Analysis of cellular and viral proteins that interact with the IE63 protein of herpes simplex virus type 1

By

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Abstract

The aim of this work was to identify proteins which interact with the herpes simplex virus type 1 (HSV-1) protein IE63 in virus infected cells. This information has allowed insights as to how IE63 can exert its various activities and will help in the understanding of how transcription, RNA processing and RNA export are co-ordinated in infected and uninfected cells.

HSV-1 is one of eight different herpesviruses to infect humans, the others are HSV-2, varicella-zoster virus, Epstein-Barr virus, human cytomegalovirus and human herpesviruses 6-8. Serological studies show that commonly throughout the world many individuals have been infected with one or more of these viruses. Although herpesviruses are generally not a problem in healthy individuals, in the immunocompromised they can cause life-threatening disease. As the immediate early (IE) protein of HSV-1, IE63 is the only HSV IE gene with homologues in all sequenced herpesviruses; studies of this essential protein, which may have similar functions throughout the *Herpesviridae*, are important to key questions about herpes biology and the development of effective therapies.

During HSV-1 infection expression of different classes of viral genes is co-ordinately regulated and sequentially ordered. The three major classes of genes, IE, early (E) and late (L), can be separated on the basis of the kinetics of their expression and requirements for ongoing viral DNA synthesis. At the core of this regulation are three of the IE proteins, IE175, IE110 and IE63. Both IE175 and IE63 are essential for viral growth and IE110, while not essential, confers growth advantage in cell culture and is essential for establishment and reactivation from latency. IE63 is thought to repress expression of viral IE and E genes, enhance expression of viral L genes, repress expression of cellular genes and is required for viral DNA replication. IE63 regulates gene expression at the level of transcriptional and post-transcriptional control. Transcriptionally it can act on its own or with IE175; post-transcriptionally IE63 inhibits splicing, redistributes

splicing factors, increases RNA 3' processing at weak virus poly (A) sites, binds RNA and shuttles between the nucleus and the cytoplasm.

Presented here are the results of co-immunoprecipitation and fusion protein pull down assays, which confirm that IE63 interacts with four cellular proteins, namely: heterogeneous ribonucleoprotein K (hnRNP K), casein kinase 2 (CK2), p32 and splicing associated protein 145 (SAP145). Further, hnRNP K, CK2 and either p32 or SAP145 are complexed together only in the presence of IE63, and interaction of IE63 with p32 or SAP145 excluded interaction with the other. Immunofluoresence demonstrated that IE63 causes p32 to redistribute from the cytoplasm to the nucleus, and that SAP145 and IE63 co-localise in punctate spots in the nucleus. Interestingly, when CK2 was co-immunoprecipitated with IE63 monoclonal antibody, this activity could phosphorylate other proteins in the co-immunoprecipitated complex including IE63, hnRNP K and p32. Phosphorylation of IE63 by CK2 increased the affinity of IE63 for hnRNP K.

A fifth protein was present in the complex. The viral thymidine kinase (vTK) was identified by Laser Mass Map Spectroscopy, and confirmed to be associated with the IE63 complex by Western blotting of the IE63 co-immunoprecipitates, and fusion protein pull downs. Whether IE63 interacts directly with vTK or indirectly as a consequence of its interaction with one of IE63's partners remains to be determined.

How these various interactions contribute to IE63 function can be inferred from the known roles for each interacting protein; but their identification points to IE63, like a growing number of proteins involved in gene regulation, being a multifunctional protein which interacts with several proteins to carry out its functions.

Interaction with transcription factors, hnRNP K and possibly p32, may account for IE63s transcriptional activity acting to enhance L gene expression and repress that of viral IE and E genes. However most of the IE63 activity is seen at the post-transcriptional level.

Inhibition of splicing (via p32 and/or SAP145), probably blocks expression of cellular genes and may also allow viral transcripts to bypass the splicing machinery before export to the cytoplasm. The redistribution of splicing factors in infected cell nuclei is most likely a reflection of the build up of viral transcripts at inactive splice sites.

Export of viral mRNA serves to enhance viral gene expression and may occur via interaction with hnRNP K or p32, alternatively interaction with these proteins may block cellular mRNA export allowing viral mRNA to saturate the export pathways. IE63 almost certainly uses more than one route to facilitate export of viral mRNA.

The affect of IE63 on viral DNA replication is likely to reflect its requirement for expression of DNA replication factors, but conceivably there could be a role for the IE63-TK interaction here.

Multiple interactions by IE63 may be facilitated by its ability to oligomerise enabling the formation of large complexes of proteins. Moreover, the particular patterns of protein:protein interaction may be dynamic, changing at different intracellular locations or times post-infection to facilitate the various functions of IE63.

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This thesis is dedicated to my Mum, Dad and Sister,
for always believing in me
and supporting me through my long education.

Contents

List of Tables and Figures

(Page numbers refer to the page previous to that containing the figure.)

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

Appendix 1

Abbreviations

^{32}P	phosphorus-32 radioisotope
^{35}S	sulphur-35 radioisotope
A	adenine or absorbance or amps
aa	amino acid
ab	antibody
APS	ammonium persulphate
ASF/SF2	alternative splicing factor / splicing factor 2
α-TIF	alpha trans-inducing factor
ATP	adenosine-5'-triphosphate
BHK	baby hamster kidney
bp	base pair
BPS	branch point sequence
BSA	bovine serum albumin
C	cytosine or carboxy (-terminal end of protein)
CAT	chloramphenicol acetyl transferase
Ci	Curie
CIP	calf intestinal phosphatase
CK2	casein kinase 2
CNS	central nervous system
CPSF	cleavage/polyadenylation specificity factor
CstF	cleavage stimulation factor
DNA	deoxyribonucleic acid
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
DTT	dithiothreitol
E	early
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
EDTA	Ethylenediaminetetra-acetic acid
FITC	fluorescein isothiocyanate
g	gram or glycoprotein

G	guanine
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 ribonuclear protein
HPV	human papilloma virus
HSV-1 / 2	herpes simplex virus type 1/ type 2
HVS	herpes virus saimiri
ICG	interchromatin granules
ICP	infected cell protein
IE	immediate early
Ig	immunoglobulin
IPTG	isopropyl thiogalactopyranoside
kb	kilo-bases
kDa	kilo-daltons
KH	hnRNP K homology
KNS	hnRNP K nuclear shuttling signal
l	litre
L	late
LAT	latency associated transcript
LBR	lamin B receptor
LMB	leptomycin B
LOX	erythroid 15-lipoxygenase
LTR	long terminal repeat
M	molar
m	milli
Mab	monoclonal antibody

MBP	maltose binding protein
MHC	major histocompatibility complex
mi	mock infected
min	minutes
MOI	multiplicity of infection
mRNA	messenger RNA
n	nano
N	amino (-terminal end of protein)
NES	nuclear export signal
NLS	nuclear localisation signal
NP40	Nonidet 40
NPC	nuclear pore complex
NS	non-structural
OD	optical density
ORF	open reading frame
ori	origin of replication
p	pico
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PF	perichromatin fibrils
pfu	plaque forming unit
PI	phosphatase inhibitors
PMSF	phenylmethylsuphonyl fluoride
pre-mRNA	precursor mRNA
PVDF	Polyvinylidene difluoride
RE	restriction endonuclease
RGG	arginine rich RNA binding motif
RNA	ribonucleic acid
RNA PolII/III	RNA polymerase II/III
RNP	ribonucleoprotein
RRE	HIV-1 Rev-response element
RT	room temperature
SAP	splicing associated protein

SDS	sodium dodecyl sulphate
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein particle
SV40	simian virus 40
T	thymine
TBP	TATA binding protein
TE	Tris EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
TF	transcription factor
TRITC	tetramethylrhodamine isothiocyanate
μ	micro
U	uridine or units
U2AF	U2 associated factor
U_L	unique long domain
U_S	unique short domain
UV	ultra-violet
V	volts
v/cTK	viral/cellular thymidine kinase
VHS	virion associated host shut off function
Vmw	apparent weight of virion polypeptide
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	Cystiene
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

Chapter 1: Introduction

Part A. The *Herpesviridae*

1A1 The family *Herpesviridae*

1A1.1 Herpesviruses are highly dispersed in nature

Nearly 100 herpesviruses have been described and most animal species including fish, amphibians, reptiles, birds and especially mammals, including cattle, pigs and man, have yielded at least one herpesvirus (Roizman, 1993). Herpesviruses of humans, their properties and the diseases they cause are shown in Table 1A1.1.

1A1.2 Definition and distinctive structural features

Herpes virions vary considerably in size from 120nm to 300nm in diameter, but all have a similar design. Classification as a member of the *Herpesviridae* is based on the architecture of the virion (Fig 1A1.2). All herpes virions consist of a core containing a double stranded linear DNA genome encased in an icosohedral capsid (Wildy *et al.*, 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 (1993).

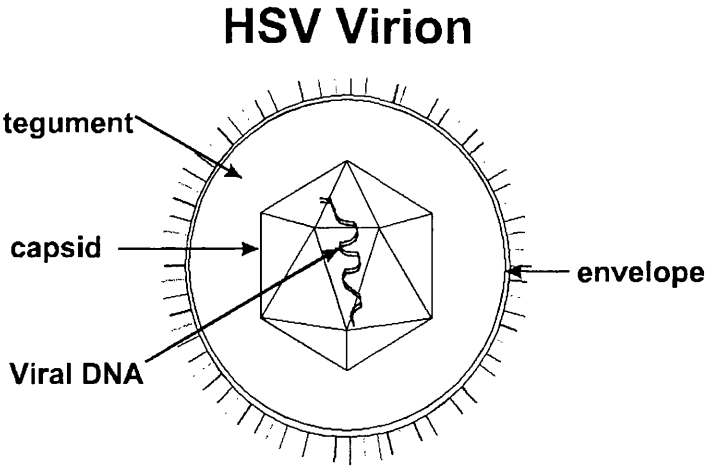
Most investigations of virion structure have been carried out with herpes simplex virus type 1 (HSV-1), but the morphology of all herpesviruses is similar. Of the 162 capsomeres making up the icosohedral capsid, 150 are hexameric and 12 are pentameric. The hexomeres occupy the faces and the edges of the capsid, while the pentamers are positioned at the vertices (Schrag *et al.*, 1989). Studies of HSV-1 capsid assembly have become more amenable with the development of an *in vitro* assembly system (Newcomb *et al.*, 1994) and a recombinant baculovirus assembly system in insect cells (Tatman *et al.*, 1994). In HSV-1, the hexons in the capsid are made from six molecules of the major capsid protein VP5 and the vertex protein VP26, the pentons are composed of VP5. A triplex

Table 1A1.1 Human herpesviruses

Common name	Subfamily	Group	G+C %	Size (kbp)	Common disease/symptoms
HSV-1	α	E	68.3	152	cold sores + other occasional symptoms
HSV-2	α	E	69	152	genital lesions
VZV	α	D	46	125	chicken pox/ shingles
EBV	γ	C	60	172	infectious mononucleosis (glandular fever), Burkitt's lymphoma + other cancers.
HCMV	β	E	57	229	mononucleosis
HHV-6	β	A	42	162	roseda
HHV-7	β	A	45	145	none known
HHV-8	γ	B	53	140.5	Kaposi's sarcoma

Abbreviations: Human herpesviruses (HHV), herpes simplex virus (HSV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), human cytomegalovirus (HCMV). Group refers to classification based on reiterated genome sequences (see Fig. 1A1.3)

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.

Taken from (Clements & Brown, 1997)

structure made up of VP19 and VP23 co-ordinates the hexons in 3-fold symmetry (Rixon, 1993; Zhou *et al.*, 1994).

The virion tegument structure and the process by which it is acquired is poorly understood. Viral structural proteins are assigned to the tegument if they are known not to be components of the capsid or envelope (Rixon, 1993). It is clear that while purified herpes DNA is capable of initiating infection, certain tegument proteins can influence the process of infection; for example, the α-*trans* inducing factor (α–TIF, VP16 or U_L48) and a virion associated host shut-off function (VHS or U_L41) (Batterson & Roizman, 1983; Read & Frenkel, 1983).

The virion envelope has a trilaminar appearance in electron micrographs (Epstein, 1962) and appears to be derived from patches of altered host cell membrane (Morgan *et al.*, 1959). The glycoprotein spikes contained in the virion envelope are approximately 8nm long and vary in number and relative amount between different herpesviruses, HSV-1 encodes at least eleven. Some have been implicated in the early stages of infection e.g. gB, gC, gD, gH, and gL. These and others also appear to have roles in immune modulation and in preventing secondary infection of cells (reviewed by Spear, 1993).

Herpesvirus DNA within the capsid is densely packaged in a liquid crystalline, phage-like manner as found with double-stranded DNA phage (Booy *et al.*, 1991).

1A1.3 Biological properties

Four significant biological properties are shared by all herpesviruses.

(i) A large array of virally encoded enzymes are 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 their natural hosts.

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

1A1.4 Classification and nomenclature of herpesviruses

Current classification

Current classification is based upon biological properties and splits the *Herpesviridae* family into three sub-families (alpha, beta and gamma) (Roizman *et al.*, 1981).

Alphaherpesvirinae

e.g. HSV-1, and varicella-zoster virus (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.

Betaherpesvirinae

e.g. human cytomegalovirus (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 secretory glands, lymphoreticular cells, kidneys and other tissues.

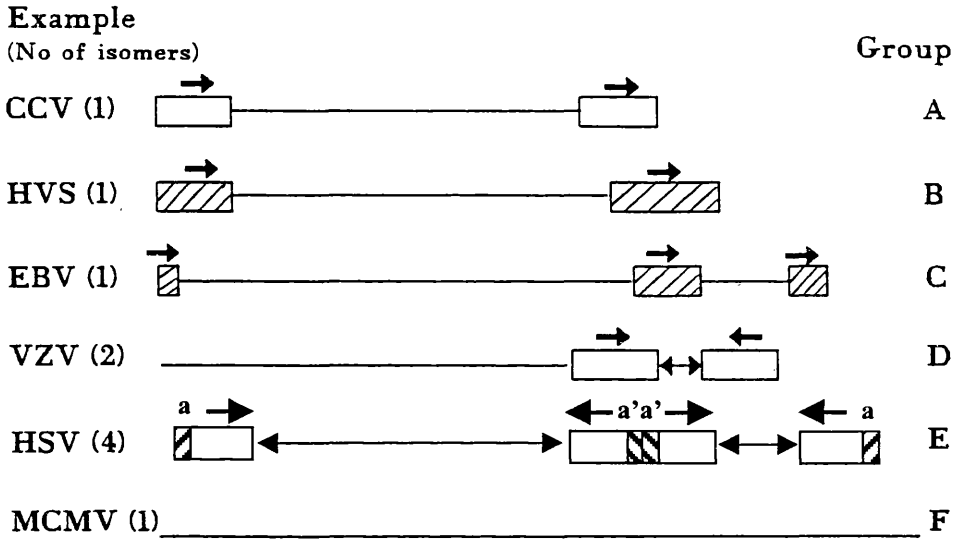
Gammaherpesvirinae

e.g. Epstein-Barr virus (EBV)
Experimentally, members of this family can only infect the same family or order to which the natural host belongs. They replicate in lymphoblastoid cells *in vitro*, and can infect some epithelial and fibroblastic cells. Latency often occurs in the lymphoid tissue. Viruses are specific for either B or T lymphocytes where lytic or latent infection is frequently found without production of infectious progeny.

Alternative Classification

The current classification is based on biological properties of the viruses; With the availability of complete nucleotide sequences for several herpesviruses it is now possible to review their classification. Classifications based on conservation of genes and gene clusters and the arrangement of these clusters relative to each other, the arrangement of terminal sequences (see Fig 1A1.4) or the presence and distribution of nucleotides which are subject to methylation have been suggested.

Fig. 1A1.5 Schematic diagram of the sequence arrangements in the six classes of *Herpesviridae*



Single lines represent unique sequences and lines with arrowheads show the sequence inversions. Repeats of >1kb are represented by open boxes and small reiterated sequences are represented by lined boxes. Arrows above boxes denote whether repeats are direct or inverted. The relative positioning of "a" sequences are indicated. See text for details.

Abbreviations: CCV: channel catfish virus, HVS: herpesvirus saimiri, EBV: Epstein-Barr virus, VZV: varicella-zoster virus, HSV: herpes simplex virus, MCMV: murine cytomegalovirus

Fortuitously, reclassification based on sequence data puts most viruses into the same subfamilies as biological properties do. Exceptions are Marek's disease virus, biologically like a gammaherpesvirus but based upon conservation and co-linearity of gene clusters, considered an alphaherpesvirus (Buckmaster *et al.*, 1988), and HHV-6, biologically a gammaherpesvirus but by sequence analysis it bears homology to a betaherpesvirus (Lindquester *et al.*, 1997).

It is debatable whether classification based purely on genes has a value if this does not reflect important and obvious biological differences, especially while the genomic sequence of all the *Herpesviridae* is not known.

1A1.5 Herpesvirus DNAs

Structure

When extracted from virions, herpesvirus DNAs are double-stranded linear molecules, which can circularise immediately upon release into host cell nuclei. Between different herpesviruses, DNA size and base composition can vary. DNA size ranges between 120 kbp to 230 kbp, base composition varies from 31 % G+C to 75% G+C. Variation in the genome size of any one herpesvirus seems to be minimal but significant, terminal and internal repeated sequences and spontaneous deletions both contribute to variation (Roizman, 1996).

Sequence arrangement in herpesvirus DNAs

Variation in the sequence arrangement of herpesvirus DNAs with respect to the presence and location of reiterated sequences is an interesting feature which can be used to split the viruses into six groups (Davison, 1993; Roizman, 1996); however, these do not correlate with evolutionary relatedness of the viruses. The genome arrangements of herpesviruses are illustrated in Fig. 1A1.4.

Genomes which contain a single unique region which is flanked by direct repeats are designated as group A genomes. Channel catfish virus has a genome of this type.

Group B genomes have multiple copies of a terminal repeat sequence at their termini. Group B genomes include the genome of herpesvirus saimiri (HVS).

EBV has a characteristic group C genome having a set of internal repeats which are unrelated to the terminal repeats.

Group D genomes have two unique regions which are flanked by inverted repeats. The repeats are not related and the repeat flanking the unique long region (U_L) is significantly shorter than that flanking the short unique region (U_S). This leads to two genomic orientations of U_S but one (either completely or predominantly) for U_L. VZV has a characteristic group D genome structure.

Group E is similar to group D except that the inverted repeats flanking the U_L region are larger and an additional repeat sequence known as the "a" sequence is found at the termini and at the junction between the L and S repeats. The presence of a larger U_L flanking repeat also means that the U_L region is freely invertable leading to the occurrence of four equimolar genomic isomers. HSV-1 and HSV-2 are examples of herpesviruses with group E genomes.

Group F genomes consist of a single unique sequence which lacks either internal or terminal repeat regions. The genome of the tree shrew herpesvirus is characteristic of this group of genomes.

1A1.2 Herpes Simplex Viruses - General features

1A2.1 Definition and serotypes

The two herpes simplex virus serotypes (HSV-1 and HSV-2) belong to the subfamily *Alphaherpesvirinae* and the genus *Simplexvirus*. As such, they are typical alphaherpesviruses, displaying structural (Fig. 1A1.2) and biological properties as detailed above (section 1A1.3). HSV-1 and HSV-2 (Dolan *et al.* 1998) DNAs share 47% - 50% base sequence homology and their genetic maps are largely co-linear. They differ in restriction endonuclease (RE) cleavage sites and apparent size of viral proteins (Roizman & Sears, 1996). Antigenic differences can be detected (Roizman *et al.*, 1984) and distinctive biological markers include virus titres, plaque size and growth in cultured cells.

Due to occasional base substitutions/deletions resulting in changes in RE sites or amino acid sequence and to variability in certain repeated sequences, epidemiologically unrelated isolates of the same HSV serotype are not identical (Chou & Roizman, 1990). Intratypical differences can be detected with monoclonal antibodies and isolates may differ in neurovirulence. However, individual strains are stable during culture *in vitro*. This property has an important diagnostic use and has been used to examine modes of transmission and epidemiology of HSV-1 and HSV-2 in human populations (Sakaoka *et al.*, 1994). Work in this thesis considers infection with the HSV-1 Glasgow isolate strain $17syn^+$ (Brown *et al.*, 1973).

1A2.2 Epidemiology, transmission, pathology and clinical features.

The natural host of HSV-1 is humans. Primary infection usually occurs in the mucosa of the mouth or throat, although HSV-1 is also capable of infecting the mucosa of the genital tract. Primary infection is usually asymptomatic but can lead to illness which is characterised by lesions in the mouth and throat, fever and a general malaise. Following replication at the site of primary infection, virus is transported by neurones to dorsal root ganglia where latency is established (Rock, 1993). Replication in the ganglia can, infrequently, lead to life threatening central nervous system (CNS) infection (Olson *et al.*, 1967) but latency generally predominates. Reactivation from latency is often associated with stress, fever, exposure to UV light, tissue damage and/or immunosuppression. Reactivated virus infection usually results in lesions of the skin in the area served by the trigeminal ganglia e.g. herpes labialis (cold sores). HSV-1 infection can also result in encephalitis, although this is usually restricted to neonatal and immuno-compromised individuals. Other rare complications can include keratitis and a disseminated infection involving organs such as the liver and adrenal glands. (Whitley, 1996).

HSV-1 infections occur world wide, all year round. In the developing world and lower socio-economic groups of the developed world some 30% of the population have sero-converted by the age of five and 70-90% by the end of adolescence. In

more affluent groups in the developed world this is delayed and sero-prevalence by 30 years of age is around 60% (Wentworth & Alexander, 1971).

1A2.3 Host immune responses

Host response to HSV-1 occurs in two phases, a non-specific containment phase and a later HSV-1 specific effector phase. Host genetic background, macrophages, natural killer cells, specific T cell sub-populations, specific antibodies and lympokine responses have been implicated as important factors in host defences against HSV-1 infections (Lopez *et al.*, 1993). The relative contribution of each depends on the experimental model system and route of inoculation.

1A2.4 Prevention and therapy

Although various vaccines against HSV-1 recurrent and primary infection have been developed (Burke, 1993) none manage to protect against cutaneous disease, neither can any known agent protect from latency. Goals for treatment of HSV-1 disease are therefore directed at reducing the time to resolve clinical symptoms, reducing the likelihood and severity of complications and reducing the time of virus shedding and therefore the likelihood of transmission.

The only non-cytotoxic agent currently available for the treatment of HSV-1 infection is acyclovir (Schaffer *et al.*, 1979), available in topical, intravenous and oral formulations. Acyclovir is phosphorylated by HSV thymidine kinase (vTK) at a rate 10^6 fold faster than by the cellular enzyme. Furthermore, acyclovir triphosphate, the active form of the drug, is recognised by HSV DNA polymerase much more readily than by the host cell polymerase. Acylovir triphosphate blocks DNA synthesis by acting as an inhibitor of polymerase activity as well as being its substrate, becoming incorporated into the growing DNA chain and causing termination of chain growth. As DNA synthesis is required for acyclovir to function, the drug cannot destroy the virus during the latent period.

1A3 HSV-1 Molecular Biology

1A3.2 The HSV-1 genome

The HSV-1 genome is 152 kbp (McGeoch *et al.*, 1986, McGeoch *et al.*, 1988, Perry & McGeoch, 1988). Like all herpes DNAs it comprises of linear, double stranded DNA (Becker *et al.*, 1968) but has an unusually high G+C content (McGeoch *et al.*, 1986, McGeoch *et al.*, 1988, Perry & McGeoch, 1988). It encodes at least 77 genes, 59 mapping to the U_L region, 13 to the U_S region and two copies of four genes to the repeated sequences. The locations of the IE genes and the latency associated transcripts (LATs) are shown in Fig. 1A3.2. The DNA arrangement for HSV-1 genome and its repeated sequences is shown in Fig. 1A1.3.

1A3.3 Viral life cycle and particles

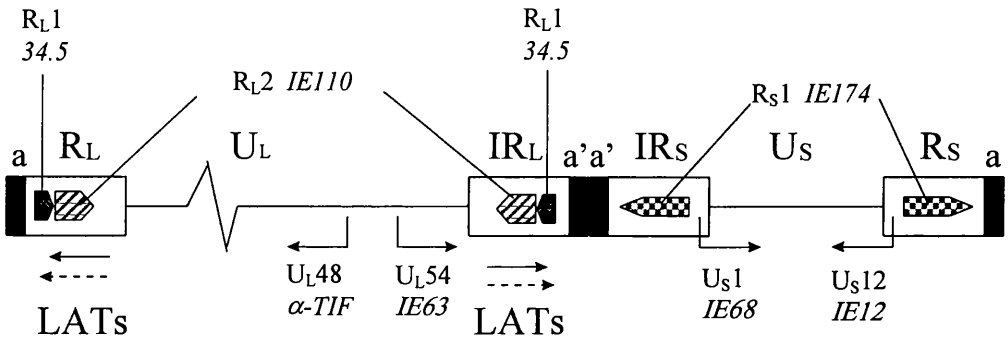
The viral life cycle is summarised in Fig. 1A3.3 (as reviewed in Roizman & Sears, 1996). From initial infection to final viral release takes approximately 18 h -20 h in fully permissive tissue culture cells. Infected cells produce three types of HSV-related particle, but only one, the virion, is capable of infecting another cell. L-particles (Szilagyi & Cunningham, 1991) lack the nucleocapsid and viral DNA, hence are non-infectious. Pre-DNA replication or PREP particles (Dargan *et al.*, 1995) are synthesised when DNA synthesis is blocked; they are similar to L particles but lack true late proteins or only possess them in minute quantities.

1A3.4 Latency and reactivation

Following primary infection, the virus is transported via retrograde axonal transport (Kristensson *et al.*, 1986) to the cell bodies of sensory neurones within the dorsal root ganglia. Here latent infection can be established. Subsequently in response to external stimuli reactivation occurs and virus passes down the axons to replicate at or near the initial site of infection.

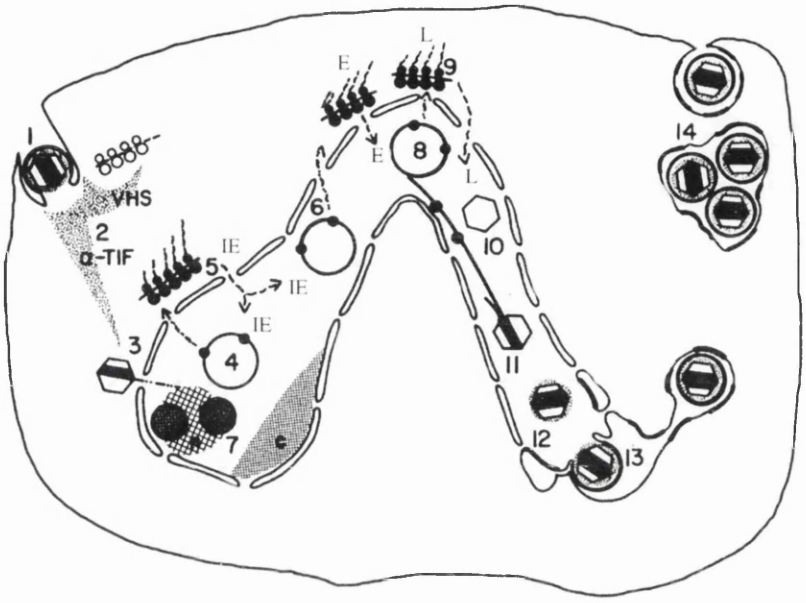
Latency can be divided into 3 stages, viz. establishment, maintenance and reactivation (reviewed in Rock, 1993).

Fig. 1A3.2 Location of Immediate Early (IE) and Latency Associated Transcripts (LATs) on HSV-1 genome



U_S and U_L are unique regions, R_L and R_S, IR_L and IR_S are reiterated inverted repeated sequences see Fig. 1A1.3. Genes are shown in plain print proteins encoded are shown in italics.

Fig.1A3.3 Schematic representation of HSV-1 replication in susceptible cells



1. The virus initiates infection by fusion of the viral envelope with the plasma membrane following attachment to the cell surface. **2.** Fusion of the membranes releases two proteins from the virion. VHS shuts off protein synthesis and α-TIF is transported to the cell nucleus. **3.** The capsid is transported to the nuclear pore where viral DNA is released into the nucleus and immediately circularises. **4.** The transcription of IE genes by RNA polymerase II is induced by α-TIF. **5.** The five α mRNAs are transported to the cytoplasm, translated and the proteins brought back to the nucleus, where they regulate E and L gene expression. **6.** A new round of transcription results in the synthesis of E proteins. **7.** At this stage in infection host cell chromatin (c) is degraded and displaced to the nuclear membrane and nucleoli become disaggregated. **8.** Viral replication is by a rolling circle mechanism, which yields head-to-tail concatemers of viral DNA. **9.** A new round of transcription/translation yields the L proteins, consisting primarily of structural proteins. **10.** The capsid proteins form procapsids. **11.** Unit length viral DNA is cleaved and packaged into the preformed capsids. **12.** Capsids containing viral DNA acquire a new protein. **13.** Viral glycoproteins and tegument proteins accumulate and form patches in cellular membranes. The capsids containing DNA attach to the underside of the membrane patches and are enveloped. **14.** The enveloped proteins accumulate in the endoplasmic recticulum and are transported to the extracellular space.
Taken from (Roizman & Sears, 1996).

Establishment

It is not clear how latency is established but there are two hypotheses,

(i) Neurones harbouring latent virus lack host factors required for transcription of viral genes

(ii) Or, within the infected neurone, a specific viral gene product arrests the viral gene expression cascade, thus leading to establishment.

Maintenance

Latent HSV genomes are maintained in a non-integrated, non-linear, endless form and have a nucleosomal structure similar to cellular chromatin (Rock & Fraser, 1983). DNA copy number in neurones is estimated to be between 10-100 (Roizman & Sears, 1987). Only a single region of the genome is transcribed into LAT RNAs, (Stevens *et al.*, 1988). These are depicted in Fig. 1A3.2. The long 8.3 kb LAT is anti-sense to the genes for ICP34.5 and IE110, the latter is a gene which may be required for reactivation from latency (Leib *et al.*, 1989a). The relevance of the LAT transcripts is not clear as mutants in these regions show that LAT RNA expression is not essential for the establishment and maintenance of latency (Sederati *et al.*, 1989, Steiner *et al.*, 1989).

Reactivation

The molecular basis for reactivation is poorly understood but viral proteins IE110 and IE65 as well as cellular proteins Oct1 and Brn3 have been implicated (Rock, 1993). Infectious virus particles are carried back to peripheral tissues by axonal transport (Cook & Stevens, 1973). LATs have also been reported to be involved in reactivation (Leib *et al.*, 1989b) but again mutants not expressing LAT RNAs can be seen to reactivate (Rodahl & Stevens, 1992). Reactivation takes 3-5 days and is triggered by stimuli such as sunlight, stress and possibly hormonal irregularities (Whitley, 1996).

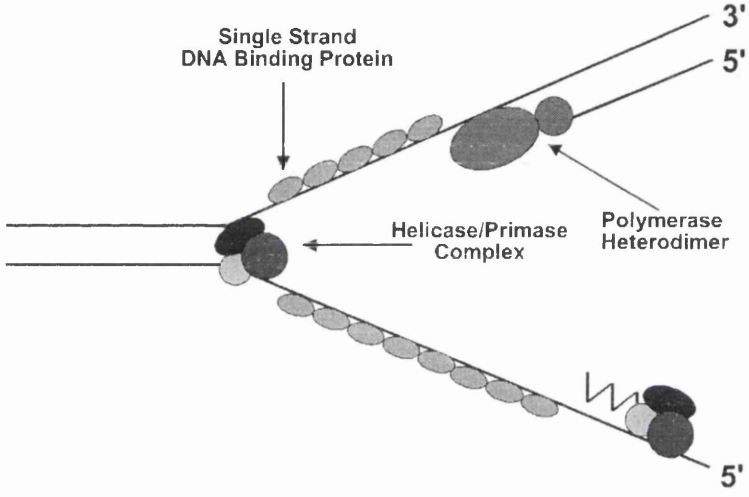
1A3.5 HSV-1 DNA replication

DNA replication functions are conserved throughout the alphaherpesviruses. For HSV-1, the complete set of seven viral genes required for replication is known (Challberg, 1991) and replication of a HSV-1 replication origin containing plasmid can be reconstituted using these proteins expressed from baculovirus vectors (Stow, 1992). A diagram showing the functions of these proteins is shown in Fig. 1A3.5. It is not known if additional host cell factors are required; however, two functions essential for cellular DNA replication, DNA topoisomerase and DNA ligase, are not encoded by HSV-1.

Viral replication typically peaks between 10 h and 20 h post-infection. It is initiated from one of three origins (ori) of replication via the binding to one of them of the ori binding protein U_L9. The helicase/primase complex (U_L5, U_L 8 and U_L 52 gene products) subsequently associates with the U_L9/ori complex and the hydrolysis of ATP creates an initiation bubble which permits association of the DNA polymerase/DNA binding protein complex (U_L30/U_L42). DNA synthesis is continuous along one strand and discontinuous along the lagging strand with the major DNA binding protein U_L29 maintaining the growing forks. Other virally encoded enzymes are also involved in nucleic acid metabolism; e.g. vTK, ribonucleotide reductase, uracil-DNA glycosylase and dUTPase (reviewed in Roizman & Sears, 1996).

Replication occurs by a rolling circle mechanism, head to tail DNA concatemers accumulate in the nucleus, at specific sites defined by host cell nuclear architecture termed viral replication sites (de Bruyn Kops & Knipe, 1994). If viral replication is blocked, the replication proteins form a punctate pattern of sites called viral pre-replicative sites (Quinlan *et al.*, 1984). Host cell replication factors co-localise with viral pre-replicative sites, which appear to be sites of assembly for viral replication factors, that under the correct conditions can progress to being active replication sites. These are dynamic structures whose patterns are reversible depending on the status of viral replication (de Bruyn Kops & Knipe, 1994). The predefined sites where viral replication foci form are suggested to correlate with ND10 sites, which are occupied by the regulatory cellular proteins PML, Sp100 and NDP55 (Maul *et al.*, 1996).

Fig. 1A3.5 Virus encoded functions directly required for HSV DNA replication



Single stranded binding protein is U_L29, the helicase/primase complex comprises $U_L5/U_L8/U_L52$, and polymerase is a dimer of U_L30/U_L42.

Taken from (Clements & Brown, 1997).

In uninfected cells, cellular RNA transcription and DNA replication have been shown to occur at the same nuclear location (Zhang *et al.*, 1994) and RNA polymerases have been implicated in the initiation of replication (Hassan & Cook, 1994).When the spatial relationship between HSV-1 replication and transcription is analysed using methods which label DNA and RNA synthesis *in vivo* it is found that viral DNA replication and transcription sites co-localise at early times in infection, with splicing machinery being largely removed from these sites. At later times more replication sites develop and while some sites have both transcription and replication occurring concurrently, others are committed solely to DNA replication (Phelan *et al.*, 1997). It is suggested that the initial number of replication/transcription sites is restricted due to the number of pre-existing ND10 sites (Maul *et al.*, 1996) and that the additional replication sites represent sites where the replicated DNA genome is in turn replicated.

1A4 HSV-1 proteins

1A4.1 General features and classification

HSV-1 expresses more than 70 distinct proteins during lytic infection; only a minority of these (30-35) are absolutely required for virus replication in cultured cells. The remainder function in a number of ways to increase host range and tissue tropism, replication and plaquing efficiency, and to control or subvert the host response to infection. Due to their high G+C content, HSV-1 proteins are often proline rich (typically 10% -14%) and thus migrate anomalously slowly in SDS-polyacrylamide gel electrophoresis (SDS-PAGE). In addition, they tend to be rich in basic amino acids (aa). It is not known if either of these characteristics has a role in HSV-1 pathogenesis.

Herpesvirus genes (and hence the corresponding proteins) can be classified into at least three regulatory classes by the kinetics of their expression and their requirement for DNA replication (Honess & Roizman, 1974; Honess & Roizman, 1975; Clements *et al.*, 1977). Immediate early (IE) (also known as α) genes can be transcribed in the absence of *de novo* viral protein synthesis and DNA replication, their expression peaks around 2 h – 4 h post-infection. IE proteins regulate expression of early and late genes. Early (E) (also known as β) genes are expressed soon after IE and peak expression is at 5 h – 7 h post-infection. E

proteins are involved in DNA metabolism and replication, their expression signals the start of viral DNA synthesis. Viral DNA synthesis is the signal for late (L) (also known as γ) gene expression. L genes can be further divided into two classes based on the stringency of their requirement for DNA synthesis. The leaky L (γ_1) class of genes are expressed (all be it at low levels) prior to DNA replication but the strict L (γ_2) genes are only expressed once viral replication has commenced. L proteins generally are structural components of the virion.

1A4.2 Protein modifications

The majority of HSV-1 proteins examined to data are post-translationally modified. These modifications include cleavage (e.g. U_L26), phosphorylation (e.g. IE63 and IE175), sulphation (glycoproteins), glycosylation (glycoproteins), myristylation (U_L11), poly(ADP)-ribosylation (IE175, U_L18) and nucleotidylation (IE175, IE68, IE63) (Roizman & Sears, 1996).

1A4.3 Protein Nomenclature

A number of systems have been used to describe the proteins of HSV-1. Namely,

(i) The order of the open reading frames (ORFS) occurring in various regions of the genome e.g. U_L19 which corresponds the protein encoded by ORF19 in the U_L region of the genome.

(ii) A system based on protein function e.g. ribonucleotide reductase.

(iii) The Glasgow system, based on the apparent molecular weight of the protein as determined by the migration of viral proteins on an SDS-PAGE. The kinetics of expression of that particular protein can then be included in its name e.g. a protein expressed with IE kinetics and migrating with an apparent molecular weight of 63 kDa, is called IE63.

(iv) The Chicago or Infected Cell Protein (ICP) system. This is distinct and represents a number corresponding to location on SDS-PAGE rather than the actual molecular weight of the protein e.g. ICP27 which corresponds to IE63.

Table 1A4.3 Glasgow and Chicago nomenclature for HSV-1 gene products expressed at immediate early times in infection

GLASGOW	CHICAGO
IE110	ICP0
IE175	ICP4
IE68	ICP22
IE63	ICP27
IE12	ICP47

In this thesis, the Glasgow system will be used to refer to IE proteins. Table 1A4.3 shows the corresponding Chicago system nomenclature for the IE genes. The majority of other proteins have or will be referred to on the basis of their ORF location or function. The exception to this are certain virion polypeptides (VP) which are numbered in their order as separated on SDS-PAGE. e.g. VP16 is 16th virus protein from the top of the gel obtained from purified virions.

1A5 HSV-1 Transcription and mRNAs

1A5.1 Transcripts

HSV-1 genes are transcribed by the host cell RNA polymerase II (RNA Pol II) (Costanzo *et al.*, 1977). Locations of the IE genes on the HSV-1 genome can be seen in Fig. 1A1.3. Viral mRNAs are capped, methylated and polyadenylated, methylation is more common in E transcripts than L ones (Hann *et al.*, 1998), alternative polyadenylation may be a mechanism of regulating gene expression (McGregor *et al.*, 1996). Only four viral transcripts IE110, IE68, IE12 and U_L15 are spliced. Transcripts sharing 5' and particularly 3' termini have been described (Wagner, 1985) and several genes appear to have multiple initiation sites e.g. (Murchie & McGeoch, 1982).

The abundance and stability of HSV-1 mRNAs varies but in general mRNAs of IE and E genes appear to be more stable than those of L genes (Roizman & Sears, 1996).

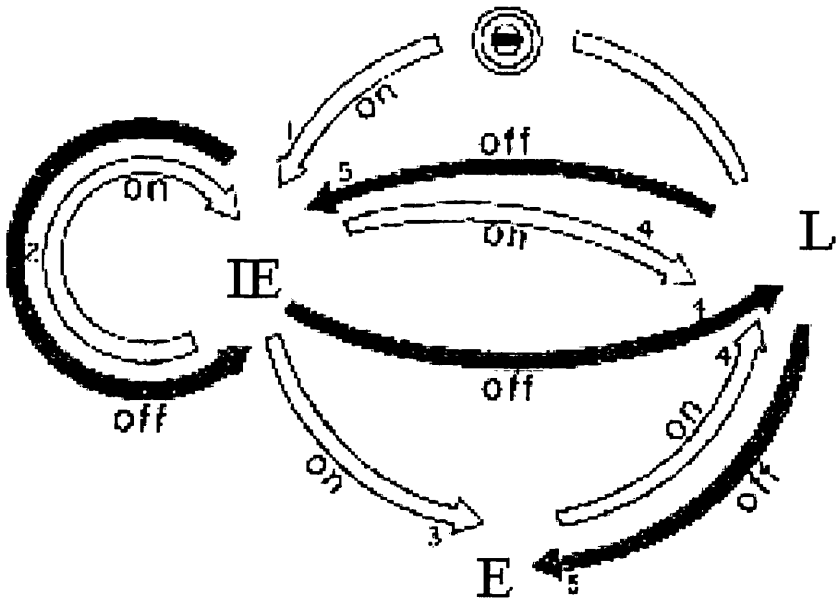
1A5.2 Regulation of gene expression

Regulation of gene expression is represented in Fig. 1A5.2a.

Induction of IE gene expression

Expression of IE genes can proceed without *de novo* viral protein synthesis, and is stimulated by the action of the virion protein α–TIF (also known as Vmw 65 or VP16). Upstream of IE promoters is a TAATGART sequence which is the *cis* acting site for α–TIF-driven expression of IE genes. The cellular factor Oct-1

Fig. 1A5.2a Schematic representation of HSV-1 gene expression



Open and filled arrows represent events in the reproductive cycle which turn gene expression on and off respectively. **1.** Turning on IE gene transcription by α-TIF packaged in the virion. **2.** Autoregulation of gene expression. **3.** Turning on E gene transcription. **4.** Turning on L gene transcription by IE and E gene products. **5.** Turning off IE and E gene expression by the products of L genes.
Taken from (Roizman & Sears, 1996)

binds to the *cis* acting site, a complex of α–TIF and another cellular protein (designated C1) then binds to the Oct-1/DNA complex and is stabilised by interactions between both Oct-1 and α–TIF and α–TIF and the GARAT sequence (reviewed in O'Hare, 1993). Binding of α-TIF to IE gene initiation sites is shown in Fig. 1A5.2b.

Regulatory functions of IE genes products

IE gene products are responsible for the regulated expression of E and L gene expression. IE175, IE110 and IE63 in particular are key to this.

IE175:

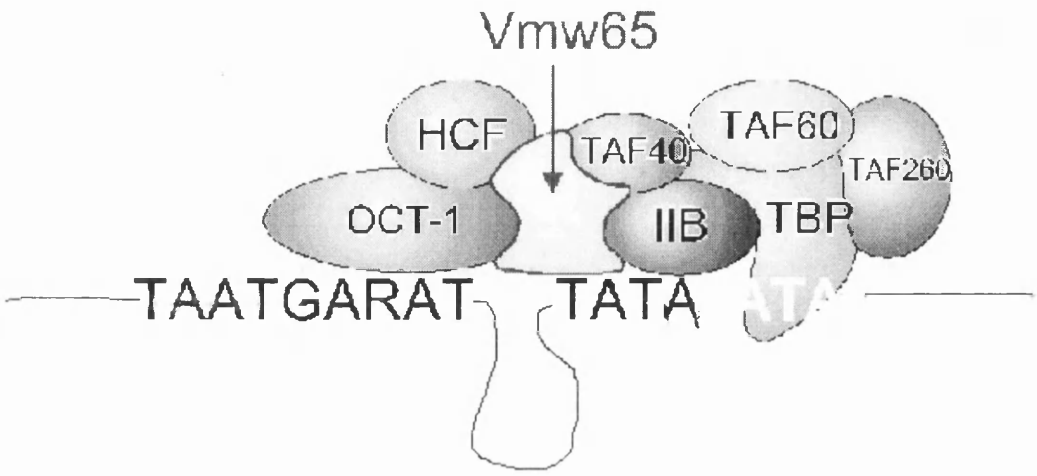
IE175 autoregulates itself, an activity which correlates with the binding of IE175 at its own transcription initiation site (Roberts *et al.*, 1988); consensus IE175 binding sites are also found in the promoter of IE110. IE175 may down regulate expression of IE110 by binding to its promoter (Faber & Wilcox, 1986).

IE175 is also strictly required for expression of E and L genes. This is taken to mean that it regulates them in a positive way. IE175 can bind to DNA, a strong consensus site of IE175 binding (Faber & Wilcox, 1986) is ATCGTCnnnnCnGnn (n is any nucleotide), but subsequent reports have revealed numerous HSV-1 binding sites which do not correspond to this consensus (Michael & Roizman, 1989).The various forms of IE175 differ in their affinity for DNA binding (Michael *et al.*, 1988). Dephosphorylated IE175 is reported to bind to IE promoters, while the phosphorylated form binds to E and L promoters (Papavassiliou *et al.*, 1991). Further evidence of IE175 involvement in transactivation of HSV-1 genes comes from its interaction with TATA binding protein and transcription factor IIB (TFIIB), both components of the RNA Pol II transcription complex (Smith *et al.*, 1993).

IE110:

IE110 is a non-essential protein but defects in this protein delay expression of E and L genes and impair viral replication (Chen & Silverstein, 1992). From transient expression experiments, IE110 is thought to enhance the activity of IE175, as well as possibly transactivating gene expression in its own right (Everett, 1984, O'Hare & Hayward, 1985, Quinlan & Knipe, 1985).

Fig. 1A5.2b Binding of α-TIF (Vmw65) to IE gene initiation sites.



Other proteins in the complex are the cellular factor Oct-1 (see text), host cell factor (HCF), transcription activating factors 40, 60 and 260 (TAF40, TAF60 and TAF260), TATA binding protein (TBP), and transcription factor IIB (IIB).
(Fig. produced by C. Loney, dept. Virology, Glasgow University.)

IE68:

IE68 appears to be dispensable to HSV-1 infection. Viral mutants in the gene for IE68, multiply in Vero and Hep-2 cells as effectively as the parent virus. However in BHK, RAT-1 cell lines and human embryonic lung cells the plating efficiency is reduced and yield is multiplicity dependent. Moreover in these cells E protein synthesis is delayed and the expression of L proteins and the number of capsids detected in the infected cells is reduced. Deletion of IE68 has no effect on viral DNA synthesis (Sears *et al.*, 1985).

IE63:

IE63 is discussed later in this thesis (1B8-1B12) but briefly, this essential IE protein activates and represses expression of viral IE and E proteins, enhances expression of viral L genes, represses expression of cellular genes and is required for viral DNA replication. The majority of its activities are thought to occur at the post-transcriptional level.

IE12:

This non-essential protein is not involved in the regulation of viral gene expression but down regulates the host immune response. Expression of sequences containing the IE12 gene cause the infected cell to be resistant to lysis by $CD8^+$ T cells, due to retention of the MHC class I molecules in the cytoplasm and a lack of peptide presentation at the cell surface (York *et al.*, 1994).

Induction of E genes

The sequence elements for expression of E genes consist of a a TATA box, a cap site (McKnight & Tijan, 1986), and binding sites for cellular factors, (for example the HSV-1 vTK gene contains binding sites for SP1 and CCAAT binding protein (CBP)), these sites are clustered upstream of the TATA box (Pande et al., 1998). Initiation of transcription requires both release from the repressed state and transactivation. Viral mutants in IE175 do not produce any E genes (Dixon & Schaffer, 1980; Watson & Clements, 1980); IE175 is almost certainly involved in

transactivation of E genes but the mechanism is not clear. The binding of IE175 to E genes requires cellular factors and is determined by both its concentration and phosphorylation (Papavassiliou *et al.*, 1991). It has been suggested that IE175 interacts with cellular factors both enhancing their binding and requiring them for its own stable DNA binding.

Induction of L genes

The structure of L genes is the least well understood and is likely to vary from gene to gene. Analyses of several genes have suggest that sequences required for efficient expression include the TATA box, and extend into the 5' transcribed non-coding domain (Dennis & Smiley, 1984, Everett & Dunlop, 1984, Guzowski & Wagner, 1993; Wagner *et.al*, 1998). Additional sequences downstream from the TATA box appear to be required by strict L genes (Greaves & O'Hare, 1989; Wagner *et.al*, 1998).

Embedded in the transcribed domains as well as in the 5' non-coding regions are IE175 binding sites (Michael *et al.*, 1988). The role of these sites is unclear as is IE175 involvement. In infected cells, IE175 is required but not sufficient for efficient and timely expression of L genes especially not of strict L genes.

One of the key observations in understanding expression of L genes has been the observation that deletions in the 5' transcribed non-coding domain of a L gene caused it to be expressed as an IE gene (Roizman & Sears, 1996). This can be explained in two ways; either the non-coding domain contains a site that allows binding of a transactivating repressor or the region may form a secondary structure which affects expression of L genes. Either way, the data suggests that the 5' transcribed region binds a repressor of some sort. This repressor must preclude expression of L genes under conditions which allow expression of IE and E genes and bind an activator which allows their expression in the absence of the repressor. As L gene expression can not occur in the absence of viral DNA synthesis, it has been suggested that replication removes the block and/or produces/modifies the transactivating factor. Alternatively removal of the block may be essential to DNA replication.

1A6 Virus effects on the host cell

1A6.1 Cytopathology

Within a few hours of HSV-1 infection, noticeable modifications of the host cell nucleus occur. Host chromatin condenses and is marginalised at the nuclear membrane. This is followed by accumulation of semi-crystalline arrays of empty viral capsids in the nucleus, leading to formation of macroscopic inclusion bodies. Other changes include re-duplication of the nuclear membrane as viral capsids associate with it, and the formation of excessive syncytia which occurs with many but not all virus strains (reviewed in Roizman & Sears, 1996).

1A6.2 Virion host shut-off function

A characteristic of HSV infection is the rapid shut-off of host macromolecular metabolism, host DNA synthesis is shut-off, host protein synthesis declines very quickly and glycosylation of host cell proteins ceases. Shut-off occurs in two phases. The first phase is the result of a virion protein activity, virion host shut-off (vhs), this activity is responsible for the destabilisation and degradation of both host and viral mRNA (Kwong & Kruper, 1988; Elgadi *et al.*, 1999; Karr & Read, 1999). The second function requires the presence of viral IE proteins such as IE68 and IE63 and is less well understood. Host shut-off confers two advantages; firstly, the host cell mRNA pool is quickly depleted allowing viral mRNAs to take over and secondly, destabilisation of viral mRNA allows rapid transition from gene expression of one class of protein to the next.

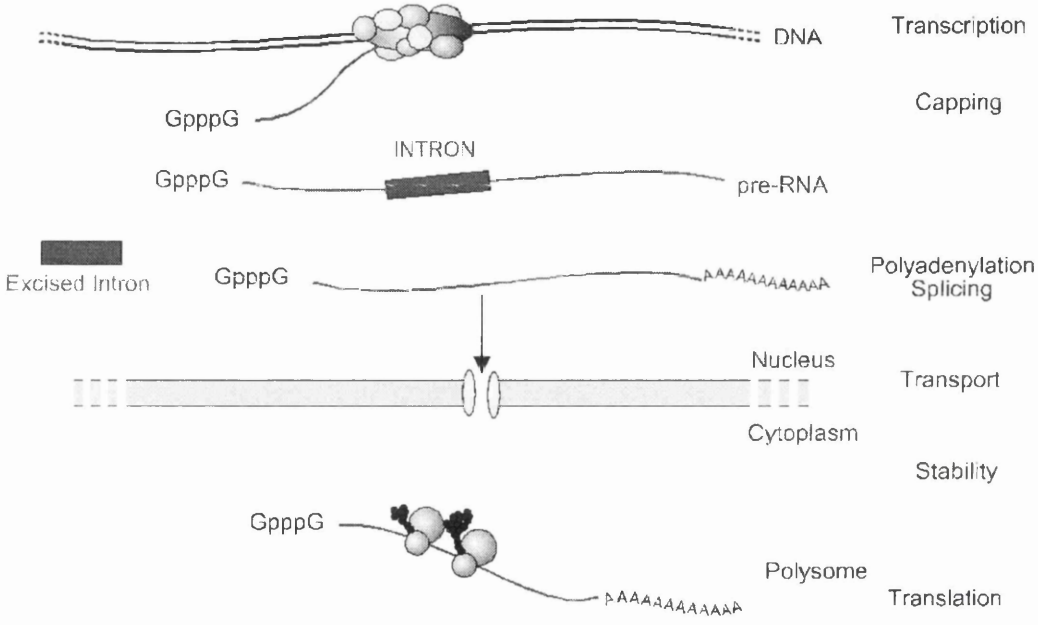
Chapter 1 Part B. From DNA to protein; IE63 can disrupt normal cellular function and plays a key role in viral gene expression.

Mammalian mRNA is transcribed from DNA as a pre-mRNA molecule. Before being translated into protein this pre-mRNA is processed. During processing, pre-mRNA generally is 5'capped (not discussed here), spliced and polyadenylated before being exported to the cytoplasm and translated into protein (Fig.1B). IE63 is known to carry out many of its viral and cellular gene regulatory functions via control of post-transcriptional processes, as well as by directly influencing transcription of viral and cellular genes. What follows is an overview of mammalian pre-mRNA processing focusing on the protein factors involved and then a description of the ways in which IE63 disrupts these processes. Regulation of transcription itself by IE63 is discussed and the functional domains of IE63 are described. Finally, post-transcriptional regulation of gene expression in several other virus systems is outlined.

1B1 hnRNPs play a key role in all stages of mRNA processing.

Newly transcribed mRNAs are associated with heterogeneous nuclear ribonuclear (hnRNP) proteins. As a family of at least 20 different proteins (Pinol-Roma *et al.*, 1988), they are located predominantly throughout the nucleus, although a subset can be detected in the cytoplasm (Dreyfuss *et al.*, 1984). hnRNP proteins remain associated with pre-mRNA until processing is complete (Dreyfuss *et al.*, 1993) and can associate with mature mRNAs during cytoplasmic transport (Pinol-Roma, 1997). By associating with pre-mRNAs, hnRNP proteins, as well as having a direct processing role, regulate the interactions of pre-mRNAs with components of the RNA processing pathway including those involved in splicing, 3' end formation and RNA export (Dreyfuss *et al.*, 1993). hnRNP proteins, like many other proteins involved in RNA processing, bind RNA via a ribonuclear protein domain (RNP) and show different sequence specificities (Dreyfuss *et al.*, 1993). Through either direct protein:RNA interactions or through co-operative binding of hnRNPs to each other, the array of hnRNP proteins bound to a given transcript is therefore determined by its RNA sequence. Hence different transcripts will be

Fig. 1B RNA processing in mammalian cells



Mammalian mRNA is transcribed from DNA as a pre-mRNA molecule. Before being translated into protein this pre-mRNA is processed. During processing pre-mRNA is spliced, polyadenylated, and 5'capped before it can be exported to the cytoplasm and translated into protein. Each of these steps can be regulated and together they allow a complex regulation of gene expression.
(Fig. produced by C. Loney, dept. Virology, Glasgow University.)

expected to associate with a unique set of hnRNP proteins, the composition of which will change during mRNA maturation directing processing events (Varani & Nagai, 1998). hnRNPs therefore play a key role in regulated gene expression.

1B2 Mammalian pre-mRNA undergoes splicing

Pre-mRNA splicing is the process by which non-coding, intervening sequences or introns are removed from pre-mRNA molecules and exon sequences are joined.

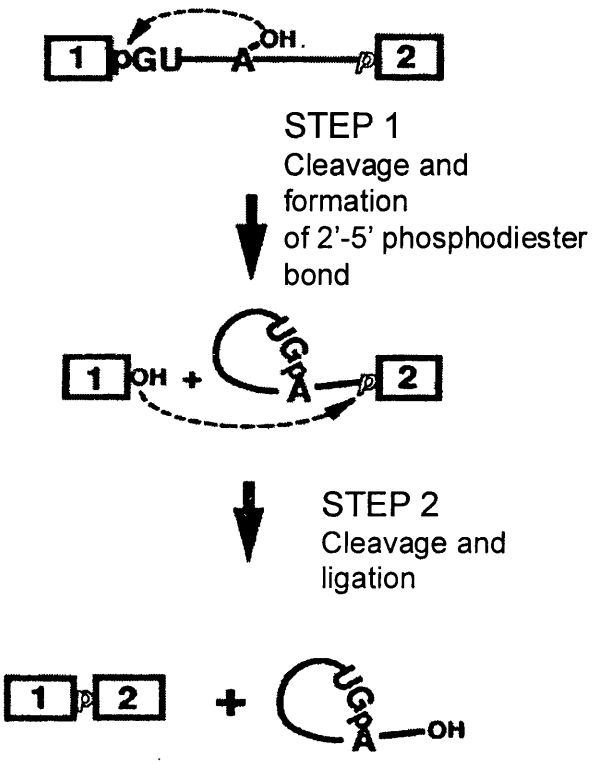
1B2.1 The splicing reaction

Pre-mRNAs are spliced in a two step reaction (Fig. 1B2.1) (Padgett *et al.*, 1984, Ruskin *et al.*, 1984). In the first step, pre-mRNA is cleaved at the 5' splice site, generating a linear first exon RNA species and an intron-second exon RNA species in a lariat formation. The lariat is the result of a 2'-5' phosphodiester bond between the G at the 5' splice site and 3' end of the intron, the point at which this bond forms is termed the branch point site (BPS). In the second step, the 3' splice site is cleaved and concomitantly the exons are ligated together, generating the spliced mRNA.

1B2.2 Mammalian pre-mRNA splicing factors

The splicing reaction is catalysed by the spliceosome, a macromolecular complex made up of five small nuclear ribonucleoprotein particles (snRNPs) and non snRNP proteins, whose number is estimated to be in the hundreds. During splicing, snRNPs and non-snRNP proteins undergo a series of interactions with each other and with pre-mRNA to cause conformational alterations which drive the reaction forward (reviewed in Green, 1991). Changes in RNA:RNA interactions between small nuclear RNAs (snRNAs) form at least part of the catalyst to splicing. Spliceosomes assemble on the pre-mRNA in a stepwise manner, forming four discrete complexes E - A - B - C (in that order) which are functional intermediates in the pathway (Fig.1B2.2). Much of the work which uncovered proteins involved in the splicing reaction has been carried out by reconstitution of an *in vitro* splicing reaction e.g. (Krainer & Maniatis, 1985) and through the use of yeast e.g. (Siliciano *et al.*, 1987) These two allow a combined genetic and biochemical approach.

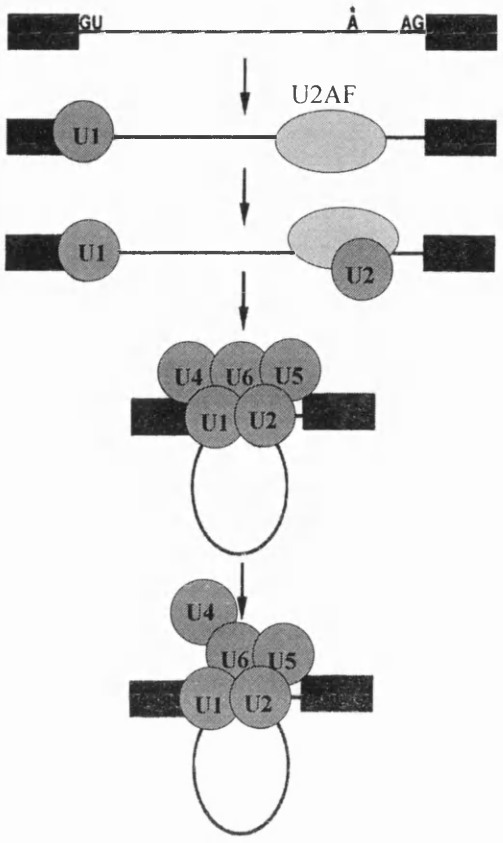
Fig. 1B2.1 The splicing reaction



Splicing occurs in a two step reaction (see text for details). Boxes represent exons 1 and 2, the single line represents intronic sequences to be removed. In step one, OH is a hydroxyl group of the A residue at the branch point site and P is the phosphate of G. In step 2, OH is the hydroxyl group of the last residue of exon 1 and ℙ is the phosphate group of the first residue of exon 2.

Taken from (Green, 1991)

Fig.1B2.2 Proteins involved in the mammalian splicing pathway



Two snRNP/pre-mRNA interactions form the basis of the spliceosome. Firstly, U1 snRNP binds to the 5' splice site while at the same time the splicing associated factor U2AF binds to the pyrimidine tract between the branch point site (BPS) and the 3' splice site, thus forming complex E. Secondly, U2 snRNP, recruited by U2AF (Gozani & Potashkin, 1998), binds to a region encompassing the BPS. This step irreversibly commits the pre-mRNA to the splicing pathway and forms complex A. Complexes A and E are also called pre-spliceosomes. Thirdly, a pre-existing (U4/U6/U5) particle enters to form complex B, and just prior to splicing a conformational change occurs that significantly destabilises the association of U4 snRNP with the complex transforming it into complex C. Following splicing, the snRNPs remain associated with the intron, and are recycled. Excised introns are degraded by a phosphodiesterase specific to the lariat structure.

GU and AG are the 5' and 3' conserved residues recognised by splicing factors, A is the residue at the branch point site.

Taken from (Green, 1991)

1B2.3 Regulation of splicing - SR proteins

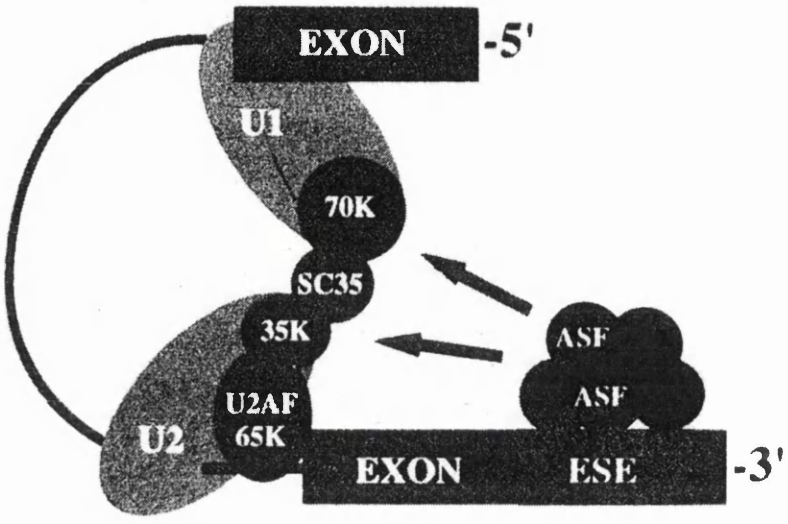
Modulation of gene expression by the use of alternative splice sites is a common mechanism. However, despite knowledge of the proteins involved in the basic splicing reaction (see above), our understanding of the proteins involved in the regulation of splicing has been limited in several ways. First, the combined biochemical and genetic methods so successfully employed to study basic splicing in yeast are not applicable, as alternative splicing appears not to occur in yeast. Second, identification of the *cis* acting sequences responsible for splicing has been difficult and therefore isolation of specific RNA binding proteins slow. Finally, only a small number of alternative splicing events have proved to be reproducible *in vitro*, adding to the problems of identifying splicing regulatory molecules.

The group of proteins most studied and implicated in splice site selection and spliceosome assembly is a family termed SR proteins (Fu, 1995). The name SR comes from the presence of a characteristic carboxy terminal serine/arginine rich domain responsible for protein:protein interactions between family members. This domain is thought to allow SR proteins to aid the recruitment and stabilisation of snRNPs with pre-mRNA at multiple and distinct points during the splicing cycle. The other distinctive feature of SR proteins is their ability to interact with RNA; which occurs via an amino terminal RNP domain. SR proteins are considered to have distinct, functionally significant RNA binding activities allowing them to recognise splicing enhancer or repressor sequences, (reviewed in Manley & Tacke, 1996).

ASF/SF2 is a typical SR protein

The way in which ASF/SF2 is involved in splicing is illustrated in Fig. 1B2.3, and is thought to be typical of SR protein control of splicing. ASF/SF2, a 33 kDa protein, has constitutive splicing activity *in vitro* (Krainer *et al.*, 1990b) and also strongly influences splice site selection in a concentration-dependent manner. Addition of ASF/SF2 to nuclear extracts, switches splice site selection of pre-mRNA bearing competing 5' splice sites towards the proximal 5' site (Ge & Manley, 1990, Krainer *et al.*, 1990a). ASF/SF2 interacts with other SR proteins, namely, a U1 specific protein of 70 kDa , the SC35 protein and the small subunit

Fig. 1B2.3 Role of ASF/SF2 in splicing



SR proteins are involved in several steps of spliceosome assembly. ASF/SF2 and SC35 connect 5' and 3' splice site complexes though a network of protein:protein interactions, stabilising and facilitating assembly of the spliceosome. ASF/SF2 contacts the 70 kDa subunit of U1 snRNP and the U2 associated protein U2AF Taken from (Manley & Tacke, 1996).

of the essential splicing factor U2AF. SC35 and ASF/SF2 are functionally interchangeable *in vitro* (Fu *et al.*, 1992). The interaction with U1 is indirect, but as ASF/SF2 also binds U2AF at the 3' splice site, it forms a link between the 5' and 3' splice sites (Wu & Maniatis, 1993). ASF/SF2 has been shown to specifically recognise RNA fragments containing intact 5' splice sites (Zuo & Manley, 1994), and to bind to and enhance expression from a purine rich bovine exonic enhancer sequence (Sun *et al.*, 1993). ASF/SF2 interacts with RNA via an RNP domain and with other proteins via an RS domain.

1B3 Polyadenylation

A poly (A) tail is found at the 3' end of most fully processed eukaryotic mRNAs. It has been proposed that this tail confers mRNA stability, promotes mRNA translational efficiency and has a role in the transport of processed mRNA from the nucleus to the cytoplasm (reviewed in Wikens *et al.*, 1997). mRNA 3' end formation occurs in a coupled two step reaction; firstly, the 3' end is cleaved and then poly (A) addition takes place. This reaction is carried out by a surprisingly large complex of multi-subunit proteins (Manley, 1995).

1B3.1 Polyadenylation elements

There are two sequence elements that specify pre-mRNA cleavage and poly (A) addition. Located 10 to 30 bases upstream of the cleavage/polyadenylation site is the sequence AAUAAA, and 20-40 bases downstream of the cleavage site is a G+U-rich motif. These two sequence motifs and their spacing from each other specify the site and strength of a poly (A) signal (Chen *et al.*, 1995, MacDonald *et al.*, 1994). Like splicing, use of alternative polyadenylation sites is a relatively common means of regulating gene expression. In addition to the AAUAAA and GU rich motifs, some mRNAs contain other genetic elements which influence the efficiency of polyadenylation, Such as in the genomes of viruses and in the human C2 complement gene and mouse calcitonin/calcitonin gene related peptide (reviewed in Proudfoot, 1996).

1B3.2 Proteins involved in polyadenylation.

A functional polyadenylation complex is illustrated in Fig.1B3, and is composed of....

Cleavage/Polyadenylation Specificity Factor (CPSF)

CPSF is made up of four polypeptides, one of which, CPSF-160, recognises AAUAAA, but can only weakly bind to it. Formation of a stable complex requires the presence of the other CPSF polypeptides and another factor CstF. Together the two factors function to recruit other components of the polyadenylation machinery to the cleavage site (Manley, 1995). Additional functions for CPSF are the co-ordination of nuclear polyadenylation with transcription (Dantonel *et al.*, 1997), participation in cytoplasmic polyadenylation (Bilger *et al.*, 1994), limitation of poly (A) tail length (Wahle, 1995) and in the interaction of proteins associated with the splicing apparatus (Lutz *et al.*, 1996).

Cleavage stimulation factor (CstF)

CstF is a heterotrimeric protein with three subunits of 77 kDa, 64 kDa, and 50 kDa. In the presence of CPSF, binding of the precursor RNA is mediated by CstF-64. CstF-64 binds specifically to the G+U-rich motif via a RNP binding domain (MacDonald *et al.*, 1994). Binding of CstF stabilises CPSF interaction with AAUAAA and is thought to determine the efficiency of polyadenylation (Takagaki *et al.*, 1989).

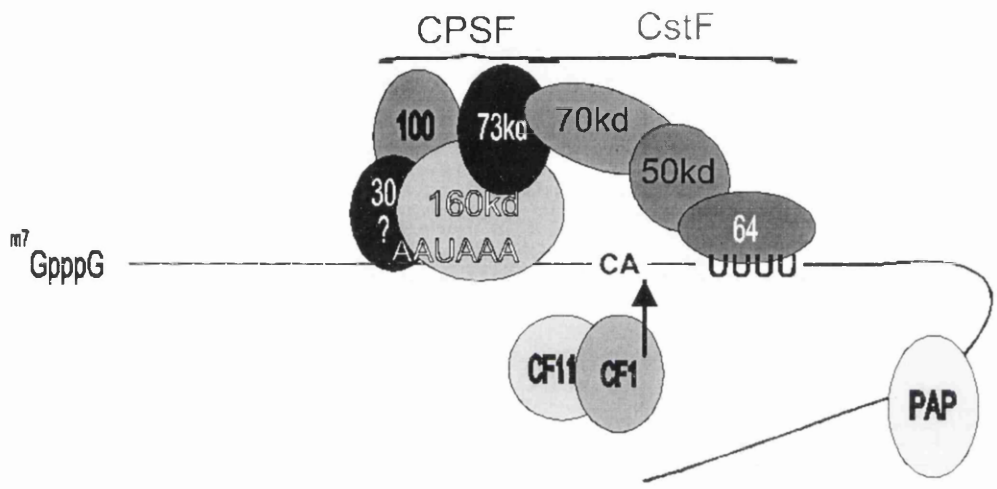
Cleavage factors I and II (CFI and CFII)

Two additional factors, CFI and CFII, are required for cleavage but not polyadenylation (Takagaki *et al.*, 1989). These factors are poorly characterised, and although endonuclease activity has not been demonstrated for any of the subunits of CFI or II, the process of elimination makes them likely to be cleavage proteins.

Poly (A) Polymerase (PAP)

PAP contains at its amino terminal the catalytic activity required for addition of the poly (A) tail (Martin & Keller, 1996). PAP interacts with one of the CPSF subunits, however these two polypeptides alone are not enough to reconstitute AAUAAA-dependent polyadenylation (Murthy & Manley, 1995).

Fig. 1B3 The polyadenylation complex



Cleavage and polyadenylation components assembled on pre-mRNA. CPSF is made up of 4 components 30 kDa,73 kDa,100 kDa and 160 kDa polypeptides, it binds to AAUAAA via the 160 kDa subunit. CstF is made up of 3 polypeptides of 70 kDa, 50 kDa and 64 kDa. Binding to UUUU sequence is via the 64 kDa subunit. CFI and CFII probably cleave RNA and PAP catalyses addition of the poly (A) tail.

(Fig. Produced by C. Loney, dept. Virology, University of Glasgow.)

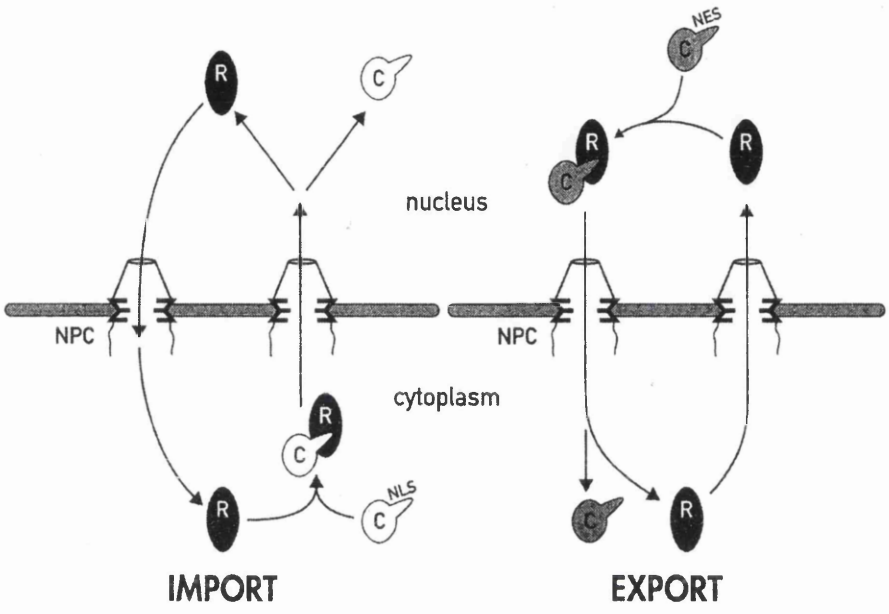
Poly (A) binding protein II (PAB II)

PAB II binds to poly (A) RNA and acts by co-operating with CPSF to hold PAP in place on the primer RNA. These interactions are disrupted after the tail has reached a distinct length and so PAB II both supports progressive synthesis of a long poly (A) tail (Bienroth *et al.*, 1993) and restricts the length of poly (A) tail to 200-300 residues (Wahle *et al.*, 1993).

1B4 Export of mRNA from the nucleus

In order to exit the nucleus, mature mRNA must pass though 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 which 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 which cause nuclear localisation are termed nuclear localisation signals (NLSs) and those which allow export of proteins are nuclear export signals (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 which then translocate through the NPC, as illustrated in Fig.1B4. Most transport processes are thought to require the small GTPase Ran. The signals, as far as we know, do not compete with each other and utilise separate pathways to/receptors in the nuclear membrane (reviewed in Izaurralde & Adam, 1998).

Fig.1B4 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. Taken from (Izaurralde & Adam, 1998).

1B4.1 Nuclear Export Signals

Rev-like NES:

This is a leucine rich sequence found not only in HIV-1 Rev, a protein which promotes the export of partially and unspliced viral RNAs (see HIV), but also in several cellular proteins including TFIIIA 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 CRM 1 (Fornerod *et al.*, 1997a). Consistent with being a receptor, CRM 1 is a Ran binding protein and interacts with a nucleoporin Nup214/CAN (Fornerod *et al.*, 1997b). The cytotoxin leptomycin B (LMB) inhibits Rev-NES mediated transport (Wolff *et al.*, 1997). In higher eukaryotes, mRNA transport is not inhibited with LMB (Fornerod *et al.*, 1997a) which strongly suggests that cellular mRNAs are not exported via this pathway.

M9 domain:

This sequence consists of a glycine rich sequence of 38 aa 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 the mRNA via the M9 domain (reviewed in Pinol-Roma, 1997). 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 an individual mRNA could depend on the pattern of associated hnRNP proteins and the type of export signals they carry.

KNS domain:

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

1B4.2 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 leads to a suggestion that the removal of other factors which 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, 1997).

1B5 Cellular location of processing events

1B5.1 Transcription, splicing and polyadenylation

The transcription machinery co-localises with nuclear structures termed perichromatic fibrials (PF) found at the boundaries of condensed chromatin domains. When cells are pulse labelled, PFs are enriched in nascent pre-mRNA radiolabelled with bromouridine or [^{3}H]-uridine and fibrial density correlates with transcriptional activity (Faken *et al.*, 1984; Faken, 1994; Van Driel *et al.*, 1995). The splicing machinery is also found in PFs (Faken *et al.*, 1984) consistent with assembly of splicing machinery initiating at the site of transcription.

Although transcription and splicing happen in PFs, only a minority of splicing factors are found there. Immunofluorescent staining for splicing factors (Faken *et al.*, 1984) (Fig. 1B9.1a) has shown them to be present in 20-50 nuclear domains or speckles set against a more diffuse nucleoplasmic pool of splicing factors. The diffuse staining corresponds to PFs, and the majority of speckles correspond in location to interchromatin granule clusters (ICGs). This data suggests that ICGs act as storage/assembly sites for splicing factors which can be recruited on demand (reviewed in Mattaj, 1994). Although speckles do not incorporate bromouridine, they do contain intron containing pre-mRNA and polyadenylated mRNA. Alternative scenarios are that the speckles have a role in the export of mRNAs or that speckles accumulate snRNPs bound to partially spliced mRNAs or excised introns after release from spliceosomes (reviewed Lamond & Earnshaw, 1998). The location of splicing factors is therefore dynamic, moving between nuclear structures. This has been illustrated by visualisation of splicing

factor ASF/SF2 fused to green fluorescent protein (GFP) in living cells (Misteli *et al.*, 1997). Organisation of the functional components within a set framework provides more opportunities for cell regulation as compared to a freely diffusible state.

1B5.2 Transport of mature mRNA from the site of processing to the NPC

There are two theories as to how mature mRNA reaches the nuclear membrane. Estimates of the rate of movement of a highly expressed hybrid gene in Drosophila salivary glands suggest that simple diffusion could account for the dispersal of mRNA (Zachar *et al.*, 1993). However, the need for structural features in mRNA for their movement (Elliot *et al.*, 1994), and the tight association of transcripts, hnRNPs, and functional processing components with the nuclear matrix argue for directed mRNA movement. mRNA can be tracked moving though the nucleus but results of these types of experiment are not consistent with one model or the other (Huang & Spector, 1991, Zachar *et al.*, 1993).

1B6 Cross talk between mRNA processing events

1B6.1 Interactions between specific processing components

The co-localisation of processing factories suggests that there is some level of cross talk between them and this is indeed the case. For example, CPSF binds to TFIID, allowing recruitment to the RNA Pol II pre-initiation complex, where, after phosphorylation of the large subunit of RNA Pol II, CPSF dissociates from TFIIA and becomes associated with the elongating RNA pol II. CPSF appears to travel with RNA pol II until the polyadenylation event (Dantonel *et al.*, 1997); perhaps the presence of poly (A) factors influences termination of elongation. Polyadenylation and transcription are therefore linked together by CPSF (Zhao *et al.*, 1999).

In a similar way to CPSF, the splicing machinery has been shown to be associated with the RNA pol II carboxy terminal domain (Steinmetz, 1997). This role for the C-terminal domain 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.

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' intron of pre-mRNA and polyadenylation are functionally linked (Niwa *et al.*, 1990).

1B6.2 Promoters can influence the post-transcriptional fate of transcripts.

One example of promoters influencing the post-transcriptional fate of mRNA is in the immunoglobulin μ gene. Transfection of the υ gene, cloned as cDNA with its introns removed, has been shown to result in very little secreted protein. This effect however is promoter-dependent, as when the endogenous promoter is replaced by a HCMV promoter, expression is comparable to that obtained when introns are included (Neuberger & Williams, 1988).

1B6.3 Multifunctional proteins

As well as interactions between components of the processing pathway, there are multifunctional proteins which 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 Ladomery, 1997). Some well characterised examples include Y box proteins, Wilm's tumour protein (WT) and hnRNP K (see Section 1C1).

Y box proteins

All Y box proteins contain a cold shock domain which is 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 also have a number of other domains, and have been 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 expression 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.

The Wilm's tumour gene (WT1)

WT1, first identified as a tumour suppresser, produces a protein, which can exist as sixteen known isoforms. It is a transcriptional activator and binds to DNA such as epidermal growth factor receptor consensus and similar sequences. The mouse cell line M15 contains at least two of the isoforms of WT1, with or without the three aa KTS. The KTS^+ form is found preferentially in nuclear speckles and co-localises with snRNPs, suggesting a role in splicing, while the KTS^- isoform preferentially co-localises with the transcription factors SP1 and TFIIB. Thus, WT1 may exert its effects at the transcriptional and post-transcriptional levels via different isoforms.

1B7 Phosphorylation and the regulation of mRNA processing

Regulation of SR protein activity and splicing factor location may be controlled at least in part by phosphorylation. SR protein specific kinases have been identified, SR protein kinase 1 (Gui *et al.*, 1994), and Clk/Sty kinase (Colwill *et al.*, 1996). Both kinases when over expressed cause redistribution of splicing factors within the nucleus. Phosphorylation is also important for functionality of splicing factors. When nuclear extracts are treated with protein phosphatase 1, the formation of spliceosomes on RNA templates is dramatically decreased (Mermound *et al.*, 1994) and in the presence of phosphatase inhibitors (Mermound *et al.*, 1992) or exogenously added hyperphosphorylated U1 70 kDa (Tazi *et al.*, 1993) spliceosomes form correctly but the splicing reaction does not proceed. A further example is ASF/SF2 which must be phosphorylated in order to function in spliceosome assembly (Xiao & Manley, 1997). Cycles of phosphorylation and dephosphorylation are likely to regulate SR protein function and in turn splicing. Similarly, other RNA processing events are probably regulated by phosphorylation.

1B8 HSV-1 protein IE63 is essential for viral replication and growth.

IE63 mutant viruses have a range of phenotypes, suggesting IE63 has several distinct functions. Some mutants show decreased levels of late viral proteins (Sacks *et al.*, 1985; McCarthy *et al.*, 1989; Rice & Knipe, 1990; McGregor *et al.*, 1996), others show a decrease in viral DNA synthesis (Rice & Knipe, 1990) promoted via a decrease in delayed E proteins involved in DNA replication; a third group show impaired host protein synthesis shut-off (McCarthy *et al.*, 1989). IE63 is thought to repress expression of viral IE and E proteins, enhance expression of viral L genes, repress expression of cellular genes and affect viral DNA replication. What makes IE63 essential is probably its requirement for the switch from E to L virus gene expression and hence the knock-on effects on viral replication. IE63 accomplishes this by regulating transcriptional and post-transcriptional events.

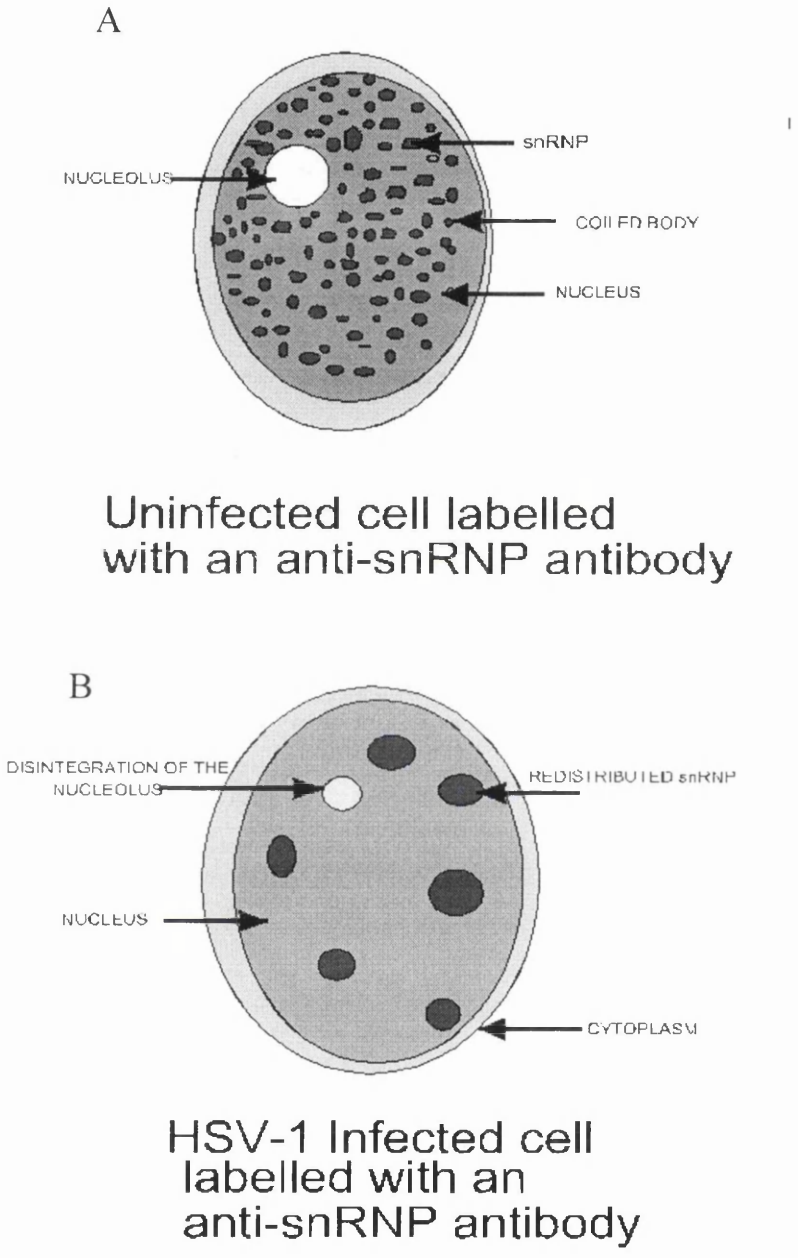
1B9 IE63 acts at the post-transcriptional level to regulate gene expression

1B9.1 IE63 redistributes splicing factors and causes inhibition of host cell splicing.

In cells infected with IE63 mutants, spliced cellular mRNA levels are higher, than in those infected with the wt virus (Hardwicke & Sandri-Goldin, 1994), this cannot be attributed to effects on transcription or RNA stability and suggests that IE63 impairs splicing function. Further, precursor mRNA from the viral genes which do require splicing (IE110 and U_L15), accumulate in the presence of IE63, suggesting that transport of the gene products is not being affected (Hardy & Sandri-Goldin, 1994).

Using indirect immunofluorescence, the nuclear distribution of host snRNP antigens is shown to alter in HSV-1 infection (Martin, *et al.* 1987), reorganisation is illustrated in Fig. 1B9.1. This redistribution is similar to that seen in uninfected cells when transcription is reduced or splicing is inhibited (Carmo-Fonseca *et al.*, 1992, O'Keefe *et al.*, 1994). During infection, the snRNP antigens form prominent clusters which condense throughout the nucleus, then migrate to the nuclear periphery. These punctate snRNP clusters correspond to clusters of interchromatin granules. IE63 co-localises with the redistributed snRNPs and its expression is necessary and sufficient to cause snRNP redistribution (Phelan *et al.*, 1993,

Fig. 1B9.1 Schematic representation of the reorganisation of cellular splicing factors during HSV-1 infection



Location of splicing factors is seen to redistribute with HSV-1 infection, from a wide spread speckled pattern to a highly punctate organisation. IE63 has been shown to be both necessary and sufficient to cause this effect. A: uninfected cells. B: infected cells 8h post infection.
Taken from (Clements & Brown, 1997).

Sandri-Goldin *et al.*, 1995). The C-terminal repressor region of IE63, which is required for inhibitory effects on splicing is also involved in redistributing snRNPs (Sandri-Goldin *et al.*, 1995).

Co-localisation suggests that IE63 interacts with one or more of the components of the splicing machinery. More direct evidence for interaction between IE63 and splicing factors has come from anti-Sm co-immunoprecipitations from wt infected cell extracts (Sandri-Goldin & Hibbard, 1996). IE63 co-immunoprecipitates with Sm containing proteins, but as Sm is an epitope common to many snRNPs, it is not possible to identify the specific partner of IE63.

As IE63 viral mutants are defective in host shut-off (Hardwicke & Sandri-Golding, 1994), it is possible that IE63 induced redistribution of splicing factors causes inhibition of splicing and this contributes to host shut-off. Alternatively, inhibition of splicing may cause redistribution of splicing factors again leading to host shut-off. While the majority of mammalian genes contain introns, HSV-1 genes, with the exception of IE110, IE68, IE12 and U_L15 do not. So this proposed mechanism of host shut-off would have little effect on viral gene expression.

Suprisingly, snRNP redistribution and splicing inhibition can be uncoupled. Redistribution is seen during infection with an IE63 mutant virus containing a base substitution at aa 480; however, splicing in cells infected with this virus is not inhibited (Sandri-Goldin *et al.*, 1995). This suggests that despite the correlation between inhibition of splicing and redistribution of splicing factors, alterations in snRNP location observed are not sufficient to inhibit splicing.

In uninfected cells, regulation of splicing activity and location of splicing factors has been shown to be dependent on phosphorylation (reviewed in Manley & Tacke, 1996). In wt HSV-1 infected cells compared to cells infected with an IE63 null mutant, studies have shown an increase in the phosphorylation of two proteins which co-immunoprecipitate with IE63 using anti-Sm serum (Sandri-Goldin & Hibbard, 1996), and one of these may be the U1 snRNP 70 kDa protein. Phosphorylation induced by the presence of IE63 therefore may contribute to the inhibition and relocalisation of splicing factors.

1B9.2 IE63 increases RNA 3' processing at inherently weak virus poly(A) sites

Like cellular gene expression, viral gene expression is regulated at the level of polyadenylation.

In vitro assays, using extracts from tissue culture cells, demonstrate that processing of the poly(A) site of the U_L38 gene (a L gene) increases due to a virus induced factor, late processing factor (LPF); however, processing at the poly (A) site of U_S12 (an IE gene) does not increase, (McLauchlan 1989). Increased usage of this site was dependent on IE63. ICP27 also causes an increased usage of certain poly (A) sites in transfected reporter genes (Sandri-Goldin *et al.* 1992).

Using *in vitro* assays (McGregor *et al.*, 1996), the processing efficiencies at poly (A) sites of two L genes U_L38 and U_L44, shown to be inherently weak processing sites, have been shown to be increased by an IE63 induced activity. In contrast, 3' processing at the poly (A) sites of selected IE and E genes, stronger processing sites, is unaffected by IE63 expression. In the same report, UV cross linking experiments demonstrated enhanced binding of the 64 kDa component of CstF to poly (A) sites of RNAs from genes of all temporal classes and this enhanced binding requires expression of IE63. Location of 64 kDa in the polyadenylation complex can be seen in Fig. 1B3.

As suggested by these data, IE63 may increase the binding of 64 kDa CstF to viral polyadenylation sites. This will not affect the use of already strong sites but will increase the efficiency of use of inherently weak L poly(A) sites. By this mechanism, IE63 therefore may regulate the switch between E and L expression, one of its known roles in infection (Rice & Knipe, 1990; Rice & Lam, 1994; Hibbard & Sandri-Goldin, 1995). Further evidence for this comes from the study of expression from the HSV-1 UL24 gene (Hann *et al.*, 1998). Two transcripts are produced from the U_L24 gene, 1.4 kb and 5.6 kb; these initiate from the same promoter but utilise separate poly (A) sites and are expressed with E and L kinetics. Accumulation of the 5.6 kb transcript, unlike that of the 1.4 kb transcript, requires IE63, Although IE63 has not been shown directly to alter poly (A) site selection, this seems a most likely scenario.

1B9.3 IE63 and nucleocytoplasmic transport of mRNAs

IE63 reduces cellular mRNA levels and causes unspliced pre-mRNAs to accumulate in the nucleus (Hibbard & Sandri-Goldin, 1995). It also results in nuclear export of some unspliced cellular mRNAs (Hibbard & Sandri-Goldin, 1995) and causes nuclear retention of intron-containing viral transcripts while intron-less transcripts are seen to be predominantly cytoplasmic (Phelan *et al.*, 1996). Further, the unspliced retained transcripts co-localise with the redistributed snRNPs and with IE63.

IE63 shuttles from the nucleus to the cytoplasm (Mears & Rice, 1998, Phelan & Clements, 1997, Soliman *et al.*, 1997) and can bind to RNA (Ingram *et al.*, 1996) and these observations have led to the suggestion that it has a role in the export of viral mRNAs. To support this, IE63 was shown to UV-cross link to intronless HSV-1 RNAs *in vivo*, while no binding was seen with two viral transcripts which undergo splicing; export of intronless HSV-1 transcripts has been shown to be reduced in infections when IE63 is not expressed (Sandri-Goldin, 1998). It has been suggested that IE63 shuttles specifically with L viral transcripts and thereby upregulates L viral protein synthesis (Soliman *et al.*, 1997), although a second report (Sandri-Goldin, 1998) observes RNA binding and shuttling of IE63 with all classes of intronless HSV-1 transcripts.

The binding of hnRNP A1 to poly(A)$^+$ RNA is significantly reduced following HSV-1 infection (Sandri-Goldin, 1998), suggesting that as well as promoting export of viral transcripts IE63 increases nuclear retention of cellular RNA.

1B10 IE63 can also affect the transcription of viral and cellular genes during infection

IE63 may act with/upon IE175 in order to up or down regulate viral/cellular mRNA transcription. IE175 and IE63 have been reported to physically interact (Pangiotidis *et al.*, 1997) but this interaction has not been independently confirmed. Despite this, a functional association between IE63 and IE175 does exist; the electrophoretic mobility of IE175 is altered in the presence of IE63 (Su & Knipe, 1989), as is its intracellular localisation (Zhu & Schaffer, 1995) and in transient expression systems IE63 can inhibit or augment the transcriptional

activity of IE175 (Su & Knipe, 1989). Since differently phosphorylated forms of IE175 have been shown to have different DNA binding activities (Papavassiliou *et al.*, 1991), IE63 may facilitate phosphorylation of IE175, thus modulating its promoter binding and activation activities.

Although most transcription effects, require expression of IE175 with IE63 acting modulate this protein; under certain conditions IE63 alone can increase the number of transcripts detected from a viral glycoprotein B promoter fused to a chloramphenicol acetyltransferase (CAT) reporter gene (Rice & Knipe, 1988). However, increased stability of mRNA in the presence of IE63 cannot be ruled out. IE63 may act directly to decrease transcription from some cellular genes, for example, histone H2b (Pangiotodis *et al.* 1997). Down regulation of H2b, is suggested to be at the level of transcription because the gene does not contain any introns or poly (A) signals.

IE63 has also been shown to have a direct role in activation of transcription, via TFIIIC, by increasing RNA Pol III transcription of *Alu* repeated sequences (Jang & Latchman, 1992). The relevance of this observation is not clear as no HSV-1 genes are known to be transcribed by RNA Pol III. However genes encoding snRNAs transcribed by RNA Pol III are encoded by other herpesviruses such as EBV and by adenovirus.

1B11 Phosphorylation sites in IE63

IE63 is known to be phosphorylated both with stable phosphate groups and with phosphates that cycle on and off during infection (Wilcox *et al.*, 1980). On SDS-PAGE, two forms of IE63 can be detected (more often only one is seen), and as many as 5 species heterogeneous with respect to charge are separated by 2D isoelectric focusing (Pereira *et al.*, 1977; Ackerman *et al.*, 1984). Phosphoamino acid analysis has shown that serine is the only IE63 amino acid phosphorylated during infection; transfected DNA expressing IE63 yielded the same phosphorylated pattern, implicating cellular kinases in this process (Zhi & Sandri-Goldin, 1999). The same study shows that Ser 114 is highly phosphorylated by protein kinase A (PKA) and that Ser at positions 16 and 18 serve as phosphorylation sites for casein kinase 2 (CK2).

Known and potential phosphorylation sites on IE63 protein are shown in Fig. 1B12b.

A role for phosphorylation of IE63 in infection is unclear; mutations in known phosphorylation sites still allow these mutant viruses to complement IE63 null mutant growth but phosphorylation of Ser 114 modulates the efficiency of nuclear import of IE63 (Zhi & Sandri-Goldin, 1999). Given the key role phosphorylation plays in the regulation of cellular RNA processing, it is likely to be important for viral RNA processing also.

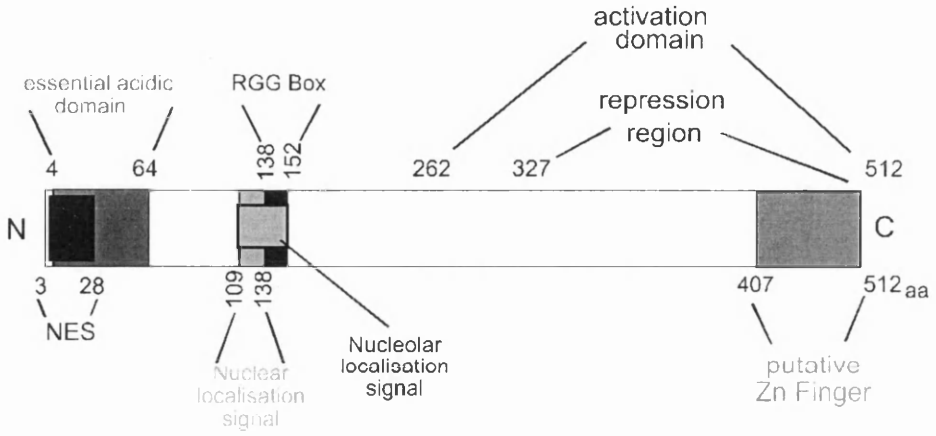
1B12 Functional domains of IE63

Mutation of various IE63 regions has allowed several separable activities to be identified with a discrete domain and these domains are featured in Figs. 1B12a and b. This dissection and reconstruction of IE63 activity can provide an understanding of how these separate activities contribute to IE63 function.

1B12.1 Essential acidic domain

At the amino terminus of IE63 (aa 4-64) and present in all the IE63 homologues (Appendix 2) lies a high concentration of acidic residues. Deletion of this domain renders the mutant virus unable to rescue an IE63 null mutant indicating its essential nature (Rice *et al.*, 1993). These mutants are defective in viral DNA replication (3-8 fold reduction), show a decrease in L gene expression and a delay in E gene expression; this function is separate from an activity in the C-terminus which also stimulates L gene expression. A number of transcriptional activators, such as HSV-1 VP16, have been shown to contain a similar acidic domain through which they stimulate transcription by interaction with one or more components of RNA Pol II (Struhl, 1991). The high degree of conservation of this domain throughout the herpesviruses further suggests its essential function (Appendix 2)

Fig. 1B12a Schematic of functional domains of IE63



IE63 contains a number of functional domains including nuclear and nucleolar localisation signals, an RNA binding domain (RGG), C terminal activation and repression regions and a nuclear export signal (NES).
(Taken from Phelan & Clements, 1998)

Fig. 1B12b Amino acid sequence of IE63

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1   MATDIDMLIDLGLDLSDSDLDEDPPEPAESRRDDLESDSSGECSSSDED  50
51  EDPHGEDGPEPILDAARPAVRPSRPEDPGVPSTQTPRPTERQGPNDPQPA 100
101 PHSVWSRLGARRPSCSPEQHGGKVARLQPPPTKAQPARGGRRGRRRGRGR 150
151 GGPGAADGLSDPRRRAPRTNRNPGGPRPGAGWTDGPGAPHGEAWRGSEQP 200
201 DPPGGQRTRGVRQAPPPLMTLAIAPPPADPRAPAPERKAPAADTIDATTR 250
251 LVLRSISERAAVDRISESFGRSAQVMHDPFGGQPFPAANSPWAPVLAGQG 300
301 GPFDAETRRVSWETLVAHGPSLYRTFAGNPRAASTAKAMRDCVLRQENFI 350
351 EALASADETLAWCKMCIHHNLPLRPQDPIIGTTAAVLDNLATRLRPFLQC 400
401 YLKARGLCGLDELCSRRRLADIKDIASFVFVILARLANRVERGVAEIDYA 450
451 TLGVGVGEKMHFYLPGACMAGLIEILDTHRQECSSRVCELTASHIVAPPY 500
501 VHGKYFYCNSLF 512
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Residues conserved through out the *alphaherpesviridae* homolgues are in bold (see Appedix 2). Potential nuclear export signals are shaded: N-terminal and C-terminal signals are Leu rich NESs, the central one has homology to the KNS domain in hnRNP K. Potential CK2 phosphorylation sites are boxed, sites for phosphorylation by other kinases are underlined: kinases examined were Casein kinase 1, PKC, PKA, Calmodulin dependent kinase 2, and Src type tyrosine kinase.

1B12.2 A Potential zinc finger, activator and repressor regions

Metal chelate affinity chromatography shows that IE63 (aa 407-512) binds zinc *in vitro* (Vaughan *et al.*, 1992) and mutation analysis (Vaughan *et al.*, 1992) demonstrates 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 has similarity to the 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).

The zinc finger containing C-terminal region is responsible for the gene activation and repression effects exerted by IE63 (Hardwicke *et al.*, 1989, McMahan & Schaffer, 1990). Later studies show that most activation is correlated with increased polyadenylation and that repression is correlated inhibition of splicing (Chapman *et al.*, 1992, Hardwicke & Sandri-Golding, 1994, McLauchlan *et al.*, 1992, Sandri-Goldin & Mendoza, 1992).

So the data which implicate or map specific protein:protein interactions correlating with activation/repression point to this domain, reinforcing the idea that protein:protein interactions are responsible for the activation and repression activities of this region. The C-terminal region is required for redistribution of snRNPs (Hibbard & Sandri-Goldin, 1995) and in anti-Sm co-immunoprecipitations the region required for co-immunoprecipitation of IE63 has been demonstrated to be aa 450-504 (Sandri-Goldin & Hibbard, 1996). Further, self interaction of IE63 requires the residues which make up the zinc finger (Zhi *et al.*, 1999).

1B12.3 Nuclear localisation signals

IE63 is a predominantly nuclear protein, it contains a classical NLS (aa 110-137) along with several weaker ones mapping to the C terminal portion of the protein. In addition, it contains a nucleolar targeting signal (aa 110-152), suggesting it interacts with the cell nucleoli (Mears *et al.*, 1995).

1B12.4 Nuclear export signal

IE63 shuttles rapidly from the nucleus to the cytoplasm (seen in a heterokaryon assay in less than 1 h Mears & Rice, 1998), and shuttling is therefore expected to be mediated by a NES or by binding to another protein which can shuttle. IE63 does not contain any sequence resembling M9, but does possess several sequences which resemble Leu-rich NESs (Fig.1B12b).

Studies of viral mutants in the heterokaryon assay, have shown that deletion of the amino terminus (aa 12-63) containing the first Leu rich NES (aa 7-20) or substitutions of aa 340 and 341 slightly decrease shuttling. However, the most deleterious mutation for shuttling is a substitution of aa 469 and 476 (PG to LE) (Mears & Rice, 1998). These mutations may affect a nearby putative Leu rich NES, could disrupt binding to a cellular shuttling molecule, or may increase nuclear retention of IE63. The possibility also exists that IE63 contains an as yet uncharacterised NES: These data suggest that IE63 shuttles via at least 2 separate pathways, one driven by the N-terminal Leu rich NES, another involving aa 469 and 476.

In contrast to the heterokaryon experiments, other studies have shown that LMB inhibits IE63 shuttling (B. Soliman, personal communication), that mutations in the N-terminal NES abolish shuttling and that this same region is able to promote shuttling when coupled to a heterologous nuclear protein (Sandri-Goldin, 1998). These data therefore suggest that shuttling is driven solely by the N-terminal Leu-rich NES.

1B12.5 RGG box and KH domains

IE63 binds directly to RNA (Ingram *et al.*, 1996) and non-specific RNA binding by IE63 has been shown (Ingram *et al.*, 1996). However in a different report IE63 is shown to bind to intronless HSV-1 RNAs while no binding is seen with two viral transcripts which undergo splicing (Sandri-Goldin, 1998).

Two types of RNA binding motif are present in IE63. The first is an RGG motif, rich in Arg and Gly residues and post-translationally modified by methylation (Mears & Rice, 1996). RGG motifs commonly mediate RNA binding in hnRNPs

and other proteins involved in pre-mRNA processing (Birney *et al.*, 1993). The RGG motif in IE63 protein overlaps with the NLS. However even when correctly localised to the nucleus, viral mutants lacking the RGG motif are defective in L gene expression (Hibbard & Sandri-Goldin, 1995).

The second type of RNA binding motif present in the C-terminal region of IE63 has homology to a series of hnRNP K homology (KH) domains. IE63 contains three KH domains and in IE63 they are required for the expression of HSV-1 L genes (B. Soliman, personal communication). These domains were first identified in hnRNP K where they constitute a unique RNA binding domain (section 1C1). KH domains are also found in other RNA binding proteins and RNA binding of the FMR1 protein from a severely retarded fragile X patient is impaired by a single point mutation in one of the KH domains (Siomi *et al.*, 1994).

1B13 IE63 and DNA replication

HSV-1 mutants with certain lesions in IE63 show a 5- to 10-fold reduction in viral DNA synthesis due to insufficient expression of most of the essential replication genes (Uprichard & Knipe, 1996). In addition, IE63 affects the localisation of the single stranded binding DNA protein (UL29), a component of the replication complex and thus promotes replication complex formation (Curtin & Knipe, 1993). Using the same methods but with an IE63 mutant virus, it has been shown that IE63 is essential for efficient DNA replication foci formation but made no difference to transcription site formation. It is possible that IE63, by redistributing the snRNPs to the nuclear periphery, facilitates formation and spatial arrangement of viral transcription and replication complexes. As splicing, splicing factors, transport routes and replication factories are associated with the nucleoskeleton in uninfected cells (Hozak *et al.*, 1993) and IE63 affects splicing, splicing factors, transport routes and replication factories, maybe IE63 alters the nucleoskeleton causing a diverse number of effects.

1B14 IE63 homologues

HSV-1 IE63 protein is the only HSV-1 IE gene which has homologues throughout the *Herpesviridae* and this suggests that aspects of the regulatory role of IE63 are maintained throughout the herpesvirus family. Comparisons of functional and structural characteristics of IE63 and its positional homologues aids understanding of each homologue's function and of regulatory protein evolution during the emergence of the different herpesvirus species.

Sequence alignments of IE63 homologues in alphaherpesviruses can be found in appendix 2.

From the N to C termini, the alphaherpesviruses consist of

- An acidic region
- A basic region
- A linker
- A conserved domain

The gammaherpes viruses have a similar structure but contain an additional N terminal region from an additional exon. The betaherpesviruses do not have this structure. There appears to be a large non-conserved C terminal extension of variable length containing an acidic domain. The amino termini are not conserved and contain basic residues. The central region is conserved and certain residues in this region are conserved in alpha-, beta-, and gamma-herpesviruses.

VZV and BHV-1 are alphaherpesviruses, HCMV is a betaherpesvirus and EBV, HVS and HHV-8 are all gammaherpesviruses.

1B14.1 VZV ORF 4

HSV-1 IE63 protein and VZV ORF 4 IE gene product - IE4 (Defechereux *et al.*, 1997), are positionally homologous (Moriuchi *et al.*, 1994). Although IE4 cannot complement an IE63-defective HSV-1 mutant (Moriuchi *et al.*, 1994), and is predominantly cytoplasmic as opposed to IE63 which is predominantly nuclear,

the amino-terminal region of IE4 can efficiently replace that of IE63 (Moriuchi *et al.*, 1995). Unlike IE63, IE4 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 IE62 (Defechereux *et al.*, 1993, Inchauspe *et al.*, 1989, Perera *et al.*, 1992). Analysis of IE4-mediated transactivation in transient transfection assays at the RNA level also suggest that IE4 is a multifunctional protein which can stimulate heterologus and autologus gene expression through more than one mechanism, one of which involves post-transcriptional regulation (Defechereux *et al.*, 1997). Unlike HSV-1 IE63 (Yao & Courtney, 1992), IE4 is a component of the VZV virion (Moriuchi *et al.*, 1994).

1B14.2 BHV-1 BICP27

The bovine herpes virus homologue of IE63 - BICP27, is expressed with E kinetics and accumulates in the nuclei of cells. Transient expression assays using target genes differing only in their poly (A) sites showed that BICP, like IE63, may be involved in increasing the processing efficiency of mRNA containing weak poly (A) sites (Singh *et al.*, 1996).

1B14.3 HCMV U_L69

Like VZV ORF 4 gene, the ORF U_L69 of HCMV, is a positional homologue of HSV-1 IE63, and is located in the HCMV virion (Winkler & Stamminger, 1996). In contrast to HSV-1 IE63, the U_L69 gene product is expressed with E-L kinetics during HCMV replicative cycle. However, it can function as a transactivator of several viral and cellular promoters (Winkler *et al.*, 1994).

1B14.4 EBV BMLF1/EB2/SM

SM is expressed at E times in EBV lytic infection. Like IE63, SM binds RNA and regulates gene expression at a post-transcriptional level, via control of splicing and RNA export (Ruvolo *et al.*, 1998). SM 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 expression of many E and L intronless viral genes may be enhanced, while expression of certain intron containing IE and latent genes may be repressed. EBV SM co-localises with SC35 and shuttles between the nucleus and the cytoplasm (Semmes *et al.*, 1998). Both the transactivation function and

shuttling are dependent on association with CRM1 (Boyle *et al.*, 1999). Homology between IE63 and SM is at the C terminal region of IE63, the region of IE63 responsible for post-transcriptional gene activation and repression activities of IE63 (Appendix 2).

1B14.5 HVS ORF 57

HVS ORF57 gene product can activate and repress expression from a range of both E and L HVS promoters; levels of mRNA do not correlate with the increase/decrease in CAT activity seen in the presence of the ORF57 gene product. In the same experiments, repression of gene expression was shown to be dependent on the presence of an intron within the gene encoding region (Whitehouse *et al.*, 1998). Like HSV-1, HVS infection results in the redistribution of SC-35 and snRNP U2 spliceosome components in the infected cell nucleus, the ORF57 gene product is enough to cause this redistribution and co-localises with the redistributed spliceosome components (Cooper *et.al.*, 1999). Taken together these data suggest that, like IE63, the ORF57 gene product of HVS regulates gene expression in a way which involves disruption of RNA splicing.

1B14.6 HHV 8 ORF 57

HHV 8 ORF57 is also a regulatory gene, it co-localises with SC-35 but unlike IE63 (Sandri-Goldin, 1998), it does not shuttle from the nucleus to the cytoplasm in the presence of actinomycin D. It has been, however, seen to shuttle in an interspecies heterokaryon assay (Bello *et al.*, 1999). This would suggest shuttling is transcription independent.

1B15 Other viruses and RNA processing

1B15.1 HIV-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. 1B15.1a). This means that gene regulation must occur at the post-transcriptional level.

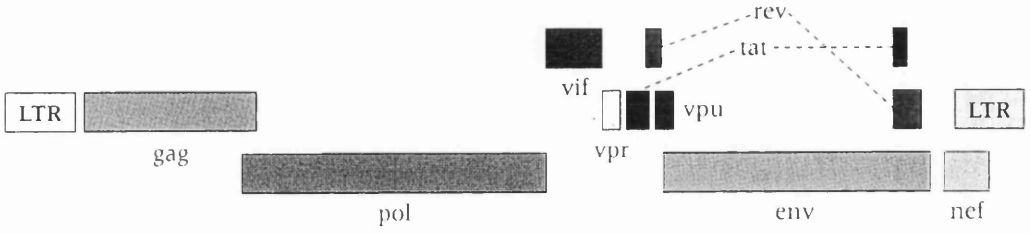
If a cellular mRNA contains an intron, interaction with a subset of splicing factors is induced , which commits the transcript to being spliced and exported or, if the signal is redundant, to degradation. This presents a problem for HIV-1 gene regulation as for production of some proteins splice sites 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, 1998b).

This problem is solved by HIV-1 Rev protein. Produced E in infection from a transcript which is fully spliced, it facilitates expression of L viral proteins produced from a primary transcript which is partially- or un-spliced. Domains of HIV-1 Rev are shown in Fig. 1B15.1b.

HIV-1 Rev and nuclear export of viral transcripts

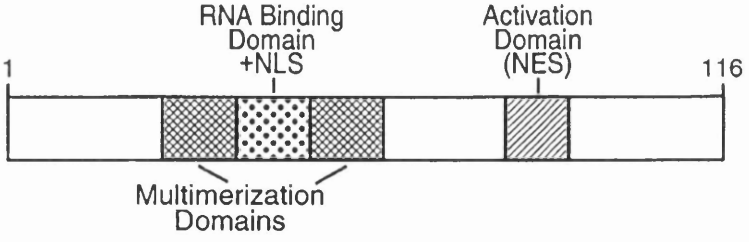
HIV-1 Rev acts to transport incompletely and unspliced HIV-1 RNAs that contain the *cis* acting Rev response element (RRE) (Malim *et al.*, 1989). This activity requires Rev to shuttle between the nucleus and the cytoplasm, exporting viral transcripts and then being reimported again. Nuclear export is mediated by a Leu-rich NES (Fig. 1B15.1b) (Fischer *et al.*, 1995; Wen *et al.*, 1995), and requires the cellular co-factor Rab (Bogerd *et al.*, 1995). The Rev-Rab-RNA complex then binds to the export receptor CRM 1 (Fornerod *et al.*, 1997a) and exits the nucleus. Once in the cytoplasm, the complex dissociates, presumably exposing the NLS, and Rev is reimported into the nucleus. HIV-1 transcripts bound to Rev are thus exported from the nucleus. This shuttling process requires the GTP bound form of Ran (Richards *et al.*, 1997). This model is consistent with the shuttling receptor model illustrated in Fig. 1B4, where the receptor is CRM1 and the NES is the Leu-rich signal in Rev.

Fig. 1B15.1a 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. 1B15.1b 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). Taken from (Cullen, 1998a)

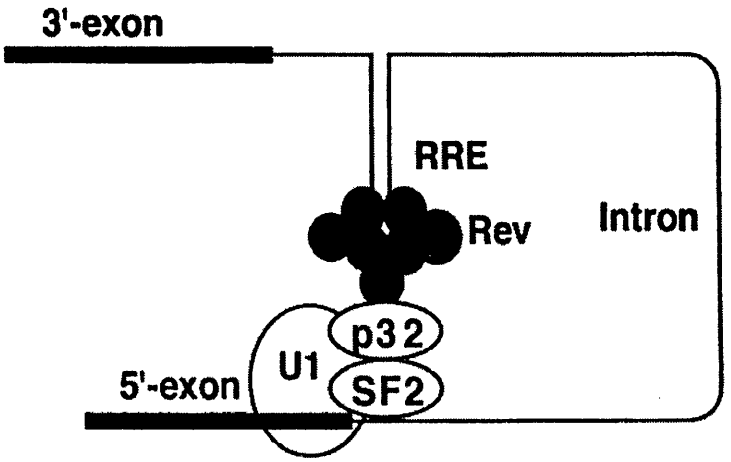
HIV-1 Rev and splicing

HIV-1 Rev has a second functional domain. A basic domain (aa 34-50) (Fig. 1B15.1b) which binds RNA, and contributes to the ability of Rev to oligomerise; this domain is also able to inhibit 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. 1B15.1c) of Rev bound to RRE interacting with p32, which is associated to ASF/SF2 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/SF2-p32 interaction has also been implicated in mRNA export via 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 of Rev shuttling.

HIV-1 does not only affect the activity of cellular splicing factors, expression of the SR protein SC35 is also seen to be upregulated during infection (Maldarelli *et al.*, 1998). *In vitro* and *in vivo* alteration of the amount of SC35 can influence the selection of alternative splice sites (Fu & Maniatis, 1992; Wang & Manely, 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 increases in SC35 alter the pattern of splicing in HIV-1 transcripts but it seems a likely scenario. If increased levels of SC35 are recruited to the spliceosome, where normally SC35/ASF/SF2 and p32 are associated, Rev could be involved in recruiting SC35 and so in the selection of viral transcript splice site (compare Figs. 1B15.1c and 1B2.3).

The effects of HIV-Rev on splicing and mRNA export which contribute to HIV gene regulation can be seen in Fig. 1B15.1d.

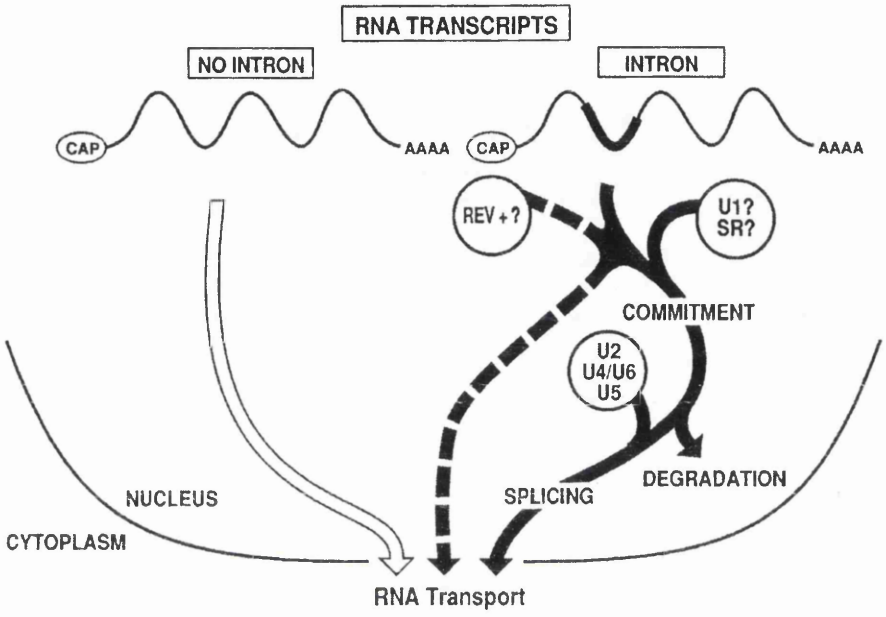
Fig.1B15.1c Putative model for p32-Rev interaction



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.

Taken from (Tange *et al.*, 1996).

Fig. 1B15.1d 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. Taken from (Cullen, 1998a).

1B15.2 Adenovirus

Although transcriptional control is important in adenovirus infection, only nine viral promoters are used by the virus and so post-transcriptional control mechanisms are needed to produce the required numbers 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, in general towards the production of smaller mRNAs (reviewed in Leppard, 1998). Two viral proteins ORF6 and 55K have been shown to be essential for the transport of viral L 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 which are not spliced, adenovirus transcripts are extensively spliced. At E times, splicing factors have a general distribution but at later times (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, it has been proposed that these foci could reflect host splicing machinery/transport pathways being saturated with viral transcripts and so "backing up" in the nucleus or that they are a result of increased recycling of splicing factors caused by the increased splicing activity or that they may be in some way related to the block on nuclear transport of host RNA. It may be the redistribution of snRNPs with viral L transcripts that acts 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.

1B15.3 Influenza

The genome of Influenza viruses consists of eight single-stranded RNAs of negative polarity, present as ribonucleoproteins. Each one is transcribed as an individual unit in the nucleus of infected cells. A significant amount of viral gene expression regulation occurs post-transcriptionally.

NS1 in splicing

NS1 has been reported to be involved in the modulation of mRNA splicing, it 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). It has been suggested that interaction of NS1 with U6 snRNA (Qiu *et al.*, 1995) inhibits

splicing by preventing base pairing between U6, U2 and U4 snRNAs and that 5' site selection is regulated by an alteration of activity of soluble splicing factors such as ASF/SF2, 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 which occurs 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 unfeasible to suggest that NS1 interacts with a similar array of proteins to disrupt/modulate splicing.

NS1 in polyadenylation and mRNA export

NS1 is required for the nuclear retention of cellular poly $(A)^+$ mRNA, where they are degraded (Katze & Krug, 1984) allowing viral polymerase access to cap snatching substrates (Katze & Krug, 1984). NS1 binds to poly (A) (Qui & Krug, 1994), to the CPSF-30 subunit (Nemeroff *et al.*, 1998) and to PAB II (Chen *et al.*, 1999), inhibiting both polyadenylation and cleavage of the 3' end of mRNA; the combined approaches cause nuclear retention of cellular mRNA. NS1 has been shown to be transdominant over the Rev-NES (Chen *et al.*, 1998b) and 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 maybe be secondary to inhibition of transport.

NS2 mediates nuclear export of viral mRNAs

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

1B15.4 Summary of viral post-transcriptional gene regulation.

In other virus systems gene expression is controlled post-transcriptionally. Examination of post-transcriptional regulation in the viral systems discussed shows that IE63 is not unusual in its effects and comparisons reveal common themes in viral disruption of RNA processing, namely,

(i) Reorganisation of snRNPs, either leading to modulation of splicing function or secondary to alterations in the activity of cellular splicing factors.

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

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

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

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

Chapter 1 Part C. Proteins found to interact with IE63 and aims of the study

This study identifies 4 cellular proteins which interact with IE63, all of which have proposed roles in RNA transcription/processing. One viral protein is also found in a complex with IE63. Properties of these 5 proteins are summarised below.

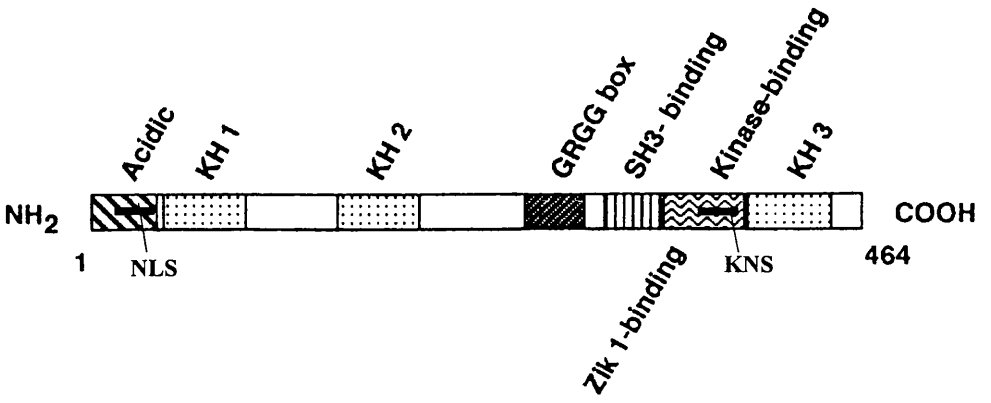
1C1 hnRNP K

hnRNP K, first discovered as a component of the hnRNP particle (Matunis *et al.*, 1992), can be found as at least five alternatively spliced isoforms (Dejgaard *et al.*, 1994). The relevance of different isoforms 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); a large fraction of hnRNP K is not associated with the hnRNP particle. Like IE63, hnRNP K is a multifunctional protein and has multiple domains. Functional domains of hnRNP K are illustrated in Fig. 1C1a.

hnRNP K binds RNA with a preference for poly (rC) RNA (Matunis *et al.*, 1992). The binding of RNA is via KH domains (Siomi *et al.*, 1994), KH (hnRNP K homology) domains were first identified as an RNA binding motif in hnRNP K, which has three copies of the conserved 65-70aa motif. Phosphorylation of hnRNP K decreases its binding to RNA (Dejgaard *et al.*, 1994).

hnRNP K contains another novel motif, the KNS. The KNS domain is a 38 aa domain which mediates bi-directional transport between the nucleus and the cytoplasm. hnRNP K shuttling, unlike that of hnRNP A1 which is mediated by the M9 motif, is transcription-independent. However, hnRNP K also contains a classic nuclear localisation signal which when deleted renders hnRNP K dependent on RNA pol II transcription for nuclear localisation. Thus hnRNP K can access a transcription-dependent pathway, raising the possibility that hnRNP K shuttles mRNA, the tie to transcription preventing wasteful reimporting of

Fig. 1C1a Domains of hnRNP K



NLS: nuclear localisation signal (aa 21-37), KH1-KH3: KH domains-novel 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: interaction site for proteins containing an SH3 domain (aa 289-315). (Taken from Bomsztyk *et al.*, 1997).

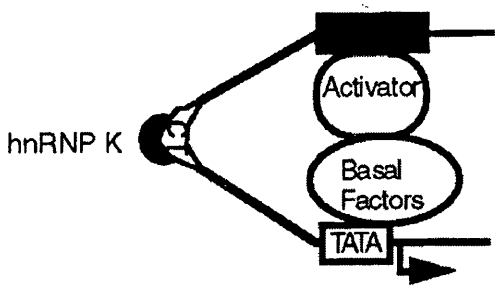
export factors. hnRNP K cannot be shown to utilise any of the known nuclear import receptors (Michael *et al.*, 1997).

hnRNP K also binds to DNA which is the preferred ligand over RNA *in vitro* (Tomonaga & Levens, 1995). This binding is sequence specific to G+C rich sequences. hnRNP K 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 is a naturally occurring C rich homopyrimidine motif, consisting of four imperfect direct repeats of the sequence CCCTCCCCA, and a fifth repeat, 9-bp downstream of the first 4, which acts as a *cis* enhancing element for c-*myc in vivo* (DesJardins & Hay, 1993). hnRNP K can interact with TBP (TATA binding protein) 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 allows bending of DNA to bring together *cis* elements facilitating transcription (Fig. 1C1b).

Using chimeric constructs and a variety of different promoters, it is suggested that hnRNP K can transactivate a number of genes (Lee *et al.*, 1996). In one case when the adenovirus E2 promoter is used, hnRNP K is seen to lower rather than activate transcription. This is reminiscent of Y-box proteins, another set of multifunctional proteins where activation or repression are promoter dependent. As well as interacting with the transcriptional activator TBP, hnRNP K interacts with a transcriptional repressor Zik1. Interaction is blocked by the binding of poly(A) RNA to hnRNP K (Denisenko *et al.*, 1996), raising the possibility that RNA binding to hnRNP K can regulate transcription.

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.*,

Fig. 1C1b Possible mechanism of action of hnRNP K



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

1995b) and *vav* (Bustello *et al.*, 1995) via SH3 domains; these domains are known to facilitate protein:protein interactions between signalling molecules. 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).

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

Finally, hnRNP K has been implicated in regulation of translation. It interacts with the elongation factor-1a (Densisenko and Bomsztyk, Personal Communication) and together with hnRNP E1, interacts with and causes translational silencing on erythroid 15-lipoxygenase (LOX) mRNA (Ostareck *et al.*, 1997). *In vitro* data suggests this is achieved by specific inhibition of 80S ribosome assembly on LOX mRNA.

hnRNP K and viruses

Hepatitis C core protein can relieve hnRNP K's repression of the human thymidine kinase (TK) gene (Hsieh *et al.*, 1998). Binding of core has been shown to be in a region of hnRNP K which may cause disruption of hnRNP K binding to other cellular factors, and this may therefore 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. Hep 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.

The progressive life cycle of the human papillomavirus, 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, over expressed 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 appears to utilise hnRNP K to control gene expression at the level of translation.

1C2 P32

P32, first isolated as a protein tightly associated with ASF/SF2 purified from HeLa cells (Krainer *et al.*, 1990b), has recently been shown to regulate RNA splicing by inhibiting phosphorylation of and RNA binding by ASF/SF2 (Peterson-Mahrt *et al.*, 1999).

Despite this, the distribution of p32, like its function, is controversial. P32 is reported to have a mitochondrial distribution (Matthews & Russell, 1998, Muta *et al.*, 1997) 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), Epstein-Barr virus EBNA I protein (Chen *et al.*, 1998a, Wang *et al.*, 1997) adenovirus polypeptide V (Matthews & Russell, 1998), and HIV-1 proteins Rev and Tat (Luo *et al.*, 1994, Tange *et al.*, 1996, Yu *et al.*, 1995b) (see HIV-1 section). Together, these interactions have suggested a role for p32 not only in splicing (Luo *et al.*, 1994, Peterson-Mahrt *et al.*, 1999, Tange *et al.*, 1996, Yu *et al.*, 1995b) but also in

nucleocytoplasmic transport (Matthews & Russell, 1998, Peterson-Mahrt *et al.*, 1999), transport to and from the mitochondria (Jiang *et al.*, 1999, Matthews & Russell, 1998) and in maintaining oxidative phosphorylation (Muta *et al.*, 1997).

1C3 SAP145

Splicing associated protein 145 (SAP145), 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. 1C3) (Champion-Arnaud & Reed, 1996).

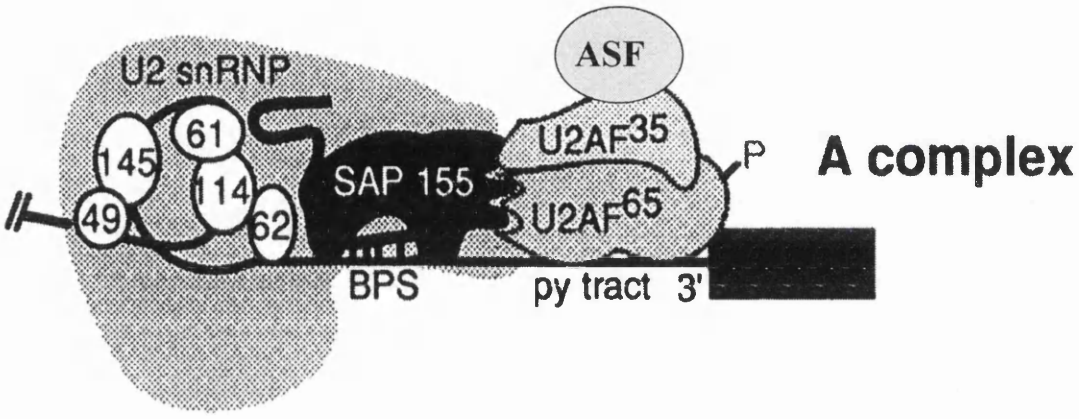
1C4 Casein Kinase II (CK2)

CK2 can use either ATP or GTP to phosphorylate serine or threonine residues that are N-terminal to acidic amino acids. CK2 is known to phosphorylate and in some cases modulate the activity of over 160 proteins (and the list is growing!), including transcription factors e.g., Sp1 (Pugh & Tijian, 1990), signalling molecules e.g. A-Raf kinase (Hageman *et al.*, 1997), ribosomal proteins e.g. L5 (Kim *et al.*, 1996), and a great number of nuclear proteins. From its substrates it is implicated in the regulation of signal transduction, transcriptional control, apoptosis, cell cycle and consequently cancer. CK2 is classically known as a heterotetrameric holoenzyme that can exist as 3 forms $\alpha_2\beta_2$ $\alpha'_2\beta_2$ or $\alpha\,\alpha'\beta_2$, α and α' are the catalytic subunits and β is the regulatory subunit. Yet 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).

CK2 and viruses

CK2 has been shown to phosphorylate a number of viral proteins (table 1C4) 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

Fig. 1C3 Position of SAP145 in U2 snRNP



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. Note ASF/SF2 is also in contact with p32 and the U1 snRNP (Fig. 1B2.3).

Table 1C4 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	PA polymerase	polymerase
Polyoma	VP1	major capsid protein
HSV	VP22	viral replication
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

phosphorylated. CK2 phosphorylation is also important in HIV-1 infection. HIV-1 Rev activates CK2 and the activated CK2 phosphorylates several cellular and viral proteins in HIV-1 infected cells (Ohtsuki *et al.*, 1998). Phosphorylation of Rev significantly reduces its binding to RRE, suggesting that CK2 may effect nuclear export of HIV-1 transcripts.

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), IE63 is also nucleotidylated (Blaho *et al.*, 1993) a modification which 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.

1C5 HSV-1 Thymidine kinase (vTK)

During synthesis of nucleotides for DNA synthesis cellular TK (cTK) uses Mg-ATP to monophosphorylate thymidine. This is a very specific reaction. HSV-1 encodes its own thymidine kinase enzyme (vTK), which shows a much broader range of phosphorylation activities. It is a deoxypyrimidine kinase, able to phosphorylate a range of nucleoside analogues, it can carry out multiple phosphorylations and can use nucleoside triphosphates other than ATP as the phosphate source. (reviewed in Evans *et al.*, 1998).

HSV TK is considered a luxury function as in normal cell culture in exponentially growing cells it is dispensable. However in serum starved resting cells, such as skin cells which HSV normally infects, it may be required, as the cell *de novo* pathway may not be active (Jamieson *et al.*, 1974). In addition TK^- HSV-1 viral mutants, are less virulent than the parental TK^+ virus (Field & Wildy, 1978). In mice, vTK is not required for DNA replication at the primary site of infection, at least when that site is the cornea. In contrast, TK^- mutants do not replicate in trigeminal ganglia (Tenser & Dunston, 1979), suggesting that vTK is required for secondary replication in the ganglia. Despite lack of replication, TK^- mutants can establish latent infection (Coen *et al.*, 1989). Reactivation from latency following superinfection with wt virus has been demonstrated, although TK^- virus could not be reactivated from explanted ganglia. Southern blotting of the reactivated virus, demonstrated *in vivo* complementation of the mutant virus with wt virus. In the

same study, LATs were detected in only ~2-5 fold higher frequencies in wt infected ganglia than in TK- virus infected ganglia. Thus vTK is not required to establish a latent infection but is needed for reactivation (Coen *et al.*, 1989).

1C6 Aims of this Project

IE63, is involved in an extraordinary range of regulatory feats and how it does this has been the subject of many studies. The key to understanding the way a protein functions is often to discover those proteins it interacts with. If these have a known role in the host cell then much can be deduced about which pathways the viral protein is affecting. The most convincing explanation for the diverse functions of IE63 is that it interacts with and modifies the behaviour of essential regulatory proteins involved in the different processes it affects.

- The primary aim of this project therefore was to identify which viral and cellular proteins IE63 interacts with.
- Secondary to this was the uncovering of the ways in which these interactions could influence IE63 activity in infection.

The data presented will be used to argue that "IE63 is a multifunctional protein which regulates HSV-1 infection though multiple protein:protein interactions".

1C7 Significance of interactions between IE63 and host cellular proteins

IE63 is a key protein in HSV-1 infection and so understanding of its action is important to the fundamental understanding of HSV-1 biology. In addition, IE63 has homologues in every member of the *Herpesviridae* so understanding its activities will have implications for the understanding of these other viruses. Any key viral protein, especially one so conserved, is a candidate for anti-viral therapy. Drugs designed to disrupt IE63 protein:protein or protein:nucleic acid interactions are an obvious potential offshoot of these kinds of study. Finally, HSV-1 via IE63 interferes with and utilises host cellular mechanisms for its own replication, therefore its study might be expected to shed considerable light on host cell processes.

Chapter 2: Materials and Methods

2A Materials

2A1 Plasmids

The following plasmids were provided by the acknowledged investigator

pGEX-27: (Mears & Rice, 1996) Provided by S. Rice, Calgary University, USA. Expresses the fusion protein GST-IE63.

pGEX-K: (Michelotti *et al.*, 1996) Provided by K. Bomsztyk, University of Washington, USA. Expresses the fusion protein GST-hnRNP K (also refered to as GST-K).

pGEX-145: (Seghezzi *et al.*, 1998) Provided by R. Reed, Harvard University, USA. Expresses the fusion protein GST-SAP145.

Various plasmids expressing MBP/GST-CK2β and truncations and GST-CK2α: (Heriche et al., 1997; Leroy *et al.*, 1999) Provided by O. Filhol. INSERM, Grenoble, France.

pCMV-63: EcoRI/BamHI fragment containing IE63 and 400 C-terminal base pairs (bp) was digested out of pEGFP63, and ligated into the EcoRI/BamHI sites in the multiple cloning site of pCMV10 (Stow *et al.*, 1993).The plasmid therefore consists of the complete open reading frame of IE63 downstream of a CMV immediate early promoter, constructed by N. Reuthmeir, Institute of Virology, Glasgow. P. Lomonte provided pEGFP63 and pCMV10.

2A2 Enzymes

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

2A3 Peptide substrate

The CK2 peptide substrate (Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu) was synthesised by Howard Marsden, MRC Virology Unit, Glasgow.

2A4 Bacteria and bacterial culture media

2A4.1 Bacteria

The *E.coli* strain DH5α was used for maintenance and propagation of plasmid DNA. Protein expression from pGEX/MBP derived plasmids utilised *E. coli* strain BL21 (Studier *et al.*, 1990). During optimisation of GST fusion protein expression use of the *E. coli* stain X_A90 was investigated.

2A4.2 Bacterial culture media

All strains were grown in L-Broth (10g NaCl, 10g Bactopeptone, 5g yeast extract in 1l water, pH 7.5) or at times in 2YT broth (5g NaCl, 16g Bactotryptone, 10g yeast extract in 1l water). Agar plates were made with 1.5% (w/v) agar in L-broth. Where necessary, media and LB agar plates were supplemented with antibiotic: 50μg/ml kanomycin (for pET28c transformed cells) or 100μg/ml ampicillin for all other plasmid containing bacteria.

2A5 Cells and tissue culture media

All cell culture media were obtained from Gibco.

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 100μg/ml streptomycin.

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.

Vero cells

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

Vero 2-2 cells

Derived from African green monkey kidney, Vero cells were grown in GMEM supplemented with 10% foetal calf serum and antibiotics as above. They were then transfected with an IE63 expressing construct encoding geneticin resistance and selected and grown in the presence of 200μg/ml G418.

2A5.4 Radioactive media

When cells were labelled, the media used were [^{35}S] l-methionine medium (Eagles medium (Eagle, 1959) containing 20% the normal methionine level, 2% new born calf serum and 30μCi/ml [^{35}S] l-methionine) or [^{32}P]-orthophosphate medium (Eagles medium containing 20% the normal phosphate level, 2% new born calf serum and 30μCi/ml [^{32}P]-orthophosphate).

Table 2A7 Antibodies used

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

Anti sera Against	Type	W. Blot	IP	IF	References	Source
PRIMARY ANTIBODIES		dilution	µl used	dilution		
IE63	Rabbit	1:100	5µl	1:600	NP	H. Marsden, MCR Virology Unit, Glasgow
IE63 (#1113)	Mouse	1:2000	5µl	1:100	(Ackerman *et al.*, 1984)	Goodwin Institute for Cancer Research, Florida
IE110 (11060)	Mouse	1:10000	ND	ND	(Everett, *et. al.*,1993b)	R. Everett, MCR Virology Unit, Glasgow
IE175 (58S)	Mouse	1:2000	5µl	ND	(Showalter, *et. al.*, 1981)	S. Silverstein, Dept. Microbiology, Columbia Uni.
IE175 (H640)	Mouse	1:2000	5µl	ND	(Ackerman *et al.*, 1984)	S. Silverstein, Dept. Microbiology, Columbia Uni.
IE175 (10176)	Mouse	1:5000	ND	ND	(Everett, *et. al.*,1993a)	R. Everett, MRC Virology Unit, Glasgow.
hnRNP A1	Mouse	1:1000	ND	ND	(Kamma, *et. al.*, 1995)	G. Dreyfuss, HH Medical Institute, Philadelphia
hnRNP C1/C2	Mouse	1:1000	ND	ND	(Kamma, *et. al.*, 1995)	G. Drefuss, HH Medical Insitute, Philadelphia
hnRNP K (#54)	Rabbit	1:10000	1µl	ND	(Van Seuningen *et al.*, 1995a)	K. Bomsztyk, Dept. Med. Washington Uni, Seattle
CK2 α subunit	Rabbit	1:2000	ND	ND	NP	O. Filhol, Inserm, Grenoble, France
CK2 β subunit	Mouse	1:2500	ND	ND	(Nastainczyk, *et. al.*, 1995)	Calbiochem, San Diego, CA
SAP 145	Rabbit	1:1000	1µl	1:150	(Seghezzi, *et. al.*, 1998)	R. Reed, Harvard Uni., Boston, USA
p32	Mouse	1:200	ND	1:50	NP	J. Scott, Dept. Virology, Glasgow Uni
Thymidine Kinase	Rabbit	1:250	ND	ND	NP	W. Summers, Yale Uni, USA
70kDa U1 snRNP (H111)	Mouse	1:1000	ND	ND	(Kastner, *et. al.*,1992)	Euro Diagnostica, Arnhem, NL
SC-35	Mouse	1:2000	ND	1:2000	(Fu, *et. al.*,1992)	Sigma
gE (#3114)	Mouse	ND	ND	1:200	(Cross *et al.*, 1987)	H. Marsden, MRC Virology Unit, Glasgow.
65K DNA Binding Protein (ZlF11)	Mouse	1:1000	ND	ND	(Schenk *et al.*, 1988)	A. MacLean, Dept. Virology, Glasgow Uni.
SECONDARY ANTIBODIES						
Anti Mouse HRP conjugate		1:1000	ND	ND		Sigma
protein A HRP conjugate		1:1000	ND	ND		Sigma
Anti Mouse FITC Conjugate		ND	ND	1:100		Sigma
Anti Rabbit TRITC Conjugate		ND	ND	1:100		Sigma
Anti Rabbit cy3 Conjugate		ND	ND	1:100		Sigma

2A6 Viruses

Viruses used were

HSV-1 strain 17^{+}
Wild type virus as described by (Brown *et al.*, 1973). Referred to as wildtype (wt) virus for the rest of this thesis.

HSV-1 27lacZ
An HSV-1 IE63 (ICP27) insertion mutant, resulting in inactivation of the IE63 gene, (Smith *et al.*, 1992). A gift from R. M. Sandri-Goldin, University California, Irvine, California.

HSV-1 gE⁻ (in1404)
HSV-1 U_S7 insertion mutant resulting in disruption of the U_S7 gene and which fails to make glycoprotein E (gE). (Johnson *et al.*, 1988). Obtained from N. Stow, MRC Virology Unit, Glasgow.

HSV-1 TK1301
HSV-1 Thymidine kinase deletion mutant (Sanders *et al.*, 1982), from D. Dargan, Institute of Virology, Glasgow.

2A7 Antisera and monoclonal antibodies

Antisera and monoclonal antibodies (Mabs) used during this investigation and their source are listed in Table 2A7.

2A8 Radiochemicals

All radiochemicals were purchased from Dupont at the following specific activities

γ–[^{32}P] ATP	6000Ci/mmol	10mCi/ml
[^{35}S] l-methionine	1175Ci/mmol	10mCi/ml
[^{32}P]-orthophosphate	8500Ci/mmol	5mCi/ml
α–[^{32}P] ATP	800Ci/mmol	10mCi/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.
Bradfords 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.
Geimsa stain	1.5%(w/v) suspension of Giemsa in glycerol, heated at 56°C for 2h and diluted with equal volume methanol.
HEPES extract buffer	50mM HEPES , 50mM NaCl, 0.1%(v/v) NP40, pH7.5.
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
50xTAE	0.2M Tris base, 0.05M EDTA (pH 8.0), pH to 8.0 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 PVDF	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 purchased from BDH Chemicals UK or from Sigma Chemical Co. unless otherwise stated in this Section or in the methods.

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

2A11 Autoclaving and glassware sterilisation

Equipment and solutions were sterilised at 15psi for 20 min by staff in wash room, Institute of Virology, Glasgow. Glassware was sterilised by baking in an oven at 180°C for at least 12 h. Heat-labile solutions were sterilised by filtration though a Whatman syringe filter (diameter 0.2μm) into a sterile tube.

2B Methods

2B1 Preparation and transformation of competent *E.coli* cells for plasmid growth and maintenance, and for protein expression.

Plasmids were grown and maintained in *E.coli* strain DH5α, and plasmids incorporating GST or MBP expression systems were transfected into *E.coli* strain BL21 or X_A90 immediately before protein expression. For all strains, the method of preparing competent *E.coli* and of transformation of plasmids into bacteria was the same.

A 1ml overnight culture was inoculated into 100ml L-broth and grown for about 3h at 37°C in a shaking incubator until the OD_{630} was approximately 0.4. The culture was then cooled on ice and cells pelleted by centrifugation at 5000rpm for 10min at 4°C in Sorvall RT 6000B refrigerated centrifuge. The cells were resuspended in 50ml cold 50mM $CaCl_2$ and left on ice for 30min. Cells were then pelleted as before and resuspended in 10ml cold 50mM $CaCl_2$, 15% glycerol. 200µl aliquots were snap frozen and stored at -70 C.

About 10ng plasmid DNA were transformed into a 200µl aliquot of competent *E.coli* which was thawed on ice. The DNA was added to the cells and the mixture incubated on ice for 30min prior to heat shock in a 42°C waterbath for 2min. 800µl of L-broth was added to the cells, which were shaken for 60min at 37°C before plating onto LB agar plates containing appropriate antibiotic (Section 2A4). Plates were incubated overnight at 37°C.

2B2 Cell Culture

2B2.1 Growth and maintenance of cells

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:versin (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 and V2-2 cells 1:5 every 4-5 days.

2B2.2 Production of virus stocks

(i) Growth of virus

HSV-1 wt strain 17+ and viral mutants, TK$^-$ and gE$^-$, were all grown in this way. Ten roller bottles of almost confluent BHK21 C13 cells (approximately 3x10^8 cells/bottle) were infected at a multiplicity of infection (MOI) of 1 in 300 and grown in appropriate media (Section 2A5) at 31°C under 5% CO_2 for 3-5 days. When cells had rounded up and were starting to detach from the plastic, virus was harvested by shaking bottles till all the cells were in the medium and centrifuging at 2000rpm for 30min at 4°C in Sorvall RT 6000B refrigerated centrifuge. The supernate was then spun at 12000rpm for 2h (Sorvall GSA rotor). The resultant pellet consisted of cell released virus and was resuspended in 1ml/bottle BHK medium, sonicated briefly to disrupt the pellet, aliquoted (10x1ml) and snap frozen. To harvest cell associated virus, the pellet from the first centrifugation was resuspended in 5ml of BHK medium and sonicated thoroughly in a sonibath. Cell debris was pelleted by centrifugation at 2000rpm for 10min (Sorvall RT 6000B refrigerated centrifuge), supernatant obtained from this spin contained cell associated virus. To increase the yield of virus, the cell pellet was re-suspended in a further 5ml of medium, sonicated and centrifuged again. Both supernates containing cell associated virus were combined, aliquoted (10x1ml) and snap

frozen. 27lacZ virus was produced in the same way but grown in the IE63 complementing cell line 2-2 (Sekulovich *et al.*, 1988).

(ii) Sterility checks

An inoculum of each virus was streaked on a blood agar plate (to test for bacterial contamination), and a brain heart infusion agar plate (to test for fungal contamination), incubated at 37°C or room temp respectively for 1 week and inspected for any growth. Mycroplasma contamination although harder to detect was regarded as present if blood agar plates discoloured.

(iii) Titration

Virus stocks were titrated on 60mm sterile dishes of confluent BHK monolayers (~10^6 cells). 1:10 Serial dilutions of virus were made (10^{-1}-10^{-8}) and 100µl of each plated out onto the monolayers. After 1h for absorption 5ml of BHK medium (Section 2A5) supplemented with methyl-cellulose to give a final concentration of 1% methyl-cellulose was added to prevent progeny virus spreading through the medium. Dishes were incubated at 37°C for 2 days and cells were Giemsa stained. Plaques were counted under dissection microscope and the titre calculated as plaque forming units/ml (pfu/ml).

2B2.3 Viral infection of cells

Almost confluent monolayers were grown on 90mm plates and infected with various viruses or left uninfected. On some occasions, infected and uninfected cells were labelled with [^{35}S] l-methionine or [^{32}P] orthophosphate. From these soluble protein extracts were made and used in various immunoprecipitation and pull down assays. With all viruses and in all cell types, infection was at a MOI of 10 and unless otherwise stated infection was for 16h. 80% confluent cells were inoculated with virus in a small volume and, after 1h for absorption, more of an appropriate growth medium was added (Section 2A5). If the cells were to be labelled, after 1h [^{35}S] l-methionine medium (150µCi) or [^{32}P]-orthophosphate

medium (150μCi) (Section 2A5) were added in place of the usual growth medium. Cells for immunofluorescence were handled in a similar way but on a smaller scale and were infected when ~60% confluent. 13mm coverslips in Linbro plate wells were seeded at $0.5x10^5$ HeLa cells per well in 1ml of normal HeLa medium and incubated overnight at 37°C prior to infection at a MOI of 10.

2B2.4 Transfection of cells

HeLa cells were transfected with pCMV-IE63 expression plasmid and used to seed coverslips for immunofluorescence or 50mm dishes to make soluble protein extracts. A $175cm^2$ confluent flask of HeLa cells (~$1x10^7$ cells) was treated with trypsin:versin (Section 2B2.1) pelleted and resuspended in 10ml serum and antibiotic free DMEM. A 100μl aliquot was taken and diluted 10x and counted. The cells were then pelleted again and resuspended in an appropriate volume, such that the concentration of the cells was $5x10^6$ per 750μl. For each electroporation, $5x10^6$ cells and 20μg of DNA were used. DNA was put in a 4mm cuvette and cells added, after incubation for 10min on ice and flicking gently to resuspend settled cells, the cells were electrophorated at 400V (Hybaid cell shock electrophorator), incubated on ice again for 10min and finally cells below the 'foam' collected with a Pasteur pipette in 5ml complete HeLa medium. Transfected cells were either plated onto 50mm dishes (5ml/dish) or were added to a 35mm dish with 3 coverslips (2.5ml/dish) and left for 24h, swirling every 10min in the first hour and changing medium after 6h, before harvesting as for infected and noninfected cells (Section 2B2.5) or fixing for immunofluorescence (Section 2B8)

2B2.5 Cell extract preparation

(i)Whole cell extracts

For immunoprecipitations and various pull down assays, soluble protein extracts were made of cells infected with various viruses or uninfected, these were sometimes labelled with [^{35}S] l-methionine or [^{32}P]-orthophosphate (Section 2B2.3 and 4). Cells were washed twice in cold PBS A and harvested in 10ml PBS A by scraping. Cells from 90mm dishes (~ 2x10^7 cells) were pelletted by centrifugation at 1000rpm for 5min at 4°C (Sorvall RT 6000B refrigerated centrifuge) and resuspended in 800µl HEPES extract buffer (50mM HEPES , 50mM NaCl, 0.1% NP40, pH7.5). The cells were lysed by sonication for 30-60sec in a soni-bath and debris pelletted by micro-centrifugation at 12000rpm for 10min at 4°C. The soluble protein extract was stored at -70°C. In order to standardise the amount of protein added to immunoprecipitations or pull downs the total protein content of soluble protein extracts was determined by standard Bradford's assay (Section 2B2.6) and if not already concentration was adjusted to 2mg/ml.

On one occasion cell extracts were treated with RNase A. 10U RNase ONE™ Ribonuclease (Promega) were added to cell extracts and after incubation at 37°C for 15 min the extracts were frozen and stored as above.

(ii) Nuclear extracts

For anti IE175 co-immunoprecipitations a nuclear extract was prepared as described by Panagiotidis (1997). After infection for 6h, nuclear extract was made by resuspending harvested cells in five packed cell volumes of buffer A (10mM HEPES, 1.5mM $MgCl_2$, 0.5mM DTT, and protease inhibitor cocktail (in place of separate protease inhibitors used by Panagiotidis)) and allowed to stand for 10min. The cells were pelleted for 10min at 2000rpm (Sorvall RT 6000B refrigerated centrifuge) and resuspended in 2 packed cell volumes of buffer A. After 10 strokes in a glass Dounce homogeniser, cells were centrifuged as before, the supernate was taken as the cytoplasmic fraction and the pellet as nuclei. The

nuclei pellet was resuspended in 3ml buffer C (20mM HEPES, 25% (v/v) glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM PMSF, 0.5mM DTT and protease inhibitor cocktail), and Dounce homogenised. This suspension was stirred gently with a magnetic stirring bar for 30min and centrifuged for 30min (15000rpm, Sorvall SS34 rotor), the resultant supernate was dialysed against 50 volumes buffer D (20mM HEPES, 25% (v/v) glycerol, 0.1M NaCl, 0.2mM EDTA, 0.5mM PMSF, 0.5mM DTT and protease inhibitor cocktail), and finally centrifuged for 20min (15000rpm Sorvall SS34 rotor). The precipitate was disregarded and supernate taken as nuclear extract.

2B2.6 Estimation of protein concentration

Estimation of protein concentration was performed using the method of Bradford (1976). 10µl of protein sample were diluted in 90µl dH_2O and mixed with 1ml of Bradfords reagent. After 10min at room temperature (RT) the absorbency of the solution was measured at 595nm, and converted to mg protein by comparison to a standard curve produced using known quantities of bovine serum albumin.

2B3 Co-immunoprecipitation experiments

To investigate protein:protein interactions, co-immunoprecipitations were carried out with several antisera. Conditions were optimised for each sera. An overview of this method is illustrated in Fig. 2B3.

Because of their high specificity, antibodies may be used to isolate specific antigens, and the proteins which interact with them 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.

Both polyclonal antisera and Mabs can be used for such co-immunoprecipitation experiments, however it is important to be aware of certain limitations, for

example an antibody may mask an interaction site or a protein:protein interaction may mask the antibody binding site. In this respect, polyclonal antisera are better but Mabs often give a cleaner result.

Mabs are non-precipitating as are most polyclonal antisera at low antigen concentrations employed, therefore a "sandwich" reagent is used to precipitate or isolate the antibody-antigen complex, and during this study *Staphylococcus aureus* protein A coupled to Sepharose was used. Mouse IgG_1 and IgG_3 bind poorly to protein A, to overcome this when co-immunoprecipitating with Mabs sheep anti-mouse IgG was routinely added.

Salt and detergent concentrations in buffers used to immunoprecipitate and to wash will affect the ability of proteins to interact and so determine the stringency of the immunoprecipitation.

Co-immunoprecipitation has been used to study many protein interactions, a classic example within herpes viruses is the discovery of glycoprotein L (gL), in which an anti peptide serum revealed a second protein which co-immunoprecipitated with gL, subsequently by Western blotting this protein was shown to be glycoprotein H (gH). It was then shown that gH required gL to be antigenically recognised, and gL depends on gH for proper post-transcriptional processing (Hutchinson *et al.*, 1992).

2B3.1 Co-immunoprecipitation using IE63 monoclonal antibody

Various cell extracts (~100µg) were mixed with 5µl H1113 Mab (and 1µl sheep anti mouse IgG) in 50µl buffer E (100mM Tris HCl, 100mM NaCl, 2mM EDTA, 2mM EGTA, 1% NP40, 0.5% Na deoxycholate, 0.5mM PMSF, pH 8) for 3h at 4°C. 75µl protein A-Sepharose (made up at 50% (w/v) and washed 2x in buffer E) was then added and mixing was for 1h. After pelleting the beads and multiple washes in buffer E, 50µl protein gel loading buffer was added, samples placed in a boiling bath for 3 min and co-immunoprecipitating proteins separated by SDS-PAGE (Section 2B5.1). After separation gels were subject to Western blot analysis (Sections 2B5.2-4). During investigation of interacting proteins different

buffers and IE63 antiserum were used (Appendix 1) but the overall method was as above. If a CK2 activity assay was to be carried out, protein gel loading buffer was not added and samples were processed as described (Section 2B6).

At one point in this thesis, IE63 co-immunoprecipitation is done under different conditions as described by Panagiotidis et al. (1997), this is the same method by which IE175 co-immunoprecipitations were carried out (see below).

2B3.2 Co-immunoprecipitation using IE175/IE63 monoclonal serum

Using the method of Panagiotidis (1997), 5µl of Mab, either H1113 against IE63 or 58S against IE175, was mixed with 200µg nuclear extract and incubated at 4°C for 2h in a final volume of 100µl binding buffer (10mM Tris-HCl, 50mM NaCl, 5% glycerol, 1mM EDTA, 4mM dithiothreitol, 0.2mg/ml ovalbumin, (pH 7,9)). 30µl protein A-Sepharose (made up as 50% (w/v) and washed 2x in binding buffer) was added and the incubation was continued for 2h at 4°C with constant mixing. The beads were washed 4x with 0.5ml binding buffer and resuspended in 40µl protein loading buffer. After boiling for 3min, samples were separated by SDS-PAGE electrophoresis and analysed by Western blotting.

2B3.3 Co-immunoprecipitation using hnRNP K anti-serum

Beads for immunprecipitation were made up by mixing protein A-Sepharose beads (made up as 50% (w/v) and washed 2x in binding buffer) with anti hnRNP K or preimmune sera in binding buffer (5mM Tris-HCl, 250mM NaCl, 1mM EDTA and 0.05% NP40, pH7.4) at 4°C for 1h (1µl serum/10µl beads). Immunoprecipitation was carried out by mixing the immune or preimmune beads with wt infected, 27lacZ infected or mock infected (mi) BHK cell extracts (~200µg) for 2h in binding buffer at 4°C. After centrifugation and multiple washes, 30µl protein gel loading buffer was added to the beads, they were boiled and precipitated proteins were separated on a 10% SDS-PAGE gel and transferred to nitrocellulose membrane for Western blotting analysis. Again for CK2 activity

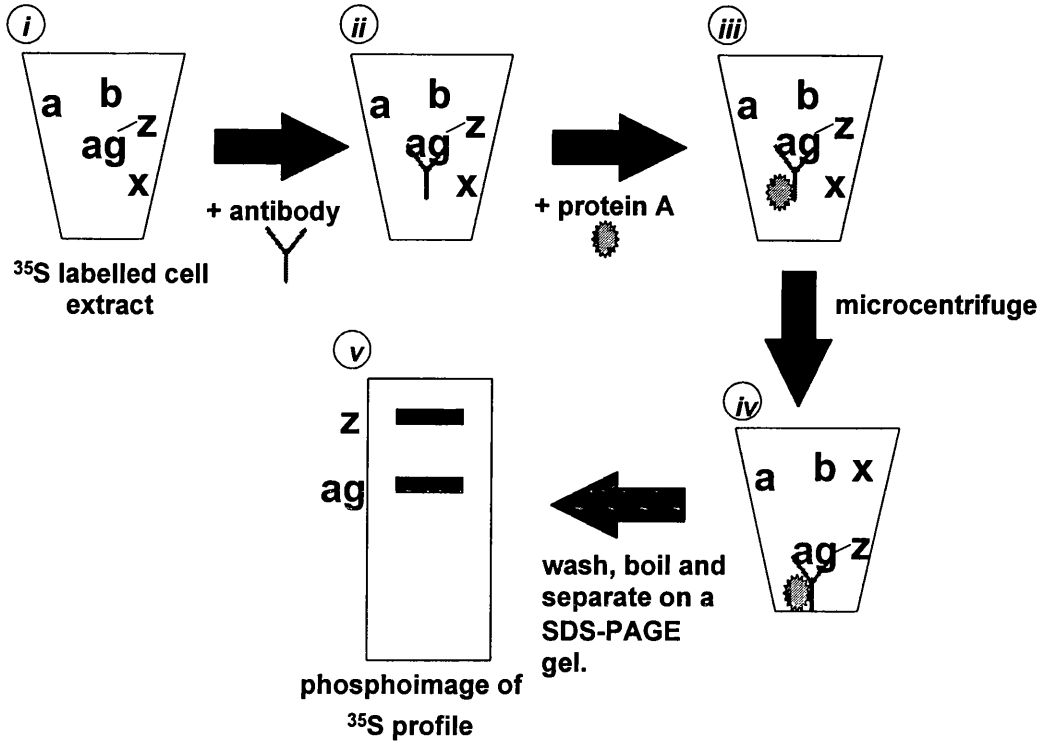


Fig. 2B3 Co-immunoprecipitation

The appropriate antibody (Y) was incubated with radiolabelled cell extract (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 phosphoimaging and an estimation of interacting protein size made by comparison with molecular weight markers simultaneously run on the gel.

assays, protein gel loading buffer was not added and samples were processed as described (Section 2B6).

2B3.4 Co-immunoprecipitation using SAP145 antiserum

As for hnRNP K co-immunoprecipitation but using 5µl of anti-SAP145 serum.

2B4 Fusion protein expression, purification and pull down assays

The Glutathione S-transferase (GST) gene fusion system can be used to identify new protein:protein interactions, or to confirm and investigate those identified in other systems. The gene for the protein of interest is cloned into a pGEX plasmid in frame with the GST gene from the parasitic helminth *Schistosoma japonicum.* Fusion proteins are expressed in *E.coli* cells containing the recombinant pGEX plasmid. A protease deficient strain of *E. coli*, BL21 is used to minimise proteolytic degradation of the fusion protein. Protein expression from a pGEX plasmid is under the control of the *tac* promoter, which is induced using the lactose analogue isopropyl β-D-thiogalactoside (IPTG). Induced cultures are allowed to express GST fusion proteins for several hours, after which cells are harvested and lysed, cellular debris is cleared by centrifugation and fusion protein purified and immobilised by binding to Glutathione which is attached to Sepharose 4B (Smith & Johnson, 1988). After fusion proteins are bound to the matrix, it is washed with buffer to remove non-specifically bound bacterial proteins and mixed with cellular extracts. The beads serve as an affinity matrix allowing proteins which interact to bind to the immobilised target fusion protein. Bound proteins can be partially purified by a simple centrifugation step, hence the name "pull down" assay. Beads are washed again to remove non-specifically bound cellular proteins and proteins analysed by SDS polyacrylamide gel electrophoresis. An overview of this method can be seen in Fig. 2B4.

During fusion protein expression several factors can be varied to maximise expression of a particular protein. Decreasing the temperature at which fusion proteins are expressed will decrease total yield but will also decrease the action of proteolytic enzymes which may degrade fusion proteins, it can also increase the solubility of the fusion protein, and alter its folding. Producing more fusion protein by increasing the IPTG concentration may increase the amount of product. Increasing the time of induction will increase how much fusion protein is expressed but will also increase the length of time proteases have to work in. As with co-immunoprecipitations, salt and detergent concentrations along with target and interacting protein concentration will effect which proteins are pulled down.

GST pull down assays have been successfully used by many workers, for example a similar approach to the one used here was used to identify an interaction between another immediate early HSV-1 protein IE110 and a novel 135kDa cellular protein (Meredith *et al.*, 1994) and was also used to look at the sequence requirements for IE110 multimerisation and interaction with the 135kDa protein (Meredith *et al.*, 1995).

Fusion protein pull downs are relatively easy to scale up after initial studies. GST has the advantage over other fusion systems of being comparatively small (compared to β-galactosidase fusions) making it easier to express large proteins as fusions (optimum size for expression in bacteria being 60kDa) and being uncharged (compared to 6 His tags), meaning GST is less likely to interfere with the interactions of and folding of IE63. Pull downs were to be scaled up to the point where bands were visible on a Coomassie stained gel, when they would be cut out and sequenced using Laser Mass Map Spectroscopy.

2B4.1 GST-IE63/hnRNP K/SAP 145

Expression of GST-IE63 was optimised, the results of which are included in this thesis (Appendix 2). What follows is the final protocol.

BL21 *E.coli* were transformed with plasmids expressing GST-hnRNP K, GST-SAP145, GST-IE63 or GST alone and grown on LB plates containing ampicillin (Section 2B1.5). Single colonies were picked and inoculated into 10ml LB broth containing ampicillin, the culture was grown overnight and next morning a 1:10 dilution into 100ml was made. After further growth at 37°C and at an OD_{630} of 0.4-0.6, fusion protein expression was induced by adding 0.2mM IPTG for 2h at 37°C. Bacteria were then harvested, by centrifugation at 3000rpm for 10 min in Sorvall RT 6000B refrigerated centrifuge, resuspended in 5ml lysis buffer (PBS 1% Triton X-100) and sonicated on ice using a soni-probe. Cell debris was separated from soluble proteins by centrifugation (15000rpm, 4°C, 20min, Sorvall SS34 rotor). Fusion proteins were purified from bacterial soluble protein lysates by binding to pre-swollen Glutathione-Sepharose 4B beads. Beads and supernate containing soluble protein lysate (5ml lysate/400µl beads), were mixed for 1h at 4°C. After washing beads 3 times with PBS 1% Triton X-100 and once with PBS, beads with protein bound were mixed with ~100µg cell extract (Section 2B2.5) for 1h at 4°C in 5ml binding buffer (50mM Hepes, 50mM NaCl, 0.1% NP40 with the addition of protease inhibitor cocktail (Boehringer Mannheim), pH7.5). Approximately equal amounts of GST and GST-hnRNP K, GST-SAP145 or GST-IE63 were used as judged by Coomassie Blue staining of an SDS-PAGE gel of the fusion proteins bound onto Glutathione-Sepharose beads. Beads were washed 4x in binding buffer and bound proteins separated on SDS PAGE gel. Bound proteins were visualised using a phosphoimaging system (Section 2B5.4) and Western blotting (Section 2B5).

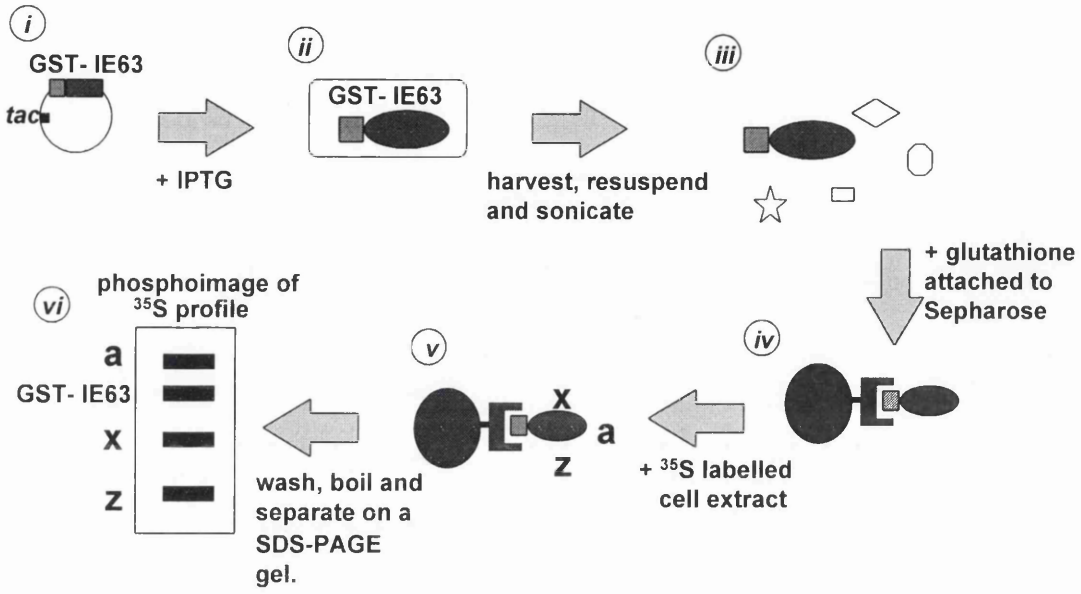


Fig. 2B4 GST pull down assay

E. coli cells strain BL21 were transformed with GST-IE63 expressing plasmid and grown to an OD_{600} of 0.4 (i). GST-IE63 expression was induced by the addition of IPTG (ii), after 2h, bacteria were harvested, resuspended in a small volume and split open by sonication (iii). GST-IE63 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-IE63 bound onto beads. After washing, the proteins which interacted with GST-IE63 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 phosphoimaging and an estimation of interacting protein size made by comparison with molecular weight markers simultaneously run on the gel.

GST-IE63 fusion protein expressed and bound onto Glutathione beads as above, was also used to look at the interaction of IE63 with itself. In this case, after the cell extract had been mixed with fusion protein at 4°C for 1h and washing with binding buffer was complete, the beads were washed overnight in 50 µl of high stringency buffer (50mM Hepes, 1M NaCl, 0.1% NP40 with the addition of protease inhibitor cocktail, pH 7.5) to elute off bound proteins. The eluted proteins were then separated on SDS- PAGE gel and IE63 visualised using Western blotting.

A further experiment with GST-IE63 was to incubate the fusion protein on beads with purified CK2 subunits. 40µl beads with GST-IE63 bound were mixed with 0.2µg CK2 α subunit or 0.2µg CK2 $\alpha_2\beta_2$ complex. This was incubated at 4°C for 1h with constant mixing, washed 3x with binding buffer and CK2 activity or physical presence detected using CK2 peptide assay or Western blotting.

2B4.2 GST and MBP CK2

GST/MBP-CK2 fusion expressing constructs were transformed into BL21 *E.coli* . A fresh colony was picked and 10ml overnight cultures set up. Next day, a 1:10 dilution into 100ml was made, cultures were grown to an OD of 0.4-0.6 and then induced for 3h with 0.3mM IPTG. The cells were harvested by centrifugation at 3000rpm for 10min (Sorvall RT 6000B refrigerated centrifuge) and resuspended in 5ml ice cold lysis buffer (10mM phosphate, 30mM NaCl, 0.25% Tween 20, 10mM EDTA, 10mM EGTA, adjusted to pH7.0). After thermal shock (-70°C to +20°C) and a 3x 2min sonication using a soni-probe, the lysate was adjusted to 0.5M NaCl and centrifuged to remove cell debris (15000rpm, 4°C, 20min, Sorvall SS34 rotor). The supernatant was then mixed with amylose resin (New England Biolabs) or Glutathione-Sepharose 4B beads, as appropriate, at 4°C for 1h. After washing 3x with PBS the beads were mixed with cell extracts (~100µg) at 4°C for 1h. Approximately equal amounts of each fusion protein were used as judged by

Coomassie Blue staining of an SDS-PAGE gel of the fusion proteins bound onto beads. Finally beads were washed 2x with PBS to remove non-specific binding and 3x with 50µl PBS containing 1M NaCl to elute off bound proteins. Eluted proteins were separated by SDS-PAGE, transferred to nitrocellulose and analysed by Western blotting for IE63.

2B4.3 Affinity chromatography using rMp32 or rMGO Sepharose columns.

Affinity columns were produced by D. Matthews (Matthews & Russell, 1998) and used to identify proteins which interact with p32. 100µl cell extract harvested 4h or 16h post infection was mixed with 40µl of p32- or glucose oxidase-Sepharose beads (rMp32/rMGO) at 4°C for 2h. After which beads were washed 10x with medium stringency washing buffer (PBS A; 200mM NaCl, 0.1% NP-40) and pelleted by micro-centrifugation (12000rpm for 1 min) before 35µl protein gel loading buffer was added and samples boiled for 3min. Samples were loaded onto SDS PAGE for analysis by Coomassie staining or Western blotting. In some cases wash buffers of higher stringency replaced the medium stringency washing buffer. (PBS A; 350mM NaCl or 500mM NaCl, 0.1% NP-40)

2B5 Western blot analysis of proteins and autoradiography

2B5.1 SDS polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

Proteins were resolved by electrophoresis though SDS polyacrylamide mini or larger gels (Laemmli, 1970) using Bio-Rad mini gel electrophoresis tanks or a large Bio Rad protean II xi cell.

Gel mixes were as follows

For 10ml	10% resolving gel	8% resolving gel	For 2ml stacking gel
H_2O	4ml	4.6ml	1.4ml
30% acrylamide mix	3.3ml	2.7ml	0.33ml
1.5M Tris (pH 8.8)	2.5ml	2.5ml	0.25ml
10% SDS	0.1ml	0.1ml	0.02ml
10% APS	0.1ml	0.1ml	0.02ml
TEMED	0.004ml	0.006ml	0.002ml

For larger gels, amounts were increased to 50ml resolving gel and 10ml stacking gel but proportions of components remained the same.

Resolving gel mixes were poured into glass plate sandwiches, overlaid with butanol and allowed to set. The butanol was washed away with water, stacking gel mixture was overlaid and a comb inserted. Protein samples were mixed with protein gel loading buffer and placed in a boiling bath for 3min prior to loading. Along with samples, 10µl Rainbow marker was also loaded and run. Gels were run at 150mV till tracking dye reached the bottom. Gels were either stained with Coomassie Blue solution (50% methanol, 7% acetic acid, 43% water, 0.02% Coomassie blue stain) for 30min and destained in protein gel destain (5% methanol, 7% acetic acid, 88% water), or transferred to nitrocellulose for Western blotting and autoradiograph analysis. Gels which weren't to be Western blotted but did contain radioactive proteins were, after staining and destaining, incubated in En^{3}hance for 30min, briefly washed with water and vacuum dried prior to exposure to Kodak X-OMAT S film.

2B5.2 Electroblotting of proteins to nitrocellulose membrane

Proteins resolved on SDS-PAGE gels were transferred to nitrocellulose (Towbin *et al.*, 1979) using a Bio-Rad transblot cell. A blotting sandwich was set up such that the gel was in contact with a sheet of nitro-celullose (Hybond-ECL Amersham) and both were sandwiched between Whatman 3mm paper of the appropriate size. This was in turn sandwiched between sponges provided by Bio-Rad and transfer carried out at 250mA for 1h.

2B5.3 Immunodetection of proteins

After transfer to nitrocellulose, the presence of specific proteins was detected using antisera. Incubation in PBS A 5% dried milk for 1h at RT, with one change of buffer after 30min, or 4°C overnight, blocked nitrocellulose membranes. They were then washed 3x 10min at RT in PBS A 0.05% Tween (PBS-T), before incubation for 1h at RT on a shaker in 20ml of appropriately diluted primary antibody in PBS-T (Table 2A.7). Following this, blots were washed 3x 10min at RT in PBS-T and protein A horseradish peroxidase conjugate or anti-mouse IgG whole molecule conjugate added in a 1/1000 dilution in PBS-T. After a 40min incubation at RT, again with shaking, blots were washed as previously and proteins were detected using the Amersham Enhanced Chemiluminescence (ECL) system. The two reagents were mixed in equal volumes and a total volume of 10ml poured onto the filter, which was agitated for 1min. The blots were wrapped in cling film and exposed to Kodak X-OMAT S film.

2B5.4 Visualisation of [^{35}S] and [^{32}P] labelled proteins by phosphoimage analysis

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

2B6 CK2 activity assays

2B6.1 Artificial peptide substrate assay for CK2 activity

The presence of CK2 protein was determined by its ability to phosphorylate an artificial peptide substrate. The final immunocomplexes were suspended in 30µl CKII reaction buffer (50mM Tris pH8.2, 20mM $MgCl_2$, 10µCi γ–[^{32}P] ATP per reaction, either with or without 0.1mM peptide substrate Arg-Arg-Arg-Glu-Glu-Glu-Thr-Glu-Glu-Glu. Reactions were carried out in a waterbath at 25°C for 30mins. After brief centrifugation the supernate was applied to a SpinZyme™

column (Pierce), which was then washed 3x with 75mM phosphoric acid. Phosphorylated peptide which bound to the column was detected by liquid scintillation counting in Beckman LS 5000 CE scintillation counter and results expressed as counts per minute (cpm). This method is illustrated in Fig. 2B6.1.

2B6.2 *In vitro* phosphorylation of co-immunprecipitated proteins

Phosphorylating ability of associated CK2 on other proteins present in the co-immunoprecipitated complex was examined using this *in vitro* phosphorylation assay. After usual washes, immunocomplexes which had been made using unlabelled cell extracts were washed with 50mM Tris pH 7.4. Complexes were then suspended in 20µl 50mM Tris pH 7.4 and to this 5µl radioactive solution (50mM Tris pH7.4, 20mM $MgCl_2$, 10µM ATP, 2.5µCi γ–[^{32}P] ATP per reaction) was added, the reactions were carried out for 15min at 25°C either in the presence or absence of 100µM DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole). Immunoprecipitated proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane. Phosphorylated proteins were visualised by phosphoimaging and immunoprecipitated proteins analysed by Western blotting for IE63 and hnRNP K.

2B6.3 Inhibition of CK2 phosphorylation

Phosphorylation could be inhibited and co-immunoprecipitated proteins analysed. Total cell extract (~100µg) was incubated with either 100µM DRB, 10U CIP or phosphatase inhibitors (30mM *p*-nitrophenyl phosphate, 10mM NaF, 0.1mM Na_3VO_4, 0.1mM Na_2MoO_4 and 10mM β-glycerophosphate) in 100µl kinase buffer (50mM Tris pH 7.4, 20mM $MgCl_2$, 10µM ATP) for 30min at 25°C, 5µl of antibody was then added and the salt concentration increased to that used in the IE63 co-immunoprecipitation. Co-immunoprecipitation was then performed as usual and precipitated proteins analysed by Western blotting for IE63 and hnRNP K.

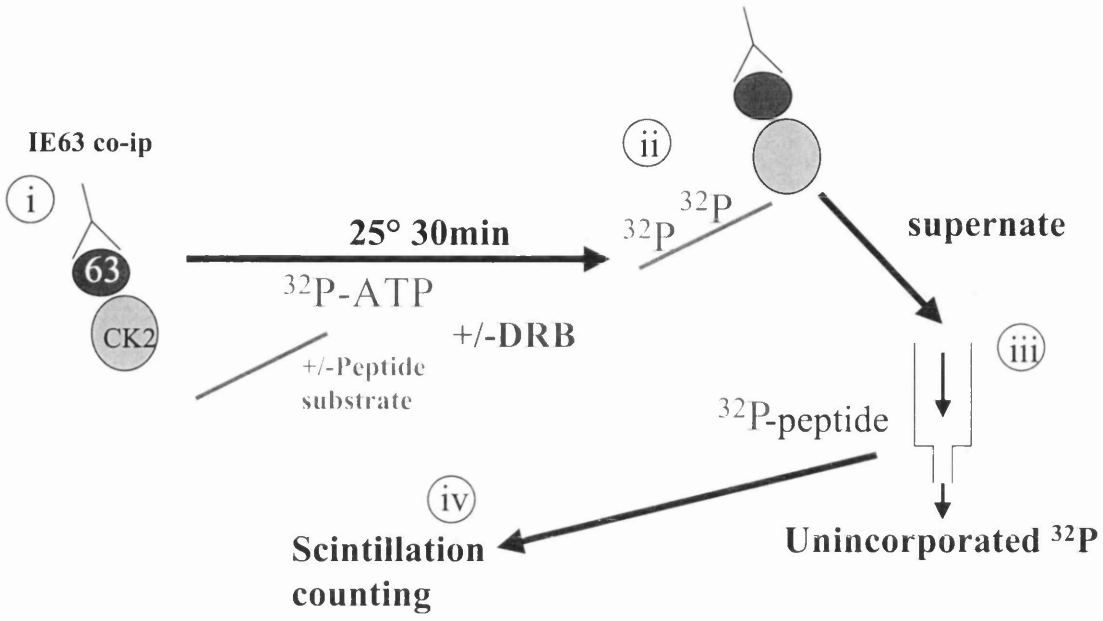


Fig 2B6.1 CK2 Peptide assay

Co-immunoprecipitates (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 co-immunoprecipitate 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 (cpm) 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.

2B6.4 Nucleotidylation assay

Nucleotidylating ability of CK2 on co-immunopreciptated proteins was investigated. Anti-IE63 co-immunoprecipitated samples or whole cell extracts were added to 50µl mixes containing 50mM Tris-HCl, 5mM $MgCl_2$, (pH 7.5) 30nM α–[^{32}P] ATP and 150µM ATP. These were incubated at 30°C for 1h and reactions terminated by addition of EDTA to 25mM. Protein gel loading buffer was added to the reactions and boiling was for 5min. Proteins were separated by SDS-PAGE electrophoresis and analysed for nucleotidylation by exposure to X-OMAT film (Kodak) at -70°C for up to 1 week.

2B7 Preparation of samples for Laser Mass Map Spectroscopy (LMMS)

An unidentified protein band pulled down by the p32 Sepharose column was identified by Laser Mass Map Spectroscopy. Samples from p32 Sepharose column pull down were separated by SDS-PAGE and transferred to PVDF nitrocellulose membrane (Amersham). This is similar to transfer to nitrocellulose (Section 2B5.2) except that membrane was first soaked 15-30sec in methanol, then 5min in H_2O, and finally in transfer buffer (50mM Tris base, 50mM glycine, 20% methanol, 0.01% SDS) for at least 5min. After transfer PVDF membrane was rinsed in dH_2O and dried overnight in a lyophilizer before staining for 1-2min in sulphrodamine stain (50mg/L sulphrodamine (Kodak), 30% methanol, 0.2% acetic acid) and washing in water. Bands of interest were excised from the membrane. Sequencing grade trypsin was rehydrated using 50mM ammonium bicarbonate to 50ng/µl. Usually, 500µl of solution was made up and stored at -20°C for up to 1 month. 2-5µl of the trypsin was added to the excised bands and left on the bench overnight. 50% ethanol/50% formic acid was added to make up the sample volume to 10µl and to extract the trypsinised protein sample from the membrane. 0.5µl of the extracted sample was loaded onto a sample strip and, after this had air dried, matrix mix (10mg/ml hydroxyannanic acid matrix, 50% acetonitrile, 0.1% TFA, 5µl insulin chains) was overlaid and again allowed to dry. The strip was finally loaded into a mass spectrometer for analysis. LASERMAT 2000 Mass Analyzer version 1.3 (Thermo Bio Analysis Ltd) software was used to interpret data produced. Simultaneously to this work a prepared sample was sent to Dr. G Kemp, St. Andrews University for LMMS.

2B8 Immunofluorescence/ Confocal microscopy

The localisation of IE63 and p32 was studied with immunofluorescence and confocal microscopy was used to look at localisation of SAP 145 and IE63. Infected, transfected or non infected cell monolayers on 13mm glass coverslips in 24-well Nunc Linbro plates, were washed 3X with PBS A and fixed for 10min at RT with 2% sucrose, 5% formaldehyde in PBS A. This solution was removed and cells washed 3x in PBS A. Cells were permeablised for 10min at RT with 0.5% NP40, 10% sucrose in PBS A. Cells were again washed 3x 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 cover slip for 60min at RT. Cells were again washed 3x in PBS and incubation with 20µl secondary antibody diluted 1:100 in PBS A carried out for 30 min at RT. For immunofluorescence, fluorescein isothiocyanate isomer I (FITC) and tetramethylrhodamine isothiocyanate (TRITC) conjugated secondary antibodies were used and for confocal microscopy cy3 conjugated antibody was used in place of TRITC. Finally cells were washed a further 3x in PBS A before a final wash in H_2O; they were air dried on the bench and mounted on glass slides with mounting media, Glycerol in PBS (Sigma).

(i) Immunofluorescence

Cells were examined on a Nikon Microphot-SA microscope with appropriate filters. Images were captured with a digital Pixel CCD digital camera and prepared for printing by using Photoshop.

(ii) Confocal Microscopy

Cells were examined with a Zeiss LSM 510 confocal microscope system, with 2 lasers giving excitation lines at 488nm (FITC) and 543nm (cy3) and a Zeiss Axioplan microscope using a 63x oil immersion objective lens, NA 1.4. Data were processed with LSM 510 software and then exported for preparation for printing using Photoshop. Roger Everett kindly assisted with the production of the confocal images presented in this thesis.

Chapter 3: Identification of proteins which interact with IE63

The aim of this project was to identify cellular and viral gene products which interact with the IE63 protein. There are many ways to investigate protein:protein interactions, in the initial part of this study co-immunoprecipitations and GST-fusion protein pull down assays were used to reveal interacting proteins of known size but of unknown identity.

The profiles of interacting proteins produced by these two methods are presented in this chapter: the establishment and optimisation of co-immunoprecipitation and GST pull down experiments is described in Appendix 1.

While pursuing the identity of these proteins, a co-worker in our laboratory discovered, using the yeast two-hybrid screen, several potential interacting proteins (Bryant *et al*, 1999). The results of the yeast two-hybrid screen are shown in table 3.

Protein interacting with IE63 in the Yeast-2-hybrid screen	*Number of clones showing sequence homology to this protein*
hnRNP K	1
CK2β subunit	51
SAP145	7
p32	6
Aly I	4
other proteins	7
sequences with no homology to any known protein in the data base	6

Table 3. Using a HeLa cell library, 2.3 x 10^6 individual clones were screened against an IE63 bait construct lacking only the N terminal 10 amino acids. 150 positives were identified, but after false positve tests only 99 remained; a futher 16 of these were not sequenced and one contained the empty vector, leaving 82 clones.

The latter part of this project was concerned with confirming and characterising the interactions suggested, and with examining the identities of two of the initially described protein bands not fished out by the two hybrid-screen (Results Chapters 4 and 5).

3A Co-immunoprecipitation assays

3A1 IE63 monoclonal antibody (H113) used to co-immunoprecipitate proteins from an [^{35}S]-methionine labelled extract of BHK cells infected with wt HSV-1

IE63 monoclonal antibody (Mab) H1113 was used to co-immunoprecipitate [^{35}S] radiolabelled proteins from wild type (wt), 27lacZ and mock-infected (mi) BHK cell extracts (Sections 2B2.3 and 2B2.5). Proteins were separated by SDS-PAGE electrophoresis and visualised by phosphoimage analysis (Sections 2B5.1 and 2B5.4). Samples were also Western blotted with IE63 Mab. Figure 3A1 panel I shows the [^{35}S]-methionine labelled profile of the cell extracts used (lanes 1-3), viral proteins can be seen as unique bands in the wt extract, some inhibition of host protein synthesis can be observed as bands in mock infected extract which are absent in the wt extract. The extract made from 27lacZ infected cells shows some viral proteins but not all, consistent with its defect being expressed during E and L phases. For example, the band at the top of the gel in wt extracts but not mi or 27lacZ infected cell extracts most likely corresponds to the major capsid protein, where as the one below probably corresponds to gB. These infected cell extracts were prepared by labelling from the 0h post-infection, this ensured that both viral and cellular proteins were labelled, however a cleaner profile of viral proteins can be obtained by labelling 3-4h post-infection, under these conditions may more differences between mi, wt and 27lacZ infected cell extracts are seen.

Lanes 4-6 show protein bands which co-immunoprecipitated with IE63 Mab; IE63 is arrowed (lane 4). Bands corresponding to proteins of approximately 180 kDa, 150 kDa, 85 kDa, ~70 kDa (doublet), 50 kDa, 44 kDa, 32 kDa (doublet) and 25 kDa were seen to specifically co-purify with IE63 (lane 4), i.e. they were absent in lanes 5 and 6.

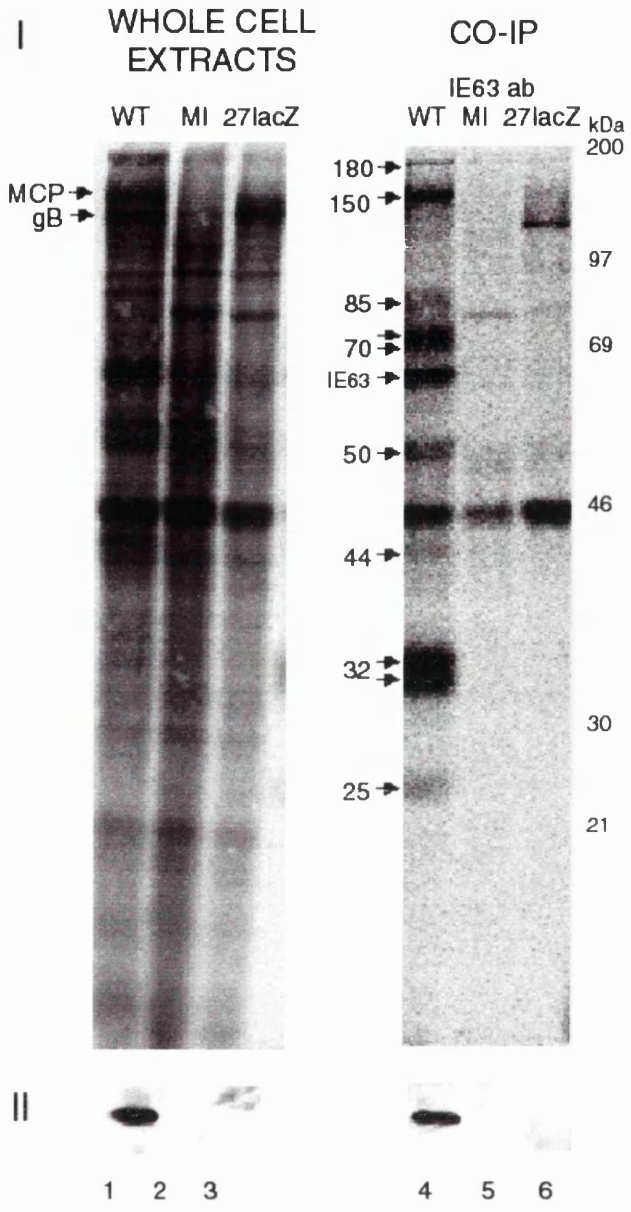


Fig 3A1. Ten proteins of known size but unknown identity co-immunoprecipitate with IE63

A co-immunoprecipitation assay was performed with IE63 Mab, H1113, and wildtype (WT), 27lacZ (27) and mock infected (MI) BHK cell extracts, the complex formed was separated on a 10% SDS PAGE gel, dried down and exposed to a phosphoimaging plate overnight.

Panel I. [^{35}S]-methionine labelled proteins co-immunoprecipitated from WT, 27 and MI extracts. Whole cell extracts used are shown in lanes 1-3, the major capsid protein (MCP) and glycoprotein B (gB) are indicated. Radiolabelled co-immunoprecipitated proteins are shown in lanes 4-6. Bands corresponding to proteins interacting specifically with IE63 are arrowed.

Panel II. Western blot for IE63 using H1113 Mab. Aliquots of the above were run on another 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted for IE63.

The Western blot in panel II shows IE63 being present in (lane 1) and immunoprecipitated from (lane 4) wt infected cell extract.

3B GST-IE63 pull down assays

3B1 Expression of GST-IE63 in BL21 *E.coli.*

Initial attempts to express GST-IE63 used a method described by Mears *et. al.* (1996) and are shown in Fig. 3B1 panel I lanes 1-4. There was no detectable full length GST-IE63 (~90 kDa), the major products being truncated forms of the fusion of around 40-50 kDa, smaller bands seen on the Coomassie gel were confirmed as IE63 truncations by the Western blot for IE63 (lane 1). As full length GST-IE63 was required, each of the variables in expression was altered in turn to increase the amount of full length product (Appendix 1). After optimisation, a workable amount of full length product was detectable both by Western blotting and Coomassie staining (Fig. 3B1 lanes 5-7). The final optimised procedure is described in Section 2B4.1.

As bacterial expression and binding to Glutathione beads varied from experiment to experiment, aliquots of the bacterial extracts were mixed with Glutathione-Sepharose beads, washed and protein analysed on SDS-PAGE gels, to ensure equivalent amounts of various fusion proteins were used.

3B2 GST-IE63 used to pull down proteins from an [^{35}S]-methionine labelled extract of BHK cells infected with wt HSV-1

When the GST-IE63 fusion protein bound to Glutathione beads was mixed with radioactive wt infected extract, washed and separated by SDS-PAGE (Section 2B4.1), the [^{35}S]-methionine profile shown in Fig. 3B2 lane 1 was obtained. Arrowed bands of 175 kDa, 145 kDa, 85 kDa, 70 kDa, 63 kDa, 50 kDa, 46 kDa, 44 kDa, 35 kDa, 32 kDa and ~25 kDa interacting with GST-IE63 and not with the control protein GST alone (lane 2) were seen.

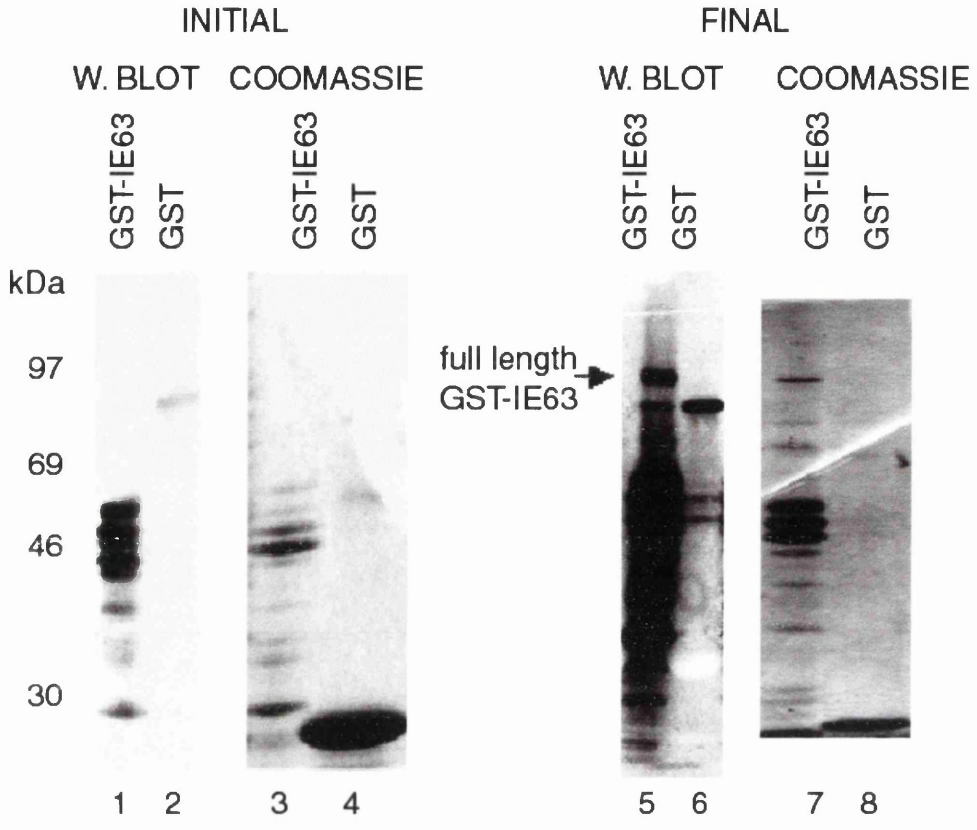


Fig. 3B1 Fusion proteins expressed from pGEX and pGEX27

Western blot for IE63 using H1113 Mab (lanes 1, 2, 5 and 6) and Coomassie stained gel (lanes 3, 4, 7 and 8) of initially expressed GST-IE63 (lanes 1 and 3) and GST alone (lanes 2 and 4) bound onto Glutathione beads, and after optimisation, of finally expressed GST-IE63 (lanes 5 and 6) and GST alone (lanes 6 and 8) bound onto Glutathione beads. GST-IE63 expression and purification was initially carried out as described by Mears and Rice (1996), optimised expression and purification conditions are outlined in Appendix 1.

The large amount of GST protein present in lane 2 obscured the identification of similar molecular weight proteins which were brought down non-specifically by GST-IE63 and conclusions about the binding of the 25 kDa, 32 kDa, 35 kDa should therefore be treated with caution.

The use of whole cell extracts ensured, as far as possible, that the interactions detected between IE63 and particular proteins were not artifacts which arose as a result of over-representation of particular cellular proteins.

When the [^{35}S] profile in Fig. 3B2 was compared to the immunoprecipitated profile (Fig. 3A1). They were very similar. It was hoped that the GST pull down assay could be scaled up more easily than the co-immunoprecipitation, and the interacting proteins identified by protein Laser Mass Map Spectroscopy. The immunoprecipitation would then provide conformation of the interactions. In fact, neither method was used to identify interacting proteins and both were used in the conformation of interactions found in the yeast-2-hybrid system.

3C Self interaction of IE63.

In certain cases a dominant negative phenotype arises when one wt and one mutant subunit interact to form a heterodimer, for example with HSV-1 ICP 4 or HIV Rev (Hope *et al.*, 1992, Shepard *et al.*, 1990). In the same way, mutations in the activation domain of IE63 produce a *trans* dominant negative phenotype during infection and in transfection experiments (Smith *et al.*, 1991). As in Fig. 3B2, a radiolabelled band of 63kDa was pulled out of infected cell extracts by GST-IE63 and not by GST alone, it was thought that this may be IE63 interacting with GST-IE63 and that IE63 may form multimers. Thus the pull down proteins were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose and Western blotted for IE63, however, due to the large amount of truncated GST-IE63 protein it was impossible to see if an extra band was present at 63 kDa with infected cell extract (data not shown). To solve this, after the usual low salt wash to remove non-specifically binding proteins, the beads were washed with a high salt wash overnight (Section 2B4.1). The eluted proteins were then separated by SDS-PAGE and Western blotted for IE63. Eluted proteins from GST-IE63 and

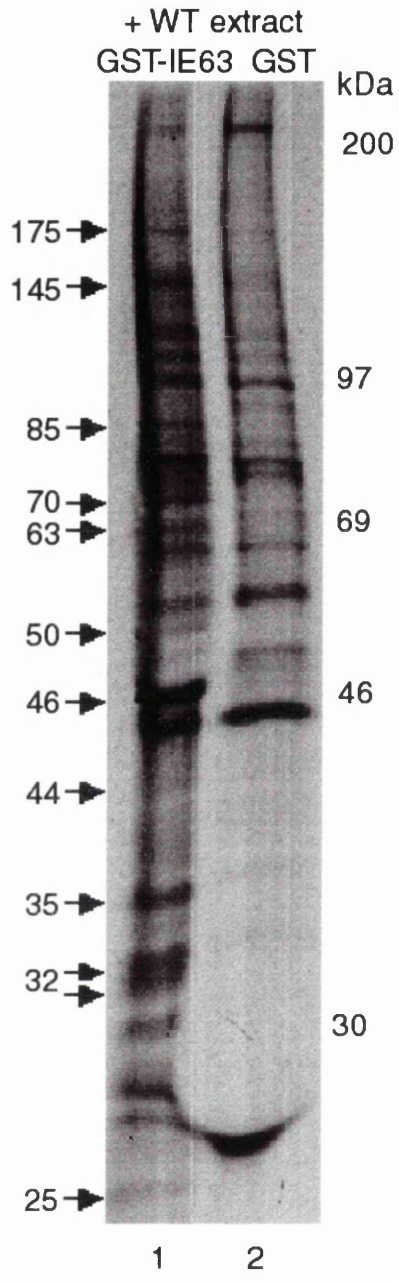


Fig. 3B2. Eleven proteins of known size but unknown identity are specifically pulled down by GST-IE63

[^{35}S]-methionine radiolabelled proteins pulled down by GST-IE63 (lane 1) and GST alone (lane 2) from wildtype (WT) infected BHK cell extracts. GST-IE63 and GST alone were expressed using final conditions and bound onto Glutathione beads, after washing with PBS beads were incubated with ~100μg cell extract for 1h. After further washes, pulled down proteins were separated on a 10% SDS-PAGE gel. Bands corresponding to proteins interacting specifically with GST-IE63 are arrowed (lane 1).

GST were compared and a band corresponding to IE63 was seen eluted from GST-IE63 (Fig. 3C lane 5); the other bands specific to lanes 5 and 6 are contaminating GST-IE63 truncations washed off the beads. IE63 is therefore capable of interacting with itself, this is also shown by Zhi *et al.*, (1999).

High molecular weight multimers were not seen on SDS-PAGE gels as these are denaturing gels, and prior to loading the samples were boiled for at least 5 min in protein gel loading buffer, which disrupts any protein:protein interactions. Use of non-denaturing gels of infected extract Western blotted for IE63 may allow visualisation of multimers and enable the number of IE63 molecules associating to be determined.

The interaction detected was between GST-IE63 and IE63 from infected cell extract, thus there is no chance that multimerisation was due to GST-GST interactions: GST itself has been reported to exist as a dimer in solution (Warholm *et al.*, 1983) which has confused the issue of studying multimerisation using GST fusion proteins.

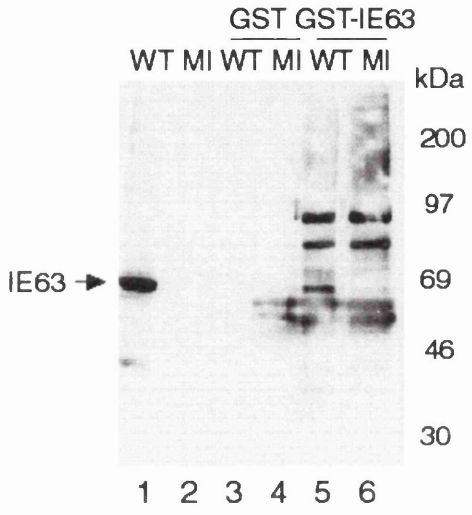


Fig. 3C IE63 interacts with itself in GST fusion protein binding assays

Western blot for IE63 using H1113 Mab. Fusion proteins bound onto Glutathione beads were incubated with 100μg protein from a BHK cell extract, after low stringency washing, specifically bound proteins were eluted with a high salt wash and separated on a 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted for IE63. Cell extracts used (lanes 1 and 2), proteins eluted from, wildtype infected (WT) extract bound onto GST (lane 3), mock infected extract (MI) bound onto GST (lane 4), WT extract bound onto GST-IE63 (lane 5) and MI extract bound onto GST-IE63 (lane 6).

Chapter 4: Interaction of IE63 with hnRNP K and CK2

The previous Chapter discussed the initial characterisation of proteins of known size but of unknown identity which interact with IE63. Concurrent with this work, a yeast two-hybrid screen was underway the results of which suggested four cellular proteins might interact with IE63 *in vivo*. This Chapter presents work which shows that two of the proteins detected, hnRNP K and CK2, interact with IE63 in virus infected cells. Experiments aimed at uncovering the implication of these interactions to infection are described.

hnRNP K was a most interesting candidate for interaction with IE63 because this protein is implicated in many of the same processes as IE63, for example, in mRNA shuttling from the nucleus to the cytoplasm, processing of pre-mRNA and regulation of gene transcription.

IE63 is known to be phosphorylated and to alter the phosphorylation of other viral proteins, but itself has never been shown to have phosphorylating ability; for this reason the association with a kinase, while interesting, was not unexpected (see Introduction Section and Discussion Section).

4A IE63 and hnRNP molecules

4A1 Interaction of IE63 with hnRNP K

4A1.1 Using the GST pull down assay

A GST pull down experiment using GST-hnRNP K and ~100μg wt infected extract (Section 2B4.1) followed by Western blotting with IE63 Mab, showed that IE63 and GST-hnRNP K interact, whereas GST and IE63 do not (Fig 4A1.1 Panel II compare lanes 3 & 4). Fusion proteins used were visualised by Coomassie staining (Panel I). [^{35}S]-methionine labelled wt extract was used and labelled proteins pulled down by GST-hnRNP K (lane 2) or GST alone (lane 1) were detected by phosphoimage analysis (Panel III). A band of 63 kDa corresponding in size to IE63 was seen along with bands of ~170kDa, 70kDa, 46kDa, and

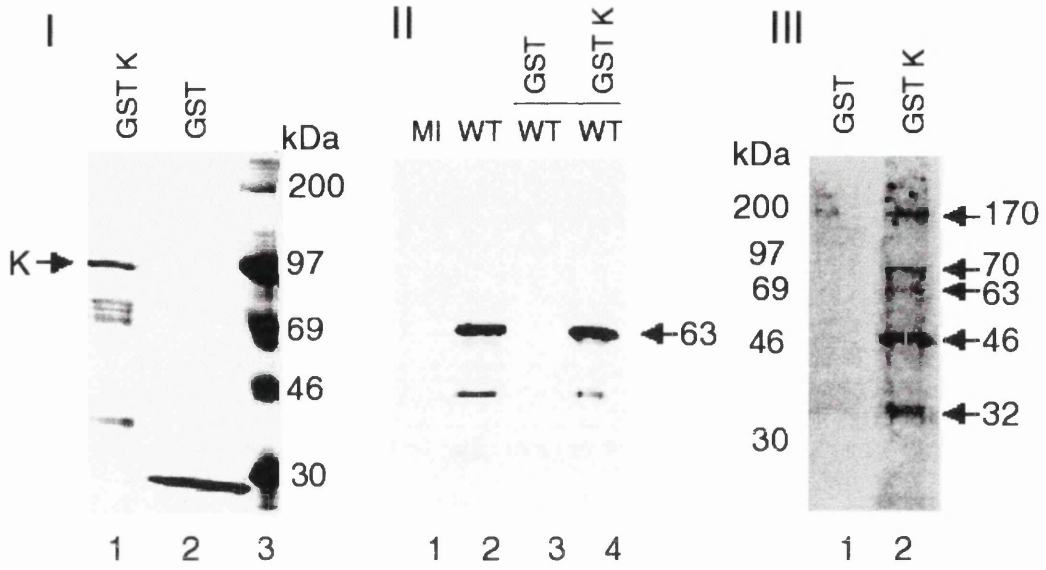


Fig. 4A1.1 Interaction of IE63 with hnRNP K in the GST fusion protein binding assay

Wild type infected (WT) or mock infected (MI) BHK cell extracts were incubated with GST or GST-hnRNP K proteins.

Panel I GST-hnRNP K (lane 1) and GST (lane 2) proteins used were run on a 10% SDS-PAGE gel and visualised by Coomassie staining.

Panel II 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 IE63 using H1113 Mab. MI (lane 1) and WT extracts (lane 2), proteins from WT extract bound to GST (lane 3) and to GST-hnRNP K (lane 4). The smaller bands in lanes 2 and 4 represent degradation products of IE63.

Panel III. [^{35}S]-methionine labelled profile of proteins pulled out of radiolabelled WT extract by GST (lane 1) or GST-hnRNP K (lane 2), Proteins were separated on 10% SDS-PAGE gel, dried down and exposed to a phosphoimaging plate overnight.

32kDa. As hnRNP K is capable of forming oligomers (Bomsztyk *et al.*, 1997), the 70kDa band (Panel III, lane 2) is likely to be GST-hnRNP K interacting with hnRNP K from the extract, or could correspond to the other 70kDa band seen in IE63 co-immunoprecipitations (Fig. 3A1). The 170kDa protein band (Panel III, lane 2) may be the same band taken to be 175kDa (or even 150 kDa) in IE63 co-immunoprecipitations/GST-IE63 pull downs (Fig. 3A1/3B2) and the 46kDa and 32 kDa bands may be the 50kDa or 44 kDa and 32kDa bands seen in these previous Figs.

4A1.2 Using Co-immunoprecipitation

Evidence for an interaction between IE63 and hnRNP K *in vivo* came from Western blotting of the IE63 co-immunoprecipitation experiments (Section 3A2) with hnRNP K antiserum. Fig 4A1.2 Panel I confirms that IE63 was precipitated by IE63 Mab from wt extract (lane 1). The same samples were Western blotted for hnRNP K (Panel II), hnRNP K was clearly co-immunoprecipitated by the IE63 Mab from wt extract, and was absent in co-immunoprecipitations from 27lacZ or mi extracts (lanes 2 and 3). Thus it is a fair conclusion that one of the bands of the 70kDa doublet seen in Fig 3A1 corresponds to hnRNP K.

hnRNP K antiserum was then used to immunoprecipitate hnRNP K (Section 2B3.3) from wt, 27lacZ and mi extracts. Fig. 4A1.2 Panel III shows that hnRNP K antiserum precipitated hnRNP K from all three extracts (lanes 1-3) while the pre-immune serum did not (lane 4). The same samples were Western blotted for IE63 (Panel IV) and IE63 was seen to be co-immunoprecipitated with hnRNP K only from the wt extract (lane 1). As a control, IE63 was not precipitated by the pre-immune serum (lane 4).

To determine if any other viral proteins were required for the interaction between IE63 and hnRNP K, co-immunoprecipitations were repeated with hnRNP K antiserum and extracts from wt infected, pCMV-63 transfected or mi cells. Transfected and wt infected extracts gave the same result namely, IE63 was co-immunoprecipitated with hnRNP K (Panel VI lanes 3 and 4). hnRNP K was immunoprecipitated with anti-hnRNP K serum from mi, wt or transfected extracts

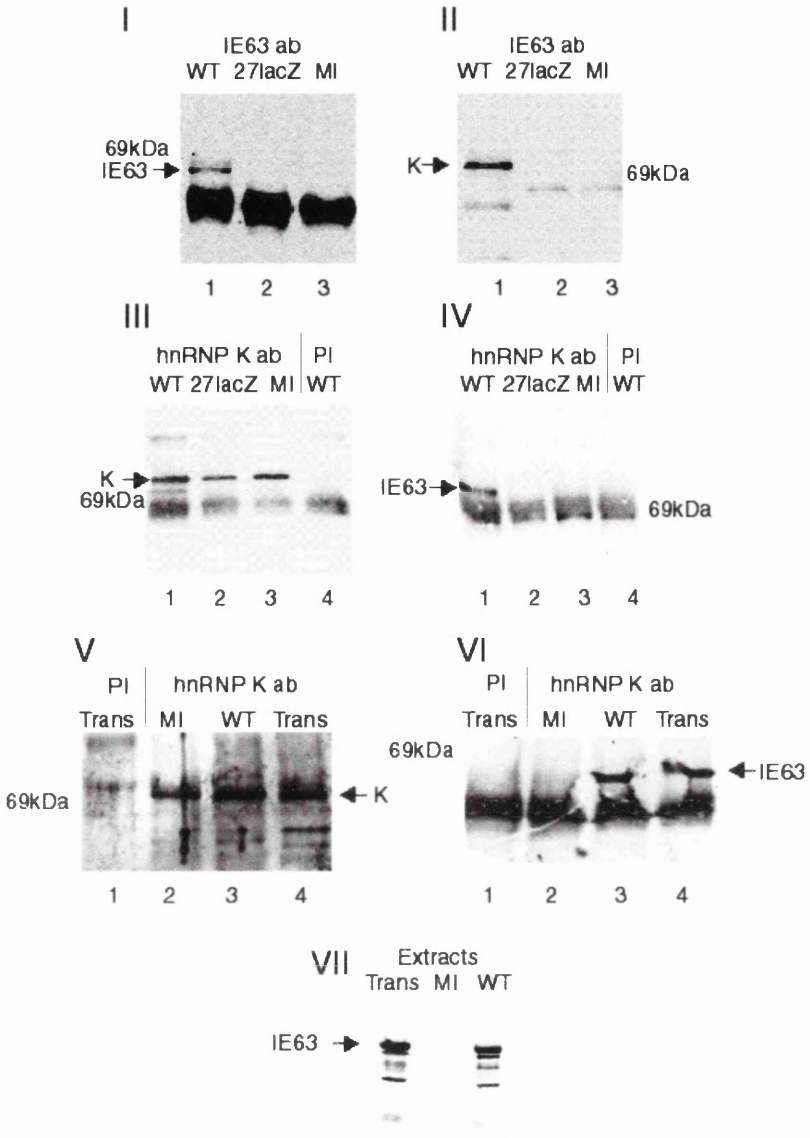


Fig.4A1.2 Co-immunoprecipitation of IE63 and hnRNP K confirms the interaction between IE63 and hnRNP K

Wild type infected (WT), 27lacZ infected (27), pCMV-63 transfected (Trans) or mock infected (MI) BHK cell extracts were co-immunoprecipitated with IE63 Mab or hnRNP K antiserum. The precipitated proteins were separated by SDS-PAGE gel electrophoresis, transferred to nitrocellulose membranes and analysed by Western blotting using hnRNP K or IE63 antibodies.

Panel I. Co-immunoprecipitation by IE63 Mab, blotted for IE63.
Panel II. Co-immunoprecipitation by IE63 Mab, blotted for hnRNP K.
Panel III. Co-immunoprecipitation by hnRNP K antiserum, blotted for hnRNP K.
Panel IV. Co-immunoprecipitation by hnRNP K antiserum, blotted for IE63.
Panel V. Co-immunoprecipitation by hnRNP K antiserum, blotted for hnRNP K.
Panel VI. Co-immunoprecipitation by hnRNP K antiserum, blotted for IE63.
Panel VII. Extracts used Western blotted for IE63.

(Panel V lanes 2-4) Again, controls with preimmune sera were used and did not precipitate hnRNP K or IE63 (Panel V lane 1 and Panel VI lane 1). Panel VII shows the mi, wt and transfected extracts Western blotted for IE63.

4A2 Using hnRNP K antiserum, the form of hnRNP K immunoprecipitated by hnRNP K antiserum was slower migrating than the form which co-immunoprecipitated with IE63 using IE63 monoclonal antibody

When the two co-immunoprecipitations, as performed above (4A1.2), were run on the same gel, and Western blotted for hnRNP K (Fig 4A2 Panel I), the form of hnRNP K co-immunoprecipitating with IE63 Mab (lane 1) migrated faster than the form immunoprecipitated by hnRNP K antiserum (lane 4). This was also seen in the [^{35}S]-methionine labelled profile of the same gel (Panel II), where hnRNP K and IE63 bands were closer together when IE63 Mab was used (Panel II lane 1) than when hnRNP K antiserum was used (Panel II lane 4).

4A3 hnRNP A_1 and hnRNP C_1/C_2 proteins do not interact with IE63

hnRNP proteins involved in pre-mRNA processing (Dreyfuss *et al.*, 1993) can, under certain conditions, be co-purified by co-immunoprecipitation (Pinol-Roma *et al.*, 1988). Because of this and as hnRNP A_1 and hnRNP C_1/C_2 are 34kDa and 41/43kDa respectively (Pinol-Roma *et al.*, 1988), similar in size to bands observed in co-immunoprecipitations with IE63 Mab (Fig 3A1), Western blots of the IE63 co-immunoprecipitations were performed. Both were negative (Fig 4A3 Panel I). Western blotting of the same reaction for IE63 (Panel II) confirmed that IE63 had been precipitated.

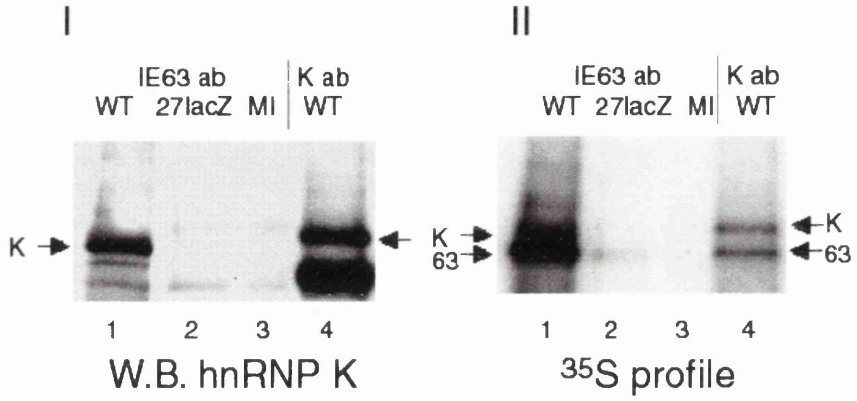


Fig. 4A2 The form of hnRNP K immunoprecipitated by hnRNP K antiserum is slower migrating than the form of hnRNP K co-immunoprecipitated with IE63 by IE63 Mab

Co-immunoprecipitations from [^{35}S]-methionine labelled wild type (WT) BHK cell extracts with IE63 Mab and hnRNP K antiserum were run on the same SDS PAGE gel, transferred to nitrocellulose and Western blotted for hnRNP K. The same piece of nitrocellulose was then exposed to a phosphoimaging plate overnight.

Panel I. Co-immunoprecipitations by IE63 Mab and hnRNP K antiserum, Western blotted for hnRNP K.

Panel II. [^{35}S]-methionine labelled profile of the Western blot shown in panel I. arrowed bands correspond to IE63 (63) and hnRNP K (K).

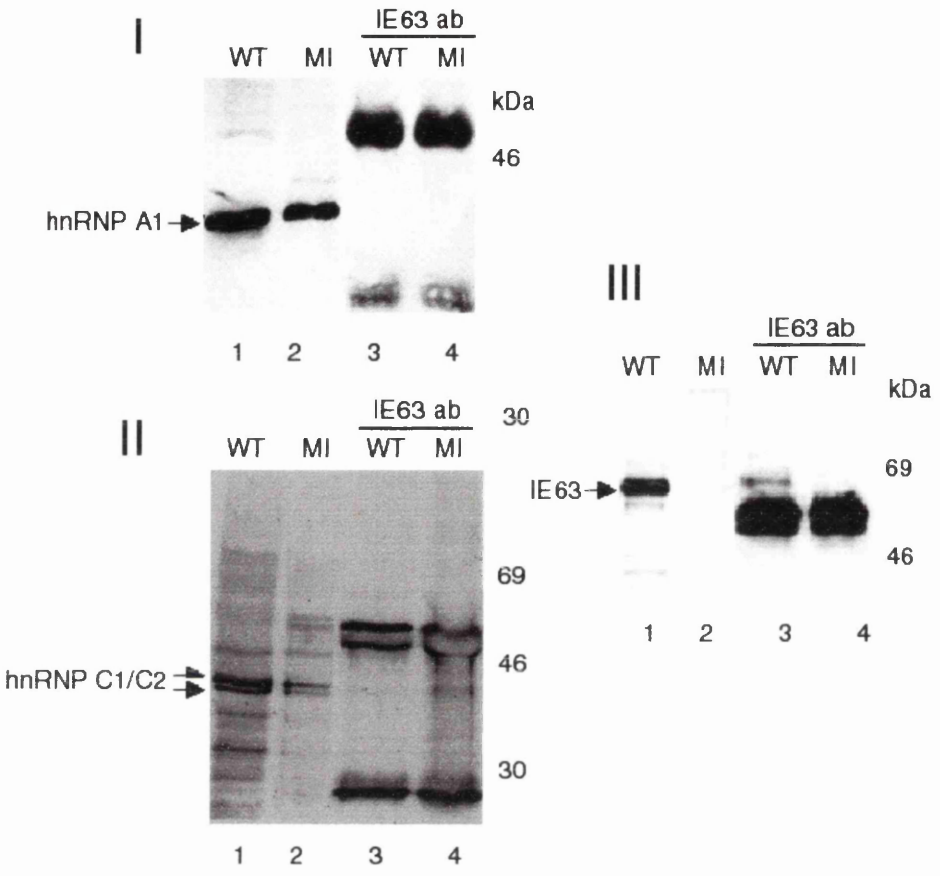


Fig.4A3 IE63 does not interact with hnRNPs A1 or C1/C2

Western blots for hnRNP A1 (Panel I), hnRNP C1/C2 (Panel II) and IE63 (Panel III) of wild type (WT) (lane 1) and mock infected (MI) (lane 2) BHK cell extracts, and of anti IE63 co-immunoprecipitates from these extracts (lanes 3 and 4). Co-immunoprecipitated proteins were separated on 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted with Mabs against hnRNP A1, hnRNP C1/C2 and IE63.

4B IE63 and CK2

4B1 IE63 interacts with CK2

CK2 is a heterotetramer consisting of two catalytic subunits (α or α') and two copies of the regulatory β subunit, α and α' are 35 kDa - 44 kDa and the β subunit is 24 kDa - 29 kDa (Dahmus, 1981, Hathaway & Traugh, 1979). The [^{35}S]-methionine labelled profile of interacting proteins identified in Fig. 3A1 contained bands of 44kDa and 25kDa which were possibly CK2. However Western blotting of this IE63 co-immunoprecipitation for either the α or β subunits of CK was negative (data not shown).

As detection of enzyme activity is a much more sensitive way to detect the presence of CK2, an assay for this activity was developed (Section 2B6.1). CK2 activity was co-immunoprecipitated from wt infected and pCMV-63 transfected extracts using IE63 Mab (Fig 4B1 Panel I lanes 5 and 8), and using hnRNP K antiserum (Panel II lanes 6 and 10). No CK2 activity was present, with either antibody, in the absence of artificial peptide substrate or in immunoprecipitates from mi or 27lacZ infected extracts or in the presence of the specific CK2 inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Zandomeni & Weinman, 1984). Detection of considerable CK2 activity implies that the negative result in Western blotting was due to the known low sensitivity of CK2 antisera, and that CK2 and IE63 do indeed interact in wt infected cell extracts.

4B2 Mapping the sites of interaction between CK2 and IE63

As the CK2β subunit was identified by the yeast two-hybrid screen, GST and MBP fusions of full length and truncated versions of CK2 β were used to map the regions of CK2 β required for interaction with IE63 (Section 2B4.2). MBP and GST fusions behave similarly, the reason for using both was the availability of constructs.

Fig. 4B2 Panel I shows the aa present in and the amounts of each fusion protein used in mapping (Section 2B4.2), visualised by Coomassie staining. An

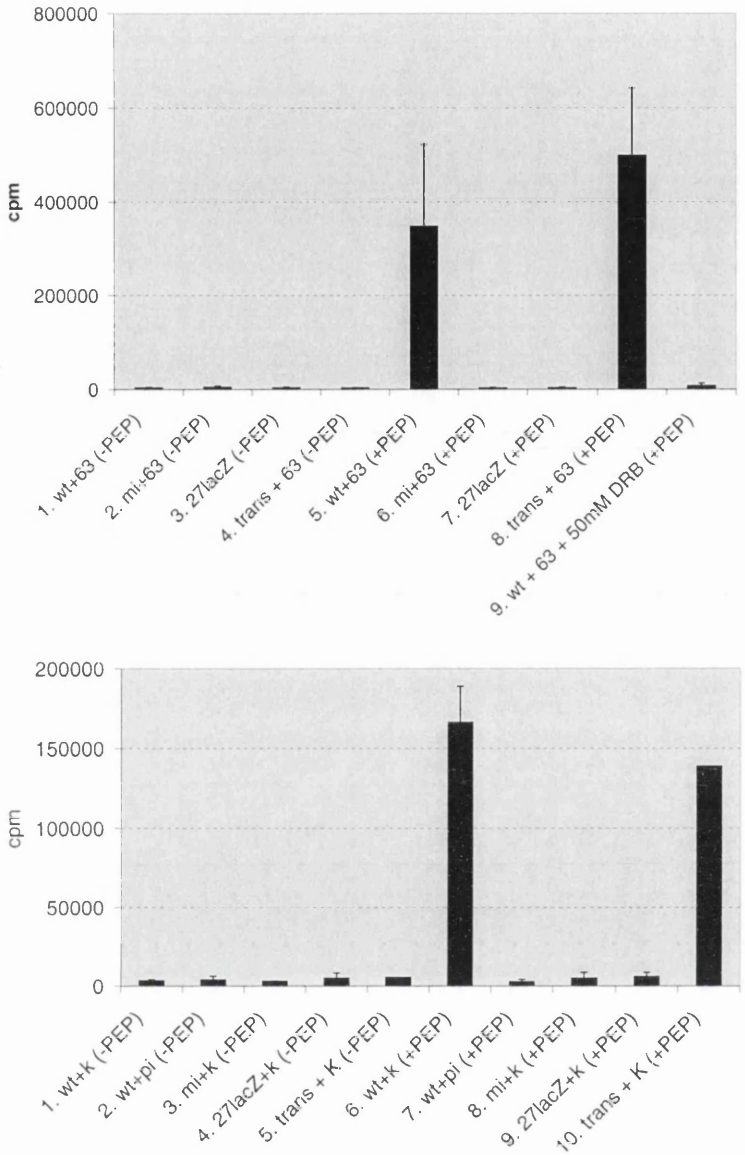


Fig. 4B1 CK2 activity is present in immunoprecipitates of wild type infected or IE63 transfected cells generated by IE63 Mab or hnRNP K antiserum

Panel I. CK2 activities present in co-immunoprecipitates generated by IE63 Mab from wild type (wt) infected, 27lacZ infected (27), pCMV-63 transfected (trans), or mock infected (mi) BHK cell extracts. Assays were performed in the absence (lanes 1-4) or presence (lanes 5-9) of the CK2 peptide substrate and in the presence of the CK2 inhibitor DRB (lane 9).
Panel II. CK2 activities present in co-immunoprecipitates generated by anti-hnRNP K or pre-immune serum of wt, 27, trans, and mi cell extracts. Assays were performed in the absence (lanes 1-5) or presence (lanes 5-10) of the CK2 peptide substrate.

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

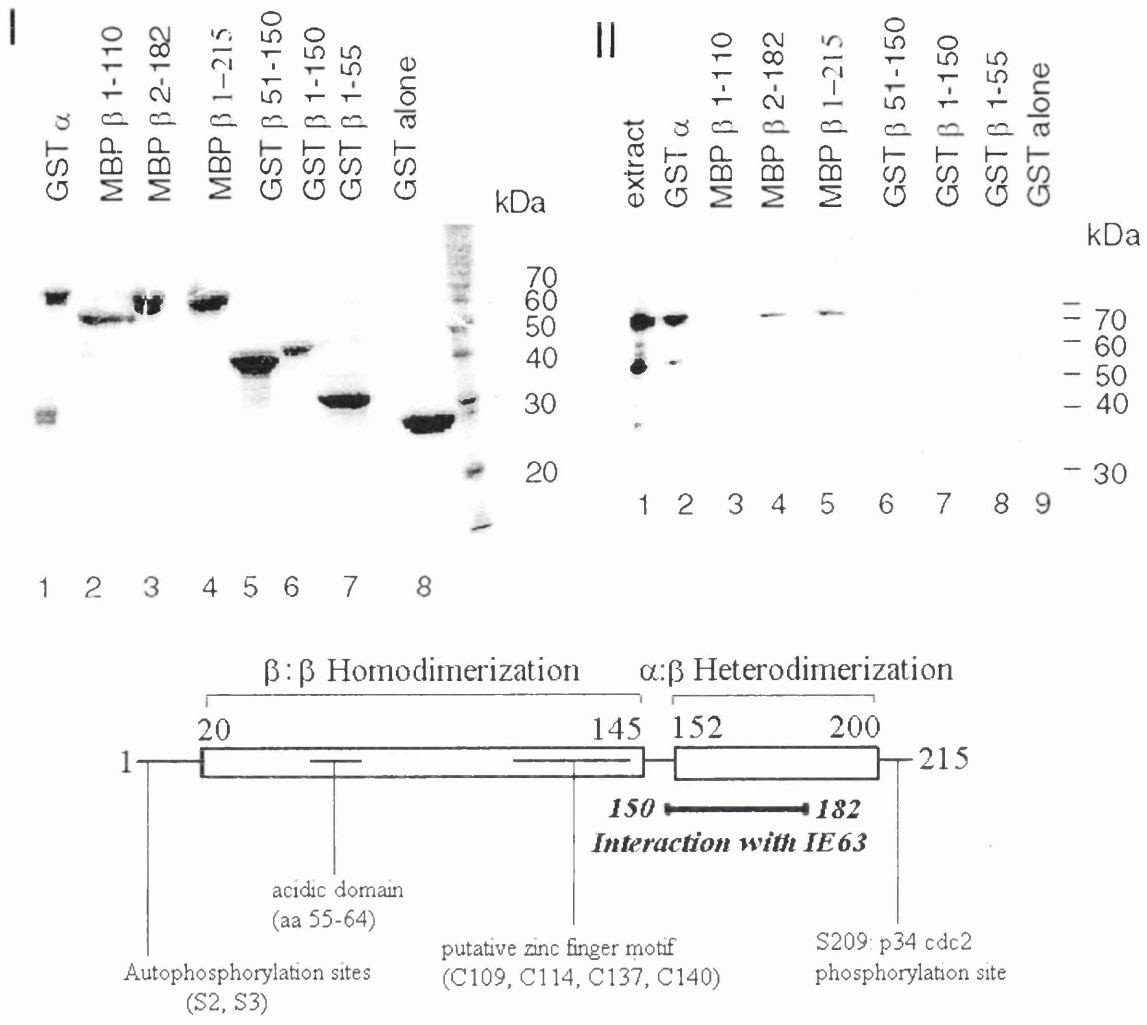


Fig 4B2. The CK2β regions involved in the interaction with IE63

GST and MBP fusion proteins of CK2β and different truncations were expressed, purified on columns and mixed with HSV-1 wild type infected cell extracts. Bound proteins were eluted, separated by SDS-PAGE and Western blotted using IE63 Mab. Numbers following β indicate aa residues present in the truncation.
Panel I. Coomassie stained gel of GST and MBP CK fusion proteins used. Proteins bound to beads were run on a 10% SDS-PAGE gel and visualised by Coomassie staining.
Panel II. Western blot of eluted proteins for IE63.
Panel III. Diagram showing regions of CK2β involved in interaction with IE63.

approximately equal amount of each fusion protein was added to wt infected cell extracts and bound proteins eluted off with high salt buffer. Eluted proteins were separated by SDS-PAGE and Western blotted for IE63. Only fusion proteins MBP β aa 2-182, and MBP β aa 1-215 bound to IE63 (Fig 4B2 Panel II lanes 4 and 5). The CK2β region necessary for IE63 interaction was therefore within aa 150-182 (Panel III), a region which overlaps with the portion required for interaction between β and α/α' subunits (Krehan & Pyerin, 1999). Other regions between aa 1 and aa 150 may be involved in the interaction but they are not sufficient for it. More complete mapping with CK2 N-terminal truncations and point mutations in aa 150-182 would be of value.

Interestingly, full length GST CK2 α fusion protein although added in the same amount as the β fusions (Panel I lane 1), pulled down far more IE63 than the β fusions did.

4B3 The CK2 α subunit can interact with IE63

Because the CK2β region mapped for interaction with IE63 overlapped with the region required for the interaction with CK2 α and as the GST-CK2 α fusion protein pulled down much more IE63 than any of the β proteins, further investigation into CK2 subunit interaction was carried out.

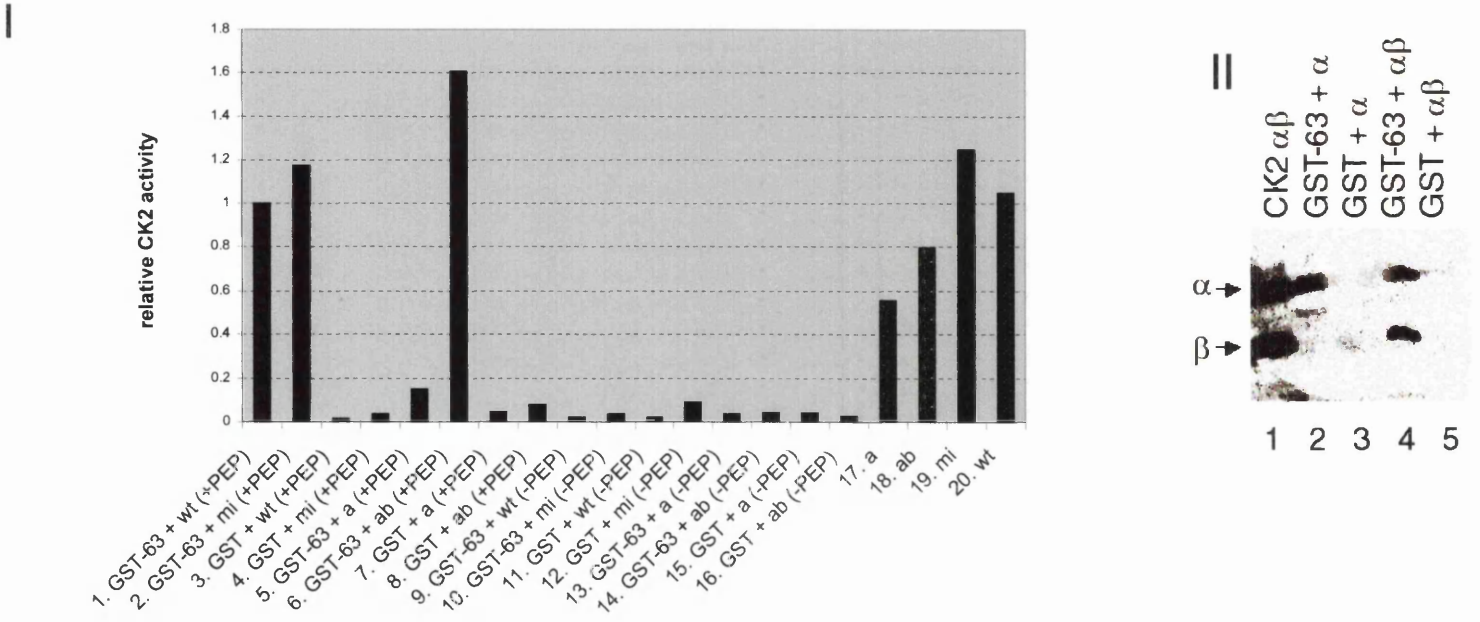
The artificial peptide assay was used to detect the presence of CK2 α binding to GST-IE63 either in the presence or absence of CK2 β subunit (Section 2B4.1 and 2B6.1). CK2 activity was, as expected, detected when GST-IE63 was mixed with wt or mi extracts (Fig 4B3 Panel I lanes 1 and 2), but not with GST alone (lanes 3 and 4). GST-IE63 mixed with purified α gave little CK2 activity (lane 5) whereas GST-IE63 plus purified $\alpha_2\beta_2$ holoenzyme was very active (lane 6). GST with either α or $\alpha_2\beta_2$ holoenzyme was inactive (lanes 7 and 8). As further controls, all reactions were repeated in the absence of the peptide substrate (lanes 9-16). In addition extracts and purified enzyme added were checked for enzymatic activity (lanes 17-20). These data therefore suggest that the interaction between IE63 and CK2 is via the β subunit of CK2.

Fig. 4B3. The CK2 α subunit interacts with IE63

GST-IE63 (GST-63) and GST fusion proteins were mixed with wild type infected BHK extract (wt), mock infected BHK extract (mi), purified CK2 α subunit (α) or CK2 holoenzyme (αβ). Proteins interacting with GST-63 or GST were assayed for CK2 activity using the peptide assay or for presence of the protein by Western blotting.

Panel I. CK2 activity as detected by phosphorylation of CK2 specific peptide substrate. Assays were performed with (lanes 1-8 and 17-20) or without (lanes 9-16) peptide substrate. Activities of extracts added are shown in lanes 17-20.

Panel II. Western blot for CK2. Proteins binding to GST or GST-63 were separated by SDS-PAGE, transferred to nitrocellulose and CK2 α and β subunit was detected by Western blotting with CK2 antisera. GST-63 mixed with purified α subunit (a) (lane 2), GST with purified α (lane 3), GST-63 with holoenzyme ($\alpha_2\beta_2$) (lane 4) and GST with ($\alpha_2\beta_2$) (lane 5).



The interaction with α subunit was also examined using Western blotting. GST-IE63 pull downs after incubation with purified CK2 subunits (α and $\alpha_2\beta_2$ (β alone was not avalible)) were separated on an SDS-PAGE gel, and Western blotted for CK2 α and β subunits. To detect the CK2 subunits with the low sensitivity CK2 antisera, a large amount of purified enzyme (0.2υg) was used. Fig 4B3 Panel II lane 2 indicates that GST-IE63 can indeed interact with α subunit of CK2, GST alone did not pull down the α subunit (lane 3). GST-IE63 pulled down both α and β subunits when incubated with holoenzyme (lane 4), GST alone pulled down neither (lane 5). From these data an interaction is suggested between IE63 and the α subunit of CK2.

4C CK2 activity in the IE63/CK2/hnRNP K complex

4C1 CK2 can phosphorylate hnRNP K and IE63

As IE63 and hnRNP K were found in a complex with CK2, the ability of CK2 to phosphorylate each of these proteins *in vitro* was investigated. CK2 activity assays (2B6.2) were set up in the presence or absence of the CK2 specific inhibitor DRB, phosphorylated proteins were separated by SDS-PAGE and detected by phosphoimaging. Using co-immunoprecipitates from wt, 27lacZ or mi cell extracts obtained with hnRNP K antiserum, addition of DRB made no difference to the phosphorylation of hnRNP K *in vitro* but considerably reduced the phosphorylation of IE63 (Fig 4C1 Panel I compare lanes 1 and 5). By contrast, using immunoprecipitates from wt infected cell extracts obtained with IE63 Mab, the *in vitro* phosphorylation of both hnRNP K and IE63 bands was consistently reduced by DRB treatment (Fig. 4C1 Panel III compare lanes 1 and 2). Western blots showed that similar amounts of IE63 and hnRNP K were present in reactions with or without DRB (panels II and IV).

Fig. 4C1. CK2 phosphorylates IE63 and a subfraction of hnRNP K *in vitro*

Co-immunoprecipitates from wild type (WT), 27lacZ (27) and mock infected (MI) BHK cell extracts generated by hnRNP K antiserum, IE63 Mab or pre-immune serum, were incubated in kinase buffer with γ-[^{32}P]-ATP , with or without the CK2 inhibitor DRB and then separated on an SDS-PAGE gel. Proteins were visualised by phosphoimaging or by Western blotting for IE63 or hnRNP K.

Panel I. Phosphoimage of phosphorylation with γ-[^{32}P]-ATP of IE63 and hnRNP K after co-immunoprecipitation with hnRNP K antiserum, in the presence (lanes 5-8) or absence (lanes 1-4) of DRB.

Panel II. Western blot of anti-hnRNP K co-immunoprecipitation (panel I) for IE63 and hnRNP K.

Panel III. Phosphoimage of phosphorylation with γ-[^{32}P]-ATP of IE63 and hnRNP K after co-immunoprecipitation with IE63 Mab, in the presence (lane 2) or absence (lane 1) of DRB.

Panel IV. Western blot for IE63 of IE63 Mab co-immunoprecipitation (panel III).

4C2 Phosphorylation of IE63 is required for, or at least increases, its interaction with hnRNP K

To examine the relevance of IE63 and hnRNP K phosphorylation, cell extracts were dephosphorylated with calf intestinal phosphatase (CIP), phosphorylation was preserved by the addition of phosphatse inhibitors (PI), or cycling phosphate addition by CK2 was inhibited by the addition of DRB (Section 2B6.3). Extracts were then co-immunoprecipitated with IE63 Mab, separated by SDS-PAGE gel electrophoresis and Western blotted for hnRNP K and IE63.

Addition of CIP increased the migration of both IE63 and hnRNP K bands (Fig.4C2 lane 3), compared to untreated proteins (lane 1), presumably due to phosphate groups removal, indicating that both proteins are phosphorylated. Two predominant bands were seen representing dephosphorylated hnRNP K and dephosphorylated IE63 but dephosphorylation of IE63 was not complete, as indicated by the smeared band. So either phosphorylation makes no difference to their interaction or enough phosphorylated IE63 was left to co-immunoprecipitate some hnRNP K. Given that, compared to untreated extracts, more IE63 was immunoprecipitated from CIP treated extracts while a similar amount of hnRNP K was co-immunoprecipitated, there was relatively less hnRNP K interacting with IE63. This adds weight to phosphorylation of IE63 being important for the interaction. The phosphorylation of hnRNP K is less likely to be important, as dephosphorylated hnRNP K was efficiently co-immunoprecipitated following CIP treatment.

The presence of phosphatase inhibitors prevented any removal of phosphate groups (lane 5) and IE63 was seen migrating similarly to the untreated sample. When hnRNP K co-immunoprecipitated with IE63 Mab from PI treated extract was examined (lane 5) there was considerably more of it than from untreated extracts (lane 1), despite there being a similar amount of IE63 precipitated. Thus we can conclude that phosphorylated IE63 has a greater affinity (or is required?) for interaction with hnRNP K. In lane 5, an even more slowly migrating hnRNP K band was seen co-precipitating with IE63 (marked *).

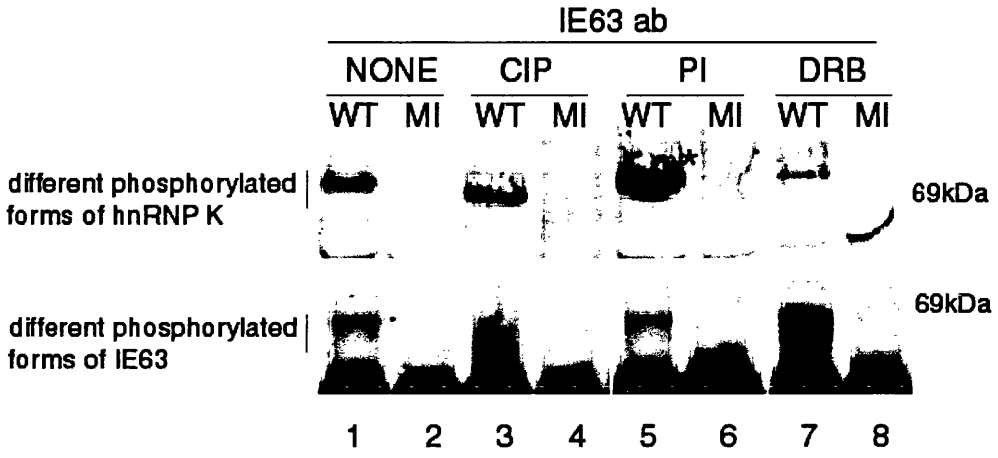


Fig 4C2. Phosphorylation by CK2 of IE63 but not hnRNP K is required for the interaction between IE63 and hnRNP K

Prior to and during co-immunoprecipitation with IE63 Mab, wild type (WT) and mock infected (MI) BHK cell extracts were treated with calf intestinal phosphatase (CIP) (lanes 3 and 4), phosphatase inhibitors (PI) (lanes 5 and 6), the CK2 specific inhibitor DRB (lanes 7 and 8), or left untreated (lanes 1 and 2). Co-immunoprecipitated proteins were separated by SDS-PAGE electrophoresis, transferred to nitrocellulose and Western blotted for IE63 and hnRNP K.

Treatment with DRB showed that less hnRNP K was co-precipitated than from untreated extract despite more IE63 being present (lane 7) as a smear which presumably was a reflection of a mix of dephosphorylated IE63 species. In other words, DRB did not completely inhibit CK2 phosphorylation. From the DRB-treated data (lanes 7 and 8), it is hard to know whether the phosphorylated proteins have a greater affinity for each other than unphosphorylated ones, or whether phosphorylation of IE63 is required but there was enough residual phosphorylated protein for some co-immunoprecipitation to occur.

Taken together the results of CIP, PI and DRB treatment suggest that the phosphorylation of IE63 by CK2 is required for (or at least increases the affinity of) the interaction between IE63 and hnRNP K, however the phosphorylation of hnRNP K has little effect on its interaction with IE63.

4C3 Phosphorylation of other proteins in the IE63 complex.

Fig. 4C3 shows all the immunoprecipitated proteins pulled out from wt extract using IE63 Mab after an *in vitro* phosphorylation assay. Comparison with the same reaction treated with DRB revealed CK2 phosphorylation of substrates other than IE63 and hnRNP K. The smallest band corresponds in size to one of the bands of the 32kDa doublet seen in a [^{35}S]-methionine labelled profile and was clearly phosphorylated by CK2. The next largest band may be the 44kDa or 50kDa band, if it is the 44kDa band then CK2α (the most likely identity of this band) is autophosphorylated: both CK2α and β have autophosphorylation sites (Dobrowolska *et al.*, 1999). Interestingly despite most autophosphorylation of CK2 occurring on the β subunit (Issinger, 1993, Litchfield *et al.*, 1991), here the putative CK2 β band at 25kDa was not autophosphorylated. IE63, hnRNP K and the upper most band also appeared to be phosphorylated by CK2.

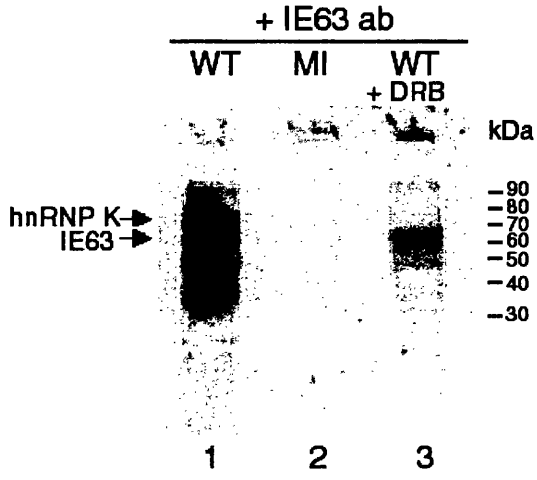


Fig. 4C3. Other proteins in the complex are phosphorylated by CK2

Phosphoimage of phosphorylation with γ-[^{32}P]-ATP of IE63 and co-immunoprecipitating proteins. Co-immunoprecipitates of wild type (WT) (lanes 1 and 3) and mock infected (MI) BHK cell extracts (lane 2) generated by anti-IE63, were incubated in kinase buffer with γ-[^{32}P]-ATP, with (lane 3) or without the CK2 inhibitor DRB (lanes 1 and 2) and then separated on an SDS-PAGE gel. Proteins were visualised by phosphoimaging after exposure to phosphoimaging plate overnight.

4C4 IE63 is not nucleotidylated by CK2

IE63 is nucleotidylated (Blaho *et al.*, 1993) a modification which has been suggested to be carried out by CK2. The products of a GST-IE63 pull down assay were incubated with α-[^{32}P]-ATP (Section 2B6.4) and nucleotidylation was detected by incorporation of [^{32}P] as seen by phosphoimaging of proteins separated by SDS-PAGE. Results (Fig. 4D2) showed firstly that nucleotidylating activity was not pulled down by GST-IE63 from wt infected (lane 3), or mi extracts (lane 4) and that purified $\alpha'_2\beta_2$ (lane 7) or $\alpha_2\beta_2$ (lane 8) could not nucleotidylate GST-IE63.

Nucleotidylation of many cellular and at least 2 viral proteins was seen when whole cell extracts had α-[^{32}P]-ATP added. The viral bands correspond in size to IE175 and to either IE68 or IE63, all of which have been reported to be nucleotidylated.

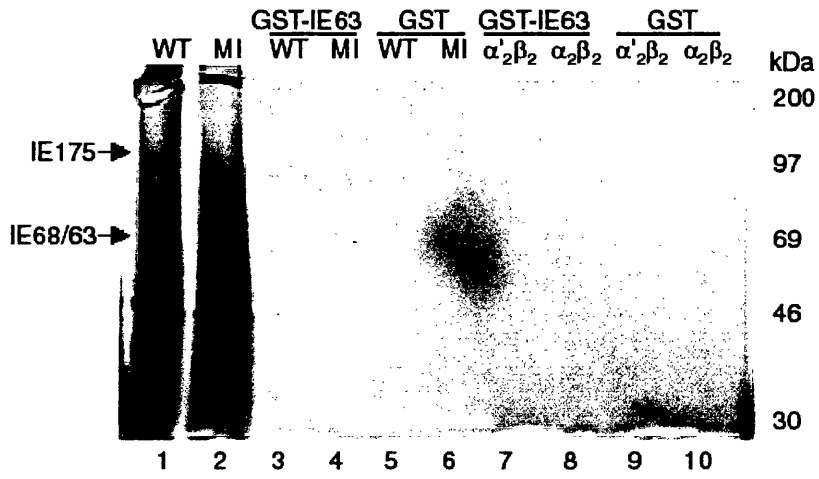


Fig. 4C4. CK2 does not nucleotidylate GST-IE63

After GST pull down assay was carried out with wild type (WT) and mock infected (MI) BHK cell extracts or with purified CK2 $\alpha_2'\beta_2$ and $\alpha_2\beta_2$. Glutathione beads with GST/GST-IE63 and interacting proteins were incubated with α-[^{32}P]-ATP, run on a 10% SDS-PAGE gel, dried down and exposed to X-OMAT film for 48h. Nucleotidylation is seen as a ^{32}P labelled band as shown by activity present within WT and MI extracts used in pull downs (lanes 1 and 2) – see text for details.

Nucleotidylation of GST-IE63 and/or interacting proteins by interacting proteins pulled out from WT extract (lane 3) and MI extract (lane 4) by GST-IE63.

Nucleotidylation of GST and/or interacting proteins by interacting proteins pulled out from WT extract (lane 5) and MI extract (lane 6) by GST.

Nucleotidylation of GST-IE63 by interacting CK2 holoenzyme, $\alpha_2'\beta_2$ (lane 7) or $\alpha_2\beta_2$ (lane 8), and of GST by $\alpha_2'\beta_2$ (lane 9) or $\alpha_2\beta_2$ (lane 10).

Chapter 5: Interaction of IE63 with p32, SAP145 and other viral proteins

Two other potential IE63 interacting proteins suggested by the yeast 2-hybrid screen were Spliceosome Associated Protein 145 (SAP145) and another protein involved in splicing, p32. This chapter confirms interactions involving IE63 with both SAP145 and p32, and examines the identity of other bands seen in the original co-immunoprecipitation profile (Fig. 3A1).

IE63s involvement in splicing inhibition made SAP145 and p32 good candidates for interaction, indeed p32 was such a good candidate that work had begun on its interaction with IE63 before results of the yeast 2-hybrid assay were known.

5A Interaction of IE63 and p32

5A1 IE63 and p32 interact to form a complex with hnRNP K and CK2

5A1.1 Using a p32 Sepharose column

A Sepharose column with p32 attached (rMp32) (Section 2B4.3) was used to look at the interaction between p32 and IE63. p32 is highly charged thus as a control glucose oxidase, a protein with a similar pI, was attached to the same type of column (rMGO) and used in parallel. After incubating rMp32 with cell extracts, Coomassie stained bands corresponding to proteins of ~63kDa, and 44kDa were seen to interact specifically with p32 (Fig. 5A1.1 panel I lane 6). Other bands seen on the gel were either not specific to rMp32 being used (compare lanes 1-3 with lanes 4-6) or the presence of IE63 (compare lanes 4 and 5 with lane 6). Interestingly a band of ~50kDa was pulled out specifically by rMp32 (compare lane 4 and 5 with lanes 1 and 2) but only from mi and 27lacZ infected cell extracts (compare lanes 4 and 5 with lane 6).

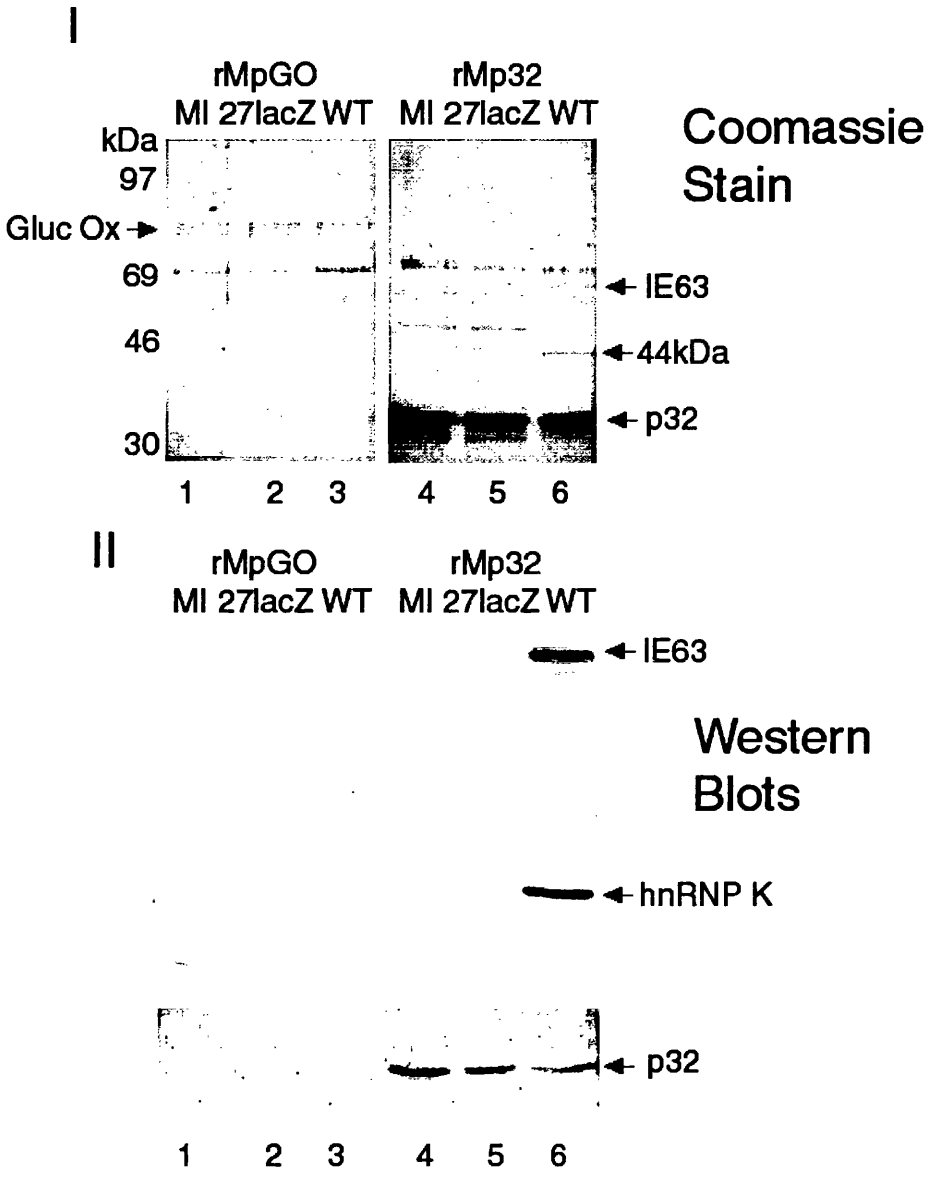


Fig 5A1.1 p32 attached to a Sepharose column binds IE63

A Sepharose column with p32 attached (rMp32) was mixed with cell extracts and the proteins interacting with it separated by SDS-PAGE electrophoresis, Proteins were visualised by Coomassie staining or Western blotting for IE63, hnRNP K and p32 of the gels, or by performing a CK2 peptide assay on the complex formed.

Panel I: Coomassie stained gel of proteins interacting with rMp32 when mixed with mock (MI) (lane 4), 27lacZ (lane 5) or wild type (WT) (lane 6) infected extracts, also proteins interacting with glucose oxidase attached to Sepharose (rMGO) when mixed with MI (lane 1), 27lacZ (lane 2) or WT (lane 3) infected extracts.

Panel II: Western blot of the above gel for IE63 (TOP), hnRNP K (MIDDLE) and p32 (BOTTOM).

panel III and IV on next page

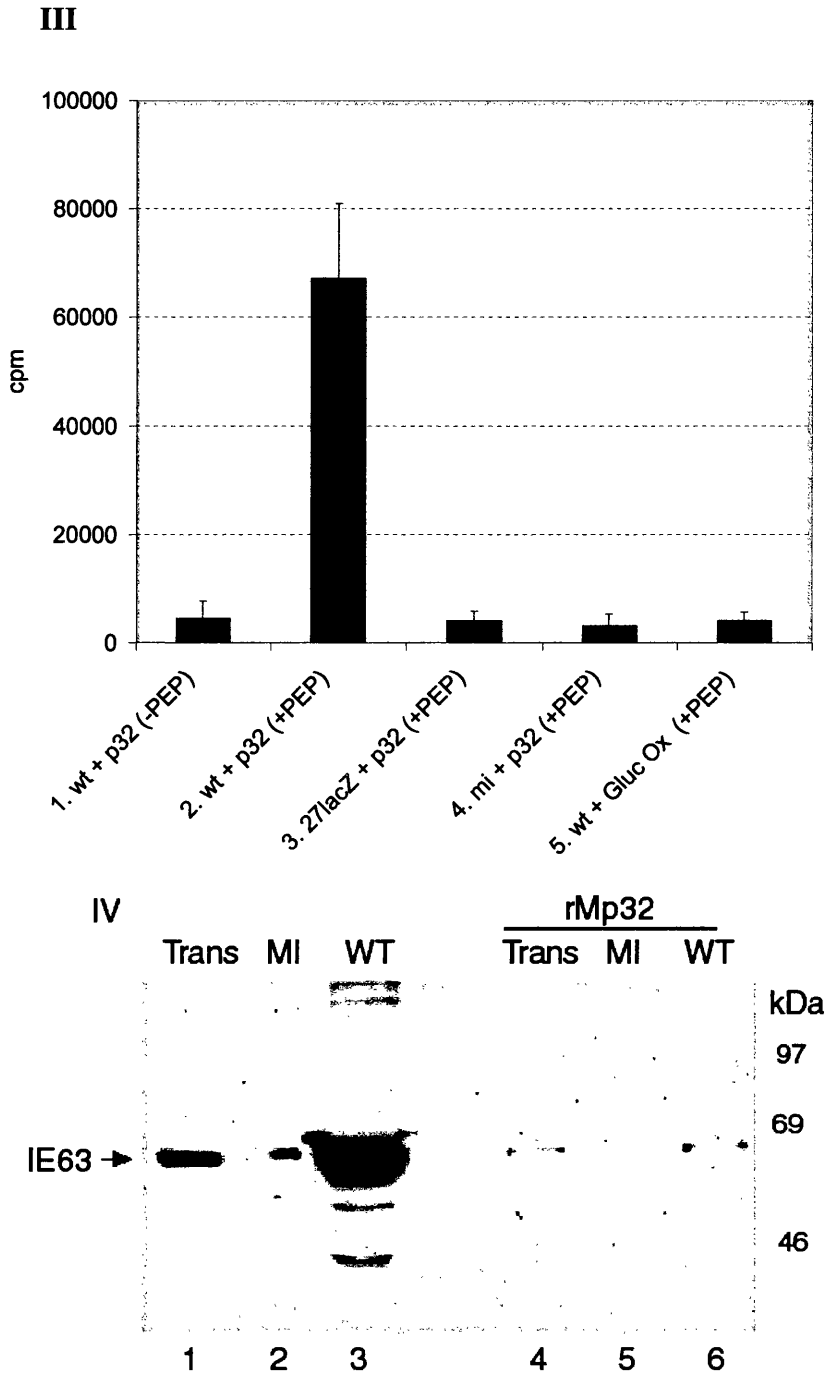


Fig 5A1.1 p32 attached to a Sepharose column binds IE63 cont.

Panel III: CK2 activity as measured by phosphorylation of an artificial peptide. Counts per minute (cpm) are proportional to activity found in pull downs with rMGO from WT extract (lane 5), and with rMp32 from WT (lanes 1 and 2), 27lacZ (lane 3), and MI (lane 4) extracts, CK2 assays were performed in the presence (lanes 2-5) or absence (lane 1) of artificial peptide.
Panel IV: Western blot for IE63 of rMp32 pull down with pCMV63 transfected (Trans) (lane 4), MI (lane 5) and WT (lane 6) cell extracts. Extracts used (lanes 1-3).

Western blotting of the same column eluates for IE63 indicated that the 63kDa band was IE63 (Fig. 5A1.1 panel II lane 6), while blotting for hnRNP K revealed that although p32 and hnRNP K did not interact in the absence of IE63 (lane 4 and 5), in wt infected extracts they did (lane 6).

Performing a CK2 peptide assay on interacting proteins pulled out on the p32 column demonstrated that CK2 was also present in the complex (Fig. 5A1.1 panel III lane 2) and that CK2 and p32 were not associated in the absence of IE63 (lanes 3 and 4). Controls without peptide (lane1) and using rMGO in a pull down with wt extract (lane 5) were used and were negative.

Use of IE63 transfected extracts (Fig.5A1.1 Panel IV lane 4) showed that IE63 alone could interact with p32 and that no other viral proteins were required.

5A1.2 Using immunoprecipitation

Further confirmation of the interaction between p32 and IE63 came from co-immunoprecipitation experiments. p32 antiserum was used to Western blot an anti-IE63 co-immunoprecipite generated as the original co-immunoprecipitation was (Section 2B3.1). IE63 precipitated itself from the wt infected extract (Fig.5A1.2 panel I lane 1) and p32 was co-immunoprecipitated with it (Panel II lane 1). As a control for cross reactivity of the IE63 Mab with p32, p32 did not precipitate from mi extract (Panel II lane 2). Thus it seems likely that one of the bands seen as a doublet at 32kDa in Fig.3A2 corresponds to p32.

5A2 IE63 causes redistribution of p32

p32 is usually seen to have a predominantly mitochondrial/cytoplasmic distribution (Muta *et al.*, 1997). Immunofluoresence was used to visualise IE63 (green) and p32 (red); infected and uninfected cells could therefore be recognised by the presence or absence of green stain, while the distribution of p32 in uninfected and infected cells could be compared by looking at the red staining. In HSV-1 infected cells, p32 appeared to be redistributed to form between one and three large foci in the perinuclear region of the cell, the nucleus was also diffusely

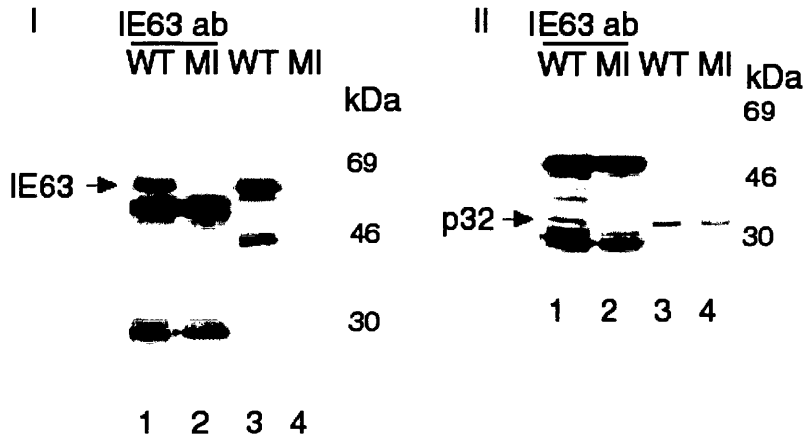


Fig. 5A1.2 IE63 and p32 co-immunoprecipitate

Co-immunoprecipitations with IE63 Mab separated on a 10% SDS-PAGE gel and Western blotted for p32 and IE63.
Panel I: Western blot for IE63 performed on anti-IE63 co-immunoprecipitates from wild type (WT) (lane 1) and mock infected (MI) (lane 2) extracts. Extracts used (lanes 3 and 4).
Panel II: Western blot for p32 performed on anti-IE63 co-immunoprecipitates from WT (lane 1) and MI (lane 2) extracts. Extracts used (lanes 3 and 4).

stained and no p32 was seen in the cytoplasm (Fig. 5B2.2 Panel I). However HSV-1 glycoprotein E (gE) is capable of binding to the IgG molecules which make up rabbit antibodies, such as the p32 one used here, and has been described as a low affinity Fc receptor (Bell *et al.*, 1990, Dubin *et al.*, 1990, Johnson *et al.*, 1988). Staining for gE (green) and p32 (red) (Panel II), revealed exact co-localisation between them due to a cross reaction between the rabbit IgG in p32 serum and gE from HSV-1 infection. Use of cells infected with gE^- virus (Panel III) and IE63 transfected cells (Panel IV) overcame the problem and revealed that IE63 alone was sufficient to cause redistribution of p32. The distribution of p32 was mitochondrial/cytoplasmic in uninfected/untransfected cells and was excluded from the nucleus. In gE^- cells and pCMV-63 transfected cells, this was altered to a diffusely nuclear pattern, although some cytoplasmic staining was still visible.

5A3 CK2 can phosphorylate p32

The ability of CK2 to phosphorylate proteins which co-immunoprecipitate with IE63 is discussed in Section 4D1 and demonstrated that a band of 32kDa was phosphorylated by an activity which was inhibited by the specific CK2 inhibitor DRB (Fig. 4D1). Examination of the amino acid sequence of p32 (Fig. 5A3) revealed that it contained 5 consensus sequences for CK2 phosphorylation. Phosphorylation of p32 by CK2 is therefore not unexpected and the 32kDa band seen to be phosphorylated by CK2 is most likely to be p32, although the phosphorylated protein could correspond to either of the bands in the 32kDa doublet.

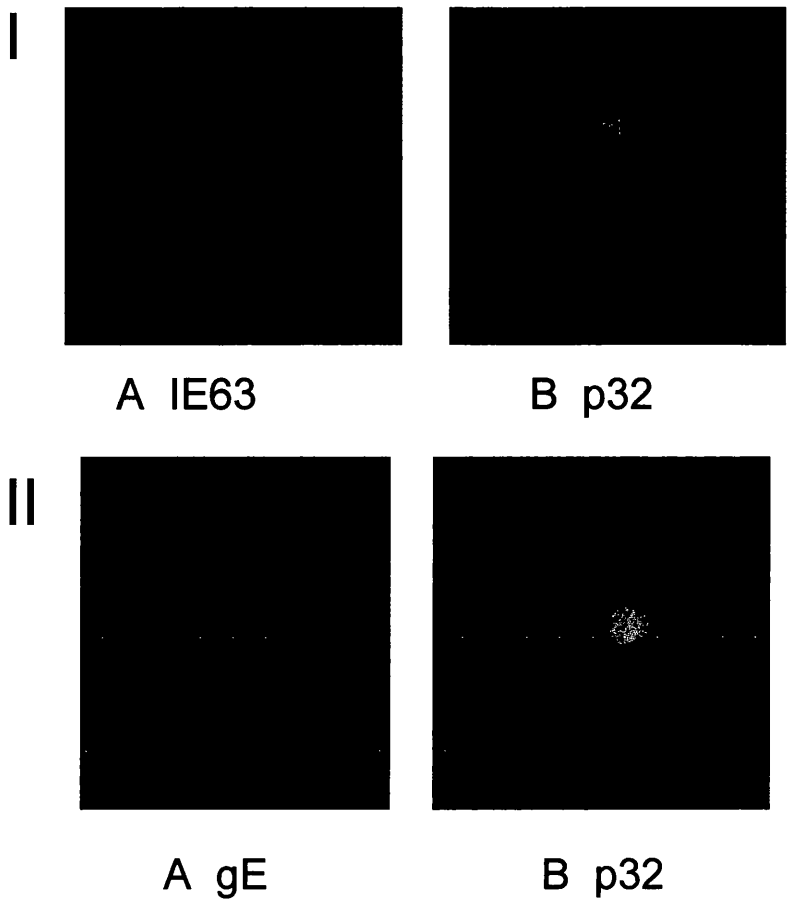


Fig. 5A2 IE63 causes p32 to redistribute in cells
Immunofluoresence was performed as described in section 2B8 on wild type (wt) infected, gE negative virus infected, pCMV63 transfected and mock infected HeLa cells.
Panel I: wt infected cells in the same field stained using IE63 (A) and p32 (B) antisera as primary antibodies and TRITC and FITC as secondary antibodies.
Panel II: wt infected cells in the same field stained using gE (A) and p32 (B) antisera as primary antibodies and TRITC and FITC as secondary antibodies.
Panels III and IV on next page.

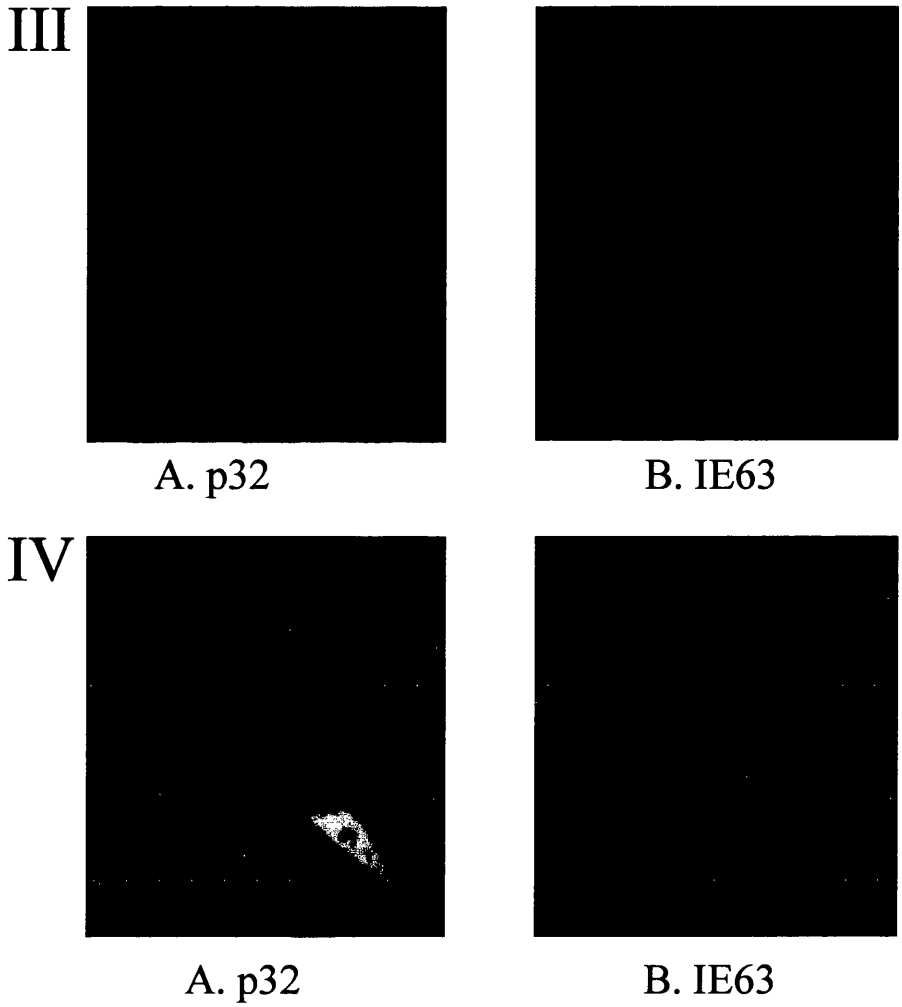


Fig. 5A2 IE63 causes p32 to redistribute in cells (CONT.)
Panel III: gE- infected HeLa cells, in the same field,
6h post-infection, stained using p32 (A) and IE63 (B) antisera
as primary antibodies and TRITC and FITC as secondary antibodies.
Panel IV: pCMV63 transfected HeLa cells, in the same field,
stained using p32 (A) and IE63 (B) antisera as primary antibodies
and TRITC and FITC as secondary antibodies

M L P L L R C V P R V L G S S V A G L R A A A P A S P F R Q L L
Q P A P R L C T R P F G L L S V R A G S E R R P G L L R P R G P C
A C G C G C G S L H **T** D G D K A F V D F L S D E I K E E R K I Q
K H K T L P K M S G G W E L E L N G T E A K L V R K V A G E K
I T V T F N I N N S I P P T F D G E E E P S Q G Q K V E E Q E P E L
T S T P N F V V E V I K N D D G K K A L V L D C H Y P E D E V G
Q E D E A E S D I F **S** I R E V S F Q **S** T G E S E W K D T N Y T L N
T D S L D W A L Y D H L M D F L A D R G V D N **T** F A D E L V E
L S **T** A L E H Q E Y I T F L E D L K S F V K S Q

Fig. 5A3 CK2 phosphorylation sites on p32

Consensus sequences for CK2 phosphorylation are shaded, amino acids predicted to be phosphorylated are shown in bold. Sites were considered as potential phosphorylation sites if a S/T residue was N terminal to a D or E residue (<4 aa away). Adapted from data in Deb & Datta (1996).

5B Interaction of IE63 with SAP145

5B1 IE63 and SAP145 interact

5B1.1 Using the GST pull down assay

GST pull down experiments using GST-SAP145 or GST-IE63 fusion proteins and ~100μg wt/mi extract, followed by Western blotting for IE63 or SAP145, showed that GST-SAP145 could pull IE63 down from infected cell extract (Fig 5B1.1 Panel I lane 5, marked with a star) and that GST-IE63 could pull SAP145 down from mi or infected cell extracts (Panel II lanes 3 and 4). As GST-IE63 pulled SAP145 out of mi and wt infected extracts, this indicates that no other viral proteins are involved in the interaction; the slight decrease in intensity of SAP145 from mi extracts was not a consistent observation. GST alone did not pull down IE63 (Panel I lane 3) or SAP145 (Panel II lanes 1 and 2). A Coomassie stained gel of the GST fusion proteins used is shown in Panel III.

5B1.2 Using co-immunoprecipitation

Further evidence for the interaction between SAP145 and IE63 came from Western blotting of anti SAP145 co-immunoprecipitates with IE63 antibody. Fig. 5B1.2 (lane 1) shows that SAP145 antiserum precipitated itself from wt extract, and that IE63 was co-immunoprecipitated with it. The pre-immune serum did not precipitate either protein (lane 2). Attempts to Western blot the original anti IE63 co-immunoprecipitation with SAP145 antibodies proved negative.

5B2 hnRNP K and SAP145 can co-immunoprecipitate but only in the presence of IE63

It was important to determine whether there was a direct SAP145-IE63 interaction or an indirect one occurring though hnRNP K. Equally it was important to know if the hnRNP K-IE63 interaction was direct or via SAP145. To study this, the interaction between SAP145 and hnRNP K was investigated. Using hnRNP K

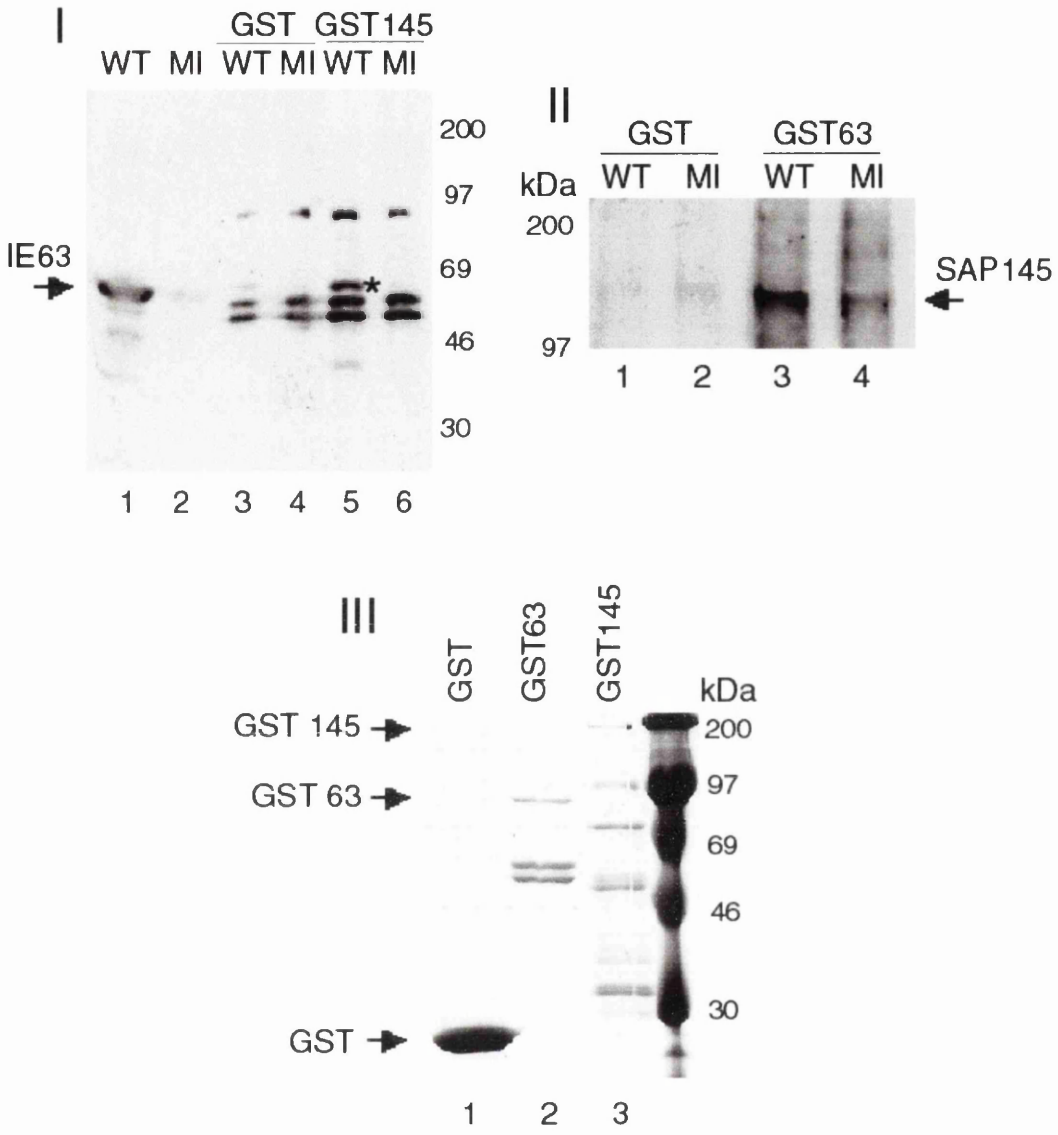


Fig. 5B1.1 GST-IE63 interacts with SAP145 and GST-SAP145 interacts with IE63

Fusion proteins were expressed bound onto beads and mixed with wild type (WT) and mock (MI) infected cell extracts. Proteins interacting were separated by SDS-PAGE electrophoresis and Western blotted for IE63 or SAP145.
Panel I: Western Blot for IE63, proteins pulled out of WT and MI extracts by GST (lanes 3 and 4), out of WT and MI extracts by GST-SAP145 (GST-145) (lanes 5and 6), and extracts used (lanes 1 and 2).
Panel II: Western blot for SAP145, proteins pulled out of WT and MI extracts by GST (lanes 1 and 2), and out of WT and MI extracts by GST-IE63 (GST-63) (lanes 3 and 4).
Panel III: Coomassie stained gel of fusion proteins used.

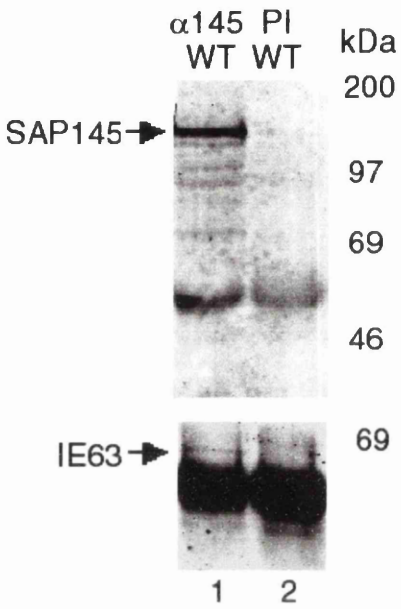


Fig. 5B1.2 SAP 145 and IE63 co-immunoprecipitate

Western blots for SAP145 and IE63. After co-immunoprecipitation from wild type infected cell extracts (WT) using SAP145 antiserum (α145) (lane 1) and pre-immune serum (PI) (lane 2), co-immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose and Western blotted for SAP145 (TOP) and IE63 (BOTTOM).

antiserum, hnRNP K was precipitated from wt, 27lacZ and mi extracts (Fig. 5B2, lanes 2-4), whereas SAP145 was only co-immunoprecipitated from wt infected extract (lane 2). Pre-immune serum precipitated neither hnRNP K or SAP145. SAP145 and hnRNP K can therefore not interact in the absence of IE63.

5B3 SAP145 and CK2 interact but only in the presence of IE63

Anti SAP145 co-immunoprecipitates from wt, 27lacZ, and mi extracts and a co-immunoprecipitate of wt extract with pre-immune serum, were assayed for the presence of CK2 activity using the peptide substrate assay. Activity was found only in co-immunoprecipitates from wt extracts (Fig. 5B3 lane 2). It follows then that SAP145 and CK2 cannot usually interact. However in infected cell extracts they can interact indirectly via IE63. A control of an anti-SAP145 co-immunoprecipitate assayed for CK2 in the absence of peptide substrate showed low activity.

5B4 SAP145 and p32 don't interact even in the presence of IE63.

The p32 column was used to examine whether SAP145 could interact indirectly with IE63 via p32. Extracts used Western blotted for IE63 and SAP145 are shown (Fig. 5B4 lanes 1-3). Proteins binding to the p32 column, were also Western blotted for IE63 and SAP145. IE63 from wt extract was seen to bind to p32 (lane 4), but SAP145 in mi (lane 5), 27lacZ (lane 6) or wt infected (lane 4) extracts was not. Therefore while IE63 and SAP145 interacted, this did not occur when IE63 was bound to p32.

5B5 IE63 and SAP145 co-localise in cells

HSV-1 infection causes a redistribution of the cellular splicing snRNPs from a widespread diffuse speckled pattern to a highly punctate organisation in the nucleus (Martin *et al.*, 1987). IE63 is both necessary and sufficient to cause this effect, and at later times post infection IE63 co-localises with the redistributed snRNPs (Phelan *et al.*, 1993; Sandri-Goldin *et al.*, 1995). SAP145 is a component of the U2 snRNP complex (Gozani *et al.*, 1994), and would be expected to be

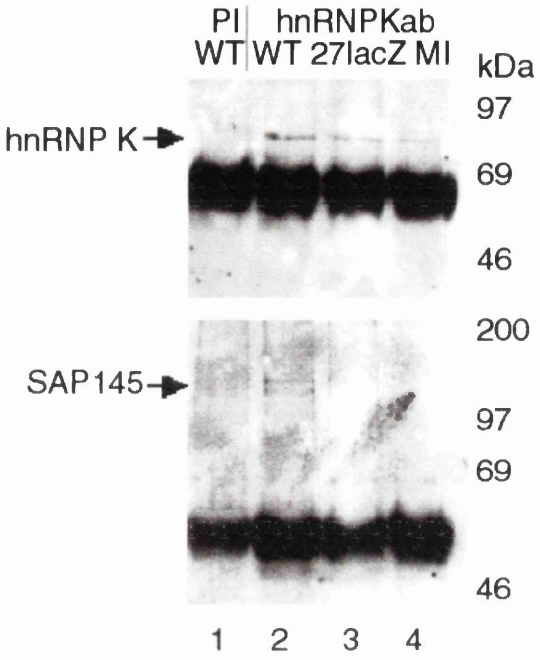


Fig. 5B2 hnRNP K and SAP145 only interact in wild type infected extract

Western blots for SAP145 and hnRNP K. After co-immunoprecipitation using hnRNP k antiserum and wild type (WT) (lane 2), 27lacZ (lane 3) or mock infected (MI) (lane 4) extracts and pre-immune serum (PI) with WT extract (lane 1), co-immunoprecipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose and Western blotted for SAP145 (BOTTOM) and hnRNP K (TOP).

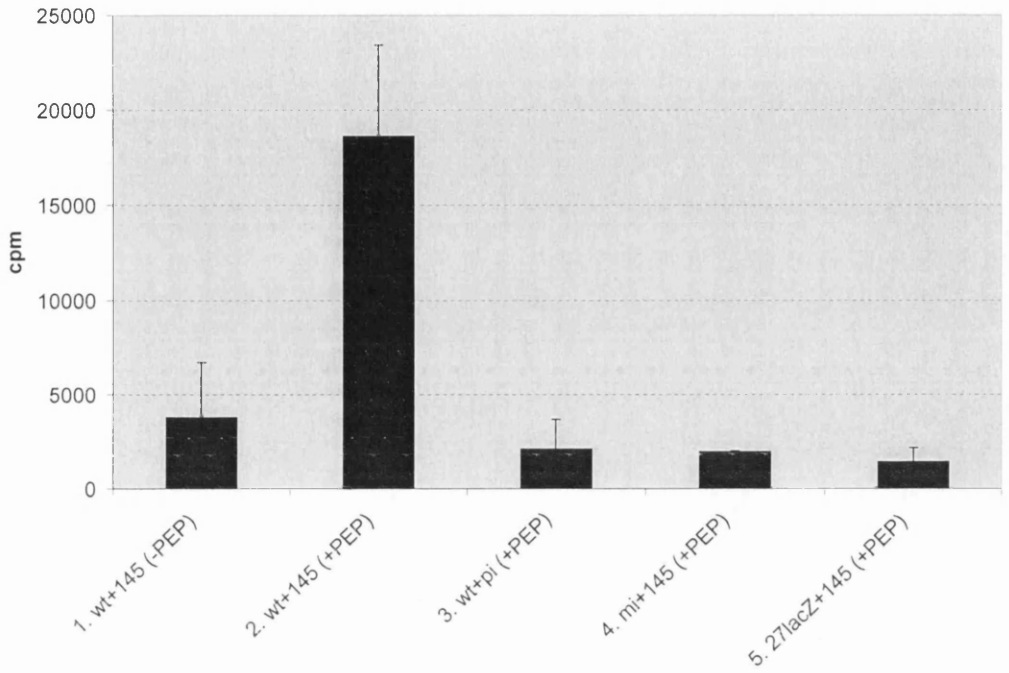


Fig. 5B3 CK2 and SAP145 only interact in the presence of IE63

CK2 activity in anti-SAP145 co-immunoprecipitates. Anti SAP145 co-immunoprecipitations were performed followed by a CK2 peptide assay. CK2 activity was determined by scintillation counting. Counts per minute (cpm) being proportional to enzyme activity. Assays were performed without peptide on co-immunoprecipitates from wild type extract (wt) (lane 1) or with peptide on co-immunoprecipitates from wt (lane 2), mock infected (mi) (lane 4), and 27lacZ infected (lane 5) extracts, or with peptide on a co-immunoprecipitate from wt extract using pre-immune serum (pi) (lane 3).

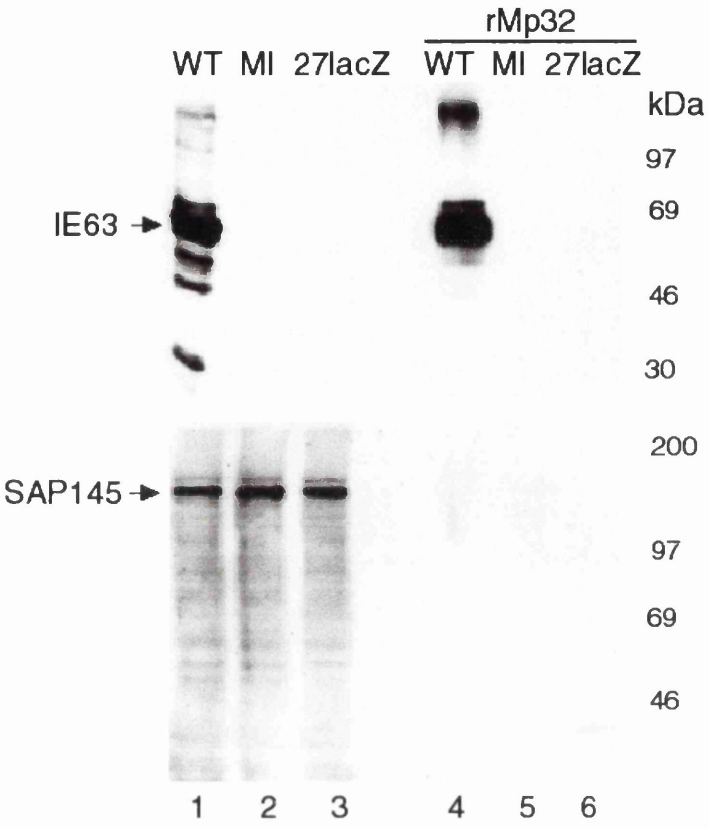


Fig. 5B4 SAP145 and p32 do not interact even in the presence of IE63

A Sepharose column with p32 attached (rMp32) was mixed with cell extracts and the proteins interacting with it separated by SDS-PAGE electrophoresis, IE63 and SAP145 were visualised by Western blotting. Extracts used (lanes 1-3), proteins interacting with rMp32 from wild type infected (WT) (lane 4), mock infected (MI) (lane 5) and 27lacZ infected (lane 6) extracts.

located with snRNPs. For this reason, the cellular location of SAP145 in infected and uninfected cells was inspected.

HeLa cells were infected with wt virus or transfected with the IE63 expressing plasmid and the localisation of IE63 and SAP145 determined. Cells were stained for IE63 (green) and SAP145 (red); infected cells and uninfected cells can then be distinguished by the presence or absence of green stain, and SAP145 location examined by looking at red stain. The distibution of SAP145 does not appear to differ between infected and uninfected cells, although it may be a little more punctate (Fig.5B5, Panel I).

Use of the confocal microscope allowed IE63 and SAP145 co-localisation to be seen in the same image, (Fig.5B5 Panels I and II). Results in IE63 transfected cells (Fig.5B5 panel III), show that no other viral factors are required for this co-localisation. Immunofluoresent staining for SAP145 (red) and SC35 (green) in wt infected cells demonstrated that they co-localise Panel IV.

5C Interaction of IE63 with other HSV-1 proteins

5C1 Interaction of IE63 with viral TK

5C1.1 The identity of a 44kDa band seen in Coomassie stained rMp32 pulldown assays.

In Fig. 5A1.1 lane 6, a densely stained band of ~44kDa was seen to be specifically interacting with rMp32 in the presence of IE63. As CK2 activity was shown to be present in the complex, one explanation was that this band was CK2α or α' subunit, however its abundance was inconsistent with CK2 only being present in very small amounts, and the amount of CK2 activity present bound to the column (Fig 5B1.1) was no greater than that co-immunoprecipitated by hnRNP K antiserum where the band was not visible by Coomassie staining. Results of rMp32 pull downs with samples at different times post infection (Fig.5C1.1), suggested that the 44kDa band was viral or at least required another viral

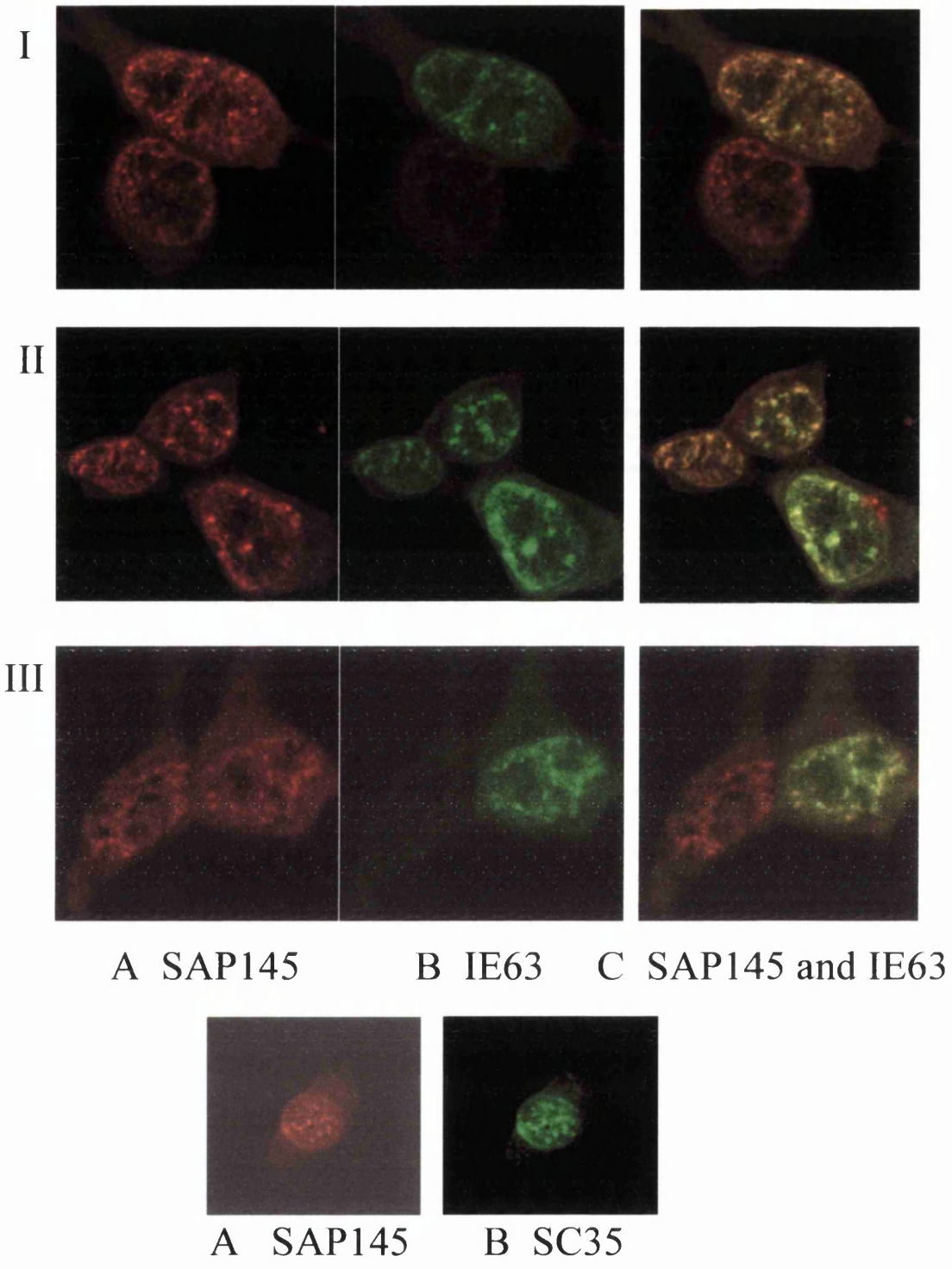


Fig. 5B5 IE63 causes the redistribution of and co-localises with SAP145
HeLa cells were infected with wild type virus or transfected with pCMV63, fixed and stained for IE63 and SAP145 then analysed by confocal microscopy. The same field is shown in each case.
Panel I: HSV-1 infected and uninfected cells stained for SAP145 (A), IE63 (B) and merged (C).
Panel II: HSV-1 infected cells stained for SAP145 (A), IE63 (B) and merged (C).
Panel III: pCMV-63 transfected cells stained for SAP145 (A), IE63 (B) and merged (C).
Panels IV: HSV-1 infected cells stained for SAP145 (A) and SC35 (B).

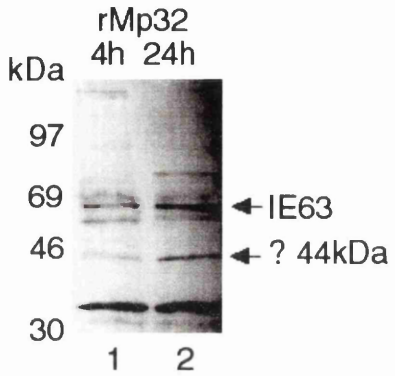


Fig 5C1.1 The 44kDa band increases in intensity between 4h and 24h post-infection

Coomassie stained gel of proteins interacting with a Sepharose column with p32 attached (rMp32) when mixed with wt (WT) extract harvested at 4h (lane 1) or 24h (lane 2) post-infection.

component for interaction with IE63. The band increased in intensity between 4h and 24h post infection (compare lanes 1 and 2).

As the protein was relatively abundant, an attempt was made to determine its identity by Laser Mass Map Spectroscopy. Simultaneously to trying to sequence the protein myself, a sample was set to St. Andrews for analysis on their machine. Sequence data obtained by Dr. G. Kemp in St. Andrews identified the band as HSV-1 TK (Kemp, unpublished data).

5C1.2 Confirmation of identity of 44kDa band as viral TK.

Confirmation came in 3 ways. Firstly the 44kDa band disappeared when TK1301 (a TK negative virus) infected extract was used in rMp32 pull downs as performed in Section 5A1 (Fig. 5C1.2 Panel I compare lanes 3 and 4).

Secondly TK was shown to interact with p32 by Western blotting, with anti TK serum. Analysis of proteins from wt extract which were bound to rMp32 or rMpGO columns, showed that p32 pulled down TK (Panel II lane 2), while TK was not seen to interact with glucose oxidase (lane 1).

Lastly anti IE63 co-immunoprecipitates were Western blotted for TK. IE63 Mab immunoprecipitated IE63 from wt infected extract (Panel III lane 1 lower), TK was co-immunoprecipitated with IE63 (lane 1 upper), mouse ascites fluid did not precipitate IE63 or TK from wt infected extracts (lane 3).

5C2 Interaction with IE175

5C2.1 Examination for an interaction between IE63 and IE175 using co-immunoprecipitation

Panagiotidis *et al* (1997) have claimed that IE63 and IE175 physically and functionally interact. This interaction seemed a good positive control for my study.

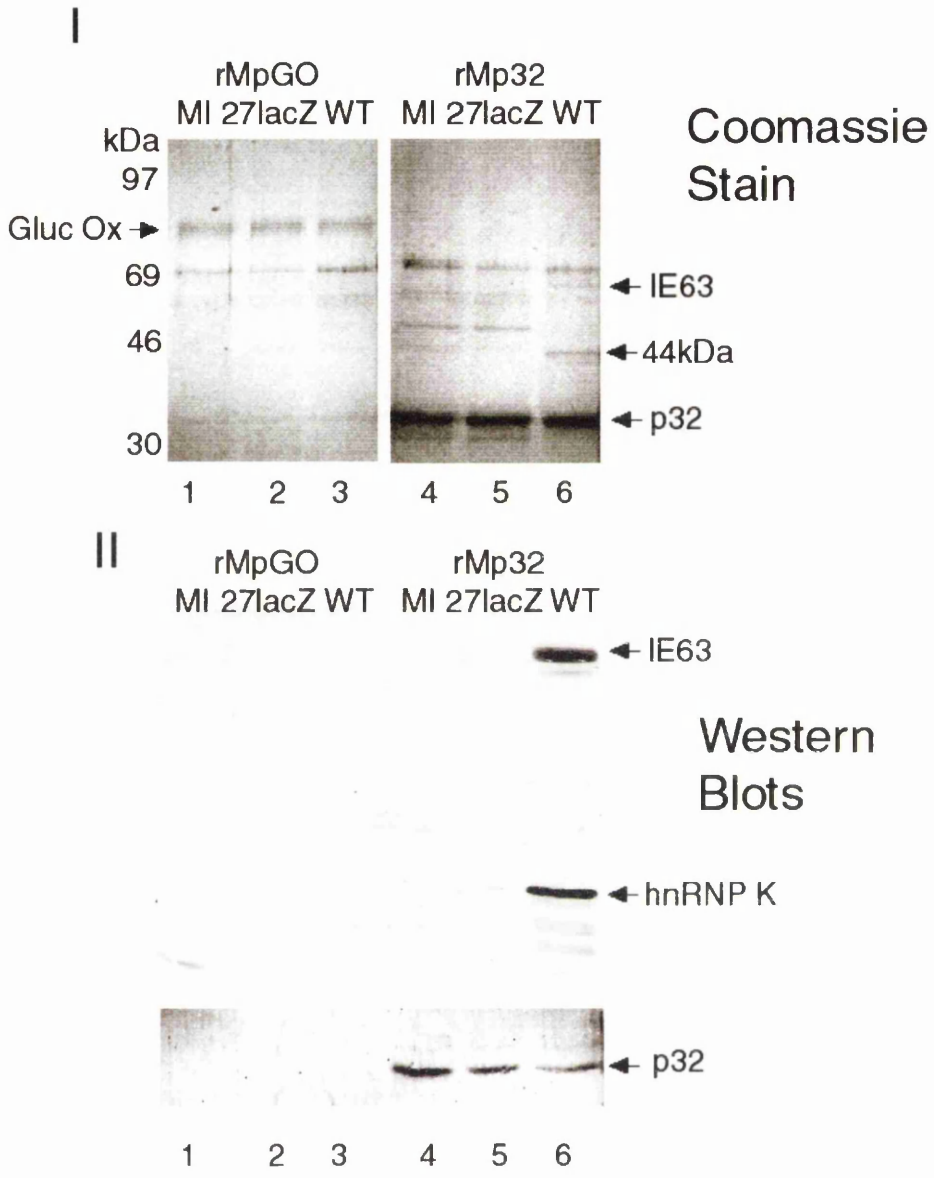


Fig 5A1.1 p32 attached to a Sepharose column binds IE63

A Sepharose column with p32 attached (rMp32) was mixed with cell extracts and the proteins interacting with it separated by SDS-PAGE electrophoresis, Proteins were visualised by Coomassie staining or Western blotting for IE63, hnRNP K and p32 of the gels, or by performing a CK2 peptide assay on the complex formed.

Panel I: Coomassie stained gel of proteins interacting with rMp32 when mixed with mock (MI) (lane 4), 27lacZ (lane 5) or wild type (WT) (lane 6) infected extracts, also proteins interacting with glucose oxidase attached to Sepharose (rMGO) when mixed with MI (lane 1), 27lacZ (lane 2) or WT (lane 3) infected extracts.

Panel II: Western blot of the above gel for IE63 (TOP), hnRNP K (MIDDLE) and p32 (BOTTOM).

panel III and IV on next page

Prior to these experiments, it had been reported that the electrophoretic mobility of IE175 was altered in the absence of IE63, however it was not clear if IE63 was directly or indirectly involved in this post-translational modification (Rice & Knipe, 1988; Su, 1989; McMahan, 1990). Interaction was also suggested from co-transfection experiments which indicated that IE63 and IE175 can alter each other's intracellular localisation (Zhu & Schaffer, 1995).

Co-immunoprecipitations, as described in Section 2B3.1, were performed with HSV-1 wt and mi whole cell extracts (Section 2B2.5i). The precipitated proteins were separated by SDS-PAGE and Western blotted for IE63 or IE175 (Fig. 5C2.1a). IE63 Mab precipitated IE63 (Panel I, lane 3) however IE175 was not detected (Panel II, lane 3).

Co-immunoprecipitations were then repeated using the conditions of Panagiotidis (1997), (Section 2B3.2) with HSV-1 wt or mi nuclear extracts (Section 2B2.5ii). As a control, mouse ascites fluid was incubated with wt nuclear extract in the same way as the Mab was. After separation on an SDS-PAGE gel and Western blotting, IE63 Mab had precipitated IE63 (Fig 5C2.1b Panel I, lane 3), but IE175 was not detected (Panel II, lane 3). Ascites fluid did not precipitate IE63 or IE175 (Panels I and II, lane 1).

IE175 Mab (58S) (Ackerman *et al.*, 1984) was used to co-immunoprecipitate IE63. The conditions published by Panagiotis were followed (Section 2B3.2) using HSV-1 wt nuclear extract, incubated with 58S Mab or mouse ascites fluid. Proteins were separated by SDS-PAGE gel electrophoresis and Western blotted for IE175, IE63 and IE110. IE175 Mab co-immunoprecipitated IE175 (Fig 5C2.1c Panel I, lane 1), but did not precipitate IE63 (Panel II, lane 1). Western blotting for IE110, another immediate early gene of HSV-1 involved in regulation of viral gene transcription, confirmed that IE175 and IE110 interact (Yao & Schaffer, 1994) (Panel III, lane 1), and served as a positive control for an IE175 co-immunoprecipitation.

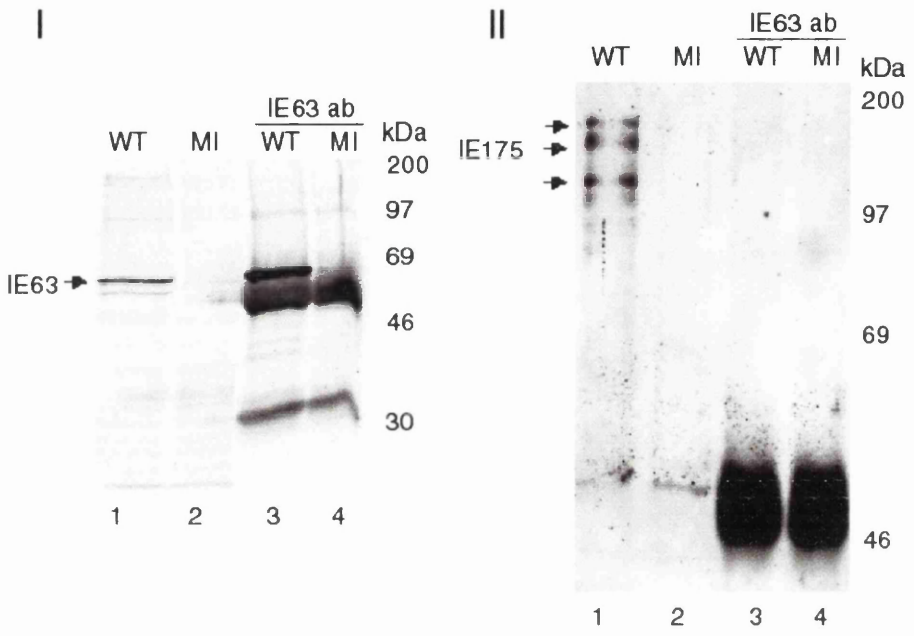


Fig 5C2.1a Using standard anti-63 co-immunoprecipitation conditions IE63 and IE175 do not interact

H1113 monoclonal antibody (Mab) was used in co-immunoprecipitation experiments under conditions in section 2B3.1, the immuno-complex precipitated was separated on 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted for IE63 or IE175

Panel I. Western blot for IE63 using H1113 Mab. Wildtype (WT) and mock infected (MI) BHK cell extracts used (lanes 1 and 2), proteins immunoprecipitated from WT extract (lane 3) and MI extract (lane 4) with IE63 Mab.

Panel II. Western blot for IE175 using a mix of 58S, H640, and 10176 Mabs. WT (lane 1) and MI cell extracts used (lane 2), proteins immunoprecipitated from WT extract (lane 3) and MI extract (lane 4) with IE63 Mab.

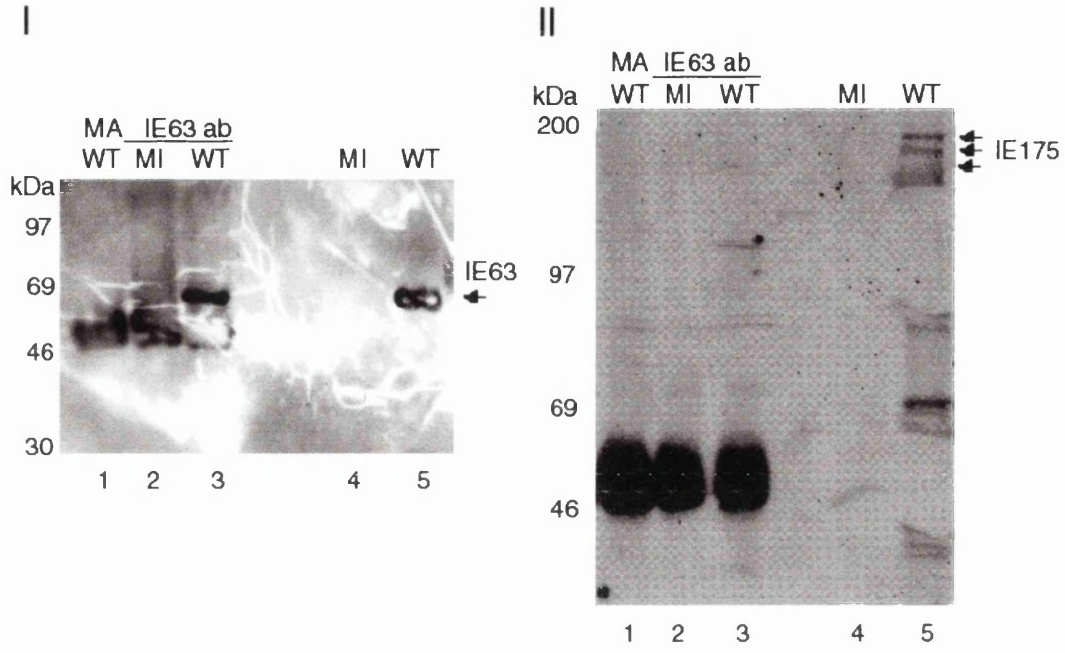
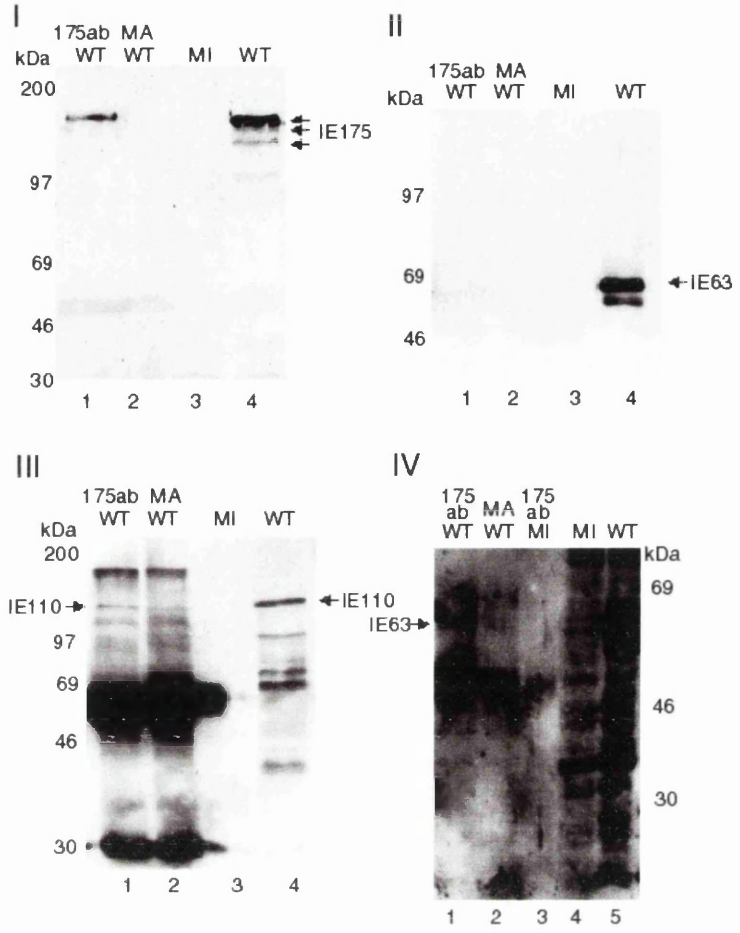


Fig 5C2.1b Using method of Panagiotidis for anti-63 co-immunoprecipitation IE63 and IE175 do not interact

IE63 H1113 monoclonal antibody (Mab) was used in co-immunoprecipitation experiments under conditions described by Panagiotidis (1997), the immuno-complex precipitated was separated on 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted for IE63 or IE175.

Panel I. Western blot for IE63 using H1113 Mab. Wildtype (WT) (lane 5) and mock infected (MI) BHK nuclear extracts used (lane 4), proteins immunoprecipitated from WT extract (lane 3) and MI extract (lane 2) with IE63 Mab, proteins immunoprecipitated from WT cell extract with mouse ascites fluid (MA) (lane 1).

Panel II. Western blot for IE175 using a mix of 58S, H640, and 10176 Mabs. WT (lane 5) and MI nuclear extracts used (lane 4), proteins immunoprecipitated from WT extract (lane 3) and MI extract (lane 2) with IE63 Mab, proteins immunoprecipitated from WT cell extract with MA (lane 1).



<u>Fig 5C2.1c</u> Using method of Panagiotidis for anti-175 immunoprecipitation IE63 and IE175 do not interact

58S monoclonal antibody (Mab) was used in co-immunoprecipitation experiments under conditions described by Panagiotidis (1997), the immuno-complex precipitated was separated on 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted for IE63, IE175, or IE110.

Panel I. Western blot for IE175 using a mix of 58S, H640, and 10176 Mabs. Wildtype (WT) (lane 4) and mock infected (MI) BHK nuclear extracts used (lane 3), proteins immunoprecipitated from WT extract with IE175 Mab (lane 1) and proteins immunoprecipitated from WT cell extract with mouse ascites fluid (MA) (lane 2).

Panel II. Western blot for IE63 using H1113 Mab. WT (lane 4) and MI nuclear extracts used (lane 3), proteins immunoprecipitated from WT extract with IE175 Mab (lane 1) and proteins immunoprecipitated from WT cell extract by MA (lane 2).

Panel III. Western blot for IE110 using 11060 Mab. WT (lane 4) and MI nuclear extracts used (lane 3), proteins immunoprecipitated from WT extract with IE175 Mab (lane 1) and proteins immunoprecipitated from WT cell extract with MA (lane 2).

Panel IV. Over exposed Western blot for IE63 using H1113 Mab. WT (lane 5) and MI nuclear extracts used (lane 4), proteins immunoprecipitated from WT extract with IE175 Mab (lane 1), proteins immunoprecipitated from WT cell extract by MA (lane 2) and proteins immunoprecipitated from MI extract with IE175 Mab (lane 3).

Severe over exposure of IE63 Western blots following IE175 co-immunoprecipitations on occasions showed a faint IE63 band when extracts of wt infected cells were incubated with IE175 Mab or with mouse ascites fluid (Panel IV lanes 1 and 2).

5C2.2 Examination for an interaction between IE63 and IE175 using GST-IE63 pull down assay

GST-IE63 and GST alone were incubated with HSV-1 wt infected extract for 1h at 4°C, Glutathione beads were washed, and interacting proteins separated by SDS-PAGE electrophoresis. IE175 was detected by Western blotting using IE175 Mab (10176) in the wt extract used (Fig 5C2.2 lane 2) but was not pulled down by GST-IE63 (lane 3).

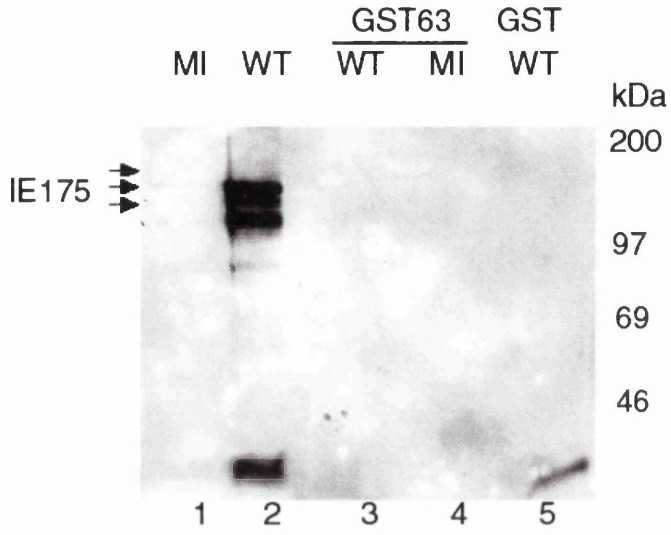


Fig 5C2.2 IE63 and IE175 do not interact in a GST fusion protein pull down assay

Western blot for IE175 using a mix of 58S, H640, and 10176 monoclonal antibodies. GST-63 and GST fusion proteins were bound onto Glutathione beads incubated with ~ 100μg BHK cell extract and washed, pulled down proteins were separated on a 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted for IE175. Wildtype (WT) and mock infected (MI) extracts used (lanes 1 and 2), Proteins pulled down by GST-63 mixed with WT extract (lane 3) and MI extract (lane 4) and GST incubated with WT extract (lane 5).

Chapter 6: Discussion

IE63 is a key regulatory protein in HSV-1 lytic infection (see Section 1B8-13) which has been demonstrated to inhibit host cell mRNA splicing (Hardwicke & Sandri-Goldin, 1994, Hardy & Sandri-Goldin, 1994), redistribute splicing factors (Martin *et al.*, 1987, Phelan *et al.*, 1993, Sandri-Goldin *et al.*, 1995), increase polyadenylation efficiency at viral poly (A) sites (McLauchlan *et al.*, 1989; McGregor *et al.*, 1996), transport viral transcripts to the cytoplasm and prevent transport of cellular transcripts (Hibbard & Sandri-Goldin, 1995, Phelan *et al.*, 1996). In addition, IE63 may regulate transcription directly (Rice & Knipe, 1988; Jang & Latchman, 1992; Pangiotidis *et al.*, 1997) and is necessary for viral replication complex formation (Curtin & Knipe, 1993). However, no convincing evidence has been presented to explain the mechanism by which IE63 participates in such a wide variety of processes. Work presented in this thesis reveals several protein:protein interactions between IE63 and host cell and viral proteins, indicating that IE63 is a multifunctional protein capable of participating in different processes through interaction with a number of partner proteins.
A separate discussion of certain experimental techniques used can be found in Appendix 1.

6A1 The interaction of IE63 with hnRNP K and CK2

6A1.1 IE63 interacts with hnRNP K and CK2

Chapter 4 presents data which confirms the interaction between IE63 and hnRNP K (Fig. 4A1.1-2), and between IE63 and CK2 (Fig.4B1). All 3 proteins were shown to co-immunoprecipitate in a complex held together by IE63 and which contains other unknown proteins; no other viral proteins were required for hnRNP K, CK2 and IE63 to associate (Fig. 4B1). A 70 kDa band and the 44 kDa and 25 kDa bands seen in the original co-immunoprecipitation (Fig. 3A1) and in the GST-IE63 pull down (Fig. 3B2) presumably correspond to hnRNP K and to CK2 α and β subunits. The interaction with hnRNP K is specific and was not found with other hnRNP proteins (Fig. 4A1.3).

6A1.2 CK2 phosphorylates IE63 and hnRNP K co-immunoprecipitated by IE63 Mab.

CK2 was seen to phosphorylate, and not to nucleotidylate (Fig. 4D2), IE63 and hnRNP K (Fig. 4C1 and 4D1) and phosphorylation may be important for protein:protein interaction in the IE63 complex (see below).

DRB always inhibited phosphorylation of IE63 and hnRNP K pulled down by IE63 Mab but the extent of this varied a little (compare Fig. 4C1 to Fig. 4D1), perhaps due to DRB instability between assays. Treatment with DRB never completely inhibited phosphorylation of IE63, implying that other kinases can phosphorylate IE63. Confirmation of phosphorylation by other kinases comes from Zhi and Sandri-Goldin (1999), and potential phosphorylation sites for several different kinases on IE63 can be seen in Fig. 1B12b.

Other co-immunoprecipitating proteins were phosphorylated by CK2 (Fig.4D1), but without positive identification of these bands this knowledge is of limited use.

6A1.3 Different forms of hnRNP K were seen co-immunoprecipitated with anti-IE63 and anti hnRNP K sera

The form of hnRNP K precipitated by hnRNP K antiserum was slower migrating (Fig.4A2) and not phosphorylated *in vitro* by CK2 (Fig.4C1) compared to the hnRNP K form co-immunoprecipitated by IE63 Mab. May be if IE63 predominantly interacts with a CK2 phosphorylated form of hnRNP K, and the hnRNP K antiserum used predominantly immunoprecipitates its non-phosphorylated form (due to specific recognition of the non phosphorylated form or this form being the major form represented in the cell), then as both forms can interact with IE63, the difference in migration seen here would be observed.

The difference in hnRNP K form precipitated may not only reflect phosphorylation, 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).While the antibody used in this study was directed against a peptide present in all four

isoforms, it is possible that the discrimination seen was between different isoforms of hnRNP K which were differentially phosphorylated by CK2.

6A1.4 Phosphorylation of IE63 is important for the interaction between IE63 and hnRNP K

Phosphorylation of hnRNP K by CK2 was not required for hnRNP K:IE63 interaction. Both CK2 phosphorylated and CK2 unphosphorylated forms of hnRNP K were co-immunoprecipitated with IE63 Mab (Figs. 4C1), and dephosphorylated hnRNP K was co-immunoprecipitated by IE63 Mab from CIP treated extracts (Fig. 4C2). However as treatment of extracts with phosphatase inhibitors increased the amount of hnRNP K co-immunoprecipitating with phosphorylated IE63, and treatment with CIP and DRB decreased the amount of hnRNP K co-immunoprecipitating with dephosphorylated IE63, the phosphorylation of IE63, may play a role in IE63:hnRNP K interaction. As dephosphorylation was not complete it is difficult to determine between phosphorylation increasing the affinity of interaction and an absolute requirement with enough residual phosphorylated form to pull down some hnRNP K.

When extracts were treated with PI and subject to IE63 immunoprecipitation an even more slowly migrating form of hnRNP K was seen (Fig. 4C2 *). Which may correspond to the form precipitated by hnRNP K antiserum (Fig. 4A2).

Phosphorylation of hnRNP K by other kinases is RNA or DNA dependent and interleukin 1 responsive. Oxidative stress increases Tyr phosphorylation of hnRNP K and this increase has been shown to increase hnRNP K association with some of its molecular partners - Lck, Vav and PKCδ (Schullery *et al.*, 1999) and Bomsztyk (Personal Communication). As hnRNP K is not normally phosphorylated by CK2 (Van Seuningen *et al.*, 1995a), the relevance of its phosphorylation in the presence of IE63 is of great interest. In the same way as oxidative stress increases phosphorylation on hnRNP K altering its molecular partners, during HSV-1 infection CK2 phosphorylation may affect hnRNP K interactions with other proteins. So far with respect to CK2 phosphorylation only

the interaction between hnRNP K and IE63 has been examined (Fig.4C2) but it is consistent with this model.

6A1.5 The CK2 subunit which interacts with IE63

Examination of the CK2 subunit which interacts with IE63 yielded contradictory data (Fig. 4B3). Use of the peptide assay suggested that the interaction of CK2 with IE63 was via the β subunit while Western blotting pointed to an interaction with the CK2 α subunit. The peptide substrate binds to the β subunit of CK2 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 CK2β is also present and this assay cannot determine if IE63 interacts with α or β, as even if IE63 interacts with purified α subunit, no CK2 activity will be detected as the peptide cannot be bound and phosphorylated. On balance, interaction with CK2 is considered to be via the α subunit. However as purified β subunit was not available (it is very unstable without the α subunit) it is not possible to rule out that IE63 can interact directly with both subunits. The presence of a 44 kDa and a 25 kDa band and of CK2 activity (as detected by the peptide assay) in the original co-immunoprecipitation (Fig. 3A2) suggests that which ever subunit directly interacts, both are present in the IE63 complex.

6A2 Relevance of the interaction of IE63 with hnRNP K

6A2.1 IE63 and hnRNP K are similar

IE63 and hnRNP K have structural and functional similarities. Both have acidic N-termini required for function, possesses methylated RGG boxes, contain repeated KH domains and have C-terminal regions which facilitate protein:protein interactions (Sections 1B12 and 1C1) . Functionally, both affect transcriptional and post-transcriptional processes, are capable of self interaction, shuttle from the nucleus to the cytoplasm and are present in multi-protein complexes (Sections 1B9-1B10 and 1C1).

These similarities suggest that IE63 and hnRNP K may access common cellular pathways. IE63 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 IE63 may be due to its interaction with hnRNP K which could play a key role in HSV-1 infection.

It has been suggested that hnRNP K can act as a molecular docking platform (see Section 1C1.1) allowing cross talk between transcription, mRNA processing and signalling. Thus, hnRNP K interaction with IE63 in infected cells suggests exciting possibilities for an IE63 regulation of these processes.

6A2.2 Transcriptional effects

IE63:hnRNP K interaction may account for the ability of IE63 to activate and repress gene expression (Panagiotidis *et al.*, 1997, Rice & Knipe, 1988). hnRNP K interacts with TBP and can activate CT-mediated transcription of c-*myc* (Michelotti *et al.*, 1996); an interaction with IE63 could redirect hnRNP K to transactivate viral genes. In support of this, 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).

IE63 binding to hnRNP K could also relieve the hnRNP K 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 (Tsieh *et al.*, 1998).

6A2.3 Shuttling effects and RNA export

IE63 may shuttle by more than one route (Section 1B1.4), while the N-terminal NES permits access to one of these routes (Sandri-Goldin, 1998), other routes are not so clear. Shuttling could be via a second C-terminal NES (Fig. 1B12b), through a novel NES or via interaction with a cellular shuttling protein. As hnRNP K can shuttle from the nucleus to the cytoplasm, interaction with IE63

may allow IE63 to piggy back on hnRNP K thereby exporting viral transcripts. Alternatively, the interaction may inhibit hnRNP K shuttling allowing IE63 to access the hnRNP K shuttling pathway. The presence of the highly conserved SADET sequence (fig. 1B12b), a sequence with high homology to part of the KNS domain of hnRNP K (Fig. 6A2.3), in the IE63 homologues of all alphaherpesviruses (Appendix 2) suggests that IE63 could hijack the hnRNP K specific export/import pathway.

Both IE63 (Ingram *et al.*, 1996) and hnRNP K (Matunis *et al.*, 1992) bind RNA directly. It is not known if hnRNP K binds to viral mRNA although HSV-1 RNA is rich in G+C (~70%) and hnRNP K has a binding preference for poly (rC) RNA.

It has been suggested that the KH domains of IE63 specifically bind late viral transcripts and facilitate their export (Soliman, T., personal communication), maybe IE63 binding to hnRNP K provides a way to selectively export these RNAs.

Fig. 6A2.3 Sequence alignment of hnRNP K and IE63

```
hnRNP K   323 YDRRGRPGDRYDGMVGFSADETWDSAIDTWSPSEWOMAY 361
IE63      338 AMRDCVLRQENFIEALASADETLAWCKMCIHHNLPLRPQ 376
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Sequence alignment showing homology between the minimally defined KNS region of hnRNP K and a region in IE63. Residues shared between proteins are shaded and residues conserved throughout the *herpesviridae* IE63 homolgues are shown in bold (see Appendix 2).

6A2.4 Translational effects

As HSV-1 infection proceeds, synthesis of viral proteins is selectively turned off, as is that of most host proteins. IE mRNAs persist in the cytoplasm late in infection when IE protein synthesis is almost completely inhibited, so translational control of viral gene expression has been suggested (Honess & Roizman, 1974; Weinheimer & McKnight, 1987; Elshiekh *et al.*, 1991; Laurent *et al.*, 1998). This level of control would occur as part of the second stage of host shut off separate from the effect by VHS protein (see Section 1A6.2). Non-

translated mature viral mRNAs have been found to be sequestered in complexes containing the 40S ribosomal subunit (Laurent *et al.*, 1998), thus initiation of translation appears to be blocked after the binding of 40S ribosomal subunits but before association with the 60S subunit; 80S ribosome assembly is therefore blocked. During HSV infection, the ribosomal subunits and some other host cell proteins involved in translation, including elongation factor 1a (ef1a), are more highly phosphorylated than in uninfected cells (Chou *et al.*, 1995, Masse *et al.*, 1990a, Masse *et al.*, 1990b).

In several systems, hnRNP K can be seen to inhibit translation of mature mRNAs by blocking 80S ribosome assembly (Collier *et al.*, 1998, Ostareck *et al.*, 1997), and hnRNP K has been shown to interact with ef1a. Interaction of hnRNP K with IE63 and CK2 may therefore play a role in HSV-1 driven inhibition of translation.

6B1 The interaction of IE63 with p32 (also involving CK2 and hnRNP K)

6B1.1 IE63 interacts with p32, CK2 and hnRNP K

IE63 was seen to interact with p32 (Fig.5A1.1) and hnRNP K and CK2 were also present in the complex. Further, the use of extracts transfected with IE63 showed that no other viral proteins were involved in the interaction. The original anti IE63 co-immunoprecipitation and GST-IE63 pull down profiles (Figs.3A2 and 3B2) showed 2 bands of approximately 33 kDa -35 kDa and Western blotting showed that p32 was co-immunoprecipitated with IE63 (Fig. 5A1.2). It seems reasonable to assume that one of the 33 kDa -35 kDa bands corresponds to p32, the identity of the other band is discussed below.

There were proteins which interacted with p32 in uninfected cells which did not interact in infected cells. This could reflect their down-regulation following infection or, more interestingly, they could be displaced by IE63 to moderate p32 activity. Without knowledge of their identity further conclusions are difficult to reach. The most prominent band is ~50kDa, none of the cellular proteins known to interact with p32 are this size.

6B1.2 p32 is phosphorylated and redistributed in the presence of IE63

IE63 expression caused a redistribution of p32 (Fig.5A2), and p32 (or a protein of similar size) was phosphorylated by co-immunoprecipitating CK2 (Fig.4D1). As p32 and CK2 were only associated with each other in infected cells, p32 would not be expected to be phosphorylated by CK2 in uninfected cells, although this was not directly examined. Inhibition of phosphorylation by DRB of the ~33 kDa band was almost complete so phosphorylation by other kinases either does not occur or occurs to a very small extent.

A doublet of bands was seen at 33 kDa -35 kDa (Fig. 3A2), one of these presumably is p32, one band of the doublet was phosphorylated (Fig. 4D1), (whether these are the same protein band has not been resolved). The known association of p32 and ASF/SF2 suggests that the second band which co-immunoprecipitated with IE63 at 33 kDa -35 kDa was ASF/SF2, however Western blotting for ASF/SF2 was unable to confirm or reject this possibility. ASF/SF2 has 3 potential CK2 phosphorylation sites (Fig. 6B1.2).

Fig. 6B1.2 Potential CK2 phosphorylation sites on ASF/SF2

MSGGGVIRGPAGNNDCRIYVGNLPPDIRTKDIEDVFYKYGAIRDIDLKNRR
GGPPFAFVEFEDPRDAEDAVYGRDGYDYDGYRLRVEFPRSGRGTGRGGG
GGGGGGAPRGRYGPPSRRSENRVVVSGLPPSGSWQDLKDHMREAGDVC
YADVYRDGTGVVEFVRKEDMTYAVRKLDNTKFRSHEGETAYIRVKVDG
PRSPSYGRSRSRSRSRSRSRSRSNSRSRSYSPRRSRGSPRYSPRHSRSRSRT

Sites were considered as potential phosphorylation sites if a S/T residue was N terminal to a D or E residue (<4 aa away). Residues which may be phosphorylated are boxed. Consensus sites shaded.

6B1.3 Is p32 a sticky protein ?

Previous studies using the yeast 2 hybrid system have shown that p32 interacts with several proteins and, coupled its high charge, it is considered to be a "sticky" protein which is pulled out non-specifically in assays. However (i) the demonstration of an interaction in different assay systems, the use of a similarly highly charged "sticky" protein (glucose oxidase) as a control in the pull down assay; (ii) the obvious redistribution of p32 in infected cells and (iii) the recent demonstration that p32 can regulate splicing (Peterson-Mahrt *et al.*, 1999), a function which IE63 clearly disrupts (Sandri-Goldin & Mendoza, 1992; Hardwicke & Sandri-Goldin, 1994; Hardy & Sandri-Goldin, 1994; Sandri-Goldin *et al.*, 1995), all point to this interaction being a specific and functionally important one.

6B2 Relevance of the interaction of IE63 with p32

6B2.1 Splicing effects

The biological functions of p32 have proved to be controversial. Originally identified as a component of the ASF/SF2 splicing factor (Krainer *et al.*, 1990b), subsequent work shows that the p33 subunit alone contains all the functional properties of a splicing factor (Mayeda *et al.*, 1992), however p32 inhibits ASF/SF2 from acting as a splicing repressor or splicing enhancer protein (Peterson-Mahrt *et al.*, 1999). The IE63/p32 interaction may therefore be the mechanism by which IE63 inhibits splicing.

The cellular location of p32 has caused confusion in the debate on p32 function; p32 has been reported to be predominantly (exclusively) mitochondrial (Muta *et al.*, 1997; Matthews & Russell, 1998), cytoplasmic (Luo *et al.*, 1994; Simos & Georgatos, 1994; Tange *et al.*, 1996; Wang *et al.*, 1997) and nuclear (Luo *et al.*, 1994; Simos & Georgatos, 1994; Tange *et al.*, 1996; Wang, 1997; Matthews, 1998). Using immunofluoresence with a standard fixation method, p32 was seen staining with a predominantly cytoplasmic distribution (Fig. 5A2). However with

HSV-1 infection and when IE63 was expressed alone, this distribution was dramatically altered and p32 was seen in the nucleus.

Although IE63 causes a redistribution of p32, there was little co-localisation of p32 and IE63 unlike previous observations for IE63 and other splicing proteins (Phelan *et al.*, 1993). Once transported to the nucleus and having disrupted splicing, p32 may perform another function.

Generally, p32 may have a predominantly cytoplasmic/mitochondrial distribution with a small amount being present in the nucleus, and this distribution may permit ASF/SF2 to function in splicing. However under specific conditions, for instance viral infection, p32 could redistribute to the nucleus and disrupt splicing activity by interacting with ASF/SF2. A similar redistribution from cytoplasm to the nucleus is observed in adenovirus infected cells, where p32 has been suggested to play a role in adenovirus splicing regulation (Matthews & Russell, 1998).

Redistribution under particular circumstances could account for the differences in p32 activity found between *in vitro* and *in vivo* studies. *In vitro* results provide evidence that p32 has the capacity to regulate splicing (Peterson-Mahrt *et al.*, 1999), but an *in vivo* example of splicing regulation is proving more difficult to find, indeed *in vivo* experiments in yeast show p32 to be important in maintaining oxidative phosphorylation (Muta *et al.*, 1997).

Disruption of splicing via p32 following HSV-1 infection would be consistent with the HIV-1 Rev:p32 interaction, in which exogenously added p32 relieves the inhibition of splicing *in vitro* exerted by the basic domain of Rev. Rev bound to RRE and interacting with p32 associated with ASF/SF2 at the splice site, would stabilise the interaction of U1 snRNP with the 5' splice site so inhibiting assembly of functional spliceosomes (Fig.1B14.1, Tange *et al.*, 1996). IE63 could act similarly in bound to HSV-1 pre-mRNA, by binding p32 altering its phosphorylation and that of ASF/SF2, so stabilising the pre-spliceosome complex.

Another HSV protein identified as interacting with p32 is the product of ORF P (Bruni & Roizman, 1996). ORF P protein co-localises with the splicing factor

SC35 (Bruni & Roizman, 1996) consistent with p32 acting as a splicing factor, however the concurrent presence of p32 was not examined. Expression of ORF P is required for the decreased accumulation of ICP0 and ICP22, two of the four HSV genes to yield spliced mRNAs (Randall & Roizman, 1997). Although not directly related to IE63 function this adds weight to the role of p32 as a splicing regulatory factor in HSV-1 infected cells.

6B2.2 Shuttling effects and RNA export

It has been suggested that IE63 utilises the same pathway for export as HIV-1 Rev (Mears & Rice, 1998, Sandri-Goldin, 1998). ASF/SF2 is one of a subset of SR proteins which shuttle from the nucleus to the cytoplasm; phosphorylation of these proteins is important for shuttling (Caceres *et al.*, 1998) and the Rev-ASF/SF2-p32 interaction has been implicated in HIV-1 mRNA transport (Gilmartin *et al.*, 1992; Yu *et al.*, 1995a; Tange *et al.*, 1996). Similarly, IE63 could interact with ASF/SF2 and p32 to promote export of viral mRNAs into the cytoplasm, either by interacting with the spliceosome, stabilising and allowing unspliced mRNAs to be exported, or by the complex directly interacting with the export machinery.

6B2.3 mRNA movement to the NPC

A cellular partner of p32, the lamin B receptor (LBR) (Nikolakaki *et al.*, 1996), is part of a subassembly of nuclear envelope proteins. During HSV-1 infection the snRNPs and the non-snRNP protein SC35 redistribute within the nucleus (Fig.1B9.1) (Martin *et al.*, 1987; Sandri-Goldin *et al.*, 1995) and migrate to the nuclear periphery (Phelan *et al.*, 1993; Sandri-Goldin *et al.*, 1995). IE63 is necessary and sufficient for this effect and co-localises with the redistributed snRNPs at later times post infection (Phelan *et al.*, 1993; Sandri-Goldin *et al.*, 1995). Copies of the motif (RS) through which most splicing factors interact (Wu & Maniatis, 1993) are present in the LBR, and the interaction of p32 with the LBR complex has lead to the suggestion that LBR acts as a transient docking site for nuclear speckles in the nuclear envelope before mRNA export takes place (Nikolakaki *et al.*, 1996). The p32/IE63 interaction may go some way to explain

the movement of the redistributed snRNPs to the nuclear periphery during the course of infection. Whether movement of speckles, docking at nuclear membrane and transport through the pore are linked has not been investigated. Maybe p32 plays a role in linking splicing regulation to export of viral mRNA.

6B2.4 Transcription effects

p32 has been identified as a transcriptional activator (Yu *et al.*, 1995b) and implicated in transcriptional regulation of viral gene expression both in HIV-1 (Yu *et al.*, 1995a) and in EBV infection (Wang *et al.*, 1997). The potential role for p32 with IE63 in HSV-1 and host gene transcriptional regulation should not be ignored.

6B2.5 Relevance of interaction of p32 with IE63 and CK2

In uninfected cells, p32 inhibits splicing in two ways, by inhibiting ASF/SF2 binding to RNA and hence its function in the initiation of pre-spliceosome formation and by blocking ASF/SF2 phosphorylation (Peterson-Mahrt *et al.*, 1999). Not only does p32 affect ASF/SF2 phosphorylation but it can itself be phosphorylated: p32 protein contains five consensus sites for CK2 phosphorylation which makes phosphorylation of p32 by CK2 a possibility (Section 6B1.2).

Cascades of phosphorylation and dephosphorylation have been suggested to direct the sequential binding and release of pre-mRNA binding proteins during the splicing reaction, and many proteins involved in splicing are known to be phosphorylated (Section 1B7, Cao *et al.*, 1997; Xiao & Manley, 1997; Kanopka *et al.*, 1998). The reorganisation of SR proteins from interchromatin granules to active splice sites has been proposed to be regulated by cycles of phosphorylation and dephosphorylation (Misteli & Spector, 1997).

Petersen-Mahrt *et al.*, 1999, report that p32 not only interacts with ASF/SF2 but also with other SR proteins and in one case this disrupts SR protein function. IE63 alters the phosphorylation of a splicing factor found in the pre-spliceosome

complex (Fig. 1B2.3), the 70 kDa component of U1 snRNP, which was more highly phosphorylated during wt infection than during 27 Lac-Z infection (Sandri-Goldin & Hibbard, 1996). Although opposite to ASF/SF2 where inhibition of phosphorylation disrupts function, this points to changes in phosphorylation of splicing factors brought about by IE63/p32 as important to the shut off of host pre-mRNA processing. It was not possible to detect a direct interaction between IE63 and U1 70 kDa (data not shown), however the interaction of p32 and IE63 could bring CK2 into contact with U1 70 kDa to alter its phosphorylation and thereby inhibit splicing.

Inhibition of splicing activity may be sufficient to cause redistribution of splicing factors as seen in HSV-1 infection, an effect shown to be due to IE63 (Phelan *et al.*, 1993). Here there may also be role for IE63/p32 induced changes in phosphorylation (direct or via a cascade).

The identity of the other band at 33 kDa -35 kDa (Fig.3A2) and whether the phosphorylated band in Fig. 4D1 is p32 or another protein needs to be resolved, but this does not alter the idea that IE63 associates simultaneously with splicing factors and a kinase creating a potential for activity of the splicing factors to be altered via phosphorylation.

6C1 The interaction of IE63 with SAP145 (also involving hnRNP K and CK2)

6C1.1 IE63 interacts with SAP145, hnRNP K and CK2, but this excludes interaction with p32

The interaction between IE63 and SAP145 was determined by both co-immunoprecipitation (Fig. 5B1.2) and GST pull down assays (Fig. 5B1.1), adding weight to its significance. However as Western blotting of the anti IE63 co-immunoprecipitate for SAP145 proved to be negative, the prominent band at the top of the original [^{35}S] profile (Fig. 3A2) is unlikely to be SAP145; the negative blot could reflect weak interaction between the two proteins or the low sensitivity of the SAP145 antibody.

hnRNP K and CK2 were also found in the complex (Figs. 5B2 and 5B3), but were not associated with SAP145 in the absence of IE63. Interestingly, the interactions of SAP145 with IE63, and of p32 with IE63 appear to be mutually exclusive (Fig. 5B4). This may explain the negative SAP145 Western blot of the anti-IE63 co-immunoprecipitate as if only a small sub population of IE63 was interacting with SAP145, or the conditions chosen for immunoprecipitation favoured the p32 interaction, combined with a weak antibody, SAP145 may not be detected. SAP145 and p32 don't normally interact in uninfected cells so interaction with IE63 does not disrupt their normal function.

6C1.2 SAP145 and IE63 co-localise in infected cells and SAP145 is not phosphorylated by CK2

Further to their interaction, SAP145 and IE63 co-localise (Fig. 5B5). SAP145 and SC35 also co-localise, consistent with co-localisation of SC35 and IE63. Fig. 4D1 does not show a phosphorylated band of this molecular weight, so it is unlikely that SAP145 is phosphorylated in infected cells. Phosphorylation in uninfected cells has not been examined.

6C2 Relevance of the interaction of IE63 with SAP145

6C2.1 Splicing effects

The defined role for SAP145 in splicing (Section 1C3, Champion-Arnaud & Reed, 1996) aids speculation about the function of its interaction with IE63. SAP145 is involved in U2 snRNP binding to the BPS and commitment to splicing; presumably IE63 interaction with SAP145 regulates this activity to block cellular pre-mRNA splicing. IE63 may block splicing by preventing the binding of SAP145 to the spliceosome complex, or by binding to SAP145 in the assembled splicing complex. Interaction with other splicing factors has been reported (Sandri-Goldin, 1998), although this work lacked controls and the immunoprecipitated profile contained a large number of non-specifically interacting proteins. Which interactions are direct with IE63 and which are as a result of splicing factors interacting with each other has not been determined.

The co-localisation of IE63 with splicing factors e.g. SC35 has been known for some time, as SAP145 is found associated with many splicing factors this interaction between IE63 and SAP145 which follows the pattern of other reported co-localisations (Phelan *et al.*, 1993) may well explain the previous findings.

Interaction of SAP145 with IE63 may primarily cause redistribution of splicing factors or may inhibit splicing and as a consequence splicing factors are redistributed.

6C3 Relevance of the lack of interaction of IE63 with SAP145 and p32

6C3.1 Splicing effects or splicing and RNA export effects

Fig. 1C3 shows the relative positions of SAP145 and ASF/SF2 at the 3' splice site; during splicing it has been proposed that p32 can modulate ASF/SF2 activity (Peterson-Mahrt *et al.*, 1999), this places p32 and SAP145 in close proximity and both interact with IE63 although these interactions appear to be mutually exclusive. Perhaps they occur at different times post-infection, redistribution of p32 occurs from early in infection whereas the co-localisation of IE63 and SAP145 is more apparent later. Or maybe the interactions involve separate sub-populations of IE63 and either both sub-populations block splicing at separate points or one blocks splicing while the other has a different role, for instance affecting export of RNA. IE63 mediated redistribution of splicing factors and inhibition of splicing can be separated from each other (Sandri-Goldin *et al.*, 1995) perhaps these two activities reflect SAP145 and p32 acting separately.

6D1 Interaction of IE63 with Viral Thymidine kinase(vTK)

6D1.1 IE63 and vTK were both present in a complex of proteins

The presence of vTK in the complex of interacting proteins was determined (Fig 5C2.2), however no attempt was made to determine if vTK was interacting directly with IE63 or indirectly via the other proteins which interact with IE63. This investigation was hindered by an inability to express vTK from virus in the absence of IE63. As IE63 is essential for vTK expression a viral mutant with this phenotype is not possible.

6D1.2 Relevance of association with vTK

Irrespective of whether the interaction is direct or indirect, vTK was detected in a complex with IE63 and this interaction may affect the functions of IE63. The enzyme vTK is involved in DNA metabolism in infected cells and in reactivation from latency (Section 1C5). IE63 affects the localisation of the single stranded binding protein U_L29, a component of the viral DNA replication complex and promotes replication complex formation (Section 1B13, Curtin & Knipe, 1993), whether this is linked to an association with vTK is not known. Maybe IE63 serves as a site of nucleation for components of the DNA replication complex so promoting replication foci formation and providing a possible route for regulation of DNA replication linked to RNA processing and/or *visa versa.*

6D2 The Interaction of IE63 with IE175

6D2.1 IE63 and IE175 do not co-immunoprecipitate

Pangiotidis *et al.* (1997), reported that IE63 and IE175 interact, however under the immunoprecipitation conditions used here IE63 and IE175 did not interact (Fig. 5C1.1). Neither, in my hands, did they interact under the conditions he used for co-immunoprecipitations with IE63 or IE175 Mabs (Fig. 5C1.2).

Small differences between the experiments which showed an interaction and those presented here were:

(i) The strain of virus used, wt HSV-1 strain KOS1.13G vs. Glasgow strain 17^+.

(ii) In the buffers used to make nuclear extract, the individual protease inhibitors were replaced by a protease inhibitor cocktail from Boehringer Mannheim

(iii) Gamma-Bind Plus Sepharose (Pharmacia) was replaced with protein A Sepharose (Sigma)

(iv) Co-immunoprecipitation used the same Mabs either H1113 for IE63 or 58S for IE175 but Western blotting for IE175 was performed with just 58S and H640 instead of a mixture of 58S, H640, and 10176 Mabs.

Strain differences between 17^+ and KOS have been noted, for example gC from KOS exhibits much less C3b binding than gC from most HSV-1 strains (Friedman *et al.*, 1986), but at the level of IE gene expression or function there are no known differences. However the relative expression levels of IE63 and IE175 may differ, perhaps contributing to a difference in sensitivity of the assay, especially if the IE63-IE175 interaction is weak.

As all extracts were stored at -70°C and kept on ice while work was carried out the difference in protease inhibitors is unlikely to be significant.

Addition of an extra Mab (10176) for Western blotting was included as attempts with 58S and H640 give a weak response even with total infected cell extract. The new mix detected the same forms of IE175 (and did so with a greater sensitivity than 58S + H640 did), so this is unlikely to account for the difference in result.

The role of the Gamma-Bind Plus Sepharose or Protein A Sepharose was to bind to the antibody, which then binds its antigen (Fig. 2B2), to which the co-precipitating protein(s) attaches. As both forms of Sepharose successfully pull out the antigen, either IE63 or IE175 depending on the antibody used, it is unlikely that either reagent would have an effect on the co-precipitating proteins.

Panagiotis *et al.* (1997), included a control with no antibody, in which neither IE63 or IE175 were precipitated so an interaction between Gamma-Bind Plus Sepharose and IE175 or IE63 can be ruled out. However, a control using an ascites fluid to detect whether IE63/IE175 reacts with unrelated mouse antibodies was not included. Here a co-immunoprecipitation with ascites fluid was included and after severe over exposure of the IE63 Western blot (Fig.5C1.3) a weak non-specific interaction between IE63 and the ascites fluid was seen.

The difference therefore may be that a stronger (maybe due to differences in strain affecting the abundance of each protein and/or the strength of their interaction ?) non-specific interaction, between the mouse ascites fluid and IE63 was detected, and that by excluding the ascites control this was seen as a specific result.

Interestingly, here only the slowest migrating form of IE175 (Fig. 5C1.3) was precipitated by the IE175 Mab 58S, while Panagiotis *et al.* (1997), with the same antibody detected at least 3 major forms; these faster migrating forms may interact with IE63. Why only the most slowly migrating form was pulled out could reflect a difference in the abundance of IE175 isoforms between different HSV-1 strains, although there is little evidence for this.

This result shows that with many protein interactions a condition can be found under which the two proteins will interact, however only if this interaction is significantly stronger than the proper controls can it be regarded as "real".

6D2.2 IE63 and IE175 do not interact in a GST pull down assay

IE63 and IE175 did not interact in a GST-IE63 pull down assay, although they did in the Panagiotis study. The difference between the GST-IE63 pull down Western blotted for IE175 reported here (Fig 5C2) and the other study, was that here whole cell extract was used in place of *in vitro* transcribed/translated IE175. Adding artificially high levels of *in vitro* synthesised IE175 may cause it to be over represented and a positive interaction with GST-IE63, as compared to a negative result when whole extract was used, is likely to be non-specific.

Further support for IE63 and IE175 not interacting comes from the absence of any published evidence confirming the interaction.

These data do not detract from the fact that IE63 and IE175 are functionally associated and together may play a role in transcription (Su & Knipe, 1989). The interaction of IE63 with CK2 may account for the change in IE175 migration on SDS-PAGE in the presence of IE63; changes in phosphorylation may alter the transcriptional activity of IE175 (see Section 1A5.2 and 1B10).

6D2.3 IE175 and IE110 do co-immunoprecipitate

Both IE110 and IE175 alone can activate/repress transcription but they have also been reported to act together to regulate gene expression at a transcriptional level (Everett, 1984, O'Hare & Hayward, 1985). When transfected and analysed by immunofluoresence, IE110 localises in punctate structures which correlate with pre-existing cellular structures (ND10 domains), co-transfection of IE110 with IE175 causes a portion of IE175 to co-localise with IE110 in these structures (Mullen *et al.*, 1995).

The IE175:IE110 interaction (Fig. 5C1.3) was included primarily as a positive control. However a literature search revealed only one other such report (Yao & Schaffer, 1994), in which far-Western blotting and GST-IE110 affinity chromatography were used. So the result presented represents the first documented evidence that IE110 and IE175 can be co-immunoprecipitated from HSV-1 infected cells.

6E IE63 as a multifunctional protein.

It is apparent that certain proteins are involved in both transcription and at least one stage of post transcriptional processing e.g. WT1, Y-box proteins, and hnRNP K (Section 1B6.3 and 1C1). These proteins are likely to function in different ways at different sites of processing and in the co-ordination of gene expression. They perform multiple roles by having distinct functional domains, and by interacting with multiple protein partners. From the protein:protein interactions demonstrated here, the distinct domain structure of IE63 and from its multiple activities it is clear that IE63 is another multifunctional protein.

Multiple interactions by IE63 may be facilitated by its ability to oligomerise (Fig. 3C), enabling the formation of large complexes of proteins. Moreover, the particular patterns of protein:protein interaction may be dynamic, changing at different intracellular locations or time points post-infection to facilitate the different functions of IE63. As seen here, the protein interactions may be exclusive of each other and involve different sub-populations of IE63. How protein:protein interactions are selectively regulated in response to location or progression of infection is unclear. Maybe the phosphorylation of IE63 or its partners, and/or the ability of IE63 to bind viral RNA, play a part, as with hnRNP K (Denisenko *et al.*, 1996; Schullery *et al.*, 1999) influencing the selection of partner proteins and conferring responsiveness to the stage of viral gene expression reached. Or IE63 could travel with the viral mRNA as it is directed from one cell location to another, affecting for instance the splicing or export machinery by modifying the appropriate factor. Direction from one event to the other may also be driven by IE63, if for instance it binds to a component of the nuclear matrix, or if one of its partners, most likely hnRNP K, determines movement.

6F Future work

These interactions suggest several ways by which IE63 could disrupt and direct RNA processing to increase viral gene expression. Future experiments will include

- Identifying which interaction is responsible for which IE63 activity by analysing the phenotype of IE63 viral mutants which do not interact with one or more of the proteins identified here.
- Inhibition of IE63 CK2 phosphorylation and/or CK2 phosphorylation of interacting proteins with chemical inhibitors or with proteins mutant in CK2 phosphorylation sites, followed by analysis of the effect on the interaction and on the process of infection.
- Determination of the hnRNP K isoform interacting with IE63, and what this form does in uninfected cells and infected cells. A way forward here is the comparison of the migration of a mixture of known hnRNP K variants with the migration of the co-immunoprecipitated form.
- Co-localisation studies of hnRNP K and IE63
- Use of IE63 in transcription assays determine effects on hnRNP K's transcriptional ability (or of p32's), analogous to the derepression of transcriptional activity exerted by the Hep C core protein.
- Investigation of the step at which splicing is halted, would help reveal the role of SAP145 in splicing inhibition, and can be determined by analysis of splicing complexes found *in vitro*.
- Determination of whether IE63 effects p32 interaction with its normal cellular partner protein ASF/SF2, through Western blotting and immunoprecipitation
- Mutation of other potential shuttling sequences in IE63, and use of mutants which don't interact with hnRNP K, analysed in the heterokaryon assay, will help elucidate other shuttling pathways IE63 may use to export viral mRNA.
- CK2 is known to phosphorylate many HSV-1 proteins it may be of interest to determine, as Rev does in HIV-1 infected cells, if interaction with IE63 increases CK2 activity.
- Identification of the other bands seen in the original IE63 co-immunoprecipitation and GST-IE63 pull down, and of the bands seen

interacting with p32 in uninfected cells which no longer do so during infection. May be done using Laser Mass Map Spectroscopy.

- Use of yeast-2-hybrid assay, and transient transfection of vTK to see if this protein interacts directly with IE63 or indirectly via a cellular factor.

Appendix 1

Investigation into and Discussion of Methods Used

A1.1 Co-immunoprecipitations

Details of theory of co-immunoprecipitations and the experimental conditions which can be varied are found in Materials and Methods.

A1.1.1 The use of various anti-IE63 antibodies available.

Unfortunately the other antibodies available namely a rabbit peptide anti-serum against the first 16 aa of IE63 and a rabbit polyclonal antiserum raised against a bacterially expressed recombinant IE63 molecule, although able to detect IE63 in Western blots did not act to immunoprecipitate IE63 from cell extracts at any of the concentrations tried (Fig. A1.1.1).

The heavy chain of the immunoprecipitating antibody and IE63 migrated around the same point on the SDS-PAGE gel, however, visualisation of the heavy chain (due to cross reactivity of immunoprecipitating antibody with secondary antibody or protein A used in Western blotting) provided a convenient control to show that similar amounts of antibody were added to each reaction.

A1.1.2 The interaction of proteins from different cell types with IE63.

In the first Results Section, a profile of BHK infected cell proteins which interact with IE63 was established (Fig. 3A1). To investigate if these interactions were similar in different cell types the experiment was repeated using radiolabelled cell extracts of HSV-1 infected HeLa or Vero cell lines. These were run on SDS-PAGE gels and compared to the profile produced from BHK cell extracts. The pattern of immunoprecipitating bands was broadly the same in all cell types (Fig. A1.1.2, corresponding bands are labelled), however the intensity of the bands did vary from experiment to experiment and in this particular case the exposure of the Hela cell immunoprecipitation had to be greatly enhanced in order to detect any

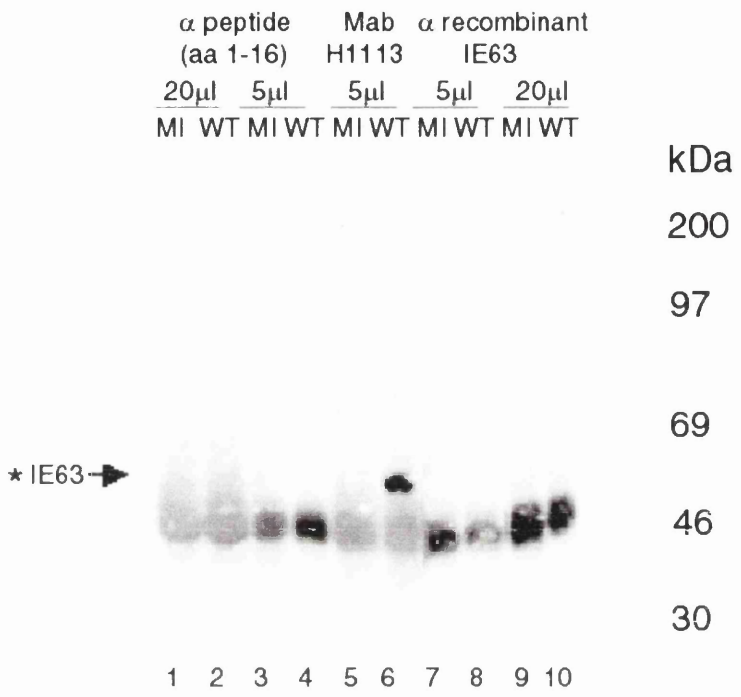
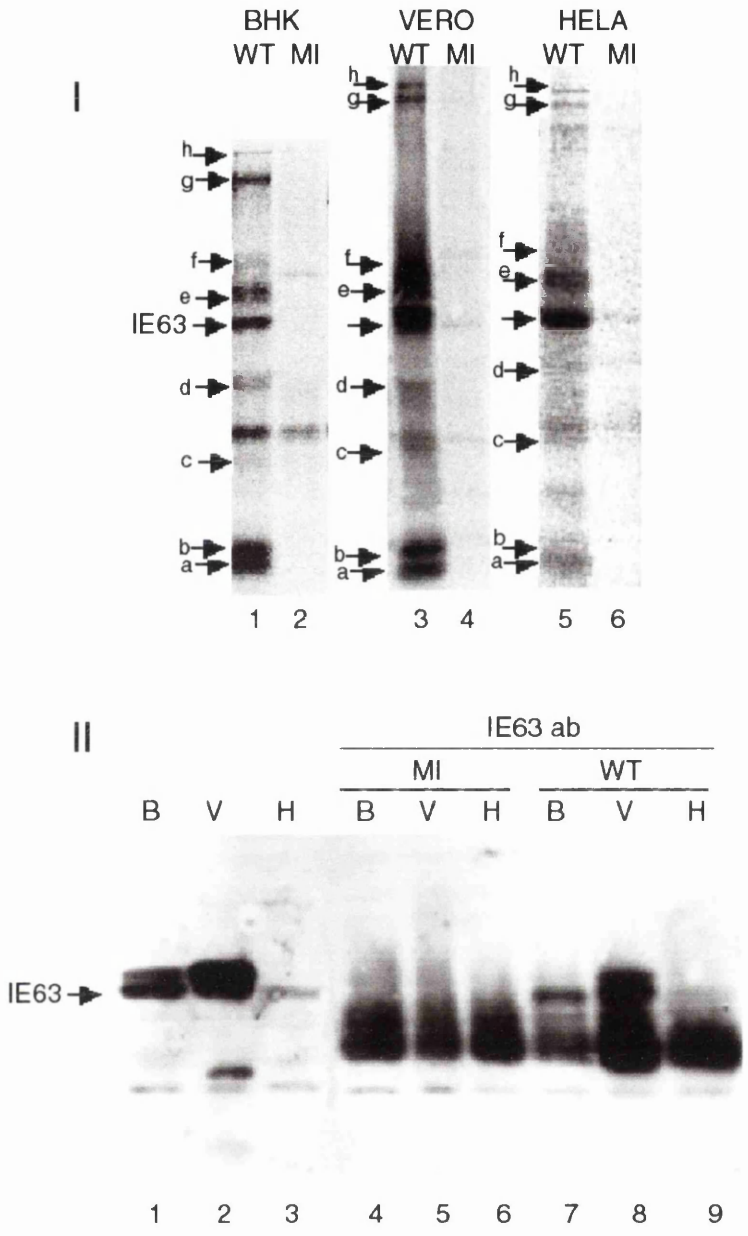


Fig A1.1.1 Only monoclonal antibody H1113 immunoprecipitates IE63

Western blot for IE63, using H1113 Mab. Co-immunoprecipitations from wildtype (WT) and mock infected (MI) BHK extracts using rabbit anti-peptide IE63 serum (peptide - lanes 1-4), IE63 Mab (H1113 - lanes 5 & 6), and the rabbit polyclonal antiserum raised against bacterially expressed IE63 (recombinant - lanes 7-10), were run on a 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted for IE63. As indicated 5μl or 20μl of each anti-sera was used to immunoprecipitate.



<u>Fig A1.1.2</u> The pattern of IE63 interacting proteins does not change in different cell lines

Panel I. [^{35}S]-methionine labelled proteins co-immunoprecipitated from various cell line extracts. [^{35}S]-methionine labelled proteins were co-immunoprecipitated, with IE63 Mab H1113, from wildtype (WT) and mock infected (MI) BHK (B-lanes 1 and 2), Vero (V-lanes 3 and 4) and HeLa (H-lanes 5and 6) cell extracts. Protein complexes formed were separated on a 10% SDS PAGE gel, dried down and exposed to a phosphoimaging plate overnight.

Panel II. Western blot for IE63 using H1113 Mab. Aliquots of the above samples were run on another 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted for IE63 (lanes 4-9). Whole cell extracts are included (lanes 1-3).

interacting bands. In the Vero, extract the bands above IE63 were more blurred than with BHK or HeLa extracts, this is likely to be a reflection of the relative amounts of proteins. The Western blot in Panel II confirms that in this case the infected Hela cells produced less IE63 than Vero or BHK cells, HeLa cells are thought to be less permissive as far as virus yield is concerned (~10 fold) but are routinely used by many labs to study virus proteins. Repetition of the co-immunoprecipitations gave profiles with somewhat different relative intensities, but always the same pattern of bands, further the immunoprecipitated bands within a particular profile showed the same relative intensity compared to each other.

Panel II also shows a band migrating slightly slower than IE63 in BHK and Vero cells (lanes 1 and 2), this looks specific and may be more phosphorylated forms of IE63. These forms were observed on occasion. They are consistent with the previous observation that two forms of IE63 can be separated by SDS-PAGE, but that more often one is seen (Ackerman *et al.*, 1984, Pereira *et al.*, 1977).

BHK cells were chosen for the rest of the study, they are routinely used in Glasgow to examine HSV-1 protein interactions and as they were routinely passaged in our lab, were easy to obtain. The exception was in immunofluorescence where the morphology of HeLa cells makes them more suitable for study.

A1.1.3 The stringency of interaction between IE63 and its co-immunoprecipitating proteins

The initial profile of protein bands which interact with IE63, was determined by immunoprecipitation with a buffer containing 100mM NaCl, a salt concentration close to the physiological salt concentration of ~90mM. The strength of each of these interactions was of interest and so co-immunoprecipitations were repeated substituting the 100mM buffer with washes of increasing salt concentration, the resultant [^{35}S] profile of bands was then inspected (Fig. A1.1.3 panel I bands labelled a to h). Bands representing specific interactions were most apparent at 100mM but at higher salt concentrations certain bands remained obvious. The 32 kDa doublet (a) showed the strongest interaction with IE63, it was still clearly

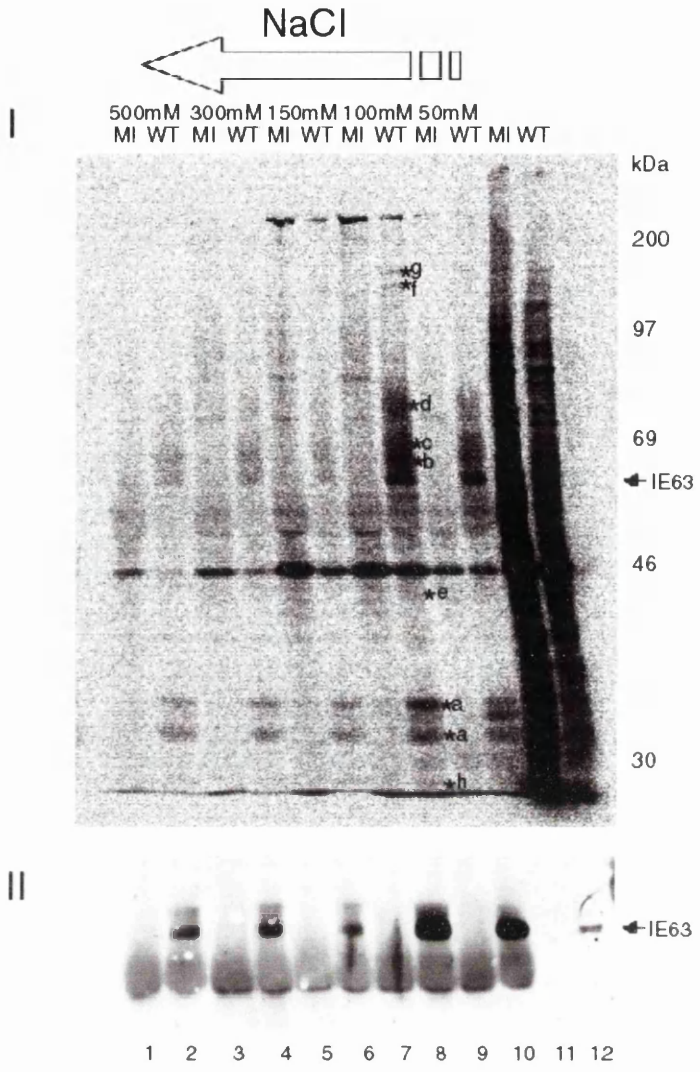


Fig. A1.1.3 Stringency of interactions between IE63 and unknown protein bands

Panel I. [^{35}S]-methionine labelled proteins co-immunoprecipitated from extracts and washed with increasing concentrations of NaCl. The co-immunoprecipitated proteins were separated on a 10% SDS-PAGE gel, dried down and exposed to a phosphoimaging plate overnight. Co-immunoprecipitation experiments were performed on wildtype (WT) and mock infected (MI) extracts using H1113 Mab with final wash conditions changed from 100mM (lanes 7 and 8) to 50mM (lanes 9 and 10), 150mM (lanes 5 and 6), 300mM (lanes 3 and 4) or 500mM (lanes 1 and 2) NaCl. Whole cell extracts used are shown in lanes 11 and 12.
Panel II. Western blot for IE63 using H1113 Mab. Aliquots of the above samples were run on another 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted for IE63 using H1113 Mab.

visible after a 500mM salt wash, the first band above IE63 ~70kDa (b) is also just discernible. The band above this one (c, also ~70kDa) remained associated up to 300mM. The bands of 85kDa (d) and 44kDa (e) were only visible at or below 150mM NaCl and the high molecular weight bands (f and g, 180kDa and 150kDa respectively) and the 25kDa band (h) disappear even at 150mM salt. In this gel, the 50kDa band cannot be distinguished.

The Western blot in Panel II shows the amounts of IE63 precipitated, as the salt concentration increases less IE63 is precipitated so care must be taken in the interpretation of these results. Loading more of the co-immunoprecipitates washed at high salt concentration to equalise the amount of IE63 present, would have solved this discrepancy. Nevertheless there is a clear difference in the affinity of interaction between IE63 and each of the other interacting proteins.

A1.2 GST-IE63 fusion protein expression

Details of theory of GST fusion protein assays and experimental conditions which can be varied are found in Materials and Methods.

A1.2.1 Initial GST-63 fusion protein expression.

Expression of GST-IE63 initially obtained can be seen in Fig.3B1 lanes 1 and 3. This was improved to the levels seen in lanes 5 and 7.

The effects of temperature, IPTG concentration, method of lysis, time of induction, as well as the ratio of beads to lysate were investigated, to ensure that the maximum amount of full length GST-63 was expressed, that all expressed protein was binding to beads and that the beads were saturated. Use of another protease deficient *E.coli* strain (X_A90) and different culture medium (2YT broth) were also investigated, neither increased yield and the results of these are not shown.

Lack of fusion protein binding to the beads might be because the protein was not expressed, because it was insoluble, because it was degraded or because it did not bind to the Glutathione beads. Thus samples were taken at different stages and run

along side the Glutathione bound proteins (Fig. A1.2.1). A very small amount of full length GST-IE63 (lanes 3 and 5 indicated by *) was detected in a sample of induced bacterial culture and in the soluble fraction of bacterial cell extract, but most of the induced protein appeared to be degraded and of the small amount expressed none bound to the Glutathione beads (lane 6).

A1.2.2 The effect of temperature

Fig. A1.2.2 shows that at 37°C some full length GST-63 is expressed and can be seen in the soluble fraction of the lysate (marked with a *, compare lanes 1 and 3). However none of this full length fusion binds to the Glutathione beads (lane 4). Repeating the experiment at 31°C or 26 °C decreased the yield. Thus protein expression continued to be at 37°C.

A1.2.3 Effect of IPTG concentration

Increasing the IPTG concentration from 0.1mM to 0.2mM slightly increased the production of fusion protein but not to the point where, even by Western blotting and probing for IE63 (Fig. A1.2.3) full length GST-IE63 could be detected binding to Glutathione beads. Increasing the IPTG concentration above 0.2mM does not increase expression and may even have a deleterious effect. Thus 0.2mM IPTG was then used.

A1.2.4 The effects of lysozyme or sonication for splitting open bacterial cells

Instead of using lysozyme as in (Mears & Rice (1996), after harvesting, bacteria were resuspended in PBS and sonicated, described more fully in Section 2B4.1. However no difference between the proteins present in the soluble fractions or insoluble fractions of the two differently produced lysates was observed (Fig. A1.2.4 compare lanes 4 and 12 and lanes 6 and 14). In contrast to this binding to the beads was more efficient after sonication, now a full length GST-IE63 fusion protein was detected and seen by Coomassie staining (lane 8 marked *).

Perhaps GST-IE63 is in different conformation after sonication allowing it to bind. Or the increased processing time (~2h) involved in lysozyme treatment

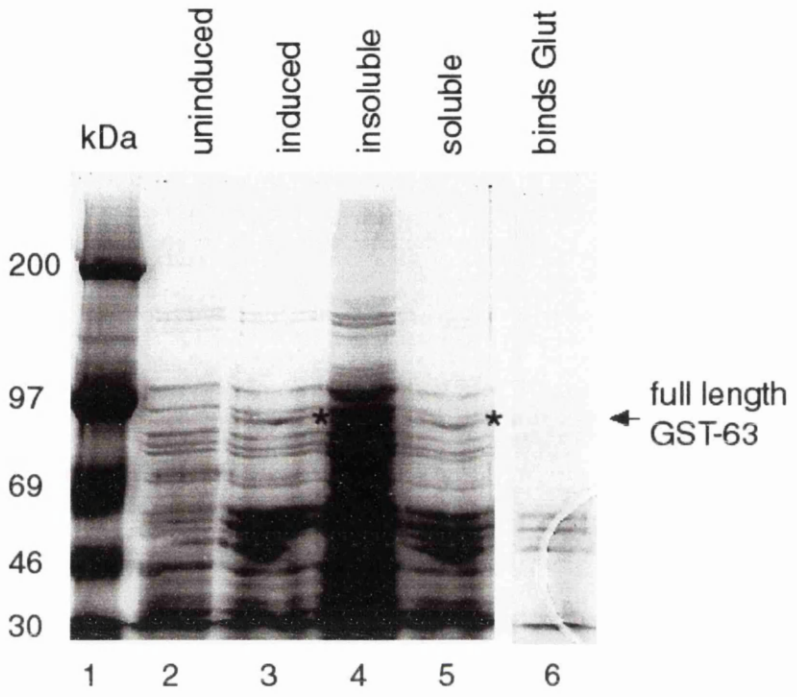


Fig. A1.2.1 A little full length GST-IE63 is expressed but does not bind Glutathione beads

Coomassie stained 10% SDS-PAGE gel of samples of bacterial culture taken before induction (lane 2), after induction with IPTG (lane 3), and of insoluble fraction (lane 4) and soluble fraction (lane 5) of the bacterial lysate produced. Proteins from the soluble fraction which bound to Glutathione beads are shown in lane 6, full length GST-IE63 indicated by *. After growing a bacterial culture, transformed with pGEX-27, to an OD_{600} of 0.4, a sample was taken before the culture was induced with IPTG. After induction a sample was taken and then the bacteria were harvested, resuspended in a small volume and lysed with lysozyme. Soluble and insoluble proteins were separated from this lysate by centrifugation and after samples were removed the soluble fraction was incubated with Glutathione beads. Again a sample was taken and all fractions were separated by SDS-PAGE electrophoresis.

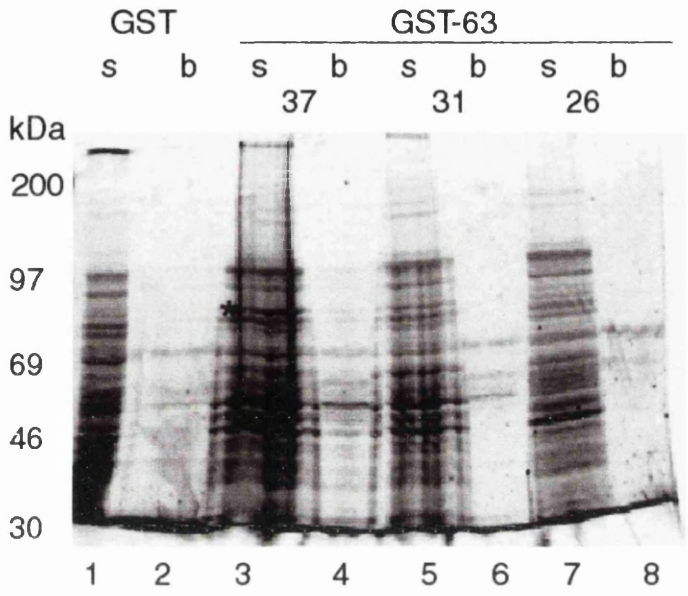


Fig. A1.2.2 Maximum expression of GST-IE63 occurs at 37°C

Coomassie stained 10% SDS-PAGE gel of the soluble protein fraction of bacterial cell lysate (s) and Glutathione bound proteins pulled out of this lysate (b). Cultures containing GST alone expressing plasmid were induced with IPTG at 37°C (lanes 1 and 2), Cultures containing GST-IE63 expressing plasmids were induced with IPTG at 37°C (lanes 3 and 4), 31°C (lanes 5 and 6) and 26°C (lanes 7 and 8). Full length GST-IE63 is marked *.

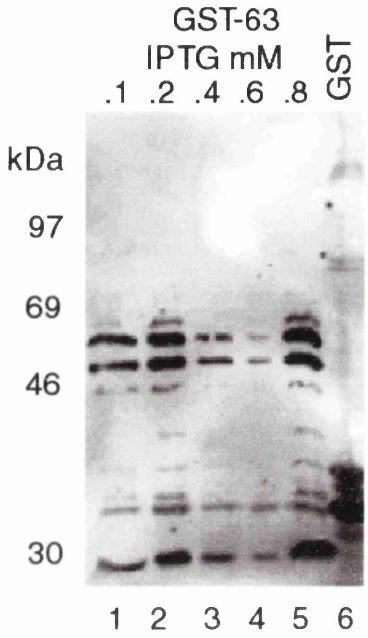


Fig. A1.2.3 Optimum IPTG concentration for GST-IE63 expression is 0.2mM

Western blot for IE63, using H1113 Mab showing GST-IE63 binding to Glutathione beads after incubation with soluble protein lysates produced from cultures induced with 0.1mM (lane 1), 0.2mM (lane 2), 0.4mM (lane 3), 0.6mM (lane 4) and 0.8mM IPTG, lane 6 shows GST protein, which had been induced with 0.1mM IPTG, bound to Glutathione beads. Proteins bound to Glutathione beads after incubation with soluble protein lysate and washing, were separated on 10% SDS-PAGE gel, transferred to nitrocellulose and Western blotted for IE63.

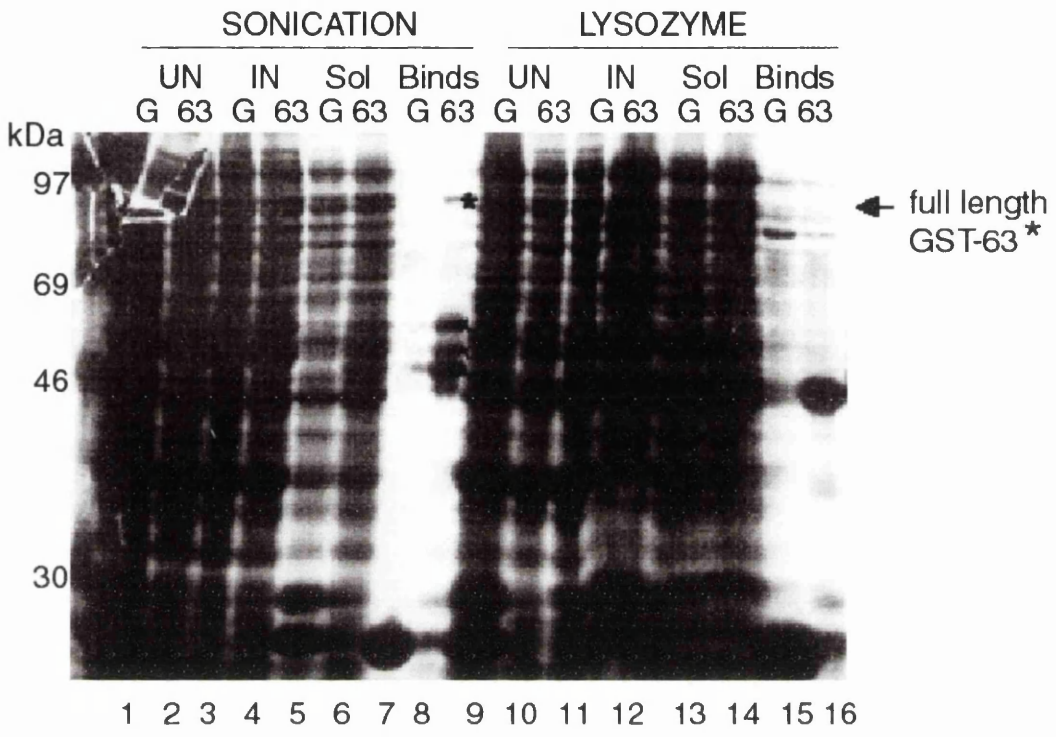


Fig. A1.2.4 Splitting open bacteria by sonication rather than using lysozyme produces full length GST-IE63 which binds to Glutathione beads.

Coomassie stained 10% SDS-PAGE gel of proteins in samples taken before induction of culture (UN), after induction of culture with 0.2mM IPTG (IN), of soluble protein lysate (Sol) extracted from cultures, and of proteins from the soluble fraction which bound to Glutathione beads (Binds). Bacteria from induced culture were lysed open by sonication (lanes 1-8) (Section 2B4.1) or using lysozyme (Mears & Rice, 1996 - lanes 9-16). In each case expression of GST-IE63 (63) is compared with expression of GST (G). Full length GST-IE63 is indicated by *.

allows enhanced proteolytic digestion. The large number of proteins on the Coomassie stained gel may have obscured a slight increase in full length GST-IE63 in the soluble fraction after sonication. Western blotting this gel for IE63 may resolve this. Sonication was used to split open the bacterial cells in optimised method.

A1.2.5 Effect of different induction times on GST-IE63 expression

Increasing the time of expression from 1.5h to 2h significantly increased the amount of GST-IE63 binding to Glutathione, however further increasing this to 3h had no further effect (marked * Fig. A1.2.5 compare lanes 6, 9 and 12). GST-IE63 protein induction was therefore carried out for 2h.

A1.2.6 Optimising ratio of beads to lysate

Having optimised expression to a degree, as there was still only a limited amount of full length GST-IE63 being produced, it was important to ensure that (i) all the GST-IE63 being expressed was being extracted from the lysate and (ii) that the Glutathione beads were saturated with GST-IE63.

Fig. A1.2.6 shows the effect of increasing the volume of beads added to 5ml of sonicated lysate. An increase from 200µl to 400µl pulled more GST-IE63 out of the lysate (lanes 2 and 3) however a further increase to 800µl (lane 4) made no difference. When the volume of bacterial lysate was increased to 10ml or 15ml (lanes 5 and 6) with 400µl beads no further fusion protein was bound.
The optimal ratio of beads to lysate was therefore 400µl beads to 5ml lysate.

A1.2.7 Final expression

At the end of this optimisation, the conditions determined and used for the remainder of the work were as described in Section 2B4.1. Only a small proportion (~ 5%) of the bound fusion protein was full length but this was a greater amount than before and it was hoped that it was enough to pull down interacting proteins. Specific truncated products were observed (Fig. 3B2) these

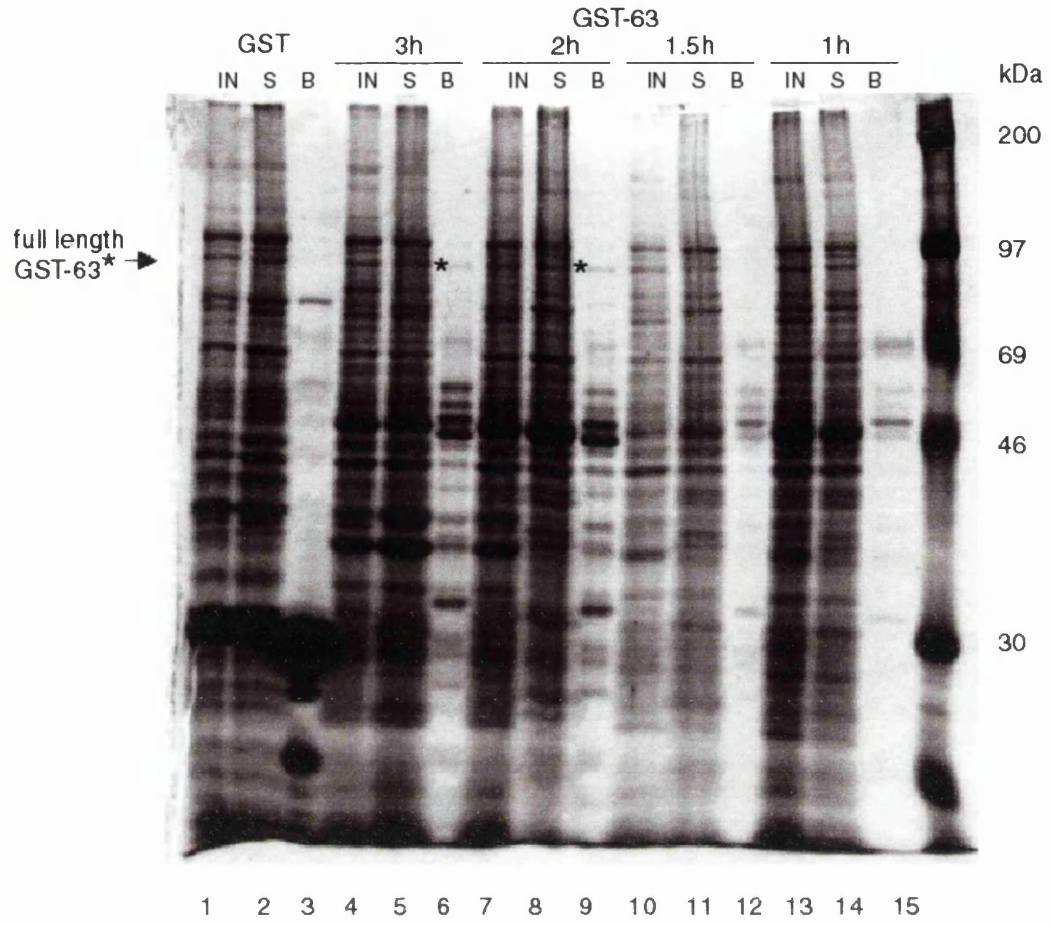


Fig. A1.2.5 Induction of GST-IE63 expression with IPTG is maximal after 2h

Coomassie stained 10% SDS-PAGE gel. Samples of IPTG induced culture containing GST expressing plasmid (IN) (lane 1), soluble protein lysate produced from this culture (S) (lane 2) and proteins from the lysate bound to Glutathione beads (B) (lane 3), were separated on a 10% SDS-PAGE gel and stained along side samples of IPTG induced expression culture containing GST-IE63 expression plasmid, induced (IN) for 1h (lane 13), 1.5h (lane 10), 2h (lane 7), and 3h (lane 4), soluble protein fraction (S) of the lysate of these cultures (lanes 14, 11, 8 and 5 respectively) and proteins bound to Glutathione beads (B) (lanes 15, 12 ,9, and 6 respectively). Full length GST-IE63 marked *. Cultures were grown to an OD_{600} 0.4, induced with 0.2mM IPTG, harvested, resuspended in 5ml PBS and sonicated, cell debris was cleared and soluble protein lysate incubated with Glutathione beads.

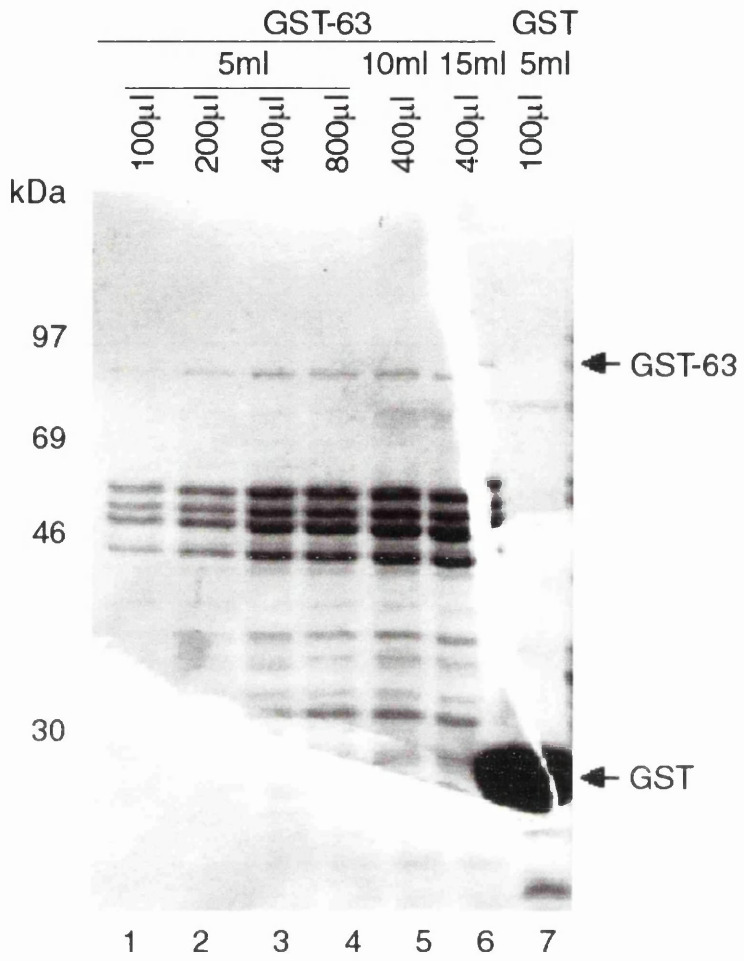


Fig. A1.2.6 Saturation of beads with GST-IE63

Coomassie stained 10% SDS-PAGE gel. 5ml soluble protein lysate extracted from IPTG induced GST-IE63 expressing cultures was incubated with increasing volumes of Glutathione beads, 100µl beads (lane 1), 200µl beads (lane 2), 400µl beads (lane 3), 800µl beads (lane 4). 10ml (lane 5) and 15ml soluble GST-IE63 lysate (lane 6) were incubated with 400µl beads and 5ml GST soluble lysate was incubated with 100µl Glutathione beads. Proteins which bound to beads after 3 washes in PBS were separated by SDS-PAGE electrophoresis.

correspond to the N-terminal portion of IE63 between aa 1 and ~aa 90-180, and are similar to the truncations reported by Mears & Rice (1996).

A1.3 Discussion of experimental techniques used

A1.3.1 Use of co-immunoprecipitation experiments: advantages and disadvantages

Co-immunoprecipitation experiments produce a good profile of proteins which interact with IE63 (Fig. 3A2) and suggest that IE63 interacts with multiple protein partners. Repeating the experiment in different cell types reveals that these interactions are universal (Fig. A1.1.2). Data from co-immunoprecipitations washed with increasing concentrations of salt, point to different strengths of interaction with different partners of IE63 (Fig. A1.1.3).

Co-immunoprecipitation from whole cell extracts is a good technique to get a representation of what is happening *in vivo*, interactions are detected in the midst of competing components, both antigen and interacting protein are found at same relative concentration as is found in the cell, complexes in their natural state are precipitated and proteins are post-transcriptionally modified correctly, so interactions dependent on this will be detected. However the protein bands were not visible even on a silver stained gel so identification by Laser Mass Map Spectroscopy or mico-sequencing was not possible. Because of the complicated nature of antibody antigen interactions it was not easy to scale up co-immunoprecipitations.

A second disadvantage of co-immunoprecipitations is that direct and indirect interactions cannot be distinguished, it should be noted that in this thesis, only the known interacting proteins and their interaction with each other and IE63 are examined, in this respect, for these proteins to be associated IE63 is required. For all the interactions, use of an IE63 expressing plasmid shows that no other viral protein is required, however an as yet unidentified cellular protein may be intermediary between IE63 and one or more of the interacting proteins reported here.

A1.3.2 Use of fusion protein pull down assays: advantages and disadvantages.

GST fusion proteins will be discussed but the same is true for MBP and Sepharose systems used.

It appears to be difficult to express a large amount of GST-IE63 particularly when compared to the amount of GST produced under the same conditions.

GST-IE63 is ~90 kDa well above the optimum size for bacterial protein expression of ~60 kDa, so premature termination of transcription or translation may be occurring, however plenty of other large fusion proteins are produced and this is unlikely to be the primary reason for truncated GST-IE63 products.

The addition of a carrier e.g. GST to a foreign protein expressed in *E. coli* is reported to stabilise expression of the fusion protein (Lee *et al.*, 1984), sometimes, however the expressed protein is degraded but the carrier is not. Moreover fusion proteins are sometimes cleaved *in vivo* at the fusion joint between the carrier and the expressed foreign protein. The appearance of GST-IE63 truncated fusions of consistent sizes suggesed there are sites within IE63 which are particularly prone to proteolytic degradation, this may be due to aberrant folding when attached to GST. Use of sonication increased the amount of full length GST-IE63 binding to beads, whether this was because sonication releases GST-IE63 in a different conformation to lysozyme treatment so leaving different sites exposed or allowing more binding to beads, or whether the increased time it takes for lysozyme treatment means that more degradationed could occur is not known.

IE63 is an essential viral protein with multiple functions during infection, it could well be toxic to bacterial cells. It was observed that as they begin to express IE63 they also start to die, and expression will never be as high as for a non-toxic protein. In comparing the density of a culture expressing GST and GST-IE63 the toxic affect of IE63 could be seen; at the same time after induction the GST culture was thicker than the GST-IE63 culture when prior to induction they were growing similarly.

Despite this problem a number of proteins can be seen to interact with GST-IE63 (Fig. 3B1), and these were of a similar size to those identified by co-immunoprecipitation. Because of the predominance of truncated products, proteins interacting with the N-terminal of IE63 may be have been over represented, however the presence of some full length IE63 in this non-quantitative assay should be enough to pull out some of all the interacting proteins.

GST fusion protein expression is a powerful tool for looking at protein:protein interactions, unlike co-immunoprecipitation, it can be scaled up to isolate proteins of unknown identity and the fusion protein can be mutated relatively easily in the pGEX vector but results must be carefully considered as this system is very much *in vitro*; the amounts of the fusion protein added are not physiological and the form in which the recombinant protein is found may differ from how it is *in vivo*. Folding may be altered by the presence of GST, masking or revealing potential sites of interaction, processing will be different in proteins expressed in *E.coli* to those expressed in mammalian cells, for example phosphorylation will be different. Any of these things many lead to false conclusions about the presence or absence of an interaction.

Although not used in this study purification of the fusion protein *in vitro* has an advantage, by mixing two purified proteins (as different types of fusion protein e.g. GST and MBP, or as a fusion protein and *in vitro* transcribed translated product) direct and indirect interactions can be distinguished.

A1.3.3 Other methods used to detect/study protein:protein interactions

Because every system has advantages and disadvantages, evidence of any protein:protein interaction must be found using more than one, examples of other procedures used to identify the protein interactions reported here, are the yeast-2-hybrid system and immunofluorescence. The yeast-2-hybrid data was completed by another worker, it is a sensitive way to screen for potential interacting proteins, giving an investigator some possibilities to confirm or discredit in the other types of assays. In addition it provides the cDNA clone of any proteins identified, and can be used to test the interaction between 2 known proteins for which the cDNA

clones are available. The yeast -2-hybrid system is limited by the stability of the proteins in yeast cells, the need for post-translational modifications which do not occur in yeast, the site of interaction not being occluded by the fusion protein or the protein itself being capable of transactivation.

Immunofluoresence is not a way to detect protein:protein interactions however it is a good way to see if reported interactions have a physiological significance, co-localisaton provides evidence that the two proteins are associated in cells, it also allows intracellular location of proteins and in the case of viral infection the position of two proteins with time post-infection can be visualised.

APPENDIX 2 : AMINO ACID CONSERVATION OF IE63 (ICP27)

Proceeding from the N to C termini, the alphaherpesviruses consist 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.

Alphaherpesviruses

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HSV-1     MATDIDMLIDLGLDLSDSDLDEDPPE.PAESRRDDLESDSSGECSSSDEDMEDPHGEDGPEPILDAARPAVRPSRPEDPG
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     PGRSAGDRLNAAAASSIADVCRRVTSSRIGEMFHGARETLTTPVKNGGFRAENS......SPWAPVLGFGSD.QFNPEAR
EHV-4     SSSRSGDRLNAAAANAIADVSKRVTSSRISDMFHGARETLTSPVKNGGFRAEHS......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
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