Studies on the interaction of HSV-1 multifunctional protein ICP27 with the nuclear pore complex proteins

A thesis presented for the Degree of Doctor of Philosophy

By

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This thesis is dedicated to:

The memory of John Barklie Clements for his intellectual and spiritual guidance,

and also to my wife and children

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Abstract

Herpes simplex virus (HSV-1) ICP27 is a multifunctional immediate early (IE) protein, which has counterparts in every herpesvirus of mammals and birds sequenced so far, indicating the importance of this key viral protein that is a potential antiviral target. It is required for expression of several early and late genes and acts at transcriptional and posttranscriptional levels. ICP27 is an RNA binding protein and inhibits pre-mRNA splicing, stimulates pre-mRNA 3' processing and affects mRNA stability. Also it has been shown that ICP27 can directly stimulate mRNA translation.

ICP27 contains both a nuclear localization signal and a nuclear export signal and shuttles between the nucleus and the cytoplasm. It interacts directly with various cellular proteins including REF and cellular mRNA export receptor TAP to export viral mRNAs. However, other transport mechanisms such as CRM1-dependent and independent pathways may also be employed. Different studies about shuttling functions suggested alternative pathways, in addition to the existing routes, for transport of proteins and mRNA in HSV-1-infected cells.

HSV-1 is a nuclear replicating virus. All transport across the nuclear envelope must occur via the nuclear pore complex (NPC), a very large, complex structure that consists of interacting fixed and mobile proteins called nucleoporins (NUPs). NUPs act to regulate the transport cargos by interaction with mRNA-bound proteins and transport receptors, karyopherins. Many features of NUPs make them the best option for interaction with ICP27 to shuttle it as a cargo-free or cargo-dependent protein. These include their transport direction through NPCs, the ability of FG-repeat domains in NUPs to undergo multiple interactions simultaneously, the transience of their binding, the unfolded structure of the FG-repeat domains and the mobility of most nucleoporins.

This work aimed to further explore the transport requirements for ICP27 and viral mRNA export. I investigated direct interaction of ICP27 with nucleoporins *in vitro* and in infected cells. NUP 62, a key FG-repeat NUP was the first nucleoporin tested in interaction with ICP27. Using fusion protein binding and co-immunoprecipitations assays with specific antibodies I show direct interaction of

these two proteins *in vitro* and in infected cells and both the N-terminus and C-terminus portions of ICP27 may be associated in this interaction. Also RNase I treatment indicated that this interaction is not mediated via RNA. Furthermore, interaction of ICP27 with nuclear FG-repeat NUP 153 that is important in cargo-free and cargo-dependent transport was investigated. Both *in vitro* pull down assay and co-immunoprecipitation experiments showed possible interaction of ICP27 and NUP 153 and again the N-terminus and the C-terminus parts of ICP27 were required for these interactions.

The only cytoplasmic nucleoporin that was tested in this Ph.D. thesis was NUP 214. FG-repeat truncates of this NUP were used in an *in vitro* experiments and showed possibility of direct involvement of ICP27 with NUP 214. *In vivo* studies confirmed this interaction. The N- and C-termini of ICP27 may participate in this interaction. Interaction studies of non FG-repeat nucleoporins such as NUP 107 and Rae1 did not show any interaction allowing me to conclude that interaction of ICP27 and nucleoporins might be via FG-repeat domains of nucleoporins.

The next aim was to investigate an involvement of a region of ICP27 that is conserved between this protein and another protein involved in nucleocytoplasmic transport, hnRNP K. This region is called the SADET region according to its amino acid sequence and is known to be essential for transport of hnRNP K. However, I could not demonstrate any association of this SADET region in interaction of ICP27 and NUPs in my study.

Finally, some RNA viruses disrupt the nucleocytoplasmic transport machinery by degradation of some nucleoporins. However, my investigations in infected and transfected cell extracts did not show any change in the level of FG-repeat nucleoporins indicating that HSV-1 does not degrade NUPS.

In conclusion, ICP27 alone or in complex with viral mRNA might interact directly with NUPs and provide a docking site to translocate in the NPC and transfer ICP27 either to the nucleus or the cytoplasm. The NUPs that are located in the cytoplasmic face of the NPC could associate with ICP27 and provide it to other NUPs, such as NUP 62, in the central gated channel and then to nuclear NUPs to dissociate in the nucleus. On the other hand, nuclear face NUPs could bind to

ICP27 in the nucleus and shuttle to the central basket and pass it through the NPC to cytoplasmic NUPs to dissociate in the cytoplasm. Alternatively it has been proposed that sometimes cargoes can bind to two transport receptor systems and may utilise both receptors simultaneously to translocate through the NPC. I suggest that in HSV-1-infected cells cargos might interact with either TAP and ICP27 or CRM1 and ICP27 simultaneously and translocate through NPC to shuttle between the nucleus and the cytoplasm.

The literature of this thesis has been stopped on 2007.

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Abbreviations

A	adenine or absorbance or amps
aa	amino acid
Ab	antibody
AD	activation domain
AIDS	acquired immune deficiency syndrome
AMP	adenosine monophosphate
APP1	activated-platelet protein-1
APS	ammonium persulphate
ARE	AU-rich element
ASF/SF2	alternative splicing factor / splicing factor 2
ATP	adenosine triphosphate
BCBL-1	cell line derived from a body cavity-based lymphoma latently infected with HHV-8
BD	DNA-binding domain
BHK	baby hamster kidney
bp	base pair
BSA	bovine serum albumin
C	cytosine or carboxy (-terminal end of protein) or cytoplasmic
CAT	chloramphenicol acetyl transferase
CBP	cap binding complex
cDNA	complementary DNA
CF	cleavage factor
CK2	casein kinase 2
CNS	central nervous system
CPE	cytopathic effects
CTD	C-terminus domain
CTL	cytotoxic T lymphocyte
DAPI	DNA-binding fluorochrome 4,6-diamidino-2-phenylindole
DFF	DNA fragmentation factor
D-MEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide

DNA	deoxyribonucleic acid
DRB	5,6-dichloro-1-β-D-ribofuranosylbenzimidazole
dsRNA	double-stranded RNA
dTMP	thymidine monophosphate
DTT	dithiothreitol
dUMP	uridine monophosphate
E	early
E. coli	*Escherichia coli*
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
ECS	export control sequence
EDTA	Ethylenediaminetetra-acetic acid
eIF	eukaryotic initiation factor
EJC	splice exon-junction complex
ER	endoplasmic reticulum
ERK	extracellular signal-regulated protein kinase
EYFP	enhanced yellow-green fluorescent protein
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FMDV	foot and mouth disease virus
g	gram or glycoprotein
G	guanine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	guanosine diphosphate
GFP	green fluorescent protein
G-MEM	Glasgow Modified Eagle's Medium
GST	glutathione-S-transferase
GTP	guanosine-5'-triphosphate
h	hour
HCMV	human cytomegalovirus
HCV	hepatitis C virus
HeLa cells	continuous cell line derived from Henrietta Lacks
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
HHV-1 to 8	human herpesvirus 1-8

HIV	human immunodeficency virus
HLA	human leukocyte antigen complex
hnRNP	heterogeneous nuclear ribonuclear protein
hPAN	human poly(A) nuclease
hpi	hours post-inoculation
HPV	human papilloma virus
HRP	horseradish peroxidase
HSV-1 / 2	herpes simplex virus type 1/ type 2
HVS	herpes virus saimiri
ICP	infected cell protein
IDU	5-Iodo-2'-deoxy-L-uridine
IE	immediate early
IF	immunofluorescence
IFA	immunofluorescence assays
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IP	immunoprecipitation
IPG	immobilised pH gradient
IPTG	isopropyl thiogalactopyranoside
kb	kilo-bases
kDa	kilo-daltons
KH	hnRNP K homology
KNS	hnRNP K nuclear shuttling signal
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
l	litre
L	late
lacZ	bacterial gene that encodes β-galactosidase
LANA	latency-associated nuclear antigen
LAT	latency associated transcript
LB	Luria-Bertani media
LMB	leptomycin B
LMW	low molecular weight marker

LPF	late processing factor
LRR	leucine-rich region
LTR	long terminal repeat
M	molar
m	milli
MAb	monoclonal antibody
MHC	major histocompatibility complex
MI	mock infected
min	minutes
miRNA	microRNA
MOI	multiplicity of infection
mRNA	messenger RNA
mRNPs	messenger ribonucleoproteins
MS2	MS2 coat protein
n	nano
N	amino (-terminal end of protein) or nuclear
NA	not applicable
ND	not determined
ND10	nuclear domain 10
NES	nuclear export signal
NK	natural killer
NLS	nuclear localisation signal
NMD	nonsense-mediated decay
NMS	normal mouse serum
NP40	Nonidet P40
NPC	nuclear pore complex
NR	neutral red
NRS	normal rabbit serum
NS	non-structural
nt	nucleotides
Nu	nucleolar
OD	optical density
ORF	open reading frame
ori	origin of replication

p	pico
PABP1	Poly (A) binding protein 1
PAGE	polyacrylamide gel electrophoresis
PAP	poly(A) polymerase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pfu	plaque forming unit
PKA	protein kinase A
PKC	protein kinase C
PML	promyelocytic leukemia protein
PMSF	phenylmethylsuphonyl fluoride
pre-mRNA	precursor mRNA
REFs	RNA and export factor binding proteins
RGG	arginine rich RNA binding motif
RNA	ribonucleic acid
RNAi	RNA interference
RNAP II	RNA polymerase II
RNase	ribonuclease
RNP	ribonucleoprotein
RRM	RNA-recognition motif
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcriptase-polymerase chain reaction
RXP	arginine-X-proline repeating motif
S	Svedberg sedimentation unit
SAP	splicing associated protein
SDS	sodium dodecyl sulphate
sec	seconds
siRNA	small interference RNA
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein particle
SR protein	Ser-Arg-rich protein
SRPK1	SR protein kinase 1
SUMO	small ubiquitin-like modifier

SV40	simian virus 40
T	thymine
TAF	TATA-binding protein associated factor
TBB	4,5,6,7-tetrabromobenzotriazole
TBP	TATA-binding protein
TE	Tris EDTA
TEMED	N, N, N', N'-tetramethylethylenediamine
TF	transcription factor
TGF	transforming growth factor
TK	thymidine kinase
TNF-α	tumour necrosis factor alpha
TOP	terminal oligopyrimidine tract
tRNA	transfer RNA
ts	temperature-sensitive
U	uridine or units
U_L	unique long domain
U_S	unique short domain
USE	upstream sequence enhancer
USP	ubiquitin-specific protease
UTR	untranslated region
UV	ultra-violet
V	volts
v/v	volume/volume
vhs	virion associated host shut off
vIRFs	viral interferon regulatory factors
Vmw	apparent weight of virion polypeptide
VSV	vesicular stomatitis virus
VZV	varicella-zoster virus
w/v	weight/volume
WB	western blott
WT	wild type
Zn	zinc
α-TIF	alpha trans-inducing factor
µ	micro

Amino acid symbols

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

1 Introduction

1.1 Herpesviridae

1.1.1 General features

The *Herpesviridae* are a family of DNA viruses that cause diseases in humans and animals and they are believed to be extremely ancient, evolving over a period of 400 million years (Thiry, Muylkens et al. 2005). *Herpesviridae* is one of the most important virus families that are involved in a wide range of significant medical and veterinary diseases. Their ubiquitous occurrence, genetic complexity, evolutionary diversity, and widely differing biological properties have motivated great research efforts worldwide, so that our understanding of these agents is now considerable (Davison and Clements 2005). The number of herpesviruses in nature is likely to exceed the approximately 130 herpesviruses identified to date (Roizman and Pellett 2001). They infect a wide range of vertebrates, including fish, amphibians, reptiles, birds, marsupials, and other mammals including humans, and at least one invertebrate, the oyster (Davison and Clements 2005). Nine herpesviruses have been isolated from humans (Table1.1). These viruses are ubiquitous and highly successful intracellular parasites, but each is usually restricted in natural infection to a single species and spreads from host to host by direct contact or by the respiratory route (Roizman and Pellett 2001; Davison 2002; Davison and Clements 2005).

Four essential biological properties of the *Herpesviridae* family that characterize them are: herpesviruses can express a number of enzymes that are involved in nucleic acid metabolism, DNA synthesis and protein handling. The next property of these viruses is replication and maturation in the nucleus. Thirdly, they induce cytopathic effects in infected cells and finally, herpesviruses can establish a latent form and reactivate in stressed cells. Latency involves stable maintenance of the viral genome in the nucleus with limited expression of a small subset of viral genes (Roizman and Pellett 2001; Davison 2002; Davison and Clements 2005).

1.1.2 Classification

The *Herpesviridae* were classified into three subfamilies on the basis of their biological properties before the DNA sequences of the individual members of the family were known (Roizman and Pellett 2001; Davison 2002; Davison and Clements 2005).

1.1.2.1 Alphaherpesvirinae

Members of this subfamily are classified in three genera: Simplexvirus (HSV-1, 2), Varicellovirus (VZV) and Mardivirus that have variable host range and a short reproductive life cycle. They are rapid spreading in culture and cause efficient destruction of infected cells. Their ability to establish latency primarily in sensory ganglia has been clear.

1.1.2.2 Betaherpesvirinae

Members of this subfamily are classified in three genera Cytomegalovirus (HCMV), Muromegalovirus (murine cytomegalovirus), and Roseolovirus (HHV-7). They have restricted host range and a long reproductive life cycle. Their infection progression is slow in culture and latency established in these genera happens in variety of tissues including secretary glands, lymphoreticular cells, kidneys, and others.

1.1.2.3 Gammaherpesvirinae

Members of this subfamily are classified in two genera include: Lymphocryptovirus (EBV) and Rhadinovirus (KSHV). Usually they are specific for either T or B-lymphocytes and a latency form has been demonstrated in lymphoid tissue. All members replicate and persist in lymphocytes and some also cause lytic infections in some types of epithelioid and fibroblastic cells. The experimental host range of these genera is limited to the family or order to which the natural host belongs.

1.1.3 Clinical features of Human Herpesviruses

Human herpesviruses are the causative agent of variety of diseases, which are a growing problem. HSV-1 is ubiquitous and causes skin/eye/mouth lesions, as well as conjunctivitis and encephalitis. Direct contact, intrauterine, neonatal and postnatal infections all occur in HSV-1 (Further information in section 12.2).

HSV-2 is the cause of most cases of genital herpes (10-15% is HSV-1) and infects roughly 20% of adults in the U.S (Whitely 2001; Whitley and Roizman 2001). Prevalence of HSV-2 is rising and neonatal infections can occur and may be severe. After initial infection, HSV-2 persists. Viruses are taken up by nerve ending and the viruses migrate rapidly in nerve fibers (axons), travelling up to the cell body of the nerve cell (the ganglion). HSV infections recur in response to signals such as sunlight, stress, colds or immune suppression (Whitely 2001; Whitley and Roizman 2001).

VZV is another alpha-herpesvirus that is spread by a respiratory route, and primary infection occurs mainly in children, where disease can be asymptomatic or may be manifested as varicella (chickenpox), which is a mild febrile illness with a blister-type rash. The virus then establishes a latent infection in the sensory ganglia of the spinal or cranial nerves, and can be reactivated by age, hormones (pregnancy) or immune suppression. When VZV reactivates, It is known as zoster (shingles), which can be painful (neuralgia) and may be severe (Arvin 2001; Mettenleiter 2002; Ku, Besser et al. 2005).

Human herpesviruses 6 and 7 (HHV-6, HHV-7) are β herpesviruses, which are relatively primitive in genetic terms, and share important common properties with HSV-1. They do not encode thymidine kinase and thus are not highly sensitive to acyclovir. Both HHV-6 and HHV-7 establish lytic infections *in vitro*, and are slow growing (like CMV that will be detailed later). HHV-6 is a pan-lymphotropic virus that infects several immune cell types, including T-cells and NK cells, as well as cells of the CNS. The virus uses CD46 as its primary cellular receptor. In terms of pathogenesis, HHV-6 has been linked to febrile illnesses, including roseola (exanthemsubitum) in infants. HHV-6 is an important pathogen in immunocompromised individuals, where it may cause pneumonia and, in bone

marrow transplant recipients, bone marrow failure. Two variants of HHV-6 exist – HHV6A and HHV6B. These differ by roughly 5% at the sequence level, but appear to have distinct biological properties, tropism and pathogenesis. Generally HHV6A is more pathogenic. Human herpesvirus 7 (HHV-7) is interesting because it has been reported that the virus uses the CD4 receptor as a component of its receptor. HHV-7 may also cause some cases of roseola and has been postulated to play a role in the pathogenesis of Pityriasis rosea. Both viruses infect almost all (>90%) of children by age 3, and persist life-long (Caserta, Mock et al. 2001; Yamanishi 2001).

Human cytomegalovirus (CMV) is a β-herpesvirus whose genome encodes a number of genes that function to downregulate the antiviral immune response. Mainly a respiratory route spreads CMV, and roughly 80% of over 35 year olds are CMV positive. It is an important cause of congenital or neonatal infections, which can be severe (fatal in 5-10% of cases), in part because of its ability to cross the placenta. It is reactivated in much the same way as other herpesviruses, and can cause pneumonia in immunosuppressed individuals, as well as retinitis. Since the virus lacks the TK gene, CMV infections are usually treated with ganciclovir (Pass 2001).

Epstein-Barr virus (EBV) is one of the most extensively characterized members of the gamma subfamily of herpesviruses and a common human virus that causes infectious mononucleosis. Symptoms of infectious mononucleosis are fever, sore throat, and swollen lymph glands. EBV remains dormant or latent in a few cells in the throat and blood for the rest of the person's life. Periodically, the virus can reactivate and is commonly found in the saliva of infected persons. Transmission of EBV requires intimate contact with the saliva of an infected person. Strong evidence links EBV to the development of a spectrum of human diseases, including Burkitt's lymphoma (BL), Hodgkin lymphomas (HL), post-transplant and AIDS-related lymphomas, nasopharyngeal carcinoma, and a small subset of T cell lymphomas, gastric carcinomas, leiomyosarcomas, follicular dendritic tumors, and other epithelial carcinomas but is probably not the sole cause of disease (Rikinson and Kieff 2001; Kutok and Wang 2006).

Kaposi's sarcoma (KS) is a mesenchymal tumour involving blood and lymphatic vessels. In children, it is believed that KS is a manifestation of primary infection, whereas in adults, it has been suggested that KS occurs after primary infection. Most evidence indicates that HHV8 is sexually transmitted among men, probably by receptive anal intercourse. Classic KS is primarily a skin disease that appears as multiple firm, purple-reddish-brown plaques and nodules on the lower extremities. It may be complicated by lymphoedema or hyperkeratosis. Under different geographical and other condition there are several clinically distinct patterns of KS. Endemic African KS has four clinically distinct patterns. KS has also been described in iatrogenic immunosuppressed organ transplant recipients. AIDS associated KS is common in this group of people (Moore and Chang 2001; Roizman and Knipe 2001; Hengge, Ruzicka et al. 2002).

1.2 Herpes simplex type 1

1.2.1 Definition

Human HSV-1 (herpes simplex virus type 1) is a double stranded DNA virus that replicates in the nucleus. After infection, its 152 kb genome, encodes about 90 transcriptional units and 84 are translated to proteins. The viral genes are expressed in a temporal co-ordinated fashion and can be divided into three classes: α (immediate early), β (delayed early) and γ (late). The virion-associated VP16 protein stimulates transcription of the five α gene and their expression does not require prior protein synthesis. α gene products regulate expression of the β proteins, which are involved in viral DNA replication and metabolism. The γ gene class, are necessary for virion assembly, maturation and egress, and can be subdivided into the γ1 genes, whose expression is stimulated by viral DNA synthesis but is not dependent on it, and the γ2 genes, whose expression shows a complete requirement for viral DNA replication (Roizman and Knipe 2001; Smith, Malik et al. 2005).

1.2.2 Clinical features and epidemiology

HSV-1 is normally associated with orofacial infections and encephalitis. Intrauterine, neonatal and postnatal infections all occur, and a common sequela of such infections is encephalitis. The illness caused by the HSV-1 virus can vary greatly, ranging from asymptomatic to severe systemic illness, which is occasionally fatal. It infects the oral mucosa, and travels up to the trigeminal ganglia in the face. In children 1-3 years of age, the major symptom of HSV-1 infection is gingivostomatitis, an infection of the gums, tongue, mouth, lips, facial area and pharynx. HSV-1 infections recur in response to signals such as sunlight, stress, colds or immune suppression. Reactivated virus infection usually results in lesions of the skin in the area served by the trigeminal ganglia e.g. herpes labialis (cold sores). Such recurrences are generally localised, but can be generalised if the host is immunocompromised (Whitely 2001; Whitley and Roizman 2001). HSV-1 infections occur throughout the world with approximately one third of the population in developing and the lower socio-economic classes of developed countries seroconverting to HSV-1 by 5 years of age, and the frequency increases to 70-80% during adolescence. In the developed world this is delayed and seroprevalence by 20-40 years of age is around 40-60% (Wentworth and Alexander 1971; Whitely 2001; Whitley and Roizman 2001). For HSV-1 infection to occur, the virus must come into contact with mucosal surfaces or grazed skin. Following replication at the site of primary infection, virus is transported by neurons to dorsal root ganglia where latency is established (Rock and Fraser 1983). HSV can affect all areas of the nervous system and cause meningitis, myelytis and radiculitis among other diseases. Other rare complications can include keratitis and a disseminated infection involving organs such as the liver and adrenal glands (Whitely 2001; Whitley and Roizman 2001).

1.2.3 Prevention and therapy

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 subpopulations, specific antibodies and lymphokine responses have been implicated as important factors in host

defences against HSV-1 infections (Lopez, Arvin et al. 1993; Xu, Screaton et al. 2001; Ellermann-Eriksen 2005). No treatment is currently available to eliminate HSV-1 infection, however a new range of antiviral agents have become available since the 1980s. Acyclovir (Zovirax), a synthetic acyclic purine-nucleoside analogue, was introduced in the early 1980s and has been shown to produce a clinical benefit in primary genital herpes when administered either intravenously or orally (Mindel, Adler et al. 1982). Acyclovir reduces the clinical severity of disease episodes, shortens their duration, prevents complications and reduces symptomatic viral shedding, but does not eliminate the infection (Kinghorn 1993). Acyclovir is a particularly effective inhibitor of HSV replication *in vitro* and *in vivo* (Schaeffer, Beauchamp et al. 1979). The efficacy and antiviral specificity of the acyclic nucleoside analogues is due in part to their selective phosphorylation in virally infected cells (Biron, Stanat et al. 1985). The HSV thymidine kinase enzyme (tk) phosphorylates acyclovir, converting it to a monophosphorylated form, which is then phosphorylated further by cellular kinases before it is incorporated into newly synthesised DNA resulting in the arrest of DNA synthesis and leading to subsequent DNA fragmentation (Reardon and Spector 1989; Clercq 2004). As DNA synthesis is required for acyclovir to function, the drug cannot destroy the virus during the latent period. In addition, valaciclovir, famciclovir, penciclovir are its oral prodrugs (Balfour 1999; Clercq 2004). The only herpesvirus vaccine licensed for human use in the US is the chickenpox vaccine for VZV, which is a live, attenuated, vaccine that is 70-90% effective. Vaccines for HCMV, HSV-2, HSV-1 and EBV are in development. Recently, three types of prophylactic vaccine are in clinical trials, an adjuvant subunit using viral surface gB and gD antigens, a replication incompetent viral mutant, and DNA vaccines (Stanberry 1995; Bernstein and Stanberry 1999; Corey, Langenberg et al. 1999; Gomi, Sunamachi et al. 2002). Additionally, genetically attenuated HSV mutants and vectors are in pre-clinical development (Whitley and Roizman 2001).

1.2.4 Structure of the virion

HSV-1 particles consist of the viral genome packaged as linear dsDNA, present in the icosahedral capsid. The capsid is roughly 100 nm in diameter and composed of 162 individual capsomers. A tegument that is unique to herpesviruses surrounds it,

and it includes proteins as well as virion RNAs that the virus needs to initiate its lifecycle in the infected cell (figure 1.1). The virion together with cellular RNAs may allow for immediate synthesis of new gene products following virus entry, setting up the cell for efficient virus replication. This particle and tegument is wrapped in a lipid-rich envelope, which includes up to 11 different viral glycoproteins and there might be as many as 1,000 copies of individual glycoproteins in the envelope (Roizman and Knipe 2001; Sciortino, Taddeo et al. 2002; Terhune, Schroer et al. 2004).

1.2.5 Viral genome

Physical characterisation studies showed the HSV-1 genome is 152 kbp. Like all herpes virus DNAs it is comprised of linear, dsDNA but has an unusually high G+C content of 68% (McGeoch, Dolan et al. 1986; McGeoch, Dalrymple et al. 1988; Perry and McGeoch 1988; Roizman and Knipe 2001).The genome is composed of long (L) and short (S) unique segments flanked by terminal repeat regions. The unique long (U_L) region is 107.9 kbp and the unique short (U_S) region is 13 kbp. The unique sequences are flanked by inverted repeats, R_L (9 kbp) and R_S (6.5 kbp) (McGeoch, Dalrymple et al. 1988). R_L and R_S are not related in sequence apart from the ‘a’ sequence (400 bp) located at the genome termini. One copy of the ‘a’ sequence is located at the S terminus, whereas the L terminus may have more than one copy. The ‘a’ sequence is also located at the L-S junction as an inverted repeat and again can be present as more than one copy (Wagner and Summers 1978). The two unique segments can invert relative to each other via the repeats such that a population of HSV DNA molecules consists of four equimolar isomers. Each gene has its own promoter to direct transcription, although some transcripts share 3' ends (Figure 1.2) (Roizman and Knipe 2001).

1.2.6 Viral life cycle

The viral life cycle is summarised in Figure 1.3. In fully permissive tissue culture cells e.g BHK cells, the entire process takes approximately 18-20 hours. This process includes attachment, entry, gene expression, assembly and egress that I will discuss briefly.

1.2.6.1 Cell attachment

In HSV-1, attachment is mediated mainly by the envelope glycoprotein C (gC), which interacts with heparin sulphate (HS) side-chains on cell surface proteoglycans (WuDunn and Spear 1989; Shieh, WuDunn et al. 1992). Heparin sulphate is the primary receptor for HSV-1 (Shieh, WuDunn et al. 1992) but perhaps not the sole receptor for HSV-1 (Gruenheid, Gatzke et al. 1993). Proposed candidates for secondary or co-receptors have included the fibroblast growth factor receptor (FGFR) (gBaird, Florkiewicz et al. 1990). Understanding the envelope glycoproteins which are involved in binding to heparin sulphate has come from the studies of viral mutants lacking functional versions of one or more of the twelve known glycoproteins (gB, gD, gH and gL) and one non envelope glycoprotein (gK) that have been found to be essential for productive infection in cell culture (Desai, Schaffer et al. 1988; Fuller and Lee 1992; Hutchinson, Browne et al. 1992; Hutchinson and Johnson 1995).

1.2.6.2 Cell penetration

After attachment, the virus then interacts with one of three possible entry receptors, via glycoprotein D (gD). These are firstly, Herpes Virus Entry Mediator (HVEM), a TNF receptor super family member (Montgomery, Warner et al. 1996). Nectin 1 and 2 are the second family of co receptors which are immunoglobulin-gene super family members that are present in both human and nonhuman cells and act as intercellular adhesion molecules. They mediate entry of all HSV-1 strains tested (Geraghty, Krummenacher et al. 1998). Thirdly, modified heparin sulphate sites generated by 3-O-sulfotransferases which mediate efficient HSV-1 entry but not HSV-2 entry (Shukla, Liu et al. 1999). Binding to these receptors triggers a conformational change in gD facilitating fusion but also gB/gH and gL that are needed also for fusion. Following penetration and internalisation, several of the tegument proteins have important functions. These include the virion host shut off (vhs) protein which indiscriminately degrades mRNA and so causes an early shut down of host protein synthesis (Kwong and Frenkel 1987). Virus capsids are transported to the nucleus where the viral DNA and at least some tegument proteins enter the nucleoplasm by an unknown mechanism. Here the course of

infection depends on whether the virus enters the lytic cycle or establishes a latent infection (Whitely 2001; Spear and Longnecker 2003).

1.2.6.3 DNA replication

In HSV-1, the complete set of seven viral genes required for replication is known and replication of a HSV-1 replication origin containing plasmid can be reconstituted using these proteins expressed from baculovirus vectors (Stow 1992). Viral replication typically peaks between 10- 20 hours post infection (hpi). It has long been thought that HSV-1 DNA rapidly circularises during productive infection of target cells, but studies by Jackson and Deluca suggest that this view may not in fact be correct (Jackson and DeLuca 2003). Circular forms of HSV DNA are not present during productive infection, and occur only in latent infection (Figure.1.4). This switch is regulated by the expression of ICP0, which prevents genome circularisation. Productive HSV-1 DNA replication most likely proceeds via a complex, multi-phase mechanism: The initial phase of herpesvirus replication occurs at specific origins of lytic-phase replication, via a putative theta-form intermediate (i.e., a replication “bubble”). Replication initiates via local unwinding of an AT-rich region within the origin of DNA replication. This is triggered by the interaction of a virally-encoded origin-binding protein (OBP; aka UL9) with specific DNA sequences at the origin that this initial phase of DNA replication uses a cellular DNA polymerase and primase. Later, the genome is believed to become nicked and to form a rolling circle template, resulting in the generation of head-to-tail concatamers containing many copies of the HSV-1 genome during the late phase of DNA replication (Lehman and Boehmer 1999). Replication during this phase occurs in an origin-independent manner. The viral helicase-primase complex protein (ICP5/8/52) then further unwinds the “open” DNA, in an ATP-dependent fashion. This allows the viral DNA polymerase to load on to the origin and to begin new DNA synthesis, using RNA primers made by the helicase-primase complex. Further studies contrary to the work of Jackson and DeLuca showed that circularization of the HSV-1 DNA occurs early in lytic infection and this process is independent of both viral genome and protein synthesis (Martinez, Sarisky et al. 1996; Lehman and Boehmer 1999; Crumpacker and Schaffer 2002; Strang and Stow 2005).

1.2.7 The lytic life cycle

The general pattern of HSV gene expression in productively infected cells was first described over 20 years ago (Figure.1.5). Herpesviruses replicate in the nucleus. Their gene expression is tightly regulated, and has two key features. First, herpesvirus proteins fall into groups, whose synthesis is coordinately regulated that is, all genes within a given group are expressed at the same time and are switched on and off by the same signals. Second, the viral protein groups are expressed in a cascade fashion starting with the immediate early (IE) group, followed by the early (E) and then the late (L) groups. This occurs because the IE proteins are needed to make the E proteins, which are, in turn, needed to make the L proteins (Honess and Roizman 1974). In HSV-1, tegument protein known as trans-activator of alpha genes (aka VP16 or UL48) initiates viral transcription, and it activates transcription from the viral immediate-early genes by acting in concert with several cellular transcription factors. VP16 cannot bind DNA directly so it is dependent on the cellular Oct-1 transcription factor and at least one other cellular factor, known as host cell factor (HCF) to form a multi-component complex on the TAATGARAT motifs which are present in all the IE gene promoters (Gaffney, McLauchlan et al. 1985; O'Hare 1993). During HSV infection, HCF binds to VP16 to promote stable interaction with Oct-1 and may act as a nuclear import factor for VP16, particularly in the early stage of infection (La Boissiere, Hughes et al. 1999). In addition, HSV infection alters the sub-cellular distribution of HCF (LaBoissiere and O'Hare 2000). These cellular factors are critical in determining whether HSV-1 expresses its lytic genes. The other key regulatory protein, which is present in the viral tegument, is *vhs* or the virion-host shutoff protein (aka UL41). *vhs* is not essential for viral replication, but it increases the efficiency of virus production, by causing the non-specific degradation of mRNAs, and by shutting off synthesis of macromolecules after infection of the cell. This allows HSV-1 to completely take over the host cell's synthetic machinery. It is important to know that *vhs*-induced mRNA degradation is indiscriminate, and so the virus must carefully regulate *vhs*. This may be achieved in part by the binding of VP16 to *vhs* at later times in viral infection after cellular mRNAs have been eliminated. Following this transactivation, the lytic gene cascade ensues. This cascade is well ordered and tightly regulated and dictates

the controlled expression of three classes of genes: the IE or α genes, the E or β genes and the L or γ genes (Honess and Roizman 1974; Whitely 2001).

1.2.7.1 Immediate early proteins

The first group of viral genes to be expressed after about 2 to 4 h post infection (hpi) is the immediate-early (α) genes, which can be expressed in the presence of inhibitors of protein synthesis. For the most part, these immediate-early proteins are regulatory proteins, which switch on the expression of other viral genes. These encode a total of 6 immediate-early (IE) genes, which are designated as ICP (infected cell proteins) 0, ICP4, ICP22, ICP27 and ICP47, plus Us1.5. Of these, only ICP4 and ICP27 are required for viral replication in tissue culture, although ICP0 deletion mutants also replicate less well than wild-type virus. All 3 of these proteins are transcriptional regulators of the viral early and late genes, and ICP4 also inhibits expression of the IE genes, which helps to shut off these genes during the later stages of viral infection (Roizman and Knipe 2001).

ICP0

ICP0 a 110 KDa multifunctional protein that plays a central role in regulating whether HSV-1 virus progresses to lytic or latent infection. It does transactivate all three classes of IE, E and late genes of HSV and is required to induce efficient lytic gene expression and consequently efficient viral replication (Warren, Devlin et al. 1977; Cai and Schaffer 1992; Cohen, Muggeridge et al. 1992);(Everett, Boutell et al. 2004; Kummer, Turza et al. 2007; Boutell, Everett et al. 2008). It is a strong and global transactivator of both viral and cellular genes and phosphorylation regulates ICP0 function (Davido, von Zagorski et al. 2005). ICP0 is phosphorylated by both viral and cellular kinases and three regions in ICP0 identified for phosphorylation that is required for maximal transactivation activity (Royston and Aurelian 1970; Boutell, Everett et al. 2008). However, it has become apparent that ICP0 acts at or before the initiation of mRNA synthesis (Jordan and Schaffer 1997). ICP0 interacts with different cellular proteins such as cyclin D3, translation elongation factor EF-1, centomeric protein CENP-C, a ubiquitin-specific protease USP7, a class II histone deacetylase and the transcription factor BMAL1 (Kawaguchi, Tanaka et al. 2001; Boutell, Canning et al. 2005). Early in infection (3 hrs), ICP0 was observed to localise in the nucleus at pre-existing nuclear sub-structures, ND10, that are also

known as PML (promyelocytic leukaemia nuclear) bodies. Within a few hours these structures are disrupted, and this disruption required the RING finger domain of ICP0 and can be inhibited by proteosome inhibitor drugs. The view from these observations is that ICP0 induces the degradation of specific cellular targets, and that this degradation is an integral component of the mechanism by which ICP0 activates viral gene expression during a productive infection (Parkinson, Lees-Miller et al. 1999; Everett 2000; Everett and Murray 2005; Boutell, Everett et al. 2008).There is a suggestion that it might have a role in reactivation from latency. Further, in certain cells such as neurons, in which ICP0 expression is deficient, latency would be more likely than productive infection (Everett 2000). It has been suggested that ICP0 regulates the balance between lytic and latent HSV-1 infection (Loiacono, Taus et al. 2003). It has been indicated that ICP0 plays a critical role in both of initiation of lytic replication and the reactivation of latent viral genomes (Boutell, Everett et al. 2008; Everett, Parada et al. 2008). It has recently shown that involvement of ICP0 and PML contribute to a cellular antiviral repression mechanism (Everett, Rechter et al. 2006). Further study also suggested ICP0 has crucial role in the viral immune escape mechanism by downregulation of CD83 expression (Kummer, Turza et al. 2007).

ICP27

ICP27 is an essential, multifunctional α protein that is required for expression of several classes of post-α genes, (Oakes and Rosemond-Hornbeak 1978; Stavraky, Rawls et al. 1983; Sacks, Greene et al. 1985; Ward and Roizman 1994; Uprichard and Knipe 1996).It acts transcriptionally and posttranscriptionally. An RNA binding protein (Mears and Rice 1996; Sokolowski, Scott et al. 2003), ICP27 inhibits pre-mRNA splicing (Hardy and Sandri-Goldin 1994; Bryant, Wadd et al. 2001; Lindberg and Kreivi 2002; Sciabica, Dai et al. 2003; Smith, Malik et al. 2005), stimulates pre-mRNA 3' and processing (McLauchlan, Phelan et al. 1992; McGregor, Phelan et al. 1996), affects mRNA stability (Brown, Nakamura et al. 1995), and shuttles between the nucleus and the cytoplasm (Phelan and Clements 1997; Mears and Rice 1998) promoting viral RNA nuclear export (Sandri-Goldin 1998; Koffa, Clements et al. 2001; Chen, Sciabica et al. 2002; Ellison, Maranchuk et al. 2005). Two recent reports have provided indirect evidence for a role in the cytoplasm. Firstly, ICP27 stimulates the polysomal association of HSV-1 VP16

RNA and the levels of VP16 protein (Ellison, Maranchuk et al. 2005). Secondly, ICP27 directly stimulates mRNA translation (Larralde, Smith et al. 2006). ICP27 will be discussed in more detail later.

ICP4

Similar to ICP27, ICP4 is an essential regulatory protein in all experimental systems (*in vitro* and *in vivo*). The RS1 gene, which is present in two copies in the viral genome, encodes this 175-kDa protein. Multiple functional domains within ICP4 have been identified, e.g. domains for transactivation, dimerisation, nuclear localisation, repression, and DNA binding. It is the major transcriptional regulator of HSV-1 (DeLuca, McCarthy et al. 1985). ICP4 is necessary to transactivate most, if not all, E and L genes (Roizman and Knipe 2001). ICP4 is a transcriptional transactivator for viral genes of every class. The DNA binding domain is absolutely required for this but there is eveidence that if is not sufficient. ICP4 binds DNA even at sequences that are degenerate compared to the consensus binding site. Both the TATA box and initiator (INR) elements are required for ICP4's activation of viral late promoters (Gu and DeLuca 1994; Kim, Zabierowski et al. 2002). Recent evidence has suggested that ICP4 may also regulate chromatin remodelling, giving another avenue for this protein to regulate gene expression (Everett, Sourvinos et al. 2003). ICP4 has also been demonstrated to repress the activity of the latency promoters, either on its own or in combination with ICP0 (Batchelor and O'Hare 1990; Goins, Sternberg et al. 1994). It has been shown that ICP4 is required for efficient HSV-1 reactivation in infected neuronal cells but ICP0 is not required for efficient stress-induced reactivation (Miller, Danaher et al. 2006).

ICP22 and US1.5

ICP22 is non-essential for viral growth but has been demonstrated to promote efficient late gene expression in a cell type dependent manner. The carboxy terminus of ICP22 is also encoded by a second overlapping transcript that has been designated as $U_S1.5$ (Carter and Roizman 1996). Analysis of ICP22 and $U_S1.5$ has shown that viruses containing a deletion of the amino terminal portion of ICP22, i.e. the domains unique to ICP22, are avirulent in experimental animal systems (Ogle and Roizman 1999). Other domains in the amino terminus have also been identified, including a putative nuclear localisation signal and a domain

that interacts with a cellular protein, p78. Domains in the carboxy portion of ICP22 common to both ICP22 and $U_S1.5$ are necessary for optimal expression of a subset of late genes, and virus mutants lacking these domains are also avirulent in animal models of infection. ICP22 has been implicated in the production of a unusually phosphorylated form of cellular RNA polymerase II (Rice, Long et al. 1995; Long, Leong et al. 1999).

ICP47

ICP47 is one of the IE gene that is not essential for replication and is a non-regulatory IE gene (Mavromara-Nazos, Ackermann et al. 1986). It is supposed that it has immunological capacity allowing virus to escape from the host defence. It is known to inhibit antigen presentation to CD8- T lymphocytes, possibly providing a mechanism by which HSV-1 escapes immune surveillance by the host (York, Roop et al. 1994) and HSV-1 modulates antigen processing at the level of the transporters of antigen processing (TAP) (Hill, Jugovic et al. 1995).

1.2.7.2 Early gene products

The next proteins to be made in the gene expression cascade are those, which are encoded by the early genes. It seems that between about 4 to 8 hpi the β proteins are expressed at peak rates. These are made before viral DNA replication, and therefore include enzymes involved in DNA metabolism (such as thymidine kinase [TK] and ribonucleotide reductase [RR]) as well as enzymes, which are involved directly in DNA replication (such as DNA polymerase and DNA helicase). The tk gene, which apparently contains all of the cis-acting regulatory elements sufficient for expression in the eukaryotic cell, is not expressed when present in the viral genome until HSV IE proteins are synthesised. It has become clear from subsequent studies that not only is ICP4 necessary for expression of the tk gene in the viral genome, but also that this transactivation is mediated through the TATA element of the tk promoter (Imbalzano, Coen et al. 1991).

1.2.7.3 Late gene products

Expression of the L genes is activated by the IE genes. They are expressed at peak times after viral DNA synthesis has commenced. These processes are

intimately linked, since inhibition of DNA replication also inhibits L gene expression and prolongs the expression of E genes. These include structural proteins, which are required to form viral particles, as well as VP16 and vhs, which become part of the tegument. Like most of the IE and E promoters, L promoters require a TATA element, and there seems to be no difference between L promoter TATA elements and other viral or eukaryotic TATA elements that account for temporal expression (Steffy and Weir 1991; Imbalzano and DeLuca 1992; Perera 2000). ICP4 is necessary for the activation of L gene expression. Further, it has been shown that the viral regulatory proteins ICP4 and ICP22 co-localise with RNA Polymerase II and viral DNA replication protein late in infection (Leopardi, Ward et al. 1997). The DNA binding protein ICP8 is required for L gene expression. The role of ICP8 in L gene expression has been difficult to separate from its role in DNA replication, since it is one of the viral proteins absolutely required for replication. Some ICP8 mutants inhibit L gene expression to a far greater extent than they inhibit viral DNA replication, suggesting a more direct role in L gene expression (Chen and Knipe 1996; McNamee, Taylor et al. 2000).

1.2.7.4 Assembly and budding

Following the expression of the L genes, the replicated viral DNA is cleaved into genome length units and packaged into the capsids. Capsids then bud through areas of the nuclear envelope that have been modified with viral glycoproteins, thus forming the viral envelope. The newly synthesised virions then pass through the ER and the cytoplasm and into the extracellular space. The observation of partially enveloped virions at cytoplasmic membranes has led to the suggestion that some virions may lose and re-acquire their envelopes during transit through the ER. The first newly formed capsids appear in the nucleus within 6 hrs of infection and the entire lytic cycle takes approximately 10 hrs (Roizman and Knipe 2001; Mettenleiter 2002).

1.2.8 The latent life cycle

HSV-1 enters sensory neurons innervating the cells of the mucosal membranes. It is then transported to the neuronal cell body where it is able to persist in an episomal state for the lifetime of the host. This is characterised by the continued

presence of the viral genome, in the absence of viral gene expression except for latency associated transcripts (LATs) (Stevens, Wagner et al. 1987; Roizman and Knipe 2001). Indeed, in the case of HSV-1, no virally encoded functions are required for establishment of the latent state. Neurons that are latently infected by HSV-1 contain 10 or more viral genomes per cell. There are two suggestions that either several viruses infect each cell or the cellular machinery replicates HSV-1 DNA during the latency period. Roizman and colleagues have shown that the HSV-1 genome contains a host dependent origin of DNA replication (ie, an origin that utilises cellular DNA polymerase) that could support the second idea. The molecular biology of HSV-1 latency remains rather unclear. Only a single viral promoter appears to remain active during latency, and this drives expression of non-coding RNA species that have been designated latency-associated transcripts. The function of these transcripts remains unclear however there is a hypothesis that on LATs function to cause a block to IE expression. Periodic reactivation of HSV-1 can occur, leading to lytic infections in the same dermatomal distribution as the initial infection. Reactivation can occur spontaneously or can be induced by stress and other signals, either directly to the neurons or to the whole organism (Stevens, Wagner et al. 1987; Roizman and Knipe 2001).

1.2.9 Virus host shut off function

HSV-1 induces early and delayed shut-off of host cell protein synthesis. Host shut-off is achieved largely through the complementary actions of two viral proteins, virion host shut-off (vhs) and ICP27. The tegument vhs that is encoded by gene UL 41 is involved in early infection host shut-off. Vhs causes the degradation of pre-existing and newly transcribed mRNA during early infection but in later infection it will be inactived by another viral protein, VP16. However, vhs does not discriminate between viral and cellular mRNAs. It shows nuclease activity and functions as an endoribonuclease in the absence of other cellular or viral proteins. Two distinct pathways defined for global shutoff include: decline in the level of host mRNA and alteration of the function of the host translational apparatus by interaction with eIF4H and eIF4B (Smibert, Popova et al. 1994; Karr and Read 1999; Doepker, Hsu et al. 2004; Taddeo and Roizman 2006; Taddeo, Zhang et al. 2006). ICP27 is the second factor that is implicated in host shutoff by inhibiting

cellular mRNA biogenesis through preventing pre-mRNA splicing and may have a direct effect on translation (Doepker, Hsu et al. 2004; Larralde, Smith et al. 2006).

1.3 The HSV-1 ICP27 protein

1.3.1 General features

ICP27 (IE63) is an immediate early (IE), multifunctional regulatory protein of 512 amino acids, which is required for HSV-1 productive infection. This 63 KDa phosphoprotein is essential for appropriate expression of viral early and late gene products and contributes to the shutoff of host protein synthesis. It has counterparts in every herpesvirus sequenced and is a potential antiviral target. ICP27 acts at both transcriptional and posttranscriptional levels (Figure 1.6) and is an RNA binding protein that inhibits pre-mRNA splicing, stimulates pre-mRNA 3' processing, affects mRNA stability, shuttles between the nucleus and the cytoplasm and promotes viral RNA nuclear export. ICP27 directly stimulates mRNA translation, interacts with various cellular proteins including, export factor REF and cellular mRNA export receptor TAP to export mRNA (Dai-Ju, Li et al. 2006; Larralde, Smith et al. 2006).

1.3.2 Transcriptional properties of ICP27

It is appears that ICP27 contributes to the transcriptional regulation of HSV-1 early and late genes (Uprichard and Knipe 1996; Jean, LeVan et al. 2001). It has been shown that ICP27 mutant viral infections severely reduced accumulation of early and late gene products (Rice, Long et al. 1995; Jean, LeVan et al. 2001; Lengyel, Guy et al. 2002). However, the mechanism by which ICP27 stimulates transcription has not been fully elucidated. ICP27 has a role in recruiting cellular RNA polymerase II (RNAP II) to sites of HSV-1 transcription by binding to the C-terminal domain of RNAP II. In cells infected with ICP27 mutant that do not interact with RNAP II, levels of the viral transcripts are reduced in comparison to wild type infection. Thus, ICP27’s role in transcription may be linked to its role in

posttranscriptional regulation (Dai-Ju, Li et al. 2006). There is certain evidence that ICP27 interacts directly with ICP8 and plays a role in the stimulation of late gene transcription (Olesky, McNamee et al. 2005).

1.3.3 Posttranscriptional properties of ICP27

Posttranscriptional activities of ICP27 are revealed below.

1.3.3.1 ICP27 inhibits splicing of host cell mRNAs

At the early stage of HSV-1 infection ICP27 is involved in the inhibition of pre-mRNA splicing (Hardwicke and Sandri-Goldin 1994; Hardy and Sandri-Goldin 1994) and has been shown to inhibit splicing in the absence of other viral factors (Lindberg and Kreivi 2002). In contrast with cellular mRNAs, most HSV-1 mRNAs are intronless except four that are intron containing and require splicing. This down regulation ability of ICP27 on splicing contributes to host cell protein synthesis shut-off (Sacks, Greene et al. 1985). However, the mechanism of splicing inhibition is not yet understood but interaction of ICP27 with various cellular proteins that are involved in splicing has been demonstrated. ICP27 interacts with small nuclear ribonucleoprotein particles (snRNPs) and co-localises with redistributed snRNPs that are directly involved in splicing reactions. This interaction is not sufficient to inhibit splicing (Martin, Barghusen et al. 1987; Phelan, Carmo-Fonseca et al. 1993; Sandri-Goldin, Hibbard et al. 1995; Sandri-Goldin and Hibbard 1996; Nilsen 2003). ICP27 interacts with different pre-mRNA processing factors including SR proteins (splicing regulatory factors) SRPK1 (SR protein kinase 1), p32 (an inhibitor of the SF2/ASF SR protein), SAP145 (spliceosome-associated protein 145, an essential splicing complex assembly factor) and mediates inhibition of splicing that could be significant part of host shut-off. The mechanism and functions of these interactions is under investigation (Gui, Tronchere et al. 1994; Bryant, Matthews et al. 2000; Graveley 2000).

1.3.3.2 ICP27 increases RNA 3' processing at weak poly (A) sites of HSV-1 mRNAs

The 3′ ends of almost all eukaryotic mRNAs and their precursors consist of homopolymeric tails of adenosine, or poly (A) that are added by poly (A) polymerase (PAP) during the process of 3′ end formation. Experimental studies demonstrated expression of ICP27 alone increases the usage of certain poly (A) sites, specifically the late genes, to stimulate RNA 3' processing (McLauchlan, Simpson et al. 1989; Sandri-Goldin and Mendoza 1992). Further, a UV cross-linking study of protein-RNA interactions showed a direct role of ICP27 in enhancing the binding of the polyadenylation factor (CstF) to viral RNA poly (A) sites from all temporal classes. CstF acts to promote and stabilise polyadenylation complex formation and virus infection in the absence of ICP27 resulted in lost binding enhancement of CstF. ICP27-induced stimulation of 3' end processing was observed in certain late genes with weak poly (A) sites but not for a number of IE and E transcripts with strong poly (A) sites (McGregor, Phelan et al. 1996).

1.3.3.3 Nucleocytoplasmic properties of ICP27

The association of ICP27 with redistributed snRNPs on intron-containing transcripts suggested an involvement of this multifunctional protein in regulation of nucleocytoplasmic RNA shuttling (Phelan, Dunlop et al. 1996). Many studies showed ICP27 shuttles between the nucleus and cytoplasm and has a direct role in nuclear export of viral RNAs. This is via its binding with intronless viral and cellular mRNAs (Phelan and Clements 1997; Soliman, Sandri-Goldin et al. 1997; Sandri-Goldin 1998; Koffa, Clements et al. 2001). It has been shown that two motifs of ICP27, an RGG box and a leucine-rich region nuclear export signal (NES) are putatively involved with RNA binding and export (Sandri-Goldin 1998; Lengyel, Guy et al. 2002). Experimental studies by using leptomycin B (LMB) that inhibits the CRM 1 export pathway and ICP27 mutants demonstrated these regions are required but not essential for nucleocytoplasmic transport of RNAs. So there must be pathways for export of viral RNAs other than the CRM 1 pathway(Phelan and Clements 1997; Soliman and Silverstein 2000; Koffa, Clements et al. 2001; Chen, Sciabica et al. 2002; Lengyel, Guy et al. 2002; Chen, Li et al. 2005; Thiry, Muylkens et al. 2005). The main cellular mRNA export pathway is nuclear export

receptor (TAP) mediated that associates with an RNA-binding protein REF, a component of the exon junction complex. It has been shown that ICP27 binds to viral mRNAs and interacts directly with REF as an adaptor protein and indirectly recruits TAP to viral mRNAs to stimulate their nuclear export through this cellular pathway (Koffa, Clements et al. 2001; Chen, Sciabica et al. 2002; Sandri-Goldin 2004). However, certain significant information about this model is unclear. A moderate effect of RGG box and REF binding site ICP27 mutants on viral gene expression, transport by the CRM 1 dependent pathway and export of some transcripts in the absence of ICP27 whose overall expression is ICP27 dependent show the TAP pathway is not the only way for export of viral RNAs (Soliman and Silverstein 2000; Lengyel, Guy et al. 2002; Pearson, Knipe et al. 2004). On the other hand, the role of the KNS domain of ICP27 in transport in not yet clarified. In addition, it is though that the tegument protein VP13/14 that contains a NES like element and shuttles may also be able to transport RNAs (Michael, Eder et al. 1997; Donnelly and Elliott 2001).

1.3.3.4 Translation properties of ICP27

A potential role of ICP27 in viral/host mRNA stability and translation was demonstrated by investigation of the role of this protein in the cytoplasm. By direct or indirect effect on translation, HSV-1 wild type ICP27 stimulates levels of the HSV-1 VP16 virion protein and polysomal association of the VP16 RNA (Ellison, Maranchuk et al. 2005). ICP27 interacts with two translation initiation factor, eIF-3G and eIF-4G as well as poly A binding protein 1 (PABP1) and shows promoting or inhibiting activity on translation via these interactions (Fontaine-Rodriguez, Taylor et al. 2004). A recent study in our laboratory showed ICP27 stimulates translation directly and this activity correlates with its ability to associate with polyribosomes in HSV-1 infected cells. So the C-terminal domain of ICP27 is essential to stimulate mRNA translation (Larralde, Smith et al. 2006).

1.3.4 ICP27 and phosphorylation

Different phosphorylation sites on ICP27 are suggested and on SDS-PAGE two forms of ICP27 could be detectable and up to 5 species by 2D isoelectric focusing (Pereira, Wolff et al. 1977). The only amino acid in ICP27 that is phosphorylated

during infection and transfection is serine. It has been shown serine 114 is highly phosphorylated by protein kinase A (PK A). But the role of phosphorylation of ICP27 in infection is unclear (Zhi and Sandri-Goldin 1999). ICP27 interacts with casein kinase 2 (CK2) and stimulates CK2 activity during infection and redistributes the holoenzyme from the nucleus to the cytoplasm (Wadd, Bryant et al. 1999; Koffa, Kean et al. 2003).

1.3.5 Putative functional domains of ICP27

Mutation studies on different regions of the 512 amino acid ICP27 protein characterised various functional sites on this protein at the N and C terminal regions (Figure 1.7).

1.3.5.1 Essential acidic domain

Amino acids 4-64 at the amino terminus of ICP27 that are present in all ICP27 homologues (Figure 1.7) contain a high concentration of acidic residues. Glutamic and aspartic acids are the two main amino acids in this region. Deletion of this domain (d1-2) suggested an essential function in viral lytic replication. This mutant is deficient for growth, defective in viral replication, shows modest decrease in late gene expression and a delay in the repression of early genes. This ICP27 mutant can repress the expression of the immediate early protein ICP4 and also some late proteins, such as ICP15 (Rice, Lam et al. 1993).

1.3.5.2 Nuclear export signal (NES)

The nuclear export signal (NES) sequence is located in the acidic region to amino acid 3-17 (Figure 1.7) and resembles a leucine-rich NES as identified in the HIV-1 Rev protein (Fischer, Huber et al. 1995). Resemblance of ICP27 NES to the HIV-1 Rev NES was shown by the relocation of ICP27 to the cytoplasm after replacing the endogenous NES with that of Rev. Also mutation of the ICP27 NES gave nuclear retention of ICP27. Similar to the Rev protein NES, in ICP27 the NES is enough to export a heterologous protein (Sandri-Goldin 1998). The viral ICP27 NES mutant is named dLeu and has similar phenotype to d1-2. It is deficient for growth and shows a decline in virus yield compared with HSV-1 wild type infection.

Both dLeu and d1-2 encode mostly nuclear ICP27. This localisation is considerably different from wild type ICP27, They are more active in protein synthesis compare to on ICP27 null mutant. These studies showed the NES and acidic domain mutants can significantly stimulate several ICP27-dependent genes *in vivo* suggesting they are not totally essential for virus replication (Lengyel, Guy et al. 2002).

1.3.5.3 Export control sequence (ECS)

Residues 31-34 in the acidic domain of ICP27 (Figure 1.7) negatively regulate the activity of the NES and are named the putative export control sequence (ECS) (Soliman and Silverstein 2000). On the other hand, a mutation of 50 aa in the acidic domain confers viral resistance to LMB, an inhibitor of the CRM-1 nuclear export pathway (Murata, Goshima et al. 2001). It has been suggested that this mutation can promote nuclear export by a negative effect on the role of the ECS (Soliman and Silverstein 2000; Murata, Goshima et al. 2001).

1.3.5.4 Nuclear and nucleolar localisation signals (NLS & NuLS)

Nuclear localisation of ICP27 is mediated mainly through a bipartite NLS, mapped at aas 110-137. Mutation at these sites is termed the d3-4 ICP27 mutant (Figure 1.7). It has been shown that there are several weaker NLSs corresponding to aas 152-512 in the C-terminus of ICP27 as infection with d3-4 and d1-5 viral mutants demonstrated predominant nuclear localisation of ICP27 (Mears, Lam et al. 1995). Also ICP27 contains a nucleolar localisation signal (NuLS) mapped to aas 110-152 composed of a NLS and an arginine-rich sequence (138-152). It was suggested to interact with the cell nucleoli and infection of cells by d4-5, d1-5, and to some extent the d3-4 ICP27, failed to localise protein efficiently to the nucleoli (Lengyel, Guy et al. 2002). This data is consistent with the idea that the RGG box (138-152) forms part of the ICP27 NuLS (Mears, Lam et al. 1995).

1.3.5.5 RNA-binding domains: RGG box and KH-like domains

The RGG box is the structural domain of ICP27 that interacts with RNA (Figure 1.7) (Mears and Rice 1996). ICP27 interacts directly with RNA (Ingram, Phelan et

al. 1996) and has been shown bind to intronless HSV-1 RNAs (Sandri-Goldin 1998). A broad range of HSV-1 mRNAs binds to ICP27 through its RGG box (Sokolowski, Scott et al. 2003). This region is rich in R and G residues and posttranscriptionally modified by methylation (Mears and Rice 1996). KH domains that were identified firstly in hnRNP K as a triple repeat (Siomi, Matunis et al. 1993) are found in the C-terminal half of ICP27 and mapped as KH1 (aa 288-350), KH2 (368-421) and KH3 (451-512). These three RNA binding motifs recognised in ICP27 have homology to a series of hnRNP K domains (KH) based on limited sequence similarity. They are conserved in *alphaherpesvirinae* suggesting their functional importance (Soliman and Silverstein 2000). Several ICP27 mutants have been made such as M11, M15 and M16 and these affect the function of the KH domains. These mutants have nuclear localisation and their shuttling abilities are reduced significantly, while M15 cannot shuttle between the nucleus and the cytoplasm (Mears and Rice 1998). Further studies showed the M15 mutant impairs binding of ICP27 with certain cellular partners and decreases the interaction of HSV-1 RNAs with ICP27 *in vivo* (Bryant, Wadd et al. 2001; Sokolowski, Scott et al. 2003; Chen, Li et al. 2005).

1.3.5.6 Putative transport sequence (SADET)

There is a nucleocytoplasmic shuttling signal (KNS) located in the hnRNP K protein (Figure 1.7) (Michael, Eder et al. 1997) that has been suggested to mediate export of RNA via a CRM1-independent pathway (Henderson and Eleftheriou 2000). A sequence of ICP27 between the KH2 and KH3 domains (SADET aa 350-354) shows some similarity to KNS and is well conserved among alpha herpesvirus homologue proteins (Figure 1.8).

1.3.5.7 Putative zinc finger, activator and repressor region

In vitro interaction of the C-terminus (aa 407-512) domain of ICP27 with zinc identified a new function of this protein (figure 1.7) (Vaughan, Thibault et al. 1992). Putative zinc fingers are implicated in protein-protein interactions (Fox, Kowalski et al. 1998; Mackay and Crossley 1998). This ability of zinc fingers to interact is shown by self-interaction of ICP27 through these motifs (Wadd, Bryant et al. 1999; Zhi, Sciabica et al. 1999), binding to spliceosome-associated protein (SAP) 145

(Bryant, Wadd et al. 2001), splicing factor SRp20 (Sciabica, Dai et al. 2003), nuclear export factor TAP (Chen, Li et al. 2005), casein kinase 2 (CK2) (Wadd, Bryant et al. 1999), RNA Polymerase II (Dai-Ju, Li et al. 2006) and poly (A) binding protein 1(Larralde, Smith et al. 2006). Thus the zinc finger domain is involved in gene activation by correlation with increasing polyadenylation and by gene repression by inhibition (Hardwicke, Vaughan et al. 1989; McMahan and Schaffer 1990; Chapman, Harris et al. 1992; McLauchlan, Phelan et al. 1992; Sandri-Goldin and Mendoza 1992; Hardwicke and Sandri-Goldin 1994). It has been suggested that ICP27 self-recognition is important for its function because point mutation of the zinc finger domain was lethal to the virus (Zhi, Sciabica et al. 1999).

1.3.5.8 SM domain

Embedded between and overlapping the KH3 domain in the C-terminal half of ICP27 (aas 426-488) is a putative SM domain that is conserved among the homologues (Figure 1.7). These proteins are involved in forming the core splicosome complex through protein-protein interaction (Kambach, Walke et al. 1999; Soliman and Silverstein 2000). In comparison to wt, HSV-1 ICP27 lacking the SM motif is deficient for viral replication, delayed in host shut-off, shows a decrease in the accumulation of IE and E protein and a delay in the appearance of L proteins. Importantly, there is a defect in host cell shut off during infection with HSV-1 ICP27 lacking the SM motif (Soliman and Silverstein 2000).

1.3.5.9 Extreme C-terminal GKYF sequence

An extreme C-terminal GKYF motif (aa 504-507) that is conserved throughout ICP27 herpesvirus homologues has a role in interaction with snRNPs (Figure 1.7). Mutation of a glycine in amino acid 504 of ICP27 led to loss of this interaction and indicated a role in protein-protein interaction (Sandri-Goldin and Hibbard 1996).

1.4 ICP27 homologues

The only HSV-1 immediate early gene, which has homologues throughout the *Herpesviridae* family, is ICP27 (Table 1.3). Although there is sequence similarity between ICP27 homologues, they show diversity in their function. The amino termini of herpesvirus ICP27 homologues are not conserved and contain acidic and basic residues however the central region is conserved within the *herpesviridae*.

1.4.1 Bovine herpesvirus 1 (BHV-1) ICP27 (BICP27)

BHV-1 ICP27 is homologue of HSV-1 ICP27 that localises in the nucleus and is expressed in the early stage of infection in contrast to HSV-1 ICP27. But like HSV-1 ICP27 it is involved in promotion of processing efficiency of mRNAs containing weak poly (A) sites (Singh, Fraefel et al. 1996).

1.4.2 Human cytomegalovirus UL69 (HCMV UL69)

The multifunctional nuclear phosphoprotein UL69 of β herpesvirus HCMV is a homologue of HSV-1 ICP27 but in contrast it is located in the virion and expressed during E and L stags of infection. It has a role in transactivating several viral and cellular promoters and functions as a nuclear shuttling protein. Unlike HSV-1 ICP27, RNA binding is not a prerequisite for UL69-mediated export (Winkler, Rice et al. 1994; Winkler and Stamminger 1996; Toth, Lischka et al. 2006).

1.4.3 Varicella zoster virus (VZV) ORF4

VZV ORF4 is an IE protein that is essential for replication of the virus and shuttles between the nucleus and cytoplasm similar to its homologue ICP27. ORF4 is present in the virion tegument and its RNA and protein are detected in latently infected human ganglia. These two proteins have considerable amino acid sequence homology and certain similar properties such as self-interaction, RNA binding for nuclear export, CRM 1 dependent pathway and KNS homology

(Moriuchi, Moriuchi et al. 1994; Baudoux, Defechereux et al. 2000; Cohen, Krogmann et al. 2005).

1.4.4 Human herpesvirus 8 (HHV-8) ORF57

HHV-8 ORF57 is an essential regulatory protein. It is expressed from one of the few spliced mRNAs of HHV-8 in the lytic cycle and is homologous to ICP27. It localises in the nucleus and shuttles between the nucleus and cytoplasm using CRM1-dependent and cellular export TAP pathways. A number of ICP27 partner proteins also interact with ORF57 including hnRNP K, CK2 and REF (Bello, Davison et al. 1999; Lukac, Kirshner et al. 1999; Malik, Blackbourn et al. 2004; Malik, Blackbourn et al. 2004; Malik and Clements 2004).

1.4.5 Herpesvirus saimiri (HVS) ORF57

The multifunctional regulatory protein ORF57 is involved in activation and repression of E and L gene expression by its C-terminal domain. It has limited homology with ICP27. Similar to ICP27, HVS ORF57 shuttles between the nucleus and cytoplasm, binds to viral RNA and exports it. It has been demonstrated that ORF57 binds to importin-α as an adaptor by its NLS and importin-β as a transport receptor, which is important for efficient viral RNA export (Whitehouse, Cooper et al. 1998; Goodwin, Hall et al. 2000; Goodwin and Whitehouse 2001).

1.4.6 Epstein- Barr virus (EBV) SM protein

EBV SM protein has about 30% homology with ICP27 protein and transactivates expression of intronless reporter gene and transrepresses intron containing genes. It is essential for virion production. SM protein binds to RNA *in vitro* and *in vivo* and shuttles between nucleus and cytoplasm. Conflicting reports showed SM protein export mediated via CRM 1 or TAP pathways (Cook, Shanahan et al. 1994; Ruvolo, Sun et al. 2004; Swaminathan 2005).

1.5 ICP27 partner proteins

ICP27 binds to several cellular proteins (summarised in Table 1.4) that are involved in its functions.

1.5.1 hnRNP K

Heterogeeous ribonucleoprotein K is a multifunctional protein with three K-homology domains (KH) that mediate DNA and RNA binding and contains both nuclear localisation (NLS) and nuclear shuttling domains (KNS). Based on these domains it was supposed that the hnRNP K protein is implicated in multiple steps of gene expression including transcription, RNA splicing and translation (Matunis, Matunis et al. 1992; Dejgaard, Leffers et al. 1994; Bomsztyk, Denisenko et al. 2004). hnRNP K co-immunoprecipitated with anti-ICP27 antibody and interaction of ICP27 with this protein was confirmed by yeast-two hybrid system (Y2-H) (Wadd, Bryant et al. 1999). The SADET sequence is perfectly conserved between KNS and ICP27 suggesting the possibility of utilisation of the same transport pathway and direct interaction with nucleoprotein complexes (Michael, Eder et al. 1997).

1.5.2 RNA export adaptor protein (REF)

REF is a part of the exon junction complex (EJC) laid down on mRNAs during splicing and involved in nonsense-mediated decay (NMD) (Gatfield, Le Hir et al. 2001). This protein shuttles between the nucleus and the cytoplasm and binds directly to both mRNA and TAP (Rodrigues, Rode et al. 2001). REF interacts with ICP27 (Chen, Sciabica et al. 2002). ICP27 recruits REF to intronless viral RNAs, indirectly recruits TAP and stimulates viral mRNAs nuclear export through a cellular mRNA export pathway (Koffa, Clements et al. 2001).

1.5.3 RNA export receptor protein TAP/NXF1

TAP is a 619-aa cellular protein that is a potential export receptor for cellular mRNA. It shuttles between nucleus and cytoplasm and associates with p15 as a cofactor that is crucial for mRNA export by TAP. The association of FG repeat

nucleoporins with TAP has been identified. TAP interacts with NUP 62, NUP 98, NUP 153 and NUP 214/CAN. It has been demonstrated TAP interacts with a constitutive transport element (CTE) that is present in the RNAs of type D retroviruses and has a role in mRNA export (Gruter, Tabernero et al. 1998; Bear, Tan et al. 1999; Bachi, Braun et al. 2000; Tan, Zolotukhin et al. 2000; Braun, Herold et al. 2001; Katahira, Straesser et al. 2002; Herold, Teixeira et al. 2003). TAP interacts with ICP27 and the C-terminus of ICP27 is required for this binding (Chen, Li et al. 2005). ICP27 recruits TAP to viral mRNAs and utilises the TAP pathway to export viral mRNAs (Koffa, Clements et al. 2001).

1.5.4 Casein kinase 2 (CK2)

CK2 is known to phosphorylate more than 200 proteins and is involved in processes such as transcriptional control, apoptosis and cell cycle regulation (Dobrowolska, Lozeman et al. 1999). ICP27 has been shown bind to CK2 β subunit by the C-terminus zinc finger and the arginine rich part of ICP27 using immunoprecipitation and Y2-H assay (Wadd, Bryant et al. 1999). Interaction of ICP27 with CK2 stimulates activity of ICP27 at early times of infection and redistributes the CK2 from the nucleus to the cytoplasm (Koffa, Kean et al. 2003).

1.5.5 Spliceosomal associated protein 145 (SAP 145)

SAP145 is one of the essential components of the spliceosome that binds to pre-mRNA. SAP145 interacts with ICP27 and causes host cell shut off by inhibiting splicing (Champion-Arnaud and Reed 1994; Bryant, Wadd et al. 2001).

1.5.6 p32

p32 was first isolated as a protein tightly associated with ASF/SF2 purified from HeLa cells (Krainer, Conway et al. 1990). p32 regulates RNA splicing by inhibiting ASF/SF2 RNA binding and phosphorylation (Petersen-Mahrt, Estmer et al. 1999). The p32 that co-immunoprecipitated with ICP27 is phosphorylated by CK2, which is precipitated with ICP27. Immunofluorescence studies showed that colocalisation of ICP27 with p32 alters the distribution of p32 to the nucleus. It seems ICP27 can recruit p32 for splicing inhibition (Bryant, Matthews et al. 2000).

1.5.7 Cellular RNA polymerase II (RNAP II) and ICP8

Cellular RNAP II transcribes all viral genes and ICP8 is a single strand DNA binding protein that down regulates transcription from the parental viral genome (Godowski and Knipe 1986).Complex formation of ICP27, ICP8 and RNAP II has been demonstrated (Zhou and Knipe 2002).The direct ICP27-ICP8 interaction plays a role in the stimulation of late gene transcription (Olesky, McNamee et al. 2005). Herpes simplex virus 1 (HSV-1) ICP27 has been shown to interact with the C-terminal domain (CTD) of RNAP II and ICP27 mutants that cannot interact fail to relocalise RNAP II to viral transcription sites, suggesting a role for ICP27 in RNAP II recruitment (Dai-Ju, Li et al. 2006).

1.6 Nucleocytoplasmic transport

1.6.1 General feature

All nucleocytoplasmic transport of macromolecules between the cytoplasm and the nucleus is mediated by at least three different classes of soluble transport receptors. These include members of the importin-β protein family (the importins and exportins, also termed karyopherins), the TAP/Mex67 family that contribute in export of mRNA and the small nuclear transport factor 2 (NFT2), which imports the small GTPase Ran into the nucleus. Cargoes shuttle between the cytoplasm and the nucleus via the nuclear pore complex (NPC). All nuclear transport factors can bidirectionally traffick through the nuclear pore complex by specific interactions with phenylalanine/glycine (FG)-rich nuclear pore complex components called nucleoporins (NUPs) (Conti and Izaurralde 2001; Kuersten, Ohno et al. 2001; Macara 2001; Weis 2002). Trafficking of cargos via the NPC is carried out by mobile transport machinery. These interaction with the NPC components which is highly selective and consume energy, could be regulated by signalling pathways and viruses (Fontoura, Faria et al. 2005).

1.6.2 Protein transport

In general proteins containing nuclear localisation signal (NLS) or nuclear export signal (NES) bind to transport receptors termed karyopherins (kaps, importins, exportins or transportins) and make a complex of transporter and cargo that dock to nucleoporins and translocates from the nuclear or cytoplasmic side of nucleopore complex (NPC). Then a small GTPase, Ran, plays a main role to interact with karyopherins and triggers the dissociation of import complexes or stabilises the assembly of export complexes to exit the nucleus (Figure 1.9) (Cole and Hammell 1998; Suntharalingam and Wente 2003; Weis 2003).

1.6.3 RNA export

mRNAs are transcribed from DNA in eukaryotic cells as pre-mRNA which is then processed to mature mRNA before being translated into protein in the cytoplasm. RNA polymerase II is the enzyme that is involved in pre-mRNA synthesis in the nucleus. Pre mRNA is processed by capping at the 5' end cleavage and polyadenylation to form the 3' end. Concomitant with these processes, splicing action place to remove non-coding intervening sequences. For translation and protein synthesis in the cytoplasm the processed mRNAs should be exported through the nuclear pore complex (NPC). In fact, traffick of mRNAs from the nucleus to the cytoplasm requires different steps including processing, packaging, recognition by export factors and accomodation through the NPC (Chen, Sciabica et al. 2002). Biochemical and genetic approaches have shown that three classes of factors are implicated for mRNA export: the adapter proteins, which bind to the mRNA directly, receptor proteins that recognise and interact to adapter proteins, and nucleoporins (NUPs) that mediate translocation and shuttling across the nuclear membrane (Komeili and O'Shea 2001; Zenklusen and Stutz 2001). There are two main export pathways identified for mRNA transport, CRM1 and TAP. CRM1 export receptor (exportin 1) is involved in export by recognition of the NES, however it is not the major contributor to mRNA export in metazoans or yeast and exports only a small subset of mRNA. CRM1 also mediates nuclear export of proteins, snRNAs and rRNAs (Fischer, Huber et al. 1995; Fornerod, Ohno et al. 1997; Cullen 2000; Weis 2003). The best candidate for mRNA export could be

TAP, a cellular receptor protein that shuttles between the nucleus and cytoplasm. It can be cross-linked to poly (A) RNA, is localised at the nuclear pores, and interacts directly with the nucleoporins. It has been shown to have a direct role in mRNA export (Figure 1.10 and table 1.6) (Segref, Sharma et al. 1997; Santos-Rosa, Moreno et al. 1998; Kang and Cullen 1999; Herold, Suyama et al. 2000; Braun, Herold et al. 2001).

1.6.4 Viral interaction with the nuclear transport machinery

Use of viruses is an invaluable tool in discovery of nuclear transport routes. Viruses, especially RNA viruses, need to control the transport machinery for successful replication in hosts. Disruption of the transport system is part of the cytopathogenic ability of viruses to prevent the host antiviral response (Fontoura, Faria et al. 2005). Poliovirus infection inhibits nuclear import of cellular proteins by targeting the karyopherinα/β1 and karyopherinβ2 pathways which are usually utilised for protein import. Thus poliovirus infection acts by degradation of certain nucleoporins such as NUP 153 and NUP 62 that result in disruption of the nuclear pore complex structure (Gustin and Sarnow 2001). The next example is the Vesiculoviral matrix protein M (VSV M) that induces a disruption in cellular mRNA export by interacting with the Rae1. Rae1 is the mRNA export factor that is essential for successful export of cellular mRNA via binding to nucleoporin 98 (Faria, Chakraborty et al. 2005). In hepatitis C, disruption of the karyopherin β3 pathway is a target for protein 5A that is suggested to interact with karyopherin β3 and inhibits host gene expression (Chung, Lee et al. 2000). In the case of DNA viruses, in human subgroup C adenovirus, two important proteins E1B and E4 Orf6 function at least in the selective export of viral mRNAs. They could block the export of cellular mRNAs and induce of viral mRNA export (Flint and Gonzalez 2003). Finally, ICP27 and its homologues in some herpesviruses cellular pathway TAP that interacts with Aly/REF and recruits viral mRNA to translocate in NPC and export it to cytoplasm. It has been shown that ICP27 does not interfere with export of cellular mRNAs, UsnRNAs or tRNAs (Koffa, Clements et al. 2001; Chen, Sciabica et al. 2002; Chen, Li et al. 2005).

1.6.5 ICP27 and viral mRNA export

Most of HSV-1 transcripts are intronless and multifunctional ICP27 is involved in the export of viral mRNAs. At later time during infection, ICP27 shuttles between the nucleus and cytoplasm. The RGG box domain is involved with viral intronless mRNA binding and the three KH-like domains are involved in RNA binding affinity and specificity (Mears and Rice 1996; Sandri-Goldin 1998; Soliman and Silverstein 2000). ICP27 binds to splicing related (SR) proteins as well as showing a direct interaction with other spliceosomal components including Aly/REF as a RNA export adaptor (Bryant, Wadd et al. 2001; Koffa, Clements et al. 2001; Chen, Sciabica et al. 2002; Sciabica, Dai et al. 2003). Amino acids 104-138 of ICP27 that have an overlap with the NLS are involved with REF interaction showing this domain is important for import of ICP27 to the nucleus and export of ICP27-bound viral RNAs to the cytoplasm. A direct role of Aly/REF in HSV-1 RNA export and and its stimulation by ICP27 has been demonstrated (Koffa, Clements et al. 2001; Chen, Sciabica et al. 2002). Recent experiments showed Aly/REF is dispensable for mRNA export in some organisms (Gatfield and Izaurralde 2002; Longman, Johnstone et al. 2003) and it is certainly possible that there is involvement of other adaptor proteins in HSV-1 RNA export (Sandri-Goldin 2004). Although the C-terminal domain of ICP27 has been shown to interact with TAP/NXF1 and this protein is required for ICP27 export, ICP27 does not facilitate export of viral intronless RNAs directly via TAP/NXF1 (Chen, Sciabica et al. 2002; Chen, Li et al. 2005). In contrast ICP27 interacts with REF, recruits TAP to viral mRNAs and stimulates their nuclear export via a cellular mRNA export pathway (Koffa, Clements et al. 2001). The TAP transport pathway is the major route for HSV-1 mRNA export to the cytoplasm but some recent evidence indicates the possibility of ICP27 independent HSV-1 RNA export and other export factors may have a role to play (Sandri-Goldin 2004) (Figure 1.11).

1.7 Nuclear pore complex (NPC)

1.7.1 General features and function

NPCs are very large protein complexes; surrounded by the two membranes of the nuclear envelope and stably anchored in the nuclear envelope. This structure is conserved in all eukaryotes (Figure 1.12). Each vertebrate NPC is an approximately 120 MDa structure consisting of about 30 different proteins termed nucleoporins (NUPs) (Table 1.5). It serves as gateways of communication between the nucleus and cytoplasm. Most NUPs are present in multiple copies per NPC and are located symmetrically on both faces and a few are on either the nuclear or cytoplasmic faces asymmetrically (Fahrenkrog and Aebi 2003; Rout, Aitchison et al. 2003; Fahrenkrog, Koser et al. 2004). The essential role of NUPs is providing docking sites for soluble transport receptors, which mediate the translocation of proteins and RNA cargoes through the nuclear pore complex. It has been shown that the central gated channel of the NPC is very stable, whereas most of peripheral components in the nuclear and cytoplasmic faces exhibited more dynamic behaviour that are consistent with their function as a structural scaffold, and adaptor as well as certain regulatory functions (Figure 1.13) (Rabut, Doye et al. 2004). Typically NUPs are organised in distinct subcomplexes (Lim and Fahrenkrog 2006). One-third of the identified vertebrate and yeast nucleoporins termed FG-repeats NUPs, are directly associated in binding to transport receptors (Stewart, Baker et al. 2001). The FG-repeats often are part of larger motifs, such as GLFG or FxFG. The FG-repeats usually are clustered in FG-rich regions, in which the individual hydrophobic repeats are separated by a hydrophilic linker (Figure 1.14). It has been shown that the FG-rich regions of some bacterially expressed recombinant nucleoporins are flexible and largely unstructured (Bayliss, Littlewood et al. 2000; Denning, Uversky et al. 2002). In addition to binding assay studies, X-ray crystallography has shown interactions between nuclear transport factors and FG repeat nucleoporins. Involvement of FG-rich nucleoporin fragments in complex with the N-terminal fragment of importin-β (Bayliss, Littlewood et al. 2000; Bayliss, Littlewood et al. 2002), the Ran nuclear import factor NTF2 (Bayliss, Leung et al. 2002), and the NTF2 like domain of TAP/NXT1 has been demonstrated (Fribourg, Braun et al. 2001). In all three structures, the interactions

appear to be primarily hydrophobic. These interactions are mediated by the phenylalanine of the FG-repeat, which is inserted into a hydrophobic pocket. In the case of TAP, the interaction of NUPs with TAP/NXT1 is mediated by a single phenylalanine of the FG sequence inside a hydrophobic pocket of the NTF2 like domain of TAP. Interestingly, both importin-β and TAP have a second nucleoporin binding region in their C-terminal part that is important for their transport activity (Bednenko, Cingolani et al. 2003). FG-repeat NUPs are natively unfolded and this property may be vital for fast transport rates through the NPCs. This form of the FG-NUP structure is postulated to pose a substantial barrier to nonreceptor cargo while providing for a high FG-density interaction zone for receptor-mediated cargo (Lim, Aebi et al. 2006; Lim and Fahrenkrog 2006). Indeed, FG-repeat regions exhibit a high degree of flexibility and mobility (Paulillo, Phillips et al. 2005), with on ability to simultaneous interact with several binding partners (Allen, Huang et al. 2001). They also promote fast molecular association and dissociation rates to facilitate transport of the proteins and RNAs via NPCs (Gilchrist, Mykytka et al. 2002). (Figure 1.14 & 1.15).

1.7.2 Nucleoporin 214/CAN

The vertebrate nucleoporin 214/CAN is a FG-repeat NUP that is located in the cytoplasmic face of the NPC (Figure 1.13). This putative oncogene NUP has three domains: the N-terminal domain of NUP 214/CAN localises to the cytoplasmic face of the NPC, the central domain associate with NUP 88 that binds to NUP 98, a mobile NUP that shuttles between nucleus and cytoplasm, and is located at the nuclear ring of the NPC near the entry to the central pore, and the C-terminal FG-repeat domain that localises in both the nuclear and cytoplasmic faces of the NPC. The multiple location of the C-terminus indicates a flexible property of the NUP 214/CAN FG-repeat domain (Paulillo, Phillips et al. 2005).The C-terminal domain of NUP 214 interacts with various transport factors and has been shown to be involved in both nuclear import and export. The importance of this NUP is shown by genetic depletion of NUP 214 in mice that causes early embryonic death. In fact, reduction in NLS-mediated protein import, and strong nuclear poly (A) RNA accumulation suggests that NUP 214 is vital for NPC function and survival (van

Deursen, Boer et al. 1996; Fornerod, van Deursen et al. 1997; Rollenhagen, Muhlhausser et al. 2003) (Figures 1.15 and 1.14).

1.7.3 Nucleoporin 153

NUP 153 is one of the large, 1476 amino acid FG-repeat nucleoporins that is located at the nuclear basket (Figure 1.13) (Pante, Bastos et al. 1994) and has a high degree of mobility and flexibility in its FG-repeat domain. It is subdivided into three distinct domains: an N-terminal domain that is anchored to the nuclear ring (Walther, Fornerod et al. 2001; Fahrenkrog, Maco et al. 2002), a zinc finger domain in centre that resides at the distal ring and a C-terminal domain with approximately 700 amino acids that contain about 40 FG-repeats with high flexibility within the NPC. This C-terminus domain can be detected at the nuclear basket and even at the cytoplasmic periphery of the central pore (Fahrenkrog, Maco et al. 2002). NUP 153 interacts with nucleic acids and several proteins. These interactions are related to transport, nuclear envelope assembly and disassembly, and cellular regulation. Also interaction of the C-terminus domain of NUP 153 with several receptor proteins has been shown (Walther, Fornerod et al. 2001). It has been demostrated that NUP 153 plays a critical role in both nuclear export and import (Ullman, Shah et al. 1999) (figures 1.16 & 1.17 & table 1.7).

1.7.4 Nucleoporin 98

NUP 98 is a member of the FG-repeat containing nucleoporins that are located on both faces of the NPC and shuttle between the nucleus and the cytoplasm (Figure 1.13) (Griffis, Altan et al. 2002). Similar to NUP 153, NUP 98 is composed of multiple domains and the N-terminal half consists of two domains of FG and GLFG repeat separated by a Rae1/Gle2 binding site. Rae1/Gle2 binds to NUP 98 at the NPC and plays a role in mRNA transport and seems to be a shuttling transport factor involved in translocation of mRNA (Pritchard, Fornerod et al. 1999). Also, Rae1/Gle2 aids in delivery of TAP to NUP 98 and the NPC (Blevins, Smith et al. 2003). NUP 98 interacts with importin-β that participates in nuclear trafficking (Griffis, Altan et al. 2002). NUP 98 is an essential component of multiple RNA export pathways and is unlikely to be involved in the nuclear import of protein (Powers, Forbes et al. 1997). NUP 98 can be expressed in two forms, NUP 98 and

NUP 98 bound to NUP 96 in complex with other NUPs that play a role in RNA export and in tethering NUP 98 and NUP 153 in the nucleus (Griffis, Xu et al. 2003). NUP 98, like NUP 214/CAN participates in nuclear export of HIV-1 Rev protein (Zolotukhin and Felber 1999) (Figure 1.17).

1.7.5 Nucleoporin 62 (p62)

NUP 62 (p62) is a major component of the NPC and directly interacts with various nuclear transport factors, importin-β, NTF2 and TAP (Paschal and Gerace 1995; Percipalle, Clarkson et al. 1997; Levesque, Bor et al. 2006). However the mechanism of involvement of these interactions in nucleocytoplasmic transport is not clear (Fukuhara, Sakaguchi et al. 2006). This FG-repeat NUP is located in the central channel near both the nucleoplasmic and cytoplasmic faces of NPCs (Figure 1.13) (Guan, Muller et al. 1995) and is implicated in transport (Finlay, Meier et al. 1991). It forms a complex with other NUPs and is constructed from two domains, a C-terminus α-helical coiled-coil domain that is probably involved in protein-protein interaction and may anchor p62 to the NPC and an N-terminal domain of xFxFG repeats, which is probably implicated in nucleocytoplasmic transport (Buss, Kent et al. 1994; Guan, Muller et al. 1995; Wiemann, Kolb-Kokocinski et al. 2005) (figure 1.15).

1.7.6 Translocation in the nuclear pore complex

Transport of shuttling proteins and RNAs is dependent on their own physical properties and the ability of them to interact with transport receptor and/or the NPC. This situation stimulates the NPC properties to suit the efficient shuttling of these molecules. Three major translocation waies are proposed for accommodation of the broad range of shuttling molecules with very different properties. First, that termed the affinity gradient model where transport receptors bind with variable affinities to different FG-containing NUPs, of which some are located at both faces of the NPC. It is based on shuttling complexes which bind to NUPs with progressively increasing affinity through the translocation route (Ben-Efraim and Gerace 2001). The virtual gating model is the second method that is based on an enzymatic event, which can be catalysed by the NPC. A Brownian action of the multiple FG-repeats creates a barrier in the NPC. The translocation

reaction of cargo is then favoured by shuttling interactions with the FG-repeats themselves or by removing the products of the enzymatic reaction which is achieved by the accessory components of the transport machinery (Rout, Aitchison et al. 2003). Finally, in the hydrophobic exclusion model, the kinetics of nuclear trafficking and the structure of NPC are integrated to accommodate molecules. FG-repeats domains can form a hydrophobic meshwork through weak interactions. Transport of receptors and cargos is achieved through structure recognition of NUP FG-repeat that allows passage through the hydrophobic environment created. melting elements with high surface hydrophobicity. Also the NPC as a flexible solution can be adapted to the translocating factors (Ribbeck and Gorlich 2002). Recent studies showed some unsolved questions about these models and some of them point to other directions (Strawn, Shen et al. 2004; Yang, Gelles et al. 2004; Zeitler and Weis 2004; Paulillo, Phillips et al. 2005). New mathematical modelling has been defined nuclear transport in a quantitative manner that could be equivalent to the previous models based on some experimental evidence (Becskei and Mattaj 2005) (figures 1.18 & 1.19).

1.8 Aims of this study

The aim of this progect was to investigate interaction of HSV-1 ICP27 with cellular nucleoporins. As reported previously, ICP27 is a multifunctional regulatory protein that has a role in transport of viral RNA using cellular components and pathways (Smith, Malik et al. 2005). ICP27 has already been implicated in nuclear export of viral RNAs. It binds to ALY/REF and is recruited to TAP and its partner NXT. TAP/NXT is essential for export of cargo because it is this heterodimer that directly interacts with components of the NPC at high affinity. However, there is another possibility that I wished to consider: ICP27 could traffic RNA and proteins through the nuclear pore complex directly by interaction with NUPs in the NPC (Mattaj and Englmeier 1998; Zenklusen and Stutz 2001; Weis 2002; Sandri-Goldin 2004; Hetzer, Walther et al. 2005).

Studies in various eukaryotic organisms suggest there must be other pathways because RNA export factor (REF) is not essential for nuclear mRNA export in *C.*

elegans (Longman, Johnstone et al. 2003) and *D. melanogaster* (Gatfield and Izaurralde 2002). For virus infection, HSV-1 ICP27 deletion d3-4 mutant virus that lacks the REF interaction domain is fully replication-competent (Lengyel, Guy et al. 2002). Although Koffa, et al, showed the HSV-1 ICP27 deletion d3-4 mutant is defective in its export ability (Koffa, Clements et al. 2001). As export of viral mRNA is necessary for viral replication this reveals an alternative pathway for mRNA export by the mutant virus. Previous studies in our laboratory on the ICP27 SADET mutant, the shuttling motif conserved between ICP27 and cellular hnRNP K protein, showed that SADET mutated ICP27 may still shuttle but was no longer able to interact with cellular nucleocytoplasmic transport proteins REF and hnRNP K. So it was proposed ICP27 could shuttle through pathways distinct from those mediated by REF and hnRNP K, possibly the CRM1 export route or via direct interaction with nucleoporins (Leiper 2004).

The main NUP in the NPC that I was interested in testing in terms of its interaction with ICP27 was NUP 62 (p62). This is because it is located in the central gated channel, directly interacts with various nuclear transport factors, importin-β, NTF2 and TAP as well as other NUPs (Paschal and Gerace 1995; Percipalle, Clarkson et al. 1997; Levesque, Bor et al. 2006) and has role in transport. However, I was also interested in testing any interaction of ICP27 with a range of NUPs. As NUPs can be divided into FG and non-FG repeat NUPS (Stewart, Baker et al. 2001), I decided to test NUPs in both categories. For the FG-repeat NUPs I selected NUPs with different locations in the NPC. As mentioned above NUP 62 is located in the central pore. However, NUP 153 has a complex localisation pattern. NUP 153 is located mostly at the nuclear pore basket but is mobile (Fahrenkrog, Maco et al. 2002). NUP 98 is also highly mobile (Griffis, Altan et al. 2002). In contrast, NUP214/CAN is found at a steady state level on the cytoplasmic face (Paulillo, Phillips et al. 2005). For the non-FG repeat NUPs I decided to use Rae 1 and NUP 107. Rae 1 is on the nuclear basket and there is evidence that it interacts with NUP98 (Blevins, Smith et al. 2003). NUP107 however, is located on the nuclear side of the nuclear membrane in complex with NUP160, NUP133, NUP 107 and NUP 96 (Fahrenkrog and Aebi 2003; Rout, Aitchison et al. 2003; Fahrenkrog, Koser et al. 2004). My choice of NUPs was constrained by the availability of good antibodies to these proteins that could work in biochemical protein-protein

interaction studies. By choosing to study a range of NUPs located throughout the NPC, I hoped to try to determine whether ICP27 trafficked through the NPC by sequential interaction with NUPs lining the pore. That is, my hypothesis was that ICP27 would act as a viral cargo loader that would be handed from NUP to NUP to facilitate direct transport through the NPC.

I also aimed to relate times of infection, production levels of ICP27 and expression, and possibly function, of the NUPs. Finally, our group had already demonstrated interaction of ICP27 with hnRNP K, a nucleocytoplasmic shuttling, RNA-binding protein. ICP27 binds hnRNP K through the SADET sequence of both proteins. hnRNP K's SADET sequence is essential for its transport between the nucleus and cytoplasm. I aimed to determine if this SADET sequence has any role in interaction with the FG-repeat NUPs.

2 Materials and Methods

2.1 Materials

2.1.1 Plasmids

The acknowledged investigators provided the following plasmids:

pGEX-27: (Mears & Rice, 1996) Provided by S. Rice, Calgary University, USA. Expresses the fusion protein GST-ICP27

pGEX-27-HIS: provided by Dr. Margy D. Koffa, Laboratory of Molecular Cell Biology, Cell Cycle and Proteomics, Greece. Inserted HIS tag at C-terminus, expresses the fusion protein HIS-ICP27

pGEX-ORF57: provided by Dr P. Malik, Edinburgh University, UK. Expresses the fusion protein GST-ORF57

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

pEYFP-27: N terminal fusion of ICP27 ORF to EYFP coding sequence, provided by J. Scott, FBLS infection and immunity, University of Glasgow.

pGEX: Amersham Ltd

pGEX6p1-RAE 1: Provided by Prof S. A. Wilson, University of Sheffield , UK. Expresses the fusion protein GST-RAE 1

pGEX6p1-p62: Provided by Prof S. A. Wilson, University of Sheffield, UK. Expresses the fusion protein GST-NUP 62

pGEX-3X-h NUP 107: Full length human NUP 107 provided by Dr. Ian Mattaj, EMBL, Germany. Expresses the fusion protein GST-NUP 107.

pTRC 2C- h NUP 153: Full length human NUP 153 provided by Dr Katie Ullman, University of Utah. Expresses the fusion protein GST-h NUP 153-6H.

GST-NUP 98 (66-515): N-terminal FG-repeat-containing domain of NUP 98 provided by Dr. Elisa Izaurralde, EMBL, Germany. Expresses the fusion protein GST-NUP 98 (66-515).

pGEX4T-CAN (1690-1894): A fragment of FG-repeat-containing domain of NUP 214/CAN provided by Dr. Elisa Izaurralde, EMBL, Germany. Expresses the fusion protein GST- NUP 214/CAN (1690-1894).

pGEX4T-CAN (1690-2090) GST-NUP 214 (1690-2090): A fragment of FG-repeat-containing domain of NUP 214/CAN provided by Dr. Elisa Izaurralde, EMBL, Germany. Expresses the fusion protein GST- NUP 214/CAN (1690-2090).

pGEXCS-NXF1/TAP: A full length TAP provided by Dr. Elisa Izaurralde, EMBL, Germany. Expresses the fusion protein GST-TAP.

pQETAP-NUP 98 (531-782): C-terminus fragment of aa 531-782 tagged with HIS in N-terminus provided by Dr. Ian Mattaj , EMBL, Germany. Expresses the fusion protein HIS-NUP 98 (531-782).

pRN3zz-CAN (1690-1894): Provided by Dr. Elisa Izaurralde, EMBL, Germany.

pRN3zz-CAN (1893-2090): Provided by Dr. Elisa Izaurralde, EMBL, Germany.

pRN3zz-hNUP 98: Provided by Dr. Elisa Izaurralde, EMBL, Germany.

2.1.2 Bacteria

The *E.coli* strain DH5α was used with transformation efficiency for maintenance and propagation of plasmid DNAs. For fusion protein expression from prokaryotic vectors I utilised *E.coli* strain BL21 (DE3) pLysS and has the advantage of being deficient in *lon* and *ompT* proteases and BL21 RP codon plus has the advantage of expression of mammalian proteins by codon usage (Stratagene).

2.1.3 Bacterial culture media

All strains were grown in L-broth (LB) (10 g NaCl, 10 g Bactopeptone, 5 g yeast extract in 1litre water, pH 7.5). Agar plates were 1.5% (w/v) agar in LB. Generally, media and LB agar plates were supplemented with antibiotic. 50 μg/ml kanamycin was used for kanamycin resistance transformed cells and 100 μg/ml ampicillin was used for all other ampicillin resistance plasmid containing bacteria.

2.1.4 Cells and Tissue culture

All cell culture media for growing cell lines were obtained from Invitrogen-Gibco. Antibiotics were added only before virus propagation (100 units/ml penicillin and 100μg/ml streptomycin) and cell lines were subcultured for less than twenty passages. HeLa cells used for transfection experiments were not treated with antibiotics at any stage.

2.1.4.1 BHK-21 (C13) cells

BHK is a fibroblastic cell line derived from baby hamster kidney cells (Macpherson and Stoker, 1962). BHK-21 cells were grown in Glasgow Modified Eagles Medium (GMEM) supplemented with 10% newborn or foetal calf serum (NBCS, FCS), 10% tryptose phosphate broth, 100 U/ml penicillin and 0.01% streptomycin.

2.1.4.2 M49 cells

A BHK derived ICP27 complementing cell line that expresses ICP27 (Lilley *et al.,* 2001). The M49 cell line was cultured in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 10% foetal calf serum (FCS), 10% tryptose phosphate broth, 1% L-glutamine, 500μg/ml of G418 (Gibco BRL) and 500μg/ml of Zeocin.

2.1.4.3 HeLa cells

An epithelial cell line derived from a human cervical carcinoma, HeLa cells were grown in Minimum Essential Medium (MEM) with Earle's salts supplemented with 10% FCS, 1% L-glutamine, 1% non essential amino acids solution, 100 U/ml penicillin and 100 μg/ml streptomycin.

2.1.4.4 Vero cells

Derived from African green monkey kidney, Vero cells were grown in DMEM supplemented with 10% FCS and 100 U/ml penicillin and 100 μg/ml streptomycin.

2.1.5 Viruses

HSV-1 strain 17^+: HSV-1 wild type strain 17^+ virus was as described (Brown et al., 1973). Referred to as wt or wt 17^+ virus.

HSV-1 vEYFP-ICP27: Recombinant HSV-1 that expresses an EYFP-ICP27 fusion protein from the IE-2 promoter was supplied by J. Scott, FBLS infection and immunity, University of Glasgow.

HSV-1 ICP27 mutants: Characteristics of ICP27 mutants used in this study are shown in Table 2.1. Mutants were provided by the acknowledged investigator.

2.1.6 Antibodies

Primary and secondary antibodies used during this investigation, the dilution used and their sources are listed in Tables 2.2 and 2.3.

2.1.7 Common solutions

30% acrylamide mix	29% w/v) acrylamide, 1% (w/v) N, N'-methylene bis-acrylamide
5X agarose gel loading buffer	1X TAE, 1% SDS (w/v), 50% Glycerol (v/v),1mg/ml bromophenol blue
Bradford's reagent	0.01% (w/v) Coomassie Brilliant Blue, 0.0003%(w/v)SDS, 4.75% (v/v) ethanol, 8.5% (v/v) phosphoric acid
Blocking buffer	5% (w/v) nonfat dry milk in PBS-T
Buffer E	100mM Tris-HCl pH 7.4, 100 mM NaCl 2mM EDTA, 1% NP40, 0.5% (w/v) Na-deoxycholate. Protease inhibitor tablets (Roche) were added before use.
Coomassie stain	0.02% (w/v) Coomassie Brilliant Blue 50% (v/v)methanol, 43%(v/v) water, 7% (v/v) acetic acid
Coomassie Destain	5% (v/v) methanol, 7% (v/v) acetic acid, 88% (v/v) water
HEPES extract buffer	50 mM HEPES pH 7.5, 50 mM NaCl,

	0.1% (v/v) Nonidet P40. Protease inhibitor tablets (Roche) were added before use.
HIS elution buffer	20 mM Tris-HCl pH 8, 100 mM NaCl, 300 mM imidazole
HIS lysis buffer	20 mM Tris-HCl pH 8, 100 mM NaCl, 20mM imidazole,0.5% NP-40. Protease inhibitor tablets (Roche) were added before use.
HIS wash buffer	HIS lysis buffer. HIS lysis buffer without NP-40. HIS lysis buffer with 1M NaCl
Lysozyme	10 mg/ml lysozyme in 25 mM Tris-HCl pH 8,0
NETN buffer	20mM Tris-HCl pH 8.0, 100 mM NaCl 1mM EDTA, 0.5% NP40, Protease inhibitor tablets (Roche) were added before use.
Giemsa stain	1.5% (w/v) suspension of Giemsa in glycerol, heated at 56^{o}C for 2 h and diluted with an equal volume of metanol

PBSA	170 mM NaCl, 3.4 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.2
PBS-T	PBSA with 0.05% (v/v) Tween 20.
3X protein gel loading buffer	150 mM Tris HCl, 300 mM dithiothreitol, 6%(w/v)SDS,0.3%(w/v) bromophenol blue, 30% (v/v) glycerol, pH 6.8
Protein gel running buffer	0.05 M Tris base, 0.05 M glycine, 0.1% (w/v) SDS
Pull down binding buffer	50mM Tris-HCl pH 8, 100 mM NaCl 0.1% NP40, Protease inhibitor tablets (Roche) were added before use.
Pull down wash buffer	50mM Tris-HCl pH 8, 150 mM NaCl 0.1% NP40. Protease inhibitor tablets (Roche) were added before use.
0.5 x TBE	0.5 x TBE (45 mM Tris base, 45 mM boric acid, 1 mM EDTA pH 8.0)
TE buffer	10 mM Tris HCl, 1 mM EDTA, pH 8.0
Towbin (blotting buffer)	25 mM Tris base, 192mM glycine, 20% (v/v) methanol

Transfer tank buffer	50 mM Tris base, 50 mM 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	0.6 mM EDTA in PBSA, 0.002% phenol red
Water	Obtained from a Ro 60 plus deioniser (Millipore, USA) and sterilised by autoclaving

2.1.8 Chemicals and Reagents

All chemicals and reagents were purchased from BDH Chemicals UK or from Sigma unless otherwise stated in this thesis.

Amersham	ECL western blotting reagents, Rainbow molecular weight markers, Hybond C nitrocellulose membrane, Glutathione Sepharose beads
Beecham Research	Ampicillin sodium B.P (Penbritin®)

Bio-Rad	Ammonium persulphate (APS), Coomassie brilliant blue, TEMED
Cayla (France)	Zeocin (phleomycin derivative)
Fluka	Formaldehyde, formamide
Invitrogen	1kb DNA ladder
Joseph Mills Ltd	Ultra pure ethanol, methanol
Kodak	X- Omat film
Prolabo	Butanol, glacial acetic acid, glycerol, isopropanol
Roche	Protease inhibitor cocktail
Qiagen	Midi and mini prep plasmid isolation kits
Active motif	Nuclear and cytoplasmic cell extract kit

2.1.9 Autoclaving and glassware sterilisation

Equipment and solutions were sterilised at 15 psi for 20 min by staff in the washroom, Institute of Virology, Glasgow or the Glasgow Biomedical Research Centre (GBRC). 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 0.2 µm) into a sterile tube.

2.2 Methods

2.2.1 Bacteria and plasmid transformation

2.2.1.1 Making competent *E.coli*

E.coli strain DH5α, was used for general plasmid transformation. *E.coli* strain BL21 (DE3) plysS or RP codon plus was used for plasmids incorporating GST or His expression systems. A 1 ml overnight culture was inoculated into 100 ml LB and grown for about 3 h 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 5000 rpm for 10 min at 4°C in a Sorvall RT 6000B refrigerated centrifuge. The cells were resuspended in 50 ml ice cold 50 mM $CaCl_2$ and left on ice for 30 min. Cells were then pelleted as before, and resuspended in 10 ml cold 50 mM $CaCl_2$, 15% glycerol. 200 µl aliquots were snap frozen in dry ice with ethanol and stored at –70^{o}C.

2.2.1.2 Transformation of plasmids

Plasmid DNA was transformed into a 200 µl aliquot of competent *E.coli,* cells which were thawed on ice. The DNA was added to the cells and the mixture incubated on ice for 30 min prior to heat shock in a 42°C water bath for 2 min. 800 µl of LB was added to the cells, which were shaken for 60 min at 37°C before plating onto LB agar plates containing appropriate antibiotic selection. Plates were incubated overnight at 37^{o}C.

2.2.1.3 Plasmid DNA extraction from transformed bacteria

A single colony from a bacterial plate or approximately 1 ml of liquid bacterial culture was used to inoculate 100 ml of LB containing the appropriate antibiotic. This was then incubated at 37^{o}C at 200 rpm. The overnight culture was spun down at 3000 rpm for 20 min in a Sorvall RT 6000B refrigerated centrifuge. Plasmid DNA was then extracted using the Qiagen midi prep kit following the

manufacturer's instructions. A typical yield of DNA using this method was 100 μg which was resuspended in 100 μl buffer E.

2.2.1.4 Electrophoretic separation and purification of DNA fragments

DNA was resolved in a horizontal slab gel of 1% (w/v) agarose in 1X TBE containing 1 μg/ml ethidium bromide and electrophoresed in a buffer of 1X TBE. Samples were loaded following addition of 5X agarose gel loading buffer. Electrophoresis was performed at 70 – 120V and DNA was visualised under UV illumination at 205 nm and photography was carried out using a camera linked to a computer running UV Products gel documentation system store 7500, version 7.12 (UV Products Ltd, Cambridge).

2.2.2 Cell culture

2.2.2.1 Passaging cells

Mammalian cells, HeLa, Vero, BHK-21 or M49 were sub cultured at 90% confluence. Trypsin solution 0.25% (w/v) in Tris-saline containing phenol red pH 7.5 was thawed from –20^{o}C and mixed with versene to yield 0.06% (v/v) trypsin /EDTA solution. T-175 flasks or roller bottles with confluent monolayers were opened in a Category 2 hood. The supernatant decanted and 10 ml or 20 ml, respectively, of versene was poured over the monolayer and decanted. The monolayer was then washed with a versene/trypsin solution and left for 2-3 min at RT. The cells were shaken into 10 ml (for a T-175 flask) or 20 ml (for roller bottle) media for further use. Cells were grown at 37 ^{o}C in humidified incubator under 5% CO_2. For continual passage, cells were split in a 1:10 ratio every 4-5 days.

2.2.2.2 Cryopreservation of cells

Confluent cell monolayers were harvested from T-175 flasks as described above and resuspended in 10 ml media. Cells were centrifuged at 2000 rpm at 4^{o}C for 5 min in a Heraeus multifuge 3 S/R refrigerated centrifuge, the media decanted and cells resuspended in 5 ml PBSA and again centrifuged for 5 min. Cells were resuspended in 4 ml of media, with 10% DMSO. Aliquots of 1 ml were transferred

into 1.5 ml cryo-vials. These were frozen overnight at –70^{o}C and moved to a liquid nitrogen freezer for long term storage.

2.2.2.3 Thawing cells

Above frozen cells were thawed quickly in a 37^{o}C water bath and resuspended in 10 ml of pre-warmed medium and centrifuged at 1000 rpm for 3 min in Heraeus multifuge 3 S/R refrigerated centrifuge and resuspended again in 25 ml of medium to seed a culture flask. This medium was changed after 6-8 h.

2.2.2.4 Transfection of cells with plasmid DNA

Cells in 35 and 60 mm dishes were transfected with ICP27 and nucleoporin expression plasmids. The day before transfection cells were plated so they were 80-90% confluent, on the day of transfection. The plasmid DNA was diluted in Optimem and in a separate Eppendorf, Lipofectamine 2000 (Invitrogen) was diluted into Optimem and incubated at RT for 5 min. The diluted plasmid DNA was then combined with the diluted Lipofectamine and incubated at RT for 20 min to allow DNA-Lipofectamine complexes to form. Medium was removed from the cells and 2 ml or 4 ml of growth medium (without serum) was added for 35 and 60 mm dishes, respectively. The DNA-Lipofectamine mix was then added directly to each plate and mixed gently. The cells were incubated at 37^{o}C for 4-5 h, after which the medium was removed and replaced with standard growth medium. The cells were incubated for 24 h post transfection.

2.2.2.5 Viral infection of cells

Almost confluent monolayers were grown on plates or flasks and infected with various viruses or left uninfected. With all viruses and in all cell types, infection was at a m.o.i of 10 unless otherwise stated for longer infection times. 80% confluent cells were incubated with virus in 100 µl per T175 flask and after 1 h adsorption 20 ml of an appropriate growth medium was added. From these infectious, soluble protein extracts were made and used in different pull down and immunoprecipitation assays.

2.2.3 Production of virus stock

2.2.3.1 Growth of virus stock

BHK cells were grown in roller bottles to a confluency of 85% and infected with 0.003 pfu/cell of wt 17^+ virus in 20 ml of media. ICP27 complementing cell line BHK M49 was used for the growth of HSV-1 ICP27 viral mutants.These were incubated at 31^{o}C for 3-5 days until CPE was complete when the cells were shaken into the medium. The cell suspension was poured into a 250 ml plastic Falcon tube and the cells pelleted by centrifugation at 2000 rpm for 30 min at 4^{o}C in Heraeus multifuge 3 S/R refrigerated centrifuge. The supernatant and cell pellet was divided into 2 individual stocks: cell associated virus and cell-released virus.

2.2.3.2 Cell associated (CA) virus

The cell pellet was resuspended in 0.5 ml medium/roller bottle, pipetted into sterile bijoux, thoroughly sonicated and centrifuged at 2000 rpm for 10 min at 4^{o}C in Heraeus multifuge 3 S/R refrigerated centrifuge to remove cell debris. The supernatant was kept on ice while the pellet was treated as above. Both supernatants were pooled to give the CA virus stock. 1 ml of this was aliquoted into 1.5 ml sterile cryo-vials and stored at –70^{o}C.

2.2.3.3 Cell released (CR) virus

The supernatant was poured into 250 ml sterile centrifuge bottles and spun at 12000 rpm (Sorvall GSA rotor) for 2 h at 4^{o}C. The supernatant was discarded and the virus pellet resuspended in 1 ml medium/roller bottle. The pellet was sonicated until homogenous and 500 µl CR aliquots transferred to 1.5 ml sterile cryo-vials and stored at –70^{o}C.

2.2.3.4 Sterility check on virus stocks

Brain heart infusion agar (BHI) plates containing 10% (v/v) horse blood (blood agar) were used to check for yeast or bacterial contamination by streaking a small

aliquot of viral stocks and incubating at 37^{o}C for 7 days. If there was no growth by 48 h, stocks were considered sterile.

2.2.3.5 Virus titration

Wild type and mutant HSV-1 ICP27 virus stocks were serially diluted 10 fold in PBSA/5% CS and 0.1 ml aliquots added to fresh monolayers of either BHK-21 or M49 cells on 60 mm plates from which the media had been removed. The plates were incubated at 37^{o}C with 5% CO_2 for 1 h to allow adsorption to the cells, before overlaying with 4 ml medium supplemented with 1% carboxymethyl cellulose. The plates were incubated at 37^{o}C for 2-3 days. The media was decanted and the monolayers fixed and stained with Giemsa/X-gal at RT for 2-24 h. After rinsing the plates with dH_2O, plaques were counted using a dissection microscope and virus titres calculated as plaque forming unit per ml (pfu/ml).

2.2.4 Protein extraction and analysis

2.2.4.1 Whole cell extract preparation

Soluble proteins extracts were made from cells infected with various viruses or uninfected for immunoprecipitations and pull down assays. Cells were washed twice in ice cold PBS A and harvested in 10ml PBS A by scraping. Cells from a 175 cm^2 flask were pelleted by centrifugation at 1000 rpm for 5min at 4°C in a Heraeus multifuge 3 S/R refrigerated centrifuge and resuspended in 800µl HEPES extract buffer. The cells were lysed by passing 5 times through a syringe with a 26-gauge needle and sonication for 30-60sec in a soni-bath and left rotating at 4°C for 30 min for solubilisation. Debris was pelleted by micro-centrifugation at 12000 rpm for 10min at 4°C in a Sorvall RT 6000B refrigerated centrifuge. The soluble protein extracts were either used directly or 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 and electrophoresed on SDS-PAGE and western blotted to examine proteins expression.

2.2.4.2 Nuclear and cytoplasmic cell extracts

For some immunoprecipitations and pull down assays a nuclear and cytoplasmic extract was prepared. After infection at different time points, nuclear and cytoplasmic extracts were made by the Active Motif nuclear extract kit (catalogue number 40010) using the manufacturer's protocol.

2.2.4.3 Estimation of protein concentration

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

2.2.4.4 SDS polyacrylamide gel electrophoresis (SDS-PAGE) of proteins

Proteins were resolved by electrophoresis through SDS-PAGE using Bio-Rad mini gel electrophoresis tanks. Gel mixes were as follows:

For 10ml	**10% resolving gel**	**6% resolving gel**	**2ml stacking gel**
dH_2O	4 ml	5.3 ml	1.4 ml
30% acrylamide mix	3.3 ml	2.0 ml	0.33 ml
1.5 M Tris (pH 8.8)	2.5 ml	2.5 ml	0.25 ml (pH 6.8)
10% SDS	0.1 ml	0.1 ml	0.02 ml
10% APS	0.1 ml	0.1 ml	0.02 ml
TEMED	0.004 ml	0.008 ml	0.002 ml

Resolving gel mixes were poured into glass plate sandwiches, overlaid with butanol and allowed to set. The butanol was washed away with dH_2O, stacking gel mixture was overlaid and a comb inserted. Protein samples were mixed with protein gel loading buffer and placed in a boiling water bath for 3 min prior to loading. Along with samples, 10 µl Rainbow marker (Amersham) was also run. Gels were electrophoresed at 150 mV until the tracking dye reached the bottom.

Gels were either stained with Coomassie Blue solution for 30 min and destained in protein gel destain or transferred to nitrocellulose membrane for Western blotting.

2.2.4.5 Western blotting and transfer of proteins to nitrocellulose membrane

To detect specific protein, proteins resolved on SDS-PAGE were transferred to nitrocellulose using a Bio-Rad Transblot cell. A blotting sandwich was set up such that the gel was in contact with a sheet of nitrocellulose (Hybond-ECL Amersham) and both were sandwiched between Whatman 3 mm papers of the appropriate size. This was in turn sandwiched between sponges provided by Bio-Rad and transfer carried out at 250 mA for 1 h.

2.2.4.6 Immunodetection of proteins

After transfer to nitrocellulose, the presence of specific proteins was detected using antisera. Incubation in PBSA, 5% dried milk overnight at 4°C blocked the nitrocellulose membranes. They were then washed for three 10 min at RT in PBSA 0.05% Tween (PBS-T), before incubation for 1-2 h at RT on a shaker in 20 ml of appropriately diluted primary antibody in PBS-T 5% dried milk (Table 2.2). Following this, blots were washed for three 10 min at RT in PBS-T, and appropriate secondary antibody added at a 1/1000 dilution in PBS-T 5% dried milk (Table 2.3). After 60 min incubation at RT, 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 poured onto the filter, which was agitated for 1 min. The blots were wrapped in cling film and exposed to Kodak X-OMAT S film.

2.2.5 Co-immunoprecipitations (CO-IP)

2.2.5.1 Immunoprecipitation and preparation of cell extracts

To investigate protein: protein interactions and their ability to detect an interaction between an antibody and its partner protein, co-immunoprecipitations were carried out with several antisera and conditions were optimised for each antibody. A

complex of antigen and antibody can be precipitated using *Staphylococcus aureus* protein A/G which interacts with IgG, coupled to Sepharose beads (Figure 2.2). Identification of the immunoprecipitated antigen and proteins, which co-purify with the target antigen, can be achieved by separation of the complex on SDS-PAGE, followed by detection of 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. 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. Generally, cells at 90% confluence were infected with wt HSV-1 and its ICP27 mutants at MOI of 10 or left uninfected as a control sample (MI). At different time points after infection cells were washed twice in PBS and harvested in 10 ml of PBS by scraping and pelleted at 1000 rpm for 5 min in a Heraeus multifuge 3 S/R refrigerated centrifuge at 4°C. HEPES extract buffer was used to lyse the cells by passing 5 times through a syringe with a 26-gauge needle and sonication for 30-60 sec in a soni-bath and left rotating at 4°C for 30 min for solubilisation. Cellular debris was removed by spinning at 12000 rpm for 10 min in a Eppendorf 5415R refrigerated centrifuge at 4°C. Protein concentration and expression profiles were measured by Bradford protein assay and fractionated on SDS-PAGE. Soluble protein extracts were either used directly or stored at -70°C.

2.2.5.2 Co-immunoprecipitation using ICP27 monoclonal antibody

Various infected and non-infected cell extracts (~100µg) were mixed with 2 µl of Mab 1113 and 2 µl of Mab 1119 or 2 µl NMS and NRS in 700 µl modified buffer E (100mM Tris HCl, 100mM NaCl, 2mM EDTA, 2mM EGTA, 1% NP40, 1% Na deoxycholate, protease inhibitor, pH 7.4) for 3h at 4°C. 100 µl protein A-Sepharose (made up at 50% (w/v) and washed 2X in buffer E) was then added and mixed for 1h. After pelleting the beads and 5X washes in buffer E at 12000 rpm 30 sec in an Eppendorf 5415R refrigerated centrifuge at 4°C, 60µl protein gel loading buffer was added, samples placed in a boiling bath for 3 min and co-immunoprecipitated

proteins separated by SDS-PAGE. After separation gels were subject to Western blot analysis.

2.2.5.3 Co-immunoprecipitation using NUP antibodies

The same protocol as described in the above section was used for co-immunoprecipitation of nucleoporins but different amounts of antibodies were added instead. In this investigation 10 µl of anti NUP 153, 12 µl of anti NUPs 414, 12 µl of anti NUP 62 and 15 µl of anti NUP 98 were used. As a control 2 µl of NMS, NRS or NGS mixed with cell extracts depending on the type of NUP antibody.

2.2.6 Expression and purification of fusion proteins and pull down assays

2.2.6.1 Expression and purification of GST fusion proteins

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. Fusion proteins are expressed in *E.coli* cells containing the recombinant pGEX plasmid and BL21 (DE3) and BL21 RP codon plus are used. 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. Cell debris was cleared by centrifugation and fusion protein purified and immobilised by binding to Glutathione attached to Sepharose 4B. After binding the matrix 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-PAGE.

GST alone, GST-ICP27, GST-ORF57, GST-TAP and different GST-NUP plasmids were transformed either into *E.coli* BL21 or BL21 RP codon plus and grown on LB plates containing 100µg/ml of ampicillin. Single colonies were inoculated into 10 ml

LB ampicilin and incubated at 37°C overnight. The culture was diluted 1:50 and grown in 100 ml LB plus ampicillin at 37°C until OD_{600} of 0.6-1 was reached. Then fusion protein expression was induced by adding 0.5-1 mM IPTG for 3 h at 37°C for GST alone and GST-ICP27 and 16 h at 29°C for other GST fusion proteins. Bacteria were harvested by centrifugation at 4000 rpm for 15 minutes in a Sorvall RT 6000B refrigerated centrifuge. Pellets were resuspended in 10 ml of NETN (modified NETN buffer for GST-ORF57 and GST-NUPs) with 0.1 volume of lysozyme and protease inhibitor and sonicated on ice using a soniprobe. Soluble protein was separated from cell debris by 40 min centrifugation at 9000 rpm in a Sorvall RT 6000B refrigerated centrifuge. The affinity chromatography system used for purification of the fusion protein from bacterial lysates involved adding pre-swollen Glutathion-Sepharose 4B beads to lysates (10 ml lysate/ 500µl), rocking for 2-4 h at 4°C to mix protein lysates and beads, followed by washing 3 times with cold PBS and once with cold PBS-T using 30 sec centrifugation at 12000 rpm in an Eppendorf 5415R refrigerated centrifuge at 4°C. Purified fusion protein was resuspended in PBS, and aliquots were stored at -20°C or -70°C. Small amounts of different stages of expression and purification, including culture before and after induction, and sonication, unbound and purified fractions were analysed on SDS-PAGE followed with either western blotting or Coomassie stain to monitor expression, concentration and purity of the fusion proteins.

2.2.6.2 Expression and purification of His tagged proteins

For expression and purification of His-ICP27 and His-NUP 98 (531-782), plasmids were transformed into *E.coli* BL21 and grown on LB and plates containing 100µg/ml kanamycin. Single colonies were inoculated into 10 ml LB with kanamycin and incubated at 37°C overnight. The culture was diluted 1:50 and grown in 100 ml LB with kanamycin at 37°C until OD_{600} of 0.6-1 was reached. Then fusion protein expression was induced by adding 0.5 mM IPTG for 3 h at 37°C. Bacteria were harvested by centrifugation at 4000 rpm for 15 minutes in a Sorvall RT 6000B refrigerated centrifuge. Pellets were resuspended in 10 ml His lysis buffer and sonicated on ice using a soniprobe. Soluble protein was separated from cell debris by a 40 min centrifugation at 9000 rpm in a Sorvall RT 6000B refrigerated centrifuge. The affinity chromatography system was used for

purification of the fusion protein from bacterial lysates by adding Ni-NTA agarose, rocking for 2-4 h at 4°C to mix protein lysates and beads, followed by 2 times wash with His wash buffer (Lysis buffer), once with His wash buffer without NP-40, once His wash buffer with 1M NaCl and once again with HIS wash buffer using 30 sec centrifugation at 12000 rpm in an Eppendorf 5415R refrigerated centrifuge at 4°C. Purified fusion protein was eluted by loading on to a column and eluted with His elution buffer in 4-5 fractions which were then aliquoted and stored at -70°C. Small amounts of different stages of expression and purification including culture before and after induction and sonication, unbound and purified fractions were analysed on SDS-PAGE followed with either western blotting or Coomassie staining to monitor expression, concentration and purity of the fusion proteins.

2.2.6.3 GST pull down assay

An equal amount of each GST fusion protein-bound beads was mixed with 100 µg cell extracts or 1 µg of purified His tagged protein. The mix was rocked in 700 µl of pull down binding buffer for 3 h at 4°C. Beads were washed 4 times in pull down wash buffer, by spinning at 12000 rpm for 30 sec in an Eppendorf 5415R refrigerated centrifuge at 4°C. After this, bound proteins were eluted in SDS protein gel loading buffer. Samples were placed in a boiling bath for 3 min and pulled down proteins separated by SDS-PAGE and visualised by western blotting (Figure 2.3).

3 Interaction of ICP27 with nucleoporin 62

3.1 Introduction

The NPC is a huge macromolecular structure that extends across the nuclear envelope, forming an entry, which regulates the flow of messenger RNA and proteins between the nucleus and the cytoplasm (Fahrenkrog and Aebi 2003; Rout, Aitchison et al. 2003; Fahrenkrog, Koser et al. 2004). Nucleoporin 62 (NUP 62) is one of the best- characterised nucleoporins localised to the nuclear pore central plug. This protein is a member of the phenylalanine-glycine (FG) repeats containing nucleoporins associated with the importin alpha/beta complex, which is involved in the import of proteins containing nuclear localization signals (Fahrenkrog and Aebi 2003; Rout, Aitchison et al. 2003; Fahrenkrog, Koser et al. 2004). Its N-terminus is believed to be involved in nucleocytoplasmic transport and binds to nuclear transfer factor 2 (NTF2) and Ran. The C-terminal end, which shows a hydrophobic heptad repeat, contains a coiled-coil structure aiding in protein–protein interactions and associates with importin-β1, transcription factor Sp1, the signaling component TRAF-3 and heat shock factor 2 (Gamper, van Eyndhoven et al. 2000). Recent studies have shown interaction of the N-terminal domain of mammalian NUP 62 with other nucleoporins of the FXFG family such as NUP 358, NUP 214 and NUP 153 and it has been proposed that some of these interactions may contribute to the movement of cargo across the nuclear pore complex (Stochaj, Banski et al. 2006). Also the functional involvement of viruses with nucleoporins has been shown in that poliovirus infection can destroy the structure of the NPC by degradation of NUP 62 (Gustin and Sarnow 2001). Work on ICP27 demonstrated its role at transcriptional and post-transcriptional levels as well as in shuttling and transport of viral mRNAs. ICP27 binds to partner proteins TAP and REF and contributes to nuclear export (Smith, Malik et al. 2005). Consequently, we were interested in seeking for interaction of ICP27 with cellular nucleoporins involved in the transport machinery. First I looked at NUP 62 and this chapter presents data that shows HSV-1 ICP27 protein interacts with NUP 62 *in vivo* in virus infected cells and *in vitro.*

3.1.1 Expression of fusion proteins and pull down assay

The study of protein interactions has been vital to the understanding of how proteins function within the cell. There are many ways to investigate protein-protein interactions. In this study GST-fusion protein pull down assays and co-immunoprecipitations were used to reveal interacting proteins. Details of the theory of GST fusion protein pull down assays and experimental conditions that can be varied are found in Materials and Methods.

3.1.2 GST-ICP27 fusion protein expression

To produce ICP27 as a GST fusion protein I used an optimised expression procedure that was established in our lab. Expression of protein from the pGEX vector is under control of the *tac* promoter and the *E.coli* BL21 strain with protease deficiency was used to minimise proteolytic degradation. Expression of some full length GST-ICP27 and various truncations was achieved. The protein was visualised by Coomassie blue staining. Figure 3.1 Panel I shows Coomassie blue stain of bound and unbound expressed GST-ICP27 (lanes 1 and 2 respectively) and purified bound fraction using a protein 200 plus LabChip kit (Agilent technologies), in order to monitor expression, concentration and purity of GST-ICP27 (lane 3). This fast protein analysis with the bioanalyser system is used allow to monitor and optimise each step of recombinant protein purification via affinity chromatography. Also Panel II demonstrates western blot of similar gels by using specific antibodies against GST (lane 1) and ICP27 (lane 2). GST-ICP27 is an unstable protein and it is difficult to obtain high yields of uncleaved fusion protein. A major cleavage product of around 50-60 kDa is always seen together with some smaller products. Regarding the specifity of antibodies using here there are more truncates of protein seen in lane 1 of Panel II Figure 3.1 because western blotting with the α- GST antibody detects fusion protein and truncated/degraded fusion protein present at low level compared to the relatively insensitive Coomassie stain.

3.1.3 Expression of HIS-ICP27

Expression of His-ICP27 has already been optimised in our lab and I have used the same procedure at 37℃ to express this protein for direct interaction assay of ICP27 with different GST-NUP fusion proteins. Figure 3.2 shows expression of His-ICP27 fractions by Coomassie blue staining and western blotting with His mAb and anti ICP27 mAb (H1113). These purified proteins were used in pull down assays to show direct interaction of ICP27 with nucleoporins.

3.1.4 GST-NUP 62 fusion protein expression

I attempted to produce significant expression of nucleoporin 62 as a GST fusion protein (gifted to us and listed in Material and Method section). An objective with this part of the study was to compare the effects of different factors upon expression of a set of human nucleoporin fusion products in *E.coli* and establish an optimised method for expression of these proteins that can be used in interaction experiment studies. Various factors might be the cause of lack of good expression of GST fusion proteins.To optimise their expression and obtain sufficient amount of purified proteins, effects of temperature, method of lysis, as well as use of protease deficient BL21-Codon plus-RP competent *E.coli* cells that enables efficient high-level expression of heterologous proteins, were also investigated. An expression plasmids of nucleoporin GST-NUP 62 was an FG-repeat human nucleoporin that is involved in protein and mRNA export, were used in optimisation experiments and the initial and final improved level of expression obtained can be seen in the next sections.

3.1.4.1 The effect of temperature on expression of the GST-NUP 62

There are numerous single case studies showing increased solubility of recombinant proteins at lower cultivation temperatures. I have seen this effect on my nucleoporin GST fusion proteins at 29℃, and prefer to carry out solubility screening at the lower temperature. Bacterial cultures were grown in 100 ml LB containing ampicillin and were induced at OD_{600} of 0.6 by 1.0 mM IPTG and incubated at 37℃ (as used usually for GST-ICP27 in our laboratory) and 29℃ (that suggested by Prof Stuart Wilson, who provided some plasmids used in this

work) and harvested after 3 and 16 hrs respectively. Pellets were lysed by sonication in lysis buffer and incubated with Gluthatione beads. Samples were analysed on 10% SDS-PAGE and stained by Coomassie blue. Results shown in Figure 3.3 demonstrated at 29℃ temperature level of full-length GST-NUP 62 expression was highest and the level and number of truncations were reduced (Panel I) in comparison with a higher cultivation temperature of 37℃ (Panel II). Thus protein expression continued to be at 29℃. GST-NUP 62 band was identified on the basis of correct molecular mass (90 KDa).

3.1.4.2 The effects of *E.coli* strain on expression of the GST-NUPs

To produce nucleoporins as GST fusion proteins, a protease deficient strain of *E.coli* (BL21) was used in usual to minimise proteolytic degradation. But because I was using human nucleoporins fused to GST and wanted to obtain significant expression of full-length GST-NUPs the strain of *E.coli* competent cell was altered to increase the amount of full-length product. Expression tests as described in Material and Methods were carried out on the strain BL-21 (DE3) Codon Plus RP (Stratagene), which has a plasmid that supplies extra tRNAs corresponding to codons that are rare in *E. coli* but common in humans. Figure 3.4 demonstrates significant efficiency of BL-21 Codon Plus RP in expression of GST-NUP62 (Panel I, lane 1 and panel II, lane 4) in comparison to the usual strain BL21 (Panel I, lane2 and panel II, lane 3). Expressed protein was western blotted against specific antibodies as shown in Panel III. Enhanced level of protein expression and reduction of truncates and degraded bands are the advantage of *E.coli* BL-21 (DE3) Codon Plus RP (Stratagene), which was used mostly in this study.

3.1.4.3 The effect of sonication and bug buster reagent on expression of GST-NUP 62

As part of my investigation to find out which method of lysis is the best to open bacterial cells to obtain efficient protein expression I have used the soni-probe and bug buster reagent master mix as detected in the Material and Method section. The expressed GST-NUP 62 bacterial pellet was lysed either by sonication or bug buster reagent. Lysates were pelleted and bound to glutathione beads and separated on 10% SDS-PAGE and western blotted with anti NUP 62 (D-20)

antibody. Results show the sonication method may lyse the bacteria better and make available more GST-NUP 62 to bind to glutathione beads. Figure 3.5 demonstrates significant difference in the level of GST-NUP 62 expression in lane 1 in contrast to lane 2 where cells were lysed by bug buster reagent. Further experiments to make the lysates were based on the sonication method.

3.1.4.4 The optimised method used for GST-NUPs expression

GST fusion nucleoporin plasmids were transformed into BL-21 (DE3) Codon Plus RP (Stratagene) and grown on LB plates containing ampicillin. Single colonies were picked and inoculated into 10 ml LB broth plus ampicilin and grown overnight. 1:50 dilution of culture was made into 100 ml LB broth containing ampicilin. Expression of nucleoporin fusion proteins was induced by adding 1.0 mM IPTG at 28^{o}C for about 16 h after obtaining OD_{600} of 0.4-0.6. Bacteria were pelleted and resuspended in modified NETN lysis buffer and solubilised. Fusion proteins were purified and bound to beads and eluted off the beads using reduced glutathione solution. Significant full-length fusion expression was obtained for several nucleoporins (NUP 62, Rae 1, NUP 98 and 214/CAN) and used in pull down assay experiments (Figure 3.6).

3.1.5 ICP27 interacts with NUP 62 in a GST pull down assay

Purified GST-ICP27 fusion protein and its homologue from KSHV GST-ORF57 purified protein and as a negative control, purified GST alone bound to glutathione beads were used to test whether ICP27 and its homologue ORF57 pulls down nucleoporin 62 from HeLa infected cell extracts. The input fusion proteins used were visualised by Coomassie blue staining and bands of approximately 90 kDa (lane 2), 80 kDa (lane 1) and 28 kDa (lane 3) were detected corresponding to purified fusion proteins GST-ICP27, GST-ORF57 and GST alone respectively (Figure 3.7 Panel I). Lower MW bands were observed showing truncations and degradation of full-length proteins. A GST pull down assay experiment was carried out using the above fusion proteins and HeLa mock infected cell extract followed by western blotting with anti NUP 62 polyclonal antiserum (N-19) that detects the N-terminus domain of nucleoporin 62 of human origin. Results showed nucleoporin 62 interacted with ICP27 (lane 2) and ORF57 (lane 3) whereas p62 did not interact

with GST alone (lane 4). Lane 1 indicates the input control band of the NUP 62 from HSV-1 17+ infected cell extract at approximately 62 kDa (Figure 3.7 Panel II).

3.1.6 NUP 62 interacts with ICP27 in a GST pull down assay

I carried out a pull down assay by using GST-NUP 62 and GST alone bound to glutathione beads and HeLa infected cell extracts with wt HSV-1 17+, ICP27 null mutant Δ27 HSV-1, CMV 27 (ICP27) lysate (which is HeLa cells transiently transfected with an ICP27 expression plasmid) and mock infected. Pulled down proteins were fractionated on SDS-PAGE and transferred to nitrocellulose membrane followed by immunoblotting by using anti-ICP27 (1113) monoclonal antibody (Figure 3.8 Panel I). In wild type (wt) HSV-1 infected cells (lane 2) and CMV 27 (lane 4) transfected HeLa samples ICP27 was pulled down by GST-NUP 62 because an approximately 63 kDa sized band was observed similar in size to the input band of ICP27 in infected wt HSV-1 cell extract (lane 1). In contrast, there was no band of this size in mock (lane 5) and Δ27 infected cell extracts (lane 3) nor in the GST alone lanes (lane 6). Roughly equivalent amounts of a 40 kDa band of GAPDH in each lane in the pull down assay was detected in Panel II. The fusion protein GST-NUP 62 and GST alone used were visualised by western blotting with anti-GST antibody and showed approximately equal protein amounts were used in this experiment (Figure 3.8 Panel III).

3.1.7 NUP 62 interacts directly with ICP27 in a GST pull down assay

Recent protein interaction studies showed interference from RNAs in some protein-protein interactions cause indirect interaction between proteins. Use of purified fusion protein in both sides of the pull down assay or RNase I treatment of samples (Material and Method) can remove interference of the RNAs in the pull down assay. To find out more abut a direct or indirect interaction between NUP 62 and ICP27, GST fusion NUP 62 and His tagged ICP27 were expressed and purified and pull down assays carried out. In order to assess the possible role of RNAs in any interaction mediated between ICP27 and nucleoporins I have used RNase I to remove RNAs from some cell extracts as described in Material and Method. Fusion

proteins GST-NUP 62 and GST alone were incubated with purified His-ICP27 followed with fractionation on SDS-PAGE and western blotting with anti-His antibody. As shown in Figure 3.9 Panel I ICP27 bound to GST-NUP 62 (lane 2) but did not bind to GST alone (lane 3). Nucleoporin 62 directly interacted and pulled down His-ICP27. However the percentage of His-ICP27 interacting with NUP 62 is low in comparison with input His-ICP27. Image quantification showed only around 0.1% of ICP27 was pulled down. Very transient interaction of nucleoporins with NUP proteins in the transport system in the NPC could result in low efficiency of apparent protein-protein interactions *in vitro*. Although the interaction *in vitro* seems low, *in vivo* other protein-protein or protein-RNA interaction may stabilise the interaction. Therefore, recombinant GST-NUP 62 full-length protein and GST alone were used in a pull down assay with HeLa cell lysates in the presence or absence of RNase I. Fusion proteins were incubated with wt HSV-1 17+ infected HeLa cell extracts and treated with and without RNase I. A western blot of the pulled down proteins was carried out with anti-ICP27 monoclonal antibody (1113). Results showed (Figure 3.9 Panel II) NUP 62 interacted with ICP27 in the presence (lane 2) and absence (lane 1) of RNase I and reveals that interaction between ICP27 and nucleoporin 62 is not RNA-mediated and they can interact directly with each other in the presence of HeLa extracts. Unbound samples of Panel II were western blotted with anti GAPDH antibody (Panel III) to demonstrate similar amount of input protein in each reaction.

3.2 Co-immunoprecipitation assays of ICP27 and NUP 62

3.2.1 Introduction

Immunoprecipitation is a frequently used method to purify specific proteins from complex samples such as cell lysates or extracts. An important characteristic of IP reactions is their potential to deliver not only the target protein but also other molecules that interact with the target. An antibody forms an immune complex with a specific target antigen such as in a lysate. It is then captured on either protein A

or G sepharose beeds. Components of the bound immune complex are eluted from beads and analysed by SDS-PAGE and often detected by western blot.

3.2.2 Using anti-ICP27 and nucleoporin 62 antibodies for co-immunoprecipitation

Western blot and immunoprecipitation assays have demonstrated the efficiency of anti-ICP27 mAbs 1113 and 1119 to detect ICP27 in different cell extracts in our laboratory. The use of various monoclonal and polyclonal antibodies against different nucleoporins was also tested and optimised to apply them for each co-immunoprecipitation assay. Profiles of protein bands, which interact either with ICP27 or nucleoporins, were established. The heavy chain of the immunoprecipitating antibodies and ICP27 and some nucleoporins migrated around the same point on the SDS-PAGE gel, however, visualisation of the heavy chain (due to cross reactivity of immunoprecipitating antibodies 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. Previous studies in our laboratory showed profiles of cell proteins, which interact with ICP27, using radiolabelled cell extracts of HSV-1 infected HeLa, Vero or BHK cell lines. The pattern of immunoprecipitating bands was broadly the same in all cell types. Many laboratories routinely use infected HeLa cells to study virus proteins. Repetition of the co-immunoprecipitations often 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. HeLa cells were chosen for the rest of the study, they are routinely used in our laboratory to examine HSV-1 protein interactions and as they were routinely passaged in our laboratory, were easy to obtain. To optimise this method for further experiments a control immunoprecipitation with beads alone was performed and ICP27 precipitated with anti ICP27 antibody (1113). Figure 3.10, lane 6 shows that beads alone, incubated in the presence of HSV-1 wild type infected cell extracts did not precipitate any ICP27. In contrast, beads with 1113 ICP27 antibody efficiently precipitated ICP27 (lane 1) while beads with anti-NUP antibody 414 did not precipitate ICP27. This demonstrates the specifity of the ICP27 antibody. ICP27 was present in the supernatant after

pelleting the α-414 beads as expected. Not all ICP27 was precipitated by 1113 antibody beads because some can still be detected in the supernatant. There are two ICP27 bands detected. These probably represent phosphorylated forms of ICP27. Because I never detected ICP27 precipitating with beads alone, I did not routinely preclear my HeLa extracts before immunoprecipitation. I understand that it is important to do this for all experiments. The following data are therefore subject to question.

3.2.3 Stringency of interaction between ICP27 and its co-immunoprecipitating nucleoporin 62

Previous studies on co-immunoprecipitation in our laboratory showed the initial profile of proteins, which interacted with ICP27, by using buffer containing 100mM NaCl, a salt concentration close to the physiological salt concentration of ~90mM. The strength of protein-protein (NUP 62/ ICP27) interactions in co-immunoprecipitations was tested substituting the 100mM buffer with washes of increasing salt concentration up to 500 mM. The resultant profile of bands was then inspected. Bands representing specific interactions were most obvious at 100mM but at higher salt concentrations bands remained (Figure 3.11). In Panel I the 63 KDa band represents ICP27 that was precipitated by its antibody at various salt concentrations in contrast normal mouse serum (NMS) that could not bring down ICP27. However NMS is not the best control to use but it is the only control I have used in this study. A better control would be to use an irrelevant antibody of the same isotope for example anti-GAPDH antibody. The 62 KDa band in Panel II showed the interaction of NUP 62 with ICP27 and it was still clearly visible after a 500mM salt wash.

3.3 RNase treatment

3.3.1 RNase I activity and use of that in protein interaction assay

It has been shown that some ICP27 interactions with its partner proteins are completely or partially RNA mediated. Each RNase has different cleavage

specificity. RNase I efficiently cleave after all four bases of single-stranded RNA, in contrast to RNase A, which only cleaves after C and U residues. RNase I degrades all RNA dinucleotide bonds leaving a 5' hydroxyl and 2', 3' cyclic monophosphate. It will degrade any RNA to a mixture of mono-, di-, and trinucleotides. It has a marked preference for single-stranded RNA over double-stranded RNA, which allows it to work well in ribonuclease protection assays. Regarding my aims to find direct interaction of ICP27 with nucleoporins I carried out the control experiments of RNase I activity to remove all RNAs and immunoprecipitation assays. In order to test achievement of RNase I activity, 100 µg of wt infected HeLa cell protein extract and mock infected cell extract was treated with RNase I at 37℃ as described in Materi al and Methods. Samples were run on an agarose gel with untreated HeLa mock infected cell extract as a control and visualised with ethidum bromide staining. Figure 3.12 Panel 1 shows the presence of RNA in lane 1 that is not treated with RNase I and remove of RNA in lanes 2 and 3 in wt and mock infected cell extracts which were treated with RNase I. Immunoprecipitation of RNase I treated cell extracts, with ICP27 antibody is shown (Figure 3.12). ICP27 from cell extracts was still bound by ICP27 antibody in the presence of RNAse I (Panel II, lane 1), while binding to normal mouse serum (NMS) was not observed (Panel II lane 2). Panel III shows that NUP 62 from RNase I treated cell extracts (input) co-precipitated with ICP27 while binding to normal mouse serum (NMS) was significantly reduced. The apparent residual binding to NMS may be nonspecific or may be the result of alteration in mobility of the antibody heavy chain band due to loading dye concentration (Figure 3.12 Panel III, lane 2). RNA residues may have no contribution to the ICP27-NUPs interaction *in vivo.* In confirmation, RNase I treatment in the pull down assay of GST fusion proteins with HeLa cell infected wt HSV-1 showed the interaction of ICP27 with nucleoporins are not RNA mediated. Figure 3.12, panel IV shows GST-NUP 62 pulled down ICP27 in the presence (Panel IV lane 2) and absence (Panel IV lane 3) of RNase I.

3.4 ICP27 and nucleoporin interactions in virus infected cells

3.4.1 ICP27 and NUP 62 co-immunoprecipitate from cell extracts

Further evidence for an interaction between ICP27 and NUP 62 *in vivo* came from western blotting of the ICP27 and NUP 62 co-immunoprecipitation experiments with various antisera. Different antibodies were used in these experiments after optimisation of the test using specific antibody and normal control serums. Results confirmed that ICP27 and NUP 62 were precipitated by each other.

3.4.1.1 ICP27 monoclonal antibodies (1113 and 1119) used to co-immunoprecipitate NUP 62 from extracts of HeLa cells

ICP27 monoclonal antibodies (mAb) 1113 and 1119 were used to co-immunoprecipitate nucleoporin 62 from wild type (wt) HSV-1, Δ27 HSV-1 and mock-infected HeLa cell extracts. I used as a control wt HSV-1 HeLa cell lysate incubated with normal mouse serum (NMS). Again, I note that this was not the best control to use in this experiment. An irrelevant antibody of same isotope (IgG) as 1113 and 1119 should have been used. Antibody, lysates and protein A beads were incubated in IP buffer at 4°C. Proteins were separated by SDS-PAGE and visualised by transfer to nitrocellulose membrane, probed with antibody and X-ray film exposure analysis. Samples were also western blotted with ICP27 Mab to show anti-ICP27 antibody pulled down ICP27 and NMS did not bring down ICP27. Figure 3.13 Panels I, II and III shows the profile of the immunoprecipitation results based on cell extracts used. As it seen in Panel I lane 1 and Panel II lane 1 anti-ICP27 antibody precipitated ICP27 and co-precipitated nucleoporin 62 respectively. Whereas ICP27 null virus infected cell extracts Δ27 (Panel I lane 2) and mock infected cell extracts (Panel I lane 3) did not precipitate with anti-ICP27 antibodies. Also ICP27 null Δ27 (Panel II lane 2) and mock-infected samples (Panel II lane 3) did not precipitate nucleoporin 62. ICP27 and NUP 62 was not precipitated by the normal mouse serum in HSV-1 17+ infected cell extract (Panels I and II lane 4). The intense lower band (about 50 KDa) is due to background from the heavy chain of the antibodies used for the immunoprecipitation. Unbound

samples and were tested by separation of the above samples on SDS-PAGE gel and western blotting with anti-FG repeat NUPs 414 antibody. The last panel of this figure (Panel III) reveals roughly equal amounts of the control unbound NUP 62 in each band in this experiment.

3.4.1.2 Monoclonal anti-FG-repeat NUPs 414 and anti NUP 62 antibodies (N-19, D-20 and H-122) used to co-immunoprecipitate HSV-1 ICP27 from extracts of HeLa cells and BHK cell infected and uninfected with HSV-1

Immunoprecipitation of wild type (wt) infected HeLa cell extract with antibodies against NUP 62 precipitated a band of about 62 kDa corresponding with NUP 62. Three anti-NUP 62 antibodies N-19, D-20 and H-122 were incubated with wt infected HeLa cell extracts and precipitated complexes were separated on SDS-PAGE and visualised by western blotting with anti-FG-repeat Nups 414 antibody. Figure 3.14 Panel I shows NUP 62 was precipitated by all of these antibodies but using anti-NUP 62 (H-122) gives the best level of NUP 62 band in this experiment in presence but not absence of the RNase I (Figure 3.14 lanes 3 and 7). The lowest level of NUP 62 band was achieved with anti-NUP 62 (D-20) and its binding was reduced after RNase I treatment (lanes 1 and 5). Levels of proteins in different lysates were indicated by western blotting of unbound samples with anti GAPDH antibody (Panel II). Reduction of interaction in lanes 5 and 6 in comparison of increased interaction in lane 7 could be due to configuration of protein and antibody binding and the position of RNAs in the complex formed.

Interaction of nucleoporin 62 with ICP27 was first investigated by co-immunoprecipitation with anti-NUP 62 (N-19) antibody due to avalibility of that during my project and ability of that to show NUP 62 in different lysates. Lysates of HeLa cells infected with wt, Δ27, M15 (a defective C-terminus point mutant, Table 2.1) virus and mock infected were incubated with this antibody and complexes separated on SDS-PAGE and analysed by western blotting with anti-ICP27 antibody (1113) and anti-NUP 62 (N-19). As a control, normal goat serum (NGS) was used with wt cell lysates. I note again that this was not the best control to use in this experiment. An irrelevant antibody of same isotope (IgG) as N-19 should have been used. Figure 3.15 Panel I shows ICP27 co-precipitated from wt and M15 infected cell extracts in the presence of RNase I (Panel I lanes 3 and 5).

While ICP27 null mutant (Δ27), did not show the corresponding band (Panel I lane 4). Also normal goat serum (NGS) did not co-precipitate ICP27 from wt infected cell extract (Panel I lane 6). In Figure 3.15 Panel II Western blotting with anti-NUP 62 (N-19) showed precipitation of NUP 62 from wt, Δ27 and M15 cell extracts incubated with anti-NUP 62 (N-19) antibody (Panel II lanes 3,4 and 5) but not with normal goat serum (Panel II lane 6). A band of 63 KDa corresponding to ICP27 is seen in lane 2 in panel I but not in the mock infected cell extract, as expected. A band of 62 KDa is seen in both the mock and 17+ infected cell extract indicating a similar amount of p62 in each.

This antibody was used for co-immunoprecipitation of ICP27 in BHK cell extracts infected with wt, Δ27 and mock infected. Figure 3.16 shows NUP 62 precipitated ICP27 in both wt infected and CMV-27 transfected BHK cells and not in Δ27 and mock infected cell extracts nor with normal goat serum (NGS). Because the NUP fusion proteins used in this project were of human origin, experiments were continued in HeLa cells. BHK cell is a standard and most commonly used cell line for growing HSV-1.

The next antibody used in co-immunoprecipitation experiments was anti-NUP 62 (H-122). Lysates of HeLa cells infected with HSV-1 wt, and mutant HSV-1 M16 (Table 2.1), Δ27 HSV-1 and mock infected were mixed with antibody. Wild type infected cell extract with normal rabbit serum (NRS) was used as a negative control. Again, I note that this was not the best control to use in this experiment. An irrelevant antibody of same isotope (IgG) as H-122 should have been used. RNase I treatment was used in addition to untreated samples. The samples were visualised with western blotting with anti-ICP27 antibody (1113) and anti-NUP 62 (H-122) to show interacting proteins. Figure 3.17 Panel I shows ICP27 co-precipitated in wt and M16 mutant infected cell extracts in the presence and absence of RNase I (Panel I) whilst ICP27 was not precipitated from the null mutant (Δ27) and mock infected cell extracts (lanes 3, 4, 8 and 9) nor from wt-infected lysate incubated with normal rabbit serum (lane 10). Western blot for nucleoporin 62 with anti-NUP 62 (H-122) antibody showed that anti-NUP 62 (H-122) antibody precipitated nucleoporin 62 from wt, M16 and Δ27 samples in RNase I treated and untreated samples (Figure 3.17 Panel II). Also normal rabbit serum

(NRS) did not precipitate NUP 62 from wt infected cell extract (Lane10). However H-122 antibody did not precipitate NUP 62 from mock infected cell extracts. A faint band can be seen in lane 4 but no band is present in lane 9. This is very surprising because H-122 antibody should recognise NUP 62 against which it was raised. To confirm that NUP 62 was able to be precipitated from mock infected cell samples a new western blot was prepared and blotted with α- 414 antibody that detects all FG-repeats nucleoporins. In this case a strong band was detected in the mock infected cell lysates. It is clear that H-122 antibody is not suitable for use in co-immunoprecipitation experiments. Fortunately the anti NUP 62 antibody N-19 was able to precipitate both NUP 62 and ICP27 from cell extracts (Figure 3.15, Panel II).

Another antibody that detects a range of FG-repeat nucleoporins was used to co-immunoprecipitate ICP27 in complex with nucleoporin 62. The anti-414 monoclonal antibody was mixed with HSV-1 wt, Δ27 and mock infected HeLa cell extracts and its ability to precipitate ICP27 was tested in the presence and absence of RNase I. Samples were analysed by western blotting with anti-ICP27 antibody (1113). Figure 3.18 Panel I shows a band of approximately 63 kDa corresponding to protein ICP27, which co-precipitated in wt infected samples even when treated with RNase I (lanes 1 and 6) while lanes containing the controls, mock infected (MI) and ICP27 null mutant (Δ27) infected cell lysates, did not show the corresponding band (Figure 3.18 Panel I). A partial band was detected in lane 7 where the Δ27 infected cell lysate was co-immunoprecipitated with anti 414 antibody. It was not seen in lane 2 without RNase I digestion. It is likely that this is due to spill-over from lane 6. Also normal mouse serum (NMS) did not co-precipitate ICP27 from wt infected cell extract (lane 4). Then samples were immunoblotted with anti-FG repeat NUPs 414 antibody and showed nucleoporin 62 as well as other tested FG repeat NUPs were precipitated by this antibody in wt, Δ27 and mock infected HeLa cell extracts (Figure 3.18 Panel II) while normal mouse serum (NMS) did not precipitated NUPs (lane 4). The additional bands are other FG-repeat nucleoporins that are detected with 414 antibody and the heavy chain of the antibody is seen at the bottom of the gel.

3.5 Mapping the interacting regions of ICP27 with NUP 62 using GST pull down assay

GST pull down assays of GST-NUP 62 were carried out and the expressed fusion protein bound to beads was mixed with HeLa cells extracts in the presence and absence of RNase I. Viral infections were carried out with the panel of the wt and ICP27 N-terminus and C-terminus mutants (Table 2.1). Samples were separated on SDS-PAGE and visualised by western blotting with anti ICP27 1113 (detects amino acids 109-138 for d3-4 mutant) and 1119 (detects amino acids 6-19 for dLeu mutant) monoclonal antibodies to detect all mutants. Anti-ICP27 1113 antibody detects all wt and mutant ICP27 proteins except d3-4 mutant in this experiment. Anti-ICP27 1119 antibody detects wt and all other mutants except dLeu. Results showed wt ICP27 and most of the mutants except ICP27 null Δ27, N-terminal mutant dLeu and C-terminal mutants M15 and M16 interacted with nucleoporin 62. Control mock-infected cell extract did not interact with GST-NUP 62 (lane 6) as well as GST alone that did not pull down ICP27 from wt infected HeLa cell extract (Figure 3.19 Panel I lane 15). Unbound samples fractionated on SDS-PAGE and western blotted with anti ICP27 antibodies showed ICP27 protein corresponding to the expected size in all wt and mutant cell extracts except Δ27 and mock infected as expected (Figure 3.19 Panel II). These data indicated that the interactive regions of ICP27 are in the initial N-terminus amino acids (dLeu mutant) and almost at the end of the C-terminus (M15 and M16 mutants) domain of ICP27. However, contrary to these data with GST pull downs I detected binding of M15 and M16 mutants to NUP 62 in immunoprecipitation experiments. This means that I cannot conclude that the ICP27 C-terminus is involved in the NUP 62 interaction. Further experiments are required to elucidate this problem.

3.6 Interaction of TAP with ICP27 and NUPs

3.6.1 Interaction of TAP with ICP27

TAP is a good control for ICP27 pull down because it interacts with both ICP27 and NUP 62. TAP/NXF1 is the most important nuclear export receptor for mRNAs in metazoans that is involved in the export of mRNA from the nucleus to the

cytoplasm. ICP27 was shown to interact with TAP/NXF1 both *in vitro* and in infected cells. It has been shown that ICP27 shuttles between the nucleus and cytoplasm via TAP and exports viral intronless mRNA. The C-terminus from residues 406 to 508 of TAP is required for the interaction with ICP27, but the N-terminal leucine-rich region from amino acids 6 to 12 also contribute in this interaction. In fact, ICP27 interacts directly with TAP/NXF1 *in vitro*. The association of ICP27 with TAP/NXF1 occurs after ICP27 has left sites of viral transcription. Therefore, ICP27 mediates viral RNA export through the TAP/NXF1 pathway (Sandri-Goldin 1998; Koffa, Clements et al. 2001; Chen, Sciabica et al. 2002; Ellison, Maranchuk et al. 2005). To confirm interaction of ICP27 with TAP, GST-TAP pull-down assays were performed with His-ICP27 and as a control GST alone. Bound samples were analyzed on SDS-PAGE followed by western blotting with anti ICP27 mAb (1113). Figure 3.20 Panel I shown GST-TAP pulled down ICP27 (lane 4) but GST alone did not interact with ICP27 (lane 3). Lane 2 shows interaction of NUP 62 with ICP27. Image quantification showed only around 0.1% of ICP27 was pulled down as before. Also the GST-TAP pull down assay test was carried out with HeLa cells infected with wt HSV-1 and its mutant Δ27. Cell extracts were incubated with GST-TAP and GST alone and were analysed on SDS-PAGE followed by western blot with anti ICP27 mAb (1113). Figure 3.20 Panel II shows ICP27 pulled down by GST-TAP (lane 3) but not with GST alone (lane 2). In comparison, ICP27 mutant Δ27 did not interact with GST-TAP (lane 4). Panel III shows expression of GST-TAP western blotted with anti-GST antibody. In Panel IV I have blotted against anti NUP 62 showing association of TAP with NUP 62.

Co-immunoprecipitation assays of TAP with ICP27 were also performed by using the anti-ICP27 antibodies 1113 and 1119 with a range of HeLa cell infected lysates and western blotted against TAP and ICP27. Results determined TAP co-precipitated with ICP27 from wt infected HeLa cells but not with the other lysates (Figure 3.21 Panel I). When membranes were western blotted against ICP27, precipitation of ICP27 with ICP27 antibody in wt and M15 infected cell extracts was observed (Figure 3.21 Panel II). This confirms previously published data from our laboratory and from the Sandri-Goldin laboratory.

4 Testing Interaction of ICP27 with nucleoporin 98, 153 and 214

4.1.1 Interaction of ICP27 with nucleoporin 98

Nucleoporin 98 is one of the best characterised FG repeat mobile nucleoporins that is placed on both sides of the nuclear pore complex and has been shown to shuttle between the nucleus and cytoplasm (Griffis, Altan et al. 2002). The ability of NUP 98 to export RNA through different pathways has been revealed (Powers, Forbes et al. 1997). It can bind to various proteins involved in nuclear transport such as HIV-1 Rev protein (Zolotukhin and Felber 1999) and importin β (Griffis, Altan et al. 2002). The N-terminal domain NUP 98 is FG-repeat rich and this contributes to protein interaction. Studies using Vesiculoviral matrix protein M (VSV M) showed that this protein binds to Rae1 and forms a complex with Rae1 and inhibits mRNA export. Rae1 is important to provide delivery of TAP to NUP 98 and also has a role in mRNA export by interaction with NUP 98 (Faria, Chakraborty et al. 2005). The ability of ICP27 in shuttling and export of viral mRNA has been shown in our laboratory and in this section I present data examining interaction of ICP27 with nucleoporin 98 in infected cells and *in vitro*.

4.1.2 ICP27 interacts with NUP 98 in a GST pull down assay

Fusion proteins GST-ICP27 and its homologue GST-ORF57 and negative control GST alone were purified, bound to glutathione beads and used to test whether ICP27 and ORF57 pulled down nucleoporin 98 from infected HeLa cell extracts. The input fusion proteins used were visualised by coomassie blue staining and bands of approximately 90 KDa (Figure 3.7 Panel I lane 2), 80KDa (lane 1) and 28KDa (lane 3) corresponding to full length purified fusion protein GST-ICP27, GST-ORF57 and GST alone respectively were observed as well as a number of degradation products. A GST pull down assay experiment was carried out using the above fusion proteins and infected HeLa cell extracts. HeLa cell nuclear and cytoplasmic extracts were prepared (Figure 5.8). Both nuclear and cytoplasmic extracts were incubated with the above fusion proteins and complexes separated by SDS-PAGE followed by western blotting with anti NUP 98 polyclonal antiserum

(L-20) that detects the N-terminus domain of nucleoporin 98 of human origin. Results showed (Figure 4.1) nucleoporin 98 in both nuclear and cytoplasmic extracts interacted with ICP27 (lanes 2 and 7) and ORF57 (lanes 4 and 8) whereas NUP 98 did not interact with GST alone (lane 3). Also lanes 1 and 6 indicate the input control band of about 98 KDa that corresponded to NUP 98 from HeLa infected cell extracts. We expect the main position of nucleoporin 98 to be in the nuclear side of the NPC. NUP 98 was observed to be present in the nuclear fraction and interacted with ICP27 and ORF57 in nuclear extracts. Some interaction was also observed with NUP 98 in cytoplasmic extracts. The lower molecular weight bands in lanes 2 and 7 may be a cross ractivity of the NUP 98 antibody with a cellular protein. However, this band was detected only in the GST-ICP27 lanes and not in the other lanes so this is difficult to explain. I should have used an irrevelent GST fusion protein to rule out nonspecific binding. In addition I should have examined binding of cytoplasmic extract to the control GST protein.

4.1.3 NUP 98 may interact with ICP27 in the pull down assay

Truncated GST-NUP 98 fusion protein containing amino acids 66-515 was expressed and purified and bound to glutathione beads (Figure 3.6 Panel II). Pull down assays were carried out using GST-NUP 98 and GST alone. These fusion proteins were interacted with HeLa cell extracts infected with wt HSV-1 17+, ICP27 null Δ27 HSV-1 and mock infected. Pulled down proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane followed by immunoblotting using anti-ICP27 (1113) monoclonal antibody (Figure 4.2 Panel I). In wild type (wt) (lane 2) infected samples ICP27 was pulled down by GST-NUP 98 (66-515) as shown by the approximately 63 KDa sized band (lane 1). In contrast, there was not any protein pulled down in mock (lane 4) and Δ27 HSV-1 infected cell extracts (lane 3) as well as GST alone with HSV-1 17+ infected extract (lane 5). The profile of unbound ICP27 protein in the pull down assay is shown by western blotting with anti-ICP27 antibody (Figure 4.2 Panel II). Also GAPDH levels in the lysates were assessed by western bloting with anti GAPDH antibody and shown in panel III.

4.1.3.1 Anti-NUP 98 antibodies (L-20 and H-300) used to try to co-immunoprecipitate ICP27 from extracts of HeLa cells infected and uninfected with HSV-1

Co-immunoprecipitation studies to find out *in vivo* any interaction between ICP27 and NUP 98 in infected cells were carried out. Co-immunoprecipitation of wild type (wt) and mutant HSV-1 infected HeLa cell extracts with antibodies against NUP 98 was tested. Two anti-NUP 98 antibodies (L-20 and H-300) were incubated with HSV-1 wt infected HeLa cell extract, M15 and M16 mutant as well as Δ27 and mock infected HeLa cell extracts. Figure 4.3 Panel I shows ICP27 was precipitated with anti-NUP 98 antibody L-20 in the presence of RNaseI. However, I was not able to compare this to precipitation in the absence of RNase I so I cannot say whether RNase reduces NUP 98 interaction with ICP27 with this antibody. Control preimmune normal goat serum did not precipitate ICP27. However, as I mentioned before in chapter 3, this is not the best control to use for the immunoprecipitation experiment. A small amount of ICP27 also co-precipitated from M15 infected cell extracts in presence of RNase I (Panel I lane 5). The significant reduction in interaction of M15 ICP27 protein with NUP 98 may reflect its reduced ability to take part in nuclear export of virus RNAs. However I did not include an input lane for M15 infected HeLa cells and it is known that M15 is replication defective. So this may explain the low level of precipitated ICP27 in this lane. As expected, ICP27 null mutant and mock infected cell extracts did not show the corresponding band (Panel I lanes 4 and 6). A band of around 63 KDa in the HSV-1 wild type infected cell extract input (lane 2) corresponds to ICP27 and acts as a positive control.

The next antibody used in a co-immunoprecipitation experiment was anti-NUP 98 (H-300). It was mixed with HeLa cell lysates infected with HSV-1 wt and HSV-1 mutants M16 and Δ27 and mock infected as well as HSV-1 wt infected cell extracts with normal rabbit serum (NRS). Also RNase I treatment was used in addition to untreated samples. Western blotting with anti-ICP27 antibody (1113) showed a possible interaction of the proteins. Figure 4.3 Panel II shows ICP27 co-precipitated in wt infected cell extracts in the presence and absence of RNase I and the approximately 63 KDa band in lanes 2 and 7 corresponded to the ICP27 protein. ICP27 also co-precipitated in M16 mutant infected cell extracts but only in the absence of RNase I. The faint band in lane 8 may be due to use of less lysate

(I do not have a control for this) in this sample or reduction of interaction by RNase I. Whilst NUP 98 H-300 antibody in the presence of ICP27 null mutant (Δ27) and mock infected cell extracts could not precipitate ICP27 (lanes 4, 5, 9 and 10). Wild type HSV-1 infected cell lysate ICP27 did not co-precipitate with pre immune rabbit serum (lane 11).

However Panel III shows that NUP 98 was not pulled down from mock infected and infected cell extracts using antibody L-20 (this was also the case for H-300). This was an unexpected result. However there is no record in the literature of these antibodies being used successfully to precipitate NUP 98. As I could not see precipitation of NUP 98 with the antibodies, this calls into question the ICP27 pull down data in Panels I and II. To explain the co-precipitation of ICP27 and NUP 98 in Panels I and II, it is possible that protein/ protein interactions such as NUP 98 and cargo like ICP27 may expose epitopes that could be hidden in the native protein. My results do not conclusively demonstrate an interaction between NUP 98 and ICP27.

4.1.4 Interaction of ICP27 with nucleoporin 153

One of the large, important FG repeat nucleoporins that is positioned in the nuclear basket is NUP 153. This 1476 amino acid nucleoporin is a structural component of the nuclear pore complex and the NPC transport system. The critical role of NUP 153 during nuclear export and import has been demonstrated (Ullman, Shah et al. 1999) and interaction of this nucleoporin with other NUPs such as NUP 62 and its contribution of them in nuclear transport has been suggested (Stochaj, Banski et al. 2006). Similar to NUP 214, the C-terminus domain of NUP 153 with many FG repeat residues is involved in interactions with various proteins that are related to the transport machinery (Walther, Fornerod et al. 2001). Since the aim of this project is to identify cellular nucleoporins which can interact with the HSV-1 ICP27, protein NUP 153 was next considered among the large subset of nucleoporins that contains long phenylalanine-glycine dipeptide-containing domains (FG repeats). It has been suggested these form a kinetic meshwork and allow transport of complexes by creating a barrier (Ribbeck and Gorlich 2001; Rout and Aitchison 2001). Also to achieve nuclear transport, proteins and RNAs bind transport-competent receptors either directly or indirectly via adaptor proteins (Mattaj and Englmeier 1998) and studies on cells infected with poliovirus have shown degradation of NUP 153 after infection with this virus in disruption of NPC (Gustin and Sarnow 2001). In this section my data suggest interaction of nucleoporin 153 with ICP27 by using protein interaction assay methods.

4.1.5 Recombinant GST-NUP 153 interacts with ICP27 in a pull down assay

Full length GST-NUP 153 fusion protein was expressed in bacteria and purified (Figure 4.4 Panel IV and V). Unfortunately many degradation products can be seen in the Coomassie-stained gel. However it is likely that the band with the lowest mobility is NUP 153 due to the correct apparent size. Western blotting of a similar gel with anti NUP 153 antibody revealed that the most upper band was indeed NUP 153. Although there is a bubble or space on the autoradiograph over the bound fraction between 70-180 KDa, the unbound fraction clearly shows a GST-NUP 153 band of around 180 KDa. This purified protein was used in a pull down

assay to test whether it is possible to bring down ICP27 from wt HSV-1 HeLa infected cell extract. RNase I treatment was used to remove the RNAs in the samples and as a control I interacted HSV-1 wt infected lysate with GST alone. SDS-PAGE followed by western blotting with anti-ICP27 antibody showed that ICP27 interacted with GST-NUP 153 but did not bind to GST alone. An approximately 63 kDa band corresponding to ICP27 was observed in the input lane (Figure 4.4 Panel I lane 3). Panel II shows unbound protein lysate used in this western blot experiment and panel III shows levels of GAPDH in the unbound samples.

4.1.5.1 Anti-NUP 153 antibody co-immunoprecipitated ICP27 from cell extracts

Specific monoclonal antibody against nucleoporin 153 was used to co-immunoprecipitate ICP27 from infected HeLa cell lysates. Antibody was mixed with wt HSV-1, ICP27 null mutant 27lac Z and Δ27 infected HeLa cell extracts and mock infected cell extracts. Samples were fractionated on 6% SDS-PAGE followed by western blotting with anti-ICP27 (1113) and anti-NUP 153 antibodies. Results indicated that anti-NUP 153 precipitated NUP 153 in all samples. Normal mouse serum did not precipitate any NUP 153 (Figure 4.5 Panel II). Western blotting with anti-ICP27 antibody showed nucleoporin 153 co-precipitated ICP27 from wt infected cell extract but not from its null mutants 27lac Z and Δ27 nor from mock infected cell extracts. Also co-immunoprecipitation with NMS did not bring down ICP27 from wt infected cell extract (Figure 4.5 Panel I) as expected.

4.1.6 Mapping the interacting regions of ICP27 with NUP 153 using GST pull down assay

Fusion protein full length GST-NUP153 was expressed and purified as described previously and HeLa cells were infected with wild type HSV-1 and ICP27 mutants representing several functional domains of ICP27 (Figure 4.6). To map the regions of ICP27 involved in the interaction with nucleoporin 153, GST-NUP 153 was used to pull down ICP27 from HSV-1 wt and ICP27 mutant-infected cell extracts. Pull down assays were followed by western blotting with both anti-ICP27

antibodies1113 and 1119. The observed pattern showed GST-NUP 153 brought down ICP27 in wt infected cell extract and interacted with both N and C terminus domain mutants of ICP27. Exceptions were dLeu a nuclear export signal mutant at very N-terminus part of ICP27 and M15 point mutation at the C terminus of ICP27. ICP27 null mutant Δ27 and mock-infected samples did not show the corresponding bands as expected (Figure 4.6 Panel I). The unbound sample membrane was stripped and western blotted with GAPDH to show the amounts of protein in the lysates (Figure 4.6 Panel III). It can be seen from the control western blot in Panel III that the amount of GAPDH is not equal in all lanes. This means that there is probably less extract loaded in lanes 5, 8 and 13. d2-3 (lane 5) showed strong interaction with NUP 153. However the weaker interaction of d6-7 may be due to lower levels of ICP27 in the reaction. Results suggest the partial contribution of some mutants that have significantly reduced interaction with NUP 153 such as d1-2 in the N-terminal domain and M16 in C-terminal part of ICP27. Unbound samples were western blotted with anti-ICP27 antibodies 1113 or 1119 and showed the levels of ICP27 protein in each sample (Figure 4.6 Panel II).

4.1.7 Interaction of ICP27 with nucleoporin 214/CAN

Nucleoporin 214/CAN is one of the cytoplasmic side FG repeat NUPs that interact with various cellular and viral factors to facilitate their transport through the nuclear pore complex. This 2090 amino acid NUP consists of three major domains including the N-terminal domain that localises to the cytoplasmic face of the NPC, the central domain associated with NUP 88 that binds to NUP 98, and is located at the cytoplasmic ring of the NPC near the entry to the central pore and the C-terminal FG-repeat domain that localises at both the nuclear and cytoplasmic face of the NPC. Multiple locations of the C-terminus indicates a flexible property of the NUP 214/CAN FG-repeat domain (Paulillo, Phillips et al. 2005).These C-terminal ~800 amino acid residues interact with various transport factors as well as with other FG repeat nucleoporins such as NUP 62 and has been shown to be involved in both nuclear import and export (Stochaj, Banski et al. 2006). The profiles of interaction between ICP27 and NUP 214/CAN proteins are presented in this part.

4.1.8 NUP 214/CAN interacts with ICP27 in the pull down assay

To test the interaction of the cytoplasmic nucleoporin 214/CAN with ICP27, I expressed and purified 2 recombinant GST fusion proteins of NUP 214 gifted by Dr. Elisa Izaurralde (Chapter 2). Both of these fusion proteins contained the C-terminal domain of NUP 214 that is FG-repeat rich. Amino acid truncation 1690-2090 was the bigger fusion protein that was observed in western blotting with an anti-GST antibody as well as anti FG-repeat NUP 414 antibody yielding an approximately 67 KDa band (Figure 3.6 Panel II). The second recombinant protein was a truncation of amino acids 1690-1894 that was used in some experiments yielding a band of an approximately 50 KDa (Figure 3.6 Panel III). Pull down assays were carried out using these GST fusion proteins and GST alone and incubated with HSV-1 wt, Δ27 and mock infected HeLa cell extracts. Samples were separated by running on SDS-PAGE and western blotted with anti-ICP27 antibody(1113). Results showed both GST-NUP 214 (1690-1894) and GST-NUP 214 (1690-2090) (Figure 4.7) interacted with ICP27 in HSV-1 wt infected cell extract samples but did not interact in Δ27 and mock infected HeLa cell extract samples (Panel I). While GST alone did not interact with ICP27 when incubated

with wt infected HeLa cell extract. This experiment also demonstrated GST-NUP 214 (1690-2090) gives a stronger band in comparison with its smaller truncation of GST-NUP 214 (1690-1894). The unbound samples of this pull down assay were analysed by running the samples and western blotting with anti-ICP27 antibody. These show the absence of ICP27 in Δ27 and mock infected HeLa cell extracts and presence of ICP27 in wt infected HeLa cell extract samples (Panel II). No band was observed in lane 6 with wild type HSV-1 infection. This was very surprising. It is possible that all the ICP27 had interacted with NUP 214 and precipitated from the extract or ICP27 had become degraded in this fraction. Western blotting with anti-GAPDH antibody showed similar levels of GAPDH in Panel III of Figures 4.7. I did not carry out the reverce experiment, using GST-ICP27 to pull down NUP 214/CAN.

4.1.8.1 ICP27 and NUP 214 co-immunoprecipitate from cell extracts

The antibody that I used in this study to co-precipitate ICP27 from cell extracts was anti-NUP 214 (N-15). Immunoprecipitation tests were carried out by mixing anti-NUP 214 (N-15) antibody with HSV-1 wt, M16 point mutant, ICP27 null Δ27 and mock infected HeLa cell extracts. Wild type HSV-1 infected cell extracts incubated with normal goat serum (NGS) was used as a control for the immunoprecipitation test. Samples were separated on SDS-PAGE followed by western blotting with anti-ICP27 (1113) antibody. As shown in Figure 4.8, ICP27 was co-precipitated by nucleoporin 214 in wt infected and M16 point mutant extract samples in the presence and absence of RNase I (lanes 1, 2, 7 and 8). The stronger band of ICP27 in M16 infected cell lysates (lane 2) in comparison to the band in HeLa cell lysates infected with wt virus is due to accidently loading of more M16 cell extract (2X) in this lane. Six lanes of Panel I were stripped and western blotted with anti FG-repeat NUP 414 antibody and showed precipitation of NUP 214 in all lanes except lane 6 where immunoprecipitation was carried out with normal goat serum. (Figure 4.8 Panel II). I did not carry out a GAPDH loading control on the unbound fraction. This should have been doen to confirm the increased loading in lane 2 Panel I.

4.1.9 Mapping the interacting regions of ICP27 with NUP 214 using a GST pull down assay

GST pull down assay was used to identify regions of ICP27 that are involved in interaction with nucleoporin 214/CAN. A truncation of GST-NUP 214 (1690-2090) that encompasses the other truncation of GST-NUP 214 (1690-1984) described previously, was used to pull down ICP27 from the panel of the ICP27 wt and mutant virus-infected cell extracts. Western blot of the membrane with anti-ICP27 antibody showed (Figure 4.9 Panel I) some of the mutant ICP27 proteins were pulled down by GST-NUP 214 (1690-2090) and there was no interaction with GST alone (Panel I lane 15). Data showed there was no binding between GST-NUP 214 (1690-2090) and ICP27 null mutant (Δ27) and mock infected HeLa cell extract (Panel I lane 2 and 3). C-terminus point mutants M15 and M16 (Panel I lane 5 and 6) and the N-terminus mutant dLeu (Panel I lane 7) were unable to interact with GST-NUP 214 (1690-2090). Other N-terminal and C-terminal mutants that interacted with GST-NUP 214 (1690-2090) gave ICP27 protein bands with different molecular weight corresponding to their mutation (Panel I). A GAPDH loading control is missing from this experiment. So I can only conclude whether a mutant interacts with NUP 214, not to what extent they may interact. Panel II shows ICP27 detected in all the lysates of HSV-1 infected cells, but not in the HSV-1 Δ27 infected, nor in mock infected cells. The Panels in Panel II are taken from different gels and gels that are differents to that on Panel I. This means that levels of ICP27 cannot be compared between the lanes. These results suggested that both the N-terminal and C-terminal part of ICP27 are involved in interaction with nucleoporin 214/CAN. The input sample of some GST-NUP 214 (1690-2090) that was used in this experiment is shown (Figure 4.9 Panel III).

5 Further study of ICP27 and nucleoporin interaction

5.1 ICP27 does not interact with non FG-repeat NUPs

5.1.1 GST-Rae 1 fusion protein expression

To optimise GST-Rae1 expression and obtain sufficient amounts of purified protein, effects of temperature and protease deficient BL21-Codon plus-RP competent *E.coli* cells that enables efficient high-level expression of heterologous proteins, were investigated. A GST-Rae1 expression plasmid (a non FG-repeat nucleoporin that is involved in mRNA export) was used in an optimisation experiment and the initial and final improved level of expression obtained can be seen in the next section.

5.1.1.1 The effect of temperature on expression of the GST-NUP Rae1

There are numerous single case studies showing increased solubility of recombinant proteins at lower cultivation temperatures. I have seen this effect on my nucleoporin GST fusion proteins at 29℃. Bacterial cultures were grown in 100 ml LB containing ampicillin and were induced at OD_{600} of 0.6 by 1.0 mM IPTG and incubated at 37℃ and 29℃ and harvested after 3 and 16 hrs respectively. Pellets were lysed by sonication in lysis buffer and incubated with gluthatione beads. Samples were analysed on 10% SDS-PAGE and stained by Coomassie blue. Results shown in Figure 5.1 demonstrated at 29℃ temperature that level of full-length GST-Rae1 expression was highest and the level and number of truncations were reduced (Panel I, lanes 1 & 2) in comparison with a higher cultivation temperature of 37℃ (Panel II, lanes 3 & 4). Thus protein expression continued to be at 29℃.

5.1.1.2 The effects of *E.coli* strain on expression of GST-Rae 1

To produce nucleoporins as GST fusion proteins, a protease deficient strain of *E.coli* (BL21) was used to minimise proteolytic degradation. Because I was using

human nucleoporins fused to GST and wanted to obtain significant expression of full-length GST-NUPs the strain of *E.coli* competent cells was altered to increase the amount of full-length product. Expression tests as described in Material and Methods were carried out on the strain BL-21 (DE3) Codon Plus RP (Stratagene), which has a plasmid that supplies extra tRNAs corresponding to codons that are rare in *E. coli* but common in humans. Figure 5.2 demonstrates significant efficiency of BL-21 Codon Plus RP in expression of GST-Rae 1 (Panel I, lane 1) in comparison to the usual strain BL21 (Panel I, lane 2). Enhanced level of protein expression and reduction of truncates and degraded bands are the advantage of *E.coli* BL-21 (DE3) Codon Plus RP (Stratagene), which was mostly used in this study. Optimised purified GST-Rae1 was analysed using a Protein 200 Plus LabChip kit (Agilent technologies) (Panel II, lane 1), and blotted with anti-GST antibody (Panel II lanes 2 & 3). This fast protein analysis with the bioanalyser system is used allow monitoring and optimisation of each step of recombinant protein purification via affinity chromatography. A similar approach was taken to purify another non FG-repeat NUP, NUP 107 (Figure 5.3, Panel I).

5.1.2 ICP27 does not interact with nucleoporin Rae1 and nucleoporin 107

Rae1 is an mRNA export factor that has a homologue Gle2 in *Saccharomyces cerevisiae.* Rae1 interacts with RNPs and mutation in the Rae1/Gle2 genes disrupts poly (A) RNA export, NPC, and nuclear envelope structure. This 41 KDa nuclear pore complex protein is a shuttling mRNA export factor that interacts with NUP 98 at an amino terminal region named the GLEBS motif or Rae1 binding site. Rae1 also binds TAP/NXF1 and a role is proposed for Rae1 in recruiting TAP to NUP 98, possibly representing the first step for interaction of mRNP export complexes to Nups (Blevins, Smith et al. 2003). It has been shown Vesicular stomatitis virus (VSV) M protein binds the mRNA export factor Rae1 and blocks mRNA export by disrupting Rae1 function (Faria, Chakraborty et al. 2005).

Nucleoporin 107 is localized to the nuclear rim and is an essential component of the nuclear pore complex (NPC). NUP 107 does not contain FG repeats that serve as docking sites for karyopherins and other transport factors. It has been shown

that depletion of mammalian NUP 107 resulted in the failure of a subset of NUPs to assemble into NPCs followed by degradation of these proteins (Ben-Efraim and Gerace 2001). To determine interaction between ICP27 and Rae1 and NUP107 pull down assays were carried out. GST-NUP107, GST-TAP (Figure 3.20, Panel III), GST-Rae 1 and GST alone were incubated with His-ICP27. Bound proteins were visualised by running on 10% SDS-PAGE and western blotting with anti-ICP27 antibody (1113). Figure 5.3 shows ICP27 was not pulled down with GST-NUP107 nor with GST-Rae 1 (Panel II, lanes 4 and 3) while GST-TAP had the ability to bring down ICP27 as expected (Panel II, lane 5). Two bands seen in lane 3 (GST-Rae 1) could be nonspecific detection of cellular proteins by the ICP27 antibody.

5.2 HSV-1 infection does not affect expression of nucleoporin

5.2.1 Introduction

Generally, transport of macromolecules through the nuclear pore complex is dependent upon interactions with the NPC, which is regulated by signalling pathways and viruses. In fact our understanding about virus-host interactions seems to supply insights into the key regulatory steps of nucleocytoplasmic shuttling, which could also provide possible targets for viral and cancer therapies (Fontoura, Faria et al. 2005). Certain RNA viruses that replicate in the cytoplasm disrupt nucleocytoplasmic trafficking. It may benefit them to inhibit cellular mRNA export and reduce the competition with the cellular machinery for replication and translation (Gustin 2003). It has been shown that poliovirus infection induces disruption of the NPC by inhibiting nuclear import of cellular proteins, which likely is due to the degradation of NUP 153, NUP 62 and probably of other nucleoporins (Gustin and Sarnow 2001). On the other hand it was demonstrated that Vesicular stomatitis virus (VSV) induces a major blockage in mRNA export by interaction between VSV M protein and the mRNA export factor Rae1 (Faria, Chakraborty et al. 2005). HCV inhibits expression by disruption of the Kapβ3/ Ran BP5 pathway.

The protein 5A (NS5A) of HCV is involved in interaction with Kapβ3 and disruption of nuclear import of ribosomal proteins (Chung, Lee et al. 2000). Also the potential ability of adenovirus in disruption of the mRNA export pathway has been demonstrated (Flint and Gonzalez 2003). HSV-1 is a DNA virus that replicates in the nucleus and its regulatory protein ICP27 can interact directly with REF/Aly that then recruits TAP to induce viral mRNA export (Koffa, Clements et al. 2001; Chen, Sciabica et al. 2002). It has been shown that HSV-1 infection of HeLa cells did not affect the nucleocytoplasmic transport of several cellular shuttling proteins but this study did not presented any data about nucleoporins (Chen, Li et al. 2005). In this project I have shown evidence to suggest direct interaction of shuttling HSV-1 protein, ICP27 with some FG-repeat nucleoporins. It was interested to examine the ability of ICP27 in interference with nucleoporins and any possible change in expression of NUPs.

5.2.2 Expression of ICP27 and mutated ICP27 in HSV-1 infected HeLa cells

A time course study of HeLa cell infection with wild type HSV-1 17+, ICP27 null (Δ27) mutant and mock infected cell extract was carried out at various times. Equivalent amounts of proteins were fractionated on 10% SDS-PAGE and western blotted with monoclonal anti ICP27 antibody (1113). Results showed ICP27 is expressed as early as 2 hours post infection (Figure 5.4, Panel II). It appears that expression of ICP27 increases after 2 hours and a high level of expression is seen at 8 h post infection at MOI=10 (lane 14), at 16 hpi with MOI=1 (lane 20) ICP27 levels eventually reduced due to cell lysis (e.g lane 29 MOI=0.1). The idea behind carrying out infections at different MOI until 48 hpi was to show expression of ICP27 in low MOI infected cells. A 63 KDa ICP27 band was detected in HSV-1 17+ infected cell extracts (lanes 2, 5, 8, 11, 14, 17, 20, 23, 26, 29). However no similar sized bands were seen in the mock infected and ICP27 null HSV-1 infected extracts (lanes 1, 3, 4, 6, 7, 9, 10, 12, 13, 15, 16, 18, 19, 21, 22, 24, 25, 27, 28, 30). The weak upper bands could be due to some cellular proteins that cross react with the anti-ICP27 antibody.

5.2.3 Expression of nucleoporins in mock, wt and HSV-1 ICP27 null mutant infected HeLa cells

A time course study of HeLa cell infection with wild type HSV-1 17+ , ICP27 null (Δ27) and mock infected cells was carried out, proteins were fractionated by running on a 6% SDS-PAGE and western blotted with monoclonal anti FG-repeat nucleoporin 414 antibody. Results showed various nucleoporins are expressed well at different MOI and at different times post infection (Figure 5.5). There is no significant difference in expression of nucleoporins in any of the cell extracts at any MOI or time post infection except at MOI=0.1 at 36 and 48 hpi (Panel III). There appears to be lower levels of FG repeat nucleoporins in lanes 22-27 but this may be due to reduced numbers of live cells even in the uninfected cell populations. The FG-repeat nucleoporins I worked on are marked. The additional bands detected could be some other cellular nucleoporins proteins that are recognised with anti FG-repeat NUPs 414 antibody but are not in my study. Infecting HeLa cells with the various HSV-1 ICP27 mutants I have used in my study and harvested and western blotted with anti FG-repeat NUPs 414 antibody showed a similar profile as above. This experiment shows that virus infection does not regulate NUPs.

5.2.4 Transient transfection study of ICP27 showed no change in expression level of endogenous nucleoporins

In addition to the previous studies on levels of expression of endogenous nucleoporins by infection with HSV-1 ICP27, I carried out transient transfection of enhanced yellow fluorescent protein ICP27 (EYFP-ICP27) plasmid, which expresses ICP27. After 24 h, transfected lysates and other control infected samples including mock, mock with optimem and lipofectamine and also GFP alone (because YFP was not available) were fractionated on 6% SDS-PAGE and western blotted with anti FG-repeat NUPs 414 antibody. Results did not show any effect of YFP-ICP27 on levels of expression of nucleoporins in tested samples in comparison with the controls seen in this experiment (Figure 5.6, Panel I). So ICP27 cannot regulate expression of nucleoporins. Also to show the presence of YFP-ICP27 and GFP alone in lysates, samples were fractionated on 10% SDS-

PAGE and western blotted with anti GFP antibody. Figure 5.6, Panel II shows the presence of ICP27 in the YFP-ICP27 transfected sample but not in the controls.

5.3 Nuclear and cytoplasmic expression of ICP27 and nucleoporins in mock, wt HSV-1 and mutated ICP27 HSV-1 infected HeLa cells

To examine the profile of nucleo-cytoplasmic expression of ICP27 and nucleoporins, infection of HeLa cells with HSV-1 wt, mutated ICP27 and mock infected cell extracts was carried out by MOI=10 at 6 and 12 hpi. Nuclear and cytoplasmic fractions were made as described by the manufacturer (Active Motif) and protein concentration estimated by Bradford assay. Samples were fractionated on 6% SDS-PAGE followed by western blotting against ICP27, NUP 98, NUP 62 and ICP4. Figure 5.7, Panel I shows ICP27 was expressed in the nucleus and cytoplasm in cells infected with wild type HSV-1. ICP27 mutant M15 was only examined at 12 hpi. It was also present in both compartments. ICP27 was not detected in mock and HSV-1 Δ27 infected cells. Expression levels of mutant M15 were reduced significantly in comparison with wild type. It has been shown that viral infection of cells with the M15 ICP27 mutant completely abrogates replication (Mears and Rice 1998) but the ability of this mutant to infect cells, express shuttling of protein and replicate DNA is similar to that of wild type HSV-1 (Mears and Rice 1998). This indicates more investigation is required to fully understand its role. The overexposed lanes 5-8 do not allow a clear comparison between ICP27 level in these experiments. Panel II shows expression levels of NUP 98 in nuclear and cytoplasmic fractions in infected and non-infected lysates. It could be seen that more nuclear expression of NUP 98 was present in most infections. Blotting with anti-NUP 62 antibody demonstrated significant nuclear expression of NUP 62 in comparison with its cytoplasmic profile (Panel III). The above membranes were stripped and western blotted against ICP4 to test expression of a viral nuclear protein (Panel IV). Unfortunately I was not able to probe for a cytoplasmic protein to verify the fractions. This experiment suggests that virus infection does not change the subcellular localisation of the NUPs.

5.4 Involvement of ICP27 SADET sequence mutants in interaction of ICP27 and nucleoporins

5.4.1 Introduction

Similar to ICP27, heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a multifunctional protein that is able to shuttle between the nucleus and the cytoplasm and with suggested role in transcription, export of mRNA as well as translation (Matunis, Matunis et al. 1992; Michael, Eder et al. 1997). An involvement of the SADET amino acid sequence that is conserved between the KNS residues of hnRNP K and the C terminus region of ICP27 has been suggested (Leiper 2004) (Figures 1.7 and 1.8). ICP27 contains a region (aa 338-378) homologous to the hnRNP K bi-directional shuttling domain KNS (Michael, Eder et al. 1997) and KNS mediated export is postulated via direct nucleoporin interaction. The HSV-1 M15 ICP27 replication defective mutant showed the importance of the positionally conserved amino acids within ICP27 in the C terminal region (Rice and Lam 1994). Claire Leiper, previously in our laboratory, made an engineered set of mutant plasmids that contain nucleotide substitution mutations in the SADET region to further investigate the structure and functions of ICP27. This work showed that certain SADET mutants altered the localisation pattern of ICP27 and also they failed to interact with the cellular export factor REF and hnRNP K. These mutants were introduced into the pCMV-27 vector (Leiper 2004). The aim of this section of the project was to evaluate involvement of ICP27 SADET mutants in interaction with nucleoporins and the nucleo-cytoplasmic transport system.

5.4.2 Expression of SADET mutant proteins in transfected HeLa cells

To test the protein expression of the ICP27 SADET mutants and to show the predicted size of the proteins. Equal amounts of wt and ICP27 SADET mutants expression plasmids (6 μg) were transfected into HeLa cells. Transfected cells were harvested after 24 hours, protein lysates prepared and Bradford assay carried out to determine protein concentration. Equal amounts of lysates were

fractionated on 10% SDS-PAGE and proteins were analysed by western blotting with the anti ICP27 1113 antibody (Figure 5.8, Panel I). All of the wt and mutant ICP27 expression constructs tested encoded a protein that migrated at a similar size to wt ICP27. The wt CMV-27 SADET showed the 63 KDa band corresponding to ICP27 and some degradation/truncation products (Panel II lane 7). Mutant proteins were expressed at a significantly lower level in comparison with wt ICP27 and the level seemed to vary among the mutants (Panel I lanes 2-7 and Panel II lanes 1-7). As a control, no protein was detected with antibody in the mock-transfected lysate (Panel I lane 1). In Panels III and IV, the above membranes were western blotted with anti GAPDH antibody and demonstrated the level of proteins used in this expression. Lanes 1-7 Panel III were exposed for a longer time than Panel IV.

5.4.3 The SADET region is not involved in the interaction of ICP27 and nucleoporins

To examine involvement of SADET mutants in interaction of ICP27 with nucleoporins, immunoprecipitation experiments were performed. Lysates from HeLa cells transfected with a range of ICP27 SADET mutants and ICP27 wild type expression plasmids were incubated with anti ICP27 1113 antibody and beads and treated with and without RNase I. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membrane and western blotted with anti ICP27 antibody 1113. RNase I untreated samples showed precipitation of ICP27 from all lysates as a 63 KDa band (Figure 5.9, Panel I). In RNase I treated samples, wt SADET (CMV-63 in Materials and Methods) and mutants GADET and SADEG showed a strong band in comparison to other mutants indicating more protein precipitation (Figure 5.10, Panel I). I cannot explain why SADET mutants have different mobilities in this experiment. Then membranes were stripped and western blotted with anti FG repeat 414 antibody and analysed to see co-immunoprecipitation of NUP 62 by ICP27 SADET mutants (Figure 5.9, Panel II). Results were difficult to interpret because of variation in NUP 62 precipitation. While the wild type SADET (CMV-63) co-immunoprecipitated NUP 62, levels of precipitated NUP 62 and hnRNP K followed those of ICP27 expression. The AGAGA mutant that is entirely changed compared to wt SADET also co-

precipitated NUP 62 (Figure 5.10, Panel II). The AGAGA mutant lysate was limiting so it was used just in the RNase I treated experiment. RNase I treatment reduced interaction of ICP27 with some mutants such as SAG and SAD as well as interaction of them with NUP 62. However there was little change in level of the wt SADET and mutants GADET and SADEG after RNase I treatment (Figure 5.10, Panel I). To show presence of ICP27 expression in the lysates and to estimate the reactive amount of protein used for this experiment, unbound samples were run on SDS-PAGE, transferred and western blotted against anti ICP27 antibody and showed ICP27 was present in these samples (Figures 5.9 and 5.10, Panels IV). The unbound sample membrane was stripped and western blotted with anti GAPDH antibody and data confirmed roughly equal amounts of protein used in this experiment (Figures 5.9 and 5.10, Panels V). ICP27 protein has a SADET sequence is partially identical to the sequence of the hnRNP K bi-directional shuttling sequence (Michael, Eder et al. 1997). The next work was designed based on the idea that this sequence from ICP27 that is conserved in its homologues has been implicated in shuttling hnRNP K. I tested whether ICP27 SADET mutants may bind hnRNP K. It could show if mutations in this region result in the loss of interaction. The rest of the ICP27 immunoprecipitation samples were fractionated by SDS-PAGE gel and western blotted against hnRNP K and showed the co-precipitation of hnRNP K by ICP27 in all wt and SADET mutants in the presence and absence of the RNase I treatment (Figures 5.9 and 5.10, Panels III). However it seems by the present results that all SADET mutants of ICP27 bound to hnRNPK. This is different to what was found previously in our laboratory and further work needs to be done to make it clear.

5.5 Time course immunoprecipitation study of wt and mutant HSV-1 infected HeLa cell extracts ICP27 and nucleoporin interaction

5.5.1 Introduction

ICP27 not only localises to the nucleus but also is capable of shuttling between the nucleus and cytoplasm, a feature that is consistent with a role in the RNA-based regulation of gene expression. Different studies have shown nuclear distribution of

ICP27 at early times of infection and both nuclear and cytoplasmic localisation at later times of infection (Phelan and Clements 1997). So it was of interest to understand the timing of nucleoporin involvement with ICP27 in infected cells by using immunoprecipitation experiments.

5.5.2 Profile of expression of ICP27 and nucleoporins in a time course immunoprecipitation study

To examine a time course of immunoprecipitation, Hela cells were infected with HSV-1 wt, ICP27 null Δ27, C-terminus mutant M16 HSV-1 and mock infected. Cells were harvested at 3, 6.5 and 9.5 hpi at MOI=10 and 16 and 22 hpi at MOI=1 and protein concentration was obtained by Bradford assay. Equal amounts of proteins were separated on 6% SDS-PAGE and transferred to nitrocellulose membrane and western blotted with anti ICP27 antibody 1113. The protein expression profile showed early expression of the wt ICP27 but not M16 mutant at 3 hpi (Figure 5.11, Panel I). This difference in level of ICP27 expression at early times of infection in comparison to Figure 5.4 could be due to experimental variation. There was no expression of ICP27 in Δ27 and mock-infected extracts at all time post infection, as expected (Figure 5.11, Panels I and II). ICP27 expression was high at 9.5 in both wt and M16 mutant. At 22 hpi with MOI=1 strong bands of wt and M16 ICP27 were also detectable (Figure 5.11, Panel II). The membranes were stripped and western blotted for FG-repeat nucleoporins using anti FG-repeat NUPs 414 antibody. The results showed a similar level of nucleoporin expression at different times within the same experiment and there was no significant change in profile of NUP expression (Figure 5.11, Panel II). Also the expression pattern of nucleoporins in different wt, Δ27, M16 and mock infected were similar. The disappearance of bands in some lanes is due to technical problems transferring the gel to membranes.

5.5.3 Wild type and M16 mutant ICP27 co-precipitated with anti FG-repeat NUPs 414 antibody

A range of immunoprecipitation and co-immunoprecipitation experiments with the above time course samples were carried out. Firstly, using anti ICP27 1113 and

1119 antibodies, I performed a time course ICP27 immunoprecipitation test. Equal amounts of lysates of HSV-1 wt, Δ27, M16 and mock infected HeLa cells extracted at 3, 6.5 and 9.5 hpi at MOI=10 and 16 and 22 hpi at MOI=1 were incubated with antibodies and protein A beads and eluted in protein sample loading buffer and fractionated on 6% SDS-PAGE. Transferred membranes were western blotted with anti ICP27 1113 antibody and exposed to X-ray film. As shown in Figure 5.12 panel I, at 3 hpi it is not clear whether ICP27 precipitated in any lysate, both RNase I treated and untreated. There are two reasons for this. Firstly, very little ICP27 is expressed at 3 hpi (Figure 5.11, Panel I). Secondly, the separation of proteins around 60 KDa on this gel is poor so the antibody heavy chain migrated in the region of the ICP27 band. The input sample shown ICP27 band because this is 8 hpi at MOI=10. At 6.5 hpi precipitation of wt and M16 ICP27 are visible under both RNase I treated and untreated condition and reveals RNA had no effect on the ICP27 precipitation (Figure 5.14, Panel II). Also 6.5 hpi samples fractionated by 10% SDS-PAGE showed clearer bands in comparison with the 6% gel (Figure 5.14, Panel III). Further infection at 9.5 hpi clearly detected wt and M16 ICP27 that precipitated in this series of experiment especially in the case of M16 mutant ICP27(Figure 5.12, Panel IV). At MOI=1 and 22 hours post infection however wt ICP27 was detected clearly but the M16 ICP27 band disappeared (Figure 5.12, Panel VI). However a repeated fractionation by 10% SDS-PAGE for 22 hpi again showed the M16 ICP27 band in this immunoprecipitation assay (Figure 5.12, Panel VII). Differences in mobility of ICP27 in IP samples and the input could be due to interference from the antibody heavy chain.

After that I carried out a co-immunoprecipitation test of ICP27 using anti FG-repeat nucleoporin antibody 414 at early and late times of infection in HeLa cells. The samples were separated on 6% SDS-PAGE transferred and western blotted with anti 414 antibody. Figure 5.13 Panels I-III show precipitation of NUPs from 3 hpi to 9.5 hpi at MOI=10 with 414 antibody. Panels IV and V show NUP precipitation at 16 and 22 hpi at MOI=1. Unfortunately because of using less input sample and technical problems there are no clear bands of NUPs in some lanes of these gels (Figure 5.13). This problem could be seen in mock samples of lanes 9 in Panels IV and V of Figure 5.13 due to less loading samples. Also a transfer problem in the western blot of Panel III made the middle of the gel unclear (Figure 5.13, Panel III).

Western blot of the above membranes with anti-ICP27 antibody showed co-immunoprecipitation of ICP27 at later times of infection in both wt ICP27 and mutant M16 (Figure 5.14, Panel I). All of these problems make this experiment very difficult to interpret. However, co-precipitation of the ICP27 in mutant M16 was significantly reduced in comparison with wt perhaps due to the inability of this mutant to complete the life cycle (Figure 5.14).

6 Discussion

6.1 ICP27 is a multifunctional HSV-1 regulatory protein

ICP27 is a multifunctional regulatory phosphoprotein, which functions at transcriptional, posttranscriptional and translational levels (Smith, Malik et al. 2005). At the posttranscriptional level ICP27 is involved in RNA processing, export and translation (McLauchlan, Phelan et al. 1992; Sciabica, Dai et al. 2003; Fontaine-Rodriguez, Taylor et al. 2004; Ellison, Maranchuk et al. 2005; Larralde, Smith et al. 2006). At late times, beginning at about 6 h after infection, ICP27 begins to shuttle between the nucleus and cytoplasm using cellular transport machinery proteins such as Aly/REF, TAP/NXF (Sokolowski, Scott et al. 2003; Chen, Li et al. 2005) and CRM1 (Sandri-Goldin 2004). The nuclear pore complexes (NPC) that consist of interacting proteins called nucleoporins is the sole gateway between the nucleus and the cytoplasm, and it mediates all trafficking between these two components (Mattaj and Englmeier 1998; Zenklusen and Stutz 2001; Weis 2002; Sandri-Goldin 2004; Hetzer, Walther et al. 2005). This project aimed to investigate the interaction of ICP27 with cellular nucleoporins and establish a new model of involvement of ICP27 with transport machinery.

6.2 Summary of interaction of ICP27 with NUPs using pull down and immunoprecipitation assays

Nuclear pore complexes are massive and comprise approximately 30 different proteins, called nucleoporins (NUPs). NUPs are involved both in transport of proteins and RNAs and can be present in the central basket, the cytoplasmic side and the nuclear side of NPCs in the nuclear membrane (Weis 2002). They are grouped into two main families, FG-repeat nucleoporins containing repetitive elements of FXFG or GLFG and non FG-repeat NUPs (Poon and Jans 2005).

There are a number of different methods that can be used to investigate protein-protein interactions. These include pull down assays and co-immunoprecipitation experiments. In this thesis I have used both techniques to investigate interaction of

HSV-1 ICP27 protein with a range of NUPs. ICP27 interacted with NUP 62 *in vitro* and *in vivo*. My data also indicated a possible interaction of NUP153 and 214/CAN with ICP27, although the data were not so complete as that with NUP 62. I was unable to determine an interaction with NUP 98 because the NUP 98 antibodies available were unable to precipitate NUP 98 itself. NUP 62, NUP153, 214/CAN and NUP98 are FG-repeat NUPs and I also tested ICP27 interaction with non-FG repeat NUPS, Rae 1 and NUP 107. It was clear that these NUPs did not interact with ICP27 indicating that this viral protein may associate specifically with certain FG repeat NUPs.

6.3 ICP27 interacts with NUP 62 directly

The mammalian protein NUP 62 is one of the best-characterised nucleoporins and there are16 copies of NUP 62 present in each NPC (Cronshaw, Krutchinsky et al. 2002). NUP 62 is organized into two domains including: an N-terminal domain of about 15 FXFG-like repeat and the C-terminal portion that contains heptad repeat sequences (Buss, Kent et al. 1994).This FXFG-like repeat nucleoporin is located in the central basket of the nuclear pore complex. It is not mobile but physically and structurally it is able to access both the nuclear and cytoplasmic sides. NUP 62 is a protein involved in transport but it also has a structural function in the NPC. *In vivo* and *in vitro* investigations demonstrated association of NUP 62 in nuclear protein import (Clarkson, Kent et al. 1996; Gamper, van Eyndhoven et al. 2000). Interaction of NUP 62 with several components of the nuclear protein import machinery has been reported. NUP 62 interacts with nuclear transport factor 2 (NTF2) and Ran (Paschal and Gerace 1995; Clarkson, Kent et al. 1996), importin-β1 (Percipalle, Clarkson et al. 1997), transcription factor Sp1 (Han, Roos et al. 1998), the signalling protein TRAF-3 and heat shock factor 2 (Gamper, van Eyndhoven et al. 2000).

In this thesis, I have defined a new interaction of ICP27 with mammalian protein NUP 62 *in vitro* and in infected cells. Immunoprecipitation data shows that the interaction of ICP27 with NUP 62 is not limited to *in vitro* experiments or overexpressing NUP 62 and can be detected in infected cells. Different lines of evidence support the idea that ICP27 is involved with NUP 62 in infected cells.

First, NUP 62 co-precipitates with ICP27 using specific anti ICP27 monoclonal antibodies in immunoprecipitation assays. Second, similar results were obtained when anti-NUP 62 antibodies were used in precipitation assays to pull down ICP27. Anti NUP 62 (N-19) and (H-122) antibodies that can detect the N-terminal and C-terminal regions of NUP 62 respectively co-precipitated ICP27 and NUP 62 from cell extracts of HeLa and BHK cells. Third, anti FG-repeat nucleoporin 414 antibody that detects a range of FG-repeat nucleoporins in the cell extracts, clearly co-precipitated ICP27 and the FG-repeat nucleoporins including NUP 62 against which it was raised. It seems the interaction of ICP27 and NUP 62 is not dependent on RNA, because incubation of cell extracts with RNase I did not affect these interactions.

In vitro evidence of association of ICP27 with NUP 62 relied on GST pull down assay data. Using purified GST-ICP27, NUP 62 was pulled down from HeLa cell extracts and using GST-NUP 62, ICP27 was pulled down from wild type HSV-1 infected HeLa cell extracts. Interestingly, ORF57, a homologue of ICP27 in HHV-8 that has a strong similarity in function including transport activities, also associates with NUP 62 in pull down assays. Using GST-ORF57, NUP 62 was pulled down from HeLa cell extracts but I was unable to carry out the reverse experiment.

I have suggested in my study that ICP27 can interact directly with NUP 62. This is supported with a series of interaction experiments using RNase I treatment and purified proteins. In a pull down assay of GST-NUP 62 with HeLa infected cell extracts ICP27 associates with NUP 62 in the presence and absence of RNase I so this interaction is not RNA mediated. Thereafter GST-NUP 62 pull down assays with purified His-ICP27 showed direct interaction between these two proteins.

I attempted to map the region of ICP27 that interacted with NUP 62. GST pull down indicated that three ICP27 mutants could no longer interact with NUP 62. These were dLeu in the N-terminus and M15 and M16, in the C-terminus. However, in contrast, co-immunoprecipitation data indicated that M15 and M16 mutants could interact with NUP 62 even in the presence of RNase I. Thus these C-terminal interactions could not be confirmed. I did not carry out a co-immunoprecipitation experiment to test interaction of ICP27 dLeu with NUP 62 so I cannot be sure that this mutant would not interact with NUP 62 *in vivo*. The

differences between my GST pull down and immunoprecipitation data could be due to there being other proteins in the ICP27-NUP 62 complex *in vivo* that can bridge an interaction between the two proteins. Future work to explain the differences I observed should focus on using co-immunoprecipitation with mutant virus infected cells. Biophysical methods such as Biacore could be used to check whether the wild type and mutated ICP27 proteins interacted directly with NUP 62. The work could be extended by high resolution immunofluorescence microscopy and electron microscopy using immuno-gold labelling of NUPs and ICP27.

Although the data set I have accumulated for ICP27 interaction with NUP 62 is the most complete of the interaction I have examined, I would like to point out that there are several problems with this set of experiments. These problems exist in all my thesis work. Firstly, it was impossible to purify GST- or His-ICP27 as completely full length proteins. There were always a large number of protein truncates and deletions present. This may be because ICP27 has a central region that is predicted to be very disordered in structure and vulnerable to protease attack. There were also problems obtaining reasonable quantities of full length NUP proteins. This means that most of the experiments were not carried out under optimal conditions. For the GST pull down experiments, I often missed out negative and positive controls. GST-TAP would have been a good control for ICP27 binding throughout the thesis. Having demonstrated that Rae 1 could not interact with ICP27, this would be a good negative control in future. I tested whether beads alone could pull down ICP27 and found no interaction. However, I did not preclear all of the extracts I used in all the experiments. This calls into question the results I obtained. Other problems existed with the mutant ICP27 virus infection data. In some cases I was not able to complete a set of experiments on the one western blot. This means that I had to piece together blots from different experiments. Because a wild type HSV-1 infected control was not present on all of these blots, I cannot compare between panels in such experiments. Levels of expression of mutant ICP27 proteins should have been compared with levels of expression of another wild type HSV-1 protein such as ICP0 or IPC4 to determine whether infections has been successful, or how much viral replication has been achieved in each case. Finally, I did not use appropriate negative controls for the co-immunoprecipitation experiments. I used normal goat or rabbit serum as a

negative control. A much better control to have used in each case would be control antibodies of the same isotype as the antibody used in the immunoprecipitation experiments but against an irrelevant protein.

If there is a direct interaction of ICP27 with NUP 62 I can propose the hypothesis that HSV-1 ICP27 protein and its HHV-8 homologue ORF57 may be involved with NUPs directly in nuclear import or export of protein and/or RNA. In the NPC, the position of NUP 62 on both the nuclear and the cytoplasmic sides and in the central gated channel (Guan, Muller et al. 1995), suggests it may be a good candidate for interaction with different proteins such as TAP as well as other nucleoporins (Stochaj, Banski et al. 2006). Interaction of the N-terminal portion of NUP 62 with NUP 153 and NUP 214 in growing cells can be dynamic and so it has been proposed that NUP 62 is closely involved with NUPs 153 and 214 in nucleocytoplasmic shuttling of macromolecules. It is clear that NUP 62 is involved in many transient interactions in the nuclear pore complex (Stochaj, Banski et al. 2006). I propose that complex formation between ICP27 and NUP 62 could provide an important structure to facilitate movement of cargo molecules between the nuclear and cytoplasmic faces of the NPC through the central gated channel during both nuclear export and import. It has been suggested that interactions of NUP 214/NUP 62 and NUP 62/NUP 153 could provide “stepping stones” to transport complexes to and from the gated channel (Stochaj, Banski et al. 2006).

6.4 HSV-1 shuttling protein ICP27 interacts with nuclear NUP 153

Nucleoporin 153 is one of the essential NUPs that is associated with both nuclear export and nuclear import (Ball and Ullman 2005). This carboxy-terminus FG-repeat nucleoporin is a glycoprotein that is enriched in amino acids serine/threonine and that can be phosphorylated and glycosylated (Denning, Patel et al. 2003; Walther, Askjaer et al. 2003). NUP 153 is a high dynamic NUP that exchanges at the nuclear pore but some evidence shows that perhaps a subset of NUP 153 molecules is immobile in the NPC (Ball and Ullman 2005). Investigation on the mobility of NUP 153 showed distinct N-terminal functional domains mediate transcription-dependent mobility (Griffis, Craige et al. 2004). It has been shown

that the N-terminal domain of NUP 153 is anchored to the nuclear ring and the long C-terminus could be structurally flexible within the nuclear pore complex and can be detectable in both the nuclear and cytoplasmic side of NPC as well as the central channel (Fahrenkrog, Maco et al. 2002). A list of various proteins and nucleic acids that interact with NUP 153 is shown in Table 1.7. The FG-repeat domain of NUP 153 interacts with importin-β (Shah, Tugendreich et al. 1998) and TAP (Bachi, Braun et al. 2000). The RNA binding domain (RBD) of NUP 153 interacts with mRNA and export of mRNA cargo through NPC could be due to direct contact or protein-protein contact with mRNA-associated proteins (Ball, Dimaano et al. 2007).

Here I have investigated an interaction of HSV-1 ICP27 with NUP 153 *in vitro* and in infected cells using immunoprecipitation and pull down assays. Confirmation of the interaction between NUP 153 and ICP27 came from co-precipitation of ICP27 with NUP 153. A complex of NUP 153 and its specific antibody, anti-NUP 153, co precipitated ICP27 from wt HSV-1 infected HeLa cell extract showing association of these two proteins in infected cells. *In vitro* corroboration of the participation of the ICP27 with NUP 153 relied on GST pull down assay data. Purified GST-NUP 153 pulled down ICP27 from wt HSV-1 ICP27 infected HeLa cell extracts and established *in vitro* association of ICP27 and NUP 153. RNAse I treatment in the pull down assay of wtild type infected HeLa cell extracts with GST-NUP 153 did not change the level of interaction and revealed that this interaction is not RNA-mediated. However, unfortunately the clone for NUP 153 was the last that I received and I did not have enough time to optimize experiments with this NUP. Optimisation of protein expression and the GST pull down and IP experiments in addition to other technique such as immunofluorescence and electron microscopy would improve the validity of this work.

My investigation to map the regions of ICP27 that associated with NUP 153 using a pull down assay of GST-NUP 153 with a range of HeLa cell lysates infected with wt HSV-1, and HSV-1 mutated in the ICP27 gene, showed possible interaction with the region of the dLeu (ΔNES) mutation in the N-terminus. dLeu ICP27 is found only in the cell nucleus so the N-terminal leucine-rich sequence must act as NES during viral infection (Lengyel, Guy et al. 2002). Another important N-terminal ICP27 region is covered by the mutation d1-2 and this contributed partially in

interaction with NUP 153. d1-2 shares part of, but not the whole of, the NES sequence and this may explain why there appeared to be a partial interaction of d1-2 with NUP 153.

In the C-terminal region of ICP27 two mutations in an essential region of the protein may participate in interaction with NUP 153. The M15 point mutation could not interact with NUP 153. However, the M16 mutation partially knocked out the ICP27-NUP 153 interaction. These data indicate that NUP 153 may also interact with the C-terminal region of ICP27.

It has been suggested that NUP 153 might be involved in disassociating nuclear import cargo-receptor complexes (Shah, Tugendreich et al. 1998). It has also been suggested that NUP 153 may play a role in receptor-independent nuclear transport, as shown for the nuclear import of the transcription factor PU.1 and the signal transducer and activator of transcription, Stat (Gould, Martinez et al. 2000; Zhong, Takeda et al. 2005). However, NUP 153 can bind to RNAs and also has role in export of proteins and RNA (Ullman, Shah et al. 1999). Very recent investigations on the mechanism of trafficking through the NPC showed interaction of NUP 153 and NUP 62. I need to verify this interaction. As I mentioned previously, NUP 62 could provide a structural mechanism for allowing sequential movement of cargo from the outside face through the central basket to the inside face of the NPC (and vice versa) during both nuclear import and export (Stochaj, Banski et al. 2006). Export of viral mRNA takes place by utilising two main cellular pathways CRM1 and TAP (Sandri-Goldin 2004). However, different investigations on the transport of proteins and RNAs indicated that there had to be alternative pathways and this is why I investigated nucleoporin and HSV-1 ICP27 interaction. Data presented here suggest indeed that NUP 153 participates in interaction with ICP27 in the NPC. The C-terminal flexible and unfolded FG-repeat domain of NUP 153 is involved in transient interaction with cargo as well as other FG-repeat NUPs (Lim, Aebi et al. 2006). Cooperation and interaction of this complex (NUP 153/ ICP27) with other nucleoporins that are located in the nuclear and cytoplasmic face of NPC may allow FG-repeat domains of NUPs to accompany and guide cargo through the NPC's central pore to its final destination (Lim, Aebi et al. 2006).

6.5 Cytoplasmic face NUP 214/CAN interacts with ICP27

The cytoplasmic filaments of the NPC have been proposed to have a role in cytoplasmic release of nuclear export complexes and in assembly and docking of nuclear import complexes (Gould, Martinez et al. 2000). NUP214/CAN is a member of the FG-repeat-containing nucleoporins in the cytoplasmic face of the NPC. NUP 214 was originally known as a putative oncogene, disrupted by chromosomal translocations linked with myeloid leukemias (Fornerod, van Deursen et al. 1997; Xylourgidis, Roth et al. 2006). Complex formation of NUP 214 with other nucleoporins such as NUP 88, NUP 358 and NUP 62 was demonstrated (Xylourgidis, Roth et al. 2006). Direct interaction of NUP214 by its FG-motif rich domain with importin-β and CRM1 has been shown and also it was proposed to function as a CRM1 inhibitor (Xylourgidis, Roth et al. 2006). However, Bernad and colleagues reported that a NUP 214-NUP 88 complex is necessary for CRM1-mediated export of a specific cargo, the 60 S preribosome, in a CRM1-FG repeat interaction-independent mechansim (Bernad, Engelsma et al. 2006). The N-terminal region of NUP 214 is positioned in the cytoplasmic side of NPC, The central coiled coils are known to interact with NUP 88 and a long and flexible C-terminal FG-repeat region localises in the nuclear and cytoplamic face of NPC and interacts with transport factors (Paulillo, Phillips et al. 2005).

In this thesis, I have shown data that suggest a new interaction of the viral HSV-1 protein ICP27 with NUP 214/CAN *in vitro* and in infected cells using co-immunoprecipitation and pull down assays. Immunoprecipitation data shows that the interaction of ICP27 with NUP 214 is not limited to *in vitro* experiments or overexpressing NUP 214 as it can be detected in infected cells. There are three lines of evidence for an interaction. First, anti NUP 214 (N-15) antibody that detects the N-terminal region of NUP 214 precipitated ICP27 and NUP 214 from wt HSV-1 infected HeLa cell extracts. Second, in the immunoprecipitation assay treatment of the complex formed with RNase I did not affect binding of these two proteins. This means the interaction of ICP27 and NUP 214 is not RNA-mediated. Third, GST pull down assays gave *in vitro* evidence of an involvement of ICP27 with NUP 214. Purified recombinant proteins GST-NUP 214 (aa 1690-1894) and

(aa 1690-2090) pulled down ICP27 from wild type HSV-1 infected HeLa cell extracts and established an *in vitro* association of ICP27 and NUP 214. Interestingly, the longer truncate of NUP 214 (aa 1690-2090) showed stronger association with ICP27 than the shorter NUP 214 (aa 1690-18940) suggesting the number of FG-repeat domains is important for the quality of interaction of ICP27 with NUP 214. There was a problem in this experiment because ICP27 was not detected in the unbound fraction although it was clearly detected in the bound fraction. This means this experiment would have to be repeated before firm conclusions can be drawn. Unfortunately we did not have any full length NUP 214 recombinant protein to compare interaction with its truncates but interaction of ICP27 and full length NUP 214 was demonstrated in infected cells in this study. Altogether, this information suggests a possible involvement of FG motif-rich domains of NUP 214 in interaction with ICP27. This FG repeat part of NUP 214 is also involved in interaction of two important transport factors importin-β and CRM1 (Fornerod, van Deursen et al. 1997; Bayliss, Littlewood et al. 2000).

Immunofluorescence studies of the flexible FG-repeat domain of NUP 214 showed that it was involved with the nucleo/cytoplasmic transport system suggesting that NUP 214 might accompany cargo-receptor complexes through the central pore (Paulillo, Phillips et al. 2005). Because it has an FG-repeat domain, NUP 214 appears to be involved with cargo import as well. It has been proposed that NUP 214's FG-repeat domain transiently accumulates at the nuclear NPC periphery in the presence of excess import cargo. Further work of this research group showed that in the case of RNA export the flexible FG-repeat region of NUP 214 is capable of accompanying the cargo through the central pore of the NPC (Paulillo, Phillips et al. 2005), underlying the importance of the FG-repeat domain. It means that during import, cargoes may be passed from an asymmetric FG-repeat nucleoporin residing NUP 214, to a symmetric FG-repeat nucleoporin, NUP 62, to an asymmetric FG-repeat nucleoporin residing on the nuclear side of the NPC, or vice versa in the context of export allowing delivery of proteins to their final destination (Paulillo, Phillips et al. 2005).

It has been defined that NUP 214 is organized into three domains and that the C-terminal domain is the FG repeat region. Consistent with interaction of NUP 214 with ICP27, I have mapped the regions of ICP27 involved in binding with NUP 214.

I demonstrated a that C-terminal residues of 465-466 (M15) and residue 488 (M16) are required for the interaction with NUP 214. However, as the C-terminal segment of NUP 214 is phosphorylated and O-glycosylated the modifications could change the binding ability of these proteins (Miller, Caracciolo et al. 1999). The longer truncate, GST-NUP 214 (1690-2090), pulled down ICP27. However, although I could show association of the FG domain of NUP 214 in interaction with ICP27 I cannot conclude that non FG domains are not involved in this interaction. Future work will be required to fully map this interaction. Immunoprecipitation experiments where cellular NUP 214 is interacted with ICP27 will give improved data compared to the GST-NUP 214 pull down experiments.

Due to the position of NUP 214 in the cytoplasmic face of NPC it could associate with ICP27 to provide it to other NUPs in central channel gate such as NUP 62 and then to nuclear NUPs such as NUP 153 and NUP 98 to dissociate in the nucleus. On the other hand, nuclear face NUPs may bind to ICP27 in nucleus and shuttle ICP27 alone or in a complex with RNA to the central basket and pass it through the NPC to NUP 214 to dissociate in cytoplasm.

6.6 Non FG-repeat NUPs Rae 1 and NUP 107 did not interact with ICP27

In this study I was interested to search for an interaction of ICP27 with non FG-repeat NUP Rae1 as a cellular shuttling protein that is involved with NUP 98 in transport activity. Result showed there may not be any association between ICP27 and Rae1 in GST pull down assays. Rae1 acts as a shuttling mRNA export factor that interacts with NUP 98 at Rae1 binding sites in N-terminal domain of NUP 98 (Pritchard, Fornerod et al. 1999). Power's group demonstrated that Rae1 may function to deliver TAP to NUP 98. This report suggested a competition of TAP and NUP 98 in binding to Rae1 (Blevins, Smith et al. 2003).

NUP 107 localises to the nuclear rim and is an essential component of the nuclear pore complex (NPC). NUP 107 does not contain FG repeats that serve as docking sites for karyopherins and other transport factors. It has been shown that depletion of mammalian NUP 107 resulted in the failure of a subset of NUPs to assemble

into NPCs followed by degradation of these proteins (Ben-Efraim and Gerace 2001). GST pull down assay using ICP27 and NUP107 showed no interaction between these two proteins supporting the idea that the FG-repeat domain of NUPs is essential for interaction with ICP27 and its shuttling activity. .

6.7 ICP27 and NUPs are in complex after 6hpi

In this study I have shown possible interaction of ICP27 with certain NUPs *in vitro* and in infected cells. Different studies have shown nuclear distribution of ICP27 at early times of infection and both nuclear and cytoplasmic localisation at later times of infection (Phelan and Clements 1997). Time course immunoprecipitation experiments showed involvement of ICP27 with nucleoporins at 6.5 hpi. As expected the low amount of expression of ICP27 at early times of infection did not show involvement of ICP27 and NUPs in the immunoprecipitation assay. At later times of infection, the level of involvement of ICP27 and FG-repeat NUPs increased and also association of ICP27 mutant M16 with NUPs appeared at 9.5 hpi. These data are consistent with the expression profile of ICP27 and NUPs and also reveals that there was no degradation of NUPs after infection with HSV-1. However further experiments such as using immunofluorescence are needed to make any firm conclusions.

6.8 ICP27 SADET residues are not involved in interaction with NUPs

Similar to ICP27, hnRNP K is a multifunctional cellular protein that shuttles between the nucleus and the cytoplasm. It is a regulatory protein that acts on gene expression at transcriptional and post-transcriptional as well as translational levels (Bomsztyk, Denisenko, and Ostrowski, 2004). Also like ICP27, hnRNP K interacts with several cellular and viral proteins. It is capable of self-interaction (Wadd, Bryant et al. 1999), and can be a component of multi-protein complexes. This protein contains a classical nuclear localization signals and a KNS domain that contains a SADET sequence that is perfectly conserved between hnRNP K KNS and ICP27 suggesting the possibility of access to common cellular transport pathways and direct interaction with the nucleoprotein complexes (Figure 1.7)

(Michael, Eder et al. 1997). hnRNP K can bind RNA and single or double stranded DNA via a GRGG box and three repeats of a motif termed the K homology domain (Siomi, Matunis et al. 1993; Bomsztyk, Denisenko et al. 2004). The KNS nucleocytoplasmic shuttling signal has been suggested to mediate export of RNA via a CRM1 independent pathway (Henderson and Eleftheriou 2000). Previous work of our laboratory on ICP27 SADET mutants showed that SADET mutated ICP27 was no longer able to interact with cellular nucleocytoplasmic transport proteins REF and hnRNP K and suggested shuttling through pathways other than those involving REF and hnRNP K, possibly through CRM1 export or via direct interaction with nucleoporins (Leiper 2004). I have investigated involvement of ICP27 SADET sequences with NUP interactions. Both RNase I treated and untreated immunoprecipitation assays showed precipitation of ICP27, but there was no distinct pattern in co-precipitation of NUP 62 and mutated ICP27s. Western blot of the above assays with anti-hnRNP K antibody showed precipitation of hnRNP K by both wild type and SADET mutant ICP27. Results with the fully changed SADET sequence, AGAGA, showed immunoprecipitation of ICP27 and NUP 62 indicating independency of interaction between ICP27 and NUPs from the SADET sequence. Claire Leiper showed in her thesis that amino acid substitution in the SADET sequence had a major impact on the localisation of ICP27 from mainly nuclear to both nuclear and cytoplasmic (Leiper 2004). In future, a good experiment would be to use these SADET mutants in immunofluorescence experiments with cells transfected with ICP27 expression constructs or cells infected with ICP27 mutant viruses to visualise co-localisation with NUPs. Interaction of ICP27 SADET mutants with hnRNP K in immunoprecipitation assays in this project is inconsistent with Leiper's results that showed mutagenesis in SADET residues leads to loss of interaction with hnRNP K in GST pull down assay of transfected BHK cells (Leiper 2004). I suggest this inconsistency could be due to using either a different experimental procedure or to the type of cell used. In this study I have used an immunoprecipitation assay that is more close to the natural system in living cells while previously a GST pull down assay was used. My experiments were in HeLa cells that may have many different properties compared to the BHK cells used previously. My results are more consistent with the report that mapped interaction sites of ICP27 with hnRNP K. amino acids242 to 397 were sufficient for interaction of ICP27 and hnRNP K and aa 166 to 242 contributed to

the interaction. In this study hnRNPK co-immunoprecipitated with anti ICP27 antibody and this interaction was confirmed by a yeast-two hybrid system (Y2-H) (Wadd, Bryant et al. 1999). Interaction of the ICP27 homologue ORF57 with hnRNP K has also been shown in our laboratory (Malik and Clements 2004). However, the resemblance between ICP27 and hnRNP K suggests that they utilise common cellular pathways, and ICP27 could prevent hnRNP K from entering into such pathways, resulting in alterations in transport of cellular RNAs. It is possible that the many gene expression-related functions of ICP27 may rely on interaction with hnRNP K and this might be a key protein in HSV-1 infection for example, acting to targeting ICP27 to transcriptionally active nuclear domains or allowing ICP27 to interact with one of hnRNP K's partner proteins.

6.9 HSV-1 infection does not regulate expression of NUPs

Infection with HSV-1 has a significant effect on nuclear function by inhibiting host RNA splicing. This results in host pre-mRNAs being held in stalled splicing complexes and thus being unable to gain access to the NPC machinery and to exit the nucleus (Hardwicke and Sandri-Goldin 1994). A feature of some RNA viruses, which replicate in the cytoplasm, including polioviruses, vesicular stomatitis virus (VSV) and influenza virus, is that they interfere with nucleocytoplasmic trafficking by different ways including degradation of NUPs (Fontoura, Faria et al. 2005; Satterly, Tsai et al. 2007). This would benefit RNA viruses because they replicate in the cytoplasmic. Inhibition of cellular mRNA export would therefore decrease competition for the translation machinery and promote translation of viral mRNAs. Also it would benefit viral replication because prevention of import might contribute to the accumulation of nuclear factors, that might be required for replication, in the cytoplasm (Fontoura, Faria et al. 2005; Satterly, Tsai et al. 2007). Interestingly, the RNA virus story indicates that nuclear pore complex components such as NUPs are the main target for inhibition of transport machinery (Gustin and Sarnow 2001; Gustin 2003). A possible role of HSV-1 ICP27 protein in disruption of nucleo-cytplasmic transport led me to determine whether ICP27 had any affect on NUP expression. Infections with wild type and mutant ICP27 HSV-1 and transient transfection studies in HeLa cells did not show any major degradation or significant

change in expression levels of FG-repeat nucleoporins. These results back up a recent study showing HSV-1 infection does not interfere with nucleocytoplasmic trafficking through the TAP and CRM1 pathways (Chen, Li et al. 2005). As I mentioned above RNA viruses are capable of interrupting NPCs. For example, VSV M protein binds to the mRNA export factor Rae1 which contributes to NUP 98-mediated inhibition of mRNA export (Faria, Chakraborty et al. 2005). My data from the interaction assays showed ICP27 failed to interact with Rae1. In contrast, upon poliovirus infection degradation of certain nucleoporins such as NUP 153 and NUP 62 occurs. This results in disruption of the nuclear pore complex structure and nucleocytoplasmic shuttling (Gustin and Sarnow 2001). Recently investigations on influenza virus infection demonstrated that the multifunctional protein NS1 that shuttles between the nucleus and the cytoplasm inhibits host mRNA nuclear export by interaction with nuclear export machinery. In fact, it has been shown that NS1 interacts with various mRNA export factors as well as NUP 98. The level of NUP 98 is depleted by NS1, indicating that NUP 98 is actively degraded during influenza virus infection. In addition, influenza virus infection actually interferes with the physical process of mRNA nuclear export (Satterly, Tsai et al. 2007). Contrasting the situation with RNA viruses, HSV-1 infection does not appear to have an impact on cellular nucleoporins to disrupt nucleocytoplasmic trafficking. Further investigation to give a clearer idea of HSV-1 ICP27 function would involve knocking out specific nucleoporins following infection with wild type and ICP27-mutant HSV-1.

6.10 General discussion

Molecular understanding of nuclear trafficking has major implications for medical science in the future. It is clear that nuclear proteins are synthesized in the cytoplasm and function in the nucleus, and RNAs are transcribed in the nucleus but translated in the cytoplasm. Therefore, cellular or viral management of macromolecular shuttling across the nuclear membrane is an important means for changing the levels and activities of such molecules as transport factors (Conti and Izaurralde 2001).

6.10.1 ICP27 may use more than one pathway to export viral mRNAs

Association of ICP27 with redistributed snRNPs on intron-containing transcripts suggested an involvement of this multifunctional protein in regulation of nucleocytoplasmic RNA shuttling (Phelan, Dunlop et al. 1996). Many studies showed ICP27 shuttles between the nucleus and cytoplasm and has a direct role in nuclear export of viral RNAs. This is via its binding with intronless viral and cellular mRNAs (Phelan and Clements 1997; Soliman, Sandri-Goldin et al. 1997; Sandri-Goldin 1998; Koffa, Clements et al. 2001). It has been shown that two motifs of ICP27, an RGG box and a leucine-rich nuclear export signal (NES) are putatively involved with RNA binding and export (Sandri-Goldin 1998; Lengyel, Guy et al. 2002). Experimental studies using leptomycin B (LMB) and ICP27 mutants demonstrated these regions are required but not essential for nucleocytoplasmic transport of RNAs. There are different pathways for export of RNAs (Phelan and Clements 1997; Soliman and Silverstein 2000; Koffa, Clements et al. 2001; Chen, Sciabica et al. 2002; Lengyel, Guy et al. 2002; Chen, Li et al. 2005; Thiry, Muylkens et al. 2005). The main cellular mRNA export pathway is nuclear export receptor (TAP)-mediated that associates with an RNA-binding protein REF, a component of the exon junction complex. It has been shown that ICP27 binds to viral mRNAs and interacts directly with REF as an adaptor protein and indirectly recruits TAP to viral mRNAs to stimulate their nuclear export through this cellular pathway (Koffa, Clements et al. 2001; Chen, Sciabica et al. 2002; Sandri-Goldin 2004).

However, certain aspects of this model are unclear. A moderate effect of the RGG box and REF binding site ICP27 mutants on viral gene expression, transport by the CRM 1 dependent pathway and export of some transcripts in absence of ICP27 whose overall expression is ICP27-dependent show the TAP pathway is not the only way for export of viral RNAs (Soliman and Silverstein 2000; Lengyel, Guy et al. 2002; Pearson, Knipe et al. 2004). Further evidence for multiple ICP27 export pathways comes from our laboratory which observed ICP27 protein export in the absence of viral RNA in the Xenopus oocyte system and this export required neither CRM1 or TAP, thus the interaction with REF is required for the nuclear

export of certain L RNAs but the protein itself can shuttle through another pathway (Koffa, Clements et al. 2001). On the other hand, other evidence for the presence, the function and the mechanism of the nature of any alternative pathway is not clear. The homology of ICP27 to the KNS domain of hnRNP K (Figure 1.7) that has transport capability, and to the viral tegument protein VP13/14 containing a NES-like element that shuttles between nucleus and cytoplasm are suggested to show their involvement in transport of RNAs, possibly by such an alternative pathway (Michael, Eder et al. 1997; Donnelly and Elliott 2001). Trafficking of most proteins and RNAs is through NES and NLS of carrier proteins (Gorlich and Mattaj 1996). The karyopherin family of transport factors (also termed importins, exportins and transportins) uses the small GTPase, Ran to provide energy for association/disassociation of the cargo to transport them through the NPC. However, energy generation is not require for translocation (Weis 2003). Movement across the NPC depends on direct interaction with NUP FG-repeat domains (Bayliss, Littlewood et al. 2000). The NPC is extremely large with the capacity to accommodate many translocating molecules. Each NPC has at least 8−16 copies of each NUP and 128 FG domains together providing thousands of individual FG repeats (Rout, Aitchison et al. 2000; Cronshaw, Krutchinsky et al. 2002). FG-NUPs are mostly large proteins, which have low overall hydrophobicity with their FG-repeat domains being natively unfolded (Denning, Uversky et al. 2002; Denning, Patel et al. 2003). Natively unfolded proteins commonly can provide multiple binding domains and so can interact simultaneously with many different partner proteins (Lim, Aebi et al. 2006).

6.10.2 Modes of transport through the NPC: possible roles of ICP27

Although much progress has been made in understanding the structure and composition of the NPC in nucleocytoplasmic transport, details on the mechanisms of how individual NUPs translocate cargo are missing (Lim, Aebi et al. 2006). Numerous attempts have been made to model the translocation mechanism. Overall four theories have been proposed for translocation of cargoes through the NPC. First, the Brownian affinity gate model. This model assumes that NUPs on the outer face of the NPC present a barrier to cargos that cannot move by

themselves. However, if these cargos interact with a transporter system that generates energy e.g GTP, the energy generated is such that the cargo/transporter can overcome the NUP barrier by interacting with NUPs and exiting through the internal pore of the NPC by Brownian motion (Rout, Aitchison et al. 2003). The next model is the affinity gradient model, which suggests FG-repeat NUPs exhibit increasing affinities with the cargo complexes (Ben-Efraim and Gerace 2001). The third model is the 'oily spaghetti' model that hypothesises that the NPC is filled with FG-NUPs that normally block passage of molecules. However, if transporter/cargo complexes gain access to the NPC they can progress through by pushing the NUPs out of the way, against the sides of the pore (Macara 2001). The last model is known as the selective phase model. This proposes that the hydrophobic FG-repeat NUPs form a mesh in the pore, which transporter- cargo molecules can get through (Ribbeck and Gorlich 2002). Recent studies on yeast transport mechanisms through the NPC have increased the complexity of the transport story. It has been shown that the asymmetric FG-repeat NUPs are dispensable for bulk transport in the NPC (Zeitler and Weis 2004). When some asymmetric yeast FG domain NUPs were removed the permeability of NPCs was not reduce. Taken together this suggested the existence of multiple translocation mechanisms (Strawn, Shen et al. 2004). Furthermore, asymmetrically positioned NUP FG domains have been found to be non essential. However, specific combinations of symmetrically localised NUPs FG domains are essential (Strawn, Shen et al. 2004).

It will be necessary to carry out several more experiments before interaction of ICP27 with NUP 62, NUP 153 and NUP 214 in infected cells is proved definitively. However, I wish to propose a new mechanism by which ICP27 may be involved in RNA or protein trafficking. ICP27 may interacts with various NUPs, both mobile and fixed, at different sites in the NPC and so can pass protein or mRNA bound to ICP27, through the NPC.

Our knowledge about the mechanism of ICP27 shuttling between the nucleus and the cytoplasm is poor. ICP27 may exit either the nucleus or the cytoplasm by more than one pathway and use different export components throughout the viral replication cycle. ICP27 can interact with the nonkaryopherin export receptor TAP that is believed to traffic the majority of mRNA molecules or with cellular

nucleocytoplasmic transport receptors belonging to the karyopherin family of soluble transport factors such as CRM1. Alternatively, as I have shown here, ICP27 may interact directly with some nucleoporins (Figure 6.1). In this scenario, ICP27-FG-repeat NUP interaction acts as a transporter machine (Figure 6.3). Interestingly, all the nucleoporins tested in this study showed at least a requirement of the nuclear export signal (NES) residues of ICP27 for interaction between NUPs and ICP27. Involvement of this region in export has been shown and also it is not the site for either TAP or CRM1 interaction to contribute in export function. It seems this site mediates a novel direct interaction of ICP27 with nucleoporins but whether this site is required for RNA export remains to be determined. New lines of studies on cargo-free shuttling have suggested NUP 153 may play a role in receptor-independent nuclear transport, as shown for the nuclear import of the transcription factor PU.1, the signal transducer and activator of transcription, Stat and the transport factor, Smad2 (Gould, Martinez et al. 2000; Xu, Kang et al. 2002; Zhong, Takeda et al. 2005). In addition to the above suggestion it has been revealed that the phosphorylation of the proteins play a role for cargo-dependent and independent transport, and unphosphorylated cellular Stat1 proteins directly interact with the FG repeat region of NUP153 and NUP 214 and provide nucleocytoplasmic translocation of Stats as a carrier-independent process. However, the phosphorylated Stat protein is then actively transported in the nucleus via importin α/β (Marg, Shan et al. 2004). The role of protein modification in ICP27 export was investigated and showed the serine at position 114, which lies within the major NLS modulates the efficiency of nuclear import is the only amino acid in the ICP27 protein that is phosphorylated during HSV-1 infection (Zhi and Sandri-Goldin 1999). It has been demonstrated that ICP27 is phosphorylated by CK2 and phosphorylation of ICP27 by CK2 is necessary for ICP27's cytoplasmic accumulation and it might regulate nucleocytoplasmic trafficking of ICP27 and/or viral RNA. Stimulation of CK2 activity by HSV-1 occurs at early times post infection and correlates with partial redistribution of the CK2 holoenzyme from the nucleus to the cytoplasm. Therefore, it is suggested that it might facilitate the HSV-1 lytic cycle by, for example, regulating trafficking of ICP27 protein and/or viral RNAs (Wadd, Bryant et al. 1999; Koffa, Kean et al. 2003). Hence, changes in the sub-cellular localisation of CK2 subunits could control ICP27's activities and distribution as well as regulate its translocation and

trafficking. In the same way nuclear import of Simian virus 40 large T antigen is enhanced by CK2 phosphorylation in HeLa cell (Xiao and Jans 1998).

6.10.3 Conclusion

To conclude the mapping results, NUPs 62, 153 and 214/CAN showed association with the ICP27 NES region (Figure 6.2 summarises the ICP27 mapping data). In the case of NUP 62, the N-terminus dLeu ICP27 mutant and the C-terminus M15 and M16 ICP27 mutants showed association. NUP 214 also interacted with the same regions of ICP27. However, for NUP 153 only partial binding of the C-terminal M16 mutant and the N-terminus mutant, d1-2 was observed. Thus the NES might be the general binding site of ICP27 for NUP interaction and different forms of FG-repeat including. Although I found some evidence for involvement of the C-terminus of ICP27 in NUP binding, the interaction varied between different experiments and there was no consistency in data with the M15 and 16 mutants. I would suggest this could be due to protein configuration changes under different experimental conditions. Consistent with this idea it has been shown that the main NUP binding domain of TAP (TAP-UBA) has half the affinity for the GLFG-repeat NUP 98 in comparison with FXFG-repeat NUPs (Grant, Neuhaus et al. 2003). In addition, another NUP binding domain of TAP, NTF2 cannot interact with GLFG motifs (Clarkson, Corbett et al. 1997). The C-terminal region of ICP27 is involved in multiple protein-protein interactions and a ICP27 self association *in vivo* has been demonstrated. It has been suggested the substitution mutations M15 and M16 may affect ICP27 multimerisation (Wadd, Bryant et al. 1999; Zhi, Sciabica et al. 1999; Koffa, Kean et al. 2003). ICP27 or NUPs might be modified posttranslationally during incubation in the extracts I used in pull down assays and these modifications could alter binding to NUPs. The N-terminus portion of NUP 62 is phosphorylated and O-glycosylated and shows that kinases and glycosyl transefrases could be potentially important to modulate these proteins (Miller, Caracciolo et al. 1999).

ICP27 shuttles and exports viral RNAs through the NPC (Smith, Malik et al. 2005). Direct interaction of ICP27 with nucleoporins might give new insights into export of viral mRNA from nucleus to the cytoplasm. Based on this model ICP27 can bind to mRNAs directly and recruit them to NUPs to transpocate them through the nuclear

pore and export them to the cytoplasm. ICP27 can bind to NUP 153 that has RNA binding ability and is located in the nuclear face of NPC. ICP27 also binds NUP 62 located in the central gated channel and could to pass it to the cytoplasmic side. My study showed interaction of ICP27 and NUPs are not RNA mediated so for export, viral mRNAs could bind directly to NUPs or indirectly via ICP27. Direct interaction of mRNA with some nucleoporins such as NUP 153, NUP 62 and Rae1 has been shown. For mRNA export, NUP 153 can function by recognizing either single stranded RNA through its RNA binding domain or by recognizing TAP, which interacts with its C-terminus domain (Ball and Ullman 2005). Therefore, I suppose that TAP could be replaced with ICP27 and provide export of mRNA. My data is not complete enough to make strong conclusions on the export function of these proteins and the RNAs and much more work requires to be done to consolidate the work and to determine details.

The molecular mechanism of transport across NPCs shows directionality, gate selectivity and energy consumption as mentioned earlier in this section and involves FG-repeat NUPs. ICP27 alone or in complex with viral mRNA might interact directly with NUPs and provide a transporter complex to translocate through the NPC and transfer ICP27 either to the nucleus or the cytoplasm. The NUPs that are located in the cytoplasmic face of the NPC could associate with ICP27 and provide it to other NUPs in the gated central channel, such as NUP 62, and then to nuclear NUPs to dissociate in the nucleus. On the other hand, nuclear face NUPs could bind to ICP27 in the nucleus and shuttle ICP27 alone or in complex with RNA, through the central basket and pass it on to cytoplasmic NUPs to dissociate in the cytoplasm. This is consistent with the current models for mechanisms of translocation. However, it has been demonstrated that individual nucleoporins can have a major function in specific nucleocytoplasmic transport pathways. This idea comes from the role of NUP 214 in CRM1-mediated nuclear protein export *in vivo* (Hutten and Kehlenbach 2006). In a study to examine the contribution of cytoplasmic NUPs in the transport machinery, depletion of NUP 214 strongly reduced nuclear protein export in comparison with depletion of NUP 358 that had only a modest effect on the same transport pathway (Hutten and Kehlenbach 2006). The association of NUP 62 with other FG-repeat nucleoporins, NUP 358, NUP 214 and NUP 153 has been shown (Stochaj, Banski et al. 2006).

However, in this study it was demonstrated that in nuclear extracts there was no direct interaction of NUP 62 and NUP 153 and they suggested NUP 153 requires an adaptor molecule to link to the NUP62 (Stochaj, Banski et al. 2006). ICP27 could be a good candidate adaptor protein in the case of its function in transport of RNA or protein.

Fahrenkrog and colleagues proposed sometimes cargoes can bind to two transport receptors and may utilise both receptors simultaneously to translocate through the NPC (Fahrenkrog, Koser et al. 2004). ICP27 can interact directly with TAP and (possibly) NUPs and there is an indirect contribution of CRM1 in transport activity of ICP27 (Lengyel, Strain et al. 2006). So I suggest that cargo might interact with either TAP and ICP27 or CRM1 and ICP27 simultaneously for translocation through NPC to shuttle between the nucleus and the cytoplasm (Figures 6.5 and 6.6). In fact, NUP directionality in transport, the ability of the FG-repeat domains to allow inter-NUP interaction, their capacity for multiple simultaneous and transient interactions, and their mobility make NUPs the best option for interaction with ICP27 to shuttle it as a cargo-free or cargo-dependent protein.

In conclusion, it is hypothesised that the multifunctional protein ICP27 regulates transport of protein and RNAs during HSV-1 life cycle via interaction with nucleoporins in addition to other export pathways previously demonstrated. There are many aspects of the interaction and function of NUPs and ICP27 interaction that could not be investigated fully in the assays performed during the course of this PhD. However it is clear that ICP27 utilises different export pathways to regulate transport of protein and RNAs during herpesvirus infection.

6.10.4 Future work

In this thesis I have provided evidence that the multifunctional regulatory HSV-1 ICP27 protein may interact with some nucleoporins during the life cycle of the virus. The findings reported in this study create a new road into elucidating the role of the NPC and NUPs as well as how individual nucleoporins contribute in transport of HSV-1 ICP27 protein and RNAs. Compared to our knowledge about the mechanism and the role of ICP27 in transport of viral RNA, our understanding about the mechanism of import and export of ICP27 is limited. A new insight into

cargo-free export of ICP27 could be obtained by removing viral RNAs from cells and using single-molecule fluorescence (SMF) techniques. SMF is a complex of four common approaches including wide-field epifluorescence microscopy, fluorescence correlation microscopy, scanning confocal microscopy and total internal reflection fluorescence microscopy that could allow visualisation of interaction of single molecules with nucleoporins in the NPC (Yang and Musser 2006). For cargo-dependent export and further investigation on RNA export, it could help to determine whether a complex of ICP27 and RNA shuttles by direct interaction with nucleoporins or using the TAP export pathway as indicated in previous studies. Future work could investigate the idea that ICP27 competes with other identified cellular pathways such as TAP and CRM1 to supply the transport machinery. Understanding the mechanism by which ICP27 mediates directional transport of RNA substrates across the NPC is an important goal for the future. In fact, to characterise molecular interactions underlying nucleocytoplasmic transport, individual nuclear export pathways need to be selectively interfered with in living cells. It has been clearly reported that some nucleoporins interact directly with RNAs. This specificity is shown for NUP 153 that interacts directly with single stranded RNAs indiscriminately. It is possible that ICP27 recruits viral mRNA to NUP 153 in the nuclear side of the NPC to interact with it, translocate and export to the cytoplasm directly or by handing to other NUPs. Future work could be based on gel shift assays to find interactions of NUP 153 with HSV-1 RNAs that also bind ICP27. Also approaches such as SELEX (systematic evolution of ligands by exponential enrichment) will be a good method to gain possible NUP 153 recognition motifs of RNAs for their ability to bind NUP 153.

Further investigation of ICP27 N-terminus and C-terminus mutants is another way that could improve this study and develop functional studies on the role of ICP27-nucleoporin interactions. Using the mutants in mapping studies or together with siRNA knock down studies of nucleoporins and transport receptors TAP or CRM1 would give a better view of the structural interactions and their function. Further mapping of the NUPs that I studied during my project to show exactly which FG domains are involved in interaction could be interesting.

Viruses have contributed to the discovery of key nuclear transport pathways. Another aspect of future interest is that, when we understand the details of nuclear

transport of ICP27, we can perhaps design drugs to target the viral mRNA transport machinery. Results may provide insights into multiple and novel key regulatory steps of nucleocytoplasmic trafficking that may also serve as potential targets for viral and cancer therapies.

References

Allen, N. P., L. Huang, et al. (2001). "Proteomic analysis of nucleoporin interacting proteins." J Biol Chem **276**(31): 29268-74.

Arvin, A. M. (2001). Varicella-Zoster virus. In Fields Virology, Philadelphia: Raven press.

Bachi, A., I. C. Braun, et al. (2000). "The C-terminal domain of TAP interacts with the nuclear pore complex and promotes export of specific CTE-bearing RNA substrates." RNA **6**(1): 136-58.

Balfour, H. H., Jr. (1999). "Antiviral drugs." N Engl J Med **340**(16): 1255-68.

Ball, J. R., C. Dimaano, et al. (2007). "Sequence preference in RNA recognition by the nucleoporin Nup153." J Biol Chem **282**(12): 8734-40.

Ball, J. R. and K. S. Ullman (2005). "Versatility at the nuclear pore complex: lessons learned from the nucleoporin Nup153." Chromosoma **114**(5): 319-30.

Batchelor, A. H. and P. O'Hare (1990). "Regulation and cell-type-specific activity of a promoter located upstream of the latency-associated transcript of herpes simplex virus type 1." J Virol **64**(7): 3269-79.

Baudoux, L., P. Defechereux, et al. (2000). "Gene activation by Varicella-zoster virus IE4 protein requires its dimerization and involves both the arginine-rich sequence, the central part, and the carboxyl-terminal cysteine-rich region." J Biol Chem **275**(42): 32822-31.

Bayliss, R., S. W. Leung, et al. (2002). "Structural basis for the interaction between NTF2 and nucleoporin FxFG repeats." Embo J **21**(12): 2843-53.

Bayliss, R., T. Littlewood, et al. (2000). "Structural basis for the interaction between FxFG nucleoporin repeats and importin-beta in nuclear trafficking." Cell **102**(1): 99-108.

Bayliss, R., T. Littlewood, et al. (2002). "GLFG and FxFG nucleoporins bind to overlapping sites on importin-beta." J Biol Chem **277**(52): 50597-606.

Bear, J., W. Tan, et al. (1999). "Identification of novel import and export signals of human TAP, the protein that binds to the constitutive transport element of the type D retrovirus mRNAs." Mol Cell Biol **19**(9): 6306-17.

Becskei, A. and I. W. Mattaj (2005). "Quantitative models of nuclear transport." Curr Opin Cell Biol **17**(1): 27-34.

Bednenko, J., G. Cingolani, et al. (2003). "Importin beta contains a COOH-terminal nucleoporin binding region important for nuclear transport." J Cell Biol **162**(3): 391-401.

Bello, L. J., A. J. Davison, et al. (1999). "The human herpesvirus-8 ORF 57 gene and its properties." J Gen Virol **80 (Pt 12)**: 3207-15.

Ben-Efraim, I. and L. Gerace (2001). "Gradient of increasing affinity of importin beta for nucleoporins along the pathway of nuclear import." J Cell Biol **152**(2): 411-7.

Bernad, R., D. Engelsma, et al. (2006). "Nup214-Nup88 nucleoporin subcomplex is required for CRM1-mediated 60 S preribosomal nuclear export." J Biol Chem **281**(28): 19378-86.

Bernstein, D. I. and L. R. Stanberry (1999). "Herpes simplex virus vaccines." Vaccine **17**(13-14): 1681-9.

Biron, K. K., S. C. Stanat, et al. (1985). "Metabolic activation of the nucleoside analog 9-[(2-hydroxy-1-(hydroxymethyl)ethoxy]methyl)guanine in human

diploid fibroblasts infected with human cytomegalovirus." Proc Natl Acad Sci U S A **82**(8): 2473-7.

Blevins, M. B., A. M. Smith, et al. (2003). "Complex formation among the RNA export proteins Nup98, Rae1/Gle2, and TAP." J Biol Chem **278**(23): 20979-88.

Bomsztyk, K., O. Denisenko, et al. (2004). "hnRNP K: one protein multiple processes." Bioessays **26**(6): 629-38.

Boutell, C., M. Canning, et al. (2005). "Reciprocal activities between herpes simplex virus type 1 regulatory protein ICP0, a ubiquitin E3 ligase, and ubiquitin-specific protease USP7." J Virol **79**(19): 12342-54.

Boutell, C., R. Everett, et al. (2008). "Herpes simplex virus type 1 ICP0 phosphorylation mutants impair the E3 ubiquitin ligase activity of ICP0 in a cell type-dependent manner." J Virol **82**(21): 10647-56.

Braun, I. C., A. Herold, et al. (2001). "Overexpression of TAP/p15 heterodimers bypasses nuclear retention and stimulates nuclear mRNA export." J Biol Chem **276**(23): 20536-43.

Brown, C., M. Nakamura, et al. (1995). "Herpes simplex virus trans-regulatory protein ICP27 stabilizes and binds to 3' ends of labile mRNA." J. Virol. **69**(11): 7187-7195.

Bryant, H. E., D. A. Matthews, et al. (2000). "Interaction between herpes simplex virus type 1 IE63 protein and cellular protein p32." J Virol **74**(23): 11322-8.

Bryant, H. E., S. E. Wadd, et al. (2001). "Herpes simplex virus IE63 (ICP27) protein interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step." J Virol **75**(9): 4376-85.

Buss, F., H. Kent, et al. (1994). "Role of different domains in the self-association of rat nucleoporin p62." J Cell Sci **107 (Pt 2)**: 631-8.

Cai, W. and P. A. Schaffer (1992). "Herpes simplex virus type 1 ICP0 regulates expression of immediate-early, early, and late genes in productively infected cells." J Virol **66**(5): 2904-15.

Carter, K. L. and B. Roizman (1996). "Alternatively spliced mRNAs predicted to yield frame-shift proteins and stable intron 1 RNAs of the herpes simplex virus 1 regulatory gene alpha 0 accumulate in the cytoplasm of infected cells." Proc Natl Acad Sci U S A **93**(22): 12535-40.

Caserta, M. T., D. J. Mock, et al. (2001). "Human herpesvirus 6." Clin Infect Dis **33**(6): 829-33.

Champion-Arnaud, P. and R. Reed (1994). "The prespliceosome components SAP 49 and SAP 145 interact in a complex implicated in tethering U2 snRNP to the branch site." Genes Dev **8**(16): 1974-83.

Chapman, C. J., J. D. Harris, et al. (1992). "Promoter-independent activation of heterologous virus gene expression by the herpes simplex virus immediate-early protein ICP27." Virology **186**(2): 573-8.

Chen, I. H., L. Li, et al. (2005). "ICP27 recruits Aly/REF but not TAP/NXF1 to herpes simplex virus type 1 transcription sites although TAP/NXF1 is required for ICP27 export." J Virol **79**(7): 3949-61.

Chen, I. H., K. S. Sciabica, et al. (2002). "ICP27 interacts with the RNA export factor Aly/REF to direct herpes simplex virus type 1 intronless mRNAs to the TAP export pathway." J Virol **76**(24): 12877-89.

Chen, Y. M. and D. M. Knipe (1996). "A dominant mutant form of the herpes simplex virus ICP8 protein decreases viral late gene transcription." Virology **221**(2): 281-90.

Chung, K. M., J. Lee, et al. (2000). "Nonstructural protein 5A of hepatitis C virus inhibits the function of karyopherin beta3." J Virol **74**(11): 5233-41.
Clarkson, W. D., A. H. Corbett, et al. (1997). "Nuclear protein import is decreased by engineered mutants of nuclear transport factor 2 (NTF2) that do not bind GDP-Ran." J Mol Biol **272**(5): 716-30.
Clarkson, W. D., H. M. Kent, et al. (1996). "Separate binding sites on nuclear transport factor 2 (NTF2) for GDP-Ran and the phenylalanine-rich repeat regions of nucleoporins p62 and Nsp1p." J Mol Biol **263**(4): 517-24.
Clercq, E. D. (2004). "ANTIVIRALS AND ANTIVIRAL STRATEGIES." Nature Reviews Microbiology **2**(9): 704-720.
Cohen, G. H., M. I. Muggeridge, et al. (1992). "Structural and functional studies of herpes simplex virus glycoprotein D." Adv Exp Med Biol **327**: 217-28.
Cohen, J. I., T. Krogmann, et al. (2005). "Varicella-zoster virus ORF4 latency-associated protein is important for establishment of latency." J Virol **79**(11): 6969-75.
Cole, C. N. and C. M. Hammell (1998). "Nucleocytoplasmic transport: driving and directing transport." Curr Biol **8**(11): R368-72.
Conti, E. and E. Izaurralde (2001). "Nucleocytoplasmic transport enters the atomic age." Curr Opin Cell Biol **13**(3): 310-9.
Cook, I. D., F. Shanahan, et al. (1994). "Epstein-Barr virus SM protein." Virology **205**(1): 217-27.
Corey, L., A. G. Langenberg, et al. (1999). "Recombinant glycoprotein vaccine for the prevention of genital HSV-2 infection: two randomized controlled trials. Chiron HSV Vaccine Study Group." Jama **282**(4): 331-40.
Cronshaw, J. M., A. N. Krutchinsky, et al. (2002). "Proteomic analysis of the mammalian nuclear pore complex." J Cell Biol **158**(5): 915-27.
Crumpacker, C. S. and P. A. Schaffer (2002). "New anti-HSV therapeutics target the helicase-primase complex." Nat Med **8**(4): 327-8.
Cullen, B. R. (2000). "Nuclear RNA export pathways." Mol Cell Biol **20**(12): 4181-7.
Dai-Ju, J. Q., L. Li, et al. (2006). "ICP27 interacts with the C-terminal domain of RNA polymerase II and facilitates its recruitment to herpes simplex virus 1 transcription sites, where it undergoes proteasomal degradation during infection." J Virol **80**(7): 3567-81.
Davido, D. J., W. F. von Zagorski, et al. (2005). "Phosphorylation site mutations affect herpes simplex virus type 1 ICP0 function." J Virol **79**(2): 1232-43.
Davison, A. J. (2002). "Evolution of the herpesviruses." Vet Microbiol **86**(1-2): 69-88.
Davison, J. A. and J. B. Clements (2005). Herpesviruses: general properties in Toply & Wilson's microbiology and microbial infections,Virology Volume 2, Hodder Arnold.
Dejgaard, K., H. Leffers, et al. (1994). "Identification, molecular cloning, expression and chromosome mapping of a family of transformation upregulated hnRNP-K proteins derived by alternative splicing." J Mol Biol **236**(1): 33-48.
DeLuca, N. A., A. M. McCarthy, et al. (1985). "Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein ICP4." J Virol **56**(2): 558-70.
Denning, D. P., S. S. Patel, et al. (2003). "Disorder in the nuclear pore complex: the FG repeat regions of nucleoporins are natively unfolded." Proc Natl Acad Sci U S A **100**(5): 2450-5.

Denning, D. P., V. Uversky, et al. (2002). "The Saccharomyces cerevisiae nucleoporin Nup2p is a natively unfolded protein." J Biol Chem **277**(36): 33447-55.

Desai, P. J., P. A. Schaffer, et al. (1988). "Excretion of non-infectious virus particles lacking glycoprotein H by a temperature-sensitive mutant of herpes simplex virus type 1: evidence that gH is essential for virion infectivity." J Gen Virol **69 (Pt 6)**: 1147-56.

Dobrowolska, G., F. J. Lozeman, et al. (1999). "CK2, a protein kinase of the next millennium." Mol Cell Biochem **191**(1-2): 3-12.

Doepker, R. C., W. L. Hsu, et al. (2004). "Herpes simplex virus virion host shutoff protein is stimulated by translation initiation factors eIF4B and eIF4H." J Virol **78**(9): 4684-99.

Donnelly, M. and G. Elliott (2001). "Nuclear localization and shuttling of herpes simplex virus tegument protein VP13/14." J Virol **75**(6): 2566-74.

Ellermann-Eriksen, S. (2005). "Macrophages and cytokines in the early defence against herpes simplex virus." Virol J **2**: 59.

Ellison, K. S., R. A. Maranchuk, et al. (2005). "Control of VP16 translation by the herpes simplex virus type 1 immediate-early protein ICP27." J Virol **79**(7): 4120-31.

Everett, R. D. (2000). "ICP0, a regulator of herpes simplex virus during lytic and latent infection." Bioessays **22**(8): 761-70.

Everett, R. D., C. Boutell, et al. (2004). "Phenotype of a herpes simplex virus type 1 mutant that fails to express immediate-early regulatory protein ICP0." J Virol **78**(4): 1763-74.

Everett, R. D. and J. Murray (2005). "ND10 components relocate to sites associated with herpes simplex virus type 1 nucleoprotein complexes during virus infection." J Virol **79**(8): 5078-89.

Everett, R. D., C. Parada, et al. (2008). "Replication of ICP0-null mutant herpes simplex virus type 1 is restricted by both PML and Sp100." J Virol **82**(6): 2661-72.

Everett, R. D., S. Rechter, et al. (2006). "PML contributes to a cellular mechanism of repression of herpes simplex virus type 1 infection that is inactivated by ICP0." J Virol **80**(16): 7995-8005.

Everett, R. D., G. Sourvinos, et al. (2003). "Recruitment of herpes simplex virus type 1 transcriptional regulatory protein ICP4 into foci juxtaposed to ND10 in live, infected cells." J Virol **77**(6): 3680-9.

Fahrenkrog, B. and U. Aebi (2003). "The nuclear pore complex: nucleocytoplasmic transport and beyond." Nat Rev Mol Cell Biol **4**(10): 757-66.

Fahrenkrog, B., J. Koser, et al. (2004). "The nuclear pore complex: a jack of all trades?" Trends Biochem Sci **29**(4): 175-82.

Fahrenkrog, B., B. Maco, et al. (2002). "Domain-specific antibodies reveal multiple-site topology of Nup153 within the nuclear pore complex." J Struct Biol **140**(1-3): 254-67.

Faria, P. A., P. Chakraborty, et al. (2005). "VSV disrupts the Rae1/mrnp41 mRNA nuclear export pathway." Mol Cell **17**(1): 93-102.

Finlay, D. R., E. Meier, et al. (1991). "A complex of nuclear pore proteins required for pore function." J Cell Biol **114**(1): 169-83.

Fischer, U., J. Huber, et al. (1995). "The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs." Cell **82**(3): 475-83.

Flint, S. J. and R. A. Gonzalez (2003). "Regulation of mRNA production by the adenoviral E1B 55-kDa and E4 Orf6 proteins." Curr Top Microbiol Immunol **272**: 287-330.

Fontaine-Rodriguez, E. C., T. J. Taylor, et al. (2004). "Proteomics of herpes simplex virus infected cell protein 27: association with translation initiation factors." Virology **330**(2): 487-92.

Fontoura, B. M., P. A. Faria, et al. (2005). "Viral interactions with the nuclear transport machinery: discovering and disrupting pathways." IUBMB Life **57**(2): 65-72.

Fornerod, M., M. Ohno, et al. (1997). "CRM1 is an export receptor for leucine-rich nuclear export signals." Cell **90**(6): 1051-60.

Fornerod, M., J. van Deursen, et al. (1997). "The human homologue of yeast CRM1 is in a dynamic subcomplex with CAN/Nup214 and a novel nuclear pore component Nup88." Embo J **16**(4): 807-16.

Fox, A. H., K. Kowalski, et al. (1998). "Key residues characteristic of GATA N-fingers are recognized by FOG." J Biol Chem **273**(50): 33595-603.

Fribourg, S., I. C. Braun, et al. (2001). "Structural basis for the recognition of a nucleoporin FG repeat by the NTF2-like domain of the TAP/p15 mRNA nuclear export factor." Mol Cell **8**(3): 645-56.

Fukuhara, T., N. Sakaguchi, et al. (2006). "Functional analysis of nuclear pore complex protein Nup62/p62 using monoclonal antibodies." Hybridoma (Larchmt) **25**(2): 51-9.

Fuller, A. O. and W. C. Lee (1992). "Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gD and gH in penetration." J Virol **66**(8): 5002-12.

Gaffney, D. F., J. McLauchlan, et al. (1985). "A modular system for the assay of transcription regulatory signals: the sequence TAATGARAT is required for herpes simplex virus immediate early gene activation." Nucleic Acids Res **13**(21): 7847-63.

Gamper, C., W. G. van Eyndhoven, et al. (2000). "TRAF-3 interacts with p62 nucleoporin, a component of the nuclear pore central plug that binds classical NLS-containing import complexes." Mol Immunol **37**(1-2): 73-84.

Gatfield, D. and E. Izaurralde (2002). "REF1/Aly and the additional exon junction complex proteins are dispensable for nuclear mRNA export." J Cell Biol **159**(4): 579-88.

Gatfield, D., H. Le Hir, et al. (2001). "The DExH/D box protein HEL/UAP56 is essential for mRNA nuclear export in Drosophila." Curr Biol **11**(21): 1716-21.

gBaird, A., R. Z. Florkiewicz, et al. (1990). "Mediation of virion penetration into vascular cells by association of basic fibroblast growth factor with herpes simplex virus type 1." Nature **348**(6299): 344-6.

Geraghty, R. J., C. Krummenacher, et al. (1998). "Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor." Science **280**(5369): 1618-20.

Gilchrist, D., B. Mykytka, et al. (2002). "Accelerating the rate of disassembly of karyopherin.cargo complexes." J Biol Chem **277**(20): 18161-72.

Godowski, P. J. and D. M. Knipe (1986). "Transcriptional control of herpesvirus gene expression: gene functions required for positive and negative regulation." Proc Natl Acad Sci U S A **83**(2): 256-60.

Goins, W. F., L. R. Sternberg, et al. (1994). "A novel latency-active promoter is contained within the herpes simplex virus type 1 UL flanking repeats." J Virol **68**(4): 2239-52.
Gomi, Y., H. Sunamachi, et al. (2002). "Comparison of the complete DNA sequences of the Oka varicella vaccine and its parental virus." J Virol **76**(22): 11447-59.
Goodwin, D. J., K. T. Hall, et al. (2000). "The carboxy terminus of the herpesvirus saimiri ORF 57 gene contains domains that are required for transactivation and transrepression." J Gen Virol **81**(Pt 9): 2253-65.
Goodwin, D. J. and A. Whitehouse (2001). "A gamma-2 herpesvirus nucleocytoplasmic shuttle protein interacts with importin alpha 1 and alpha 5." J Biol Chem **276**(23): 19905-12.
Gorlich, D. and I. W. Mattaj (1996). "Nucleocytoplasmic transport." Science **271**(5255): 1513-8.
Gould, V. E., N. Martinez, et al. (2000). "A novel, nuclear pore-associated, widely distributed molecule overexpressed in oncogenesis and development." Am J Pathol **157**(5): 1605-13.
Grant, R. P., D. Neuhaus, et al. (2003). "Structural basis for the interaction between the Tap/NXF1 UBA domain and FG nucleoporins at 1A resolution." J Mol Biol **326**(3): 849-58.
Graveley, B. R. (2000). "Sorting out the complexity of SR protein functions." Rna **6**(9): 1197-211.
Griffis, E. R., N. Altan, et al. (2002). "Nup98 is a mobile nucleoporin with transcription-dependent dynamics." Mol Biol Cell **13**(4): 1282-97.
Griffis, E. R., B. Craige, et al. (2004). "Distinct functional domains within nucleoporins Nup153 and Nup98 mediate transcription-dependent mobility." Mol Biol Cell **15**(4): 1991-2002.
Griffis, E. R., S. Xu, et al. (2003). "Nup98 localizes to both nuclear and cytoplasmic sides of the nuclear pore and binds to two distinct nucleoporin subcomplexes." Mol Biol Cell **14**(2): 600-10.
Gruenheid, S., L. Gatzke, et al. (1993). "Herpes simplex virus infection and propagation in a mouse L cell mutant lacking heparan sulfate proteoglycans." J Virol **67**(1): 93-100.
Gruter, P., C. Tabernero, et al. (1998). "TAP, the human homolog of Mex67p, mediates CTE-dependent RNA export from the nucleus." Mol Cell **1**(5): 649-59.
Gu, B. and N. DeLuca (1994). "Requirements for activation of the herpes simplex virus glycoprotein C promoter in vitro by the viral regulatory protein ICP4." J Virol **68**(12): 7953-65.
Guan, T., S. Muller, et al. (1995). "Structural analysis of the p62 complex, an assembly of O-linked glycoproteins that localizes near the central gated channel of the nuclear pore complex." Mol Biol Cell **6**(11): 1591-603.
Gui, J. F., H. Tronchere, et al. (1994). "Purification and characterization of a kinase specific for the serine- and arginine-rich pre-mRNA splicing factors." Proc Natl Acad Sci U S A **91**(23): 10824-8.
Gustin, K. E. (2003). "Inhibition of nucleo-cytoplasmic trafficking by RNA viruses: targeting the nuclear pore complex." Virus Res **95**(1-2): 35-44.
Gustin, K. E. and P. Sarnow (2001). "Effects of poliovirus infection on nucleo-cytoplasmic trafficking and nuclear pore complex composition." Embo J **20**(1-2): 240-9.

Han, I., M. D. Roos, et al. (1998). "Interaction of the transcription factor Sp1 with the nuclear pore protein p62 requires the C-terminal domain of p62." J Cell Biochem **68**(1): 50-61.

Hardwicke, M. A. and R. M. Sandri-Goldin (1994). "The herpes simplex virus regulatory protein ICP27 contributes to the decrease in cellular mRNA levels during infection." J Virol **68**(8): 4797-810.

Hardwicke, M. A., P. J. Vaughan, et al. (1989). "The regions important for the activator and repressor functions of herpes simplex virus type 1 alpha protein ICP27 map to the C-terminal half of the molecule." J Virol **63**(11): 4590-602.

Hardy, W. R. and R. M. Sandri-Goldin (1994). "Herpes simplex virus inhibits host cell splicing, and regulatory protein ICP27 is required for this effect." J Virol **68**(12): 7790-9.

Henderson, B. R. and A. Eleftheriou (2000). "A comparison of the activity, sequence specificity, and CRM1-dependence of different nuclear export signals." Exp Cell Res **256**(1): 213-24.

Hengge, U. R., T. Ruzicka, et al. (2002). "Update on Kaposi's sarcoma and other HHV8 associated diseases. Part 1: epidemiology, environmental predispositions, clinical manifestations, and therapy." Lancet Infect Dis **2**(5): 281-92.

Herold, A., M. Suyama, et al. (2000). "TAP (NXF1) belongs to a multigene family of putative RNA export factors with a conserved modular architecture." Mol Cell Biol **20**(23): 8996-9008.

Herold, A., L. Teixeira, et al. (2003). "Genome-wide analysis of nuclear mRNA export pathways in Drosophila." Embo J **22**(10): 2472-83.

Hetzer, M. W., T. C. Walther, et al. (2005). "Pushing the envelope: structure, function, and dynamics of the nuclear periphery." Annu Rev Cell Dev Biol **21**: 347-80.

Hill, A., P. Jugovic, et al. (1995). "Herpes simplex virus turns off the TAP to evade host immunity." Nature **375**(6530): 411-5.

Honess, R. W. and B. Roizman (1974). "Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins." J Virol **14**(1): 8-19.

Hutchinson, L., H. Browne, et al. (1992). "A novel herpes simplex virus glycoprotein, gL, forms a complex with glycoprotein H (gH) and affects normal folding and surface expression of gH." J Virol **66**(4): 2240-50.

Hutchinson, L. and D. C. Johnson (1995). "Herpes simplex virus glycoprotein K promotes egress of virus particles." J Virol **69**(9): 5401-13.

Hutten, S. and R. H. Kehlenbach (2006). "Nup214 is required for CRM1-dependent nuclear protein export in vivo." Mol Cell Biol **26**(18): 6772-85.

Imbalzano, A. N., D. M. Coen, et al. (1991). "Herpes simplex virus transactivator ICP4 operationally substitutes for the cellular transcription factor Sp1 for efficient expression of the viral thymidine kinase gene." J Virol **65**(2): 565-74.

Imbalzano, A. N. and N. A. DeLuca (1992). "Substitution of a TATA box from a herpes simplex virus late gene in the viral thymidine kinase promoter alters ICP4 inducibility but not temporal expression." J Virol **66**(9): 5453-63.

Ingram, A., A. Phelan, et al. (1996). "Immediate early protein IE63 of herpes simplex virus type 1 binds RNA directly." J Gen Virol **77 (Pt 8)**: 1847-51.

Jackson, S. A. and N. A. DeLuca (2003). "Relationship of herpes simplex virus genome configuration to productive and persistent infections." Proc Natl Acad Sci U S A **100**(13): 7871-6.

Jean, S., K. M. LeVan, et al. (2001). "Herpes simplex virus 1 ICP27 is required for transcription of two viral late (gamma 2) genes in infected cells." Virology **283**(2): 273-84.

Jordan, R. and P. A. Schaffer (1997). "Activation of gene expression by herpes simplex virus type 1 ICP0 occurs at the level of mRNA synthesis." J Virol **71**(9): 6850-62.

Kambach, C., S. Walke, et al. (1999). "Crystal structures of two Sm protein complexes and their implications for the assembly of the spliceosomal snRNPs." Cell **96**(3): 375-87.

Kang, Y. and B. R. Cullen (1999). "The human Tap protein is a nuclear mRNA export factor that contains novel RNA-binding and nucleocytoplasmic transport sequences." Genes Dev **13**(9): 1126-39.

Karr, B. M. and G. S. Read (1999). "The virion host shutoff function of herpes simplex virus degrades the 5' end of a target mRNA before the 3' end." Virology **264**(1): 195-204.

Katahira, J., K. Straesser, et al. (2002). "Complex formation between Tap and p15 affects binding to FG-repeat nucleoporins and nucleocytoplasmic shuttling." J Biol Chem **277**(11): 9242-6.

Kawaguchi, Y., M. Tanaka, et al. (2001). "Herpes simplex virus 1 alpha regulatory protein ICP0 functionally interacts with cellular transcription factor BMAL1." Proc Natl Acad Sci U S A **98**(4): 1877-82.

Kim, D. B., S. Zabierowski, et al. (2002). "The initiator element in a herpes simplex virus type 1 late-gene promoter enhances activation by ICP4, resulting in abundant late-gene expression." J Virol **76**(4): 1548-58.

Kinghorn, G. R. (1993). "Genital herpes: natural history and treatment of acute episodes." J Med Virol **Suppl 1**: 33-8.

Koffa, M. D., J. B. Clements, et al. (2001). "Herpes simplex virus ICP27 protein provides viral mRNAs with access to the cellular mRNA export pathway." Embo J **20**(20): 5769-78.

Koffa, M. D., J. Kean, et al. (2003). "CK2 protein kinase is stimulated and redistributed by functional herpes simplex virus ICP27 protein." J Virol **77**(7): 4315-25.

Komeili, A. and E. K. O'Shea (2001). "New perspectives on nuclear transport." Annu Rev Genet **35**: 341-64.

Krainer, A. R., G. C. Conway, et al. (1990). "Purification and characterization of pre-mRNA splicing factor SF2 from HeLa cells." Genes Dev **4**(7): 1158-71.

Ku, C.-C., J. Besser, et al. (2005). "Varicella-Zoster Virus Pathogenesis and Immunobiology: New Concepts Emerging from Investigations with the SCIDhu Mouse Model." J. Virol. **79**(5): 2651-2658.

Kuersten, S., M. Ohno, et al. (2001). "Nucleocytoplasmic transport: Ran, beta and beyond." Trends Cell Biol **11**(12): 497-503.

Kummer, M., N. M. Turza, et al. (2007). "Herpes simplex virus type 1 induces CD83 degradation in mature dendritic cells with immediate-early kinetics via the cellular proteasome." J Virol **81**(12): 6326-38.

Kutok, J. L. and F. Wang (2006). "Spectrum of Epstein-Barr Virus-Associated Diseases." Annu. Rev. Pathol. Mech. Dis **1**: 375-404.

Kwong, A. D. and N. Frenkel (1987). "Herpes simplex virus-infected cells contain a function(s) that destabilizes both host and viral mRNAs." Proc Natl Acad Sci U S A **84**(7): 1926-30.

La Boissiere, S., T. Hughes, et al. (1999). "HCF-dependent nuclear import of VP16." Embo J **18**(2): 480-9.

LaBoissiere, S. and P. O'Hare (2000). "Analysis of HCF, the cellular cofactor of VP16, in herpes simplex virus-infected cells." J Virol **74**(1): 99-109.

Larralde, O., R. W. Smith, et al. (2006). "Direct stimulation of translation by the multifunctional herpesvirus ICP27 protein." J Virol **80**(3): 1588-91.

Lehman, I. R. and P. E. Boehmer (1999). "Replication of herpes simplex virus DNA." J Biol Chem **274**(40): 28059-62.

Leiper, C. (2004). Characterisation of mutants in the herpes simplex virus-1 ICP27 regulatory protein. IBLS. Glasgow, University of Glasgow. **PhD:** 200.

Lengyel, J., C. Guy, et al. (2002). "Mapping of functional regions in the amino-terminal portion of the herpes simplex virus ICP27 regulatory protein: importance of the leucine-rich nuclear export signal and RGG Box RNA-binding domain." J Virol **76**(23): 11866-79.

Lengyel, J., A. K. Strain, et al. (2006). "ICP27-dependent resistance of herpes simplex virus type 1 to leptomycin B is associated with enhanced nuclear localization of ICP4 and ICP0." Virology **352**(2): 368-79.

Leopardi, R., P. L. Ward, et al. (1997). "Association of herpes simplex virus regulatory protein ICP22 with transcriptional complexes containing EAP, ICP4, RNA polymerase II, and viral DNA requires posttranslational modification by the U(L)13 proteinkinase." J Virol **71**(2): 1133-9.

Levesque, L., Y. C. Bor, et al. (2006). "Mutations in tap uncouple RNA export activity from translocation through the nuclear pore complex." Mol Biol Cell **17**(2): 931-43.

Lim, R. Y., U. Aebi, et al. (2006). "From the trap to the basket: getting to the bottom of the nuclear pore complex." Chromosoma **115**(1): 15-26.

Lim, R. Y. and B. Fahrenkrog (2006). "The nuclear pore complex up close." Curr Opin Cell Biol **18**(3): 342-7.

Lindberg, A. and J. P. Kreivi (2002). "Splicing inhibition at the level of spliceosome assembly in the presence of herpes simplex virus protein ICP27." Virology **294**(1): 189-98.

Loiacono, C. M., N. S. Taus, et al. (2003). "The herpes simplex virus type 1 ICP0 promoter is activated by viral reactivation stimuli in trigeminal ganglia neurons of transgenic mice." J Neurovirol **9**(3): 336-45.

Long, M. C., V. Leong, et al. (1999). "ICP22 and the UL13 protein kinase are both required for herpes simplex virus-induced modification of the large subunit of RNA polymerase II." J Virol **73**(7): 5593-604.

Longman, D., I. L. Johnstone, et al. (2003). "The Ref/Aly proteins are dispensable for mRNA export and development in Caenorhabditis elegans." Rna **9**(7): 881-91.

Lopez, C., A. Arvin, et al. (1993). Immunity to herpesvirus infections in human.In the Human Herpesviruses, New York:Raven press.

Lukac, D. M., J. R. Kirshner, et al. (1999). "Transcriptional activation by the product of open reading frame 50 of Kaposi's sarcoma-associated herpesvirus is required for lytic viral reactivation in B cells." J Virol **73**(11): 9348-61.

Macara, I. G. (2001). "Transport into and out of the nucleus." Microbiol Mol Biol Rev **65**(4): 570-94, table of contents.

Mackay, J. P. and M. Crossley (1998). "Zinc fingers are sticking together." Trends Biochem Sci **23**(1): 1-4.

Malik, P., D. J. Blackbourn, et al. (2004). "Functional co-operation between the Kaposi's sarcoma-associated herpesvirus ORF57 and ORF50 regulatory proteins." J Gen Virol **85**(Pt 8): 2155-66.

Malik, P., D. J. Blackbourn, et al. (2004). "The evolutionarily conserved Kaposi's sarcoma-associated herpesvirus ORF57 protein interacts with REF protein and acts as an RNA export factor." J Biol Chem **279**(31): 33001-11.

Malik, P. and J. B. Clements (2004). "Protein kinase CK2 phosphorylation regulates the interaction of Kaposi's sarcoma-associated herpesvirus regulatory protein ORF57 with its multifunctional partner hnRNP K." Nucleic Acids Res **32**(18): 5553-69.

Marg, A., Y. Shan, et al. (2004). "Nucleocytoplasmic shuttling by nucleoporins Nup153 and Nup214 and CRM1-dependent nuclear export control the subcellular distribution of latent Stat1." J Cell Biol **165**(6): 823-33.

Martin, T. E., S. C. Barghusen, et al. (1987). "Redistribution of nuclear ribonucleoprotein antigens during herpes simplex virus infection." J Cell Biol **105**(5): 2069-82.

Martinez, R., R. T. Sarisky, et al. (1996). "Herpes simplex virus type 1 alkaline nuclease is required for efficient processing of viral DNA replication intermediates." J Virol **70**(4): 2075-85.

Mattaj, I. W. and L. Englmeier (1998). "Nucleocytoplasmic transport: the soluble phase." Annu Rev Biochem **67**: 265-306.

Matunis, E. L., M. J. Matunis, et al. (1992). "Characterization of the major hnRNP proteins from Drosophila melanogaster." J Cell Biol **116**(2): 257-69.

Mavromara-Nazos, P., M. Ackermann, et al. (1986). "Construction and properties of a viable herpes simplex virus 1 recombinant lacking coding sequences of the alpha 47 gene." J Virol **60**(2): 807-12.

McGeoch, D. J., M. A. Dalrymple, et al. (1988). "The complete DNA sequence of the long unique region in the genome of herpes simplex virus type 1." J Gen Virol **69 (Pt 7)**: 1531-74.

McGeoch, D. J., A. Dolan, et al. (1986). "Complete DNA sequence of the short repeat region in the genome of herpes simplex virus type 1." Nucleic Acids Res **14**(4): 1727-45.

McGregor, F., A. Phelan, et al. (1996). "Regulation of herpes simplex virus poly (A) site usage and the action of immediate-early protein IE63 in the early-late switch." J Virol **70**(3): 1931-40.

McLauchlan, J., A. Phelan, et al. (1992). "Herpes simplex virus IE63 acts at the posttranscriptional level to stimulate viral mRNA 3' processing." J Virol **66**(12): 6939-45.

McLauchlan, J., S. Simpson, et al. (1989). "Herpes simplex virus induces a processing factor that stimulates poly(A) site usage." Cell **59**(6): 1093-105.

McMahan, L. and P. A. Schaffer (1990). "The repressing and enhancing functions of the herpes simplex virus regulatory protein ICP27 map to C-terminal regions and are required to modulate viral gene expression very early in infection." J Virol **64**(7): 3471-85.

McNamee, E. E., T. J. Taylor, et al. (2000). "A dominant-negative herpesvirus protein inhibits intranuclear targeting of viral proteins: effects on DNA replication and late gene expression." J Virol **74**(21): 10122-31.

Mears, W. E., V. Lam, et al. (1995). "Identification of nuclear and nucleolar localization signals in the herpes simplex virus regulatory protein ICP27." J Virol **69**(2): 935-47.
Mears, W. E. and S. A. Rice (1996). "The RGG box motif of the herpes simplex virus ICP27 protein mediates an RNA-binding activity and determines in vivo methylation." J Virol **70**(11): 7445-53.
Mears, W. E. and S. A. Rice (1998). "The herpes simplex virus immediate-early protein ICP27 shuttles between nucleus and cytoplasm." Virology **242**(1): 128-37.
Mettenleiter, T. C. (2002). "Herpesvirus Assembly and Egress." J. Virol. **76**(4): 1537-1547.
Michael, W. M., P. S. Eder, et al. (1997). "The K nuclear shuttling domain: a novel signal for nuclear import and nuclear export in the hnRNP K protein." Embo J **16**(12): 3587-98.
Miller, C. S., R. J. Danaher, et al. (2006). "ICP0 is not required for efficient stress-induced reactivation of herpes simplex virus type 1 from cultured quiescently infected neuronal cells." J Virol **80**(7): 3360-8.
Miller, M. W., M. R. Caracciolo, et al. (1999). "Phosphorylation and glycosylation of nucleoporins." Arch Biochem Biophys **367**(1): 51-60.
Mindel, A., M. W. Adler, et al. (1982). "Intravenous acyclovir treatment for primary genital herpes." Lancet **1**(8274): 697-700.
Montgomery, R. I., M. S. Warner, et al. (1996). "Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family." Cell **87**(3): 427-36.
Moore, P. S. and Y. Chang (2001). Kaposi's Sarcoma-Associated Herpesvirus. In Fields Virology, Philadelphia: Raven press.
Moriuchi, H., M. Moriuchi, et al. (1994). "Varicella-zoster virus open reading frame 4 protein is functionally distinct from and does not complement its herpes simplex virus type 1 homolog, ICP27." J Virol **68**(3): 1987-92.
Murata, T., F. Goshima, et al. (2001). "A single amino acid substitution in the ICP27 protein of herpes simplex virus type 1 is responsible for its resistance to leptomycin B." J Virol **75**(2): 1039-43.
Nilsen, T. W. (2003). "The spliceosome: the most complex macromolecular machine in the cell?" Bioessays **25**(12): 1147-9.
O'Hare, P. (1993). "The virion transactivator of herpes simplex virus." Seminars in Virology **4**(3): 145-155.
Oakes, J. E. and H. Rosemond-Hornbeak (1978). "Antibody-mediated recovery from subcutaneous herpes simplex virus type 2 infection." Infect Immun **21**(2): 489-95.
Ogle, W. O. and B. Roizman (1999). "Functional anatomy of herpes simplex virus 1 overlapping genes encoding infected-cell protein 22 and US1.5 protein." J Virol **73**(5): 4305-15.
Olesky, M., E. E. McNamee, et al. (2005). "Evidence for a direct interaction between HSV-1 ICP27 and ICP8 proteins." Virology **331**(1): 94-105.
Pante, N., R. Bastos, et al. (1994). "Interactions and three-dimensional localization of a group of nuclear pore complex proteins." J Cell Biol **126**(3): 603-17.
Parkinson, J., S. P. Lees-Miller, et al. (1999). "Herpes simplex virus type 1 immediate-early protein vmw110 induces the proteasome-dependent degradation of the catalytic subunit of DNA-dependent protein kinase." J Virol **73**(1): 650-7.

Paschal, B. M. and L. Gerace (1995). "Identification of NTF2, a cytosolic factor for nuclear import that interacts with nuclear pore complex protein p62." J Cell Biol **129**(4): 925-37.

Pass, R. F. (2001). Cytomegalovirus. In Fields Virology, Philadelphia: Raven press.

Paulillo, S. M., E. M. Phillips, et al. (2005). "Nucleoporin domain topology is linked to the transport status of the nuclear pore complex." J Mol Biol **351**(4): 784-98.

Pearson, A., D. M. Knipe, et al. (2004). "ICP27 selectively regulates the cytoplasmic localization of a subset of viral transcripts in herpes simplex virus type 1-infected cells." J Virol **78**(1): 23-32.

Percipalle, P., W. D. Clarkson, et al. (1997). "Molecular interactions between the importin alpha/beta heterodimer and proteins involved in vertebrate nuclear protein import." J Mol Biol **266**(4): 722-32.

Pereira, L., M. H. Wolff, et al. (1977). "Regulation of herpesvirus macromolecular synthesis. V. Properties of alpha polypeptides made in HSV-1 and HSV-2 infected cells." Virology **77**(2): 733-49.

Perera, L. P. (2000). "The TATA motif specifies the differential activation of minimal promoters by varicella zoster virus immediate-early regulatory protein IE62." J Biol Chem **275**(1): 487-96.

Perry, L. J. and D. J. McGeoch (1988). "The DNA sequences of the long repeat region and adjoining parts of the long unique region in the genome of herpes simplex virus type 1." J Gen Virol **69 (Pt 11)**: 2831-46.

Petersen-Mahrt, S. K., C. Estmer, et al. (1999). "The splicing factor-associated protein, p32, regulates RNA splicing by inhibiting ASF/SF2 RNA binding and phosphorylation." Embo J **18**(4): 1014-24.

Phelan, A., M. Carmo-Fonseca, et al. (1993). "A herpes simplex virus type 1 immediate-early gene product, IE63, regulates small nuclear ribonucleoprotein distribution." Proc Natl Acad Sci U S A **90**(19): 9056-60.

Phelan, A. and J. B. Clements (1997). "Herpes simplex virus type 1 immediate early protein IE63 shuttles between nuclear compartments and the cytoplasm." J Gen Virol **78 (Pt 12)**: 3327-31.

Phelan, A., J. Dunlop, et al. (1996). "Herpes simplex virus type 1 protein IE63 affects the nuclear export of virus intron-containing transcripts." J Virol **70**(8): 5255-65.

Poon, I. K. and D. A. Jans (2005). "Regulation of nuclear transport: central role in development and transformation?" Traffic **6**(3): 173-86.

Powers, M. A., D. J. Forbes, et al. (1997). "The vertebrate GLFG nucleoporin, Nup98, is an essential component of multiple RNA export pathways." J Cell Biol **136**(2): 241-50.

Pritchard, C. E., M. Fornerod, et al. (1999). "RAE1 is a shuttling mRNA export factor that binds to a GLEBS-like NUP98 motif at the nuclear pore complex through multiple domains." J Cell Biol **145**(2): 237-54.

Rabut, G., V. Doye, et al. (2004). "Mapping the dynamic organization of the nuclear pore complex inside single living cells." Nat Cell Biol **6**(11): 1114-21.

Reardon, J. E. and T. Spector (1989). "Herpes simplex virus type 1 DNA polymerase. Mechanism of inhibition by acyclovir triphosphate." J Biol Chem **264**(13): 7405-11.

Ribbeck, K. and D. Gorlich (2001). "Kinetic analysis of translocation through nuclear pore complexes." Embo J **20**(6): 1320-30.

Ribbeck, K. and D. Gorlich (2002). "The permeability barrier of nuclear pore complexes appears to operate via hydrophobic exclusion." Embo J **21**(11): 2664-71.

Rice, S. A. and V. Lam (1994). "Amino acid substitution mutations in the herpes simplex virus ICP27 protein define an essential gene regulation function." J Virol **68**(2): 823-33.

Rice, S. A., V. Lam, et al. (1993). "The acidic amino-terminal region of herpes simplex virus type 1 alpha protein ICP27 is required for an essential lytic function." J Virol **67**(4): 1778-87.

Rice, S. A., M. C. Long, et al. (1995). "Herpes simplex virus immediate-early protein ICP22 is required for viral modification of host RNA polymerase II and establishment of the normal viral transcription program." J Virol **69**(9): 5550-9.

Rikinson, A. B. and E. Kieff (2001). Epstein-Barr virus. In Fields Virology, Philadelphia: Raven press.

Rock, D. L. and N. W. Fraser (1983). "Detection of HSV-1 genome in central nervous system of latently infected mice." Nature **302**(5908): 523-5.

Rodrigues, J. P., M. Rode, et al. (2001). "REF proteins mediate the export of spliced and unspliced mRNAs from the nucleus." Proc Natl Acad Sci U S A **98**(3): 1030-5.

Roizman, B. and D. M. Knipe (2001). Herpes simplex viruses and their replication, Philadelphia: Raven press.

Roizman, B. and P. E. Pellett (2001). The family Herpesviridae:A Brief introduction. In Fields Virology, Philadelphia: Raven press.

Rollenhagen, C., P. Muhlhausser, et al. (2003). "Importin beta-depending nuclear import pathways: role of the adapter proteins in the docking and releasing steps." Mol Biol Cell **14**(5): 2104-15.

Rout, M. P. and J. D. Aitchison (2001). "The nuclear pore complex as a transport machine." J Biol Chem **276**(20): 16593-6.

Rout, M. P., J. D. Aitchison, et al. (2003). "Virtual gating and nuclear transport: the hole picture." Trends Cell Biol **13**(12): 622-8.

Rout, M. P., J. D. Aitchison, et al. (2000). "The yeast nuclear pore complex: composition, architecture, and transport mechanism." J Cell Biol **148**(4): 635-51.

Royston, I. and L. Aurelian (1970). "The association of genital herpesvirus with cervical atypia and carcinoma in situ." Am J Epidemiol **91**(6): 531-8.

Ruvolo, V., L. Sun, et al. (2004). "Functional analysis of Epstein-Barr virus SM protein: identification of amino acids essential for structure, transactivation, splicing inhibition, and virion production." J Virol **78**(1): 340-52.

Sacks, W. R., C. C. Greene, et al. (1985). "Herpes simplex virus type 1 ICP27 is an essential regulatory protein." J Virol **55**(3): 796-805.

Sandri-Goldin, R. M. (1998). "ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif." Genes Dev **12**(6): 868-79.

Sandri-Goldin, R. M. (2004). "Viral regulation of mRNA export." J Virol **78**(9): 4389-96.

Sandri-Goldin, R. M. and M. K. Hibbard (1996). "The herpes simplex virus type 1 regulatory protein ICP27 coimmunoprecipitates with anti-Sm antiserum, and the C terminus appears to be required for this interaction." J Virol **70**(1): 108-18.

Sandri-Goldin, R. M., M. K. Hibbard, et al. (1995). "The C-terminal repressor region of herpes simplex virus type 1 ICP27 is required for the redistribution of small nuclear ribonucleoprotein particles and splicing factor SC35; however, these alterations are not sufficient to inhibit host cell splicing." J Virol **69**(10): 6063-76.

Sandri-Goldin, R. M. and G. E. Mendoza (1992). "A herpesvirus regulatory protein appears to act post-transcriptionally by affecting mRNA processing." Genes Dev **6**(5): 848-63.

Santos-Rosa, H., H. Moreno, et al. (1998). "Nuclear mRNA export requires complex formation between Mex67p and Mtr2p at the nuclear pores." Mol Cell Biol **18**(11): 6826-38.

Satterly, N., P. L. Tsai, et al. (2007). "Influenza virus targets the mRNA export machinery and the nuclear pore complex." Proc Natl Acad Sci U S A **104**(6): 1853-8.

Schaeffer, H., L. Beauchamp, et al. (1979). "p.9-(2-hydroxyethoxymethyl) guanine activity against viruses of the herpes group." Nature **272**: 583-585.

Schmitt, I. and L. Gerace (2001). "In vitro analysis of nuclear transport mediated by the C-terminal shuttle domain of Tap." J Biol Chem **276**(45): 42355-63.

Sciabica, K. S., Q. J. Dai, et al. (2003). "ICP27 interacts with SRPK1 to mediate HSV splicing inhibition by altering SR protein phosphorylation." Embo J **22**(7): 1608-19.

Sciortino, M. T., B. Taddeo, et al. (2002). "Of the three tegument proteins that package mRNA in herpes simplex virions, one (VP22) transports the mRNA to uninfected cells for expression prior to viral infection." Proc Natl Acad Sci U S A **99**(12): 8318-23.

Segref, A., K. Sharma, et al. (1997). "Mex67p, a novel factor for nuclear mRNA export, binds to both poly(A)+ RNA and nuclear pores." Embo J **16**(11): 3256-71.

Shah, S., S. Tugendreich, et al. (1998). "Major binding sites for the nuclear import receptor are the internal nucleoporin Nup153 and the adjacent nuclear filament protein Tpr." J Cell Biol **141**(1): 31-49.

Shieh, M. T., D. WuDunn, et al. (1992). "Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans." J Cell Biol **116**(5): 1273-81.

Shukla, D., J. Liu, et al. (1999). "A novel role for 3-O-sulfated heparan sulfate in herpes simplex virus 1 entry." Cell **99**(1): 13-22.

Singh, M., C. Fraefel, et al. (1996). "Identification and characterization of BICP27, an early protein of bovine herpesvirus 1 which may stimulate mRNA 3' processing." J Gen Virol **77 (Pt 4)**: 615-25.

Siomi, H., M. J. Matunis, et al. (1993). "The pre-mRNA binding K protein contains a novel evolutionarily conserved motif." Nucleic Acids Res **21**(5): 1193-8.

Smibert, C. A., B. Popova, et al. (1994). "Herpes simplex virus VP16 forms a complex with the virion host shutoff protein vhs." J Virol **68**(4): 2339-46.

Smith, R. W., P. Malik, et al. (2005). "The herpes simplex virus ICP27 protein: a multifunctional post-transcriptional regulator of gene expression." Biochem Soc Trans **33**(Pt 3): 499-501.

Sokolowski, M., J. E. Scott, et al. (2003). "Identification of herpes simplex virus RNAs that interact specifically with regulatory protein ICP27 in vivo." J Biol Chem **278**(35): 33540-9.

Soliman, T. M., R. M. Sandri-Goldin, et al. (1997). "Shuttling of the herpes simplex virus type 1 regulatory protein ICP27 between the nucleus and cytoplasm mediates the expression of late proteins." J Virol **71**(12): 9188-97.

Soliman, T. M. and S. J. Silverstein (2000). "Herpesvirus mRNAs are sorted for export via Crm1-dependent and -independent pathways." J Virol **74**(6): 2814-25.
Soliman, T. M. and S. J. Silverstein (2000). "Identification of an export control sequence and a requirement for the KH domains in ICP27 from herpes simplex virus type 1." J Virol **74**(16): 7600-9.
Spear, P. G. and R. Longnecker (2003). "Herpesvirus entry: an update." J Virol **77**(19): 10179-85.
Stanberry, L. R. (1995). "Herpes simplex virus vaccines as immunotherapeutic agents." Trends Microbiol **3**(6): 244-7.
Stavraky, K. M., W. E. Rawls, et al. (1983). "Sexual and socioeconomic factors affecting the risk of past infections with herpes simplex virus type 2." Am J Epidemiol **118**(1): 109-21.
Steffy, K. R. and J. P. Weir (1991). "Mutational analysis of two herpes simplex virus type 1 late promoters." J Virol **65**(12): 6454-60.
Stevens, J. G., E. K. Wagner, et al. (1987). "RNA complementary to a herpesvirus alpha gene mRNA is prominent in latently infected neurons." Science **235**(4792): 1056-9.
Stewart, M., R. P. Baker, et al. (2001). "Molecular mechanism of translocation through nuclear pore complexes during nuclear protein import." FEBS Lett **498**(2-3): 145-9.
Stochaj, U., P. Banski, et al. (2006). "The N-terminal domain of the mammalian nucleoporin p62 interacts with other nucleoporins of the FXFG family during interphase." Exp Cell Res **312**(13): 2490-9.
Stow, N. D. (1992). "Herpes simplex virus type 1 origin-dependent DNA replication in insect cells using recombinant baculoviruses." J Gen Virol **73 (Pt 2)**: 313-21.
Strang, B. L. and N. D. Stow (2005). "Circularization of the herpes simplex virus type 1 genome upon lytic infection." J Virol **79**(19): 12487-94.
Strawn, L. A., T. Shen, et al. (2004). "Minimal nuclear pore complexes define FG repeat domains essential for transport." Nat Cell Biol **6**(3): 197-206.
Suntharalingam, M. and S. R. Wente (2003). "Peering through the pore: nuclear pore complex structure, assembly, and function." Dev Cell **4**(6): 775-89.
Swaminathan, S. (2005). "Post-transcriptional gene regulation by gamma herpesviruses." J Cell Biochem **95**(4): 698-711.
Szilvay, A. M., K. A. Brokstad, et al. (1997). "Oligomerization of HIV-1 Rev mutants in the cytoplasm and during nuclear import." Virology **235**(1): 73-81.
Taddeo, B. and B. Roizman (2006). "The virion host shutoff protein (UL41) of herpes simplex virus 1 is an endoribonuclease with a substrate specificity similar to that of RNase A." J Virol **80**(18): 9341-5.
Taddeo, B., W. Zhang, et al. (2006). "The U(L)41 protein of herpes simplex virus 1 degrades RNA by endonucleolytic cleavage in absence of other cellular or viral proteins." Proc Natl Acad Sci U S A **103**(8): 2827-32.
Tan, W., A. S. Zolotukhin, et al. (2000). "The mRNA export in Caenorhabditis elegans is mediated by Ce-NXF-1, an ortholog of human TAP/NXF and Saccharomyces cerevisiae Mex67p." Rna **6**(12): 1762-72.
Terhune, S. S., J. Schroer, et al. (2004). "RNAs are packaged into human cytomegalovirus virions in proportion to their intracellular concentration." J Virol **78**(19): 10390-8.

Thakurta, A. G., G. Gopal, et al. (2004). "Conserved nuclear export sequences in Schizosaccharomyces pombe Mex67 and human TAP function in mRNA export by direct nuclear pore interactions." J Biol Chem **279**(17): 17434-42.

Thiry, E., B. Muylkens, et al. (2005). "Recombination in the alphaherpesvirus bovine herpesvirus 1." Vet Microbiol.

Toth, Z., P. Lischka, et al. (2006). "RNA-binding of the human cytomegalovirus transactivator protein UL69, mediated by arginine-rich motifs, is not required for nuclear export of unspliced RNA." Nucleic Acids Res **34**(4): 1237-49.

Ullman, K. S., S. Shah, et al. (1999). "The nucleoporin nup153 plays a critical role in multiple types of nuclear export." Mol Biol Cell **10**(3): 649-64.

Uprichard, S. L. and D. M. Knipe (1996). "Herpes simplex ICP27 mutant viruses exhibit reduced expression of specific DNA replication genes." J Virol **70**(3): 1969-80.

van Deursen, J., J. Boer, et al. (1996). "G2 arrest and impaired nucleocytoplasmic transport in mouse embryos lacking the proto-oncogene CAN/Nup214." Embo J **15**(20): 5574-83.

Vaughan, P. J., K. J. Thibault, et al. (1992). "The herpes simplex virus immediate early protein ICP27 encodes a potential metal binding domain and binds zinc in vitro." Virology **189**(1): 377-84.

Wadd, S., H. Bryant, et al. (1999). "The multifunctional herpes simplex virus IE63 protein interacts with heterogeneous ribonucleoprotein K and with casein kinase 2." J Biol Chem **274**(41): 28991-8.

Wagner, M. J. and W. C. Summers (1978). "Structure of the joint region and the termini of the DNA of herpes simplex virus type 1." J Virol **27**(2): 374-87.

Walther, T. C., P. Askjaer, et al. (2003). "RanGTP mediates nuclear pore complex assembly." Nature **424**(6949): 689-94.

Walther, T. C., M. Fornerod, et al. (2001). "The nucleoporin Nup153 is required for nuclear pore basket formation, nuclear pore complex anchoring and import of a subset of nuclear proteins." Embo J **20**(20): 5703-14.

Walther, T. C., H. S. Pickersgill, et al. (2002). "The cytoplasmic filaments of the nuclear pore complex are dispensable for selective nuclear protein import." J Cell Biol **158**(1): 63-77.

Ward, P. L. and B. Roizman (1994). "Herpes simplex genes: the blueprint of a successful human pathogen." Trends Genet **10**(8): 267-74.

Warren, K. G., M. Devlin, et al. (1977). "Isolation of Herpes simplex virus from human trigeminal ganglia, including ganglia from one patient with multiple sclerosis." Lancet **2**(8039): 637-9.

Weis, K. (2002). "Nucleocytoplasmic transport: cargo trafficking across the border." Curr Opin Cell Biol **14**(3): 328-35.

Weis, K. (2003). "Regulating access to the genome: nucleocytoplasmic transport throughout the cell cycle." Cell **112**(4): 441-51.

Wentworth, B. B. and E. R. Alexander (1971). "Seroepidemiology of infectious due to members of the herpesvirus group." Am J Epidemiol **94**(5): 496-507.

Whitehouse, A., M. Cooper, et al. (1998). "The immediate-early gene product encoded by open reading frame 57 of herpesvirus saimiri modulates gene expression at a posttranscriptional level." J Virol **72**(1): 857-61.

Whitely, R. J. (2001). Herpes simplex viruses. In Fields Virology. Philadelphia, Philadelphia: Raven press.

Whitley, R. J. and B. Roizman (2001). "Herpes simplex virus infections." The Lancet **357**(9267): 1513-1518.

Wiemann, S., A. Kolb-Kokocinski, et al. (2005). "Alternative pre-mRNA processing regulates cell-type specific expression of the IL4I1 and NUP62 genes." BMC Biol **3**: 16.

Winkler, M., S. A. Rice, et al. (1994). "UL69 of human cytomegalovirus, an open reading frame with homology to ICP27 of herpes simplex virus, encodes a transactivator of gene expression." J Virol **68**(6): 3943-54.

Winkler, M. and T. Stamminger (1996). "A specific subform of the human cytomegalovirus transactivator protein pUL69 is contained within the tegument of virus particles." J Virol **70**(12): 8984-7.

WuDunn, D. and P. G. Spear (1989). "Initial interaction of herpes simplex virus with cells is binding to heparan sulfate." J Virol **63**(1): 52-8.

Xiao, C. Y. and D. A. Jans (1998). "An engineered site for protein kinase C flanking the SV40 large T-antigen NLS confers phorbol ester-inducible nuclear import." FEBS Lett **436**(3): 313-7.

Xu, L., Y. Kang, et al. (2002). "Smad2 nucleocytoplasmic shuttling by nucleoporins CAN/Nup214 and Nup153 feeds TGFbeta signaling complexes in the cytoplasm and nucleus." Mol Cell **10**(2): 271-82.

Xu, X. N., G. R. Screaton, et al. (2001). "Virus infections: escape, resistance, and counterattack." Immunity **15**(6): 867-70.

Xylourgidis, N., P. Roth, et al. (2006). "The nucleoporin Nup214 sequesters CRM1 at the nuclear rim and modulates NFkappaB activation in Drosophila." J Cell Sci **119**(Pt 21): 4409-19.

Yamanishi, K. (2001). Human Herpes virus6 and Human herpesvirus 7. In Fields Virology, Philadelphia: Raven press, 2785-2799.

Yang, W., J. Gelles, et al. (2004). "Imaging of single-molecule translocation through nuclear pore complexes." Proc Natl Acad Sci U S A **101**(35): 12887-92.

Yang, W. and S. M. Musser (2006). "Visualizing single molecules interacting with nuclear pore complexes by narrow-field epifluorescence microscopy." Methods **39**(4): 316-28.

York, I. A., C. Roop, et al. (1994). "A cytosolic herpes simplex virus protein inhibits antigen presentation to CD8+ T lymphocytes." Cell **77**(4): 525-35.

Zeitler, B. and K. Weis (2004). "The FG-repeat asymmetry of the nuclear pore complex is dispensable for bulk nucleocytoplasmic transport in vivo." J Cell Biol **167**(4): 583-90.

Zenklusen, D. and F. Stutz (2001). "Nuclear export of mRNA." FEBS Lett **498**(2-3): 150-6.

Zhi, Y. and R. M. Sandri-Goldin (1999). "Analysis of the phosphorylation sites of herpes simplex virus type 1 regulatory protein ICP27." J Virol **73**(4): 3246-57.

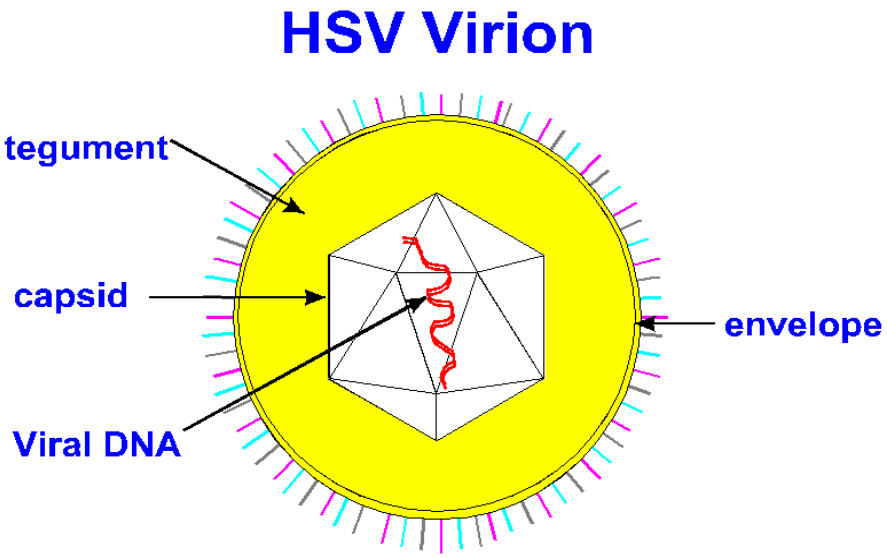
Zhi, Y., K. S. Sciabica, et al. (1999). "Self-interaction of the herpes simplex virus type 1 regulatory protein ICP27." Virology **257**(2): 341-51.

Zhong, H., A. Takeda, et al. (2005). "Carrier-independent nuclear import of the transcription factor PU.1 via RanGTP-stimulated binding to Nup153." J Biol Chem **280**(11): 10675-82.

Zhou, C. and D. M. Knipe (2002). "Association of herpes simplex virus type 1 ICP8 and ICP27 proteins with cellular RNA polymerase II holoenzyme." J Virol **76**(12): 5893-904.

Zolotukhin, A. S. and B. K. Felber (1999). "Nucleoporins nup98 and nup214 participate in nuclear export of human immunodeficiency virus type 1 Rev." J Virol **73**(1): 120-7.

(a)



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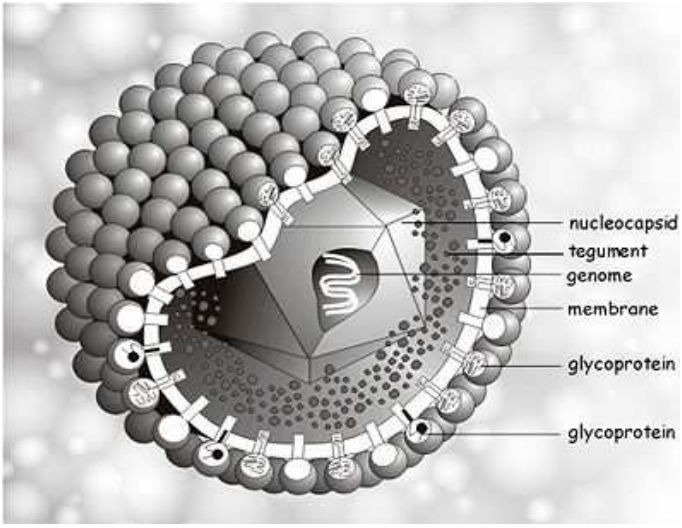


Figure 1.1: HSV virion, a. The tegument surrounds the double stranded DNA in an icosahedral capsid. The viral envelope containing glycoprotein spikes surrounds it (Clements and Brown, 1997). **b.** Schematic representation of the HSV virion. Reproduced from Marko Reschke *et al.,* 1997.

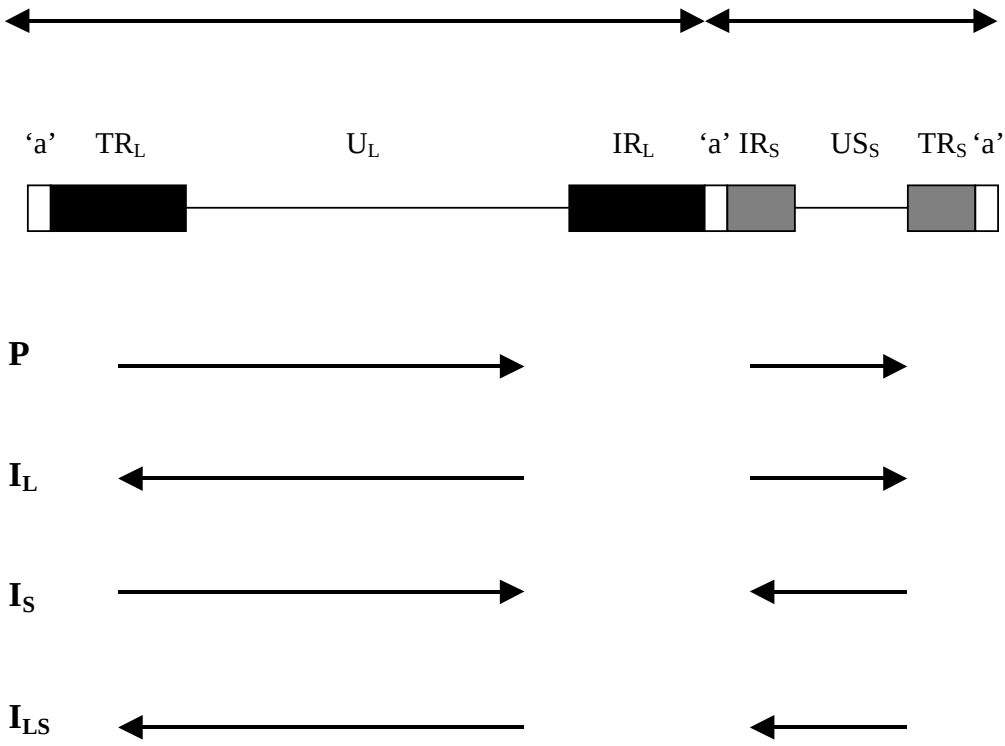


Figure 1.2. Schematic diagram of the HSV-1 genome:

The organization of the HSV-1 genome, showing the unique long and short regions (U_L and Us, respectively), which are covalently linked and flanked by the terminal and internal repeats (TR and IR, respectively). The 'a' sequence is present at each end of the genome and in an inverted orientation at the L-S junction. The L and S regions can invert relative to each other to form four isomers (Hayward et al., 1975). The orientation of the four isomers is shown by the arrows.

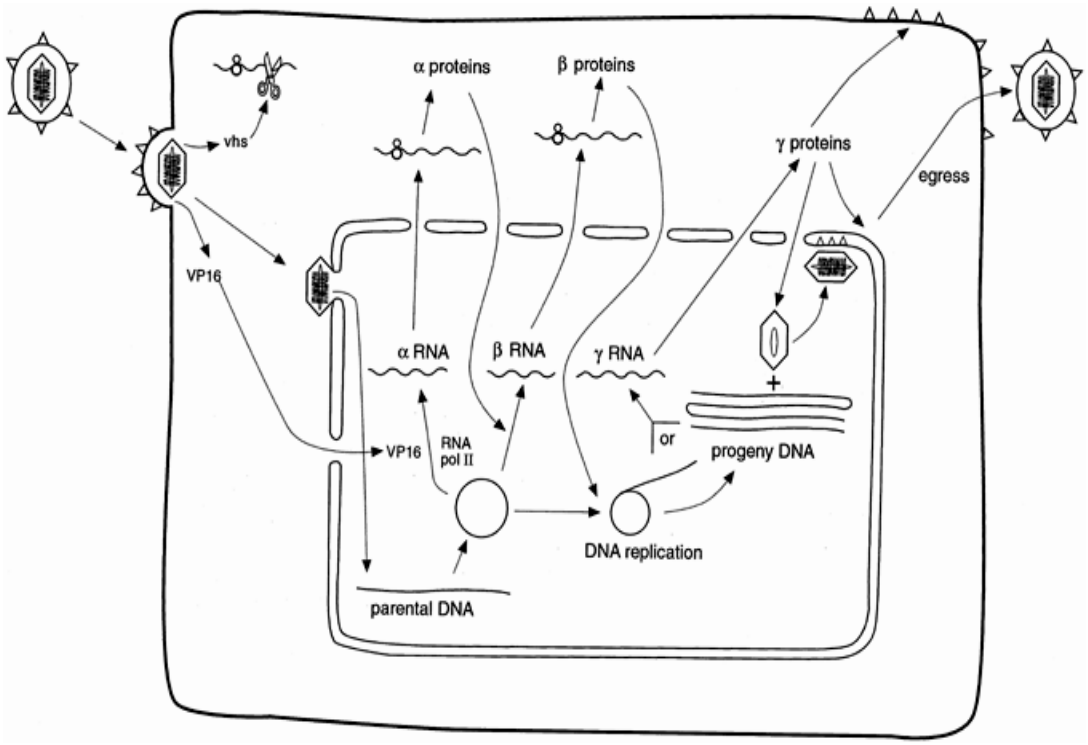


Figure 1.3: Diagram of the replication cycle of HSV.

In the upper left of the figure**,** in the first step of replication, the HSV virion attaches to the cell membrane. Next there is fusion of the virus envelope with the plasma membrane and the capsid and tegument proteins are release into the cytoplasm. vhs protein in the tegument causes cellular mRNAs degradation. In the second step, the viral DNA enters the nucleus by shuttling of the capsid through the NPC. In next step, replication and transcription of viral DNA take place. Virus gene expression follows a cascade pathway with the immediate early (IE) or α, the early (E) or β and the late (L) or γ genes expressed sequentially making regulatory and structural proteins. Virus assembly is next step in the HSV life cycle. The virion is enveloped by budding through cellular membranes, and finally, from the plasma membrane of the cell allowing the virion to exit from the cell (Whitely, 2001).

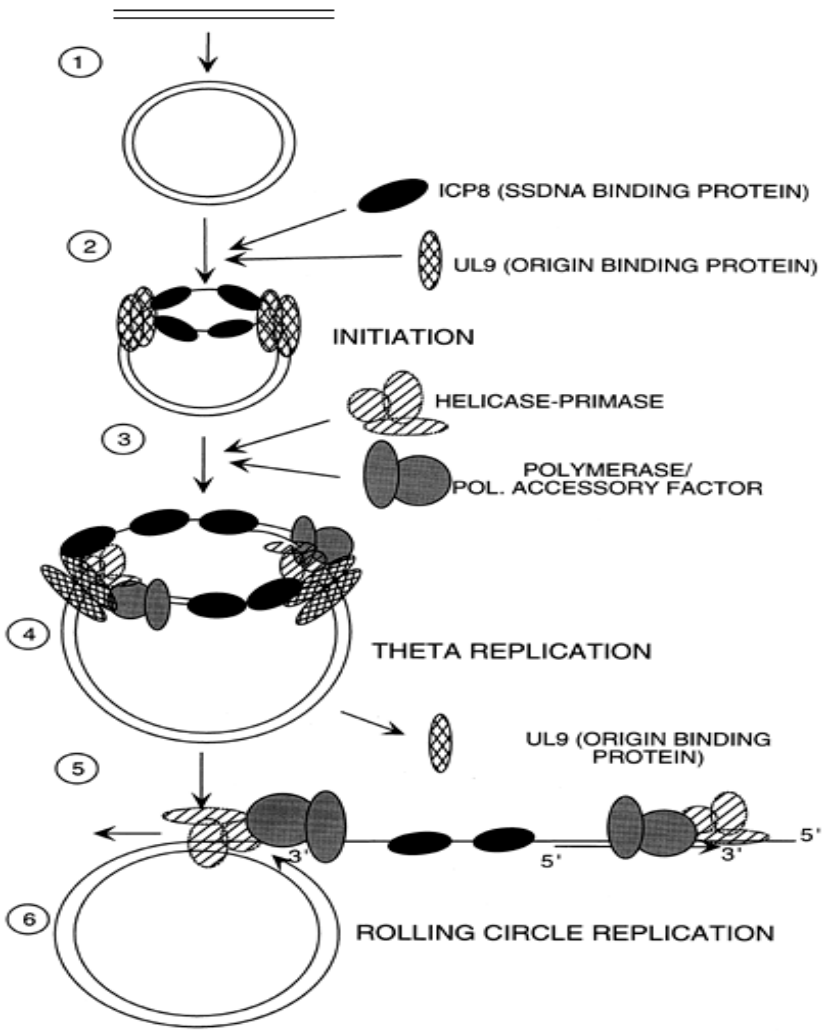


Fig.1.4: Diagram of a model of HSV DNA replication, with the following steps:

1. Circularisation of DNA occurs upon entry into the nucleus. 2. UL9 begins to unwind the DNA by binding to either ***ori***l or ***oris*** and then recruits ICP8 to the unwound single-stranded DNA. 3. Five viral DNA replication proteins are recruited to the replication fork by UL9 and ICP8. 4. A complex of the helicase–primase proteins and the viral polymerase at each replication fork initiates rounds of theta-form replication. 5. An unknown mechanism switches replication of DNA from theta to rolling-circle mode. UL9 is not necessary for this step because it is not origin dependent. 6. Replication of DNA through the rolling-circle mode produces concatamers of viral DNA, which are cleaved into monomeric molecules during packaging. Diagram courtesy of E. McNamee from (Whitely, 2001).

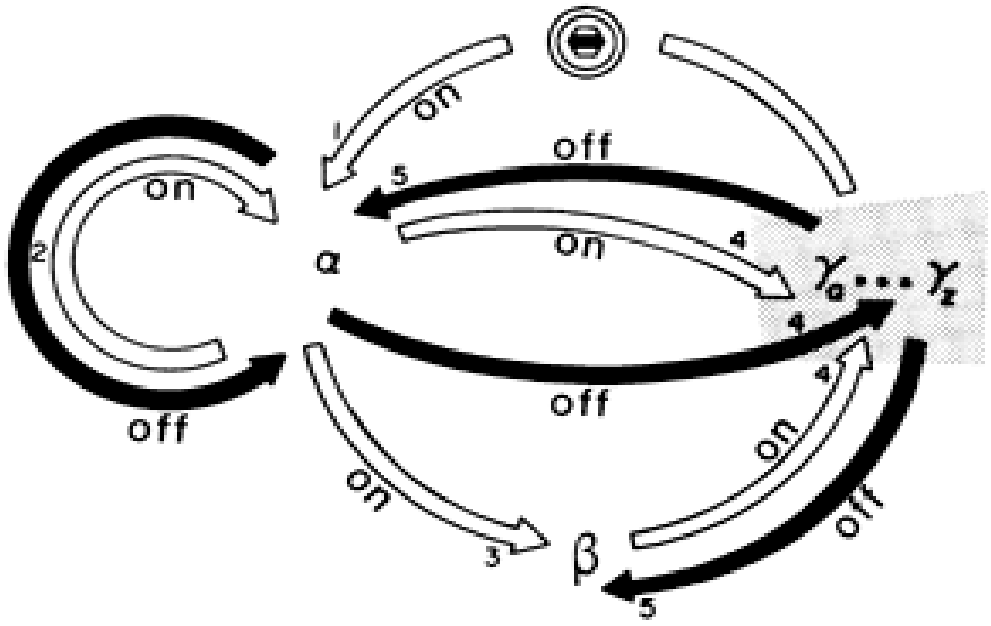


Fig.1.5: Schematic representation of the regulation of HSV gene expression. Open and filled arrows showing events in the reproductive cycle of HSV that switch gene expression “on” and “off,” respectively. 1. α-TIF switches on IE or α gene transcription. 2. Autoregulation of α gene expression. 3. Switching on of E or β gene transcription. 4. Switching on of L or γ gene transcription by α and β gene products through transactivation of γ genes. The heterogeneity is shown as a continuum in which inhibitors of viral DNA synthesis are shown to have minimal effect on γa gene expression but totally preclude the expression of γz genes. 5. γ genes late in infection product switches off of α and β gene expression (Whitely, 2001).

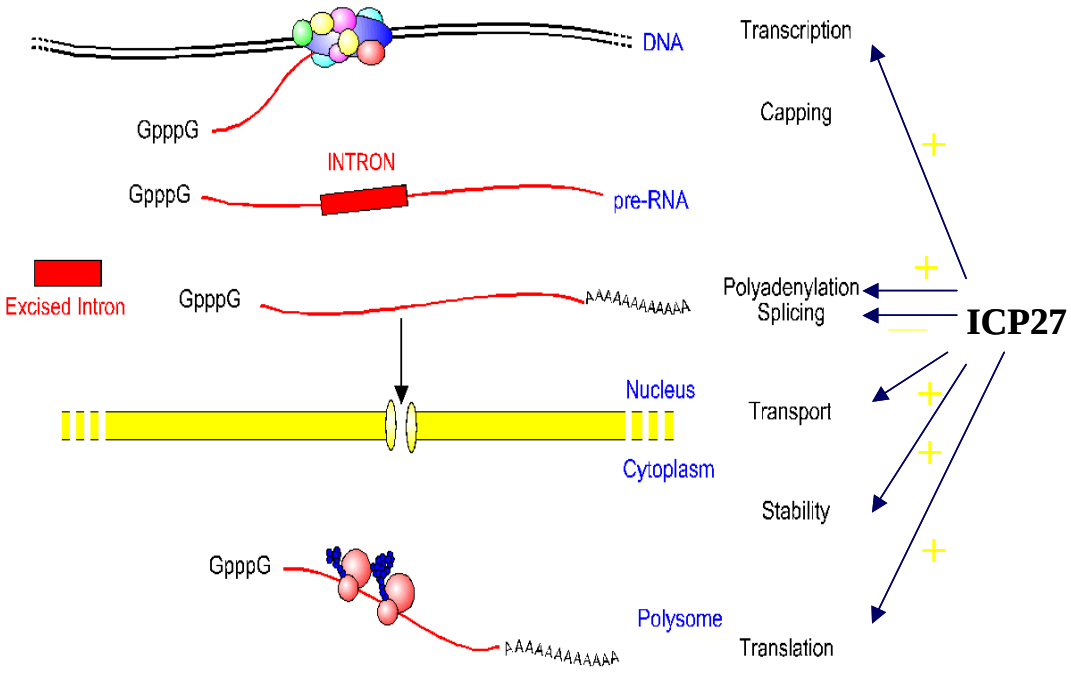


Figure 1.6: Levels of ICP27 action. ICP27 acts at transcriptional and posttranscriptional levels and positively regulates mRNA processing, include: levels of action capping, polyadenylation, transport, RNA stability, negatively regulates splicing action and recently, translation (Leiper, 2004).

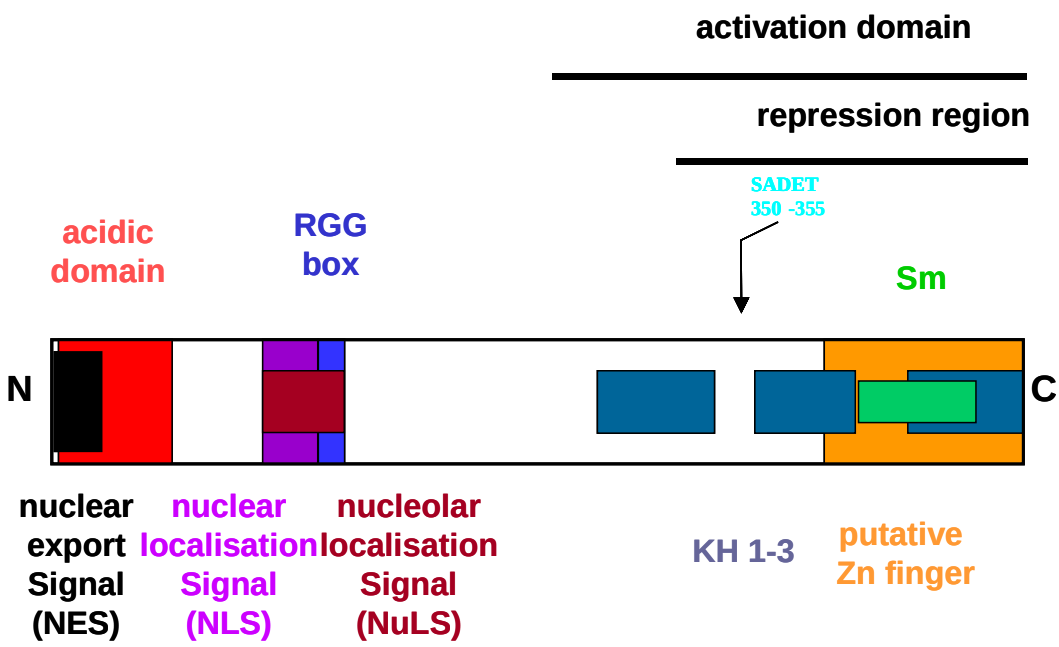


Figure 1.7: Cartoon of ICP27 highlighting its functional domains.

Sequence alignment of hnRNP K and ICP27

hnRNP K 323 YDRRGRPGDRYDGMVGFSADETWDSAIDTWSPSEWQMAY 361
ICP27 338 AMRDCVLRQENFIEALASADETLAWCKMCIHHNLPLRPQ 376

Figure 1.8: Sequence alignment of hnRNP K and ICP27: showing homology between the minimally defined KNS region of hnRNP K and a region in ICP27.

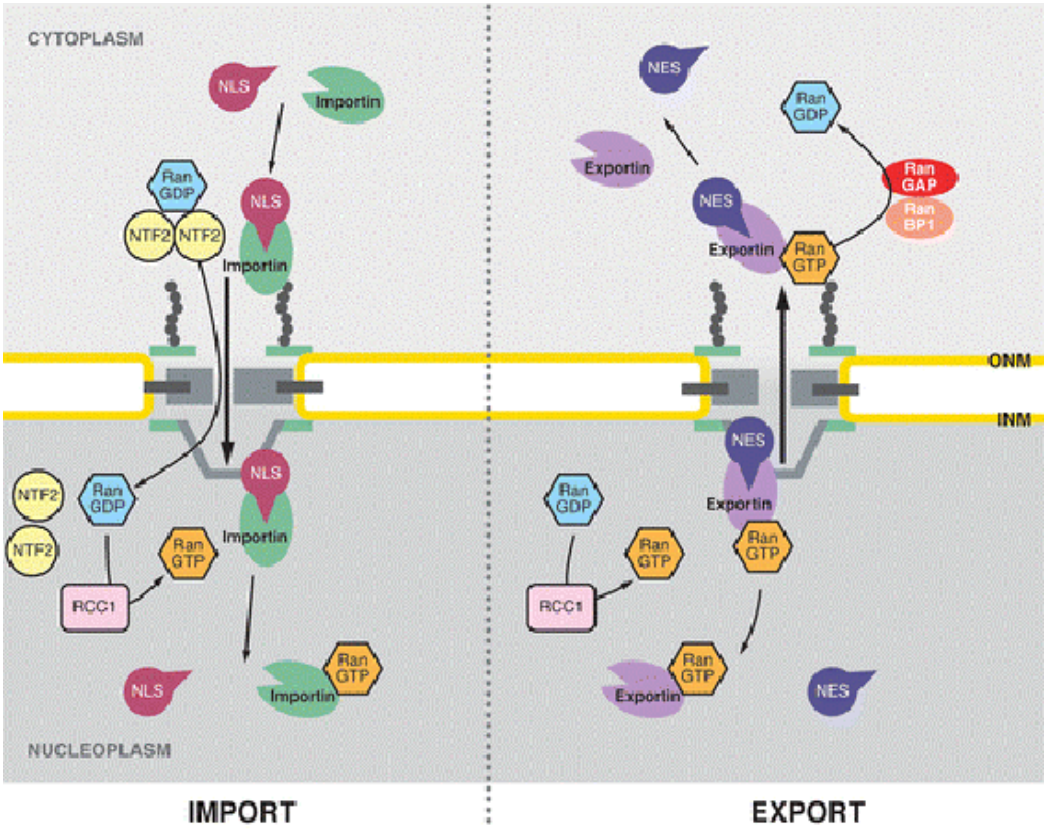


Figure 1.9: Import and export cycles mediated by Importins and Exportins. Importins bind to cargo molecules with NLS in the cytoplasm and mediate interactions with the NPC to translocate into the nucleus, and then RanGTP binds to importin for cargo release and recycling. The export cycle is similar but with the crucial difference that RanGTP induces cargo binding to an export receptor in the nucleus (Hetzer, Walther, and Mattaj, 2005).

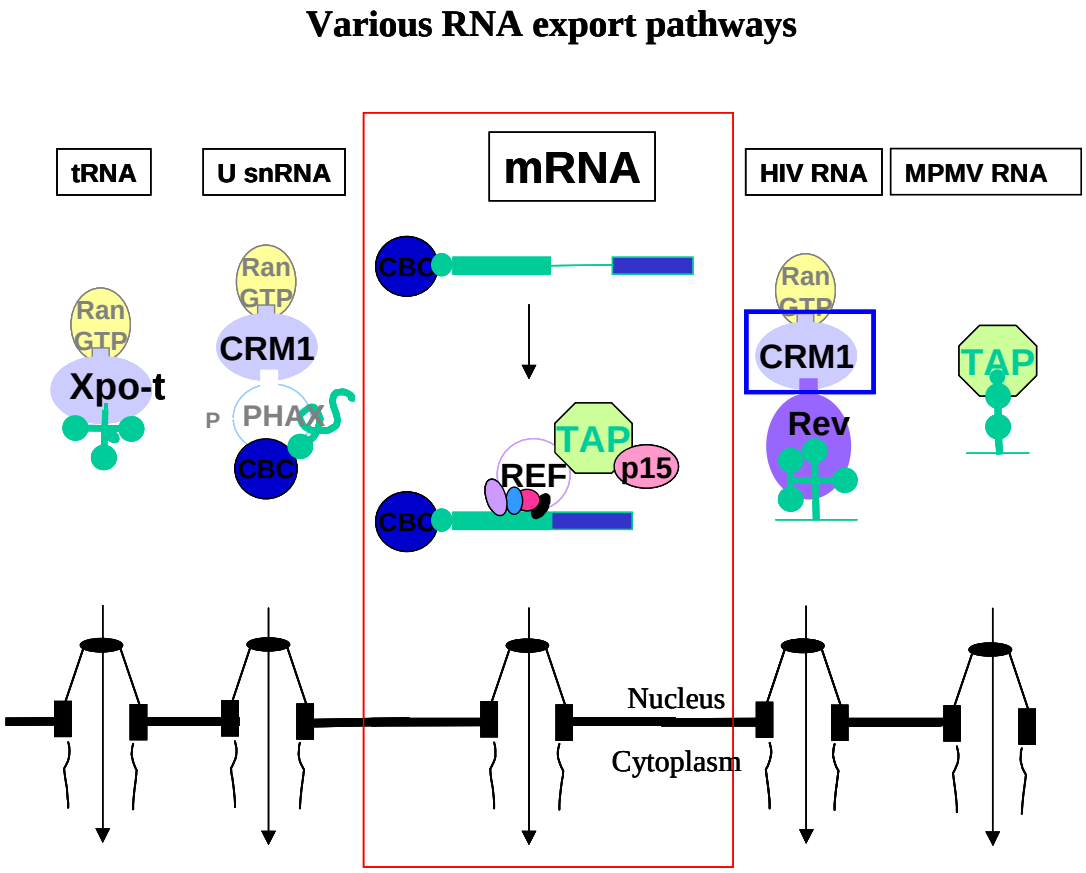


Figure 1.10: Various RNA export pathways: The diagram shows different types of RNAs utilising various export pathways for receptor-export adaptor molecules such as exportin, PHAX-CRM-1, REF-TAP, Rev-CRM-1 and CTE-TAP for export to the cytoplasm; MPMV: Mason Pfizer Monkey virus (Conti and Izaurralde, 2001; Cullen, 2000; Stutz and Rosbash, 1998).

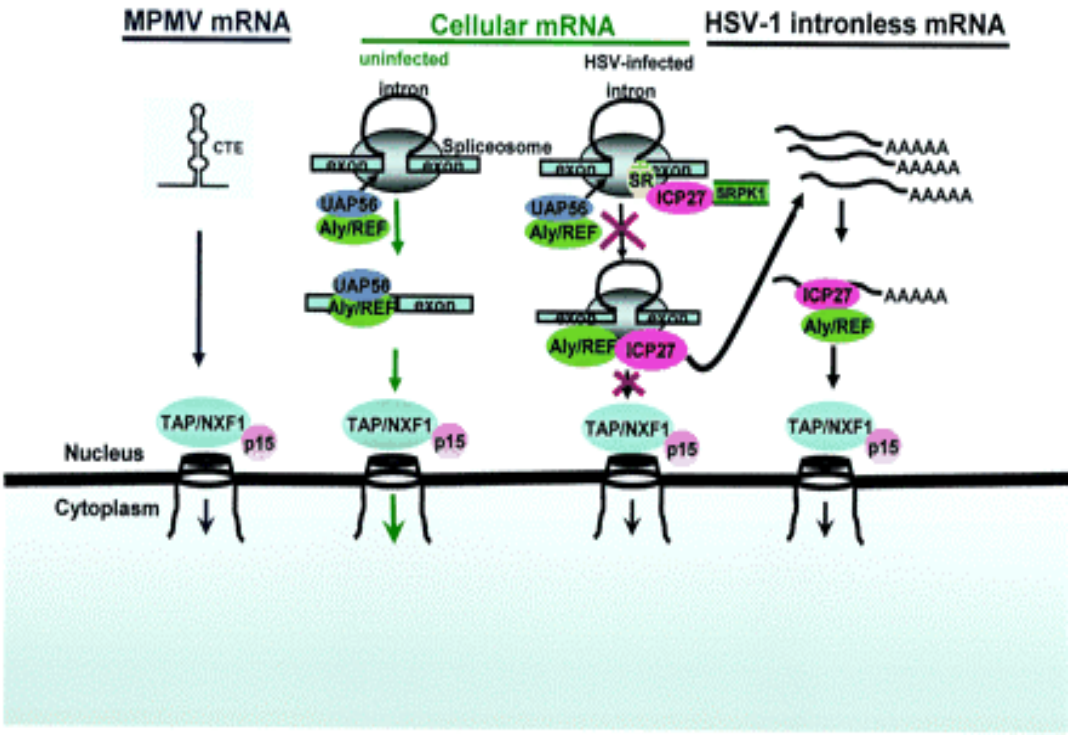


Figure1.11: HSV-1 utilises cellular pathways to export viral spliced mRNAs. The cellular TAP/NXF1 pathway is used to export unspliced mRNA encoded by simple retroviruses and intronless mRNAs encoded by HSV-1. At the left, in Mason-Pfizer monkey virus (MPMV), the CTE interacts directly with TAP/NXF1/p15, and exports mRNAs. At the right, in HSV-1-infected cells, ICP27 interacts with Aly/REF and recruits it to sites of HSV-1 transcription. ICP27 binds viral mRNAs, and the ICP27-Aly/REF-RNA complex is directed to TAP/NXF1 (Sandri-Goldin, 2004).

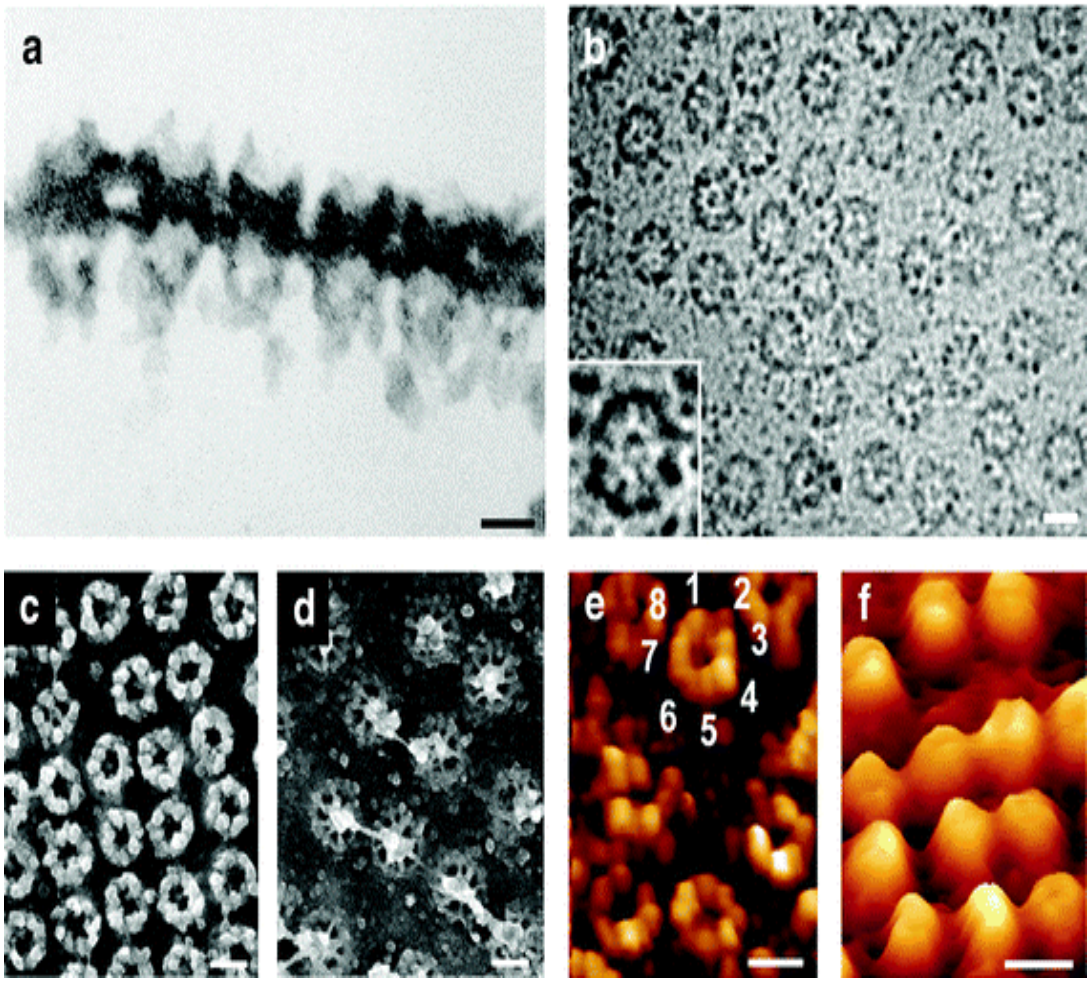


Figure 1.12: Imaging the nuclear pore complex (NPC) by various microscopy techniques. Image of a thin-section of embedded nuclear envelope (NE) captured by transmission electron microscopy (TEM). **b** Image of the NE embedded in thick amorphous ice taken by energy-filtered electron microscopy (EFTEM). c. the cytoplasmic and **d** the nuclear face of the NE. **e**, **f** Scanning force microscopy (SFM) images of the cytoplasmic (**e**) and the nuclear face (**f**) of the NE. Scale bars in panels **a**–**f**, 100 nm (Lim, Aebi, and Stoffler, 2006).

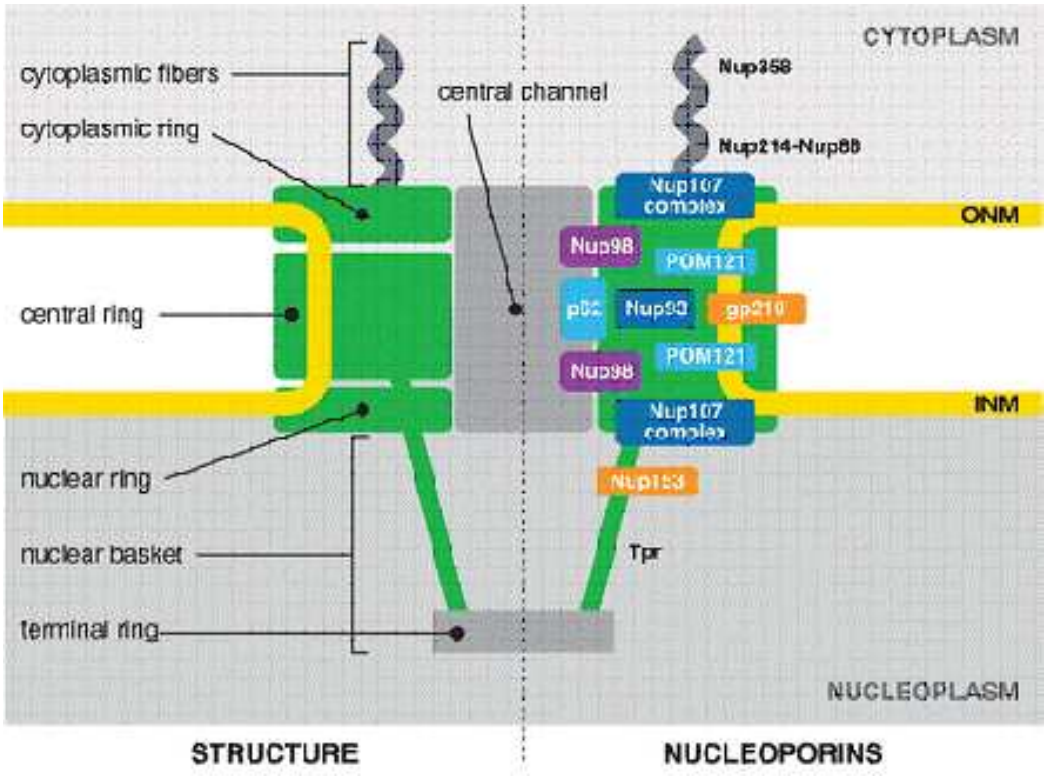


Figure 1.13: Diagramme of a vertebrate NPC showing possible architecture. The cytoplasmic, central and nuclear rings are shown in *green*. The inner nuclear membrane (INM) and outer nuclear membrane (ONM) (*yellow*) come together at the sites of NPC insertion. Cytoplasmic and nuclear filaments are attached to the ring structures. The nuclear filaments form a basket structure topped by a terminal ring (*grey*). Nucleoporins are located throughout the NPC as shown. The colour code represents the three dynamic classes of nucleoporins: Structural scaffold NUPs are shown in *blue*, mobile NUPs in *purple* and the highly dynamic NUPS in *orange.* (Hetzer, Walther, and Mattaj, 2005).

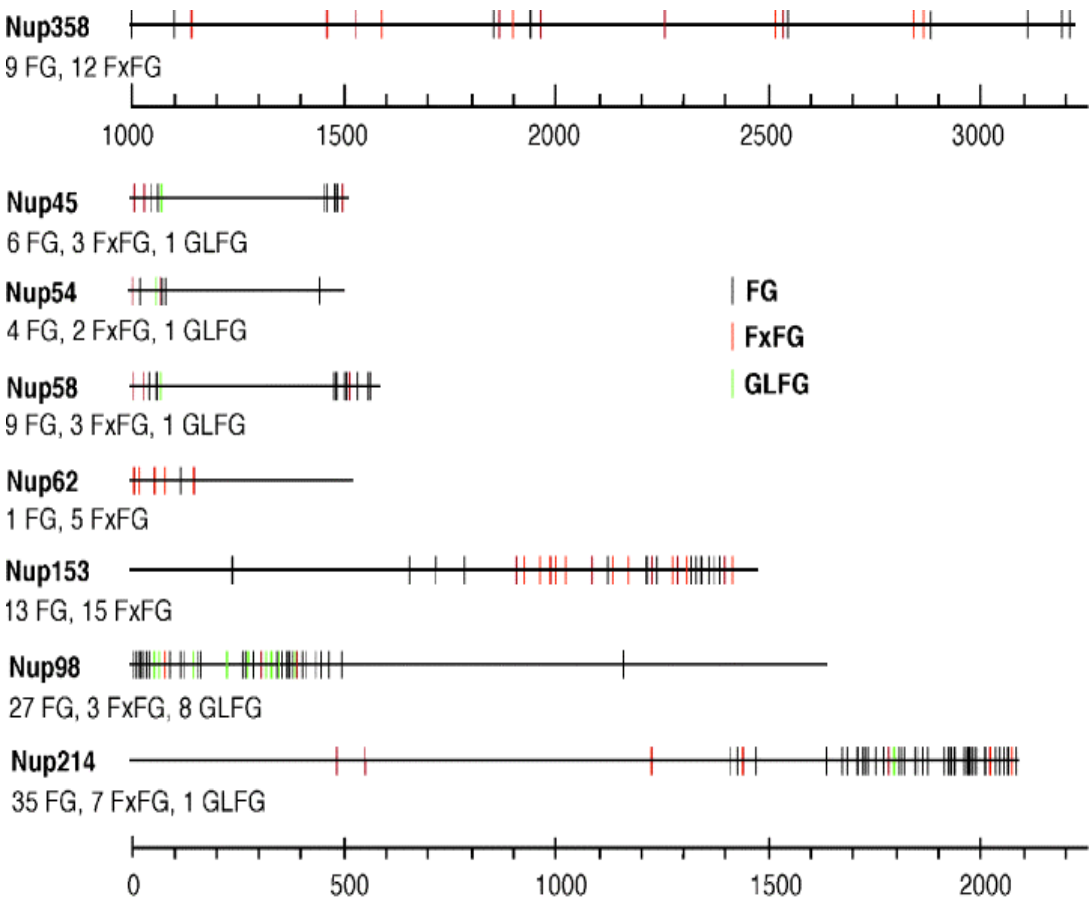


Figure 1.14: Distribution of FG repeats on mammalian nucleoporins. The C-terminal region (amino acids 1000–3224) of human NUP 358, and full-length NUP 45 (rat), NUP 54 (human), NUP 58 (rat), NUP 62 (human), NUP 98 (human), NUP 153 (human) and NUP 214(human) are shown. The FG-repeat sequences are indicated by coloured bar. FxFG-repeats, red bars, GLFG repeats are green bars. (Bednenko, Cingolani, and Gerace, 2003).

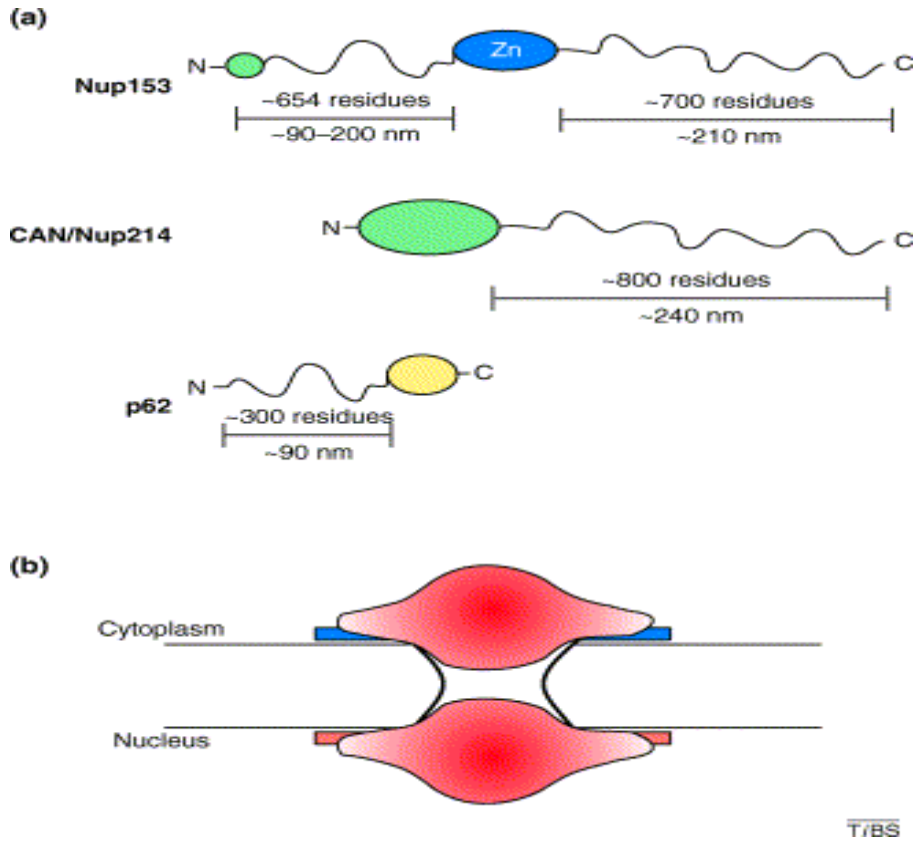


Figure 1.15: Domain size and organisation and spatial distribution in the NPC of FG-repeat nucleoporins. (a) A comparison of the size and structure of the FG-repeat nucleoporins NUP 153, NUP 214/CAN and NUP 62. The FG-repeat portions of the NUPs are generally unfolded in their native state. The C-terminal portions of NUP 153 and NUP 214/CAN and the N-terminal part of NUP 62 might be flexible for interaction with cargos. **(b)** Immunogold EM indicated high densities of FG repeat NUPs in the cytoplasmic and nuclear periphery of the central basket (deep red colour) but a decreased density towards the centre of the central pore and the extremity of the NPC (pale red to white shading) (Fahrenkrog, Koser, and Aebi, 2004).

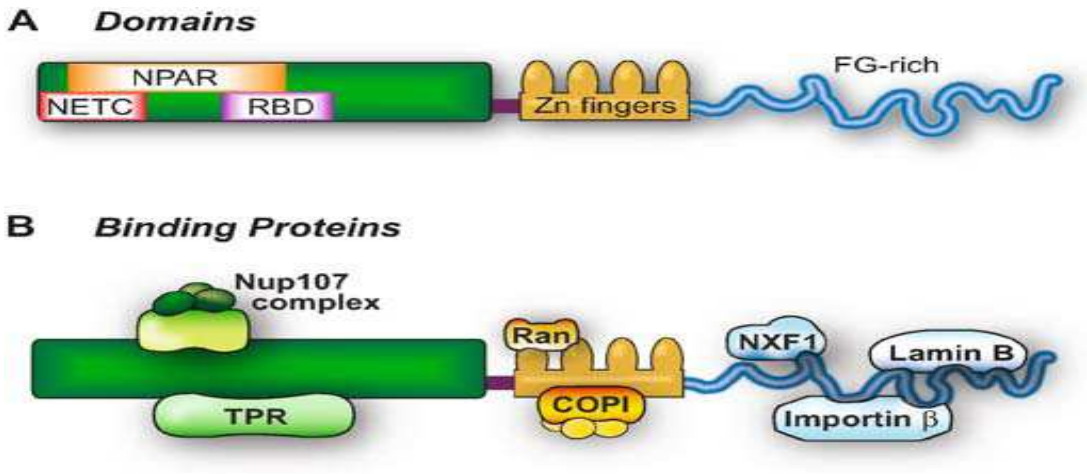


Figure 1.16: NUP 153 organization. **A**. The architecture of NUP 153 shows three main domains. 1. The N-terminal region that displays three functional components: the nuclear envelope targeting cassette (NETC; 2–144), the nuclear pore associating region (NPAR; 39–339), and an RNA binding domain (RBD; 250–400). 2. The central zinc finger domain (650–880) with four zinc fingers. 3. The C-terminal region (881–1475) that contains FG-rich motifs. **B.** A range of proteins that may interact with NUP 153. Their interacting regions are indicated (Ball and Ullman, 2005).

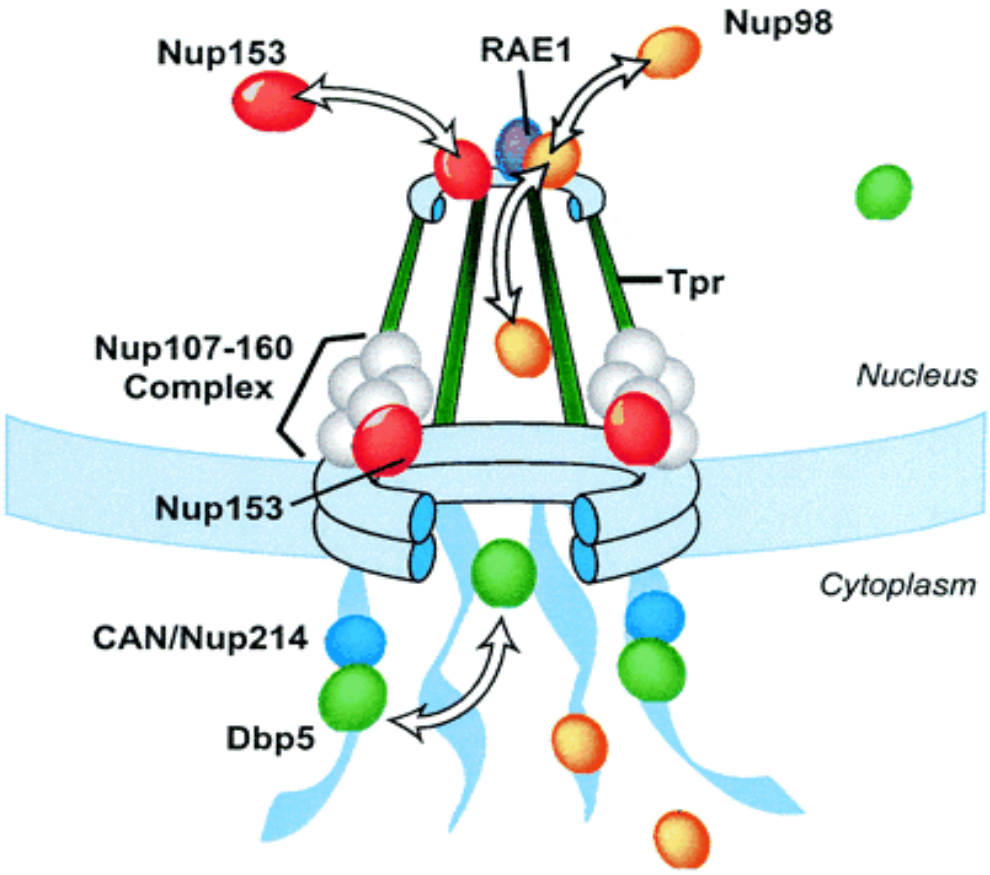


Figure 1.17: Location and mobility of NUPs and related factors involved in mRNA export. There is a stable and mobile population of NUP 153 each located in different sites of the NPC. NUP 98 is a highly mobile NUP that cooperates with components of the NPC on both the nuclear and cytoplasmic sides. NUP 98 binds to RAE1 in mRNA export. Some stable NUPs such as those of the NUP 107-160 complex can be found in association with both NUP 153 and NUP 98. Association of NUP 153 with the nuclear basket component Tpr is necessary for its localisation. NUP 214/CAN is localised to the filaments on the cytoplasmic face of the NPC. This NUP is thought to be a docking site for transport of RNAs and proteins such as the dynamically-located DEAD box helicase, Dbp5 (Dimaano and Ullman, 2004).

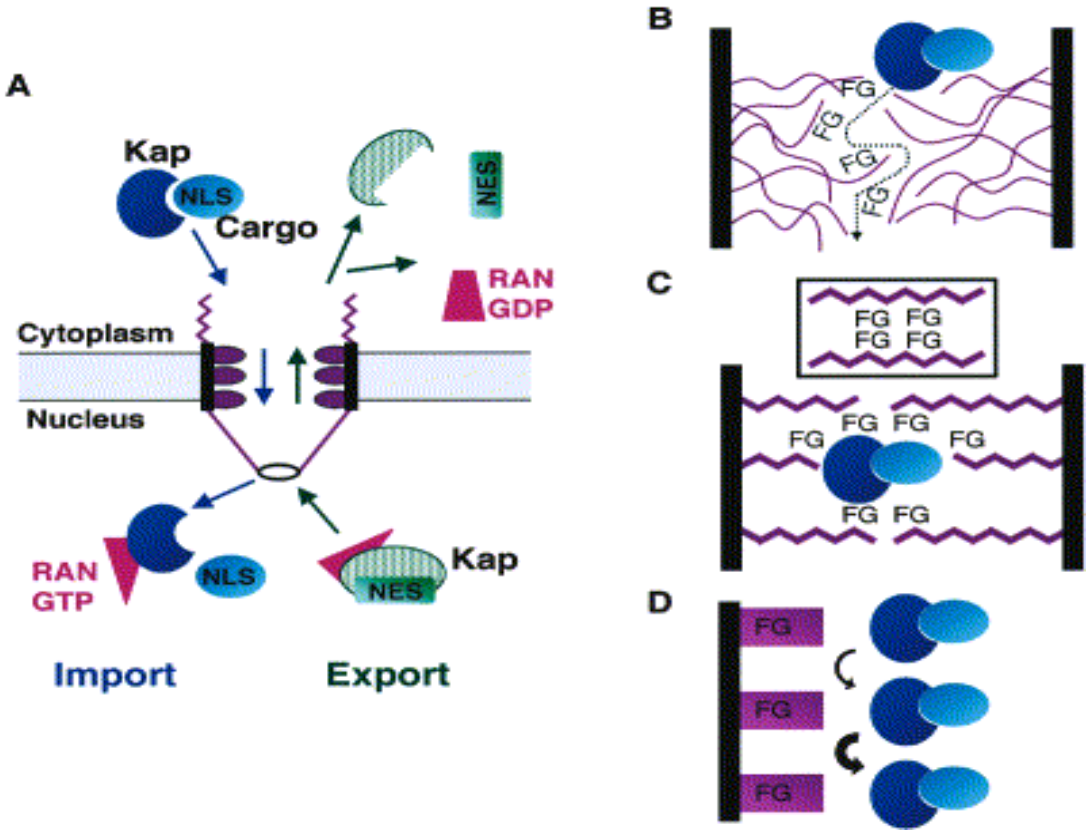


Figure 1.18: Models for NPC translocation: (**A**) On the left, the karyopherin (Kap)-dependent transport pathways is shown and described in Figure 1.9. On the right, different translocation models are shown. (**B**) The FG NUPs form a filamentous network that excludes most large molecules. FG-repeat interactions may allow movement through the network and the NPC. (**C**) The FG-repeat NUPs may interact weakly and cause the central chanel to be hydrophobic. The FG binding sites on the cargo receptors facilitate selective passage through the hydrophobic environment. (**D**) The FG-repeat NUPs may provide a series of sequential binding sites of increasing affinity for receptor-cargo complex (Suntharalingam and Wente, 2003).

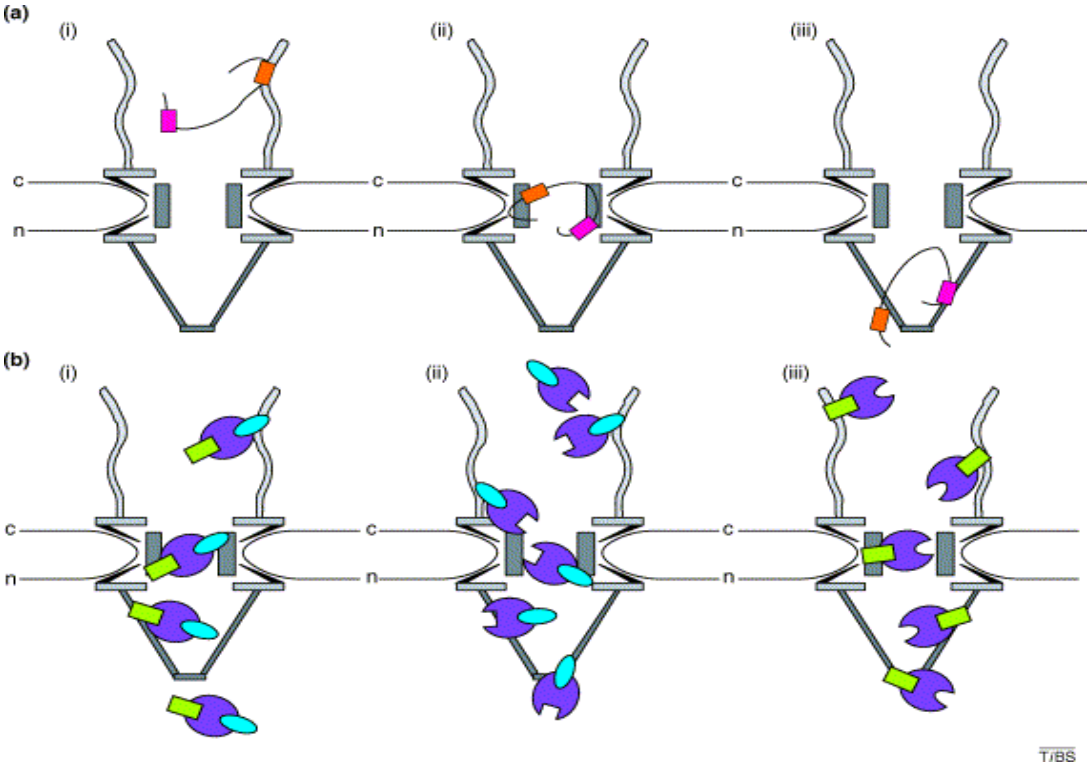


Figure 1.19: Schematic diagram shows proposed mechanisms of NPC translocation of transport receptors and cargoes. (a) A model showing translocation of a cargo receptor with two nucleoporin-binding sites. For import in the cytoplasmic periphery of the NPC the receptor can use the first FG-repeat-binding site (orange for NUP interaction. For translocation through the central basket it may use both of its FG-binding sites (orange and pink). Its second site (pink) may be implicated in accessing the nuclear side of the NPC. **(b)** A transport receptor (purple) that may interact with two transport factors (blue and green) could use both receptors simultaneously one or other to translocate through the central basket. c, cytoplasm; n, nucleus (Fahrenkrog, Koser, and Aebi, 2004).

Table1.1: Human Herpesviruses

Designatin	Common name	Sub Fam	Genoe Size (KB)	Disease
HHV-1	Herpes simplex virus Type 1	α	152	Oral, Ocular lesions(fever blisters),Encephalitis
HHV-2	Herpes simplex virus Type 2	α	152	Genital, Anal lesions (severe fatal neonatal!)
HHV-3	Varicella zoster virus	α	125	Chickenpox, Shingles
HHV-4	Epstein-Bar virus	γ(LCV)	184	Infectious mononucleosis(IM), Tumors
HHV-5	Cytomegalovirus	β	248	IM, Congenital infections, Infection in allograft recipients, possible role in atherosclerosis and restenosis
HHV-6A	Human herpesvirus 6A	β	159-170	Some infection in allograft recipient and AIDS patients (Pneumonia, Marrow failure, encephalitis)
HHV-6B	Human herpesvirus 6B	β	162-168	Roseola in infants, infection in allograft recipients and AIDS patients,possible role in multiple sclerosis
HHV-7	Human herpesvirus-7	β	145	Some case of Roseola
HHV-8	Human herpesvirus-8 Kaposi's sarcoma-associated virus	γ(Rhad	170/ 210	Tumors, Kaposi's sarcoma, some B cell tumors

Nine human Herpesviruses, classification, G+C% content genomic size and disease associated with them. (LCV: Lymphocryptovirus, Rhad: Rhadinovirus) (Field Virology,Fig72-3,2001).

Table 1. 2: Nucleolar localisation signals (NuLs)

Protein	Function	NuLs	Reference
MDV MEQ	Transcriptional transactivator	RRRKRNDAARRRRRKQ	Liu *et al.*, 1997
HIV-1 Rev	Nucleocytoplasmic targetting of intron containing viral transcripts	RRNRRRRWRERQRQ	Cochrane *et al.*, 1990
HIV-1 Tat	Transcriptional transactivator	GRKKRRQRRRAHQN	Dang and Lee, 1989
HTLV-1 Rex	Nucleocytoplasmic targetting of intron containing viral transcripts	PKTRRRPRRSQRKRPPTP	Nosaka *et al.*, 1989
PRRV N protein	Nucleocapsid protein	RGKGPGKKNKKKNPEK	Rowland *et al.*, 1999
Hsp 70	Stress response protein	FKRKHKKDISQNKRAVRR	Dang and Lee, 1989
Topoisomerase 1	Unwinding supercoiled DNA	RESIDUES 157-199 K RICH	Mo *et al.*, 2000
FXR2P	Associates with FMRP, ribosomes and RNA	RPQRRNRSRRRRFR	Tamanini *et al.*, 2000

This Table lists several examples of NuLs. Abbreviations: MDV: Mareks disease virus; MEQ: MDV Eco Q; HIV-1: Human immunodeficiency virus type 1; HTLV-1: Human T cell lymphotropic virus type 1; PRRV: Porcine reproductive and respiratory syndrome virus; Hsp: heat shock protein; FMRP: Fragile X mental retardation protein; FXR2P: FMRP related protein 2.

Table 1.3: HSV-1 ICP27 homologues

Herpes virus	Subfamily	ICP27 homologue	Reference
α-Herpes virus	Varicella-Zoster virus (VZV)	ORF4	Inchauspe, Nagpal et al 1989
	Equine herpes virus type 1 (EHV-1)	UL3	Zhao, Holden et al 1992
β-Herpes virus	Human cytomegalovirus (CMV)	UL69	Chee, Bankier et al 1990
γ-Herpes virus	Eptein-Bar virus (EBV)	SM	Cook, Shanahan et al 1994
	Herpes virus samiri (HVS)	ORF57	Nicholas, Gompels et al 1988
	Human herpes virus 8 (HHV8)/ Kaposi's sarcoma associated virus(KSHV)	ORF57	Bello, Davison et al 1999

Table 1.4. HSV-1 ICP27 partner proteins

Protein	Activity	Method	Reference
hnRNP K	Transcription activator, regulate translation	Yeast-two hybrid screen in vivo binding studies	Wadd, Bryant et al 1999
Casein kinase 2	Serine/threonin protein kinase	Yeast-two hybrid screen in vivo binding studies	Wadd, Bryant et al 1999
Common sm protein	Protein component of snRNP	in vivo binding studies with anti sm serum	Sondri-Goldin and Hibbard 1996
SAP 145	Protein component of U2 snRNP	Yeast-two hybrid screen in vivo binding studies	Bryant, Wadd et al 2001
TAP	Cellular export receptor	Yeast-two hybrid screen in vivo binding studies	Chen, Sciabica et al 2002
REF	Cellular RNA export factor	Yeast-two hybrid screen in vivo binding studies	Koffa, Clements et al 2001
RNA pol II holoenzym	Catalyses synthesis of RNA	in vivo binding studies	Zhou and Knipe 2002
P32	Cellular (mainly mitochonderial) multifunctional protein	Yeast-two hybrid screen in vivo binding studies	Bryant, Matheows et al 2000
SRp20	Member of the SR protein family splicing factors	Yeast-two hybrid screen in vivo binding studies	Scibica, Dai et al 2003
SRPK1	SR protein kinase1, highly specific for serin/arginine dipeptides	in vivo binding studies	Scibica, Dai et al 2003

Table showing a list of ICP27 partner proteins and their activity, methods of investigation and references.

Table 1.5: List of nucleoporins in different species: vertebrate, yeast, worm and fly

Vertebrate	S. cerevisiae	C. elegans	D. melanogaster
Nup358		npp-9	Nup358
Nup214	yNup159	npp-14	Dnup214
gp210	---	npp-12	Gp210
Nup205	yNup192	npp-3	CG11953
Tpr	Mip1/Mip2	R07G3.3	---
Nup188	yNup188	---	---
Nup160	yNup120	npp-6	CG4738
Nup155	yNup170/yNup157	npp-8	CG4579
Nup153	yNup1/2, yNup60	npp-7	NUP153
Nup133	yNup133	npp-15	CG6958
POM121	---	---	---
Nup107	yNup84	npp-5	CG6743
Nup98	yNup145-N	npp-10	Nup98
Nup96	ynup145-C	npp-10	Nup96
Nup98	yNic96	npp-13	CG11092
Nup88	yNup82	---	Mbo
Nup85/75	---	npp-2	---
p62/Nup62	yNsp1	npp-11	Nup62
Nup58	yNup49	---	Nup58
Nup54	yNup57	npp-1	Nup54
Nup50	---	npp-16	---
Nup45	---	npp-4	---
Nup43	---	C09G9.2	CG11875
Nup37	---	---	---
Nup35	---	npp-19	CG6773
Sec13	ySec13	npp-20	CG8722
Seh1	ySeh1	npp-18	---
RAE1	---	npp-17	---
Gle1	yGle1	---	---
Gle2	yGle2(Rae1)	---	---
hCG1	yNup42(Rip)ddNdc1	---	---
---	POM152, Nup60,POM34	---	---
---	Ndc1, yNup85,yNup53, yNup59	---	---
---	yNup59, cdc31	---	---

The vertebrate nucleoporins are listed according to their molecular weight.
The *S. cerevisiae, C. elegans, and D. melanogaster* homologues are listed.

Table 1.6: mRNA export factors

Name	Alternative name	Role and/or function
CRM1	Exportin	Nuclear export receptor for proteins and some mRNAs
GTPase Ran		Cofactor for CRM1
NES	Nuclear export sequence	Short leucine-rich sequence in nuclear export proteins that interacts with CRM1
LMB	Leptomycin B	Drug that inhibits binding of NES to CRM1 by covalent modification at a conserved cysteine residue
CTE	Constitutive transport element	Highly structured RNA sequence in unspliced retrovirus mRNA that binds to TAP
TAP	NXF1	The major nuclear mRNA export receptor in metazoans; homologue of Mex67p in yeasts
p15	NXT1	Heterodimeric binding partner of TAP/NXF1
EJC		Multiprotein complex that is deposited upstream of exon-exon junctions during splicing; EJC proteins have roles in mRNA export, localization, and surveillance
Aly	REF	An mRNA export adaptor protein that is part of the EJC; the yeast homologue is termed Yra1
UAP56		Splicing factor required for spliceosome assembly and for the recruitment of Aly/REF to the EJC; the yeast homologue is Sub2p

List of mRNA export factors and their alternative names and respective activities

Table 1.7: NUP 153 partner proteins

Protein transport	RNA transport	NPC/ lamina	Membrane remodeling	Transcription factors	Cellular regulation	Nucleic acid
Importin-β RanGTPase Importin-α2 Kapβ3/RanBP5 Transportin-1 Crm1/Exportin-1 Importin-7/RanBP7 Nup50/Npap60 NTF2	NXF1/TAP Exportin-t Exportin-5 eIF5A	Nup160-complex Tpr Lamin B	COPI	Smad2 Stat1 PU.1	Caspase-3 (inferred) SENP2 (SUMO protease)	DNA RNA

Table showing NUP 153 interaction with nucleic acids and proteins. These interactions are listed and grouped within functional categories related to transport, nuclear envelope assembly and disassembly, and cellular regulation.

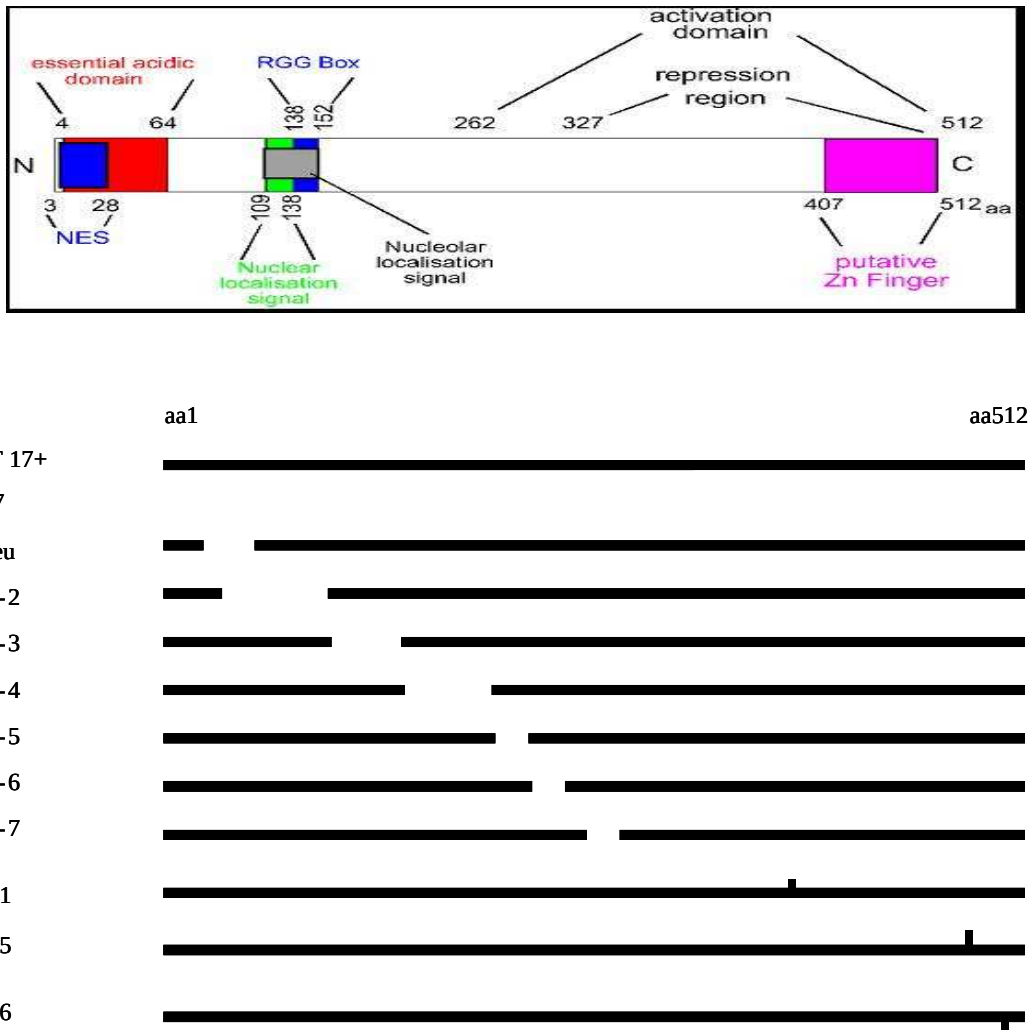


Figure 2.1: ICP27 deletion, truncation and substitution mutants. A schematic representation of the 512-amino-acid protein, ICP27 showing positions of the various mutations used in this study.

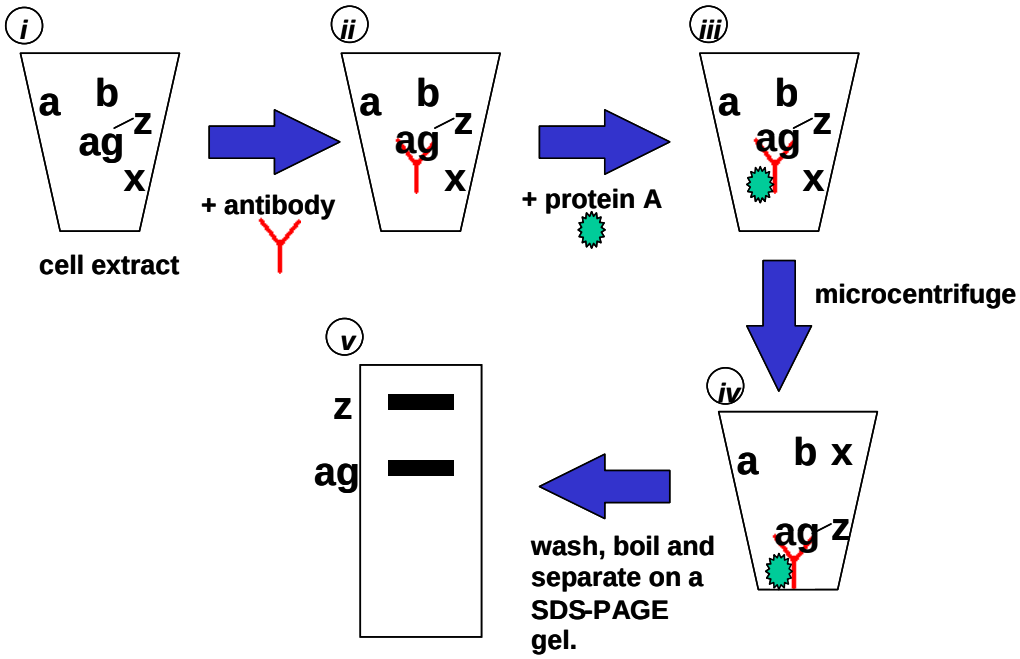


Figure 2.2: Co-immunoprecipitation assay: The appropriate antibody is incubated with cell extracts (i). Antibody binds to its antigen, and also to any protein antigen bound to it. Protein A-Sepharose is added to form a complex of antibody, antigen and interacted proteins (iii). This complex is precipitated by centrifugation (iv). Complexes are washed and boiled to break protein:protein interactions and proteins are separated by SDS-PAGE gel electrophoresis (v) (Bryant et al., 2000).

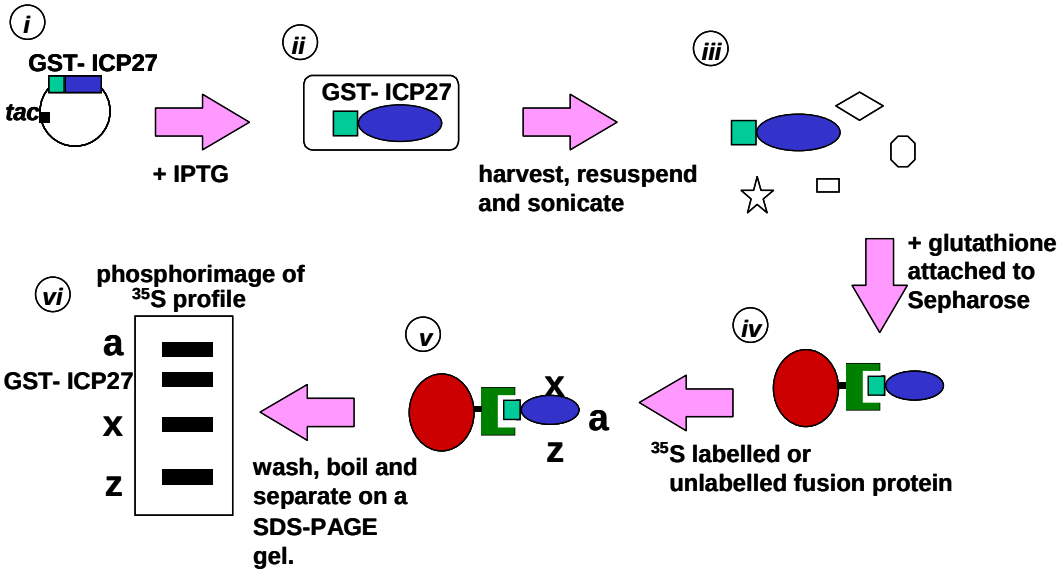


Figure 2.3: GST pull down assay: *E.coli* transfected with GST-ICP27 (I) are induced with IPTG (ii) to allow protein expression and lysates prepared (iii). GST-ICP27 is purified by addition of glutathione attached to Sepharose beads (iv). The pull down assay is performed by adding infected cell extracts to purified GST-ICP27 (v). Complexes are washed and boiled to break protein:protein interactions and proteins are separated by SDS-PAGE (Bryant et al., 2000)

Table 2.1 Characteristics of HSV-1 ICP27 mutants used

Virus	Mutation		Amino acid(s) affected	Nuclear localization	Growth in BHK-21 cells	Reference
	Type	Funtional domain(s)				
Δ27	Deletion	All	1-512	ND	Defective	(Rice & Knipe, 1990)
dLeu	Deletion	NES	6-19	N>C	Deficient	(Lengyel et al., 2002)
d1-2	Deletion	Acidic	12-63	N, Shuttling reduced	Deficient	(Rice et al., 1993)
d2-3	Deletion	ND	64-109	ND	Deficient	(Aubert et al., 2001)
d3-4	Deletion	NLS	109-138	C>N	Deficient	(Mears et al., 1995)
d4-5	Deletion	RGG box	139-153	N, not Nu	Defective	(Mears et al., 1995)
d5-6	Deletion	ND	154-173	ND	Competent	(Mears & Rice, 1996)
d6-7	Deletion	ND	174-200	ND	Competent	(Aubert et al., 2001)
M11	Point	KH1	340-341	N, Shuttling reduced	Defective	(Rice & Lam, 1994)
M15	Point	Zinc finger	465-466	N, No Shuttling	Defective	(Rice & Lam, 1994)
M16	Point	Zinc finger	488	N, Shuttling reduced	Defective	(Rice & Lam, 1994)

Abbreviations: ND, not determined; N, nuclear; Nu, nucleolar; C, cytoplasmic

Table 2.2: Primary antibodies used in this study (NA, not applicable; WB, western blot; IF, immunoflorecense; dil, dilution**)**

Immunogen	Host/Isotype	IF dil	WB dil	References	Source
GAPDH	Mouse / IgG2b	NA	1:10 000	Cat No. 4300	Ambion
GFP	Rabbit/ IgG	NA	1:2500	Cat No. sc-8334	Santa Cruz Biotechnology
GST	Goat	NA	1:10 000	Cat No. 27-4577-01	Amersham Bioscience
hnRNP K (P-20)	Goat / IgG	NA	1:200	Cat No. sc-16554	Santa Cruz Biotechnology
HSV-1 ICP27 (1113)	Mouse/IgG1	1:400	1:2 000	(Ackermann et al,1984)	Goodwin Institute for Cancer Research, Florida
HSV-1 ICP27(1119)	Mouse/IgG1	1:400	1:2 000	(Lengyel et al., 2002)	Goodwin Institute for Cancer Research, Florida
Normal goat serum	Goat	NA	NA	Cat No. sc-2028	Santa Cruz Biotechnology
Normal mouse serum	Mouse/ IgG	NA	NA	Cat No. sc-2025	Santa Cruz Biotechnology
Normal rabbit serum	Rabbit/ IgG	NA	NA	Cat No. sc-2027	Santa Cruz Biotechnology
NUP 62 (N-19)	Goat /IgG	NA	1:500	Cat No. sc-1916	Santa Cruz Biotechnology
NUP 62 (D-20)	Goat /IgG	NA	1:250	Cat No. sc-6078	Santa Cruz Biotechnology
NUP 62 (H-122)	Goat /IgG	1:50	1:100	Cat No. sc-25523	Santa Cruz Biotechnology
NUP 98 (C-16)	Goat /IgG	NA	1:100	Cat No. sc-14155	Santa Cruz Biotechnology
NUP 98 (L-20)	Goat /IgG	NA	1:100	Cat No. sc-14153	Santa Cruz Biotechnology
NUP 98 (H-300)	Rabbit/IgG	1:50	1:100	Cat No. sc-30112	Santa Cruz Biotechnology
NUP 153	Mouse /IgG1	1:250	1:250	Cat No. MMS-102P	Covance
NUP 214 (N-15)	Goat /IgG	NA	1:100	Cat No. sc-26055	Santa Cruz Biotechnology
NUPs 414	Mouse /IgG1	1:2000	1:2000	Cat No. MMS-120R	Covance
TAP/NXF1 (G-12)	Mouse /IgG1	NA	1:100	Cat No. sc-28377	Santa Cruz Biotechnology
TAP/NXF1 (H-120)	Rabbit /IgG	NA	1:1000	Cat No. sc-25768	Santa Cruz Biotechnology

Table 2.3: Secondary antibodies used in this study

Antiserum against	Conjugated	WB dilution	References	Source
Goat	HRP	1:1 000	Cat No. A9452	Sigma
Mouse	HRP	1:1 000	Cat No. A9044	Sigma
Rabbit	HRP	1:1 000	Cat No. A0542	Sigma

(WB, western blot)

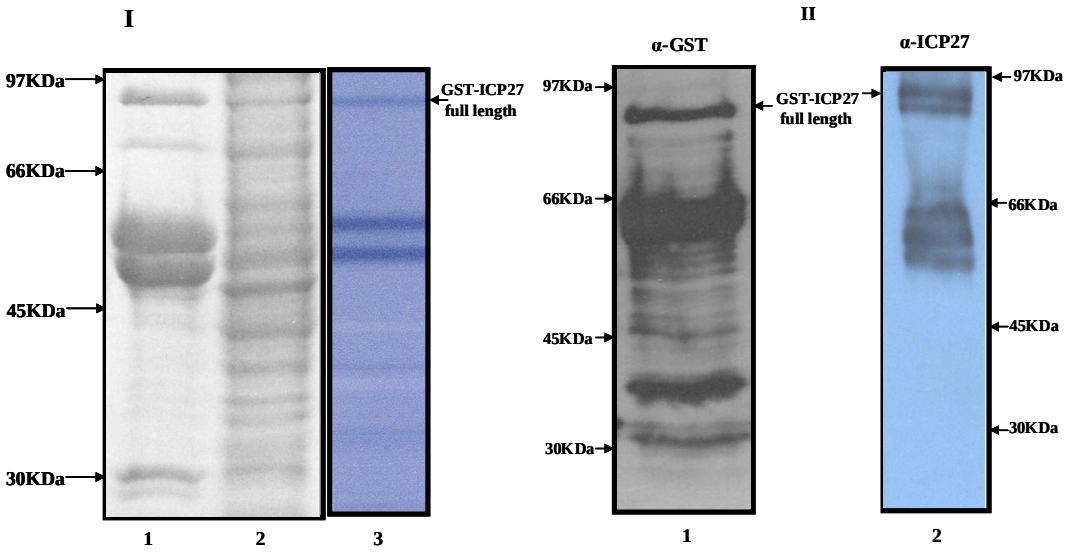


Figure 3.1: GST-ICP27 protein expression in BL21 cells

Coomassie blue stained and western blot SDS-PAGE showing GST-ICP27 full length fusion protein bound onto and eluted from glutathione 4B sepharose beads. GST-ICP27 was expressed in *E.coli* strain BL21 at 37°C and bound to GST-beads. Expressed protein was mixed with SDS loading buffer and boiled and separated by 10% SDS-PAGE gel and Bionalyzer.

Panel I: Gels were stained with Coomassie blue showing bound proteins (lane 1) and unbound proteins (lane 2) and purified fractions were analysed using a Protein 200 Plus LabChip kit (Agilent technologies) (lane 3), in order to monitor expression, protein concentration and purity.

Panel II: Similar SDS PAGE gels to that shown in Panel II were western blotted using anti GST antibody (lane 1) and anti ICP27 (1113) antibody (lane 2) to show expression and purity of the GST-ICP27.

Full length GST-ICP27 is marked. The smaller bands represent degradation products/truncations of GST-ICP27.

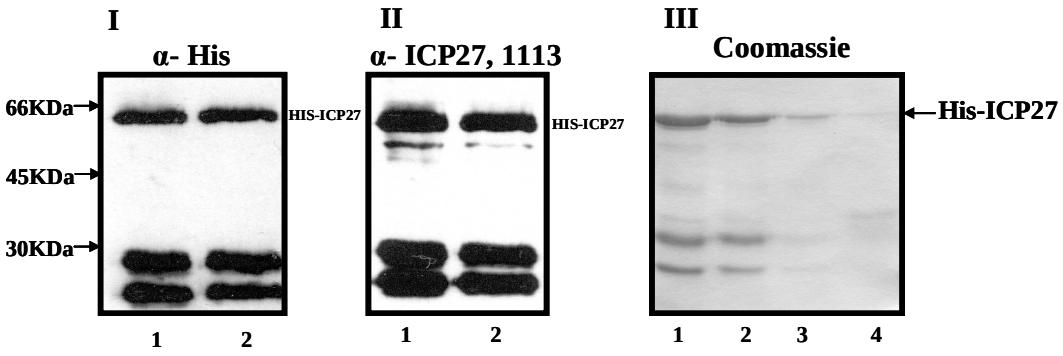


Figure 3.2: *In vitro* expressed His-ICP27 protein

Panel I: Western blot of 10% SDS-PAGE gel with anti His Ab showing the purified His tagged protein from the bacterial lysates bound to Ni-NTA Agarose beads to elute 6xHis-tagged proteins. Lanes 1 and 2, first and second fractions of eluted His-ICP27. Full length ICP27 is marked and lower bands are its truncations.

Panel II: Western blot of 10% SDS-PAGE gel with anti ICP27 Ab (1113) showing the purified His tagged protein from the bacterial lysates bound to Ni-NTA Agarose beads to elute 6xHis-tagged proteins. Lanes 1 and 2, first and second fractions of eluted His-ICP27. Full length ICP27 marked and lower bands are its truncations.

Panel III: Coomassie blue stained 10% SDS-PAGE gel showing the purified His tagged protein from the bacterial lysates bound to Ni-NTA Agarose beads to elute 6xHis-tagged proteins. Lanes 1, 2, 3 and 4, four fractions of His-ICP27. Full length ICP27 marked and lower bands are its truncations.

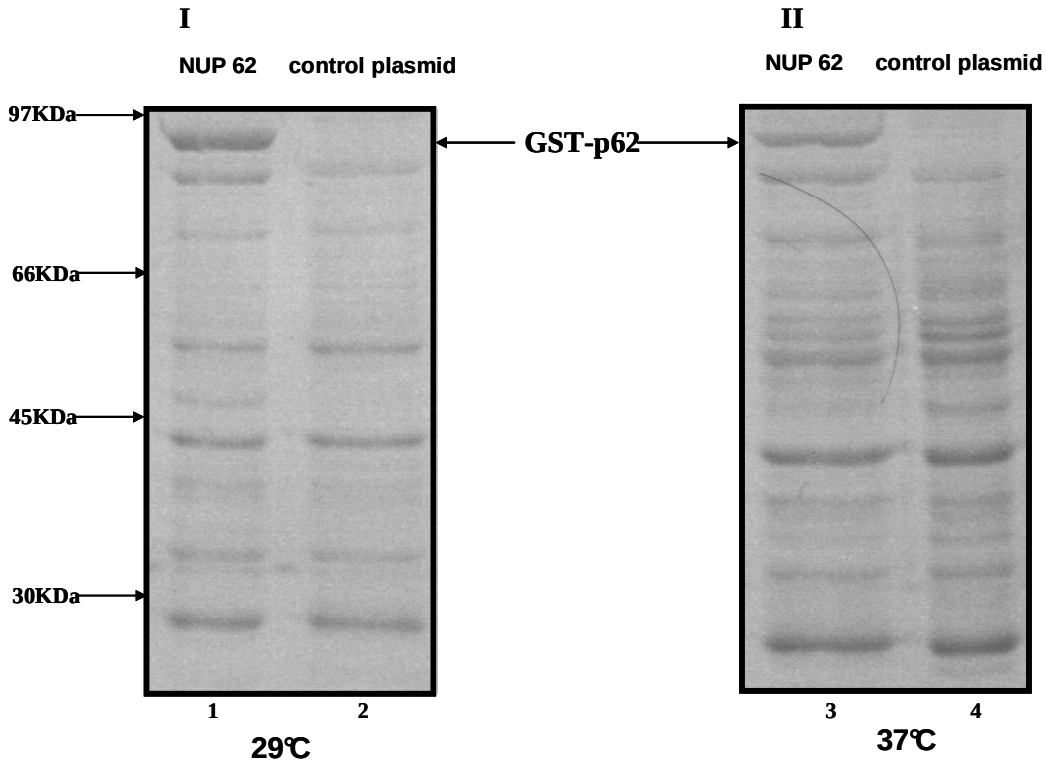


Figure 3.3: Optimising expression of GST-NUP 62

Coomassie blue stained 10% SDS-PAGE gel showing purified proteins from the bacterial lysates bound to Glutathione 4B sepharose. Full length GST-NUP 62 is marked.

Panel I, purified GST fusion protein from bacterial cultures transformed with a GST-NUP 62 expression plasmid and pUC (negative control) were induced with IPTG at 29°C (lane 1 GST-NUP 62 and lane 2 negative control pUC plasmid).

Panel 2, purified GST fusion bound protein from bacterial cultures transformed with a GST-NUP 62 expression plasmid and pUC (negative control) were induced with IPTG at 37°C (lane 1 GST-NUP 62 and lane 2 negative control pUC plasmid).

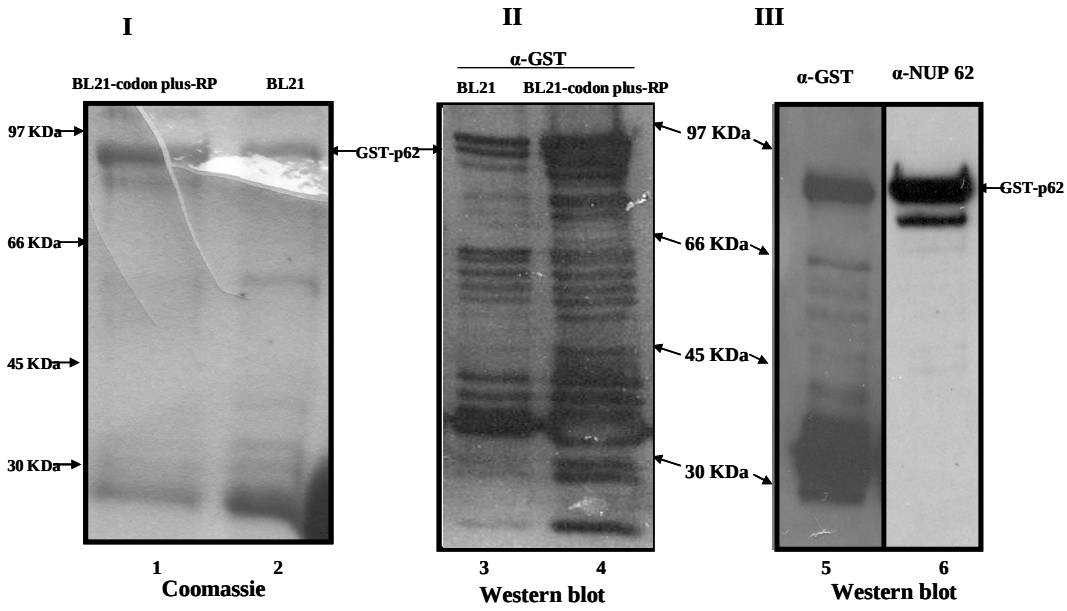


Figure 3.4: Improved expression of GST-NUP 62 obtained with using *E.coli* strain BL21-Codon plus-RP competent cells

Coomassie blue stained and western blotted 10% SDS-PAGE gel showing the purified protein from the bacterial lysates bound to Glutathione 4B sepharose. Panel I, bacterial cultures of BL21 and BL21-codon plus-RP competent cells E.coli containing GST-NUP 62 expressing plasmids were induced with IPTG at 29°C (lane 1 BL21-codon plus-RP and lane 2 BL21).

Panel II, bacterial cultures of BL21 and BL21-codon plus-RP competent cells E.coli containing GST-NUP 62 expressing plasmids were induced with IPTG at 29°C and blotted with anti-GST antibody (lane 3 BL21 and lane 2 BL21-codon plus-RP).

Panel III, Purified GST-NUP 62 expressed in BL21-codon plus-RP E.coli at 29°C and blotted with anti-GST antibody (lane 5) and anti-NUP 62 antibody (lane 6). Full length GST-NUP 62 is marked.

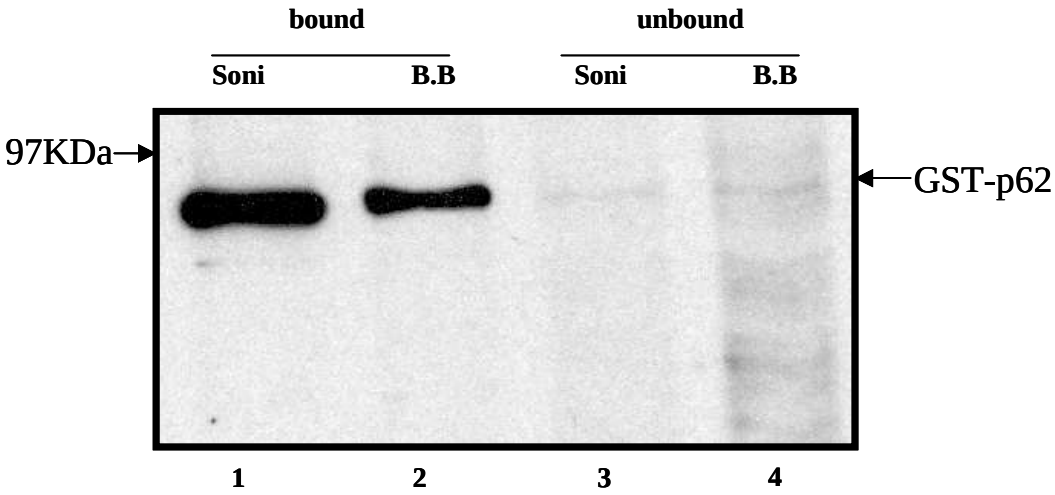


Figure 3.5: Lysis of bacteria by sonication is better than using bug buster master mix to produce GST-NUP 62 which binds to glutathione beads

Using sonication and bug buster master mix methods to assay their effect on the level of GST-NUP 62 full length expression. Bacteria from induced cultures were lysed by sonication (lanes 1 & 3) and using bug buster master mix (lanes 2 & 4). Lysates were mixed with glutathione beads and eluted by boiling in SDS loading buffer. Purified GST fusion bound protein and unbound proteins were separated by 10% SDS-PAGE and visualised by western blotting with anti NUP 62 (D-20) goat antibody.

Lane 1. Sonicated bound full length GST-NUP 62. Lane 2. Bug buster lysed bound full length GST-NUP 62. Lane 3. Sonicated unbound GST-NUP 62. Lane 4. Bug buster lysed unbound GST-NUP 62.

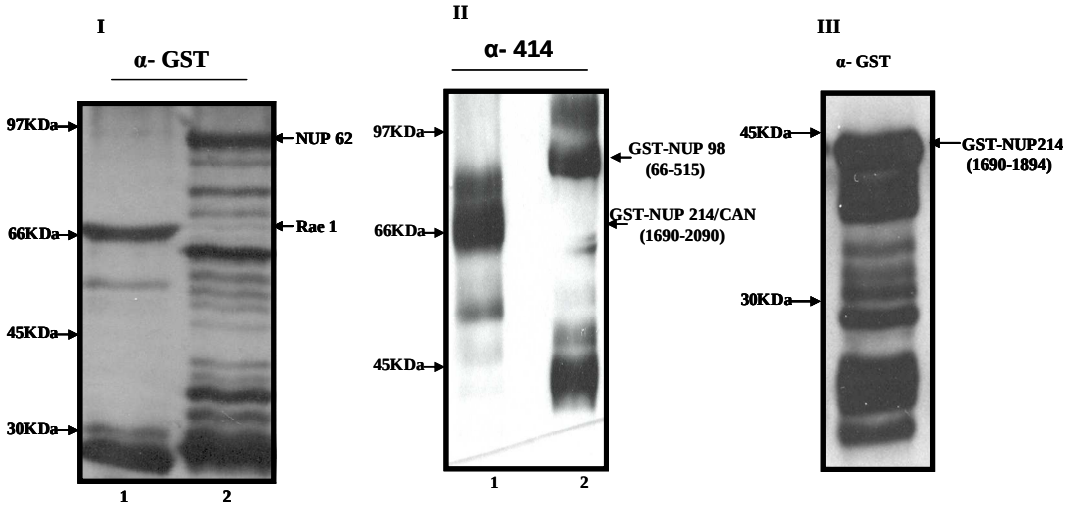


Figure 3.6: Optimised expression of GST fusion nucleoporins

Expression of GST fusion NUPs optimised. Bacterial cultures of BL21-codon plus-RP competent E.coli containing GST fusion nucleoporin expressing plasmids were induced with IPTG at 29°C, protein lysates were prepared and separated on 10% SDS-PAGE followed by western blotting.

Panel I: Western blot of GST fusion proteins against GST antibody (lane 1 GST-Rae 1 and lane 2 GST-NUP 62). Full length protein bands at around 60 & 90 KDa were detected representing GST-Rae 1 and GST-NUP 62 respectively and lower bands indicating truncations and degraded fusion proteins.

Panel II: Western blot of GST fusion proteins against anti FG-repeat NUPs 414 Ab (lane 1 GST-NUP 214/CAN (1690-2090) and lane 2 GST-NUP 98 (66-515). Protein bands at around 67 & 86 KDa representing GST-NUP 214/CAN (1690-2090) and GST-NUP 98 (66-515) respectively and lower bands indicating truncations and degraded fusion proteins

Panel III: Western blot of GST-NUP 214/CAN (1690-1894) against anti GST antibody. Protein bands at around 50 KDa representing GST-NUP 214/CAN (1690-1894) and lower bands indicating truncations and degraded fusion protein.

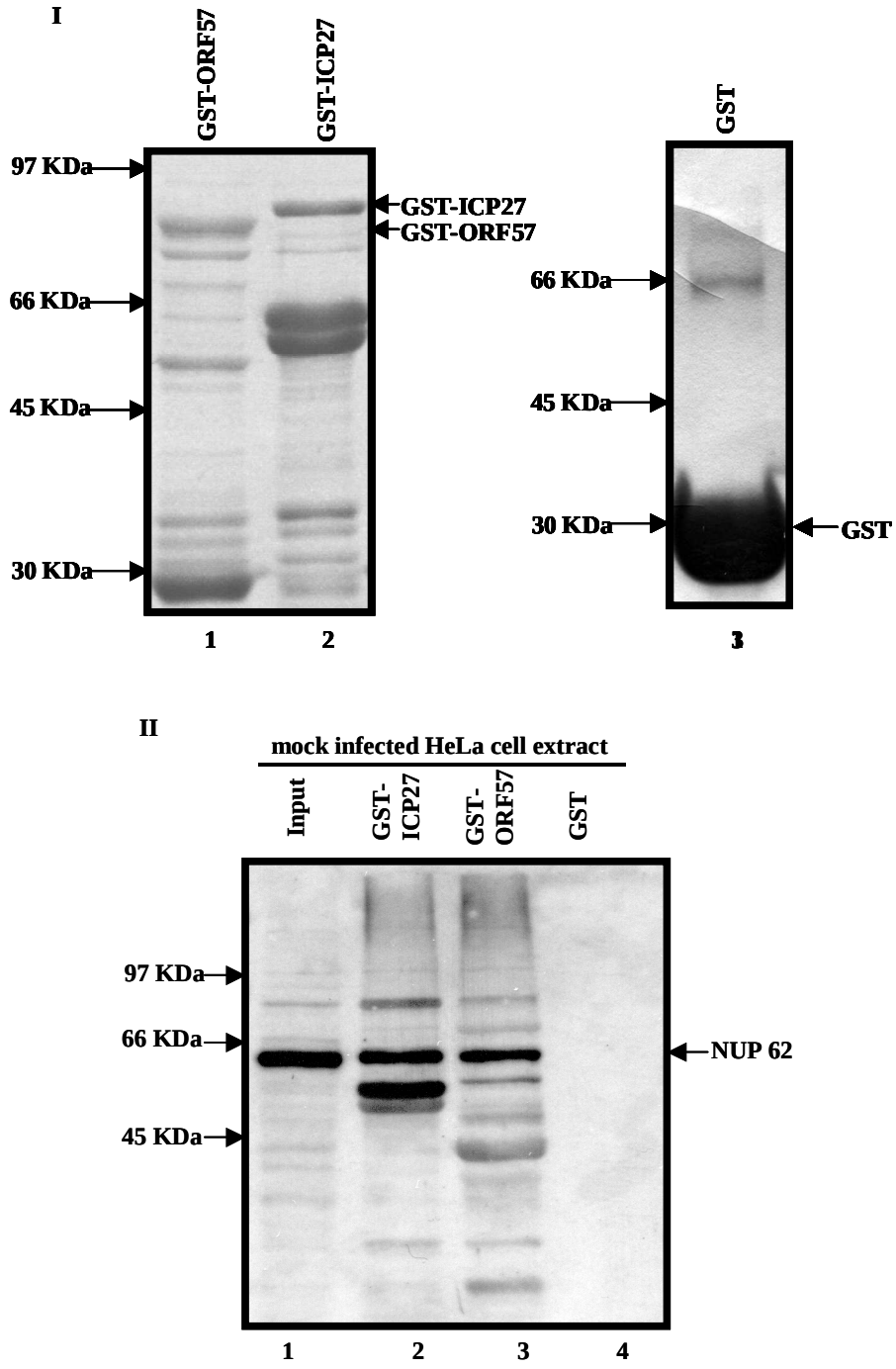


Figure 3.7: ICP27 and its homologue ORF57 interact with NUP 62 in the GST pull down assay.

Panel I: Coomassie stain of 10% SDS-PAGE of expression of GST-ORF57 (lane 1), GST-ICP27 (lane 2) and GST alone (lane 3) used in this pull down assay.

Panel II: Western blotting with anti NUP 62 (N-19) antibody. Lane 1, input 10% of sample of mock infected cell lysate; lane 2, GST-ICP27; lane 3, GST-ORF57; lane 4, GST alone.

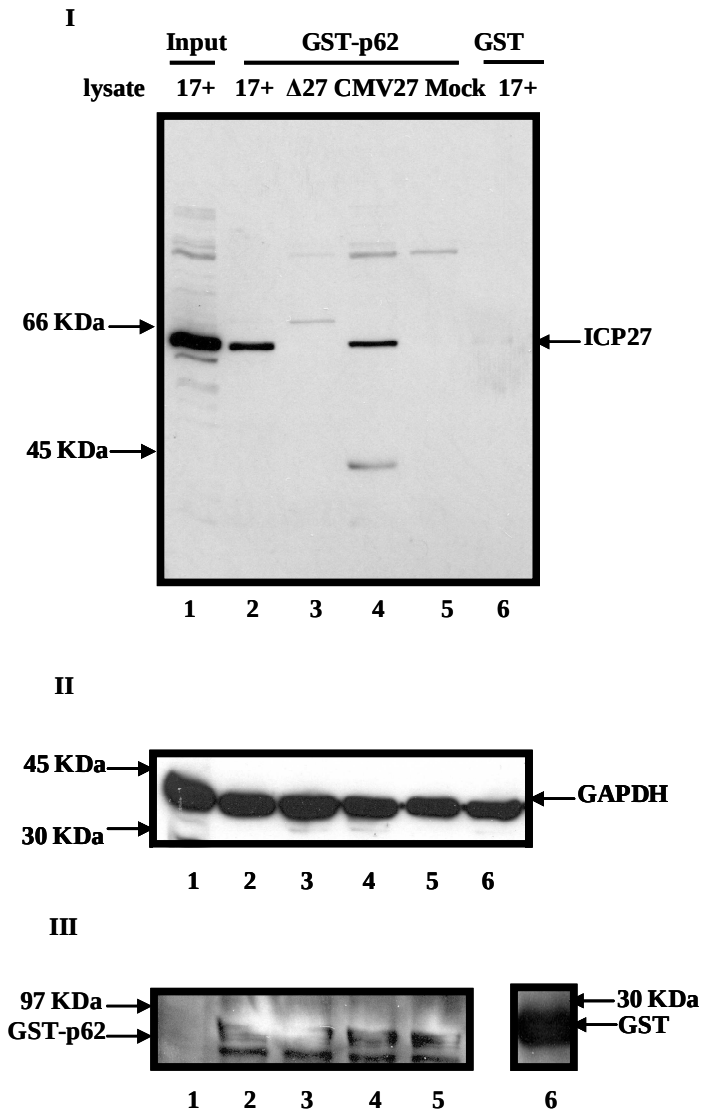

I
Input
GST-p62
GST
lysate
17+
17+
Δ27
CMV27
Mock
17+
66 KDa
45 KDa
ICP27
1
2
3
4
5
6
II
45 KDa
30 KDa
GAPDH
1
2
3
4
5
6
III
97 KDa
GST-p62
30 KDa
GST
1
2
3
4
5
6

Figure 3.8: NUP 62 interacts with ICP27 in the pull down assay.

Full length GST-NUP 62 expressed and purified and incubated with extracts of HeLa cells infected (MOI=10) with wt HSV-1 17+, Δ27, CMV27 (transfected with plasmid CMV27) and mock infected.

Panel I: Pulled down proteins fractionated on SDS-PAGE and transferred to nitrocellulose membrane followed by immunoblotting using anti-ICP27 (1113) monoclonal antibody. Lane 1, showing input that is 10% of HSV-1 wt infected cell extract lysate; lane 2, wild type infected cell extract incubated with GST-NUP 62; lane 3, Δ27 infected cell extract incubated with GST-NUP 62; lane 4, CMV27 transfected cell extract incubated with GST-NUP 62; lane 5, mock infected cell extract incubated with GST-NUP 62; lane 6, wt infected cell extract incubated with GST alone.

Panel II: Unbound samples run on 10% SDS-PAGE and western blotted against GAPDH by using anti GAPDH antibody and showed roughly equal amount protein used in this experiment.

Panel III: Unbound samples stripped and immunoblotted against GST-NUP 62 by using anti GST antibody showed input level of GST-NUP62 in lanes 2, 3, 4, 5 and GST alone in lane 6. Lane 1 indicates just input of wt infected cell extract and so does not show any GST fusion protein.

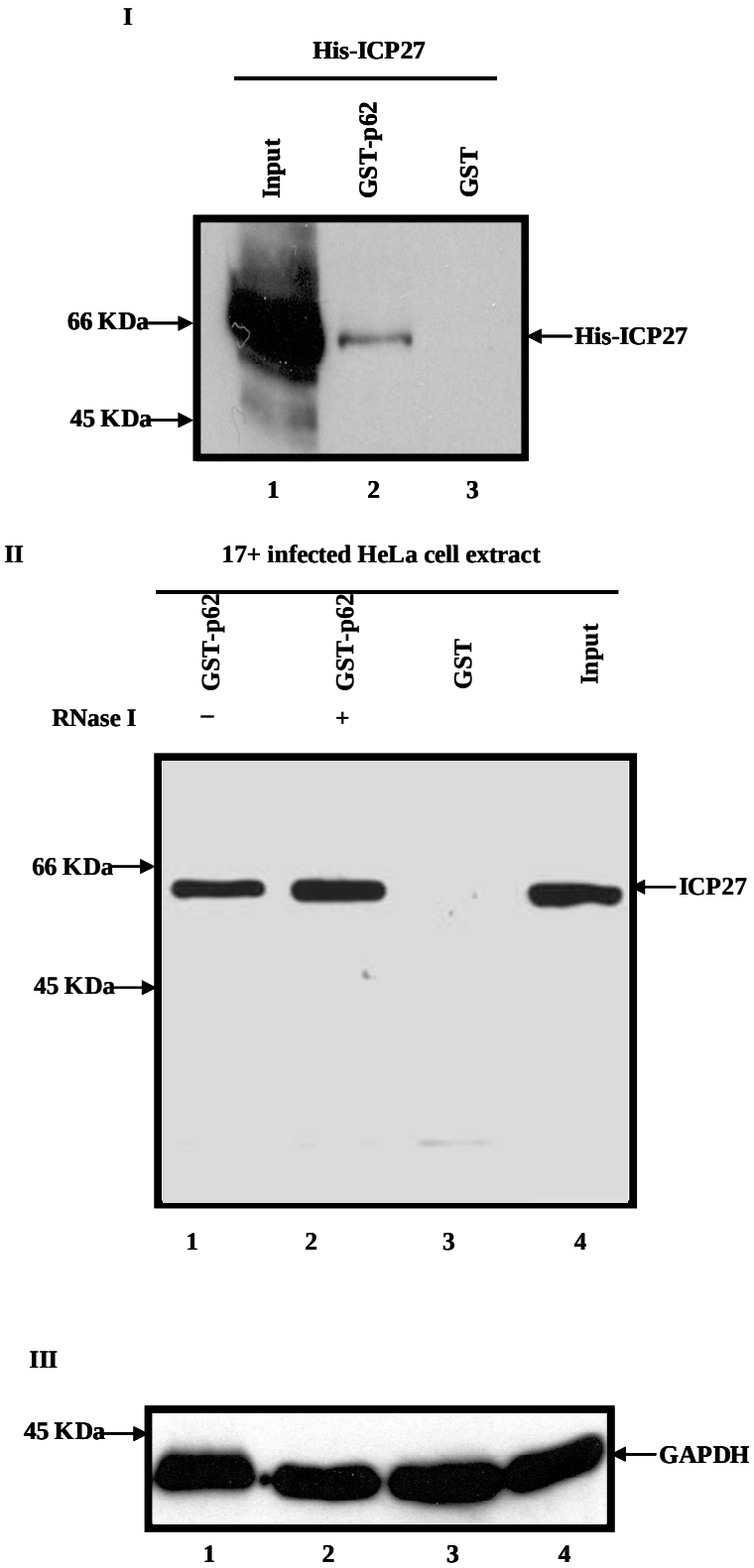

I
His-ICP27
Input
GST-p62
GST
66 KDa
45 KDa
His-ICP27
1
2
3
II
17+ infected HeLa cell extract
GST-p62
GST-p62
GST
Input
RNase I
–
+
66 KDa
45 KDa
ICP27
1
2
3
4
III
45 KDa
GAPDH
1
2
3
4

Figure 3.9: NUP 62 interacts directly with ICP27 in the pull down assay. GST fusion NUP 62 and His-tagged ICP27 were expressed and purified and infected cell extracts (MOI=10) were prepared and pull down assays carried out.

Panel I: GST-NUP 62 and GST alone were incubated with purified His-ICP27 followed with fractionation on a 10% SDS-PAGE and western blotting with anti-His antibody. Lane 1, showing input that is 10% of His-ICP27; lane 2, His-ICP27 incubated with GST-NUP 62; lane 3, His-ICP27 incubated with GST alone. Image quantification of the band in lane 2 showed 0.1% density in comparison with input.

Panel II: RNase I was used in pull down assay to remove RNAs from interaction complex. GST-NUP 62 and GST-alone incubated with wt 17+ HSV-1 infected HeLa cell extracts in the presence (lane 2) and absence (lane 1) of RNase I. Lane 3, GST alone incubated with wt infected cell extract. Lane 4, 10µl input wt infected cell extract ICP27.

Panel III: Above unbound complex formed samples western blotted with anti GAPDH antibody.

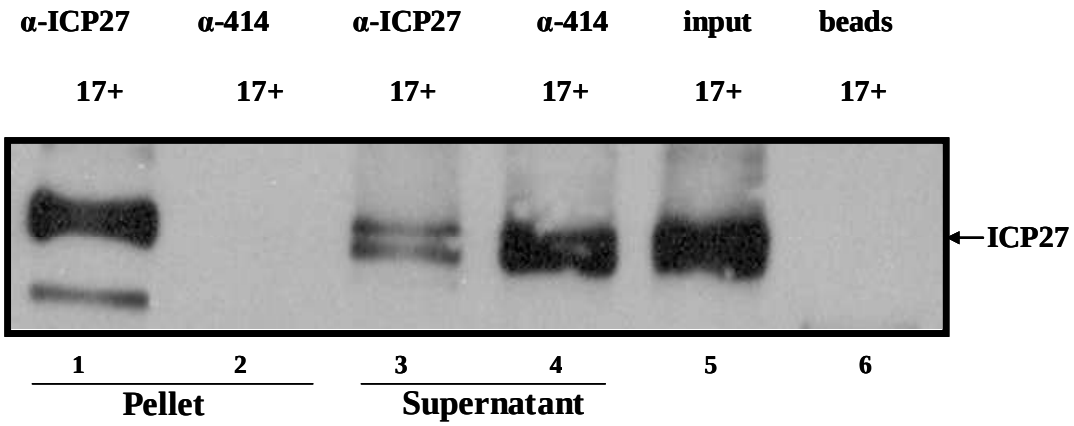


Figure 3.10: Testing immunoprecipitation of ICP27.

17+ wt HSV-1 infected extracts (MOI=10) immunoprecipitated by using anti-ICP27 Ab and anti-FG NUP 414 antibody and beads alone. Samples analysed by 10% SDS-PAGE and western blotted against anti-ICP27 (1113) antibody.

Lane 1, IP with HSV-1 17+ HeLa infected cell lysate and 1113 antibody. Lane 2, IP with HSV-1 17+ HeLa infected cell lysate and anti FG repeat 414 control antibody. Lane 3, supernatant of IP with HSV-1 17+ HeLa infected cell lysate and 1113 antibody. Lane 4, supernatant of IP with HSV-1 17+ HeLa infected cell lysate and anti FG repeat 414 control antibody. Lane 5, showing input that is 10% of HSV-1 17+ HeLa infected cell lysate . Lane 6 shows that beads alone, incubated in the presence of HSV-1 wild type infected cell extracts did not precipitate any ICP27.

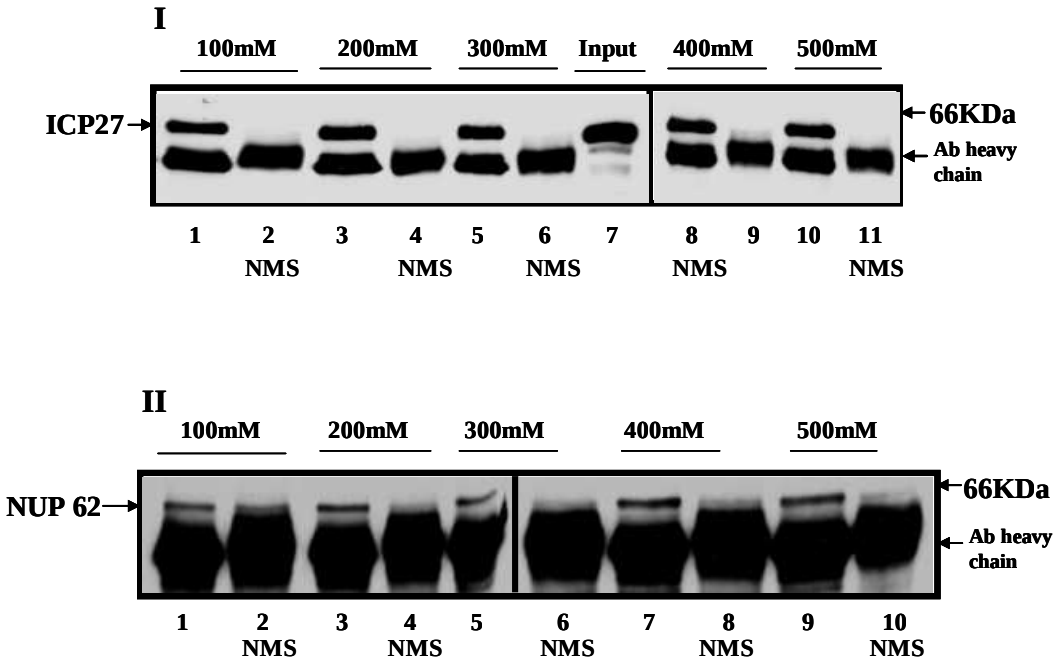


Figure 3.11: Stringency of interaction between ICP27 and nucleoporin NUP 62 in co-immunoprecipitation assay

Co-immunoprecipitation assay carried out to check stringency of interaction of ICP27 with nucleoporin 62: Mock and wt HSV-1 HeLa cell infected extracts (MOI=10) immunoprecipitated by using anti-ICP27 Ab and normal mouse serum (NMS) then washed with various concentration of salt buffer from 100 to 500 mM. Samples were analysed by 10% SDS-PAGE.

Panel I, western blot with anti-ICP27 Ab shows ICP27 protein immunoprecipitated by anti-ICP27 Ab in the range of 100-500mM salt. Lane 1 at 100mM salt. Lane 3 at 200nmM salt. Lane 5 at 300mM salt. Lane 8 at 400mM salt. Lane 10 at 500mM salt. Lanes 2, 4, 6, 9 and 11 for NMS at 100-500 mM salt. Lane 7 showing input that is 10% of HSV-1 wt infected cell extract lysate

Panel II, western blot with 414 antibody shows ICP27 co-immunoprecipitated nucleoporin p62 at concentration of 100 to 500mM in lanes 1, 3, 5, 7 and 9 but NMS antibody in lanes 2, 4, 6, 8 and 10 did not co-immunoprecipitated NUP 62 at the level of 100-500 mM salt.

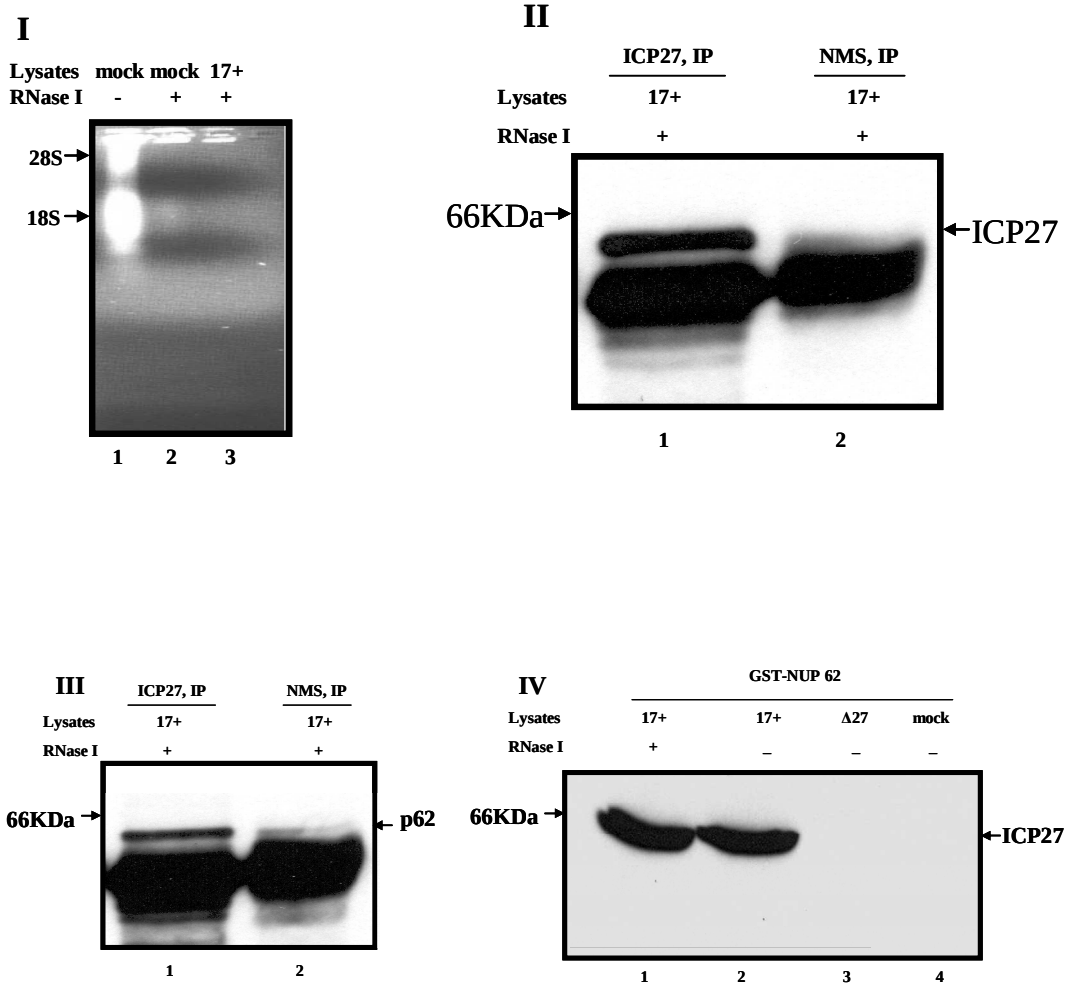

I
Lysates mock mock 17+
RNase I - + +
28S
18S
1 2 3
II
ICP27, IP
NMS, IP
Lysates 17+ 17+
RNase I + +
66KDa
ICP27
1 2
III
ICP27, IP
NMS, IP
Lysates 17+ 17+
RNase I + +
66KDa
p62
1 2
IV
GST-NUP 62
Lysates 17+ 17+ Δ27 mock
RNase I + – – –
66KDa
ICP27
1 2 3 4

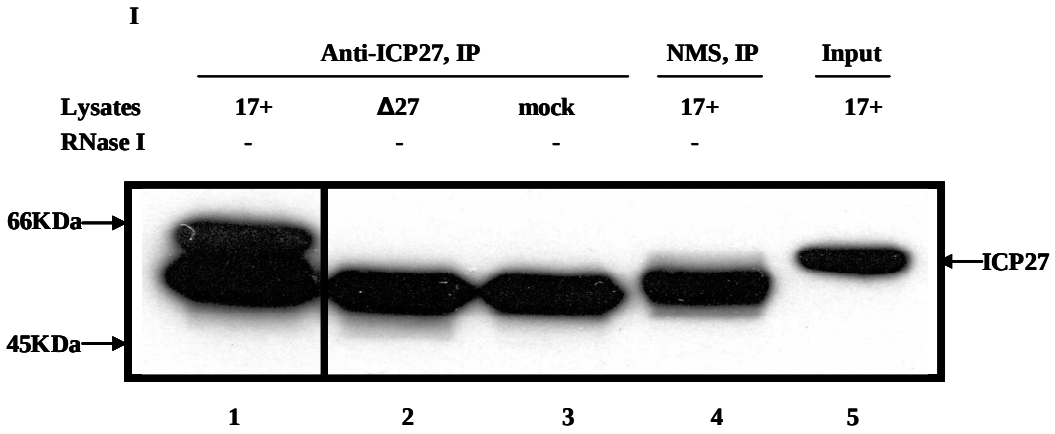
Figure 3.12: RNase I treatment of HeLa mock and wt HSV-1 infected cell extracts and protein interaction assay experiments

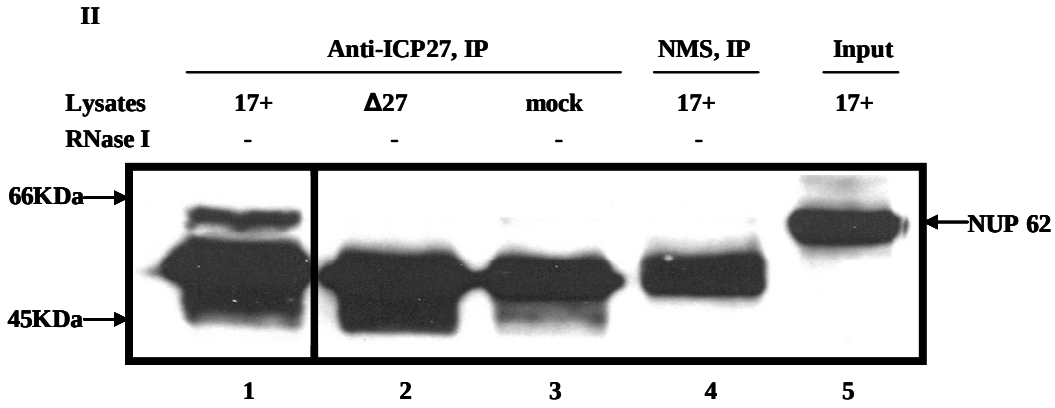
Panel I: RNase treatment HeLa infected cell extracts. 100 µg protein of HeLa cell extracts infected (MOI=10) with 17+ wt HSV-1 and mock infected, treated with 100 U RNase I for 45 minutes at 37℃, were analysed by agarose gel electrophoresis to check RNA integrity. Untreated mock infected cell extract was included as a control to show native RNA. Lane 1 RNase I untreated HeLa mock infected. Lane 2 RNase I treated HeLa mock infected cell extract. Lane 3 RNase I treated HeLa 17+ wt HSV-1 infected cell extract.

Panel II: RNase I treatment used in an immunoprecipitation assay of ICP27/ICP27. Immunoprecipitation test of HeLa infected wt HSV-1 cell extracts carried out with anti ICP27 Ab (1113) and normal mouse serum then treated with RNase I as described in Materials and Methods and western blotted with 1113 mAb. Lane 1 ICP27 bound to the antibody and immunoprecipitated. Lane 2. ICP27 did not bind to normal mouse serum.

Panel III: RNase I treatment used in on immunoprecipitation assay of ICP27/NUP 62. The ICP27 precipitation membrane from the previous experiment was stripped and western blotted with 414 antibody. Lane 1 NUP 62 bound to ICP27 and co-immunoprecipitated. Lane 2 NUP 62 did not bind to normal mouse serum.

Panel IV: RNase I treatment used in a GST pull down assay. HSV-1 wt HeLa infected cell extracts mixed with GST-NUP 62 in the presence and absence of RNase I treatment and western blotted with 1113 mAb. Lane 1 GST-NUP 62 pulled down ICP27 in the presence of RNase I. Lane 2 GST-NUP 62 pulled down ICP27 in absence of RNase I. Lane 3 Δ27 in absence of RNase I. Lane 4 mock infected in absence of RNase I


I
Anti-ICP27, IP
NMS, IP
Input
Lysates
17+
Δ27
mock
17+
17+
RNase I
-
-
-
-
66KDa
45KDa
ICP27
1
2
3
4
5


II
Anti-ICP27, IP
NMS, IP
Input
Lysates
17+
Δ27
mock
17+
17+
RNase I
-
-
-
-
66KDa
45KDa
NUP 62
1
2
3
4
5

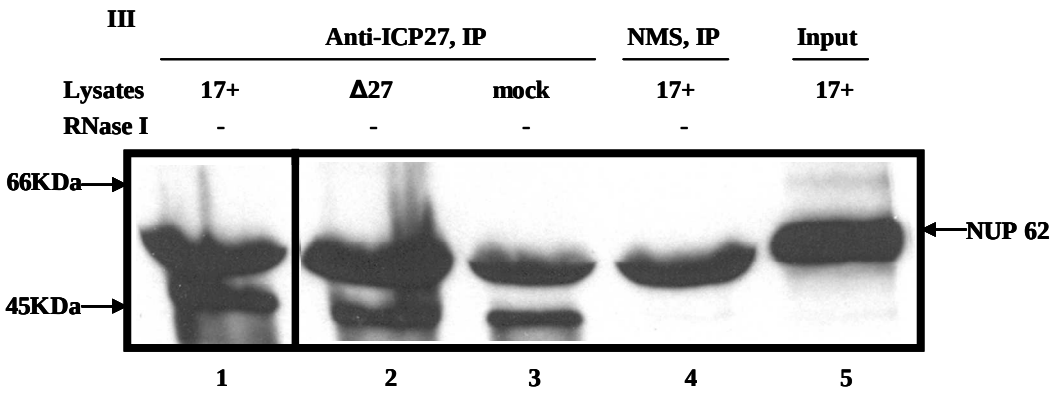

III
Anti-ICP27, IP
NMS, IP
Input
Lysates
17+
Δ27
mock
17+
17+
RNase I
-
-
-
-
66KDa
45KDa
NUP 62
1
2
3
4
5

Figure 3.13: Anti ICP27 monoclonal antibodies used to co-immunoprecipitate nucleoporin 62 from extracts of HeLa cells infected and uninfected with wt and mutant HSV-1.

Co-immunoprecipitation of nucleoporin 62 from wild type (wt) HSV-1, Δ27 and mock-infected HeLa cell extracts (MOI=10).

Panel I: Immunoprecipitated complexes fractionated on a 10% SDS-PAGE gel and separated and analysed by western blotting with anti ICP27 antibody. ICP27 precipitated from wt sample lane 1, but not with lane 2, Δ27 and lane 3, mock infected. NMS control test did not precipitate ICP27 from wt infected sample (lane 4). Lane 5, showing input that is 10% of HSV-1 wt infected cell extract lysate.

Panel II: Above membrane stripped and western blotted against NUP 62

Panel III: Unbound precipitated samples fractionated on a 10% SDS-PAGE gel and western blotted with anti 414 antibody.

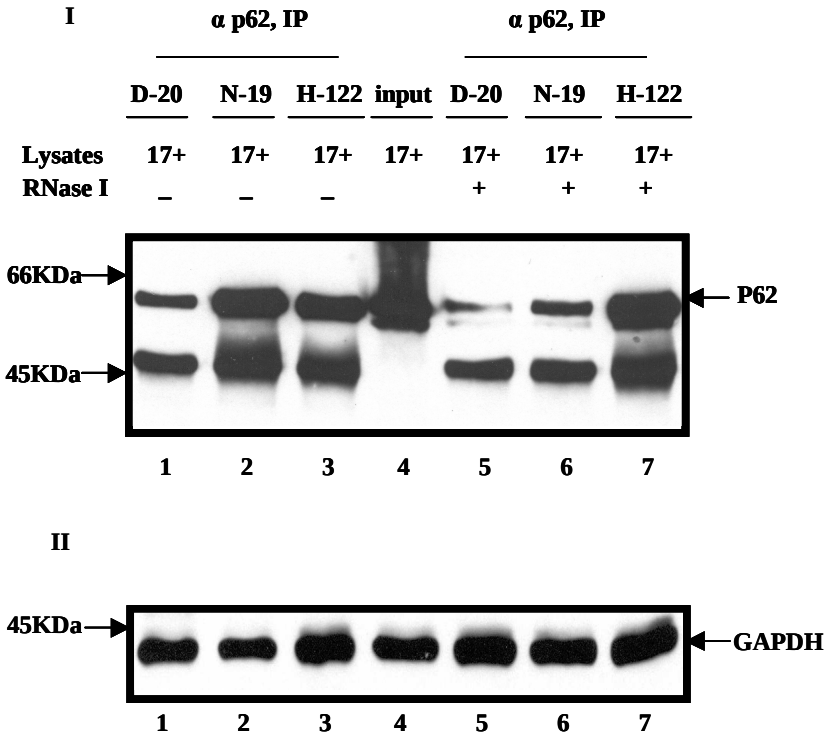
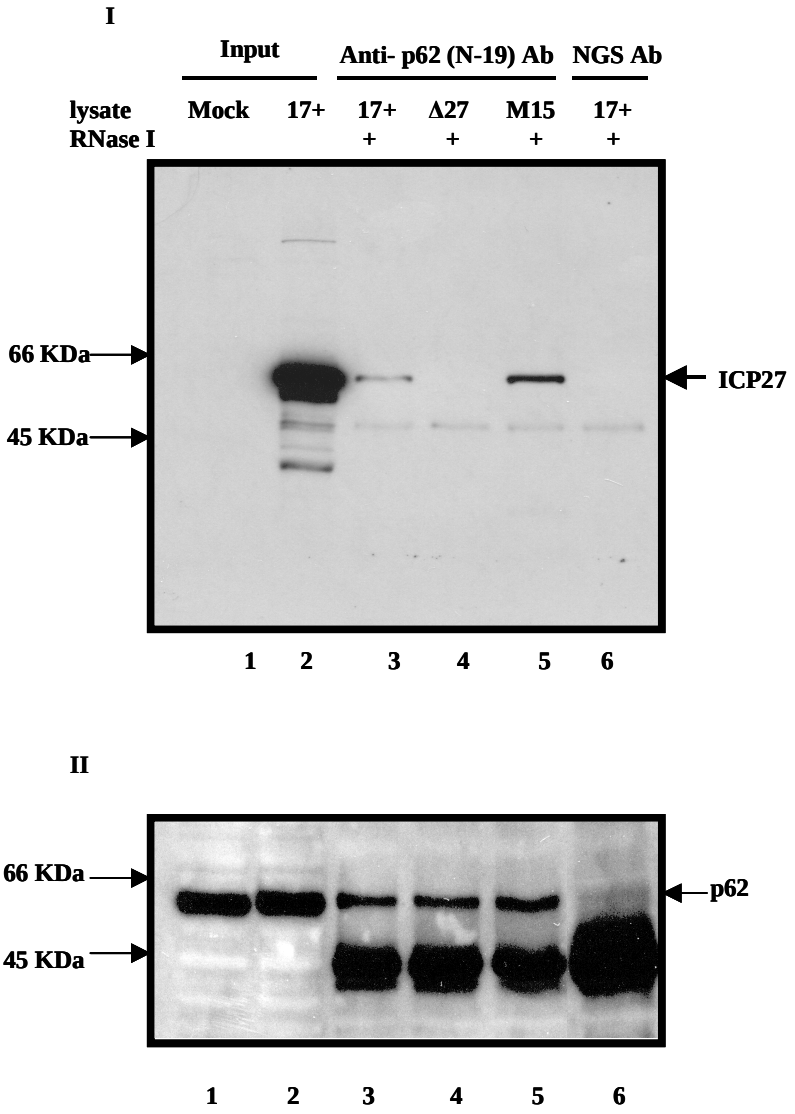


Figure 3.14: Anti nucleoporin 62 antibodies (N-19, D-20 and H-122) used to immunoprecipitate NUP 62 from extracts of HeLa cells infected with wt HSV-1.

Three anti-NUP 62 antibodies incubated with wt infected HeLa cell extracts (MOI=10) and separated on 10% SDS-PAGE and visualised by western blotting with anti-FG-repeat Nups 414 antibody.

Panel I showed NUP 62 precipitated by all of these antibodies in the presence (lanes 5-7) and absence (lanes 1-3) of RNase I. Lane 4 showing input that is 10% of HSV-1 wt infected cell extract lysate.

Panel II showing level of GAPDH in lysates.


I
Input
Anti- p62 (N-19) Ab
NGS Ab
lysate
Mock
17+
17+
Δ27
M15
17+
RNase I
+
+
+
+
66 KDa
45 KDa
ICP27
1 2 3 4 5 6
II
66 KDa
45 KDa
p62
1 2 3 4 5 6

III

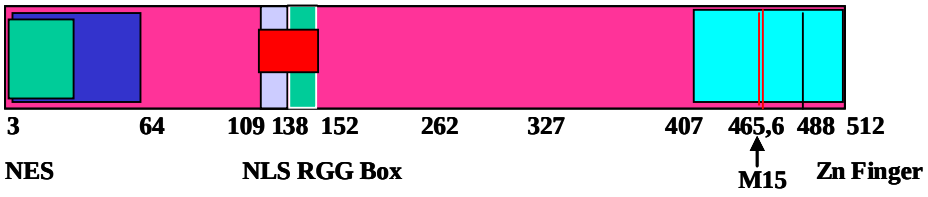

3
64
109 138
152
262
327
407
465,6
488
512
NES
NLS RGG Box
M15
Zn Finger

Figure 3.15: Anti nucleoporin 62 antibody (N-19) used to co-immunoprecipitate HSV-1 ICP27 from extracts of HeLa cells infected and uninfected with HSV-1.

Co-immunoprecipitation experiment in the presence of RNase I using anti-NUP 62 (N-19) antibody and HeLa cells lysates infected (MOI=10) with wt, M15 and Δ27 HSV-1 were incubated with antibody as well as wt infected extract with normal goat serum (NGS). Samples were fractionated on a 10% SDS-PAGE and visualised with western blotting with anti-ICP27 antibody (1113) and anti-NUP 62 (N-19) to show interacting proteins.

Panel I: The complexes formed visualised with western blotting with anti-ICP27 antibody (1113). Lane 1 showing input that is 10% of mock infected cell, lane 2 showing input that is 10% of wt infected cell extract lysate, lane 3 wt, Lane 4 Δ27, lane 5 M15, lane 6 wt control with NGS

Panel II: Above membrane stripped and western blotted with anti-NUP 62 (N-19) and showed anti NUP 62 antibody precipitated NUP 62 from all lysates (lanes 3, 4 and 5) except NGS sample (lane 6).

Panel III: Schematic representative of the location of the M15 ICP27 mutants.

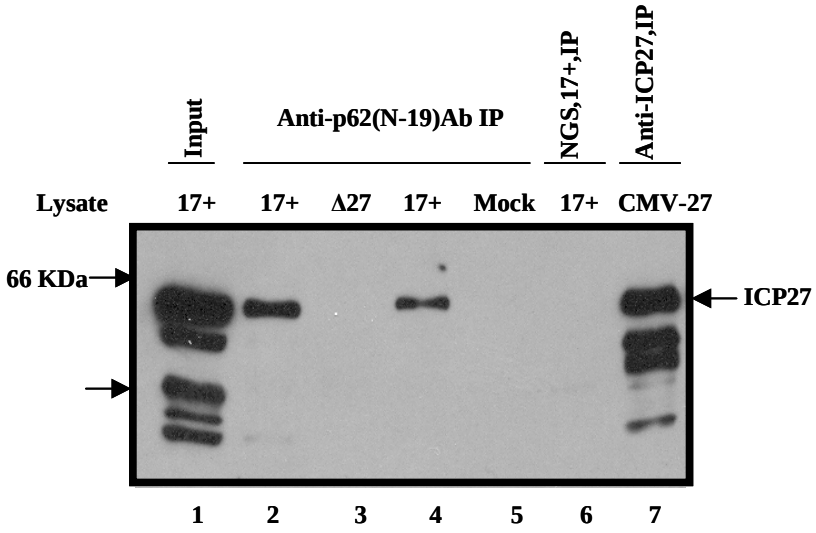
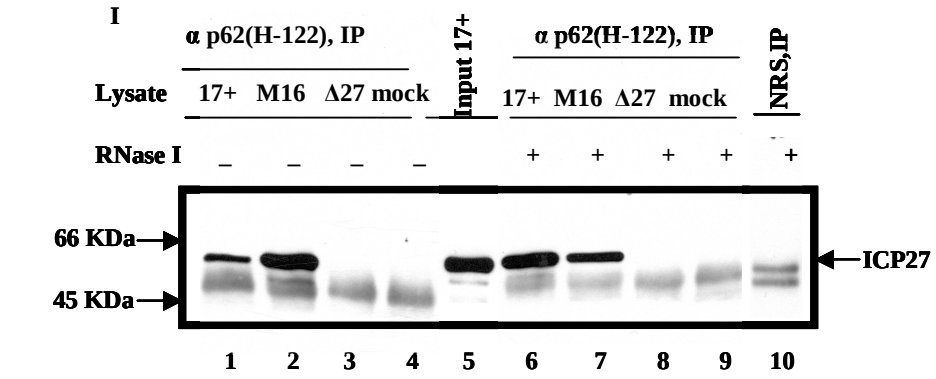
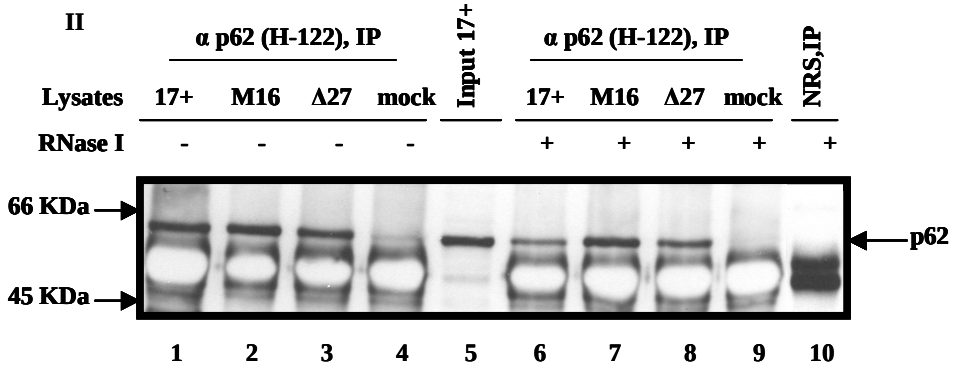
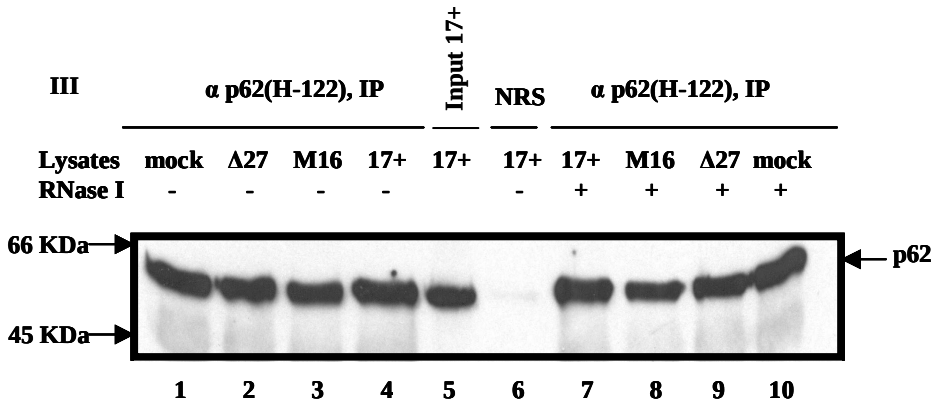


Figure 3.16: Anti-NUP 62 (N-19) antibody co-immunoprecipitates ICP27 from wt HSV-1 17+ infected extracts of BHK cells.

Co-immunoprecipitation experiment using anti-NUP 62 (N-19) and BHK cell infected with HSV-1 wt and Δ27 mutant (MOI=10) and transfected pCMV-27 (ICP27) lysates. Samples were incubated with antibody as well as HSV-1 wt infected cell extract with normal goat serum (NGS). Samples were fractionated on a 10% SDS-PAGE and visualised with western blotting with anti-ICP27 antibody (1113) and showed NUP 62 co-precipitated ICP27 from wt infected and transfected cell extracts (lanes2 and 4) but did not co-precipitate in Δ27 (lane 3). Also NGS did not show any ICP27 band (lane 6). Lane 1 showing input that is 10% of HSV-1 wt infected cell extract lysate. Lane 7 showed immunoprecipitation of ICP27 from pCMV-27 transfected BHK cell and western blot with anti ICP27 antibody indicated ICP27 band in size of 63 KDa and some truncates of that.


I
α p62(H-122), IP
Input 17+
α p62(H-122), IP
NRS,IP
Lysate
17+ M16 Δ27 mock
17+ M16 Δ27 mock
RNase I
66 KDa
45 KDa
ICP27
1 2 3 4 5 6 7 8 9 10


II
α p62 (H-122), IP
Input 17+
α p62 (H-122), IP
NRS,IP
Lysates
17+ M16 Δ27 mock
17+ M16 Δ27 mock
RNase I
66 KDa
45 KDa
p62
1 2 3 4 5 6 7 8 9 10


III
α p62(H-122), IP
Input 17+
NRS
α p62(H-122), IP
Lysates
mock Δ27 M16 17+ 17+ 17+ 17+ M16 Δ27 mock
RNase I
66 KDa
45 KDa
p62
1 2 3 4 5 6 7 8 9 10

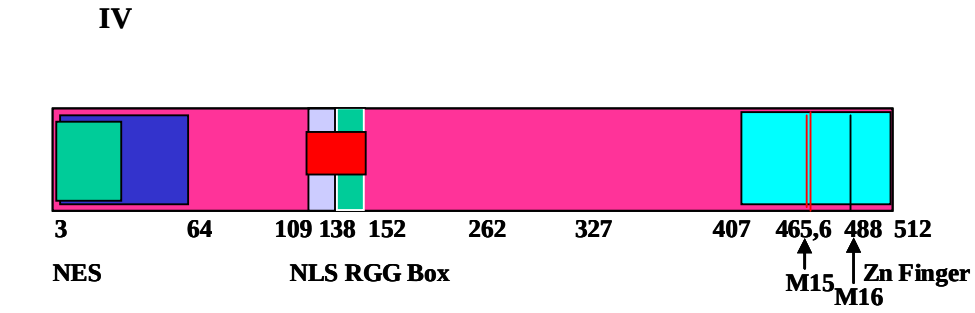

IV
3 64 109 138 152 262 327 407 465,6 488 512
NES
NLS RGG Box
M15
M16
Zn Finger

Figure 3.17: Anti nucleoporin 62 antibody (H-122) used to co-immunoprecipitate HSV-1 ICP27 from extracts of HeLa cells infected and uninfected with HSV-1.

Co-immunoprecipitation experiment was carried out by using anti-NUP 62 (H-122). HeLa cell infected with HSV-1 wt and HSV-1 mutants M16, Δ27 (MOI=10) and mock infected lysates mixed with antibody as well as wt infected extract with normal rabbit serum (NRS). Samples were run on 10% SDS-PAGE gel and samples visualised with western blotting with anti-ICP27 antibody (1113) and anti-NUP 62 (H-122) to show interacting proteins. Samples were untreated or treated with RNase I to remove RNA from interaction.

Panel I: The complexes formed were visualised with western blotting with anti-ICP27 antibody (1113) and showed NUP 62 co-precipitated ICP27 from wt and M16 infected cell extracts without RNase I treatment (lanes 1 and 2) and with RNase I treatment (lanes 6 and 7) but did not co-precipitate from Δ27 and mock infected cells in both conditions (lanes 3, 4, 8 and 9). Also NRS did not show any ICP27 band (lane 10). Lane 5 showing input that is 10% of HSV-1 wt infected cell extract lysate.

Panel II: Above membrane was stripped and western blotted with anti-NUP 62 (H-122) and showed antibody precipitated NUP 62 from all lysates (lanes 1, 2, 3, 4, 6, 7, 8 and 9) except NRS sample (lane 10). A single band in lane 5 at about 62 KDa corresponds to the size of NUP 62.

Panel III: **To confirm panel II and presence of NUP 62 in all lanes, samples were separated on SDS PAGE and western blotted with anti FG-repeat NUPs 414 antibody and showed NUP 62 levels in all lysates used in this experiment.**

Panel IV: Schematic representative of the position of M15 and M16 ICP27 mutants.

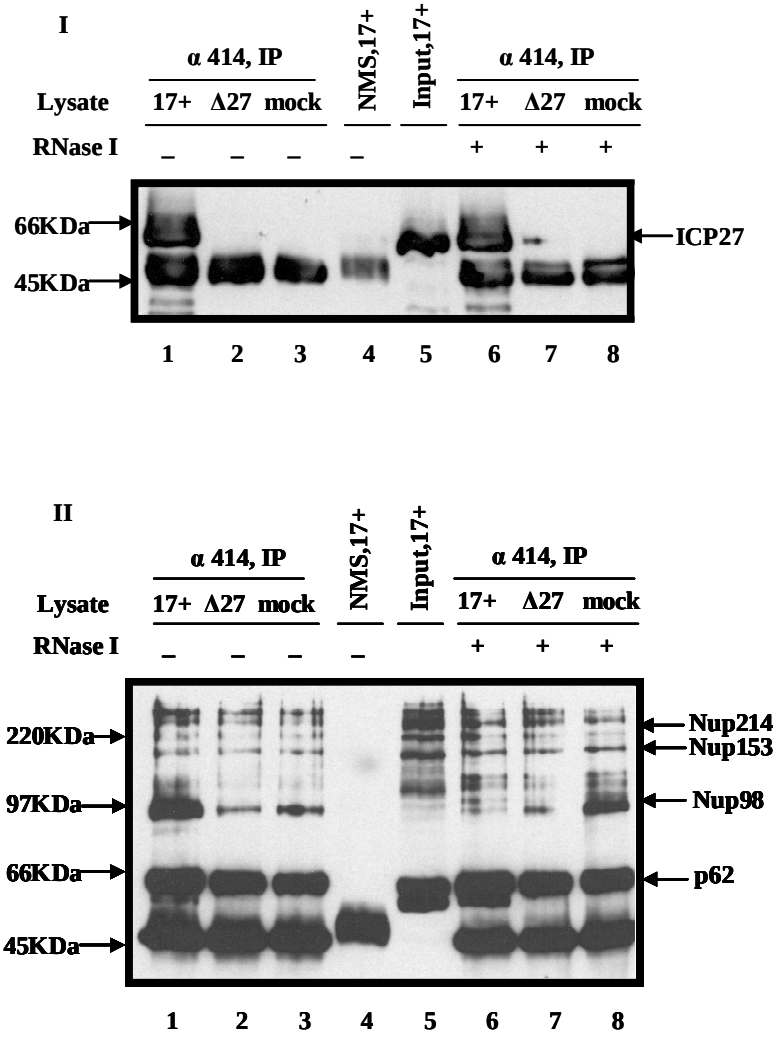


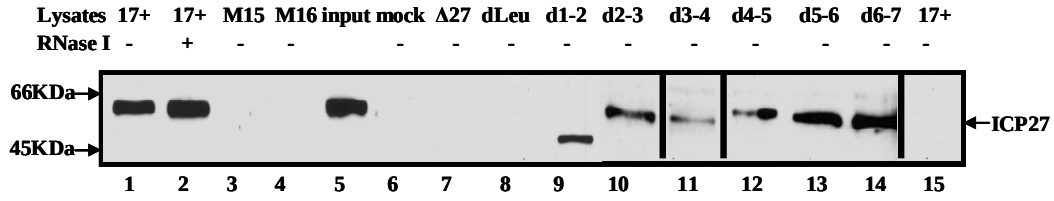
Figure 3.18: Monoclonal anti FG-repeat NUPs 414 used to co-immunoprecipitate HSV-1 ICP27 from extracts of HeLa cells infected and uninfected with HSV-1.

To examine co-immunoprecipitation of ICP27 with FG-repeat nucleoporins anti-414 monoclonal antibody was mixed with HSV-1 wt and mutant Δ27 (MOI=10) and mock infected HeLa cell extracts as well as HSV-1 wt infected cell extract with normal goat serum (NMS) and tested in presence and absence of RNase I. Samples were run on 6% SDS-PAGE and analysed by western blotting with anti-ICP27 antibody (1113) and anti 414 antibody.

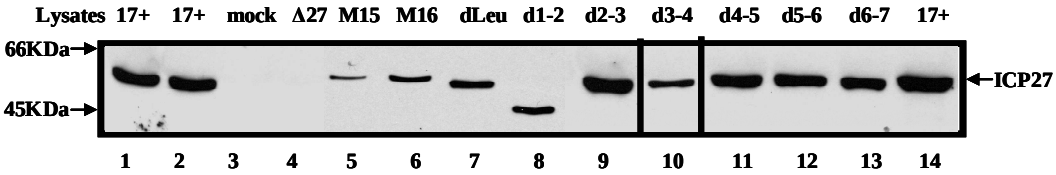
Panel I: The complexes formed visualised with western blotting with anti-ICP27 antibody (1113) and showed FG repeat nucleoporins co-precipitated ICP27 from wt infected cell extracts in both without and with RNase I treatment (lanes 1 and 6) but did not co-precipitate with Δ27 in both conditions (lanes 2 and 7). Also NMS did not show any ICP27 band (lane 4). Lane 5 showing input that is 10% of HSV-1 wt infected cell extract lysate.

Panel II: Above membrane was stripped and western blotted with anti FG repeat 414 antibody and showed anti 414 antibody precipitated a range of FG repeat nucleoporins from all lysates (lanes 1, 2, 3, 6, 7 and 8) except NMS sample (lane 4). The various bands in lane 5 correspond to different FG repeat nucleoporins including NUP 214, NUP153, NUP 98 and NUP 62.

I Bound



II Unbound



III

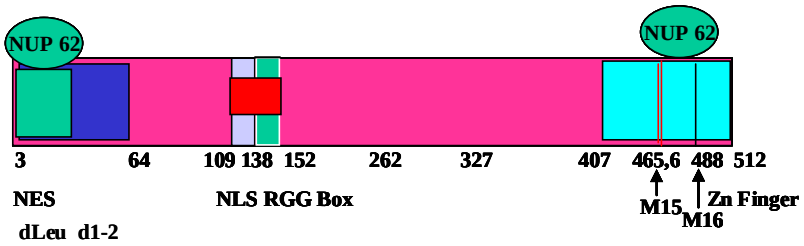


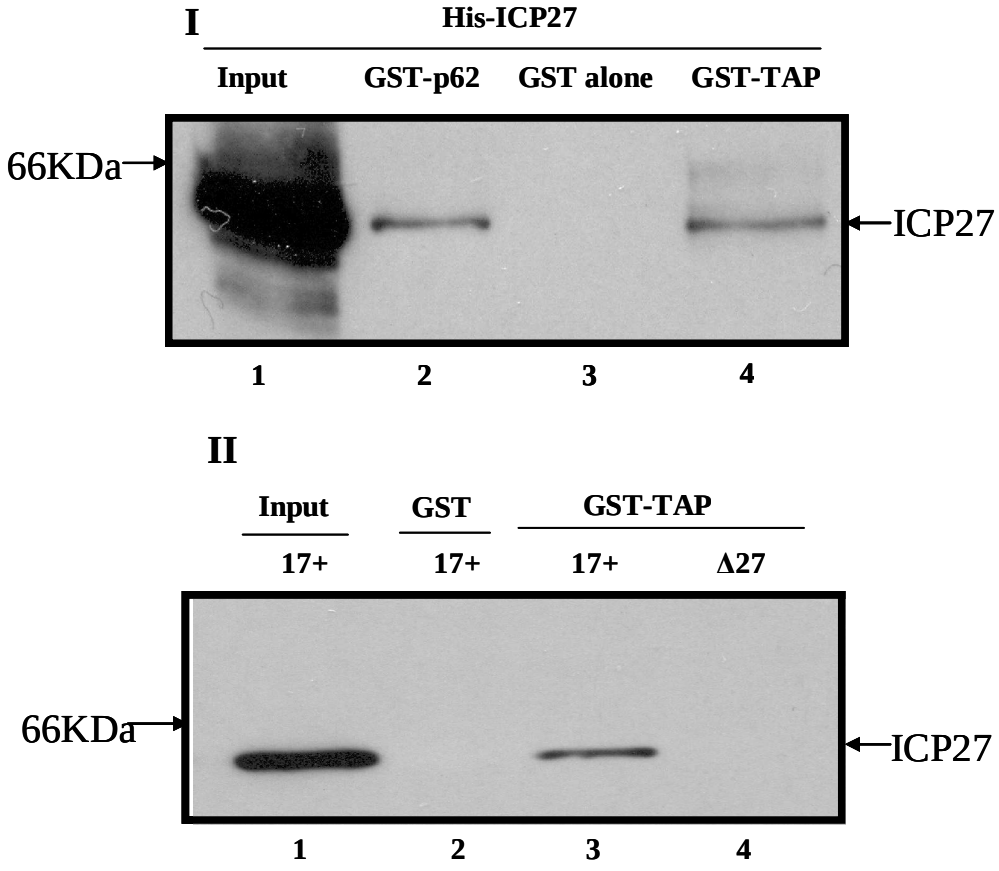
Figure 3.19: Mapping the interacting regions of ICP27 with NUP 62 using GST pull down assay.

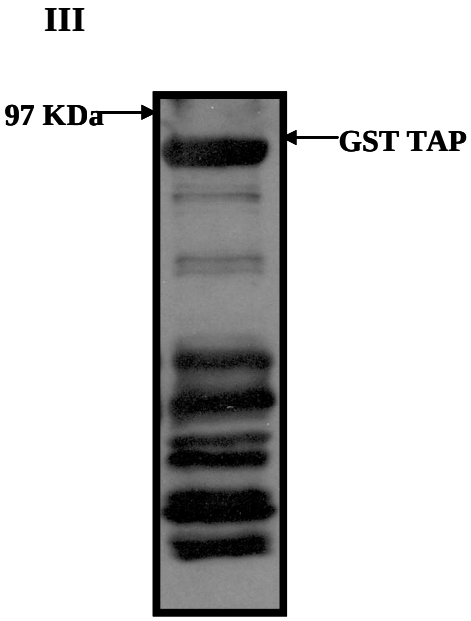
Series of HSV-1 wt and mutants ICP27 HeLa cell extracts (MOI=10) incubated with full length fusion protein GST-NUP 62. Pulled down complexes were separated by SDS-PAGE and analysed by western blot.

Panel I: Bound pulled down proteins western blotted with anti ICP27 antibodies 1113 and 1119 NUP 62 pulled down ICP27 from wt, d1-2, d2-3, d3-4, d4-5, d5-6 and d6-7 (lanes 1, 2, 9, 10, 11, 12, 13, 14 respectively) but not from M15, M16, Δ27, mock, dLeu and GST alone (3, 4, 6, 7, 8 and 15 respectively). Lane 2 shows RNase I treated sample and lane 5 showing input that is 10% of HSV-1 wt infected cell extract lysate.

Panel II: Unbound pulled down proteins western blotted with anti ICP27 mix up 1113 and 1119 and showed presence and amount of ICP27 in different cell extracts (lanes 1, 2 and 5-14) and absence of ICP27 in Δ27, mock infected HeLa cell extracts (lanes 3 and 4).

Panel III: Schematic diagram of ICP27 showing the position of the mutants


I
His-ICP27
Input
GST-p62
GST alone
GST-TAP
66KDa
ICP27
1
2
3
4
II
Input
GST
GST-TAP
17+
17+
17+
Δ27
66KDa
ICP27
1
2
3
4


III
97 KDa
GST TAP

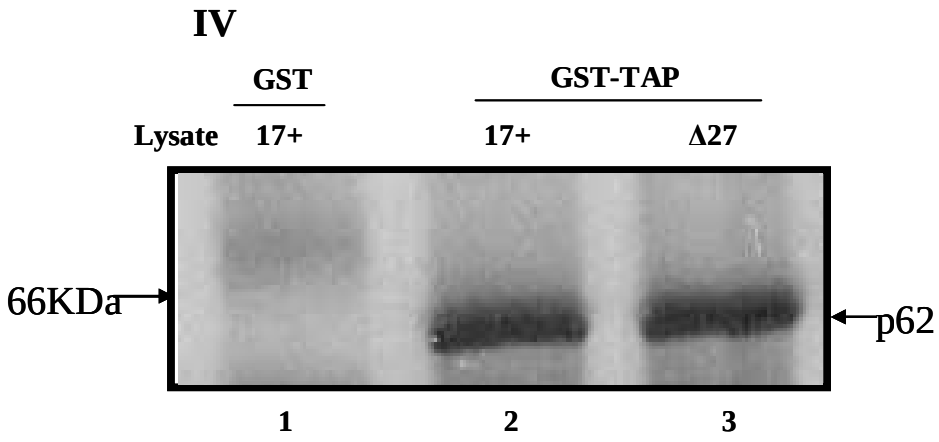


Figure 3.20: GST-TAP interacts *in vitro* with His-tagged ICP27 and pulls down ICP27 from HeLa wt HSV-1 infected cell extracts

Pull down assay and western blot with antibodies.
Panel I: Lysates from *E.coli* expressing GST-TAP, GST alone, GST-NUP 62 and His-ICP27 were incubated with glutathione agarose beads or with Ni-NTA agarose beads. Bound purified GST fusion proteins and GST alone were mixed and incubated with purified His-ICP27 and were eluted with SDS-sample buffer and analyzed on SDS-PAGE followed by western blotting with anti-ICP27 Ab (1113). Lane 1 showing input that is 10% of lysate His-ICP27. Lane 2 GST-NUP 62 pulled down ICP27. Lane 3 GST alone did not pull ICP27 down. Lane 4 GST-TAP pulled down ICP27. The position of ICP27 is indicated. This is the same blot as in Panel I in Figure 3.9.
Panel II: GST-TAP pull-down assays were performed with equal amount of protein from HeLa infected cell extracts (MOI=10). Samples were analyzed on SDS-PAGE followed by western blot with anti-ICP27 Ab (1113). Lane 1, HeLa HSV-1 17+ infected cell extract input. Lane 2, GST alone with HeLa HSV-1 17+ infected cell extract. Lane 3, GST-TAP with HeLa HSV-1 17+ infected cell extract. Lane 4, GST-TAP with HeLa Δ27 infected cell extract.
Panel III: Lysates from *E.coli* expressing GST-TAP, were incubated with glutathione agarose beads beads. Bound purified GST-TAP fusion protein analyzed on SDS-PAGE followed by western blotting with anti-GST Ab. Significant expression of GST-TAP was achieved and indicated. Bands lower down shows smaller truncate of that protein.
Panel IV: Samples analyzed on SDS-PAGE followed by western blot with anti-NUP 62 Ab showed level of NUP 62 in above experiment. Lane 1, GST alone with HeLa HSV-1 17+ infected cell extract. Lane 2, GST-TAP with HeLa HSV-1 17+ infected cell extract. Lane 3, GST-TAP with HeLa Δ27 infected cell extract.

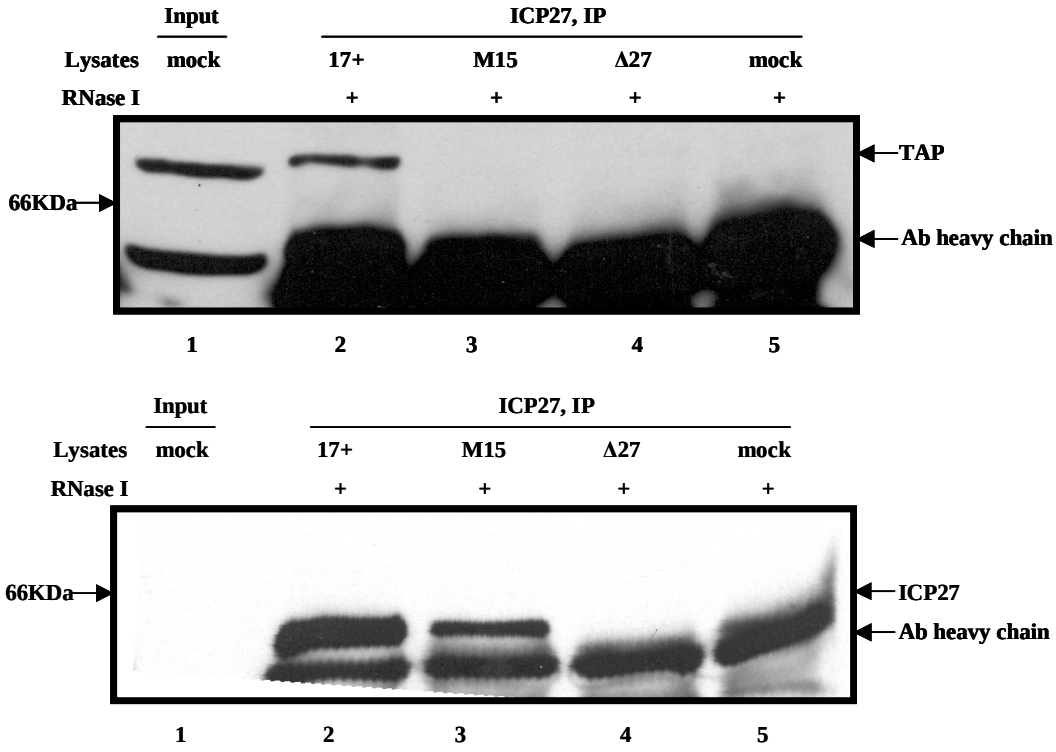


Figure 3.21: ICP27 and TAP co-immunoprecipitate from HeLa cell extracts

ICP27 co-precipitated TAP: Immunoprecipitation test was carried out by mixing anti-ICP27 antibodies 1113 and 1119 with HSV-1 wt and HSV-1 mutant ICP27 M15 and HSV-1 null Δ27 and mock infected HeLa cell extracts (MOI=10). Complexes formed were separated on a 6% SDS-PAGE gel followed by western blotting with anti-TAP and ICP27 antibodies.

Panel I shows co-precipitation of TAP in 17+ infected HeLa cell extracts in the presence of RNase I (lane 2) by ICP27 but no co precipitation with M15, Δ27 and mock infected (lanes 3,4 and 5). Input, 10% of mock infected HeLa cell extract corresponding to TAP around 75 KDa in lane 1 and the lower band could be due cellular protein that crosses reaction with this antibody.

Panel II shows precipitation of ICP27 in 17+ and M15 infected HeLa cell extracts in the presence of RNase I (lane 2 and 3) by its antibody but no co precipitation in Δ27 and mock infected cell extracts (lanes 4 and 5).

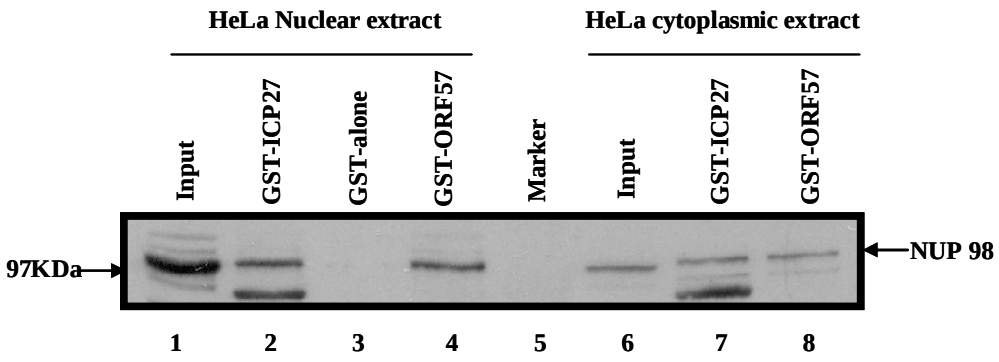
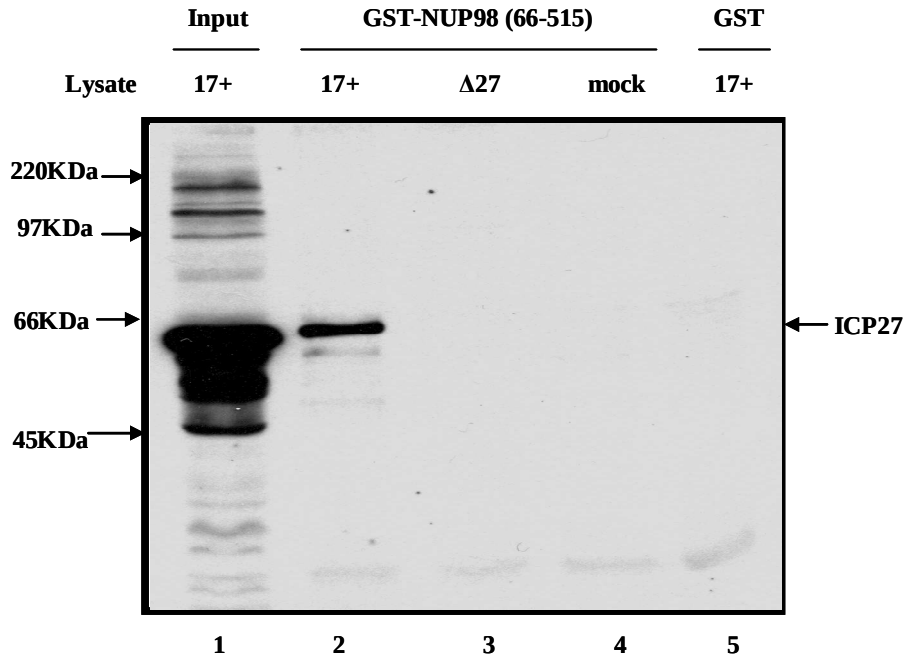


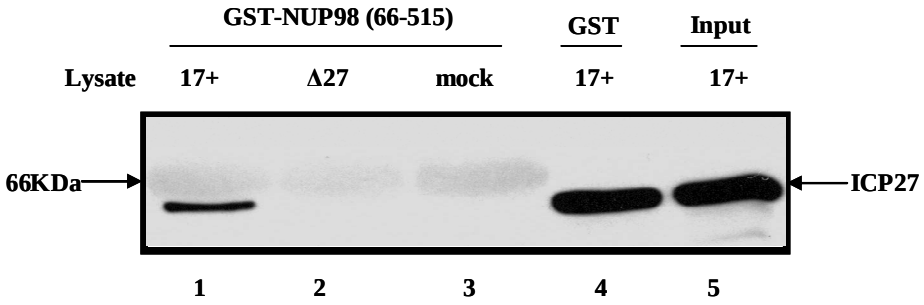
Figure 4.1: ICP27 and its homologue ORF57 interact with NUP 98 in the GST pull down assay.

Nuclear and cytoplasmic HeLa cell extracts were used in a GST pull down with GST-ICP27, GST-ORF57 and GST alone and were western blotted with anti NUP 98 (L-20) antibody. GST-ICP27 interacted with nuclear (lane 2) and cytoplasmic (lane 7) NUP 98 and did not interact with GST in nuclear extracts (lane 3). Also GST-ORF57 interacted with nuclear (lane 4) and cytoplasmic (lane 8) NUP 98. Lane 5 is a Rainbow marker lane. Lane 1 showing input that is 10% of mock infected cell extract lysate

I


Input
GST-NUP98 (66-515)
GST
Lysate
17+
17+
Δ27
mock
17+
220KDa
97KDa
66KDa
45KDa
ICP27
1
2
3
4
5

II


GST-NUP98 (66-515)
GST
Input
Lysate
17+
Δ27
mock
17+
17+
66KDa
ICP27
1
2
3
4
5

III

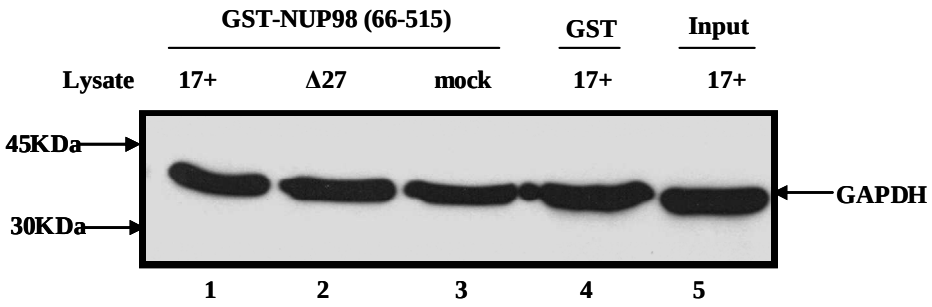

GST-NUP98 (66-515)
GST
Input
Lysate
17+
Δ27
mock
17+
17+
45KDa
30KDa
GAPDH
1
2
3
4
5

Figure 4.2: NUP 98 interacts with ICP27 in the pull down assay.

GST-NUP 98 (66-515) and GST alone expressed and purified and mixed with lysates of HeLa cells infected with wt HSV-1 and mutant HSV-1 and used in pull down assay

Panel I: GST-NUP 98 (66-515) and GST incubated with HSV-1 wt and ICP27 null mutant Δ27 and mock infected HeLa cell extract (MOI=10) and analysed by 10% SDS-PAGE and western blotted with anti ICP27 antibody 1113. The blot showed interaction of NUP 98 with wt ICP27 (lane 2) but not with Δ27 and mock infected cell extracts nor with GST alone (lanes 3, 4 and 5). Lane 1 showing input that is 10% of HSV-1 wt infected cell extract lysate

Panel II: Unbound samples run on SDS-PAGE and western blotted against ICP27, showed presence of ICP27 in lysates of HeLa cell infected with HSV-1 wild type used in this pull down assay.

Panel III: Above membrane stripped and western blotted with anti GAPDH antibody.

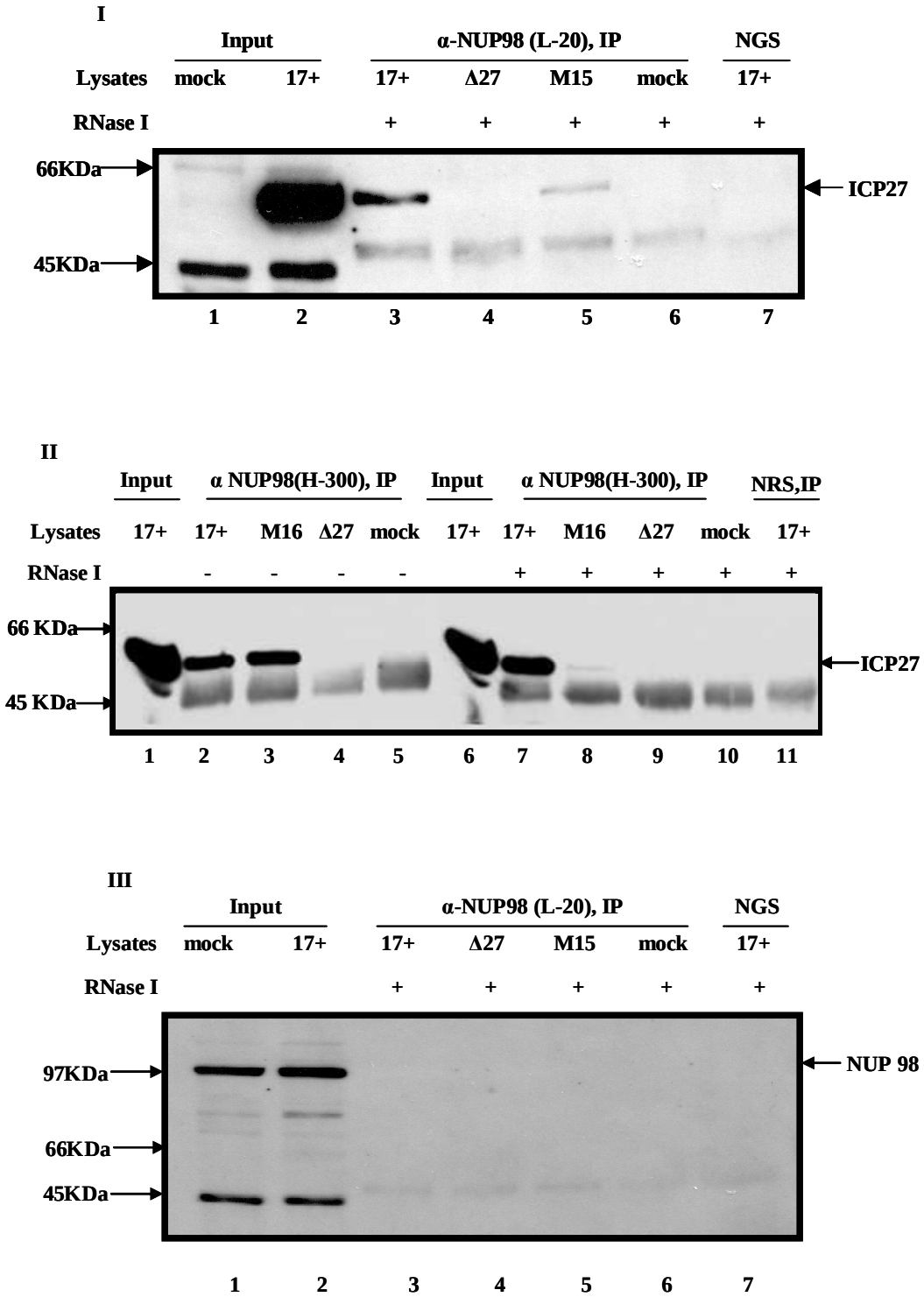


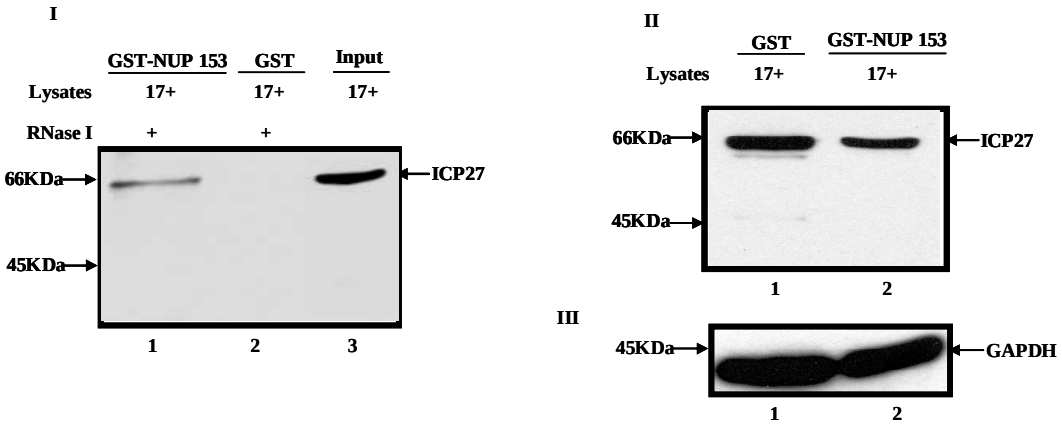
Figure 4.3: ICP27 co-immunoprecipitated from cell extracts by using anti NUP 98 antibodies.

Panel of HSV-1 wt and mutant ICP27 HeLa infected cell lysates (MOI=10) incubated with anti NUP 98 for co-immunoprecipitation experiments.

Panel I: Anti NUP 98 (L-20) goat antibody used for immunoprecipitation followed by SDS-PAGE and western blotted with anti ICP27 1113 antibody. As shown in HSV-1 wt 17+ (lane 3) and point mutant M15 (lane5) infected cell lysates, ICP27 was co-precipitated but not in Δ27 and mock infected cell lysates (lanes 4 and 6). Lanes 1 and 2 shows input mock and HSV-1 17+ infected cell lysates showing the position of ICP27. Also normal goat serum did not precipitate ICP27 from HSV-1 wt 17+ infected cell (ane 7).

Panel II: Anti NUP 98 (H-300) rabbit antibody used for immunoprecipitation followed by SDS-PAGE and western blotted with Anti ICP27 1113 antibody. As shown in both RNase I treated and untreated HSV-1 wt 17+ (lanes 2 and 7) and another point mutant M16 (lanes 3 and 8) infected cell lysates, ICP27 co-precipitated but not in Δ27 and mock infected cell lysates (lanes 4 and 6). Lanes 1 and 6 showing input that is 10% of HSV-1 wt infected cell extract lysate. Also normal rabbit serum did not precipitate ICP27 from wt 17+ infected cell lysate (lane11).

Panel III: Anti NUP 98 (L-20) goat antibody used for immunoprecipitation followed by SDS-PAGE and western blotted with anti NUP 98 (L-20) antibody. As shown anti NUP 98 (L-20) antibody did not precipitate NUP 98 from infected and uninfected cells.


I
GST-NUP 153
GST
Input
Lysates
17+
17+
17+
RNase I
+
+
66KDa
45KDa
ICP27
1
2
3
II
GST
GST-NUP 153
Lysates
17+
17+
66KDa
45KDa
ICP27
1
2
III
45KDa
GAPDH
1
2

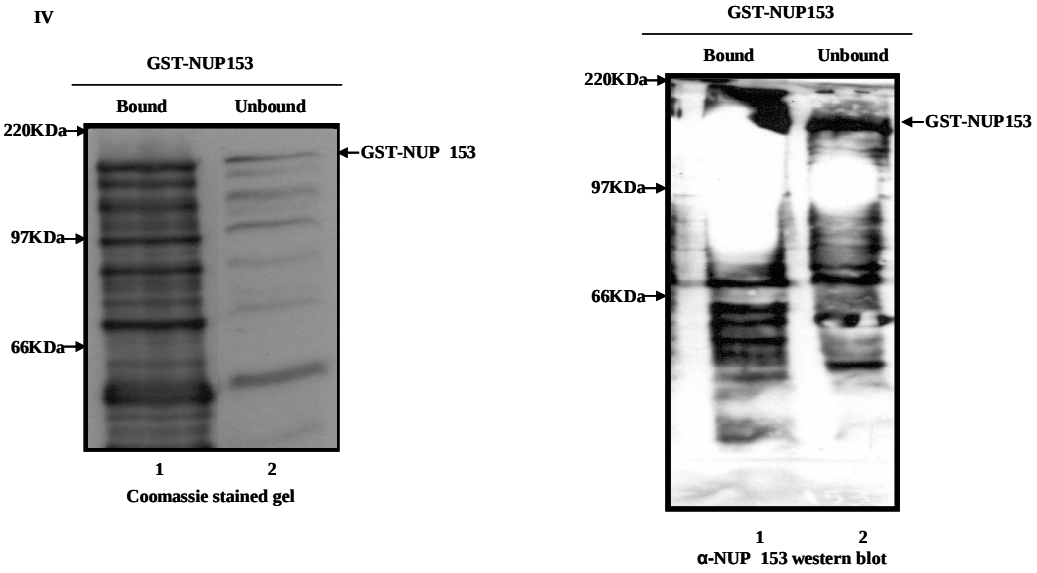

IV
GST-NUP153
Bound
Unbound
220KDa
97KDa
66KDa
GST-NUP 153
1
2
Coomassie stained gel
V
GST-NUP153
Bound
Unbound
220KDa
97KDa
66KDa
GST-NUP153
1
2
α-NUP 153 western blot

Figure 4.4: Recombinant GST-NUP 153 interacts directly with ICP27 in the pull down assay.

GST-NUP 153 incubated with wt HSV-1 17+ HSV-1 infected HeLa cell extracts (MOI=10) followed by RNase I traetment.

Panel I: Pull down assay samples were run on 10% SDS-PAGE and western blotted with anti ICP27 antibody 1113 and showed interaction of ICP27 and NUP 153 in the presence (lane 1) of RNase I. Lane 3 showing input that is 10% of HSV-1 wt infected cell extract lysate. ICP27 was not precipitated with GST alone (lane 2).

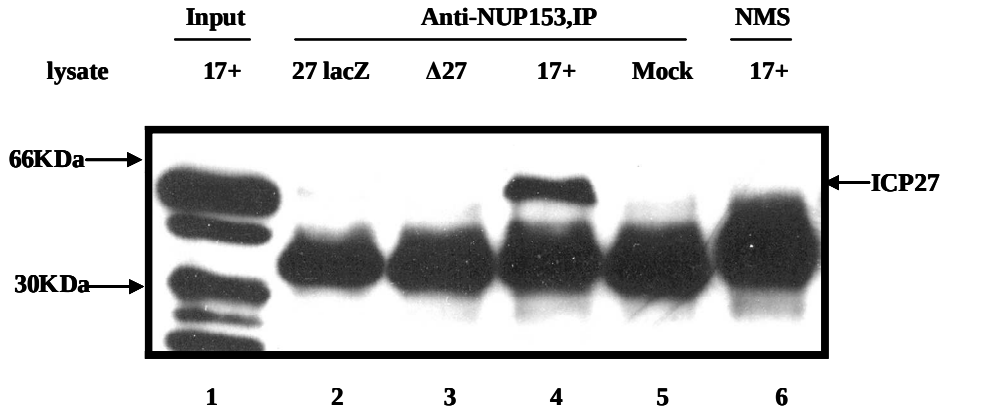
Panel II: Unbound samples of this pull down assay run on 10% SDS-PAGE and western blotted with anti ICP27 antibody 1113 that showed ICP27 in both GST alone (lane 1) and GST-NUP 153 (lane 2) pull down samples.

Panel III: Above unbound membrane stripped and western blotted with anti-GAPDH antibody. Lane 1, HSV-1 wt infected cell extract incubated with GST alone; lane 2, HSV-1 wt infected cell extract incubated with GST-NUP 153.

Panel IV: Coomassie blue stained 6% SDS-PAGE gel showing the expression and purification of GST-NUP 153. Lane 1 bound purified GST-NUP 153 fusion protein; lane 3, unbound purified GST-NUP 153 fusion protein.

Panel V: Western blotted 6% SDS-PAGE gel showing the expression and purification of GST-NUP 153 using anti NUP 153 antibody. Lane 1 bound purified GST-NUP 153 fusion protein; lane 3, unbound purified GST-NUP 153 fusion protein.

I


Input
Anti-NUP153,IP
NMS
lysate
17+
27 lacZ
Δ27
17+
Mock
17+
66KDa
30KDa
ICP27
1
2
3
4
5
6

II

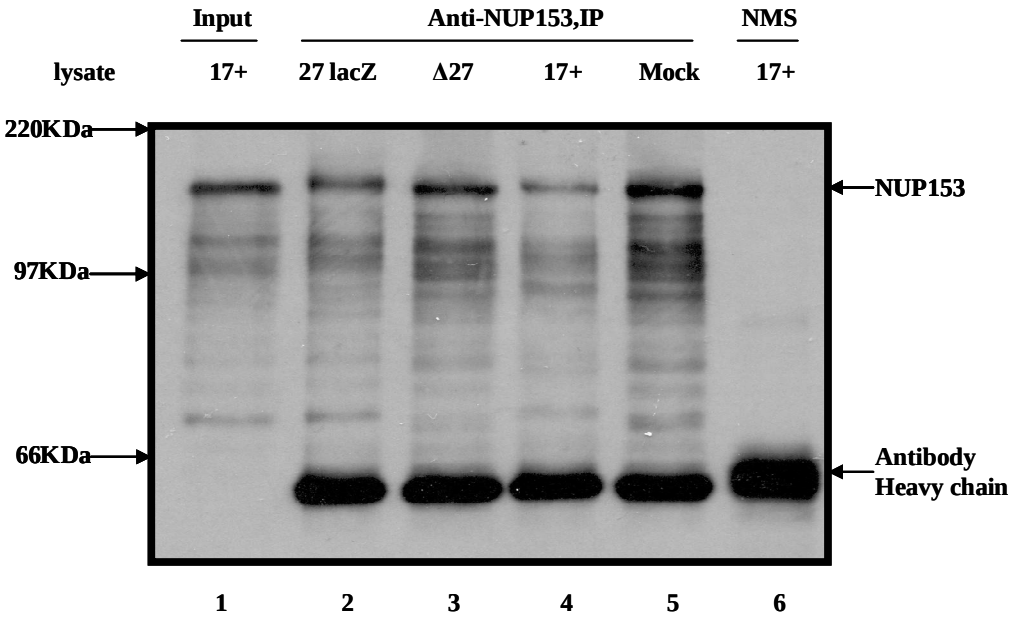

Input
Anti-NUP153,IP
NMS
lysate
17+
27 lacZ
Δ27
17+
Mock
17+
220KDa
97KDa
66KDa
NUP153
Antibody
Heavy chain
1
2
3
4
5
6

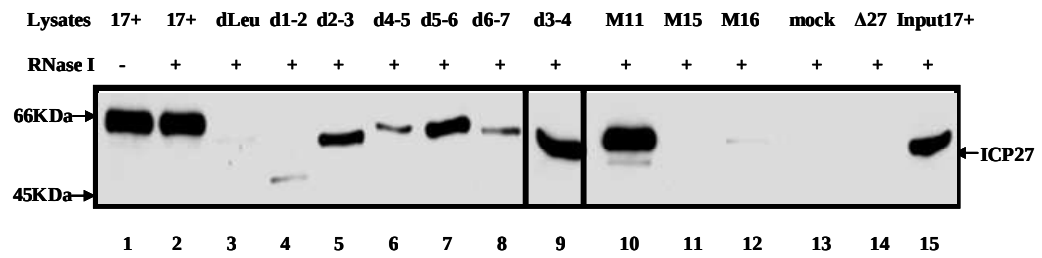
Figure 4.5: Anti-NUP 153 antibody co-immunoprecipitated ICP27 from HSV-1 infected cell extracts.

Specific monoclonal antibody against nucleoporin 153 was used to co-immunoprecipitate ICP27 from HSV-1 infected cell lysates (MOI=10).

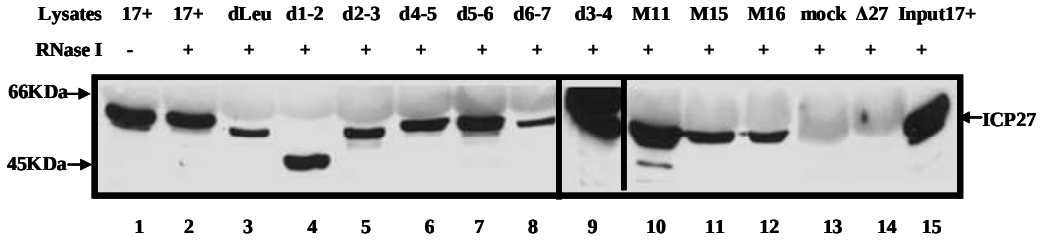
Panel I: Range of HSV-1 wt and mutant HeLa infected cell lysates immunoprecipitated with anti NUP 153 antibody and run on a 6% SDS-PAGE gel and followed by western blotting with anti-ICP27 antibody (1113). Data demonstrated nucleoporin 153 co-precipitated ICP27 from HSV-1 wt infected cell extracts (lane 4) but not from its null mutants 27lac Z (lane 2) and Δ27 (lane 3) and mock infected cell extracts (lane 5). Also co-immunoprecipitation with NMS did not bring down ICP27 from wt infected cell extract (lane 6). Lane 1 showing input that is 10% of HSV-1 wt infected cell extract lysate

Panel II: Anti-NUP 153 antibody precipitated NUP 153 in all samples (lanes 1, 2, 3, 4, 5) except (lane 6) where normal mouse serum (NMS) was used as a control antibody and approximately a 153 KDa band corresponding to nucleoporin 153 was detected with mock input sample (lane 1). The lower bands about 55 KDa represent heavy chains of the antibody (lanes 2, 3, 4, 5, 6).

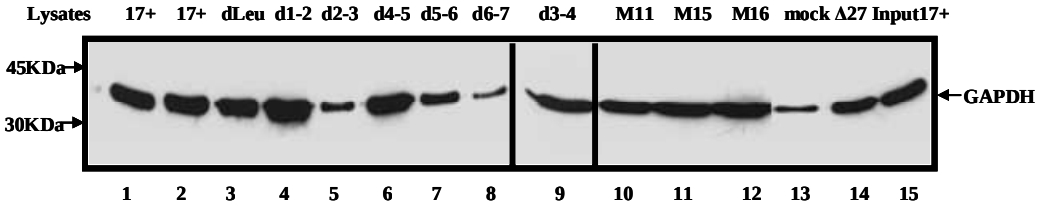
I


Lysates 17+ 17+ dLeu d1-2 d2-3 d4-5 d5-6 d6-7 d3-4 M11 M15 M16 mock Δ27 Input17+
RNase I - + + + + + + + + + + + + + +
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

II


Lysates 17+ 17+ dLeu d1-2 d2-3 d4-5 d5-6 d6-7 d3-4 M11 M15 M16 mock Δ27 Input17+
RNase I - + + + + + + + + + + + + + +
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

III


Lysates 17+ 17+ dLeu d1-2 d2-3 d4-5 d5-6 d6-7 d3-4 M11 M15 M16 mock Δ27 Input17+
45KDa
30KDa
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

IV

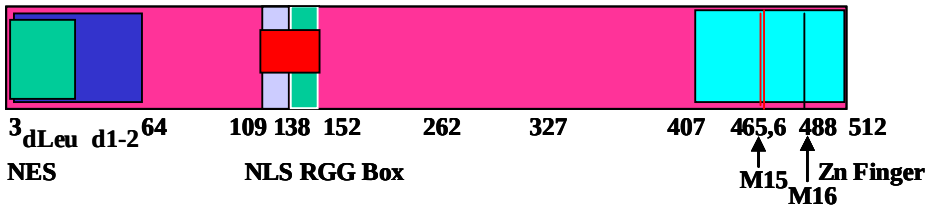

3 dLeu d1-2 64 109 138 152 262 327 407 465,6 488 512
NES
NLS RGG Box
M15
M16
Zn Finger

Figure 4.6: Mapping the interacting regions of ICP27 with NUP 153 using GST pull down assay.

Series of wt and ICP27 mutant HeLa HSV-1 infected cell extracts (MOI=10) incubated with fusion protein GST-NUP153 in the presence of RNase I and pulled down and separated by 10% SDS-PAGE gel and analysed by western blot.

Panel I: Bound pulled down proteins western blotted with anti ICP27 antibodies1113 and 1119 and showed NUP 153 pulled down ICP27 from wt, d2-3, d3-4, d4-5, d5-6, d6-7 and partially d1-2 and M16 (lanes 1, 2, 4, 5, 6, 7, 8, 9, 10 and 12 respectively) but not with Δ27, mock, dLeu and M15 (13, 14, 3 and 11 respectively). Lane 1 shows RNase I non-treated sample of wt infected cell extract and lane 15 showing input that is 10% of HSV-1 wt infected cell extract lysate.
Panel II: Unbound pulled down proteins western blotted with anti ICP27 antibodies 1113 and 1119 and showed presence and amount of ICP27 in different cell extracts (lanes 1-12 and 15) and absence of ICP27 in Δ27, mock infected HeLa cell extracts (lanes 13 and 14)

Panel III: Unbound samples membrane stripped and western blotted with anti GAPDH antibody showed the relative concentrations of the proteins, lanes 5, 8 and 13 showing low amount of the protein because of using less lysate.

Panel IV: Schematic representative of ICP27 mutants.

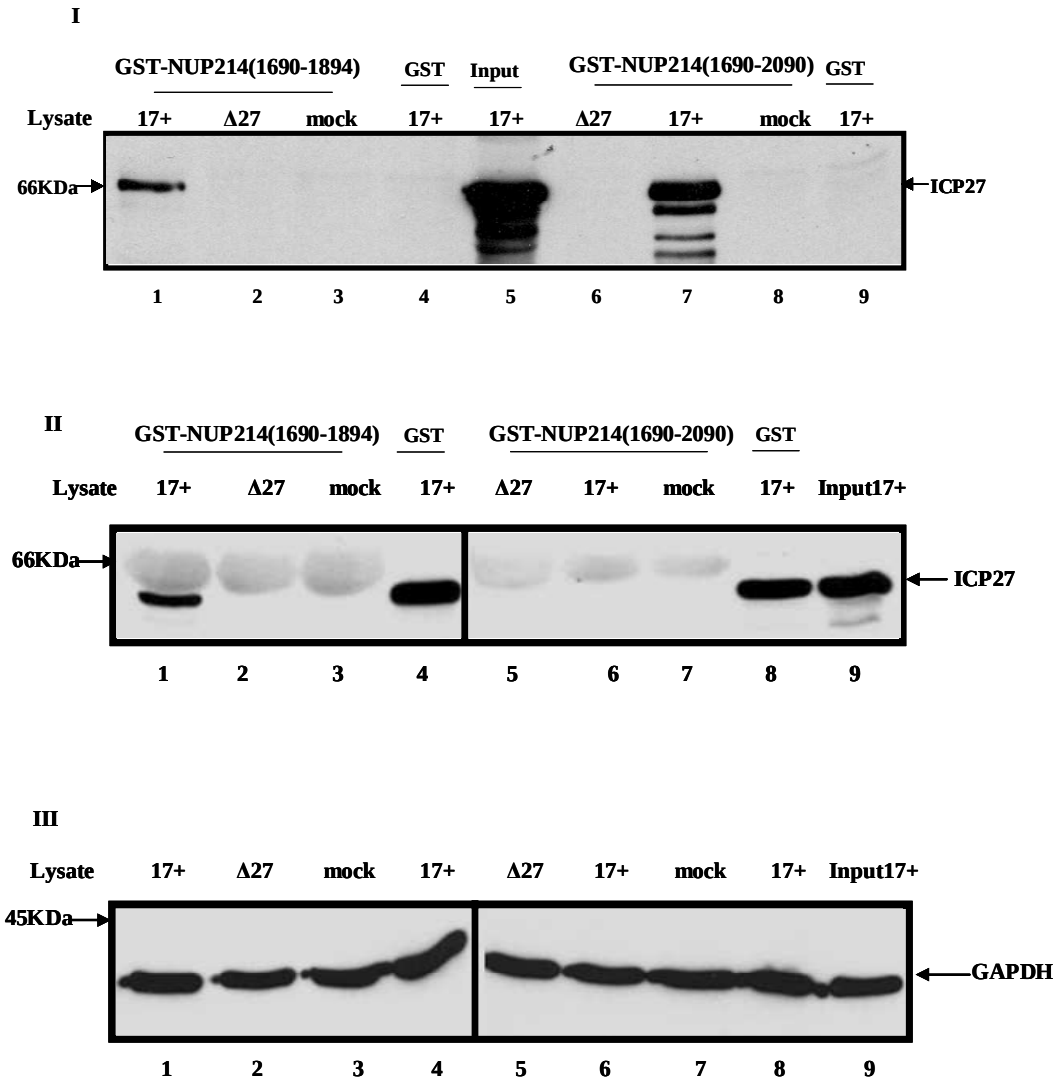

I
GST-NUP214(1690-1894)
GST
Input
GST-NUP214(1690-2090)
GST
Lysate
17+
Δ27
mock
17+
17+
Δ27
17+
mock
17+
66KDa
ICP27
1 2 3 4 5 6 7 8 9
II
GST-NUP214(1690-1894)
GST
GST-NUP214(1690-2090)
GST
Lysate
17+
Δ27
mock
17+
Δ27
17+
mock
17+
Input17+
66KDa
ICP27
1 2 3 4 5 6 7 8 9
III
Lysate
17+
Δ27
mock
17+
Δ27
17+
mock
17+
Input17+
45KDa
GAPDH
1 2 3 4 5 6 7 8 9

Figure 4.7: NUP 214/CAN interacts with ICP27 in the pull down assay.

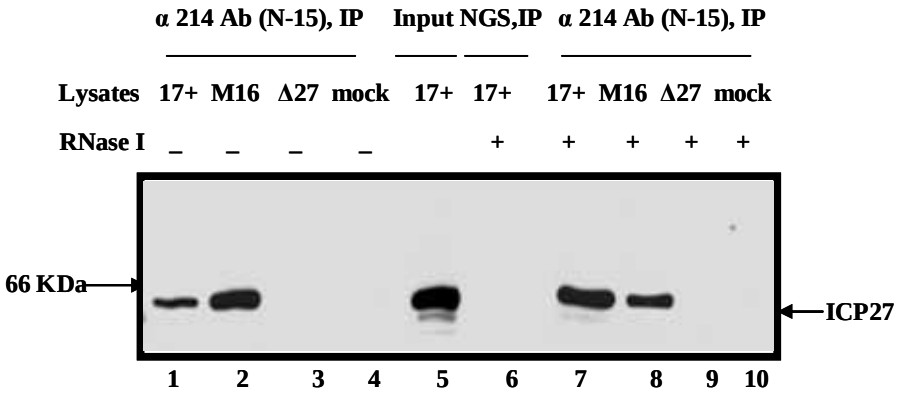
Purified GST fusion protein NUP214/CAN (aa 1690-1894), (aa 1690-2090) and GST alone incubated with wt 17+ HSV-1 and mutant ICP27 HSV-1 (MOI=10) and mock infected HeLa cell extracts and separated on 10% SDS-PAGE and analysed by using anti ICP27 antibody.

Panel I: Western blot with anti ICP27 1113 antibody showed ICP27 pulled down by NUP 214 in HSV-1 17+ infected cell lysate (lanes 1 and 7) but not with HSV-1 Δ27 and mock infected cell lysates (lanes 2, 3, 6 and 8). Also GST alone did not bring down ICP27 from HSV-1 wt 17+ cell extract (lanes 4 and 9). Lane 5 showing input that is 10% of HSV-1 wt infected cell extract lysate.

Panel II: Unbound cell extracts of this experiment fractionated on a gel and western blotted with anti ICP27 1113 antibody showing the presence of ICP27 in HSV-1 17+ infected cell sample (lane 2) and absence in Δ27 and mock infected cell lysates (lanes 3, 4, 7 and 9). Lanes 4 and 8 presents unbound ICP27 in interaction with GST alone. Lane 6 did not presents unbound ICP27 in HSV-1 17+ infected could be due to very efficient interaction with NUP 214.

Panel III: The above panel II membrane western blot reprobed with anti GAPDH antibody.

I


α 214 Ab (N-15), IP
Input
NGS,IP
α 214 Ab (N-15), IP
Lysates 17+ M16 Δ27 mock 17+ 17+ 17+ M16 Δ27 mock
RNase I _ _ _ _ + + + + +
66 KDa
ICP27
1 2 3 4 5 6 7 8 9 10

II

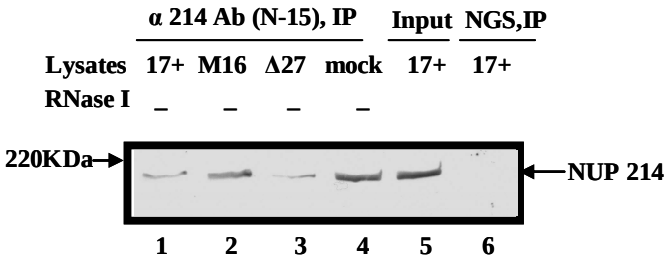

α 214 Ab (N-15), IP
Input
NGS,IP
Lysates 17+ M16 Δ27 mock 17+ 17+
RNase I _ _ _ _
220KDa
NUP 214
1 2 3 4 5 6

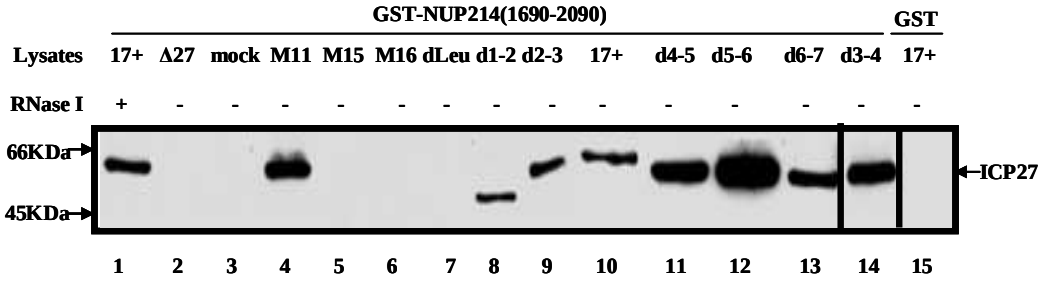
Figure 4.8: ICP27 and NUP 214/CAN co-immunoprecipitate from HeLa cell extracts.

Immunoprecipitation test carried out by mixing anti-NUP 214 (N-15) antibody with HSV-1 wt, M16 point mutant, ICP27 null Δ27 (MOI=10) and mock infected HeLa cell extracts. Also HSV-1 wt infected cell extract incubated with normal goat serum (NGS) was used as a control.

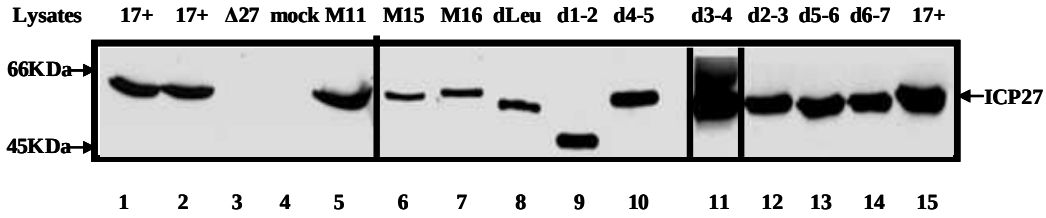
Panel I: Complexes formed were separated on a 10% SDS-PAGE followed by western blotting with anti-ICP27 (1113) antibody. Lanes 1 and 7, co-precipitation of ICP27 in HSV-1 17+ infected cell extract in the absence and presence of RNase I; lanes 2 and 8, co-precipitation of HSV-1 mutant M16 ICP27 under non RNase I treated and RNase I treated condition respectively; lanes 3,4,9 and 10, demonstrates there are no protein precipitated in HSV-1 Δ27 and mock infected cell extracts; lane 6, no precipitation of ICP27 by normal goat serum; lane 5, showing input that is 10% of HSV-1 wt infected cell extract lysate.

Panel II: The first six lanes of Panel I were stripped and western blotted with anti-FG repeat NUP 414 antibody showing precipitation of NUP 214. Lane 1, 17+ HSV-1 infected cell extract in absence of RNase I; lane 2, HSV-1 mutant M16 ICP27 infected cell extract under non-RNase I treatment; lane 3, HSV-1 Δ27 infected cell extract in absence of RNase I; lane 4, mock infected cell extract in absence of RNase I, lane 5, showing input that is 10% of HSV-1 wt infected cell extract lysate; lane 6, 17+ HSV-1 infected cell extract incubated with NGS in presence of RNase I.

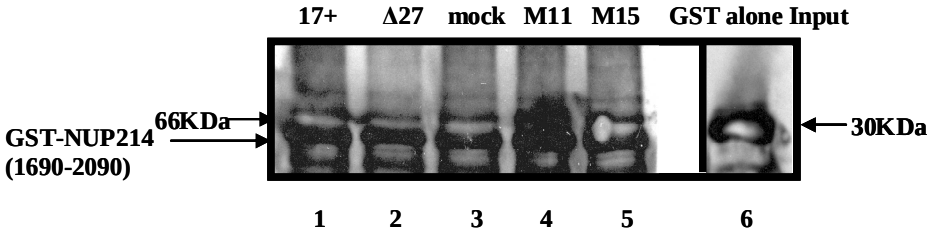
I


GST-NUP214(1690-2090)
GST
Lysates 17+ Δ27 mock M11 M15 M16 dLeu d1-2 d2-3 17+ d4-5 d5-6 d6-7 d3-4 17+
RNase I + - - - - - - - - - - - - - -
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

II


Lysates 17+ 17+ Δ27 mock M11 M15 M16 dLeu d1-2 d4-5 d3-4 d2-3 d5-6 d6-7 17+
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

III


17+ Δ27 mock M11 M15 GST alone Input
GST-NUP214
(1690-2090)
66KDa
30KDa
1 2 3 4 5 6

IV

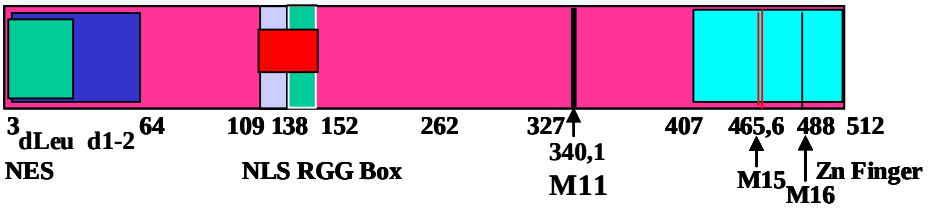

3 dLeu d1-2 64 109 138 152 262 327 340,1 407 465,6 488 512
NES
NLS RGG Box
M11
M15
M16
Zn Finger

Figure 4.9: Mapping the interacting regions of ICP27 with NUP 214 using GST pull down assay.

Fusion protein GST-NUP 214 (1690-2090) was used to pull down ICP27 from a panel of the HSV-1 ICP27 wt and mutated viruses.

Panel I: HeLa cells were infected with wt HSV-1 and HSV-1 ICP27 mutant viruses (MOI=10). Cell extracts were incubated with GST-NUP 214 (1690-2090) and complexes formed were separated on a 10% SDS-PAGE gel and western blotted with either anti ICP27 antibody 1113 or 1119. GST-NUP 214 (1690-2090) interacted with wt ICP27 in the presence (lane 1) and absence (lane 10) of RNase I. HSV-1 Mutant ICP27 M11, d1-2, d2-3, d4-5, d5-6, d6-7 and d3-4 (lanes 4, 8, 9, 11, 12, 13 and 14 respectively) also interacted with GST-NUP 214. HSV-1 ICP27 mutants Δ27, M15, M16 and dLeu (lanes 2, 5, 6 and 7 respectively) did not interact. Also mock-infected cell extract did not show any ICP27 band (lane 3). GST alone in lane 16 did not pull down ICP27 from 17+ HSV-1 infected cell extract.

Panel II: Unbound samples run on 10% SDS-PAGE gel and western blotted with anti ICP27 1113 and 1119 antibodies and demonstrated presence of ICP27 wt and mutants in the samples except in Δ27 and mock infected cell extracts.

Panel III: First five lanes of the membrane from Panel 1 were stripped and western blotted with anti GST antibody showing presence and similar amounts of input GST-NUP 214 (1690-2090) (lanes 1-5) and GST alone (lane 6) used in this experiment.

Panel IV: Schematic representative of ICP27 mutants.

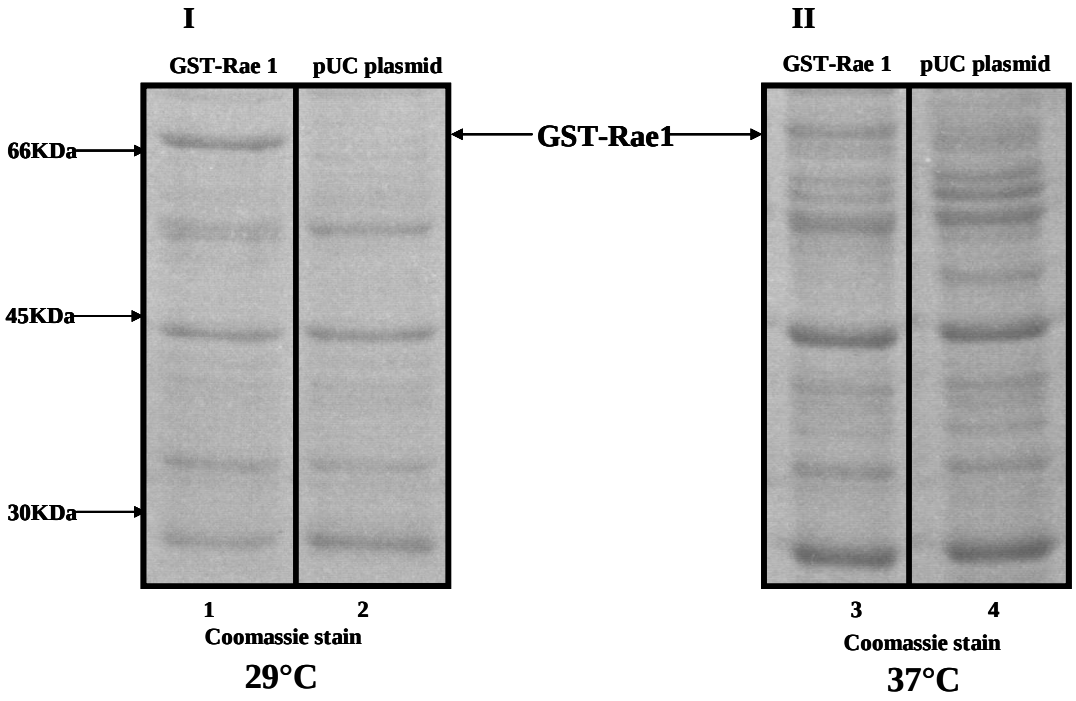

I
II
GST-Rae 1
pUC plasmid
GST-Rae 1
pUC plasmid
66KDa
45KDa
30KDa
GST-Rae1
1
2
3
4
Coomassie stain
Coomassie stain
29°C
37°C

Figure 5.1: Optimum expression of GST-Rae1 obtained at 29°C

Coomassie blue stained 10% SDS-PAGE gel showing the purified proteins from the bacterial lysates bound to Glutathione 4B sepharose. Full length GST-Rae 1 is marked.

Panel I, bound, purified GST fusion protein from bacterial cultures containing GST-Rae 1 and negative control no GST-Rae 1 expressing plasmids were induced with IPTG at 29°C (lane 1 GST-Rae 1 and lane 2 negative control pUC plasmid).

Panel II, bound, purified GST fusion protein from bacterial cultures containing GST-Rae 1 and negative control no GST-Rae 1 expressing plasmids were induced with IPTG at 37°C (lane 1 GST-Rae 1 and lane 2 negative control pUC plasmid).

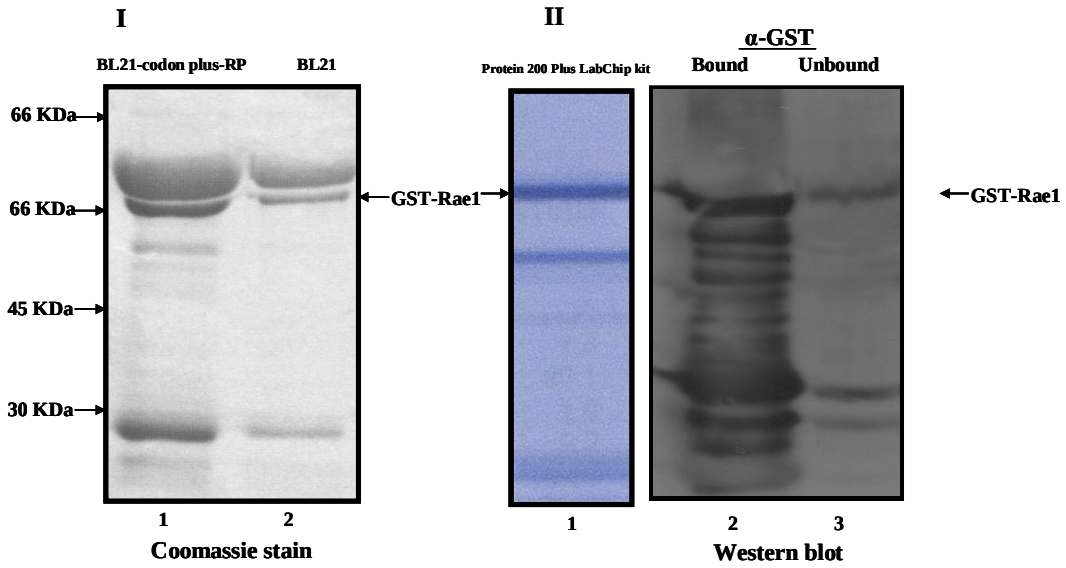


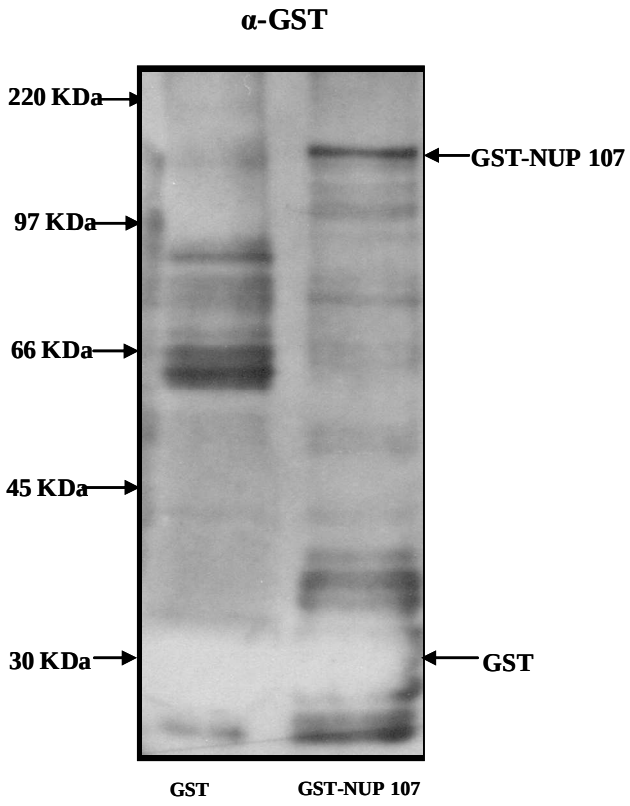
Figure 5.2: Maximum expression of GST-Rae1 obtained using BL21-Codon plus-RP competent *E.coli* cells

10% SDS-PAGE gel showing the purified protein from the bacterial lysates bound to Glutathione 4B sepharose.

Panel I, bacterial cultures of BL21 and BL21-codon plus-RP competent E.coli cells containing GST-Rae1 expressing plasmids were induced with IPTG at 29°C (lane 1 BL21-codon plus-RP and lane 2 BL21).

Panel II, Purified GST-Rae1 from BL21-codon plus-RP was analysed using a Protein 200 Plus LabChip kit (Agilent technologies) (lane 1), and western blotting with anti-GST antibody (lane 2, bound to beads. lane 3 unbound to beads) in order to monitor expression, protein concentration and purity. Full length GST-Rae1 is marked.

I


α-GST
220 KDa
97 KDa
66 KDa
45 KDa
30 KDa
GST-NUP 107
GST
GST
GST-NUP 107

II

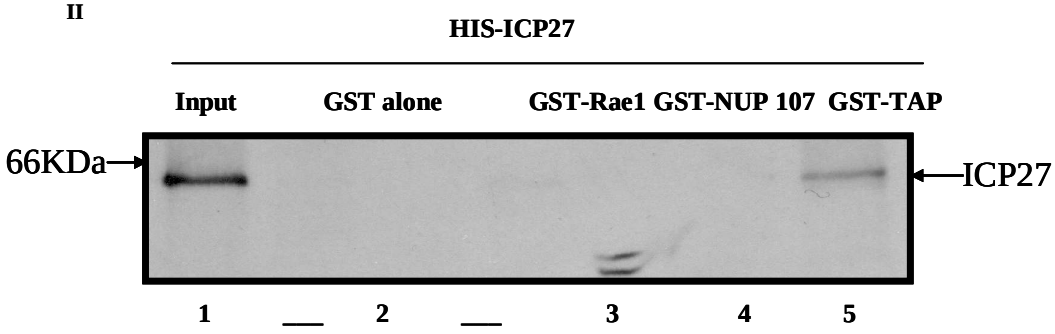
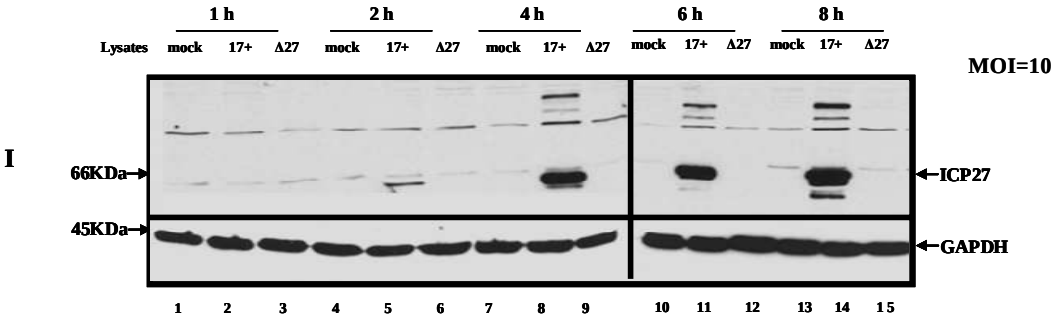
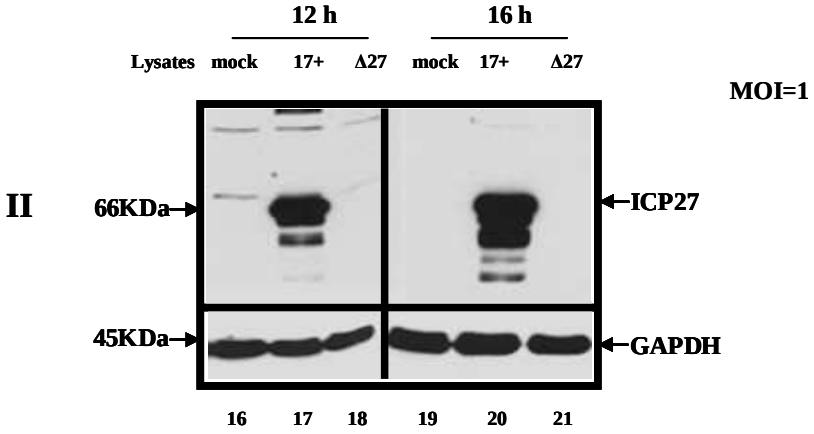

HIS-ICP27
Input
GST alone
GST-Rae1
GST-NUP 107
GST-TAP
66KDa
ICP27
1
2
3
4
5

Figure 5.3: GST-Nucleoporin 107 and GST- Rae 1 do not interact with ICP27 from His-ICP27.

Panel I: Western blot of purified GST-NUP 107 fusion protein and GST alone with anti-GST antibody. Lane 1 shows expression of GST alone and lane 2 expression of GST-NUP 107.

Panel II: Lysates from *E. coli* expressing GST-NUP 107, GST-Rae 1, GST alone, GST-TAP and His-ICP27 were incubated with glutathione agarose beads or with Ni-NTA agarose beads respectively. Bound purified GST fusion proteins and GST alone were mixed and incubated with purified His-ICP27 and were eluted with SDS-sample buffer and analyzed on SDS-PAGE followed by western blotting with anti-ICP27 antibody (1113).
Lane 1, showing input that is 10% of His-ICP27 input; lane 2, GST-alone did not pull down ICP27; lane 3, GST-Rae 1 did not pull ICP27 down; lane 4, GST-NUP 107 could not pull down ICP27; lane 5, But GST-TAP pulled down ICP27. The position of ICP27 is indicated.


I
1 h
2 h
4 h
6 h
8 h
Lysates mock 17+ Δ27 mock 17+ Δ27 mock 17+ Δ27 mock 17+ Δ27 mock 17+ Δ27
MOI=10
66KDa
45KDa
ICP27
GAPDH
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


II
12 h
16 h
Lysates mock 17+ Δ27 mock 17+ Δ27
MOI=1
66KDa
45KDa
ICP27
GAPDH
16 17 18 19 20 21

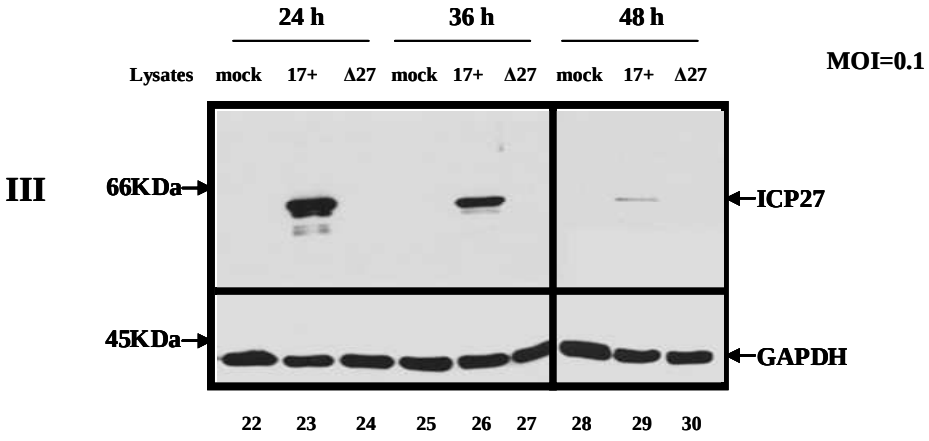
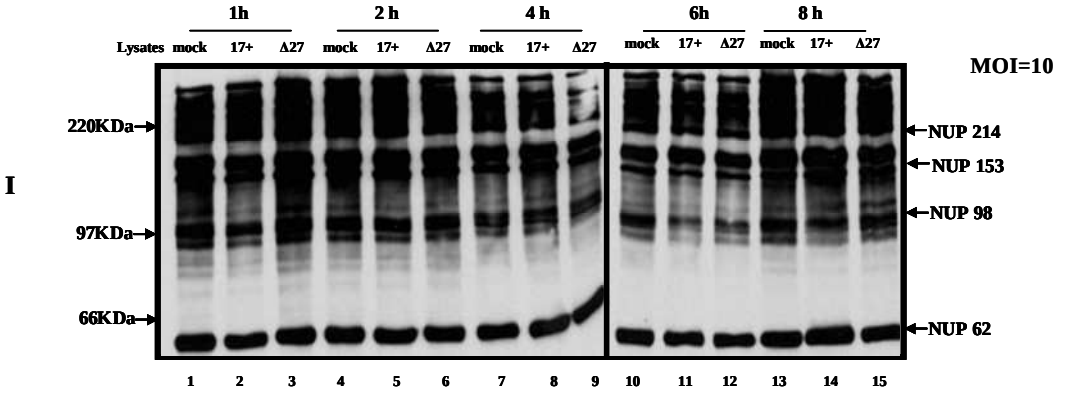
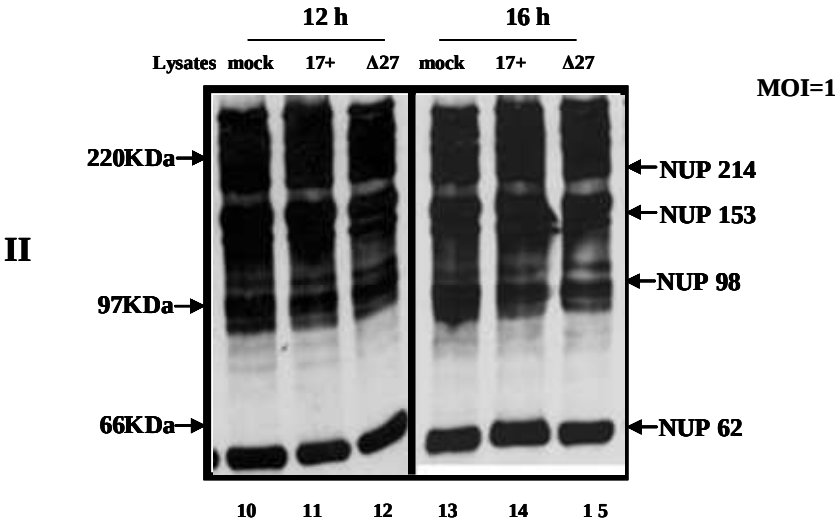

III
24 h
36 h
48 h
Lysates mock 17+ Δ27 mock 17+ Δ27 mock 17+ Δ27
MOI=0.1
66KDa
45KDa
ICP27
GAPDH
22 23 24 25 26 27 28 29 30

Figure 5.4: ICP27 is expressed as early as 2 hours post infection during a time course study

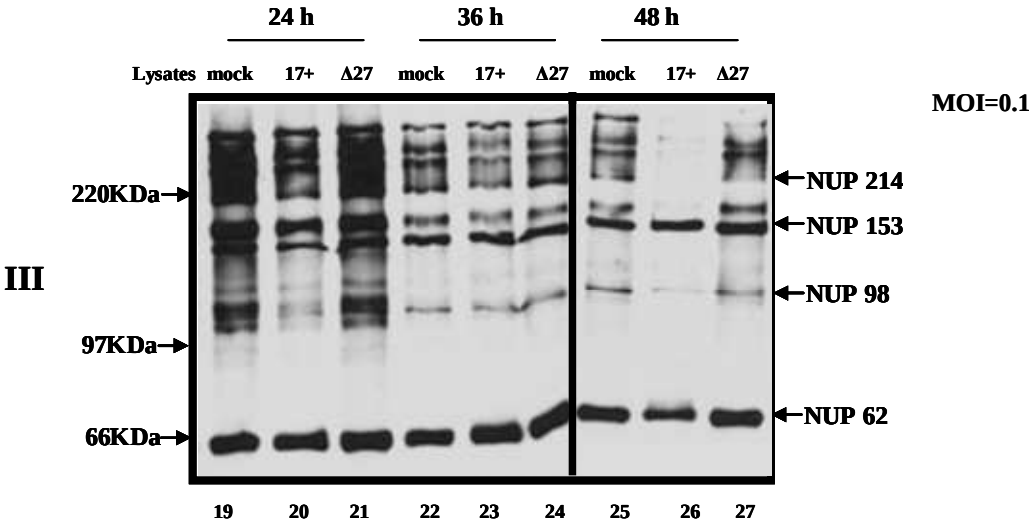
Panels I, II and III Shows western blots for ICP27 with monoclonal anti-ICP27 Ab (H1113) in HeLa infected cell extracts at various times and MOI after infection with wt HSV-1, ICP27 null Δ27 virus and mock infected cells. HeLa cells were grown in 175 mm flasks and infected with wt HSV-1 strain 17+, Δ27 HSV-1 and mock infected with an MOI=10 at 1-8 hpi, MOI=1 at 12-16 hpi and MOI=0.1 at 24-48 hpi. Samples were collected at various time points post-infection, cells pelleted, lysed in Hepes buffer and then in SDS loading buffer, separated by 10% SDS-PAGE and visualised by western blotting with anti-ICP27 monoclonal antibody (H1113). Then membranes were stripped and western blotted with anti GAPDH antibody. Samples used in lanes: 1, 4, 7, 10, 13, 16, 19, 22, 25 and 28 were mock infected cell extracts. Lanes 2, 5, 8, 11, 14, 17, 20, 23, 26 and 29 were HSV-1 17+ infected cell extracts. Lanes 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 wereHSV-1 Δ27 infected cell extracts.


1h
2 h
4 h
6h
8 h
Lysates mock 17+ Δ27 mock 17+ Δ27 mock 17+ Δ27 mock 17+ Δ27 mock 17+ Δ27
MOI=10
220KDa
97KDa
66KDa
NUP 214
NUP 153
NUP 98
NUP 62
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

I


12 h
16 h
Lysates mock 17+ Δ27 mock 17+ Δ27
MOI=1
220KDa
97KDa
66KDa
NUP 214
NUP 153
NUP 98
NUP 62
10 11 12 13 14 1 5

II

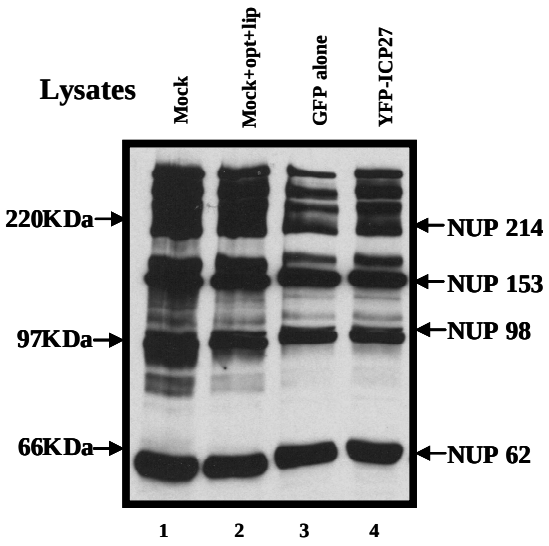

24 h
36 h
48 h
Lysates mock 17+ Δ27 mock 17+ Δ27 mock 17+ Δ27
MOI=0.1
220KDa
97KDa
66KDa
NUP 214
NUP 153
NUP 98
NUP 62
19 20 21 22 23 24 25 26 27

III

Figure 5.5: Various nucleoporins expressed during a time course study of HeLa cells infected by wt HSV-1 and ICP27 null mutant virus

Panels I, II and III Show western blots for nucleoporins with monoclonal anti FG-repeat 414 Ab in HeLa cells infected at various times and various MOI after infection with wt HSV-1, ICP27 null (Δ27) virus and mock infected cells that demonstrates expression of nucleoporins. HeLa cells were grown in 175 mm flasks and infected with wt HSV-1 strain 17+, Δ27 HSV-1 and mock infected cells by MOI=10 at 1-8 hpi, MOI=1 at 12-16 hpi and MOI=0.1 at 24-48 hpi. Samples were collected at various time points post-infection, cells pelleted, lysed in Hepes buffer and then in SDS loading buffer, separated by 10% SDS-PAGE and visualised by western blotting with anti-414 monoclonal antibody. Samples used in lanes: 1, 4, 7, 10, 13, 16, 19, 22, 25 and 28 were mock infected cell extracts. Lanes 2, 5, 8, 11, 14, 17, 20, 23, 26 and 29 were HSV-1 17+ infected cell extracts. Lanes 3, 6, 9, 12, 15, 18, 21, 24, 27 and 30 were HSV-1 Δ27 infected cell extracts.

I


Lysates
Mock
Mock+opt+lip
GFP alone
YFP-ICP27
220KDa
97KDa
66KDa
NUP 214
NUP 153
NUP 98
NUP 62
1
2
3
4

II

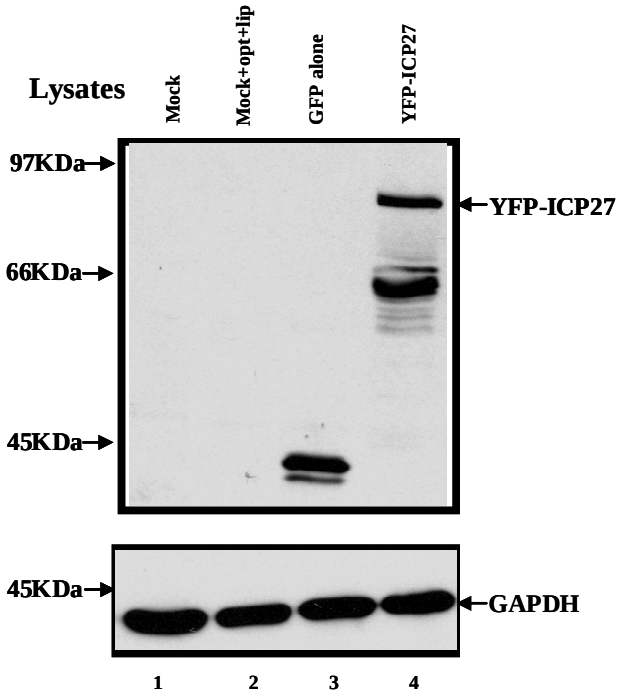
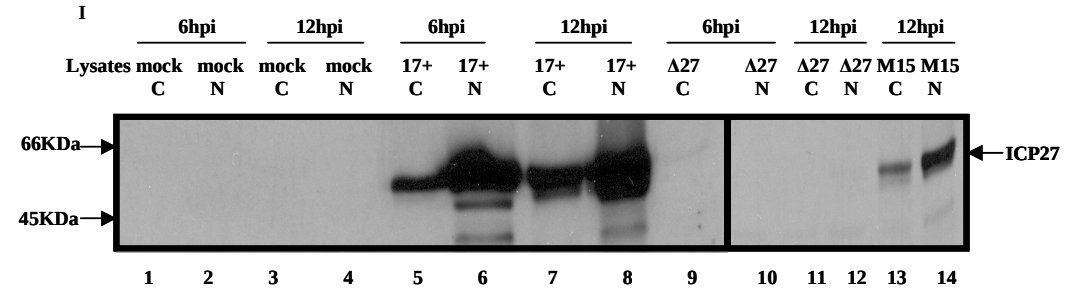

Lysates
Mock
Mock+opt+lip
GFP alone
YFP-ICP27
97KDa
66KDa
45KDa
YFP-ICP27
45KDa
GAPDH
1
2
3
4

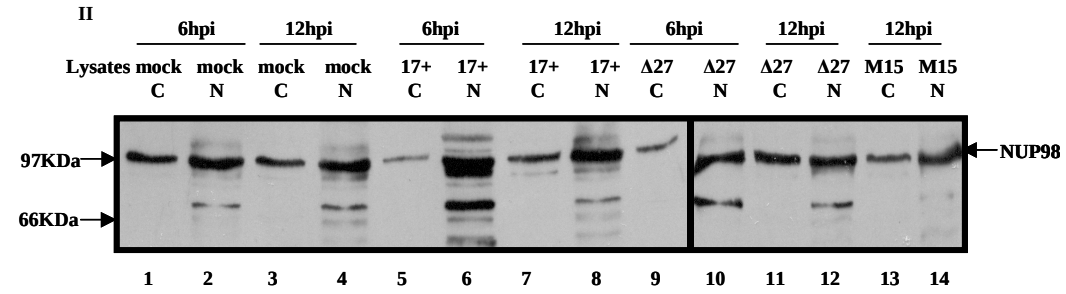
Figure 5.6: Transient co-transfection study of ICP27 showed no change in expression level of nucleoporins.

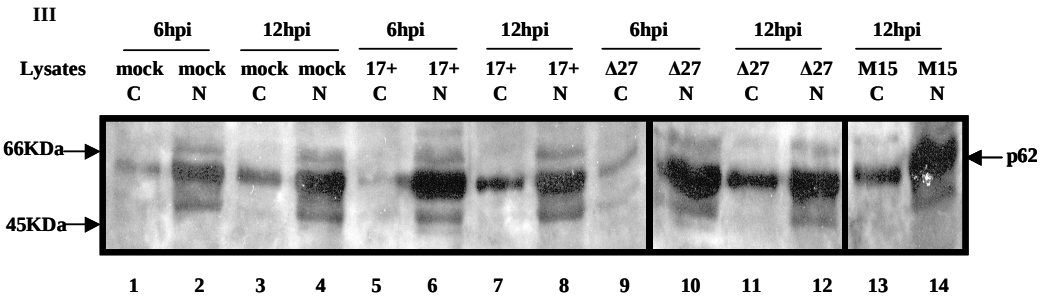
Transient transfection of enhanced yellow fluorescent protein ICP27 (EYFP-ICP27) plasmid, which expresses ICP27 in HeLa cells. After 24 hpi, infected lysates were run on 6% SDS-PAGE and western blotted with specific antibodies.

Panel I: Expression level of NUPs after western blotting with anti FG-repeat NUPs 414 antibody in control samples (lanes 1, 2 and 3) and transfected sample (lane 4).

Panel II: Samples visualised by western blotting with anti GFP antibody to test presence of ICP27 in the transfected sample as it shown appropriate GFP and YFP-ICP27 (lanes 3 and 4).
Stripped membrane immunoblotted against GAPDH indicate equal amount proteins used in this experiment shown in the bottom of this panel.


I
6hpi
12hpi
6hpi
12hpi
6hpi
12hpi
12hpi
Lysates mock mock mock mock 17+ 17+ 17+ 17+ Δ27 Δ27 Δ27 Δ27 M15 M15
C N C N C N C N C N C N C N
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9 10 11 12 13 14


II
6hpi
12hpi
6hpi
12hpi
6hpi
12hpi
12hpi
Lysates mock mock mock mock 17+ 17+ 17+ 17+ Δ27 Δ27 Δ27 Δ27 M15 M15
C N C N C N C N C N C N C N
97KDa
66KDa
NUP98
1 2 3 4 5 6 7 8 9 10 11 12 13 14


III
6hpi
12hpi
6hpi
12hpi
6hpi
12hpi
12hpi
Lysates mock mock mock mock 17+ 17+ 17+ 17+ Δ27 Δ27 Δ27 Δ27 M15 M15
C N C N C N C N C N C N C N
66KDa
45KDa
p62
1 2 3 4 5 6 7 8 9 10 11 12 13 14

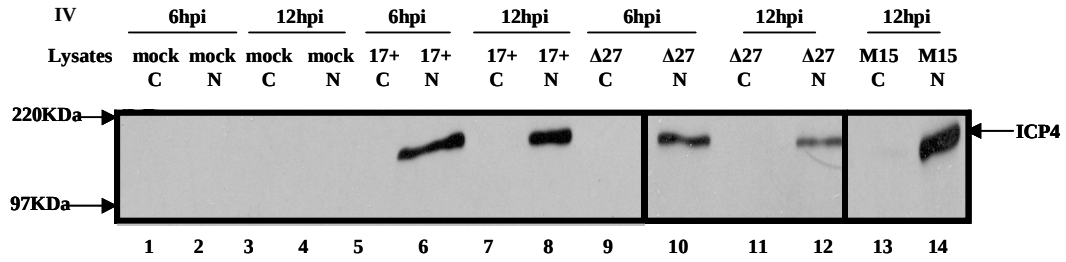

IV
6hpi
12hpi
6hpi
12hpi
6hpi
12hpi
12hpi
Lysates mock mock mock mock 17+ 17+ 17+ 17+ Δ27 Δ27 Δ27 Δ27 M15 M15
C N C N C N C N C N C N C N
220KDa
97KDa
ICP4
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Figure 5.7: Nuclear and cytoplasmic expression of ICP27 and nucleoporins during a time course study of HeLa cells mock infected and infected with wt HSV-1 and ICP27 mutants

Panels I, II, III and IV Shows western blots for ICP27, nucleoporins and ICP4 in HeLa infected cells at a 6 and 12 h after infection with wt HSV-1, ICP27 null Δ27 virus, M15 and mock infected cells that demonstrate distribution of nuclear and cytoplasmic expression of these proteins. HeLa cells were grown in a 175 mm flask and infected with wt HSV-1 strain 17+, Δ27, M15 and mock infected by MOI=10 at 6 and 12 hpi. Samples were collected at various time points post-infection, cells were pelleted, and nuclear and cytoplasmic fractions prepared according to the manufacturer's instructions (Active Motif). Proteins were separated by 10% SDS-PAGE and visualised by western blotting with different antibodies. Then membranes were stripped and western blotted with anti-ICP4 antibody. Samples used in lanes: 1-4 were mock infected (MI) cell extract. Lanes 5-8 were 17+ infected cell extract. Lanes 9-12 were Δ27 infected cell extract. Lanes 13 and 14 were M15 just at 12 hpi.

Panel I: Nuclear and cytoplasmic expression of ICP27 western blotted with anti-ICP27 antibody 1113.

Panel II: Nuclear and cytoplasmic expression of NUP 98 western blotted with anti-NUP 98 (L-20) antibody.

Panel III: Nuclear and cytoplasmic expression of NUP 62 western blotted with anti-NUP 62 (N-19) antibody.

Panel IV: Nuclear and cytoplasmic expression of ICP4 western blotted with anti-ICP4 antibody showed expression of this protein in wt, M15 and Δ27.

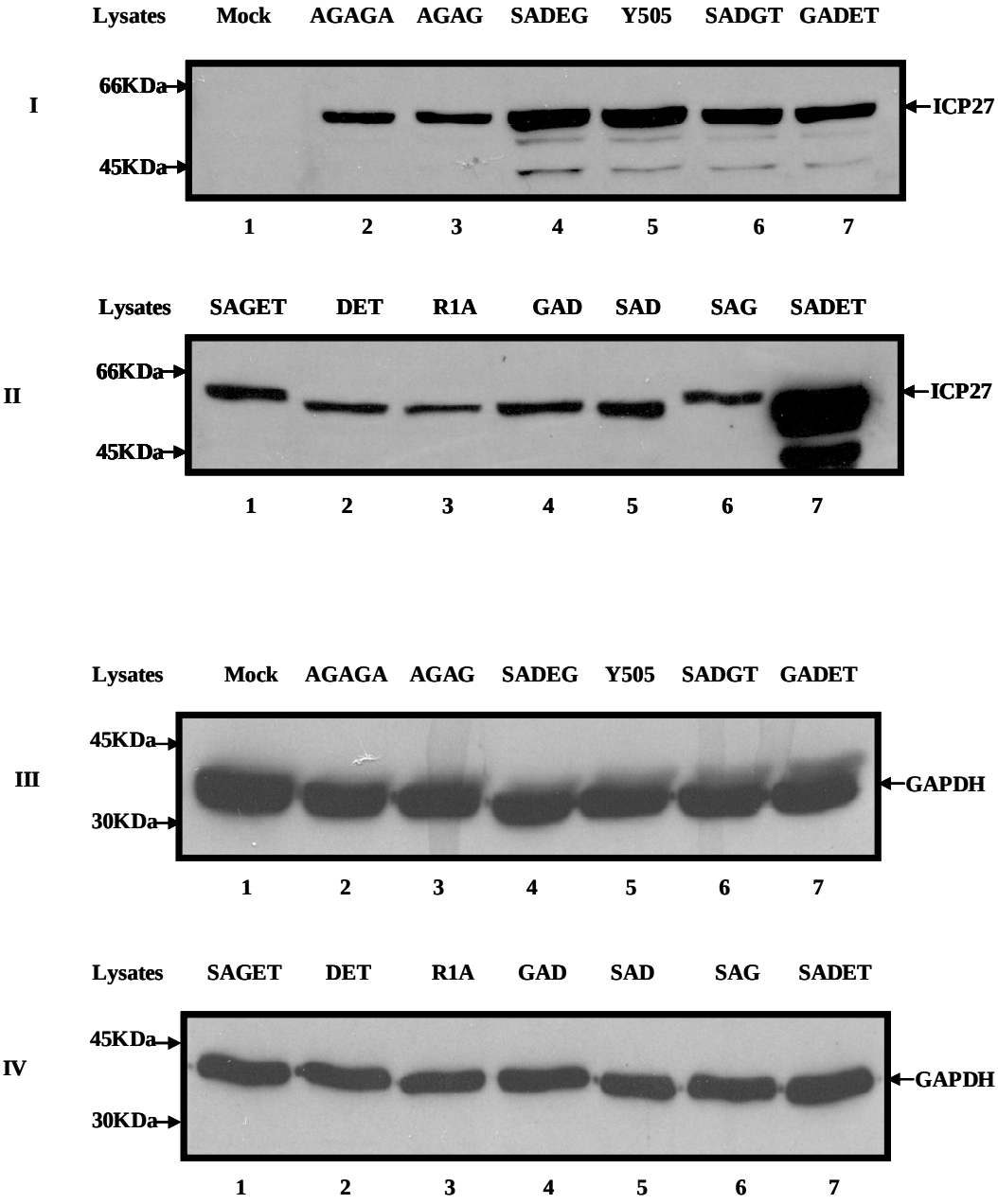

I
Lysates Mock AGAGA AGAG SADEG Y505 SADGT GADET
66KDa
45KDa
ICP27
1 2 3 4 5 6 7
II
Lysates SAGET DET R1A GAD SAD SAG SADET
66KDa
45KDa
ICP27
1 2 3 4 5 6 7
III
Lysates Mock AGAGA AGAG SADEG Y505 SADGT GADET
45KDa
30KDa
GAPDH
1 2 3 4 5 6 7
IV
Lysates SAGET DET R1A GAD SAD SAG SADET
45KDa
30KDa
GAPDH
1 2 3 4 5 6 7

Figure 5.8: Expression profile of wt and SADET mutant ICP27.

To show the profile of protein expression and predicted size of the proteins, HSV-1 wt ICP27 and ICP27 SADET mutant were transfected into HeLa cells. Transfected cells were harvested after 24 hours and Bradford assay carried out to determine protein concentration. Equal amounts of lysates were fractionated on 10% SDS-PAGE and western blotted with antibodies shown. 6µg DNA used for transfection regarding 60 mm dishes.

Panel I: Transferred membranes western blotted with anti ICP27 antibody showing varying expression levels of SADET mutant ICP27s (lanes 2-7).
Panel II: Another transferred membrane western blotted with anti ICP27 antibody showing varying expression levels of SADET mutant ICP27s (lanes 1-7).

Panel III: Above membrane (Panel I) stripped and western blotted against GAPDH showed roughly similar amount of proteins used in each lane (lanes 1-7).
Panel IV: Above membrane (Panel II) stripped and western blotted against GAPDH showed roughly similar amount of proteins used in each lane (lanes 1-7).

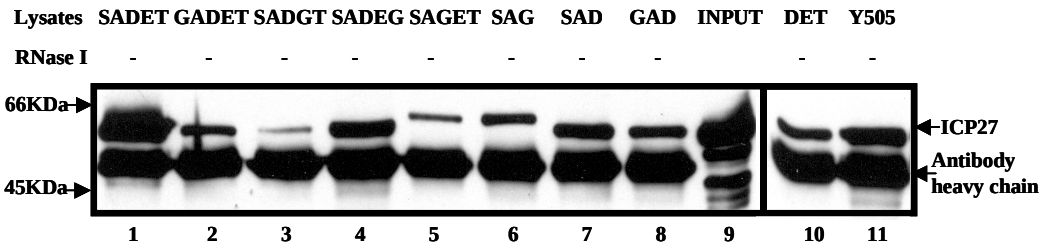
Table 5.1: ICP27 SADET mutants and their amino acid changes used

Mutants	Amino acid changes
SADET	WT
AGAGA	SADET to AGAGA
AGAG	SADET to AGAGT
SADEG	T to G
Y505	Y to A
SADGT	E to G
GADET	S to G
SAGET	D to G
DET	SA deletion
R1A*	--------*
GAD	S to G and ET deletion
SAD	ET deletion
SAG	D to G and ET deletion

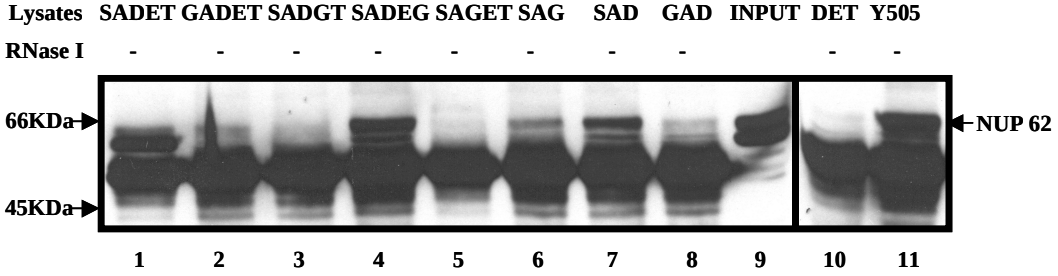
All of the above mutations were introduced into the expression plasmid pCMV-27 that contains the ICP27 ORF under the control of a HCMV IE promoter (Leiper, 2004).

* Removed from further experiments because of lack of details that was impossible to get.

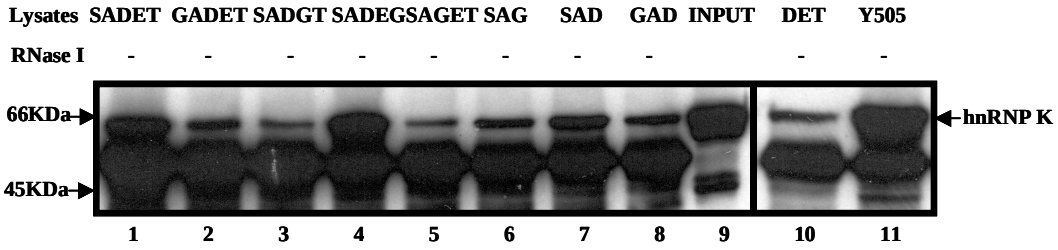
I


Lysates SADET GADET SADGT SADEG SAGET SAG SAD GAD INPUT DET Y505
RNase I - - - - - - - - - -
66KDa
45KDa
ICP27
Antibody heavy chain
1 2 3 4 5 6 7 8 9 10 11

II


Lysates SADET GADET SADGT SADEG SAGET SAG SAD GAD INPUT DET Y505
RNase I - - - - - - - - - -
66KDa
45KDa
NUP 62
1 2 3 4 5 6 7 8 9 10 11

III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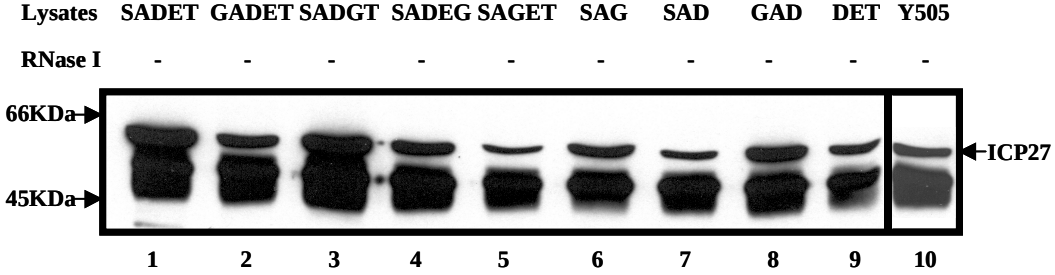
RNase I - - - - - - - - - -
66KDa
45KDa
hnRNP K
1 2 3 4 5 6 7 8 9 10 11

IV


Lysates SADET GADET SADGT SADEG SAGET SAG SAD GAD DET Y505
RNase I - - - - - - - - - -
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9 10

V

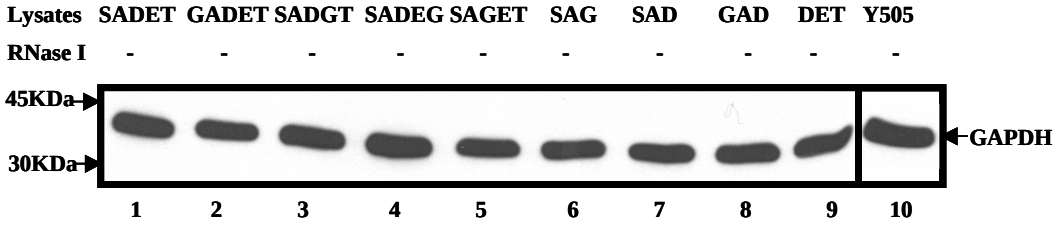

Lysates SADET GADET SADGT SADEG SAGET SAG SAD GAD DET Y505
RNase I - - - - - - - - - -
45KDa
30KDa
GAPDH
1 2 3 4 5 6 7 8 9 10

Figure 5.9: SADET region involvement in interaction of ICP27 and nucleoporins by using immunoprecipitation assay under non RNase I treatment condition.

Immunoprecipitation experiments performed with a range of cell lysates 24 h after transfection. Plasmids expressing ICP27 SADET mutants were transfected into HeLa cells. Lysates were prepared after 24 h and incubated with anti ICP27 antibody. Bound and unbound samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane and western blotted with specific antibodies.

Panel I: Membranes western blotted with anti ICP27 antibody showed precipitation of ICP27 in all samples.

Panel II: Above membranes stripped and western blotted with anti NUP 414 antibody showed co-precipitation of NUP 62 in some of the samples (lanes 1, 4, 6, 7 and 11) but not with others (lanes 2, 3, 5, 8 and 10).

Panel III: Rest of samples run and visualised by western blotting with anti hnRNP K antibody showed co-precipitation of hnRNP K by all the wt and SADET ICP27 mutants.

Panel IV: Unbound samples run and western blotted with anti ICP27 antibody showed presence of ICP27 in all samples.

Panel V: Above unbound samples membranes stripped and western blotted against GAPDH showed equal amount proteins used in this experiment.

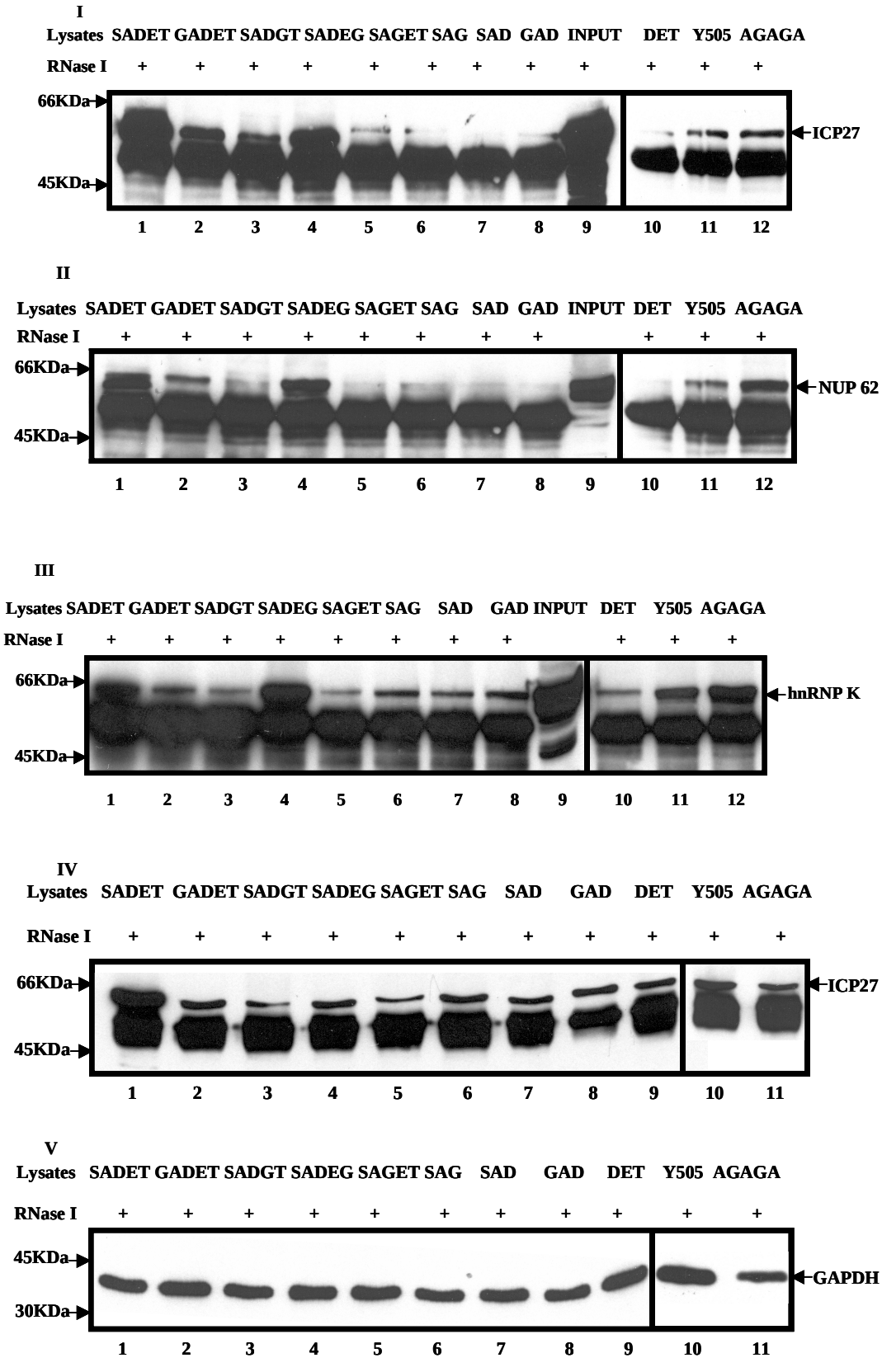

I
Lysates SADET GADET SADGT SADEG SAGET SAG SAD GAD INPUT DET Y505 AGAGA
RNase I + + + + + + + + + + + +
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9 10 11 12
II
Lysates SADET GADET SADGT SADEG SAGET SAG SAD GAD INPUT DET Y505 AGAGA
RNase I + + + + + + + + + + + +
66KDa
45KDa
NUP 62
1 2 3 4 5 6 7 8 9 10 11 12
III
Lysates SADET GADET SADGT SADEG SAGET SAG SAD GAD INPUT DET Y505 AGAGA
RNase I + + + + + + + + + + + +
66KDa
45KDa
hnRNP K
1 2 3 4 5 6 7 8 9 10 11 12
IV
Lysates SADET GADET SADGT SADEG SAGET SAG SAD GAD DET Y505 AGAGA
RNase I + + + + + + + + + + + +
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9 10 11
V
Lysates SADET GADET SADGT SADEG SAGET SAG SAD GAD DET Y505 AGAGA
RNase I + + + + + + + + + + + +
45KDa
30KDa
GAPDH
1 2 3 4 5 6 7 8 9 10 11

Figure 5.10: SADET region involvement in interaction of ICP27 and nucleoporins by using immunoprecipitation assay under RNase I treatment condition.

Immunoprecipitation experiments performed with a range of cell lysate after 24 h of to transfection. Plasmids expressing ICP27 SADET mutants were transfected into HeLa cells. Lysates were prepared after 24 h and incubated with anti ICP27 antibody and treated with RNase I. Bound and unbound samples were separated by 10% SDS-PAGE gel and transferred to nitrocellulose membrane and western blotted with specific antibodies.

Panel I: Membranes western blotted with anti ICP27 antibody showed precipitation of ICP27 in most of samples.

Panel II: Above membranes stripped and western blotted with anti NUP 414 antibody showed co-precipitation of NUP 62 to some of the samples (lanes 1, 2, 3, 4, 11 and 12) but not with others (lanes 5, 6, 7, 8 and 10).

Panel III: Rest of samples run and visualised by western blotting with anti hnRNP K antibody showed co-precipitation of hnRNP K by all the wt and SADET ICP27 mutants.

Panel IV: Unbound samples run and western blotted with anti ICP27 antibody showed presence of ICP27 in all samples.

Panel V: Above unbound samples membranes stripped and western blotted against GAPDH showed equal amount proteins used in this experiment.

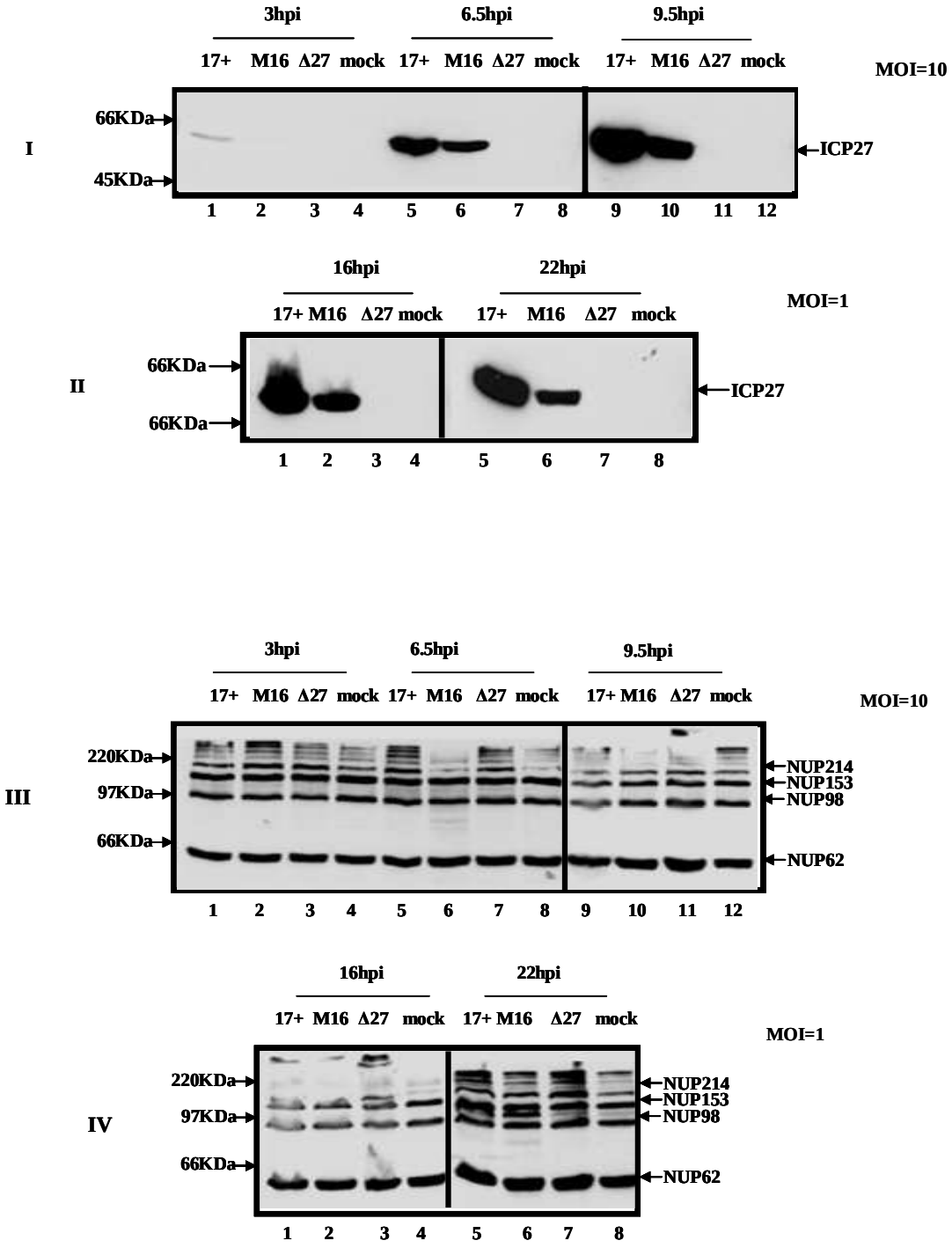

3hpi
6.5hpi
9.5hpi
17+ M16 Δ27 mock 17+ M16 Δ27 mock 17+ M16 Δ27 mock
MOI=10
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9 10 11 12
I
16hpi
22hpi
17+ M16 Δ27 mock 17+ M16 Δ27 mock
MOI=1
66KDa
66KDa
ICP27
1 2 3 4 5 6 7 8
II
3hpi
6.5hpi
9.5hpi
17+ M16 Δ27 mock 17+ M16 Δ27 mock 17+ M16 Δ27 mock
MOI=10
220KDa
97KDa
66KDa
NUP214
NUP153
NUP98
NUP62
1 2 3 4 5 6 7 8 9 10 11 12
III
16hpi
22hpi
17+ M16 Δ27 mock 17+ M16 Δ27 mock
MOI=1
220KDa
97KDa
66KDa
NUP214
NUP153
NUP98
NUP62
1 2 3 4 5 6 7 8
IV

Figure 5.11: Time course study of expression of ICP27 and FG repeat nucleoporins in HeLa cells infected with wt and mutant HSV-1.

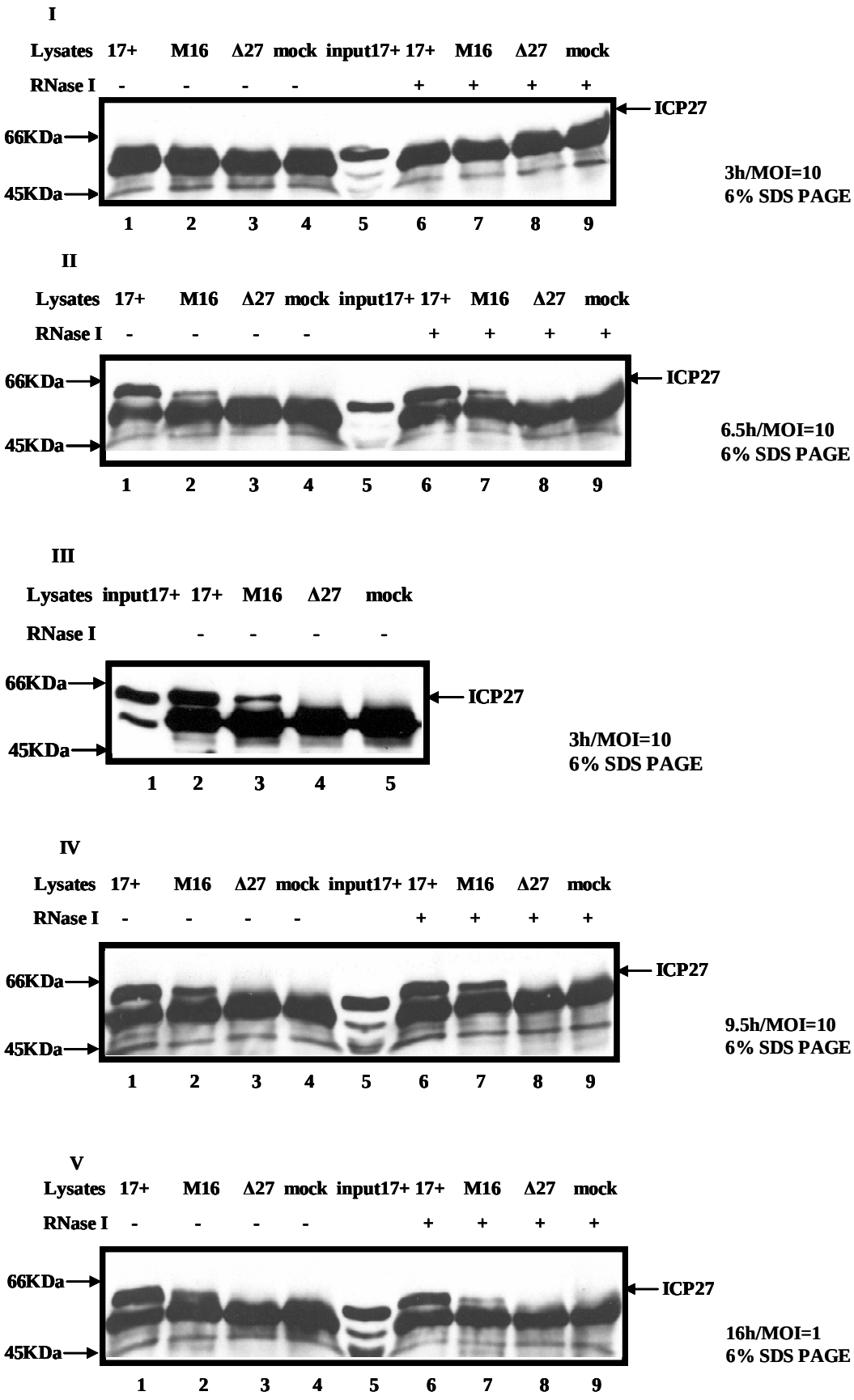
Hela cells were infected with wt HSV-1 and HSV-1 mutants Δ27 and M16 or mock infected. Cells were harvested at 3, 6.5, 9.5 (MOI=10), 16 and 22 (MOI=1) hpi and protein concentration obtained by Bradford assay. Equal amounts of protein were fractionated on 6% SDS-PAGE and transferred to nitrocellulose membrane and western blotted with anti ICP27 antibody 1113 and anti FG-repeat NUPs 414.

Panel I: Membranes were western blotted against ICP27 and showed expression of wt ICP27 from 3 hpi (lane 1) that increased after 6.5 and 9.5 hpi at MOI=10 (lanes 5,9) but expression of M16 ICP27 is shown from 6.5 hpi and enhanced until 9.5 hpi (lanes 6, 10). Also Δ27 and mock-infected samples did not show any ICP27 expression.

Panel II: Shows expression of wt ICP27 and M16 at 16 hpi (lane 1) that increased until 22 hpi at MOI=1 (lanes 5). Also Δ27 and mock-infected samples did not show any ICP27 expression.

Panel III: Above membranes (Panel I) were stripped and western blotted against FG-repeat NUPs showing roughly similar levels of nucleoporins in infected samples at MOI=10 and different time of infection (lanes 1-12).

Panel IV: Above membranes (Panel II) were stripped and western blotted against FG-repeat NUPs showing roughly similar levels of nucleoporins in infected samples at MOI=1 and different time of infection (lanes 1-8).


I
Lysates 17+ M16 Δ27 mock input17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
ICP27
66KDa
45KDa
1 2 3 4 5 6 7 8 9
3h/MOI=10
6% SDS PAGE
II
Lysates 17+ M16 Δ27 mock input17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9
6.5h/MOI=10
6% SDS PAGE
III
Lysates input17+ 17+ M16 Δ27 mock
RNase I - - - -
66KDa
45KDa
ICP27
1 2 3 4 5
3h/MOI=10
6% SDS PAGE
IV
Lysates 17+ M16 Δ27 mock input17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9
9.5h/MOI=10
6% SDS PAGE
V
Lysates 17+ M16 Δ27 mock input17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9
16h/MOI=1
6% SDS PAGE

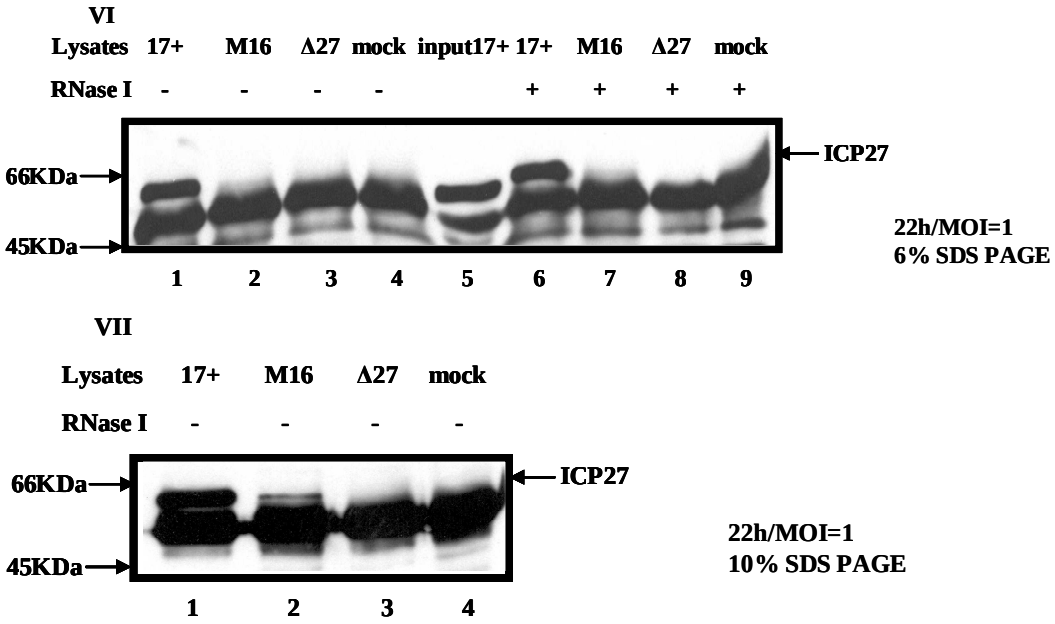
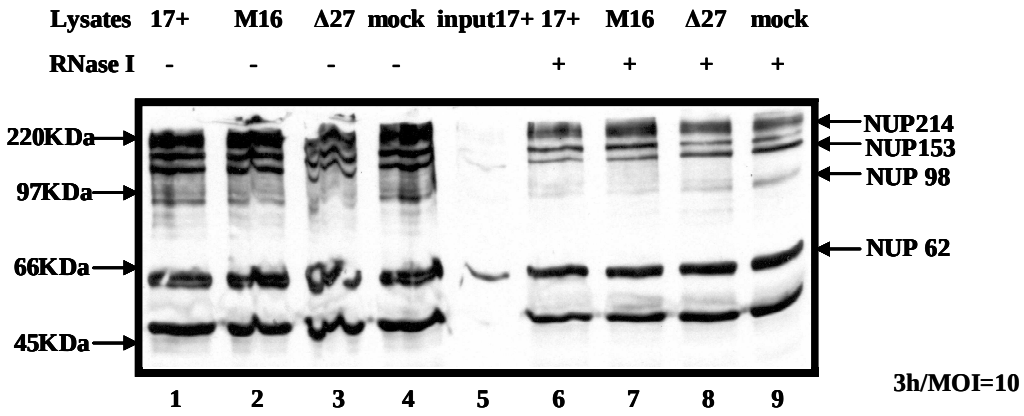

VI
Lysates 17+ M16 Δ27 mock input 17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9
22h/MOI=1
6% SDS PAGE
VII
Lysates 17+ M16 Δ27 mock
RNase I - - - -
66KDa
45KDa
ICP27
1 2 3 4
22h/MOI=1
10% SDS PAGE

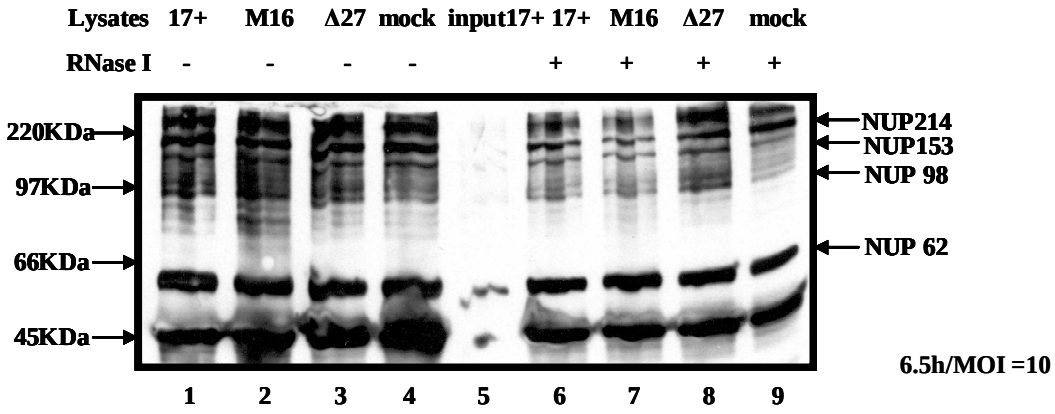
Figure 5.12: Immunoprecipitation profile of HSV-1 wild type and mutant ICP27 HeLa infected cell extracts at different post infection times. **Equal amounts of proteins from HeLa cell extracts infected with HSV-1 wt, M16, Δ27 and mock infected at different times were incubated with anti ICP27 antibody. Samples were either treated or untreated with RNase I and fractionated on 6% or 10% SDS-PAGE and transferred to nitrocellulose membranes and western blotted with anti ICP27 antibody.**

Panel I: Precipitation of ICP27 at 3 hours post infection on 6% SDS-PAGE gel.
Panel II: Precipitation of ICP27 at 6.5 hours post infection on 6% SDS-PAGE gel.
Panel III: Precipitation of ICP27 at 6.5 hours post infection on 10% SDS-PAGE gel and RNase I absence.
Panel IV: Precipitation of ICP27 at 9.5 hours post infection on 6% SDS-PAGE gel.
Panel V: Precipitation of ICP27 at 16 hours post infection on 6% SDS-PAGE gel
Panel VI: Precipitation of ICP27 at 22 hours post infection on 6% SDS-PAGE gel.
Panel VII: Precipitation of ICP27 at 22 hours post infection on 10% SDS-PAGE gel and RNase I absence.
The input lane in each case was a lysate of HeLa cells infected with wild type HSV-1 17+ for 8hpi at on MOI=10 and it is 10% of HSV-1 wt infected cell extract lysate.

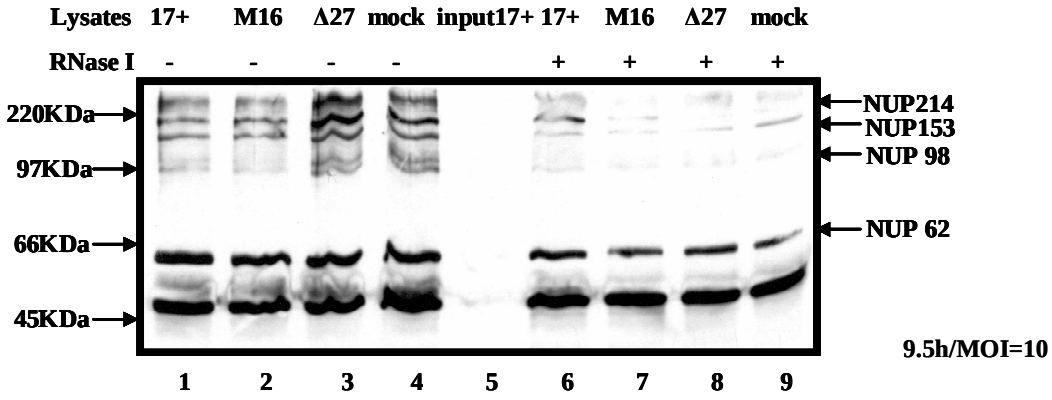
I


Lysates 17+ M16 Δ27 mock input17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
220KDa
97KDa
66KDa
45KDa
NUP214
NUP153
NUP 98
NUP 62
1 2 3 4 5 6 7 8 9
3h/MOI=10

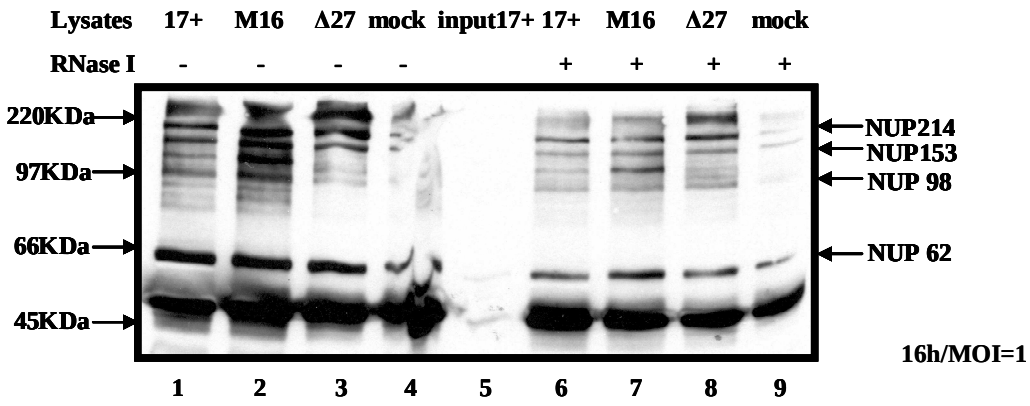
II


Lysates 17+ M16 Δ27 mock input17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
220KDa
97KDa
66KDa
45KDa
NUP214
NUP153
NUP 98
NUP 62
1 2 3 4 5 6 7 8 9
6.5h/MOI =10

III


Lysates 17+ M16 Δ27 mock input17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
220KDa
97KDa
66KDa
45KDa
NUP214
NUP153
NUP 98
NUP 62
1 2 3 4 5 6 7 8 9
9.5h/MOI=10

IV


Lysates 17+ M16 Δ27 mock input17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
220KDa
97KDa
66KDa
45KDa
NUP214
NUP153
NUP 98
NUP 62
16h/MOI=1
1 2 3 4 5 6 7 8 9

V

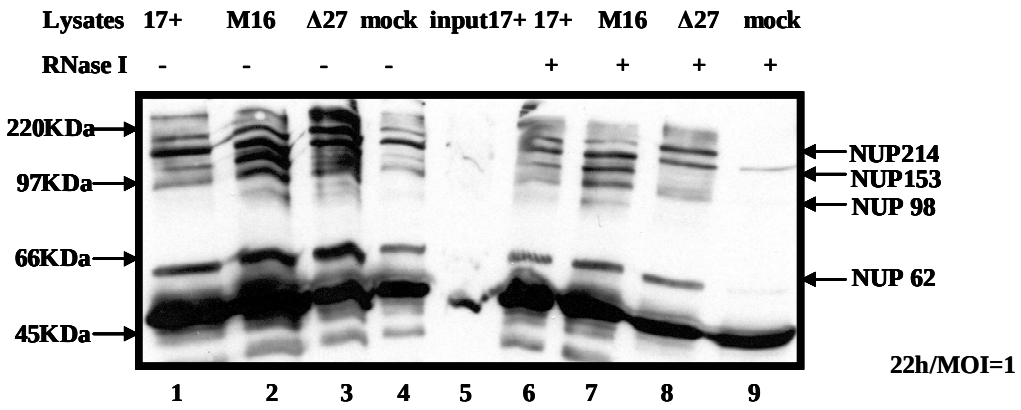

Lysates 17+ M16 Δ27 mock input17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
220KDa
97KDa
66KDa
45KDa
NUP214
NUP153
NUP 98
NUP 62
22h/MOI=1
1 2 3 4 5 6 7 8 9

Figure 5.13: Immunoprecipitation test with anti FG-repeat NUPs 414 used in time course study.

Equal amount of proteins from HeLa cells infected with HSV-1 wt and HSV-1 mutants M16 and Δ27 and mock infected at different times were incubated with anti 414 antibody. Samples were either treated or untreated with RNase I fractionated on 6% SDS-PAGE and transferred to nitrocellulose membranes and western blotted with anti 414 antibody. Less amounts of input used in this gels. 6 of HSV-1 wt infected cell extract lysate used as a input.

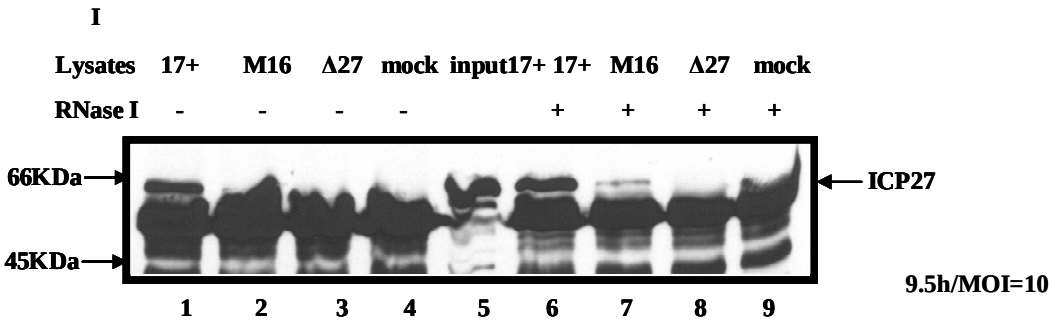
Panel I: Precipitation of FG repeat nucleoporins showed at 3 hours post infection.

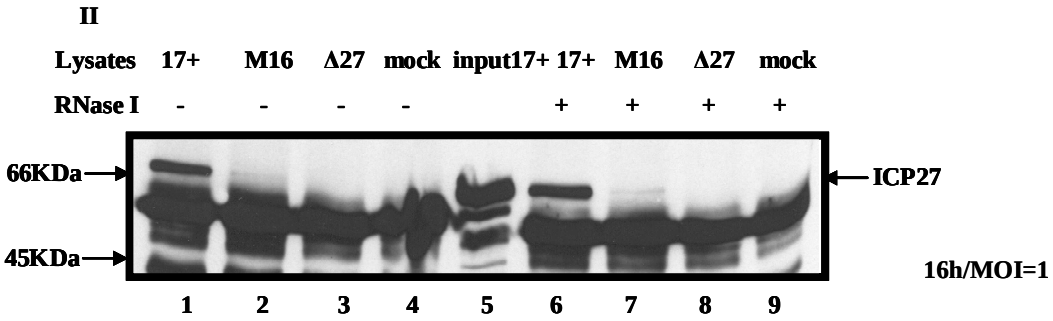
Panel II: Precipitation of FG repeat nucleoporins showed at 6.5 hours post infection

Panel III: Precipitation of FG repeat nucleoporins showed at 9.5 hours post infection

Panel IV: Precipitation of FG repeat nucleoporins showed at 16 hours post infection

Panel V: Precipitation of FG repeat nucleoporins showed at 22 hours post infection


I
Lysates 17+ M16 Δ27 mock input17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9
9.5h/MOI=10


II
Lysates 17+ M16 Δ27 mock input17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9
16h/MOI=1

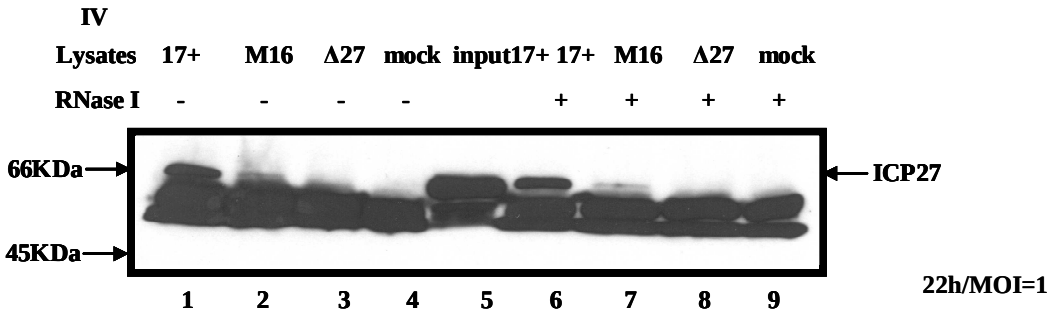

IV
Lysates 17+ M16 Δ27 mock input17+ 17+ M16 Δ27 mock
RNase I - - - - + + + +
66KDa
45KDa
ICP27
1 2 3 4 5 6 7 8 9
22h/MOI=1

Figure 5.14: Co-immunoprecipitation profile of wild type and mutants ICP27 HeLa infected cells at different post infection time.

Equal amounts of proteins from HeLa cells infected with HSV-1 wt and HSV-1 mutants M16 and Δ27 and mock infected at different times were incubated with anti FG-repeat 414 antibody. Samples were either treated or untreated with RNase I, fractionated on 6% SDS-PAGE and transferred to nitrocellulose membranes and western blotted with anti ICP27 antibody.10% of HSV-1 wt infected cell extract lysate used for input.

Panel I: Co-precipitation of ICP27 showed at 9.5 hours post infection.

Panel II: Co-precipitation of ICP27 showed at 16 hours post infection.

Panel III: Co-precipitation of ICP27 showed at 22 hours post infection.

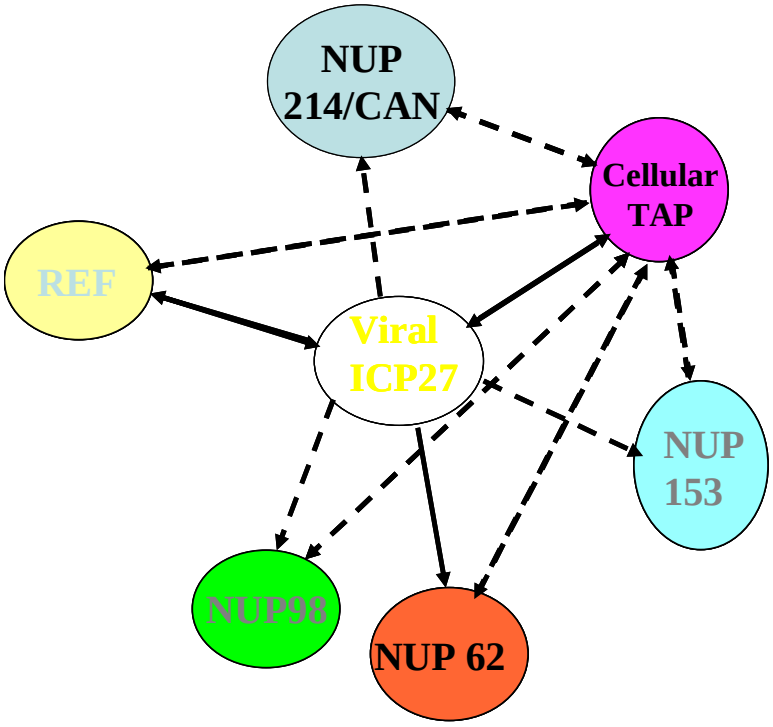


Figure 6.1: Schematic diagram of interaction of ICP27 with nucleoporins.

ICP27 may interacs with several FG-repeat nucleoporins NUP 62, NUP 153, NUP 214/CAN. It interacts with TAP that is associated with these nucleoporins in the nucleo-cytoplasmic transport machinery. The solid lines represent proved interactions and the dotted lines represents possible interactions.

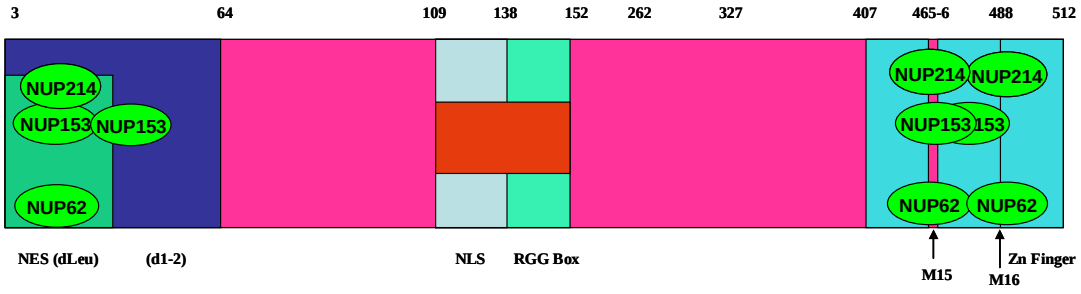


Figure 6.2: Regions of ICP27 involved in interaction with nucleoporins.

Mapping studies of ICP27 interaction with different NUPs showed that:
NUP 62 interacts with dLeu, M15 and M16 regions of ICP27
NUP 153 interacts with dLeu and M15 regions of ICP27 with contribution of d1-2 and M16
NUP 214 interacts with dLeu, M15 and M16 regions of ICP27

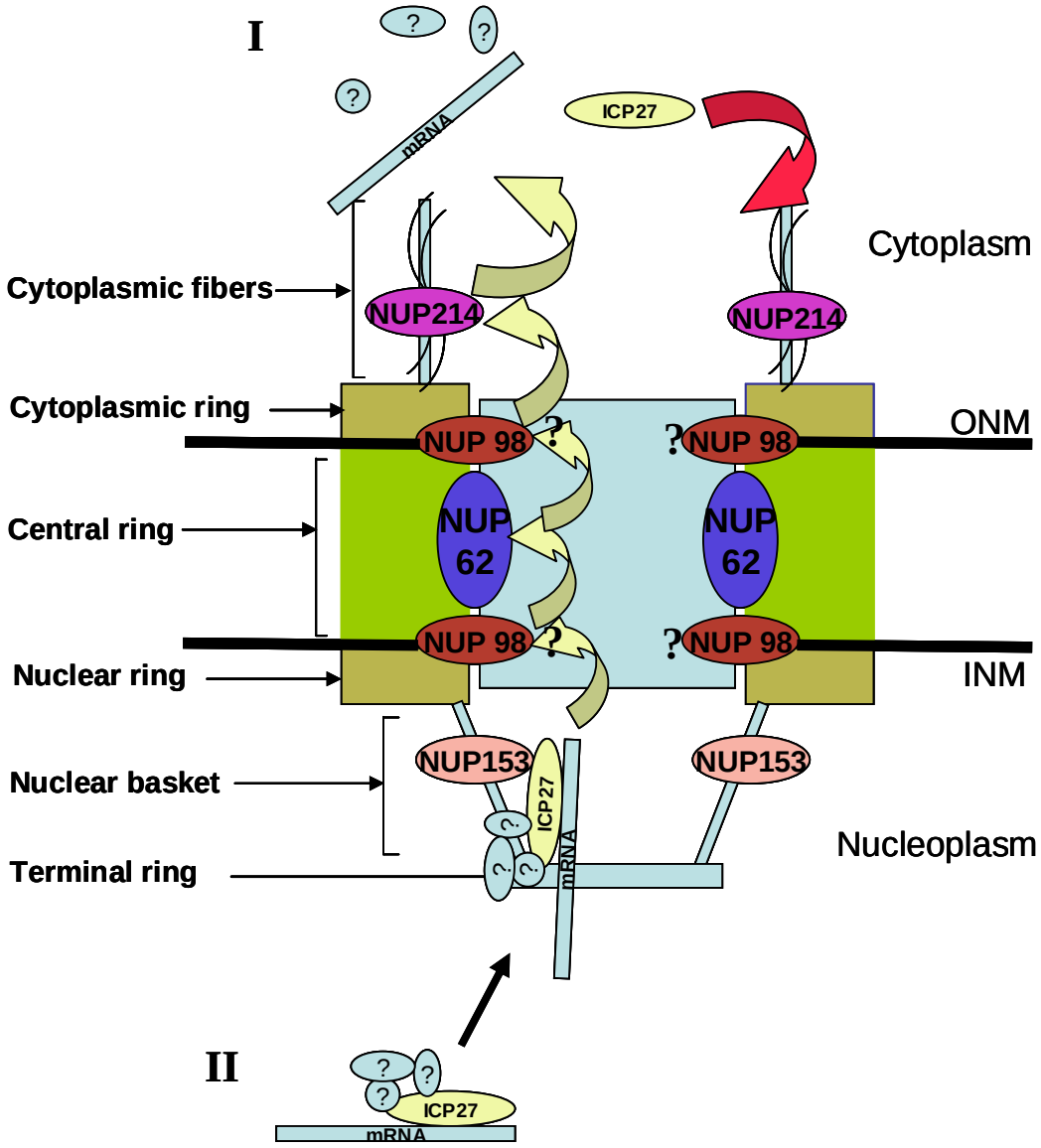


Figure 6.3: Proposed involvement of ICP27 and NUPs in transport of cargo-dependent ICP27. I: The ICP27/mRNA complex binds first to NUP 153. It is then handed to the other FG-repeat NUPs such as NUP 98 and NUP 62 to translocate through the central pore, and pass to the cytoplasmic face of the NPC by interaction with cytoplasmic face NUPs such as NUP 214. Finally, dissociation of the complex occurs in the cytoplasm. II: Representation of the complex formed of ICP27 with viral RNA that might interact with NUP 153 FG-repeat-binding site to position in the nuclear periphery of the NPC.

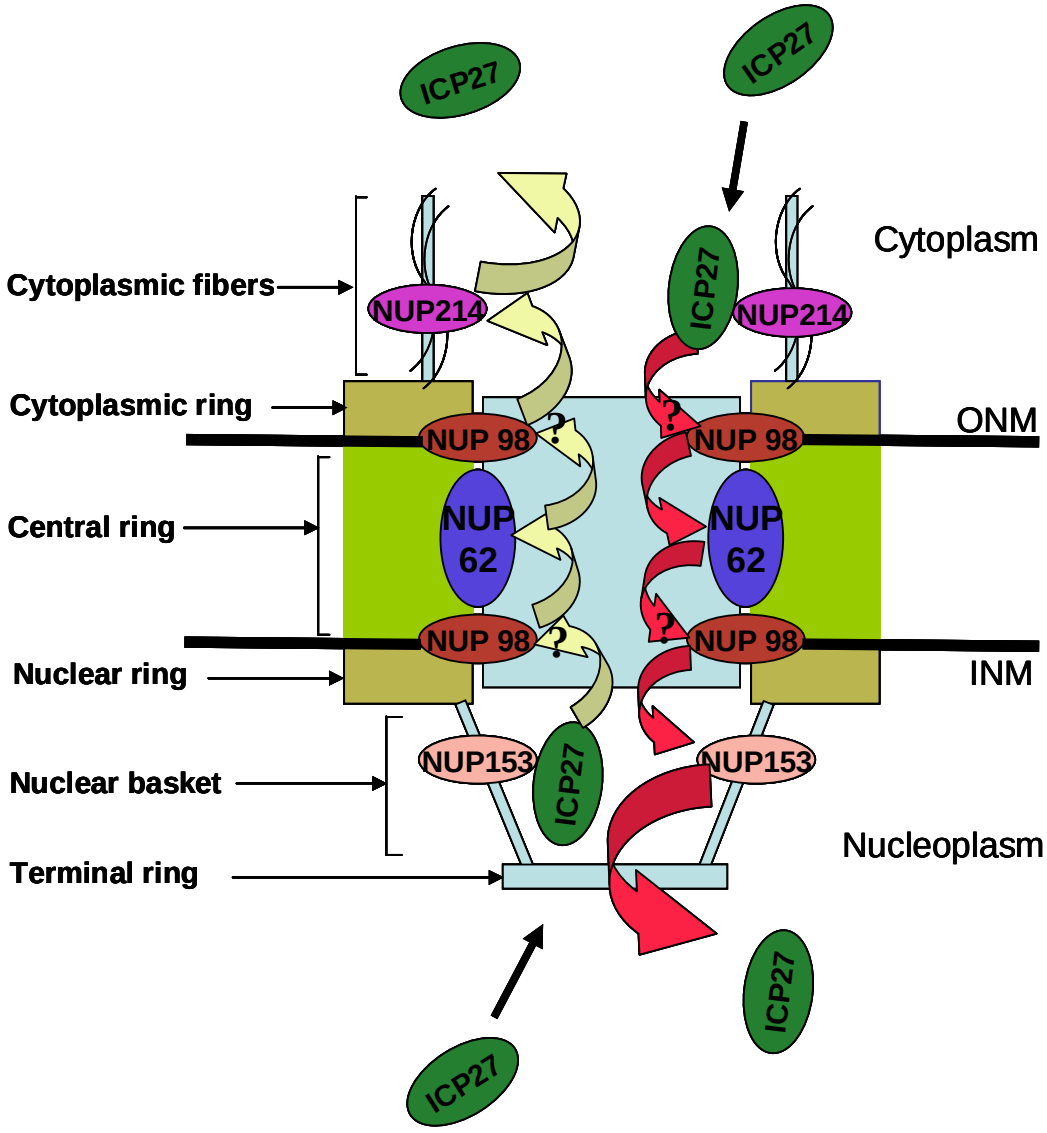


Figure 6.4: A model of ICP27 nucleocytoplasmic trafficking: Possible unphosphorylated ICP27 could constitutively shuttle between the nucleus and the cytoplasm via direct interactions with the nucleoporins. This carrier-independent translocation pathway facilitates export and import of the ICP27 through the NPC.

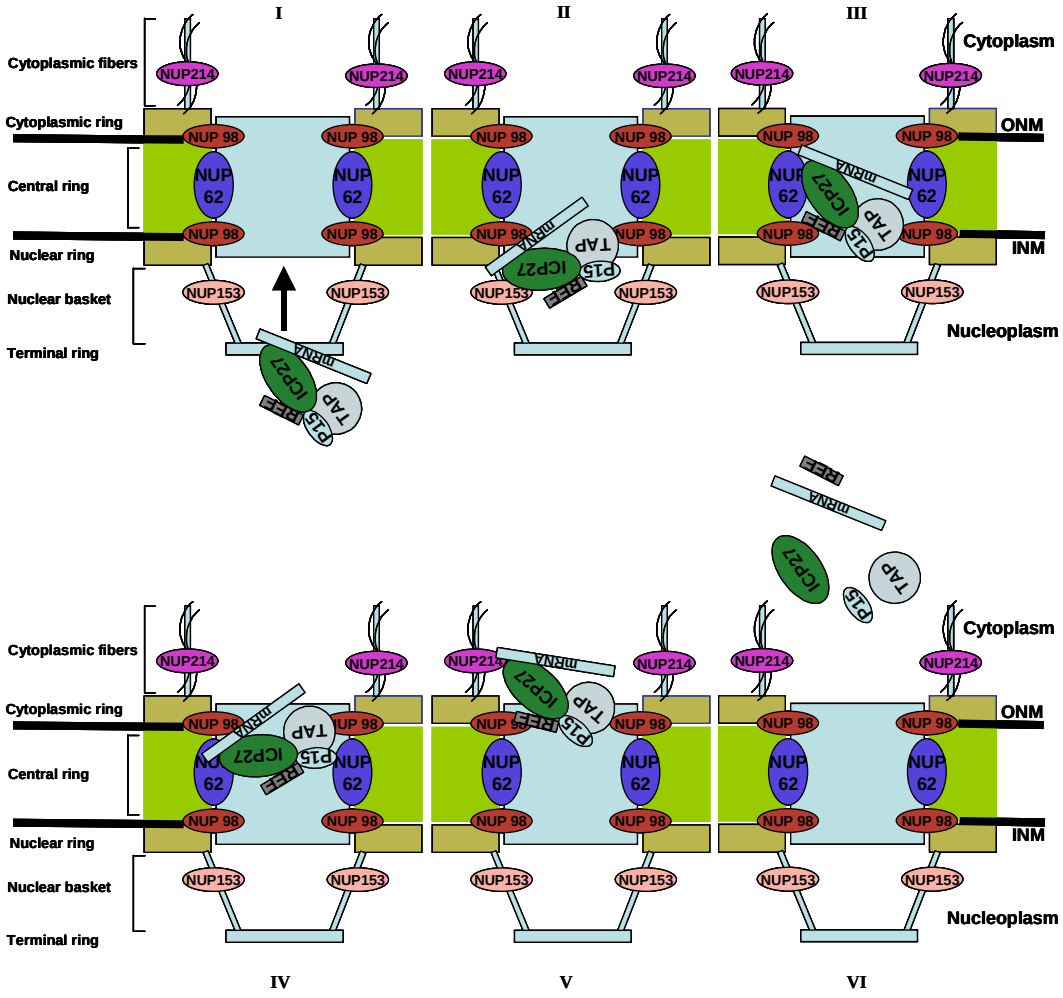


Figure 6.5: Possible association of ICP27 and TAP in interaction with nucleoporins for transport of cargoes.

A complex of cargo that can interact with ICP27 that is in complex with TAP could translocate through the nuclear pore complex and mediate transport between the nucleus and the cytoplasm (**I-VI**).

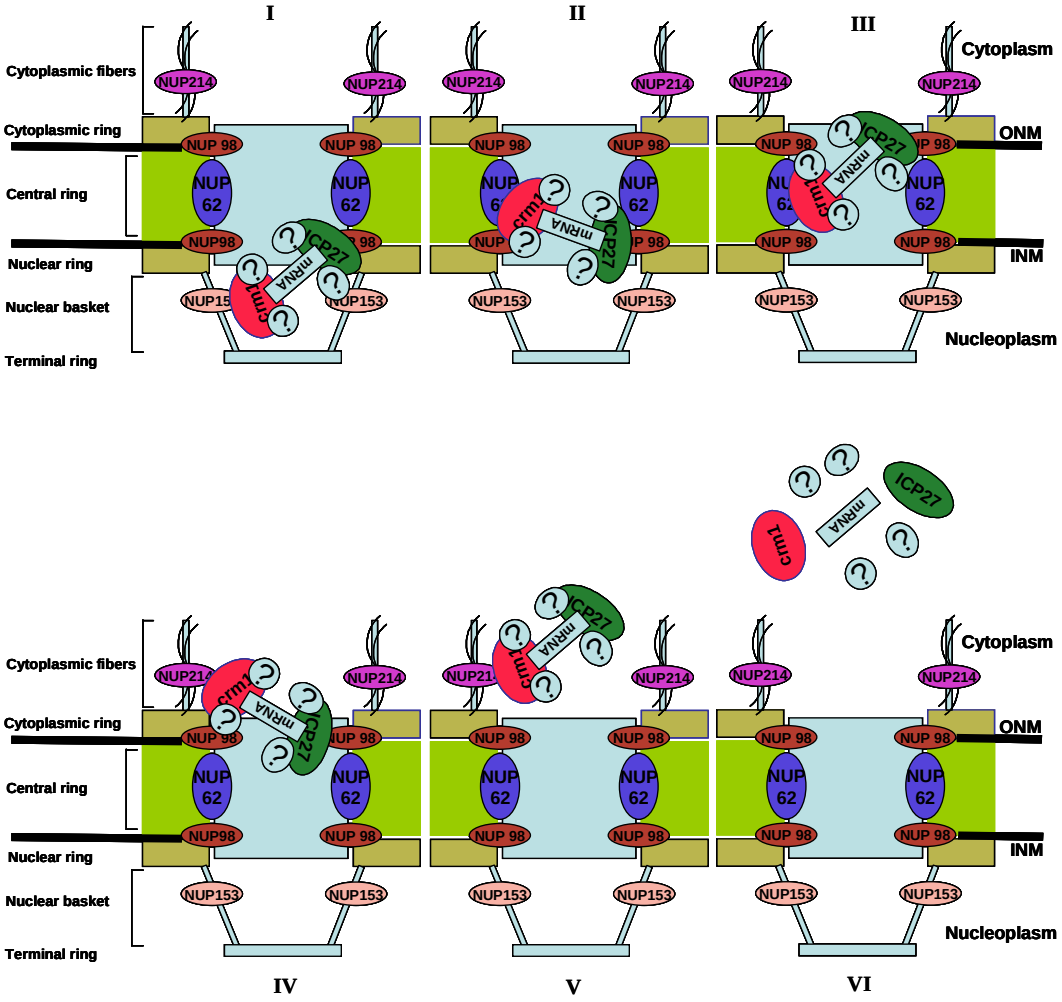


Figure 6.6: Possible association of ICP27 and CRM1 in interaction with nucleoporins for export of cargoes.

A cargo complex could interact with two transport factors ICP27 and CRM1 simultaneously and translocate through the nuclear pore complex to transport between the nucleus and the cytoplasm (**I-VI**).