57 and GST protein expression: Coomassie blue stained gel showing GST-57 (FL and small) fusion proteins and GST bound onto glutathione beads. GST-fusion proteins were expressed in *E.coli* strain BL21 and bound to GST-beads. Aliquots of expressed proteins were mixed with SDS loading buffer, separated by SDS-PAGE. Gel was stained with Coomassie blue. The smaller bands in lanes 1 and 2 represent degradation products/truncations of GST-57 (FL and small respectively). Shown in **lanes: 1.** GST-57 full length (FL) **2.** GST-57 (small) **3.** GST alone.

products was detectable by Coomassie brilliant blue staining (Fig. 3A2 Panel I lane 1). GST-57 small was also expressed (Fig. 3A2 Panel I lane 2) and both were used in pull down assays.

3A3 Proteins from ^{35}S-labelled extracts of BCBL-1 cells pull down by GST-57

GST-57 or GST fusion proteins bound to glutathione beads were mixed with ^{35}S-labelled TPA-induced BCBL-1 cell extracts (Sections 2B3.5 and 2B3.7), beads washed and bound proteins analysed by SDS-PAGE. A typical ^{35}S-labelled profile obtained is shown in Fig. 3A3 Panel I lanes 1 & 2. Arrowed bands of ~180 kDa, 150 kDa, 110 kDa 85 kDa, 70 kDa, 50 kDa, 44-42 kDa (doublet), 35 kDa, 30 kDa, 25 kDa and 20 kDa strongly interacting with GST-57 (lane 1) and less or not with the control protein GST alone (lane 2) are shown.

Known sizes of these proteins in the light of ICP27 interactions gave some idea about their identities and these were tested individually by Western blotting. The largest band ~150 kDa could be SAP145, another band ~110 kDa could be ORF50, a band ~70 kDa could be hnRNP K, ~50 kDa could be ORF57 itself, 44-42 kDa could be CK2 α and α' subunits, ~30 kDa could be REF and the small band ~25 kDa could be the CK2 β subunit. Each of these possibilities is discussed separately in the following Chapters. This left proteins of ~180 kDa, and ~85 kDa, ~35 kDa and ~20 kDa still unidentified for which there are no probable candidates yet.

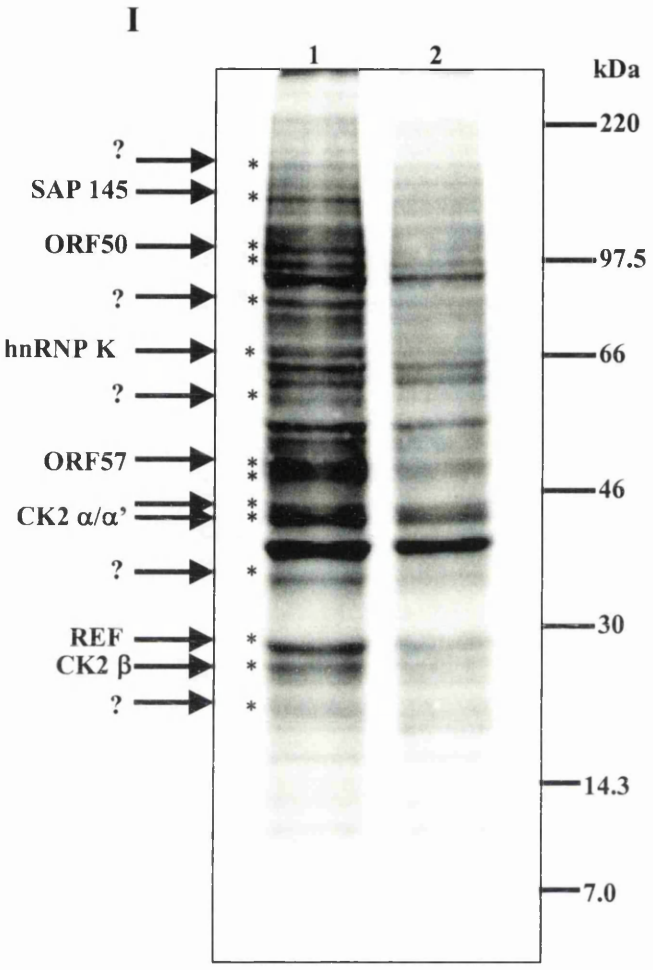
3B Coimmunoprecipitation assays

Because of their high specificity, antibodies may be used to isolate specific antigens and other proteins which interact with these from complex protein mixtures such as cell lysates. Identification of the immunoprecipitated antigen and proteins which co-purify with the target antigen can be achieved by separation of the complex on an SDS-PAGE gel, followed by detection of radiolabelled proteins by autoradiography or non-labelled proteins by Western blotting.

3B1 ORF57 interacting proteins from KSHV positive BCBL-1 cells in a coimmunoprecipitation assay using anti-ORF57 peptide Ab

Anti-ORF57 peptide polyclonal antibody (Table 2A7) was used to immunoprecipitate ^{35}S-labelled proteins from uninduced or TPA-induced BCBL-1 cell extracts to obtain the profile of interacting proteins. Proteins were analysed by SDS-PAGE and visualised by phosphorimage analysis and autoradiography. Samples were also Western blotted with

Fig 3A3. GST-57 pull down profile from ^{35}S-labelled BCBL-1 cell extracts



Panel I. Phosphorimager analysis showing [^{35}S]-methionine radiolabelled proteins pulled down by GST-57 and GST alone from TPA-induced BCBL-1 cell extracts. GST-57 and GST proteins were expressed and bound onto glutathione beads, after washing with PBS a pull down assay was performed with ~200μg [^{35}S]-labelled TPA-induced BCBL-1 cell extracts. The pulled down complex formed was separated on a 10% SDS-PAGE gel, dried down and exposed to a phosphorimaging plate overnight. Bands corresponding to proteins interacting specifically with GST-57 are arrowed (lane 1). Pull downs included in **lanes: 1.** TPA-induced BCBL-1 cell extracts + GST-57 (FL) **2.** TPA-induced BCBL-1 cell extracts + GST alone.

anti-ORF57 Ab. Several proteins were obtained in the immunoprecipitation profile (Fig. 3B1 Panel I). As they are of known size and unknown identity screening with plausible candidate antibodies should help identify them. Figure 3B1 Panel I shows a typical ^{35}S-labelled profile of the immunoprecipitates obtained from uninduced and TPA-induced BCBL-1 cell extracts (lanes 1 & 2). Proteins can be seen as bands in the TPA-induced extract lane which are absent or less intense in the uninduced extracts and induced extracts immunoprecipitated with pre-immune serum. Cell extracts were prepared by labelling from 0-24 h post-TPA induction, to ensure that both viral and cellular proteins were labelled.

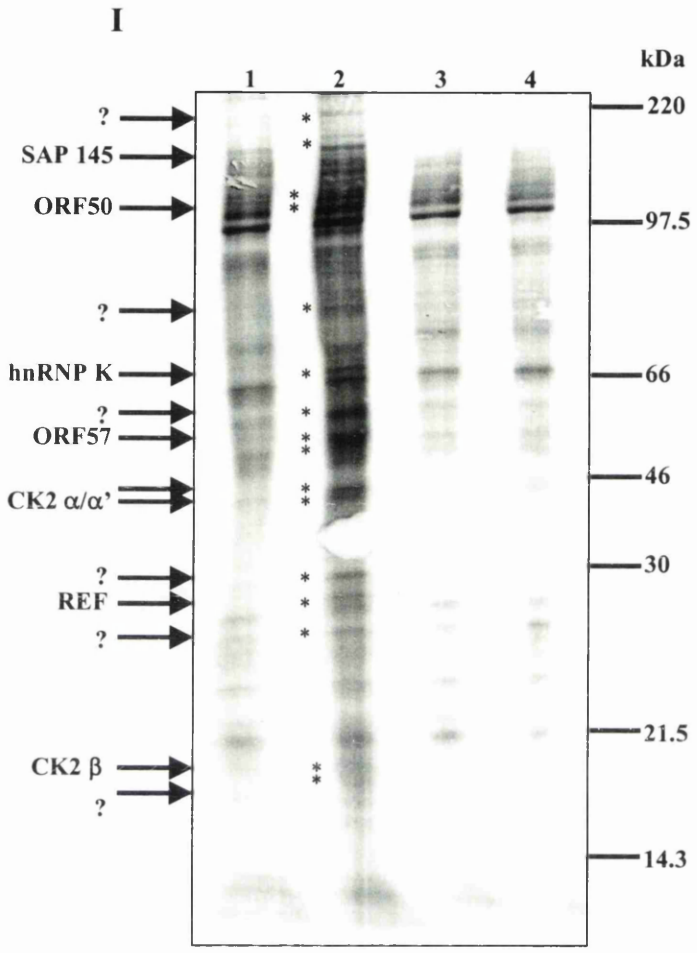
Bands corresponding to proteins of approximately 180 kDa, 150 kDa, 110 kDa, 85 kDa, 70 kDa, 50 kDa, 44 kDa, 35kDa, 30 kDa 25 kDa and 20 kDa appeared to specifically coprecipitate with ORF57 (lane 2). Educated guesses based on size still left proteins of ~180 kDa, and ~85 kDa, ~35 kDa and ~20 kDa for which no probable candidate proteins are available yet.

Western blotting using anti-ORF57 Ab on the same extracts showed ORF57 present in input TPA-induced and absent from uninduced BCBL-1 cell extracts (Figure 3B1 Panel II lanes 1 & 2). Because of the size of ORF 57 protein (~50-52 kDa) and availability of only one species of antiserum (rabbit) it was difficult to show that ORF57 was present in coimmunoprecipitates from TPA-induced cell extracts. The heavy chain of the antibody added to immunoprecipitates is of the same size as ORF57 which in Western blots gives a stronger signal than ORF 57 band in input TPA-induced cell extracts (due to its interaction with the anti-rabbit secondary antibody) thus masking the presence of an ORF57 band in immunoprecipitates (see Fig. 3B1 Panel III lanes 3 to 6).

When the ^{35}S-labelled profile obtained in GST pull downs (Fig. 3A3 Panel I) was compared to the coimmunoprecipitations profile (Fig. 3B1 Panel I) the sizes of interacting proteins were quite similar. The GST pull down assay could be scaled up more easily than the coimmunoprecipitations for the identification of interacting candidate proteins.

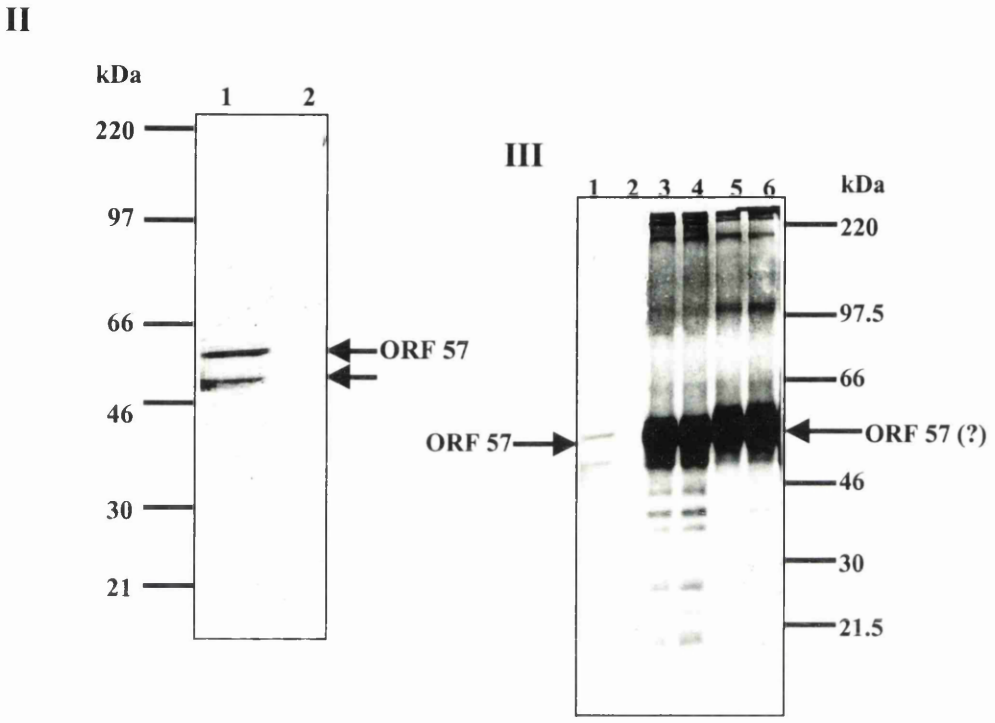
The coimmunoprecipitation technique was used with Ab to another IE lytic viral protein (ORF50) on the same BCBL-1 cell extracts. Anti-ORF50 Ab gave a prominent band of ~110 kDa corresponding to ORF 50 in the Western blots from TPA-induced

Fig 3B1. Anti-ORF57 Ab coimmunoprecipitation profile from ^{35}S-labelled BCBL-1 cells



Panel I. Phosphorimager analysis showing [^{35}S]-labelled proteins coimmunoprecipitated from uninduced and TPA-induced BCBL-1 cell extracts using anti-ORF57 Ab. A coimmunoprecipitation assay was performed with anti-ORF57 Ab or pre immune serum and [^{35}S]-labelled uninduced and TPA-induced BCBL-1 cell extracts. The complex formed was separated on a 10% SDS-PAGE gel, dried down and exposed to a phosphorimaging plate overnight. Immunoprecipitations included in **lanes: 1.** Uninduced BCBL-1 extract + anti-ORF57 Ab **2.** TPA-induced BCBL-1 extract + anti-ORF57 Ab **3.** Uninduced BCBL-1 extract + Pre-immune serum **4.** TPA-induced BCBl-1 extract + Pre-immune serum.

Fig 3B1. ORF57 protein is present in TPA-induced BCBL-1 cell extracts used for coimmunoprecipitations



A coimmunoprecipitation assay was performed with anti-ORF57 Ab or pre immune serum and [^{35}S]-labelled uninduced and TPA-induced BCBL-1 cell extracts, the complex formed was separated on SDS-PAGE gel, transferred to nitrocellulose membrane and Western blotted for ORF57 using anti-ORF57 Ab.

Panel II. Western blot for ORF57 with anti-ORF57 Ab of BCBL-1 cell extracts showing that ORF57 protein is present in TPA-induced BCBL-1 cell extracts used for coimmunoprecipitations. Aliquots of the input BCBL-1 cell extracts were run on a 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted for ORF57. Samples used in **lanes: 1**. Input TPA-induced BCBL-1 extract **2.** Input uninduced BCBL-1 extract.

Panel III. Western blot for ORF57 with anti-ORF57 Ab showing that in Western blots ORF57 protein from anti-ORF57 Ab (rabbit) coimmunoprecipitates is masked by anti-rabbit secondary Ab. Aliquots of the input BCBL-1 cell extracts and anti-ORF57 Ab coimmunoprecipitates were run on a 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted for ORF57 using anti-ORF57 Ab. Immunoprecipitations included in **lanes: 1.** Input TPA-induced BCBL-1 extract **2.** Input uninduced BCBL-1 extract **3.** TPA-induced BCBL-1 extract+ pre immune serum **4.** Uninduced BCBL-1 extract + pre immune serum **5.** TPA-induced BCBL-1 extract + anti-ORF57 Ab **6.** Uninduced BCBL-1 extract + anti-ORF57 Ab.

immunoprecipitates and whole cell BCBL-1 cell extracts. With anti-ORF57 Ab ORF50 protein was precipitated as confirmed by Western blotting using anti-ORF50 Ab (see Chapter 8).

3C Self-interaction of ORF57

A dominant negative phenotype can arise when one wt and one mutant protein interact to form a non-functional dimer, for example with KSHV ORF50, HSV-1 ICP4 or HIV Rev (Shepard *et al.*, 1990; Hope *et al.*, 1992; Lukac *et al.*, 1999). HSV-1 ICP27 can produce a *trans* dominant negative phenotype during viral infection and in transfection experiments due to mutations in the activation domain (Smith *et al.*, 1991) and forms dimers in solution (Wadd *et al.*, 1999; Zhi *et al.*, 1999). A radiolabelled band of ~50kDa was pulled down from TPA-induced BCBL-1 cell extracts by GST-57 and not by GST alone (Fig. 3A3 Panel I) suggesting the possibility that this could be ORF57 interacting with GST-57, and that ORF57 may form dimers or multimers.

The GST-57 pull down assay was performed using ^{35}S-labelled *in vitro* transcribed-translated ORF57 protein. Bands corresponding to ^{35}S-labelled *in vitro* synthesised ORF57 (FL) and (small) were eluted from GST-57 (FL) whereas nothing bound to GST alone (Fig. 3C Panel I compare lanes 1 & 5 with 2 & 4). Thus ORF57 is capable of interacting with itself and the interaction domain appears to lie at its C terminus as GST-57 (small) was also capable of binding to ^{35}S-labelled-ORF57 (small) and not to GST alone (Fig. 3C Panel II compare lanes 3 with 2).

GST protein itself has been reported to exist as a dimer in solution (Warholm *et al.*, 1983) but the interaction detected here was between GST-57 and ^{35}S-labelled-ORF57 protein synthesised *in vitro* without any GST-tag, thus this dimerisation could not be due to a GST-GST interaction.

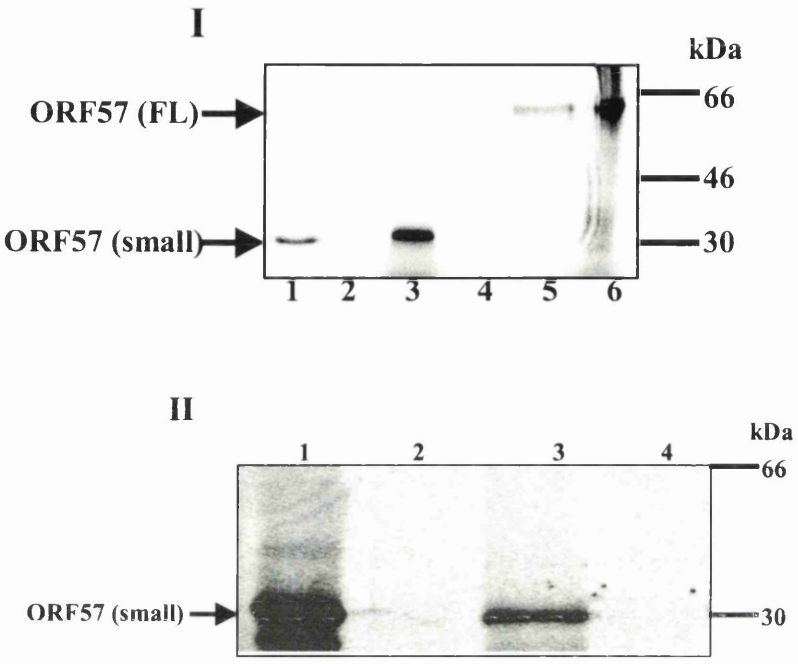
3D ORF57 does not interact with ICP27

Since ICP27 has been shown to self-interact in the solution (Smith *et al.*, 1991) and forms dimers in solution (Wadd *et al.*, 1999; Zhi *et al.*, 1999) and ORF57 also interacted with itself, pull down assays were performed to determine if they could interact with each other. ICP27 and ORF57 share approximately 35% sequence homology and in functional assays ORF57 was shown to partially substitute for ICP27 (Silverstein S., personal communication). An *in vitro* pull down assay was performed using GST-57 (FL or small)

with GST-ICP27 or GST alone, mixed either with ^{35}S-labelled *in vitro* synthesised ORF57 (FL and small) or ICP27 (Section 2B4.3).

On phosphorimager analysis, a band corresponding to labelled ORF57 (FL) was eluted from GST-57 (FL) (Fig. 3D Panel I lane 6) and labelled ORF57 (FL) was eluted from GST-57 (small) (Fig. 3D Panel I lane 8). Neither GST-57 (FL) nor GST-57 (small) showed any binding to ^{35}S-labelled-ICP27 (compare with lanes 3 & 4) and nothing bound to GST alone (lanes 7 & 9). GST-ICP27 did not bind either ^{35}S-labelled ORF57 (FL) or ^{35}S-labelled ORF57 (small) (lanes 10 & 11) but did pull down labelled ICP27 (lane 5).

Fig 3C1. ORF57 interacts with itself in GST fusion protein binding assays

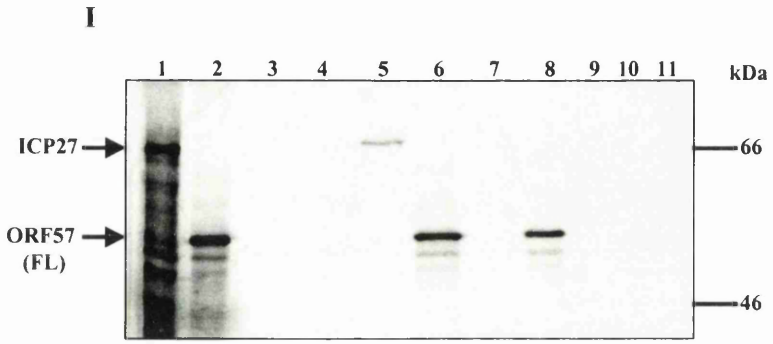


GST-57 (FL and small) fusion proteins and GST bound onto glutathione beads were incubated with ^{35}S-labelled-ORF57 (FL and small), after washing, specifically bound proteins were eluted with a high salt wash and separated on SDS-PAGE gel, gel was dried and analysed by phosphorimaging.

Panel I. Phosphorimager analysis showing that GST-57 (FL) binds both full length and small (an N-terminal deletion fragment) ORF57. Pull downs included in **lanes: 1.** GST-57 (FL) + ^{35}S-labelled-ORF57 (small) **2.** GST alone + ^{35}S-labelled-ORF57 (small) **3.** Input ^{35}S-labelled-ORF57 (small) **4.** GST alone + ^{35}S-labelled-ORF57(FL) **5.** GST-57 (FL) + ^{35}S-labelled-ORF57 (FL) **6.** Input ^{35}S-labelled-ORF57 (FL).

Panel II. Phosphorimager analysis showing that ORF57 self interaction involves the C terminus as GST-57 (small) was capable of binding to ORF57 small (an N-terminal deletion fragment of ORF57). Pull downs included in **lanes: 1.** Input ^{35}S-labelled-ORF57 (small) **2.** GST alone + ^{35}S-labelled-ORF57 (small) **3.** GST-57 (small) + ^{35}S-labelled-ORF57 (small) **4.** GST alone + ^{35}S-labelled-ORF57 (FL).

Fig 3D. ORF57 interacts with itself but not with HSV-1 ICP27



Panel I. Phosphorimager analysis showing that ORF57 interacts with itself but not with HSV-1 ICP27. GST-57 (FL and small) and GST-ICP27 fusion proteins or GST bound onto glutathione beads were incubated with ^{35}S-labelled-ORF57 (FL and small) and ^{35}S-labelled-ICP27, after washing, specifically bound proteins were eluted with a high salt wash and separated on SDS-PAGE gel, gel was dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** Input ^{35}S-labelled-ICP27 **2.** Input ^{35}S- labelled-ORF57 (FL) **3.** GST-57 (FL) + ^{35}S-labelled-ICP27 **4.** GST-57 (small) + ^{35}S-labelled-ICP27 **5.** GST-ICP27 + ^{35}S-labelled-ICP27 **6.** GST-57 (FL) + ^{35}S-labelled-ORF57 (FL) **7.** GST alone + ^{35}S-labelled-ORF57 (FL) **8.** GST-57 (small)+ ^{35}S- labelled-ORF57 (FL) **9.** GST alone + ^{35}S-labelled-ICP27 **10.** GST-ICP27 + ^{35}S-labelled-ORF57 (FL) **11.** GST-ICP27 + ^{35}S-labelled-ORF57 (small).

Chapter 4: Interaction of ORF57 with hnRNP K

Using the yeast-2-hybrid system, *in vitro* binding and immunoprecipitation assays (Wadd *et al.*, 1999) showed that ICP27 interacts with hnRNP K. I therefore investigated if ORF 57 behaves in a similar fashion. This Chapter presents data, which shows that hnRNP K interacts with ORF57 *in vitro* and in virus-infected cells. Experiments were aimed at determining the implication of this interaction for the virus life cycle.

HnRNP K, first identified as a component of the hnRNP particle (Matunis *et al.*, 1992) was an interesting candidate for interaction with ORF57 because it has similar properties to ICP27 for example, at the post-transcriptional level in RNA binding and shuttling from the nucleus to the cytoplasm, and also at transcriptional level in the regulation of gene expression (see Introduction and Discussion Sections).

4A ORF57 and hnRNP K

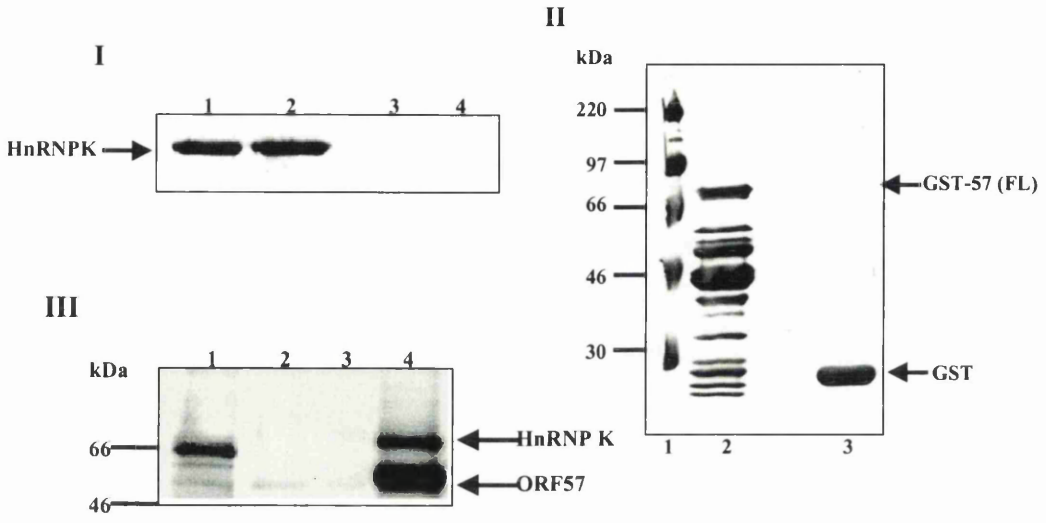
4A1 Interaction of ORF57 with hnRNP K

4A1.1 ORF57 interacts with hnRNP K in the GST pull down assay

A GST pull down experiment, using GST-57 (FL) or GST alone with protein from induced or uninduced BCBL-1 cell extracts (Section 2B4.3) followed by Western blotting (Section 2B6.2 & 3) with hnRNP K polyclonal antiserum (against a synthetic peptide representing C-terminal aa 452-464 conserved in murine and human hnRNP K, Table 2A7) showed that GST-57 and hnRNP K interact, whereas GST and hnRNP K do not (Fig. 4A1.1 Panel I compare lanes 1 & 2 with 3 & 4). The fusion proteins used were visualised by Coomassie brilliant blue staining (Fig. 4A1.1 Panel II) and approximately equal protein amounts were used. Extracts from ^{35}S-labelled TPA-induced BCBL-1 cell extracts were pulled down by GST-57 or GST alone and interaction detected by phosphorimager analysis (Section 2B6.4) (Fig. 4A1.1 Panel III). A band of ~65-70 kDa corresponding in size to hnRNP K in whole cell extract was visible in lanes 1 & 4 and GST-57 pulled down ORF57 only in lane 4. GST alone did not pull down either hnRNPK or ORF57 (lanes 2 & 3).

Thus, GST-57 pulled down hnRNP K from BCBL-1 cell extracts; under these conditions hnRNP K is present in its native conformation and concentration and was not added artificially as a fusion protein.

Fig 4A1.1. GST-57 pulls down hnRNP K from BCBL-1 cell extracts



Panel I. Western blot for hnRNP K with anti-hnRNP K Ab showing that GST-57 pulls down hnRNP K from TPA-induced and uninduced BCBL-1 cell extracts. TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST or GST-57 proteins. After incubation with extracts, proteins pulled down were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose and Western blotted for hnRNP K using anti-hnRNP K Ab. Pull downs included in **lanes: 1.** TPA-induced BCBL-1 extract + GST-57 **2.** Uninduced BCBL-1 extract +GST-57 **3.** TPA-induced BCBL-1 extract + GST **4.** Uninduced BCBL-1 extract + GST.

Panel II. Expression of GST-57 and GST proteins: Coomassie brilliant blue stained gel. GST-57 and GST proteins used were run on a 10% SDS-PAGE gel and visualised by Coomassie staining. The smaller bands in lane 2 represent degradation products/truncations of GST-57. Shown in **lanes: 1.** Protein gel marker **2.** GST-57 **3.** GST alone.

Panel III. Phosphorimager analysis showing that GST-57 pulls down hnRNP K and ORF57 from TPA-induced and uninduced and TPA-induced BCBL-1 cell extracts respectively. TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST or GST-57 proteins. After incubation with extracts, proteins pulled down were separated by SDS-PAGE electrophoresis, dried down and exposed to a phosphorimaging plate overnight. Pull downs included in **lanes: 1.** GST-57 + 35S-labelled uninduced BCBL-1 cell extracts **2.** GST alone + 35S-labelled uninduced BCBL-1 cell extracts **3.** GST alone + 35S-labelled TPA-induced BCBL-1 cell extracts **4.** GST-57 + 35S-labelled TPA-induced BCBL-1 cell extracts.

4A1.2 hnRNP K also interacts with ORF57 in the GST pull down assay

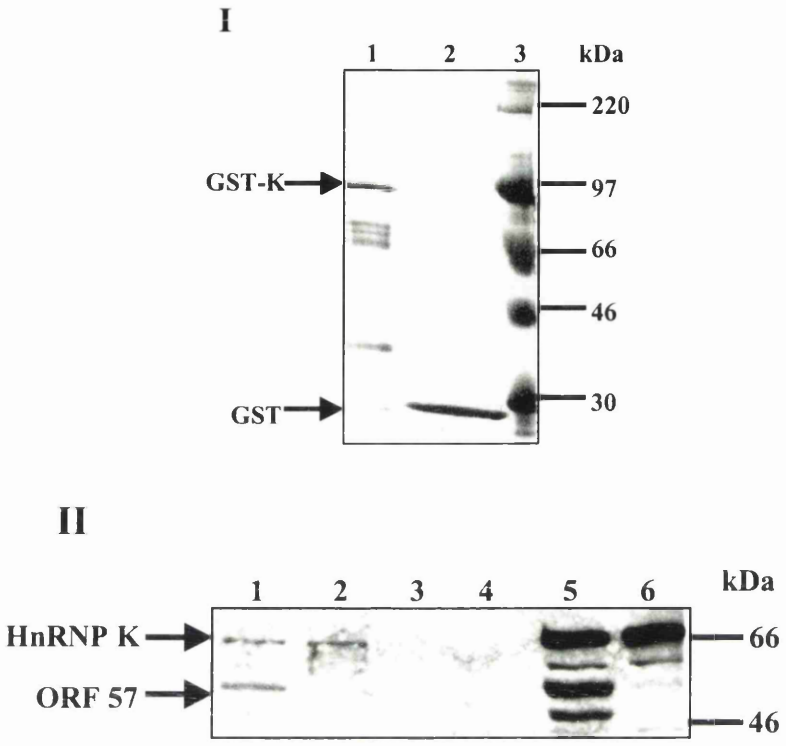
GST-K (full-length) or GST alone bound to glutathione Sepharose beads was used to pull down interacting proteins from ~200μg protein from TPA-induced or uninduced BCBL-1 cell extracts and anti-ORF57 Ab was used in immunoblotting. Fusion proteins used were visualised by Coomassie brilliant blue staining (Fig. 4A1.2 Panel I). In TPA-induced BCBL-1 cell extracts with GST-K, a band corresponding to ORF 57 (~50-52 kDa) was present in the cell extracts whereas the GST control lane was negative for a band of similar size (Fig. 4A1.2 Panel II compare lower band in lane 1 with lanes 3 & 4). As hnRNP K is capable of self-interaction (Bomsztyk *et al.*, 1997), the 66-70 kDa band is likely to be hnRNP K from the extracts interacting with GST-K (Fig. 4A1.2 Panel II compare upper band in lanes 1 & 2 with lanes 5 & 6).

4A1.3 ORF57 and hnRNP K coimmunoprecipitate from cell extracts

Confirmation of an interaction between ORF57 and hnRNP K came from Western blotting with hnRNP K antiserum of ORF57 Ab immunoprecipitates. HnRNP K was coimmunoprecipitated only from ^{35}S-labelled TPA-induced BCBL-1 cell extracts and was absent in coimmunoprecipitates from uninduced cell extracts (Fig. 4A1.3 Panel I compare lanes 1 with 2 & 3). Whereas immunoprecipitation followed by Western blotting with anti-hnRNP K Ab gave an hnRNP K band from BJAB and TPA-induced as well as uninduced BCBL-1 cell extracts (Fig. 4A1.3 Panel II compare lanes 1, 2, & 3 with 4)). Thus, it is a fair conclusion that the band of ~65-70 kDa seen in the ^{35}S-labelled protein profile of immunoprecipitated proteins using anti-ORF57 Ab, corresponded to hnRNP K (Fig. 3B1Panel I lane 2).

The presence of ORF57 in immunoprecipitates with ORF57 Ab could not be confirmed by immunoblotting for ORF57 as the heavy chain of IgG was of similar size (~45-50 kDa) and because rabbit antibodies were used both for immunoprecipitations and immunoblotting due to unavailability of any other species of ORF57 antibody. However phosphorimager analysis of the ^{35}S-labelled protein profile with anti-ORF57 Ab immunoprecipitates demonstrated a ~50-52kDa band present only in TPA-induced BCBL-1 cell extracts, absent in the uninduced extracts and in immunoprecipitates with pre-immune serum (Fig. 4A1.3 Panel III compare lane 2 with 1, 3, & 4), which corresponded in size to the ORF57 band present in control input BCBL-1 cell extracts by ORF57 Ab immunoblot analysis (Shown in Chapter 3).

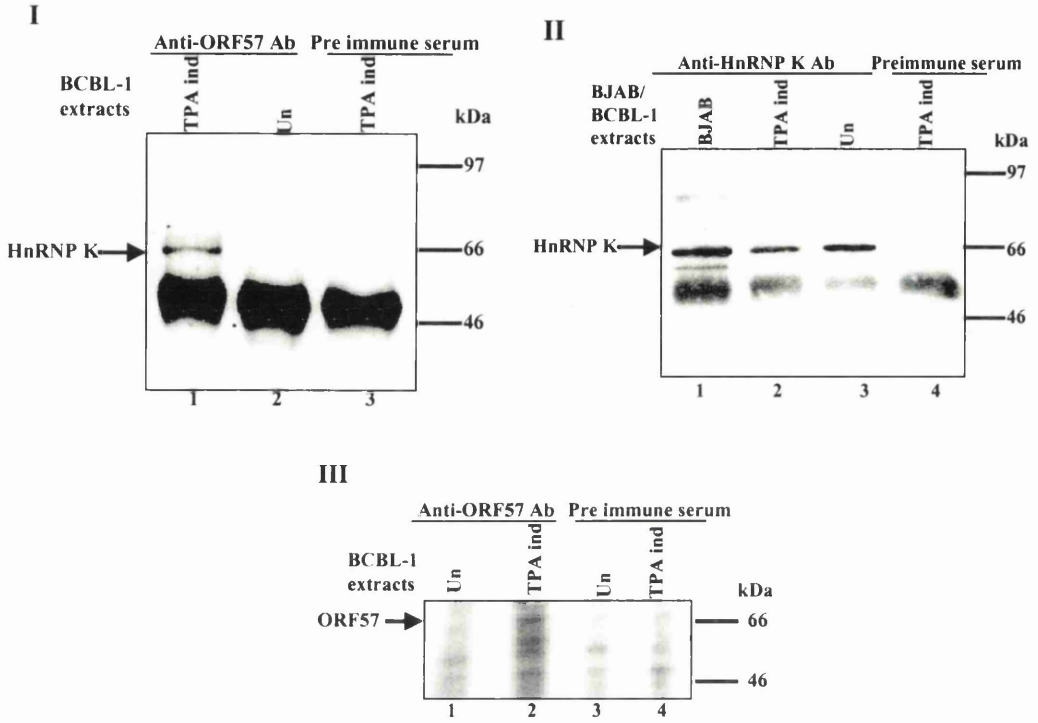
Fig 4A1.2. GST-K pulls down ORF57 from virus-infected BCBL-1 cells



Panel I. Expression of GST-K and GST proteins: Coomassie brilliant blue stained gel. GST-hnRNP K and GST proteins used were run on a 10% SDS-PAGE gel and visualised by Coomassie staining. The smaller bands in lane 1 represent degradation products/truncations of GST-hnRNP K. Shown in **lanes: 1.** GST-K **2.** GST alone **3.** Protein gel marker.

Panel II. Western blot with anti-ORF57 plus anti-hnRNP K Ab showing that GST-K pulls down hnRNP K and ORF57 from TPA-induced and uninduced and TPA-induced BCBL-1 cell extracts respectively. TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST-K or GST proteins. After incubation with extracts, proteins pulled down were, along with aliquots of the extracts used, separated by SDS-PAGE electrophoresis, transferred to nitrocellulose and Western blotted for hnRNP K and ORF57 using anti-hnRNP K plus anti-ORF57 Ab. Pull downs included in **lanes: 1.** Induced BCBL-1 extract + GST-K **2.** Uninduced BCBL-1 extract + GST-K **3.** Induced BCBL-1 extract + GST alone **4.** Uninduced BCBL-1 extract + GST alone **5.** Input induced BCBL-1 extract **6.** Input uninduced BCBL-1 extract.

Fig 4A1.3. ORF57 Ab coimmunoprecipitates hnRNP K from TPA-induced BCBL-1 cell extracts



A coimmunoprecipitation assay was performed with anti-ORF57 or anti-hnRNP K Ab, and [^{35}S]-labelled uninduced and TPA-induced BCBL-1 cell extracts. The complex formed was separated on a 10% SDS-PAGE gel, and either transferred to nitrocellulose and Western blotted for ORF57 or dried down and exposed to a phosphorimaging plate overnight.

Panel I. Western blot for hnRNP K with anti-hnRNP K Ab showing that anti-ORF57 Ab coimmunoprecipitates hnRNP K from TPA-induced BCBL-1 cell extracts. The complex formed was separated on a 10% SDS-PAGE gel, and transferred to nitrocellulose and Western blotted for ORF57. Immunoprecipitations included in **lanes: 1.**TPA-induced BCBL-1 extracts + anti-ORF57 Ab **2.** Uninduced BCBL-1 extracts + anti-ORF57Ab **3.** TPA-induced BCBL-1 extracts + pre immune serum.

Panel II. Western blot for hnRNP K with anti-hnRNP K Ab showing that anti-hnRNP K Ab coimmunoprecipitates hnRNP K from TPA-induced and uninduced BCBL-1 cell extracts. The complex formed was separated on a 10% SDS-PAGE gel, and transferred to nitrocellulose and Western blotted for ORF57. Immunoprecipitations included in **lanes: 1.** BJAB cell extracts + anti-hnRNP K Ab **2.** TPA-induced BCBL-1 extracts + anti-hnRNP K Ab **3.** Uninduced BCBL-1 extracts + anti-hnRNP K Ab **4.** TPA-induced BCBL-1 extracts + pre immune serum.

Panel III. Phosphorimager analysis showing presence of ORF57 in anti-ORF57 Ab coimmunoprecipitation. The complex formed was separated on a 10% SDS-PAGE gel, and dried down and exposed to a phosphorimaging plate overnight. Immunoprecipitations included in **lanes: 1.** Uninduced BCBL-1 extracts + anti-ORF57Ab **2.** TPA-induced BCBL-1 extracts + anti-ORF57 Ab **3.** Uninduced BCBL-1 extracts + pre immune serum **4.** TPA-induced BCBL-1 extracts + pre immune serum.

To determine if other viral proteins were required for the interaction between ORF57 and hnRNP K, coimmunoprecipitations were done with hnRNP K antiserum and extracts from pEGFP-ORF57 transfected, GFP alone transfected or untransfected cells. When pKS3 (GFP-gORF57) or GFP alone transfected HeLa cell extracts were immunoprecipitated with hnRNP K Ab and Western blotted with anti-GFP Mab this gave a band corresponding in size to GFP-gORF57 only in the transfected pKS3 lane and not in the GFP alone lane (Fig. 4A1.3 Panel IV compare lanes 2 & 3). Western blotting with anti-hnRNP K Ab showed that hnRNP K was immunoprecipitated in the appropriate samples (lanes 2 & 3) and immunoprecipitations using preimmune sera did not precipitate either ORF57 or hnRNP K (Fig. 4A1.3 Panel V lanes 5 & 6).

4A2 The hnRNP K protein pulled down with the GST-57 was a rapidly migrating form

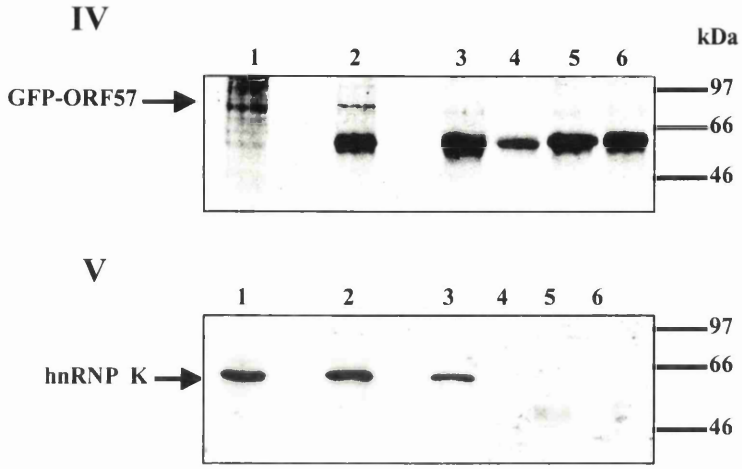
When the GST-57 pull down samples (using GST-57 fusion protein with BCBL-1 TPA-induced and uninduced BCBL-1 whole cell extracts) were analysed on the same gel (10%) with input BCBL-1 whole cell extracts, and Western blotted for hnRNP K, hnRNP K protein pulled down with GST-57 appeared to be a rapidly migrating form than the majority of hnRNP K present in whole cell extracts (Fig. 4A2 Panel I compare lanes 1 & 2 with 5 & 6). On running a higher percentage gel (12%) longer for better resolution, the hnRNP K form pulled down with GST-57 migrated faster than the major form present in input whole cell extracts (Fig. 4A2 Panel II compare lanes 3 & 4 with 5 & 6). This could be a less phosphorylated or differently modified form of hnRNP K.

4A3 HnRNP K aa 240-337 are involved in interaction with ORF57 protein

4A3.1 Mapping the interacting regions of hnRNP K-ORF57 using the GST pull down assay

GST fusions of hnRNPK deletion mutants (GST-KΔ3/KΔ7/KΔ12/KΔ31) were used to map regions of hnRNP K required for interaction with ORF57. Fig. 4A3.1 Panel I a & I b shows the aa present, and the amounts of each fusion protein expressed and purified on glutathione columns as visualized by Coomassie brilliant blue staining. Approximately equal amounts of each fusion protein or GST alone were added to ^{35}S-labelled-ORF57 (FL) protein and the pull down assay was performed. Only fusion proteins GST-KΔ3 and GST-KΔ31 deletion mutants bound to ORF57 *in vitro* (lanes 4 & 6), whereas no binding was detected with GST-KΔ7 and GST-KΔ12 deletion mutants or GST alone (Fig. 4A3.1 Panel II lanes 3, 5 & 2). These data indicate that the interactive region is located between

Fig 4A1.3. ORF57 coimmunoprecipitates with anti-hnRNP K Ab

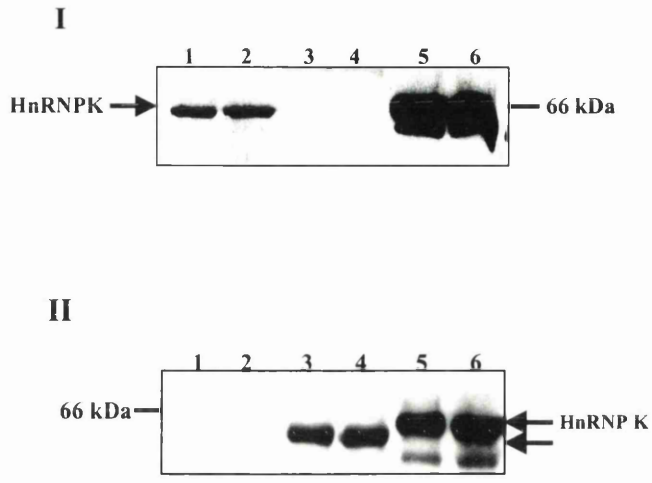


A coimmunoprecipitation assay was performed with anti-hnRNP K Ab or pre immune serum and GFP-ORF57 transfected HeLa cells. The complex formed was separated on a 12% SDS-PAGE gel, and transferred to nitrocellulose and Western blotted for GFP or hnRNP K.

Panel IV. Western blot for GFP-ORF57 with anti-GFP Ab showing that GFP-ORF57 coimmunoprecipitates by anti-hnRNP K Ab from GFP-ORF57 transfected HeLa cells. Immunoprecipitations included in **lanes: 1.** Input GFP-ORF57 transfected HeLa extracts **2.** GFP-ORF57 + HeLa extract + anti-hnRNP K Ab **3.** GFP alone transfected HeLa extracts + anti-hnRNP K Ab **4.** Untransfected HeLa extracts + protein-A agarose beads only **5.** GFP-ORF57 + HeLa extract + pre immune serum **6.** HeLa extract alone + pre immune serum.

Panel V. Western blot for hnRNP K with anti-hnRNP K Ab showing that hnRNP K coimmunoprecipitates with anti-hnRNP K Ab from both transfected and untransfected HeLa cells. Immunoprecipitations included in **lanes: 1.** Input GFP-ORF57 transfected HeLa extracts **2.** GFP-ORF57 + HeLa extract + anti-hnRNP K Ab **3.** GFP alone transfected HeLa extracts + anti-hnRNP K Ab **4.** Untransfected HeLa extracts + protein-A agarose beads only **5.** GFP-ORF57 + HeLa extract + pre immune serum **6.** HeLa extract alone + pre immune serum.

Fig 4A2. GST-57 pulls down hnRNP K from BCBL-1 cell extracts



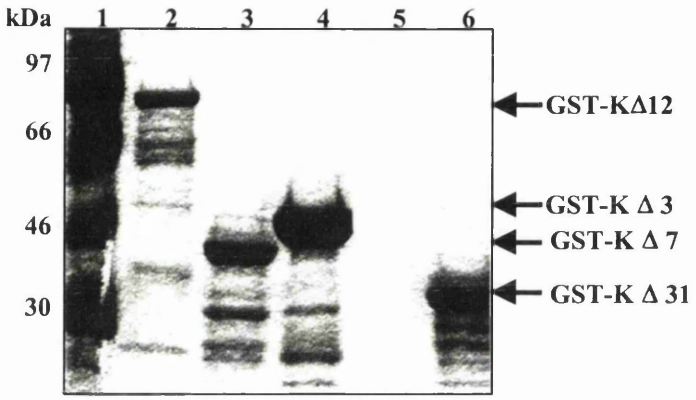
TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST or GST-57 proteins. After incubation, proteins pulled down were, along with input BCBL-1 cell extracts, separated by SDS-PAGE electrophoresis, transferred to nitrocellulose and Western blotted for hnRNP K using anti-hnRNP K Ab.

Panel I. Western blot (10% gel) for hnRNP K with anti-hnRNP K Ab showing that GST-57 pulls down hnRNP K from TPA-induced and uninduced BCBL-1 cell extracts. Pull downs included in **lanes: 1.**TPA-induced BCBL-1 extract + GST-57 **2.** Uninduced BCBL-1 extract +GST-57 **3.** TPA-induced BCBL-1 extract + GST **4.** Uninduced BCBL-1 extract + GST **5.** Input TPA-induced BCBL-1 extract **6.** Input uninduced BCBL-1 extract.

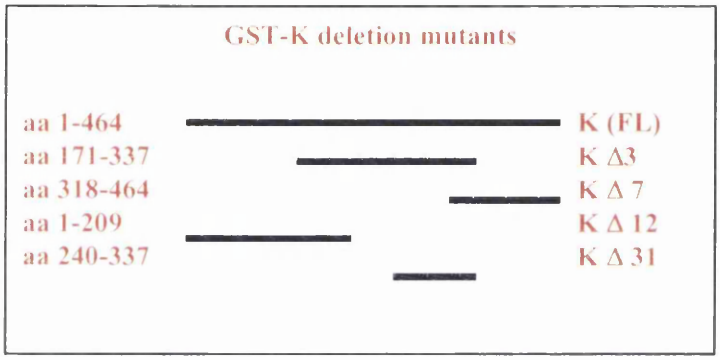
Panel II. Western blot (12% gel) for hnRNP K with hnRNP K Ab showing that GST-57 pulls down hnRNP K from TPA-induced and uninduced BCBL-1 cell extracts. Pull downs included in **lanes: 1.** Uninduced BCBL-1 extract + GST alone **2.** TPA-induced BCBL-1 extract + GST alone **3.** Uninduced BCBL-1 extract + GST-57 **4.** TPA-induced BCBL-1 extract + GST-57 **5.** Input uninduced BCBL-1 extract **6.** Input TPA-induced BCBL-1 extract.

Fig 4A3.1. GST-K deletion protein expression

I a



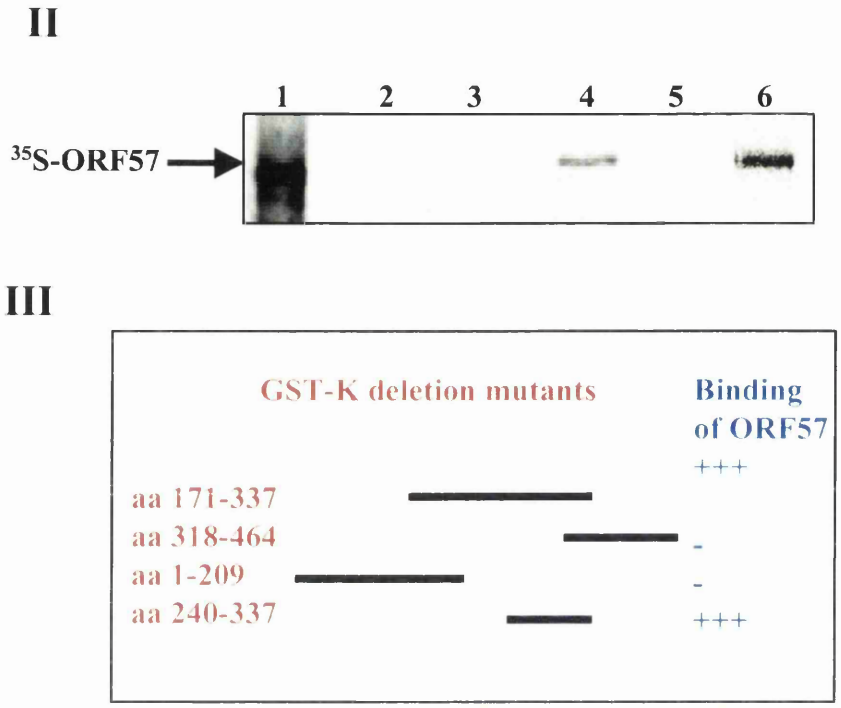
I b



Panel I *a*. GST-K deletion protein expression: Coomassie blue stained gel. GST-K deletions used were run on a 10% SDS-PAGE gel and visualised by Coomassie staining. The smaller bands in lanes 2, 3, 4 and 6 represent degradation products/truncations of GST-hnRNP K fusion protein deletions. Shown in **lanes: 1.** Protein gel marker **2.** GST-K12 deletion (aa 1-209) **3.** GST-K3 deletion (aa 171-337) **4.** GST-K7 deletion (aa 318-464) **5.** Empty **6.** GST-K31 deletion (aa 240-337).

Panel I *b*. Schematic representation of hnRNP K protein deletions

Fig 4A3.1. Mapping the ORF57 binding regions on hnRNP K: Two GST-K deletions interact with *in vitro* transcribed-translated ORF57



Panel II. Phosphorimager analysis showing that two GST-K deletions interacts with *in vitro* transcribed-translated ORF57. GST-K fusion protein deletions bound onto glutathione beads were incubated with ^{35}S-labelled-ORF57 (FL), after washing, specifically bound proteins were eluted and separated on a 10% SDS-PAGE gel, dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** Input ^{35}S- labelled-ORF57 (FL) **2.** ^{35}S-labelled-ORF57 (FL) + GST alone **3.** ^{35}S-labelled-ORF57 (FL) + GSTKΔ7 (aa 318-464) **4.** ^{35}S- labelled-ORF57 (FL) + GSTKΔ3 (aa 171-337) **5.** ^{35}S- labelled-ORF57 (FL) + GSTKΔ12 (aa 1-209) **6.** ^{35}S- labelled-ORF57 (FL) + GSTK Δ 31 (aa 240-337).

Panel III. Representation of *in vitro* synthesised ORF57 binding to hnRNP K protein deletions

aa 240 and 337 (i.e. KΔ31) and suggest that *in vitro* hnRNP K protein can directly interact with ORF57. The GST-K truncation mapping is represented diagrammatically in Fig. 4A3.1 Panel III.

4A3.2 Mapping the interacting regions of hnRNP K using ORF57 from virus-infected cells

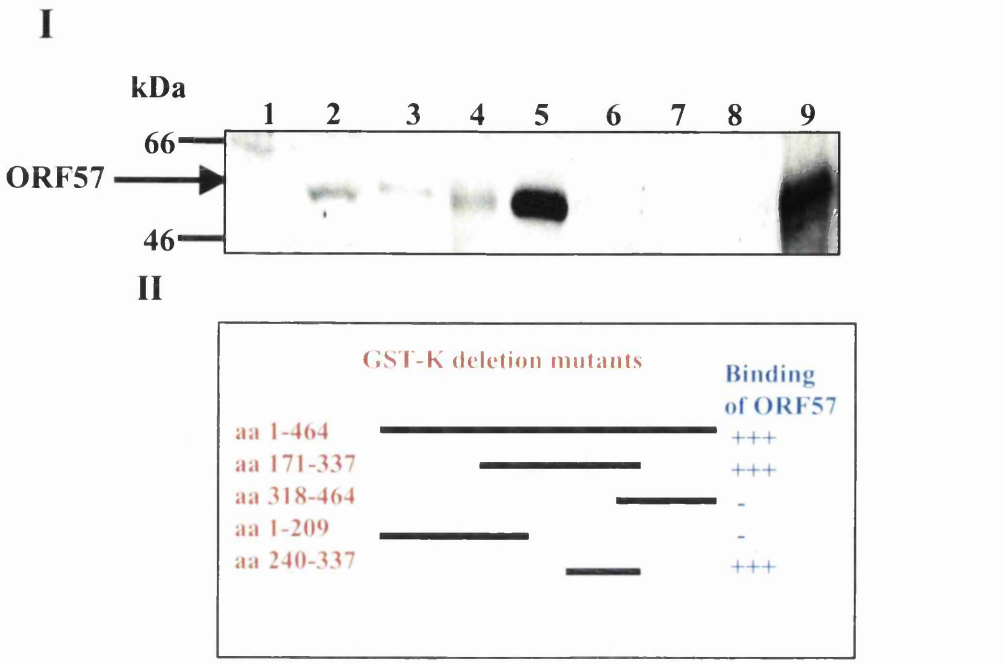
GST fusions of full-length and deletion mutants of hnRNP K were used to confirm the regions of hnRNP K required for interaction using ORF57 from virus-infected cells. Approximately equal amounts of GST-K (FL) or deletion mutants of GST-K fusion proteins or GST alone (Fig. 4A3.1 Panel I a & I b) were added to ~200μg protein from TPA-induced and uninduced BCBL-1 cell extracts and a pull down assay was performed. Eluted proteins were separated by SDS-PAGE and Western blotted for ORF57. Only fusion proteins GST-K (FL) (Fig. 4A3.2 Panel I lanes 3 & 5) and deletion mutants GST-KΔ3 and GST-KΔ31 (Panel I lanes 2 & 4) bound to ORF57, whereas no binding was detected with GST-KΔ7, GST-KΔ12 or GST (Panel I lanes 6, 7 & 1). When less than 50% of GST-K (FL) was used with TPA-induced BCBL-1 cell extracts to confirm the specificity of interaction, less than half of the ORF57 was pulled out (lane 3) in comparison to lane 5 (Fig. 4A3.2 Panel I). These data confirmed that the minimum region for ORF57 interaction is located between hnRNP K aa 240 and 337 as defined by the GST-KΔ31. This is represented in Fig. 4A3.1 Panel II and the domain region of hnRNP K protein involved in interaction with ORF57 is shown in Fig. 4A3.2 Panel III.

4A4 ORF57 protein aa 17-215 and 329-387 are involved in interaction with hnRNP K protein

4A4.1 Mapping the interacting regions of ORF57 using GST pull downs

GST-hnRNP K and ^{35}S-labelled-ORF57 (FL) and deletion mutants (pcDNA4-ORF57 containing aa 1-455, 17-455, 1-215, 181-328, 329-455 and 387-455) were used in GST pull down assay. Fig. 4A4.1Panel I shows the aa present, and the expression of each ^{35}S-labelled protein as visualized by phosphorimager analysis. A typical gel of one such analysis is shown in Fig. 4A4.1Panel II. Only ^{35}S-labelled-ORF57 (FL) and deletion mutants aa 17-455, 1-215 and 329-455 bound to GST-K *in vitro* (Fig. 4A4.1Panel II a lanes 2, 1, & 3 and Fig 4A4.1Panel II b lane 2), whereas no detectable binding was detected with deletion mutants aa 181-328, 387-455 or with GST alone (Fig. 4A4.1 Panel II a lanes 4, 5 & 6 and Panel II b lanes 1, 3 & 4). Use of equivalent amounts of fusion proteins GST-hnRNP K and GST alone in the GST pull down assays was confirmed by

Fig 4A3.2. ORF57 from BCBL-1 cells binds to aa 240-337 of hnRNP K

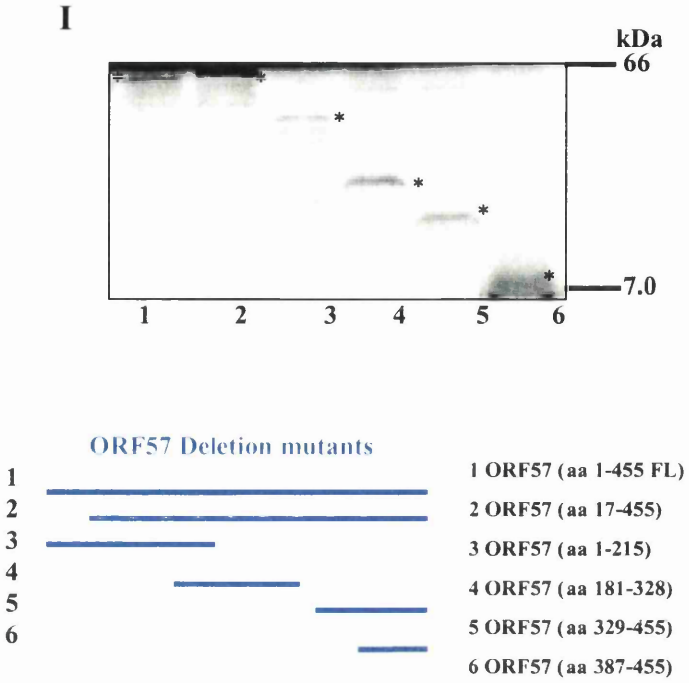


Panel I. Western blot for ORF57 with anti-ORF57 Ab showing that ORF57 from TPA-induced BCBL-1 cells binds to aa 240-337 of hnRNP K. TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST or GST-K fusion protein deletions. After incubation, proteins pulled down were, along with input BCBL-1 cell extracts, separated by SDS-PAGE electrophoresis, transferred to nitrocellulose and Western blotted for hnRNP K using anti-hnRNP K Ab. Pull downs included in **lanes: 1.** TPA-induced BCBL-1 extract + GST alone **2.** TPA-induced BCBL-1 extract + GST KΔ31 **3.** TPA-induced BCBL-1 extract + GST-K FL (less than 50%) **4.** TPA-induced BCBL-1 extract + GST KΔ3 **5.** TPA-induced BCBL-1 extract + GST-K (FL) **6.** TPA-induced BCBL-1 extract + GST KΔ7 **7.** TPA-induced BCBL-1 extract + GST KΔ12 **8.** Input uninduced BCBL-1 extract (30%) **9.** Input induced BCBL-1 extract (30%).

Panel II. Representation of GST-K deletions binding to ORF57 from virus-infected cells

Panel III. Representation of ORF57 binding to hnRNP K protein

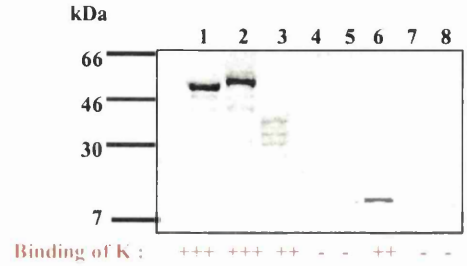
Fig 4A4.1. ORF57 deletion mutants



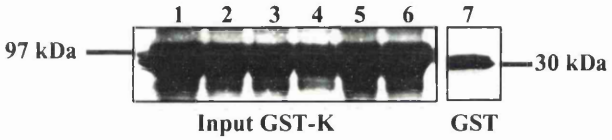
Panel I. Phosphorimager analysis showing ^{35}S-labelled-ORF57 protein deletions. ^{35}S-labelled-ORF57 protein deletions were synthesised *in vitro* using pcDNA-ORF57 recombinants (FL and deletions) and analysed by phosphorimaging. Shown in **lanes: 1.** ^{35}S-labelled-ORF57 (aa 17-455) **2.** ^{35}S-labelled-ORF57 (aa 1-455 FL) **3.** ^{35}S-labelled-ORF57 (aa 1-215) **4.** ^{35}S-labelled-ORF57 (aa 181-328) **5.** ^{35}S-labelled-ORF57 (aa 329-455) **6.** ^{35}S-labelled-ORF57 (aa 387-455).

Fig 4A4.1. HnRNP K binds to aa 17-181 and aa 329-387 of ORF57

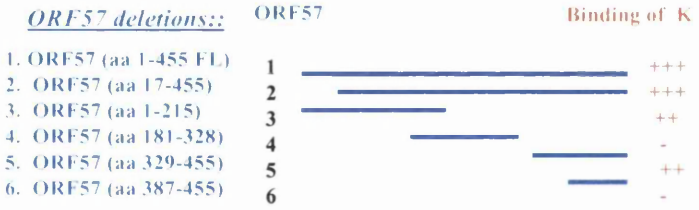
II



III



IV



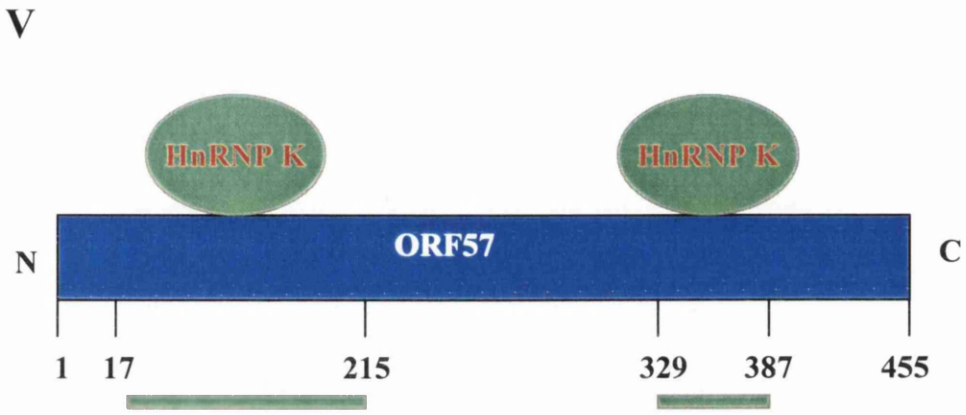
GST-K fusion or GST protein bound onto glutathione beads were incubated with ^{35}S-labelled-ORF57 (FL and deletions). After washing, specifically bound proteins were eluted and separated on a 10% SDS-PAGE gel and either dried and analysed by phosphorimaging or transferred to nitrocellulose and Western blotted for GST-K or GST using anti-GST Ab.

Panel II. Phosphorimager analysis showing that hnRNP K binds to aa 17-181 and aa 329-387 of ORF57. Pull downs included in **lanes: 1.** ^{35}S-ORF57 (aa 17-455) + GST-K **2.** ^{35}S-ORF57 (aa 1-455 FL) + GST-K **3.** ^{35}S-ORF57 (aa 1-215) + GST-K **4.** ^{35}S-ORF57 (aa 181-328) + GST-K **5.** Rainbow marker **6.** ^{35}S-ORF57 (aa181-328) + GST-K **7.** ^{35}S-ORF57 (aa 387-455) + GST-K **8.** ^{35}S-ORF57 (aa 1-455 FL) + GST alone.

Panel III. Western blot for GST-K and GST with anti-GST Ab on samples loaded in Panel II

Panel IV. Representation of hnRNP K binding to ORF57 protein deletions

Fig 4A4.1. HnRNP K binds to aa 17-181 & aa 329-387 of ORF57



Panel V. Representation of hnRNP K binding regions of ORF57 protein

Western blotting the other half of the eluted samples and immunodetection with anti-GST Ab (Fig. 4A4.1 Panel III lanes 1-7 respectively). These data indicated that there are two interactive regions for hnRNP K on ORF57 involving aa 17-215 and aa 329-387. However the binding with aa 17-455 was stronger than with either aa 1-215 or aa 329-455, indicating that other regions are also required for interaction. Thus *in vitro* ORF57 protein could interact with both N- and C-terminal regions of hnRNP K independently but both regions might contribute to the interaction with the full protein. Binding of hnRNP K to ORF57 is represented diagrammatically in Fig 4A4.1 Panel IV & V.

4A5 ORF57 protein partially co-localises with hnRNP K within cells

HnRNP K usually has a predominantly nuclear distribution but depending on the cell type may also have a cytoplasmic distribution. Confocal microscopy was used to visualise ORF57 (green) and hnRNP K (red) in transfected mammalian cells. On overlay, both proteins co-localise partially (Fig. 4A5 Panel I and Panel II). In GFP tagged ORF57-transfected BHK and HeLa cells, both proteins were predominantly nuclear but also occupied different subdomains, for example, hnRNP K can be seen at the rim of the nucleus (Panel I and Panel II).

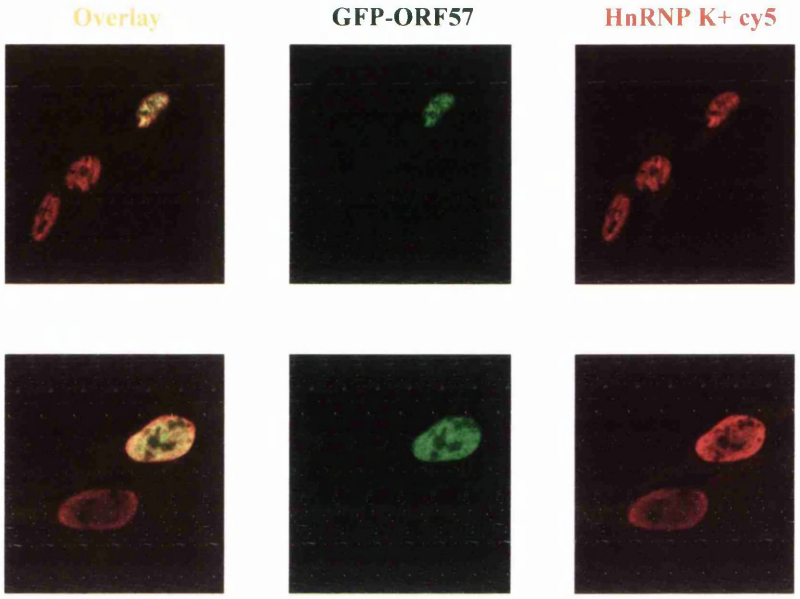
4B A role for the hnRNP K-ORF57 protein interaction in gene expression?

4B1 The CT-element bearing promoter driving a luciferase reporter

HnRNP K has high affinity binding to single-stranded polypyrimidine-rich sequences and has been shown to interact with a C-rich DNA sequence, termed the CT element (CCCTCCCCA) located upstream of the *c-myc* gene (Takimoto *et al.*, 1993) and at other places. Overexpressed hnRNP K and TBP synergistically activated transcription of a CT element-dependent reporter gene *in vivo* (Michelotti *et al.*, 1996). As ORF57 has been suggested to have roles at transcriptional/posttranscriptional levels similar to hnRNP K, it was tested how the coexpression of hnRNP K with ORF57 affects expression of a luciferase reporter gene with a CT element promoter. 293 cells were transfected with ORF57 and FLAG-tagged hnRNP K expression constructs and with a plasmid encoding the luciferase reporter gene driven by a synthetic promoter containing three repeats of the CT element either wild-type (CT3 wt) or a mutated version (CT3 mut) present upstream of the minimal *c-fos* promoter (Michelotti *et al.*, 1996). In this assay Flag-hnRNP K alone did not affect luciferase expression in 293 cells (Fig. 4B1Panel I lane 2). ORF57 activated

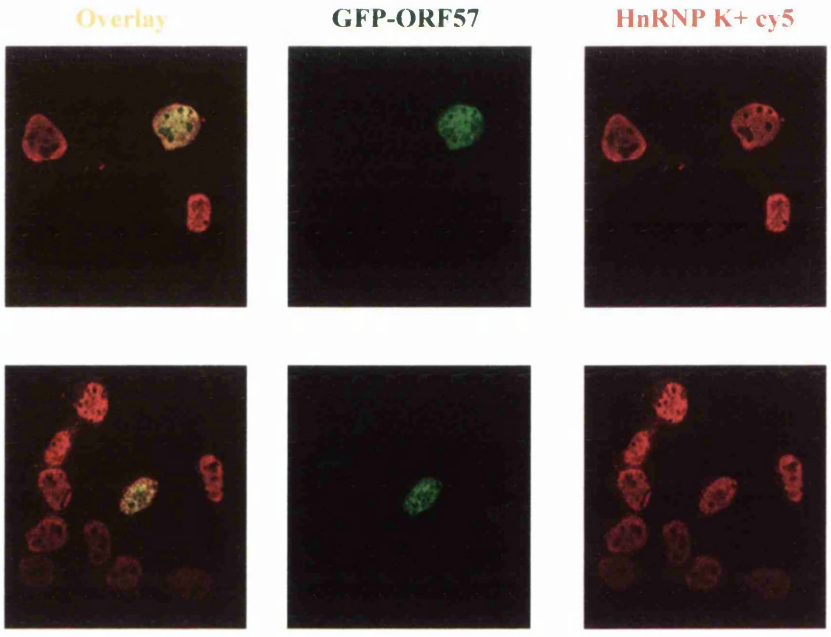
Fig 4A5. ORF57 partially colocalises with hnRNP K in BHK and HeLa cells

I



BHK cell transfected with GFP-ORF 57

II



Hela cells transfected with GFP-ORF57

Panels I and II. ORF57 colocalises with with hnRNP K in punctate spots in the cell nucleus, hnRNP K is present at the nuclear rim.

transcription from the CT3 wild type promoter element some two fold but did not affect transcription from the CT3-mut promoter (lane 3 & 7). When both proteins were co-expressed, the sequence-specific trans-activation effect of ORF57 from the wild-type (CT3) element was reduced (Fig. 4B1 Panel I lane 4).

Similar results were obtained in HeLa cells (Fig. 4B1 Panel II). Interestingly, in this cell line Flag-hnRNP K slightly activated transcription from the CT3-wt promoter (lane 2) but not from the CT3-mut promoter (lane 6) while ORF57 did not activate transcription from the CT3-wt promoter (lane 3). The sequence-specific trans-activation effect of hnRNP K protein on the CT3-wt promoter was reduced when both ORF57 and hnRNP K were co-expressed (lane 4). As Flag-hnRNP K activated the CT element in a sequence specific manner in HeLa but not in 293 cells this indicates the involvement of cell-specific factors in the process.

Regulation of the activation effect of one protein's activity by a partner protein from the CT3-wt promoter suggests that hnRNP K and ORF57 are functionally linked. Although as hnRNP K protein is abundant in cells, the endogenous protein levels may have blunted the observed effects. Addition of ORF50 protein, a transactivator for various KSHV promoters, and switch for inducing lytic virus cycle, did not have any effect on expression from the CT3-wt promoter in 293 cells (Fig. 4B1 Panel III lane 4) and addition of hnRNP K with ORF50 had no effect (lane 5). Both ORF57 and ORF50 together activated expression only from the CT3-wt promoter (lane 6) that was reduced on addition of hnRNP K (lane 7) to similar levels as with ORF57 alone.

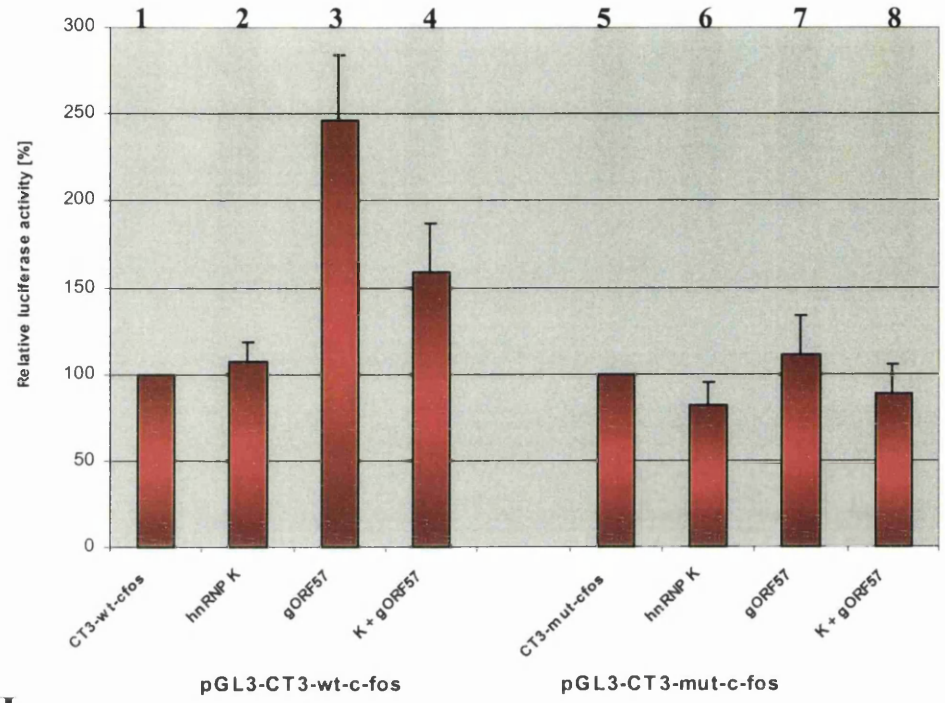
4C Discussion

4C1 ORF57 interacts with hnRNP K

These data show that hnRNP K interacts with ORF57 in GST pull down assays and both proteins coimmunoprecipitate from transfected HeLa cells. ORF57 interacts with a rapidly migrating form of hnRNP K that could be a differentially phosphorylated form. Primary transcripts of hnRNP K are alternatively spliced to generate four variants and changes in the relative proportions of variants are associated with alterations in cell proliferation (Dejgaard *et al.*, 1994). Thus the form detected could be an alternatively spliced variant of hnRNP K. While the antibody used in this study was directed against a peptide present in all four isoforms, the discrimination seen may be between different isoforms differentially phosphorylated by a kinase associated with ORF57. It is known

Fig 4B1 Panel I and Panel II. Effect of addition of both hnRNP K and ORF57 together on gene expression from the CT3-wt/mut-c-fos promoter in 293 (Panel I) and HeLa (Panel II) cells

I



II

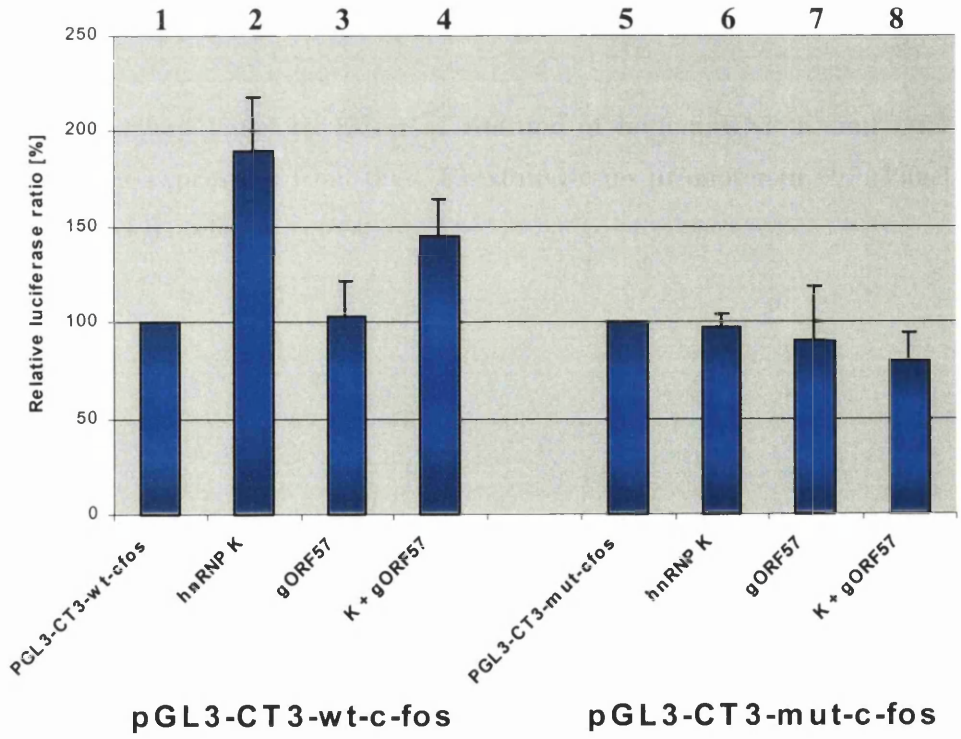
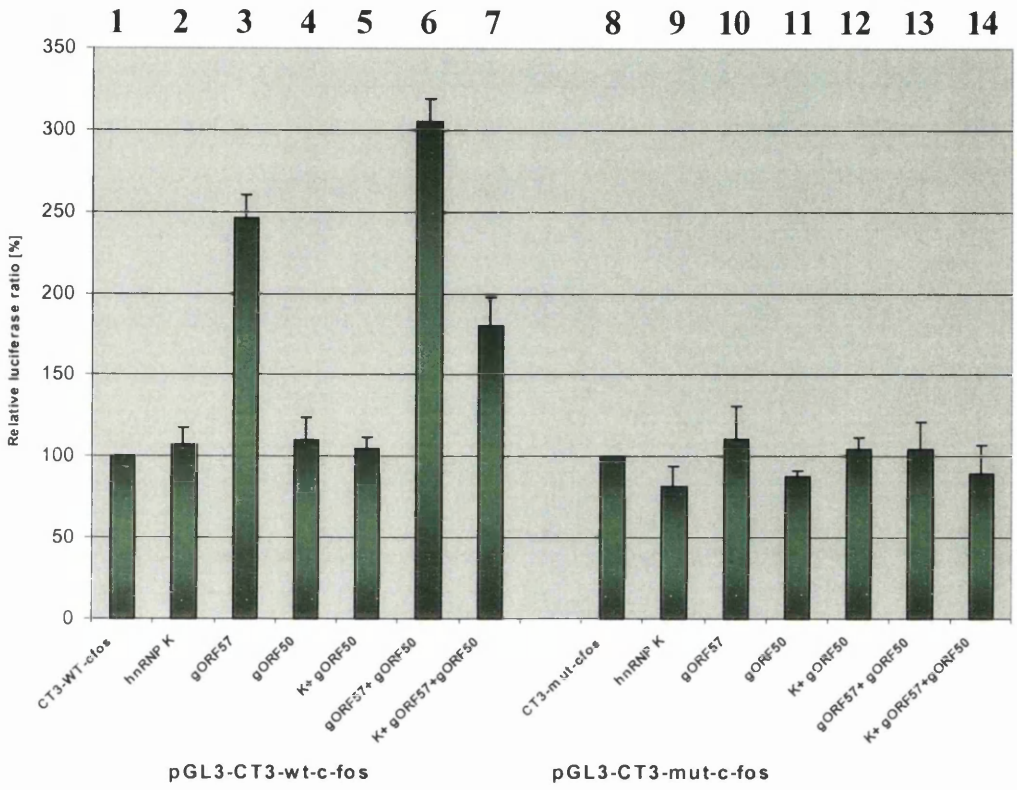


Fig 4B1 Panel III. Effect of addition of ORF50 alone or together with hnRNP K and ORF57 on gene expression from the CT3-wt/mut-c-fos promoter in 293 cells

III



Panel III. Effect of addition of ORF50 alone or together with hnRNP K and ORF57 on gene expression from the CT3-wt/mut-c-fos promoter in 293 cells.

Fig 4B1 Panels I, II and III. Effect of addition of ORF57 alone or together with hnRNP K and ORF50 on gene expression from the CT3-wt/mut-c-fos promoter in 293 cells. The DNA mixture consisted of either 0.25μg of the pGL3-CT3 wt-c-fos or pGL3-CT3mut-c-fos luciferase reporter plasmid and either 0.75μg FLAG-hnRNP K expression vector or 0.85μg ORF57 expression vector (pKS3) or 0.85μg ORF50 expression vector or a combination of these recombinants per well in transfections. 10 ng of pRL-SV40 plasmid (Promega) was cotransfected to normalize transfection efficiency in a dual luciferase assay. The total amount of plasmid DNA transfected in each sample was held constant by adding the appropriate amount of the corresponding pcDNA3.1 parental plasmid without the insert, and was brought to a total of 2.0μg (for 293 cells) and 1.5μg (for HeLa cells). Cells were harvested 48 h after transfection, frozen at -70°C for at least 2 h and luciferase activity was measured using the Dual Luciferase^{R} Reporter Assay Kit.

that phosphorylation of K diminishes its binding affinity to RNA (Dejgaard *et al.*, 1994). Thus the phosphorylation status of hnRNP K may be crucial for its interaction with ORF57 throughout the virus life cycle, and alteration in the phosphorylation status of partner proteins could determine the extent of the interaction. It is interesting that hnRNP K interacts with and represses the C/EBP$^{\beta}$, transcription factor (Miau *et al.*, 1998), whose co-activator Nopp140, is phosphorylated by and interacts with CK2.

Amino acids 240-337 of hnRNP K are sufficient for interaction with ORF57 and this is the same region which is required for interaction with ICP27 (Wadd *et al.*, 1999). This region, denoted KI, was previously found to be responsible for binding of other K protein partners such as protein kinase C δ, Zik 1, Eed, and YB-1 (Denisenko *et al.*, 1996; Denisenko & Bomsztyk, 1997; Schullery *et al.*, 1999; Shnyreva *et al.*, 2000). This region contains the GRGG box, the SH3 binding region and part of KNS domain of hnRNP K protein. The GRGG box binds to RNA, the SH3 region binds to proteins with SH3 domains whereas KNS is implicated in bi-directional movement of protein between the nucleus and cytoplasm.

Mapping data for ORF57 indicated two interactive regions for hnRNP K involving aa 17-215 and aa 329-387. No interaction was found with the middle region (aa 215-328) and the C-terminal 68 amino acids. This suggested that, *in vitro*, ORF57 protein could interact at both N- and C-terminal parts with hnRNP K independently. This requirement of two regions of interaction has been shown for the hnRNP K partner protein YB-1, (Shnyreva *et al.*, 2000) also a multifunctional protein implicated in various stages of gene expression. As both hnRNP K and ORF57 are involved in several processes, their protein:protein interactions may be dynamic, changing at different intracellular locations or times post infection to facilitate the various ORF57 functions. Perhaps the phosphorylation status of ORF57 or its partners plays a part (Denisenko *et al.*, 1996; Schullery *et al.*, 1999) influencing the selection of partner proteins at particular stages in the viral life cycle.

4C2 Significance of interaction of ORF57 with hnRNP K

4C2.1 Similarities between ORF57 and hnRNP K

There are indications that hnRNP K and ORF57 are similar proteins. They are of similar size, ORF57 consisting of 455 amino acids and hnRNP K of 464 amino acids. Functionally, ORF57 and hnRNP K both affect transcriptional and post-transcriptional

processes, are phosphorylated, capable of self interaction, shuttle from the nucleus to the cytoplasm and are present in multi-protein complexes. HnRNP K protein is involved in many steps of transcriptional regulation, interacting with general and sequence-specific transcription factors and binding sequence-specific polypyrimidine-rich DNA motifs within promoter regions. It has been proposed that K protein acts as a scaffold or architectural transcription factor that promotes assembly of sequence-specific transcription factors and basal transcription machinery on promoter elements (Michelotti *et al.*, 1996). The transcription effects of hnRNP K protein are cell type-dependent, and hnRNP K protein could act as scaffold or docking platform for multiprotein complexes that include transcription factors. Acting as such, it may respond to changes in the extracellular environment at sites of nucleic acid-dependent processes and get involved in various processes of gene expression (Schullery *et al.*, 1999; Ostrowski *et al.*, 2000).

These similarities suggest that ORF57 and hnRNP K may access common cellular pathways. ORF57 could prevent hnRNP K from accessing these pathways thereby inhibiting competition or subvert its activities directing them for use in viral rather than cellular gene expression. Alternatively, many of the functions ascribed to ORF57 may be due to its interaction with hnRNP K, which could play a key role in KSHV infection. ORF57-hnRNP K interaction may account for the ability of ORF57 to activate and repress gene expression. Depending on the cell type and intracellular environment, binding of ORF57 to hnRNP K could also relieve hnRNP K mediated suppression of gene activity, similar to the way in which expression of hepatitis C virus core protein relieves hnRNP K suppression of the cellular TK promoter (Hsieh *et al.*, 1998).

4C2.2 Effect of the ORF57-hnRNP K interaction at the transcriptional level

ORF57 protein homologues such as ICP27 of HSV-1 and Mta of EBV have been shown to modulate transcription. HnRNP K interacts with the general transcriptional factor TBP (Michelotti *et al.*, 1996; Shnyreva *et al.*, 2000) and cotransfection of hnRNP K and TBP can activate CT-mediated transcription of c-*myc* (Michelotti *et al.*, 1996). TBP-mediated activation of transcription is regulated by a variety of gene-specific transcription factors (Wu *et al.*, 1998) and the hnRNP K-TBP-ORF57 complex, may be required for regulating viral transcription. Tandem copies of a C+T-rich DNA sequence, similar to known hnRNP K DNA binding sites, are present in an HSV-1 genome location, which has been proposed to act as a transcriptional regulator of viral IE genes (Quinn *et al.*, 1998).

When both ORF57 and hnRNP K proteins are coexpressed, the transcriptional activation

of the CT element-containing promoter was reduced. Both ORF57 and hnRNP K could bind an as yet unidentified common transcription factor (X, such as transcription factor TBP for hnRNP K) and the interactive region of ORF57 is the same for both hnRNP K and X; suggesting that when present together hnRNP K and X may compete for ORF57. Gene-specific activation of transcription, particularly from CT element containing promoters, may be activated by increasing concentrations of either the hnRNP K-X, or the ORF57-X complexes, explaining why the reporter gene expression was activated when either ORF57 or hnRNP K was transfected alone. Coexpression of ORF57 and hnRNP K could favour the formation of a transcriptionally inactive ORF57-hnRNP K complex, resulting in the decreased reporter gene expression. Similar effects have been shown for hnRNP K and YB-1 interaction with TBP (Shnyreva *et al.*, 2000) and for Sp1 and C/EBP β transcription factors where the interaction with hnRNP K protein abolished their trans-activation ability (Miau *et al.*, 1998; Du *et al.*, 1998).

4C2.3 Effects of the ORF57-hnRNP K interaction at the nucleocytoplasmic shuttling and RNA export level

KSHV ORF57 is shown to be a nuclear protein expressed during lytic cycle that post-transcriptionally activates the expression of reporter genes (Gupta *et al.*, 2000; Kirshner *et al.*, 2000). Since hnRNP K also is involved in post-transcriptional regulation it is possible that by interacting with ORF57 either it affects the stability,or processing or export of viral RNAs is increased. As hnRNP K can shuttle from the nucleus to the cytoplasm, interaction with ORF57 may allow ORF57 to piggyback on it thereby exporting viral transcripts. Alternatively, the interaction may inhibit hnRNP K shuttling allowing ORF57 to access the hnRNP K shuttling pathway. KSHV RNA has ~53% G+C content in LUR and ~84% in TR regions and hnRNP K has a binding preference for poly (rC) RNA. The ORF57 homologue ICP27 (Ingram *et al.*, 1996) and hnRNP K (Matunis *et al.*, 1992) both bind RNA directly but it is not yet known if hnRNP K can bind to herpesvirus mRNAs.

4C2.4 Effect of the ORF57-hnRNP K interaction at the translational level

In several systems, hnRNP K can be seen to inhibit translation of mature mRNAs by blocking 80S ribosome assembly (Ostareck *et al.*, 1997; Collier *et al.*, 1998), and it has been shown to interact with efla. Interaction of hnRNP K with ORF57 and/or CK2 may play a role at the translational level to control KSHV-driven regulation of translation.

Chapter 5: Interaction of ORF57 with protein kinase CK2

ICP27 is known to be phosphorylated and to alter the phosphorylation of other viral proteins, but itself does not have phosphorylating activity. Using the yeast-2-hybrid system, *in vitro* binding assays and immunoprecipitations (Wadd *et al.*, 1999), ICP27 was found to be associated with CK2. I therefore determined if ORF57 behaves in a similar fashion. This Chapter presents data showing that CK2 interacts with ORF57 *in vitro* and in virus-infected cells. Experiments were aimed at determining the implication of this interaction for the virus life cycle.

CK2 is a heterotetramer consisting of two catalytic subunits (α or α') and two copies of the regulatory subunit (β); CK2 α and α' are 44 kDa and 40/42 kDa, and the CK2 β subunit is 24 kDa - 29 kDa (Hathaway & Traugh, 1979; Dahmus, 1981). To find out whether ORF57 interacted with and was phosphorylated by CK2, *in vitro* binding assays, CK2 peptide activity assays *in vitro* and virus-infected cell phosphorylation experiments were performed.

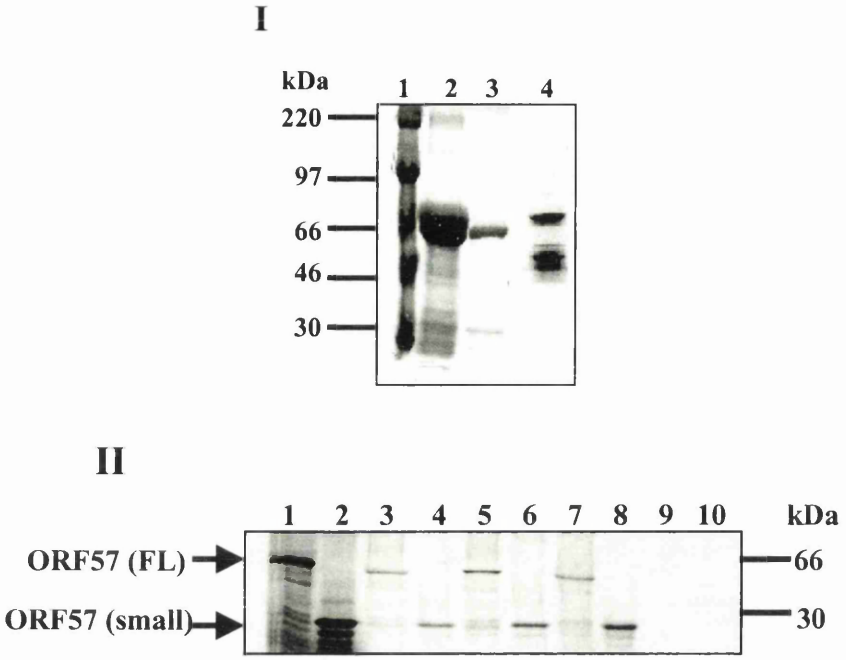
5A ORF57 interaction with CK2

5A1 ORF57 interacts with CK2

5A1.1 Using the in vitro pull down assay

The ^{35}S-labelled profile of ORF57 interacting proteins (Fig. 3B1 Panel I lane 2) contained bands of 44, 42 kDa and 25 kDa which could possibly be CK2. To detect interaction using GST-CK2 α or α' and MBP-CK2 β subunit fusion proteins (Section 2B4.5), pull down assays were performed with ^{35}S-labelled-ORF57 (FL) and ^{35}S-labelled-ORF57 (small) (Section 2B4.9). The fusion proteins used were visualised by Coomassie brilliant blue staining (Fig. 5A1.1 Panel I) and equivalent amounts of fusion proteins were used. Bound proteins were eluted from the beads, heated to 100°C in protein gel sample buffer for 5 min and analysed by SDS-PAGE (Section 2B6.1). The gel was dried and exposed to a phosphorimager screen (Section 2B6.4). ORF57 was pulled down by both the α and α'catalytic subunits (Fig. 5A1.1 Panel II lanes 3, 4 and 5, 6) and the regulatory β subunit (lanes 7 and 8) but not by GST alone (lanes 9 and 10). Both ^{35}S-labelled-ORF57 (FL) and ORF57 (small, N-terminal Δ aa 1-180) bound CK2 subunits α or α' and β, suggesting that

Fig 5A1.1. ORF57 interacts with *in vitro* synthesised CK2 α and β subunits



Panel I. Expression of protein kinase CK2 α, CK2 α' and CK2 β subunit proteins: Coomassie blue stained gel. GST-CK2 α, GST-CK2 α' and MBP-CK2 β fusion proteins used were run on a 10% SDS-PAGE gel and visualised by Coomassie staining. The smaller bands in lanes 2, 3, and 4 represent degradation products/truncations of GST-CK2α, GST-CK2 α' and MBP-CK2 β fusion protein. Shown in **lanes: 1.** Protein gel marker **2.** GST-CK2 α (FL) **3.** GST-CK2 α' (FL) **4** MBP-CK2 β (FL).

Panel II. Phosphorimager analysis showing ORF57 interacts with *in vitro* synthesised CK2 α and β subunits. GST-CK2 α, GST-CK2 α' and MBP-CK2 β fusion proteins bound onto glutathione beads were incubated with ^{35}S-labelled-ORF57 (FL and small), after washing, specifically bound proteins were eluted and separated on a 10% SDS-PAGE gel, dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** Input *in vitro* synthesised ^{35}S-labelled-ORF57 (FL) **2.** Input *in vitro* synthesised ^{35}S-labelled-ORF57 (small) **3.** GST-CK2 α+^{35}S-labelled-ORF57 (FL) **4.** GST-CK2 α + ^{35}S-labelled-ORF57 (small) **5.** GST-CK2 α' + ^{35}S-labelled-ORF57 (FL) **6.** GST-CK2 α' + ^{35}S-labelled-ORF57 (small) **7.** MBP-CK2 β + ^{35}S-labelled-ORF57(FL) **8.** MBP-CK2 β + ^{35}S-labelled-ORF57 (small) **9.** GST alone + ^{35}S-labelled-ORF57 (FL) **10.** MBP alone + ^{35}S-labelled-ORF57 (FL).

the first 180 aa of ORF57 are not required for interaction.

5A1.2 Using the pull down assay with virus-infected cells

Using GST-CK2 α or α' and MBP-CK2 β fusion proteins with ~200μg protein from TPA-induced and uninduced BCBL-1 cell extracts (Section 2B4.3), pull down assays were performed. The fusion proteins used were visualised by Coomassie brilliant blue staining (Fig. 5A1.1 Panel I). The SDS-PAGE gel was transferred to a nitrocellulose membrane followed by Western blotting (Section 2B6.2 & 3) with CK2 α or β subunit rabbit polyclonal antisera.

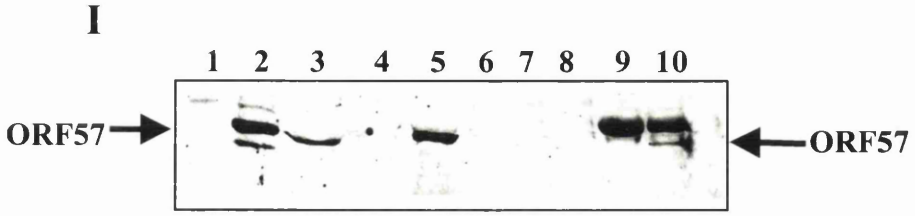
Results demonstrated that CK2 α/α' and β subunits interact with ORF57 from BCBL-1 cells, whereas GST did not (Fig. 5A1.2 Panel I compare lanes 3, 5 and 10 with 8). The ORF57 from TPA-induced BCBL-1 cell extracts bound to both α, α' and β subunits (lanes 3, 5 and 10) whereas GST alone and MBP alone did not (Fig. 5A1.2 Panel I lanes 7 and 8). A protein from MBP-CK2β fusion protein preparation interacts and pulls down with both uninduced and TPA-induced BCBL-1 cell extracts and cross-reacts with anti-ORF57 Ab, giving a non-specific band ~55 kDa running slightly higher than ORF57 band in both TPA-induced and uninduced BCBL-1 cells (lanes 10 & 9). This protein band was absent in MBP alone lane (lane 8) and could be a truncated product of MBP-CK2β fusion protein not present in MBP alone, which interacts non-specifically with BCBL-1 cells.

5A1.3 ORF57 binds directly to both the α and β subunits of CK2

ICP27 showed an interaction with the β-subunit in the yeast-two-hybrid screen; and as ICP27 was pulled down from HSV-1 infected cell extracts with recombinant CK2 α also, this interaction was regarded as bridged by β subunit (Wadd *et al.*, 1999). To examine if the ORF57-CK2 interaction is mediated only by subunit β or also by CK2 subunits α/α', a pull down assay was performed. Both ORF57 and CK2 were used as recombinant fusion proteins expressed from prokaryotic vectors.

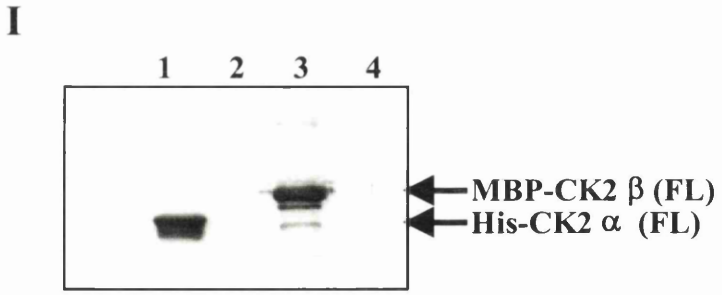
GST-57 recombinant protein bound glutathione beads (Section 2B4.3) or GST protein alone beads were mixed with equivalent amounts of purified recombinant MBP-CK2 β subunit or His-CK2 α subunit protein (Section 2B4.5), and pull down assays were performed using the anti-CK2 β or α subunit Abs for detection. Results showed that recombinant ORF57 bound the CK2 α and the CK2 β subunit (Fig. 5A1.3 lane 1 & 3) whereas GST alone did not bind either α or β subunits (Fig. 5A1.3 lane 2 & 4).

Fig 5A1.2. CK2 α, α' and β subunits interact with ORF57 from virus-infected BCBL-1 cell extracts



Panel I. Western blot for ORF57 with anti-ORF57 Ab. CK2 α, α' and β subunits interact with ORF57 from virus-infected BCBL-1 cell extracts. TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST, MBP or GST-CK2 α, GST-α' and MBP-β proteins. After incubation with extracts, proteins pulled down were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose and Western blotted for ORF57 using anti-ORF57 Ab. Pull downs included in **lanes: 1.** Uninduced BCBL-1 extract **2.** TPA-induced BCBL-1 extract **3.** TPA-induced BCBL-1 extract + GST-CK2 α **4.** Uninduced BCBL-1 extract + GST-CK2 α **5.** TPA-induced BCBL-1 1 extract + GST-CK2 α' **6.** Uninduced BCBL-1 extract + GST-CK2 α' **7.** TPA-induced BCBL-1 extract + GST alone **8.** TPA-induced BCBL-1 extract + MBP alone **9.** Uninduced BCBL-1 extract + MBP-CK2 β **10.** TPA-induced BCBL-1 extract + MBP-CK2 β.

Fig 5A1.3. ORF57 binds directly to both CK2 α and β subunits



Panel I. Western blot for CK2 α and β with anti-CK2 α plus anti-CK2 β Abs: GST-57 or GST binding to His-CK2 α or MBP-CK2 β subunits shows ORF57 binds directly to both CK2 α and β subunits. GST-57 or GST fusion proteins were incubated with His-CK2 α, and MBP- CK2β proteins. After incubation, proteins pulled down were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose and Western blotted for CK2 α and β with anti-CK2 α plus anti-CK2 β Abs. Pull downs included in **lanes: 1.** His-CK2 α + GST-57 (FL) **2.** His-CK2 α + GST alone **3.** MBP-CK2 β + GST-57 (FL) **4.** MBP-CK2 β + GST alone.

5A2 N- and C-terminal regions of CK2 α, and aa 150-182 of CK2 β are involved in interaction with ORF57 protein

5A2.1 Mapping regions of the CK2 α subunit involved in interaction with ORF57 protein

^{35}S-labelled CK2 subunit α full-length (aa 1-391) and deletion mutants (aa 1-265 and aa 266-391) were synthesised *in vitro* (Section 2B4.9) and used to map regions of CK2 α required for interaction with ORF57. Fig. 5A2.1 panel I shows the aa present and the expression of each *in vitro* transcribed/translated CK2 α protein as visualized by phosphorimaging (Section 2B6.4). Approximately equal amounts of GST-57 (FL or small) or GST alone (Section 2B4.3) were added and the pull down assay was performed.

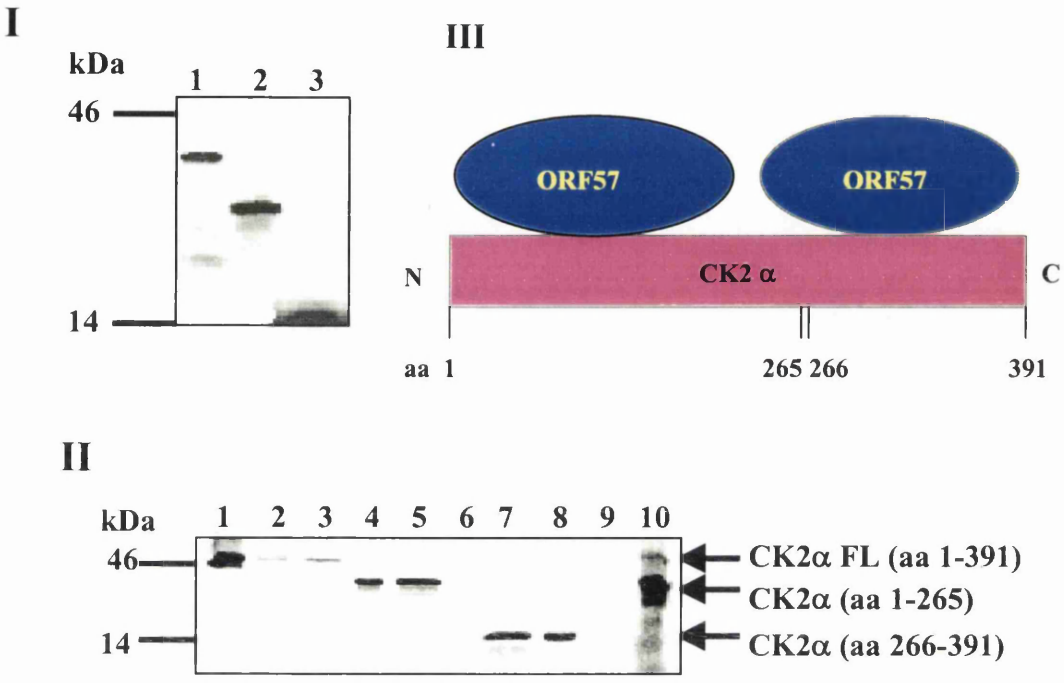
The GST-57 (FL and small) proteins bound to full-length CK2 α (Fig. 5A2.1 Panel II lanes 2 & 3), as did CK2 α (aa 1-265), and the CK2 α (aa 266-391), (lanes 4, 5, 7 & 8) whereas no binding was detected with GST alone (lanes 6 & 9). Lanes 1 and 10 show 20 % of the input CK2 α subunit (aa 1-391) and the N terminus (aa 1-265) respectively (Fig. 5A2.1 Panel II). Thus both the N- and C-terminal regions of CK2 α are involved in interaction with ORF57. Fig. 5A2.1 Panel III shows a diagrammatic representation of these interactions.

5A2.2 Mapping regions of the CK2 β subunit involved in interaction with ORF57 protein

GST-/MBP- fusions of full-length and deletions of CK2 β (Section 2B4.5) were used to map regions of CK2 β required for interaction with ORF57. Fig. 5A2.2 Panel I shows the aa present in and a typical expression of each MBP-CK2 β fusion protein (FL and deletion mutants) purified on amylose columns, and Fig. 5A2.2 Panel II shows the typical expression of GST-CK2 β fusion proteins (FL and deletion mutants) purified on glutathione Sepharose columns, as visualised by Coomassie brilliant blue staining. Approximately equal amounts of each fusion protein or GST/MBP alone were added to the ^{35}S-labelled-ORF57 (FL or small) and the pull down assays were performed.

Only fusion proteins MBP-CK2 β (aa 1-215) and deletion MBP-CK2 β (aa 1-182) bound to full-length ORF57 (lanes 2 & 3), whereas no binding was detected with MBP-CK2 β (aa 1-110), GST-CK2 β (aa 1-150), GST-CK2 β (aa 51-150) and GST-CK2 β (aa 1-55) in

Fig 5A2.1. Mapping of CK2 α: ORF57 can interact independently with both N- and C- terminal domains of CK2 α

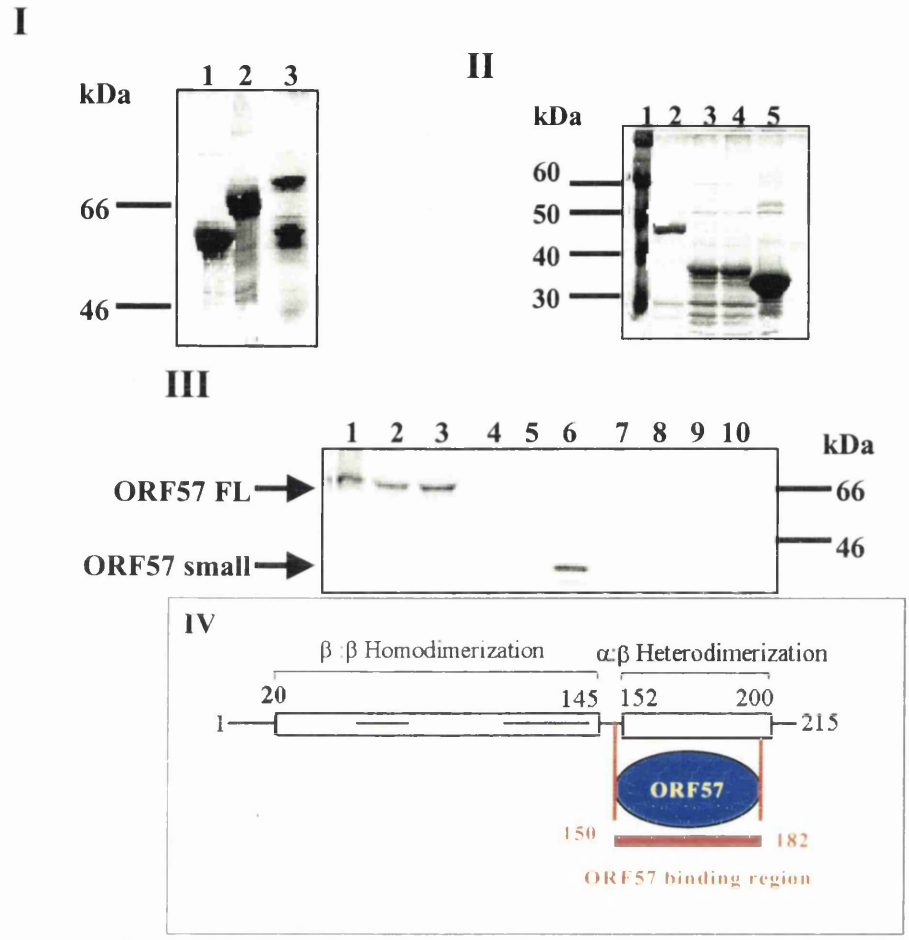


Panel I. Phosphorimager analysis showing expression of *in vitro* synthesised CK2 α full-length and its deletion mutants. ^{35}S-labelled- CK2 α FL and deletion proteins were synthesized *in vitro* using pET-CK2 α (FL and small) recombinants and analysed by phosphorimaging and autoradiography. Shown in **lanes: 1.** ^{35}S-labelled-CK2 α FL (aa 1-391) **2.** ^{35}S-labelled-CK2 α (aa 1-265) **3.** ^{35}S-labelled-CK2 α (aa 266-391).

Panel II. Phosphorimager analysis showing *in vitro* synthesised CK2 α interacts with ORF57. GST-57 and GST fusion proteins bound onto glutathione beads were incubated with ^{35}S-labelled-CK2 α (FL and deletions), after washing, specifically bound proteins were eluted and separated on a 10% SDS-PAGE gel, dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** Input *in vitro* synthesised ^{35}S-labelled-CK2 α FL (aa 1-391) **2.** ^{35}S-labelled-CK2 α FL (aa 1-391) + GST-57 (FL) **3.** ^{35}S-labelled-CK2 α FL (aa 1-391) + GST-57 (small) **4.** ^{35}S-labelled-CK2 α (aa 1-265) + GST-57 (FL) **5.** ^{35}S-labelled-CK2 α (aa 1-265) + GST-57 (small) **6.** ^{35}S-labelled-CK2 α (aa 1-265) + GST alone **7.** ^{35}S-labelled-CK2 α (aa 266-391) + GST-57 (FL) **8.** ^{35}S-labelled-CK2 α (aa 266-391) + GST-57 (small) **9.** ^{35}S-labelled-CK2 α FL (aa 1-391) + GST alone **10.** Input *in vitro* synthesised ^{35}S-labelled-CK2 α (aa 1-265).

Panel III. Representation of ORF57 binding regions of CK2 α

Fig 5A2.2. CK2 β binds aa 180-215 of ORF57



MBP-CK2 β and GST-CK2 β (FL and deletion) fusion proteins used were run on a 10% SDS-PAGE gel and visualised by Coomassie staining. The smaller bands in lanes represent degradation products/truncations of MBP-CK2 β and GST-CK2 β proteins.

Panel I. Expression of MBP-CK2 β (FL) and its deletions: Coomassie blue stained gel. Shown in **lanes: 1.** MBP-CK2 β (aa 1-110) **2.** MBP-CK2 β (aa 1-182) **3.** MBP-CK2 β FL (aa 1-215).

Panel II. Expression of GST-CK2 β deletions: Coomassie blue stained gel. Shown in **Lanes: 1.** Protein gel marker **2.** GST-CK2 β (aa 1-150) **3.** GST-CK2 β (aa 51-150) **4.** GST-CK2 β (aa 51-150) **5.** GST-CK2 β (aa 1-55).

Panel III. Phosphorimager analysis showing CK2 β binds aa 180-215 of ORF57. MBP-CK2 β, GST-CK2 β, MBP and GST fusion proteins bound onto maltose/glutathione beads were incubated with ^{35}S-labelled-ORF57 (FL and small). After washing, specifically bound proteins were eluted and separated on a 10% SDS-PAGE gel, dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** Input *in vitro* synthesised ^{35}S-labelled-ORF57 (FL) **2.** ORF57 (FL) + MBP-CK2 β FL (aa 1-215) **3.** ORF57 (FL) +MBP-CK2 β (aa 1-182) **4.** ORF57 (FL) + MBP-CK2 β (aa 1-110) **5.** ORF57 (FL) + MBP alone **6.** ^{35}S-labelled-ORF57 small (aa 181-455) +MBP-CK2 β FL (aa 1-215) **7.** ORF57 (FL) + GST-CK2 β (aa 1-150) 8. ORF57 (FL) + GST-CK2 β (aa 51-150) 9. ORF57 (FL) + GST-CK2 β (aa 1-55) 10. ORF57 (FL) + GST.

Panel IV. Representation of ORF57 binding regions of CK2 β

lanes 4, 7, 8 and 9 respectively (Fig. 5A2.2 Panel III). The fusion protein MBP-CK2 β (aa 1-215) was also able to interact with ORF57 small (lane 6). MBP alone (lane 5) and GST alone (lane 10) did not bind full-length ORF57 (Fig. 5A2.2 Panel III). Thus the interactive region for ORF57 is located between aa 150 and 182 of CK2 β. Other regions between aa 1 and 150 may be involved but they are not sufficient. More detailed mapping with N-terminal truncations of CK2 β and point mutations in residues 150-182 would provide information on the exact amino acids required for this interaction. The region of CK2 β protein involved in interaction with ORF57 is shown in Fig. 5A2.2 Panel IV.

5A3 Multiple regions of ORF57 protein interact with CK2 α; aa 387-455 with CK2 α' and aa 181-215 with CK2 β

5A3.1 Mapping regions of ORF57 involved in interaction with CK2 α

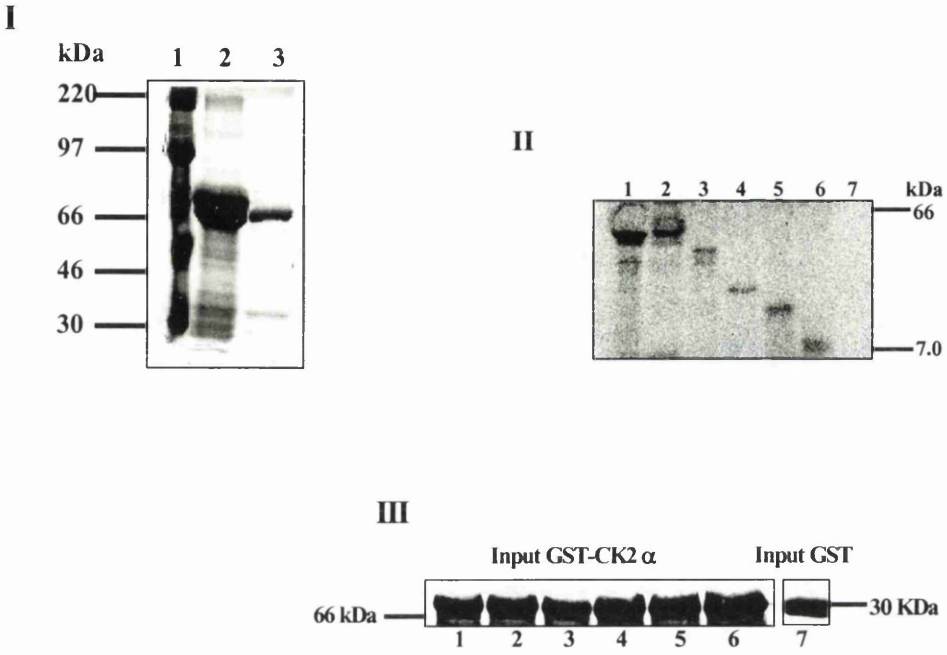
GST-CK2 α or GST alone, with ^{35}S-labelled-ORF57 (FL) or deletion mutants (pcDNA4-ORF57 containing aa 1-455, 17-455, 1-215, 181-328, 329-455 and 387-455) were used to map ORF57 regions required for interaction with CK2 α. Fig. 4A4.1 Panel I shows the aa present and a typical expression of each ^{35}S-labelled-ORF57 protein, as visualised by phosphorimager analysis. The fusion proteins used were visualised by Coomassie brilliant blue staining (Fig. 5A3.1 Panel I), and approximately equal amounts of GST-CK2 α or GST alone were added to ^{35}S-labelled-ORF57 (FL or deletion mutants) and the pull down assays were performed.

Labelled ORF57 (FL) and all deletion mutants aa 17-455, 1-215 181-328, 329-455 and 387-455 bound GST-CK2 α *in vitro* (Fig. 5A3.1 Panel II lanes 1-6), whereas no binding was detected with GST alone (lane 7). Equivalent amounts of the fusion protein GST-CK2 α and GST alone were used in the pull down assay as confirmed by Western blotting the other half of the eluted samples, and immunodetection with anti-GST antibody (Fig. 5A3.1 Panel III lanes 1-6 and 7 respectively). These data indicated a requirement for multiple regions of ORF57 for interaction with CK2 α involving regions throughout the ORF57 protein. No interaction and binding was observed between GST alone and the full-length ORF57 demonstrating specificity of the interactions.

5A3.2 Mapping regions of ORF57 involved in interaction with CK2 α'

GST-CK2 α' or GST alone with ^{35}S-labelled-ORF57 full-length or deletion mutants

Fig 5A3.1. Several regions of catalytic subunit CK2 α interact with ORF57



Panel I. Expression of CK2 α and α' GST-fusion proteins: Coomassie blue stained gel. GST-CK2 α and GST-CK2 α' (FL) fusion proteins used were run on a 10% SDS-PAGE gel and visualised by Coomassie staining. The smaller bands in lanes 2 and 3 represent degradation products/truncations of GST-CK2 α and GST-CK2 α' proteins respectively. Shown in **lanes: 1.** Protein gel marker **2.** GST-CK2 α (FL) **3.** GST-CK2 α' (FL).

Panel II. Phosphorimager analysis showing that several regions of catalytic subunit CK2 α interact with ORF57. GST-CK2 α (FL) or GST fusion proteins bound onto glutathione beads were incubated with ^{35}S-labelled-ORF57 (FL and deletions). After washing, specifically bound proteins were eluted and separated on a 20% SDS-PAGE gel, dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** ^{35}S-labelled-ORF57 (aa 17-455) + GST-CK2 α (FL) **2.** ^{35}S-labelled-ORF57 (aa 1-455 FL)+ GST-CK2 α (FL) **3.** ^{35}S-labelled-ORF57 (aa 1-215) + GST-CK2 α (FL) **4.** ^{35}S-labelled-ORF57 (aa 181-328) + GST-CK2 α (FL) **5.** ^{35}S-labelled-ORF57 (aa 329-455) + GST-CK2 α (FL) **6.** ^{35}S-labelled-ORF57 (aa 387-455) + GST-CK2 α (FL) **7.** ^{35}S-labelled-ORF57 (aa 1-455 FL) + GST alone.

Panel III. Western blot for GST-CK2 α and GST with anti-GST Ab on pull down samples loaded in Panel II. Pulled down proteins were eluted and separated on a SDS-PAGE gel, and Western blotted for GST-CK2 α (FL) or GST using anti-GST Ab.

(pcDNA4-ORF57 containing aa 1-455, 17-455, 1-215, 181-328, 329-455 and 387-455) were used to map ORF57 regions required for interaction with CK2 α'. Fig. 4A1.1 Panel I shows the aa present and a typical expression of each ORF57 protein, as visualized by phosphorimager analysis. The fusion proteins used were visualised by Coomassie brilliant blue staining (Fig. 5A3.1 Panel I) and approximately equal amounts of GST-CK2 α' fusion protein or GST alone were added to labelled-ORF57 (FL or deletion mutants) protein and the pull down assays were performed.

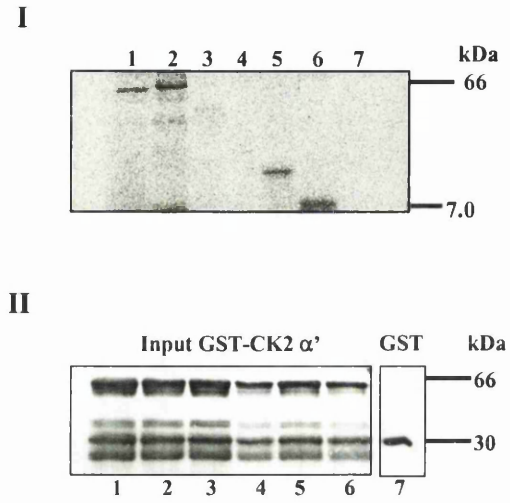
Labelled-ORF57 full-length and deletion mutants aa 17-455, 329-455 and 387-455 bound to GST-CK2 α' *in vitro* (Fig. 5A3.2 Panel I lanes 2, 1, 5 and 6) whereas, no binding was detected with deletion mutants aa 1-215 and 181-328 (lanes 3 and 4) and GST alone (lane 7). Use of similar amounts of the GST alone and GST-CK2 α' were determined by Western blotting the other half of the eluted samples, and immunodetection with anti-GST antibody (Fig. 5A3.2 Panel II lanes 1-6 and 7 respectively). Thus the minimum region of ORF57 sufficient for interaction with CK2 α' involves C terminus aa 387-455. No interaction was found with the N terminus (aa 1-215) and middle region (aa 181-328). These data indicated that there is an absolute requirement for the C terminus region of ORF57 for interaction with CK2 α'. No interaction and binding was observed between the GST alone and the full-length ORF57 demonstrating the specificity of the interaction.

5A3.3 Mapping regions of ORF57 involved in interaction with CK2 β

MBP-CK2 β or MBP alone, with ^{35}S-labelled-ORF57 full-length or deletion mutants (pcDNA4-ORF57 containing aa 1-455, 17-455, 1-215, 181-328, 329-455 and 387-455) were used to map regions of ORF57 required for interaction with MBP-CK2 β. Fig. 4A4.1 Panel I shows the aa present and a typical expression of each ORF57 protein, as visualized by phosphorimager analysis. The fusion proteins used were visualised by Coomassie brilliant blue staining (Fig. 5A3.3 Panel I), and approximately equal amounts of MBP-CK2 β or MBP alone were added to labelled-ORF57 (FL or deletion mutants) and the pull down assays were performed (section 2B4.9).

ORF57 full-length and deletion mutants aa 17-455, 1-215 and 181-328 bound to MBP-CK2 β *in vitro* (Fig. 5A3.3 Panel II a lanes 2, 1, 3 and 4 and Panel II b lanes 1, 2 & 3), whereas no binding was detected with ORF57 aa 329-455 and 387-455 (Panel II a lanes 5 & 6 and Panel II b lanes 4 & 5) and with MBP alone (Panel II a lane 7). Use of similar amounts of MBP-CK2 β and MBP alone in the pull down assay was confirmed (Fig. 5A3.3 Panel III lanes 1-6 and 7 respectively) by Western blotting the other half of

Fig 5A3.2. CK2 α' interacts with C-terminal aa 387-455 of ORF57

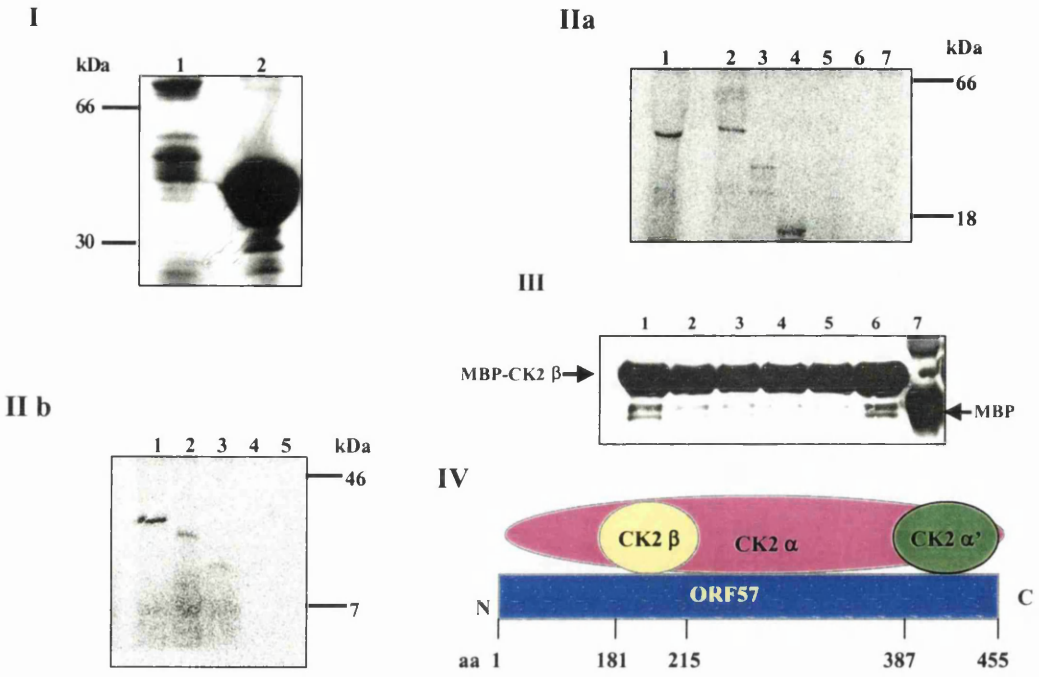


GST-CK2 α' (FL) or GST fusion proteins bound onto glutathione beads were incubated with ^{35}S-labelled-ORF57 (FL and deletions). After washing, specifically bound proteins were eluted and separated on a 20% SDS-PAGE gel, and either dried and analysed by phosphorimaging or transferred to nitrocellulose and Western blotted for GST-CK2 α' (FL) or GST using anti-GST Ab.

Panel I. Phosphorimager analysis showing that CK2 α' interacts with C-terminal aa 387-455 of ORF57. Pull downs included in **lanes: 1.** ^{35}S-labelled-ORF57 (aa 17-455) + GST-CK2 α' (FL) **2.** ^{35}S-labelled-ORF57 (aa 1-455 FL)+ GST-CK2 α' (FL) **3.** ^{35}S-labelled-ORF57 (aa 1-215) + GST-CK2 α' (FL) **4.** ^{35}S-labelled-ORF57 (aa 181-328) + GST-CK2 α' (FL) **5.** ^{35}S-labelled-ORF57 (aa 329-455) + GST-CK2 α' (FL) **6.** ^{35}S-labelled-ORF57 (aa 387-455) + GST-CK2 α' (FL) **7.** ^{35}S-labelled-ORF57 (aa 1-455 FL) + GST alone.

Panel III. Western blot for GST-CK2 α' and GST with anti-GST Ab on pull down samples loaded in Panel II.

Fig 5A3.3. CK2 β interacts with C-terminal aa 181-215 of ORF57



Panel I. Expression of MBP-CK2 β: Coomassie blue stained gel. MBP-CK2 β (FL) and MBP fusion proteins used were run on a 10% SDS-PAGE gel and visualised by Coomassie staining. The smaller bands in lanes 1 and 2 represent degradation products/truncations of MBP-CK2 β and MBP proteins respectively. Shown in **lanes: 1.** MBP-CK2 β FL (aa 1-215) **2.** MBP alone.

Panel II. Phosphorimager analysis showing CK2 β interacts with C-terminal aa 181-215 of ORF57. MBP-CK2 β (FL) or MBP fusion proteins bound onto maltose beads were incubated with ^{35}S-labelled-ORF57 (FL and deletions). After washing, specifically bound proteins were eluted and separated on SDS-PAGE gel, and either dried and analysed by phosphorimaging or transferred to nitrocellulose and Western blotted for GST-CK2 α' (FL) or GST using anti-GST Ab.

Panel II a. Phosphorimager analysis. Pull downs included in **lanes: 1**. ^{35}S-labelled-ORF57 (aa 17-455) + MBP-CK2 β (FL) **2.** ^{35}S-labelled-ORF57 (aa 1-455 FL)+ MBP-CK2 β (FL) **3.** ^{35}S-labelled-ORF57 (aa 1-215) + MBP-CK2 β (FL) **4.** ^{35}S-labelled-ORF57 (aa 181-328) + MBP-CK2 β (FL) **5.** ^{35}S-labelled-ORF57 (aa 329-455) + MBP-CK2 β (FL) **6.** ^{35}S-labelled-ORF57 (aa 387-455) + MBP-CK2 β (FL) **7.** ^{35}S-labelled-ORF57 (aa 1-455 FL) + MBP alone.

Panel II b. Phosphorimager analysis. Pull downs included in **lanes: 1**. ^{35}S-labelled-ORF57 (aa 17-455) + MBP-CK2 β (FL) **2.** ^{35}S-labelled-ORF57 (aa 1-215) + MBP-CK2 β (FL) **3.** ^{35}S-labelled-ORF57 (aa 181-328) + MBP-CK2 β (FL) **4.** ^{35}S-labelled-ORF57 (aa 329-455) + MBP-CK2 β (FL) **5.** ^{35}S-labelled-ORF57 (aa 387-455 FL) + MBP-CK2 β (FL).

Panel III. Western blot for MBP-CK2 β and MBP with anti-MBP Ab on samples loaded in Panel II a

Panel IV. Representation of CK2 subunits binding regions on ORF57

the eluted samples, and immunodetection with anti-MBP antibody (Table 2A7). Thus the minimum region required for interaction with CK2 β involves ORF57 aa 181-215. No interaction was observed between CK2 β and the C-terminal region of ORF57, and between MBP alone and full-length ORF57 demonstrating the specificity of the interaction. Fig. 5A3.3 Panel IV shows a diagrammatic representation of the various regions of ORF57 involved in interaction with both the catalytic (α/α') and regulatory (β) CK2 subunits.

5A4 ORF57 protein partially co-localises with CK2 within cells

CK2 has a nuclear and cytoplasmic distribution depending on the cell type used and the stage of cell cycle. ORF57 in TPA-induced BCBL-1 cells and pKS3 (GFP-gORF57) or pKS4 (GFP-cORF57) transfected cells shows a nuclear distribution. Confocal microscopy was used to visualise the locations of ORF57 and CK2 in transfected mammalian cells. Cells were stained for CK2 β subunit using β-c rabbit Ab (Table 2A7) whereas transfected ORF57 was visible due to green fluorescence of GFP. CK2 (red) being a cellular protein was present in all the cells whereas ORF57 transfected cells could be recognised by the presence of green fluorescence of GFP-ORF57 (Fig 5A4 Panels I, II and III).

In BHK and HeLa cells both proteins were nuclear but also occupied different domains. CK2 was also present in cytoplasm of some cells (CK2 mock in Panel I, II and III). ORF57 was only nuclear and on overlay, both proteins show partial punctate co-localisation with each other suggesting their involvement in various activities at some point in the cell cycle. (Fig 5A4 Panels II and III). Addition of DRB did not alter the distribution of transfected ORF57 protein in either cell types whereas CK2 became nuclear (CK2 mock in Panel I, II and III) and as a control for DRB, distribution of hnRNP A1 did not alter and it remained nuclear in presence or absence of DRB (Panel I).

5B CK2 activity in the ORF57-CK2 complex

5B1 ORF57 is phosphorylated by CK2

5B1.1 ORF57 protein has CK2 consensus sites

After confirming the physical association of CK2 with ORF57, the next objective was to find out if ORF57 could be phosphorylated by CK2. The ORF57 protein aa sequence was scanned for possible motifs (including PROSITE patterns, PROSITE profiles and pfam

Fig 5A4 Panel I. CK2 and hnRNP A1 localisation in BHK cells in the presence or absence of DRB

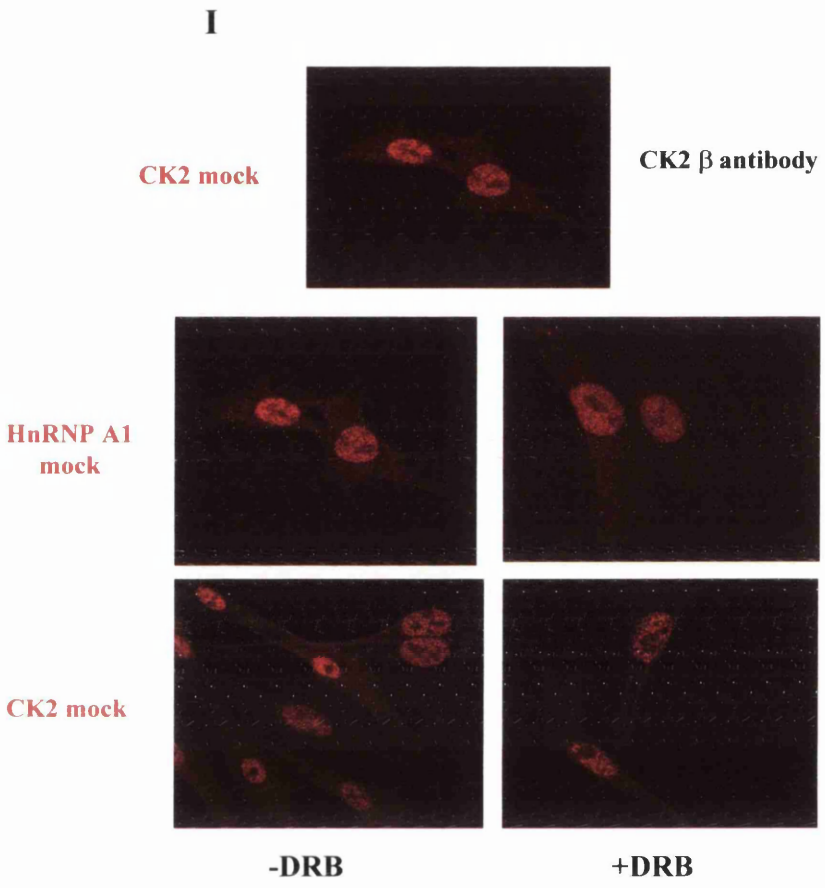
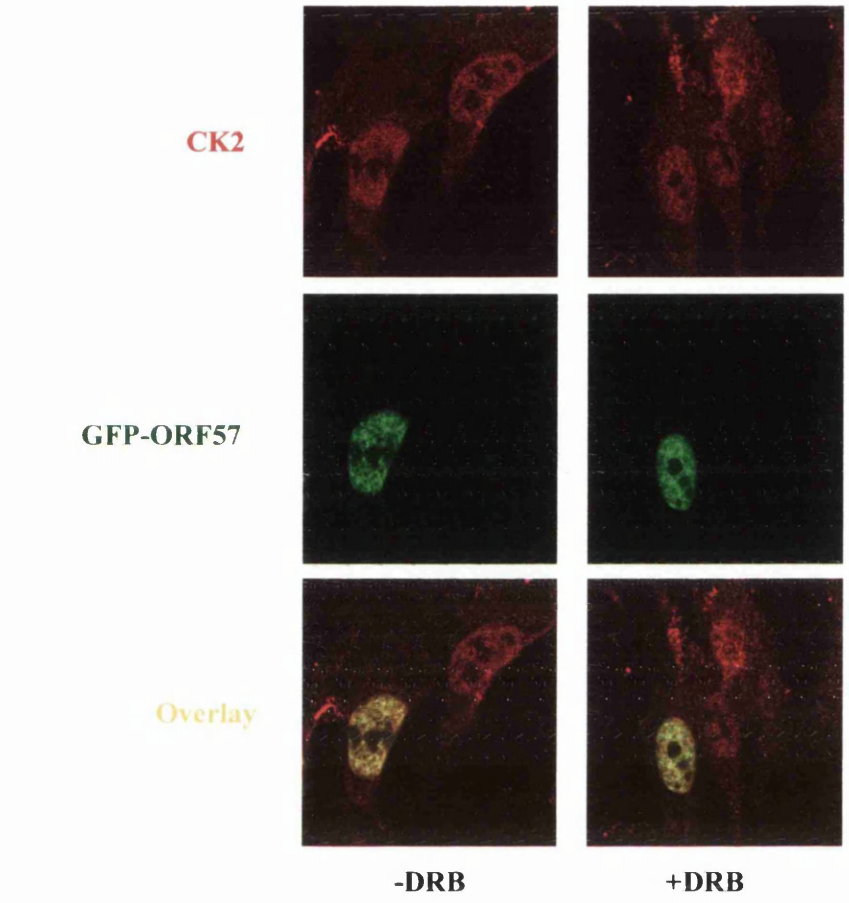


Fig 5A4 Panel II. GFP-ORF57 transfected in BHK cells partially colocalises with CK2

II



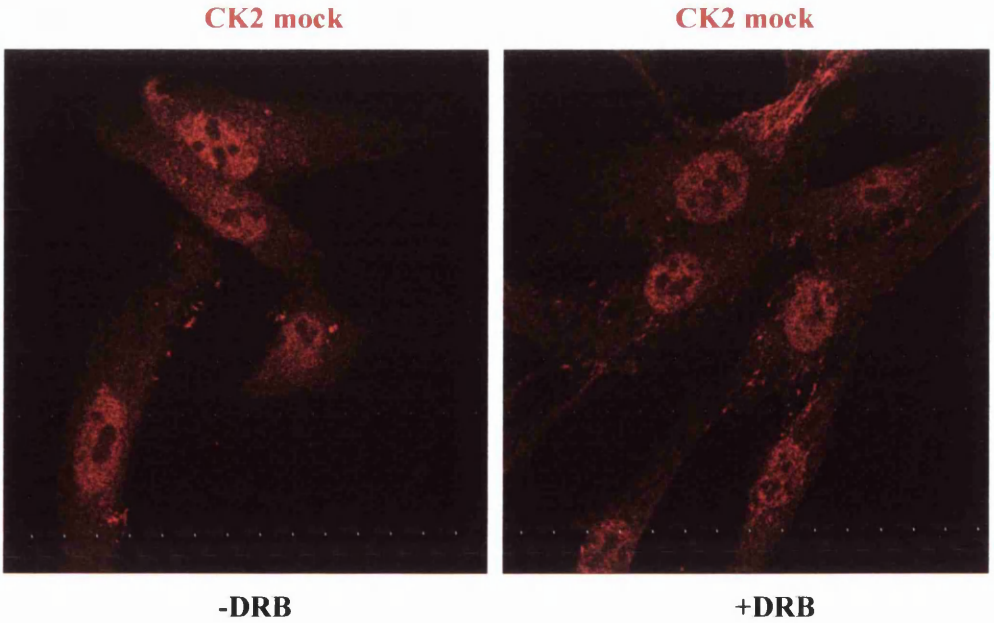
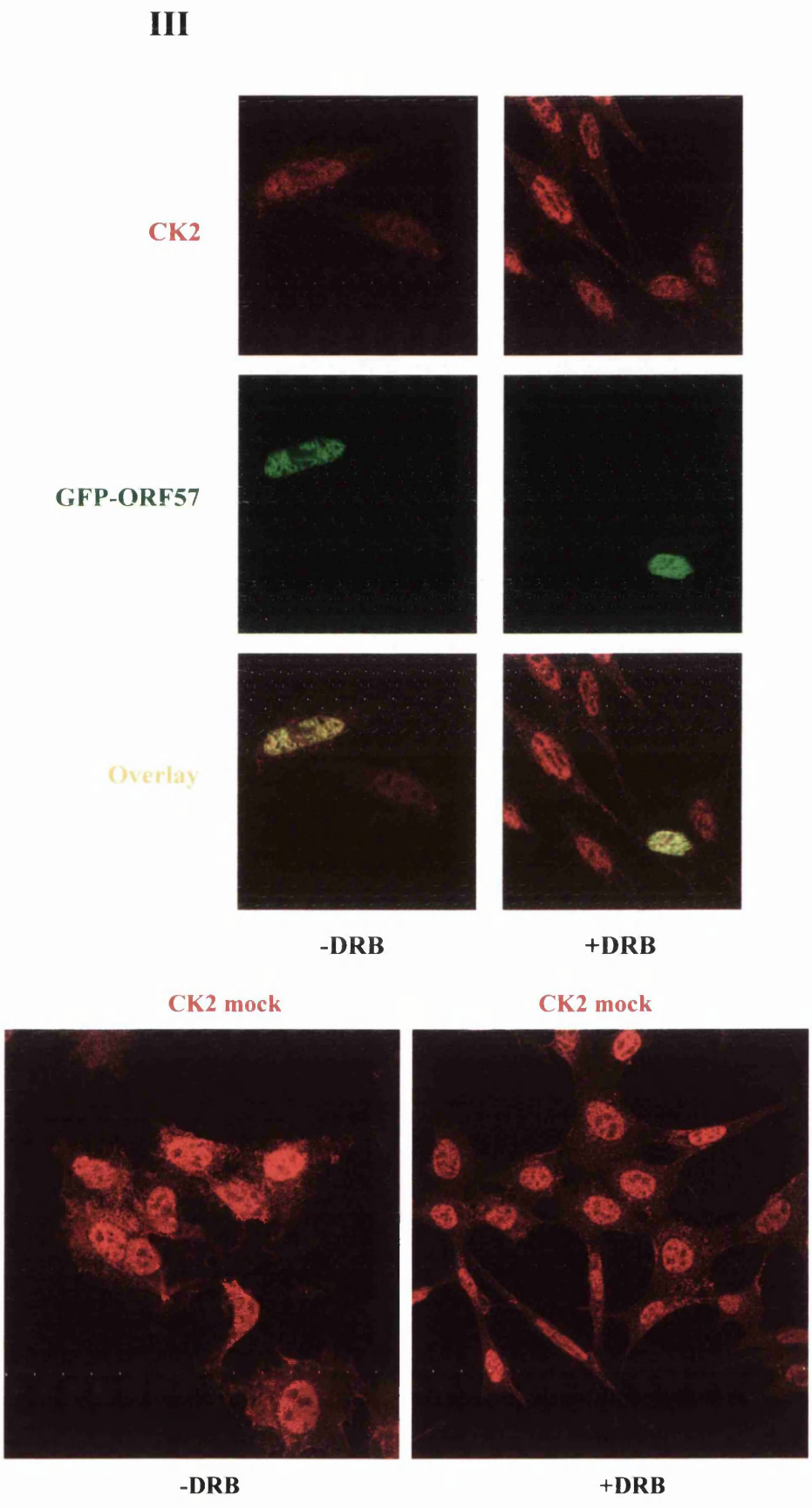


Fig 5A4Panel III. GFP-ORF57 transfected in HeLa cells partially colocalises with CK2



collection) using the website: *Http://Expasy.cbr.nrc.ca/cgi-bin/scanprosite* and *Http://hits.isb-sib.ch/cgi-bin/PFSCAN* (Falquet *et al.*, 2002) and various motifs and sites for several phosphorylation kinases were obtained which are colour coded and conserved residues in all gamma-2 herpesviruses are shown in bold (Fig. 5B1.1 Panel I). ORF57 aa sequence included six putative consensus sites for CK2 phophorylation (Fig. 5B1.1 Panel II). Phosphorylation of ORF57 by CK2 is therefore not unexpected.

5B1.2 ORF57 is phosphorylated in vitro by CK2

Analysis of the CK2 phosphorylation consensus sites in ORF57 protein indicated four consensus sites in the N-terminal portion (aa 1-180) and two sites in the C-terminal portion (Fig. 5B1.1 Panel II). Thus, ORF57 fusion protein with a deletion of 180 aa at the N terminus (GST-57 small), in comparison to full-length ORF57, should have only two possible CK2 consensus sites. The phosphorylating ability of CK2 on GST-57 (both FL and small, see Fig 3A2 Panel I) or GST alone was examined using the *in vitro* phosphorylation assay (Section 2B7.1). GST-57 (FL or small) or GST coated Sepharose beads, were mixed with of human recombinant CK2 holoenzyme, then CK2 reactions (Section 2B7.1) were carried out in the presence or absence of the specific CK2 inhibitor DRB (Zandomeni & Weinmann, 1984). The reaction mix was analysed by SDS-PAGE. The gel was either dried and exposed to the phosphoImager screen to analyse phosphorylated proteins or transferred to a nitrocellulose membrane for immunoblotting.

Results demonstrated that recombinant CK2 holoenzyme phosphorylated GST-57 *in vitro* (Fig. 5B1.2 Panel I) and GST-57 full-length was phosphorylated to a greater extent than the N-terminal deletion mutant GST-57 small (lanes 4 & 1 respectively). The phosphorylation of ORF57 *in vitro* was CK2 specific as addition of DRB to the reaction mix considerably reduced the phosphorylation of full-length GST-57 (FL and small) and larger truncations of GST-57 (FL) (lanes 5 & 2). Absence of recombinant CK2 holoenzyme added to GST-57 (FL) fusion caused failure to phosphorylate ORF57 (lane 3). GST-ICP27 was used as a positive control for the reaction and, as expected, was phosphorylated by CK2 (lane 6) and was inhibited by DRB (lane 7). GST alone, was phosphorylated apparently also by kinases other than CK2, because DRB inhibited phosphorylation of GST to a lesser extent (Fig. 5B1.2 Panel II lanes 3 & 4) compared to phosphorylation of GST-57 (Panel II lanes 1 & 2). The relative amounts of GST-57 protein in the phosphorylation reactions were determined by Western blotting the phosphorylated samples with anti-ORF57 Ab; the protein level of GST-57 (FL) in the absence of DRB was similar to that in the presence of DRB (Panel III lanes 1 & 2).

Fig 5 B1.1 Panel I. Various proposed motifs on ORF57

I

Motif scan with the KSHV ORF57 amino acid sequence

[1]MVQAMIDMDIMKGILEDSVSSSEFDESRDDETDAPTLEDEQLSEPA
EPPADERIRGTQSAQGIPPPLGRIPKKSQGRSQLRSEIQFCSPLSRP
RSPSPVNRYGKKIKFGTAGQNTRPPPEKRPRRRPRDRLQYGRTTRGG
QCRAAPKRATRRPQVNCQRQDDDVRQGVSDAVKKLRLPAS[180]MIIDG
ESPRFDDSIIPRHHGACFNVFIPAPPSHVP**E**VFTDRDITALIRAGGK
DDELIN**K**KISAKKIDHLHRQMLSFVTSRH**N**QAY**W**VSC**R**RETAAAGGL
QTLGAFVE**E**QMT**W**AQTVVRHGGWFDEKDI**D**IILDTAIFVCNAFVTRF
RLLHLS**C**VFDKQSELALIKQVAYLVAMGNRLVEACNLLGEVKLNFRG
GLLLAFVLTIPGMQSRRSISARGQELFRTLLEY**Y**R**PG**DVMGLLNVIV
ME**H**HSLCRNSE**C**AAATRAAMGSAKFNK**G**LF**F**YPLS[455]

1. **PKC PHOSPHORYLATION SITE:** Protein kinase C phosphorylation site
Number of matches: 10
   1. aa's 136-138 TTR
   2. aa's 150-152 TRR
   3. aa's 187-189 SPR
   4. aa's 219-221 TDR
   5. aa's 242-244 SAK
   6. aa's 442-444 SAK
   7. aa's 258-260 TSR
   8. aa's 268-270 SCR
   9. aa's 388-390 SRR
   10. aa's 393-395 SAR
2. **cAMP PHOSPHORYLATION SITE**: cAmp- and cGMP-dependent protein kinase phosphorylation site
Number of matches: 3
   1. aa's 147-150 KRAT
   2. aa's 239-242 KKIS
   3. aa's 270-273 RRET
3. **TYROSINE PHOSPHORYLATION SITE**: Tyrosine kinase phosphorylation site
   1. aa's 125-133 RRPRDRLQY
4. **MYRISTYL**: N-myristoylation site
Number of matches: 6
   1. aa's 56-61 GTQSAQ
   2. aa's 140-145 GQCRAA
   3. aa's 167-172 GVSDAV
   4. aa's 200-205 GACFNV
   5. aa's 277-282 GGLQTL
   6. aa's 374-379 GLLLAF
5. **AMIDATION**: Amidation site
   1. aa's 102-105 YGKK
6. **LEUCINE ZIPPER**: Leucine zipper pattern
   1. aa's 343-364 LIKQVAYLVAMGNRLVEACNLL
7. **ARGININE RICH**: Arginine rich region
   1. aa's 122-152 RPRRRPRDRLQYGRTTRGGQCRAAPKRATRR
8. **CK2 PHOSPHORYLATION SITE**: See Fig 5B1.1 Panel II

*** Bold residues are conserved among ORF57 homologues.**
*** Each motif is coded with the same colour as marked in the legends below.**

Fig 5B1.1 Panel II. **CK2 consensus phosphorylation sites in ORF57**

II

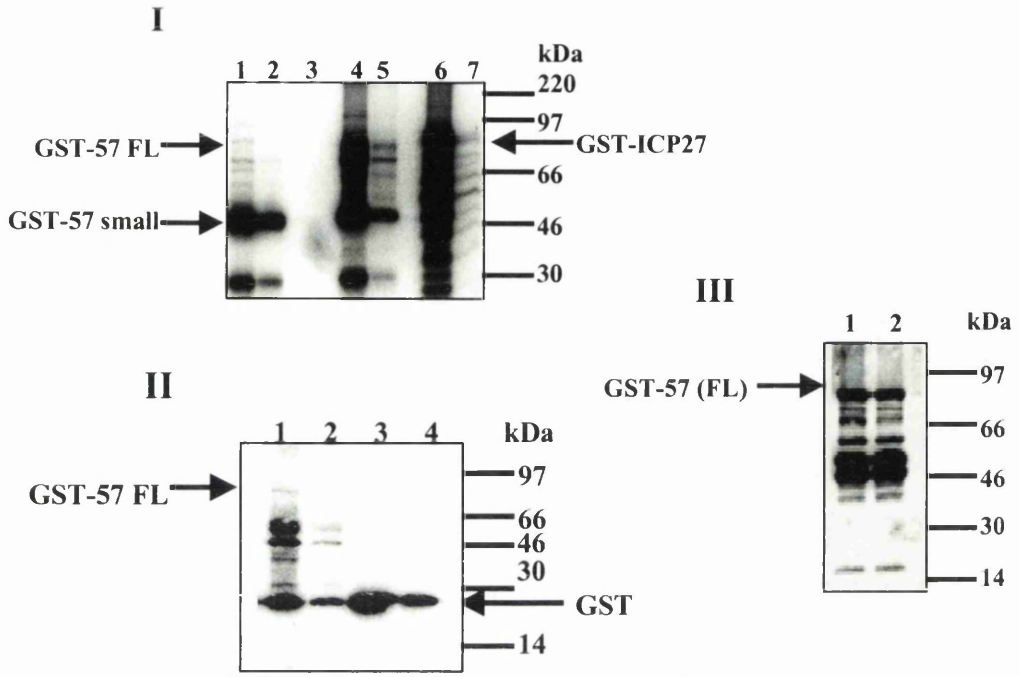
CK2 consensus phosphorylation sites in the KSHV ORF57 amino acid sequence

[1]MVQAMIDMDIMKGILEDSVSSSEFDESRDDETDAPTLED
EQLSEPAEPPADERIRGTQSAQGIPPPLGRIPKKSQGRSQ
LRSEIQFCSPLSRPRSPSPVNRYGKKIKFGTAGQNTRPPP
EKRPRRRPRDRLQYGRTTRGGQCRAAPKRATRRPQVNCQR
QDDDVRQGVSDAVKKLRLPAS[180]MIIDGESPRFDDSIIPR
HHGACFNVFIPAPPSHVP**E**VFTDRDITALIRAGGKDDELI
N**K**KISAKKIDHLHRQMLSFVTSRH**N**QAY**W**VSC**R**RETAAAG
GLQTLGAFVE**E**QMT**W**AQTVVRHGGWFDEKDI**DI**ILDTAIF
VCNAFVTRFRLLHLS**C**VFDKQSELALIKQVAYLVAMGNRL
VEACNLLGEVKLNFRGGLLLAFVLTIPGMQSRRSISARGQ
ELFRTLLEY**Y**R**PG**DVMGLLNVIVME**H**HSLCRNSE**C**AAATR
AAMGSAKFNK**G**LF**F**YPLS[455]

1. aa 20-23 SSSE
2. aa 22-25 SEFD
3. aa 27-30 SRDD
4. aa 36-39 TLED
5. aa 219-222 TDRD
6. aa 402-405 TLLE

*** Bold residues in the ORF57 aa sequence are conserved amongst ORF57 homologues**
*** CK2 sites are given in red**

Fig 5B1.2. ***In vitro*** **phosphorylation of ORF57 by CK2**



Fusion protein beads GST-57 (FL and small) or GST alone were mixed with human recombinant CK2 holoenzyme from *E. coli* ($\alpha_2\beta_2$ holoenzyme) in phosphorylation kinase buffer. Reactions were carried out for 30 min at 25°C either in the presence or absence of 100 μM 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (DRB) a CK2 inhibitor that acts *in vitro* and *in vivo*. GST-ICP27 protein was used as a positive control substrate and GST protein coated beads were used as a negative control. After incubation, protein gel loading buffer was added, samples were heated for 5 min at 100°C and proteins were run on by SDS-PAGE, gel was either dried and analysed by phosphorimaging or transferred to nitrocellulose and Western blotted for GST-57 and GST using anti-GST Ab.

Panel I. Phosphorimager analysis showing *in vitro* phosphorylation of ORF57 (FL and small) by CK2. Phosphorylation reactions included in **lanes: 1.** GST-57 (small, aa 181-455) + recombinant CK2 + ^{32}P-γ-dATP **2.** GST-57 (small aa 181-455) + recombinant CK2 + ^{32}P-γ-dATP + DRB **3.** GST-57 (FL, aa 1-455) + no recombinant CK2 + ^{32}P-γ-dATP **4.** GST-57 (FL, aa 1-455) + recombinant CK2 + ^{32}P-γ-dATP **5.** GST-57 (FL, aa 1-455) + recombinant CK2 + ^{32}P-γ-dATP + DRB **6.** GST-ICP27 + recombinant CK2 + ^{32}P-γ-dATP **7.** GST-ICP27 + recombinant CK2 + ^{32}P-γ-dATP + DRB.

Panel II. Phosphorimager analysis showing that ORF57 gets phophorylated *in vitro* by CK2. Phosphorylation reactions included in **lanes: 1.** GST-57 (FL) + recombinant CK2 + ^{32}P-γ-dATP **2.** GST-57 (FL) + recombinant CK2 + ^{32}P-γ-dATP + DRB **3.** GST alone + recombinant CK2 + ^{32}P-γ-dATP **4.** GST alone + recombinant CK2 + ^{32}P-γ-dATP + DRB.

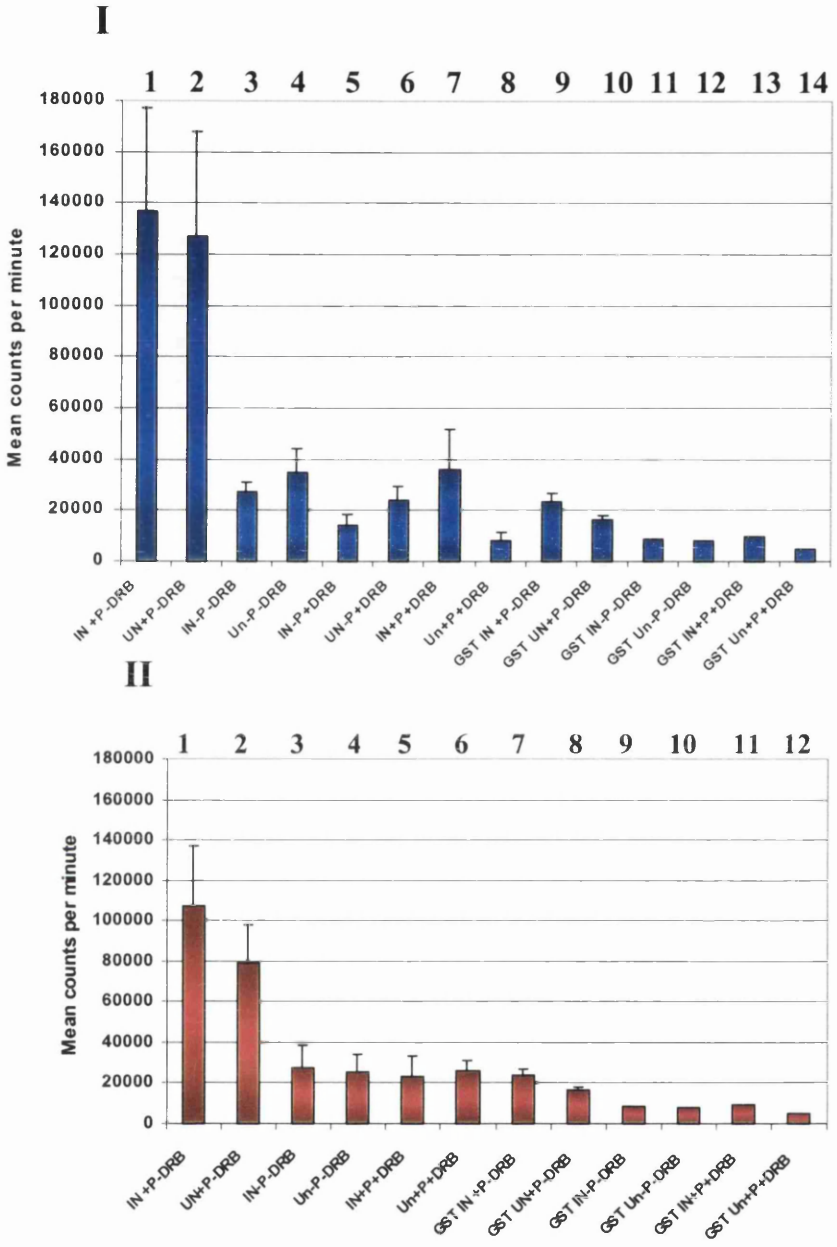
Panel III. Western blot for GST-57 with anti-ORF57 Ab showing that similar amounts of GST-57 were used for *in vitro* phosphorylation. Phosphorylation reactions included in **lanes: 1.** GST-57 (FL) + recombinant CK2 + ^{32}P-γ-dATP **2.** GST-57 (FL) + recombinant CK2 + ^{32}P-γ- dATP + DRB.

5B2 CK2 activity is present in the ORF57 pull down complex using a CK2 specific peptide substrate assay

Detection of enzyme activity is a sensitive way of CK2 estimation in the complex and assays to determine CK2 phosphorylation of an artificial peptide substrate were performed (Wadd *et al.*, 1999). The peptide substrate binds to the CK2 β subunit where it is held for the α subunit to phosphorylate. Thus, the peptide substrate assay will not show CK2 activity catalysed by the α subunit unless the CK2 β subunit is also present in the complex. The GST pull down assay using TPA-induced and uninduced BCBL-1 cell extracts was followed by a peptide substrate assay for CK2 activity (Section 2B7.2). An average of three independent experiments performed in duplicates showed that in this assay, GST-57 (FL) gave higher levels of CK2 activity (Fig. 5B2 Panel I lanes 1 & 2) in comparison to GST-57 (small) (Fig. 5B2 Panel II lanes 1 & 2). Much reduced CK2 activity was present with either GST-57 in the absence of peptide substrate (lanes 3 & 4) or in the presence of DRB (lanes 5 & 6) and in pull down samples from GST alone beads (Fig. 5B2 Panel I lanes 9-14 & Panel II lanes 7-12). Positive confirmation of pulled down proteins in the assay using GST-57 fusion protein was obtained by confirming the presence of hnRNP K in the complexes by immunoblotting for this partner protein (Fig. 5B2 Panel III).

CK2 activity was coimmunoprecipitated from pKS3 (pEGFP-gORF57 FL) and pKS1 (pEGFP-ORF57 small) transfected 293 cell extracts using anti-hnRNP K Ab (Fig. 5B2 Panel IV) and pKS3 showed higher levels of CK2 activity in comparison to pKS1 (Fig. 5B2 Panel IV lanes 5 & 2). Little CK2 activity was present with hnRNP K Ab in the absence of peptide substrate (lanes 1 & 4) or in the presence of DRB (lanes 3 & 6) and in immunoprecipitates with pre-immune serum (lanes 10, 12 & 13). Detection of considerable CK2 activity from ORF57 transfected cells implies that CK2 and ORF57 do interact in the cells and activity using anti-hnRNP K Ab demonstrates that hnRNP K is present in the complex with ORF57-CK2. Very little CK2 activity was present with hnRNP K Ab in ORF50 alone transfected 293 cell extracts in the absence of peptide substrate (lanes 7) or in its presence (lanes 8) implying that anti-hnRNP K Ab does not immunoprecipitate ORF50 alone or any CK2 activity associated with ORF50. Detection of considerable CK2 activity with anti-hnRNP K Ab from ORF57+ORF50 cotransfected cells (lanes 9) implies that hnRNP K is present in the complex with ORF57-CK2 and gORF50 may or may not be a part of this immunoprecipitated complex. Thus these data

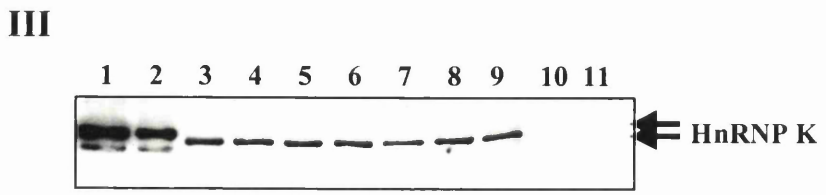
Fig 5B2 Panels I and II. CK2 activity pulled down with GST-57 (FL) (Panel I), GST-57 small (Panel II) or GST alone from BCBL-1 cells



Panels I and II. CK2 activity in GST-57 (FL and small) pull downs. GST-57 (FL and small) pull downs were followed by a CK2 assay. Assays were performed without substrate peptide on pull downs from TPA induced and uninduced BCBL-1 extracts (**lanes 3** and **4**) or with peptide on pull downs from TPA induced (**lane 1**) and uninduced (**lane 2**), BCBL-1 extracts, or with/without peptide on pull downs from TPA induced and uninduced BCBL-1 cell extracts using GST alone (**lanes 9-14** and **7-12**), in presence or absence of DRB.

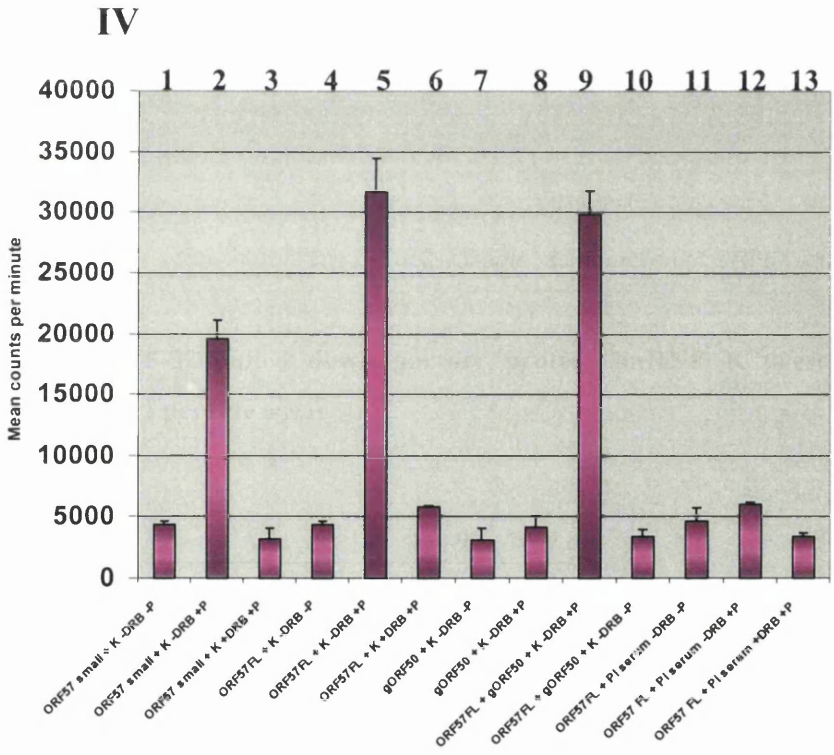
CK2 activity is proportional to counts per minute of γ-[^{32}P]-ATP incorporated into the peptide substrate.

Fig 5B2 Panel III. GST-57 pulled down partner protein hnRNP K present in samples used for the CK2 peptide assay



Panel III. Western blot anti-hnRNP K Ab on GST-57 (FL) pull down samples used for the CK2 peptide assay shown in Fig 5B2 Panel I. Pull downs included in **lanes: 1.** Input uninduced BCBL-1 cell extracts **2.** Input TPA induced BCBL-1 cell extracts **3.** GST-57 (FL) + TPA induced BCBL-1 cell extracts **4.** GST-57 (FL) + uninduced BCBL-1 cell extracts **5.** GST-57 (FL) + TPA induced BCBL-1 cell extracts **6.** GST-57 (FL) + uninduced BCBL-1 cell extracts **7.** GST-57 (FL) + TPA induced BCBL-1 cell extracts **8.** GST-57 (FL) + uninduced BCBL-1 cell extracts **9.** GST-57 (FL) + TPA induced BCBL-1 cell extracts **10.** GST alone + TPA induced BCBL-1 cell extracts **11.** GST alone + uninduced BCBL-1 extracts.

Fig 5B2 Panel IV. CK2 activity in ORF57 (small or FL), ORF50, or ORF57+ORF50 transfected 293 cells generated by anti-hnRNP K Ab immunoprecipitates



Panel IV. CK2 activity in anti-hnRNP K Ab coimmunoprecipitates. Anti-hnRNP K Ab coimmunoprecipitates were followed by a CK2 assay. Assays were performed either in presence or absence of DRB, without peptide substrate or with peptide on coimmunoprecipitates from GFP-ORF57 (FL or small), ORF50 or ORF57 plus ORF50 transfected 293 cell extracts.

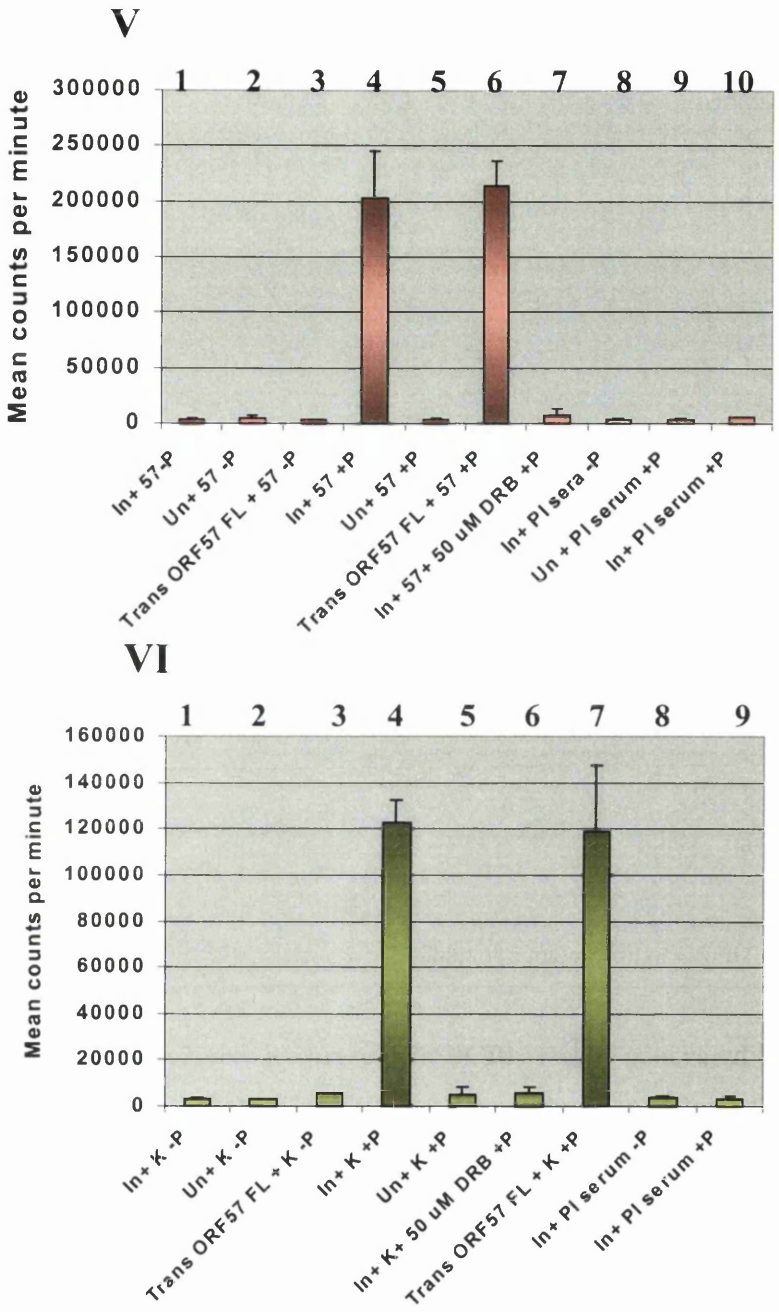
showed that no other viral proteins are required for hnRNP K, CK2 and ORF57 to associate.

CK2 activity was coimmunoprecipitated from TPA-induced and pKS3 transfected BCBL-1 cell extracts using anti-ORF57 (Fig. 5B2 Panel V lanes 4 & 6) and anti-hnRNP K antisera (Fig. 5B2 Panel VI lanes 4 & 7). No CK2 activity was present with either antibody in the absence of peptide substrate (lanes 1, 2 & 3) or in immunoprecipitates from uninduced cell extracts (lane 5) or in the presence of the specific CK2 inhibitor DRB (Panel V lanes 7 & Panel VI lanes 6) and with pre-immune serum (Panel V lanes 8, 9, & 10 and Panel VI lanes 8 & 9). Detection of CK2 activity with anti-ORF57 Ab implies that CK2 and ORF57 interact in the TPA-induced cell extracts and presence of activity using anti-hnRNP K Ab demonstrates that hnRNP K is part of the complex with ORF57-CK2. All three proteins (ORF57-hnRNP K-CK2) coimmunoprecipitated in a complex held together by ORF57 (Fig. 5B2 Panel VI), as there was no detectable CK2 activity with anti-hnRNP K Ab from uninduced cell extracts. Thus the 66 kDa band, the 44 kDa-42 kDa and the 25 kDa bands seen in the original coimmunoprecipitation (Fig. 3B1) presumably correspond to hnRNP K and to CK2 α/α' and β subunits.

5B3 The CK2 specific inhibitor DRB reduces ORF57 phosphorylation in virus-infected cells

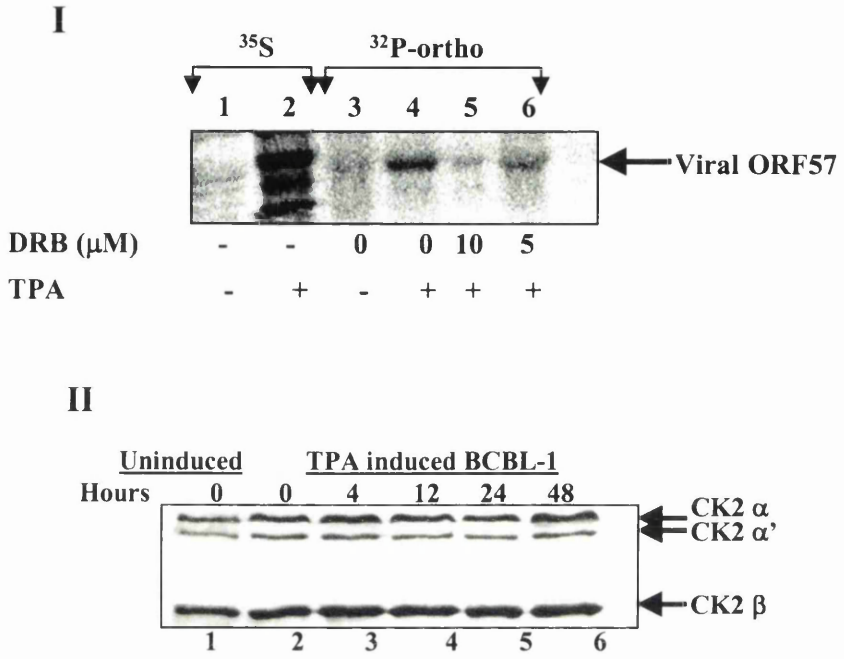
Uninduced, TPA-induced or pKS3 (pEGFP-gORF57)/pKS4 (pEGFP-cORF57) transfected BCBL-1 cells were labelled with either ^{35}S-methionine or ^{32}P-orthophosphate for 6 h. Low, non toxic, doses of DRB, were applied to living cells to test for effects on ORF57 phosphorylation. In some experiments [^{32}P]-orthophosphate labelling was done for 6 h in the presence or absence of DRB, and then immunoprecipitations were performed on cell extracts using the ORF57Ab. Phosphorylation of ORF57 in TPA-induced BCBL-1 cells was reduced in the presence of 5μM DRB (Fig. 5B3 Panel I compare lanes 4 and 6) and the inhibition was greater with 10μM DRB (compare lanes 4 & 5) but was not abolished completely (even at 100μM DRB concentration, data not shown) suggesting that other kinases besides CK2 can phosphorylate ORF57. The ^{35}S-labelled extracts showed the presence of other labelled proteins also immunoprecipitated with anti-ORF57 Ab (lane 2). Western blot analysis on the ORF57 transfected samples showed that ORF57 accumulation was not affected by DRB, excluding the possibility that the reduction of phosphorylation was due to DRB inhibition of transcription. Fig. 5B3 Panel II shows that after TPA induction during the course of lytic replication in BCBL-1 cells, levels of CK2 protein subunits (α, α' and β) do not change in comparison

Fig 5B2 Panels V and VI. CK2 activity present in BCBL-1 cells generated by anti-ORF57 Ab (Panel V) and anti-hnRNP K Ab (Panel VI) coimmunoprecipitates



Panels V and VI. CK2 activity in anti-ORF57 (Panel V) and anti-hnRNP K (Panel VI) Ab coimmunoprecipitates. Anti-ORF57 or anti-hnRNP K Ab coimmunoprecipitates were followed by a CK2 assay. Assays were performed either in presence or absence of DRB, without peptide substrate on coimmunoprecipitates from TPA-induced BCBL-1 cell extracts or with peptide on coimmunoprecipitates from TPA-induced, uninduced, and GFP-gORF57 transfected BCBL-1 cell extracts, or with peptide on coimmunoprecipitations from TPA-induced, and uninduced BCBL-1 cell extracts using pre immune serum.

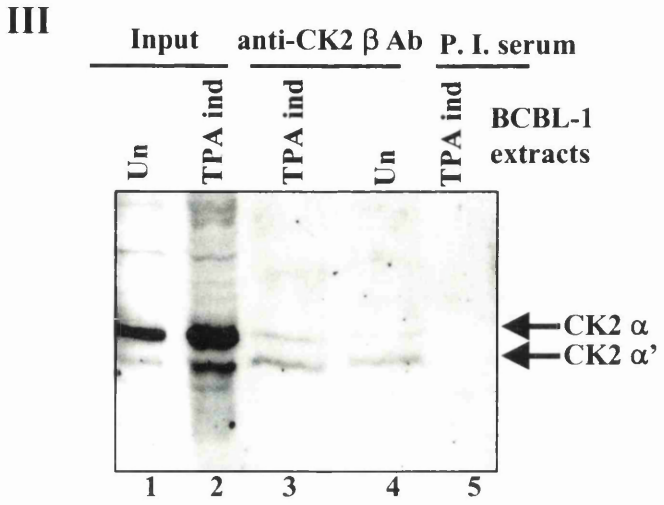
Fig 5B3. DRB inhibits *in vivo* phosphorylation of ORF57 by CK2 in BCBL-1 cells



Panel I. Phosphorimager analysis on anti-ORF57 Ab immunoprecipitates from labelled BCBL-1 cells showing that DRB inhibits *in vivo* phosphorylation of ORF57 by CK2 in BCBL-1 cells. Uninduced or TPA-induced BCBL-1 cells were labelled with either [^{35}S]-methionine (lanes 1-2) or [^{32}P]-orthophosphate (lanes 3-6) for 6h in the presence or absence of DRB. Total extracts from cells uninduced (lanes 1 and 3), TPA-induced (lanes 2 and 4-6) in the absence (lanes 1 to 4) or presence of 5 μM (lane 6) or 10 μM (lane 5) of DRB, were used for immunoprecipitation of ORF57. Anti-ORF57 Ab was used followed by SDS-PAGE electrophoresis and gel was analysed by phosphorimaging. A band migrating at 50-52 kDa corresponding to full-length ORF57 is shown. Phosphorylation reactions included in **lanes: 1.** Uninduced BCBL-1 cells + ^{35}S-methionine **2.** TPA-induced BCBL-1 cells + ^{35}S-methionine **3.** Uninduced BCBL-1 cells+ 0 μM DRB + ^{32}P-orthophosphate **4.** TPA-induced BCBL-1 cells+ 0 μM DRB+ ^{32}P-orthophosphate **5.** TPA-induced BCBL-1 cells+ 10 μM DRB+ ^{32}P-orthophosphate **6.** TPA-induced BCBL-1 cells+ 5 μM DRB+ ^{32}P-orthophosphate.

Panel II. Western blot with anti-CK2 α plus β Abs on BCBL-1 cells at various times post-TPA induction showing that CK2 subunit (α, α' and β) protein levels are not altered in TPA-induced cells in comparison to uninduced BCBl-1. Total extracts from uninduced and TPA-induced cells were used for analysing protein levels of CK2. Cell extracts from various time points post TPA-induction were analysed by SDS-PAGE electrophoresis and gel was transferred to nitrocellulose and Western blotted for CK2 α, α' and β using anti-CK2 α plus β Abs. Shown in **lanes: 1.** Uninduced BCBL-1 cells **2.** TPA-induced BCBL-1 cells at 0 h post-induction **3.** TPA-induced BCBL-1 cells at 4 h post-induction **4.** TPA-induced BCBL-1 cells at 12 h post-induction **5.** TPA-induced BCBL-1 cells at 24 h post-induction **6.** TPA-induced BCBL-1 cells at 48 h post-induction.

Fig 5B3 Panel III. CK2 β Ab coimmunoprecipitates both CK2 α and α' from virus-infected TPA induced BCBL-1 at levels similar to uninduced BCBL-1 cells



Panel III. Western blot for CK2 α protein with anti-CK2 α Ab showing that CK2 β Ab coimmunoprecipitates both CK2 α and α' from virus-infected TPA-induced BCBL-1 at levels similar to uninduced BCBL-1 cells. Total extracts from uninduced and TPA-induced cells were used for immunoprecipitation of CK2. Anti-CK2 β Ab was used followed by SDS-PAGE electrophoresis and gel was transferred to nitrocellulose and Western blotted for CK2 α and α' using anti-CK2 α Ab. Immunoprecipitates included in **lanes: 1.** Input uninduced BCBL-1 extract **2.** Input TPA-induced BCBL-1 extract **3.** TPA-induced BCBL-1 extract + anti-CK2 β Ab (rabbit) **4.** Uninduced BCBL-1 extract + anti-CK2 β Ab (rabbit) **5.** TPA-induced BCBL-1 extract + pre immune serum.

to uninduced cells. Composition of the CK2 holoenzyme was not altered in virus-infected cells as immunoprecipitations performed at 6 h post-TPA induction using anti-CK2 β polyclonal Ab (β-c) showed both α and α' subunits were immunoprecipitated at similar levels from TPA-induced and uninduced BCBL-1 cell extracts (Fig. 5B3 Panel III compare lanes 3 & 4).

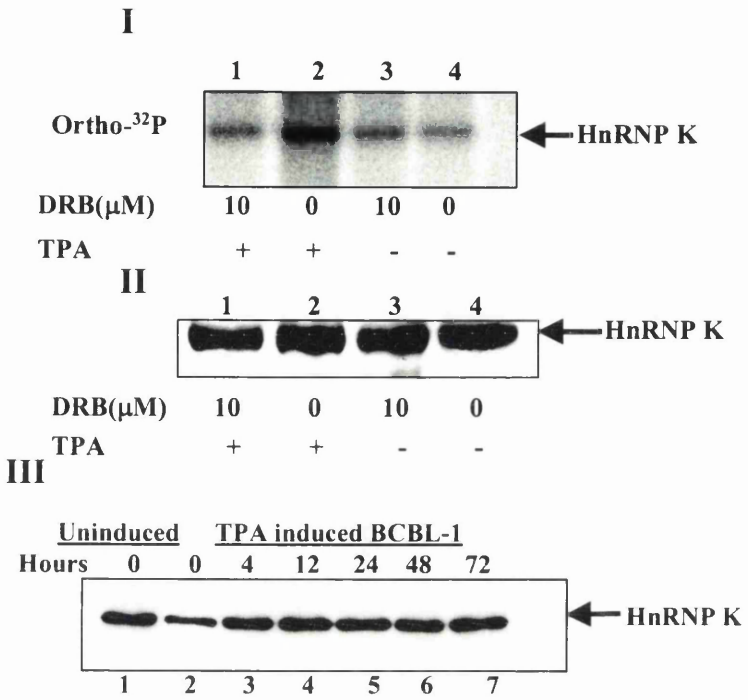
The presence of 44 kDa, 42 kDa and a 25 kDa bands in the anti-ORF57 Ab coimmunoprecipitates (Fig. 3B1), Western blotting of TPA-induced BCBL-1 cells for α/α' and β subunits (Fig. 5B3 Panel II) and the presence of CK2 activity in pull down and coimmunoprecipitated complexes (as detected by the peptide substrate assay) suggests that both catalytic (α/α') and regulatory (β) subunits of CK2 are present in the ORF57 complex.

5B4 Phosphorylation of hnRNP K is altered in virus-infected TPA-induced cells

hnRNP K interacts with inducible kinases (Van Seuningen *et al.*, 1995a; Schullery *et al.*, 1999) and via phosphorylation regulates its interactions with protein and RNA partners (Ostrowski *et al.*, 2000; Ostrowski *et al.*, 2001) But as hnRNP K is not normally phosphorylated by CK2 in uninfected cells (Van Seuningen *et al.*, 1995a) the relevance of its phosphorylation in the presence of ORF57 in virus-infected cells is of great interest. Therefore, the effect of DRB on the phosphorylation status of hnRNP K in virus-infected cells was examined. TPA-induced and uninduced BCBL-1 cells were labelled with [^{32}P]-orthophosphate for 6 h, in the presence or absence of 10μM DRB. Extracts from labelled TPA-induced and uninduced BCBL-1 cells were immunoprecipitated with hnRNP K Ab followed by SDS-PAGE, Western blot analysis and autoradiography.

Fig. 5B4 Panel I shows that the hnRNP K band immunoprecipitated with anti-hnRNP K Ab is phosphorylated and, interestingly, its phosphorylation is inhibited by DRB in the TPA-induced BCBL-1 cells (lane 1) in comparison to the DRB untreated cells (lane 2) but not in the uninduced cells (lane 3 and 4). Western blot analysis using the anti-hnRNP K Ab performed on the same membrane demonstrated similar amounts of hnRNP K protein (Fig. 5B4 Panel II). Fig. 5B4 Panel III shows that after TPA induction of BCBL-1 cells, the levels of hnRNP K protein remain similar to uninduced cells. Thus in the TPA-induced BCBL-1 cells, CK2 phosphorylated ORF57 (Fig. 5B3 Panel I), and hnRNP K (Fig. 5B4 Panel I), and DRB decreased this phosphorylation. Association of ORF57 with CK2 and hnRNP K together in a complex could explain the altered phosphorylation status of hnRNP K in virus-infected cells.

Fig 5B4. The phosphorylation pattern of hnRNP K is altered in TPA induced BCBL-1 cells



Uninduced or TPA-induced BCBL-1 cells were labelled with [^{32}P]-orthophosphate for 6h in the presence or absence of DRB. Total extracts from cells uninduced, TPA-induced in the absence or presence of 10 μM of DRB, were used for immunoprecipitation of hnRNP K. Anti-hnRNP K Ab was used followed by SDS-PAGE electrophoresis and gel was either dried and analysed by phosphorimaging or transferred to nitrocellulose and Western blotted for hnRNP K using anti-hnRNP K Ab.

Panel I. Phosphorimager analysis on anti-hnRNP K Ab coimmunoprecipitates from BCBL-1 cells showing that phosphorylation pattern of hnRNP K is altered in TPA-induced BCBL-1 cells. A band migrating at 66 kDa corresponding to full-length hnRNP K is shown. Immunoprecipitates included in **lanes: 1.** TPA-induced BCBL-1 cells + 10 μM DRB **2.** TPA-induced BCBL-1 cells + 0 μM DRB **3.** Uninduced BCBL-1 cells + 10 μM DRB **4.** Uninduced BCBL-1 cells + 0 μM DRB.

Panel II. Western blot for hnRNP K with anti-hnRNP K Ab on anti-hnRNP K Ab coimmunoprecipitates from BCBL-1 cells showing that in presence or absence of DRB, hnRNP K protein levels remains similar in coimmunoprecipitates from ORF57 transfected and untransfected BCBL-1 cells. Immunoprecipitates included in **lanes: 1.** TPA-induced BCBL-1 cells +10μM DRB **2.** TPA-induced BCBL-1 cells +0μM DRB **3.** Uninduced BCBL-1 cells + 10μM DRB **4.** Uninduced BCBL-1 cells +0μM DRB.

Panel III. Western blot for hnRNP K with anti-hnRNP K Ab on BCBL-1 cell extracts at various times post-TPA induction showing that hnRNP K protein levels are not altered in TPA-induced cells in comparison to uninduced BCBl-1. Total extracts from uninduced and TPA-induced cells were used for analysing hnRNP K protein levels. Cell extracts from various time points post TPA-induction were analysed by SDS-PAGE electrophoresis and gel was transferred to nitrocellulose and Western blotted for hnRNP K using anti-hnRNP K Ab. Shown in **lanes: 1.** Uninduced BCBL-1 cells **2.** TPA-induced BCBL-1 cells at 0 h post-induction **3.** TPA-induced BCBL-1 cells at 4 h post-induction **4.** TPA-induced BCBL-1 cells at 12 h post-induction **5.** TPA-induced BCBL-1 cells at 24 h post-induction **6.** TPA-induced BCBL-1 cells at 48 h post-induction **7.** TPA-induced BCBL-1 cells at 72 h post-induction.

5B5 The CK2 inhibitor DRB reduces interaction of ORF57 with hnRNP K

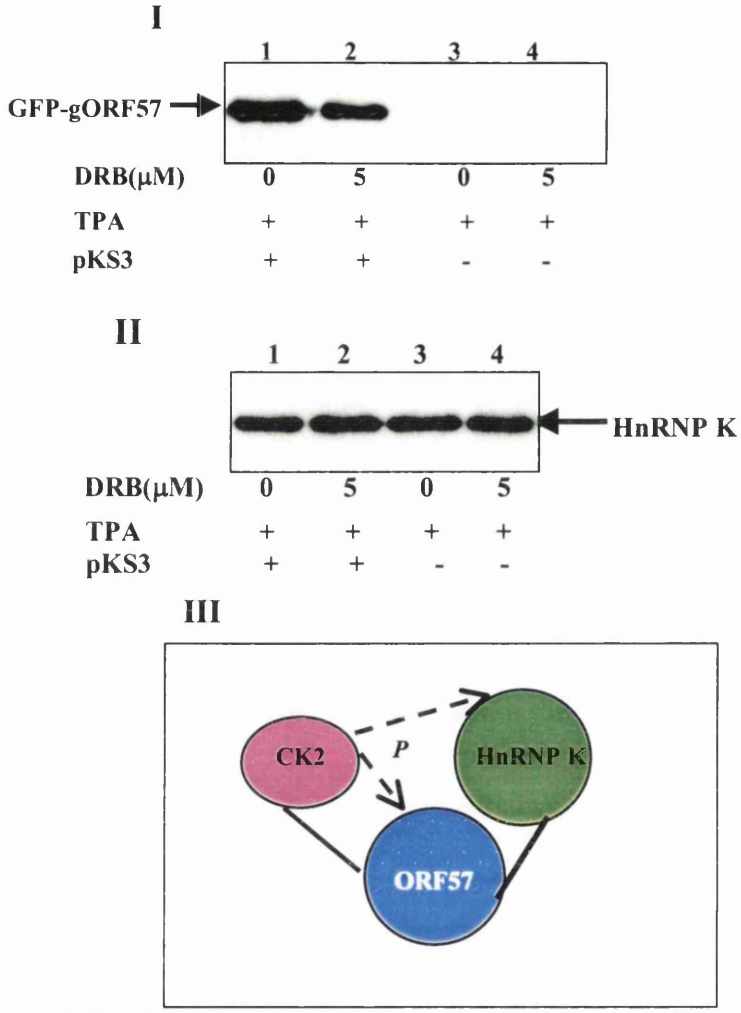
The effect of DRB on the interaction of ORF57 with hnRNP K was examined. BCBL-1 cells were either transfected with plasmid pKS3 (pEGFP-gORF57) and induced with TPA or untransfected and TPA-induced and grown in the presence or absence of 5μM DRB. Cell extracts were immunoprecipitated with the anti-hnRNP K Ab followed by SDS-PAGE and Western blotting. Immunoblotting with the anti-GFP Mab (Table 2A7) showed ORF57 is coimmunoprecipitated with anti-hnRNP K Ab only from the pKS3 transfected TPA-induced cell extracts (Fig. 5B5 Panel I lane 2) and addition of DRB reduced this binding (lane 2) whereas nothing was visible in untransfected TPA-induced cells (lanes 3 & 4). In the presence of DRB, the amount of ORF57 protein interacting with hnRNP K was consistently reduced by ~2-fold. Western blotting using anti-hnRNP K Ab showed that hnRNP K protein was immunoprecipitated in approximately equal amounts from all the cell extracts (Fig. 5B5 Panel II lanes 1-4).

CK2 phosphorylates ORF57 and hnRNP K to some extent in the virus-infected cells (Section 5B3 and 4) and phosphorylation of ORF57 by CK2 is either necessary or increases its interaction with hnRNP K. Thus phosphorylation of ORF57 may play an important role in the ORF57-hnRNP K interaction. Since ORF57 phosphorylation was not completely inhibited by DRB, it is difficult to determine between phosphorylation increasing the affinity of this interaction and an absolute requirement, with enough residual phosphorylated ORF57 present to pull down some hnRNP K. Fig. 5B5 Panel III depicts a representation of the relationship between ORF57, hnRNP K and CK2.

5C Phosphorylation of other proteins in the ORF57 complex

All the coimmunoprecipitated proteins from TPA-induced BCBL-1 cell extracts using ORF57 Ab after *in vitro* phosphorylation assay are shown in Fig. 5C1. Comparison of the same reaction performed in presence of DRB (compare lane 2 with lane 1) revealed CK2 phosphorylation of substrates other than ORF57 and hnRNP K. The smallest band corresponds in size to the 25/32kDa band seen in a ^{35}S-labelled profile could be autophosphorylated CK2 β, the next largest band may be the 44/42 kDa autophosphorylated CK2 α/α' as both CK2 α and β have autophosphorylation sites (Dobrowolska *et al.*, 1999); most CK2 autophosphorylation occurs on the β subunit (Issinger, 1993) (Litchfield *et al.*, 1991). ORF57, hnRNP K and a larger band

Fig 5B5. DRB reduces interaction of ORF57 with hnRNP K



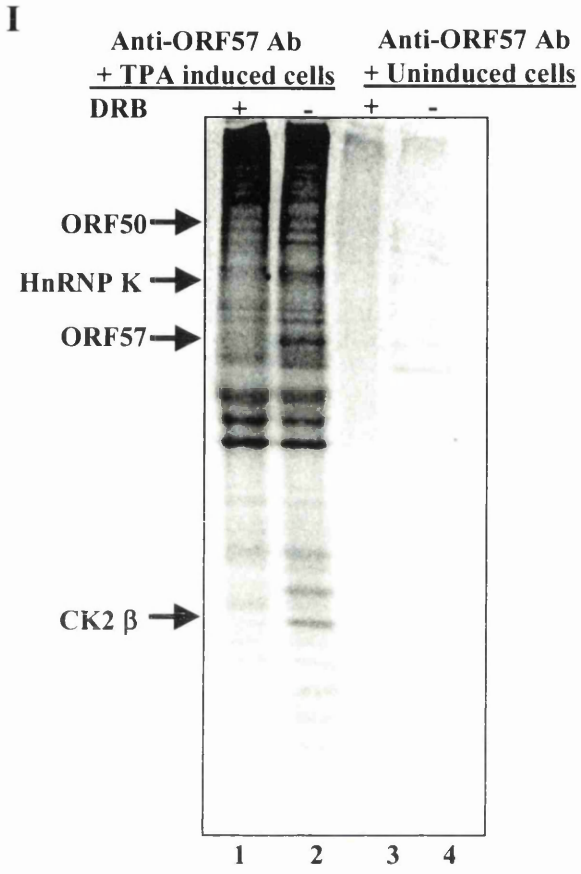
TPA-induced (lanes 1-4) pKS3 (GFP-gORF57) transfected BCBL-1 cells were labelled with [^{32}P]-orthophosphate for 6h in the presence or absence of DRB. Total extracts from cells untransfected (lanes 3 and 4), pKS3 transfected (lanes 1 and 2) in the absence (lanes 1 and 3) or presence of 5 μM (lanes 2 and 4) of DRB, were used for immunoprecipitation of GFP-ORF57. Anti-hnRNP K Ab was used for coimmunoprecipitation followed by SDS-PAGE electrophoresis and Western blotting.

Panel I. Western blot for GFP-ORF57 with anti-GFP Ab on anti-hnRNP K Ab coimmunoprecipitates from BCBL-1 cells showing that DRB reduces interaction of ORF57 with hnRNP K. Coimmunoprecipitates included in **lanes: 1.** pKS3 transfected + TPA-induced BCBL-1 cells + 0 μM DRB **2.** pKS3 transfected + TPA-induced BCBL-1 cells + 5 μM DRB **3.** Untransfected + TPA-induced BCBL-1 cells + 0 μM DRB **4.** Untransfected + TPA-induced BCBL-1 cells + 5 μM DRB.

Panel II. Western blot for hnRNP K with anti-hnRNP K Ab on anti-hnRNP K Ab coimmunoprecipitates from BCBL-1 cells showing that in presence or absence of DRB, hnRNP K protein levels remains similar in coimmunoprecipitates from ORF57 transfected and untransfected BCBL-1 cells. Coimmunoprecipitates included in **lanes: 1.** pKS3 transfected + TPA-induced BCBL-1 cells + 0 μM DRB **2.** pKS3 transfected + TPA-induced BCBL-1 cells + 5 μM DRB **3.** Untransfected + TPA-induced BCBL-1 cells + 0 μM DRB **4.** Untransfected + TPA-induced BCBL-1 cells + 5 μM DRB.

Panel III. Representation of the relationship between ORF57, CK2 and hnRNP K

Fig 5C1. Phosphorylation of other proteins in the anti-ORF57 Ab coimmunoprecipitates



Panel I. Phosphorimager analysis on anti-ORF57 Ab coimmunoprecipitates from BCBL-1 cells after *in vitro* phosphorylation showing phosphorylation of other proteins in the anti-ORF57 Ab coimmunoprecipitates. Anti-ORF57 Ab coimmunoprecipitates from TPA-induced or uninduced BCBL-1 cells were phosphorylated *in vitro* using [^{32}P]-ATP in presence or absence of DRB for analysing *in vitro* phosphorylation of coimunoprecipitated ORF57 and its partner proteins. *In vitro* phsophorylated proteins were analysed by SDS-PAGE electrophoresis and autoradiography. Coimmunoprecipitates included in **lanes: 1.** TPA-induced BCBL-1 cells + DRB **2.** TPA-induced BCBL-1 cells + No DRB **3.** Uninduced BCBL-1 cells + DRB **4.** Uninduced BCBL-1 cells + No DRB.

that could be ORF50, which has been shown to have CK2 sites (Lukac *et al.*, 1999) also appeared to be phosphorylated by CK2. Phosphorylation in presence of DRB (lane1) indicates activity of kinases other than CK2.

5D Discussion

This Chapter presents data that confirms the interaction between ORF57 and CK2 (see Section 5A) and also phosphorylation of ORF57 by CK2 that is reduced by DRB an inhibitor of CK2 activity (see Section 5B). ORF57 interacts with both the catalytic (α/α') and regulatory (β) subunits directly (see Section 5A1.3). Binding of ORF57 is in the CK2 β region aa 150-182, containing the α/α' and β heterodimerisation domain (aa 152-200) (Krehan & Pyerin, 1999) and is the same region required for interaction with ICP27 in yeast-two hybrid interactions (Wadd *et al.*, 1999).

CK2 α protein can interact *in vitro* with the N-terminal, central and C-terminal regions of ORF57 protein independently, indicating the presence of several interactive regions on both partners. CK2 α' subunit can interact *in vitro* with the C-terminal part of ORF57 independent of interaction with any other region indicating the absolute requirement of this region. Binding with CK2 α' at the ORF57 C terminus does not rule out its phosphorylation of the ORF57 N terminus as binding regions and residues involved in phosphorylation may not necessarily be the same. ORF57 protein can interact *in vitro* with CK2 β aa 181-215 independent of the involvement of other regions.

No specific roles for the different α and α' subunits have been shown so far (Guerra & Issinger, 1999), although differences in autophosphorylation ability between $\alpha_2\beta_2$ and $\alpha'_2\beta_2$ holoenzymes were identified (Dobrowolska *et al.*, 1999) and in fully differentiated neurons CK2 holoenzyme consists of the α' subunit alone (Faust & Montenarh, 2000). HIV type 1 protein Rev also interacts with both CK2 α and β subunits through two helical regions joined by a loop (D'Agostino *et al.*, 2000) and p38 MAP kinase directly interacts with the α and β subunits to activate the holoenzyme through what appears to be an allosteric mechanism (Sayed *et al.*, 2000).

The *in vitro* phosphorylation assay utilising the N-terminal deletion mutant suggested that main targets for CK2 phosphorylation reside in the N-terminal 180 aa of ORF57 although this truncation was phosphorylated to a lesser extent than ORF57 FL (see Section 5B1.2). Phosphorylation of ORF57 was significantly decreased in presence of the CK2 inhibitor

DRB (shown in Fig. 5B3 Panel I) and this phosphorylation may be important for protein:protein interactions in the ORF57 complex (see Section 5B4). However treatment with DRB never completely inhibited ORF57 phosphorylation, implying that other kinases can phosphorylate the protein. ORF57, and phosphorylation by other kinases at potential phosphorylation sites (refer to Fig. 5B1.1) need to be studied in detail. HnRNP K also was present in a complex with ORF57-CK2, was phosphorylated by CK2 and DRB reduced its interaction with ORF57. All three proteins (ORF57/hnRNP K/CK2) were shown to coimmunoprecipitate in a complex held together by ORF57 which also contains unknown proteins, and the CK2 peptide assay done on pKS3 transfected cells showed that only ORF57 was required for this association (shown in Fig. 5B2 Panel III).

CK2 phosphorylated hnRNP K and ORF57 in virus-infected cells. HnRNP K is not normally phosphorylated by CK2 (Van Seuningen *et al.*, 1995a) so interaction with ORF57 may cause an alteration in its substrate preference, changing its kinase partner. It could be this CK2 phosphorylated form of hnRNP K that interacts with the ORF57. HnRNP K pulled down with ORF57 was a rapidly migrating form (see Section 4A2) and this possibly less phosphorylated form may interact preferentially with ORF57. Slower migrating hnRNP K forms (predominant in the uninfected cells) are not phosphorylated by CK2 and may not interact with ORF57; their phosphorylation is not inhibited by addition of DRB. Phosphorylation of hnRNP K by kinases other than CK2 is RNA or DNA dependent and interleukin 1 responsive. Oxidative stress increases its Tyr phosphorylation and its association with certain molecular partners - Lck, Vav and PKCδ (Schullery *et al.*, 1999). Similarly during KSHV infection CK2 phosphorylation may affect hnRNP K interactions with other proteins. The CK2 phosphorylation of other KSHV proteins requires to be examined.

The sub-cellular location of CK2 is suggested to be key to its function (Faust & Montenarh, 2000). CK2 has been proposed to play a role during cell division by shifting its location between the cytoplasm and nucleus with the CK2 α' subunit present in the nucleus during G1-phase, and in the cytoplasm during S-phase (Yu *et al.*, 1991). As CK2 is a multifunctional protein kinase, changes in its sub-cellular location following KSHV infection due to interaction with shuttling viral proteins and an alteration of its substrate preferences could serve to facilitate the viral lytic cycle. CK2 activity is also induced by stress, mediated via a direct interaction with the p38 mitogen activated kinase (Sayed *et al.*, 2000). The functional significance of CK2 interaction with ORF57 in KSHV infection is not clear yet, but it may regulate ORF57 nucleocytoplasmic shuttling and/or

export of viral RNAs. It could also be that phosphorylation of ORF57 and its partner proteins by CK2 is required for either export of ORF57 protein on its own or its export with the viral RNA cargo. In case of UsnRNA export, the export adaptor PHAX is first phosphorylated in the nucleus and then exported with RNA to the cytoplasm; for the assembly of the export complex phosphorylation of PHAX protein is essential (Ohno *et al.*, 2000).

Future work with ORF57 deletion mutant viruses and/or ORF57 point mutants in potential CK2 phosphorylation sites followed by analysis of the effect on the interaction and on the process of infection in presence or absence of phosphorylation inhibitors will provide more information about the roles of ORF57/CK2/hnRNP K interaction. It will be interesting to see if interaction with ORF57 stimulates and activates CK2 activity in KSHV infected cells as HIV-1 Rev is reported to activate CK2 *in vitro* and ICP27 does in HSV-1 infected cells (Koffa, M., personal communication).

Chapter 6: Interaction of ORF57 with RNA and export factor binding proteins (REFs)

Using the yeast-2-hybrid assay, the HSV-1 ICP27 protein, interacted with REF1-I (a homologue of mouse ALY-1 or a yeast homologue of Yra1p protein) from a human HeLa cell cDNA library. I therefore determined if ORF57 was associated with REF as a partner protein. During the course of this study it was confirmed using *in vitro* binding assays and *Xenopus laevis* oocytes microinjections (Koffa *et al.*, 2001) that ICP27 was associated with REFs and provides viral mRNAs with access to a cellular mRNA export pathway. To find out whether ORF57 interacted with REF and if ORF57 itself was acting as an RNA export factor, *in vitro*, and *in vivo* binding assays and RNA export assays were performed. This Chapter presents data that shows that ORF57 interacts with REFs under *in vitro* conditions and in virus-infected cells. Experiments were aimed at determining the implication of this interaction for the KSHV life cycle. REFs are a conserved family of proteins required for cellular mRNA export (Strasser & Hurt, 2000; Stutz *et al.*, 2000; Rodrigues *et al.*, 2001) they have sequence similarity to hnRNPs and bind RNA non-specifically (Stutz *et al.*, 2000). Only fully processed RNAs are exported, pre-mRNAs and excised introns stay in the nucleus. Splicing is required for efficient mRNA export as a result of coupling between the splicing and the mRNA export machineries.

6A ORF57 interacts with REF

6A1 In GST-pull down assays

6A1.1 In vitro transcribed-translated ORF57 interacts with REF in the GST pull down assay

The ^{35}S-labelled immunoprecipitated profile of ORF57 interacting proteins (Fig. 3B1 Panel I lane 2) contained a band of 28/30 kDa, which could be possibly REF. Beads carrying recombinant GST-REF2-I (Section 2B4.6) or GST alone were assayed for binding to *in vitro* synthesized ^{35}S-labelled-ORF57 (FL and small). The fusion proteins used were visualised by Coomassie brilliant blue staining (Fig. 6A1.1 Panel I lane 2 & 9) and equivalent amounts of proteins were used in pull down assays. Samples eluted twice with reduced glutathione buffer were heated to 100°C in SDS gel sample buffer for 5 min, analysed by SDS-PAGE (Section 2B6.1) and the gel was dried and exposed to a phosphorimager screen (Section 2B6.4). The GST-REF2-1 pulled down ORF57 full-

length and small whereas GST alone did not (Fig. 6A1.1 Panel II compare lanes 7 & 8 with 5 & 6). Thus, it is concluded that REF interacts with *in vitro* synthesized ORF57.

6A1.2 ORF57 from virus-infected cells interacts with REF in the GST pull down assay

GST pull down assays using fusion protein GST-REF2-I or GST alone, bound to glutathione Sepharose beads were used to identify interacting proteins from TPA-induced and uninduced BCBL-1 cell extracts (Section 2B4.4). Samples eluted with reduced glutathione buffer were run on SDS-PAGE (Section 2B6.1), transferred to a nitrocellulose membrane and anti-ORF57 Ab was used for immunoblotting (Section 2B6.2 & 3). The fusion proteins used were visualised by Coomassie brilliant blue staining (Fig. 6A1.1 Panel I) and equivalent amounts of fusion proteins were used. Western blotting with anti-ORF57 Ab showed that from TPA-induced BCBL-1 cell extracts, GST-REF2-I pulled down a band ~50-52 kDa corresponding to ORF57 in input whole cell extracts whereas the uninduced BCBL-1 cell extract lane and GST control lanes were negative for a band of similar size (Fig. 6A1.2 Panel I compare upper band in lane 3 with lane 2 and lanes 4, 5, & 6). The anti-ORF57 Ab cross-reacted with a protein from the GST-REF2-I preparation, which pulled down and interacted with BCBL-1 extracts, as a smaller band below the ORF57 protein was found in both TPA-induced and uninduced BCBL-1 extracts lanes (see lower band in lanes 3 & 4). This protein band was absent in GST alone lanes (lanes 5 & 6) and could be a truncated product of GST-REF2-I fusion protein not present in GST alone, which interacts non-specifically with BCBL-1 cells.

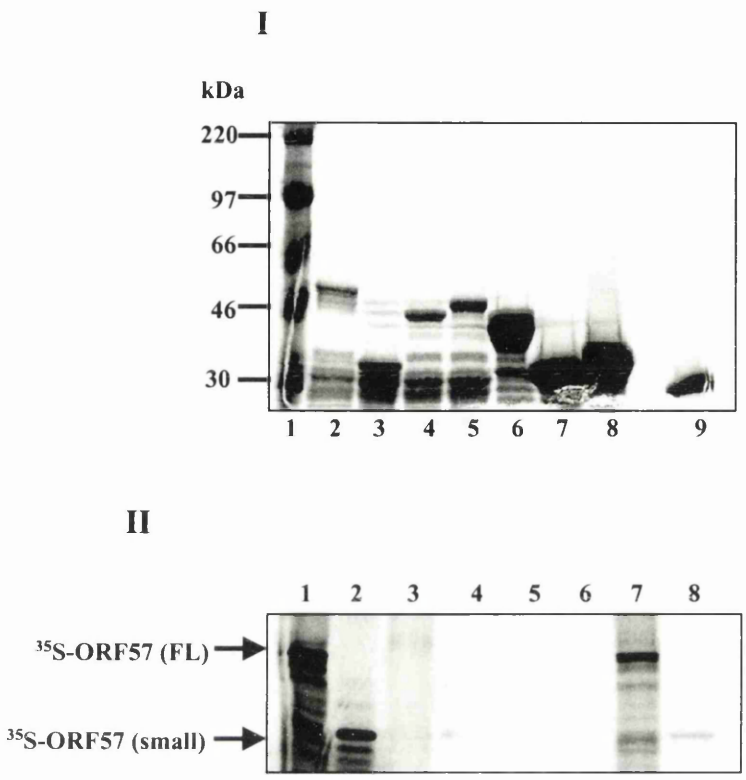
6A1.3 GST-57 interacts with REF from virus-infected cells

Using glutathione beads carrying recombinant GST-57 (FL) (Section 2B4.3) or GST alone (see Fig 4A1.1 Panel II), with protein from TPA-induced and uninduced BCBL-1 cell extracts (Section 2B4.3) followed by Western blotting with anti-REF rabbit Ab (KJ70), showed that GST-57 and REF interact, whereas GST alone and REF do not (Fig. 6A1.3 Panel I compare lanes 1 & 2 with 3 & 4). The fusion proteins used were visualised by Coomassie brilliant blue staining (see Fig. 4A1.1 Panel II) and equivalent amounts of fusion proteins were used. A band of ~28-30 kDa, corresponding in size to REF in whole cell extract, was visible on Western blot in uninduced and TPA-induced BCBL-1 cells (compare lane 1 with 3 & 4).

6A2 ORF57 and REF coimmunoprecipitate from virus-infected cell extracts

Confirmation of an interaction between ORF57 and REF came from immunoblotting of the anti-ORF57 Ab immunoprecipitates (Section 2B5.2) from virus-infected cell extracts.

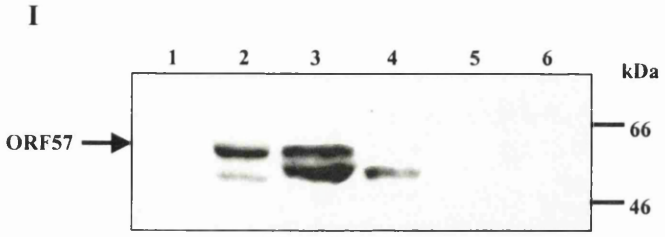
Fig 6A1.1 Panels I and II. ORF57 synthesised *in vitro* interacts with REF-2-I



Panel I. Expression of GST-REF2-I and GST proteins: Coomassie blue stained gel. GST-REF2-I (FL), its deletions and GST proteins used were run on a 10% SDS-PAGE gel and visualised by Coomassie staining. The smaller bands in lanes 2-9 represent degradation products/truncations of GST-REF (FL) and its deletions. Shown in **lanes: 1.** Protein gel marker **2.** GST-REF2-I (aa 1-218 FL) **3.** GST-REF2-I (aa 1-74) **4.** GST-REF2-I (aa 1-152) **5.** GST-REF2-I (aa 1-198) **6.** GST-REF2-I (aa 74-218) **7.** GST-REF2-I (aa 75-152) **8.** GST-REF2-I (aa 153-218) **9.** GST alone.

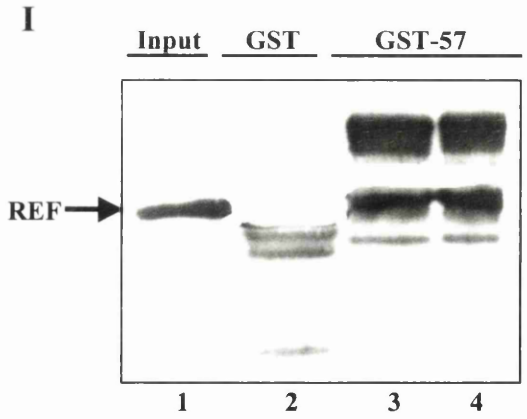
Panel II. Phosphorimager analysis showing that ORF57 synthesised *in vitro* interacts with REF-2-I. GST-REF2-I and GST fusion proteins bound onto glutathione beads were incubated with ^{35}S-labelled-ORF57 (FL and small), after washing, specifically bound proteins were eluted with reduced glutathione buffer and separated on a SDS-PAGE gel, gel was dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** Input ^{35}S-labelled-ORF57 (FL) **2.** Input ^{35}S- labelled-ORF57 (small) **3.** GST-REF2-I + ^{35}S-labelled-ORF57 (FL)-first elution **4.** GST-REF2-I + ^{35}S-labelled-ORF57 (small) -first elution **5.** GST alone + ^{35}S-labelled-ORF57 (FL) -second elution **6.** GST alone + ^{35}S-labelled-ORF57 (small) -second elution **7.** GST-REF2-I + ^{35}S-labelled-ORF57 (FL) -second elution **8.** GST-REF2-I + ^{35}S-labelled-ORF57small -second elution.

Fig 6A1.2. GST-REF2-I pulls down ORF57 from virus-infected cells



Panel I. Western blot for ORF57 with anti-ORF57 Ab showing that GST-REF2-I pulls down ORF57 from virus-infected cells. TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST-REF2-I or GST proteins. After incubation with extracts, proteins pulled down were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted for ORF57 using anti-ORF57 Ab. Pull downs included in **lanes: 1.** Input uninduced BCBL-1 extract **2.** Input TPA-induced BCBL-1 extract **3.** TPA-induced BCBL-1 extract + GST-REF2-I **4.** Uninduced BCBL-1 extract + GST-REF2-I **5.** TPA-induced BCBL-1 extract + GST alone **6.** Uninduced BCBL-1 extract + GST alone.

Fig 6A1.3. GST-57 pulls down REF from BCBL-1 cells



Panel I. Western blot for REF with anti-REF (KJ70) Ab showing that GST-57 pulls down REF from BCBL-1 cells. TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST-57 or GST proteins. After incubation with extracts, proteins pulled down were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted for REF using anti-REF Ab. Pull downs included in **lanes: 1.** Input TPA-induced BCBL-1 extract **2.** TPA-induced BCBL-1 extract + GST alone **3.** TPA-induced BCBL-1 extract + GST-57 **4.** Uninduced BCBL-1 extract + GST-57.

When the immunoprecipitates using anti-ORF57 Ab with TPA-induced and uninduced BCBL-1 cell extracts were Western blotted with anti-REF Ab, REF was coimmunoprecipitated with ORF57 from TPA-induced BCBL-1 cell extracts (Fig. 6A2 Panel I lane 4) but not from uninduced cell extracts (lane 3) or pre-immune serum immunoprecipitates (lanes 5 & 6). Whereas using anti-REF Ab, REF was immunoprecipitated from both TPA-induced as well as uninduced cell extracts (Fig. 6A2 Panel II lanes 3 & 4). Thus it is a fair conclusion that the band of ~28-30 kDa seen in the ^{35}S-labelled protein profile of immunoprecipitated proteins using ORF57 Ab, corresponded to REF (Fig. 3B1 Panel I lane 2). Phosphorimager analysis of ^{35}S-labelled proteins from immunoprecipitates demonstrated that there was an ~50-52 kDa band present only in TPA-induced BCBL-1 cell extracts and absent in uninduced cell extracts (see Fig. 4A1.3 Panel III compare lanes 1 & 2) which corresponded in size to ORF57 band present in control BCBL-1 whole cell extracts by anti-ORF57 Ab immunoblot analysis, indicating that ORF57 was immunoprecipitated using ORF57 antibody.

6A3 TAP/NXF1 factor coimmunoprecipitates with ORF57/REF from virus-infected cell extracts

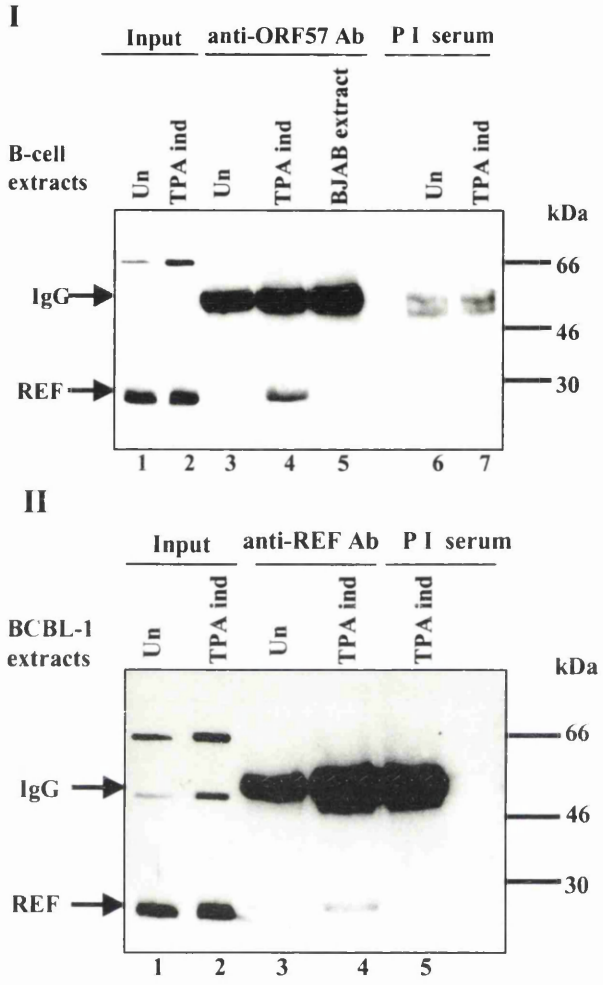
Since REF proteins have been shown to interact with the TAP/NXF1 complex (Strasser & Hurt, 2000; Stutz *et al.*, 2000; Rodrigues *et al.*, 2001), TAP was also tested for its presence in the immunoprecipitate complex. Western blot analysis showed that anti-ORF57 Ab coimmunoprecipitated TAP protein from TPA-induced and not from uninduced BCBL-1 cell extracts (Fig. 6A3 Panel I compare lanes 3 with 2). RNAse treatment did not disrupt these interactions (lane 4). Thus, it is unlikely that the ORF57/REF/TAP complex represents binding of these proteins tethered on RNA. Glutathione beads carrying GST-57 (see Fig 4A1.1 Panel II) pulled down both REF (Section 6A1.3) and TAP protein from both TPA-induced and uninduced BCBL-I cell extracts (Fig. 6A3 Panel II lanes 2 & 3) whereas GST alone did not interact with TAP (lane 4).

6A4 ORF57 forms a ternary complex with REF and TAP: Is the association of TAP with ORF57 direct or indirect?

6A4.1 Investigation using the GST pull down assay

^{35}S-labelled-ORF57 synthsized *in vitro* using the rabbit reticulocyte lysate system was tested for its ability to bind recombinant GST-TAP (Section 2A1) or GST protein on beads (Section 2B4.9). A weak interaction of ORF57 with TAP was observed (Fig. 6A4.1

Fig 6A2 Panels I and II. Anti-ORF57 Ab coimmunoprecipitates REF from TPA-induced BCBL-1 extracts (Panel I) and anti-REF Ab immunoprecipitates REF from both TPA-induced and uninduced BCBL-1 extracts (Panel II)

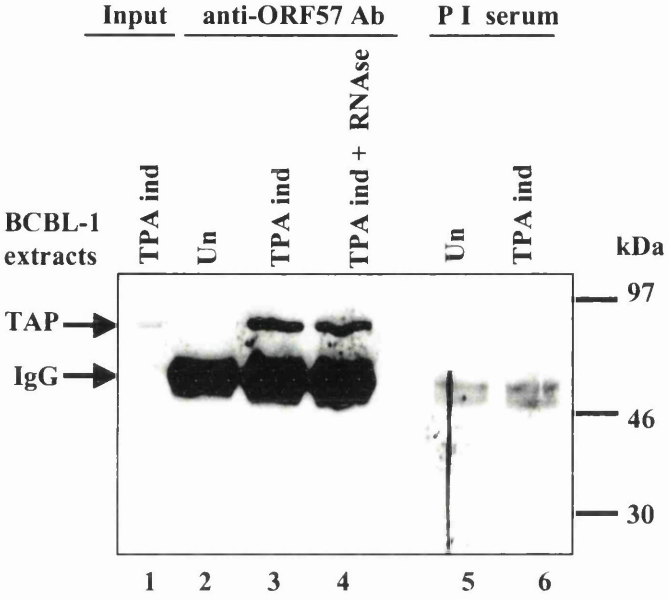


A coimmunoprecipitation assay was performed with anti-ORF57 or anti-REF Ab or pre immune serum and B-cell extracts (BJAB-KSHV negative cells and uninduced and TPA-induced BCBL-1). The complex formed was separated on SDS-PAGE gel, transferred to nitrocellulose and Western blotted for REF using anti-REF Ab.

Panel I. Western blot for REF with anti-REF (KJ70) Ab showing that anti-ORF57 Ab coimmunoprecipitates REF from TPA-induced BCBL-1 extracts. Coimmunoprecipitates included in **lanes: 1.** Input uninduced BCBL-1 extract **2.** Input TPA-induced BCBL-1 extract **3.** Uninduced BCBL-1 extract + anti-ORF57 Ab **4.** TPA-induced BCBL-1 extract + anti-ORF57 Ab **5.** BJAB (KSHV negative) cell extract + anti-ORF57 Ab **6.** Uninduced BCBL-1 extract + pre immune serum **7.** TPA-induced BCBL-1 extract + pre immune serum.

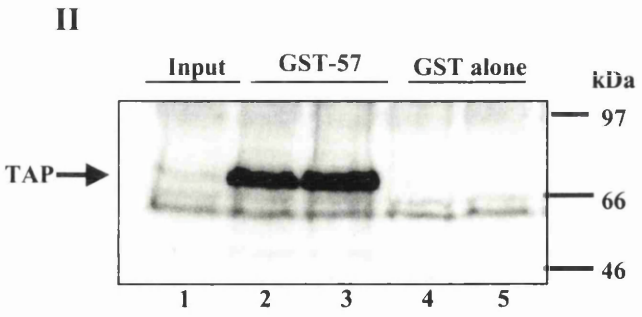
Panel II. Western blot for REF with anti-REF (KJ70) Ab showing that anti-REF Ab immunoprecipitates REF from both TPA-induced and uninduced BCBL-1 extracts. Immunoprecipitates included in **lanes: 1.** Input uninduced BCBL-1 extract **2.** Input TPA-induced BCBL-1 extract **3.** Uninduced BCBL-1 extract + anti-REF Ab **4.** TPA-induced BCBL-1 extract + anti-REF Ab **5.** TPA-induced BCBL-1 extract + pre immune serum.

Fig 6A3 Panel I. Anti-ORF57 Ab coimmunoprecipitates TAP from BCBL-1 cells



Panel I. Western blot for TAP with anti-TAP (KJ60) Ab showing that anti-ORF57 Ab coimmunoprecipitates TAP from BCBL-1 cells. A coimmunoprecipitation assay was performed with anti-ORF57 or pre immune serum and uninduced and TPA-induced BCBL-1 cell extracts. The complex formed was separated on SDS-PAGE gel, transferred to nitrocellulose and Western blotted for TAP using anti-TAP Ab. Coimmunoprecipitates included in **lanes: 1.** Input TPA-induced BCBL-1 extract **2.** Uninduced BCBL-1 extract + anti-ORF57 Ab **3.** TPA-induced BCBL-1 extract + anti-ORF57 Ab **4.** TPA-induced BCBL-1 extract + anti-ORF57 Ab + RNAse **5.** Uninduced BCBL-1 extract +pre immune serum **6.** TPA-induced BCBL-1 extract+ pre immune serum.

Fig 6A3 Panel II. GST-57 pulls down TAP from BCBL-1 cells



Panel II. Western blot for TAP with anti-TAP (KJ60) Ab showing that GST-57 pulls down TAP from BCBL-1 cells. TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST-57 or GST proteins. After incubation with extracts, proteins pulled down were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted for TAP using anti-TAP Ab. Pull downs included in **lanes: 1.** Input TPA-induced BCBL-1 extract **2.** Uninduced BCBL-1 extract + GST-57 **3.** TPA-induced BCBL-1 extract + GST-57 **4.** Uninduced BCBL-1 extract + GST alone **5.** TPA-induced BCBL-1 extract + GST alone.

Panel I lane 7) and the addition of 5 μg recombinant His-REF2-I stimulated the recruitment of ORF57 to GST-TAP (Fig. 6A4.1 Panel I lane 4). RNAse treatment did not abolish ORF57/REF/TAP complex formation (lane 5). This suggests that ORF57 can form a complex that contains REF and TAP proteins. It is possible that endogenous REF proteins present in the rabbit reticulocyte lysate mediates the interaction between TAP and ORF57 observed in lanes 4 and 5.

6A4.2 Investigation using a coimmunoprecipitation assay

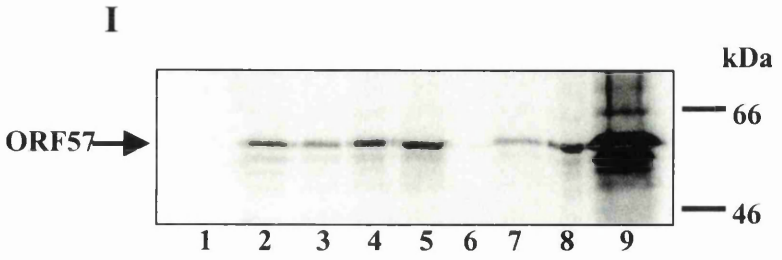
Purified GST-TAP or equivalent amounts of GST alone were mixed with ^{35}S-labelled-ORF57, anti-TAP rabbit Ab or pre-immune serum was used for coimmunoprecipitations (Section 2B5.5). Some reactions were carried out in presence of RNAse added at the first step during mixing of the two proteins and analysed by SDS-PAGE and phosphorimaging. Anti-TAP Ab, brought down *in vitro* synthesized ORF57 mixed with GST-TAP (Fig. 6A4.2 Panel I lane 4) but did not bring down ORF57 from GST alone (Fig. 6A4.2 Panel I lane 2) and pre-immune sera did not precipitate ORF57 from either GST-TAP or GST alone (Fig. 6A4.2 Panel I lanes 6, 5 & 7). RNAse treatment did not abolish complex formation (lane 3). This suggests that ORF57 can form a complex with TAP protein though it is not clear if this interaction is direct or indirect as endogenous REF proteins present in the rabbit reticulocyte lysate could mediate the interaction. Formation of a ternary complex using *E. coli*-derived GST-ORF57, His-REF and His-TAP will provide more information about the exact nature of this complex.

6A5 Antibodies to the RBD of REF do not prevent its binding to ORF57

ICP27 requires the RNP-type RNA binding domain (RBD) of REF for its interaction (Koffa *et al.*, 2001). Anti-REF rabbit antibodies (KJ70 & KJ56, raised against recombinant RBD of murine REF1-II produced in *E. coli*) prevented binding of REF to RNA but not to TAP (Rodrigues *et al.*, 2001) both of which interactions require N- and C-terminal variable regions of REF for binding. ^{35}S-labelled-REF synthsized *in vitro* was incubated with affinity-purified anti-REF (KJ70) Ab. After a 1 h incubation period, samples were divided and assayed for binding to immobilized GST-ORF57 or GST alone on glutathione agarose beads or to protein-A Sepharose beads. Bound fractions were analysed by SDS-PAGE and phosphorimager analysis.

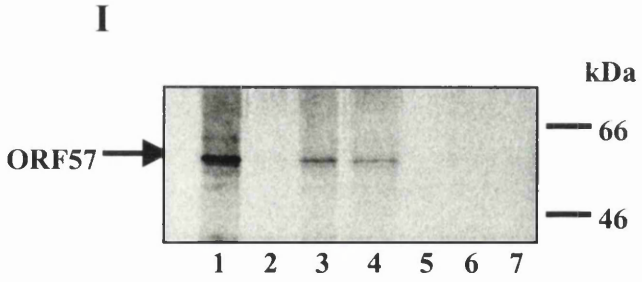
Preincubation of REF2-I with the antibody did not prevent its interaction with ORF57 (Fig. 6A5 Panel I lane 5) although REF2-I must have bound the antibody because it could be selected on protein-A Sepharose beads (Fig. 6A5 Panel I lane 2). GST alone did not

Fig 6A4.1. ORF57 interacts with REF and TAP but might not bind TAP directly



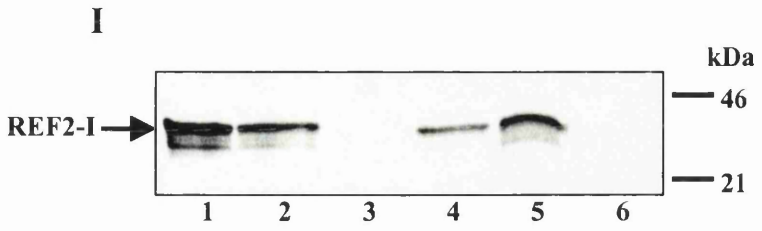
Panel I. Phosphorimager analysis showing that ORF57 interacts with REF and TAP but might not bind TAP directly as addition of REF strengthens ORF57-TAP interaction thus ORF57-TAP interaction could be mediated via REF protein present in rabbit reticulocyte lysate. GST-REF2-I, His-REF2-I, GST-TAP, and His-thioredoxin (control) or GST (control) fusion proteins bound onto agarose beads were incubated with ^{35}S-labelled-ORF57 (FL) after washing, specifically bound proteins were eluted and separated on a SDS-PAGE gel, gel was dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** ^{35}S-ORF57 (FL) + His-thioredoxin (control) coated beads **2.** ^{35}S-ORF57 (FL) + GST-REF2-I coated beads **3.** ^{35}S-ORF57 (FL) + His-REF2-I coated beads **4.** ^{35}S-ORF57 (FL) + His-REF2-I purified + GST-TAP coated beads **5.** ^{35}S-ORF57 (FL) + His-REF2-I purified + GST-TAP coated beads + RNAse I **6.** ^{35}S-ORF57 (FL) + GST alone coated beads **7.** ^{35}S-ORF57 (FL) + GST-TAP alone coated beads **8.** ^{35}S-ORF57 (FL) + His-REF2-I coated beads + RNAse I **9.** Input *in vitro* synthesised ^{35}S-labelled-ORF57 (FL).

Fig 6A4.2. GST-TAP coimmunoprecipitates ORF57



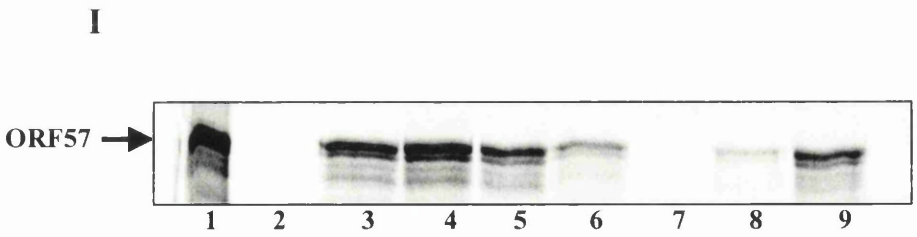
Panel I. Phosphorimager analysis showing that anti-TAP Ab coimmunoprecipitates ORF57 with GST-TAP. A coimmunoprecipitation assay was performed with anti-TAP Ab or pre immune serum using GST-TAP or GST and ^{35}S-labelled-ORF57 (FL) protein. The complex formed was separated on SDS-PAGE gel, gel was dried and analysed by phosphorimaging. Coimmunoprecipitates included in **lanes: 1.** Input *in vitro* synthesised ^{35}S-labelled-ORF57 (FL) **2.** ^{35}S-ORF57 (FL) + GST alone + anti-TAP (KJ60) Ab +protein-A Sepharose **3.** ^{35}S-ORF57 (FL) + GST-TAP + RNAse I + anti-TAP (KJ60) Ab +protein-A Sepharose **4.** ^{35}S-ORF57 (FL) + GST-TAP + anti-TAP (KJ60) Ab +protein-A Sepharose **5.** ^{35}S-ORF57 (FL) + GST-TAP + RNAse I +pre immune serum + protein-A Sepharose **6.** ^{35}S-ORF57 (FL) + GST-TAP+ pre immune serum +protein-A Sepharose **7.** ^{35}S-ORF57 (FL) + GST alone + pre immune serum +protein-A Sepharose.

Fig 6A5. Antibodies to REFs RBD do not prevent its binding to ORF57



Panel I. Phosphorimager analysis showing that antibodies to REFs RBD do not prevent its binding to ORF57. ^{35}S-labelled-REF2-I synthsized *in vitro* was incubated with affinity-purified anti-REF (KJ70) Ab. After a 1 h incubation period, samples were divided and assayed for binding to immobilized GST-ORF57 or GST alone on glutathione agarose beads or to protein-A Sepharose beads. Bound fractions were analysed by SDS-PAGE and phosphorimager analysis. Pull downs included in **lanes: 1.** Input *in vitro* synthesised ^{35}S-labelled-REF2-I **2.** ^{35}S-labelled-REF2-I+ anti-REF (KJ70) Ab + protein-A Sepharose beads **3.** ^{35}S-labelled-REF2-I+ GST alone on glutathione agarose beads **4.** ^{35}S-labelled-REF2-I+ GST-57 FL on glutathione agarose beads+RNAse I **5.** ^{35}S-labelled-REF2-I+ GST-57 FL on glutathione agarose beads + anti-REF (KJ70) Ab **6.** ^{35}S-labelled-REF2-I+ GST alone + anti-REF (KJ70) Ab +protein-A Sepharose beads.

Fig 6A6.1 Panel I. ORF57 binds the N- and C- terminal domains of REF2-1 but not the RBD



Panel I. Phosphorimager analysis showing that ORF57 binds the N- and C- terminal domains of REF2-1 but not the RBD. GST-REF2-I FL and its deletions fusion proteins or GST alone on glutathione agarose beads were added to ^{35}S-labelled-ORF57 (FL) protein and the pull down assays were performed. The complex formed was separated on SDS-PAGE gel, gel was dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** Input *in vitro* synthesised ^{35}S-labelled-ORF57 (FL) **2.** ^{35}S-ORF57 (FL) + GST alone **3.** ^{35}S-ORF57 (FL) + GST-REF2-I (aa 74-218) **4.** ^{35}S-ORF57 (FL) + GST-REF2-I (aa 153-218) **5.** ^{35}S-ORF57 (FL) + GST-REF2-I (aa 1-198) **6.** ^{35}S-ORF57 (FL) + GST-REF2-I (aa 1-152) **7.** ^{35}S-ORF57 (FL) + GST-REF2-I RBD (aa 75-152) **8.** ^{35}S-ORF57 (FL) + GST-REF2-I (aa 1-74) **9.** ^{35}S-ORF57 (FL) + GST-REF2-I FL (aa 1-218).

bind REF2-I either without or with addition of anti-REF antibody (Fig. 6A5 Panel I lanes 3 & 6). This suggests that the RBD might not be involved in binding to ORF57.

6A6 The variable regions of REF are required for interaction with ORF57 protein

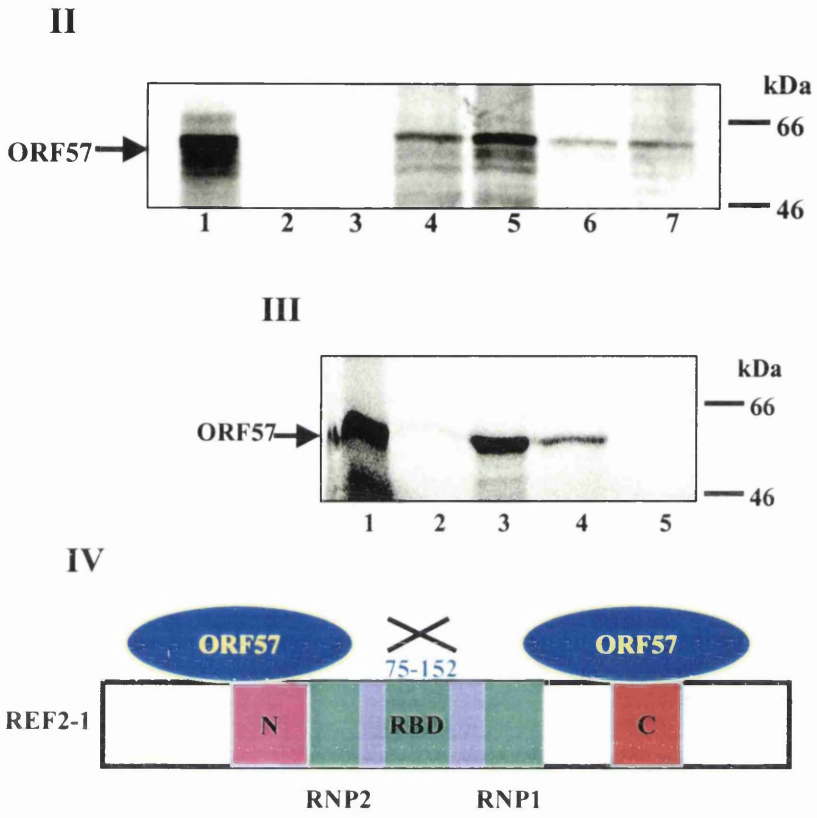
6A6.1 Mapping the interacting regions of REFs using the GST pull down assay

GST- fusions of full-length and deletion mutants of REF2-I were used to map regions required for interaction with ORF57. Fig. 6A1.1Panel I shows the aa present, and the amounts of each fusion protein purified on GST columns, as visualized by Coomassie brilliant blue staining. Approximately equal amounts of each GST-REF2-I (Stutz *et al.*, 2000) fusion protein or GST alone on glutathione agarose beads were added to ^{35}S-labelled-ORF57 (FL) protein and the pull down assays were performed (Section 2B4.9). REF2-I FL aa 1-218 (Fig. 6A6.1Panel I lane 9), or fragments that contained aa 1-74 (lane 8), aa 1-152 (lane 6), aa 1-198 (lane 5), aa 74-218 (lane 3), or aa 153-218 (lane 4) bound to ORF57 whereas the fragment containing aa 74-152 (lane 7) did not. This indicates that the central RBD of REF2-I, containing aa 74-152, which resembles an RNP-type RNA binding domain (Rodrigues *et al.*, 2001) is not required for interaction with ORF57. No binding was detected with GST alone (lane 2) and RNAse treatment did not disrupt the interaction with full-length or a fragment containing aa 1-198 of REF2-I (Fig. 6A6.1Panel II lanes 5 & 7). These data indicated that the REF region located between aa 1-74 and aa 153-218 (i.e.at the N and C terminus) is required for interaction with ORF57.

To further characterize the binding of ORF57 to REF proteins in general, its binding properties to various fragments derived from REF2-I and REF1-II proteins were examined. REF2-I and REF1-II exhibit 95% identity in their RBD and 100% in the conserved RNP boxes (Stutz *et al.*, 2000). However they differ at multiple positions within the C-vr region and, REF1-II lacks the N-vr region present in REF2-I (see Introduction Section 1C3). ^{35}S-labelled-ORF57 (FL) protein was assayed for binding to glutathione agarose beads coated with recombinant full-length REF1-II (aa 1-163) or, REF1-II deletion fragments (aa 14-102 and 103-163) fused to GST.

ORF57 bound to the beads coated with recombinant full-length REF1-II (Fig. 6A6.1Panel III lane 3) and the C-terminal REF1-II deletion fragment aa 103-163 (Fig. 6A6.1Panel III lane 4) indicating that the N-terminal variable region is not strictly required for binding. The RBD domain of REF1-II (aa 14-102) that shares 95% identity with REF2-I, also did not exhibit any detectable ORF57-binding activity (Fig. 6A6.1Panel III lane 2). These

Fig 6A6.1 Panels II, III, and IV. ORF57 binds REFs in general (i.e. also interacts with REF1-II) but not via RNA



Panel II. Phosphorimager analysis showing that ORF57 binds to the REF2-1 but not via RNA. GST-REF2-I (FL) and its C–terminal deletion (aa1-198) fusion protein or GST alone on glutathione agarose beads were added to ^{35}S-labelled-ORF57 (FL) protein and with or without RNAse treatment the pull down assays were performed. The complex formed was separated on SDS-PAGE gel, gel was dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** Input *in vitro* synthesised ^{35}S-labelled-ORF57 FL **2.** ^{35}S-ORF57 (FL) + GST alone **3.** ^{35}S-ORF57 (FL) + GST alone + RNAse I **4.** ^{35}S-ORF57 (FL) + GST-REF2-I FL (aa 1-218) **5.** ^{35}S-ORF57 (FL) + GST-REF2-I FL (aa 1-218) + RNAse I **6.** ^{35}S-ORF57 (FL) + GST-REF2-I (aa 1-198) **7.** ^{35}S-ORF57 (FL) + GST-REF2-I (aa 1-198) + RNAse I.

Panel III. Phosphorimager analysis showing that ORF57 binds REFs in general (i.e. also interacts with REF1-II). ^{35}S-labelled-ORF57 (FL) protein was assayed for binding to glutathione agarose beads coated with recombinant full-length REF1-II (aa 1-163) or, REF1-II deletion fragments (aa 14-102 and 103-163) fused to GST or, GST alone. The complex formed was separated on SDS-PAGE gel, gel was dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** Input *in vitro* synthesised ^{35}S-labelled-ORF57 (FL) **2.** ^{35}S-ORF57 (FL) + GST-REF1-II RBD (aa 14-102) **3.** ^{35}S-ORF57 (FL) + GST-REF1-II FL (aa 1-163) + RNAse I **4.** ^{35}S-ORF57 (FL) + GST-REF1-II C-terminus (aa 103-163) **5.** ^{35}S-ORF57 (FL) + GST alone.

Panel IV. ORF57 binds the N- and C- terminal domains of REF2-1 but not the RBD

interactions are specific as GST alone did not show any binding to ORF57 (lane 5). Thus, ORF57 bound both REF1-II, REF2-I and to all fragments carrying at least one variable region and a conserved box. This was true of deletion fragments containing aa 103-163 from REF1-II (Fig. 6A6.1Panel III lane 4) which had C-vr region (residues 103-128) and the conserved box (residues 129-163), and aa 1-74 or 153-218 from REF2-I (Fig. 6A6.1Panel I lanes 8 & 4). Diagrammatic representation of the REF regions interacting with ORF57 is shown in Fig. 6A6.1Panel IV. Thus, either of the N- or C-terminal variable regions with at least one conserved box of various REFs is sufficient for ORF57 binding. These REF regions also correspond to those that bind TAP and RNA.

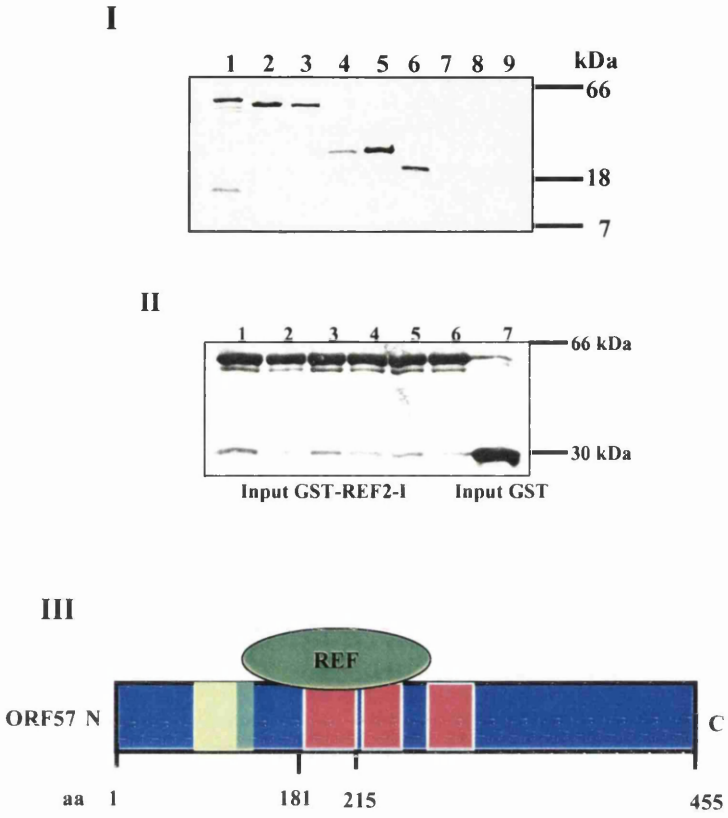
6A7 The N-terminal aa of ORF57 are involved in interaction with REF protein

6A7.1 Mapping the REF interacting regions of ORF57 using the GST pull down assay

To map the regions of ORF57 required for interaction with REFs, glutathione agarose beads coated with GST-REF2-I or GST alone and ^{35}S-labelled-ORF57 FL (aa 1-455) and deletion mutants containing aa 17-455, 1-215, 181-328, 329-455 and 387-455 were used. Fig. 4A4.1Panel I shows the aa present and a typical expression of each labelled-ORF57 protein as visualised by phosphorimager analysis. Equal amounts of GST-REF2-I or GST alone were added and the pull down assays were performed (Section 2B4.9). Eluted proteins were separated by SDS-PAGE by loading the samples on two gels and one gel was dried and exposed to phosphorimager screen while the other gel was transferred to a nitrocellulose membrane for Western blotting.

ORF57 (FL) and deletion mutants aa 17-455, 1-215 and 181-328 bound to beads carrying GST-REF2-I (Fig. 6A7.1 Panel I lanes 1, 2 & 3, 4 & 5, and 6) whereas ORF57 deletion mutants aa 329-455 and aa 387-455 showed no detectable binding nor did ORF57 (FL) bind to GST alone (Fig. 6A7.1 Panel I. lanes 7, 8 & 9). The amounts of GST-REF2-I and GST alone used for the pull down assay were confirmed by Western blotting the other half of the eluted samples, and immunodetection with anti-GST antibody (Fig. 6A7.1 Panel II lanes 1-7 respectively). These data indicated that the N-terminal region of ORF57 (aa 1-328) interacted and bound to REF and *in vitro* minimum interactive region with REF could lie between aa 181-215 of ORF57. No interaction was found with the ORF57 C-terminal region (aa 329-455) demonstrating the specificity of the interaction. A diagrammatic representation of ORF57 regions interacting with REF is shown in Fig. 6A7.1Panel III.

Fig 6A7.1 Panel I, II, and III. REF interacts with N-terminal aa 181-215 of ORF57



Glutathione agarose beads coated with GST-REF2-I or GST alone and ^{35}S-labelled-ORF57 FL (aa 1-455) and its deletion mutants were used in a pull down assay to map the regions of ORF57 required for interaction with REF. Eluted proteins were separated by SDS-PAGE by loading the samples on two gels, one gel was dried and exposed to phosphorimager screen and the other gel was transferred to a nitrocellulose membrane for Western blotting.

Panel I. Phosphorimager analysis showing that REF interacts with N-terminal aa 181-215 of ORF57. Pull downs included in **lanes: 1.** ^{35}S-labelled-ORF57 (aa 1-455 FL) + GST-REF2-I **2.** ^{35}S-labelled-ORF57 (aa 17-455)+ GST-REF2-I **3.** ^{35}S-labelled-ORF57 (aa 17-455)+ GST-REF2-I 4. ^{35}S-labelled-ORF57 (aa 1-215) + GST-REF2-I 5. ^{35}S-labelled-ORF57 (aa 1-215) + GST-REF2-I **6.** ^{35}S-labelled-ORF57 (aa 181-328) + GST-REF2-I **7.** ^{35}S-labelled-ORF57 (aa 329-455) + GST-REF2-I **8.** ^{35}S-labelled-ORF57 (aa 387-455) + GST-REF2-I **9.** ^{35}S-labelled-ORF57 (aa 1-455 FL)+ GST alone.

Panel II. Western blot for GST-REF and GST with anti-GST Ab to show equal loading of GST-REF fusion protein used for mapping the regions of ORF57 required for interaction with REF. Pull downs included in **lanes: 1.** ^{35}S-labelled-ORF57 (aa 1-455 FL) + GST-REF2-I **2.** ^{35}S-labelled-ORF57 (aa 17-455) + GST-REF2-I **3.** ^{35}S-labelled-ORF57 (aa 1-215) + GST-REF2-I **4.** ^{35}S-labelled-ORF57 (aa 181-328) + GST-REF2-I **5.** ^{35}S-labelled-ORF57 (aa 329-455) + GST-REF2-I **6.** ^{35}S-labelled-ORF57 (aa 387-455) + GST-REF2-I **7.** ^{35}S-labelled-ORF57 (aa 1-455 FL)+ GST alone.

Panel III. Representation of REF binding region of ORF57 protein

6A8 ORF57 protein co-localises with REF protein within cells

Human REF exhibits a nuclear distribution, with a widespread staining in the nucleoplasm and sites of highest concentration in speckled domains (Wichmann *et al.*, 1999; Zhou *et al.*, 2000). Confocal microscopy was used to visualise the location of both proteins. ORF57 in TPA-induced BCBL-1 cells, pKS3 (GFP-gORF57) or pKS4 (GFP-cORF57) transfected cells (this study), and other mammalian cells shows a nuclear distribution (Kirshner *et al.*, 2000). Both proteins could not be seen in TPA-induced BCBL-1 cells due to lack of availability of a different species of antibody other than rabbit. Thus GFP-tagged ORF57 and c-*myc*-REF2-1 were transfected by electroporation in BCBL-1 cells, cells were then TPA-induced and stained for REF using anti-c-*myc* rabbit Ab (Table 2A7) whereas transfected GFP-ORF57 was visible due to green fluorescence of GFP.

The distribution of REF in c-*myc*-REF2-I transfected (red fluorescence) and TPA-induced BCBL-1 cells was nuclear similar to endogenous REF in untransfected cells. GFP-ORF57 (green) had nuclear localisation similar to ORF57 in TPA-induced untransfected BCBL-1 cells. On overlay both proteins ORF57 and REF co-localized with each other in speckled domains (Fig. 6A8 Panel I).

6A9 REF interacts with hnRNP K and forms a complex with ORF57 and hnRNP K in infected cells

6A9.1 REF interacts with hnRNP K from virus-infected cells in the GST pull down assay

Since ORF57 interacts with both hnRNP K and REF, it was investigated whether hnRNP K and REF could interact with each other. The fusion proteins used (GST-REF2-I /GST alone) were visualised by Coomassie brilliant blue staining (see Fig. 6A1.1 Panel I) and equivalent amounts were used. Pull downs from TPA-induced and uninduced BCBL-1 cells using GST-REF2-I or GST alone were immunoblotted for hnRNP K (Section 6A1.2). HnRNP K was selected on beads coated with GST-REF2-I from both TPA-induced and uninduced BCBL-1 cells (Fig. 6A9 1 Panel I lanes 3 & 4) but not with GST alone (Fig. 6A9 1 Panel I lanes 5 & 6). As hnRNP K was pulled down from uninduced BCBL-1 cells where no lytic cycle viral proteins were present, REFs and hnRNP K do not require viral ORF57 protein to form a complex.

Fig 6A8. ORF57 partially colocalises with REF in TPA induced BCBL-1 cells

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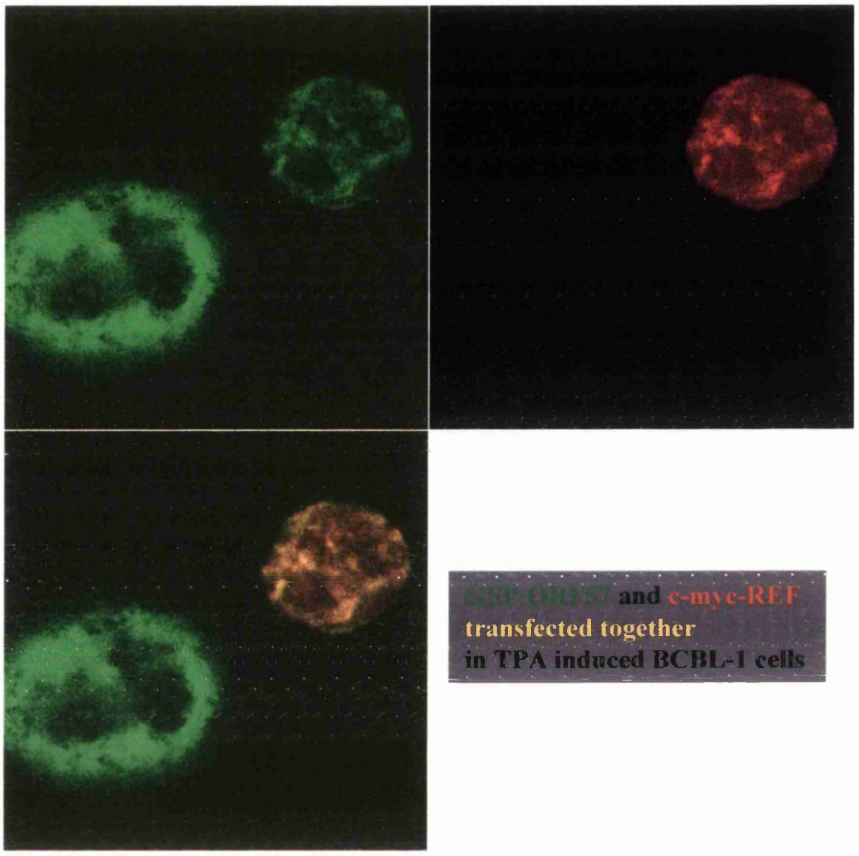
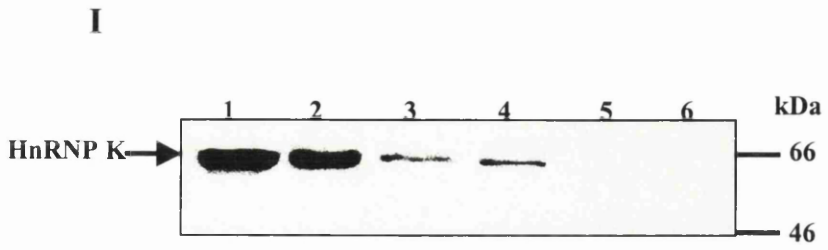


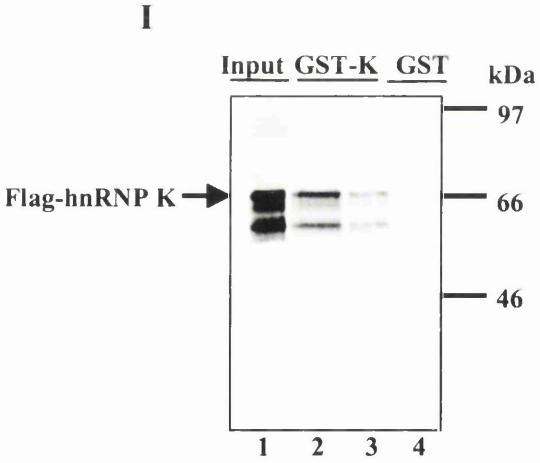
Panel I. ORF57 tagged with GFP colocalises with myc-REF in the nucleus of BCBL-1 cells

Fig 6A9.1. GST-REF2-I pulls down hnRNP K from virus-infected cells



Panel I. Western blot for hnRNP K with anti-hnRNP K Ab showing that GST-REF2-I pulls down hnRNP K from virus-infected cells. Pull downs from TPA-induced and uninduced BCBL-1 cells using GST-REF2-I or GST alone were separated by SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted for hnRNP K using anti-hnRNP K Ab . Pull downs included in **lanes: 1.** Uninduced BCBL-1 extract **2.** TPA-induced BCBL-1 extract **3.** TPA-induced BCBL-1 extract + GST-REF2-1 **4.** Uninduced BCBL-1 extract + GST-REF2-1 **5.** TPA-induced BCBL-1 extract + GST alone **6.** Uninduced BCBL-1 extract + GST alone.

Fig 6A9.2. GST-REF2-I interacts with *in vitro* synthesised hnRNP K



Panel I. Phosphorimager analysis showing that GST-REF2-I interacts with *in vitro* synthesised hnRNP K. GST-REF2-I (FL) or GST alone on glutathione agarose beads were added to ^{35}S-labelled-Flag-hnRNP K protein with or without RNAse treatment and the pull down assay was performed. The complex formed was separated on SDS-PAGE gel, gel was dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** Input *in vitro* synthesised ^{35}S-labelled-Flag-hnRNP K **2.** ^{35}S-labelled-Flag-hnRNP K+ GST-REF2-I + RNAse **3.** ^{35}S-labelled-Flag-hnRNP K+ GST-REF2-I **4.** ^{35}S-labelled-Flag-hnRNP K+ GST alone.

6A9.2 REF interacts with in vitro transcribed-translated hnRNP K in the GST pull down assay

Beads carrying recombinant GST-REF2-I or GST alone were assayed for binding to *in vitro* synthesized ^{35}S-labelled-Flag-hnRNP K. The fusion proteins used were visualised by Coomassie brilliant blue staining (see Fig. 6A1.1 Panel I) and similar amounts of fusion proteins were used. GST-REF2-1 pulled down Flag-hnRNP K whereas GST alone did not (Fig. 6A9.2 Panel I compare lanes 3 & 4). RNAse treatment did not disrupt this interaction (Fig. 6A9.2 Panel I lane 2). This further demonstrates that REF and hnRNP K can associate in the absence of any viral protein.

6A9.3 HnRNP K interacts with in vitro transcribed-translated REF in pull down assay

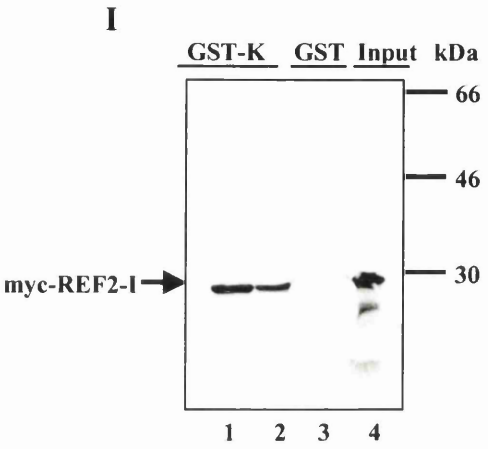
To confirm the binding of REF to hnRNP K, beads carrying recombinant GST-K or GST alone were assayed for binding to *in vitro* synthesized ^{35}S-labelled-myc-REF2-I. The fusion proteins used were visualised by Coomassie brilliant blue staining (see Fig. 4A1.2 Panel I) and similar amounts of fusion proteins were used. GST-K pulled down ^{35}S-labelled-myc-REF2-I whereas GST alone did not (Fig. 6A9.3 Panel I compare lanes 2 & 3). RNAse treatment did not disrupt this interaction (Fig. 6A9.3 Panel I lane 1).

6A10 Y14 interacts with ORF57 and forms a complex with ORF57 and TAP/NXF1 in infected cells

6A10.1 Y14 interacts with ORF57 from virus-infected cells in the GST pull down assay

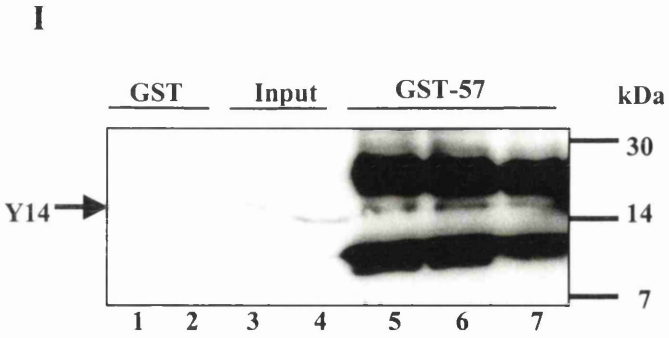
Y14, an hnRNP-like protein that preferentially associates with spliced mRNAs, has been suggested to load with REF/TAP, on the pre-mRNAs during splicing and coprecipitate with spliceosomes (Kataoka *et al.*, 2000). Beads carrying recombinant GST-57 (Section 2B4.3) or GST alone, with protein from TPA-induced and uninduced BCBL-1 cell extracts (Section 2B4.3) followed by Western blotting with anti-Y14 rabbit Ab, showed that GST-57 and Y14 interact, whereas GST alone and Y14 do not (Fig. 6A10.1 Panel I compare lanes 6 & 7 with 1 & 2). The fusion proteins used were visualised by Coomassie brilliant blue staining (see Fig. 4A1.1 Panel II) and similar amounts of fusion proteins were used. GST-57 pulled down Y14 from TPA-induced as well as uninduced BCBL-1 cell extracts (lanes 6 & 7); anti-Y14 Ab cross-reacts with two bands from GST-57 fusion protein preparation above and below the Y14 band in the BCBL-1 input control (compare lanes 5, 6, & 7 with 3 & 4). RNAse treatment did not disrupt this interaction (Fig. 6A10.1 Panel I lane 5). These data demonstrate that ORF57 and Y14 can associate in TPA-induced BCBL-1 cells thus REF/TAP/Y14 may complex with ORF57 protein.

Fig 6A9.3. GST-K interacts with *in vitro* synthesised ^{35}S-labelled-mycREF2-I



Panel I. Phosphorimager analysis showing that GST-K interacts with *in vitro* synthesised ^{35}S-labelled-mycREF2-I. GST-K or GST alone on glutathione agarose beads were added to ^{35}S-labelled-myc-REF2-I protein with or without RNAse treatment and the pull down assay was performed. The complex formed was separated on SDS-PAGE gel, gel was dried and analysed by phosphorimaging. Pull downs included in **lanes: 1.** ^{35}S-labelled-myc-REF2-I + GST-K + RNAse **2.** ^{35}S-labelled-myc-REF2-I + GST-K **3.** ^{35}S-labelled-myc-REF2-I + GST alone **4.** Input ^{35}S-labelled-myc-REF2-I.

Fig 6A10.1. GST-57 pulls down Y14 from virus-infected cells



Panel I. Western blot for Y14 with anti-Y14 Ab showing that GST-57 pulls down Y14 from virus-infected cells. Pull downs from TPA-induced and uninduced BCBL-1 cells using GST-57 or GST alone were analysed by SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted for Y14 using anti-Y14 Ab . Pull downs included in **lanes: 1.** TPA-induced BCBL-1 extract + GST alone **2.** Uninduced BCBL-1 extract + GST alone **3.** Input uninduced BCBL-1 extract **4.** Input TPA-induced BCBL-1 extract **5.** TPA-induced BCBL-1 extract + GST-57 + RNAse **6.** TPA-induced BCBL-1 extract + GST-57 **7.** Uninduced BCBL-1 extract + GST-57.

6B RNA export assays

Recently, an assay that allows examination of TAP-mediated stimulation of RNA nuclear export in cultured cells was reported (Braun *et al.*, 2001). This is more sensitive assay using the pCMV128 (CAT-RRE) reporter plasmid (Fig. 6B Panel I) than the assay using plasmid pDM138 (Herold *et al.*, 2000). Plasmid pDM138 harbours the CAT coding sequence inserted into an intron which is not efficiently spliced, and cells transfected with this plasmid retain the unspliced pre-mRNA in the nucleus, yielding only trace levels of CAT enzyme activity (Huang *et al.*, 1991). Plasmid pCMV128 is similar but has a HCMV promoter instead of an SV40 promoter (McDonald *et al.*, 1992). The basal level of CAT activity in cells transfected with pCMV128 is ten fold higher than in cells transfected with pDM138 (Braun *et al.*, 2001). Transfection with this reporter plasmid yields low levels of CAT enzyme activity as the CAT coding sequence present within the single intron is spliced out and stays in the nucleus. However co-transfection with recombinant plasmids expressing proteins which allow nuclear retention to be bypassed and promote export of unspliced transcripts will lead to an increase in CAT activity. Overexpression of TAP with pCMV128, in the absence of exogenous p15 resulted in a significant but modest increase of CAT activity (~7-fold) (Herold *et al.*, 2000; Braun *et al.*, 2001). Furthermore, co-expression of TAP/p15 heterodimers pCMV128 caused a 44-fold increase in CAT enzyme activity with an increase in expression of TAP levels. RNAse protection analysis confirmed that TAP/p15 heterodimers enhanced CAT gene expression by allowing the unspliced transcripts to enter the cytoplasm (Braun *et al.*, 2001). These results also indicated that in cells overexpressing TAP, p15 becomes limiting, and *vice versa*, and thus no large pools of free TAP or p15 exist *in vivo*.

6B1 Overexpression of ORF57 promotes the nuclear exit of inefficiently spliced pre-mRNAs and has a low RNA export ability in 293 cells

Increased accumulation of cytoplasmic poly $(A)^+$ mRNA compared to nuclear levels of target CAT mRNA in ORF57 transfected cells (Gupta *et al.*, 2000), ORF57 nucleocytoplasmic shuttling ability (Bello *et al.*, 1999) and RNA binding activity of its homologues suggests possible involvement in mRNA nuclear export. The fact that ORF57 interacts with REF proteins strengthens this. Therefore, pKS3 (pEGFP-gORF57) was co-transfected with a constant amount of pCMV128 reporter plasmid and β-gal plasmid (to normalise the transfection efficiency) into human 293 and 293-T cells following the published procedure (Braun *et al.*, 2001) (see Section 2B9.2). Protein levels were analysed by Western blotting. Overexpression of the plasmid encoding ORF57

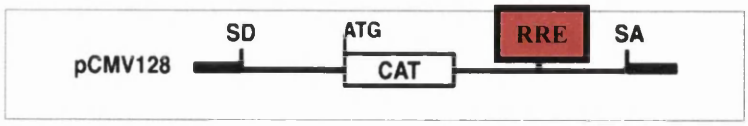
resulted in a modest increase in CAT activity (Fig. 6B1 Panel I). ORF57 [pKS3, GFP-ORF57 (FL)] increased CAT expression by four- to five-fold compared to the expression levels obtained with pCMV128 alone or with empty GFP vector (pEGFP-C1). CAT activity was reduced to 2.5- to 3-fold using the ORF57 N-terminal (aa 1-180) deletion mutant pKS1 compared to GFP alone. Therefore, ORF57 stimulates nuclear export of unspliced pre-mRNAs that are otherwise inefficiently exported. The level of induction may reflect the lower levels of ORF57 FL (pKS3) protein (Fig. 6B1 Panel II) compared to GFP alone with plasmids transfected at the similar concentration.

6B2 ORF57 as a RevM10-fusion further triggers nuclear export when directly tethered to RRE containing RNA

In the above assay, stimulation of RNA export by a test protein could depend on its ability to bind the target RNA or RNA-associated proteins. Thus the effect of tethering ORF57 directly to pCMV128 pre-mRNA was tested. ORF57 was fused to the C terminus of HIV RevM10 protein. RevM10 is an export defective mutant and, unlike wild-type Rev, cannot promote the export of RNAs bearing the HIV RRE as it carries two point mutations in its NES (Malim *et al.*, 1989a; Cullen, 1998). But RevM10 has a functional NLS and an intact RRE-binding domain and can target fusion proteins to RRE-bearing RNAs (Malim *et al.*, 1989a; Cullen, 1998; Guzik *et al.*, 2001). Vectors expressing Rev alone, RevM10 or RevM10-ORF57 fusion protein were co-transfected into 293 or 293-T cells with pCMV128. The interaction of these proteins with pCMV128 is shown diagrammatically in Fig. 6B2 panels I and II. Overexpression of the RevM10-ORF57 fusion increased CAT expression by 6- to 8-fold compared to the level obtained with pCMV128 vector+RevM10 alone. This was observed in several independent experiments and Fig. 6B2 Panel III shows the data from three experiments. Addition of GFP empty vector (pEGFP-C1) did not further activate nuclear RNA export from RevM10 (lane 2) or from the RevM10-ORF57 fusion (lane 5) and ORF57 FL when transfected with RevM10 (lane 3), stimulated CAT to similar levels as with pCMV128 alone. Co-expression of ORF57 with the RevM10-ORF57 fusion further stimulated nuclear RNA export up to 12- to 14-fold (lane 6). Western blotting with anti-HA Ab (an HA tag is present in Rev/RevM10 and RevM10-ORF57 fusion vectors) showed that expression levels of RevM10-ORF57 and RevM10 were similar and did not alter with addition of GFP empty vector or GFP-gORF57 expression plasmid (Fig. 6B2 Panel IV).

Fig 6B. CAT assay reporter plasmid construct (pCMV128 CAT-RRE)

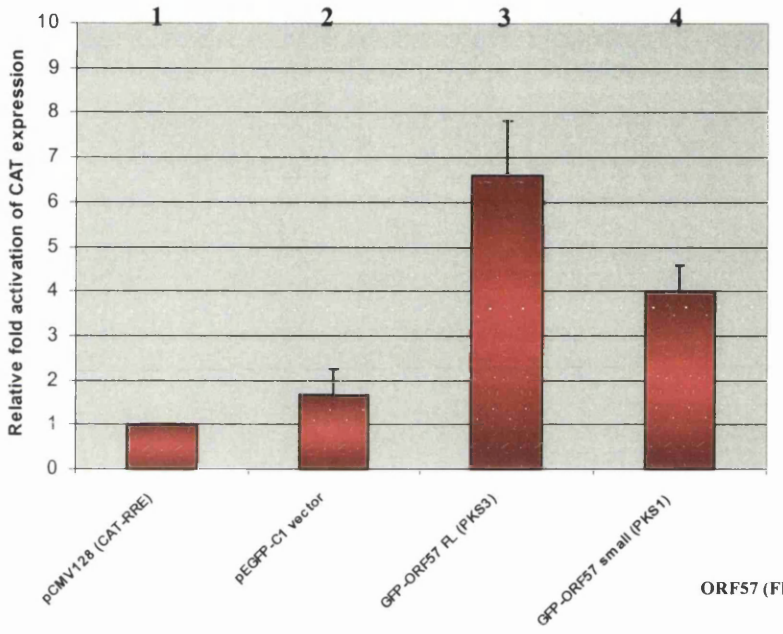
I



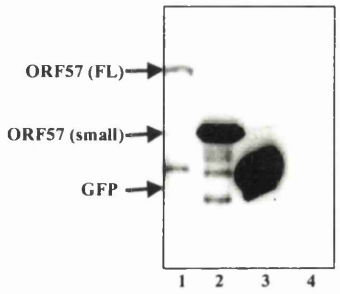
Panel I. Plasmid pCMV128 showing splice doner (SD); splice acceptor (SA); chloramphenicol acetyl transferase (CAT) reporter gene; Rev response element (RRE) binding site.

Fig 6B1. Overexpression of ORF57 stimulates RNA export in 293 cells

I



II

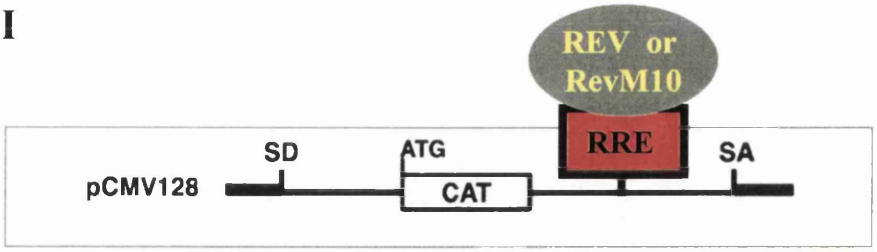


Panel I. Overexpression of ORF57 stimulates RNA export in 293 cells. Human 293 cells were transfected with a mixture of plasmids including β-gal, pCMV128 (CAT-RRE) and either GFP alone or fused to ORF57 full length or small (N-terminal deletion mutant). Cells were collected 48 h posttransfection and β-gal and CAT activities were determined from three separate experiments.

Panel II. Western blot for GFP-ORF57 and GFP with anti-GFP Ab showing expression of GFP-ORF57 (FL and small) and GFP alone (pEGFP-C1). Transfections with various plasmids used for CAT assay were analysed by SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted. Shown in **lanes: 1.** GFP-gORF57 FL (pKS3) **2.** GFP-ORF57 small (pKS1) **3.** GFP empty vector (pEGFP-C1) **4.** Untransfected 293 cell extracts.

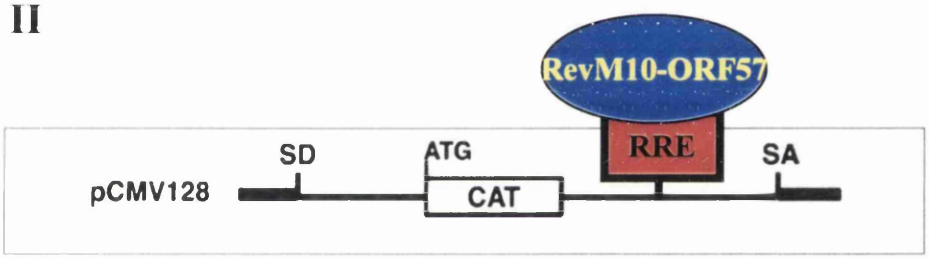
Fig 6B2 Panels I and II. CAT assay construct for Rev, RevM10 or RevM10-ORF57 fusion protein binding

I



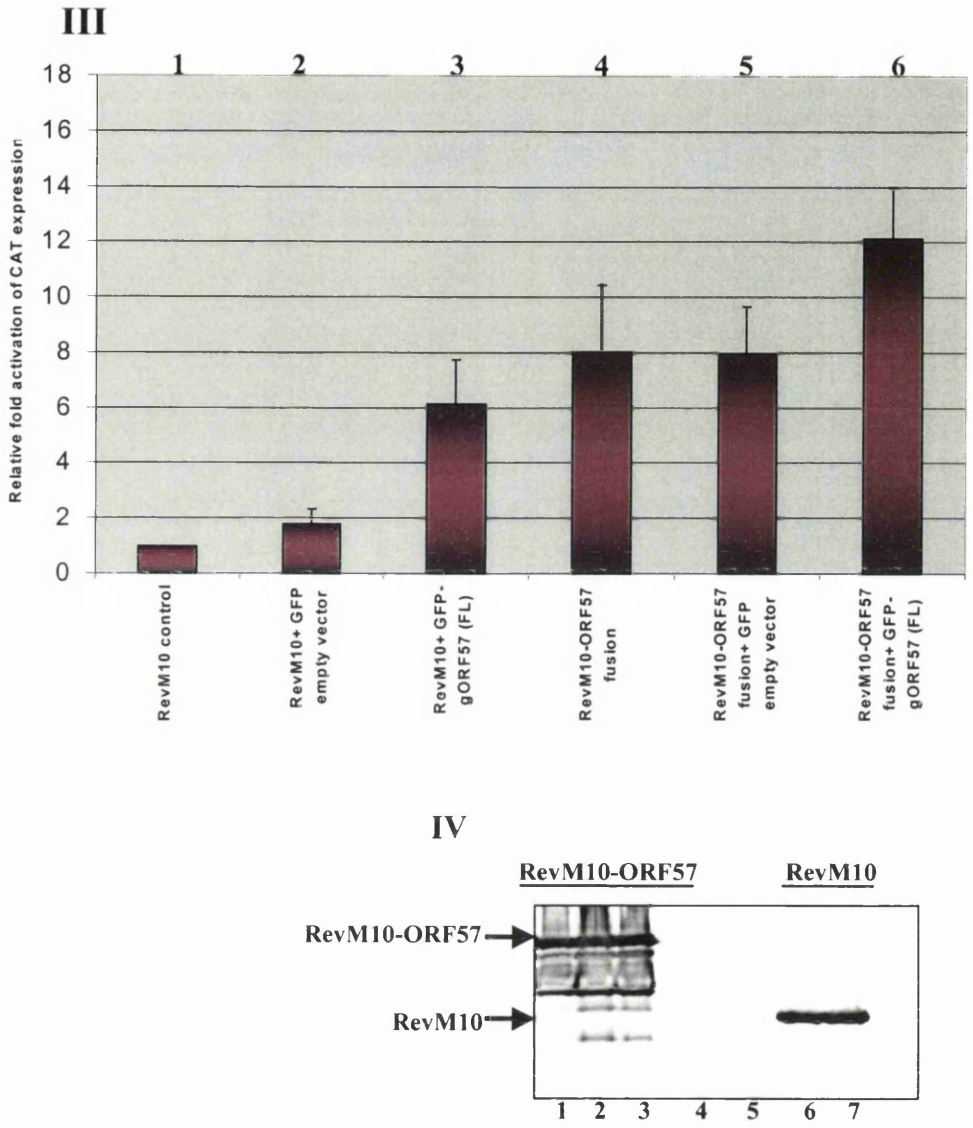
Panel I. Plasmid pCMV128 showing splice doner (SD; splice acceptor (SA); chloramphenicol acetyl transferase (CAT) reporter gene; Rev or RevM10 Rev protein binding to the Rev response element (RRE) binding site.

II



Panel II. Plasmid pCMV128 showing splice donor (SD); splice acceptor (SA); chloramphenicol acetyl transferase (CAT) reporter gene; RevM10-ORF57 fusion protein binding to the Rev response element (RRE) binding site.

Fig 6B2 Panels III and IV. ORF57 triggers nuclear export further as a RevM10 fusion tethered to the RNA export cargo



Panel III. ORF57 triggers nuclear export further as a RevM10 fusion tethered to the RNA export cargo. 293 cells were transfected with a mixture of plasmids including β-gal, pCMV128 and either GFP alone or fused to ORF57 full length (pKS3). Cells were collected 48 h posttransfection and β-gal and CAT activities were determined from three separate experiments.

Panel IV. Western blot for HA-RevM10-ORF57 and HA-RevM10 with anti-HA Ab showing that expression levels of RevM10-ORF57 and RevM10 are similar. Transfections with various plasmids used for CAT assay were analysed by SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted. Samples used in **lanes: 1.** RevM10-ORF57 fusion expression plasmid **2.** RevM10-ORF57 + GFP empty vector (pEGFP-C1) **3.** RevM10-ORF57 + GFP-gORF57 (FL) **4.** Untransfected 293 cell extracts **5.** pCMV128 reporter plasmid **6.** RevM10 expression plasmid **7.** RevM10+ GFP-gORF57 (FL).

As a positive control, Rev was included in the study and activated export of CAT-RRE RNAs by 130 to 160-fold (Fig. 6B2 Panel V). Interestingly, co-transfection of ORF57 with Rev protein reduced the activation of CAT expression by Rev to 80 to 110-fold. Western blotting with anti-HA Ab for Rev expression levels in absence (lane 1) or presence of ORF57 (lane 2) showed that this reduction in CAT activation was not due to reduced Rev levels and ORF57 protein could be regulating Rev export activity (Fig. 6B2 Panel VI).

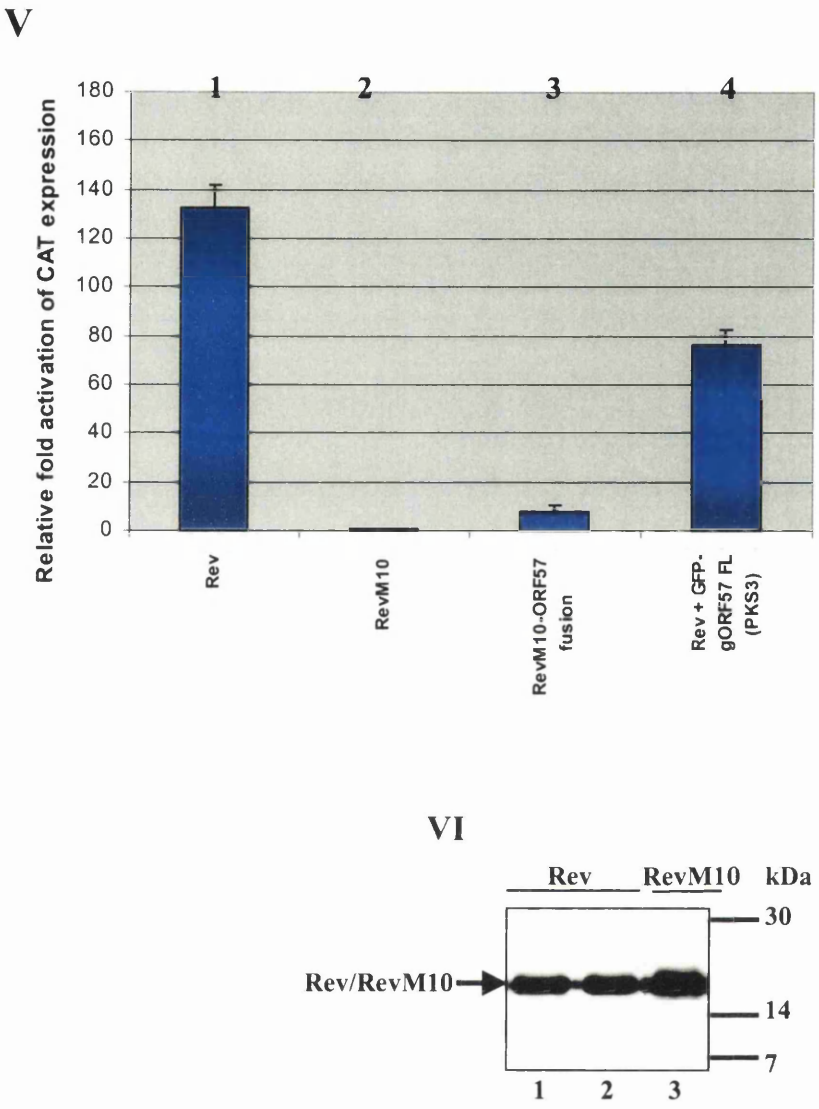
Recently Guzik *et al.* and Braun *et al.* reported that expression of TAP alone as a RevM10-fusion (RevM10-TAP) moderately stimulated CAT activity (10- to 15-fold) with pCMV128 but its co-expression with its co-factor p15/NXT1, due to formation of TAP+p15 heterodimers, helped to increase CAT activity up to ~250-fold (Braun *et al.*, 2001; Guzik *et al.*, 2001). Fig 6B2 Panel VII shows that addition of ORF57 (RevM10-ORF57 fusion) to transfections with TAP+p15 reduced CAT activity (35- to 50-fold) compared to activity obtained with TAP+p15 and RevM10 (45- to 70-fold). This was observed in several independent experiments and Fig. 6B2 Panel VII shows the average of three experiments. Addition of REF to ORF57 and TAP +p15 transfections did not increase the CAT activity more than TAP+p15 together (data not shown). Fig. 6B2 Panel VIII shows reduction in CAT activity from TAP+p15 in presence of ORF57 (RevM10-ORF57) was not due to reduced protein expression levels of TAP or p15 which were unaffected when co-transfected with ORF57.

6B3 RevM10-ORF57 mediated RNA export utilizes a CRM1-independent nuclear export pathway

Rev export is dependent on a leucine-rich NES, shown to interact with the export receptor CRM1 (Fornerod *et al.*, 1997a; Stade *et al.*, 1997). The Rev-CRM1 complex interacts with RanGTP, as well as with several nucleoporins and these interactions are believed to be crucial to Rev-mediated export (Bogerd *et al.*, 1998; Askjaer *et al.*, 1999; Zolotukhin & Felber, 1999). CRM1-mediated export is specifically inhibited by LMB and Rev-mediated export is inhibited by this drug (Wolff *et al.*, 1997). In contrast, ICP27-mediated RNA export has been reported to be LMB-insensitive in *Xenopus laevis* oocytes, indicating that it gains access to a CRM1-independent export pathway (Koffa *et al.*, 2001).

To examine effects of LMB on export mediated by the RevM10-ORF57 fusion protein, 293 cells were co-transfected with the RevM10-ORF57 and pCMV128. At 24 h

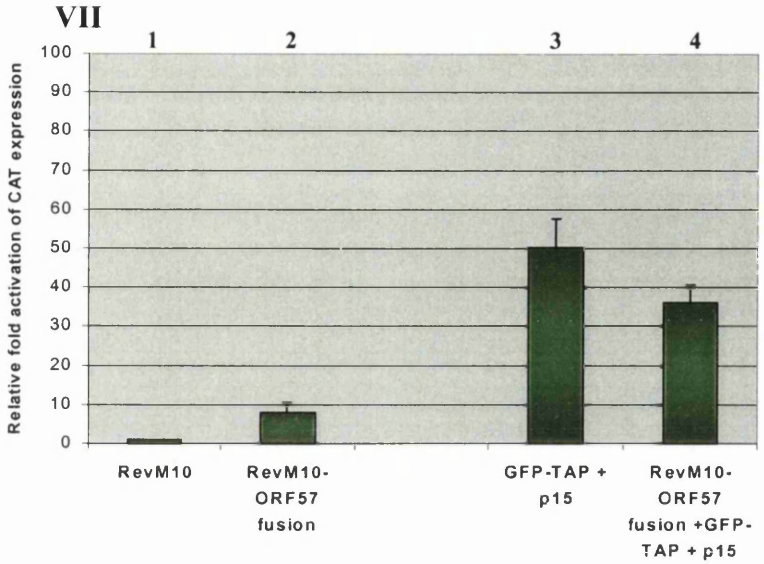
Fig 6B2 Panels V and VI. ORF57 triggers nuclear export as a RevM10-fusion tethered to the RNA export cargo but reduces Rev-mediated export

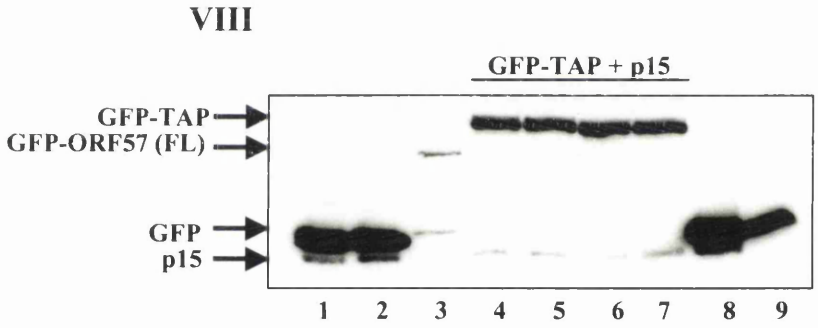


Panel V. ORF57 triggers nuclear export as a RevM10-fusion tethered to the RNA export cargo but reduces Rev-mediated export. 293 cells were transfected with a mixture of plasmids including β-gal, pCMV128, Rev, RevM10, RevM10-ORF57 fusion and either GFP alone or fused to ORF57 full length (pKS3). Cells were collected 48 h posttransfection and β-gal and CAT activities were determined from three separate experiments.

Panel VI. Western blot for Rev and RevM10 with anti-HA Ab showing that expression levels of Rev and RevM10 are similar. Transfections with various plasmids used for CAT assay were analysed by SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted. Samples used in **lanes: 1.** Rev expression plasmid **2.** Rev + GEP-gORF57 (FL) **3.** RevM10 expression plasmid.

Fig 6B2 Panels VI and VII. ORF57 seems to compete with TAP/p15 heterodimers for nuclear RNA export components





Panel VII. ORF57 seems to compete with TAP/p15 heterodimers for nuclear RNA export components. 293 cells were transfected with a mixture of plasmids including β-gal, pCMV128, RevM10, RevM10-ORF57 fusion and GFP-TAP+ zzp15 or GFP alone to keep the amounts of vector backbone constant. Cells were collected 48 h posttransfection and β-gal and CAT activities determined from 3 experiments.

Panel VIII. Western blot with anti-GFP Ab showing expression of TAP+p15 alone, and in presence of RevM10-ORF57. Transfections with various plasmids used for CAT assay were analysed by SDS-PAGE gel, transferred to a nitrocellulose membrane and immunoblotted. Samples used in **lanes: 1.** RevM10 expression plasmid + GFP empty vector (pEGFP-C1) **2.** RevM10-ORF57 + GFP empty vector (pEGFP-C1) **3.** GFP-gORF57 (FL) **4.** GFP-TAP + p15 **5.** RevM10-ORF57 fusion + GFP-TAP + p15 **6.** RevM10 + GFP-TAP + p15 **7.** RevM10-ORF57 fusion + REF2-I + GFP-TAP + p15 **8.** Rev expression plasmid + GFP empty vector (pEGFP-C1) **9.** pCMV128 reporter alone+ GFP empty vector (pEGFP-C1).

post-transfection, the medium was replaced with a medium containing 5nM LMB or an equivalent amount of ethanol used to solubilise the LMB. Cells were harvested 24 h after addition of LMB and assayed for CAT activity. As a positive control, cells were transfected with Rev-expressing plasmid and pCMV128 in presence of LMB; RevM10-expressing plasmid with pCMV128 in presence of LMB was used as negative control. The result of three experiments are shown in Fig. 6B3 Panel I. The nuclear RNA export ability of Rev was inhibited more than five-fold by LMB in these experiments as reported previously (Wolff *et al.*, 1997; Otero *et al.*, 1998). In presence of LMB, only a very small decrease in CAT export was observed in cells transfected with the RevM10-ORF57 fusion protein. Fold inhibition was calculated taking the ratio of the CAT activity obtained with no drug to the CAT activity of the LMB-treated samples. These results indicate that the ORF57 fusion protein promotes export of pCMV128-CAT-RRE RNA through a CRM1-independent pathway.

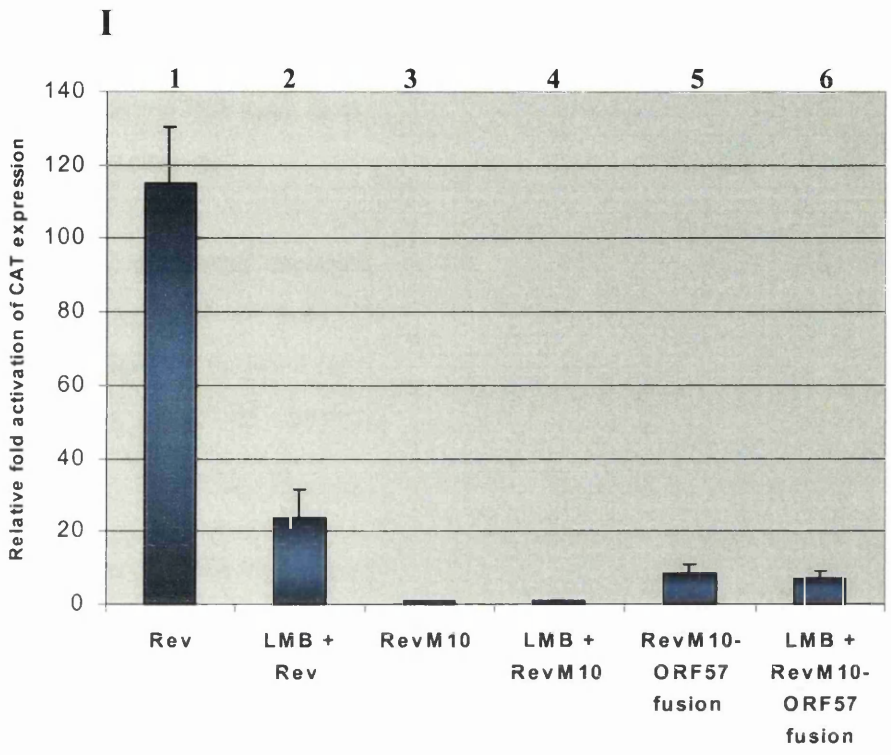
This study provides evidence for a role of ORF57 in nuclear mRNA export. ORF57 fused to an export-incompetent Rev protein can substitute for Rev and promote nucleocytoplasmic export of intron-containing RNA through a CRM1-independent pathway.

6C Discussion

This Chapter presents data that demonstrate the interaction between ORF57 and REF and shows that ORF57 acts as RNA export factor. ORF57 interacts directly with REF in pull down assays and coimmunoprecipitations and forms a complex with REF and TAP proteins in KSHV-infected cells. Unlike ICP27 (Koffa *et al.*, 2001), the REF RBD is not required for interaction with ORF57 and either one or other of its variable regions (with at least one conserved box) is sufficient for its binding to ORF57. ORF57 also interacts with more than one REF and, as REF1-II lacks the N-vr region, this shows that the N-terminal variable region of REFs is not strictly required for interaction. The ORF57-binding regions on REF are similar to those that bind TAP and RNA. The N-terminal aa of ORF57 are required for interaction with REF, the minimum interacting region of ORF57 is aa 181-215 although aa 17-181 could also be required.

Although the possibility of important functions other than export is not excluded for ORF57, the conservation of ORF57 homologues in the Herpesviridae family, their structural organization and the observation that at least three of its homologues

Fig 6B3. RNA export by ORF57 is leptomycin B insensitive



Panel I. ORF57-mediated export uses a CRM 1-independent pathway. Transfections of 293 cells were performed with plasmids expressing Rev, RevM10 or RevM10-ORF57 fusion proteins. At 24h posttransfection 5nM LMB was added to one half of the samples and ethanol solvent was added to the other half (controls). Samples were collected 12 h later and CAT and β-gal activities were measured.

(ICP27, EBV Mta and HVS ORF57) have a role in RNA export (Boyle *et al.*, 1999; Goodwin *et al.*, 1999; Farjot *et al.*, 2000; Koffa *et al.*, 2001) suggests that this protein is likely to participate, directly or indirectly in the export of viral mRNAs to the cytoplasm. Moreover it has recently been shown for the herpesviruses HSV-1 and EBV-1 that some viral RNA export is mediated by CRM1-independent pathways (Farjot *et al.*, 2000; Koffa *et al.*, 2001) suggesting that these herpesvirus proteins may shuttle through a distinct and as yet unknown mechanism.

Overexpression of a plasmid encoding ORF57 increased export of CAT mRNA. The ability of ORF57 to export the pre-mRNA was reduced using the N-terminal (aa 1-180) deletion mutant implying the need for a functional full-length protein. Therefore, ORF57 stimulates nuclear export of unspliced pre-mRNAs that are otherwise inefficiently exported.

ORF57 tethered to an RNA export cargo as a RevM10-ORF57 fusion further increased CAT expression compared to the expression levels obtained with ORF57 unfused to RevM10 or RevM10 alone. These data suggest that ORF57 was able to complement the NES defect in RevM10, to a certain extent. The higher CAT activity when the ORF57 was fused to RevM10- is likely to be due to direct binding of fusion protein to the CAT pre-mRNA via the RRE, or by binding via other intermediate RNA binding proteins.

Co-transfection of the RevM10-ORF57 fusion and full-length ORF57 further stimulated nuclear RNA export suggesting that ORF57 may need to dimerise to export RNAs efficiently. Interestingly ORF57 has been shown to self-interact in this study (see Section 3C) and HSV-1 ICP27 has also been reported to dimerise (Wadd *et al.*, 1999; Zhi *et al.*, 1999) an event suggested to play an important role in its various functions.

When ORF57 was co-transfected with a Rev expression construct it reduced the activation of CAT expression by Rev. Thus ORF57 seems to negatively regulate nuclear RNA export by Rev protein, indicating that at some stage they may share common export components. However LMB experiments showed that ORF57 nuclear export is not CRM1 dependent, and unlike Rev protein, it does not require this bridging adaptor to access the nuclear pores. The nuclear pore complex component Rip1p, (an FG-repeat nucleoporin) interacted with the Rev NES via CRM1 in a yeast-two-hybrid interaction (Neville *et al.*, 1997) and RevNES-CRM1-Rip1 is the export route followed by the Rev protein-mRNA cargo during HIV-1 infection. It is possible that both ORF57 and Rev

share a similar second step of RNA export by competing for other nuclear pore components which Rev accesses through CRM1.

ICP27 associates with viral mRNAs and recruits the TAP adaptor via its interaction with REF proteins, allowing the otherwise inefficiently exported HSV1 mRNAs to access the TAP-mediated export pathway (Koffa *et al.*, 2001). TAP utilizes a CRM1-independent pathway (Gruter *et al.*, 1998; Kang & Cullen, 1999) and its interaction with cellular RNAs is thought to be mediated by REFs (Strasser & Hurt, 2000; Stutz *et al.*, 2000). TAP is directly implicated in the export of simian type D retroviral RNAs bearing the CTE by interacting with CTE RNA and nucleoporins (Gruter *et al.*, 1998; Braun *et al.*, 1999; Kang & Cullen, 1999; Kang *et al.*, 2000; Ryan & Wente, 2000). Thus, TAP forms a different class of export receptors, which interact directly with components of the NPC.

Interestingly, in the RNA export assay, addition of ORF57 with TAP in presence of its co-factor p15 resulted in reduced levels of CAT activity compared to the activity obtained with TAP +p15 only. Addition of REF to the ORF57 transfections did not increase CAT activity more than that obtained with TAP+p15, suggesting that REF is not the limiting factor for ORF57-mediated RNA export.

Braun *et at.*, (2001) reported using the same RNA export assay that overexpression of TAP alone resulted in a 6-8-fold increase in CAT activity which was elevated to 40-fold in presence of p15 due to heterodimer formation. TAP alone, expresses at lower levels in the absence of p15 and formation of heterodimers with p15 increase the stability of TAP protein. Using a TAP-RevM10 fusion, CAT activity increased up to 250-fold in presence of p15 in comparison to 12-15-fold obtained with RevM10-TAP fusion alone (Braun *et al.*, 2001). For TAP, addition of p15 enhances CAT expression at the level of RNA export resulting in a 10-fold relative increase in the cytoplasmic levels of RRE mRNA (Guzik *et al.*, 2001). For ORF57, such a co-factor (as for TAP, formation of heterodimers by p15) has not yet been identified which might increase its stability and export ability.

ORF57 may modulate the efficiency of REF association with mRNAs and provide specificity and efficiency to the binding of viral mRNAs. In the export assay for the RevM10-ORF57 fusion tethered directly to the CAT pre-mRNA, RNA binding specificity provided by ORF57 will not be of significance as it is already tethered to the RNA export cargo. Any reduction in CAT activity after addition of ORF57 to TAP+p15 co-transfection may imply that ORF57 or TAP/p15 utilize some common export

components resulting in saturation of the export pathway.

A yeast-two-hybrid screen in our laboratory showed a strong interaction between Rip-1 and ICP27 whereas in the same screen ICP27 failed to interact with CRM1 (Wadd, 2000). Unlike Rev, the herpesvirus proteins (ORF57, ICP27, Mta) may not need a bridging protein such as CRM1 to link with the NPC but rather might directly interact with NPC components to facilitate docking of the mRNA export cargo. Koffa *et al.* (2001) demonstrated ICP27 protein export in the absence of viral RNA if re-import of the protein was inhibited. This export required neither CRM1 nor TAP suggesting that RNA-independent ICP27 export uses another as yet unidentified export receptor or ICP27 itself is capable of nuclear export and acts as an export adaptor.

A C-terminal fragment of Nup214 (Δcan) including the FG repeat-rich region has a transdominant negative phenotype and inhibits Rev function in the CAT-RRE system (Bogerd *et al.*, 1998; Kang & Cullen, 1999). When the TAP binding sequence CTE replaced the RRE, TAP also induced the cytoplasmic accumulation of CAT-CTE intron-containing RNAs, but overexpression of Δcan had no effect (Bogerd *et al.*, 1998; Kang & Cullen, 1999). ORF57 homologue EBV Mta protein does not inhibit expression of intron-containing genes but activates nuclear export of intron-containing polyadenylated RNA possessing suboptimal splice sites (Buisson *et al.*, 1999; Farjot *et al.*, 2000). Mta associates *in vivo* with the C-terminal fragment (Δcan) of Nup214 (Boyle *et al.*, 1999) that could be direct or possibly indirect via CRM1. Overexpression of Δcan gave a dramatic inhibition of both Rev- and Mta-dependent transactivation (Farjot *et al.*, 2000). Although TAP function is insensitive to LMB, it binds Nup214 both *in vitro* and in yeast cells (Kang & Cullen, 1999; Katahira *et al.*, 1999). These results suggest a mechanism for Mta distinct from the Rev pathway but also involving Nup214 (without interaction with CRM1) which could be an essential component in Mta-mediated nuclear export pathway (Farjot *et al.*, 2000). Boyle *et al.*, (1999) based on *in vivo* association of Mta with Δcan predicted this protein to be capable of interacting with nucleoporins or other structural nuclear proteins directly (with or without the involvement of CRM1) (Boyle *et al.*, 1999).

Different nucleoporins with repeat sequences line the entire nuclear pore channel, from the nuclear basket to the cytoplasmic pore filaments. ORF57 with bound RNAs could pass through nuclear pores via direct multiple contacts to FG-repeat nucleoporins and

direct contact between ORF57 and nucleoporins (such as Rip1p/Nup214/Nup42p/hCG1 or others) could be one of the last steps during ORF57-mediated nuclear mRNA export.

REF and TAP play an important part in cellular mRNA export. Since ORF57 binds directly to REF and complexes containing ORF57/REF/and TAP are formed *in vitro* and found in KSHV-infected cells (see Section 6A) the interaction of ORF57 with these cellular export factors might have a regulatory effect. ORF57 could have an inhibitory effect on their mRNA export activity or hijack their roles to promote viral mRNA export. Facilitating an alteration of TAP activity would enable ORF57 to fully utilise an export pathway which it may share with TAP. It is clear from this study that ORF57 mediated RNA export shares certain components of the nuclear export pathway utilised by cellular mRNAs but it may exploit more than one export route at different stages of infection. This hypothesis is further supported by the fact that HSV-1 recombinant *d*3-4, lacking the REF interacting region of ICP27 (aa 109-138) was only partially defective in nuclear accumulation and for growth in Vero cells (Mears *et al.*, 1995). The *d*3-4 mutant showed a 9- to 34-fold defect in growth in Vero cells compared to wt HSV-1 strain (KOS1.1). This suggests that although the sequences deleted in *d*3-4, which include a putative NLS and REF binding site, are required for optimal viral growth, their deletion is not lethal. Perhaps in KSHV infected cells at the early stages of infection, ORF57 accesses the TAP mRNA pathway through REF but at later stages could act independently of TAP.

Chapter 7: Interaction of ORF57 with SAP145 and not with p32 protein

Two other cellular proteins interacting with ICP27 identified by the yeast 2-hybrid screen and confirmed by binding assays were SAP145 and another protein involved in splicing, p32 (Bryant *et al.*, 2000; Bryant *et al.*, 2001). The possible involvement of ICP27 in splicing inhibition and host cell shut off provided reasonable basis for interaction with SAP145 and p32. To find out if ORF57 interacted with the SAP145 and p32, *in vitro* and *in vivo* binding assays were performed. The data presented here shows that SAP145 interacts with ORF57 *in vitro* and in virus-infected cells however ORF57 failed to interact with p32. The implications of these interactions are discussed.

7A ORF57 and SAP145

SAP145, an essential component of the SF3b subunit, binds to pre-mRNA (Staknis & Reed, 1994) and it is one of the seven splicing-associated polypeptides found within the U2 snRNP (Gozani *et al.*, 1994), where it acts to tether the U2 snRNA to the branch site in the intron of pre-mRNA (Champion-Arnaud & Reed, 1994).

7A1 Interaction of ORF57 with SAP145 in GST pull down assays

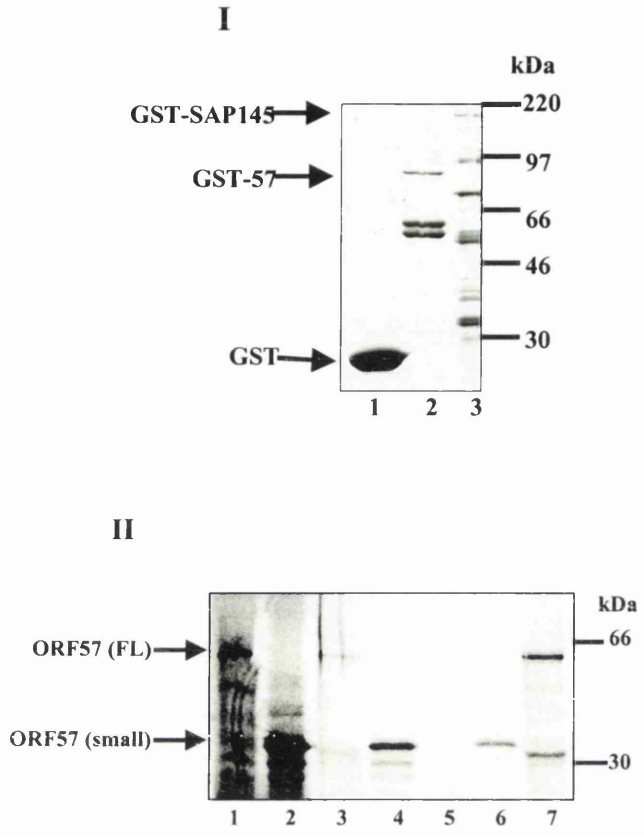
7A1.1 SAP145 interacts in vitro with ORF57

GST pull down assays using GST-SAP145 or GST with ^{35}S-labelled-ORF57 were performed. Fusion proteins used were visualised by Coomassie blue staining (Fig. 7A1.1 Panel I lanes 3 & 1) and equivalent amounts of fusion proteins, GST-SAP145 (Section 2B4.4) or GST alone, were added to ^{35}S-labelled-ORF57 (FL) or ^{35}S-labelled-ORF57 (small) protein. Samples eluted twice with reduced glutathione buffer were heated to 100°C in SDS gel buffer for 5 min, analysed by SDS-PAGE (Section 2B6.1) and the gel was dried and exposed to a phosphorimager screen (Section 2B6.4). The fusion protein GST-SAP145 bound ^{35}S-labelled-ORF57 (FL and small) protein (Fig. 7A1.1 Panel II lanes 3 & 7 and 4 & 6) and GST alone did not pull down ORF57 FL (lane 5).

7A1.2 SAP145 interacts with ORF57 from virus-infected cells

GST-SAP145 or GST alone bound to glutathione Sepharose beads were used to pull down interacting proteins from TPA-induced and uninduced BCBL-1 cell extracts (Section 2B4.4). Western blotting with anti-ORF57 Ab showed that in TPA-induced

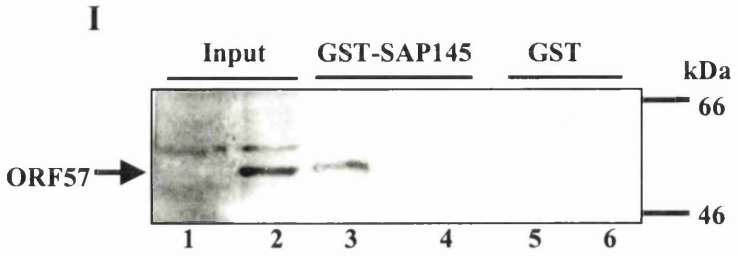
F ig 7A1.1. GST-SAP145 interacts with *in vitro* synthesised ORF57



Panel I. GST-SAP145, GST-ORF57 and GST expression: Coomassie blue stained gel. GST-SAP145, GST-57 and GST proteins used were run on a SDS-PAGE gel and visualised by Coomassie staining. The smaller bands in lanes 2 and 3 represent degradation products/truncations of full length GST-SAP145 and GST-57 respectively. Shown in **lanes: 1**. GST alone **2.** GST-57 protein **3.** GST-SAP145 protein.

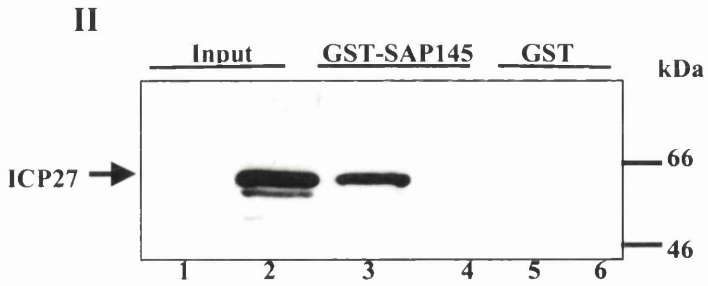
Panel II. Phosphorimager analysis showing that GST-SAP145 interacts with *in vitro* synthesised ORF57. GST-SAP145 and GST fusion proteins bound onto glutathione beads were incubated with ^{35}S-labelled-ORF57 (FL and small), after washing, specifically bound proteins were eluted with reduced glutathione buffer and separated on a SDS-PAGE gel, gel was dried and analysed by phosphorimaging. Pull downs included in **lanes:**
1. Input ^{35}S-labelled-ORF57 (FL) **2**. Input ^{35}S- labelled-ORF57 (small) **3**. GST-SAP145 + ^{35}S-labelled-ORF57 (FL)-first elution **4**. GST-SAP145 + ^{35}S-labelled-ORF57 (small)-first elution**5.** GST alone + ^{35}S-labelled-ORF57 (FL)-second elution **6**. GST-SAP145 + ^{35}S-labelled-ORF57 (small)-second elution7. GST-SAP145 + ^{35}S-labelled-ORF57 (FL)-second elution.

Fig 7A1.2 Panel I. GST-SAP145 pulls down ORF57 from virus-infected BCBL-1 cells



Panel I. Western blot for ORF57 with anti-ORF57 Ab showing that GST-SAP145 pulls down ORF57 from virus-infected BCBL-1 cells. TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST-SAP145 or GST proteins. After incubation with extracts, proteins pulled down were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted for ORF57 using anti-ORF57 Ab. Pull downs included in **lanes: 1**. Input uninduced BCBL-1 extract **2**. Input TPA-induced BCBL-1 extract **3**. TPA-induced BCBL-1 extract + GST-SAP145 **4**. Uninduced BCBL-1 extract + GST-SAP145 **5**. TPA-induced BCBL-1 extract + GST alone **6**. Uninduced BCBL-1 extract + GST alone.

Fig 7A1.2 Panel II. GST-SAP145 pulls down ICP27 from virus-infected BHK cells



Panel II. Western blot for ICP27 with anti-ICP27 Ab showing that GST-SAP145 pulls down ICP27 from virus-infected BHK cells. HSV-1 wt-infected and mock-infected BHK cell extracts were incubated with GST-SAP145 or GST proteins. After incubation with extracts, proteins pulled down were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted for ICP27 using anti-ICP27 Ab. Pull downs included in **lanes: 1.** Input mock-infected BHK extract **2**. Input wt-infected BHK cell extract **3**. wt-infected BHK cell extract + GST-SAP145 **4**. Mock-infected BHK extract + GST-SAP145 **5**. wt-infected BHK cell extract + GST alone **6**. Mock-infected BHK extract + GST alone.

BCBL-1 cell extracts (Fig. 7A1.2 Panel I lane 3), GST-SAP145 pulled down a band corresponding to ORF 57 (~50-52 kDa) present in whole cell TPA-induced BCBL-1 extracts (Panel I lane 2) whereas uninduced cell extracts and GST-SAP145 (lane 4) and GST alone (lanes 5 & 6) were negative for a band of similar size. As an ORF57 loading control a portion of input uninduced and TPA-induced BCBL-1 cell extracts used for pull down assays were loaded in lanes 1 & 2 respectively. Under these conditions ORF57 is present in its native conformation and concentration in whole cell extracts and was not added artificially as fusion protein.

As a positive control GST-SAP145 pulled down ICP27 from wt HSV-1 infected BHK cell extracts (Fig. 7A1.2 Panel II lane 3) and GST alone (negative control) did not interact with wt infected or mock infected cell extracts (lanes 5 & 6).

7A1.3 GST-57 protein interacts with SAP145 in the reciprocal pull down assay

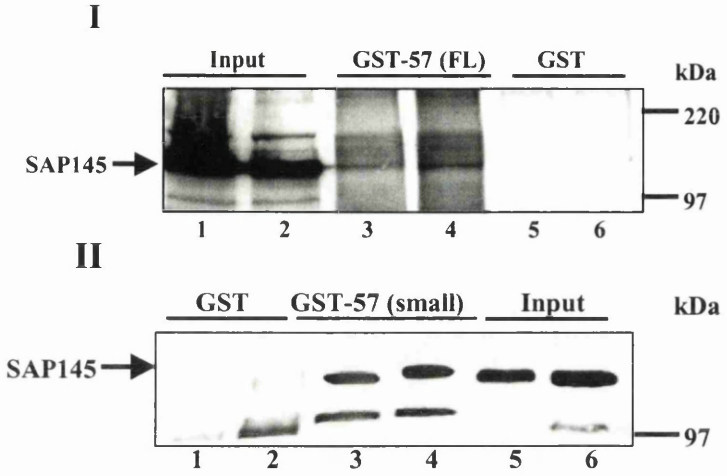
GST-57 fusion protein or GST alone bound to glutathione Sepharose beads were used to pull down interacting proteins from TPA-induced and uninduced BCBL-1 cell extracts (Section 2B4.4) and equivalent amounts of these were used for pull downs. Western blotting with anti-SAP145 Ab showed that GST-57 (FL) pulled down a band corresponding in size to SAP145 from TPA-induced and uninduced cell extracts (Fig. 7A1.3 Panel I lanes 3 & 4). GST alone did not pull down SAP145 from TPA-induced and uninduced cell extracts (Fig. 7A1.3 Panel I lanes 5 & 6 and Panel II lanes 1 & 2). GST-57 (small) was also capable of interaction with SAP145 from both TPA-induced and uninduced BCBL-1 cell extracts (Fig. 7A1.3 Panel II lanes 3 & 4). This indicates that no other lytic viral proteins are required for this interaction and the region of SAP145 interaction must lie at the ORF57 C terminus. For SAP145 loading controls, a portion of total input uninduced and TPA-induced BCBL-1 cell extracts were used (Panel I lanes 1 & 2 and Panel II lanes 6 & 5).

As a positive control GST-ICP27 pulled down SAP145 from HSV-1 wt infected BHK cell extracts (Fig. 7A1.3 Panel III lane 2) and GST alone (negative control) did not interact with SAP145 from wt infected or mock infected BHK cell extracts (lanes 3 & 4).

7A2 ORF57 protein coimmunoprecipitates SAP145 from virus-infected cell extracts

Confirmation of interaction between SAP145 and ORF57 was provided by Western blotting of anti-ORF57 Ab and anti-SAP145 Ab coimmunoprecipitates from TPA-induced and uninduced BCBL-1 cell extracts with SAP145 Ab. (Section 2B5.1 & 2B5.4). Fig. 7A2 Panel I lanes 3 & 4 shows that anti-SAP145 Ab immunoprecipitated SAP145 from both TPA-induced and uninduced BCBL-1 cell extracts, whereas anti-ORF57 Ab

Fig 7A1.3 Panels I and II. GST-57 (FL) (Panel I) and GST-57 small (Panel II) pulls down SAP145 from virus-infected BCBL-1 cells

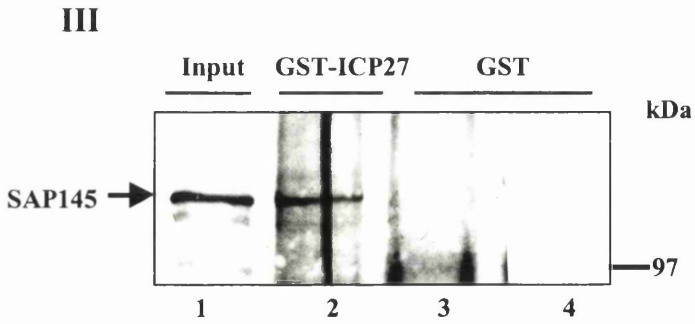


TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST-57 (FL and small) or GST proteins. After incubation with extracts, proteins pulled down were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted for SAP145 using anti-SAP145 Ab.

Panel I. Western blot for SAP145 with anti-SAP145 Ab showing that GST-57 (FL) pulls down SAP145 from virus-infected BCBL-1 cells. Pull downs included in **lanes: 1**. Input uninduced BCBL-1 extract **2**. Input TPA-induced BCBL-1 extract **3.** TPA-induced BCBL-1 extract + GST-57 (FL) **4**. Uninduced BCBL-1 extract + GST-57 (FL) **5**. TPA-induced BCBL-1 extract + GST alone **6**. Uninduced BCBL-1 extract + GST alone.

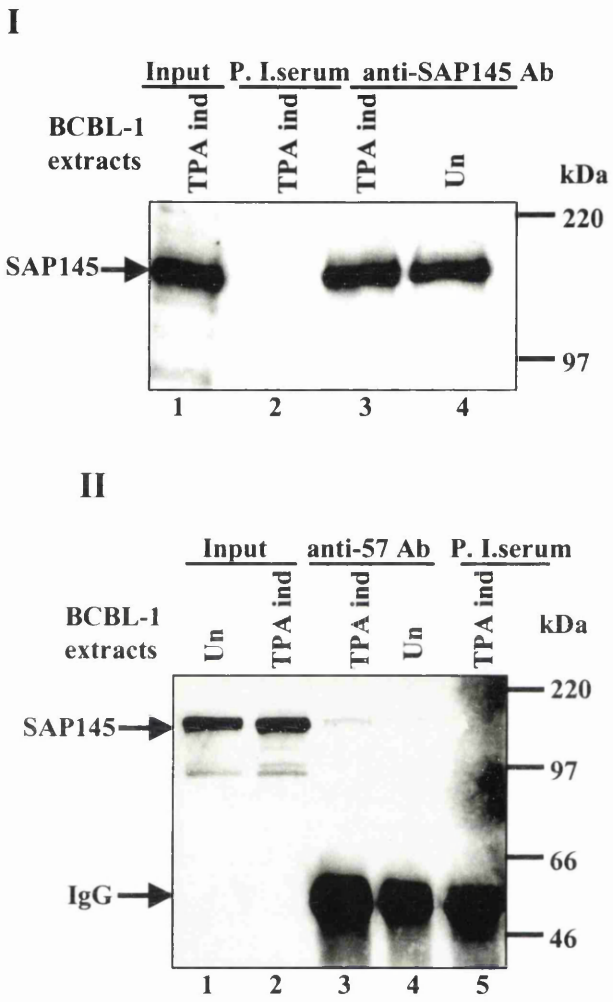
Panel II. Western blot for SAP145 with anti-SAP145 Ab showing that GST-57 small pulls down SAP145 from BCBL-1 cells. Pull downs included in **lanes: 1**. TPA-induced BCBL-1 extract + GST alone **2**. Uninduced BCBL-1 extract + GST alone **3.** TPA-induced BCBL-1 extract + GST-57 (small) **4**. Uninduced BCBL-1 extract + GST-57 (small) **5**. Input TPA-induced BCBL-1 extract **6**. Input uninduced BCBL-1 extract.

Fig 7A1.3. GST-ICP27 pulls down SAP145 from HSV-1 infected BHK cells



Panel III. Western blot with anti-SAP145 Ab. HSV-1 wt-infected and mock-infected BHK cell extracts were incubated with GST-ICP27 or GST proteins. After incubation with extracts, proteins pulled down were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted for SAP145 using anti-SAP145 Ab. Pull downs included in **lanes: 1**. Input wt-infected BHK cell extract **2**. wt-infected BHK cell extract + GST-ICP27 **3**. wt-infected BHK cell extract + GST alone **4**. Mock-infected BHK extract + GST alone.

Fig 7A2 Panels I and II. Anti-SAP145 Ab immunoprecipitates SAP145 from both TPA-induced and uninduced BCBL-1 (Panel I) and anti-ORF57 Ab coimmunoprecipitates SAP145 from TPA-induced BCBL-1 only (Panel II)



A coimmunoprecipitation assay was performed with anti-SAP145 Ab (Panel I) or anti-ORF57 Ab (Panel II) or pre immune serum and uninduced and TPA-induced BCBL-1 cell extracts. The complex formed was separated on SDS-PAGE gel, transferred to nitrocellulose and Western blotted for SAP145 using anti-SAP145 Ab.

Panel I. Western blot for SAP145 with anti-SAP145 Ab showing that anti-SAP145 Ab immunoprecipitates SAP145 from both TPA-induced and uninduced BCBL-1. Coimmunoprecipitates included in **lanes: 1**. Input TPA-induced BCBL-1 extract **2**. TPA-induced BCBL-1 extract + pre immune serum **3**. TPA-induced BCBL-1 extract + anti-SAP145 Ab **4**. Uninduced BCBL-1 extract + anti-SAP145 Ab.

Panel II. Western blot for SAP145 with anti-SAP145 Ab showing that anti-ORF57 Ab coimmunoprecipitates SAP145 from TPA-induced BCBL-1 cells only. Coimmunoprecipitates included in **lanes: 1.** Input uninduced BCBL-1 extract **2**. Input TPA-induced BCBL-1 extract **3**. TPA-induced BCBL-1 extract + anti-ORF57 Ab **4**. Uninduced BCBL-1 extract + anti-ORF57 Ab **5**. TPA-induced BCBL-1 extract + pre immune serum.

coimmunoprecipitated SAP145 only from the TPA-induced BCBL-1 extracts and not from the uninduced BCBL-1 cell extracts (Fig. 7A2 Panel II compare lanes 3 & 4). The pre-immune serum did not immunoprecipitate SAP145 from TPA-induced BCBL-1 cell extracts (Fig. 7A2 Panel I lane 2 & Panel II lane 5).

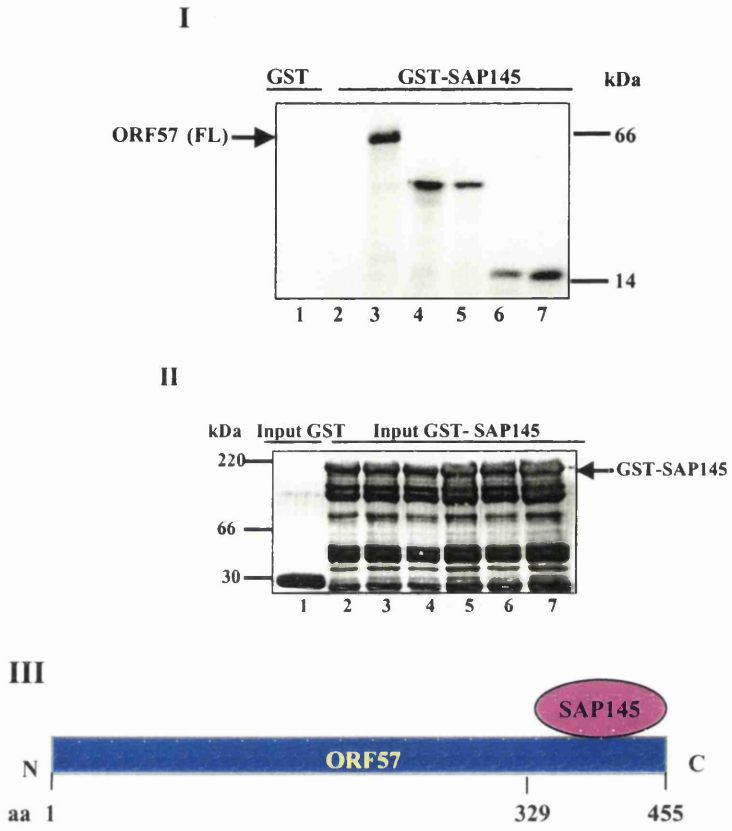
7A3 Mapping regions of ORF57 involved in interaction with SAP145

GST-SAP145 fusion protein or GST alone and ^{35}S-labelled-ORF57 FL (aa 1-455) (from pGBKS-ORF57, FL) or deletion mutants containing aa 181-455 (from pGBKS-ORF57, small), 1-215 and 329-455 (from pcDNA-ORF57) were used to map regions of ORF57 required for interaction with SAP145 in pull down assays. Fig. 3A1 Panel II and 4A4.1 Panel I shows a typical expression of each ^{35}S-labelled *in vitro* synthesised ORF57 protein, as visualised by phosphorimager analysis. The ^{35}S-labelled-ORF57 FL (aa 1-415) and deletion mutants (aa 181-455, and 329-455) bound to GST-SAP145 (Fig. 7A3 Panel I lanes 3, 4 & 5, and 6 & 7) whereas, no binding was detected with N-terminal deletion mutant aa 1-215 (lane 2) and of labelled-ORF57 FL to GST alone (lane 1). Use of similar amounts of the fusion proteins GST-SAP145 or GST alone was confirmed by Western blotting and immunodetection with anti-GST Ab (Fig. 7A3 Panel II lanes 1-7 respectively). The minimum interactive region of ORF57 for SAP145 involves aa 329-455 (Fig. 7A3 Panel III). Fine mapping with point mutants in the C terminus region of ORF57 would provide further information of the exact residues involved in the interaction.

7A4 HnRNP K and SAP145 associate only in the presence of ORF57

The interaction between SAP145 and hnRNP K was investigated. GST-K fusion protein (see Fig 4A1.2 Panel I) was used in pull down assays with TPA-induced and uninduced BCBL-1 cell extracts. Western blotting with anti-SAP145 Ab showed that SAP145 bound to GST-K only in the TPA-induced BCBL-1 cell extracts (Fig. 7A4 Panel I lane 1) not in uninduced cell extracts (Panel I lane 2), and SAP145 from TPA-induced or uninduced BCBL-1 cell extracts did not bind GST alone (Panel I lanes 3 & 4). Immunoblotting of the same membrane with anti-ORF57 Ab plus anti-hnRNP K Ab showed that GST-K also pulled down ORF57 only in the TPA-induced BCBL-1 cell extracts whereas hnRNP K was pulled down from both TPA-induced and uninduced cells (Fig. 7A4 Panel II lanes 1 & 2). Therefore, hnRNP K can associate with SAP145 but only in the presence of ORF57 and ORF57 must be associated with hnRNP K and SAP145 simultaneously in certain complexes.

Fig 7A3. SAP145 interacts with C-terminal aa 329-455 of ORF57



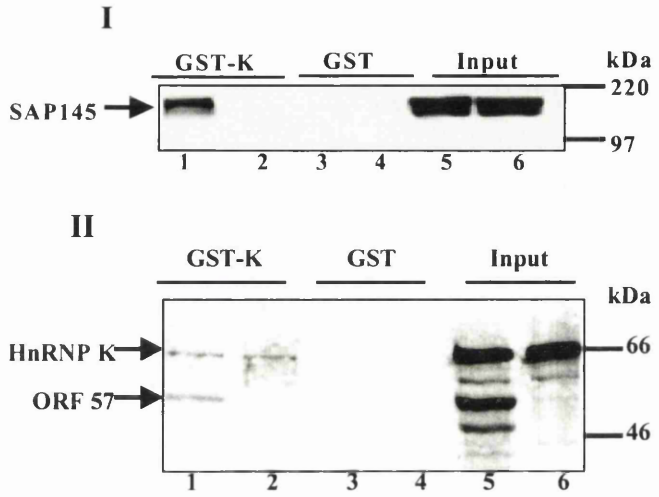
Glutathione agarose beads coated with GST-SAP145 or GST alone and ^{35}S-labelled-ORF57 (FL, small and its N- and C-terminal deletion mutants) were used in a pull down assay to map the regions of ORF57 required for interaction with SAP145. Eluted proteins were separated by SDS-PAGE by loading the samples on two gels, one gel was dried and exposed to phosphorimager screen and the other gel was transferred to a nitrocellulose membrane for Western blotting.

Panel I. Phosphorimager analysis showing that SAP145 interacts with C-terminal aa 329-455 of ORF57. Pull downs included in **lanes: 1.** ^{35}S-labelled-ORF57 (aa 1-455 FL) + GST-alone **2.** ^{35}S-labelled-ORF57 (aa 1-215) + GST- SAP145 **3.** ^{35}S-labelled-ORF57 (aa 1-455, FL) + GST-SAP145 **4.** ^{35}S-labelled-ORF57 (aa 181-455, small) + GST-SAP145 **5.** ^{35}S-labelled-ORF57 (aa 181-455, small) + GST-SAP145 **6.** ^{35}S-labelled-ORF57 (aa 329-455) + GST- SAP145 **7.** ^{35}S-labelled-ORF57 (aa 329-455) + GST-SAP145.

Panel II. Western blot for GST-SAP145 and GST with anti-GST Ab to show equal loading of GST-SAP145 fusion protein used for mapping the regions of ORF57 required for interaction with SAP145. Pull downs included in **lanes: 1.** ^{35}S-labelled-ORF57 (aa 1-455 FL) + GST-alone **2.** ^{35}S-labelled-ORF57 (aa 1-215) + GST- SAP145 **3.** ^{35}S-labelled-ORF57 (aa 1-455, FL) + GST-SAP145 **4.** ^{35}S-labelled-ORF57 (aa 181-455, small) + GST-SAP145 **5.** ^{35}S-labelled-ORF57 (aa 181-455, small) + GST-SAP145 **6.** ^{35}S-labelled-ORF57 (aa 329-455) + GST- SAP145 **7.** ^{35}S-labelled-ORF57 (aa 329-455) + GST-SAP145.

Panel III. Representation of SAP145 binding region of ORF57 protein

Fig 7A4. GST-K pulls down SAP145 from TPA-induced BCBL-1 cells



Panel I. Western blot for SAP145 with anti-SAP145 Ab showing that GST-K pulls down SAP145 from TPA-induced BCBL-1 cells. TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST-K or GST proteins. After incubation with extracts, proteins pulled down were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted for SAP145 using anti-SAP145 Ab. Pull downs included in **lanes: 1.** TPA-induced BCBL-1 extract + GST-K **2.** Uninduced BCBL-1 extract + GST-K **3.** TPA-induced BCBL-1 extract + GST alone **4.** Uninduced BCBL-1 extract + GST alone **5.** Input TPA-induced BCBL-1 extract **6.** Input uninduced BCBL-1 extract.

Panel II. Western blot with anti-ORF57 plus anti-hnRNP K Ab showing that GST-K pulls down ORF57 from TPA-induced BCBL-1 cells. TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST-K or GST proteins. After incubation with extracts, proteins pulled down were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted for ORF57 and hnRNP K using anti-ORF57 plus anti-hnRNP K Ab. Pull downs included in **lanes: 1.** TPA-induced BCBL-1 extract + GST-K **2.** Uninduced BCBL-1 extract + GST-K **3.** TPA-induced BCBL-1 extract + GST alone **4.** Uninduced BCBL-1 extract + GST alone **5.** Input TPA-induced BCBL-1 extract **6.** Input uninduced BCBL-1 extract.

7A5 ORF57 is required for association of SAP145 with CK2

Fig. 5B2 Panel IV & VI shows that CK2 was detected in hnRNP K coimmunoprecipitates but only when ORF57 was present (i.e. either in ORF57 transfected 293 or TPA-induced BCBL-1 cell extracts). Thus SAP145 -hnRNP K association in the presence of ORF57 could occur as an indirect interaction via CK2. Anti-SAP145 Ab and pre-immune rabbit serum coimmunoprecipitates of TPA-induced or uninduced BCBL-1 cell extracts were assayed for CK2 activity using the peptide substrate activity assay. CK2 activity was coimmunoprecipitated with SAP145 only in the presence of ORF57 from TPA-induced BCBL-1 cell extracts or from ORF57 transfected cells (Fig. 7A5 lane 2 & 4). Assays performed in the absence of peptide or in the presence of peptide with uninduced BCBl-1 cell extracts or with pre-immune serum gave no CK2 activity (Fig. 7A5 lanes 1, 3 and 5, 6). Thus CK2 was found with SAP145 only in the presence of ORF57 and ORF57 must be able to form a complex with CK2 and SAP145 simultaneously in some complexes.

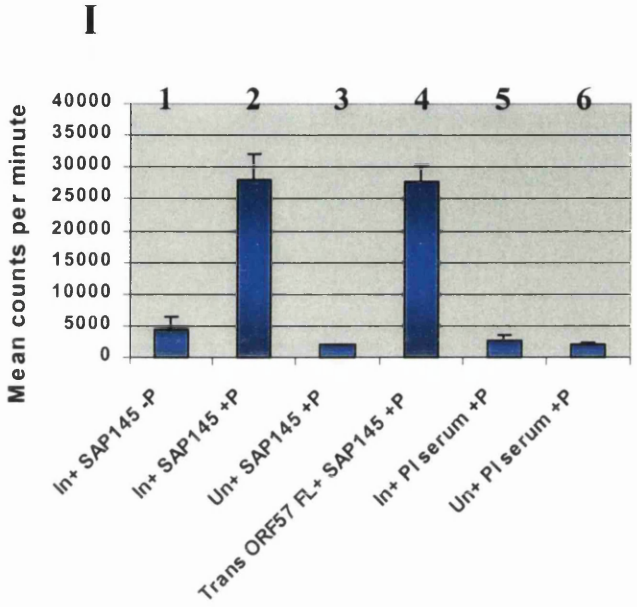
7A6 Discussion

7A6.1 Significance of the ORF57-SAP145 interaction for viral gene expression

This Chapter presents data that demonstrate the interaction between ORF57 and SAP145. ORF57 interacts directly with SAP145 in pull down assays and coimmunoprecipitations and forms a complex with SAP145, hnRNP K and CK2 proteins in KSHV-infected cells. SAP145 acts to tether the U2 snRNA to the branch point sequence in the intron of pre-mRNA. ORF57 might play a role in splicing inhibition either by preventing the necessary step of SAP145 binding to the spliceosome complex, or by binding to SAP145 in the assembled splicing complex and preventing further involvement of SAP145 in the downstream steps of splicing complex formation. The co-localisation of ORF57 with another splicing factor SC35 has been reported (Bello *et al.*, 1999).

Viral pre-mRNA containing either inefficiently spliced introns or intronless, depending on their primary sequence and/or their length, may not be able to recruit export factors efficiently and thus cannot efficiently compete with cellular mRNAs for binding to export pathway components. ORF57 has been shown to interact with RNA export factor, REF (see Chapter 6) which can bind RNAs non-specifically in splicing-dependent and/or independent manner. REF proteins are components of a multiprotein EJC implicated in the export of cellular mRNAs. REF proteins have been reported to interact with spliceosomes. It is possibile that ORF57 may modulate the efficiency of the REF

Fig 7A5. ORF57 is required for association of CK2 & SAP145 and anti-SAP145 Ab brings down CK2 activity only from TPA-induced or ORF57 (FL) transfected cells



Panel I. CK2 activity in anti-SAP145 Ab coimmunoprecipitates. Anti-SAP145 coimmunoprecipitations were followed by a CK2 assay. Assays were performed without substrate peptide on coimmunoprecipitates from TPA-induced BCBL-1 cell extracts (**lane 1**) or with peptide on coimmunoprecipitates from TPA-induced (**lane 2**), uninduced (**lane 3**), and GFP-gORF57 transfected (**lane 4**) BCBL-1 cell extracts, or with peptide on coimmunoprecipitations from TPA-induced (**lane 5**), and uninduced (**lane 6**) BCBL-1 cell extracts using pre immune serum.

CK2 activity is proportional to counts per minute of γ-[^{32}P]-ATP incorporated into the peptide substrate.

association with various mRNAs. Interaction of ORF57 with SAP145 could directly or indirectly recruit ORF57 protein to mRNPs and facilitate RNA nuclear export post-splicing. Alternatively, ORF57 via SAP145 may cause inhibition of host cell splicing leaving cellular mRNAs unsuitable/inaccessible for nuclear export thus reducing the competition for viral mRNAs for export factors such as REF. Inhibition of host cell splicing could result in larger pools of REFs (or other export factors) available for viral mRNA nuclear export. Thus this interaction between SAP145/ORF57 demonstrates physical association between components of the splicing machinery and a viral protein involved in transcriptional/posttranscriptional regulation and associated with other proteins involved in nuclear export process (such as REF and/or TAP).

The possibility of selective inhibition of splicing by ORF57 might be important because ORF57-mediated cytoplasmic accumulation of unspliced mRNAs is likely not to be its only role. An action for ORF57 in splicing inhibition is not yet clear. No consistent effect of ORF57 on KSHV-promoter driven reporter genes was found in the presence or absence of intronic sequences (Kirshner *et al.*, 2000). The SV40 T intron and a synthetic intron derived from β-globin gene failed to display regulation by ORF57. Moreover, an authentic KSHV gene (ORF50) bearing its native intron was not down regulated by ORF57 (Kirshner *et al.*, 2000) arguing against an ORF57-encoded activity that globally impairs splicing or represses expression from intron-containing genes. One report involving co-transfection experiments performed with or without intron-containing reporter constructs showed a slight decrease in reporter activity in presence of ORF57 however the inhibition was not as marked as with ICP27 or EBV Mta (Gupta *et al.*, 2000). However together with ORF50, ORF57 up-regulated a intronless PAN promoter construct reporter 10-fold more efficiently than its intron-bearing derivative relative to a two-fold up-regulation from ORF57 alone (Kirshner *et al.*, 2000).

The available data on ORF57 is against a general down regulation of intron-bearing sequences however, they do not exclude that this phenomenon could be viral gene specific. Introns in the target gene can interfere with ORF57-mediated activation (Gupta *et al.*, 2000) and such an effect might be important in selectively enhancing expression of KSHV. Investigation of pre-mRNA splicing inhibition process either in the presence of transiently expressed ORF57 in cells or by addition of recombinant ORF57 protein to *in vitro* splicing extracts would help analyse the possible role of ORF57 in splicing inhibition and help determine the step at which splicing may be halted.

7B ORF57 and p32

The p32 protein, initially isolated as a protein tightly associated with ASF splicing factor purified from HeLa cells (Krainer *et al.*, 1990) can regulate RNA splicing by inhibiting ASF RNA binding and phosphorylation (Petersen-Mahrt *et al.*, 1999). The location of p32 within cells and interactions with several cellular and viral proteins have suggested a role for p32 in splicing, nucleocytoplasmic transport to and from mitochondria and in maintaining oxidative phosphorylation. To find out whether ORF57 interacted with the p32, *in vitro* and *in vivo* binding assays were performed.

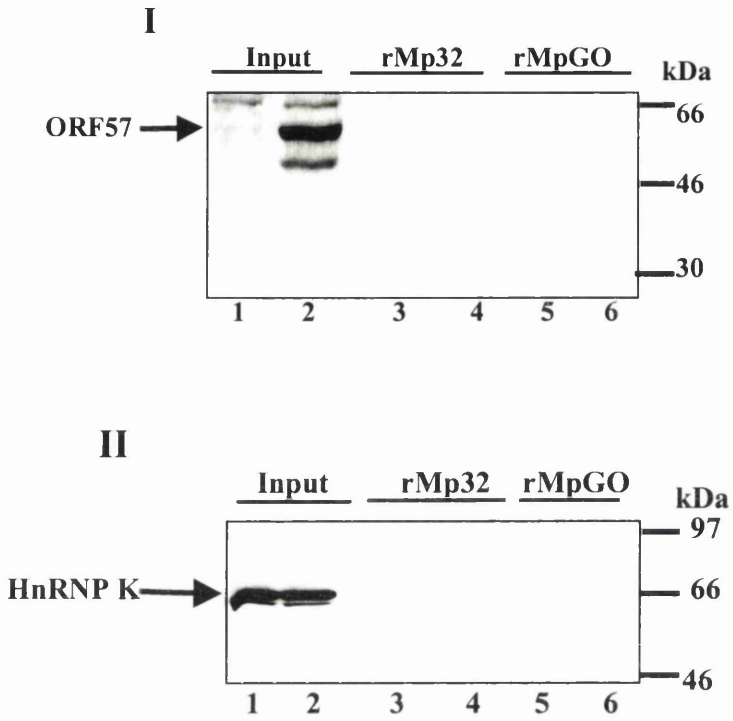
7B1 Investigation of an interaction between ORF57 with p32

7B1.1 ORF57 and p32 do not interact using a p32 Sepharose column pull down assay

A Sepharose column with p32 attached (rMp32) (Section 2B4.8) was used to investigate the interaction. As p32 is a highly charged protein, glucose oxidase, a protein with a similar isoelectric charge (pI), was attached to the same type of column (rMpGO) and used as a control. After incubating rMp32 or rMpGO with protein from TPA-induced and uninduced BCBL-1 cell extracts, pull down assays were performed. Immunoblotting with anti-ORF57 Ab showed that p32 did not pull down any band corresponding to the size of ORF57 in the TPA-induced BCBL-1 cell extracts (Fig. 7B1.1 Panel I lane 3) whereas in the control input BCBL-1 cell extracts ORF57 was present in TPA-induced BCBL-1 extracts (Fig. 7B1.1 Panel I lanes 2). rMpGO also failed to pull down ORF57 from BCBL-1 cell extracts (Panel I lanes 5 & 6).

Western blotting of the same column eluates for hnRNP K revealed that although ORF57 and hnRNP K usually interact in the TPA-induced cell extracts, rMp32 pull downs were negative for the presence of hnRNP K also (Fig. 7B1.1 Panel II lanes 3 & 4), rMpGO also failed to pull down hnRNP K from BCBL-1 cell extracts (Panel II lanes 5 & 6). Immunoblotting of the membrane with anti-p32 mouse Mab showed that p32 was present in the input BCBL-1 cell extract lanes and in rMp32 pull down samples (Fig. 7B1.1 Panel III lanes 1, 2 and 3, 4) and absent in rMpGO pull down samples (Fig. 7B1.1 Panel III lanes 5 & 6). As a positive control rMp32 pulled down ICP27 from wt HSV-1 infected BHK cell extracts (Fig. 7A1.2 Panel IV lane 3) and the rMpGO (negative control) did not interact with wt infected or mock infected cell extracts (lanes 5 & 6).

Fig 7B1.1 Panels Iand II. P32 does not pull down ORF57 (Panel I) and hnRNP K (Panel II) from TPA-induced BCBL-1 cells

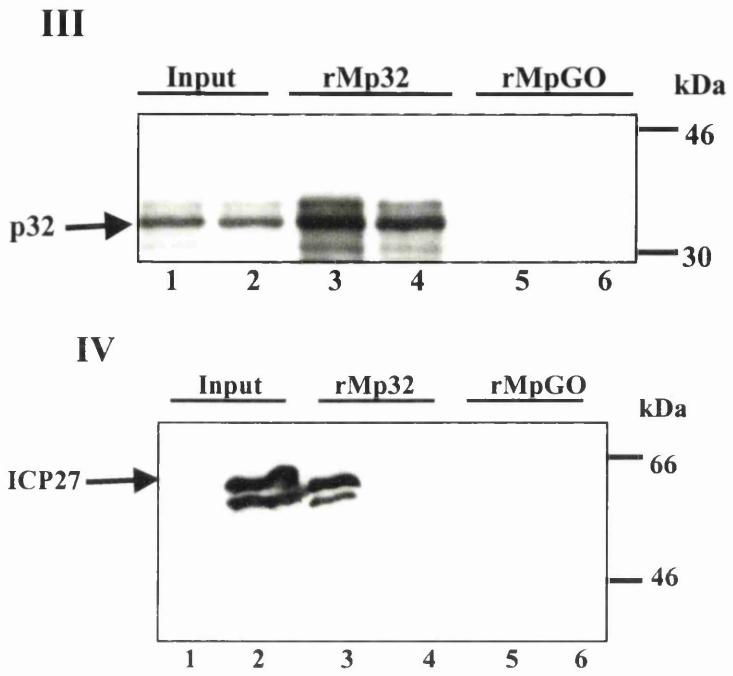


P32 attached (rMp32) or glucose oxidase attached (rMpGO) Sepharose columns were used to incubate rMp32 or rMpGO proteins with TPA-induced and uninduced BCBL-1 cell extracts, and pull down assays were performed. After incubation, proteins pulled down were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted with appropriate Ab.

Panel I. Western blot for ORF57 with anti-ORF57 Ab showing that p32 does not pull down ORF57 from BCBL-1 cells. Pull downs included in **lanes: 1**. Input uninduced BCBL-1 extract **2**. Input TPA-induced BCBL-1 extract **3.** TPA-induced BCBL-1 extract + rMp32 **4**. Uninduced BCBL-1 extract + rMp32 **5**. TPA-induced BCBL-1 extract + rMpGO **6**. Uninduced BCBL-1 extract + rMpGO.

Panel II. Western blot with anti-hnRNP K Ab showing that p32 does not pull down hnRNP K from BCBL-1 cells. Pull downs included in **lanes: 1.** Input uninduced BCBL-1 extract **2.** Input TPA-induced BCBL-1 extract **3.** TPA-induced BCBL-1 extract + rMp32 **4.** Uninduced BCBL-1 extract + rMp32 **5.** TPA-induced BCBL-1 extract + rMpGO **6.** Uninduced BCBL-1 extract + rMpGO.

Fig 7B1.1 Panels III and IV. P32 pulls down itself from BCBL-1 cells (Panel III) and ICP27 from HSV-1 infected cells (Panel IV)



Panel III. Western blot with anti-p32 Mab showing that p32 pulls down itself from BCBL-1 cells. P32 attached (rMp32) or glucose oxidase attached (rMpGO) Sepharose columns were used to incubate rMp32 or rMpGO proteins with TPA-induced and uninduced BCBL-1 cell extracts, and pull down assays were performed. After incubation, proteins pulled down were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted for p32 using anti-p32 Ab. Pull downs included in **lanes: 1.** Input uninduced BCBL-1 extract **2.** Input TPA-induced BCBL-1 extract **3.** TPA-induced BCBL-1 extract + rMp32 **4.** Uninduced BCBL-1 extract + rMp32 **5.** TPA-induced BCBL-1 extract + rMpGO **6.** Uninduced BCBL-1 extract + rMpGO.

Panel IV. Western blot with anti-ICP27 Ab showing that p32 pulls down ICP27 from HSV-1 infected BHK cells. P32 attached (rMp32) or glucose oxidase attached (rMpGO) Sepharose columns were used to incubate rMp32 or rMpGO proteins with HSV-1 wt-infected or mock-infected BHK cell extracts, and pull down assays were performed. After incubation, proteins pulled down were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted for ICP27 using anti-ICP27 Ab. Pull downs included in **lanes: 1.** Input mock-infected BHK extract **2.** Input wt-infected BHK cell extract **3.** wt-infected BHK cell extract + rMp32 **4.** Mock-infected BHK extract + rMp32 **5.** Wt-infected BHK cell extract + rMpGO **6.** Mock-infected BHK extract + rMpGO.

Performing a CK2 peptide assay on pulled down samples from the rMp32 column demonstrated that CK2 activity was not present in the pull down complexes. Controls without peptide substrate and rMpGO column pull downs samples were also used and found negative (data not shown). The i*n vitro* phosphorylation assay performed on the rMp32 column pull downs using TPA-induced and uninduced BCBL-1 cell extracts in absence or presence of DRB also showed lack of kinase activity associated with pull downs, again demonstrating that ORF57 and CK2 did not form a complex with p32 in virus-infected or uninfected cells (Fig. 7B1.1 panel V lanes 1, 2 & 3, 4).

7B1.2 ORF57 and p32 do not coimmunoprecipitate from virus-infected cells

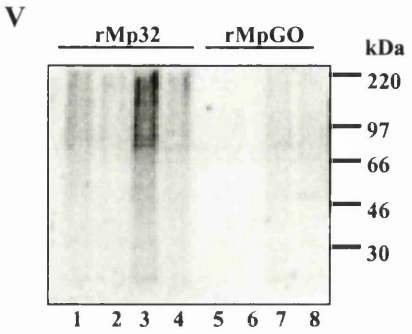
Confirmation of the lack of an interaction between p32 and ORF57 was obtained from coimmunoprecipitates with Anti-ORF57 Ab. Western blotting with anti-p32 Mab on anti-ORF57 Ab coimmunoprecipites showed that p32 protein was not coimmunoprecipitated by anti-ORF57 Ab from the TPA-induced BCBL-1 cell extracts (Fig.7B1.2 panel I compare lane 4 with 1 & 2). As a control, pre-immune serum coimmunoprecipitates, were also negative for the presence of p32 (Fig.7B1.2 Panel I lanes 5 & 6).

7B2 Discussion

7B2.1 Significance of the lack of interaction of ORF57 with p32

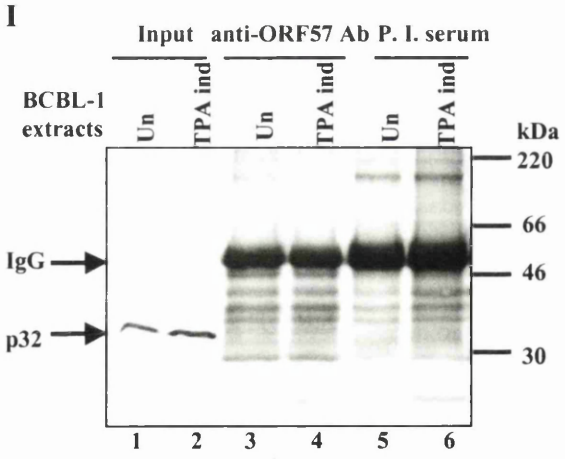
The interaction between ORF57 and p32 was investigated by both affinity column pull down assays and coimmunoprecipitations. ORF57, unlike ICP27, did not show an interaction with p32 under these conditions. In the context of the studies it is helpful to find an absence of p32 interaction with ORF57 i.e. not having to explain the role played by a p32/ORF57 interaction in KSHV-infected cells. The biological function (s) of p32 remain controversial because it has been difficult to reconcile all of the findings on protein interactions and variable observations on compartmentalisation. Originally identified as a component of the ASF splicing factor (Krainer *et al.*, 1990), subsequent work showed that the p33 subunit alone contained all the functional properties of a splicing factor (Mayeda *et al.*, 1992), however p32 does inhibit ASF from acting as a splicing repressor or splicing enhancer protein (Petersen-Mahrt *et al.*, 1999). P32 has been reported to be predominantly (exclusively) mitochondrial (Muta *et al.*, 1997; Matthews & Russell, 1998; van Leeuwen & O'Hare, 2001), cytoplasmic (Simos & Georgatos, 1994; Luo *et al.*, 1994; Tange *et al.*, 1996; Wang *et al.*, 1997) and also nuclear (Simos & Georgatos, 1994; Luo *et al.*, 1994; Tange *et al.*, 1996; Wang *et al.*, 1997; Matthews & Russell, 1998).

Fig 7B1.1 Panel V. P32 does not pull down CK2 kinase activity from TPA-induced BCBL-1 cells



Panel V. Phosphorimager analysis showing that p32 does not pull down CK2 kinase activity from TPA-induced BCBL-1 cells. An *in vitro* phosphorylation assay was performed on rMp32/rMpGO-affinity column pull down proteins from uninduced and TPA-induced BCBL-1 cell extracts in presence or absence of DRB. Pull downs used for *in vitro* phosphorylation reactions included in **lanes: 1.** TPA-induced BCBL-1 extract + rMp32 **2.** TPA-induced BCBL-1 extract + rMp32 + DRB **3.** Uninduced BCBL-1 extract + rMp32 **4.** Uninduced BCBL-1 extract + rMp32 + DRB **5.** TPA-induced BCBL-1 extract + rMpGO **6.** TPA-induced BCBL-1 extract + rMpGO + DRB **7.** Uninduced BCBL-1 extract + rMpGO **8.** Uninduced BCBL-1 extract + rMpGO + DRB.

Fig 7B1.2. Anti-ORF57 Ab does not coimmunoprecipitate p32 from TPA-induced BCBL-1 cells



Panel I. Western blot for p32 with anti-p32 Mab of anti-ORF57 Ab coimmunoprecipitates showing that anti-ORF57 Ab does not coimmunoprecipitate p32 from TPA-induced BCBL-1 cells. A coimmunoprecipitation assay was performed with anti-ORF57 Ab or pre immune serum and uninduced and TPA-induced BCBL-1 cell extracts. The complex formed was separated on SDS-PAGE gel, transferred to nitrocellulose and Western blotted for p32 using anti-p32 Mab. Coimmunoprecipitates included in **lanes: 1.** Input uninduced BCBL-1 extract **2.** Input TPA-induced BCBL-1 extract **3.** Uninduced BCBL-1 extract + anti-ORF57 Ab **4.** TPA-induced BCBL-1 extract + anti-ORF57 Ab **5.** Uninduced BCBL-1 extract + pre immune serum **6.** TPA-induced BCBL-1 extract + pre immune serum.

Although KSHV ORF57 protein is homologous to ICP27, it differs in some important aspects from its HSV-1 counterpart. ICP27 expression leads to a strong inhibition of RNA splicing, an effect considered to contribute to the shutoff of host protein synthesis and thus provide advantageous conditions for the export of viral RNAs most of which (delayed early and late mRNAs) are unspliced. Whereas, several examples of spliced KSHV mRNAs have already been documented, including the multiply spliced transcripts for K15/LAMP expressed at low levels in latency, which are strongly up regulated during lytic growth (Glenn *et al.*, 1999) and delayed early genes for K14/74 (Kirshner *et al.*, 1999; Talbot *et al.*, 1999), KbZIP (Lin *et al.*, 1999; Gruffat *et al.*, 1999) and the late gene encoding K8.1 (Chandran *et al.*, 1998; Raab *et al.*, 1998) and ORF29 (Renne *et al.*, 1998). These data do not support a strong inhibition of RNA splicing throughout the KSHV lytic cycle.

The ICP27/p32 interaction in HSV-1 may be the mechanism by which HSV-1 globally inhibits splicing whereas lack of a ORF57/p32 interaction in KSHV could render ORF57 selective in intron-recognition of only certain transcripts bearing introns while ignoring others. It seems that although ICP27 and ORF57 are homologous they do not have completely similar activities and differ in some important aspects from each other.

Chapter 8: Interaction of ORF57 with the KSHV transcriptional activator ORF50

KSHV IE proteins ORF57 and ORF50 are essential for lytic virus replication (Sarid *et al.*, 1998; Sun *et al.*, 1999). ORF50 is a transcription activator capable of inducing KSHV genes and analogous to transactivators HVS ORF50 and EBV BRLF-1 (Russo *et al.*, 1996; Whitehouse *et al.*, 1997a; Zhu *et al.*, 1999a), and is sufficient to induce the switch from latent to lytic virus replication (Lukac *et al.*, 1998; Sun *et al.*, 1998). Like its HVS counterpart (Thurau *et al.*, 2000), KSHV ORF50 can activate viral promoters, including that of ORF57 (Lukac *et al.*, 1999). ORF50 protein is extensively phosphorylated *in vivo*, and reportedly contains consensus phosphorylation sites for CK2 and protein kinase C (Lukac *et al.*, 1999). Protein kinase C is activated by reagents (phorbol esters) that can reactivate KSHV replication (Renne *et al.*, 1998).

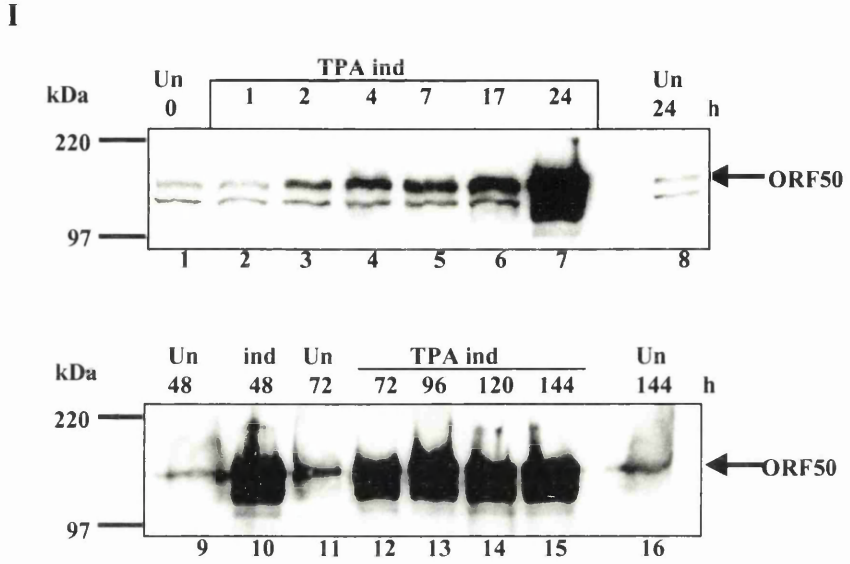
ORF57 was shown to synergise with ORF50 in activation of gene expression but no molecular basis for this synergy was demonstrated (Kirshner *et al.*, 2000). Using the *in vitro* binding assays and immunofluorescence, the HSV-1 homologous proteins ICP27 and ICP4 respectively have been reported to interact (Zhu & Schaffer, 1995; Panagiotidis *et al.*, 1997). I therefore determined if ORF57 was associated with ORF50 as a partner protein and had any effect on its function. This Chapter presents data indicating that ORF50 interacts with ORF57 *in vitro* and in KSHV infected cells. Experiments were aimed at determining the implication of this interaction for the virus life cycle using ORF50 promoter-based luciferase reporter assays.

8A Investigation of interaction between ORF57 and ORF50

8A Expression kinetics of ORF50

Western blotting of uninduced and TPA-induced BCBL-1 cells collected at various times after induction, using anti-ORF50 Ab, showed that some ORF50 protein is present in uninduced cells (Fig 8A see lanes 1, 8, 9, 11 and 16) presumably as a result of small number of uninduced cells undergoing spontaneous reactivation. ORF50 protein is expressed early and its concentration begins to increase between 1 -2 h (Fig 8A compare lanes 2 & 3) after TPA induction. Anti-ORF50 Ab identifies two bands in BCBL-1

Fig 8A. ORF50 protein is expressed early and increases from 2 h post TPA induction in BCBL-1 cells



Panel I. Western blot for ORF50 with anti-ORF50 Ab showing that ORF50 protein is expressed early and increases from 2 h post TPA induction in BCBL-1 cells. BCBL-1 suspension cultures, grown in 30 mm dishes, almost reaching confluency ($1x10^6$/ml), were split at $0.2x10^6$ cells/ml (1:5) and induced using TPA at 20ng/ml final concentration on the same day or left uninduced. Samples were collected at indicated time points post-induction, cells were pelleted, lysed in SDS loading buffer, separated by SDS-PAGE, and analysed by Western blotting for ORF50 with anti-ORF50 polyclonal antibody. Shown in **lanes: 1.** 0 h uninduced BCBL-1 extract **2.** 1 h TPA-induced BCBL-1 extract **3.** 2 h TPA-induced BCBL-1 extract **4.** 4 h TPA-induced BCBL-1 extract **5.** 7 h TPA-induced BCBL-1 extract **6.** 17 h TPA-induced BCBL-1 extract **7.** 24 h TPA-induced BCBL-1 extract **8.** 24 h uninduced BCBL-1 extract **9.** 48 h uninduced BCBL-1 extract **10.** 48 h TPA-induced BCBL-1 extract **11.** 72 h uninduced BCBL-1 extract **12.** 72 h TPA-induced BCBL-1 extract **13.** 96 h TPA-induced BCBL-1 extract **14.** 120 h TPA-induced BCBL-1 extract **15.** 144 h TPA-induced BCBL-1 extract **16.** 144 h uninduced BCBL-1 extract.

extracts (lanes 2-7); an upper band corresponding to ORF50 (~110-120 kDa) and a second lower band (~100kDa) which is also visible in uninduced cells (lanes 1 and 8). After TPA induction, the intensity of the upper band increases with time (compare lanes 2-7, 10, and 12-15), indicating that the lower band may not be a viral protein and could be a cross-reacting cellular protein.

8A1 ORF57 interacts with ORF50

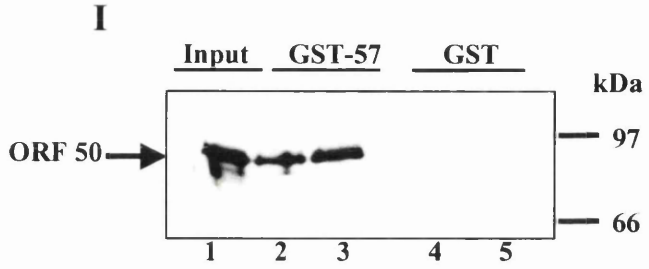
8A1.1 Using in vitro synthesised ORF50 in a GST pull down assay

The ^{35}S-labelled profile of proteins coimmunoprecipitated with ORF57 (Fig. 3B1 Panel I lane 2) contained a band of ~110-120 kDa which could be ORF50. To examine the binding of ORF57 to ORF50, beads carrying either recombinant GST-57 (Section 2B4.3) or GST alone were assayed for binding to *in vitro* synthesized ^{35}S-labelled-FLc50 (Section 2B4.9). Fusion proteins used were visualised by Coomassie brilliant blue staining (see Fig. 4A1.1 Panel II) and equivalent amounts of these were used. Eluted samples were heated to 100°C in SDS gel sample buffer for 5 min, analysed by SDS-PAGE (Section 2B6.1) and the gel was dried and exposed to a phosphorimager screen (Section 2B6.4). GST-57 pulled down a band of ~90kDa corresponding to *in vitro* synthesized ORF50 whereas GST alone did not (Fig. 8A1.1 Panel I lanes 2 & 4). As RNAse treatment did not disrupt this interaction (Fig. 8A1.1 Panel I lane 3), it is unlikely that ORF57/50 interaction represents interaction of these proteins tethered on RNA.

8A1.2 Using the GST pull down assay with virus-infected cell extracts

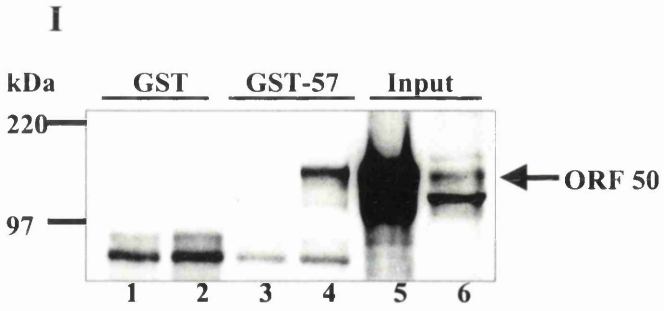
A pull down experiment using GST-57 fusion protein and TPA-induced and uninduced BCBL-1 cell extracts (Section 2B4.4) followed by SDS-PAGE and Western blotting (Section 2B6.2 & 3) with ORF50 polyclonal antiserum (a kind gift from G. Hayward, Table 2A7), showed that ORF50 and GST-57 interact, whereas GST alone and ORF50 do not. GST-57 pulled down ORF50 from TPA-induced BCBL-1 cell extracts (Fig. 8A1.2 panel I lane 4 see upper band) and not from uninduced BCBL-1 cell extracts (lane 3) which show a small amount of ORF50 due to some cells undergoing spontaneous reactivation (lane 6). An intense band of ~110 kDa, corresponding in size to ORF50, was seen in induced whole cell extracts (lane 5); GST alone did not pull down ORF50 from the cell extracts (lanes 1 & 2). Anti-ORF50 Ab stains two bands in input BCBL-1 cell extracts (lanes 5 & 6); an upper ORF50 band (~110-120 kDa) and a band lower than ORF50 (~100kDa) also visible in uninduced cells which is a cross-reacting cellular band.

Fig 8A1.1. GST-57 interacts with *in vitro* synthesised ORF50



Panel I. Phosphorimager analysis showing that GST-57 interacts with *in vitro* synthesised ORF50. The GST-57 (FL) or GST (control) (5μg protein) pull down assay was performed using *in vitro* transcribed-translated ^{35}S-labelled-ORF57 (FL) protein (5μl). Pulled down proteins were separated by SDS-PAGE, gel was dried and exposed to phosphorimager screen for analysis of labelled proteins. Pull downs included in **lanes: 1.** Input *In vitro* synthesised ^{35}S-labelled-FLc50 **2.** *In vitro* synthesised ^{35}S-labelled-FLc50 + GST-57 **3.** *In vitro* synthesised ^{35}S-labelled-FLc50 + GST-57 + RNAse **4.** *In vitro* synthesised ^{35}S-labelled-FLc50 + GST alone **5.** *In vitro* synthesised ^{35}S-labelled-FLc50 + GST alone+ RNAse.

Fig 8A1.2. GST-57 pulls down ORF50 from TPA-induced BCBL-1 cells



Panel I. Western blot with anti-ORF50 Ab showing that GST-57 pulls down ORF50 from TPA-induced BCBL-1 cells. The GST-57 (FL) or GST (control) (5μg protein) pull down assay was performed using TPA-induced and uninduced BCBL-1 cell extracts (200 μg). Pulled down proteins were separated by SDS-PAGE, transferred to nitrocellulose and Western blotted for ORF50 using anti-ORF50 Ab. Pull downs included in **lanes: 1.** Uninduced BCBL-1 extract + GST alone **2.** TPA-induced BCBL-1 extract + GST alone **3.** Uninduced BCBL-1 extract + GST-57 **4.** TPA-induced BCBL-1 extract + GST-57 **5.** Input TPA-induced BCBL-1 extract **6.** Input uninduced BCBL-1 extract.

Thus, GST-57 was capable of interacting with ORF50 from infected cell extracts. These conditions are closer to the *in vivo* situation as ORF50 is present in its native conformation and concentration in whole cell extracts.

8A2 ORFs 57 and 50 gene products coimmunoprecipitate from infected cell extracts

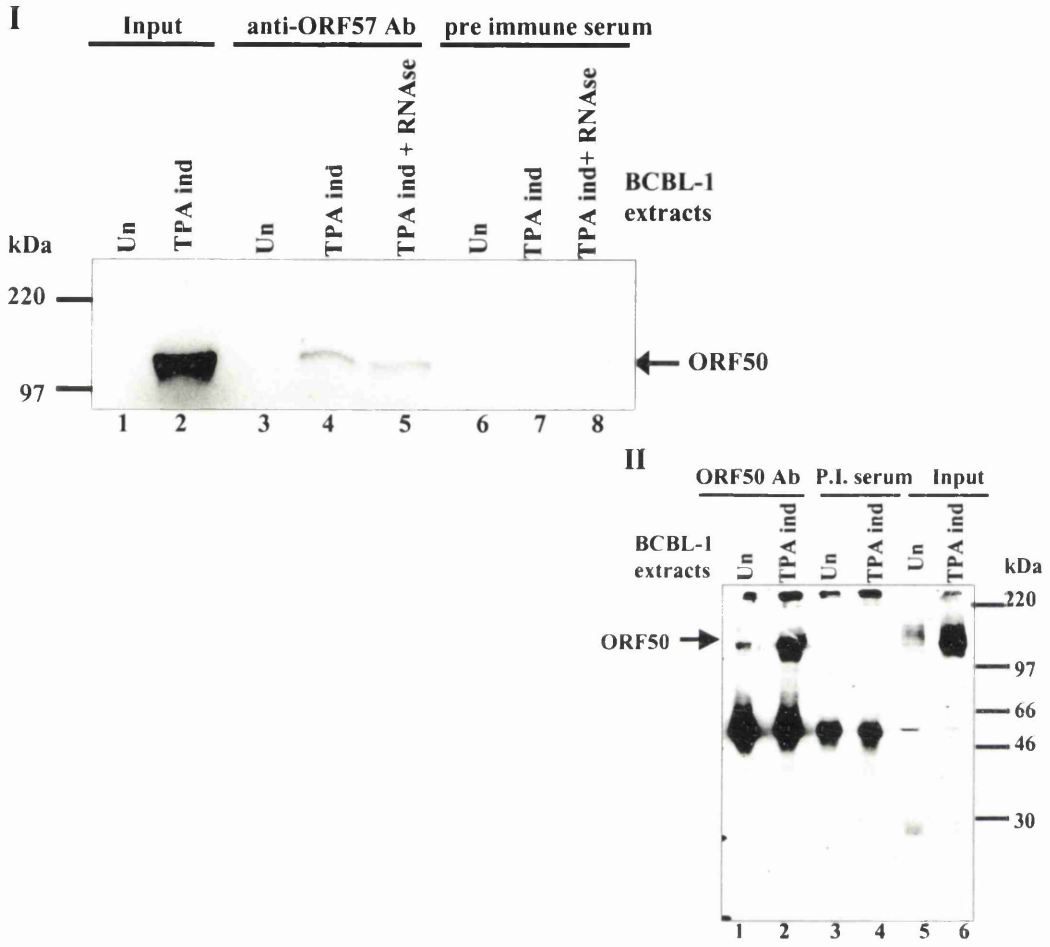
Confirmation of an interaction in infected cells came from Western blotting of the anti-ORF57 coimmunoprecipitates (Section 2B5.2) with ORF50 rabbit polyclonal antiserum. The samples immunoprecipitated with anti-ORF57 Ab were Western blotted with ORF50 Ab. A ~110-120 kDA band of ORF50 was present in TPA-induced BCBL-1 cell extracts (Fig. 8A2 Panel I, upper band is immunoprecipitated in lane 4 in comparison to control input BCBL-1 cell extracts from lane 2) that was absent in uninduced BCBL-1 cells (lane 3) and using pre-immune serum (Fig. 8A2 Panel I lanes 6, 7 & 8). RNAse treatment did not disrupt this interaction (Fig. 8A2 Panel I lane 5). Thus the band of ~110kDa seen in the ^{35}S profile of immunoprecipitated proteins using ORF57 Ab could well correspond to ORF50 (Fig. 3B1 Panel I lane 2).

Anti-ORF50 Ab coimmunoprecipitates followed by Western blotting with anti-ORF50 Ab confirmed the presence of immunoprecipitated ORF50 protein in the TPA-induced and uninduced BCBL-1 cell extracts (Fig. 8A2 Panel II lanes 1 & 2) whereas pre-immune sera did not precipitate ORF50 (lanes 3 & 4). Phosphorimager analysis on anti-ORF50 Ab coimmunoprecipitates from labelled BCBL-1 cell extracts showed a band of ~50-52kDa was present in the TPA-induced cell extract lane and absent in the uninduced lane along with some other unidentified bands (Fig. 8A2 Panel III lanes 2 & 1). On comparison this corresponded in size to ORF57 band present in the input whole cell extracts, when the same membrane was Western blotted with ORF57 Ab (Chapter 3 Fig 3B1 Panel II). Thus ORF57 was also immunoprecipitated using ORF50Ab.

8A3 ORF57 partially colocalises with ORF50 protein within cells

ORF50 has a nuclear distribution in TPA stimulated BCBL-1 cells (Lukac *et al.*, 1998). Confocal microscopy was used to visualise the location of the two proteins. In BCBL-1 cells 48 h after TPA induction, viral ORF50 had a nuclear localisation (Fig 8A3 Panel I). In TPA-induced BCBL-1 cells viral ORF57 and in mammalian cells transfected with pKS3 (GFP-gORF57), or pKS4 (GFP-cORF57) ORF57 protein showed a nuclear distribution (Fig 8A3 Panel II a & II b). A GFP-tagged ORF57 was used in these studies, as both endogenous viral proteins could not be visualised together due to lack of availability of a primary antibody of a species other than rabbit. Expression vector

Fig 8A2 Panels I and II. ORF50 is coimmunoprecipitated by anti-ORF57 Ab from TPA-induced BCBL-1 cells (Panel I) and anti-ORF50 Ab coimmunoprecipitates ORF50 from TPA-induced BCBL-1 cells (Panel II)

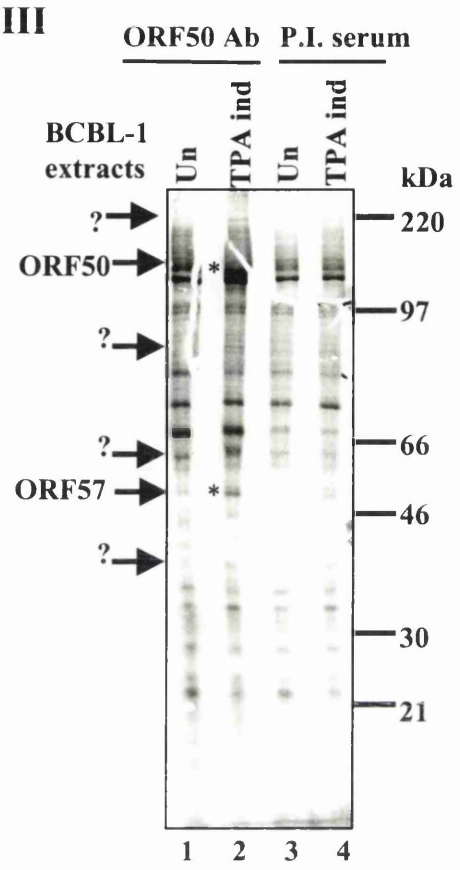


A coimmunoprecipitation assay was performed with anti-ORF57 (Panel I) or anti-ORF50 (Panel II) Ab or pre immune serum and uninduced and TPA-induced BCBL-1 cell extracts. The complex formed was separated on SDS-PAGE gel, transferred to nitrocellulose and Western blotted for ORF50 using anti-ORF50 Ab.

Panel I. Western blot for ORF50 with anti-ORF50 Ab showing that ORF50 is coimmunoprecipitated by anti-ORF57 Ab from TPA-induced BCBL-1 cells. Coimmunoprecipitates included in **lanes: 1.** Input uninduced BCBL-1 extract **2.** Input TPA-induced BCBL-1 extract **3.** Uninduced BCBL-1 extract + anti-ORF57 Ab **4.** TPA-induced BCBL-1 extract +anti-ORF57 Ab **5.** TPA-induced BCBL-1 extract +anti-ORF57 Ab + RNAse **6.** Uninduced BCBL-1 extract + pre immune serum **7.** TPA-induced BCBL-1 extract + pre immune serum **8.** TPA-induced BCBL-1 extract + pre immune serum + RNAse.

Panel II. Western blot for ORF50 with anti-ORF50 Ab showing that ORF50 is coimmunoprecipitated by anti-ORF50 Ab coimmunoprecipitates ORF50 from TPA-induced BCBL-1 cells. Coimmunoprecipitates included in **lanes: 1.** Uninduced BCBL-1 extract + anti-ORF50 Ab **2.** TPA-induced BCBL-1 extract +anti-ORF50 Ab **3.** Uninduced BCBL-1 extract + pre immune serum **4.** TPA-induced BCBL-1 extract + pre immune serum **5.** Input uninduced BCBL-1 extract **6.** Input TPA-induced BCBL-1 extract.

Fig 8A2 Panel III. Anti-ORF50 Ab coimmunoprecipitates profile



Panel III. Phosphorimager analysis showing anti-ORF50 Ab coimmunoprecipitation profile. A coimmunoprecipitation assay was performed with anti-ORF57 (Panel I) or anti-ORF50 Ab or pre immune serum and ^{35}S-labelled-uninduced and -TPA-induced BCBL-1 cell extracts. The complex formed was separated on SDS-PAGE gel, gel was dried and exposed to phosphorimager screen for analysis of labelled proteins. Coimmunoprecipitates included in **lanes: 1.** Uninduced BCBL-1 extract + anti-ORF50 Ab **2.** TPA-induced BCBL-1 extract +anti-ORF50 Ab **3.** Uninduced BCBL-1 extract + pre immune serum **4.** TPA-induced BCBL-1 extract + pre immune serum

GFP-ORF57 was transfected by electroporation in BCBL-1 cells, which were TPA-induced for 48 h and stained for ORF50 using anti-ORF50 rabbit Ab (red fluorescence Table 2A7). Transfected GFP-ORF57 was visible due to its green fluorescence.

Two ORF50 staining patterns are evident in BCBL-1 cells, both occur at almost a similar frequency. In one pattern ORF50 is evenly distributed throughout the nucleus. In the second pattern, the ORF50 distribution is "ring-like" and there is reduced localisation in the centre (Fig 8A3 Panel I). The ring-like staining profile was also seen in Z stack confocal images.

GFP-ORF57 expression pattern was more uniform throughout the nucleus and on overlay partial colocalisation was only detected in nuclei showing the ring-like pattern of ORF50 expression (Fig 8A3 Panel III).

8A4 ORF50 is pulled down by hnRNP K in a complex with ORF57 from TPA-induced BCBL-1 cell extracts

GST-K (full-length) or GST alone bound to beads was used to pull down interacting proteins from TPA-induced and uninduced BCBL-1 cell extracts (Section 2B4.4). Eluted samples were separated by SDS-PAGE (Section 2B6.1), transferred to nitrocellulose membranes and anti-ORF50 Ab was used in immunoblotting. The fusion proteins were visualised by Coomassie brilliant blue staining (see Fig. 4A1.2 Panel I) and equivalent amounts were used. In TPA-induced BCBL-1 cell extracts with GST-K a band corresponding to ORF50 was present whereas the GST control lane was negative for a band of similar size (Fig. 8A4 Panel I compare upper band in lanes 2 & 4 with l).

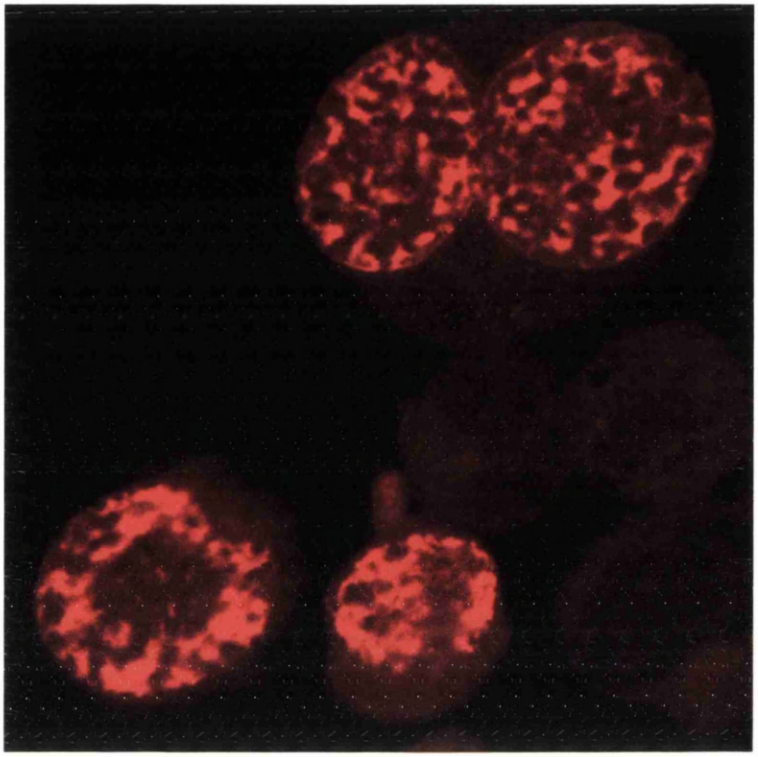
Western blotting with a mixture of anti-ORF57 plus anti-hnRNP K Abs showed that GST-K pulled down ORF57 only from the TPA-induced cells (Fig. 8A4 Panel II lane 1) and cellular hnRNP K from both TPA-induced and uninduced cells (Fig. 8A4 Panel II compare lanes lanes 1 & 2). HnRNP K is capable of self interaction (Bomsztyk *et al.*, 1997) and the 66-70 kDa band with anti-hnRNP K Ab is likely to be hnRNP K from the cellular extracts interacting with GST-K.

8A5 hnRNP K associates with *in vitro* synthesised ORF50

Beads carrying recombinant GST-K (Section 2B4.4) or GST alone were assayed for binding to *in vitro* synthesized ^{35}S-labelled-FLc50. GST-K pulled down a band of

Fig 8A3 Panel I. Expression of viral ORF50 in TPA induced BCBL-1cells

I

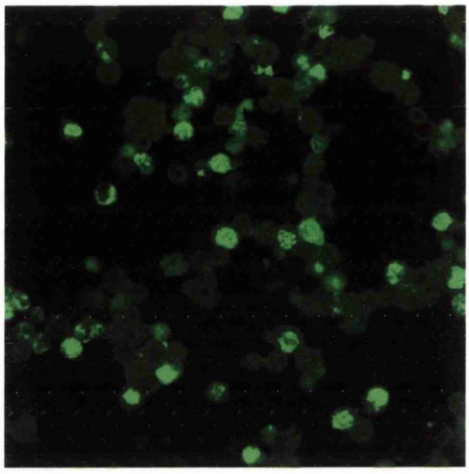


Panel I. Viral ORF50 protein in the nucleus of TPA induced BCBL-1 cells 48h post-induction

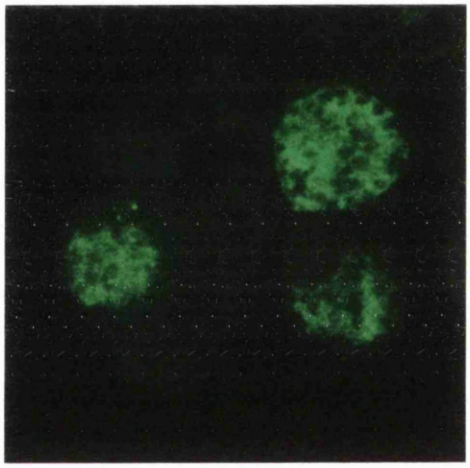
Fig 8A3 Panel II *a*. Expression of viral ORF57 and GFP-ORF57 in TPA induced and uninduced BCBL-1 cells

II a

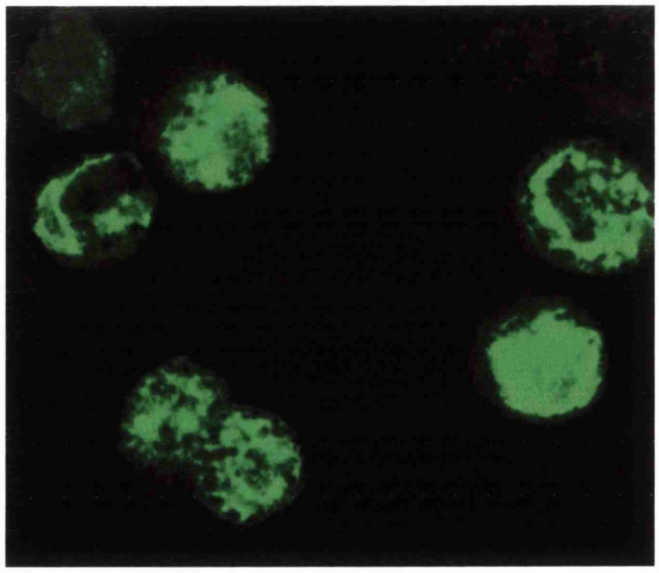
Viral ORF57 protein in 48 h TPA induced BCBL-1 cells



Viral ORF57 seen in the nucleus of TPA induced BCBL-1 cells

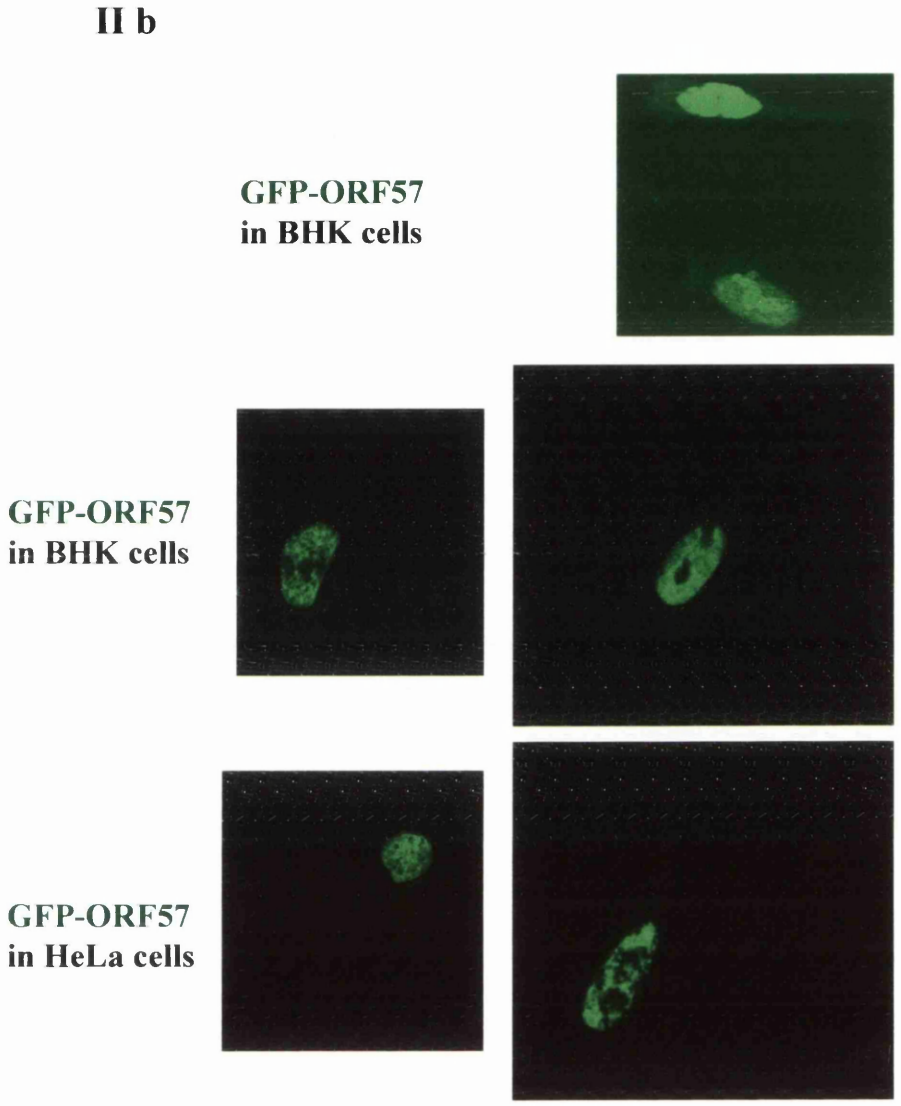


Electroporated GFP-ORF57 seen in the nucleus of uninduced BCBL-1 cells



Panel II *a*. Viral ORF57 or electroporated GFP-ORF57 in TPA induced BCBL-1 cells

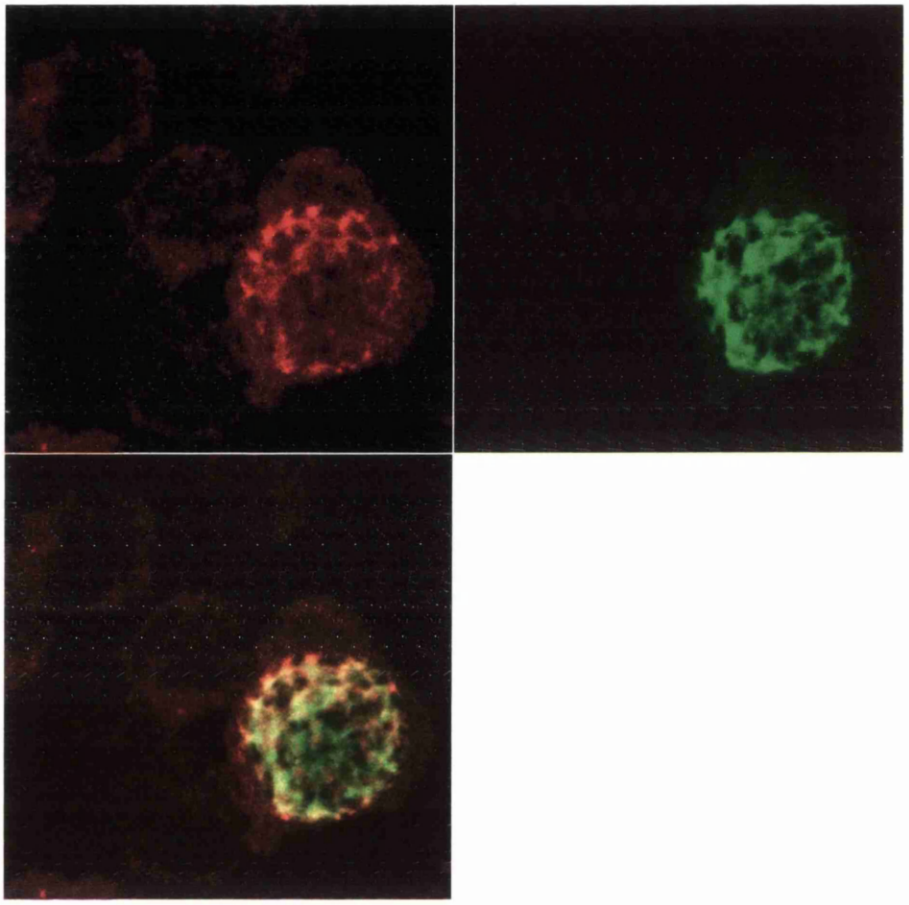
Fig 8A3 Panel II *b*. Expression of transfected GFP-ORF57 in mammalian cells



Panel II *b*. GFP-ORF57 transfected in mammalian cells (BHK and HeLa)

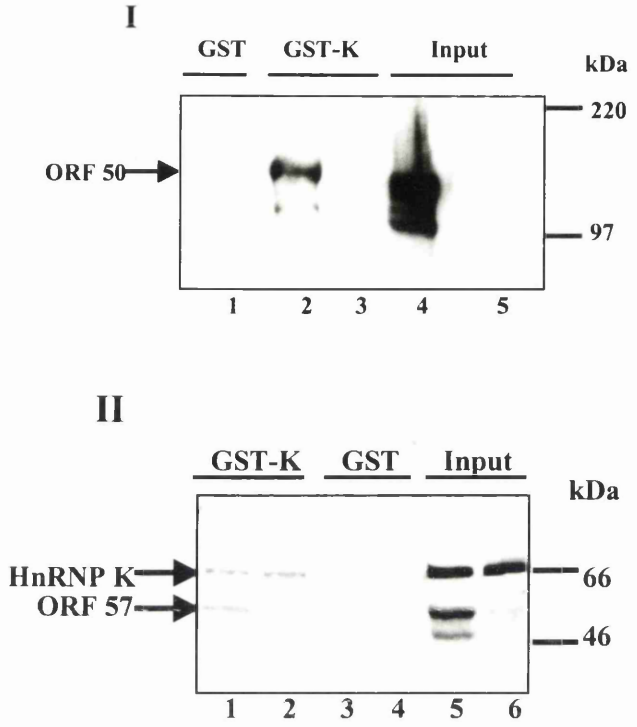
Fig 8A3 Panel III. Expression of viral ORF50 and electroporated GFP-ORF57 together in TPA induced BCBL-1cells

III



Panel III. Viral ORF50 and electroporated GFP-ORF57 protein together in the nucleus of TPA induced BCBL-1 cells 48 h post-induction

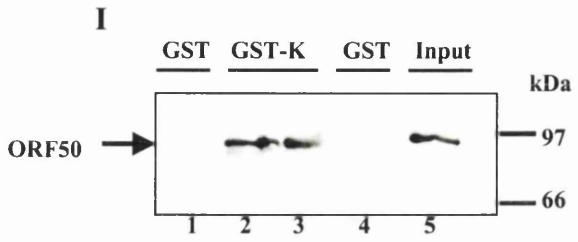
Fig 8A4. GST-K pulls down ORF50 from TPA-induced BCBL-1 cells



Panel I. Western blot for ORF50 with anti-ORF50 Ab showing that GST-K pulls down ORF50 from TPA-induced BCBL-1 cells. The GST-K or GST (control) (5μg protein) pull down assay was performed using TPA-induced and uninduced BCBL-1 cell extracts (200 μg). Pulled down proteins were separated by SDS-PAGE, gel was transferred to nylon membrane and Western blotted for ORF50 using anti-ORF50 Ab. Pull downs included in **lanes: 1.** TPA-induced BCBL-1 extract + GST alone **2.** TPA-induced BCBL-1 extract + GST-K **3.** Uninduced BCBL-1 extract + GST-K **4.** Input TPA-induced BCBL-1 cell extract **5.** Input uninduced BCBL-1 cell extract.

Panel II. Western blot with mixture of anti-ORF57 plus anti-hnRNP K Ab showing that GST-K pulls down ORF57 from TPA-induced BCBL-1 cells. TPA-induced and uninduced BCBL-1 cell extracts were incubated with GST-K or GST proteins and after incubation, pulled down proteins were separated by SDS-PAGE gel, transferred to nitrocellulose and Western blotted for ORF57 and hnRNP K using anti-ORF57 plus anti-hnRNP K Ab. Pull downs included in **lanes: 1.** TPA-induced BCBL-1 extract + GST-K **2.** Uninduced BCBL-1 extract + GST-K **3.** TPA-induced BCBL-1 extract + GST alone **4.** Uninduced BCBL-1 extract + GST alone **5.** Input TPA-induced BCBL-1 extract **6.** Input uninduced BCBL-1 extract.

Fig 8A5. GST-K interacts with *in vitro* synthesised ORF50 protein



Panel I. Phosphorimager analysis showing that GST-K interacts with *in vitro* synthesised ORF50 protein. The GST-K or GST (control) (5μg protein) pull down assay was performed with *in vitro* transcribed-translated ^{35}S-labelled-ORF50 protein (5μl). Pulled down proteins were separated by SDS-PAGE, and gel was dried and exposed to phosphorimager screen for analysis of labelled. Pull downs included in **lanes: 1.** *In vitro* synthesised ^{35}S-labelled-FLc50 + GST + RNAse **2.** *In vitro* synthesised ^{35}S-labelled-FLc50 + GST-K + RNAse **3.** *In vitro* synthesised ^{35}S-labelled-FLc50 + GST-K **4.** *In vitro* synthesised ^{35}S-labelled-FLc50 + GST alone **5.** Input *In vitro* synthesised ^{35}S-labelled-FLc50.

~90kDa corresponding to *in vitro* synthesized ORF50 whereas GST alone did not (Fig. 8A5 Panel I lanes 3 & 4.) and RNAse treatment did not disrupt this interaction (Panel I lane 2).

The binding between ORF50 and hnRNP K could be direct or mediated through a cellular protein present in the RRL used for the synthesis of protein *in vitro*, but does not require ORF57 though all three proteins can be present in a complex in virus-infected cells. As hnRNP K and ORF50 both are DNA-binding proteins and also bind several proteins, this interaction between ORF50 and hnRNP K could be either direct or mediated by protein:protein and/or DNA:protein interactions.

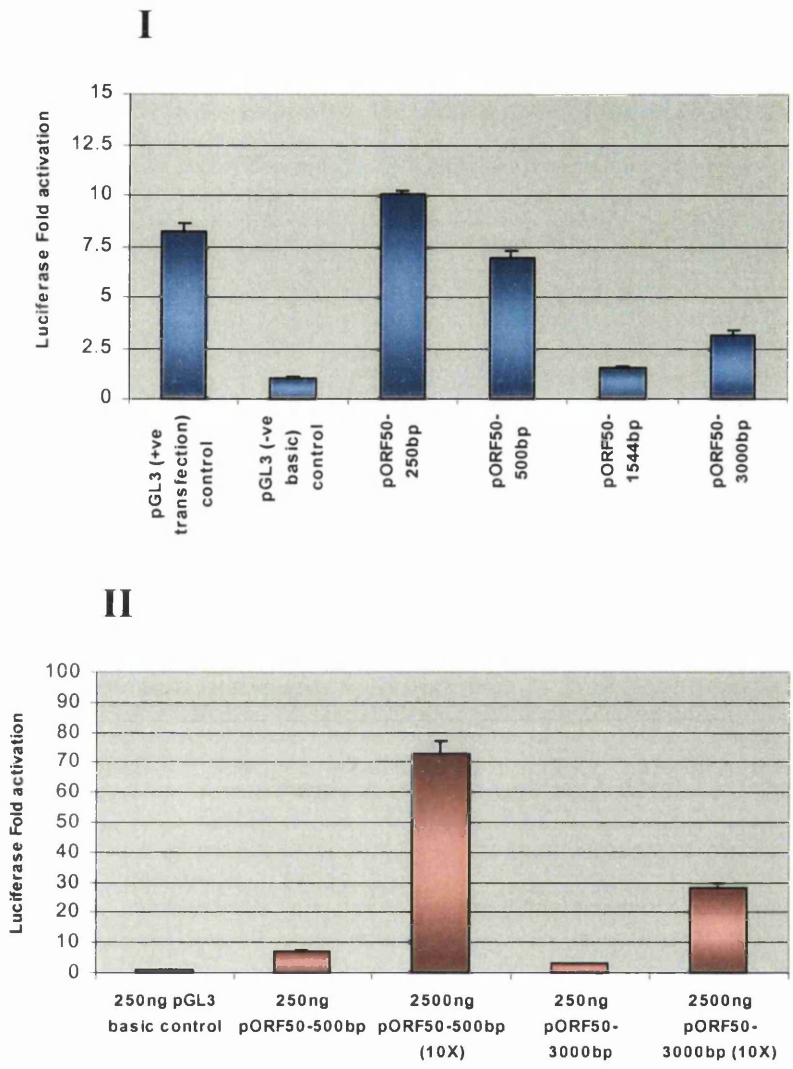
8B ORF57 modulates ORF50 activity

To investigate the functional interaction between ORF50 and ORF57, effects on the pORF50 promoter of ORF50 alone, ORF57 alone and ORF50-ORF57 together were measured.

8B1.1 The pORF50 promoter

To map the ORF50 promoter, various lengths of the putative promoter region upstream of the ORF50 transcription start site were cloned in front of the coding sequence for the *Firefly* luciferase gene in the pGL3-basic vector (Promega) (kindly provided by D J Blackbourn). These pORF50 constructs were responsive to TPA or sodium butyrate which are inducers of KSHV lytic replication (D J Blackbourn, unpublished results). Luciferase reporter constructs containing -3000bp, -1544bp, -500bp and -250bp regions upstream of the ORF50 start site were used in this study. Intrinsic activity of the pORF50 promoter in these constructs was measured by luciferase expression in 293 cells transfected with these constructs alone or pGL3 basic (control vector) using Polyfect (Section 2B9.3) and fold activation was calculated. Transfection of pORF50-3000bp and pORF50-1544bp resulted in 3.1- and 1.6 -fold enhancement of luciferase expression respectively whereas pORF50-500bp and pORF50-250bp resulted in 7.0- and 10.0-fold activation over pGL3, demonstrating that ORF50 upstream regions had intrinsic promoter activity (Fig. 8B1.1 Panel I). For further studies, -500bp and -3000bp promoter fragments were used. These promoter region constructs upstream of ORF50 gene activated luciferase expression in a dose dependent manner and use of a 10-fold concentration of pORF50-500bp and 3000bp constructs resulted in approximately 10-fold higher luciferase levels (Fig. 8B1.1 Panel II).

Fig 8B1.1 Panels I and II. Various lengths of the putative promoter region upstream of ORF50 gene activate luciferase expression variably and in a dose dependent manner



293 cells were transfected with a mixture of plasmids including pGL3 (positive) control for transfection, pGL3 basic (negative) control, pORF50-250bp/500bp/1544bp/3000bp promoter constructs, pRL-SV40 Renilla luciferase,) and pcDNA empty vector to keep the amounts of DNA constant. Cells were collected 48 h post-transfection and luciferase activity was determined from three separate experiments using Dual-Luciferase Reporter Assays (Promega) and normalised with pRL-SV40.

Panel I. Various lengths of putative promoter region upstream of ORF50 gene activate luciferase expression variably

Panel II. Promoter constructs upstream of the ORF50 gene activate luciferase expression in a dose dependent manner

8B1.2 ORF57 alone regulates the activity of pORF50 promoter

Published data have shown that ORF50 positively regulates its own expression to a varying degree (Seaman *et al.*, 1999; Deng *et al.*, 2000; Wang *et al.*, 2001b). And ORF57 + ORF50 synergistically upregulated the expression of several delayed early genes (Kirshner *et al.*, 2000). To determine the effect of ORF57 on the pORF50 promoter, ORF57 expression plasmid pcDNA4-gORF57 or the pcDNA3 control vector were transfected into 293 cells with either pORF50-500bp or 3000bp reporter plasmids. A control plasmid pRL-SV40 that constitutively expresses *Renilla* luciferase was included in each transfection to obtain the normalised luciferase activity ratio. Cells were harvested 48 h post-transfection and Dual-Luciferase Reporter Assays (Promega) were performed. The pORF50-500bp promoter was activated 7.8-fold by ORF57 (Fig. 8B1.2 Panel I & II) whereas the pORF50-3000bp promoter was activated 4.2-fold (Fig. 8B1.2 Panel III).

To test the dose-dependence, a fixed amount of pORF50-500bp or 3000bp (250 ng) was co-transfected with increasing amounts of pcDNA4-gORF57 into 293 cells. Decreasing amounts of pcDNA3 empty vector were included so that the total amount of pcDNA3 vector remained the same. Normalised *Firefly* luciferase activity increased with the amount of pcDNA4-gORF57 (0 to 500 ng) for the pORF50-500bp promoter construct (Fig. 8B1.2 Panel I) and higher doses (40 ng to 4000 ng) gave progressively higher pORF50 promoter activity from both 500bp and 3000bp promoters before reaching a plateau at 600 ng (Fig. 8B1.2 Panel II & III). Fold activation was calculated based on the promoter reporter plasmid, which was normalised to one. These results demonstrate that ORF57 expression activated the ORF50 promoter and it did not activate the SV40 promoter in plasmids pGL3-control and pRL-SV40.

Co-transfection of various ORF57 deletion mutants (pcDNA4-ORF57) with a fixed amount of pORF50-500bp or pORF50-3000bp (250ng) showed that full-length ORF57 protein is important for the activation of luciferase expression. Decreasing amounts of pcDNA3 were also included in each sample so that the total amount of pcDNA3 vector remained the same (Fig. 8B1.2 Panel IV & V). Use of ORF57 deletion mutants showed that an activation domain resides at the C-terminal region (aa 329-455) that generated the highest activation of luciferase (108% and 130%) from both the promoter constructs (500bp and 3000bp respectively). Removal of this activation domain (aa 329-387) appears to result in reducing the activation from aa 387-455 (65% and 84%). A repression

Fig 8B1.2 Panel I. Expression from pORF50-500bp promoter is activated by increasing doses of ORF57

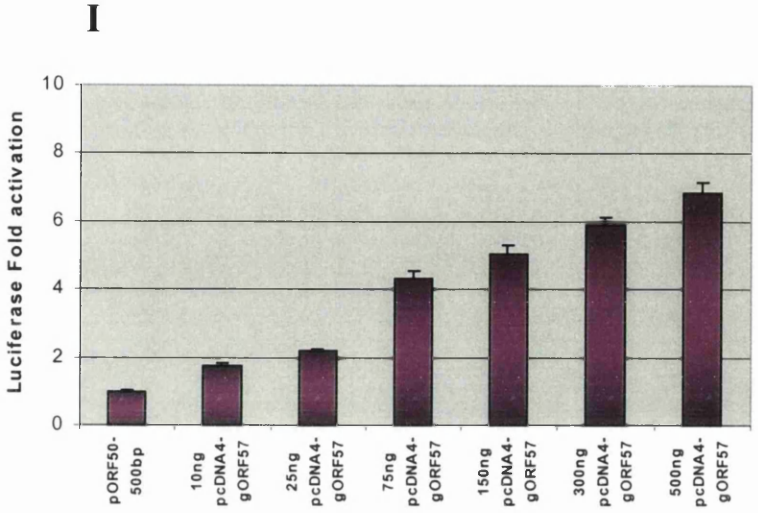
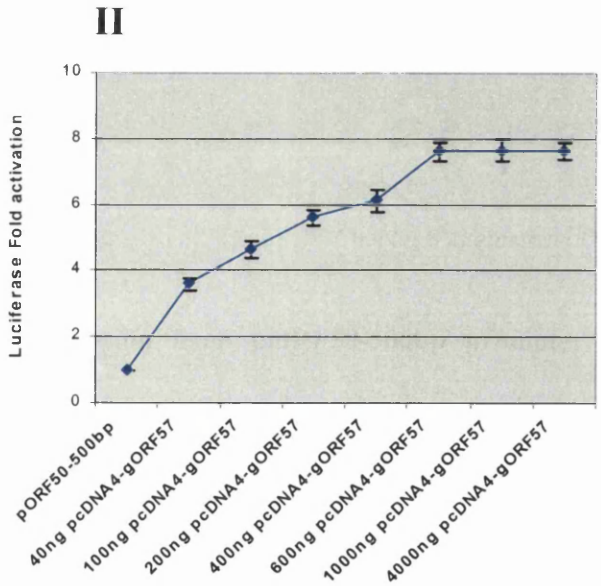
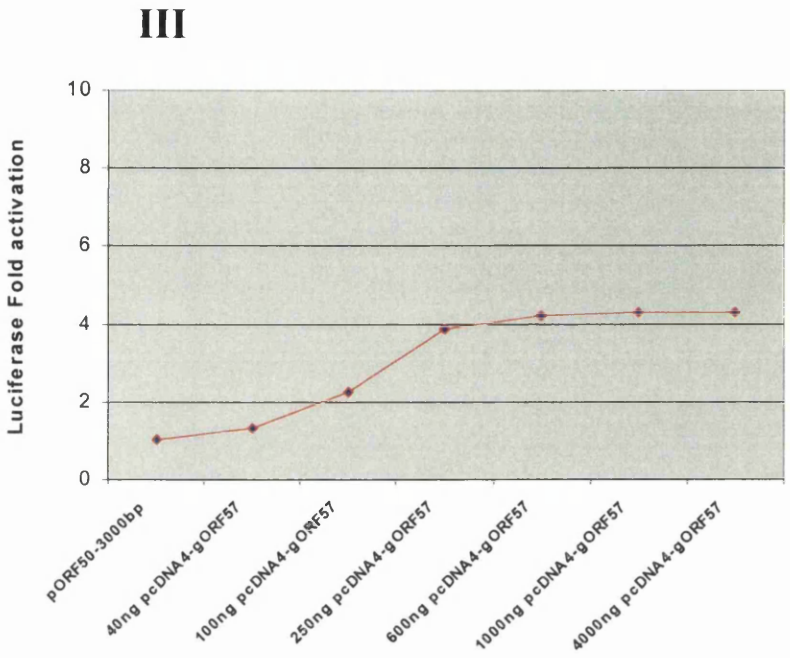


Fig 8B1.2 Panel II. Expression from pORF50-500bp promoter is activated by increasing doses of ORF57 but reaches saturation at higher concentrations



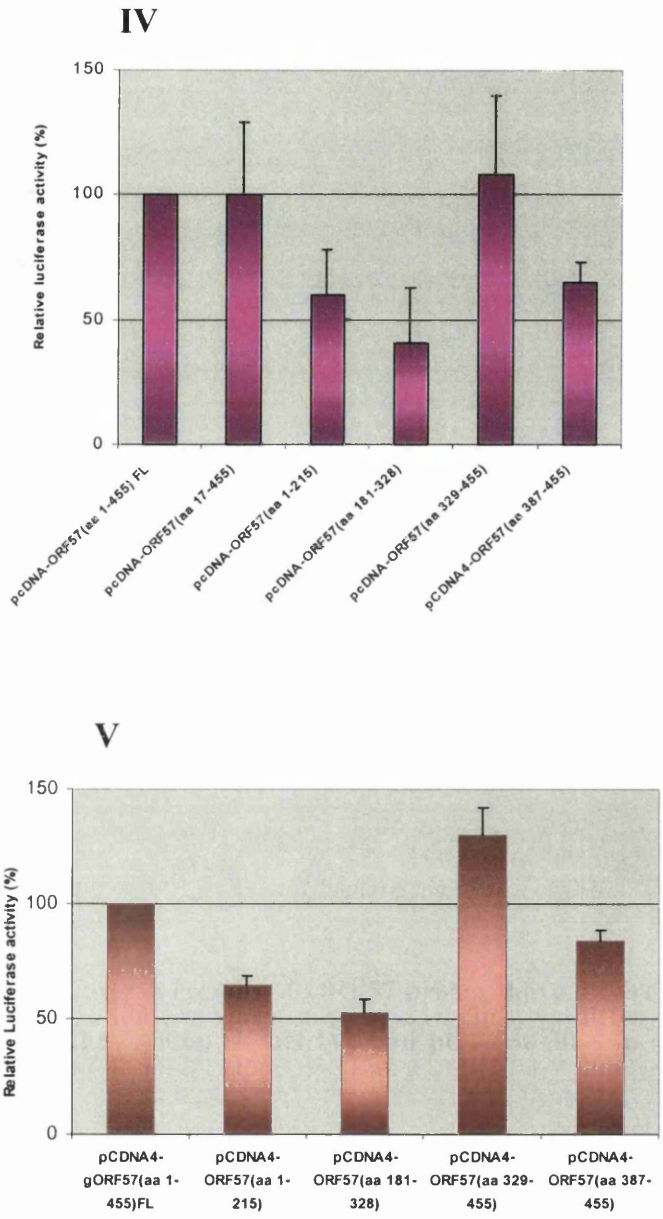
293 cells were transfected with a mixture of plasmids including pGL3 control, pORF50-500bp promoter construct, pRL-SV40 Renilla luciferase, and increasing doses of pcDNA4-gORF57, or pcDNA empty vector to keep the amounts of DNA constant. Cells were collected 48 h post-transfection and luciferase activity was determined from three separate experiments using Dual-Luciferase Reporter Assays (Promega) and normalised with pRL-SV40.

Fig 8B1.2 Panel III. pORF50-3000bp promoter is also activated but to a lesser degree than pORF50-500bp by increasing doses of ORF57 and reaches saturation at higher concentrations



293 cells were transfected with a mixture of plasmids including pGL3 control, pORF50-3000bp promoter construct, pRL-SV40 Renilla luciferase, and increasing doses of pcDNA4-gORF57, or pcDNA empty vector to keep the amounts of DNA constant. Cells were collected 48 h post-transfection and luciferase activity was determined from three separate experiments using Dual-Luciferase Reporter Assays (Promega) and normalised with pRL-SV40.

Fig 8B1.2 Panels IV and V. Various regions of ORF57 protein have different effects on expression from the pORF50-500bp (Panel IV) and pORF50-3000bp (Panel V) promoters



293 cells were transfected with a mixture of plasmids including pGL3 control, pORF50-500bp or pORF50-3000bp promoter construct, pRL-SV40 Renilla luciferase, and pcDNA4-gORF57 (600ng), or various deletion mutants of pcDNA4-gORF57 (600ng) and pcDNA empty vector to keep the amounts of DNA constant. Cells were collected 48 h post-transfection and luciferase activity was determined from three separate experiments using Dual-Luciferase Reporter Assays (Promega) and normalised with pRL-SV40. The luciferase activity value of ORF57 full length was taken as 100% and relative values were calculated for other ORF57 deletions and plotted on the graph.

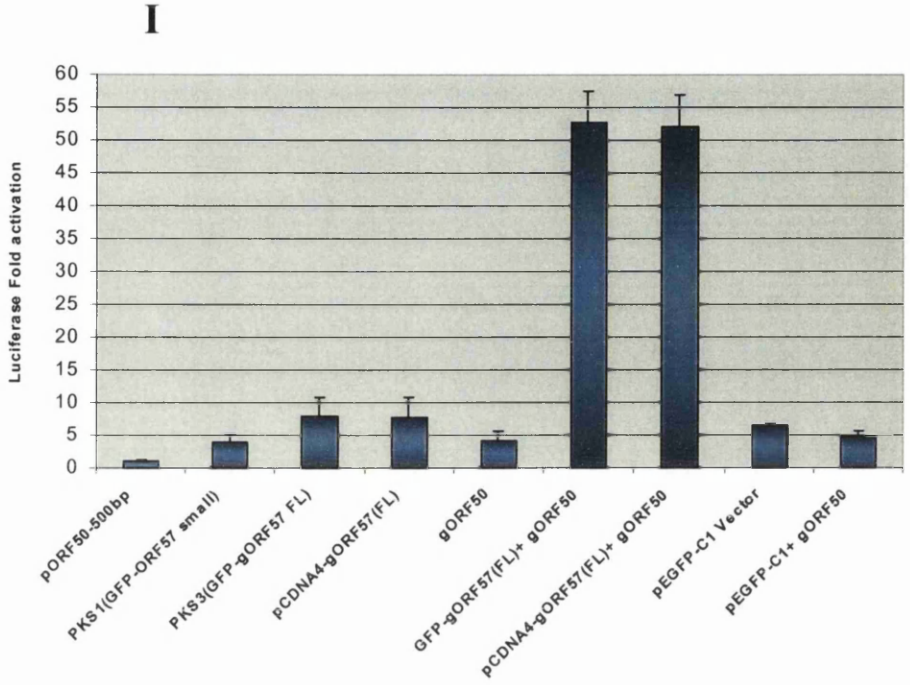
domain also likely to be present at the N terminus as the N-terminal (aa 1-215) and middle region (aa 181-328) were less important and aa 181-328 produced the lowest levels of activation (41% and 53%) and removal of this region causes an increase in activity from the C terminus region (aa 329-455).

8B1.3 ORF57 together with ORF50 further upregulates the activity of pORF50

To investigate whether ORF57 functionally interacts with ORF50 in the control of gene expression from the ORF50 promoter region, 293 cells were transfected with pORF50 promoter reporter constructs and expression vectors making ORF50 and ORF57 proteins. An amount of ORF50 expression plasmid DNA (500 ng) giving maximum activation of the luciferase expression from the pORF50 promoter (as shown previously and in our hands) was used in transfections with ORF57 expression plasmid. ORF57 expressing plasmids at a concentration giving maximum activation (600 ng) with ORF50 expression vector gave enhanced expression from the pORF50 promoter (Fig. 8B1.3 Panel I & II). A fixed amount of pORF50-500bp or pORF50-3000bp (250ng) was cotransfected with pcDNA4-gORF57 or pKS3 or pKS1 (GFP-gORF57 FL or small) and pcDNA3-gORF50 into 293 cells. Decreasing amounts of pcDNA3 (and/or GFP expression vector) were included so that the total amount of pcDNA3 (or GFP expression vector) DNA remained the same. With addition of ORF57+ORF50, normalised *Firefly* luciferase activity further increased by 12.7-fold compared to ORF50 alone and increased 6.7-fold compared to ORF57 alone from pORF50-500bp (Fig. 8B1.3 Panel I). The pKS3 (GFP-ORF57 FL) and pcDNA-gORF57 plasmids alone activated expression up to 7.8-fold whereas pKS1 (GFP-ORF57 small) induced expression only by 3.8-fold, again demonstrating the requirement for a full-length ORF57 protein, and gORF50 increased expression from its own promoter by 4.1-fold. pEGFP-C1 alone also activated expression (up to 6-fold) but did not show any synergistic activation with gORF50. Cotransfection of ORF57 with ORF50 synergistically upregulated expression up to 52-fold from pORF50 promoter (Fig. 8B1.3 Panel I).

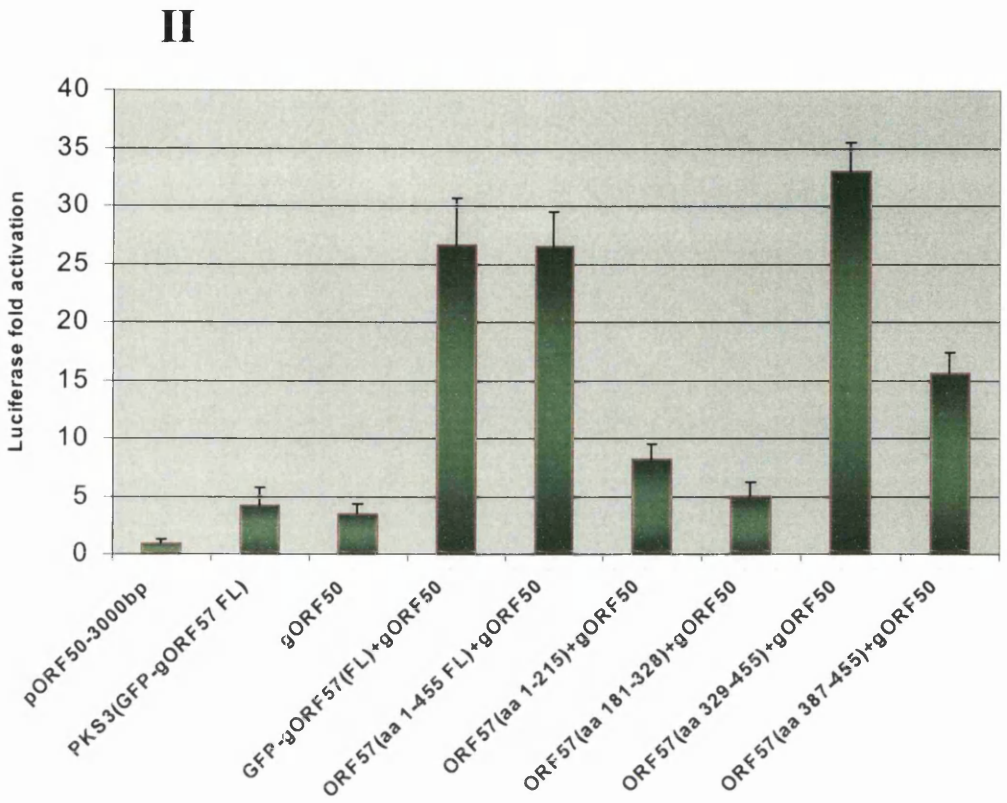
Similar effects but lower levels of luciferase induction (7.9 fold) relative to pORF50-500bp were obtained with the pORF50-3000bp construct (Fig. 8B1.3 Panel II). With ORF57+ORF50, luciferase activity was further increased by 7.9-fold compared to ORF50 alone and increased 6.3-fold compared to ORF57 alone. The ORF57 alone activated the luciferase expression up to 4.2-fold and gORF50 was able to increase expression from its own promoter by 3.4-fold. Cotransfection of pKS3 (GFP-ORF57 FL) and pcDNA4-gORF57 with ORF50 synergistically upregulated the ORF50-mediated expression from

Fig 8B1.3 Panel I. ORF57 synergistically augments ORF50-mediated expression from the pORF50-500bp promoter



293 cells were transfected with a mixture of plasmids including pGL3 control, pORF50-500bp promoter construct, pRL-SV40 Renilla luciferase, pEGFP-C1 control (GFP empty vector), pcDNA-gORF50 (500ng), GFP-ORF57 small (600ng), GFP-ORF57 FL (600ng), pcDNA4 gORF57 (600ng), or pcDNA4/GFP empty vector to keep the amounts of DNA constant. Cells were collected 48 h post-transfection and luciferase activity was determined from three separate experiments using Dual-Luciferase Reporter Assays (Promega) and normalised with pRL-SV40.

Fig 8B1.3 Panel II. ORF57 protein and its deletion mutants synergistically augments ORF50-mediated expression from the pORF50-3000bp promoter



293 cells were transfected with a mixture of plasmids including pGL3 control, pORF50-3000bp promoter construct, pRL-SV40 Renilla luciferase, pEGFP-C1 control (GFP empty vector), pcDNA-gORF50 (500ng), GFP-ORF57 FL (600ng), pcDNA4-gORF57 FL (600ng) and various ORF57 deletion mutants (600ng), or pcDNA4/GFP alone empty vector to keep the amounts of DNA constant. Cells were collected 48 h post-transfection and luciferase activity was determined from three separate experiments using Dual-Luciferase Reporter Assays (Promega) and normalised with pRL-SV40.

its own promoter pORF50 up to 26.6-fold (Fig. 8B1.3 Panel II). Various deletion mutants of ORF57 activated ORF50-mediated expression from pORF50-3000bp to different extents indicating the requirement of particular regions (C terminus) of ORF57 protein.

To test the dose-dependence of ORF57 activation increasing amounts of pcDNA4-gORF57 (0 to 900 ng) were cotransfected into 293 cells with a fixed amount of pORF50-500bp promoter plasmid (250 ng) and 500 ng of ORF50 expressing plasmid DNA giving maximum activation. Up to 13-fold increase over the activity from ORF50 alone (and a further 6.6-fold increase in activity over ORF57 alone) was observed (Fig. 8B1.3 Panel III). To test the dose-dependence of ORF57 activation over a broader 10-fold range, a fixed amount of pORF50-500bp or pORF50-3000bp (250 ng) was cotransfected with increasing amounts (40 ng to 4000 ng) of pKS3 (GFP-gORF57 FL) into 293 cells and decreasing amounts of pEGFP-C1 were included in each sample so that the total amount of pcDNA3 and/or GFP vector DNA remained the same. Normalised *Firefly* luciferase activity increased up to 13 fold with the amount of pKS3 (40 ng to 600 ng) from the pORF50-500bp promoter but higher doses (600 ng to 4000 ng) did not result in any further increase (Fig. 8B1.3 Panel IV). Using the pORF50-3000bp promoter, normalised *Firefly* luciferase activity increased with the amount of pKS3 (40 ng to 600 ng) but resulted in lower levels of activation, i.e. up to 7.9-fold over ORF50 alone (and 6.3-fold increase in activity over ORF57 alone) again higher doses of pKS3 (600ng to 4000ng) gave no further increase (Fig. 8B1.3 Panel V). These results demonstrate that ORF57 further augments activity from pORF50 promoter in a dose dependent manner, and it did not activate the SV40 promoter in plasmids pGL3-control and pRL-SV40.

8B1.4 HnRNP K does not augment ORF50 activity and with ORF57+ ORF50 no augmentation was observed from the interferon stimulatory response promoter: ORF57 regulation of ORF50 activity is specific to the ORF50 promoter

Cotransfection of a vector expressing FLAG-tagged hnRNP K with the ORF50 expression vector and pORF50 constructs (500bp and 3000bp) did not result in activation of luciferase expression (Fig. 8B1.4 Panel I) indicating that hnRNP K can not substitute for ORF57 in this assay.

Cotransfection of ORF57+ORF50 with the interferon stimulatory response element promoter construct, pISREluc did not stimulate luciferase expression in the absence of IFN-α, and ORF57 alone or ORF50 alone did not activate luciferase expression (Fig. 8B1.4 Panel II). However the pISRE promoter was functional since it responded to the

Fig 8B1.3 Panel III. In the presence of a constant amount of ORF50, expression from the pORF50-500bp promoter is further activated by ORF57 in a dose dependent manner

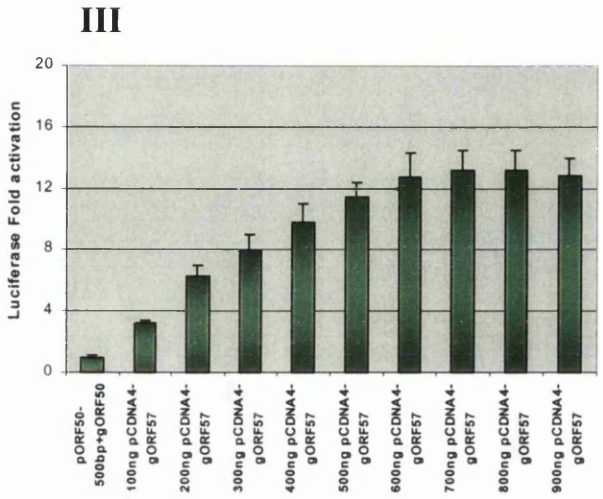
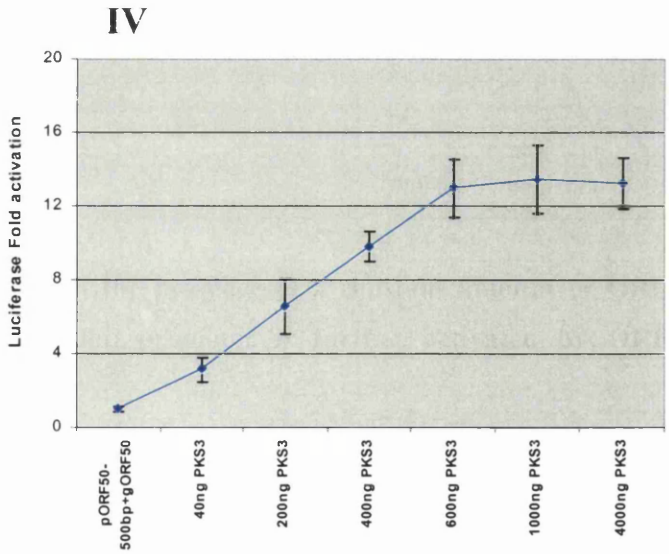
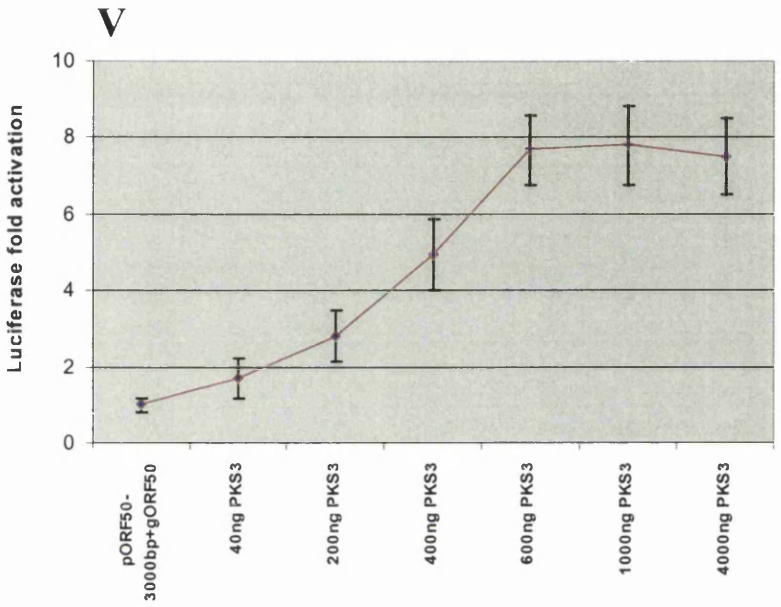


Fig 8B1.3 Panel IV. In presence of a constant amount of ORF50, expression from the pORF50-500bp promoter is further activated by increasing doses of ORF57 but reaches saturation at higher concentrations



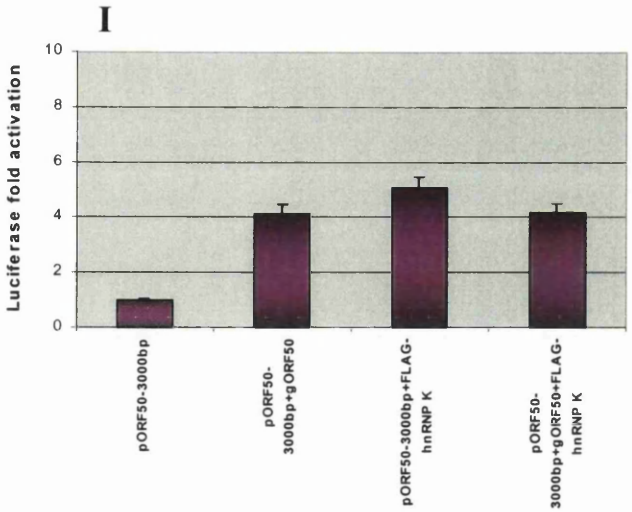
293 cells were transfected with a mixture of plasmids including pGL3 control, pORF50-500bp promoter construct, pRL-SV40 Renilla luciferase, pcDNA-gORF50 (500ng) and increasing doses of GFP-ORF57 FL (pKS3), or pcDNA4/GFP alone empty vector to keep the amounts of DNA constant. Cells were collected 48 h post-transfection and luciferase activity was determined from three separate experiments and normalised with pRL-SV40.

Fig 8B1.3 Panel V. In the presence of a constant amount of ORF50, expression from the pORF50-3000bp promoter is further activated by ORF57 in a dose dependent manner but reaches saturation at higher concentrations



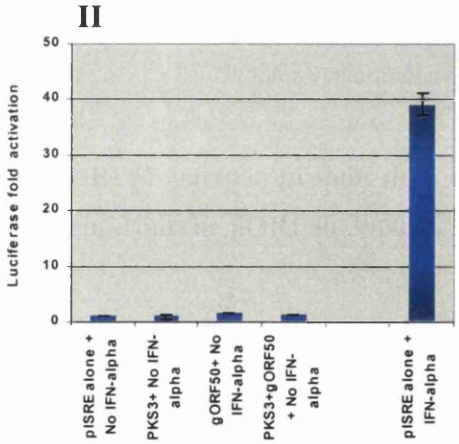
293 cells were transfected with a mixture of plasmids including pGL3 control, pORF50-3000bp promoter construct, pRL-SV40 Renilla luciferase, pcDNA-gORF50 (500ng) and increasing doses of GFP-ORF57 FL (pKS3), or pcDNA4/GFP alone empty vector to keep the amounts of DNA constant. Cells were collected 48 h post-transfection and luciferase activity was determined from three separate experiments using Dual-Luciferase Reporter Assays (Promega) and normalised with pRL-SV40.

Fig 8B1.4 Panel I. HnRNP K, an ORF57 partner protein does not synergistically induce expression with ORF50 protein from the pORF50-3000bp promoter



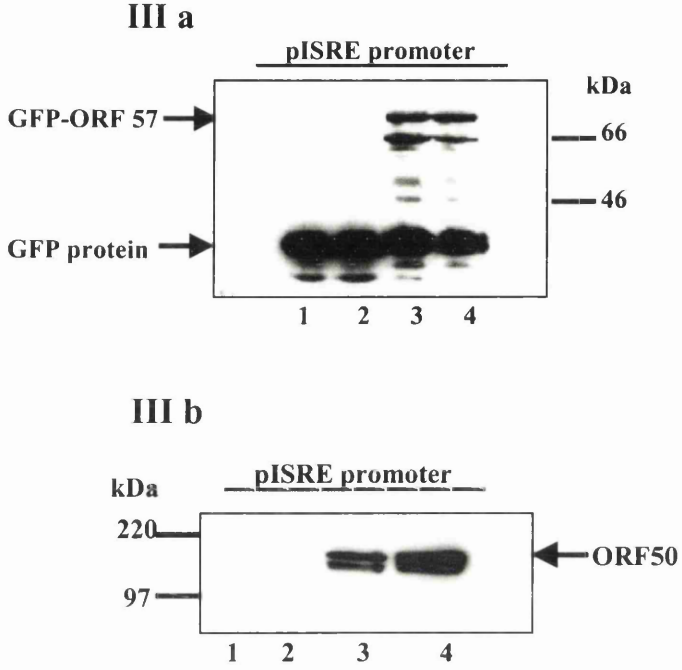
293 cells were transfected with a mixture of plasmids including pGL3 control, pORF50-3000bp promoter construct, pRL-SV40 Renilla luciferase, pcDNA-gORF50 (500ng) and hnRNP K expression vector (FLAG-hnRNP K 500ng), or pcDNA4 empty DNA to keep the amounts of DNA constant.

Fig 8B1.4 Panel II. ORF57 and ORF50 interaction does not result in synergistic activation from the pISRE promoter



A promoter containing interferon stimulatory response element linked to luciferase (pISRE) was used to test the specificity of ORF57+ORF50 effects on a heterologous promoter. 293 cells were transfected with a mixture of plasmids including pGL3 control, pISRE promoter construct, pRL-SV40 Renilla luciferase, pcDNA-gORF50 and GFP-ORF57 FL (pKS3), or pcDNA4/GFP empty vector to keep the amounts of DNA constant. 200 units/ml IFN-α was added 5 h post-transfection to transfected pISRE promoter construct in duplicates in three separate experiments.

Fig 8B1.4 Panel III *a* and *b*. Levels of ORF57 and ORF50 in the absence or presence of IFN-α



293 cells were transfected with a mixture of plasmids including pGL3 control, pISRE promoter construct, pRL-SV40 Renilla luciferase, pcDNA-gORF50 and GFP-ORF57 FL (pKS3), or pcDNA4/GFP empty vector to keep the amounts of DNA constant. Interferon-alpha (IFN-α) was added to some pISRE transfected cells at 200 units/ml 5 h post-transfection. Cells were collected, lysed in SDS-PAGE sample boiling buffer, separated by SDS-PAGE and gel was transferred to nylon membrane and Western blotted for ORF50 using anti-ORF50 Ab.

Panel III *a*. Western blot for GFP-ORF57 with anti-GFP Ab showing levels of GFP-ORF57 and GFP in presence and absence of IFN-α. Transfections included in **lanes: 1.** pISRE control transfected in 293 cells **2.** pISRE control +IFN-α **3.** pISRE + GFP-gORF57+ IFN-α **4.** pISRE + GFP-gORF57 + No IFN-α.

Panel III *b*. Western blot for ORF50 with anti-ORF50 Ab showing levels of gORF50 in presence and absence of IFN-α. Transfections included in **lanes: 1.** pISRE control transfected in 293 cells **2.** pISRE control +IFN-α **3.** pISRE + gORF50+ IFN-α **4.** pISRE + gORF50 + No IFN-α.

addition of IFN-α at 5 h post-transfection. Analysis of protein levels by Western blotting with anti-GFP Ab and anti-ORF50 Ab showed that ORF57 and ORF50 expression did not alter significantly in presence of IFN-α (Fig. 8B1.4 Panel III a and III b lanes 3 & 4).

8C Discussion

This Chapter presents data which show that KSHV ORF57 interacts with ORF50 in GST pull down assays and coimmunoprecipitations. Confocal microscopy showed that ORF57 colocalised partially with ORF50 present in a ring-like staining pattern whereas at other nuclear locations both proteins seem to have a different distribution. The ring-like structure could be the sites of viral transcription or could be sites where ORF50 is held after it comes into contact with ORF57 and/or other IE proteins. ICP27 has been shown to physically and functionally interact with ICP4 (Panagiotidis *et al.*, 1997). Interaction also was suggested from cotransfection experiments, which indicated that ICP27 could alter intracellular localisation of ICP4, and the C-terminal half of ICP27 was shown to be required for this inhibitory effect (Zhu & Schaffer, 1995). Although ICP4 localises to both the nuclei and cytoplasm of cells infected with either wt HSV-1 or an ICP0 null mutant virus, this protein was present exclusively in the nuclei of cells infected with an ICP27 null mutant virus, suggesting that ICP27 was able to inhibit the nuclear localisation of ICP4 during virus infection. The electrophoretic mobility of ICP4 was altered in the absence of ICP27, but it was unclear if ICP27 was directly or indirectly involved in this posttranslational modification (altered phosphorylation of ICP4) (Rice & Knipe, 1988; Su & Knipe, 1989; McMahan & Schaffer, 1990). Thus ICP27 and ICP4 are functionally associated and together may play a role in transcription (Su & Knipe, 1989). A similar interaction has been reported between VZV ORF4 and ORF62 that are homologues and analogues of KSHV ORF57 and ORF50 respectively (Spengler *et al.*, 2000).

Interaction with ORF57 could also modify ORF50 posttranslationally such as by phosphorylation mediated by CK2. ORF50 protein possesses CK2 consensus sites (Lukac *et al.*, 1999) but no interaction has been shown between it and CK2. Localisation and an association of ORF57 and ORF50 with different nuclear subdomains could be dynamic and relate to the various activities both proteins are involved in, the cell type and stage of the lytic virus cycle.

Precise boundaries for the ORF50 promoter region have not been mapped. However,

Sakakibara *et al.,* (2001) have identified Oct-1 binding sites as being required for maximum ORF50 activation (Sakakibara *et al.*, 2001). The ORF50 upstream region has intrinsic promoter activity (Seaman *et al.*, 1999; Deng *et al.*, 2000; Wang *et al.*, 2001b). The pORF50-500bp promoter construct was activated 7.8-fold by expression of ORF57 and the pORF50-3000bp promoter construct by 4.2-fold. Full-length ORF57 is required to enhance the activation of expression from pORF50 perhaps due to a requirement for cellular cofactors binding to different domains of the ORF57 protein. These results demonstrate that ORF57 can activate the pORF50 promoter independent of a B cell-specific factor, other virus-specific factors and from a plasmid lacking chromatin structure.

Similar to published results, ORF50 regulated its own activity by enhancing expression 4.1-fold from pORF50. Seamen *et al.,* (1999) reported that in BCBL-1 cells an ORF50 promoter construct, containing 655 bp fragment upstream of the ORF50 coding region, was activated 2.7-fold by ORF50 protein. Wang *et al.,* (2001) used a pORF50-1566bp construct and showed that in CV-1 cells the promoter was activated 7.1-fold by ORF50 while in 293, 3T3 and BJAB cells activation levels were lower. However Deng *et al.,* (2000) using an ORF50-3000bp construct (obtained from BC-1 cells), reported that in 293 cells with ORF50 protein this promoter was activated 40.3-fold, 144.4-fold in BCBL-1 cells and 38.7-fold in KS-1 cells. Thus ORF50 protein was capable of inducing expression using constructs with promoter sequences 5' to its transcription start site. This is similar to EBV Rta (an ORF50 homologue), which is capable of inducing activity from its own promoter that involves a non-binding mechanism (Zalani *et al.*, 1992).

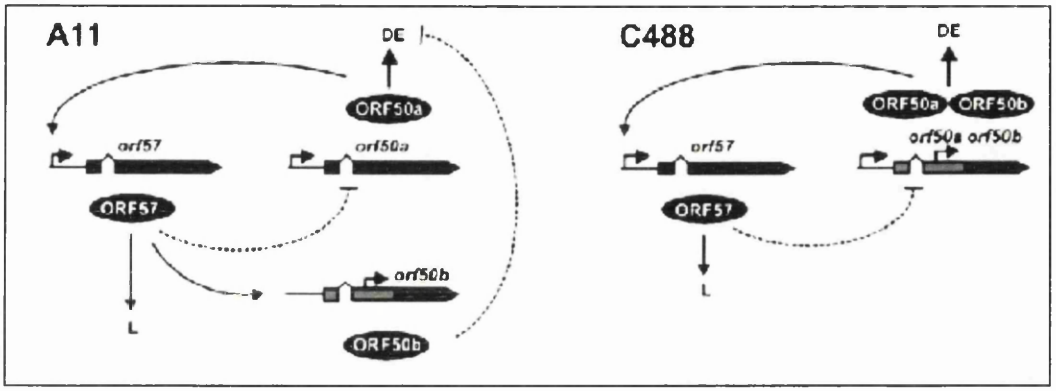
Together with ORF50, ORF57 further augmented activity by 13-fold from pORF50-500bp and 7.9 fold from pORF50-3000bp as compared to ORF50 alone. Upregulated luciferase levels from pORF50 with the C terminus of ORF57 in the presence of ORF50, indicated that this region, which appears to have activation and repression properties, could interact with ORF50 or a bridging cellular protein. The HVS ORF57 gene C terminus contains a hydrophobic domain required for transactivation and an extreme C-terminal zinc finger-like domain required for transrepression (Goodwin *et al.*, 2000).

Lack of any activation from the pORF50 promoter using ORF50+hnRNP K proteins, and from pISRE promoter using ORF57+ORF50 proteins suggested that the synergistic effect of ORF57+ORF50 might be specific for the ORF50 promoter.

ORF57 can modulate effects of ORF50 on expression from some (e.g., PAN) but not all (e.g., TK) ORF-50 responsive promoters but no mechanism for this synergy was shown (Kirshner *et al.*, 2000). The synergistic effect was suggested not to be due to upregulation of ORF50 expression but rather to a posttranslational enhancement of the transcriptional activity of ORF50 as the ORF50 mRNA and protein levels remain unaltered after addition of ORF57. In this study ORF50 was expressed from a HCMV promoter (Kirshner *et al.*, 2000) and it was shown that ORF57 does not affect reporter gene expression from a HCMV promoter (Gupta *et al.*, 2000; Kirshner *et al.*, 2000). Thus, no effects on ORF50 mRNA and protein levels after addition of ORF57 were measured and the ORF57-mediated effect on ORF50 was thought to be at the posttranslational level (Kirshner *et al.*, 2000). The ORF57 augmentation of ORF50-inducible activity observed in these studies, used the pORF50 promoter, and the effect could be due to either transcriptional-posttranscriptional/or even posttranslational regulation. Thus it is proposed that ORF57, in addition to its role as a posttranscriptional regulator of virus gene expression, may also modulate transcription either through its ability to modulate the activity (such as DNA binding) of ORF50 or through direct interactions with KSHV regulatory regions.

For HVS strain A11 two different mRNAs are transcribed from ORF50 gene: a long, spliced transcript (ORF50a), which can be detected early in infection and causes strong transactivation, and a shorter, unspliced transcipt (ORF50b), which only weakly transactivates promoters of other HVS genes (Nicholas *et al.*, 1991; Whitehouse *et al.*, 1997a; Whitehouse *et al.*, 1997b; Whitehouse *et al.*, 1998a). The HVS ORF57 gene product in strain A11 repressed the transactivating capability of ORF50a protein, but activated that of ORF50b protein (Whitehouse *et al.*, 1998a). The levels of mRNA transcribed from genes without an intron were not affected by ORF57 protein suggesting that HVS ORF57 regulates gene expression through a posttranscriptional mechanism (Whitehouse *et al.*, 1998b). ORF50a protein was shown to regulate ORF57 gene expression. Early in virus replication, ORF57 gene is expressed at basal levels and thereafter is proposed to be transactivated by the ORF50a protein, based on an increase in ORF57/ORF50 RNA levels (Whitehouse *et al.*, 1998a). These observations led to a feedback mechanism proposal in HVS (Fig 8C1), whereby ORF50a is downregulated by the ORF57 product which it specifically transactivates, while ORF50b is upregulated by ORF57 product: together both act on delayed early genes giving enhanced gene expression. Simultaneously, ORF57 gene was proposed to be downregulating itself by the same intron-dependent mechanism as represses ORF50a (Whitehouse *et al.*, 1998a).

Fig 8C1. Comparative model of gene regulation in HVS strains A11 and C488



Comparative model of gene regulation in the virus strains A11 and C488. The Figure compares the published observations for strain A11 (Whitehouse *et al.*, 1997a,b; 1998a,b) with the results on virus strain C488 (Thurau *et al.*, 2000). In strain C488, ORF50 seems to play a more central role in the regulation of virus gene expression, since the mechanism of transactivation blockade by ORF50b is not active. Stimulatory effects are indicated as arrows, inhibitory functions as dashed lines.

A marked sequence divergence was observed in the ORF50 gene between HVS strains A11 and C488. For strain C488, in contrast to strain A11, the unspliced ORF50b form also transactivated the ORF57 promoter as well as ORF50a (Thurau *et al.*, 2000). HVS strain A11-ORF50b is a poor transactivator and a mechanism of competitive downregulation of target genes at delayed times of infection has been postulated (Whitehouse *et al.*, 1998a). This type of negative regulation is unlikely for strain C488, in which ORF50b is fully functional and where ORF50 function is not switched off by ORF57-mediated splicing regulation and proposed downregulation of ORF50a. As the replication kinetics of strains A11 and C488 are indistinguishable, downregulation of ORF50 by ORF57 may not be critical for C488 replication (see Fig 8C1) (Thurau *et al.*, 2000). Whitehouse *et al.* (1998) believed that a third, as yet unidentified, gene may be responsible for the transactivation of A11 ORF50a between 15-18 h post-infection. The levels of HVS ORF57 and ORF50 RNA were simultaneously increased dramatically between 18-21 h post-infection and these proteins could be positively regulating each other to ensure enhanced viral gene expression at that time.

KSHV ORF57 augments the activity of ORF50, and similarly to HVS A11 (at 15 h post-infection), ORF50 is transactivated further from its basal levels by the ORF57 gene product, resulting in activated expression of both ORF50 and ORF57 simultaneously, which could then act synergistically on promoters for delayed early and late genes. Interaction of ORF57 and ORF50 could play a vital role in controlling latent and lytic cycles of KSHV by acting as sensors of the conditions for lytic viral replication in infected cells, through interactions with various cellular proteins.

Chapter 9: General Discussion

9A Multifunctional nature of the ORF57 protein

Certain multifunctional proteins can exert effects on transcription, RNA processing, RNA transport and translation. They have affinity for several protein partners and for nucleic acid, both DNA and/or RNA. These include: hnRNP K, YB-1 and WT1. Such biochemically promiscuous multifunctional proteins are thought to co-ordinate different stages of gene expression. ORF57 protein was shown to be implicated in several stages of gene expression. The present study shows interaction of ORF57 with several cellular proteins known to be involved in different processes. Their interaction with ORF57 suggests a regulatory role for ORF57 by either enhancing their activities or subverting their normal activities to facilitate viral gene expression. ORF57 is capable of self-interaction (see Section 3C) that could help it to form large multiprotein complexes.

9A1.1 Interaction of ORF57 with HnRNP K

ORF57 and hnRNP K proteins have multiple interactive domains. HnRNP K contain two different types of nucleic acid binding domains, the KH domains and the RGG cluster, and the KI region responsible for protein-protein interaction. Similar to hnRNP K, ORF57 interacts with several proteins involving different regions and domains. These data indicate that K and ORF57 protein may simultaneously interact with more than one of their partners to form multiple protein:protein or protein:nucleic acid complexes. It is plausible that the interaction of hnRNP K+ORF57 with one of their partners (either nucleic acid or proteins) may be the regulating factor for interaction with another partner. Competitive studies *in vitro* using one of the partner proteins or defined sequence RNA and DNA substrates can demonstrate their effect on the strength of this interaction.

HnRNP K has been suggested to act as a molecular docking platform allowing cross talk between transcription, mRNA processing and signalling (Bomsztyk *et al.*, 1997). Its interaction with ORF57 in KSHV-infected cells suggests exciting possibilities for ORF57-mediated regulation of these processes. As both proteins are involved in transcription/post-transcriptional processing and nuclear transport it is plausible that these processes are regulated by interaction of ORF57 and hnRNP K. ORF57 might be hijacking some hnRNP K activities or diverting them towards viral gene expression.

9A1.2 Interaction of ORF57 with protein kinase CK2

Binding of CK2 α *in vitro* with N-terminal, central and C-terminal regions of ORF57 protein independent of the interaction with other regions could be due to specific folding of the interacting regions of either or both proteins. A crystal structure of the ORF57 protein could elucidate the nature of these multiple ORF57 interactive regions with both catalytic (α/α') and regulatory (β) subunits of CK2.

9A1.3 Interaction of ORF57 with REF

Some ORF57 partners identified here could facilitate its interaction with mRNPs, including RNA binding proteins hnRNP K and REF. ORF57 could shuttle via a novel NES and this study does not support a CRM1 dependent export route (see Section 6B3), or alternatively via interaction with a cellular shuttling protein.

REF can form multimers *in vitro* (Virbasius *et al.*, 1999; Rodrigues *et al.*, 2001). And perhaps REF binding to ORF57 requires dimer formation by one or both proteins. Data presented here and published studies demonstrate that the REF variable regions, particularly the C-vr region, are involved in its binding to ORF57 (this study), TAP, and RNA as well as in dimer formation (Rodrigues *et al.*, 2001). Due to a lack of detailed structural information on ORF57 and incomplete structural information on REF, it is difficult to speculate on how the same regions of REF are involved in interactions with ORF57/TAP or RNA. Although in KSHV-infected cells the nature of these interactions could vary depending on the cell type, cell cycle stage and protein modifications. As ORF57 seems to negatively regulate nuclear RNA export by Rev or TAP proteins, this indicates that either ORF57 itself acts as an export factor or at some stage in the export process these proteins share common export components.

REF proteins coprecipitate with spliceosomes and Y14, an hnRNP-like protein that preferentially associates with spliced mRNAs, interacts with TAP (Kataoka *et al.*, 2000). REF/TAP/ Y14 have all been suggested to load on the pre-mRNAs during splicing but have not yet been reported to be present together in a complex. In this study, Y14 interacted with ORF57 *in vitro* and was present in a complex with ORF57/REF/TAP from KSHV-infected BCBL-1 cells.

9A1.4 Interaction of ORF57 with SAP145

HSV-1 infection inhibits host cell pre-mRNA splicing (Hardy & Sandri-Goldin, 1994), and ICP27 is implicated in this process (Hardwicke & Sandri-Goldin, 1994). Although

effects of ORF57 on KSHV host cell splicing are not well characterised, few differences on gene activation from intronless and intron-containing genes have been observed (Gupta *et al.*, 2000; Kirshner *et al.*, 2000) and these are not as strong and universal as with ICP27. In the case of KSHV although transcript-mapping information is not exhaustive, several mRNAs are spliced. It is difficult to speculate at this stage what the effect of ORF57 is on splicing in KSHV-infected cells. But it is predicted that inhibition of host cell splicing would favour the export of viral RNAs.

The interaction between SAP145/REF/TAP/Y14 and ORF57 could couple components of the splicing machinery, a viral protein involved in transcriptional/posttranscriptional regulation and proteins involved in nuclear RNA export.

9A1.5 Interaction of ORF57 with KSHV ORF50

The mechanism of activation of ORF50 responsive elements in viral promoters is not fully understood. It is not clear whether ORF50, an ORF50 interacting IE viral protein or a cellular protein could tether a common factor to responsive elements in various KSHV promoters to mediate transcriptional activation. ORF50 may activate viral promoters not only directly by DNA binding but also indirectly by piggybacking onto cellular DNA binding proteins or altering their activity and/or abundance and one plausible candidate is the transcriptional regulator hnRNP K. The transactivation domain of HVS ORF50 has been shown to bind TBP (Hall *et al.*, 1999). Rta of EBV also binds TBP through its activation domain (Manet *et al.*, 1993) and ICP4, the major transcriptional regulatory protein of HSV-1 forms a tripartite complex with TBP and TFIIB (Smith *et al.*, 1993; Gu *et al.*, 1995). If ORF50 also interacts with TBP that induces/represses transcription then its interaction with ORF57 (and indirectly hnRNP K) could be important for transcriptional effects.

HnRNP K binds TBP and overexpressed hnRNP K and TBP synergistically activated transcription of a CT element-dependent reporter gene *in vivo* (Michelotti *et al.*, 1996). ORF57 interacts with both hnRNP K and ORF50, which could be interacting with other transcription factors (for e.g. TBP), regulating transcription under different cellular environments. Interaction of ORF57 with hnRNP K could favour the formation of transcriptionally inactive complexes (ORF57-hnRNP K) thus making more cellular transcription cofactors available to ORF50. The possibility of ORF50-TBP interaction and whether ORF50 and ORF57-associated hnRNP K bind to DNA requires further investigation.

ORF50 protein is extensively phosphorylated *in vivo*, and proposed to contain several serine/threonine-rich consensus phosphorylation sites for protein kinase CK2 and protein kinase C (Lukac *et al.*, 1999). Thus phosphorylation of ORF50 and ORF57 could play an important role in controlling the latent and lytic cycles of KSHV. Similar to VZV, ORF62 where a hypophosphorylated form of ORF62 interacts with ORF4 (Spengler *et al.*, 2000) it could be the less phosphorylated form of ORF50 which interacts with ORF57 at the onset of the lytic cycle resulting in activation from ORF50 promoters whereas later in the lytic cycle, ORF57-associated CK2 might modify ORF50 phosphorylation resulting in no further activation from pORF50 promoters.

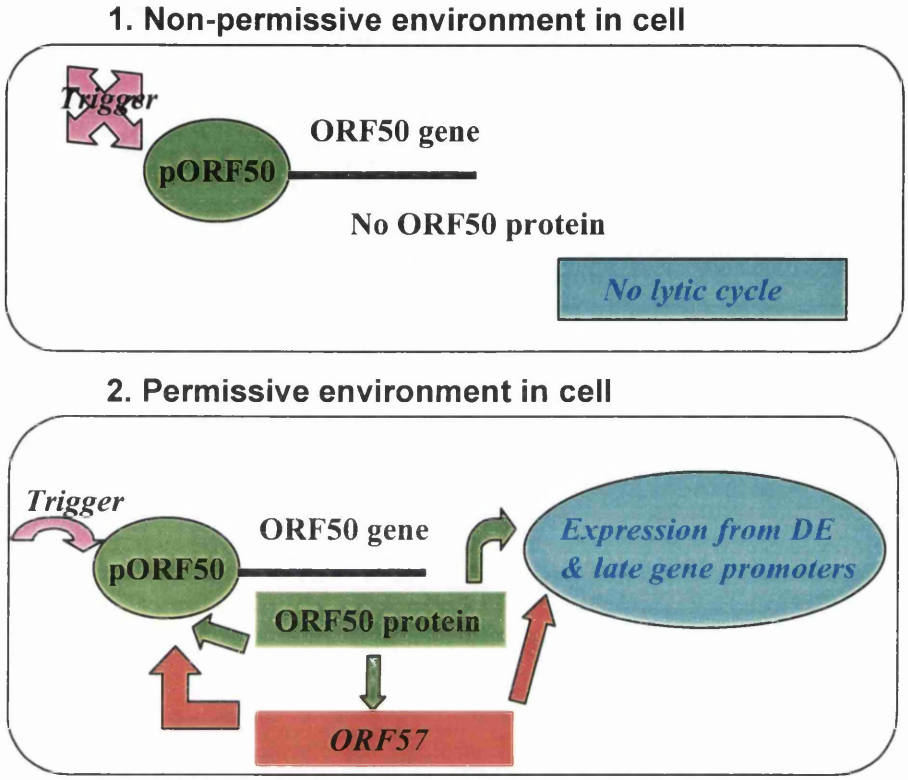
Activation of gene expression from the pORF50 promoter in presence of ORF57 could be due to ORF57-mediated upregulation of transcripts reflecting more stabilized mRNAs, improved mRNA processing and more efficient viral mRNA export by accessing/recruiting cellular mRNA export factors.

A model of regulation of gene expression in KSHV can be proposed (summarised in Fig 9A1.5) in which the virally encoded gene products ORF57 and ORF50 are central components. When the environment within the cell is non-conducive to virus replication, ORF50 is not expressed and the virus does not enter the lytic cycle. Under permissive conditions, ORF50 positively auto-regulates its expression and causes a switch from latent to lytic replication. ORF57 is then expressed to augment ORF50 activity and ORF57+ORF50 together have a synergistic effect on expression from delayed early and late viral genes thereby promoting lytic viral replication (Fig 9A1.5). Regulation of ORF50 by ORF57 could play an important role in the cascade of viral gene expression by establishing a positive feedback loop allowing the virus to amplify the environmental stimuli involved in the reactivation phenomenon from latency.

9B Cross talk between events involved in gene regulation

Recent studies in yeast have identified a link between transcription and mRNA export (Lei *et al.*, 2001). This study revealed that Yra1 (the yeast homologue of REF) is recruited to mRNA during transcription (Lei *et al.*, 2001) and mRNA export factors REF and UAP56 couple the machineries that function in splicing and export of mRNA (Strasser & Hurt, 2000; Stutz *et al.*, 2000; Zhou *et al.*, 2000; Gatfield *et al.*, 2001; Jensen *et al.*, 2001; Luo *et al.*, 2001; Strasser & Hurt, 2001). A recent study has suggested that a

Fig 9A1.5. Proposed model of ORF50 and ORF57 gene regulation at the beginning of the KSHV lytic cycle



Model of gene regulation in KSHV. ORF50 and ORF57 seems to play a central role in the regulation of virus gene expression. ORF50 action is indicated by green arrows and ORF57 action by red arrows.

conserved TREX ("transcription/export") complex might be a principal mediator in coupling transcription to export (Strasser *et al.*, 2002). The TREX complex is specifically recruited to activated genes during transcription and travels the entire length of the gene with RNA polymerase II suggesting that it physically links proteins that function in both mRNA export and transcription. The remarkable conservation of the TREX complex from yeast to humans reveals the importance of the association of mRNA export and transcription components in the same complex (Strasser *et al.*, 2002). ICP27 has recently been shown to coimmunoprecipitate with RNA polymerase II (Zhou & Knipe, 2002).

It has emerged in recent years that RNA splicing and 3' end formation are not strictly posttranscriptional events and are believed to occur cotranscriptionally. Both reactions are not only capable of influencing each other's efficiency and specificity but are also tightly linked to transcription. The phosphorylated CTD of RNA polymerase II provides key molecular contacts with RNA processing factors involved in splicing and 3' end formation throughout transcriptional elongation and termination (Proudfoot *et al.*, 2002).

Here, ORF57 has been shown to interact with: (i) a viral transcriptional activator ORF50 which causes the switch from latent to lytic cycle, (ii) a cellular transcription factor hnRNP K which has also been implicated in mRNA export and translation, (iii) a constitutive member of the splicing complex SAP145, (iv) REF, a known RNA binding protein and export factor, and finally (v) a protein kinase CK2 which has the ability to phosphorylate ORF57 and certain of its associated proteins.

This study raises several areas for further investigation. The various partner proteins of ORF57 comprise proteins capable of carrying out a diverse range of functions. ORF57 could facilitate cross talking between different stages of viral gene expression, coupling the transcription complex with the splicing machinery and RNA export apparatus. In KSHV infected cells, the common contact link for these various protein:protein and protein:nucleic acid interactions could be via ORF57 that could play a pivotal role in the viral life cycle.

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